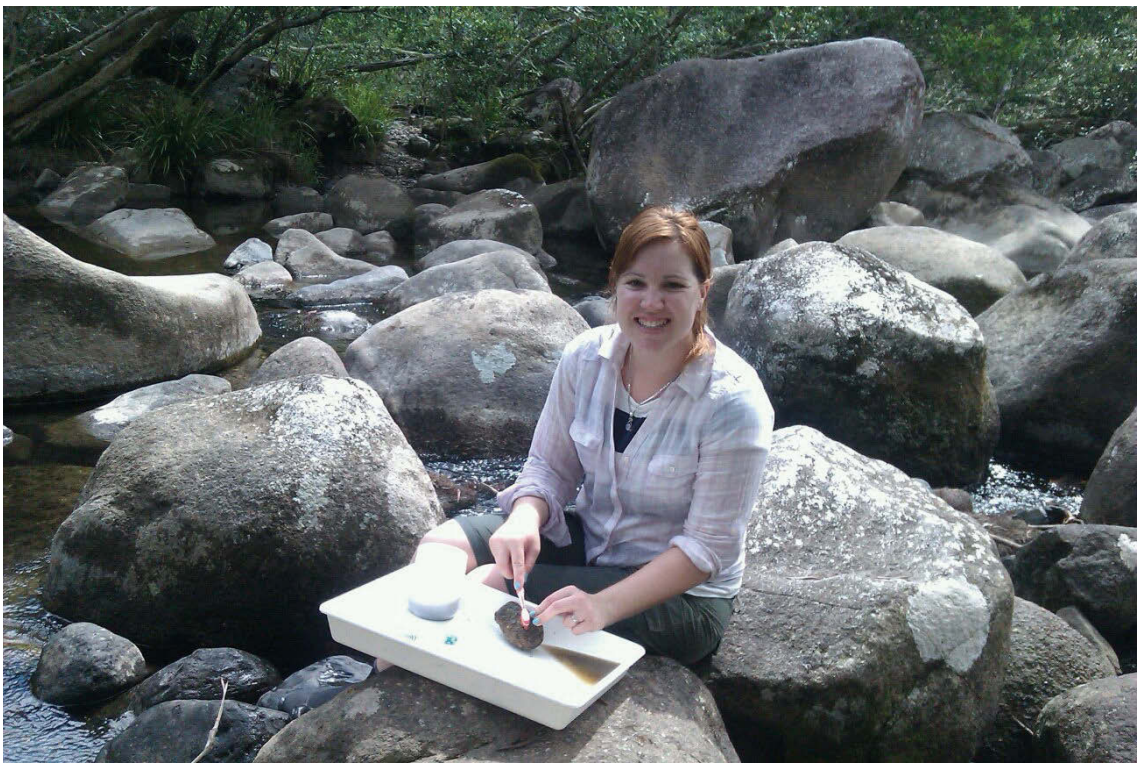
# Benthic diatoms as indicators of herbicide toxicity in rivers



*The author scrubbing rocks*

Rebecca J. Wood

A thesis in fulfilment of the requirements for the degree of Doctor of Philosophy

July 2017

Ecosystem Security Team

School of Life Sciences

University of Technology Sydney

## Certificate of original authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text. This research is supported by an Australian Government Research Training Program Scholarship.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature:

Date:

23/07/17

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# Preface

This thesis consists of seven chapters. Chapters 2 to 5 are written as peer reviewed journal articles and have been published in scientific journals. Chapter 6 will be submitted to a journal for peer review. They are included in this this thesis as they were when accepted by the relevant journal and therefore some minor differences may occur from the final published manuscripts. Publication details and contributions of co-authors are detailed below.

**Chapter 2:** Wood, R.J., Mitrovic, S.M., Kefford, B.J., 2014. Determining the relative sensitivity of benthic diatoms to atrazine using rapid toxicity testing: A novel method. *Science of The Total Environment* 485–486, 421-427.

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**Chapter 3:** Wood, R.J., Mitrovic, S.M., Lim, R.P., Kefford, B.J., 2016. How benthic diatoms within natural communities respond to eight common herbicides with different modes of action. *Science of The Total Environment* 557–558, 636-643.

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**Chapter 4:** Wood, R.J., Mitrovic, S.M., Lim, R.P., Kefford, B.J., 2016. The influence of reduced light intensity on the response of benthic diatoms to herbicide exposure. *Environmental Toxicology and Chemistry* 35, 2252–2260.

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drafting of manuscript: R.J.W., revising manuscript: all.

**Chapter 5:** Wood, R.J., Mitrovic, S.M., Lim, R.P., Kefford, B.J., 2017. Chronic effects of atrazine exposure and recovery in freshwater benthic diatoms from two communities with different pollution histories. *Aquatic Toxicology* 109, 200-208.

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## List of Abbreviations

| | |
|---|---|
| DO | dissolved oxygen |
| EC | electrical conductivity |
| FRP | filterable reactive phosphorus |
| GBR | Great Barrier Reef |
| GLM | generalized linear model |
| NOx | oxidised nitrogen |
| PICT | pollution induced community tolerance |
| PSII | photosystem 2 inhibiting |
| SPEAR | SPEcies At Risk |
| SSD | species sensitivity distribution |
| TEF | toxic equivalency factor |
| TEQ | toxic equivalency quotient |
| TIS | toxicant induced succession |
| TSS | total suspended solids |

## Abstract

Agricultural herbicides are common pollutants of freshwater environments and pose a potential threat to aquatic biota. Assessing the impacts of herbicide pollution on primary producers such as benthic diatoms is essential in protecting freshwater ecosystems from degradation. Benthic diatoms are highly responsive to changes in environmental conditions and changes in community composition can be used to assess the ecological health of rivers. This thesis aims to investigate the impact of herbicide toxicity on benthic diatoms and to determine whether benthic diatoms are suitable indicators of herbicide toxicity in rivers that flow into the Great Barrier Reef (GBR). This was achieved through a series of scientific studies, each addressing key questions regarding the effects of herbicides on benthic diatoms.

Benthic diatoms exposed to herbicides in rapid toxicity tests showed varying sensitivity to herbicides, some taxa being highly sensitive whilst others were unaffected by herbicide exposure. The relative sensitivity of the diatom taxa was consistent between herbicides with differing modes of action and was not altered under reduced light intensities. Prior pollution of the collection site was influential in determining response of diatom communities to herbicide exposure; the diatom community from a highly polluted agricultural stream was less affected than the community collected from a reference site with no history of prior exposure. My thesis identifies individual diatom taxa that are most at risk of herbicide toxicity and also taxa that are tolerant and able to thrive under high herbicide concentrations. This study found that benthic diatom communities within the GBR catchment were affected by herbicide toxicity, showing a decline in sensitive taxa with increasing contamination of the site, after the wet season. Diatom communities were also influenced by other environmental variables such as nutrients and salinity and separating the individual effects of herbicides will require further research.

My thesis demonstrates the effects of herbicide toxicity on benthic diatoms at both the species and community levels. Each study in this thesis provides new insights into the effects of herbicide exposure on natural benthic diatom communities and contributes to the field of aquatic ecotoxicology. As a whole, my thesis illustrates the great potential that benthic diatoms have to assess agricultural impacts, including herbicides in rivers of the GBR catchment area.

# Chapter 1 Introduction

## 1.1 Scope and need for this study

Herbicides have been identified as a contributing pollutant of agricultural runoff that has degraded ecosystems of the Great Barrier Reef (GBR) (Brodie et al., 2012; Davis et al., 2013; Lewis et al., 2009). Widespread herbicide usage in agricultural regions of the GBR catchment area has been linked to herbicide contamination of waterways flowing into the reef (Brodie et al., 2012; Davis et al., 2013). Herbicides applied in the paddock are mobilized by rainfall events and irrigation waters, resulting in adjoining rivers receiving frequent concentrations of pollutants (O'Brien et al., 2016; Shaw et al., 2010; Smith et al., 2012). Herbicide pollution has the potential to adversely impact aquatic biota, especially phototrophs such as benthic diatoms (Magnusson et al., 2008; Magnusson et al., 2010). Better understanding the ecological risk of herbicide pollution to the GBR ecosystems is a key research goal set out in the Reef Scientific Consensus Statement (Waterhouse et al., 2017).

Concentrations of herbicides over the recommended trigger values for ecological protection are frequently detected in rivers of the GBR catchment area (Lewis et al., 2009). Clearly there is a need for monitoring tools that can complement chemical monitoring to provide ecologically relevant information on herbicide toxicity (Davis et al., 2013). Biomonitoring programs have the potential to provide information on the ecological effects of herbicide exposure (Liess et al., 2008). Additionally, biomonitoring is relatively inexpensive and can be performed over numerous sites and at varied time scales (Liess et al., 2008). Whilst there are a number of biological monitoring tools available, few are designed specifically for the measurement of herbicide impacts. Photosynthetic organisms such as diatoms have great potential as an indicator of herbicide impacts due to their physiological similarities to the herbicides' target organism, their broad distribution, and their fast response time (Bellinger et al., 2006; Burns and Ryder, 2001; Debenest et al., 2010). However, a diatom based monitoring index designed to detect herbicide impacts does not exist and there is a lack of information on the traits of different diatom taxa that contribute to their herbicide sensitivity, especially for Australian species (Magnusson et al., 2008). There is a need for further research on the sensitivity of individual freshwater benthic diatom species,

and to better understand how diatoms within natural benthic diatom communities respond to herbicide exposure.

## 1.2 Diatoms

### *1.2.1 Biology and Ecology*

Diatoms are a diverse group of microalgae with silicified cell walls, made up of two valves that fit together like the base and lid of a petri-dish, forming the frustule with girdle bands between each valve (Figure 1.1). These valves have distinctive patterning and varied morphology, which is the foundation of identification and is the subject of debate within taxonomic research (Mann, 1999) (Figure 1.2).

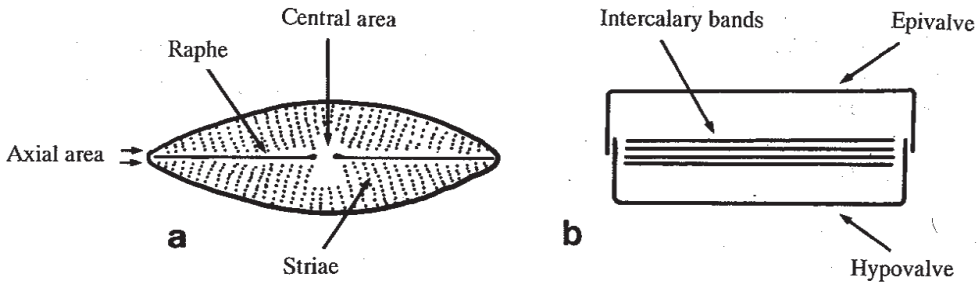


**Figure 1.1** A diagram of a typical pennate diatom a) valve view b) girdle view (Gell et al., 1999).

**Figure 1.2** A diagram of a diatom frustules (Round et al., 1990).

The life cycle of diatoms is unique, and while most diatoms follow a general diplontic pattern of asexual reproduction during the diploid phase, there is much variation of sexual reproductive methods between species (Mann, 1993). A majority of the life cycle is spent utilising asexual growth (Figure 1.3), where one daughter cell inherits the smaller inner valve (hypovalve) of the mother cell, leading to successively smaller cell sizes (Mann and Droop, 1996). This size reduction is then followed by the sexual production of an auxospore; a much larger cell, resulting in the restoration of cell size (Chepurnov and Mann, 2004; Mann, 1993).

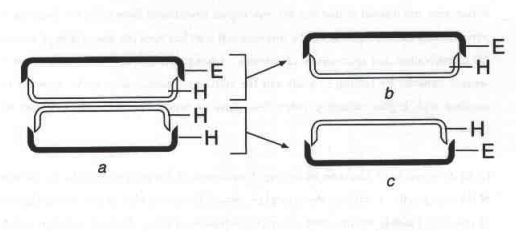


**Figure 1.3** Asexual cell division E=epivalave H=hypovalve a) dividing diatom b) one daughter cell the same size as parent c) the other daughter cell smaller than parent (John, 2000).

Diatoms are primary producers that provide a vital energy resource in lotic ecosystems, which is the foundation of the aquatic food web (Burns and Ryder, 2001). Diatoms are ecologically widespread, occurring in freshwater and marine aquatic environments and occupying various habitats; attached bottom dwelling (benthic) or free floating (planktonic) (Mann, 1999). Diatoms are a part of the complex 'biofilm' or periphyton communities that colonize benthic surfaces in the photic zone of aquatic ecosystems (Lowe and Laliberte, 1996). Periphyton form films or mats covering submerged surfaces such as rocks (epilithon), sand (epipsammon), wood (epixylon) and other plants (epiphyton) (Allan and Castillo, 2007). These microscopic communities are dominated by species of diatoms, green algae and cyanobacteria, but also contain red algae, fungi, bacteria and other unicellular organisms. Diatoms play a very important role in the maintenance of freshwater ecosystems, providing a food source for herbivores, stabilization of the river bed, and the recycling of nutrients, especially carbon and silica (Bate et al., 2007; Chessman et al., 1999b).

### *1.2.2 Toxic effects of herbicides on diatoms*

The effects of herbicides on phototrophs, such as benthic diatoms, are similar to those of the herbicides' target species due to the structural and functional similarities between them (Tlili et al., 2011). Herbicides are classified by their mode of action, which will determine the mechanism of toxicity to an organism. Most commonly herbicides work by disrupting the function of photosystem II, resulting in the inhibition of photosynthesis (Debenest et al., 2010). Other types of herbicides also affect cell growth, nutrient absorption and protein synthesis (Debenest et al., 2010; DeLorenzo et al., 2001).

The toxic effects of herbicides to diatoms have been studied in a number of laboratory based experiments (Gustavson et al., 2003; Magnusson et al., 2008; Magnusson et al., 2010) and in mesocosm studies (Tlili et al., 2011; Villeneuve et al., 2011; Wendt-Rasch et al., 2004) as well as *in situ* studies (Dorigo et al., 2010b; Morin et al., 2009). Herbicide exposure can reduce photosynthetic productivity, and in turn have an impact on biomass production (Jüttner et al., 2003; Villeneuve et al., 2011). Diatoms were growth inhibited by the herbicides atrazine, simazine, hexazinone, tebuthurion and glyphosate in laboratory based toxicity tests (Peterson et al., 1997; Peterson et al., 1994). Short term studies of diatom response have found decreased photosynthetic activity with increasing concentrations of herbicide exposure (Gustavson et al., 2003; Laviale et al., 2011; Magnusson et al., 2008). Sensitivity is variable between species; some taxa have been shown to be more tolerant to exposure than others (Larras et al., 2012; Magnusson et al., 2010). Community structure can be altered following exposure to herbicides, resulting in increased dominance of tolerant species and reduced abundance of more sensitive species (Debenest et al., 2009; Dorigo et al., 2010a; Magnusson et al., 2012; Pesce et al., 2010). The influence of environmental factors such as light availability, pH, salinity, and temperature can alter the toxicity of herbicides to diatoms (Guasch et al., 1998; Larras et al., 2013a; Larras et al., 2014b). Diatom sensitivity to herbicides is a rapidly growing field of research; however, there is a need for more studies to address the lack of sensitivity data for individual freshwater benthic diatom species and to determine how the relative sensitivity of the diatom taxa varies within natural communities (Magnusson et al., 2010).

### *1.2.3 Suitability as indicators*

Diatoms are ideal indictor organisms due to their widespread distribution and abundance, their importance as a food source at the base of the aquatic food web and their accessibility (Bate et al., 2007; Gustavson et al., 2003). Diatoms respond quickly to changes in water quality and can be used as an early warning sign of environmental stress (Sabater et al., 2007). Additionally, diatoms possess many traits that make them suitable as indicators, such as rapidly responding to changes in environmental conditions due to their short generation time, ease of collection, sessile habit, and they are sensitive to a wide range of disturbances and pollutants (Burns and Ryder, 2001; Dela-Cruz et al., 2006). For these reasons diatoms are often chosen to monitor ecological effects in aquatic ecosystems (Jüttner et al., 2003; Kelly et al., 1998; Wunsam et al., 2002). Diatoms have been shown to be useful as indicators of water quality generally (Chessman, 1985; Chessman et al., 1999b; Chessman, 1986; Philibert et al., 2006) and as indicators of inorganic pollutants (Dela-Cruz et al., 2006) and of anthropogenic disturbance (Bunn et al., 1999; Sheldon and Walker, 1997; Sonneman et al., 2001). However, the ability to use diatoms as an indicator for herbicide toxicity has not been established and further research is needed to assess their suitability for this purpose.

## 1.3 Potential threat to aquatic ecosystems

The worldwide growth of agricultural productivity has involved an industrialization of food production coinciding with the use of substantial quantities of synthetic pesticides including herbicides and insecticides (Ecobichon, 2001; Jones, 2005). Herbicides can be transported from the site of application in the paddock through spray drift and accidental spills, or indirectly via surface run-off or ground water leaching, leading to the contamination of surrounding terrestrial and aquatic ecosystems and the exposure of non-target organisms (DeLorenzo et al., 2001; Ma et al., 2006). When introduced into aquatic environments herbicides can have detrimental impacts on the aquatic biota (Graymore et al., 2001). Additionally, direct impacts on the biota may lead to indirect trophic cascade effects, for example effects to freshwater algae that form the basis of the aquatic food chain (Morin et al., 2009).

The potential impact of herbicides on the aquatic environment is dependent on many factors. The herbicide's mode of action will determine the effect it can have on the biota (DeLorenzo et al., 2001) and organisms with similar physiology to that of the chemical's target organism will be the most at risk (Tlili et al., 2011). There are several factors that may increase the risk for an organism exposed to herbicides including:

- life stage (Hutchinson et al., 1998),
- duration of exposure (Ahlers et al., 2006),
- biomagnification (Borgå et al., 2001),
- presence of additional stressors (Deneer, 2000; Magnusson et al., 2010),
- population density (Liess, 2002),
- history of exposure (Landis et al., 1996) and importantly;
- concentration (Schäfer et al., 2011c).

The highest concentrations of herbicides in rivers and streams are often associated with the first rainfall events after application, which can mobilize herbicides due to their aqueous solubility (Graymore et al., 2001; Tlili et al., 2011). The persistence of herbicides in the environment is determined by the chemical properties of the compound, as well as environmental conditions, rate of photo-degradation, adsorption by sediment and organic matter and uptake by biota (Schäfer et al., 2011c).

The toxic effects of herbicide exposure on aquatic biota has been observed with algae (Fairchild et al., 1998; Magnusson et al., 2010; Tlili et al., 2011), seagrasses (Haynes et al., 2000b), microorganisms (Schäfer et al., 2011b), mangroves (Duke et al., 2005) and corals (Jones, 2005; Shaw et al., 2008). Investigating the toxic impacts of herbicides to the aquatic biota is a field of ongoing research and ecological imperative. There has been insufficient research into the influence of multiple stressors, species interactions and community level effects and environmental factors on the toxicity of herbicides to the aquatic biota (DeLorenzo et al., 2001; Magnusson et al., 2010; Schäfer et al., 2011c).

## 1.4 Bioindicators

The use of bioindicators in water quality monitoring schemes has become commonplace in Australia (Smith et al., 1999) and elsewhere, especially in Europe and North America (Fore and Grafe, 2002; Kelly et al., 1998; Kelly and Whitton, 1995; Passy et al., 2004). Bioindicators are used as ecological assessment tools in environmental management, owing to their ability to provide information on ecosystem responses to pollutants and to demonstrate impacts via trophic interactions. Bioindicators can be used to determine the risk associated with various environmental contaminants including nutrient pollution (Bellinger et al., 2006; Philibert et al., 2006), sewage (Vermeirssen et al., 2010), mine associated drainage (Verb and Vis, 2005) and pesticides (Schäfer et al., 2011b). Various methods have been developed to facilitate bioassessment using different organisms as indicators, most commonly fish, invertebrates and diatoms (Bellinger et al., 2006; Chessman et al., 1999b).

Increasingly, the use of bioindicators is being recognized as an essential tool for monitoring ecosystem health and resilience. Traditional laboratory toxicity testing using single species tests is not representative of the variability of sensitivity shown by different freshwater taxa (Gustavson et al., 2003). Toxicant guidelines are often based on the responses of a small number of species in laboratory toxicity tests and may not actually reflect whole community responses (Kefford et al., 2005). Additionally, laboratory derived toxicity values do not take into account the interactions between pollutants in the field, and the compounding effects this may have on the biota (Morin et al., 2009). For these reasons bioassessment is a valuable diagnostic tool in the assessment of pollutant related toxicity and measurement of in-field effects (Liess et al., 2008).

## 1.5 Biotic indices and SPEAR

Indices are a common method for the interpretation of biological data. Examples of indices used with diatoms include; the trophic diatom index (TDI) (Kelly et al., 2001), the diatom species index for Australian rivers (DSIAR) (Chessman et al., 2007) and the eutrophication pollution diatom index (EPI-D) (Dell'Uomo, 1996). Indices are used to predict effects of an environmental stressor on an indicator community and compare that to observations in the field. The use of biotic indices provides an important link to

determine the ecological response of organisms to a particular stressor. This is useful for chemical toxicants such as herbicides, where field concentrations are difficult to determine due to delivery in pulse flow events and the interactions of various environmental factors may be influencing an organism's exposure and response (Liess et al., 2008). Whilst generalized indices of stream health have been widely adopted (Kelly et al., 1998; Rimet, 2005), those focusing specifically on pesticides are a recent development (Liess and Ohe, 2005). One such example is the trait based SPEcies At Risk (SPEAR), which uses macroinvertebrates to determine effects of pesticides - $SPEAR_{pesticides}$ - (mostly insecticides) (Liess and Ohe, 2005). Traits-based biomonitoring indices such as $SPEAR_{pesticides}$ can show the causal link between exposure to a specific stressor and its effects on a population (Culp et al., 2011). Exposed populations display a shift in the distribution of traits towards those that enhance tolerance to a particular stressor, a mechanism resulting in community composition change (Culp et al., 2011). This results in the replacement of sensitive taxa within a community with more tolerant ones, a concept known as pollution induced community tolerance (PICT) (Blanck, 2002). However, linking traits to environmental conditions is complex; single trait responses may only reflect the response of the most dominant taxon in a community and traits are not always consistent within taxonomic groups (Pilière et al., 2016). Additionally, the importance of individual traits on a species ability to adapt may depend on the interaction of multiple traits and their environmental context (Verberk et al., 2013).

The $SPEAR_{pesticides}$ index has been used successfully for pesticides in south-east Australia (Schäfer et al., 2011b) and in Europe (Schafer et al., 2007). However, herbicides are less toxic to animals relative to photosynthetic organisms, and as $SPEAR_{pesticides}$ uses macroinvertebrates, it is less able to detect toxicity from herbicides than insecticides and fungicides (the latter often being toxic to a wide range of organisms). As herbicides are likely to be more toxic to phototrophs such as benthic diatoms there is potential for the SPEAR approach to be adapted to utilise diatoms to assess the ecological impacts of herbicide toxicity in rivers.

## 1.6 Study area

The GBR is a world heritage listed area that has immense ecological value and economic importance for Australia (Smith et al., 2012). There are a number of anthropogenic processes that are having detrimental impacts to the GBR including overfishing, destructive fishing practices, coral bleaching associated with high water temperature, and land based pollutants such as sediments, nutrients and herbicides (Brodie et al., 2012). Terrestrial, coastal and marine ecosystems are inextricably linked, and land use changes affecting water quality in the catchment area have flow on effects for the reef (Smith et al., 2012). Agriculture in the catchment areas of the GBR has been increasing since the late 1800s especially in the sugar cane industry and cattle grazing, which currently account for at least 75% of their land use (Johnson and Ebert, 2000; Lewis et al., 2009; Smith et al., 2012). Run-off from agricultural land transports sediments and land based pollutants into the GBR Lagoon in great volumes via flood plumes, especially during periods of heavy rainfall and high flow events (Devlin and Schaffelke, 2009). The improvement of water quality from diffuse agricultural run-off is a key issue concerning the health of the GBR Marine Park and herbicides have been identified as a significant contributor to the degradation of water quality entering the reef (Brodie et al., 2012; Lewis et al., 2009; Smith et al., 2012).

Residues from agricultural herbicides have been detected in various areas of the GBR Marine Park and its catchments including rivers and streams (Davis et al., 2008; Mitchell et al., 2005), estuaries (Devlin and Schaffelke, 2009), sediments (McMahon et al., 2005), sea grasses (Haynes et al., 2000a), and marine samples (Davis et al., 2008; Lewis et al., 2009). Herbicide discharge into the GBR Lagoon can be linked to specific herbicide intensive land uses, with the highest concentrations originating from catchment areas with greatest sugar cane cultivation (Davis et al., 2008; Lewis et al., 2009). Studies have repeatedly observed concentrations of herbicides in the GBR catchment area exceeding Australian water quality guidelines for ecological protection (ANZECC, 2000; Davis et al., 2008; Lewis et al., 2009; Mitchell et al., 2005; Smith et al., 2012). The most commonly reported herbicides detected at high concentrations are the photo system II inhibitors (PSII) atrazine, hexazinone, diuron and 2,4-D (Davis et al., 2008; Mitchell et al., 2005). The annual estimated load of photo system II herbicides into the GBR Marine Park is 30 metric tons and as other pesticides are used

in the region would be an underestimation of total pesticide loads (Brodie et al., 2012). Herbicide exposure poses a threat to the long-term health of the GBR ecosystem. Freshwater ecosystems of the GBR catchment are especially at risk of toxic impacts due to the elevated concentrations of herbicides detected in rivers associated with agriculture (Davis et al., 2013; Smith et al., 2012). There is a need for further research into the impacts of herbicide exposure to freshwater communities in the GBR catchment and for better methods of monitoring toxic impacts (Davis et al., 2008; Lewis et al., 2009; Schaffelke et al., 2005).

## 1.7 Thesis aims and overview

The main aim of my thesis is to assess the impact of herbicide toxicity on benthic diatoms at the species and community levels and to determine whether benthic diatoms are suitable indicators of herbicide toxicity in rivers that flow into the GBR.

Each chapter of my thesis addresses key scientific questions that contribute to the broader understanding of the impact of herbicides to benthic diatoms in rivers and towards the development of a diatom based biomonitoring index that can assess herbicide impacts in rivers. The specific objectives of each chapter are outlined below.

Chapter 2: The focus of this chapter is to address the lack of sensitivity data for Australian freshwater diatom taxa. Producing these sensitivity data using traditional methods to conduct single species toxicity tests would be lengthy and cultures of many local taxa may not be available. Rapid toxicity testing methods can provide approximate sensitivity data on a large number of species in a relatively short period of time and using less resources (Kefford et al., 2005). This study developed a new method to expose benthic diatom communities collected in the field to herbicides in short laboratory based toxicity tests. Using this method, sensitivity data can be derived for multiple taxa in one rapid toxicity test, thus speeding up the process of collecting toxicity data with new species. By rapidly collecting toxicity data for many species, a specific aim of this thesis is achievable, namely to determine the relative herbicide sensitivities of multiple freshwater diatom taxa from a natural benthic community.

Chapter 3: Herbicide pollution in rivers is the result of agricultural runoff consisting of mixtures of various herbicides, with differing modes of toxic action. The potential for herbicides with differing modes of action to alter the response of benthic diatoms within

natural communities to exposure has not been established. The aim of this study is to investigate whether the relative sensitivities of benthic diatom taxa differ between eight commonly used herbicides with three differing modes of action.

Chapter 4: Peak herbicide concentrations in rivers typically occur during high flow events, along with increased loads of suspended solids. It is therefore likely that peak herbicide concentrations in rivers co-occur with reduced light conditions to the benthic community. Benthic diatoms may respond differently to herbicide exposure under altered light conditions and it is important to determine whether light and herbicide toxicity interact at both the species and community levels. The specific aims of this chapter are i) to determine whether reduced light conditions can alter the relative herbicide sensitivity of diatoms and ii) to assess the influence of prior pollution history on the response of diatoms to herbicides.

Chapter 5: Elevated concentrations of herbicides can persist in rivers for lengthy periods (weeks to months). Consequently, benthic diatoms may be exposed to herbicides under chronic exposure conditions over multiple generations. Chronic exposure to herbicides could alter the relative sensitivity of benthic diatoms and it is therefore important to assess diatom sensitivity over a range of exposure durations. This chapter aims to investigate the responses of individual diatom taxa as well as the community to herbicides over a 12 day exposure period and to identify diatom taxa with the potential to recover during chronic herbicide exposure scenarios.

Chapter 6: This chapter utilises the sensitivity data produced in Chapters 2-5 as well as sensitivity data obtained from literature to develop a new diatom based biomonitoring index - $SPEAR_{herbicides}$. The impacts of herbicide pollution in sites across the GBR catchment area are considered and effects on benthic diatom communities at monitored sites are compared to that at reference sites.

Chapter 7: In this chapter, the findings of Chapters 2 to 6 are discussed and overall conclusions are drawn. Recommendations for management and overarching outcomes are discussed along with suggested future research directions.

# Chapter 2: Determining the relative sensitivity of benthic diatoms to atrazine using rapid toxicity testing: A novel method

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*Bluewater Creek, QLD, Australia.*

## 2.1 Abstract

Herbicides pose a potential threat to aquatic ecosystems, especially to phototrophic organisms such as benthic diatoms. Benthic diatoms may be a valuable indicator of the toxic impacts of herbicides in aquatic systems. However, this requires information on the herbicide sensitivity of a wide range of freshwater benthic diatom taxa. Unfortunately this information is only available for a limited number of species as current methods of developing new algae toxicity tests on individual taxa are lengthy and costly. To address this issue, we developed a new rapid toxicity test method to test natural benthic communities, from which the relative herbicide sensitivity of many individual taxa can be derived. This involved the collection of natural benthic communities from rocks *in situ*, which were placed directly into laboratory toxicity tests. Sensitivity data for several diatom genera in a 48 hour exposure toxicity test were produced, without the need for cultures or multiple site visits. After exposure to the highest treatment of atrazine (500 μg L−1) there were significant declines of healthy cells in the most sensitive genera: *Gomphonema* declined by 74%, *Amphora* by 62%, *Cymbella* by 54% and *Ulnaria* by 34% compared to the health of cells in the control treatment. In contrast, the genera, *Eunotia*, *Achnanthidium* and *Navicula*, had no statistically significant decline in cell health. This method can identify the diatom taxa most at risk of herbicide toxicity within the natural benthic diatom community. The rapid toxicity testing method presented is a simple and effective method to obtain sensitivity data for multiple taxa within a natural benthic diatom community in a relatively short period of time.

## 2.2 Introduction

Herbicide contamination of freshwater ecosystems poses a potential threat to primary producers, such as benthic diatoms, and they may be a valuable indicator community for toxic impacts (DeLorenzo et al., 2001). Benthic diatoms are ubiquitous and respond rapidly to environmental conditions, therefore changes in community composition due to herbicide toxicity may reflect past herbicide concentrations (Burns and Ryder, 2001). Herbicide exposure in streams typically occurs as pulses associated with diffuse agricultural runoff, and as a result, routine (i.e. calendar based) sampling of herbicides will most likely underestimate herbicide concentration and thus toxicity (Davis et al., 2013). In order to address this, chemical monitoring needs to include event based sampling after rainfall and during floods to estimate the peak concentration of herbicides and/or include the use of passive samplers to estimate the average concentration. However, these measures require multiple site visits, increasing the cost of monitoring. Furthermore, with any chemical monitoring there is uncertainty as to the ecological risk of the chemicals observed and the chemicals detected may not be the entire suite of chemicals present in the field (Magnusson et al., 2008). Consequently, there is a need for biomonitoring tools that give an integrated response to chemicals over time, and freshwater benthic diatoms may be a cost effective and ecologically relevant solution for herbicides (Debenest et al., 2009; Morin et al., 2009).

Linking field effects to any one particular stressor in the environment can be problematic due to the range of variables that can alter community structure and the influence of multiple stressors (Morin et al., 2009; Schafer et al., 2007). However, Schäfer et al. (2011a) proposed a conceptual model for trait based biomonitoring indices that link exposure to a specific stressor with community composition changes in the field, such as the SPEcies At Risk (SPEAR) index (Liess and Ohe, 2005). The $SPEAR_{pesticides}$ index has been developed using macroinvertebrates to describe changes in the proportion of sensitive taxa within a community, relative to the intensity of pesticide stress (Liess and Ohe, 2005). The key trait used in $SPEAR_{pesticides}$ is the sensitivity of macroinvertebrate taxa to organic toxicants (Liess and Ohe, 2005; Schafer et al., 2007). $SPEAR_{pesicides}$ has been used successfully in Europe and also in Southeast Australia, to link pesticide exposure (mostly insecticides and fungicides) to field effects (Liess et al., 2008; Schäfer et al., 2011c). However, $SPEAR_{pesticides}$ is less effective at

predicting herbicide toxicity as it uses macroinvertebrates as indicators which respond more strongly to insecticides and fungicides (Schäfer et al., 2011b). Benthic diatoms may be a more suitable indicator community to assess herbicide toxicity, especially photosystem II inhibitors (PSII), as their phytotoxic effects have been established (Debenest et al., 2010; Magnusson et al., 2010; Magnusson et al., 2012).

The principle impediment to developing a biomonitoring index for herbicides, based on the community composition of diatoms (or other primary producers) is lack of information on how particular taxa respond to herbicides (Culp et al., 2011; Morin et al., 2009; Roubeix et al., 2011b). Although some information exists on the toxicity of herbicides to a few freshwater benthic diatom species (Debenest et al., 2009; Larras et al., 2012; Magnusson et al., 2010; Tang et al., 1997), for any particular region, there are very few taxa with herbicide sensitivity data (Magnusson et al., 2012). This is in part due to the time constraints and costs of current standard toxicity tests which involve the use of single species cultures to determine individual sensitivities. Cultures of most species are unavailable and obtaining sensitivity data for numerous species by standard toxicity testing methods would be very time consuming. A new method that can produce sensitivity data for a number of local taxa in a relatively short period of time would be ideal for obtaining the required data for a traits-based monitoring index that can detect herbicide toxicity in rivers (Culp et al., 2011). We followed the rapid toxicity approach which aims to determine herbicide toxicity to multiple taxa from a multispecies community in a relatively short period of time (Hickey et al., 2009; Kefford et al., 2005). Other studies either use single species cultures to produce this sensitivity data for individual taxa (Larras et al., 2013b; Magnusson et al., 2010; Roubeix et al., 2011b), or use community level measures of health such as photosynthetic inhibition that cannot determine which taxa within the community are contributing to the sensitivity (Magnusson et al., 2012; Proia et al., 2011; Prosser et al., 2013).

This paper establishes a new method to determine the relative herbicide sensitivity of field derived freshwater benthic diatom taxa using rapid toxicity tests. These tests aim to produce relative sensitivity data for several freshwater diatom taxa in one 48 hour test (see Kefford et al., 2003). The current study utilises a new approach to place benthic

diatoms collected *in situ* directly into rapid toxicity tests that can determine the relative sensitivity of the individual diatom taxa from within the freshwater benthic community.

## 2.3 Materials and Methods

### *2.3.1 Diatom collection locations*

Diatoms were collected from Bluewater Creek (−19.14385, 146.26817) on the 18th of May 2012. The creek is located in North Queensland, Australia, at the base of Paluma State Forest near the town of Bluewater and is surrounded by eucalypt woodland. The stream substrate at the sample site is mostly large boulders, cobbles and pebbles, with a mean channel width of 7 m and highly diverse habitats present including deep and shallow pools, falls, runs and shallow riffles. The study site was chosen as there is no agriculture and only recreational activities occurring upstream of the site. The site is therefore considered a reference site for agricultural impacts such as herbicide pollution.

### *2.3.2 Sampling of natural benthic diatom communities*

Pebbles and cobbles (approximately 5–25 cm in the longest axis) from the stream bed were chosen at random from various areas of a 50 m section of the stream bed and placed in trays for scrubbing. Multiple areas within a 50 m stretch of stream bed were sampled in order to include a variety of habitat types; riffles, pools and falls, for the purpose of obtaining the greatest possible number of taxa in a composite site collection. Areas which were stagnant pools and also very shallow areas likely to have been recently dried out were avoided to minimise collection of dead material. The benthic diatoms were removed from the rocks by scrubbing with a soft bristle toothbrush, using a squirt bottle with site water to wash off the detached material into a collection tray. The detached benthic diatoms were collected into a 500 mL plastic sample container as a composite sample, which was stored in the dark at site water temperature (21 ± 1 °C) for transportation to the lab.

### *2.3.3 Rapid toxicity tests*

The benthic diatoms were exposed over 48 h to atrazine to determine the relative sensitivities of the taxa within the community. Tests were conducted in a controlled temperature laboratory at 24 ± 2 °C at a light intensity of 20 μmol $m^{-2}$ $s^{-1}$ (±10%), under a 12:12 hour light:dark cycle. After transportation to the lab the experiment was

initiated within 4 h of sampling and included a 1 hour acclimatisation period to stabilise the temperature to that of the room.

The solution containing the removed benthic diatom community from Bluewater Creek was homogenised by gentle shaking and divided into 1 mL aliquots randomly assigned to 18 x 40 mL test vials by pipette. The test vials were then made up to a final volume of 20 mL with site water and spiked with a known atrazine herbicide concentration depending on treatment. The atrazine stock solution was prepared by dissolving analytical grade atrazine (Sigma Aldrich, CAS 1912-24-9) in site water using a carrier of 99% ethanol to increase the solubility of atrazine (2% v:v) with the maximum final volume of ethanol in the treatments being 0.05% (Magnusson et al., 2010). An ethanol control treatment with a final volume of 0.05% ethanol was included and compared to a site water only control after 48 hours to eliminate carrier effects. All herbicide treatments were compared to the ethanol control. An additional control treatment at the start of the experiment (t=0) was also prepared to indicate the diatom community and health at the start of the experiment. The experiment had a static water supply, without renewal of water or agitation for the duration of the test period as is common in algal bioassays (Larras et al., 2012; Magnusson et al., 2008). Diatoms were exposed to atrazine concentrations of 50, 200 and 500 μg $L^{-1}$, which were shown to elicit a response in the sensitive taxa from trial tests (data not shown). These concentrations correspond with estimated mixture toxicities of PSII herbicides regularly detected at polluted sites within the study region which exceeded the ANZECC (2000) atrazine 95 % trigger value for ecological protection (13 μg $L^{-1}$) for 30 consecutive days and reached a maximum of 807 μg $L^{-1}$ atrazine equivalent concentrations ($TEQ_{CP}$) (Smith et al., 2012). However, in less polluted sites in this region associated with agricultural land use the mean atrazine concentrations are typically lower but still frequently exceed the 99 % freshwater ecological trigger value (0.07 μg $L^{-1}$) (Lewis et al., 2009). All treatments and controls were replicated thrice. Spiked water samples were also prepared in the same manner as each herbicide test treatment (50, 200, 500 μg $L^{-1}$) to be analysed for determination of the actual atrazine concentrations, which were within 15% of the nominal values (Supplementary Table S1). Analysis of atrazine concentrations (1 ug $L^{-1}$ limit of detection) were determined by chemical analysis (LC–MS/MS) by Eurofins Agroscience Testing Pty Ltd a National Association of Testing Authorities (NATA) accredited laboratory.

### *2.3.4 Preservation of samples*

After the exposure period (48 h) the contents of each replicate test vial were preserved with 3 drops of Lugol's iodine solution. The lids of the glass test vials were replaced and agitated to loosen the algae and ensure uniform preservation for later identification. After the preserved samples had settled, 10 mL of liquid was poured from each test vial, and the settled benthic diatoms were carefully transferred into a 10 mL sample storage container.

### *2.3.5 Identification of diatoms*

Diatoms were identified by observation under an Olympus BX50 light microscope. Sub samples were taken from each replicate and observed in a Lund cell at a 400× magnification. Counting was conducted in random transects along the Lund cell until a total of at least 100 cells were counted and identified per replicate, which was sufficient for enumerating the common taxa in the sample; rare taxa that did not occur in every replicate were not included in analysis. Benthic diatoms were identified to the genus level using the following international (Cox, 1996; Round et al., 1990) and Australian (Gell et al., 1999; Sonneman et al., 2000) keys.

### *2.3.6 Health status of diatoms*

The growth rates of the various diatoms differs substantially, and is very slow for some benthic taxa with doubling rates as low as 0.1-0.3 $d^{-1}$, this would make estimation of growth rate via cell counts difficult and lengthy (Admiraal, 1976; Gould and Gallagher, 1990). Therefore we have used a method of health classification similar to the live cell counts performed in other studies (Debenest et al., 2009; Pohlon et al., 2010; Proia et al., 2011), except data is recorded on a per taxa basis and includes identification of taxa as well as classification of health. The health status of the diatom cells was recorded as the number of diatom cells per genus that were either ‘healthy’ or ‘unhealthy’. Cells were classified depending on the condition of the Lugols stained cell contents and chloroplasts. If chloroplasts appeared more than 50% intact then it was classed as a healthy cell, and if the chloroplasts were <50% intact or absent or the frustule was broken then it was classed as unhealthy (Supplementary Table S2). Broken frustules were only counted if more than 50% of the valve was left intact and could be identified. Diatom community composition was calculated using only the healthy cells in order to

determine the effects on the live benthic community. The percentage of healthy cells in each treatment was calculated as a proportion of the total number of cells counted in that treatment per genus.

### *2.3.7 Statistical analysis*

We assessed the effects of herbicide concentration on the health of diatoms using a generalized linear model (GLM). Concentration response of the diatom genera was performed using GLM on binary health data (healthy/unhealthy) with a logit link function. The model estimated the likelihood that a diatom cell would be healthy based on the concentration of exposure (50, 200, 500 μg $L^{-1}$) compared to the ethanol control and was carried out on a per taxon basis. Where atrazine exposure resulted in a significant decline in diatom cell health the EC50 was calculated with nominal concentrations using probit analysis (Finney, 1971).

The health of the cells was also assessed at the start (t=0) and the end (48 h) of the experiment to insure the stability of control health and to eliminate any carrier effects. It was important to determine the background level of health for each genus, as this was expected to differ depending on the successional stage of the benthic diatom community at the time of collection (Davie et al., 2012). The background health of test controls (48 h) was assessed using GLM as described above, compared to the start of experiment controls (t=0) as the reference parameter. Background health, concentration response and EC50 calculations were computed using SPSS 18 statistical package (SPSS 18).

Non-metric multi-dimensional scaling (MDS) ordination was conducted to examine community compositional changes of the healthy benthic diatom community among treatment groups at Bluewater Creek. MDS was conducted from the Bray Curtis index of similarity on untransformed community composition data. Only the community composition data for the healthy cells was used in the MDS for the common taxa (taxa which were observed at least once in every sample). A one way ANOSIM was used to determine the differences in the healthy diatom community between treatments. SIMPER analysis was performed to determine which taxa contributed to the differences between groups. Multivariate statistical analysis was performed using PRIMER v6 (Clarke and Gorley, 2006).

## 2.4 Results

### *2.4.1 Background health within control groups in rapid toxicity tests*

The background health of diatoms remained relatively consistent between controls across most genera from Bluewater Creek throughout the experiment. No carrier effect was observed for any of the taxa in the study (Supplementary Table S3). There were no differences between health of cells at the start of the experiment (t=0) and the ethanol controls (48 h) across all diatom genera (Supplementary Table S3).

### *2.4.2 Concentration response and relative sensitivity of the diatom genera*

Differences in the relative atrazine sensitivity between benthic diatom genera were observed (Figure 2.1). The most tolerant genera did not show a significant change in the health of cells with herbicide exposure: *Navicula*, *Eunotia* and *Achnanthidium* (Table 2.1). Diatoms from the genus *Navicula*, showed no concentration response to atrazine treatments and were the most tolerant in the benthic diatom community (Table 2.1 and Figure 2.1a). The most sensitive diatom genera within the benthic community were *Gomphonema*, *Ulnaria*, *Cymbella* and *Amphora*, all of which showed a significant concentration response at the highest treatment of 500 μg $L^{-1}$ (Table 2.1). This was equivalent to a decline relative to the control by 74% in *Gomphonema*, 62% in *Amphora*, 54% in *Cymbella* and 34% in *Ulnaria* (Table 2.1). *Gomphonema* (Figure 2.1b) displayed a significant threshold concentration response to atrazine exposure and was the most sensitive taxa with an EC50 of 43 μg L−1 (Table 2.1). The genera *Ulnaria*, *Cymbella* and *Amphora* responded with significant dose–response relationships to atrazine exposure (Figure 2.1c, e and f).

### *2.4.3 Community effects of herbicide exposure*

The non-metric MDS ordination (stress = 0.09) showed a gradient of change in community composition of healthy benthic diatoms from the control groups to the highest herbicide exposure groups (Supplementary Figure S4). The separation of the highest concentration treatment is evident and the ANOSIM results were significant overall (Global R= 0.361, p-value = 0.005); however, the pairwise comparisons were not significant. The differences in community composition observed can be attributed to a decline in the most sensitive taxa and the increase of tolerant taxa after herbicide

exposure (Table 2.1). The genera that had the greatest influence on the differences between the communities were *Amphora*, *Navicula* and *Ulnaria*, with each genus contributing approximately 19% to the Bray–Curtis dissimilarity between groups.

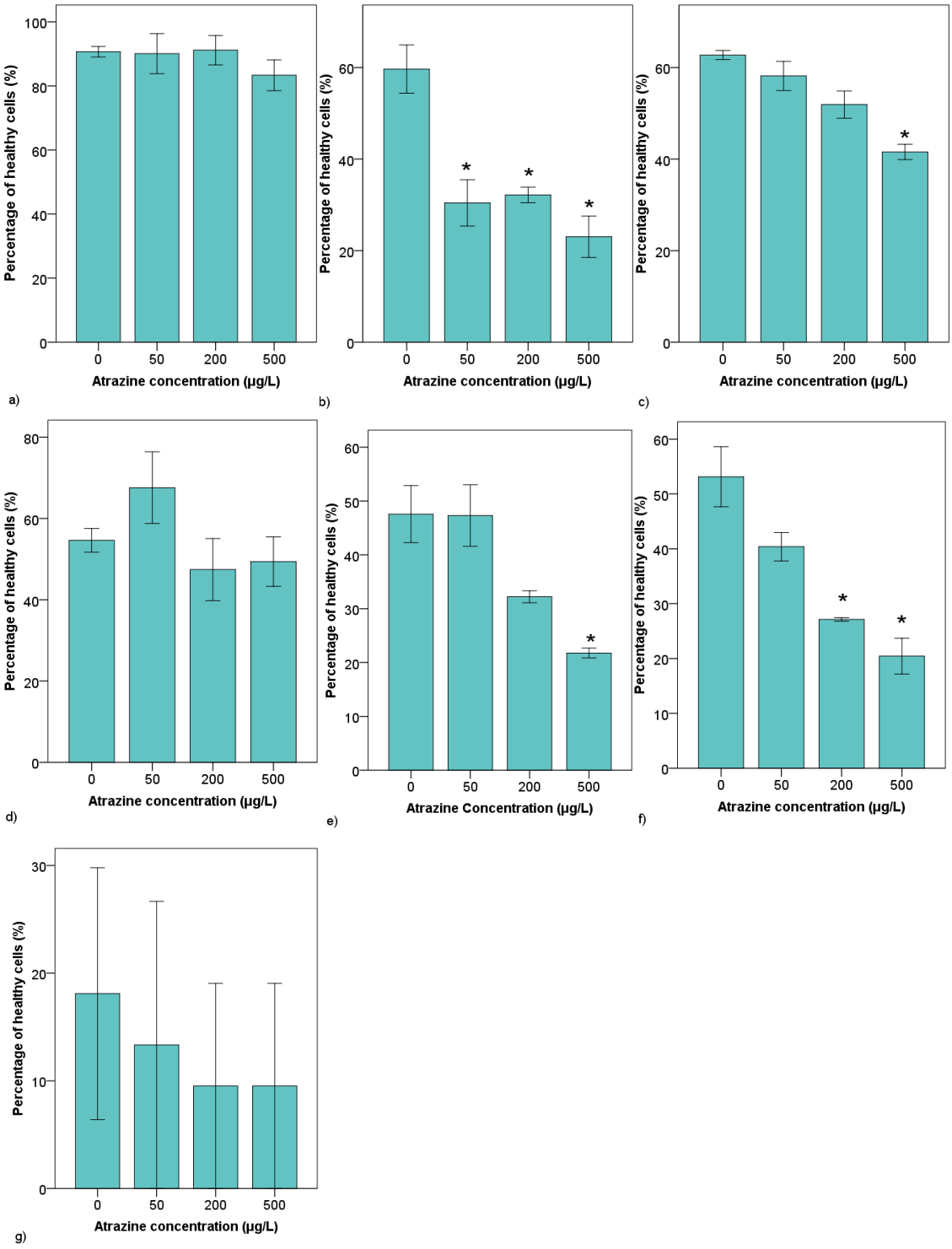


**Figure 2.1** Effects of atrazine on the health (%) of diatom cells by genus a) *Navicula*, b) *Gomphonema*, c) *Ulnaria*, d) *Achnanthidium*, e) *Cymbella*, f) *Amphora* and g) *Eunotia* at 48 h of exposure (Error bars represent ± 1 SE). Treatments marked * are statistically different from ethanol controls at alpha 0.05

**Table 2.1** Concentration response of the diatom genera using the generalized linear model (GLM). Effects of herbicide concentration on the health of diatom cells at each treatment level (50, 200, 500 µg $L^{-1}$ atrazine) at 48 h of exposure compared to ethanol controls (no herbicide). Percentage of healthy cells per treatment, percentage composition of the healthy benthic diatom community, EC50 and EC10 values. – Not calculable.

| Genus | Concentration (µg $L^{-1}$) | Sig. | Healthy cells (% ± SE) | Community composition (%) | EC50 (µg $L^{-1}$) | EC10 (µg $L^{-1}$) |
|---|---|---|---|---|---|---|
| *Navicula* | 0 | - | 91 ± 1.6 | 13 ± 3.2 | - | - |
| | 50 | 0.808 | 90 ± 6.2 | 17 ± 0.9 | | |
| | 200 | 0.905 | 91 ± 4.6 | 17 ± 0.9 | | |
| | 500 | 0.403 | 83 ± 4.8 | 18 ± 2.1 | | |
| *Ulnaria* | 0 | - | 63 ± 1.0 | 40 ± 1.6 | 1200 | 84 |
| | 50 | 0.434 | 58 ± 3.2 | 40 ± 1.3 | | |
| | 200 | 0.059 | 52 ± 3.0 | 42 ± 2.8 | | |
| | 500 | 0.000 | 42 ± 1.7 | 43 ± 1.3 | | |
| *Gomphonema* | 0 | - | 55 ± 6.9 | 14 ± 2.0 | 43 | 0.12 |
| | 50 | 0.013 | 24 ± 6.1 | 10 ± 0.9 | | |
| | 200 | 0.010 | 24 ± 2.0 | 11 ± 0.7 | | |
| | 500 | 0.001 | 15 ± 2.8 | 9.2 ± 1.2 | | |
| *Achnanthidium* | 0 | - | 55 ± 3.0 | 8.5 ± 1.0 | - | - |
| | 50 | 0.437 | 68 ± 8.9 | 10 ± 1.1 | | |
| | 200 | 0.429 | 47 ± 7.6 | 12 ± 1.8 | | |
| | 500 | 0.500 | 49 ± 6.1 | 16 ± 1.9 | | |
| *Eunotia* | 0 | - | 18 ± 12 | 1.9 ± 1.3 | - | - |
| | 50 | 0.367 | 13 ± 13 | 1.0 ± 1.0 | | |
| | 200 | 0.485 | 9.5 ± 9.5 | 1.1 ± 1.1 | | |
| | 500 | 0.485 | 9.5 ± 9.5 | 1.2 ± 1.2 | | |
| *Cymbella* | 0 | - | 48 ± 5.3 | 9.2 ±1.29 | 420 | 75 |
| | 50 | 0.995 | 47 ± 5.7 | 7.5 ± 0.6 | | |
| | 200 | 0.193 | 32 ± 1.1 | 6.8 ± 0.5 | | |
| | 500 | 0.027 | 22 ± 0.9 | 4.6 ± 0.4 | | |
| *Amphora* | 0 | - | 53 ± 5.5 | 13 ± 2.9 | 240 | 14 |
| | 50 | 0.119 | 40 ± 2.6 | 14 ± 3.1 | | |
| | 200 | 0.002 | 27 ± 0.3 | 9.8 ± 1.2 | | |
| | 500 | 0.000 | 20 ± 3.3 | 8.2 ± 1.6 | | |

## 2.5 Discussion

A new method was established to determine the relative herbicide sensitivity of diatoms within a natural benthic community using rapid toxicity testing. The relative sensitivity of multiple diatom genera from a diverse field derived sample was determined from one 48 hour exposure test. This method is quicker and less costly than traditional methods of testing diatoms and algae which involve establishing cultures of each taxa and then testing each species individually (Brain et al., 2012a; Larras et al., 2012; Magnusson et al., 2008; Magnusson et al., 2010; Moro et al., 2012; Nelson et al., 1999; Peterson et al., 1997; Tang et al., 1997). The method used in this study is based on the rapid toxicity approach previously used with invertebrates (Hickey et al., 2009; Kefford et al., 2005; Kefford et al., 2003). With the application of multiple rapid toxicity tests, herbicide sensitivity data for many taxa can be produced in a short period of time. This method is advantageous for the development of a traits based index, which would require sensitivity data for as many local taxa as possible.

We previously tested the use of an artificial substrate method for the collection of field derived natural benthic diatom communities (Guasch and Sabater, 1998; Laviale et al., 2011; Proia et al., 2011) for use in rapid toxicity tests. However, a number of sampling cages containing glass slides (Supplementary Figure S5) were lost or buried by substrate during the colonisation period due to the extremity of flow events in the study region, and the remaining substrates had highly variable densities of diatom growth. Another method using pebble substrates collected *in situ* was also tested. Unfortunately, we observed a very low density of diatoms on the small pebbles collected during the study, and since the purpose of retrieving pebbles from the field was to obtain a natural benthic community containing as many taxa as possible, this method was deemed unsuitable. Furthermore the diatom flora of small pebbles may only represent taxa that are rapid colonisers and may not reflect the general diatom community at a site due to the frequent movement and burial thereby resetting the colonisation process (Davie et al., 2012). These approaches were abandoned in favour of the scrubbing method of benthic diatom collection described in this study, which was quicker, requiring no prior site visits, and less expensive, requiring no specialised equipment. The results derived from this method showed limited variation of healthy cells in the controls over the test period (Supplementary Table S3), validating this method for comparisons between

treatments and controls and enabling the relative sensitivities of multiple taxa in a natural benthic diatom community to be determined from one 48 hour rapid test.

This study identified differences in the herbicide sensitivity of freshwater diatom genera within a natural benthic community. Identifying taxa by genus was necessary for the determination of cell health and to avoid uncertainty associated with identifying to the species level from live material. It is possible that that the individual species contributing to the genus tested here might not be representative of other members of the genus which were not tested, potentially leading to contradictory results. For example, Larras et al. (2012) found that *Gomphonema parvulum* was relatively tolerant to atrazine, whereas in this study *Gomphonema* was the most sensitive. However, a study by Growns (1999) found that genus and species level identification were similar at predicting impacts of river regulation because of the small number of species in a majority of diatom genera. Further studies should investigate whether this is the case for herbicide impacts and whether relative herbicide sensitivity differs between members of the same genera from within natural benthic communities. As field derived benthic diatom community samples could not be stored for any length of time, it is not possible to repeat the experiment to determine whether the diatom responses vary. However, similar experiments with independently collected diatoms conducted over the course of this thesis were consistent and suggest that the experiments are repeatable.

In this study *Navicula* was the most tolerant genus to atrazine exposure and other genera such as *Ulnaria, Gomphonema, Cymbella* and *Amphora* were relatively more sensitive. *Navicula* are considered in the literature to be tolerant of both nutrient and herbicide pollution (Chalifour and Juneau, 2011; Guasch and Sabater, 1998). However, Magnusson et al. (2010) found that photosynthetic inhibition occurred in the estuarine diatom *Navicula* sp. at atrazine concentrations much lower than the exposures in this study. The concentrations of atrazine in the current study (50-500 μg $L^{-1}$) exceed the field measured peak concentrations which regularly reach 10 μg $L^{-1}$ in rivers that flow into the Great Barrier Reef (GBR) (Brodie et al., 2012; Lewis et al., 2012; Smith et al., 2012). However, considering that PSII herbicides such as atrazine often occur in mixtures of two or more and that their toxicity is additive (Magnusson et al., 2010), recent studies within the study region have shown that the estimated mixture toxicity (TEQ) of PSII herbicides exceeded the atrazine trigger value for ecological protection

(13 μg $L^{-1}$) for 30 consecutive days and reached atrazine equivalent concentrations of up to 807 μg $L^{-1}$ (O'Brien et al., 2016; Smith et al., 2012). This justifies the ecological relevance of using concentrations up to 500 μg $L^{-1}$ in identifying which taxa are most at risk of herbicide toxicity in field derived communities.

Open questions include: (1) whether the diatoms cells which appeared healthy were physiologically impaired and (2) whether those individuals regarded as unhealthy would recover following the cessation of herbicide exposure. Prosser et al. (2013) observed rapid recovery of quantum yield from periphyton communities after the cessation of atrazine exposure with ≥ 95% recovery within 48 hours and other studies have observed similar rapid recovery in quantum yield (Brain et al., 2012a; Laviale et al., 2011). However, as those studies did not investigate changes in cell health, it is uncertain what relevance they have for the recovery of diatoms classified as unhealthy in the current study. Indeed other studies (Dorigo et al., 2010b; Magnusson et al., 2012) have observed much slower recovery of periphyton community structure following exposure to herbicides in the field (Morin et al., 2010). Such studies suggest that alternative approaches, for example the changes in cell health used in the current study, should also be investigated. Indeed, the ability of certain diatoms to recover after herbicide exposure may be an important trait for consideration alongside sensitivity in the development of a traits-based monitoring index using diatoms (Gustavson et al., 2003). The results of the relative sensitivity by diatom genera is meant as a means for ranking the relative sensitivities of the taxa or for classifying their sensitivity (e.g. sensitive or tolerant) and not as an indication of what atrazine concentration will or will not harm diatom taxa in nature where exposure periods might be different and might co-occur with other stressors.

## 2.6 Conclusions

The current study developed a new method of producing sensitivity data for a range of individual diatom taxa from within a natural benthic community in a short period of time. The rapid toxicity tests provided consistent control data with a low variability in the health of cells per genera at the start of the experiment, which was suitable for determining the differences in the relative sensitivity of diatom genera to atrazine exposure. This method can deliver sensitivity data for multiple taxa from the one 48 hour test, without the need for cultures or multiple site visits, and will be useful for the production of herbicide sensitivity data that can be used for a new traits based index that can detect herbicide toxicity using benthic diatoms. These results could also be used to make species sensitivity distributions (SSDs) based on communities of diatoms that occur in specific regions. We thus recommend the use of this method for conducting rapid toxicity testing of diatoms. Future studies should investigate the differences in sensitivity between members of the same genera from within natural benthic communities, the effects of herbicidal mode of action on relative sensitivity and the effects of light on PSII sensitivity in freshwater diatoms.

**Supplementary data**

Supplementary data for Chapter 2 in available in Appendix A.

# Chapter 3: How benthic diatoms within natural communities respond to eight common herbicides with different modes of action

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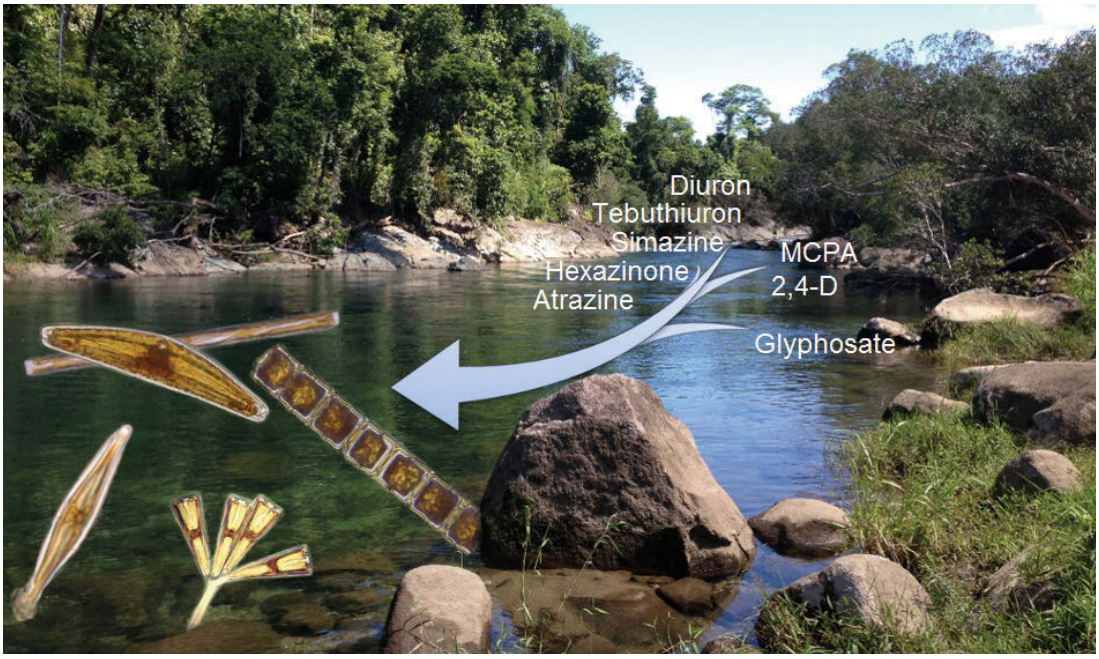

## 3.1 Abstract

Herbicides are common pollutants of rivers in agricultural regions. These contaminants include various types of chemicals with different modes of toxic action. Herbicides can have toxic effects on freshwater benthic diatoms, the base of the aquatic food web. We examined the effects of (non-mixture) herbicide exposure to the health of diatoms for eight common herbicides with three different modes of action; the photosystem II (PSII) inhibitors: atrazine, simazine, hexazinone, tebuthiuron and diuron; two auxinic herbicides: MCPA and 2,4-D; and the EPSP synthase inhibitor: glyphosate. Benthic diatoms within riverine communities were exposed to each herbicide in rapid toxicity tests at concentrations of 50, 200 and 500 μg $L^{-1}$. The most sensitive taxa were *Gomphonema* spp. and *Encyonema gracilis*. *Navicula cryptotenella* was the most tolerant to herbicide exposure. There was no significant effect of the different herbicide modes of action at the community level. Herbicide mode of action did not alter which taxa were most sensitive within the community and sensitivity rankings of the dominant diatom taxa were similar for each of the eight herbicides. The consistency of the results between herbicides suggests that freshwater benthic diatoms may be suitable in situ indicators for detecting the toxicity of herbicides with differing modes of action.

## 3.2 Introduction

Freshwater benthic diatoms are important phototrophic organisms of lotic and lentic freshwater environments and are often the dominant primary producers in rivers. Diatoms are widely recognised as effective bioindicators as they are ubiquitous, diverse and highly responsive to changes in environmental conditions (Rimet, 2012). Agricultural herbicides are common pollutants of freshwater environments and can alter the growth and physiology of freshwater benthic diatoms, as well as their community structure and diversity (Debenest et al., 2010; DeLorenzo et al., 2001; Magnusson et al., 2012; Morin et al., 2009). Benthic diatoms are among the first aquatic biota to respond to toxicant exposure and their response is affected by past exposure, which makes them potential indicators of herbicide toxicity in rivers (Rimet and Bouchez, 2011; Sabater et al., 2007). Assessing the ecological effects of herbicide pollution is essential in protecting freshwater ecosystems from degradation and monitoring changes in the benthic diatom community may enable the early detection of toxic impacts (DeLorenzo et al., 2001; Ricart et al., 2009). Sensitivity of freshwater benthic diatoms to herbicides differs between taxa (Debenest et al., 2009; Larras et al., 2012; Roubeix et al., 2011b) and this trait has the potential to be used to link shifts in community composition with herbicide toxicity in rivers (Schäfer et al., 2011b). However, herbicide sensitivity of many common freshwater benthic diatom species is unknown and this is a barrier to the development of a biomonitoring tool capable of diagnosing herbicide toxicity in the field (Roubeix et al., 2011b).

Herbicides are often detected in waterways with agricultural activity in their catchments (Davis et al., 2013; Lewis et al., 2009) and rivers are frequently contaminated with more than one type of herbicide (Magnusson et al., 2010). Herbicides can be classified by their modes of action, the biochemical mechanism by which they act on organisms. Photosystem II inhibitors (PSII) act on the PSII reaction centre by blocking electron transport and halting photosynthesis, ultimately resulting in oxidative stress and cell death (Debenest et al., 2010; Rutherford and Krieger-Liszkay, 2001). In agricultural regions of tropical North Queensland, Australia, the PSII herbicides atrazine and diuron are the two most commonly detected herbicides and account for approximately 90% of the annual herbicide load, although other PSII herbicides are also regularly detected including hexazinone, tebuthiuron and simazine (Davis et al., 2012; Lewis et al., 2009).

Despite the focus on monitoring these priority PSII contaminants there are other types of herbicides used routinely which are less frequently monitored, for example glyphosate (Davis et al., 2008). Glyphosate is a herbicide which is used for broad-spectrum weed control and acts through the inhibition of the enzyme EPSP synthase, resulting in chlorophyll degradation and reduced photosynthesis (Baylis, 2000; Malik et al., 1989). Another major group of herbicides that is used in the GBR catchment are the auxinic herbicides, including 2,4-D and MCPA, which mimic natural plant growth hormones causing uncontrolled growth and deformation in broadleaf weeds (Grossmann, 2010).

It has not been established whether the relative sensitivity (i.e. which taxa are sensitive/tolerant relative to other taxa) of individual freshwater benthic diatoms is affected by herbicides with differing modes of action. Benthic diatoms in agricultural rivers are exposed to a variety of herbicides, including those with differing modes of action. If diatoms are to be utilised as bioindicators of herbicide toxicity in rivers it is crucial to determine whether herbicide mode of action could alter the relative sensitivity of diatom taxa within natural communities (Wood et al., 2014). It is important for ecotoxicological studies to assess both the response of the benthic diatom community as well as how individual species respond to herbicide toxicity.

The current study examines the response of individual diatom taxa from within a field derived benthic community to eight commonly used herbicides. The herbicides chosen were five PSII inhibitors: atrazine, simazine, hexazinone, tebuthiuron and diuron; two auxinic herbicides: MCPA and 2,4-D; and the EPSP synthase inhibitor: glyphosate. The aim of this study was to determine whether there were differences in the response of the benthic diatom community and the individual diatom taxa to herbicides from the aforementioned three modes of action, and whether the relative sensitivities of the diatom taxa differ for each of these herbicides. To achieve these aims we needed a method (Kefford et al., 2005; Wood et al., 2014) designed to determine the response of multiple species of diatoms within a natural community, rather than estimate the absolute level of herbicide exposure that particular taxa can persist with. The response of each diatom taxon within the community was then compared across the eight herbicides to determine whether their relative sensitivities are the same or different.

## 3.3 Materials and Methods

The rapid toxicity test method used followed that of Wood et al. (2014), as described below, except that eight herbicides were tested in an orthogonal design, rather than one in Wood et al. (atrazine) (2014). The raw data for atrazine are the same as used in Wood et al. (2014), with new data for the other herbicides. Taxonomic identification of the diatoms has been refined to the species level in the current study (Genus in Wood et al. 2014). The genus *Gomphonema* is now divided into two distinct taxa; *Gomphonema gracile* and *Gomphonema* spp. (which in this study comprised *Gomphonema parvulum* and *Gomphonema minutum*). The genus *Amphora* in Wood et al. (2014) has been verified as *Encyonema gracilis* (*Amphora and Encyonema* are related taxa).

### *3.3.1 Study site and diatom collection*

Diatoms were collected on the 18$^{th}$ of May 2012 from Bluewater Creek, Queensland, Australia (19°14.406'S, 146°26.873'E). Bluewater Creek is part of the small coastal catchment area of Black River Basin, which flows directly into the Great Barrier Reef World Heritage Area (GBRWHA). The study site is located at the base of Paluma State Forest with no agricultural activity in the upper catchment and only recreational activities occurring upstream of the site. The study site is considered to have negligible herbicide pollution based on the surrounding land uses. Diatoms were collected by scrubbing rock substrates with a soft bristled brush and washing the detached diatoms into a collection jar. Substrates were collected for scrubbing along a 50 m length of the stream channel including various habitats such as edges, runs, riffles and pools. The composite site sample of detached benthic diatoms was transported to the laboratory in the dark at 21±1°C for the commencement of testing.

### *3.3.2 Rapid toxicity tests*

#### *3.3.2.1 Preparation of test diatoms*

The toxicity tests were initiated within four hours of sampling, including a one-hour acclimatisation period to stabilise the temperature of the samples to that of the test room. The detached benthic diatoms were homogenised by gentle shaking and 1 mL of this homogenised solution was pipetted into 40mL glass test vials. Unfiltered river water was added to each vial up to a final volume of 20 mL and spiked with a

predetermined concentration of each herbicide; atrazine, simazine, hexazinone, tebuthiuron, diuron, MCPA, 2,4-D and glyphosate. The test vials containing the benthic diatoms and individual herbicides were incubated for 48 hours in a controlled temperature laboratory set at 24 ± 2 °C, and light intensity of 20 μmol $m^{-2}$ $s^{-1}$ (±10%) on a 12 hour light/dark cycle.

#### *3.3.2.2 Preparation of test solution*

The eight herbicides were PESTANAL analytical grade products (all ≥ 99% pure) sourced from Sigma Aldrich; atrazine (CAS 1912-24-9), hexazinone (CAS 51235-04-2), tebuthiuron (CAS 34014-18-1), simazine (CAS 122-34-9), diuron (CAS 330-54-1), MCPA (CAS 94-74-6), 2,4-D (CAS 94-75-7) and glyphosate (CAS 1071-83-6). Herbicide solutions were prepared by dissolving appropriate weights of each herbicide in 10 mL 99% ethanol to increase their solubility except for simazine, which was dissolved in 25 mL ethanol, due to its low solubility and glyphosate, which was dissolved in milli-Q water. Three nominal test concentrations (50, 200 and 500 μg $L^{-1}$) of each herbicide were made by diluting appropriate volumes of stock solution with river water from the collection site (20 mL final volume). These concentrations were chosen to determine the relative sensitivity of the common taxa within the benthic community and were shown to elicit a response in the sensitive taxa whilst leaving the most tolerant taxa unaffected (Wood et al., 2014). These are also environmentally realistic concentrations for the region, where atrazine equivalent concentrations of up to 807 μg $L^{-1}$ for PSII mixtures have been recorded (Davis et al., 2013; Smith et al., 2012). Two control treatments (no herbicide) were prepared: a river water control (site water only) and a carrier control with ethanol equal to the maximum final concentration in the treatments (0.05% ethanol, 99.05% river water). The field collected diatom community includes live cells as well as unhealthy and even dead diatom cells, and the proportion of unhealthy or dead cells will vary depending on the successional stage of the benthic community (Wood et al., 2014). Consequently, an additional river water control treatment was prepared to indicate the health of diatoms at the start of the experiment (0 h) and the health of cells in each treatment and control was assessed as described below. All treatments and controls were replicated three times. Separate and concurrent test solutions of each herbicide were prepared as described above and stored in a freezer overnight in the dark before being sent to Eurofins Agroscience Testing Pty Ltd, a

National Association of Testing Authorities (NATA) accredited laboratory for verification of herbicide test concentrations by chemical analysis (LC–MS/MS).

### *3.3.3 Preservation, identification, and health classification of diatoms*

At the end of the 48 h exposure period the diatoms were preserved with Lugol's solution and identified under an Olympus BX50 light microscope (Olympus) at 400x magnification. At least 300 diatom cells were identified per treatment level (100 per replicate test vial), which was sufficient to capture the dominant taxa within the community. Each cell was identified to the lowest taxonomic group possible using various taxonomic keys (Cox, 1996; Gell et al., 1999; Sonneman et al., 2000). The health status of each cell identified was recorded as either 'healthy' or 'unhealthy' by visual inspection of the cells as per Wood et al. (2014); intact cells with chloroplasts present were regarded as healthy, whereas empty, broken, misshaped or cells with abnormal cell contents were considered unhealthy. This method, while it permits the health of cells to be assessed, generally does not permit verification of species level identification of diatoms, which requires cleaning of benthic samples and loss of cell contents. Consequently, samples from the river water control groups at 0 h and 48 h were sent for taxonomic identification to Dr. Jennie Fluin at the University of Adelaide to verify identifications from live material. These samples were cleaned and mounted on permanent slides for identification using an Olympus BH-2 (Olympus) light microscope at 1000x magnification. The diatom species list can be found in Supplementary Table S1.

### *3.3.4 Statistical analysis*

A generalized linear model (GLM) was used to determine the statistical significance of the effect of three factors on diatom health: concentration, mode of action and herbicide nested within mode of action (hereafter herbicides (mode of action). The GLM also tested the significance of two interaction terms: concentration * mode of action and concentration * herbicides (mode of action). The three herbicide modes of action were; PSII inhibitors (atrazine, simazine, hexazinone, tebuthiuron and diuron), auxinic herbicides (MCPA and 2,4-D) and the EPSP synthase inhibitor (glyphosate). The GLM was calculated using absolute binary health data (healthy vs. unhealthy) with logit link function on a per taxon basis and compared the likelihood of cells being identified as

unhealthy depending on treatment. Herbicide concentration response of each taxon was determined statistically in separate GLMs for each individual herbicide to compare the health of cells at each exposure concentration (50, 200, 500 μg $L^{-1}$) to that of the carrier controls. GLM was also used to compare cell health between the start and end of the experiment and to eliminate carrier effects.

The average response across herbicide treatments was used to rank the relative sensitivity of the diatom taxa; this was calculated by subtracting the percentage of healthy cells at each treatment concentration from the percentage of healthy cells in the carrier control, and taking the mean of this difference between concentrations (50, 200, 500 μg $L^{-1}$), for each herbicide on a per taxon basis. The EC10 and EC50 values were calculated using probit analysis on health data (proportion of healthy cells per taxon) with nominal herbicide concentrations (Finney, 1971). GLM and probit analysis were computed using the IBM SPSS 21 statistical package (SPSS 21).

Community level effects on the benthic diatoms were assessed using a three factor permutational multivariate analysis of variance (PERMANOVA). The PERMANOVA assessed the effect of concentration, mode of action, herbicide (mode of action) and their interactions with concentration effects (as per the GLMs design). PERMANOVA and non-metric multidimensional scaling (nMDS) ordination was created from resemblance matrices of Bray–Curtis similarities in order to visualise the difference between concentration treatments on the community. Community analysis was conducted on untransformed health data (proportion of healthy cells per taxon) using the PRIMER 6 software package (Clarke and Gorley, 2006). Taxa with <5% relative abundance were excluded from the analysis.

## 3.4 Results

### *3.4.1 Herbicide analysis and health of diatoms in the controls*

For all taxa, there was no significant difference ($p > 0.05$) between the carrier control, the river water control (48 h) and the background control (0 h) indicating that there were no carrier effects and the level of healthy cells did not change over the duration of the test (Supplementary Table S2). Most taxa had levels of health in the controls above 47% except for *Eunotia* cf. *incisa*, which had very low background health levels (>33.3%; Supplementary Table S2).

The measured concentrations in the herbicide treatments deviated up to 16% from nominal concentrations (50, 200, 500 μg $L^{-1}$) except for diuron treatments, which was 38% less than the nominal concentrations (Supplementary Table S3). The following results are presented in terms of nominal concentrations.

### *3.4.2 Herbicide concentration effects and the influence of herbicide mode of action*

There was a very strong effect of herbicide concentration ($p < 0.005$) on cell health for all diatom taxa except *Eunotia* cf. *incisa* (Fig. 1, Supplementary Table S4). The effect of herbicide mode of action on the health of diatom cells was statistically significant for only *G. gracile* ($p = 0.021$). *G. gracile* was more sensitive to the PSII inhibitors compared to the auxinic herbicides and the EPSP synthase inhibitor (Fig.1c). Within the mode of action groups there was a difference between the individual herbicides effects in two taxa; *Gomphonema* spp. ($p = 0.041$) and *Ulnaria ulna* ($p = 0.025$). There were no significant interactions between concentration and mode of action for any taxa ($p = 0.477– 0.998$ see Supplementary Table S4), neither were there any interactions for concentration and herbicide (mode of action) ($p = 0.445–0.966$), indicating that the effects of concentration were consistent across the different modes of action and herbicides within the modes of action.

Herbicide concentration response differed substantially between diatom taxa. *Gomphonema* spp. and *E. gracilis* showed a significant decline in health compared to that of the controls across all herbicides and concentrations (Fig. 1a and b) and had the lowest EC50 values across all herbicides (Supplementary Tables S4 & S5). *G. gracile* showed a significant decline ($p < 0.05$) in health with exposure to the PSII inhibiting herbicides at some concentrations, however there was no significant response ($p > 0.05$) to the herbicides MCPA, 2,4-D or glyphosate (Fig. 1c, Supplementary Table S6). The health of *U. ulna* declined significantly ($p < 0.05$) with exposure to each of the herbicides (Fig. 1d, Supplementary Table S7). *Cymbella* sp. displayed a decline in health to most herbicides however this was significant ($p < 0.05$) only in atrazine, hexazinone and diuron at 500 μg $L^{-1}$ (Fig. 1e, Supplementary Table S8). There was no apparent effect of herbicide concentration in *Eunotia* cf. *incisa* cells (Fig. 1g; Supplemenary Table S9). However, due to the very low level of *Eunotia* cf. *incisa* baseline health in the control treatments (Supplementary Table S2), it is difficult to

discern effects of herbicide concentration from controls (Fig. 1g). The health of *Achnanthidium minutissimum* was not affected by most herbicides, the only significant decline was after exposure to 2,4-D at the highest concentration treatment (Fig. 1f; Supplementary Table S10). The health of *Navicula cryptotenella* cells were not affected by any of the herbicides at the lowest concentration treatment (50 μg $L^{-1}$) and only showed a significant ($p < 0.05$) overall concentration response in two herbicides, diuron and glyphosate (Fig. 1h; Supplementary Table S11). However, even after exposure to 500 μg $L^{-1}$ diuron the proportion of healthy *N. cryptotenella* cells remained high (71%); therefore *N. cryptotenalla* appears to be a relatively tolerant species regardless of herbicide.

### *3.4.3 Relative sensitivity of the diatoms*

The relative sensitivities of diatom taxa were similar between herbicides (Fig. 2). *Gomphonema* spp. and *E. gracilis* were always the most sensitive taxa. *G. gracile* was 3rd most sensitive to the PSII inhibitors, but was ranked as relatively less sensitive to non PSII herbicides (Fig. 2). *U. ulna* and *Cymbella* sp. had intermediate sensitivity compared to other taxa. N. cryptotenella was always ranked among most tolerant of diatom taxa. *A. minutissium* and *Eunotia* cf. *incisa* were relatively tolerant to all the herbicides but their ranking was variable (Fig. 2).

### *3.4.4 Effects on the benthic diatom community*

Herbicide concentration had a significant effect on the health of benthic diatoms at the community level (p=0.001). The nMDS plot shows a distinct separation of the community between control and herbicide concentration treatments (Supplementary Figure S14). There was no effect of mode of action (p= 0.182) or herbicide (mode of action) (p = 0.167). There was no interaction of effects; concentration effects were consistent between modes of action (p = 0.287) and between herbicides (mode of action) (p= 0.374).

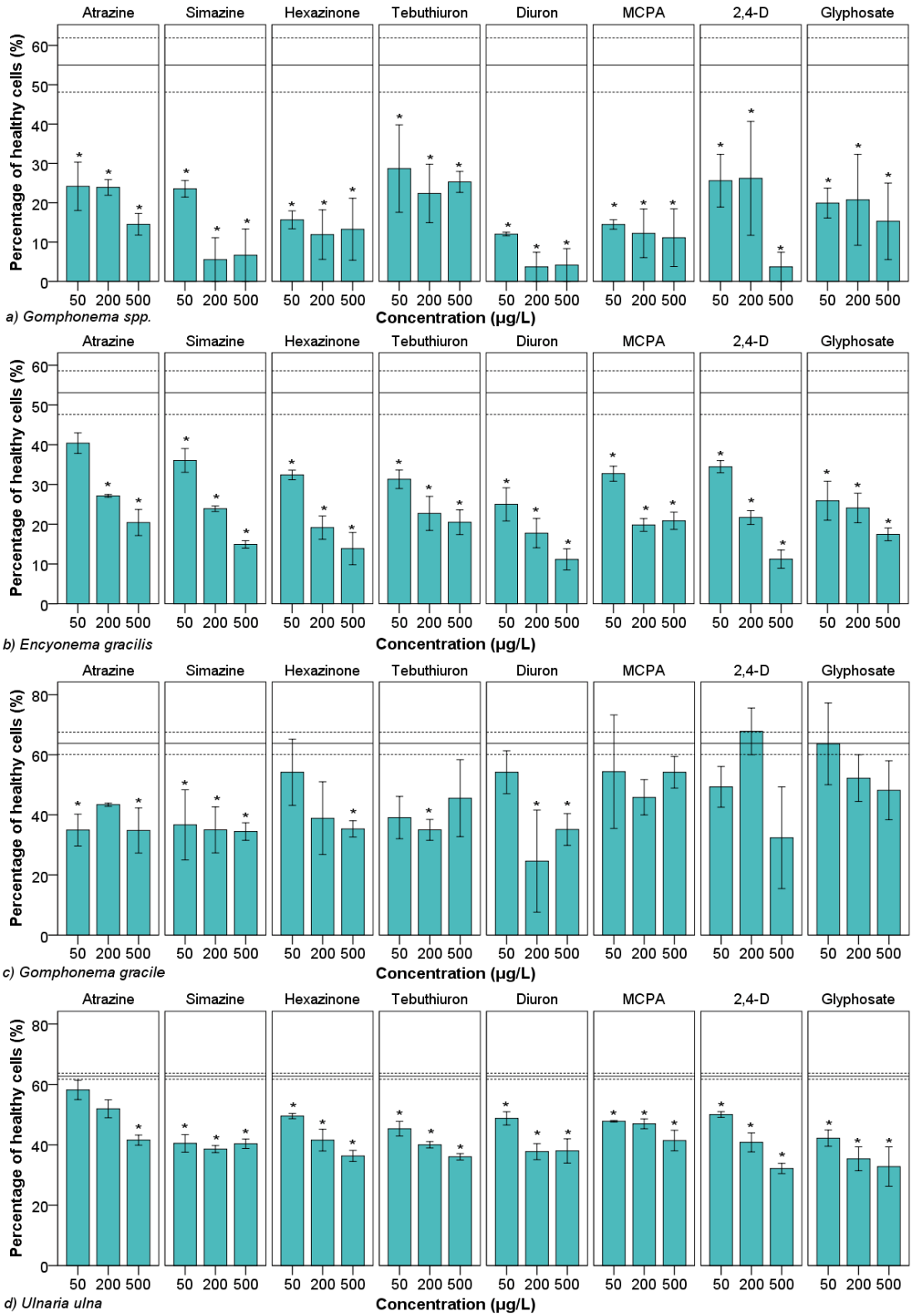


**Figure 3.1** Effect of eight herbicides on the health of diatom cells for each diatom taxon; a) *Gomphonema* spp., b) *E. gracilis,* c) *G. gracile,* d) *Cymbella* sp., e) *U. ulna,* f) *A. minutissimum,* g) *E. cf. incisa* and h) *N. cryptotenella*. Mean response of the carrier control is shown as a solid horizontal line with 95% confidence intervals shown as dashed lines. Error bars represent Standard Error (±SE). * Indicates statistically significant difference ($p<0.05$) from carrier control using GLM analysis.

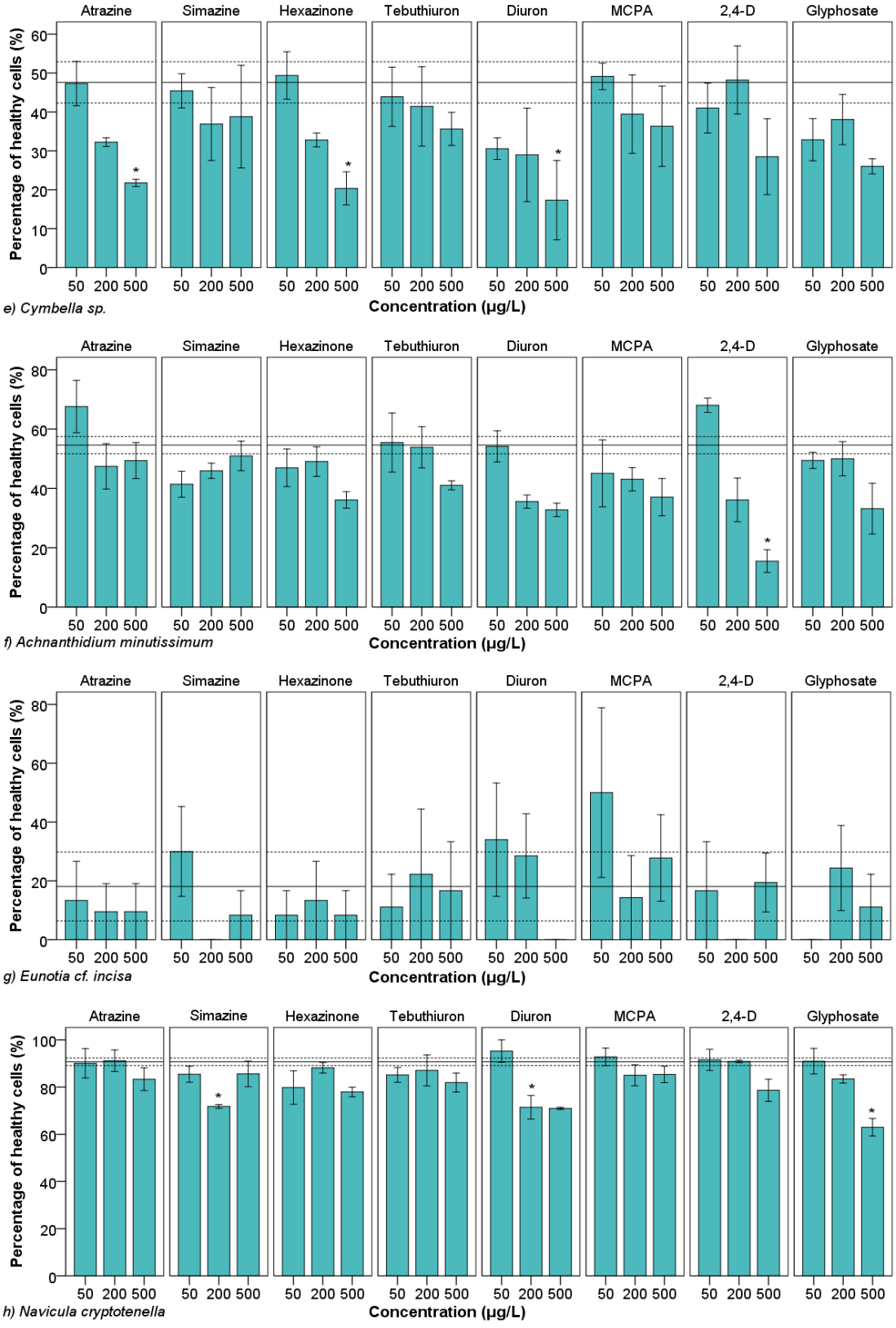


**Figure 3.1** (Continued)

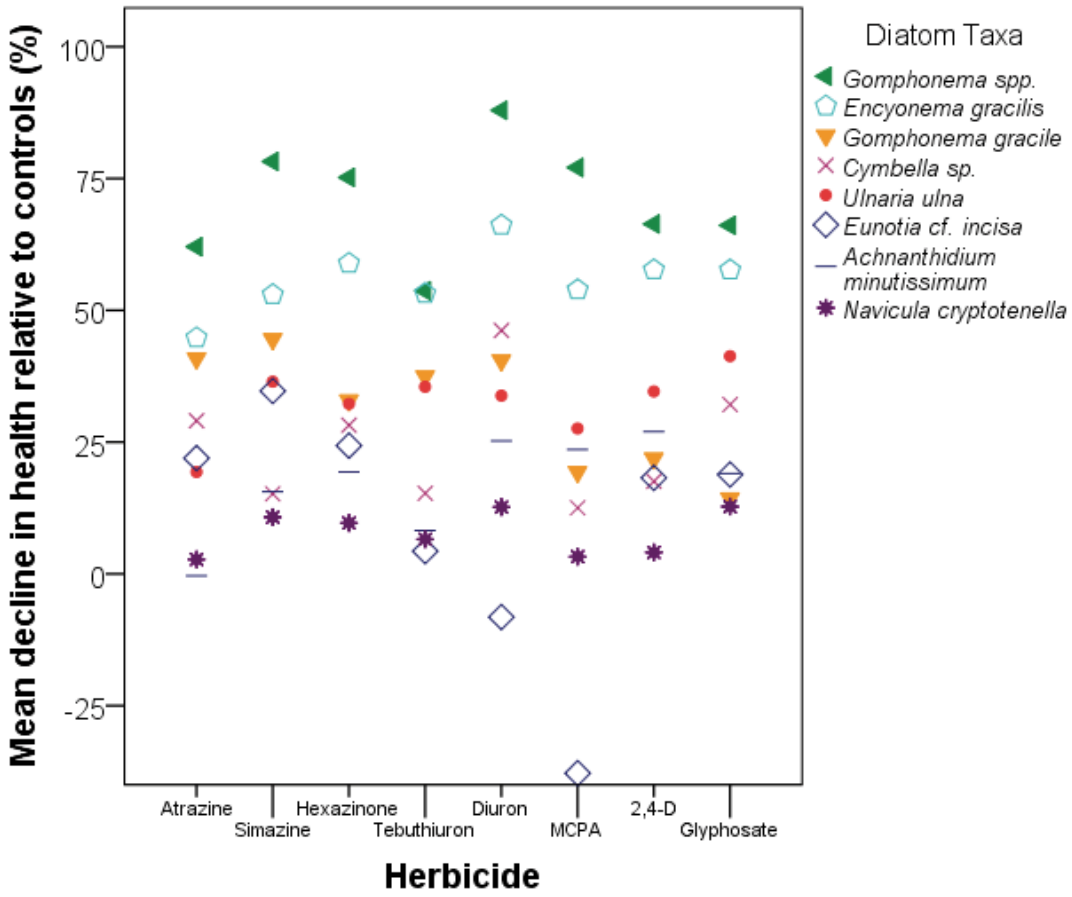


**Figure 3.2** Ranking of the diatom taxa based on the mean change in proportion of healthy cells across all test concentrations (50, 200, 500 µg $L^{-1}$) relative to controls for eight herbicides: atrazine, simazine, hexazinone, tebuthiuron, diuron, MCPA, 2,4-D, and glyphosate. The greater difference from controls corresponds with the most sensitive taxa.

## 3.5 Discussion

We assessed the response of benthic diatoms to eight different herbicides commonly found as contaminants in rivers within the GBR catchment area, North Queensland, Australia (Lewis et al., 2009). The eight herbicides chosen account for a vast majority of the herbicide load in the region, with the PSII inhibitors atrazine and diuron together accounting for approximately 90% of the measured loads within rivers of the GBR catchment area (Davis et al., 2012). The diatom community showed a significant response to herbicide concentration, and the effects of concentration were consistent between the eight different herbicides. We found evidence of an influence of mode of action on diatom health in just one taxon, *Gomphonema gracile*. There was also a difference in the effect of the different herbicides within modes of action in two taxa: *G*. spp and *Ulnaria ulna*. Whilst specific herbicide or mode of action is important for some taxa, these taxa are in the minority and there were no effects of mode of action or different herbicides at the community level. These taxa were a representative assemblage from our study site rather than a sample of species selected for standard toxicity testing. The relative sensitivities of the dominant diatoms in our community was consistent between eight common herbicides including three differing herbicidal modes of action and is not likely to be altered by different combinations of these herbicides at the concentrations tested.

*Gomphonema gracile* was the only taxon differentially affected by herbicide mode of action, with increased sensitivity to the PSII herbicides compared to the other modes of action. Other studies have reported that the PSII inhibitors are more phytotoxic to diatoms than herbicides with other modes of action (Larras et al., 2012) and diuron is reported as the most toxic of the PSII inhibitors (Magnusson et al., 2010). However, our study found that despite the different toxicities of the herbicides, the relative sensitivities of the majority of diatom taxa within this benthic community were retained. The most sensitive diatom taxa - *Gomphonema* spp. (consisting of *G. parvulum* and *G. minutum*) and *Encyonema gracilis* - showed significant declines in health to all herbicides. At the lowest exposure concentrations in the study, 50 μg $L^{-1}$, only these two most sensitive taxa showed a significant decline in health. At 200 μg $L^{-1}$, *Ulnaria ulna* and *Gomphonema gracile* also declined in health, although for *G. gracile* this was only the case for the PSII herbicides. In contrast, the tolerant taxa *Navicula cryptotenella* and

*Achnanthidium minutissimum* were relatively unaffected by herbicide exposure at any concentration used.

Our results demonstrate that the highest herbicide concentrations detected in the study region are likely to have adverse impacts on benthic diatoms. The ECx values calculated in the current study show that the most sensitive taxon; *G.* spp. (EC10 ≤ 5 μg $L^{-1}$ for all eight herbicides) could be affected by elevated concentrations of herbicides detected in polluted rivers of the study region (O'Brien et al., 2016). Peak concentrations of atrazine recorded during flooding events in the GBR catchment area often exceed 10 μg $L^{-1}$ and reach up to 27 μg $L^{-1}$, whilst peak concentrations of diuron reached 8.5 μg $L^{-1}$ and 2,4-D peaked at 9.5 μg $L^{-1}$ (Davis et al., 2013). In a single flooding event the mixture of PSII herbicides in river water at the most polluted sites can result in much higher mixture toxicities; with calculated atrazine equivalent concentrations up to 807 μg $L^{-1}$ (Smith et al., 2012). The EC50 values calculated in the current study ranged from 44 μg $L^{-1}$ to >500 μg $L^{-1}$ which is quite high for the most sensitive freshwater benthic diatom taxa compared to that of other studies (Larras et al., 2012; Magnusson et al., 2010; Roubeix et al., 2011b), although these studies used other end-points and exposure durations making comparisons problematic.

Our results are similar to that of other studies on diatoms with herbicide sensitivity varying greatly between individual diatom taxa, and the consistency of these results between herbicides emphasises their suitability as ecological indicators of herbicide toxicity in rivers (Larras et al., 2012; Roubeix et al., 2011b). Larras et al. (2012) conducted a study on 11 benthic diatom species and found that relative sensitivity was similar between the PSII herbicides diuron, terbutryn, isoproturon and atrazine, but differed for metolachlor, an inhibitor of long chain fatty acids. Species sensitivity distributions (SSD) showed that the motile diatom species, such as *Craticula accomoda, Eolimna minima, Mayamaea fossalis* and *Nitzschia palea* were most tolerant regardless of herbicide type (Larras et al., 2012). This may suggest a general trend in diatom sensitivity exists, perhaps related to phylogeny (Larras et al., 2014a). However, the *Gomphonema* taxa in the current study showed different sensitivities; *Gomphonema* spp. was relatively more sensitive than *Gomphonema gracile* to all herbicides tested. We note that *Gomphonema* spp. consisted of *G. parvulum* and *G. minutum,* in similar proportions (Supplementary Table S1). Other studies have reported that *G. parvulum* is

a relatively tolerant taxon (Larras et al., 2012; Pérès et al., 1996). Therefore, it is possible that the contribution of *G. minutum* to *Gomphonema* spp. in the current study is resulting in a more sensitive taxon than if we had been able to assess the health of *G. parvulum* and *G. minutum* separately. This raises the question whether genus level identification of diatom taxa would reduce the diagnostic capabilities of a diatom-based index for herbicide pollution. In the case of the community we studied, it would not significantly alter the relative sensitivity of the taxa within the community as the relative sensitivities of both *Gomphonema* spp. and *G. gracile* within this community were both more sensitive relative to the most tolerant taxon in the study, *Navicula cryptotenella*. Nevertheless, the intra-genus variation highlights the possibility that, depending on the dominant species present within the benthic community, the relative ranking of diatoms classified at the genus level could differ. This highlights the need for sensitivity data to be determined for a wide range of benthic diatom taxa that can lead to a better understanding of their relative sensitivities in natural communities (Roubeix et al., 2011b).

Some studies have highlighted the relationship between trophic mode of diatoms and tolerance to PSII herbicides (Debenest et al., 2009; Larras et al., 2012; Pérès et al., 1996). These studies suggest that the capability of some species of diatoms to obtain energy from alternate sources by switching their trophic mode from autotroph to heterotroph, allows them to cope with reduced photosynthetic efficiency, thereby making them more tolerant to PSII inhibitors. Heterotrophy is common in many *Navicula* species and they are one of the most herbicide tolerant taxa in the current study and also in others (Guasch et al., 1998; Larras et al., 2012; Ricart et al., 2009; Schmitt-Jansen and Altenburger, 2005). However, the heterotrophic capabilities of diatoms vary greatly between taxa and biological data on this trait are limited (Hellebust and Lewin, 1977). More information would be needed for both heterotrophy traits and sensitivity in order to establish whether heterotrophy can be used to infer herbicide tolerance in benthic diatom taxa.

In conclusion, herbicide mode of action did not alter the response of benthic diatoms at the community level. We observed a trend in relative herbicide sensitivity of the diatom taxa that was similar across herbicides with differing modes of action. The herbicidal mode of action did not change which benthic diatom taxa were most sensitive, but did

alter the sensitivity of one taxon in this study, *G. gracile*. The taxa most sensitive to herbicide toxicity were *Gomphonema* spp. and *Encyonema gracilis,* whilst the most tolerant was *Navicula cryptotenella*. The current study tested diatoms collected *in situ* to investigate the response of benthic diatoms to herbicides within an actual riverine community. The response of benthic diatoms to herbicide toxicity in the field could also be influenced by environmental factors such as light (Wood et al., 2016a), temperature (Larras et al., 2013a), flow regime (Villeneuve et al., 2011) and nutrient inputs (Guasch et al., 1998; Morin et al., 2015) and warrants further investigation. The sensitivity data produced is highly relevant to understanding the response of diatoms to herbicide exposure in the field and enhances knowledge of how individual diatom taxa respond to herbicides within complex biofilm communities. This data could contribute to the development of a diatom based biomonitoring index similar to the SPEAR index (Liess and Ohe, 2005) which uses macroinvertebrates as indicators of pesticide toxicity by calculating changes in the proportion of sensitive taxa in the community. The consistency of the results across the herbicides tested in the current study shows that freshwater benthic diatoms are a promising bioindicator for herbicide toxicity in rivers, including herbicides of differing modes of action.

**Supplementary data**

Supplementary data for Chapter 3 in available in Appendix B.

# Chapter 4 The influence of reduced light intensity on the response of benthic diatoms to herbicide exposure

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*Turbid conditions in Barratta Creek, QLD, Australia.*

## 4.1 Abstract

Herbicide pollution events in aquatic ecosystems often co-occur with increased turbidity and reduced light intensity. It is therefore important to determine whether reduced light intensity can influence herbicide toxicity, especially to primary producers such as benthic diatoms. Benthic diatoms collected from four rivers were exposed to herbicides in 48 h rapid toxicity tests under high light (100 µmol $m^{-2}$ $s^{-1}$) and low light (20 µmol $m^{-2}$ $s^{-1}$) intensities. The effects of two herbicides (atrazine and glyphosate) were assessed on 26 freshwater benthic diatom taxa. There was no significant interaction of light and herbicide effects at the community level or on the majority (22 of 26) of benthic diatom taxa. This indicates that low light levels will likely have only a minor influence on the response of benthic diatoms to herbicides.

## 4.2 Introduction

Benthic diatoms are key primary producers in aquatic ecosystems and are ecologically important as the base of the food web (Roubeix et al., 2011a). Herbicide pollution is frequently detected in rivers, estuaries and in coastal plumes as a result of diffuse agricultural run-off (Davis et al., 2013) including waterways draining into the Great Barrier Reef (Lewis et al., 2009; Smith et al., 2012). Phototrophic organisms such as freshwater benthic diatoms can be sensitive to herbicides, showing reduced growth and altered community structure in response to herbicide exposure (Debenest et al., 2009; Guasch et al., 1998; Roubeix et al., 2010). Monitoring the changes in the benthic diatom community may be a way to assess the ecological effects of herbicides in aquatic ecosystems as benthic diatoms are quick to respond to changes in environmental conditions (Rimet and Bouchez, 2011). Identifying which species are at risk of herbicide toxicity is an important step towards linking community compositional changes to herbicide impacts in the field (Larras et al., 2014a). In order to predict how the benthic diatom community will respond to herbicide pollution events, more information on the sensitivity of individual benthic diatom species to herbicides is required (Roubeix et al., 2011b). Additionally, there are numerous environmental factors that have the potential to alter the response of benthic diatoms to herbicide exposure, for example temperature (Larras et al., 2013a), grazing pressure (Muñoz et al., 2001), nutrients and light (Guasch et al., 1998; Guasch and Sabater, 1998).

Agricultural run-off is associated not only with herbicide delivery to rivers but also increased turbidity, therefore peak herbicide concentrations could co-occur with reduced light intensities (Dorigo et al., 2004; Kroon et al., 2012; Oliver et al., 2010). It is therefore important to understand the combined effects of low light intensities and herbicide exposure to freshwater benthic diatoms. Light is a major determinant of primary production and benthic community composition (Lange et al., 2011). Photoinhibition under high light intensity or growth limitation under low light intensity can be a stressor that may influence the subsequent ability of benthic diatoms to tolerate additional stress events such as herbicide exposure (Bonnineau et al., 2012). Moreover, many common agricultural herbicides are photosystem II (PSII) inhibiting, which act by halting photosynthetic reactions (Rutherford and Krieger-Liszkay, 2001). Since the mode of action of PSII herbicides is light dependent, low light conditions during

exposure may decrease the sensitivity of phototrophs (Brain et al., 2012b). Studies are needed to elucidate the influence of light and herbicide effects, their potential interactive effects and to determine whether these may alter the sensitivity of individual taxa within the benthic diatom community.

In this study natural benthic diatom assemblages collected from four rivers were exposed to two commonly used herbicides (atrazine and glyphosate) in 48 h rapid toxicity tests, under two experimental light intensities; high light (100 μmol $m^{-2}$ $s^{-1}$) and low light (20 μmol $m^{-2}$ $s^{-1}$). The two herbicides have different modes of action; atrazine is a PSII inhibitor and glyphosate inhibits EPSP synthase. The purpose of this study was to determine 1) if there was an interaction between herbicide effects and light intensity on diatom cell health in a range of freshwater benthic taxa, 2) whether relative herbicide sensitivity of the diatom taxa within the exposed communities is altered when exposed under different light intensities, 3) whether interactions differed between herbicides with different modes of action, and 4) if there was an interaction between light and herbicide effects at the community level. We hypothesized that there would be an interaction between light intensity and herbicide effects for the PSII inhibitor, atrazine, but not the non-PSII inhibitor, glyphosate.

## 4.3 Materials and Methods

### *4.3.1 Study sites and benthic diatom collection*

Natural benthic diatom communities were collected from four rivers that drain into the Great Barrier Reef World Heritage Area (GBRWHA); Alligator Creek (19°25.777'S, 146°56.599'E), Barratta Creek (19°42.416'S, 147°08.850'E), Liverpool Creek (17°43.432'S, 145°55.999'E) and Gowrie Creek (18°26.856'S, 145°50.873'E). These sites represent a continuum of herbicide exposure from minimal (Alligator), to moderate (Liverpool and Gowrie), to high (Barratta) (Davis et al., 2012; Davis et al., 2013; Kroon et al., 2012; Lewis et al., 2009), so as to include diatom communities with different levels of prior herbicide exposure. Collections occurred between 24$^{th}$ October and 5$^{th}$ November 2012. The sites are very similar in light conditions (Supplementary Table S1) and at all sites the water was clear at the time of diatom collection. Benthic diatoms were obtained by scrubbing rocky substrates with soft bristled brushes into a composite site sample from a 20m reach of river bed, as per Wood et al. (2014). Samples were

stored at a water temperature similar to that of the collection site and transported to the laboratory for rapid toxicity tests.

### *4.3.2 Rapid toxicity tests*

On arrival at the laboratory, diatoms were acclimatized to controlled laboratory temperature conditions for one hour (24 ± 2 °C at 20 μmol $m^{-2}$ $s^{-1}$ ±10%), after which the toxicity tests commenced. The experimental details of the rapid toxicity tests are described in Wood et al. (2014) and thus will be only summarised here.

#### *4.3.2.1 Exposure of diatoms*

The benthic diatom communities collected from each site were exposed to herbicides in separate concurrent rapid toxicity tests. The detached benthic diatoms were homogenised by gentle shaking and 1 mL was pipetted into 30mL glass test vials and made up to a final volume of 10 mL with river water spiked with a predetermined herbicide concentration. In the first set of tests, diatoms from Alligator Creek and Barratta Creek were exposed to nominal concentrations of 50, 200 and 500 μg $L^{-1}$ of either atrazine or glyphosate. In the second set of tests, diatoms from Gowrie Creek and Liverpool Creek were exposed to only atrazine at nominal concentrations of 20, 50, 200 and 500 μg $L^{-1}$. These concentrations (50, 200 and 500 μg $L^{-1}$) were shown to elicit a response in the sensitive taxa (Wood et al., 2014). We sought to determine the relative sensitivities of a number of taxa within the benthic community. The fact that some taxa remained unaffected at the concentrations used, while others were affected at the lowest concentration tested, shows that the concentrations used were appropriate for this aim. Additionally, these concentrations are environmentally realistic in creeks flowing through the region; concentrations of 27 μg $L^{-1}$ atrazine have been recorded at the polluted Barratta Creek (Davis et al., 2013) with a mixture toxicity calculated for PSII herbicides at up to 807 μg $L^{-1}$ (atrazine equivalent concentration) (Smith et al., 2012). The diatoms were exposed under two experimental light regimes; low light intensity at 20 μmol $m^{-2}$ $s^{-1}$ (±10%) or high light intensity at 100 μmol $m^{-2}$ $s^{-1}$ (±10%) for 48 hours with a 12 hour light:dark cycle. These light intensities were chosen to represent below (i.e. 20 μmol $m^{-2}$ $s^{-1}$) and above or near optimal (i.e. 100 μmol $m^{-2}$ $s^{-1}$) light intensities for most benthic diatom species reported in literature (Admiraal, 1976; Tuji, 2000). These light intensities were consistent with intensities measured in the field, which

predominantly occurred in partly to fully shaded conditions (Supplemental Data, Table S1). Peak herbicide concentrations in rivers typically occur following rainfall when turbidity is elevated and sunlight is reduced due to cloud cover. So the highest observed light conditions on the day of sampling (fine weather) are unlikely to occur during peak herbicide exposure in these rivers. Therefore we have tested the diatoms at two light levels; optimal light levels for benthic diatoms (high light) and at sub-optimal light levels (low light). The lighting conditions in the laboratory were achieved with lamps (Pierlite 36W/ 840 4000k) suspended at various heights above the bench to provide the required light intensity. Light intensity was measured in air just above the test containers using a PAR meter (LI-190 Quantum Sensor).

#### *4.3.2.2 Preparation of herbicide solutions*

Analytical grade atrazine and glyphosate (99% pure) Pestanal brand products were sourced from Sigma Aldrich. Stock solutions were prepared by dissolving atrazine in 10 mL 99% ethanol. Glyphosate was dissolved in 10 mL river water. Nominal concentrations (20, 50, 200 and 500 μg $L^{-1}$) of each herbicide were prepared by diluting appropriate volumes of stock solution of each herbicide with river water. Three control treatments (no herbicide) were prepared: (1) a river water control at the start of the test (0 h); and two at the end of the test period (48 h): (2) a river water only control; (3) and a carrier control (river water + 0.05% ethanol). The 0 h control treatment was required to assess the background health of the field collected diatom community (as naturally there can be many dead or unhealthy cells) and the carrier control was to determine if the concentration of the carrier used (ethanol) was having an effect (Wood et al., 2014). In each case the river water used was the water from the study site collected with the diatoms. This ensured that the measures of diatom sensitivity to the herbicides were for exposure in environmentally realistic water. The physico-chemical parameters of each site are reported in Supplemental Data, Table S2. All herbicide and control treatments were replicated three times and 48 h treatments and controls were placed in each experimental light intensity (high and low) in an orthogonal design. Test solutions of each herbicide concentration were sent to Eurofins Agroscience Testing Pty Ltd, a National Association of Testing Authorities (NATA) accredited laboratory, for chemical analysis (LC–MS/MS) to determine the accuracy of their respective nominal concentrations.

### *4.3.3 Preservation, identification and health status of diatoms*

Diatoms were preserved with Lugol's solution at the end of the 48 h exposure period. Diatoms were identified under an Olympus BX50 light microscope (Olympus) at 400x magnification to the lowest possible taxonomic level using the keys of Cox (1996), Gell et al. (1999) and Sonneman et al. (2000). Each identified cell was classified as either "unhealthy" if the cell was empty or broken or the chloroplasts were abnormal or otherwise "healthy" if the integrity of the cell wall and its contents are intact (as per Wood et al. (2014), see Supplemental Data, Table S3 for examples). Diatom cells smaller than 10 μm in length were excluded from analysis due the difficulty of identification and health classification. *Achnanthidium minutissimum* was the only taxon occurring at > 5% relative abundance that could not be assessed due to its size. The preservation with Lugol's solution which permits observations of the cells' health typically does not allow for species level identification, although this was possible for some taxa. So samples from the 0 h control were cleaned and mounted on permanent slides for identification using an Olympus BH-2 light microscope at 1000x magnification by Dr Jennie Fluin, University of Adelaide. Genus level identifications from live material that were confirmed to be monospecific (sp.) were distinguished from genera that included multiple species that were indistinguishable (spp.). The complete species list is given in Supplemental Data, Table S4.

### *4.3.4 Statistical analysis*

As no taxon was present at multiple sites in sufficient numbers, the data from each site were analysed separately. We used a generalized linear model (GLM) to assess the effects of herbicide concentration and light intensity and also the interaction of these two factors on cell health on a per taxon basis. The two herbicides (atrazine/glyphosate) were analysed separately using binary health data (healthy vs. unhealthy) with a logit link function. Treatments were compared to the control parameter (48 h) corresponding to the same light intensity and herbicide carrier medium used: glyphosate treatments (river water only control) and atrazine treatments (river water + 0.05% ethanol control). Where there was a significant overall concentration response a further GLM analysis was conducted on each herbicide and light factor individually to determine at which concentration the effect was significant. The health of the diatom taxa in each of the control treatments was also compared using GLM to assess their health at the end of the

test period (48 h) and to eliminate solvent effects (carrier control) compared to that of the controls at the beginning of the test (0 h). GLM analysis and graphs were computed using the SPSS 18 statistical package.

We have treated the controls separately as we had different controls for atrazine (due to atrazine requiring a carrier to increase its solubility), whereas glyphosate did not (river water only). While combining the controls would increase the statistical power of the tests, it would not account for (any) effect of the carrier. The results were also analysed with controls combined (river water and ethanol) and this resulted in 3 (not 4) taxa with significant interactions between light and concentration effects. This was due to a marginally significant result changing to a non-significant response for *Gomphonema clevei*. Regardless of how the controls are treated, it did not change the overall conclusion. Given that it produces an even more conservative result (in terms that there were more taxa with interactions), the results are reported with separate control treatments for glyphosate (river water control) and atrazine (ethanol control).

Community level effects were analysed using PERMANOVA to assess the effect of herbicide concentration, light intensity and their interaction on the benthic diatom communities. Community analysis was calculated using untransformed health data (proportion of cells per taxon), with each study site treated separately and each herbicide analysed separately. Taxa with <5% relative abundance were excluded from the analysis. Pairwise PERMANOVA tests were used to test within factors (concentration) to see which treatments were significantly different. Community compositional analysis was conducted using the PRIMER 6 software package (Clarke and Gorley, 2006).

## 4.4 Results

### *4.4.1 Measured herbicides in test treatments*

The measured concentrations of atrazine in the test solutions were always within 18% of nominal concentrations (mean of 8.75 %); however, the glyphosate concentrations in the test solutions were up to 46% greater than that of nominal concentrations (mean of 30.75 %) (see Supplemental Data, Table S5).

### *4.4.2 Background health among control groups and measured herbicide concentrations in test solutions*

The health of diatoms was consistent between control treatments for most taxa (see Supplemental Data, Table S6). There were no significant effects of the solvent carrier on all taxa tested. Higher health status in the control at 48 h compared to that at the beginning of the test were recorded for *Gomphonema gracile, Gomphonema clevei* and *Fragillaria* sp. (Gowrie Ck and Liverpool Ck). The health of *Ulnaria ulna* (Barratta Ck) varied over time and treatment. Some taxa had low percentages of health in all controls (<30% healthy); viz. *Cymbella* sp., *Cymbella aspera*, *Encyconema* sp., *Epithemia adanata* and *Ulnaria ulna* (Alligator Ck), due to their low health status at the time of field collection (0 h).

### *4.4.3 Light and herbicide effects in diatom taxa*

The effect of herbicide concentration on cell health was significant ($p<0.05$) for 15 out of 26 taxa from the four sites for atrazine, and 7 out of 16 taxa from two sites (Barratta and Alligator Creeks) for glyphosate (Table 4.1). There was also an effect of light intensity on the health of cells of *Fragillaria* sp. (Gowrie Ck), *Navicula* cf. *cryptotenella* (Liverpool Ck) and *Navicula* cf. *radiosa* (Liverpool Ck) (Table 4.1). For the majority of taxa there was no interaction between herbicide concentration and light intensity on the health of diatoms cells (Table 4.1). This interaction occurred in 4 out of 26 taxa: *Ulnaria ulna* (Barratta Ck), *Gomphonema clevei* (Alligator Ck), *Fragillaria* sp. (Liverpool Ck), and *Navicula* cf. *cryptotenella* (Liverpool Ck). For two of these taxa, *Ulnaria ulna* and *Gomphonema clevei,* herbicide sensitivity was dependent on light level in atrazine but not in glyphosate exposure. Glyphosate was not tested on taxa from Gowrie and Liverpool Creek samples (Table 4.1).

**Table 4.1** Effect of herbicide concentration, light intensity and their interaction on the cell health of diatom taxa from each of the communities using GLM analysis.

| | Atrazine | | | Glyphosate | | |
|---|---|---|---|---|---|---|
| **Taxa** | **Conc.** | **Light Level** | **Interaction** | **Conc.** | **Light Level** | **Interaction** |
| Alligator Creek: | | | | | | |
| *Adlafia* cf. *bryophila* | 0.461 | 0.925 | 0.717 | 0.451 | 0.994 | 0.094 |
| *Cymbella aspera* | 0.014 | 0.474 | 0.620 | 0.024 | 0.999 | 0.998 |
| *Epithemia* cf. *adanata* | 0.157 | 0.358 | 0.809 | 0.253 | 0.646 | 0.818 |
| *Epithemia* cf. *cistula* | <0.0001 | 0.609 | 0.708 | <0.0001 | 0.512 | 0.392 |
| *Eunotia* cf. *minor* | 0.080 | 0.854 | 0.408 | 0.255 | 0.372 | 0.438 |
| *Gomphonema clevei* | <0.0001 | 0.284 | 0.036 | 0.001 | 0.075 | 0.812 |
| *Gomphonema gracile* | 0.008 | 0.482 | 0.594 | 0.131 | 0.717 | 0.376 |
| *Gomphonema truncatum* | <0.0001 | 0.064 | 0.650 | 0.003 | 0.075 | 0.346 |
| *Navicula* cf. *cryptotenella* | 1.000 | 1.000 | 1.000 | 0.999 | 1.000 | 0.858 |
| *Ulnaria ulna* | 0.004 | 0.122 | 0.743 | <0.0001 | 0.128 | 0.213 |
| Barratta Creek: | | | | | | |
| *Mayamaea atomus* | 0.920 | 0.272 | 0.860 | 1.000 | 1.000 | 1.000 |
| *Melosira varians* | 0.007 | 0.064 | 0.644 | 0.498 | 0.999 | 0.850 |
| *Pleurosira* sp. | 0.010 | 0.220 | 0.751 | 0.041 | 0.924 | 0.088 |
| *Navicula* cf. *cryptocephala* | 0.984 | 0.999 | 0.913 | 0.979 | 0.999 | 0.784 |
| *Navicula schroeteri* | 0.810 | 0.726 | 0.754 | 0.608 | 0.853 | 0.895 |
| *Navicula* cf. *subtillissima* | 0.517 | 0.800 | 0.576 | 0.931 | 0.293 | 0.732 |
| *Ulnaria ulna* | <0.0001 | 0.100 | <0.0001 | 0.001 | 0.067 | 0.161 |
| Gowrie Creek: | | | | | | |
| *Cocconeis placentula* | 0.017 | 0.605 | 0.706 | | | |
| *Fragillaria* sp. | <0.0001 | 0.040 | 0.451 | | | |
| *Gomphonema* spp. | <0.0001 | 0.440 | 0.050 | | | |
| *Navicula* cf. *cryptocephala* | 0.294 | 0.999 | 0.583 | | | |
| *Navicula* cf. *cryptotenella* | 0.958 | 0.999 | 0.884 | | | |
| *Nitzschia paleaceae* | 0.116 | 0.126 | 0.135 | | | |
| *Ulnaria ulna* | <0.0001 | 0.595 | 0.768 | | | |
| Liverpool Creek: | | | | | | |
| *Cymbella* sp. | 0.967 | 0.999 | 0.990 | | | |
| *Encyconema* sp. | 0.415 | 0.430 | 0.712 | | | |
| *Fragillaria* sp. | 0.015 | 0.999 | 0.001 | | | |
| *Gomphonema* cf. *minutum* | 0.173 | 0.999 | 0.842 | | | |
| *Navicula* cf. *cryptotenella* | 0.277 | 0.001 | 0.020 | | | |
| *Navicula* cf. *radiosa* | 0.919 | 0.034 | 0.421 | | | |
| *Navicula* cf. *rhynchocephala* | 0.999 | 1.000 | 0.646 | | | |
| *Nitzschia paleaceae* | 0.990 | 1.000 | 1.000 | | | |
| *Pinnularia viridus* | 0.099 | 1.000 | 0.756 | | | |
| *Ulnaria ulna* | 0.001 | 0.570 | 0.320 | | | |

The interaction of light and atrazine concentration led to *Gomphonema clevei* being more sensitive to atrazine under low light intensity (Figure 4.1a). There was a significant decline in healthy cells ($p<0.05$) after exposure to 200 and 500 μg $L^{-1}$ atrazine under low light intensity; however, under high light intensity its response to atrazine exposure was not significant ($p>0.05$) (Figure 4.1a). Conversely, the interaction of effects on *Ulnaria ulna* led to greater sensitivity to atrazine under high light intensity, with a significant decline ($p<0.05$) in healthy cells at all three concentrations of atrazine under high light intensity (Figure 4.1b). In contrast, the percentage of healthy cells of *Fragillaria* sp. and *Navicula* cf. *cryptotenella* increased under low concentrations of atrazine exposure and high light intensity (Supplemental Data, Figures S7 & S8). Despite the interaction effects of light intensity and herbicide on *Navicula* cf. *cryptotenella*, there was no significant concentration response at low light intensity ($p>0.05$), indicating that this taxon is relatively tolerant to herbicides.

### *4.4.4 Relative sensitivity of the diatom taxa*

Taxa that were sensitive relative to other taxa (hereafter sensitive taxa) showed a significant concentration response ($p<0.05$) to herbicide exposure while taxa tolerant relative to other taxa tested (hereafter tolerant taxa) showed no such response. These responses are exemplified by *Epithemia* cf. *cistula* (Alligator Ck) for the former and *Navicula schroeteri* (Barratta Ck) for the latter (Figure 4.2a & b).

The diatom taxa were classified as either sensitive or tolerant based on their responses to herbicide exposure in the rapid toxicity tests (Table 4.2). Individual responses of the sensitive taxa can be found in the Supplemental Data, Figures S9-S16 and tolerant taxa in Supplemental Data, Figures S17-S29. Some taxa could not be classified due to their low health status in the control or inconsistent trends between treatment concentrations (Supplemental Data, Figures S30-S36). Of the eight taxa classified as sensitive, six of them were found at the relatively unpolluted site, Alligator Creek, which represented 67% of the classified taxa present (Table 4.2). Whereas, sites with a history of agricultural activity and herbicide exposure had lower proportions of sensitive taxa; Barratta Creek had 50% sensitive taxa, Liverpool had 44% and Gowrie had 43% (Table 4.2).

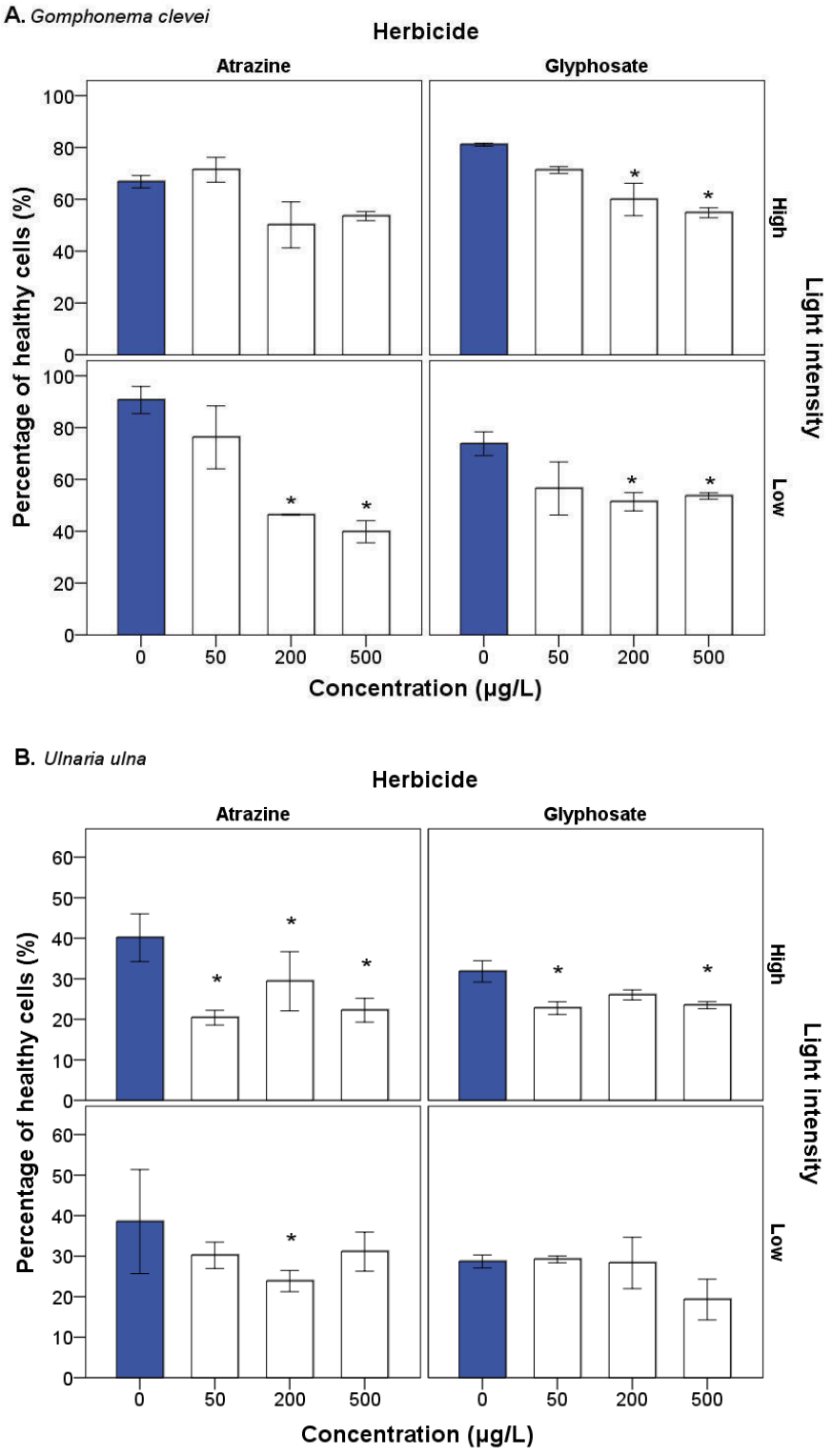


**Figure 4.1** Percentage of healthy diatom cells for two taxa; A. *Gomphonema clevei* from Alligator Ck and B. *Ulnaria ulna* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 or 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$), compared to controls (shaded bars). * indicates statistical difference ($p<0.05$) compared to controls in GLM analysis. The responses of other taxa are shown in Supplemental Data, Figures S7-S36.

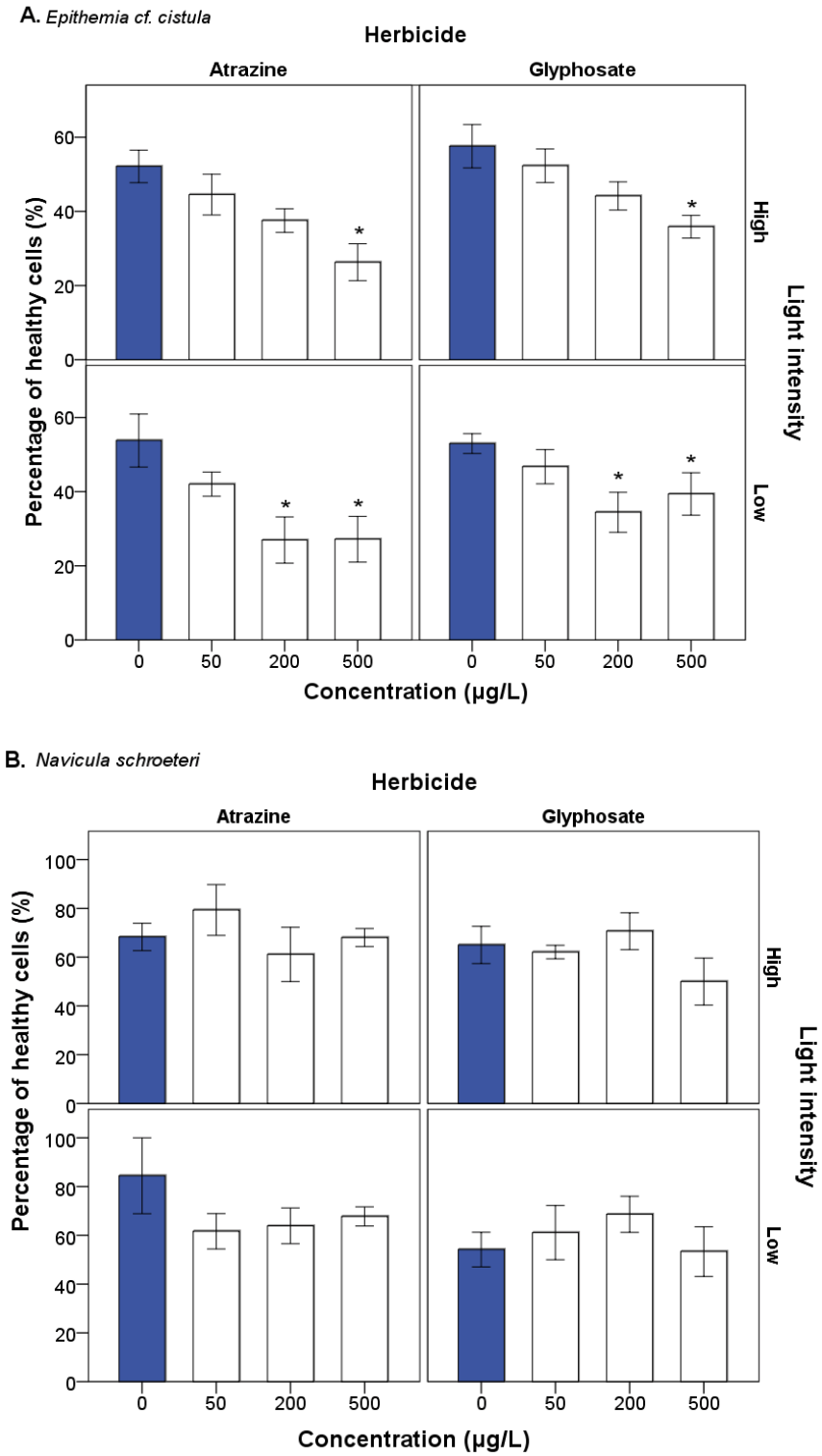


**Figure 4.2** Percentage of healthy diatom cells for two taxa; A. *Epithemia* cf. *cistula* from Alligator Ck and B. *Navicula 57chroeterii* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$), compared to controls (shaded bars). * indicates statistical difference ($p<0.05$) compared to controls in GLM analysis. The responses of other taxa are shown in Supplemental Data, Figures S7-S36.

**Table 4.2** Classification of diatom taxa as sensitive or tolerant based on responses in rapid toxicity tests to atrazine and glyphosate and occurrence of taxa from counts within control communities at t=0 (Supplemental Data, Table S4). Sites listed in order of increasing herbicide exposure of the collection site, from Alligator Creek (minimal), Liverpool and Gowrie Creeks (moderate) to Barratta Creek (high).

| **Diatom Taxon** | **Tolerant/ Sensitive** | **Alligator Creek** | **Liverpool Creek** | **Gowrie Creek** | **Barratta Creek** |
|---|---|---|---|---|---|
| *Adlafia* aff. *bryophila* | T | x | | | |
| *Cymbella aspera* | S | x | | | |
| *Epithemia adnata* | T | x | | | |
| *Epithemia cistula* | S | x | | | |
| *Gomphonema clevei* | S | x | x | | |
| *Gomphonema gracile* | S | x | | | x |
| *Gomphonema minutum* | S | | x | x | x |
| *Gomphonema parvulum* | S | | x | x | x |
| *Gomphonema truncatum* | S | x | | | |
| *Mayamaea atomus* | T | | x | x | x |
| *Navicula cryptocephala* | T | | | x | x |
| *Navicula cryptotenella* | T | x | x | x | |
| *Navicula schroeterii* | T | | | | x |
| *Navicula subtillissima* | T | | | | x |
| *Navicula* aff. *rhynchocephala* | T | | x | | |
| *Nitzschia paleaceae* | T | | x | x | |
| *Pinnularia viridis* | T | | x | | |
| *Ulnaria ulna* | S | x | x | x | x |
| Percentage of sensitive taxa: | | 67% | 44% | 43% | 50% |
| Percentage of tolerant taxa: | | 33% | 56% | 57% | 50% |

### *4.4.5 Community level responses to atrazine and glyphosate exposure*

There was no interaction between light and herbicide concentration at the community level in any of the four benthic diatom communities tested in this study. The Alligator Creek benthic diatom community was significantly affected by atrazine concentration ($p=0.001$), whereas there was no effect of light ($p=0.335$) and no interaction between these two parameters ($p=0.440$). The atrazine concentration effect was significant at all atrazine concentration treatments compared to that of the controls ($p=0.019$, 0.001, 0.001 for 50, 200 and 500 μg $L^{-1}$ respectively). Glyphosate concentration also had a significant effect on community composition ($p=0.002$) at 200 μg $L^{-1}$ ($p= 0.007$) and 500 μg $L^{-1}$ ($p=0.002$). There was no difference between the communities at different light intensities ($p=0.213$) and no interaction between light and glyphosate concentration ($p=0.420$).

The diatom community from Barratta creek was affected by atrazine concentration at 500 μg $L^{-1}$ ($p=0.007$), but not affected by light intensity ($p=0.748$) and there was no interaction between these two parameters ($p=0.385$). The community was not significantly affected by glyphosate concentration ($p=0.123$). However, there was an effect of light intensity at the community level in the glyphosate treatments ($p=0.028$), but no interaction between these two parameters ($p=0.607$).

The diatom community at Gowrie Creek was effected by atrazine concentration ($p=0.005$) but not by light intensity ($p=0.094$) and there was no interaction between these two parameters ($p= 0.376$). Atrazine effects were significant at 200 ($p= 0.002$) and 500 μg $L^{-1}$ ($p=0.004$). There were no community level effects at Liverpool Creek due to light intensity ($p=0.084$) or atrazine concentration ($p=0.131$), and there was no significant interaction between these two parameters ($p=0.884$).

## 4.5 Discussion

### *4.5.1 Light effects and interactions with herbicide effects*

For the majority of freshwater benthic diatom taxa in this study (22/26) and for the entire community, light intensity did not affect herbicide toxicity. In most cases the health of the diatoms after herbicide exposure was not altered by either high light (100 μmol $m^{-2}$ $s^{-1}$) or low light (20 μmol $m^{-2}$ $s^{-1}$) intensities. There was a significant interaction between light intensity and herbicide toxicity in only 4 out of 26 diatom taxa. *Ulnaria ulna* (Barratta Ck) was more sensitive to atrazine under high light intensity, whereas *Gomphonema clevei* (Alligator Creek) was more sensitive to atrazine under low light intensity. For *Fragillaria* sp. (Liverpool Creek) and *Navicula* cf. *cryptotenella* (Liverpool Creek) the interaction of low concentrations of atrazine and high light intensity was favourable to cell health. However, in no case did sensitive taxa become tolerant, or *vice versa* when exposed to herbicides under lowered light intensity.

Light and herbicide interactions in individual taxa were observed only for atrazine the PSII herbicide, but not glyphosate the EPSP synthase inhibitor. This may be attributed to the mechanism of toxicity of atrazine, which directly targets the light reactions that occur during photosynthesis (Brain et al., 2012b; Millie et al., 1992). The toxicity of PSII herbicides such as atrazine require light (Rutherford and Krieger-Liszkay, 2001) and it has been shown that atrazine can be less effective under low light intensities (Brain et al., 2012b). Interestingly, we found that sensitivity to atrazine decreased in *Ulnaria ulna*, but increased in *Gomphonema clevei* under low light intensity. Increased sensitivity to atrazine under low light intensity was also observed in the marine diatom *Phaeodactylum tricornutum* (Mayasich et al., 1986). Deblois et al. (2013) found that the planktonic diatoms *Fragilaria crotonensis* and *Aulacoseira granulata* var. *angustissima* were more sensitive to atrazine under low light intensity after acclimation to low light conditions, whereas the opposite trend was observed when acclimated to high light intensity. Studies on green algae report increasing atrazine sensitivity under high light intensity exposure (Deblois et al., 2013; Fischer et al., 2010; Mayasich et al., 1986). The cellular and photoregulatory mechanisms responsible for these different interactions between taxa are not well understood (Deblois et al., 2013; Fischer et al., 2010). Indeed, the range of responses highlights the need to investigate interactions of light intensity and herbicide toxicity at the individual, population and community levels.

### *4.5.2 Relative sensitivity of the diatom taxa and pollution history of the study sites*

The various diatom taxa within the benthic communities differed in their responses to herbicide exposure. Sensitive taxa showed significant declines in health, some in a dose response manner, whilst other taxa were unaffected by herbicide exposure even at the highest concentration treatment of 500µg $L^{-1}$. Ten taxa were classified as tolerant to herbicide exposure, and 8 taxa relatively sensitive to herbicides (Table 4.2). Of these, a larger proportion of the sensitive taxa were found at the unpolluted Alligator Creek site (67% sensitive taxa), compared to the other three sites with histories of agricultural activity and herbicide pollution with lower proportions of sensitive taxa (44 - 50% sensitive taxa). Liverpool Creek had the lowest proportion of sensitive taxa (44%) and this community was also unaffected by atrazine exposure. The occurrence of sensitive/tolerant taxa in field derived communities could be used to indicate herbicide toxicity, for example in the development of a diatom based index of herbicide toxicity. Changes in the distribution of particular traits within the community, such as herbicide sensitivity, could be linked to herbicide impacts at a particular site. This approach is applied by the SPEcies At Risk (SPEAR) index, which uses macroinvertebrates to identify pesticide toxicity (mainly insecticides) (Liess and Ohe, 2005). The development and assessment of a similar index using diatoms as indicators of herbicide impacts should be the subject of further studies.

### *4.5.3 Community level responses*

The response of diatoms to herbicide exposure at the community level also depended on prior exposure histories of the sites. The diatom community at the reference site (Alligator Creek) was the most sensitive to herbicide effects. It was the only site affected by atrazine at all concentrations tested. There were also significant effects in the Alligator Creek community at glyphosate concentrations of 200 µg $L^{-1}$ and 500 µg $L^{-1}$. However, the community at the polluted site (Barratta Creek) was only affected by atrazine at the highest concentration (500 µg $L^{-1}$) and was not significantly affected by glyphosate at the concentrations tested. The diatom community at the moderately impacted Gowrie Creek site was also affected by atrazine concentrations at 200 µg $L^{-1}$ and 500 µg $L^{-1}$ and the Liverpool Creek community was not affected by atrazine at the concentrations tested. The communities at the sites with a history of herbicide

contamination and intense agricultural impacts were more tolerant to herbicide effects. Prior herbicide exposure can lead to toxicant induced succession (TIS), where exposed communities exhibit a shift in community composition towards more tolerant taxa (Blanck, 2002; Schmitt-Jansen and Altenburger, 2005). This can result in communities becoming more tolerant to subsequent toxicant exposures, or pollution induced community tolerance (PICT) (Dorigo et al., 2010b; Magnusson et al., 2012). Whilst we did not aim to investigate TIS or PICT, our findings are consistent with these concepts.

Our results show that there was little effect of reduced light intensity on the toxicity of these two herbicides. The only effect of light intensity at the community level was at Barratta Creek, which showed a significant difference between the high and low intensity light treatments. There were no interactions between light and herbicide effects at the community level at any of the sites. However, adaptations to environmental conditions such as light intensity have been found to alter the sensitivity of periphyton to herbicides resulting in changes in benthic diatom community composition (Bonnineau et al., 2012; Guasch et al., 1997; Guasch and Sabater, 1998). The ability of individual diatom taxa to adapt to light availability may influence their response to herbicide exposure, especially in the case of PSII herbicides which act directly on photosynthesis (Laviale et al., 2010). Indeed this may give motile benthic diatom taxa, such as *Navicula cryptotenella*, a competitive advantage in situations where reduced light and herbicide stress co-occur. Diatoms that are motile have the capability to avoid stress and seek more favourable environmental conditions within the biofilm, therefore light limitation is less of a problem (Passy, 2007). Whereas, the attached diatoms such as *Gomphonema truncatum*, may be more impacted by the combined effects of reduced light and herbicide toxicity. These differences in species traits and sensitivity may be important in field situations where the additive toxicity of multiple PSII herbicides results in high atrazine equivalent concentrations (Magnusson et al., 2010) and light intensity regimes differ to that of a constant laboratory setting (Laviale et al., 2010).

## 4.6 Conclusions

Our experiments are designed to determine the influence of light intensity on the relative sensitivity of benthic diatoms, not as a simulation of the influence of multiple environmental stressors under field conditions. As we are measuring diatoms within natural benthic communities inter-specific interactions could also indirectly affect the

responses of the diatom taxa. However, since we are measuring changes in the health of diatom cells it is likely that the measured responses are at least partly the result of physiological effects. We observed interactions between light and atrazine effects in only a minority of taxa in this study. Despite the interaction of light and atrazine exposure in a few taxa, our results indicate that the relative sensitivity of benthic diatoms is not likely to be altered by reduced light intensity.

Community level responses to these two herbicides (atrazine and glyphosate) with different modes of action were not light dependent. The toxic effects of the herbicides in the benthic community differed between sites according to the prior pollution history of the sites, with the reference site, Alligator Creek, being most sensitive. Our results show that the influence of reduced light intensity during exposure is unlikely to change the identification of which taxa are most sensitive to herbicides, nor to alter community level responses to herbicide exposure. These findings are important for the use of diatom-based indices of herbicide toxicity in biomonitoring and validate the use of these experimental light intensities to identify the relative sensitivity of the benthic diatom taxa within natural communities.

**Supplemental data**

The supplemental data for Chapter 4 available in Appendix C

# Chapter 5 Chronic effects of atrazine exposure and recovery in freshwater benthic diatoms from two communities with different pollution histories.

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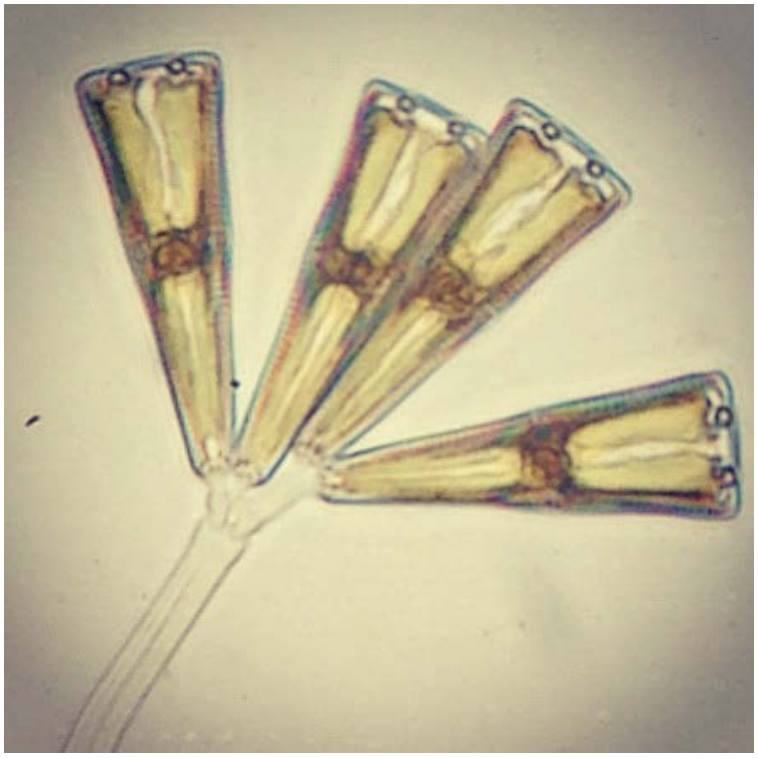

*Gomphonema truncatum*

## 5.1 Abstract

Diffuse agricultural runoff into rivers can result in contamination with herbicides for prolonged periods of time. Chronic exposure to herbicides has the potential to alter toxic impacts in primary producers such as benthic diatoms. Determining how individual diatom taxa respond to herbicide exposure over varied exposure durations is essential for assessing herbicide impacts. This study investigated the responses of various benthic diatom taxa and effects at the community level over 12 days of atrazine exposure. Diatom communities were collected from two sites with differing exposure histories; a relatively unpolluted site (Alligator Creek) and an agricultural stream (Barratta Creek) known to be polluted by atrazine and other herbicides. Diatom community composition and the proportion of healthy cells per taxon were assessed at 0, 2, 3, 6, 9 and 12 days of atrazine exposure. Pollution history altered the response of the diatom community to atrazine exposure. In the Alligator Creek diatom community there was a shift in composition towards more tolerant taxa and the loss of sensitive taxa in atrazine exposed treatments. The sensitive taxon (*Gomphonema truncatum*) was consistently affected by atrazine toxicity. Conversely, the polluted Barratta Creek diatom community was not strongly affected by atrazine exposure. Our study shows that during chronic atrazine exposure some taxa demonstrated the ability to recover despite initial toxicity response. Recovery could be an important trait for understanding the ecological effect of herbicide exposure on diatom species in nature and in applied circumstances such as biomonitoring indices.

## 5.2 Introduction

The contamination of aquatic ecosystems with herbicides is a major issue of concern in agricultural regions worldwide. Herbicide pollution in rivers often occurs when high concentration pulses enter waterways from agricultural runoff coinciding with high rainfall events (Solomon et al., 1996). However, in the highly polluted Barratta Creek peak concentrations of atrazine (12.3 μg $L^{-1}$) occurred during periods of low flow coinciding with the end of the sugar cane harvesting period and elevated concentrations of herbicides continued for several continuous months of the year (O'Brien et al., 2016). Additionally, low-level herbicide concentrations have been detected year round in other rivers (Shaw et al., 2010; Smith et al., 2012). As a result, freshwater organisms are likely to be exposed to herbicides under both acute and chronic exposure scenarios (Dorigo et al., 2004; Smith et al., 2012). Both short and long term herbicide exposure have the potential to alter the structure and function of primary producers such as benthic diatom communities (Larras et al., 2012; Magnusson et al., 2008; Magnusson et al., 2012; Ricart et al., 2009; Rimet and Bouchez, 2011). In order to assess potential herbicide impacts it is essential to investigate the responses of individual diatom taxa as well as the community to herbicides over a range of exposure durations.

Exposure to herbicides over different durations will likely alter the potential physiological and ecological effects to the benthic diatom community (Gustavson et al., 2003). Under short-term exposure (defined here as ≤ 96 hours) herbicide toxicity has been shown to alter photosynthesis (Magnusson et al., 2010), growth (Larras et al., 2012) and cell health (Wood et al., 2014; Wood et al., 2016a; Wood et al., 2016b) of benthic diatoms. Whereas, longer exposure of diatoms to herbicides can result in increased herbicide tolerance through physiological acclimation (Roubeix et al., 2011b; Tiam et al., 2015) and taxa that are initially impaired may have the ability to recover and subsequently outcompete others that are slower to, or cannot, recover (Carder and Hoagland, 1998; Magnusson et al., 2008; Magnusson et al., 2012). Alternatively, cells which do not develop tolerance to herbicide exposure can become affected with subsequent exposure as stress from the herbicide builds up (Nelson et al., 1999). Freshwater benthic diatoms have doubling rates of approximately 0.1 – 2.2 $d^{-1}$ (Admiraal, 1976; Gould and Gallagher, 1990) and longer exposure periods will involve multi-generational exposure, which can increase toxicity (Kefford et al., 2008; Rose et

al., 2002). Multigenerational exposure can also lead to the selection of better adapted individuals, resulting in genetic adaptation, which leads to increased tolerance and in turn resistance (Stachowski-Haberkorn et al., 2013). At the community level, herbicide exposure can exert a selective pressure that results in the dominance of more tolerant taxa to the detriment of sensitive taxa (Blanck, 2002). This compositional shift can alter sensitivity at the community level resulting in increased pollution tolerant communities (Magnusson et al., 2012; Pesce et al., 2010; Tlili et al., 2011). Establishing how herbicide exposure duration affects freshwater diatoms and whether the same taxa that are sensitive to short-term exposure are also similarly affected by longer exposure durations is critical in understanding the effects of herbicides on the benthic diatom community.

The current study investigated the response of freshwater benthic diatoms within natural diatom communities to the photosystem II (PSII) inhibiting herbicide atrazine, over 12 day continuous exposure laboratory experiments. Diatom communities were collected from two locations with differing pollution histories; Alligator Creek, a relatively unpolluted reference site, and Barratta Creek, an agriculturally impacted stream known to be polluted by herbicides, including atrazine and other PSII herbicides (Davis et al., 2008; O'Brien et al., 2016). Within these benthic communities the number of healthy diatom cells per taxon was assessed on day 0 (prior to exposure), 2, 3, 6, 9 and 12 of atrazine exposure. The aim was to determine whether exposure duration alters the effect of atrazine on specific diatom taxa and to identify taxa capable of recovery in the presence of atrazine. Additionally, we assessed chronic effects at the community level using changes in species composition and relative abundance.

## 5.3 Materials and Methods

### *5.3.1 Study sites and diatom collection*

Benthic diatoms were collected from Alligator Creek and Barratta Creek on the 24$^{th}$ of October 2012. These rivers flow into the Great Barrier Reef Marine Park (GBRMP), a World Heritage listed area. The GBRMP catchment covers 424,000 km$^2$ and includes 35 smaller coastal catchments of which these rivers are included. The collection sites are located in the dry tropical climatic region approximately 30km (Alligator) and 70km (Barratta) south west of Townsville, Queensland, Australia.

Alligator Creek originates in Bowling Green Bay National Park and flows through the Ramsar listed wetland, Bowling Green Bay (ANCA, 2001). Diatom samples were collected from a sampling site (19°25.777'S, 146°56.599'E) in the upper catchment of the river at the base of the National Park, with no agricultural activity upstream. The river is approximately 8 metres wide with mostly large cobbles, boulders and bedrock substrate. The upstream and surrounding vegetation is dense eucalypt forest and rainforest. There is only recreational activities upstream of the sampling location, therefore herbicide impacts at the collection site are considered negligible (Lewis et al., 2009).

Barratta Creek is located in the lower Burdekin River region, an agricultural district supporting extensive sugarcane farming (Davis et al., 2008). Barratta Creek also drains into Bowling Green Bay, a wetland listed in Australia's National Directory of Important Wetlands and a Ramsar wetland of international significance (ANCA, 2001). The collection location (19°42.416'S, 147°08.850'E) is situated in the upper Barratta Creek catchment, with predominantly agricultural land uses upstream (grazing, mixed horticulture and sugarcane) (Davis et al., 2008). The collection site has a narrow sparsely vegetated corridor of small eucalypt trees and grasses on either side of the approximately 5 metre wide river channel comprising a rocky substrate with gravel and sand embankments. This location is highly impacted by agricultural herbicides such as atrazine, diuron, hexazinone 2,4-D and MCPA, which are used in the sugarcane industry (Davis et al., 2008; Davis et al., 2012). Concentrations of the PSII herbicides, atrazine and diuron exceeded the ecological protection guidelines for several continuous months of the year, with maximum recorded concentrations of 12.3 μg $L^{-1}$ atrazine and 12.8 μg $L^{-1}$ diuron (O'Brien et al., 2016). Mixtures of multiple PSII herbicides are frequently detected at Barratta Creek and their toxic effects to primary producers such as benthic diatoms have been shown to be additive (Magnusson et al., 2010). The mixture toxicity of PSII herbicides has been estimated by calculating the toxic equivalency quotient (TEQ) which adds the concentrations of PSII inhibitors in a mixture after applying a toxic equivalency factor (TEF) based on the response of the freshwater alga *Chlorella pyrenoidosa* ($TEQ_{CP}$). The estimated toxicity of PSII herbicide mixtures at Barratta Creek has exceeded the atrazine trigger value for ecological protection (13 μg $L^{-1}$) for 30 consecutive days (Smith et al., 2012) with a maximum $TEQ_{CP}$ atrazine equivalent concentration of 807 μg $L^{-1}$. Therefore, the

potential toxicity of herbicide exposure in Barratta Creek at these high atrazine equivalent concentrations should be examined.

The benthic diatoms were collected by scrubbing pebbles and cobbles from the bottom of the riverbed with a soft bristled toothbrush to remove the attached diatoms. Rocky substrates were sampled from various locations within an approximately 20 m reach of river including riffles, pools and edge zones. The detached benthic diatoms were washed into trays and pooled into a composite sample per site. These samples were stored in the dark and transported directly to the laboratory at the same temperature as that of the site water. Water quality conditions at the time of diatom collection are summarised in Supplementary Table S1 and published information is available for Barratta Creek coinciding with our study from that of O'Brien et al. (2016) and its previous condition from Davis et al. (2008 and for Alligator Creek (Lewis et al., 2009).

### *5.3.2 Determination of atrazine concentrations at the sampling locations*

Grab water samples were taken at each of the collection sites at the time of diatom collection. The water was collected into solvent rinsed, 1 L amber glass bottles, transported on ice and placed in a freezer overnight in the dark at 4°C before being sent for measurement of atrazine concentrations by liquid chromatography tandem mass spectrometry (LC–MS/MS) at Eurofins Agroscience Testing Pty Ltd.

*Toxicity tests*

The collecting, transport and toxicity testing of benthic diatoms followed the method described in Wood et al. (2014), except that the test used in the current study was over a longer duration (12 days rather than 2 days). The live benthic diatom samples were allowed to acclimatise to conditions of the temperature-controlled laboratory for one hour before commencement of the toxicity tests, which commenced within 3 hours of diatom collection from the field. Two toxicity tests were conducted simultaneously with diatoms collected from each site under the same experimental conditions. The toxicity tests were conducted over 12 days at 24°C under a light intensity of 100 μmol $m^{-2}$ $s^{-1}$ on a 12 h light/dark cycle. For the test treatments 1 mL subsamples of the benthic diatoms were pipetted into 30 mL test vials with river water from the corresponding site to a final volume of 10 mL. Herbicide exposure treatments were spiked with atrazine at predetermined nominal concentrations of 50, 200 and 500 μg $L^{-1}$. These concentrations

were shown to elicit a response in the sensitive taxa (Wood et al., 2014; Wood et al., 2016a; Wood et al., 2016b) and despite being higher than the measured peak concentrations of atrazine recorded at Barratta Creek (12.3 μg $L^{-1}$), correspond with estimated mixture toxicities of PSII herbicides frequently detected in the study region (Smith et al., 2012). Atrazine stock solutions were prepared by dissolving analytical grade atrazine (Pestanal) in 10 mL 99% ethanol. Two control treatments were prepared for each site (no atrazine) that is river water controls (using water from the respective collection sites) and carrier controls with 0.05 % ethanol in site water (final ethanol concentration equal to the maximum concentration in the atrazine treatments). All treatments were replicated three times on days 2, 3, 6, 9 and 12 of the experiment in an orthogonal design. An additional control treatment was prepared for each site at 0 h using river water only to assess the health of the diatoms at the start of the experiment prior to atrazine exposure. Test solutions of each herbicide concentration were sent to Eurofins Agroscience Testing Pty Ltd a National Association of Testing Authorities (NATA) accredited laboratory, for chemical analysis (LC–MS/MS) to determine the accuracy of their respective nominal concentrations.

### *5.3.3 Preservation, identification and health classification of diatom cells*

At the conclusion of each test period (0, 2, 3, 6, 9 and 12 days) the diatoms in the test vials were preserved with Lugol's solution. The diatom cells in these samples were later counted and identified under an Olympus BX50 (Olympus) light microscope at 400x magnification to the lowest taxonomic level possible (mostly at species level) using the taxonomic keys of Cox (1996), Gell et al. (1999) and Sonneman et al. (2000). At least 100 diatom cells per replicate sample were counted (300 cells per treatment). The diatom cells were also classified as either "healthy" or "unhealthy" by visual inspection of each cell; intact cells with chloroplasts present were regarded as healthy, whereas empty, broken, misshapen cells or those with abnormal cell contents were considered unhealthy (Wood et al., 2014). This method allows the health of cells at the time of preservation to be assessed; however, it is not always possible to verify species level identification of diatoms from live material. Consequently, preserved samples from the day 0 control treatment from both Alligator Creek and Barratta Creek were also analysed by Dr Jennie Fluin, University of Adelaide to verify the species level identifications. These samples were cleaned and mounted on permanent slides for

identification using an Olympus BH-2 (Olympus) light microscope at 1000x magnification. The full taxon list can be found in Supplementary Table S2.

### *5.3.4 Statistical analysis*

The effect of atrazine on the number of healthy cells of the common diatom taxa was assessed using a generalized linear model (GLM). GLM was calculated on untransformed binary health data (healthy/unhealthy) using a logit link function. The proportion of healthy cells in the ethanol controls was compared to that of the river water controls using GLM to assess carrier effects; where no effect was detected they were combined for further analysis and if effects were detected then the atrazine treatments were compared to that of the ethanol controls in order to adjust for the carrier effects. The control treatments were compared across days (0, 2, 3, 6, 9, 12) to check for successional effects (changes in proportion of healthy cells overtime) in each taxon. Atrazine toxicity effects were assessed using GLM on a per taxon basis each day, by comparing atrazine treated groups (50, 200, 500 μg $L^{-1}$) to that of the corresponding control for that day to account for possible successional effects. The results were represented graphically by calculating the percentage of healthy cells per taxon (number of healthy cells/total cells counted). The GLMs were analysed in SPSS Statistics 22 (IBM).

Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis similarities was used to assess the effect of atrazine concentration through time (2 factor design) and the interaction of these factors on benthic diatom community composition. A one factor PERMANOVA test was then performed to determine atrazine concentration effects in the diatom community on each experimental day (2, 3, 6, 9 and 12). Homogeneity of multivariate dispersion test (PERMDISP) was performed to determine whether the group dispersion (average distance to the group centroid) differed across treatments and days. Untransformed relative abundance data for the common diatom taxa (>5 % in at least one sample) were used for all community level analysis. SIMPER analysis was used to determine the diatom taxa driving the differences between the groups. Non-metric multidimensional scaling (nMDS) ordination was performed using group centroids to visualise the trajectory of change between benthic diatom communities across atrazine concentrations and time. All multivariate analysis was performed using PRIMER v6 (Clarke and Gorley, 2006).

## 5.4 Results

### *5.4.1 Measured atrazine concentrations and control communities*

The diatom communities present at the two study sites differed in their species compositions. The community from Alligator Creek consisted of *Gomphonema truncatum* (20%) and *Gomphonema gracile* (19%), *Epithemia* cf. *cistula* (18%) and *Gomphonema clevei* (9%); whereas the Barratta Creek Community was dominated by *Ulnaria ulna* (43%), followed by *Pleurosira* sp. (23%), *Melosira varians* (10%) and *Navicula schroeterii* (9.3%) (see Supplementary Table S2 and Figures S2-S3).

The Barratta Creek river water sample had an atrazine concentration of 13 μg $L^{-1}$, while that of Alligator Creek water sample was below the detection limit (< 1 μg $L^{-1}$). The measured concentrations of atrazine in the Barratta Creek toxicity test treatments were slightly higher than that of Alligator Creek due to the background levels of atrazine in the site water; however, measured concentrations were within 18% of nominal concentrations (mean of 9%) (Supplementary Table S1).

### *5.4.2 Community level responses to atrazine exposure*

The response of the benthic diatom community to atrazine exposure differed through time and between the two communities (Figure 5.1A & B). The diatom community composition from Alligator Creek differed significantly between atrazine concentration treatments ($p = 0.001$, Pseudo-F = 3.4), through time ($p = 0.001$, Pseudo-F = 4.8) and their interaction ($p = 0.003$, Pseudo-F = 1.8). The multivariate dispersion between samples differed with time ($p = 0.001$) but not between concentration treatments ($p = 0.63$) (Supplementary Figure S4). The change in trajectory of the Alligator Creek diatom community composition through time and with atrazine exposure is illustrated in the nMDS plot (Figure 5.1A). Atrazine effects within individual day groups were significant at the end of the experiment, i.e., on day 12 overall ($p = 0.014$, Pseudo F = 2.0), and not on the other days ($p > 0.05$, Supplementary Table S3).

The Barratta Creek community differed significantly with time ($p = 0.001$, Pseudo-F = 21) and with atrazine concentration ($p = 0.026$, Pseudo-F = 1.8) and the two factors showed a significant interaction ($p = 0.025$, Pseudo-F = 1.4). These results indicate that time had a much stronger effect on the Barratta Creek benthic diatom community compared to atrazine concentration (Pseudo-F of 21 versus 1.4). Community

compositional change followed a similar trajectory between the treatments over the 12 day exposure period (Figure 5.1B). The multivariate dispersion differed significantly between days ($p = 0.002$) and between concentration treatments ($p = 0.036$); this can be seen in the spread of samples in the nMDS plot (Supplementary Figure S1). The effect of atrazine on the individual days was only slightly significant on day 2 ($p = 0.043$, Supplementary Table S3) and not on the other days ($p>0.05$).

Changes in relative abundance of the dominant diatom taxa at Alligator Creek are shown in Supplementary Figure S2. SIMPER analysis showed the diatom taxa that contributed most to the differences between treatments in the Alligator Creek Community (Supplementary Table S4). *Gomphonema truncatum* dominated the control treatment communities increasing in abundance over the course of the experiment to 45% on day 12 (Table S4). Whereas in all atrazine treatments on day 12, *Gomphonema truncatum* declined in relative abundance to just 1.7% at 50 μg $L^{-1}$, 25% at 200 μg $L^{-1}$and 15% at 500 μg $L^{-1}$ (Supplementary Table S4). *Cymbella aspera* also declined in relative abundance in the higher atrazine concentration treatments, 200 and 500 μg $L^{-1}$ atrazine to 0.75% and 2.8%, respectively compared to that of the control treatment (8.3%) on day 12. *Navicula cryptotenella* increased in relative abundance in atrazine treatments, from control treatment levels of 7.0% to dominate the highest atrazine concentration treatment of 500 μg $L^{-1}$ at 31% on day 12 (Supplementary Table S4).

Within the Barratta Creek community, the relative abundance of *Ulnaria ulna* declined in all treatments over the course of the experiment, from 43% on day 0 to 16% on day 12 (Supplementary Figure S3 and Table S5). *Navicula cryptocephala* was the dominant taxon by the end of the exposure period, increasing in relative abundance from 4.0% on day 0, to 23% by day 12 (Supplementary Figure S3 and Table S5). *Mayamaea atomus* and *Cyclotella* sp. were poorly represented on day 0 (both < 2%); however, by day 12 they had increased in relative abundance to 13% and 17%, respectively (Supplementary Figure S3 and Table S5).

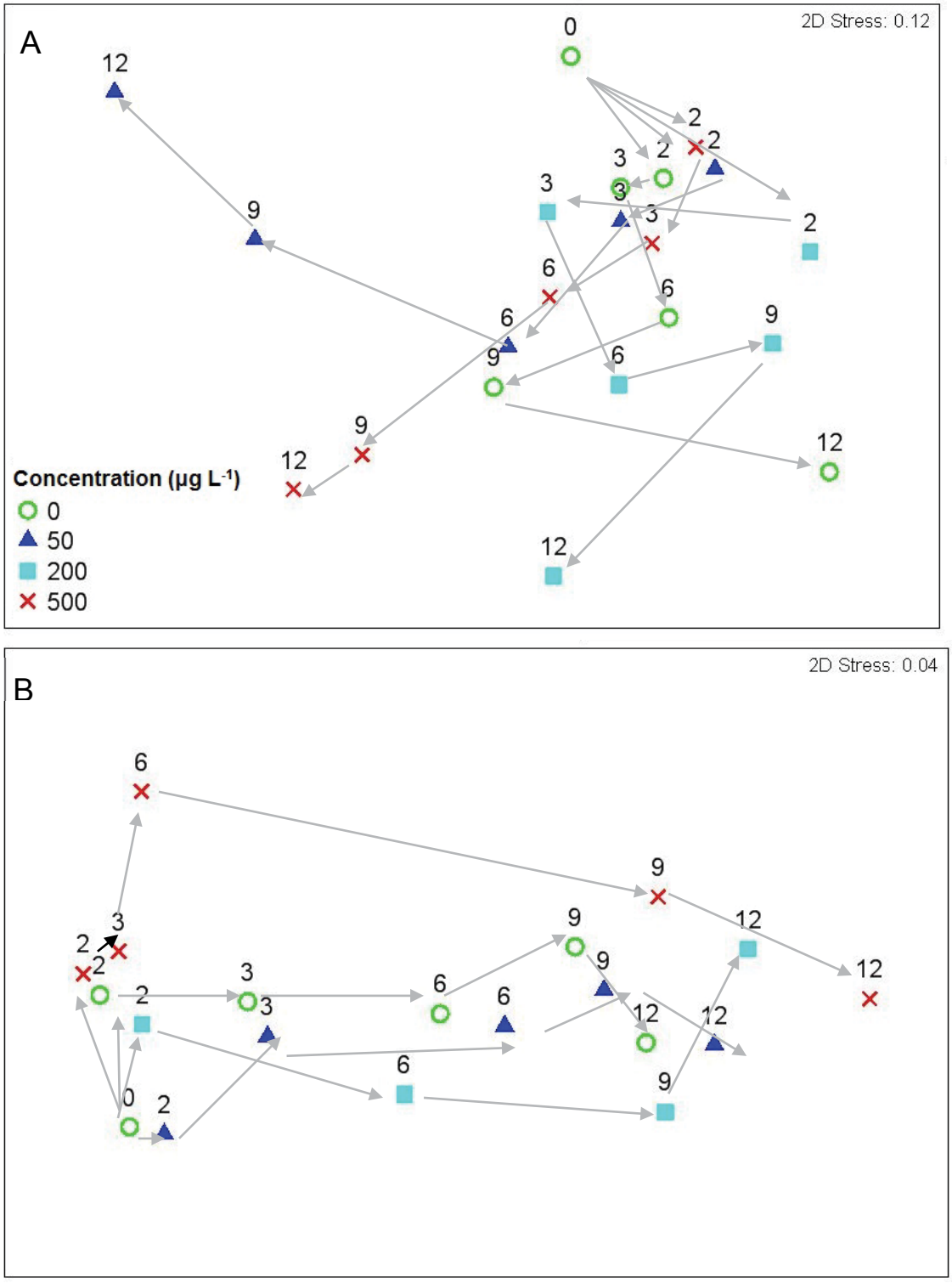


**Figure 5.1** nMDS of the community composition of healthy benthic diatoms at a) Alligator Creek and b) Barratta Creek for each atrazine concentration treatment (50, 200 & 500 µg $L^{-1}$) and the control treatment (no atrazine) over the 12 day exposure period. Numbers indicate the duration (days) since commencement of exposure.

### *5.4.3 Effect of atrazine toxicity, exposure duration and recovery of diatom taxa*

The effect of atrazine exposure varied between the diatom taxa and depended on exposure duration; these different types of responses are summarised in Table 5.1. Some diatoms were negatively affected by atrazine exposure whilst others showed no significant effects. Additionally, several taxa from each of the communities were able to recover at least partially from atrazine exposure by the end of the experiment (Table 5.1).

In the Alligator Creek diatom community the most sensitive taxon was *Gomphonema truncatum* (Figure 5.2H), which was significantly affected by atrazine exposure over the 12 day period ($p<0.05$, Supplementary Table S6). There was a significant successional effect within the control treatment in *Gomphonema truncatum* ($p<0.05$, Supplementary Table S6). The proportion of healthy cells of *Gomphonema truncatum* declined in atrazine treatments on days 2, 3 and 6 at 200 μg $L^{-1}$ and 500 μg $L^{-1}$ compared to that of the control treatment (Figure 5.2H). On day 9 the proportion of healthy cells declined at 50 μg $L^{-1}$ and 500 μg $L^{-1}$ and its was still negatively affected on day 12 at all atrazine concentrations (Figure 5.2H). There was no significant difference in the proportion of healthy *Cymbella aspera* cells in the control treatment over the course of the experiment (Figure 5.2B, Supplementary Table S6). *Cymbella aspera* was negatively affected by atrazine exposure on days 2 and 6 in the 500 μg $L^{-1}$ treatment and also affected at treatments above 200 μg $L^{-1}$ on day 9; however, on day 12 there was no longer a significant difference between the control and atrazine treatments. The proportion of healthy cells of *Ulnaria ulna* in the control treatment declined throughout the course of the experiment with a significant successional effect ($p<0.05$, Supplementary Table S6). *Ulnaria ulna* (Figure 5.2K) was significantly affected by atrazine on day 2 at all concentrations ($p<0.05$); however, the decline was not significant after day 3 ($p>0.05$, Supplementary Table S6). From day 6 the proportion of healthy cells was higher in the lowest concentration treatments (50 μg $L^{-1}$) compared to that of the control treatment; however, these effects were difficult to discern due to very low proportion of healthy cells in the control treatment (Figure 5.2K).

**Table 5.1** Responses of the diatom taxa to atrazine exposure at various exposure durations.

| Diatom Taxon | Response |
|---|---|
| **Alligator Creek:** | |
| *Gomphonema truncatum* | Negatively affected initially above 200 µg $L^{-1}$. Remaining affected at 12 days at all concentrations.<br>No recovery. |
| *Cymbella aspera*<br>*Epithemia* cf. *cistula*<br>*Gomphonema clevei*<br>*Ulnaria ulna* | Negatively affected initially above 200 µg $L^{-1}$for *E. cistula* and *G. clevei*, and at all concentrations for *U. ulna*. Remaining affected at 9 days for *C. aspera.*<br>Recovery in all treatments by day 12. |
| *Gomphonema gracile*<br>*Eunotia* cf. *minor* | Negative effects of atrazine on day 3 of exposure (500 µg $L^{-1}$), but subsequent recovery in all treatments. |
| *Adlafia* cf. *bryophila*<br>*Epithemia* cf. *adanata*<br>*Navicula cryptotenella*<br>*Nitzschia sigmoidea* | No significant effect. |
| **Barratta Creek:** | |
| *Ulnaria ulna* | Negatively affected initially at all concentrations. Lasting negative effects at high concentrations (500 µg $L^{-1}$).<br>Recovery in lowest concentration treatment (50 µg $L^{-1}$.) by day 9. |
| *Melosira varians*<br>*Pleurosira* sp. | Negatively affected by high concentration treatments (500 µg $L^{-1}$) on days 2 and 3 for *M. varians*, and on day 3 for *Pleurosira.*<br>Recovery in all atrazine treatments by day 12. |
| *Amphora* spp.<br>*Cymbella* sp.<br>*Gomphonema* spp.<br>*Gyrosigma* sp.<br>*Pinnularia* sp.<br>*Nitzschia* spp. | No significant effect. |
| *Cyclotella* sp.<br>*Mayamaea atomus*<br>*Navicula cryptocephala*<br>*Navicula schroeterii* | Negatively affected at 50 µg $L^{-1}$ on day 3 for *Cyclotella*, at 500 µg $L^{-1}$ on day 6 for *M. atomus* and *N. cryptocephala*, at 500 µg $L^{-1}$ on day 9 for *Cyclotella* and *N. schroeterii* and at 50 µg $L^{-1}$ on day 12 for *M. atomus.*<br>Recovery and positive affects above 200 µg $L^{-1}$ on day 12. |
| *Navicula subtillissima* | No negative effects.<br>Positive effects at various concentrations after day 9 of exposure. |

Many of the sensitive taxa that initially showed a decline in the proportion of healthy cells from atrazine exposure recovered fully by the end of the experiment (day 12). For *Epithemia* cf. *cistula* the control treatment showed a significant successional effect ($p<0.05$, Supplementary Table S6). The health of *Epithemia* cf. *cistula* was negatively affected by atrazine exposure on days 2 and 3 above 200 μg $L^{-1}$ and also on day 6 (200 μg $L^{-1}$), but showed no significant negative effects after 9 days of atrazine exposure (Figure 5.2D). The proportion of healthy cells of *Eunotia* cf. *minor* showed no successional effect in the control treatment ($p>0.05$, Supplementary table S6) and was affected by atrazine on day 3 (500 μg $L^{-1}$); however, no effects of atrazine were discernable at longer exposure durations due to high standard error of the treatments (Figure 5.2E). *Gomphonema clevei* showed a significant successional effect in the control treatment over the course of the experiment, with a drop in the health of control treatment on days 9 and 12 ($p<0.05$, Supplementary Table S3). The proportion of healthy *Gomphonema clevei* cells (Figure 5.2F) was significantly lower in atrazine treatments on day 2 (200 & 500 μg $L^{-1}$) and on day 3 (500 μg $L^{-1}$); however, the negative effects of atrazine were no longer evident after day 6 at all concentrations tested (Figure 5.2F, Supplementary Table S6). The proportion of healthy *Gomphonema gracile* cells declined significantly in the control treatment over the course of the experiment ($p<0.05$, Supplementary Table S6). In atrazine treatments, there was lower proportion of healthy cells of *Gomphonema gracile* on day 3 (500 μg $L^{-1}$); however, the negative effects of atrazine were no longer significant after day 6 and on day 9 atrazine treatments had higher proportions of healthy cells than that of the control treatment (Figure 5.2G).

The proportion of healthy cells of some diatom taxa within the Alligator Creek community was unaffected by atrazine over the duration of the experiment; these were: *Adlafia* cf. *bryophila*, *Navicula cryptotenella* and *Nitzschia sigmoidea* (Figures 5.2A, I and J). *Epithemia* cf. *adanata* (Figure 5.2C) was not significantly affected by atrazine exposure; however, the proportion of healthy cells in the control treatment was low throughout the experiment making it difficult to determine effects (Figure 5.2C).

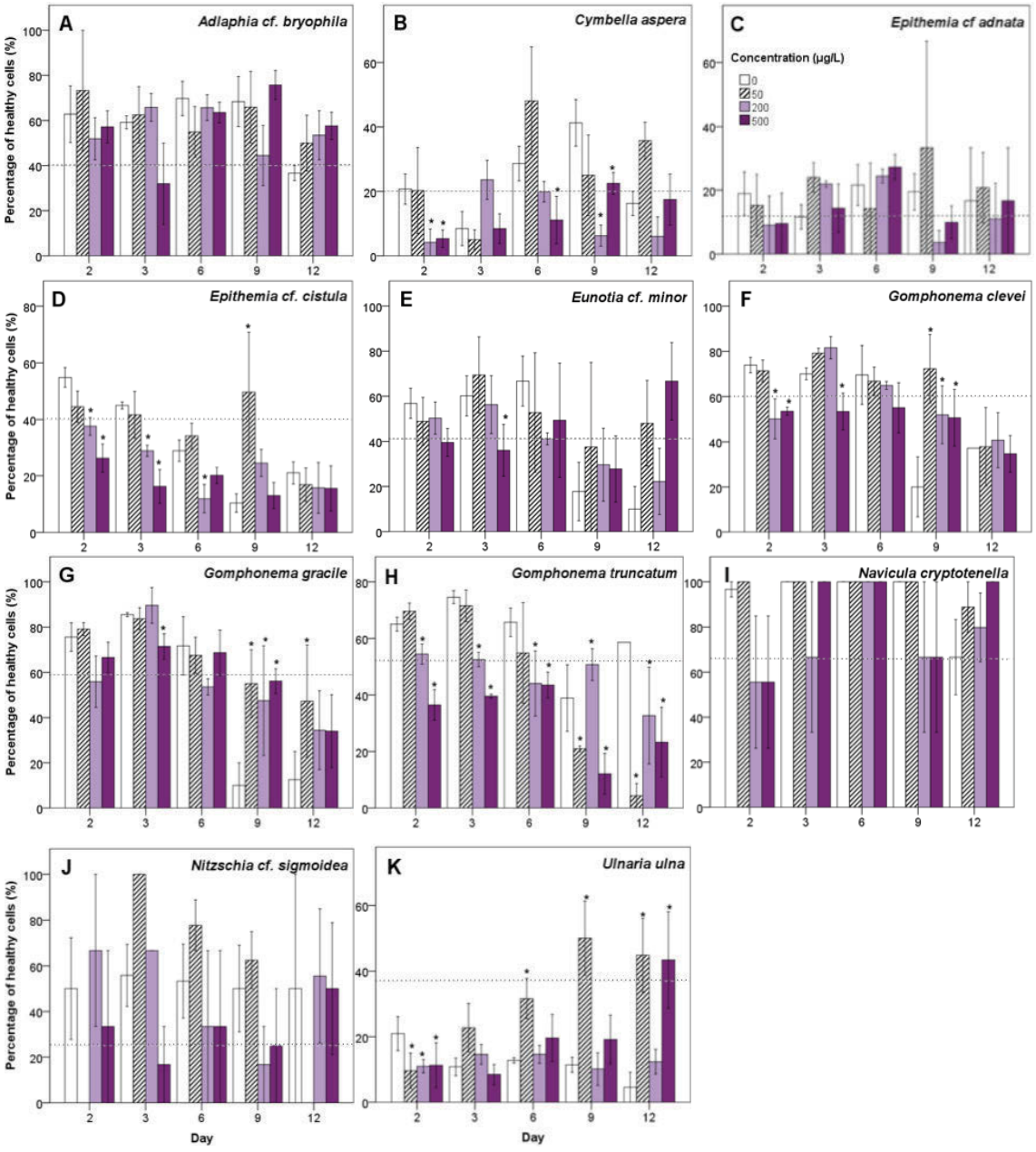


**Figure 5.2** Changes in the percentage of healthy cells (mean ± SE, n = 3) for the benthic diatom taxa from Alligator Creek over the 12 day experiment. * indicates statistically significant difference ($p<0.05$) from the control treatment for each time period based on GLM analysis on binary health data. Dotted horizontal line indicates background health of the control treatment at the start of the experiment (day 0)

Diatoms from Barratta Creek showed varied responses to atrazine exposure with most taxa showing potential for recovery at longer exposure durations (>6 days). Within the control treatment there was a successional effect in the proportion of healthy cells of *Ulnaria ulna* ($p<0.05$, Supplementary Table S6). On day 2 of exposure *Ulnaria ulna* (Figure 5.3O) had lower proportions of healthy cells all atrazine treatments (50, 200 and 500 μg $L^{-1}$) compared to that of the control treatment. However, at longer exposures on days 9 and day 12 the negative effects of atrazine exposure were only significant at the highest atrazine concentration (500 μg $L^{-1}$). The proportion of healthy cells of *Melosira varians* was consistent in the control treatment across the days ($p>0.05$, Supplementary Table S6). *Melosira varians* was affected at high concentrations of atrazine (500 μg $L^{-1}$) on days 2 and 3; however, there was no negative effect of atrazine in any treatment on day 9 and on day 12 there was increased proportions of healthy cells in the 200 μg $L^{-1}$ treatment (Figure 5.3H). *Pleurosira* sp. showed a significant successional effect in the control treatment over the course of the experiment ($p<0.05$, Supplementary Table S6). The proportion of healthy cells of *Pleurosira* sp. was negatively affected by atrazine exposure at 500 μg $L^{-1}$ on day 3, but had recovered by day 12 in all atrazine treatments (Figure 5.3N).

There was no significant effect of atrazine exposure on *Gomphonema* spp. (Figure 5.3E) or *Pinnularia* sp. (Figure 5.3M) over the course of the experiment and the proportion of healthy cells in the control treatment was also consistent ($p>0.05$, Supplementary Table S6). There were some taxa - *Amphora* spp. (Figure 5.3B), *Cymbella* sp. (Figure 5.3D), *Gyrosigma* sp. (Figure 5.3F) and Nitzschia spp. (Figure 5.3L) where although there was no detectable effect of the atrazine treatments, these taxa had very low proportions of healthy cells in the control treatment making it difficult to detect significant effects between treatments and the effect of the treatments on these taxa remains uncertain.

Other diatom taxa showed highly varied responses to atrazine exposure depending on exposure duration. The proportion of healthy cells of *Cyclotella* sp. increased in the control treatment over the course of the experiment ($p<0.05$, Supplementary Table S6). *Cyclotella* sp. showed negative effects of atrazine on day 3 at 50 μg $L^{-1}$ and on day 9 at 500 μg $L^{-1}$, however there were higher proportions of healthy cells compared to that of the control treatment in atrazine treatments on day 6 at 50 μg $L^{-1}$ and on day 12 at 500 μg $L^{-1}$ (Figure 5.3C). The proportion of healthy *Mayamaea atomus* cells varied in the

control treatment between the days ($p<0.05$, Supplementary Table S6) and was negatively affected by atrazine at 500 μg $L^{-1}$ on day 6; however, by day 12 it had recovered to control treatment levels at concentrations above 200 μg $L^{-1}$ (Figure 5.3G). *Navicula cryptocephala* did not show any successional effects in the control treatment ($p>0.05$, Supplementary Table S6). *Navicula cryptocephala* was negatively affected by atrazine on day 6 at 500 μg $L^{-1}$, but on day 12 the proportion of healthy cells was significantly higher at 500 μg $L^{-1}$ atrazine than that of the control treatment (Figure 5.3I). The proportion of healthy cells of *Navicula schroeterii* was consistent in the control treatment throughout the experiment ($p>0.05$, Supplementary Table S6). There were negative effects of atrazine exposure in *Navicula schroeterii* on day 9 at 500 μg $L^{-1}$; however, on day 12 there was a higher proportion of healthy cells at lower concentrations and no effect at 500 μg $L^{-1}$ (Figure 5.3J). *Navicula subtillissima* showed consistent proportions of healthy cells in the control treatment throughout the experiment ($p>0.05$, Supplementary Table S6). There were no negative effects of atrazine and the proportion of healthy cells was significantly higher at 500 μg $L^{-1}$ than that of the control treatment on day 12 (Figure 5.3K).

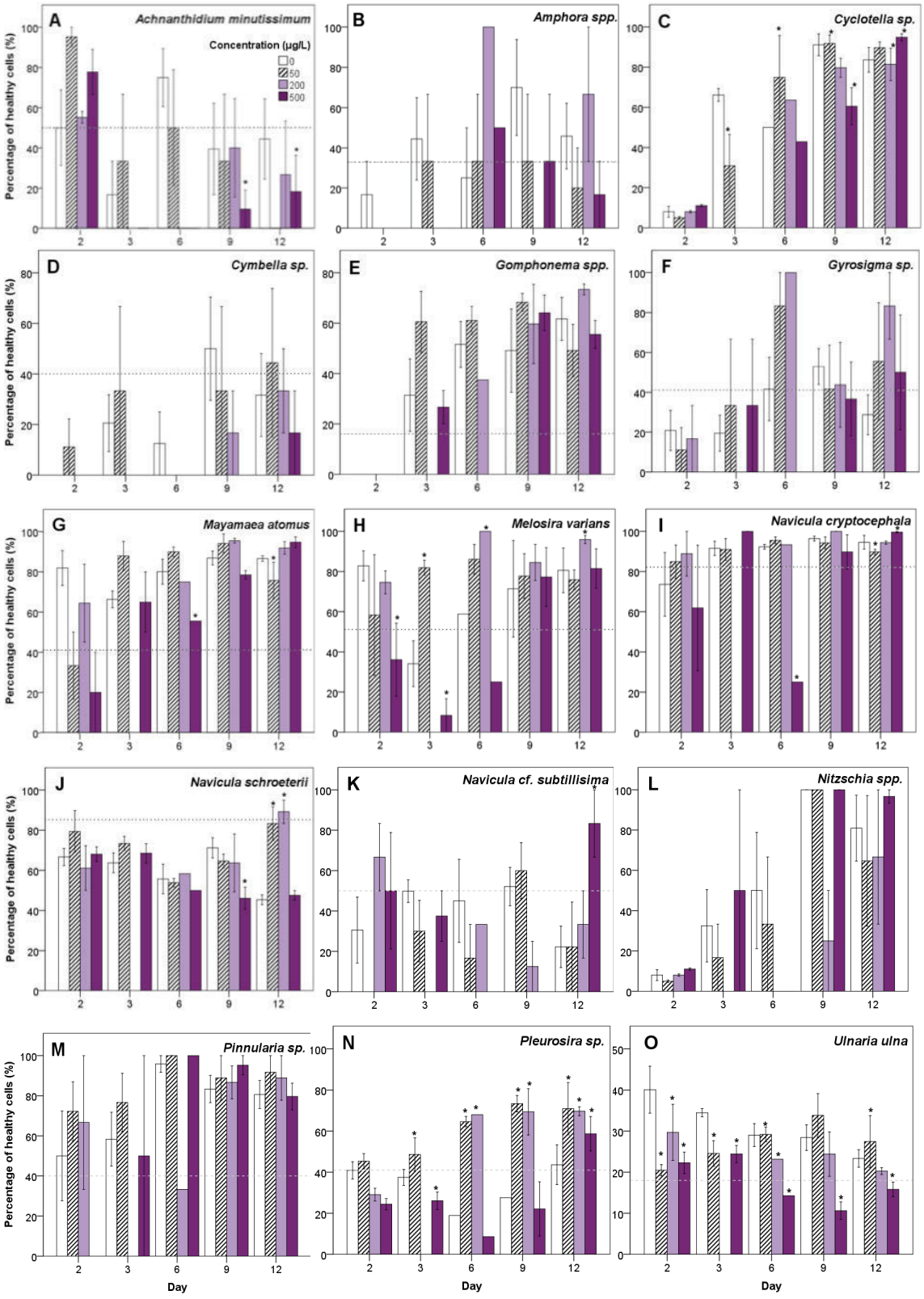


**Figure 5.3** Changes in the percentage of healthy cells (mean ± SE, n=3) for the benthic diatom taxa from Barratta Creek over the 12 day experiment. * indicates statistically significant difference ($p<0.05$) from the control treatment for each time period based on GLM analysis on binary health data. Dotted horizontal line indicates background health of the control treatment at the start of the experiment (day 0).

## 5.5 Discussion

### *5.5.1 Effects of atrazine on the diatom communities and the influence of prior pollution*

The response of the benthic diatom community to atrazine exposure over the 12 days differed between the two sites. The diatom community from the impacted Barratta Creek showed a strong effect of succession over the study period, whereas community composition was not strongly altered by atrazine exposure. The initial diatom community at Barratta Creek contained a higher percentage of herbicide tolerant taxa (50%), compared with the reference site Alligator Creek (33%) (Wood et al., 2016b). Diatom taxa previously classified as tolerant (Wood et al., 2016b) including *Navicula cryptocephala*, *Navicula schroeterii, Navicula subtillissima* and *Mayamaea atomus* were found only in the Barratta Creek diatom community (Supplementary Table S2). Concentrations of atrazine in the site water (13 μg $L^{-1}$) at the time of diatom collection indicate that the Barratta Creek benthic community is highly likely to have been pre-exposed to herbicides before commencement of the current study. Furthermore, Barratta Creek is highly polluted by PSII herbicides; mixtures of up to seven PSII inhibiting herbicides were frequently detected throughout 2011-2012, as well as herbicides with other modes of action (MCPA), and fungicides and insecticides (O'Brien et al., 2016; Smith et al., 2012).

Before exposure, the diatom community at Alligator Creek was dominated by *Gomphonema truncatum.* This species has been classified as sensitive to herbicides by Wood et al. (2016b and was not present at the herbicide polluted Barratta Creek (Supplementary Table S2). The results of the present study are consistent with this classification, showing a significant decline in the proportion of healthy cells with both acute and chronic atrazine exposure. *Gomphonema truncatum* is classified as a meso-eutrophic species, occupying fresh to brackish waters of pH >7, with high dissolved oxygen requirements and is tolerant of moderate to high nutrient pollution (Dela-Cruz et al., 2006; Van Dam et al., 1994). The decline of *Gomphonema truncatum* might be a potential indicator of herbicide toxicity in nutrient enriched rivers. In contrast, *Navicula cryptotenella* was not negatively affected by atrazine exposure at the concentrations tested and was dominant in all atrazine treatments by the end of the experiment. These

results are supported by previous studies that found *Navicula cryptotenella* to be tolerant to herbicide exposure (Ricart et al., 2009; Wood et al., 2016a; Wood et al., 2016b). *Navicula cryptotenella* is generally reported as being tolerant of organic pollution and is favoured in high nutrient conditions (Lange et al., 2011).

Under chronic exposure to toxicants, such as herbicides, more tolerant taxa are favoured and are able to persist (Roubeix et al., 2011b). This selection pressure allows tolerant taxa to outcompete the more sensitive taxa leading to subsequent restructuring of the community and increased community tolerance, known as pollution induced community tolerance (PICT) (Blanck, 2002; Dorigo et al., 2004). Increased herbicide tolerance in periphyton communities subjected to chronic herbicide exposure has been demonstrated in other studies (Magnusson et al., 2012; Schmitt-Jansen and Altenburger, 2005; Tlili et al., 2011). However, communities that have already undergone PICT may have lost the ability to adapt and further herbicide exposure may not induce community compositional changes (Andrus et al., 2015; Dalton et al., 2015; King et al., 2016; Tlili et al., 2008). Prior exposure history is an important determinant of response of the diatom community to herbicide exposure (Kim Tiam et al., 2014). Indeed, the diatom community collected from the polluted Barratta Creek showed a similar community structure between atrazine exposed and control treatments at the end of the 12 day experiment (Figure 5.1B). Our findings suggest that the community at the polluted Barratta Creek site had already undergone selection and restructuring so that individuals and species unable to persist under chronic atrazine exposure may have already been eliminated. This was in contrast to the community at the unpolluted Alligator Creek site, which demonstrated selection pressure with atrazine exposure resulting in the decline of species that are relatively more sensitive to atrazine, such as *Cymbella aspera* and *Gomphonema truncatum*.

### *5.5.2 Exposure duration and recovery of diatoms*

The effect of atrazine on the freshwater benthic diatoms in the present study varied between taxa from the two sites and with exposure duration (Table 1). Many of the taxa initially affected by atrazine showed the potential for recovery, at least partially over time. For these taxa, the exposure duration was important in determining their sensitivity relative to other taxa in the community. For example, in the Barratta Creek community, *Melosira varians* was able to recover to similar proportions of healthy cells

as that of the control treatment within 9 days of atrazine exposure. Other studies on periphyton communities (Gustavson et al., 2003; Lawrence et al., 2015) and single benthic diatom species (Coquille et al., 2015) have also found that herbicide toxicity responses were dependent on exposure duration. However, the present study highlights how these effects vary in different benthic diatom taxa within field collected periphyton communities. The ability to recover is potentially an important factor shaping the response of benthic diatoms to chronic herbicide exposure and may be a trait worthy of consideration in the development of diatom indices of herbicide pollution (Schäfer et al., 2011a). More studies are needed to establish how the diatom taxa vary in their ability to recover when exposed to herbicides to better understand community responses to toxicant exposure in the field.

Some diatom taxa showed positive effects in atrazine treatments compared to that of the control treatment at various times through the experiment (Table 2). This mostly applied to diatoms such as *Cyclotella* sp., *Melosira varians, Pleurosira sp., Navicula schroeterii* and *Navicula* cf. *subtillissima* from the polluted Barratta Creek site. Clearly these opportunistic taxa are able to thrive despite exposure to high concentrations of atrazine. It is possible that with the decline of more sensitive taxa within the community these taxa are able to benefit from a lack of competition and are advantaged when exposed to herbicides compared with the control treatment. Mechanisms for recovery and tolerance in benthic diatoms are not well understood. Some studies have noted hormesis in response to herbicide exposure (Proia et al., 2011; Roubeix et al., 2011b; Tlili et al., 2008), otherwise known as the "greening effect"; when algae are able to increase their concentrations of light harvesting pigments in response to exposure to sub-lethal doses of herbicides in order to compensate for the inhibition of photosynthesis (Cedergreen et al., 2007; Ricart et al., 2009). Coquillé et al. (2015) showed that chlorophyll fluorescence in the diatom *Gomphonema gracile* was stimulated after exposure to metolachlor at low concentrations (<10 µg $L^{-1}$); however, this was inhibited at higher concentrations. Our results showed that *Gomphonema gracile* was able to recover from atrazine effects on day 3 (500 µg $L^{-1}$), without any significant negative effects after 6 days of continual atrazine exposure. Although our study did not measure fluorescence, rather the presence of healthy cells containing chloroplasts, it is possible that the increased proportions of healthy cells seen in herbicide exposed treatments could be

linked to the greening effect in certain taxa (Proia et al., 2011; Roubeix et al., 2011b; Tlili et al., 2008).

The present study assessed the response of benthic diatoms within natural communities to atrazine over 12 day continuous exposures. Toxicity response was measured as the proportion of healthy cells on a per taxon basis. Healthy cells are live and functional at the time of preservation, whereas unhealthy cells were obviously dead (did not contain chloroplasts or were broken) or did not have intact chloroplasts and were therefore unlikely to be viable cells capable of recovery or reproduction. The measure used is similar to other studies that assess diatom population dynamics using live/dead cell ratios (Coquille et al., 2015; Tiam et al., 2015). The use of natural field-derived communities for lab-based experiments has presented some limitations in the present study. Some diatom taxa in this study had very low levels of health in controls, for eg., *Amphora* spp., reflecting the natural succession of the diatom community from which they were derived. Diatom health in the control treatment also varied over the 12 day experiment in some taxa, making it difficult to discern any effect of atrazine exposure. We consider the sensitivity data produced in the current study to be a direct measure of species herbicide sensitivity traits. Traits data such as this can be used to assess the biological condition of rivers (Stevenson, 2014), or as an indication of particular stressor impacts, i.e. herbicide pollution (Morin et al., 2015; Rimet and Bouchez, 2011).

## 5.6 Conclusions

Our results indicate that the response of benthic diatom communities to atrazine exposure varied with exposure duration and concentration and was influenced by the prior exposure history of the site and their species assemblages. The reference community (from Alligator Creek) displayed a shift in diatom community composition towards more tolerant taxa when exposed to atrazine. However, in the polluted Barratta Creek community, atrazine exposure was not a strong factor in driving community compositional change over the course of the experiment, suggesting that its prior exposure to herbicides has already restructured the community through the development of PICT. The effect of chronic atrazine exposure on several of the benthic diatom taxa in this study varied with exposure duration. The current study identifies diatom taxa that were capable of recovery during prolonged atrazine exposure despite their initial toxicity response.

## Supplementary Material

The supplementary material for Chapter 5 is available in Appendix D

# Chapter 6 Benthic diatoms as indicators of herbicide toxicity in rivers - a new SPEcies At Risk ($SPEAR_{herbicides}$) index

*Finch Hatton Creek, QLD, Australia.*

## 6.1 Abstract

Benthic diatom communities are used widely as indicators of river health due to their rapid response to changes in water quality. The ability for diatom based indices to detect eutrophication has been well established; however, an index designed specifically to detect herbicide impacts is yet to be established. Herbicide contamination of rivers is common in agricultural regions and poses a threat to aquatic ecosystems. This study developed a new biomonitoring index ($SPEAR_{herbicides}$) that uses benthic diatom communities to detect the toxic impacts of herbicide pollution in rivers. The effect of diffuse agricultural runoff on benthic diatoms within 14 rivers in the Great Barrier Reef catchment was assessed including herbicides, nutrients, total suspended solids and salinity. The $SPEAR_{herbicides}$ index showed that the proportion of herbicide sensitive taxa within the communities declined with increasing herbicide toxicity of the sites. The impacts of herbicide toxicity on the diatom community were only apparent after the wet season. $SPEAR_{herbicides}$ was also strongly correlated with nutrients (FRP, ammonia, NOx) as well as TSS and EC. Further research is necessary to elucidate the effects of herbicides on benthic diatom communities within a multiple stressor environment.

## 6.2 Introduction

Benthic diatoms are important biological components of freshwater ecosystems and can be used to assess the ecological health of rivers (Kelly et al., 1998; Van Dam et al., 1994). The ability to use changes in benthic diatom communities as indicators of declining water quality and anthropogenic impacts has been well established; for eutrophication (Bellinger et al., 2006), urbanisation (Newall and Walsh, 2005) and inorganic pollution (Dela-Cruz et al., 2006). These indices utilise the differing sensitivities of the diatom taxa (usually to nutrients) to indicate trophic impacts in rivers (Rimet 2012). However, previous studies have found that diatom indices of trophic pollution in rivers were not suitable for detecting the impacts of herbicide toxicity (Blanco and Bécares, 2010; Morin et al., 2009). Therefore, there is a need for a biomonitoring index that is designed to detect the impacts of herbicides in rivers (Larras et al., 2017).

Herbicide pollution in rivers is an issue of concern worldwide, especially in agricultural regions. Herbicides can have impacts on aquatic phototrophic organisms and their ability to inhibit photosynthesis and growth in benthic diatoms has been demonstrated (Debenest et al., 2009; Tlili et al., 2011). Exposure of benthic diatom communities to herbicides can result in the loss of sensitive species, thus altering species composition (Magnusson et al., 2012; Schmitt-Jansen and Altenburger, 2005). Although most agricultural herbicides are not designed to affect invertebrates, fish and other aquatic biota, they may be altered indirectly as a result of impacts on primary producers (Rohr and Crumrine, 2005). As benthic diatoms are often the dominant primary producers in shallow streams and rivers (Chessman et al., 2009) and are sensitive to herbicide exposure (Wood et al., 2016a; Wood et al., 2016b; Wood et al., 2017), they have the potential to be used as biomonitors of herbicide pollution.

A traits-based approach, such as the SPEcies At Risk (SPEAR) index (Liess and Ohe, 2005), has the potential to be utilised to separate the effects of herbicides on diatom communities from other stressors. SPEAR has been successfully used with stream macroinvertebrates affected by stressors such as salinity (Schäfer et al., 2011a) and pesticides (Beketov et al., 2013; Schäfer et al., 2011b). SPEAR indicates the fraction of sensitive taxa in a community based on traits that make them at risk from the stressor of interest, such as their physiological sensitivity to the stressor as determined by toxicity

tests. Changes in the proportion of sensitive taxa (SPEAR taxa) in the community can then be linked to the impacts of that stressor in the field. The SPEAR approach has the potential to be adapted to utilise diatoms to detect herbicide impacts.

This study investigates the effect on herbicide toxicity on benthic diatom communities within rivers of the GBR catchment area. A novel diatom based $SPEAR_{herbicides}$ index is developed that utilises benthic diatoms to assess herbicide impacts in rivers. We have classified the diatom species as either SPEcies At Risk (SPEAR) from herbicide toxicity or not at risk (notSPEAR). The new $SPEAR_{herbicides}$ index was then tested in 14 river sites that flow into the Great Barrier Reef (GBR) in Australia with sampling after the dry and wet seasons for two successive years. These sampling sites were located in river reaches that are impacted by varying levels of agricultural intensity, from upstream catchments devoted to conservation with no agriculture or grazing to high intensity of crops such as sugarcane. We predict that any effect of herbicides on the diatom community would be more evident immediately after the wet season (i.e. May 2012 and April 2013) than before the wet season.

## 6.3 Methods

### *6.3.1 Study sites and study design*

Benthic diatom communities were collected from 14 sites within the Great Barrier Reef (GBR) catchment area (Figure 1); see Supplementary Table S1 for latitudes and longitudes of the sites. These rivers are located across coastal catchment areas in freshwater reaches (above tidal influence) that drain directly into the GBR Marine Park Area. Ten sites, marked by red circles in Figure 1, are considered contaminated by herbicides to various degrees and are part of the Reef Water Quality Protection Plan (RWQPP) monitoring programme, which measures pesticide, nutrient, suspended solids attributes and discharge of the rivers (Smith et al., 2012). The remaining four sites, marked by green triangles in Figure 1, have no agricultural or urban areas present upstream, but have nature conservation and recreational activities and are thus extremely unlikely to have any significant pesticide contamination. These four sites are included as reference sites.


**Figure 6.1** Map of the Great Barrier Reef catchment region and study sites. Green triangles are the reference sites and red circles are the monitored sites. Catchment regions marked in different colours.

The climate of the study region is characterised by the summer (monsoonal) wet season, typically December – March where most of the annual rainfall occurs and this rain is often intense leading to elevated discharges in the region's rivers (Waterhouse et al., 2012). The wet season coincides with peak concentrations of herbicides in most rivers of the region (Lewis et al., 2009). Our sampling regime was designed to accord with two successive wet seasons (2011/2012 and 2012/2013). Sites were sampled immediately before and immediately after these wet seasons i.e. in November 2011, May 2012, September 2012 and April 2013.

### *6.3.2 Diatom sampling, preservation and identification*

At each study site and sampling occasion natural diatom communities were collected. Attached benthic diatoms were removed by scrubbing collected substrates with a toothbrush and scraping with a sharpened knife. Various submerged substrata from the edge habitat within a 20 m length of river were sampled. Submerged pebbles and cobbles were the preferred substrates. If rocky substrates were unavailable then leaves and other submerged objects such as branches were used. The detached benthic material from various substrates were combined into one composite sample per site in a 20 mL vial and preserved in 70% ethanol (Chessman et al., 1999a).

The preserved diatom samples were stored for later identification by Dr. Jennie Fluin from the University of Adelaide, to species level where possible. Diatom samples were cleaned and mounted on permanent slides for identification using an Olympus BH-2 (Olympus) light microscope at 1000x magnification. Ten transects of the mounted slide were counted and up to 600 diatom cells were identified per sample.

### *6.3.3 Environmental data*

Water temperature (˚C), dissolved oxygen (DO), electrical conductivity (EC) and pH were recorded *in situ* at the same time and location as the diatom sample collection (Table 6.1). Herbicide, nutrient, discharge and total suspended solids (TSS) data across the 10 monitored sites were provided by the Queensland Department of Science, Information Technology and Innovation (DSITI) from water quality monitoring data collected as part of the RWQPP. The selected environmental variables used in this study are presented in Table 6.1. Discharge data were available for two of the reference sites (Bluewater Creek and Finch Hatton Creek), which are within 12 km downstream of the

diatom collection sites. Herbicide and nutrient data were not available at the four reference sites. Herbicide concentrations were assumed to be negligible at all reference sites based on the absence of agricultural land uses upstream of these sites and prior sampling did not detect herbicides at these sites; Bulgun Creek (Lewis et al., 2009), Finch Hatton Creek (Lewis et al., 2009; Mitchell et al., 2005). Likewise anthropogenic nutrient sources and land use impacts affecting these variables were considered negligible in the catchments of the reference sites. Therefore, missing data for these reference sites were assumed to be half the minimum values of the monitored sites.

Water quality data from the RWQPP were collected using both manual grab sampling techniques and automated samplers. Sampling occurred every few hours to daily intervals during high flow events and at a reduced frequency (usually monthly) during low or base-flow conditions. Nutrient concentrations were analysed using Flow Injection Analysis (colourimetric techniques) at the Science Division Chemistry Centre (Dutton Park, Queensland), a National Association of Testing Authorities, Australia (NATA) accredited laboratory. Herbicide concentrations in the water samples were analysed using solid phase extraction followed by liquid chromatography-mass spectrometry (LC-MS) at Queensland Health Forensic and Scientific Services (Coopers Plains, Queensland), also a NATA accredited laboratory.

In order to summarize the environmental data collected at each site, the mean concentrations of nutrients (oxidised nitrogen (NOx), ammonia, filterable reactive phosphorus (FRP)), discharge and TSS were calculated over the 60 days prior to the diatom sampling using the RWQPP data. Data was also available to calculate averages over longer time periods (6 months and 12 months), however these variables were highly correlated with the 60 day values so have not been included in the results. Analysis using these time frames did not change the conclusions of this study.

**Table 6.1** Summary of environmental variables, the limits of reporting and the abbreviation used in analysis.

| Variable | LOR | Abbreviation | Source of data |
|---|---|---|---|
| *Herbicides (μg $L^{-1}$) including:* | | | |
| Atrazine | 0.01 | ATR | RWQPP |
| Ametryn | 0.01 | AME | RWQPP |
| Diuron | 0.01 | DIU | RWQPP |
| Hexazinone | 0.01 | HEX | RWQPP |
| Prometryn | 0.01 | PRO | RWQPP |
| Simazine | 0.01 | SIM | RWQPP |
| Tebuthiuron | 0.01 | TEB | RWQPP |
| *Nutrients (mg $L^{-1}$)* | | | |
| Ammonia as N | 0.002 | Ammonia | RWQPP |
| Oxidised Nitrogen | 0.001 | NOx | RWQPP |
| Filterable Reactive Phosphorus | 0.001 | FRP | RWQPP |
| *Other Water Quality* | | | |
| Discharge ($m^3$ $s^{-1}$) | - | Discharge | RWQPP |
| Total Suspended Solids (mg $L^{-1}$) | 1 | TSS | RWQPP |
| Electrical Conductivity (μS $cm^{-1}$ @ 25˚C) | - | EC | RWQPP & *in situ* |
| Water Temperature (°C) | - | Temp | *in situ* |
| pH | - | pH | *in situ* |
| Dissolved Oxygen (mg $L^{-1}$) | - | DO | *in situ* |

### *6.3.4 Calculation of $SPEAR_{herbicides}$ index*

The SPEAR index is a measure of the relative abundance of sensitive taxa in the community. The $SPEAR_{herbicides}$ index is calculated as per the invertebrate SPEAR index described in Schäfer et al. (2011a):

$$SPEAR_{herbicides} = \frac{\sum_{i=1}^{n} \log (x_i+1)\, y}{\sum_{i=1}^{n} \log (x_i+1)}$$

Where $n$ is the number of diatom taxa in a sample, $x_i$ is the abundance of taxon $i$ and $y$ is 1 if the taxon is classified as a SPEcies At Risk (SPEAR), otherwise $y$ is 0.

The SPEAR value can then be converted to a percentage to indicate the proportion of sensitive taxa in the community.

### *6.3.5 Diatom sensitivity data*

We compiled a list of freshwater benthic diatom taxa and their sensitivities to herbicides based on their responses in studies from the scientific literature. The literature search was conducted on the 12th of October 2016 using the 'Web of Science' search of all databases for the term "freshwater diatom herbicide sensitivity". The search returned 40 journal articles from which the sensitivity data were derived. Articles were excluded if they 1) had been performed at the community level with no species sensitivity data, 2) did not contain data for at least three freshwater benthic diatom taxa, 3) were reviews of the literature or 4) field based studies with no herbicide exposure treatment. We also excluded publications by the current authors (Wood et al., 2014; Wood et al., 2016a; Wood et al., 2016b; Wood et al., 2017) as these studies will be used to derive further sensitivity data in this study. From the remaining nine studies, classifications of sensitivity for 28 freshwater benthic diatom taxa were obtained (Supplementary Table S2). Taxa were classified as sensitive or tolerant based on their relative sensitivities to herbicide exposure compared with the other taxa in the study. In this list there were two taxa (*Eolimna minima* and *Nitzschia palea*) for which different studies gave conflicting sensitivity assessments; in both cases there was one study in disagreement with at least two others, so we used the sensitivity classification identified by the majority of studies.

Further sensitivity data for 31 local freshwater diatom taxa were collected from the rapid toxicity tests performed by Wood et al. (2014; 2016a and b; in press) (Supplementary Table S3).

### *6.3.6 Classification of SPEAR taxa*

A total of 289 taxa was found in the samples collected from the 16 sites (Supplementary Table S3). Each taxon was classified as either SPEAR or notSPEAR based on its sensitivity to herbicides. For 39 taxa, sensitivity data were available for the taxon in question (Supplementary Table S2 &S3). The sensitivities of the remaining taxa were extrapolated from the sensitivity data of related taxa in the database. Data were first extrapolated at the Genus level, then at the Order level. If there were several species within a Genus with differing sensitivity classifications then the dominant classification was applied to other species within that Genus. Conflicting classifications at the Order level did not occur. Where there was no sensitivity data available at the Order level, they were excluded from the index (this applied to 6% of taxa, which collectively accounted for 2.7% of individuals observed). The complete taxon list and SPEAR classification is given in Supplementary Table S4.

In the case of one taxon, *Gomphonema parvulum* there was conflicting sensitivity data. Based on acute (≤ 96 h) exposure, Larras et al. (2012) reported *G. parvulum* as tolerant, whereas Wood et al. (2016b) reported *G.* spp. (including *G. parvulum* and *G. minutum*) as sensitive. However, in a recent study by Wood et al. (in press), *G.* spp. (including *G. parvulum* and *G. minutum*) was shown to be more tolerant to chronic exposures (12 d) of herbicides and had high potential for recovery from exposure; therefore, we have classified both these taxa as tolerant in this study.

### *6.3.7 Calculating the PSII mixture toxicity*

The effects of herbicides with similar modes of action in a mixture has been shown to be additive (Magnusson et al., 2010). In order to estimate the effects of herbicide mixtures in a given sample the toxic equivalency quotient (TEQ) was calculated. The concentrations of PSII inhibitors in a sample can be added together after each component has a toxic equivalency factor (TEF) applied, using the equation derived by Safe (1998):

$$TEQ = \sum C_i \times TEF_i$$

Where $C_i$ = the concentration of the individual herbicides and $TEF_i$ = the toxic equivalency factor of the individual herbicides.

The TEF for each herbicide was derived from Ma et al. (2006) for the freshwater microalgal species, *Scenedesmus obliquus* ($TEQ_{SO}$). The TEQso values (toxic equivalent quotient for *S. obliquus*) were calculated for each site following the method in Smith et al. (2012), using the RWQPP data for the herbicides - atrazine, diuron, ametryn, simazine and prometryn. The 95th percentile was then calculated for $TEQ_{SO,}$ across three time frames preceding the diatom samplings (i.e. 60 days, 6 months and 12 months prior).

### *6.3.8 Statistical analysis*

The data set was divided into two groups - end of the dry season (i.e. the November 2011 and September 2012 sampling occasions) and after the wet season (i.e. the May 2012 and April 2013 sampling occasions), hereafter referred to as the dry and wet season sampling. For each data set (Dry and Wet) linear regression analysis was conducted to model the relationship between $SPEAR_{herbicides}$ and $TEQ_{SO}$ (60 days). Linear regression was also performed to assess the relationship of $SPEAR_{herbicides}$ to the individual environmental variables. Analysis of covariance (ANCOVA) was performed to test the significance of the regression slope, as well as to determine whether this was consistent between the two sampling years.

Canonical correspondence analysis (CCA) with automatic stepwise model building by permutation tests was performed to construct a model that best explains the relationship of the benthic diatom community to the measured environmental variables. Variable selection was performed on the combined data sets (Dry and Wet data) using all available predictors; DO (*in situ*), pH (*in situ*), EC (*in situ*), Temperature (*in situ*), $TEQ_{SO}$ (60 days), Discharge (60 days), TSS (60 days), Ammonia (60 days), NOx (60 days) and FRP (60 days). All environmental variables were checked for normality and homoscedasticity and were log transformed where appropriate prior to analysis. Variables that were muticolinear were excluded from the analysis (VIF > 10). The CCA included only diatom taxa with greater than 5% relative abundance and occurred in at least two samples. The diatom relative abundance data were root transformed prior to analysis to down-weight the common taxa. CCA, Regression analysis and ANCOVA were performed using the statistical software R, using packages - stats (R Core Team., 2017) and vegan (Jari Oksanen et al., 2017).

## 6.4 Results

### *6.4.1 SPEAR index and herbicide toxicity of the sites*

The calculated herbicide mixture toxicity (60 day 95$^{th}$ percentile $TEQ_{SO}$) of the monitored sites reached a maximum of 209 μg $L^{-1}$ atrazine equivalent concentrations in the dry season samples and a maximum of 43 μg $L^{-1}$ in the wet season samples. The maximum concentrations occurred at Barratta Creek in the dry season and Sandy Creek in the wet season. The proportion of herbicide sensitive taxa within the diatom communities declined with increasing herbicide toxicity of the sites in the samples collected after the wet season rainfall (Table 6.2, Figure 6.2). $SPEAR_{herbicides}$ was significantly negatively correlated with herbicide toxicity of the sites after the wet season across all the three time scales (Table 6.2, Figure 6.2). However, there was no significant relationship between $SPEAR_{herbicides}$ and the $TEQ_{SO}$ of the sites after the dry season over any of the time frames (Table 6.2, Figure 6.2). There were no differences in $SPEAR_{herbicides}$ between the two sampling years (Table 6.2).

**Table 6.2** Results of ANCOVA for relationship of $SPEAR_{herbicides}$ to calculated mixture toxicity, expressed as log $TEQ_{SO}$ 95$^{th}$ percentile, after the dry and wet seasons over two sampling years. Bold type indicates statistical significance ($p<0.05$).

| | | | | Regression coefficient p value | |
|---|---|---|---|---|---|
| **Season** | **Model $R^2$** | **Model p value** | **F** | **$TEQ_{SO}$** | **Year** |
| Dry | -0.094 | 0.905 | 0.100 | 0.697 | 0.823 |
| Wet | 0.209 | **0.023** | 4.43 | **0.012** | 0.180 |

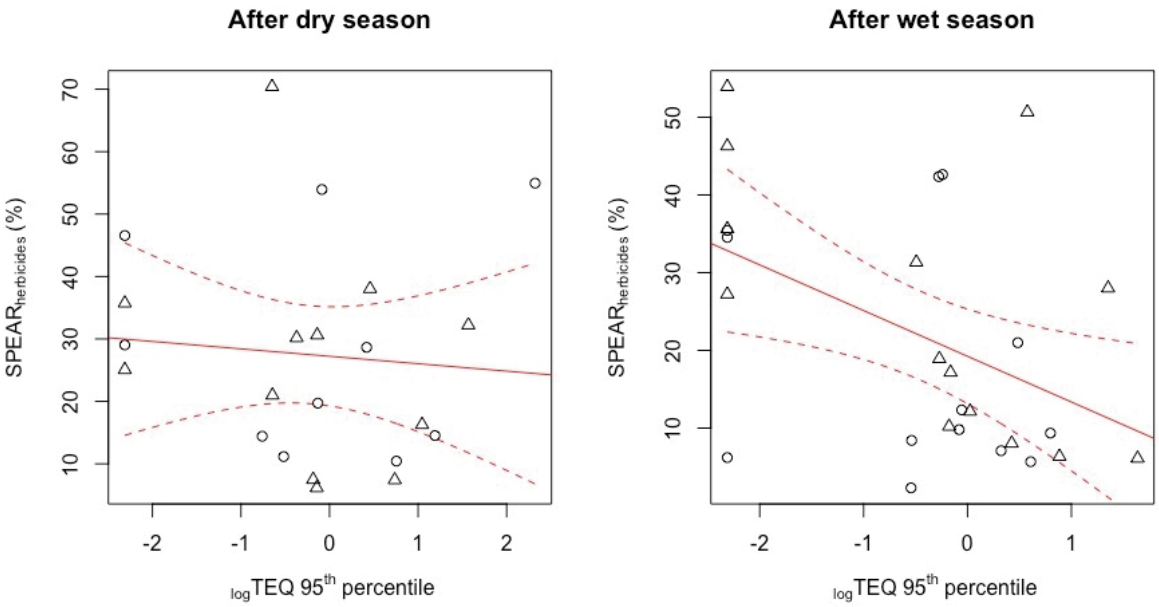


**Figure 6.2** Diatom community characterised as $SPEAR_{herbicides}$ (%) against calculated mixture toxicity expressed as log $TEQ_{SO}$ 95$^{th}$ percentiles calculated over 60 days prior to sampling the dry season and wet season rainfall events. Circles = samples relevant for the 2011/12 wet season, triangles = 2012/13 wet season.

### *6.4 Influence of environmental variables on the diatom community*

The CCA model constructed from the measured environmental variables that best described diatom community composition of the sites is shown in Figure 6.3. All measured environmental variables were considered in the model. The predictors chosen in the optimal model were TEQ, Ammonia, FRP, TSS, Discharge and Temperature (Figure 2). The measured environmental variables explained 23% of the variance of species distributions (total inertia = 6.9). The CCA axes represent 53% and 29% of the variance, respectively. The reference sites were not closely related to gradients of TEQ, Ammonia, FRP, TSS, Discharge and Temperature (Figure 6.3A). The diatom species most related to the highest herbicide contaminated sites were - *Epithemia sorex* (ESOR), *Diadesmis confervacea* (DCOF), *Luticola goeppertiana* (LGOE), *Melosira* spp. (MELO), *Nitzschia perminuta* (NIPM), *Stauroneis anceps* (STAN) and *Tabularia fasciculata* (TFAS) (Figure 6.3B).

The $SPEAR_{herbicides}$ index was not significantly correlated ($p > 0.05$) with discharge, pH or temperature in either dry or wet seasons (Table 6.3). $SPEAR_{herbicides}$ showed a significant positive correlation with DO after the wet season (Table 6.3, Figure 6.4A). $SPEAR_{herbicides}$ was negatively correlated with ammonia (Figure 6.4B), NOx (Figure 6.4C), EC (Figure 6.4D) and FRP (Figure 6.4E) after the wet season but not after the dry season (Table 6.3). There was also a statistically significant negative correlation between $SPEAR_{herbicides}$ and TSS for both dry and wet seasons (Table 6.3, Figure 6.4F). These results were consistent in both sampling years for all variables ($p<0.05$).

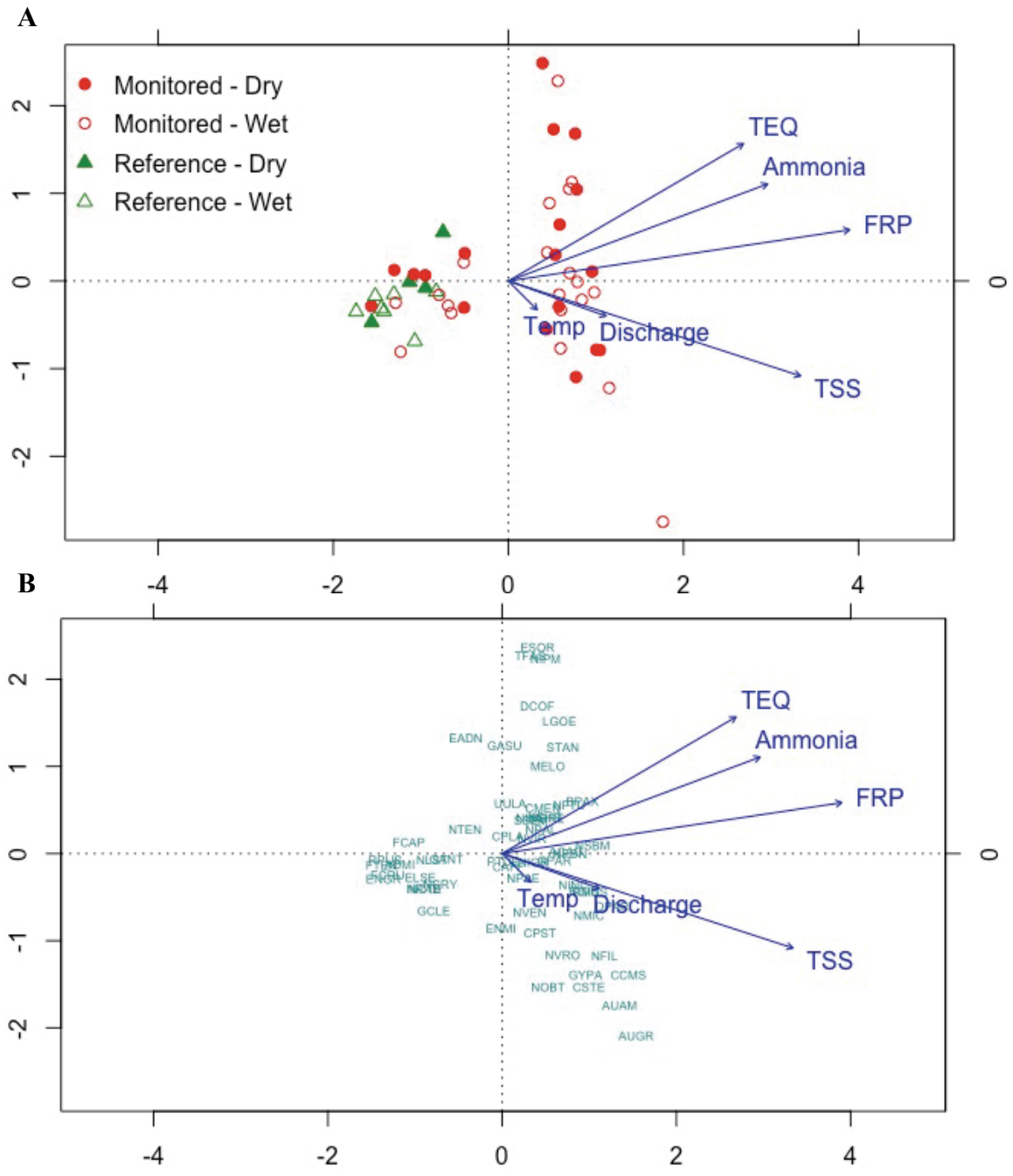


**Figure 6.3** Canonical correspondence analysis (CCA) plots of diatom community composition with points corresponding to a) site or b) diatom species. Blue arrows represent the relationship of the environmental variables with the diatom community. Diatom species names corresponding to the unique four letter diatom codes are listed in Supplementary Table S4.

**Table 6.3** Results of ANCOVA analysis for relationship of SPEAR to the environmental variables for dry and wet season data. – indicates that the parameter was incalculable.

| Variable | Season | $R^2$ | F | p |
|---|---|---|---|---|
| Discharge ($m^3$ $s^{-1}$) | Dry | -0.097 | 0.069 | 0.763 |
| | Wet | 0.125 | 2.85 | 0.050 |
| pH | Dry | -0.101 | 0.035 | 0.872 |
| | Wet | 0.0768 | 1.952 | 0.123 |
| Temp (°C) | Dry | -0.083 | 0.198 | 0.561 |
| | Wet | -0.008 | 0.901 | 0.466 |
| DO ($mg\ L^{-1}$) | Dry | -0.095 | 0.094 | 0.709 |
| | Wet | 0.247 | 5.266 | **0.007** |
| Ammonia ($mg\ L^{-1}$) | Dry | -0.077 | 0.252 | 0.506 |
| | Wet | 0.294 | 6.4 | **0.003** |
| NOx ($mg\ L^{-1}$) | Dry | -0.085 | 0.180 | 0.581 |
| | Wet | 0.147 | 3.242 | **0.035** |
| EC ($\mu S\ cm^{-1}$ @ 25°C) | Dry | 0.085 | 1.979 | 0.063 |
| | Wet | 0.383 | 9.067 | **0.001** |
| FRP ($mg\ L^{-1}$) | Dry | 0.022 | 1.237 | 0.136 |
| | Wet | 0.447 | 11.51 | **<0.001** |
| TSS ($mg\ L^{-1}$) | Dry | 0.107 | 2.264 | **0.048** |
| | Wet | 0.219 | 4.643 | **0.011** |

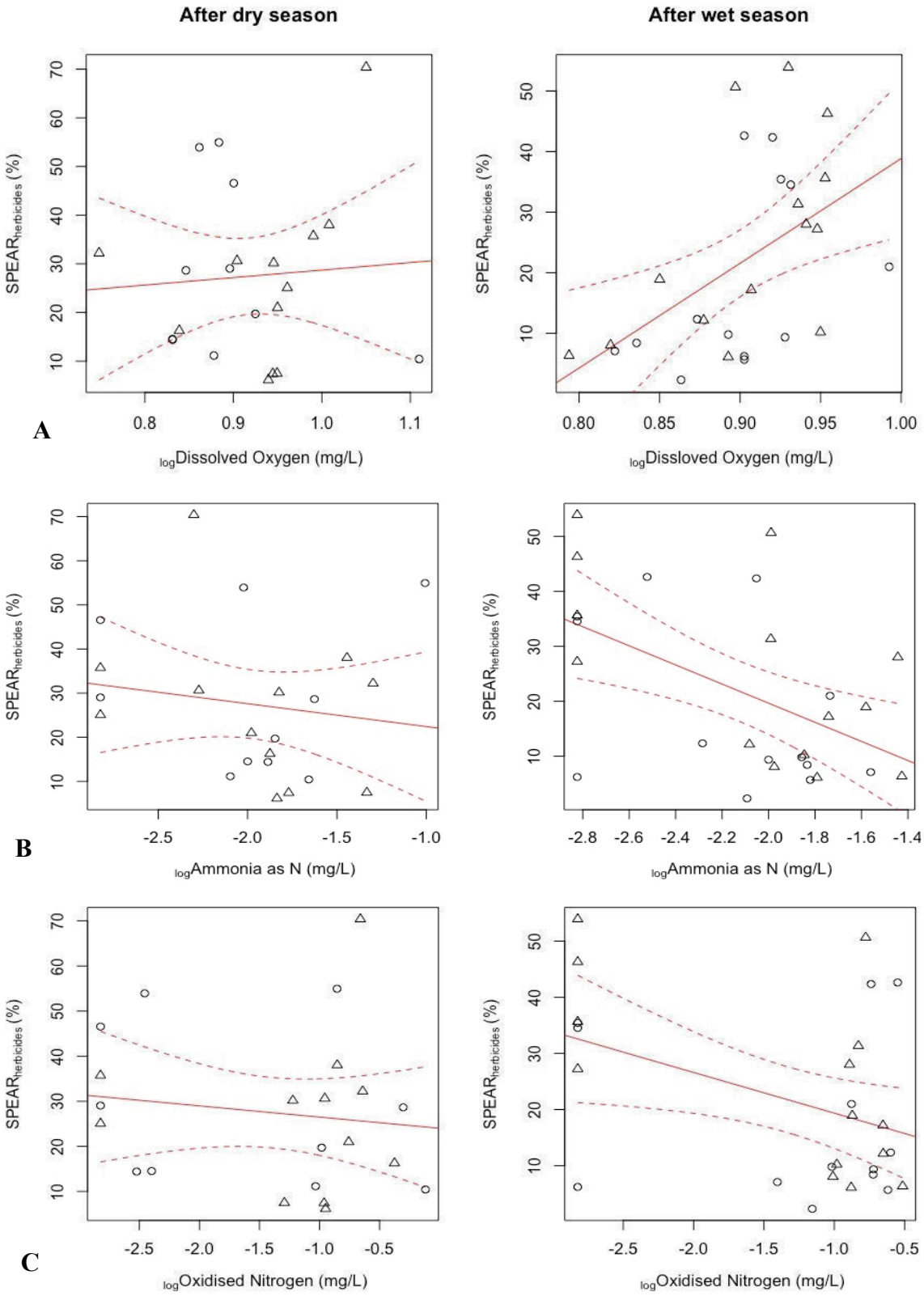


**Figure 6.4** Diatom community characterised as $SPEAR_{herbicides}$ (%) against the environmental variables; a) DO, b) Ammonia, c) NOx, d) EC, e) FRP and f) TSS. Circles = samples relevant for the 2011/12 wet season, triangles = 2012/13 wet season.

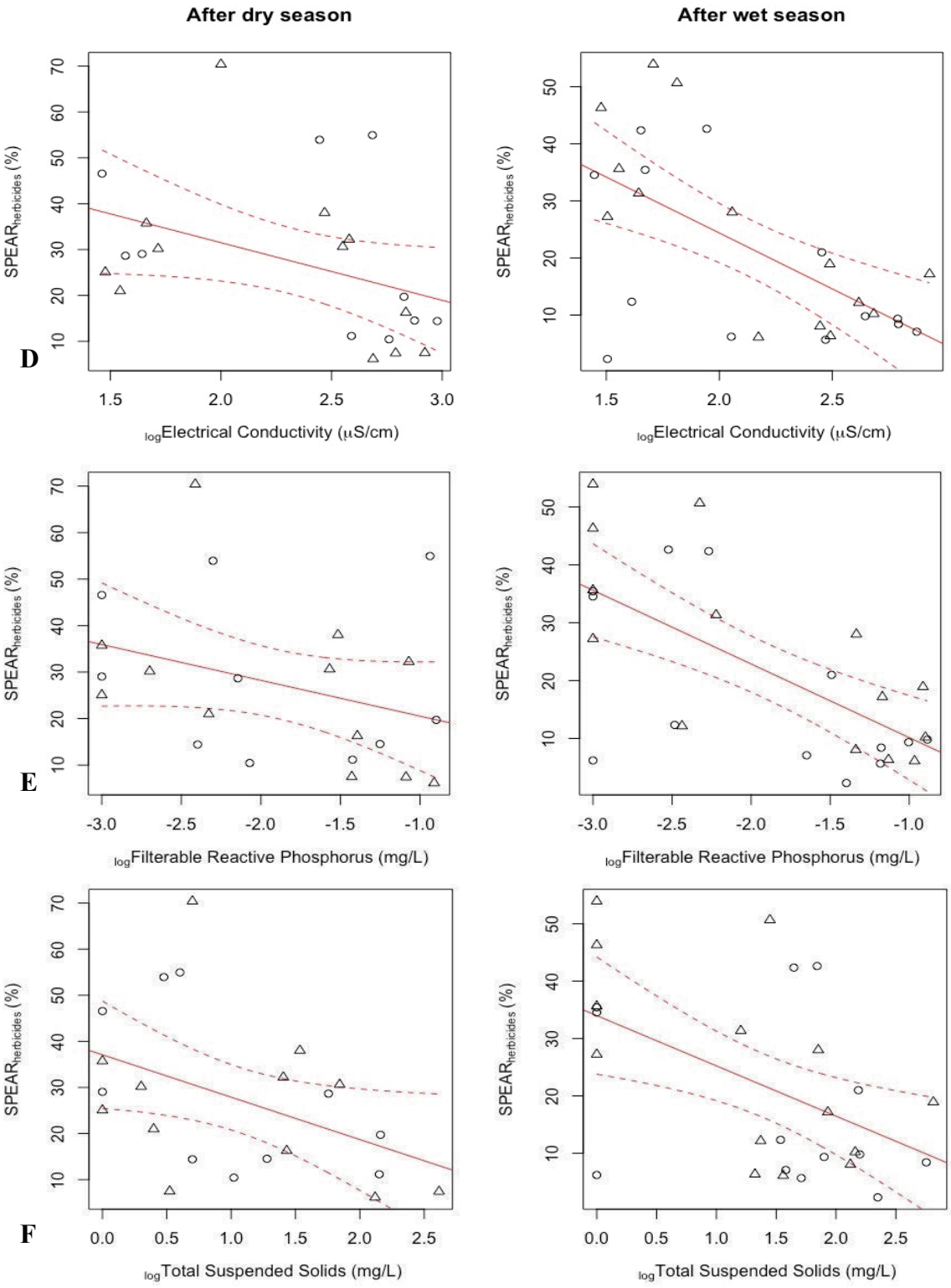


**Figure 6.4** (Continued)

## 6.5 Discussion

### *6.5.1 Influence of environmental variables on diatom community*

In the study region of the GBR catchments, agricultural land development has led to increased loads of suspended sediments, nutrients and herbicides in rivers (Kroon et al., 2012; Waterhouse et al., 2012). Diatom community composition is influenced by changes in water quality and by environmental stressors (Lange et al., 2011; Rimet, 2012). At our study sites the main factors influencing the diatom community were herbicide toxicity ($TEQ_{SO}$), Ammonia, FRP and TSS, reflecting the impacts of agriculture on the diatom community (Figure 2). Diatom community composition was significantly influenced by FRP concentrations, which ranged from 0.002 - 0.135 mg $L^{-1}$. This finding is consistent with other studies that have found diatom community composition to be influenced by agricultural impacts and changes in nutrient concentrations (Sonneman et al., 2001; Stevenson et al., 2008).

### *6.5.2 Effects of herbicide toxicity and $SPEAR_{herbicides}$*

$SPEAR_{herbicides}$ was significantly negatively correlated with herbicide toxicity ($TEQ_{SO}$) across the study sites after two wet seasons. This indicates that the relative abundance of herbicide sensitive diatom species declined with increasing herbicide toxicity. As we hypothesised, this decline in herbicide sensitive diatom taxa was only observed after the wet season rains in both years and not after the two dry seasons. The impacts of herbicide toxicity on the diatom community thus appeared to recover during the dry season, when herbicide concentrations are generally lower (Davis et al., 2012). Other studies have also found that the diatom community has the ability to recover after exposure to herbicides (Dorigo et al., 2010b; Proia et al., 2011). Therefore, timing the collection of diatom communities immediately after peak herbicide concentrations is important for the use of $SPEAR_{herbicides}$ as a biomonitoring tool.

### *6.5.3 Occurrence of tolerant taxa*

Of the diatom taxa strongly related to the sites at the highest gradient of herbicide toxicity, most were classified as tolerant for the calculation of the $SPEAR_{herbicides}$ index - *Diadesmis confervacea*, *Epithemia sorex*, *Luticola goeppertiana*, *Melosira* spp., *Nitzschia perminuta* and *Stauroneis anceps. Diadesmis confervacea*, *Luticola*

*goeppertiana*, *Nitzschia perminuta* and *Stauroneis anceps,* are also considered to be tolerant to organic pollution and indicators of eutrophic conditions (Kelly and Whitton, 1995; Van Dam et al., 1994). However, *Epithemia* is considered highly sensitive to elevated nutrient conditions preferring phosphorus concentrations below 0.01 mg $L^{-1}$ (Kelly et al., 2001) and occurring exclusively at pH > 7 (Van Dam et al., 1994).

### *6.5.4 Multiple stressor effects*

The effect of herbicides on the diatom community has the potential to be altered by environmental factors such as nutrients, light and temperature (Bonnineau et al., 2012; Dalton et al., 2015; Larras et al., 2013a; Wood et al., 2016b). The exposure of benthic communities to herbicides may also be altered by river flow either directly by dilution or indirectly by changing biofilm structure (Ponsatí et al., 2016). However, seasonal hydrographical and climatic variations may be more predominant in shaping diatom communities than exposure to herbicides under field conditions (Andrus et al., 2015). It is often difficult to distinguish the effects of herbicides from other co-occurring pollutants, especially nutrients in agricultural regions (Guasch et al., 1998; Larras et al., 2017; Roubeix et al., 2010). This makes diagnosis of the potentially toxic impacts of herbicide pollution on aquatic ecosystems problematic (Debenest et al., 2010; Morin et al., 2009).

$SPEAR_{herbicides}$ was negatively correlated with $TEQ_{SO}$; however, it was also negatively correlated with nutrients (FRP, ammonia, NOx) as well as TSS and EC. It was therefore difficult to distinguish the effects of herbicide toxicity from other agricultural impacts at the sites. The $SPEAR_{herbicides}$ index was most strongly negatively correlated with FRP, EC and TEQ after the wet season rains, showing that the effects of these factors are dependent on seasonal discharge regime. Concentrations of nutrients and EC in rivers tend to co-increase with increased herbicide loads due to agricultural land practices (Schäfer et al., 2011a). Salinity has an influence on the diatom species present in the diatom community, with individual diatom taxa showing affinities for particular ions in freshwater (Potapova and Charles, 2003; Wilson et al., 1994). Previous studies have shown that diatom relative abundances shift along conductivity gradients, for example a study of diatom communities at 1109 sites across the US found that optimal EC for individual diatom species ranged from 40 to 902 μS $cm^{-1}$ (Wilson et al., 1994). The measured EC at our study sites ranged from 47 - 1400 μS $cm^{-1}$, with the median value

of the sites being 362 µS $cm^{-1}$. A number of other studies have found EC to be a dominant covariate influencing diatom community structure (Blinn and Bailey, 2001; Chessman and Townsend, 2010; Sonneman et al., 2001). However, studies on the effects of salinity as a single stressor on diatom community composition have reported effects at EC levels much higher than the ranges observed in the current study (> 2500 µS $cm^{-1}$) (Cañedo-Argüelles et al., 2017; Rotter et al., 2013).

### *6.5.5 Suggested future research*

Further research is needed to elucidate possible reasons for $SPEAR_{herbicides}$ being correlated with nutrients, EC and herbicides despite it being based on the herbicide sensitivity of diatom taxa. One possibility is that high nutrient concentrations and/or EC might be a better indicator of high herbicide stress than the measurements of herbicides alone. A second explanation is that as elevated nutrient concentrations and EC tend to co-occur with herbicides, species of diatoms that thrive in elevated nutrient concentration and/or EC environments have also been exposed to herbicides and thus have evolved greater tolerance of herbicides than those species that prefer low nutrient concentration and/or EC environments. Future research should in particular consider the effect of nutrients, salinity and/or herbicides on diatom communities and whether diatom species exposed to nutrients, salinity and/or herbicides develop co-tolerance to these stressors. Mesocosm studies could be used to link changes in herbicide concentrations with the response of $SPEAR_{herbicides}$ and provide further evidence of the combined effects of herbicides, salinity and nutrients on benthic diatom communities.

## 6.6 Conclusions

The proportion of sensitive taxa in the benthic diatom community declined with increasing herbicide toxicity of the sites. Our results demonstrated that $SPEAR_{herbicides}$ is capable of detecting agricultural impacts to rivers, including the effects of herbicide toxicity, nutrient pollution and increased salinity on benthic diatom communities. Further research is required to elucidate the specific impact of herbicides on benthic diatom communities in a multi-stressor context.

**Supplementary Material**

The supplementary material for Chapter 6 is available in Appendix E

# Chapter 7: General Discussion and Conclusions

## 7.1 Discussion

This thesis presents an investigation into the impacts of herbicides on freshwater benthic diatoms in rivers of the GBR. An outline of the conceptual framework of my thesis is depicted in Figure 7.1. Presented in this thesis is a novel method from which the relative species sensitivities of many benthic diatom taxa within natural communities can be derived (Chapter 2). Utilising this method a series of specific scientific questions has been answered; i) is the relative sensitivity of diatoms altered by exposure to herbicides with differing modes of action (Chapter 3), ii) do benthic diatoms respond differently to herbicide exposure under reduced light intensity (Chapter 4), does prior pollution of the site influence diatom response to herbicide toxicity (Chapter 4 & 5) and iii) how does diatom sensitivity to atrazine differ as exposure period increases from 2 to 12 days at both the species and community levels (Chapter 5). Finally, I describe the effects of herbicide pollution on benthic diatom communities from rivers within the GBR catchment area (Chapter 6).

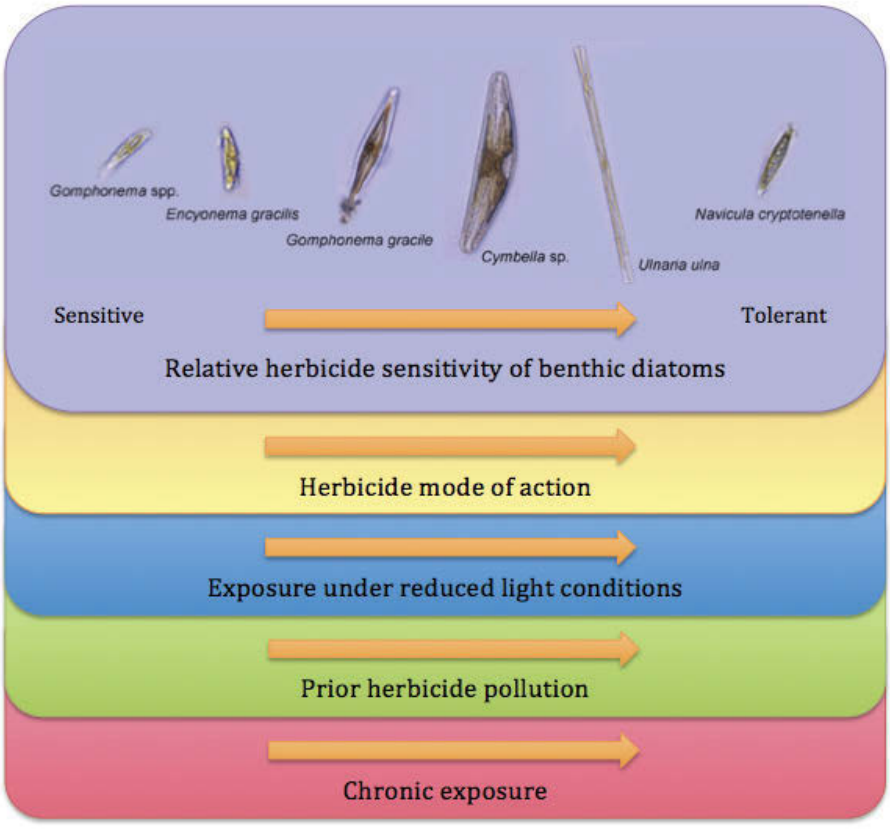


**Figure 7.1** Conceptual diagram of thesis findings.

### *7.1.1 Relative sensitivity of diatoms within natural communities*

Changes in the proportion of sensitive taxa in the diatom community can give an indication of the biological condition of a river (Stevenson, 2014). Unfortunately, the scarcity of information available regarding which diatom taxa are most sensitive to herbicide pollution has been a limitation to the use of benthic diatoms as indicators of herbicide impacts (Morin et al., 2009). Benthic diatoms are incredibly diverse and for any one region there are only a handful of taxa for which relative sensitivity data exist due to the time and labour intensive nature of single species ecotoxicological studies (Larras et al., 2014a). The literature on herbicide sensitivity of freshwater benthic diatom species from tropical regions is even scarcer (Magnusson et al., 2010; Magnusson et al., 2012). It was therefore necessary to investigate the relative sensitivity of as many local benthic diatom taxa from the study region as possible. Chapter 2 addresses this issue, providing a new and rapid approach to obtain sensitivity data for benthic diatoms within field derived natural communities. The results of this chapter align with that of other studies demonstrating how herbicide sensitivity differs between taxa (Larras et al., 2012; Roubeix et al., 2011b), highlighting the need for further sensitivity data to be collected on local diatom species. Additionally, this chapter provides a method that allows the relative sensitivity of diatoms to be determined from naturally derived benthic communities, as well as delivering community level data with ecological relevance.

### *7.1.2 Increasing environmental relevance of sensitivity data*

Advancing the understanding of how benthic diatoms within natural communities respond to herbicide exposure is a primary goal of my thesis. Furthermore, it was important to investigate whether the observed differences in relative sensitivity of diatoms could be altered by exposure to herbicides with differing modes of toxic action. In the field herbicide exposure most commonly occurs when mixtures of various chemicals are washed into rivers from adjoining agricultural pastures. This means that aquatic organisms such as benthic diatoms could be exposed to multiple herbicides including those with different modes of action. I therefore wanted to determine whether the results from Chapter 2 would be consistent across as variety of herbicides commonly detected at field sites in our study region. In Chapter 3 the rapid toxicity approach developed in Chapter 2 was utilised to assess the relative sensitivity of benthic

diatoms to eight herbicides with differing modes of action. The results of Chapter 3 showed a consistent trend in diatom relative sensitivity across eight common herbicides with different modes of action. Herbicide mode of action did not alter which diatoms were most sensitive in the community. Although we have no data on the effect of simultaneous exposure to herbicide mixtures, other studies have generally found the effects are additive (Faust et al., 1993; Magnusson et al., 2010), implying that mixtures of herbicides will not alter the results. This suggests that benthic diatoms can be utilised as indicators of herbicide impacts across a broad range of common herbicides.

Chapter 4 investigates whether trends in diatom sensitivity are consistent under reduced light conditions. Peak herbicide concentrations in rivers often co-occur with periods of increased turbidity and reduced light availability (Kroon et al., 2012). Previous studies have shown that light could have an interactive effect on herbicide sensitivity in algae (Bonnineau et al., 2012; Deblois et al., 2013; Guasch and Sabater, 1998); however, there were no studies assessing the influence of reduced light intensity during exposure to the relative sensitivity of freshwater benthic diatoms. It was therefore important to determine whether these factors could interact with the response of benthic diatoms to herbicide exposure. The interactive effects of reduced light and herbicide exposure on benthic diatoms were assessed at the species and community levels. My results showed that for the majority of diatom taxa there was no interaction and relative sensitivity remained consistent regardless of reduced light conditions during exposure. These findings indicate that the identification of which taxa are most sensitive to herbicides is unlikely to be altered by reduced light conditions during exposure.

### *7.1.3 Chronic exposure and prior pollution history*

Some rivers within the GBR study region are highly polluted with agricultural herbicides, for example Barratta Creek, where concentrations of herbicides are elevated for several months of the year continuously (O'Brien et al., 2016). Chronic herbicide pollution can have deleterious impacts on diatom communities, with the potential to alter community composition as well as photosynthesis (Larras et al., 2012; Magnusson et al., 2008; Magnusson et al., 2012; Ricart et al., 2009; Rimet and Bouchez, 2011). Studies assessing diatom sensitivity to herbicides are often conducted under short duration exposure scenarios (< 96 h) (Larras et al., 2013b), whereas studies at longer exposure durations often focus on community level effects rather than effects at the

individual level (Gustavson et al., 2003; Tlili et al., 2011). Exposure duration may alter effects on diatoms at the individual, population and community levels. Investigating differences in sensitivity over longer exposure durations (> 96 h) is important in order to better understand how diatoms cope with varied exposure in field situations. The fifth chapter of my thesis investigates how benthic diatoms respond to herbicide exposure over varied exposure durations at the individual and community levels. The herbicide toxicity response of diatoms varied with exposure duration with some taxa demonstrating the ability to recover from negative impacts. Other studies have demonstrated the ability for diatoms to recover at the community level (Laviale et al., 2011; Prosser et al., 2013); however, the results of Chapter 5 highlight the differing abilities of the individual diatom taxa with regards to recovery during chronic exposure. These findings are important in the context of assessing the impacts of chronic herbicide exposure in the field and in applied circumstances such as biomonitoring indices. This illustrates the importance of diatom species assemblage in the response and recovery of diatoms to herbicide pollution.

Prior exposure of the diatom community can influence its response to subsequent exposures. The results of Chapters 4 and 5 found that prior pollution of history of the collection site had an influence on benthic diatom responses to herbicide exposure. The diatom community at the polluted Barratta Creek contained a higher proportion of tolerant taxa compared to that of the reference site. These tolerant taxa were able to remain healthy and persist despite exposure to high concentrations of atrazine in toxicity tests (500 ug $L^{-1}$). Barratta Creek diatom community composition was also less affected by herbicide exposure over 12 days, whereas the reference community from Alligator Creek showed changes in community structure as a result of exposure to atrazine. This is consistent with studies demonstrating toxicant induced succession (TIS), where exposed communities show a shift in community composition towards more tolerant taxa (Blanck, 2002; Schmitt-Jansen and Altenburger, 2005); this can result in higher tolerance to subsequent exposures, known as pollution induced community tolerance (PICT) (Dorigo et al., 2010b; Magnusson et al., 2012).

### *7.1.4 Effects of herbicides in field communities within GBR*

Declining water quality within the GBR catchment is a consequence of agricultural land use that has resulted in increased loads of sediments, nutrients and herbicides in rivers

(Kroon et al., 2012). In Chapter 6, a new diatom based $SPEAR_{herbicides}$ index is proposed to monitor the effects of herbicide pollution in rivers. The index revealed how varying water quality, including herbicides, nutrients and suspended solids, influenced the benthic diatom community at 14 sites within the GBR catchment area. $SPEAR_{herbicides}$ showed that the proportion of sensitive taxa in the community declined with increasing toxicity of the sites; however, it was not possible to distinguish from the influence of other environmental factors as benthic diatom community composition was also strongly influenced by changes in nutrients (especially FRP), salinity (EC) and by suspended solids (TSS). Isolating the specific effects of herbicides on benthic diatoms in this multiple stressor context should be the focus of further research.

## 7.2 Further research

### *7.2.1 Diatom sensitivity and traits*

The $SPEAR_{herbicides}$ index requires traits based data for numerous taxa in order to classify them as either sensitive (SPEcies At Risk) or tolerant (not SPEcies At Risk) to herbicides. Chapters 2 to 5 produced sensitivity data for 31 local diatom taxa, a further 28 taxa were classified as either sensitive or tolerant based on a literature search (Chapter 6). Ideally, there should be herbicide sensitivity data for more taxa, covering each Genus so that Order level extrapolations of sensitivity are not necessary. It would also be advantageous to obtain further herbicide sensitivity data for additional diatom taxa that are not currently available, including rare taxa. In addition, several other traits were considered for incorporation into the $SPEAR_{herbicides}$ index; however, they were excluded due to scarcity of data; heterotrophic ability (facultative heterotrophy), motility and recovery (recovery to prolonged exposure). Further studies should investigate the occurrence of these traits in diatom species in relation to their herbicide sensitivity.

### *7.2.2 Validating $SPEAR_{herbicides}$ in the field*

The results of Chapters 2 to 5 demonstrate that benthic diatoms are suitable indicator organisms for herbicide toxicity; they showed variable responses between taxa and they responded rapidly to exposure in toxicity tests. However, the results of Chapter 6 indicate that there is still research needed to increase the confidence in the $SPEAR_{herbicides}$ index in detecting the effects of herbicides in the field. Mesocosm

experiments could establish causality between the response of the $SPEAR_{herbicides}$ and herbicide concentrations in field collected diatom communities. Mesocosm experiments could also establish whether other environmental factors such as FRP and EC also influence $SPEAR_{herbicides}$.

Chapter 6 found that the relationship between $SPEAR_{herbicides}$ and $TEQ_{SO}$ differed between the wet and dry seasons. A seasonal sampling study of the diatom flora at selected field sites could establish trends in $SPEAR_{herbicides}$ and to show how quickly the diatom community recovers at polluted sites following the ending of significant herbicide pollution.

Additionally, the effectiveness of the new $SPEAR_{herbicides}$ index to regions outside the GBR catchment area has not been tested and further studies are recommended to establish the transferability of the index both within Australia and globally.

## 7.3 Management Implications

The protection of freshwater ecosystems from impacts of herbicide pollution is an important task for environmental managers. Herbicide pollution is a significant issue concerning the health and sustainability of the GBR ecosystem (Lewis et al., 2012). Managing and responding to herbicide pollution is of utmost importance to address declining water quality within GBR catchments (Brodie et al., 2017; Waterhouse et al., 2012). The results of this thesis give a better understanding of which diatom species are tolerant and which are sensitive to herbicide exposure. Benthic diatoms are responsive to herbicide exposure and could be used as a biomonitoring tool to indicate the ecological effects of herbicide pollution in rivers. They could be used with other lines of ecological and ecotoxicological evidence to indicate the status of rivers and streams. In particular field collected benthic diatom communities could be exposed to river water samples in laboratory based ecotoxicological bioassays to assess the effects of river contamination. This could provide additional support in weight of evidence assessments of the impacts of herbicide pollution in rivers that flow into the GBR.

The research presented in this thesis demonstrates that benthic diatoms are sensitive to herbicides and changes in diatom community composition can be linked to agricultural impacts in rivers within GBR catchments. The $SPEAR_{herbicides}$ index shows great potential as a monitoring tool capable of diagnosing the impacts of herbicides in rivers.

$SPEAR_{herbicides}$ enables the ecological condition of the river to be assessed from just one site visit. This would be extremely useful to assess impacts in rivers which are difficult or costly to access, or where chemical monitoring is not undertaken as a screening tool. $SPEAR_{herbicides}$ could be utilised to complement chemical monitoring programs and add further valuable information on the biological response of benthic diatom communities to pollution in rivers.

## 7.4 Conclusions

The research presented in my thesis contributes to a broader understanding of the effects of herbicide toxicity on benthic diatoms and the impact of herbicide pollution in rivers. Freshwater benthic diatoms are responsive to herbicide pollution, with some species being highly sensitive to exposure. My thesis identifies individual diatom taxa that are most at risk of herbicide toxicity and also taxa that are tolerant and able to thrive in highly toxic conditions. Benthic diatom communities exposed to herbicides in rapid toxicity tests demonstrated a shift in community composition, with sensitive taxa declining and the most tolerant taxa dominating contaminated treatments. The reported trend in relative sensitivity of the diatom taxa was consistent between herbicides with differing modes of action and under reduced light conditions.

Prior pollution at the collection site was influential in determining response of diatom communities to herbicide exposure, highlighting the importance of diatom community composition in determining community level effects of herbicides. Benthic diatom communities within the GBR catchment were affected by herbicide toxicity, showing a decline in sensitive taxa with increasing contamination of the site. However, the effects of herbicides on the diatom community were only apparent after the wet season, suggesting recovery during the dry season. Diatom communities were also influenced by other environmental variables such as nutrients and salinity. Further research is needed to clarify the individual effects of herbicides in the presence of multiple stressors.

My thesis demonstrates the effects of herbicide toxicity on benthic diatoms within natural benthic communities. This collection of studies illustrate that benthic diatoms have great potential to assess the impacts of agricultural pollutants, including herbicides in rivers of the GBR catchment area.

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# APPENDIX A

**Supplementary Material for Chapter 2**

**Supplementary Table S1** Duplicate water analysis of nominal and measured atrazine concentrations for each rapid toxicity test treatment, taken at start of experiment.

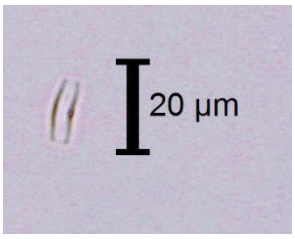
| **Nominal concentrations (µg $L^{-1}$)** | **Measured concentrations (µg $L^{-1}$)** |
|---|---|
| 50 | 48 & 46 |
| 200 | 180 & 170 |
| 500 | 440 & 420 |

**Supplementary Table S2** Health classification of diatoms

**Diatoms from Bluewater Creek**

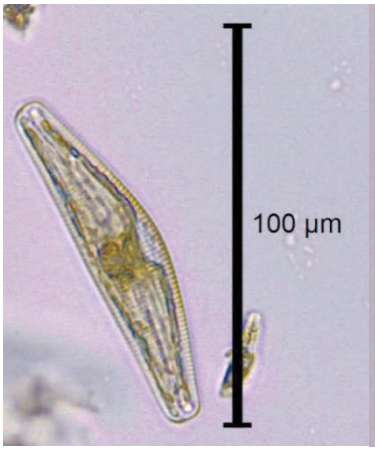
*Achnanthidium:*


Healthy cell

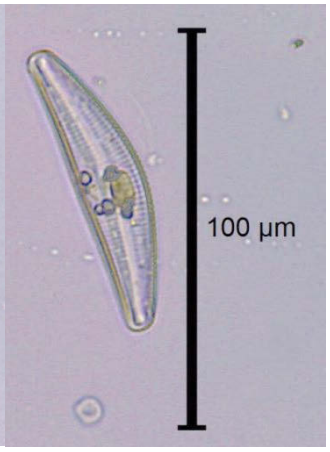



Unhealthy empty cell

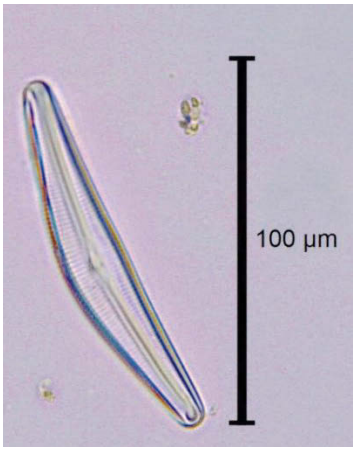
*Cymbella:*




Healthy cell




Unhealthy cell with abnormal chloroplast

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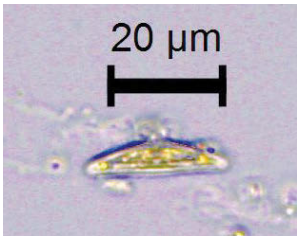
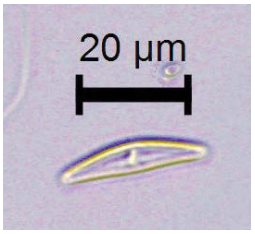
*Amphora:*

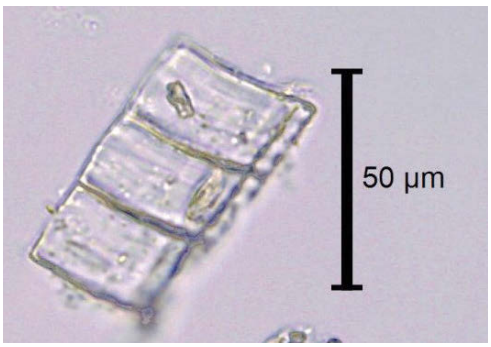



Healthy cell



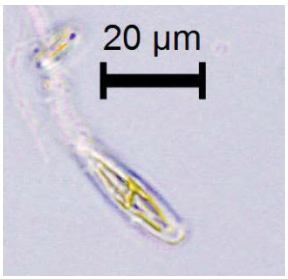

Unhealthy empty cell

*Eunotia:*




Unhealthy empty cells

*Gomphonema:*

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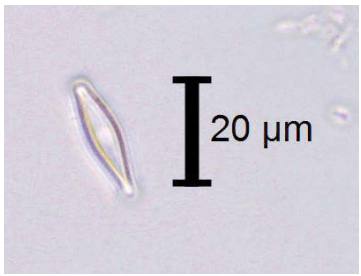
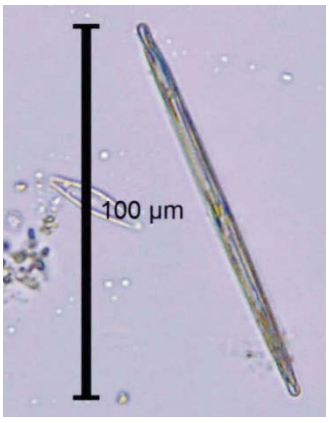



Unhealthy empty cell

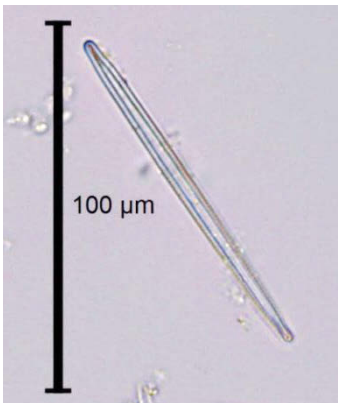
*Navicula:*


Healthy cell

*Ulnaria:*



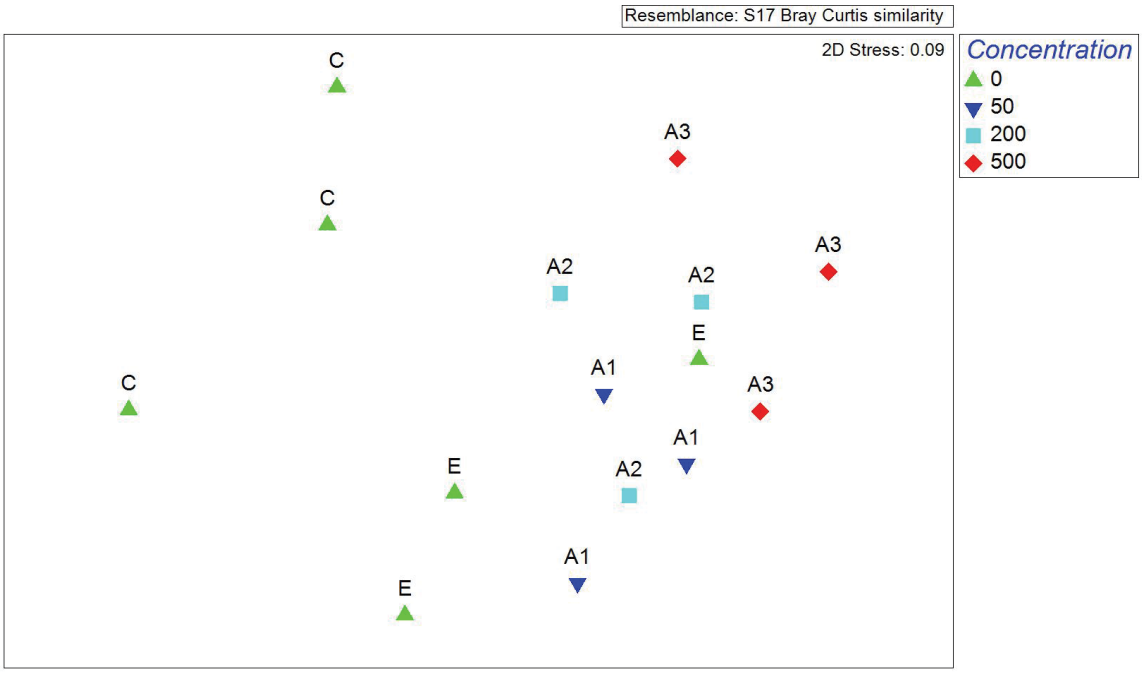

Healthy cell




Unhealthy empty cell

**Supplementary Table S3** GLM results for background health within test control treatments; E48 – 48 hour ethanol controls, C48 – 48 hour site water only controls, compared to site water controls at t = 0.

| Genus/ Treatment | B | Std. Error | Wald Chi-Square | df | Sig. |
|---|---|---|---|---|---|
| *Navicula* | | | | | |
| E48 | 0.320 | 0.966 | 0.110 | 1 | 0.740 |
| C48 | 20.551 | 25128.089 | 0.000 | 1 | 0.999 |
| *Ulnaria* | | | | | |
| E48 | -0.032 | 0.275 | 0.014 | 1 | 0.906 |
| C48 | 0.347 | 0.295 | 1.386 | 1 | 0.239 |
| *Gomphonema* | | | | | |
| E48 | -0.182 | 0.555 | 0.108 | 1 | 0.743 |
| C48 | 0.709 | 0.700 | 1.026 | 1 | 0.311 |
| *Achnanthidium* | | | | | |
| E48 | 0.642 | 0.483 | 1.767 | 1 | 0.184 |
| C48 | 1.460 | 0.631 | 5.354 | 1 | 0.021 |
| *Eunotia* | | | | | |
| E48 | -0.262 | 1.333 | 0.039 | 1 | 0.844 |
| C48 | -21.873 | 56188.123 | 0.000 | 1 | 1.000 |
| *Cymbella* | | | | | |
| E48 | -0.533 | 0.452 | 1.391 | 1 | 0.238 |
| C48 | -0.405 | 0.636 | 0.406 | 1 | 0.524 |
| *Amphora* | | | | | |
| E48 | 0.179 | 0.332 | 0.291 | 1 | 0.590 |
| C48 | -0.112 | 0.366 | 0.094 | 1 | 0.759 |




**Supplementary Figure S4** MDS ordination of healthy benthic community composition in the 48 hour atrazine exposure treatments, from Bluewater Creek diatom communities (Global R = 0.361, p-value = 0.005). Control treatments: C = Control, E = Ethanol Control, and atrazine treatments: A1 = 50 µg $L^{-1}$, A2 = 200 µg $L^{-1}$, A3 = 500 µg $L^{-1}$.

a)  b) 

**Supplementary Figure S5** a) an artificial substrate cage attached to the stream bed, b) view of the cage with removable glass slides on the detachable cage drawer insert.

# APPENDIX B

**Supplementary Material for Chapter 3**

**Supplementary Table S1** List of benthic diatom species from Bluewater Creek within control treatments; C0 = river water controls at t = 0. C48 = 48 hour river water controls. These samples were counted for verification of species identifications and not used in analysis.

| **Diatom Taxon** | CO | C48 |
|---|---|---|
| *Achnanthidium minutissimum* | 179 | 205 |
| *Brachysira vitrea* | 0 | 1 |
| *Encyonema gracilis* | 64 | 41 |
| *Eunotia incisa* | 0 | 1 |
| *Eunotia* sp. | 1 | 0 |
| *Fragilaria capucina* var. *capucina* | 0 | 8 |
| *Fragilaria tenera* | 16 | 20 |
| *Gomphonema gracile* | 1 | 0 |
| *Gomphonema minutum* | 3 | 9 |
| *Gomphonema parvulum* | 9 | 3 |
| *Navicula cryptotenella* | 2 | 0 |
| *Urosolenia* sp. | 2 | 1 |
| *Skelotonema* sp. | 11 | 12 |
| *Ulnaria ulna* | 8 | 1 |
| *Unknown* sp. | 6 | 3 |

**Supplementary Table S2** Background health within control treatments; E48 = 48 hour ethanol controls, C48 = 48 hour controls and T0 = controls at t = 0. P values derived from GLM analysis.

| Diatom Taxon | Treatment | Percent healthy cells (±SE%) | P value |
|---|---|---|---|
| *Navicula cryptotenella* | T0 | 87.5 ± 7.2 | 0.946 |
| | E48 | 90.7 ± 1.6 | |
| | C48 | 100.0 | |
| *Ulnaria ulna* | T0 | 64.2 ± 4.0 | 0.301 |
| | E48 | 62.7 ± 1.0 | |
| | C48 | 70.7 ± 2.0 | |
| *Gomphonema gracile* | T0 | 78.0 ± 3.1 | 0.145 |
| | E48 | 63.8 ± 3.7 | |
| | C48 | 50.8 ± 7.9 | |
| *Gomphonema spp.* | T0 | 62.3 ± 4.6 | 0.425 |
| | E48 | 55.0 ± 6.9 | |
| | C48 | 76.7 ± 5.1 | |
| *Achnanthidium minutissimum* | T0 | 34.8 ± 7.6 | 0.063 |
| | E48 | 54.6 ± 2.9 | |
| | C48 | 74.4 ± 6.7 | |
| *Eunotia cf. incisa* | T0 | 33.3 ± 33.3 | 0.981 |
| | E48 | 18.1 ± 11.7 | |
| | C48 | 0.00 | |
| *Cymbella sp.* | T0 | 60.7 ± 4.8 | 0.492 |
| | E48 | 47.6 ± 52.9 | |
| | C48 | 46.8 ± 7.1 | |
| *Encyonema gracilis* | T0 | 50.5 ± 2.2 | 0.750 |
| | E48 | 53.1 ± 5.5 | |
| | C48 | 46.7 ± 3.7 | |

**Supplementary Table S3** Duplicate water analysis of nominal and measured atrazine concentrations for each rapid toxicity test treatment, taken at start of experiment.

| **Herbicide** | **Nominal concentrations ($\mu g\ L^{-1}$)** | **Measured concentrations ($\mu g\ L^{-1}$)** |
|---|---|---|
| Atrazine | 50 | 48 & 46 |
| | 200 | 180 & 170 |
| | 500 | 440 & 420 |
| Simazine | 50 | 53 & 43 |
| Hexazinone | 200 | 170 & 170 |
| | 500 | 420 & 420 |
| Tebuthiuron | 200 | 202 & 193 |
| Diuron | 500 | 330 & 310 |
| MCPA | 50 | 51 & 45 |
| 2,4-D | 200 | 170 & 170 |
| | 500 | 480 & 480 |
| Glyphosate | 50 | 44 & 53 |
| | 200 | 252 & 177 |

**Supplementary Table S4** Results of GLM testing the effects of herbicide concentration, mode of action (MOA), herbicide type and their interaction on the health of each benthic diatom taxon. P values derived from GLM analysis.

| Diatom Taxon | Concentration | MOA | Herbicide (MOA) | Concentration* MOA | Concentration* Herbicide (MOA) |
|---|---|---|---|---|---|
| *Navicula cryptotenella* | <0.001 | 0.346 | 0.886 | 0.731 | 0.835 |
| *Ulnaria ulna* | <0.001 | 0.068 | 0.025 | 0.772 | 0.516 |
| *Gomphonema gracile* | 0.004 | 0.021 | 0.849 | 0.495 | 0.833 |
| *Gomphonema* spp. | <0.001 | 0.628 | 0.041 | 0.928 | 0.712 |
| *Achnanthidium minutissimum* | <0.001 | 0.311 | 0.647 | 0.477 | 0.445 |
| *Eunotia* cf. *incisa* | 1.000 | 1.000 | 0.803 | 0.998 | 0.966 |
| *Cymbella* sp. | <0.001 | 0.367 | 0.296 | 0.911 | 0.959 |
| *Encyonema gracilis* | <0.001 | 0.976 | 0.268 | 0.981 | 0.956 |

**Supplementary Table S5** Effect of herbicide exposure on the health of *Gomphonema* spp. Cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| **Herbicide** | **Concentration (µg $L^{-1}$)** | **P value** | **Percent healthy cells (±SE%)** | **EC50 (µg $L^{-1}$)** | **EC10 (µg $L^{-1}$)** |
|---|---|---|---|---|---|
| Atrazine | overall | 0.004 | | 44 | 0.1 |
| | 50 | 0.013 | 24 ± 6.1 | | |
| | 200 | 0.010 | 24 ± 2.0 | | |
| | 500 | 0.001 | 15 ± 2.8 | | |
| Simazine | overall | <0.001 | | 34 (0,77) | 3 (0,18) |
| | 50 | 0.005 | 24 ± 2.1 | | |
| | 200 | 0.000 | 5.6 ± 5.6 | | |
| | 500 | 0.007 | 6.7 ± 6.7 | | |
| Hexazinone | overall | 0.001 | | <0.01 | <0.01 |
| | 50 | 0.001 | 16 ± 2.3 | | |
| | 200 | 0.001 | 12 ± 6.3 | | |
| | 500 | 0.003 | 13 ± 7.9 | | |
| Tebuthiuron | overall | 0.024 | | 56 | <0.01 |
| | 50 | 0.024 | 29 ± 11 | | |
| | 200 | 0.007 | 23 ± 7.4 | | |
| | 500 | 0.013 | 25 ± 2.7 | | |
| Diuron | overall | <0.001 | | 3 | 0.04 |
| | 50 | 0.002 | 12 ± 0.5 | | |
| | 200 | 0.002 | 3.7 ± 3.7 | | |
| | 500 | 0.002 | 4.2 ± 4.2 | | |
| MCPA | overall | 0.001 | | 0.3 | <0.01 |
| | 50 | 0.004 | 15 ± 1.2 | | |
| | 200 | 0.008 | 12 ± 6.2 | | |
| | 500 | 0.002 | 11 ± 7.4 | | |
| 2,4-D | overall | 0.004 | | 55 | 5 |
| | 50 | 0.029 | 26 ± 6.7 | | |
| | 200 | 0.036 | 26 ± 15 | | |
| | 500 | 0.002 | 3.7 ± 3.7 | | |
| Glyphosate | overall | 0.005 | | 5.3 | <0.01 |
| | 50 | 0.006 | 20 ± 3.8 | | |
| | 200 | 0.018 | 21 ± 12 | | |
| | 500 | 0.004 | 15 ± 9.7 | | |

**Supplementary Table S6** Effect of herbicide exposure on the health of *Encyonema gracilis* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| **Herbicide** | **Concentration (µg $L^{-1}$)** | **P value** | **Percent healthy cells (±SE%)** | **EC50 (µg $L^{-1}$)** | **EC10 (µg $L^{-1}$)** |
|---|---|---|---|---|---|
| Atrazine | overall | 0.001 | | 240 (82, 1000) | 14 (0, 55) |
| | 50 | 0.119 | 40 ± 2.6 | | |
| | 200 | 0.002 | 27 ± 0.3 | | |
| | 500 | <0.001 | 20 ± 3.3 | | |
| Simazine | overall | <0.001 | | 150 (45, 280) | 8 (0, 33) |
| | 50 | 0.024 | 36 ± 2.9 | | |
| | 200 | <0.001 | 24 ± 0.7 | | |
| | 500 | <0.001 | 15 ± 1.0 | | |
| Hexazinone | overall | <0.001 | | 97 (7, 210) | 4 (0, 22) |
| | 50 | 0.009 | 32 ± 1.2 | | |
| | 200 | <0.001 | 19 ± 2.9 | | |
| | 500 | <0.001 | 14 ± 4.0 | | |
| Tebuthiuron | overall | <0.001 | | 120 | 0.5 |
| | 50 | 0.009 | 31 ± 2.3 | | |
| | 200 | <0.001 | 23 ± 4.3 | | |
| | 500 | <0.001 | 21 ± 3.1 | | |
| Diuron | overall | <0.001 | | 47 | 0.9 |
| | 50 | 0.001 | 25 ± 4.2 | | |
| | 200 | <0.001 | 18 ± 3.7 | | |
| | 500 | <0.001 | 11 ± 2.7 | | |
| MCPA | overall | <0.001 | | 1200 | 0.7 |
| | 50 | 0.014 | 33 ± 2.0 | | |
| | 200 | <0.001 | 20 ± 1.6 | | |
| | 500 | <0.001 | 21 ± 2.2 | | |
| 2,4-D | overall | <0.001 | | 110 (22, 200) | 10 (0.1, 36) |
| | 50 | 0.026 | 34 ± 1.5 | | |
| | 200 | <0.001 | 22 ± 1.8 | | |
| | 500 | <0.001 | 11 ± 2.3 | | |
| Glyphosate | overall | <0.001 | | 71 | 0.1 |
| | 50 | 0.004 | 26 ± 4.9 | | |
| | 200 | 0.001 | 24 ± 3.7 | | |
| | 500 | <0.001 | 17 ± 1.6 | | |

**Supplementary Table S7** Effect of herbicide exposure on the health of *Gomphonema gracile* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| **Herbicide** | **Concentration (µg $L^{-1}$)** | **P value** | **Percent healthy cells (±SE%)** | **EC50 (µg $L^{-1}$)** | **EC10 (µg $L^{-1}$)** |
|---|---|---|---|---|---|
| Atrazine | overall | 0.040 | | >500 | <0.01 |
| | 50 | 0.014 | 35 ± 5.3 | | |
| | 200 | 0.127 | 43 ± 0.5 | | |
| | 500 | 0.011 | 35 ± 7.5 | | |
| Simazine | overall | 0.034 | | 290 | <0.01 |
| | 50 | 0.032 | 37 ± 12 | | |
| | 200 | 0.008 | 35 ± 7.6 | | |
| | 500 | 0.039 | 34 ± 2.9 | | |
| Hexazinone | overall | 0.140 | | >500 (b.e. 890) | 9 |
| | 50 | 0.240 | 54 ± 11 | | |
| | 200 | 0.100 | 39 ± 12 | | |
| | 500 | 0.024 | 35 ± 2.7 | | |
| Tebuthiuron | overall | 0.143 | | - | - |
| | 50 | 0.100 | 39 ± 7.1 | | |
| | 200 | 0.024 | 35 ± 3.5 | | |
| | 500 | 0.188 | 46 ± 13 | | |
| Diuron | overall | 0.036 | | 395 | 11 |
| | 50 | 0.536 | 54 ± 7.1 | | |
| | 200 | 0.011 | 25 ± 17 | | |
| | 500 | 0.033 | 35 ± 5.3 | | |
| MCPA | overall | 0.580 | | - | - |
| | 50 | 0.434 | 54 ± 19 | | |
| | 200 | 0.171 | 46 ± 5.9 | | |
| | 500 | 0.415 | 54 ± 5.3 | | |
| 2,4-D | overall | 0.233 | | >500 | 260 |
| | 50 | 0.281 | 49 ± 6.8 | | |
| | 200 | 0.945 | 68 ± 7.8 | | |
| | 500 | 0.082 | 32 ± 17 | | |
| Glyphosate | overall | 0.448 | | >500 | 95 |
| | 50 | 0.878 | 63 ± 14 | | |
| | 200 | 0.281 | 52 ± 7.8 | | |
| | 500 | 0.169 | 48 ± 9.8 | | |

**Supplementary Table S8** Effect of herbicide exposure on the health of *Cymbella* sp. Cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 μg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| Herbicide | Concentration (μg $L^{-1}$) | P value | Percent healthy cells (±SE%) | EC50 (μg $L^{-1}$) | EC10 (μg $L^{-1}$) |
|---|---|---|---|---|---|
| Atrazine | overall | 0.095 | | 420 | 75 |
| | 50 | 0.995 | 47 ± 5.7 | | |
| | 200 | 0.193 | 32 ± 1.1 | | |
| | 500 | 0.027 | 22 ± 0.9 | | |
| Simazine | overall | 0.857 | | >500 | 110 |
| | 50 | 0.924 | 45 ± 4.3 | | |
| | 200 | 0.437 | 37 ± 9.4 | | |
| | 500 | 0.644 | 39 ± 13 | | |
| Hexazinone | overall | 0.062 | | 420 | 90 |
| | 50 | 0.847 | 49 ± 6.1 | | |
| | 200 | 0.175 | 33 ± 1.8 | | |
| | 500 | 0.027 | 20 ± 4.3 | | |
| Tebuthiuron | overall | 0.767 | | >500 | 140 |
| | 50 | 0.937 | 44 ± 7.6 | | |
| | 200 | 0.593 | 41 ± 10 | | |
| | 500 | 0.333 | 36 ± 4.3 | | |
| Diuron | overall | 0.102 | | 440 | 2 |
| | 50 | 0.177 | 31 ± 2.8 | | |
| | 200 | 0.112 | 29 ± 12 | | |
| | 500 | 0.019 | 17 ± 10 | | |
| MCPA | overall | 0.547 | | >500 | 180 |
| | 50 | 0.794 | 49 ± 3.4 | | |
| | 200 | 0.615 | 39 ± 10 | | |
| | 500 | 0.230 | 36 ± 10 | | |
| 2,4-D | overall | 0.354 | | >500 | 170 |
| | 50 | 0.712 | 41 ± 6.4 | | |
| | 200 | 0.982 | 48 ± 8.8 | | |
| | 500 | 0.098 | 28 ± 9.8 | | |
| Glyphosate | overall | 0.331 | | >500 | 3 |
| | 50 | 0.257 | 33 ± 5.4 | | |
| | 200 | 0.308 | 38 ± 6.5 | | |
| | 500 | 0.081 | 26 ± 1.9 | | |

**Supplementary Table S9** Effect of herbicide exposure on the health of *Ulnaria ulna* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 μg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| **Herbicide** | **Concentration (μg $L^{-1}$)** | **P value** | **Percent healthy cells (±SE%)** | **EC50 (μg $L^{-1}$)** | **EC10 (μg $L^{-1}$)** |
|---|---|---|---|---|---|
| Atrazine | overall | 0.002 | | >500 (b.e. 1240) | 85 (0, 190) |
| | 50 | 0.434 | 58 ± 3.2 | | |
| | 200 | 0.059 | 52 ± 3.0 | | |
| | 500 | 0.000 | 42 ± 1.7 | | |
| Simazine | overall | <0.001 | | >500 | <0.01 |
| | 50 | 0.000 | 41 ± 2.9 | | |
| | 200 | 0.000 | 39 ± 1.2 | | |
| | 500 | 0.000 | 40 ± 1.6 | | |
| Hexazinone | overall | <0.001 | | >500 (b.e. 1000) | 9 (0, 43) |
| | 50 | 0.027 | 50 ± 0.9 | | |
| | 200 | 0.000 | 42 ± 3.6 | | |
| | 500 | 0.000 | 36 ± 1.9 | | |
| Tebuthiuron | overall | <0.001 | | >500 (b.e. 1400) | 1 |
| | 50 | 0.002 | 45 ± 2.4 | | |
| | 200 | 0.000 | 40 ± 1.0 | | |
| | 500 | 0.000 | 36 ± 1.1 | | |
| Diuron | overall | <0.001 | | >500 | 3 |
| | 50 | 0.011 | 49 ± 2.2 | | |
| | 200 | 0.000 | 38 ± 2.7 | | |
| | 500 | 0.000 | 38 ± 4.0 | | |
| MCPA | overall | 0.002 | | >500 | 0.6 |
| | 50 | 0.007 | 48 ± 0.3 | | |
| | 200 | 0.004 | 47 ± 1.6 | | |
| | 500 | 0.000 | 42 ± 3.4 | | |
| 2,4-D | overall | <0.001 | | >500 (b.e. 570) | 15 (0.2, 46) |
| | 50 | 0.020 | 50 ± 1.0 | | |
| | 200 | 0.000 | 40 ± 3.2 | | |
| | 500 | 0.000 | 32 ± 1.7 | | |
| Glyphosate | overall | <0.001 | | >500 (b.e. 830) | 0.1 |
| | 50 | 0.000 | 42 ± 2.7 | | |
| | 200 | 0.000 | 35 ± 4.0 | | |
| | 500 | 0.000 | 33 ± 6.5 | | |

**Supplementary Table S10** Effect of herbicide exposure on the health of *Eunotia cf. incisa* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| Herbicide | Concentration (µg $L^{-1}$) | P value | Percent healthy cells (±SE%) | EC50 (µg $L^{-1}$) | EC10 (µg $L^{-1}$) |
|---|---|---|---|---|---|
| Atrazine | overall | 0.773 | | - | - |
| | 50 | 0.367 | 13 ± 13 | | |
| | 200 | 0.485 | 9.5 ± 9.5 | | |
| | 500 | 0.485 | 9.5 ± 9.5 | | |
| Simazine | overall | 0.623 | | 330 | 77 |
| | 50 | 0.866 | 18 ± 12 | | |
| | 200 | 1.000 | 30 ±15 | | |
| | 500 | 0.190 | 8.3 ± 8.3 | | |
| Hexazinone | overall | 0.586 | | 460 | 320 |
| | 50 | 0.910 | 8.3 ± 8.3 | | |
| | 200 | 0.757 | 13 ± 13 | | |
| | 500 | 0.167 | 8.3 ± 8.3 | | |
| Tebuthiuron | overall | 0.847 | | - | - |
| | 50 | 0.910 | 11 ± 11 | | |
| | 200 | 0.398 | 22 ± 22 | | |
| | 500 | 0.766 | 17 ± 17 | | |
| Diuron | overall | 0.919 | | - | - |
| | 50 | 0.497 | 34 ± 19 | | |
| | 200 | 0.571 | 28 ± 14 | | |
| | 500 | 0.999 | 0.0 ± 0.0 | | |
| MCPA | overall | 0.799 | | - | - |
| | 50 | 0.325 | 50 ± 29 | | |
| | 200 | 0.621 | 14 ± 14 | | |
| | 500 | 0.796 | 28 ± 15 | | |
| 2,4-D | overall | 0.771 | | - | - |
| | 50 | 0.292 | 17 ± 17 | | |
| | 200 | 1.000 | 0.0 ± 0.0 | | |
| | 500 | 0.883 | 19 ± 10 | | |
| Glyphosate | overall | 0.862 | | - | - |
| | 50 | - | 0.0 ± 0.0 | | |
| | 200 | 0.943 | 24 ± 14 | | |
| | 500 | 0.591 | 11 ± 11 | | |

**Supplementary Table S11** Effect of herbicide exposure on the health of *Achnanthidium minutissimum* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

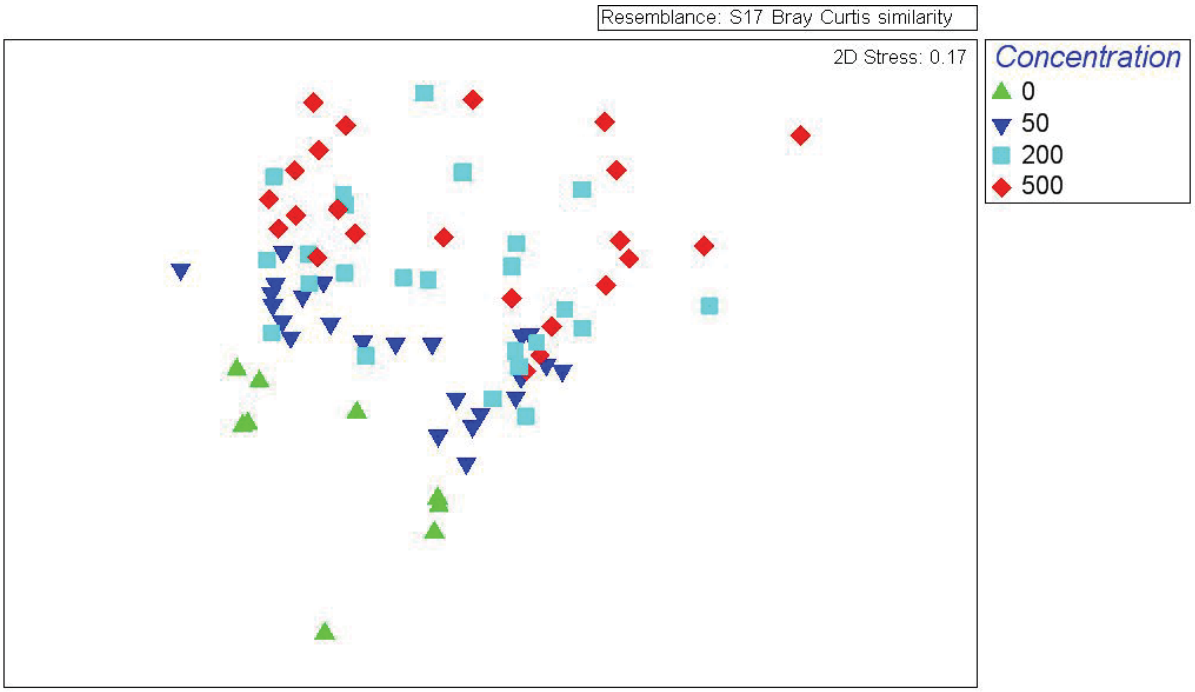
| Herbicide | Concentration (µg $L^{-1}$) | P value | Percent healthy cells (±SE%) | EC50 (µg $L^{-1}$) | EC10 (µg $L^{-1}$) |
|---|---|---|---|---|---|
| Atrazine | overall | 0.407 | | >500 | 330 |
| | 50 | 0.437 | 68 ± 8.8 | | |
| | 200 | 0.429 | 47 ± 7.6 | | |
| | 500 | 0.500 | 49 ± 6.1 | | |
| Simazine | overall | 0.614 | | - | - |
| | 50 | 0.213 | 41 ± 4.4 | | |
| | 200 | 0.386 | 46 ± 2.6 | | |
| | 500 | 0.786 | 51 ± 5.0 | | |
| Hexazinone | overall | 0.472 | | >500 | 53 |
| | 50 | 0.490 | 47 ± 6.3 | | |
| | 200 | 0.573 | 49 ± 5.0 | | |
| | 500 | 0.114 | 36 ± 2.8 | | |
| Tebuthiuron | overall | 0.440 | | >500 | 260 |
| | 50 | 0.911 | 55 ± 10 | | |
| | 200 | 0.879 | 54 ± 6.9 | | |
| | 500 | 0.189 | 41 ± 1.5 | | |
| Diuron | overall | 0.171 | | >500 | 56 |
| | 50 | 0.911 | 54 ± 5.3 | | |
| | 200 | 0.114 | 36 ± 2.2 | | |
| | 500 | 0.074 | 33 ± 2.3 | | |
| MCPA | overall | 0.470 | | >500 | 6 |
| | 50 | 0.374 | 45 ± 11 | | |
| | 200 | 0.320 | 43 ± 3.9 | | |
| | 500 | 0.123 | 37 ± 6.3 | | |
| 2,4-D | overall | <0.001 | | 320 (170, 500) | 120 (11, 200) |
| | 50 | 0.276 | 68 ± 2.4 | | |
| | 200 | 0.123 | 36 ± 7.4 | | |
| | 500 | 0.001 | 15 ± 3.8 | | |
| Glyphosate | overall | 0.394 | | > 500 | 210 |
| | 50 | 0.672 | 49 ± 2.7 | | |
| | 200 | 0.569 | 50 ± 5.8 | | |
| | 500 | 0.092 | 33 ± 8.5 | | |

**Supplementary Table S12** Effect of herbicide exposure on the health of *Navicula cryptotenella* cells. P values derived from GLM analysis comparing treatments overall and at each treatment level (50, 200, 500 µg $L^{-1}$) with carrier controls. EC50 & EC10 values calculated using probit analysis. – not calculable.

| **Herbicide** | **Concentration (µg $L^{-1}$)** | **P value** | **Percent healthy cells (±SE%)** | **EC50 (µg $L^{-1}$)** | **EC10 (µg $L^{-1}$)** |
|---|---|---|---|---|---|
| Atrazine | overall | 0.825 | | >500 | >500 |
| | 50 | 0.808 | 90 ± 6.2 | | |
| | 200 | 0.905 | 91 ± 4.6 | | |
| | 500 | 0.403 | 83 ± 4.8 | | |
| Simazine | overall | 0.140 | | >500 | 33 |
| | 50 | 0.482 | 86 ± 3.4 | | |
| | 200 | 0.041 | 72 ± 0.8 | | |
| | 500 | 0.400 | 87 ± 5.4 | | |
| Hexazinone | overall | 0.428 | | >500 | >500 |
| | 50 | 0.230 | 80 ± 7.1 | | |
| | 200 | 0.691 | 88 ± 2.2 | | |
| | 500 | 0.151 | 78 ± 2.0 | | |
| Tebuthiuron | overall | 0.721 | | >500 | >500 |
| | 50 | 0.456 | 85 ± 3.2 | | |
| | 200 | 0.476 | 87 ± 6.5 | | |
| | 500 | 0.250 | 82 ± 4.0 | | |
| Diuron | overall | 0.049 | | >500 (b.e. 1700) | >500 (b.e.130) |
| | 50 | 0.841 | 95 ± 4.8 | | |
| | 200 | 0.040 | 71 ± 5.0 | | |
| | 500 | 0.055 | 71 ± 0.5 | | |
| MCPA | overall | 0.698 | | >500 | >500 |
| | 50 | 0.942 | 93 ± 3.7 | | |
| | 200 | 0.423 | 85 ± 4.5 | | |
| | 500 | 0.423 | 85 ± 3.5 | | |
| 2,4-D | overall | 0.328 | | >500 | 430 |
| | 50 | 0.887 | 92 ± 4.5 | | |
| | 200 | 0.969 | 91 ± 0.5 | | |
| | 500 | 0.173 | 78 ± 4.7 | | |
| Glyphosate | overall | 0.029 | | >500 (b.e. 850) | 230 |
| | 50 | 0.750 | 91 ± 5.4 | | |
| | 200 | 0.356 | 83 ± 1.8 | | |
| | 500 | 0.013 | 63 ± 3.7 | | |

**Supplementary Table S13** ANOSIM results for community level effects of herbicide concentration treatment (0, 50, 200, 500 μg $L^{-1}$) on the benthic diatom community.

| Herbicide group | Treatments | R statistic | P value |
|---|---|---|---|
| PSII inhibitors | Global | 0.264 | 0.001 |
| | 0, 50 | 0.342 | 0.003 |
| | 0, 200 | 0.562 | 0.001 |
| | 0, 500 | 0.692 | 0.001 |
| | 50, 200 | 0.059 | 0.112 |
| | 50, 500 | 0.218 | 0.008 |
| | 200, 500 | 0.005 | 0.368 |
| Auxinic herbicides | Global | 0.371 | 0.001 |
| | 0, 50 | 0.383 | 0.005 |
| | 0, 200 | 0.533 | 0.001 |
| | 0, 500 | 0.66 | 0.002 |
| | 50, 200 | 0.113 | 0.141 |
| | 50, 500 | 0.248 | 0.019 |
| | 200, 500 | 0.033 | 0.277 |
| ESPS inhibitor glyphosate | Global | 0.588 | 0.001 |
| | 0, 50 | 0.622 | 0.009 |
| | 0, 200 | 0.525 | 0.009 |
| | 0, 500 | 0.852 | 0.005 |
| | 50, 200 | 0.074 | 0.5 |
| | 50, 500 | 0.667 | 0.1 |
| | 200, 500 | -0.037 | 0.6 |




**Supplementary Figure S14** nMDS plot showing community level response of benthic diatoms to herbicides. Control communities are represented by green triangles (0 µg $L^{-1}$); the herbicide concentration treatments; 50 µg $L^{-1}$, 200 µg $L^{-1}$, 500 µg $L^{-1}$, are indicated by dark blue triangles, pale blue squares and red diamonds respectively.

# APPENDIX C

**Supplementary Material for Chapter 4**

**Table S1** Light intensities at the study sites measured in the air at the water surface of diatom collection locations using PAR meter (LI-190 Quantum Sensor).

| Site | Shading | Light intensity (µmol $s^{-1}$ $m^{-2}$) |
|---|---|---|
| Alligator Creek | Full Shade | 78 |
| | Partial | 382 |
| | Direct sunlight | 916 |
| Barratta Creek | Full Shade | 65 |
| | Partial | 246 |
| | Direct sunlight | 1832 |
| Gowrie Creek | Full Shade | 92 |
| | Partial | 515 |
| | Direct sunlight | 775 |
| Liverpool Creek | Full Shade | 176 |
| | Partial | 560 |
| | Direct sunlight | 804 |

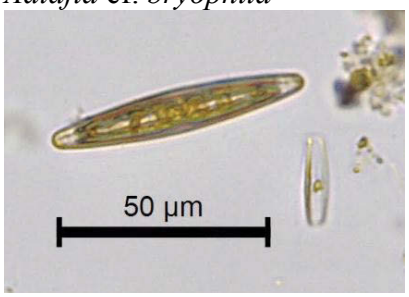
**Table S2** Physico-chemical parameters at the diatom collection sites at the time of sampling.

| Site | Temperature (°C) | pH | Dissolved Oxygen (mg/L) | Conductivity (µS/cm) |
|---|---|---|---|---|
| Alligator Creek | 28.3 | 7.6 | 8.93 | 95.2 |
| Barratta Creek | 25.0 | 7.7 | 4.71 | 575.3 |
| Gowrie Creek | 25.3 | 7.6 | 9.14 | 75.9 |
| Liverpool Creek | 27.2 | 7.5 | 8.72 | 47.1 |

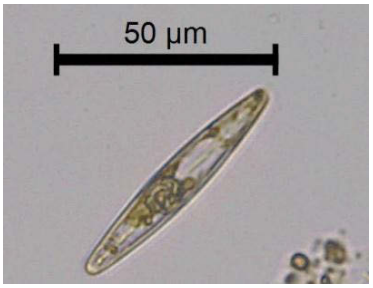
**Table S3** List of taxa and health classification of diatoms

**Diatoms from Alligator Creek:**

*Adlafia* cf. *bryophila*




Healthy cell




Unhealthy cell

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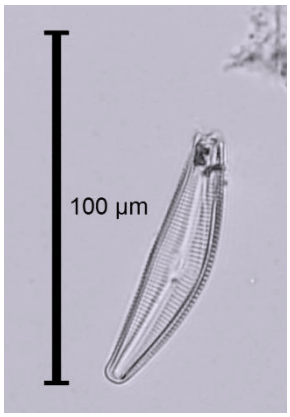
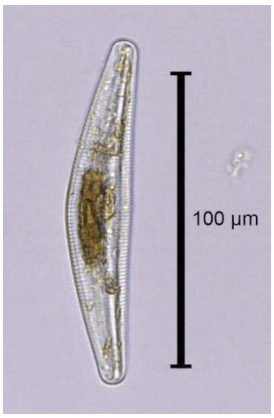
*Cymbella aspera*


Healthy cell




Unhealthy cell




Unhealthy cell

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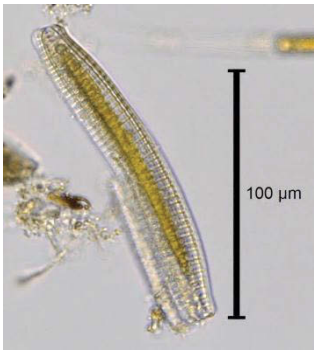
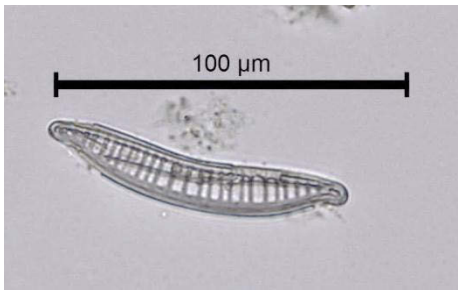
*Epithemia* cf. *adanata*

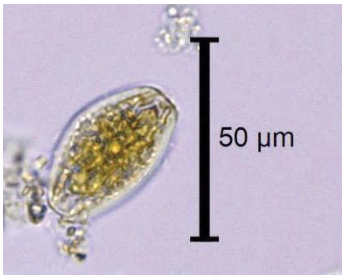



Healthy cell

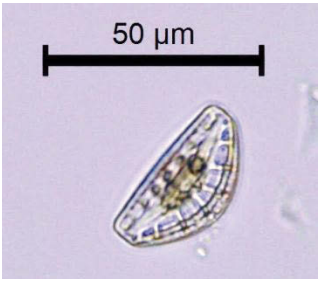



Unhealthy cell

*Epithemia* cf. *cistula*




Healthy cell




Unhealthy cell

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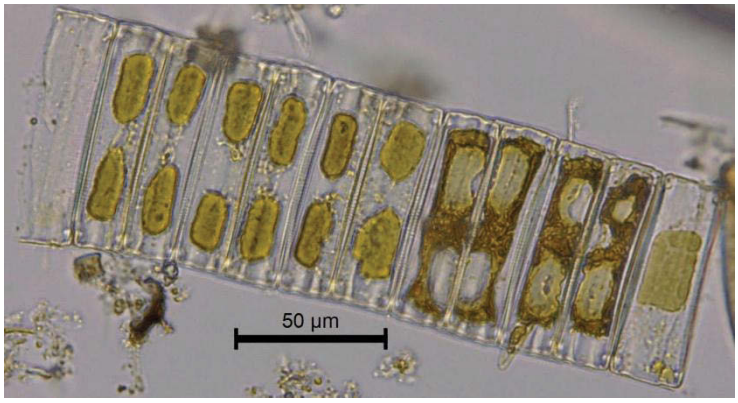
*Eunotia* cf. *minor*




Healthy and unhealthy cells

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*Gomphonema clevei*


Healthy cell


Unhealthy cell

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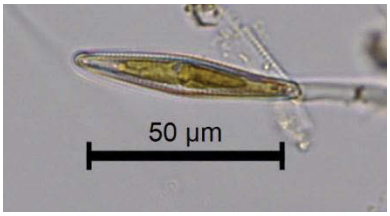
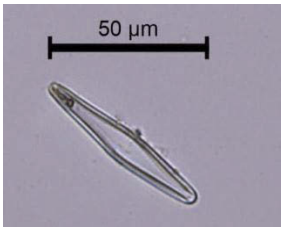
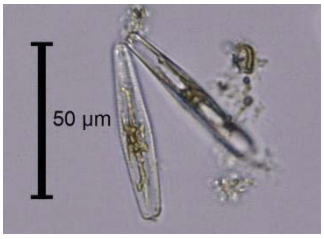
*Gomphonema gracile*




Healthy cell

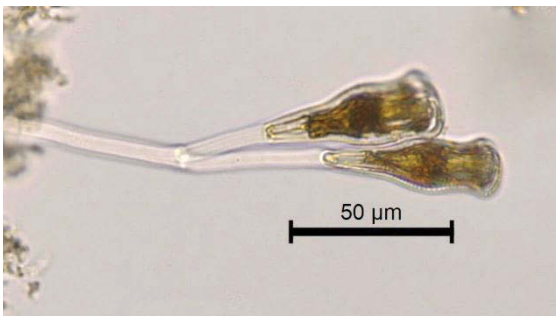



Unhealthy cell

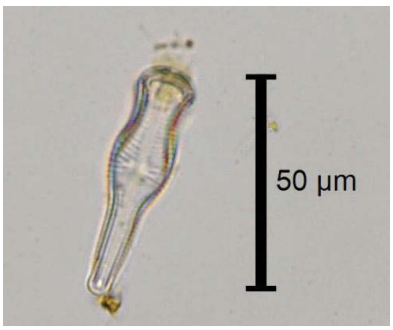



Unhealthy cell

*Gomphonema truncatum*




Healthy cell




Unhealthy cell

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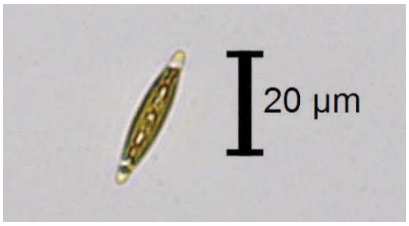
*Navicula* cf. *cryptotenella*




Healthy cell

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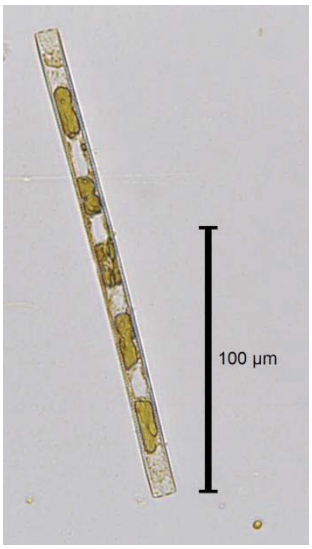
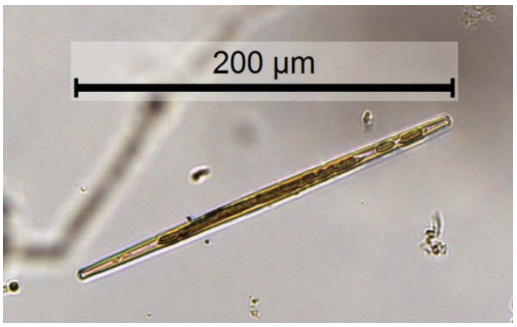
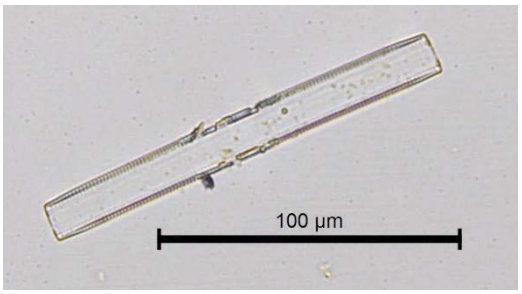
*Ulnaria ulna*




Healthy cell




Healthy cell




Unhealthy cell

**Diatoms from Barratta Creek:**

*Mayamaea atomus*


Healthy cell

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*Melosira varians*


Healthy and unhealthy cells

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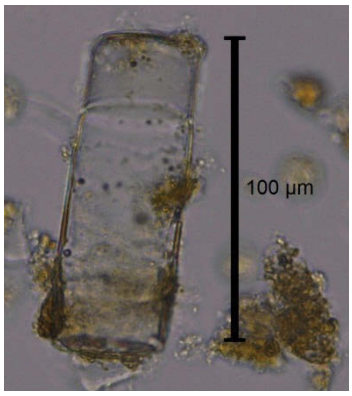
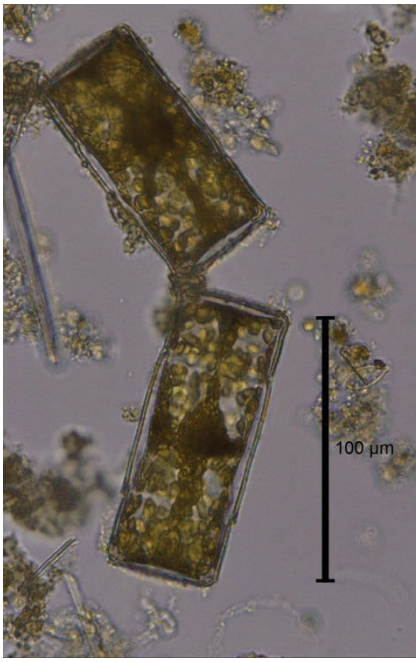
*Pleurosira* sp.

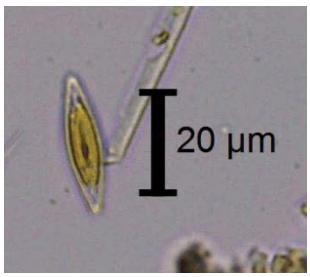



Unhealthy cell




Healthy cells

*Navicula* cf. *cryptocephala*




Healthy cell

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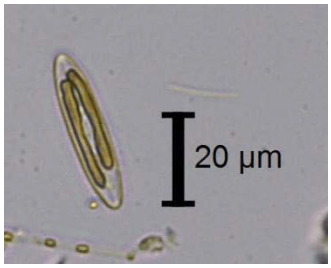
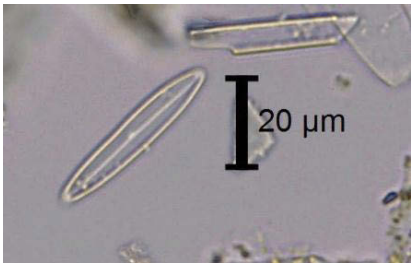
*Navicula schroeterii*




Healthy cell




Unhealthy cell

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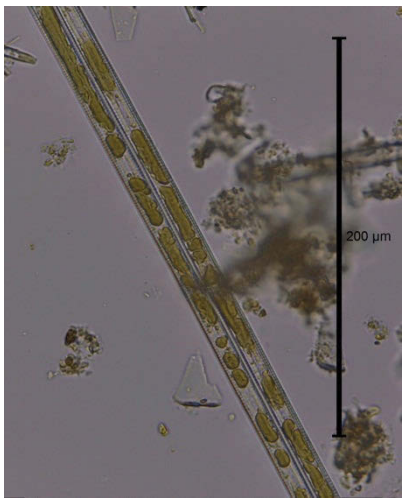
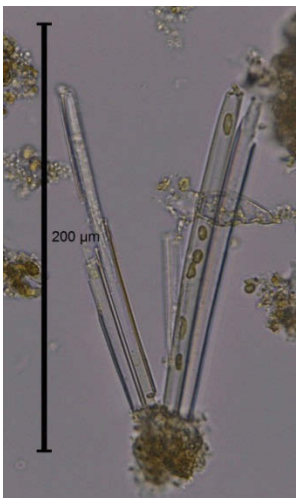
*Navicula* cf. *subtillissima*

Unhealthy cell

*Ulnaria ulna*




Healthy cells




Unhealthy cells

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**Diatoms from Gowrie Creek**

*Cocconeis placentula*


Healthy cell

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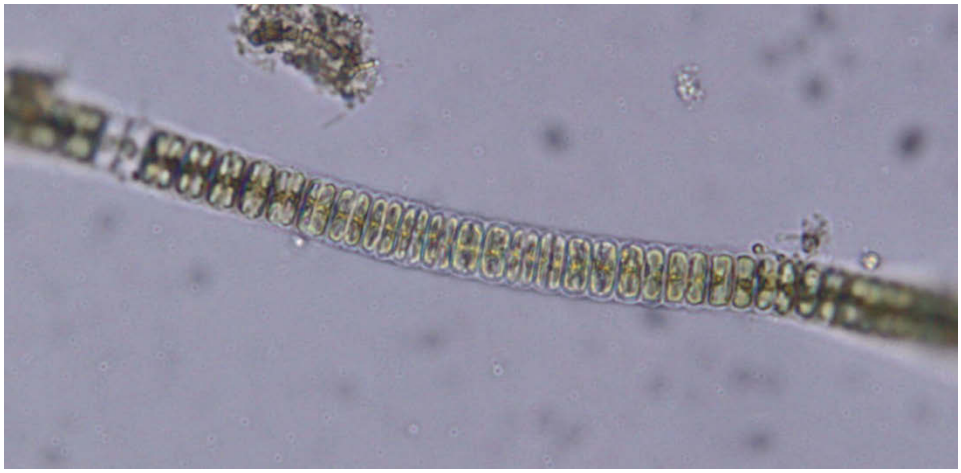
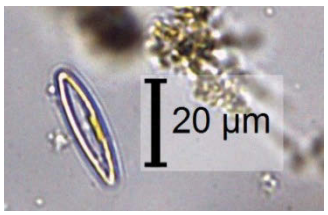
*Fragillaria* sp.

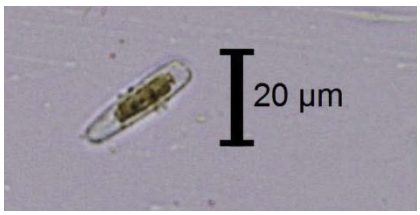


Healthy and unhealthy cells

*Gomphonema spp.*




Unhealthy cell




Healthy cell

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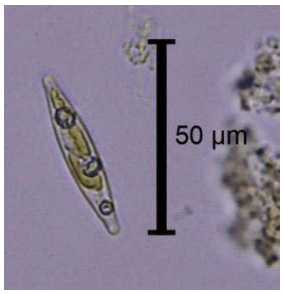
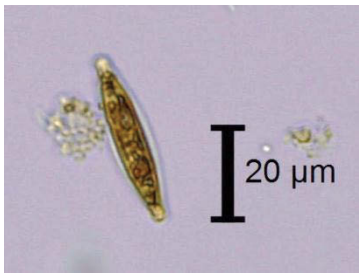
*Navicula* cf. *cryptotenella*




Unhealthy cell




Healthy cell

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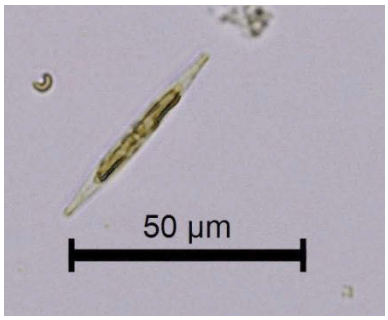
*Nitzchia paleaceae*




Healthy cell

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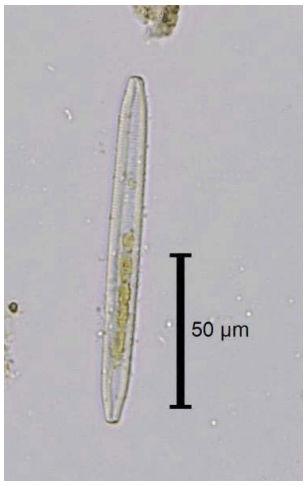
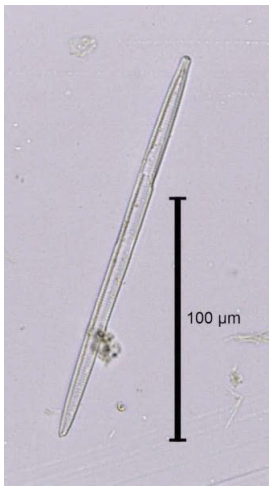
*Ulnaria ulna*




Unhealthy cell




Unhealthy cell

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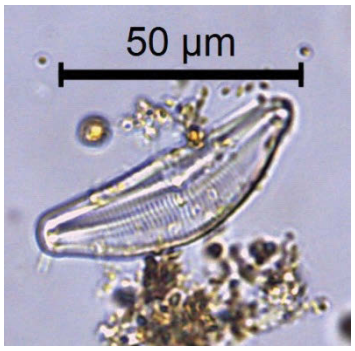
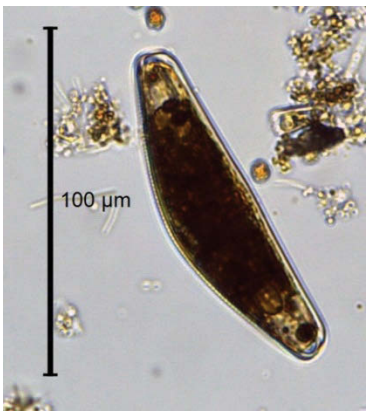
**Diatoms from Liverpool Creek:**

*Cymbella sp.*




Unhealthy cell




Healthy cell

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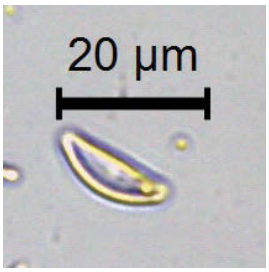
*Encyconema sp.*


Healthy cell




Unhealthy cell

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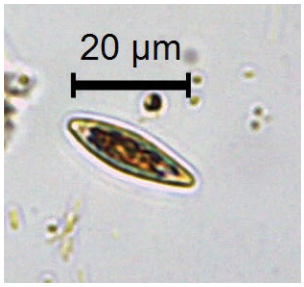
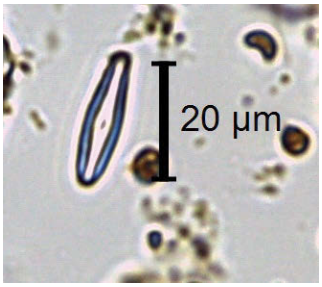
*Gomphonema* cf. *minutum*




Healthy cell




Unhealthy cell

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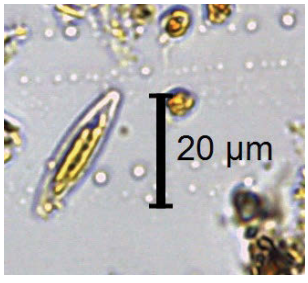
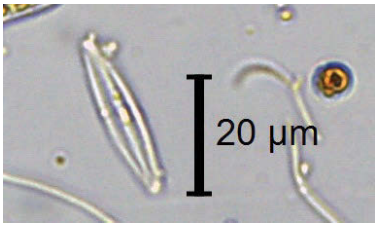
*Navicula cryptotenella*

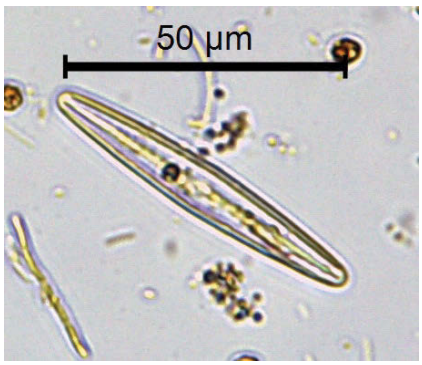



Healthy cell




Unhealthy cell

*Navicula* cf. *radiosa*




Unhealthy cell

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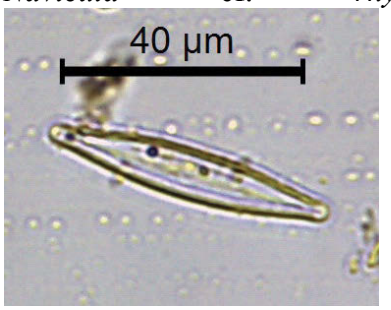
*Navicula* cf. *rhynchocephala*




Unhealthy cell


Healthy cell

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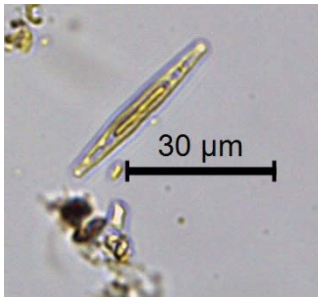
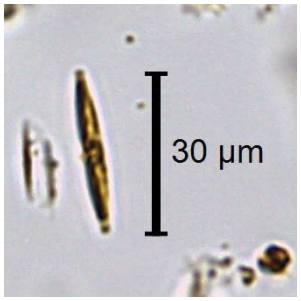
*Nitzschia* paleaceae




Unhealthy cell




Healthy cell

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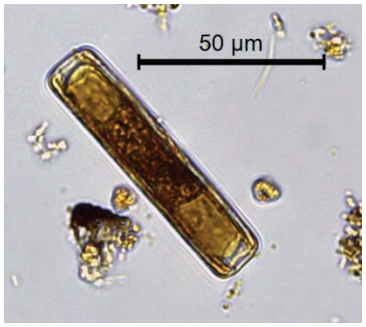
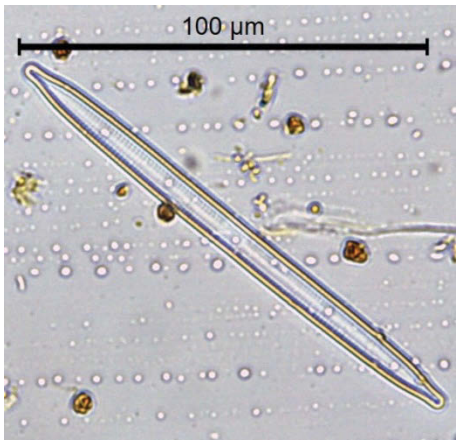
*Pinnularia* *viridus*




Healthy cell

*Ulnaria* *ulna*




Unhealthy cell

**Table S4** Complete species list and diatom cell count from each collection site within samples taken at the start of the experiment (0 h).

| **Diatom taxon:** | **Alligator Creek** | **Barratta Creek** | **Gowrie Creek** | **Liverpool Creek** |
|---|---|---|---|---|
| *Achnanthes exigua* | 0 | 0 | 4 | 0 |
| *Achnanthes oblongella* | 0 | 0 | 4 | 0 |
| *Achnanthidium minutissimum* | 116 | 7 | 49 | 104 |
| *Achnanthidium* sp. | 0 | 0 | 0 | 3 |
| *Adlafia* aff. *bryophila* | 19 | 0 | 0 | 0 |
| *Amphora* aff. *coffeaeformis* | 0 | 1 | 0 | 0 |
| *Amphora pediculus* | 0 | 2 | 21 | 0 |
| *Cocconeis placentula* | 0 | 2 | 18 | 3 |
| *Cymbella aspera* | 2 | 0 | 0 | 0 |
| *Diadesmis confervacea* | 0 | 0 | 59 | 0 |
| *Diploneis elliptica* | 0 | 2 | 0 | 0 |
| *Encyonema gracilis* | 0 | 0 | 6 | 0 |
| *Encyonema minuta* | 17 | 2 | 12 | 86 |
| *Eolimna subminuscula* | 4 | 0 | 0 | 0 |
| *Epithemia adnata* | 3 | 0 | 0 | 0 |
| *Epithemia cistula* | 15 | 0 | 0 | 0 |
| *Epithemia sorex* | 1 | 0 | 0 | 0 |
| *Eunotia bilunaris* v. *mucophila* | 0 | 0 | 0 | 1 |
| *Eunotia* sp. | 2 | 0 | 0 | 0 |
| *Fragilaria capucina* var. *capucina* | 11 | 0 | 7 | 14 |
| *Gomphonema clevei* | 101 | 0 | 0 | 1 |
| *Gomphonema gracile* | 9 | 4 | 0 | 0 |
| *Gomphonema minutum* | 0 | 42 | 25 | 53 |
| *Gomphonema parvulum* | 0 | 15 | 7 | 2 |
| *Gomphonema* sp. | 9 | 0 | 0 | 0 |
| *Gomphonema truncatum* | 3 | 0 | 0 | 0 |
| *Luticola goeppertiana* | 0 | 3 | 0 | 0 |
| *Mayamaea atomus* | 0 | 22 | 6 | 2 |
| *Melosira varians* | 0 | 5 | 0 | 0 |
| *Navicula cryptocephala* | 0 | 2 | 4 | 0 |
| *Navicula cryptotenella* | 19 | 0 | 7 | 20 |
| *Navicula decussis* | 0 | 0 | 5 | 4 |
| *Navicula schroeterii* | 0 | 6 | 0 | 0 |
| *Navicula subtillissima* | 0 | 2 | 0 | 0 |
| *Navicula viridula* | 0 | 0 | 3 | 0 |
| *Navicula* aff. *rhynchocephala* | 0 | 0 | 0 | 2 |
| *Nitzschia inconspicua* | 6 | 0 | 0 | 0 |
| *Nitzschia paleaceae* | 0 | 0 | 52 | 2 |
| *Nitzschia* sp. | 2 | 0 | 0 | 0 |
| *Pinnularia viridis* | 0 | 0 | 0 | 1 |
| *Planothidium lanceolatum* | 0 | 0 | 11 | 4 |
| *Pleurosira* sp. | 0 | 99 | 0 | 0 |
| *Surirella* sp. | 0 | 0 | 0 | 1 |
| *Ulnaria ulna* | 2 | 115 | 11 | 4 |
| Total | 341 | 331 | 311 | 307 |

**Table S5** Duplicate water analysis of herbicide concentrations in rapid toxicity test treatments taken at start of experiment (0 h).

| **Herbicide** | **Nominal concentrations (µg $L^{-1}$)** | **Measured concentrations (µg $L^{-1}$)** |
|---|---|---|
| Atrazine | 20 | 19 & 20 |
| | 50 | 44 & 41 |
| | 200 | 170 & 180 |
| | 500 | 450 & 500 |
| Glyphosate | 50 | 73 & 66 |
| | 200 | 240 & 250 |

**Table S6** GLM results for background health within test control treatments; 48 h ethanol controls, 48 h site water only controls, compared to site water controls at 0 h. Statistical significance at alpha 0.05 is indicated in bold type.

| **Genus** | ***p* value** |
|---|---|
| Barratta Creek: | |
| *Mayamaea atomus* | 0.877 |
| *Melosira varians* | 0.897 |
| *Pleurosira sp.* | 0.109 |
| *Navicula cf. cryptocephala* | 0.976 |
| *Navicula schroeteri* | 0.382 |
| *Navicula cf. subtillissima* | 0.720 |
| *Ulnaria ulna* | **<0.001** |
| Alligator Creek: | |
| *Adlafia* aff. *bryophila* | 0.149 |
| *Cymbella aspera* | 0.979 |
| *Epithemia cf. adanata* | 0.963 |
| *Epithemia cf. cistula* | 0.067 |
| *Eunotia cf.minor* | 0.233 |
| *Gomphonema clevei* | **0.011** |
| *Gomphonema gracile* | **0.026** |
| *Gomphonema truncatum* | 0.108 |
| *Navicula cf. cryptotenella* | 0.739 |
| *Ulnaria ulna* | 0.398 |
| Gowrie Creek: | |
| *Cocconeis placentula* | 0.624 |
| *Fragillaria* sp. | **<0.001** |
| *Gomphonema* spp. | 0.789 |
| *Navicula cf. cryptocephala* | 0.287 |
| *Navicula cf. cryptotenella* | 1.000 |
| *Nitzschia paleaceae* | 0.949 |
| *Ulnaria ulna* | 0.083 |
| Liverpool Creek: | |
| *Cymbella sp.* | 0.701 |
| *Encyconema sp.* | 0.991 |
| *Fragillaria sp.* | **<0.001** |
| *Gomphonema cf. minutum* | 0.199 |
| *Navicula cf. cryptotenella* | 0.459 |
| *Navicula cf. radiosa* | 0.260 |
| *Navicula cf. rhynchocephala* | 0.775 |
| *Nitzschia paleaceae* | 1.000 |
| *Pinnularia viridus* | 0.160 |
| *Ulnaria ulna* | 0.544 |

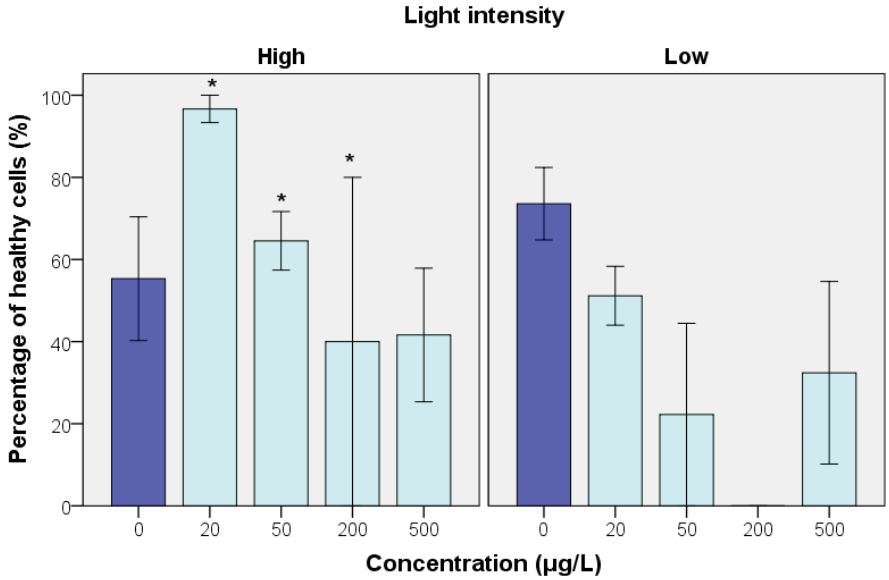



**Figure S7** Percentage of healthy diatom cells for *Fragillaria* sp. from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

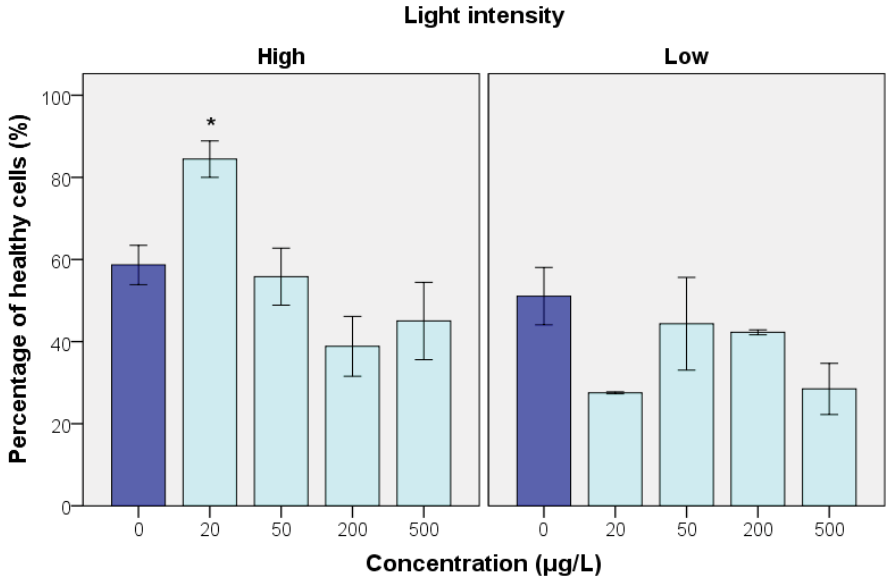



**Figure S8** Percentage of healthy diatom cells for *Navicula* cf. *cryptotenella* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

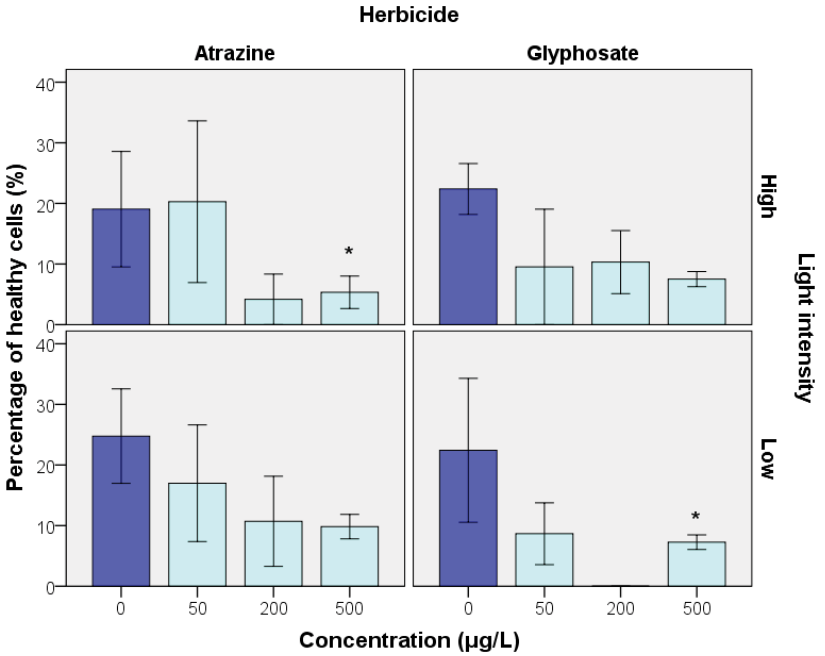



**Figure S9** Percentage of healthy diatom cells for *Cymbella aspera* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

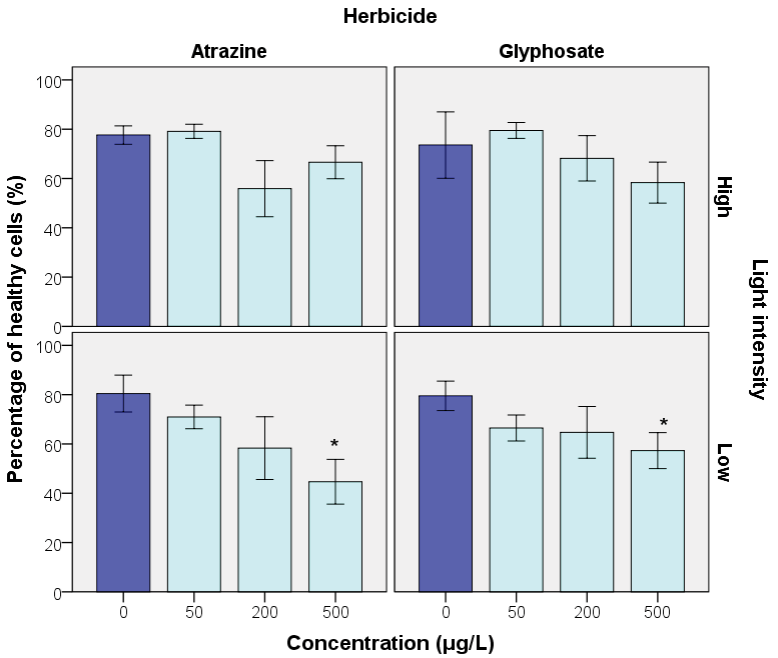



**Figure S10** Percentage of healthy diatom cells for *Gomphonema gracile* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

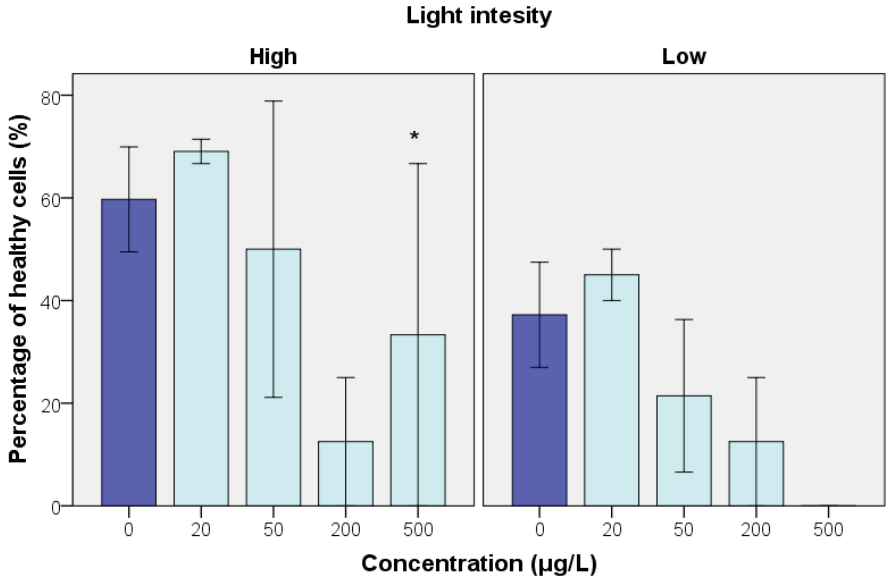



**Figure S11** Percentage of healthy diatom cells for *Gomphonema* cf. *minutum* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

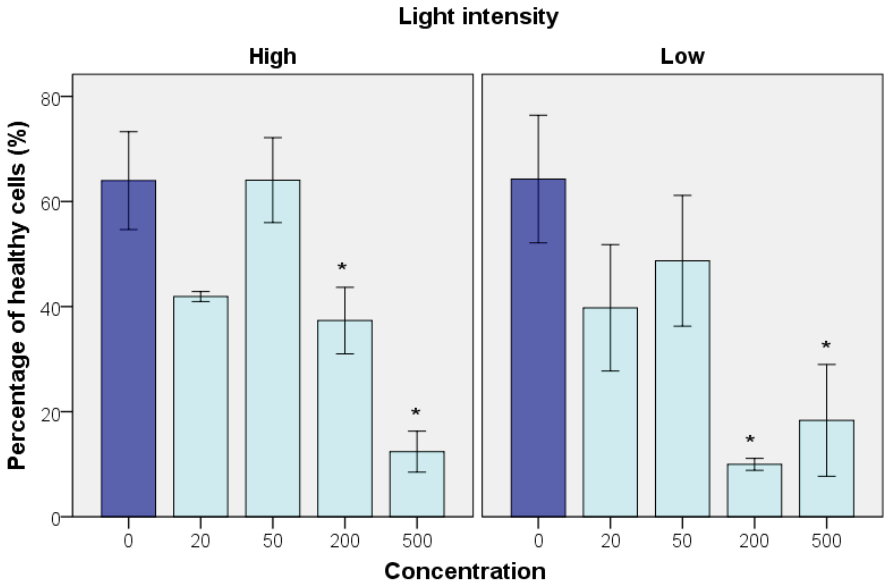



**Figure S12** Percentage of healthy diatom cells for *Gomphonema* spp. from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

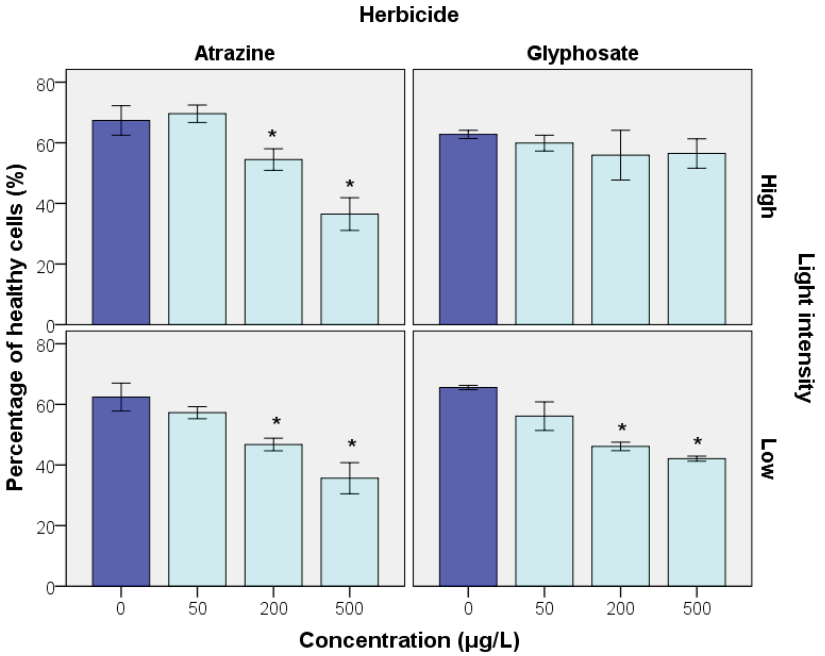



**Figure S13** Percentage of healthy diatom cells for *Gomphonema truncatum* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

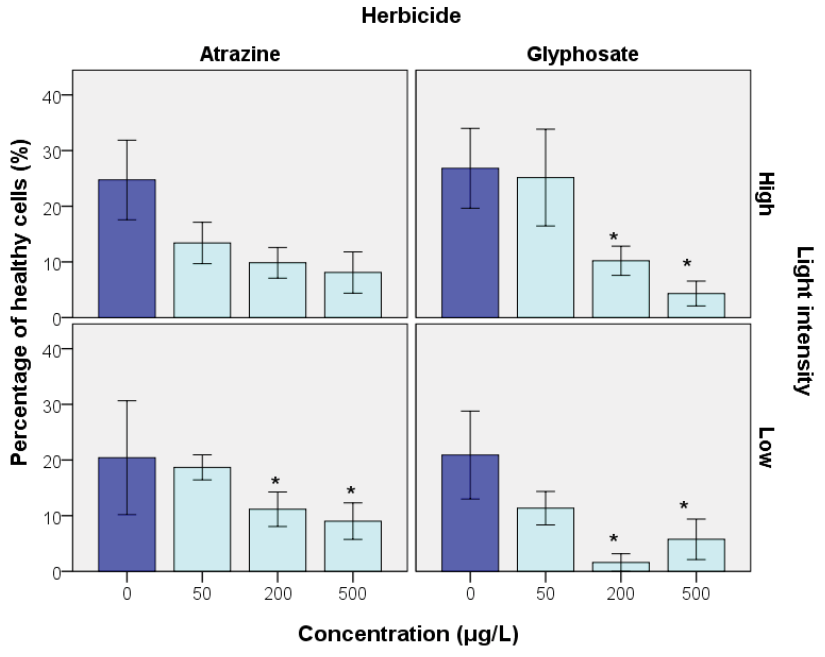



**Figure S14** Percentage of healthy diatom cells for *Ulnaria ulna* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

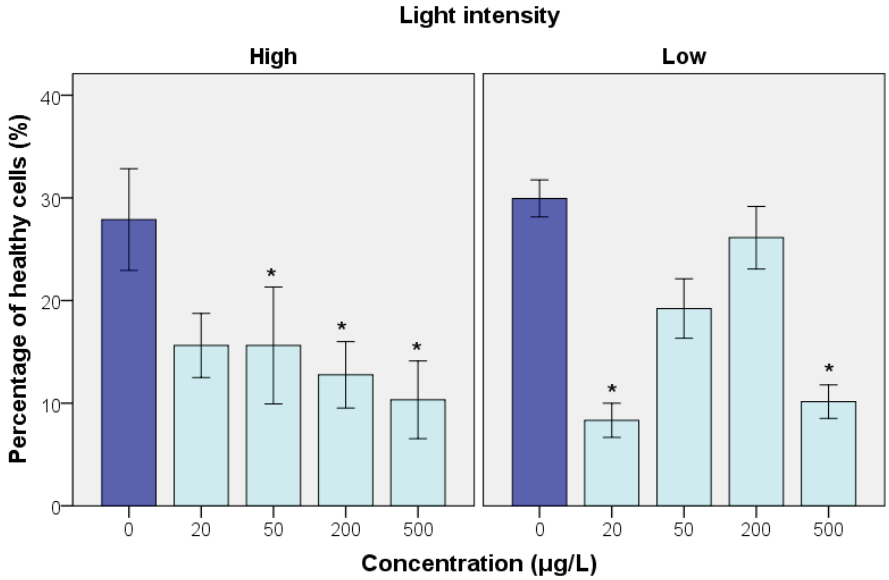



**Figure S15** Percentage of healthy diatom cells for *Ulnaria ulna* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

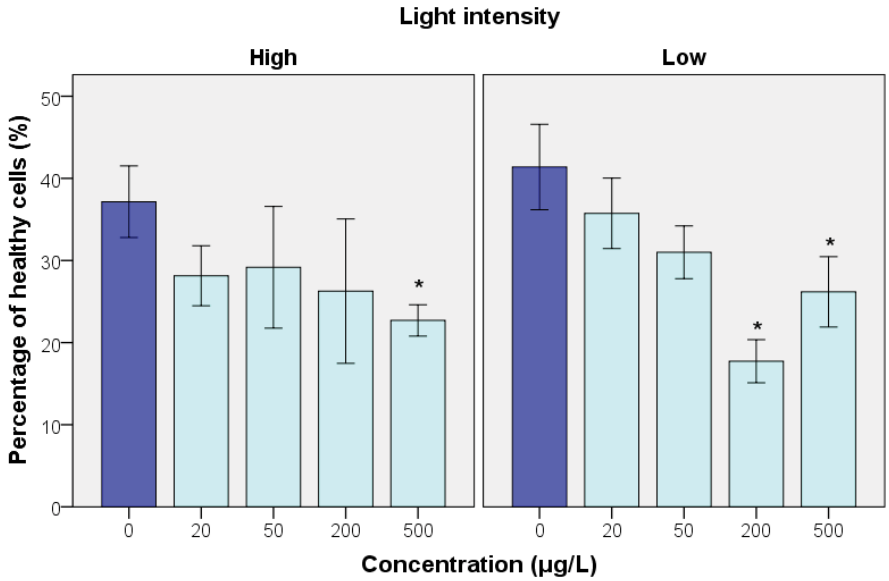



**Figure S16** Percentage of healthy diatom cells for *Ulnaria ulna* from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

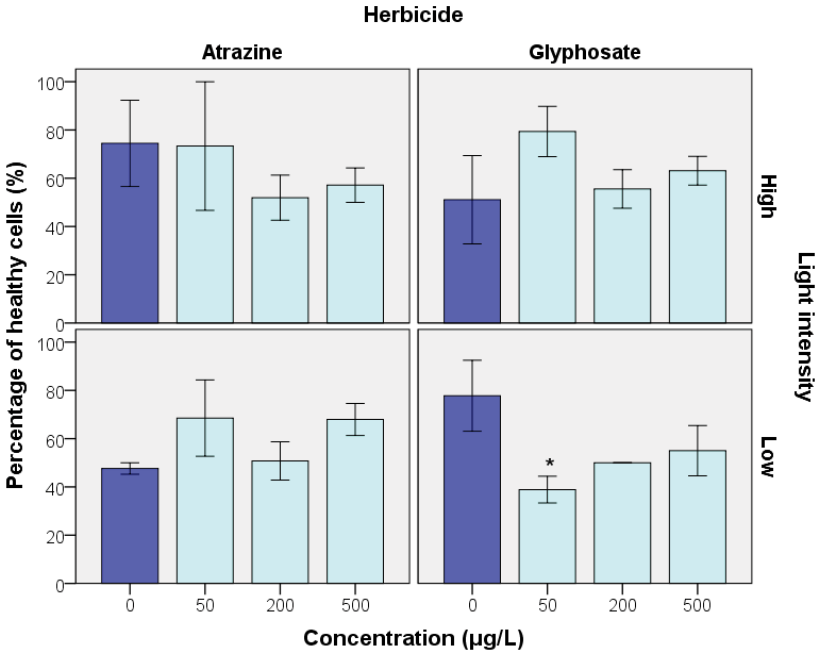



**Figure S17** Percentage of healthy diatom cells for *Adlafia* aff. *bryophila* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

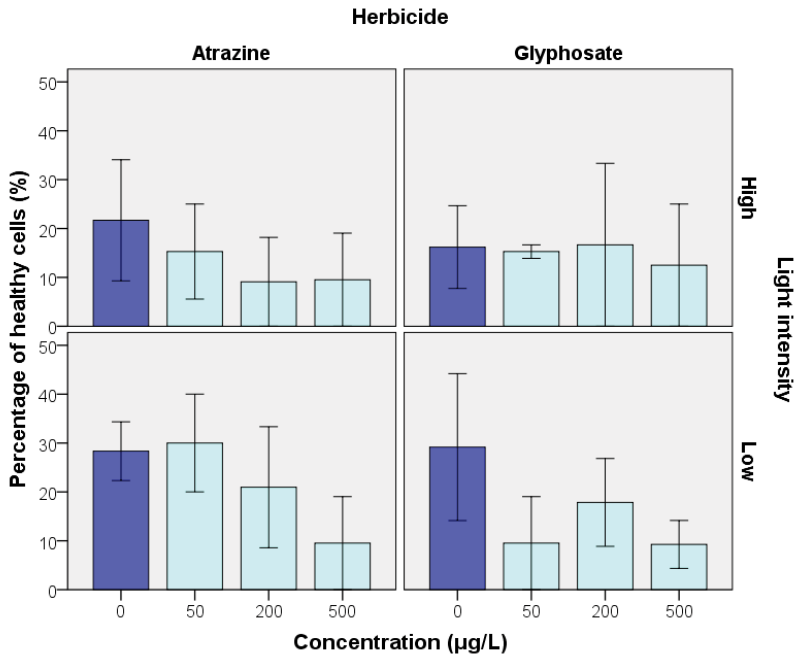



**Figure S18** Percentage of healthy diatom cells for *Epithemia* cf. *adanata* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

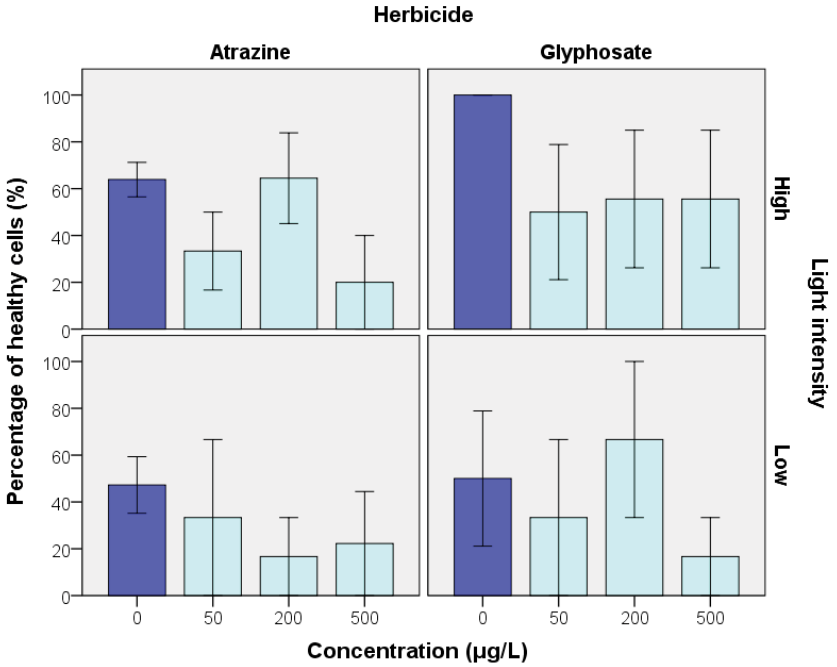



**Figure S19** Percentage of healthy diatom cells for *Mayamaea atomus* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

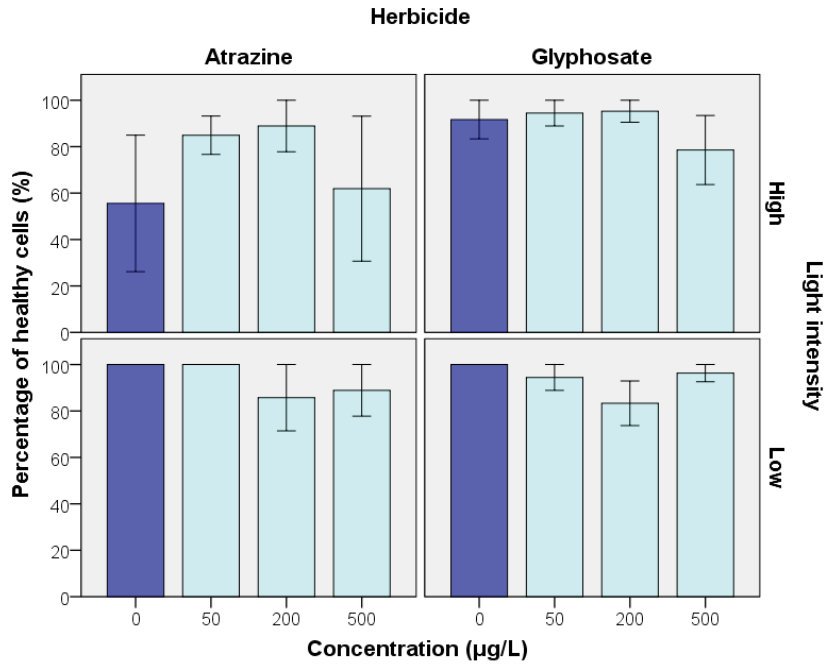



**Figure S20** Percentage of healthy diatom cells for *Navicula* cf. *cryptocephala* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

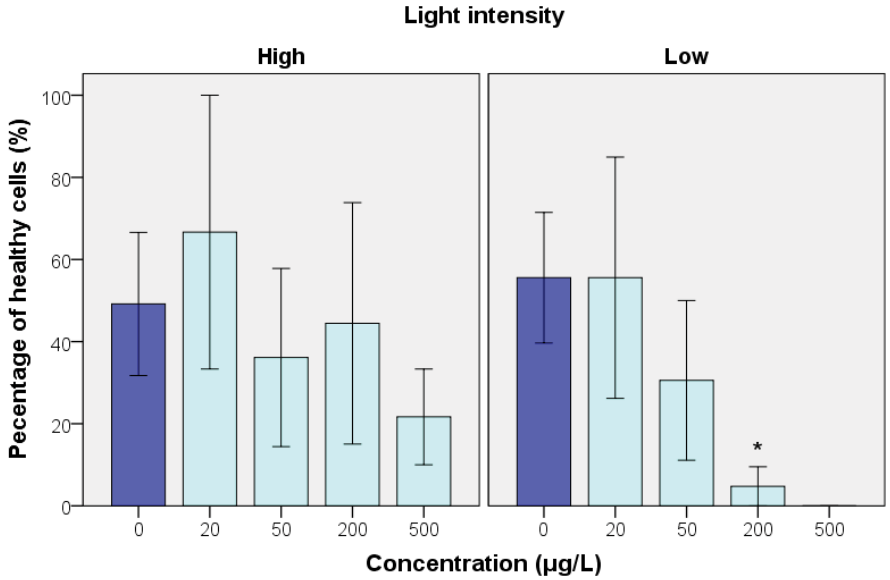



**Figure S21** Percentage of healthy diatom cells for *Navicula* cf. *cryptocephala* from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

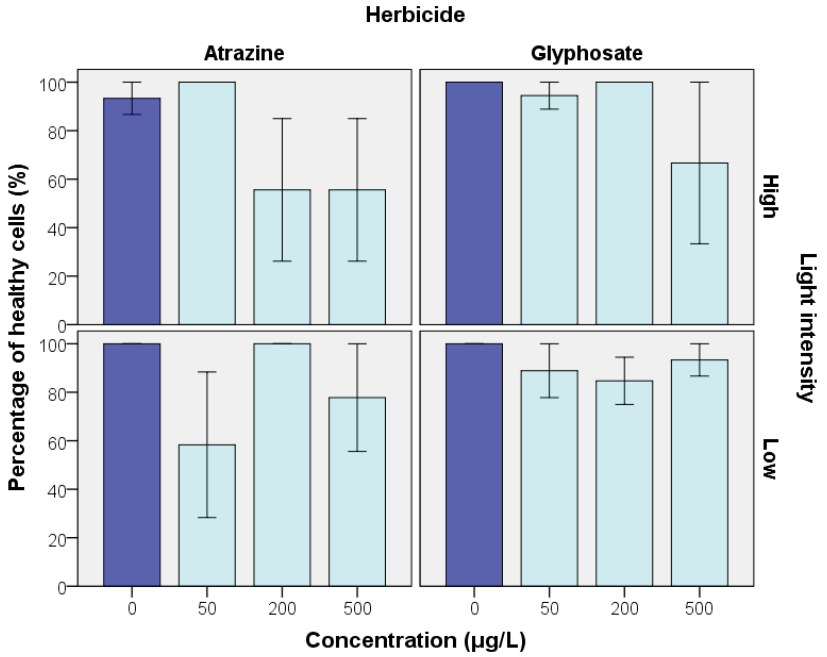



**Figure S22** Percentage of healthy diatom cells for *Navicula* cf. *cryptotenella* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

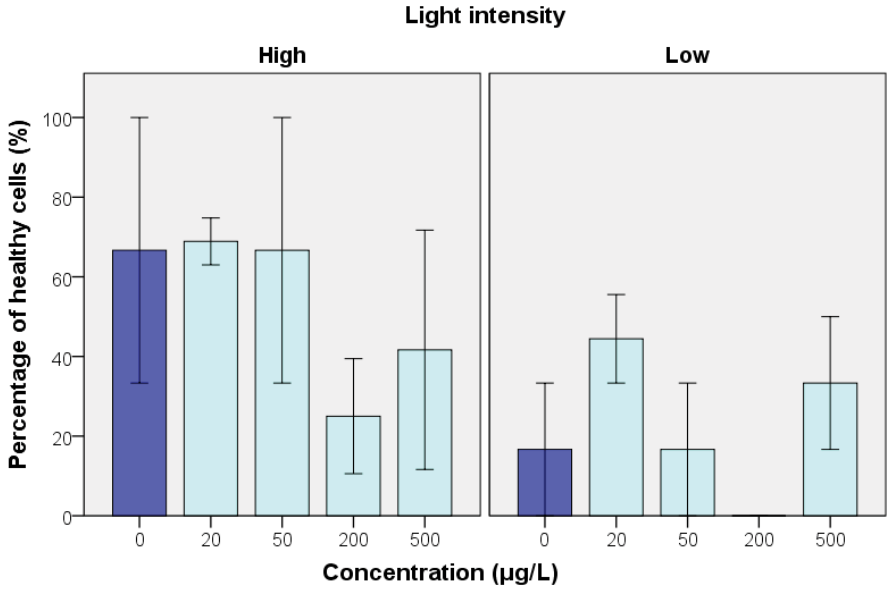



**Figure S23** Percentage of healthy diatom cells for *Navicula* cf. *cryptotenella* from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

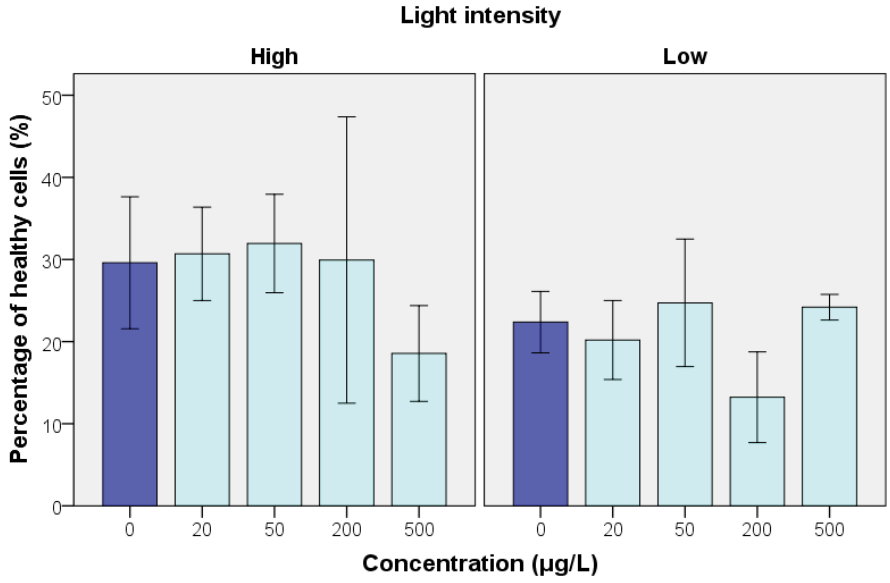



**Figure S24** Percentage of healthy diatom cells for *Navicula* cf. *radiosa* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

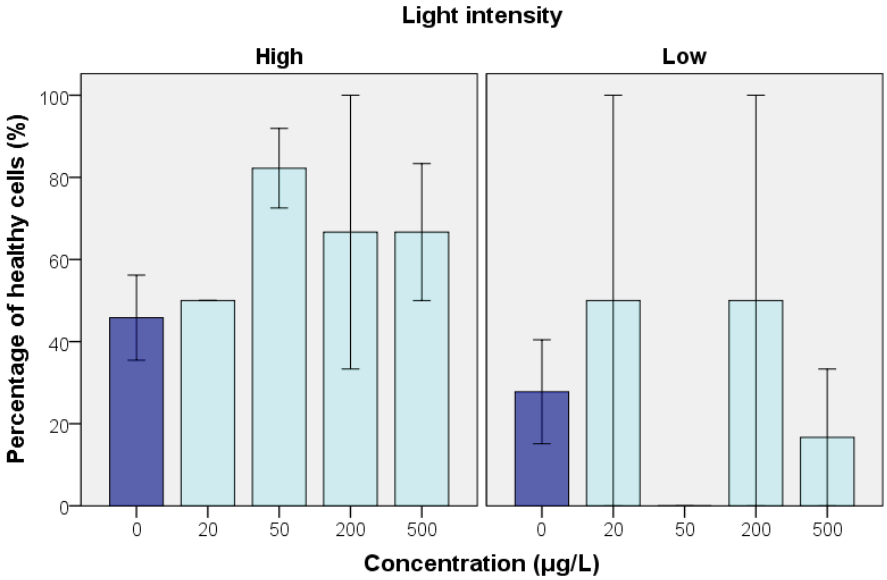



**Figure S25** Percentage of healthy diatom cells for *Navicula* cf. *rhynchocephala* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

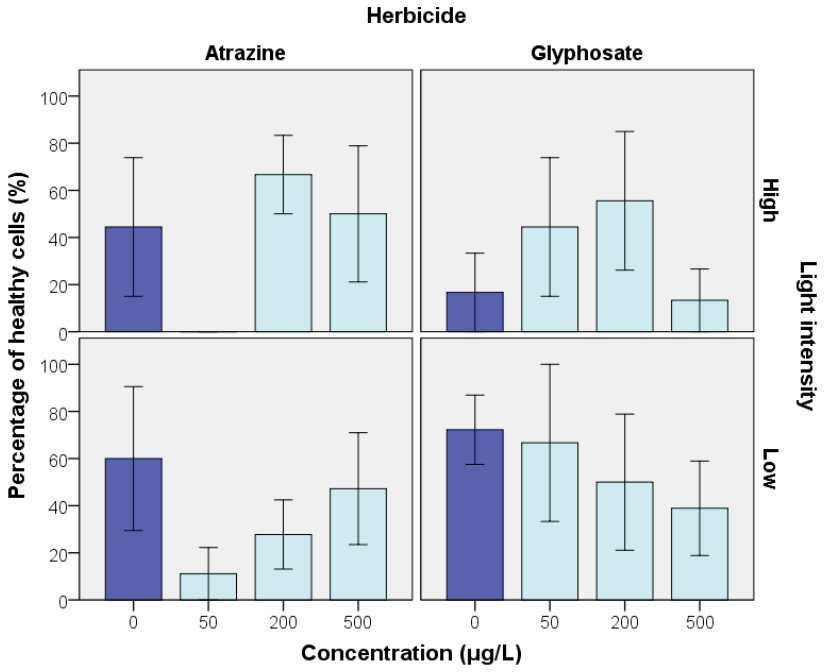



**Figure S26** Percentage of healthy diatom cells for *Navicula* cf. *subtillissima* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

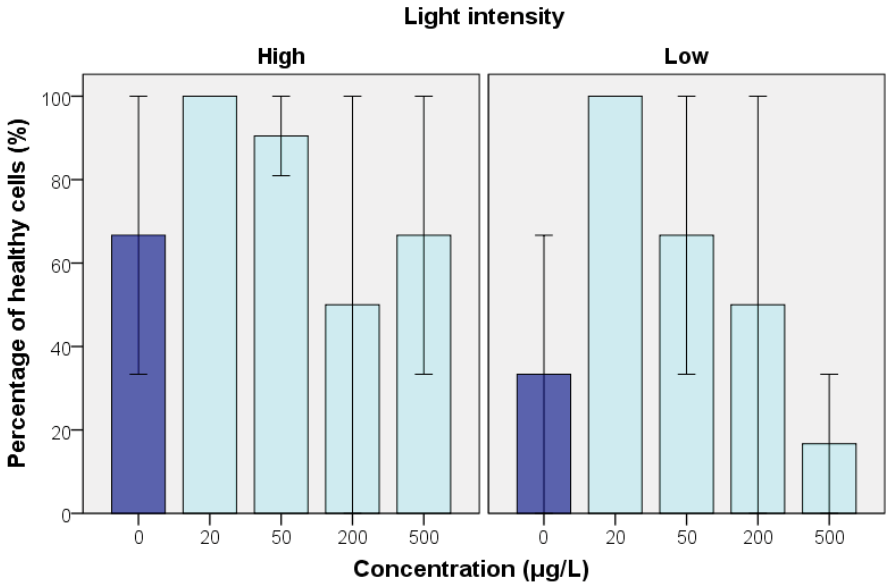



**Figure S27** Percentage of healthy diatom cells for *Nitzschia paleaceae* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

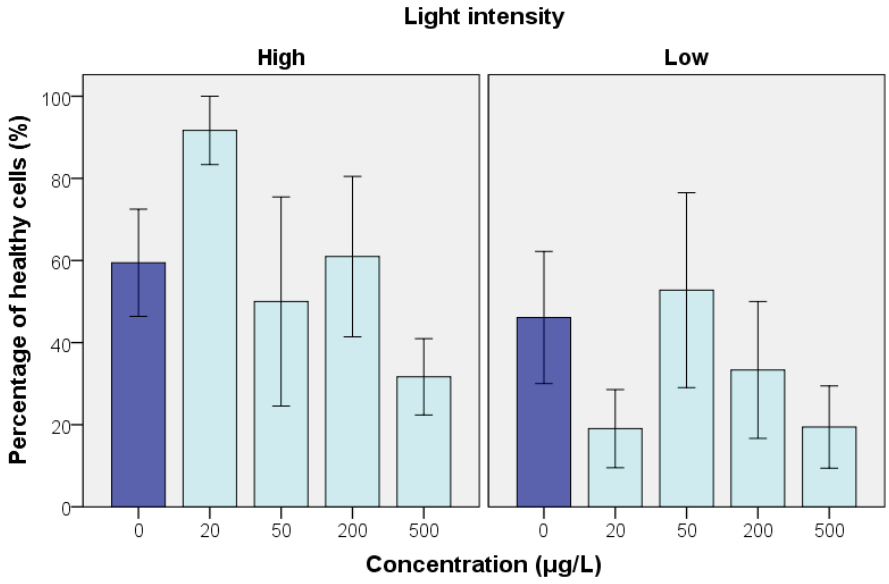



**Figure S28** Percentage of healthy diatom cells for *Nitzschia paleaceae* from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

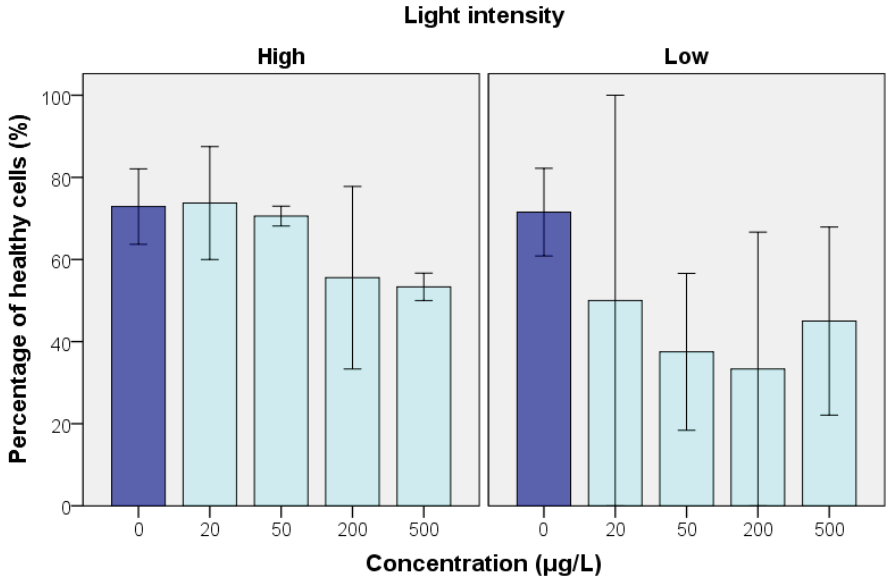



**Figure S29** Percentage of healthy diatom cells for *Pinnularia viridus* from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

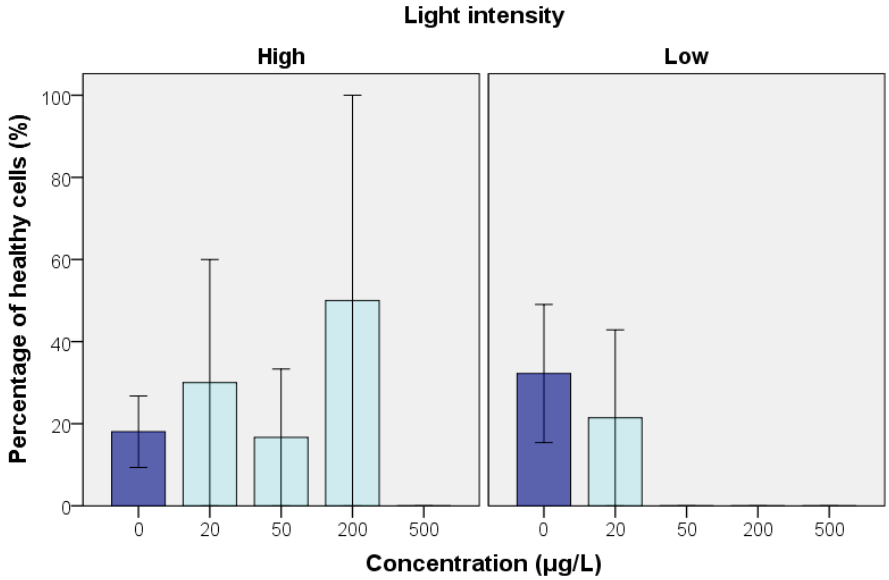



**Figure S30** Percentage of healthy diatom cells for *Cymbella* sp. from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

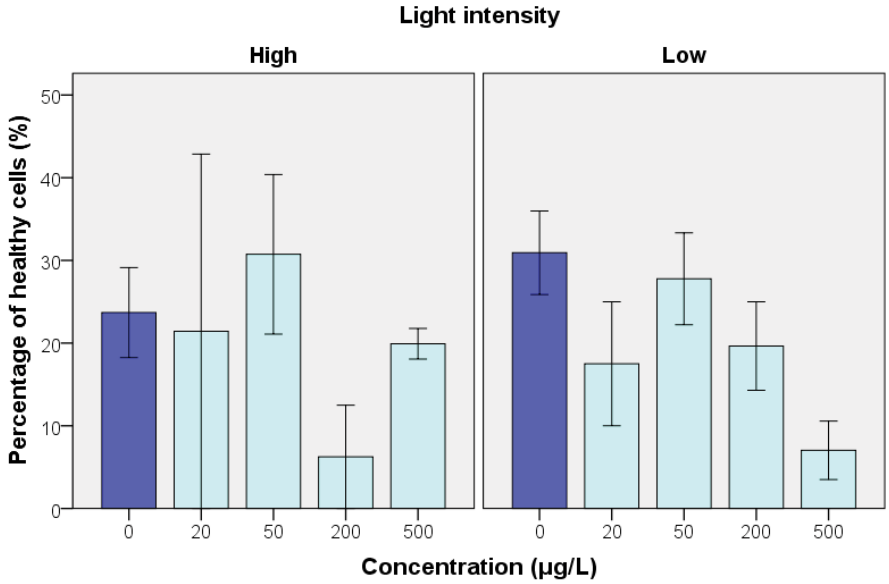



**Figure S31** Percentage of healthy diatom cells for *Encyconema* sp. from Liverpool Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

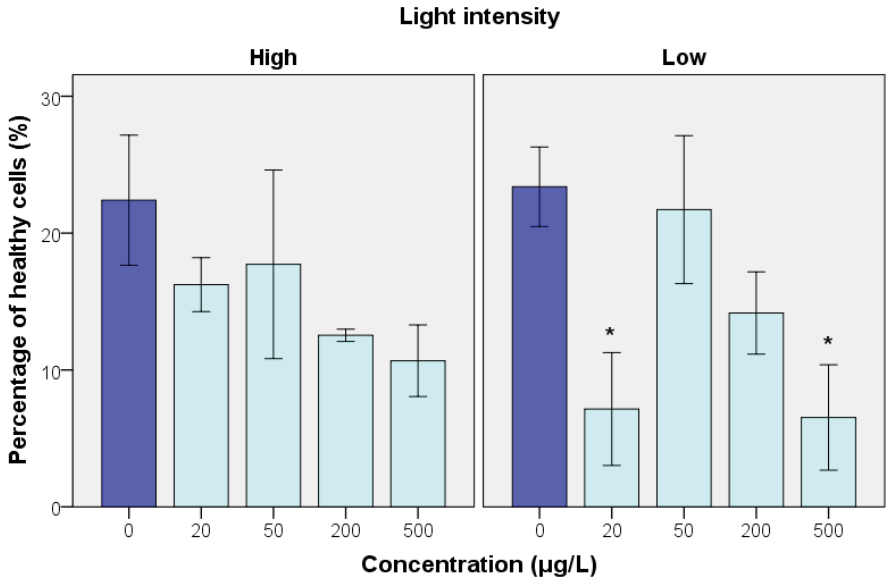



**Figure S32** Percentage of healthy diatom cells for *Cocconeis placentula* from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

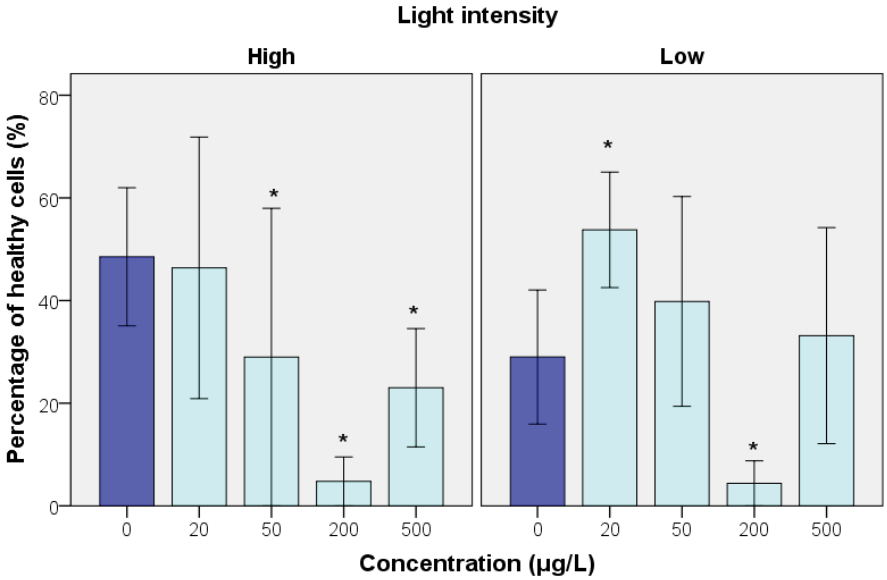



**Figure S33** Percentage of healthy diatom cells for *Fragillaria* sp. from Gowrie Ck, after 48 hr exposure to atrazine (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol m-2 $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

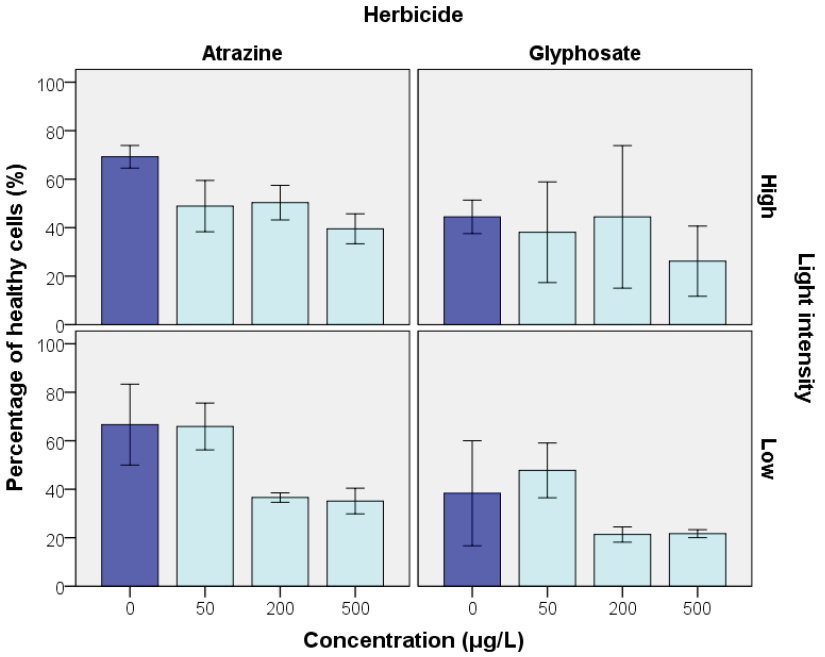



**Figure S34** Percentage of healthy diatom cells for *Eunotia* cf. *minor* from Alligator Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

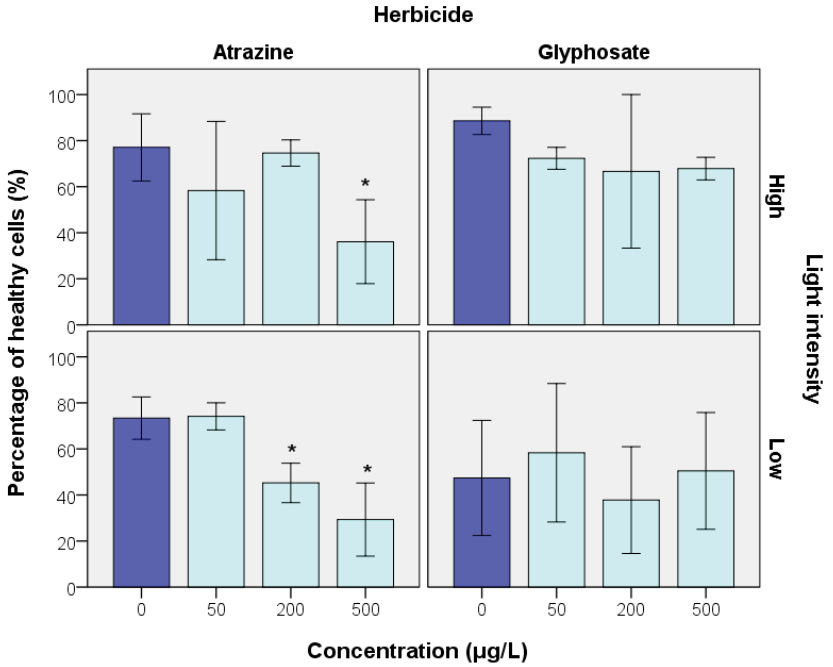



**Figure S35** Percentage of healthy diatom cells for *Melosira varians* from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

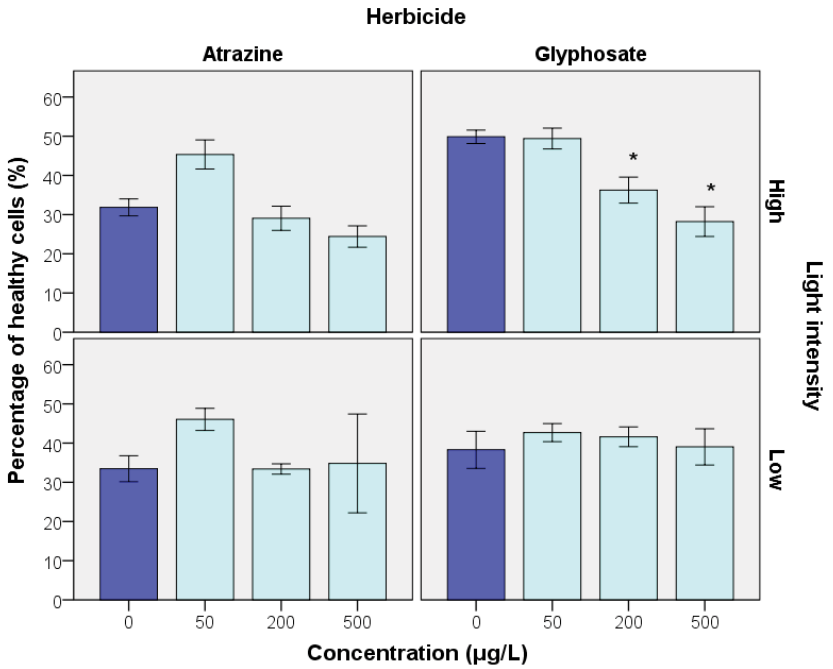



**Figure S36** Percentage of healthy diatom cells for *Pleurosira* sp. from Barratta Ck, after 48 hr exposure to either atrazine or glyphosate (50, 200 and 500 µg $L^{-1}$), at low and high light intensities (20 µmol $m^{-2}$ $s^{-1}$ and 100 µmol $m^{-2}$ $s^{-1}$).

* indicates statistical difference (p = <0.05) compared to controls in GLM analysis.

# APPENDIX D

**Supplementary Material for Chapter 5**

**Supplementary Table S1** Water quality measurements taken at time of diatom collection and measured atrazine concentrations in river water and toxicity test treatments.

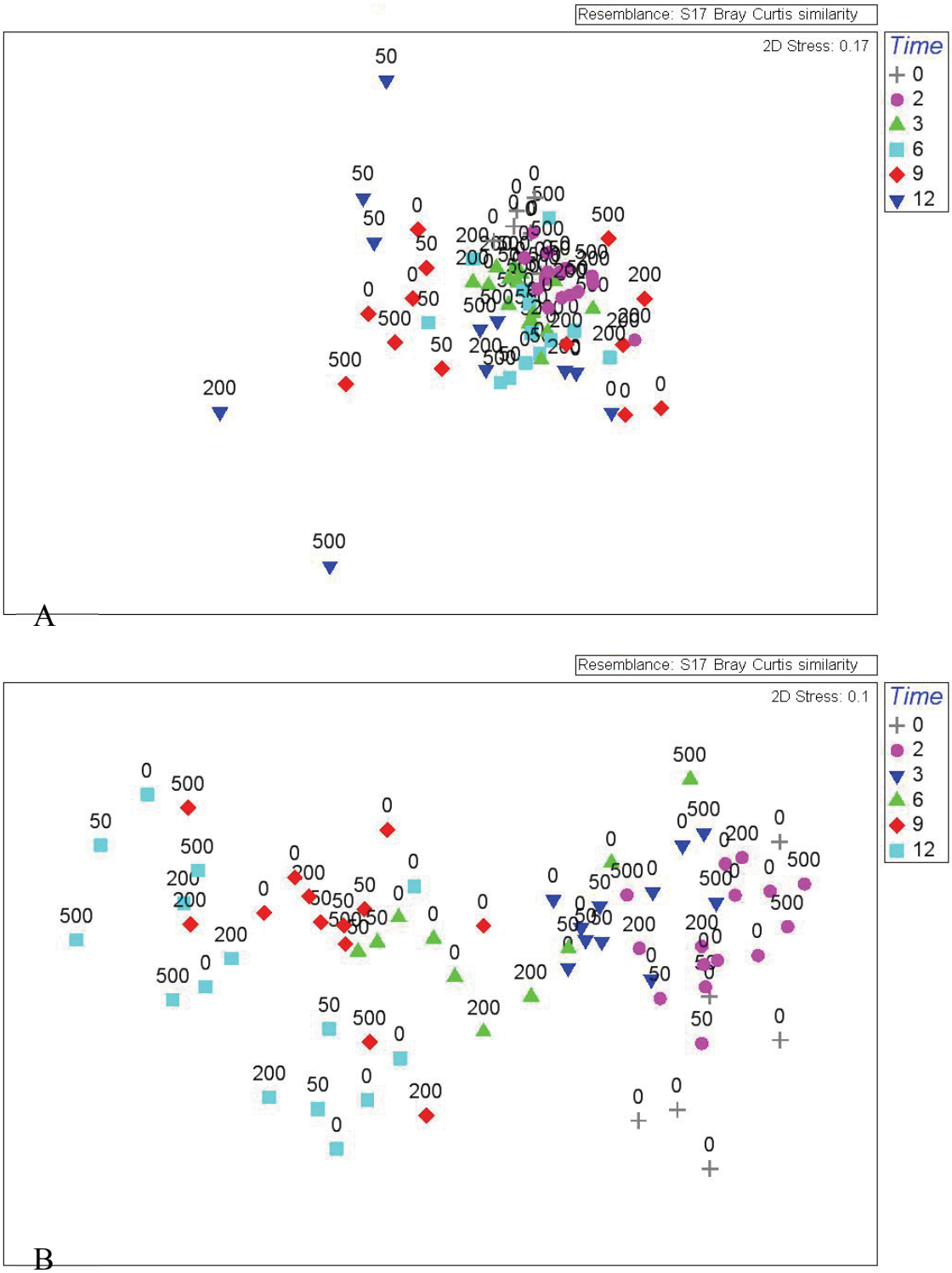
| | **Alligator Creek** | **Barratta Creek** |
|---|---|---|
| Water temperature (°C) | 28.7 | 25.0 |
| pH | 7.55 | 7.7 |
| Dissolved Oxygen (mg $L^{-1}$) | 8.83 | 5.33 |
| Conductivity (µS $cm^{-1}$ @ 25 °C) | 95.7 | 557 |
| Light at water surface (µmol $m^{-2}$ $s^{-1}$) | 78 - 916 | 65 - 1832 |
| Atrazine in river water (1 µg $L^{-1}$) | < LOR (1 µg $L^{-1}$) | 13 |
| Nominal test treatment 50 µg $L^{-1}$ | 44 & 41 | 51 & 54 |
| Nominal test treatment 200 µg $L^{-1}$ | 170 & 180 | - |
| Nominal test treatment 500 µg $L^{-1}$ | 450 & 500 | - |

**Supplementary Table S2** Species list and diatom cell count from Barratta and Alligator Creeks within samples sent for species verification taken at the start of the experiment (day 0).

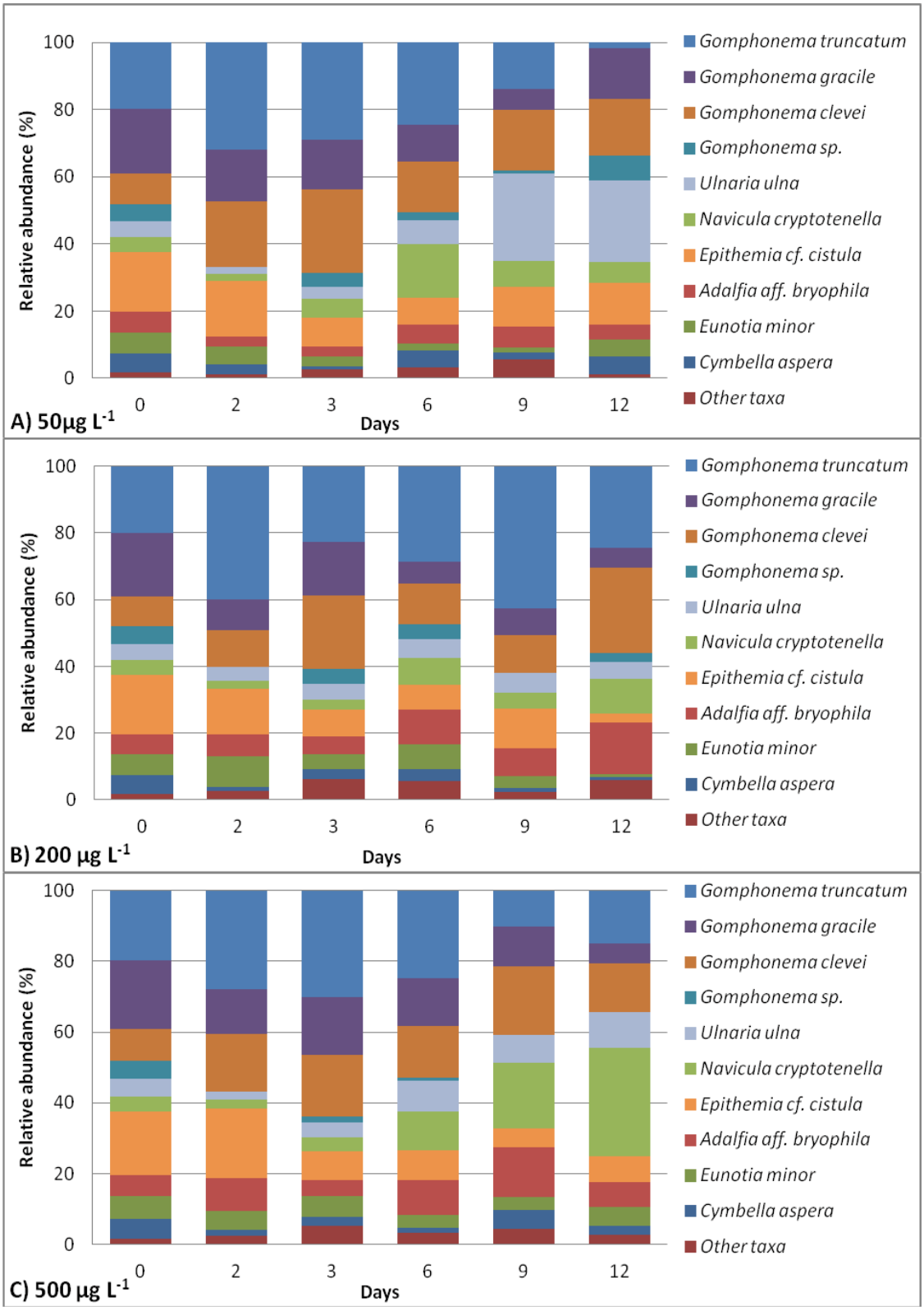
| **Diatom taxon** | **Alligator Creek** | **Barratta Creek** |
|---|---|---|
| *Achnanthidium minutissimum* | 116 | 7 |
| *Adlafia* aff. *bryophila* | 19 | 0 |
| *Amphora* aff. *coffeaeformis* | 0 | 1 |
| *Amphora pediculus* | 0 | 2 |
| *Cocconeis placentula* | 0 | 2 |
| *Cymbella aspera* | 2 | 0 |
| *Diploneis elliptica* | 0 | 2 |
| *Encyonema minuta* | 17 | 2 |
| *Eolimna subminuscula* | 4 | 0 |
| *Epithemia adnata* | 3 | 0 |
| *Epithemia cistula* | 15 | 0 |
| *Epithemia sorex* | 1 | 0 |
| *Eunotia* sp. | 2 | 0 |
| *Fragilaria capucina* var. *capucina* | 11 | 0 |
| *Gomphonema clevei* | 101 | 0 |
| *Gomphonema gracile* | 9 | 4 |
| *Gomphonema minutum* | 0 | 42 |
| *Gomphonema parvulum* | 0 | 15 |
| *Gomphonema* sp. | 9 | 0 |
| *Gomphonema truncatum* | 3 | 0 |
| *Luticola goeppertiana* | 0 | 3 |
| *Mayamaea atomus* | 0 | 22 |
| *Melosira varians* | 0 | 5 |
| *Navicula cryptocephala* | 0 | 2 |
| *Navicula cryptotenella* | 19 | 0 |
| *Navicula schroeterii* | 0 | 6 |
| *Navicula subtillissima* | 0 | 2 |
| *Nitzschia inconspicua* | 6 | 0 |
| *Nitzschia* sp. | 2 | 0 |
| *Pleurosira* sp. | 0 | 99 |
| *Ulnaria ulna* | 2 | 115 |
| Total | 341 | 331 |

**Supplementary Table S3** PERMANOVA results for the atrazine concentration effects on the diatom community on each experimental day (main test) and at each concentration compared to the control treatment (pair-wise tests). * indicates statistical significance ($p<0.05$).

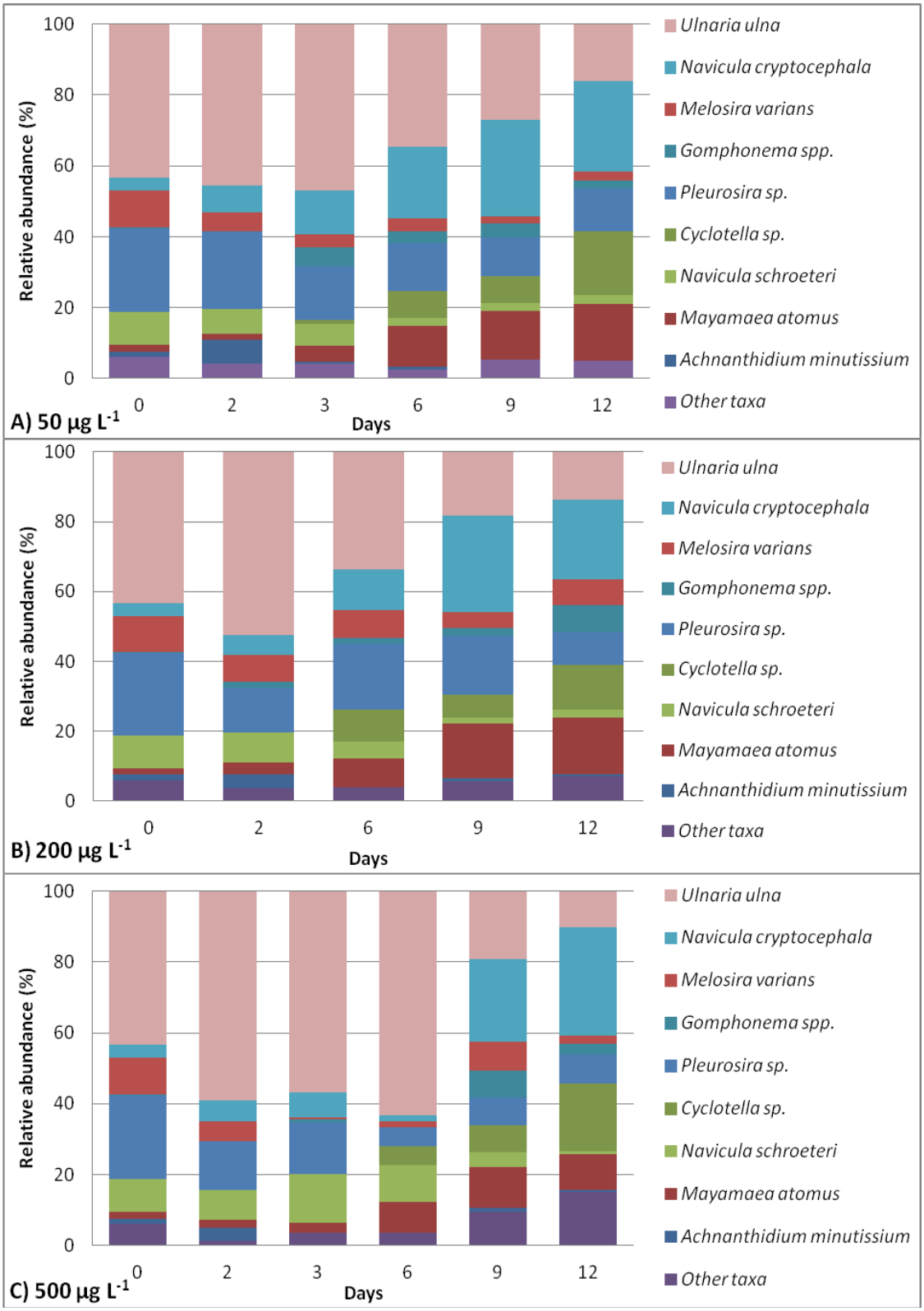
| **Alligator Creek** | **Concentration** | **p-value** | **Pseudo-F** | **df** | **Unique permutations** |
|---|---|---|---|---|---|
| Day 2 | Main test | 0.061 | 1.72 | 3 | 999 |
| | 50 | 0.635 | - | - | 84 |
| | 200 | 0.039* | - | - | 84 |
| | 500 | 0.435 | - | - | 84 |
| Day 3 | Main test | 0.481 | 0.97 | 3 | 998 |
| | 50 | 0.390 | - | - | 84 |
| | 200 | 0.160 | - | - | 84 |
| | 500 | 0.782 | - | - | 84 |
| Day 6 | Main test | 0.163 | 1.42 | 3 | 996 |
| | 50 | 0.071 | - | - | 56 |
| | 200 | 0.192 | - | - | 21 |
| | 500 | 0.025* | - | - | 56 |
| Day 9 | Main test | 0.110 | 1.68 | 3 | 997 |
| | 50 | 0.286 | - | - | 21 |
| | 200 | 0.331 | - | - | 56 |
| | 500 | 0.347 | - | - | 56 |
| Day 12 | Main test | 0.014* | 2.03 | 3 | 956 |
| | 50 | 0.095 | - | - | 10 |
| | 200 | 0.386 | - | - | 10 |
| | 500 | 0.092 | - | - | 10 |
| **Barratta Creek** | | **p-value** | **Pseudo-F** | **df** | **Unique permutations** |
| Day 2 | Main test | 0.043* | 2.02 | 3 | 998 |
| | 50 | 0.029* | - | - | 84 |
| | 200 | 0.299 | - | - | 84 |
| | 500 | 0.413 | - | - | 84 |
| Day 3 | Main test | 0.335 | 1.21 | 2 | 909 |
| | 50 | 0.925 | - | - | 84 |
| | 200 | - | - | - | - |
| | 500 | 0.314 | - | - | 28 |
| Day 6 | Main test | 0.156 | 1.76 | 3 | 956 |
| | 50 | 0.841 | - | - | 35 |
| | 200 | 0.767 | - | - | 15 |
| | 500 | 0.186 | - | - | 5 |
| Day 9 | Main test | 0.125 | 1.41 | 3 | 995 |
| | 50 | 0.665 | - | - | 35 |
| | 200 | 0.193 | - | - | 35 |
| | 500 | 0.368 | - | - | 35 |
| Day 12 | Main test | 0.275 | 1.28 | 3 | 998 |
| | 50 | 0.733 | - | - | 84 |
| | 200 | 0.273 | - | - | 84 |
| | 500 | 0.098 | - | - | 84 |




**Supplementary Figure S1** nMDS plot showing differences between samples across concentration treatments (50, 200 and 500 µg $L^{-1}$) and through time (days) at A – Alligator Creek and B – Barratta Creek




**Supplementary Figure S2** Relative abundance of the common diatom taxa from Alligator Creek over the course of the experiment within atrazine treatments: A) 50 µg $L^{-1}$, B) 200 µg $L^{-1}$ and C) 500 µg $L^{-1}$ and the control treatment (day 0). Diatom taxa combined in 'other taxa' category are *Epithemia* cf. *adanata*, *Nitzschia* cf. *sigmoidea* and *Pinnularia* sp.




**Supplementary Figure S3** Relative abundance of the common diatom taxa from Barratta Creek over the course of the experiment within atrazine treatments: A) 50 µg $L^{-1}$, B) 200 µg $L^{-1}$ and C) 500 µg $L^{-1}$ and the control treatment (day 0). Diatom taxa combined in 'other taxa' category are *Gyrosigma* sp., *Fragilaria* sp. *Pinnularia* sp. *Nitzschia* spp. and *Navicula* cf. *subtillisima*.

**Supplementary Table S4** SIMPER results Alligator Creek – relative abundance (%) of taxa most contributing to the difference between concentration treatment groups (50, 200 and 500 µg $L^{-1}$) and the control treatment on each day of exposure (2, 3, 6, 9, 12).

**Day 2**

**0 ug/L & 50ug/L Average dissimilarity = 17.75**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema clevei* | 15.58 | 19.51 | 3.04 | 1.68 | 17.10 | 17.10 |
| *Gomphonema truncatum* | 27.45 | 31.85 | 2.79 | 1.32 | 15.74 | 32.85 |
| *Gomphonema gracile* | 13.63 | 15.18 | 1.77 | 1.47 | 9.96 | 42.81 |
| *Eunotia minor* | 6.32 | 5.37 | 1.57 | 1.28 | 8.84 | 51.65 |

**0 ug/L & 200ug/L Average dissimilarity = 24.81**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 27.45 | 39.85 | 6.20 | 1.54 | 25.00 | 25.00 |
| *Gomphonema clevei* | 15.58 | 10.79 | 3.09 | 1.26 | 12.46 | 37.46 |
| *Gomphonema gracile* | 13.63 | 9.38 | 3.01 | 1.26 | 12.12 | 49.58 |
| *Epithemia cf. cistula* | 17.26 | 13.80 | 2.29 | 1.58 | 9.22 | 58.80 |

**0 ug/L & 500ug/L Average dissimilarity = 19.68**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema clevei* | 15.58 | 16.20 | 2.77 | 1.67 | 14.07 | 14.07 |
| *Adlalfia cf. bryophila* | 3.77 | 9.17 | 2.70 | 2.30 | 13.73 | 27.80 |
| *Gomphonema gracile* | 13.63 | 12.72 | 2.66 | 1.49 | 13.53 | 41.33 |
| *Gomphonema truncatum* | 27.45 | 27.85 | 1.93 | 1.43 | 9.82 | 51.15 |

**Day 3**

**0 ug/L & 50ug/L Average dissimilarity = 18.73**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 28.54 | 28.92 | 3.50 | 1.56 | 18.66 | 18.66 |
| *Gomphonema clevei* | 18.84 | 24.94 | 3.13 | 1.33 | 16.71 | 35.37 |
| *Epithemia cf. cistula* | 11.22 | 8.77 | 2.36 | 1.40 | 12.59 | 47.96 |
| *Gomphonema gracile* | 18.93 | 14.73 | 2.27 | 1.65 | 12.13 | 60.09 |

**0 ug/L & 200ug/L Average dissimilarity = 20.77**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 28.54 | 22.65 | 4.88 | 1.84 | 23.49 | 23.49 |
| *Gomphonema gracile* | 18.93 | 16.22 | 2.10 | 1.09 | 10.11 | 33.59 |
| *Gomphonema clevei* | 18.84 | 21.90 | 2.05 | 1.06 | 9.89 | 43.49 |
| *Navicula cryptotenella* | 5.62 | 2.97 | 1.91 | 1.45 | 9.21 | 52.70 |

**0 ug/L & 500ug/L Average dissimilarity = 19.88**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 28.54 | 30.19 | 3.09 | 1.41 | 15.53 | 15.53 |
| *Gomphonema clevei* | 18.84 | 17.41 | 2.16 | 1.28 | 10.85 | 26.38 |
| *Epithemia cf. cistula* | 11.22 | 8.15 | 2.14 | 1.21 | 10.77 | 37.15 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Gomphonema gracile* | 18.93 | 16.11 | 2.04 | 1.35 | 10.24 | 47.39 |

**Day 6**

**0 ug/L & 50ug/L Average dissimilarity = 26.73**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 36.41 | 24.31 | 6.52 | 1.17 | 24.40 | 24.40 |
| *Navicula cryptotenella* | 6.14 | 15.65 | 4.92 | 1.72 | 18.41 | 42.82 |
| *Gomphonema clevei* | 20.09 | 14.79 | 3.23 | 1.41 | 12.07 | 54.88 |
| *Epithemia cf. cistula* | 5.83 | 7.91 | 2.04 | 1.69 | 7.65 | 62.53 |

**0 ug/L & 200ug/L Average dissimilarity = 29.23**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 36.41 | 28.53 | 6.57 | 1.34 | 22.48 | 22.48 |
| *Gomphonema clevei* | 20.09 | 12.15 | 3.97 | 2.00 | 13.59 | 36.07 |
| *Adlalfia cf. bryophila* | 5.13 | 10.24 | 2.61 | 1.33 | 8.93 | 45.00 |
| *Navicula cryptotenella* | 6.14 | 7.98 | 2.60 | 1.47 | 8.90 | 53.90 |

**0 ug/L & 500ug/L Average dissimilarity = 29.45**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 36.41 | 24.89 | 5.76 | 1.75 | 19.55 | 19.55 |
| *Navicula cryptotenella* | 6.14 | 10.82 | 3.88 | 1.29 | 13.19 | 32.74 |
| *Gomphonema gracile* | 9.87 | 13.27 | 3.30 | 1.59 | 11.22 | 43.96 |
| *Ulnaria ulna* | 4.21 | 8.80 | 3.25 | 1.91 | 11.03 | 54.99 |

**Day 9**

**0 ug/L & 50ug/L Average dissimilarity = 43.25**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 24.58 | 13.82 | 10.83 | 1.28 | 25.05 | 25.05 |
| *Ulnaria ulna* | 8.65 | 26.13 | 8.74 | 4.51 | 20.21 | 45.26 |
| *Gomphonema clevei* | 17.21 | 18.25 | 6.03 | 2.95 | 13.94 | 59.19 |
| *Gomphonema gracile* | 11.90 | 6.08 | 5.05 | 1.98 | 11.67 | 70.87 |

**0 ug/L & 200ug/L Average dissimilarity = 43.85**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 24.58 | 42.68 | 13.72 | 1.83 | 31.28 | 31.28 |
| *Gomphonema clevei* | 17.21 | 11.12 | 5.74 | 1.37 | 13.10 | 44.38 |
| *Gomphonema gracile* | 11.90 | 8.00 | 4.95 | 1.38 | 11.30 | 55.68 |
| *Epithemia cf. cistula* | 6.34 | 11.75 | 3.72 | 1.14 | 8.48 | 64.16 |

**0 ug/L & 500ug/L Average dissimilarity = 43.87**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 24.58 | 10.35 | 11.44 | 1.14 | 26.08 | 26.08 |
| *Navicula cryptotenella* | 8.61 | 18.62 | 7.88 | 1.48 | 17.96 | 44.04 |
| *Gomphonema clevei* | 17.21 | 19.14 | 6.19 | 1.66 | 14.11 | 58.14 |
| *Gomphonema gracile* | 11.90 | 11.24 | 4.53 | 2.27 | 10.33 | 68.48 |

**Day 12**

**0 ug/L & 50ug/L Average dissimilarity = 61.31**

| Species | Abundance | Abundance | Av. | Diss/SD | Contrib% | Cum. |
|---|---|---|---|---|---|---|

| | 0 ug/L | 50 ug/L | Diss | | | % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 44.60 | 1.74 | 21.43 | 13.44 | 34.95 | 34.95 |
| *Ulnaria ulna* | 3.85 | 24.03 | 10.09 | 1.95 | 16.46 | 51.41 |
| *Gomphonema gracile* | 1.90 | 14.95 | 7.16 | 1.28 | 11.67 | 63.08 |
| *Epithemia cf. cistula* | 8.28 | 12.06 | 5.35 | 1.22 | 8.72 | 71.81 |

**0 ug/L & 200ug/L Average dissimilarity = 43.16**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 44.60 | 24.54 | 10.35 | 1.05 | 23.97 | 23.97 |
| *Gomphonema clevei* | 12.07 | 25.41 | 7.16 | 1.62 | 16.59 | 40.56 |
| *Adlalfia cf. bryophila* | 6.38 | 15.31 | 6.74 | 0.86 | 15.62 | 56.18 |
| *Cymbella aspera* | 8.33 | 0.75 | 3.95 | 1.06 | 9.15 | 65.34 |

**0 ug/L & 500ug/L Average dissimilarity = 48.75**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Gomphonema truncatum* | 44.60 | 14.92 | 14.84 | 2.54 | 30.44 | 30.44 |
| *Navicula cryptotenella* | 7.01 | 30.51 | 11.75 | 0.96 | 24.10 | 54.54 |
| *Cymbella aspera* | 8.33 | 2.76 | 3.53 | 1.14 | 7.24 | 61.78 |
| *Gomphonema clevei* | 12.07 | 13.70 | 3.44 | 1.45 | 7.06 | 68.85 |

**Supplementary Table S5** SIMPER results Barratta Creek – relative abundance (%) of taxa most contributing to the difference between concentration treatment groups (50, 200 and 500 µg $L^{-1}$) and the control treatment on each day of exposure (2, 3, 6, 9, 12).

**Day 2**

**0 ug/L & 50ug/L Average dissimilarity = 17.75**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 58.86 | 45.27 | 6.79 | 2.27 | 29.88 | 29.88 |
| *Pleurosira* sp. | 16.47 | 21.90 | 3.21 | 1.38 | 14.10 | 43.98 |
| *Achnanthidium minutissimum* | 1.08 | 6.70 | 2.81 | 1.46 | 12.37 | 56.35 |

**0 ug/L & 200ug/L Average dissimilarity = 24.81**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 58.86 | 52.59 | 4.10 | 1.52 | 23.76 | 23.76 |
| *Pleurosira* sp. | 16.47 | 12.89 | 3.13 | 1.34 | 18.14 | 41.90 |
| *Navicula cryptocephala* | 3.23 | 5.50 | 1.79 | 1.18 | 10.34 | 52.24 |

**0 ug/L & 500ug/L Average dissimilarity = 19.68**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 58.86 | 59.15 | 3.61 | 1.34 | 18.91 | 18.91 |
| *Melosira varians* | 8.20 | 5.50 | 3.38 | 2.01 | 17.68 | 36.59 |
| *Pleurosira* sp. | 16.47 | 13.91 | 3.15 | 1.49 | 16.48 | 53.07 |

**Day 3**

**0 ug/L & 50ug/L Average dissimilarity = 18.73**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 49.40 | 46.52 | 2.73 | 1.06 | 17.29 | 17.29 |
| *Navicula cryptocephala* | 10.59 | 12.19 | 2.21 | 1.44 | 13.96 | 31.25 |
| *Melosira varians* | 3.39 | 3.66 | 1.76 | 1.37 | 11.11 | 42.36 |
| *Pleurosira* sp. | 14.75 | 15.00 | 1.68 | 1.41 | 10.64 | 52.99 |

**0 ug/L & 500ug/L Average dissimilarity = 19.88**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 49.40 | 56.93 | 4.89 | 1.64 | 22.75 | 22.75 |
| *Navicula schroeterii* | 6.18 | 13.67 | 4.50 | 1.33 | 20.95 | 43.71 |
| *Navicula cryptocephala* | 10.59 | 6.94 | 2.58 | 1.67 | 12.01 | 55.72 |

**Day 6**

**0 ug/L & 50ug/L Average dissimilarity = 26.73**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 35.53 | 34.52 | 4.57 | 1.13 | 21.49 | 21.49 |
| *Navicula cryptocephala* | 17.57 | 19.76 | 3.06 | 1.23 | 14.37 | 35.86 |
| *Pleurosira* sp. | 11.70 | 13.51 | 2.85 | 1.79 | 13.42 | 49.28 |
| *Mayamaea atomus* | 9.32 | 11.64 | 2.54 | 1.71 | 11.95 | 61.23 |

**0 ug/L & 200ug/L Average dissimilarity = 29.23**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Navicula cryptocephala* | 17.57 | 11.68 | 3.92 | 1.47 | 16.75 | 16.75 |
| *Pleurosira* sp. | 11.70 | 18.57 | 3.92 | 1.66 | 16.73 | 33.48 |
| *Ulnaria ulna* | 35.53 | 33.36 | 3.00 | 0.97 | 12.83 | 46.31 |
| *Cyclotella* sp. | 4.84 | 9.05 | 2.85 | 1.31 | 12.18 | 58.48 |

**0 ug/L & 500ug/L Average dissimilarity = 29.45**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Ulnaria ulna* | 35.53 | 62.07 | 13.27 | 2.96 | 34.42 | 34.42 |
| *Navicula cryptocephala* | 17.57 | 1.72 | 7.92 | 2.34 | 20.56 | 54.98 |
| *Pleurosira* sp. | 11.70 | 5.17 | 3.27 | 0.98 | 8.47 | 63.45 |

**Day 9**

**0 ug/L & 50ug/L Average dissimilarity = 43.25**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| Ulnaria ulna | 25.64 | 26.95 | 3.52 | 1.61 | 17.29 | 17.29 |
| Mayamaea atomus | 10.86 | 13.45 | 2.66 | 2.38 | 13.09 | 30.38 |
| Pleurosira sp. | 10.56 | 10.89 | 2.55 | 1.67 | 12.55 | 42.93 |
| Navicula cryptocephala | 27.31 | 26.81 | 1.70 | 1.65 | 8.36 | 51.29 |

**0 ug/L & 200ug/L Average dissimilarity = 43.85**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| Ulnaria ulna | 25.64 | 18.19 | 5.02 | 1.24 | 17.63 | 17.63 |
| Navicula cryptocephala | 27.31 | 27.49 | 4.66 | 2.16 | 16.37 | 34.00 |
| Pleurosira sp. | 10.56 | 16.64 | 4.09 | 1.27 | 14.38 | 48.38 |
| Mayamaea atomus | 10.86 | 15.45 | 3.16 | 3.09 | 11.11 | 59.49 |

**0 ug/L & 500ug/L Average dissimilarity = 43.87**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| Ulnaria ulna | 25.64 | 19.28 | 4.02 | 1.13 | 14.68 | 14.68 |
| Navicula cryptocephala | 27.31 | 23.26 | 3.90 | 1.98 | 14.26 | 28.95 |
| Pleurosira sp. | 10.56 | 7.75 | 3.37 | 1.34 | 12.30 | 41.25 |
| Melosira varians | 3.26 | 8.03 | 3.12 | 1.17 | 11.39 | 52.64 |

**Day 12**

**0 ug/L & 50ug/L Average dissimilarity = 61.31**

| Species | Abundance 0 ug/L | Abundance 50 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Navicula cryptocephala* | 18.76 | 24.73 | 6.80 | 1.25 | 21.37 | 21.37 |
| *Cyclotella sp.* | 17.54 | 17.79 | 4.92 | 1.67 | 15.46 | 36.83 |
| *Ulnaria ulna* | 20.12 | 15.88 | 4.39 | 1.30 | 13.80 | 50.64 |

**0 ug/L & 200ug/L Average dissimilarity = 43.16**

| Species | Abundance 0 ug/L | Abundance 200 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Navicula cryptocephala* | 18.76 | 22.37 | 5.56 | 1.44 | 17.44 | 17.44 |
| *Ulnaria ulna* | 20.12 | 13.40 | 4.51 | 1.55 | 14.12 | 31.56 |
| *Cyclotella sp.* | 17.54 | 12.51 | 4.25 | 2.10 | 13.32 | 44.88 |
| *Mayamaea atomus* | 10.75 | 15.93 | 3.30 | 1.42 | 10.36 | 55.24 |

**0 ug/L & 500ug/L Average dissimilarity = 48.75**

| Species | Abundance 0 ug/L | Abundance 500 ug/L | Av. Diss | Diss/SD | Contrib% | Cum. % |
|---|---|---|---|---|---|---|
| *Navicula cryptocephala* | 18.76 | 30.39 | 6.58 | 1.68 | 19.61 | 19.61 |
| *Ulnaria ulna* | 20.12 | 10.18 | 5.43 | 1.50 | 16.19 | 35.80 |
| *Cyclotella sp.* | 17.54 | 19.01 | 4.82 | 1.40 | 14.38 | 50.18 |

**Supplementary Table S6** GLM results showing p-values for successional effects (across all days), and effect of atrazine concentration at each exposure day.

| Taxa | Successional effect | Overall concentration effect | | | | |
|---|---|---|---|---|---|---|
| | | Day 2 | Day 3 | Day 6 | Day 9 | Day 12 |
| **Alligator Creek:** | | | | | | |
| *Adlafia cf. bryophila* | 0.017* | 0.878 | 0.578 | 0.935 | 0.141 | 0.771 |
| *Cymbella* cf. *aspera* | 0.243 | 0.044* | 0.149 | 0.002* | 0.001* | 0.027* |
| *Epithemia* cf. *adanata* | 0.542 | 0.319 | 0.538 | 0.837 | 0.200 | 0.494 |
| *Epithemia cf. cistula* | <0.001* | <0.001* | <0.001* | 0.015* | <0.001* | 0.678 |
| *Eunotia* cf. *minor* | 0.141 | 0.435 | 0.152 | 0.472 | 0.934 | 0.101 |
| *Gomphonema gracile* | <0.001* | 0.353 | 0.189 | 0.727 | 0.893 | 0.003* |
| *Gomphonema clevei* | <0.001* | 0.006* | 0.001* | 0.278 | 0.001* | 0.339 |
| *Gomphonema truncatum* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| *Navicula cryptotenella* | 0.540 | 0.585 | / | / | / | 0.607 |
| *Nitzschia sigmoidea* | 0.940 | 1.000 | 0.891 | 0.980 | 0.826 | 0.960 |
| *Pinnularia sp.* | 0.677 | - | 0.982 | 1.000 | 0.522 | 1.000 |
| *Ulnaria ulna* | 0.028* | 0.017* | 0.257 | 0.011* | <0.001* | <0.001* |
| **Barratta Creek:** | | | | | | |
| *Achnanthidium* sp. | 0.054 | 0.169 | 0.463 | 1.000 | 0.168 | 0.065 |
| *Amphora* spp. | 0.379 | 1.000 | 1.000 | 0.982 | 1.000 | 0.280 |
| *Cyclotella* sp. | <0.001* | - | 0.091 | 0.007* | <0.001* | 0.002* |
| *Cymbella* sp. | 0.831 | 1.000 | 0.994 | 1.000 | 0.996 | 0.949 |
| *Gomphonema* spp. | 0.205 | 0.290 | 0.080 | 0.145 | 0.430 | 0.003* |
| *Gyrosigma* sp. | 0.639 | 0.982 | 0.814 | 0.268 | 0.975 | 0.434 |
| *Mayamaea atomus* | 0.008* | 0.766 | 0.222 | 0.103 | 0.003* | <0.001* |
| *Melosira varians* | 0.073 | 0.050* | 0.005* | 0.012* | 0.162 | 0.023* |
| *Navicula schroeterii* | 0.182 | 0.579 | 0.477 | 0.992 | 0.058 | 0.047* |
| *Navicula subtillissima* | 0.449 | 0.867 | 0.796 | 0.874 | 0.958 | 0.207 |
| *Navicula cryptocephala* | 0.576 | 0.867 | 0.754 | 0.006* | 0.373 | 0.002* |
| *Nitzschia* sp. | 0.120 | - | 0.976 | - | - | 0.852 |
| *Pinnularia* sp. | 0.205 | 0.965 | 0.246 | 0.496 | 0.869 | 0.761 |
| *Pleurosira* sp. | 0.045* | 0.018* | <0.001* | <0.001* | <0.001* | <0.001* |
| *Ulnaria ulna* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |

Note: - incalculable taxa absent
/ all cells healthy

# APPENDIX E

## Supplementary Material for Chapter 6

**Supplementary Table S1** Locations of the study sites

| Site Name | Catchment | Location |
|---|---|---|
| Burnett River | Burnett | -25.04928, 152.09853 |
| Comet River | Fitzroy | -23.61247, 148.55138 |
| Fitzroy River | Fitzroy | -23.38111, 150.51691 |
| Sandy Creek | Plane | -21.28368, 149.01983 |
| Pioneer River | Pioneer | -21.14407, 149.07528 |
| Finch Hatton Creek | Pioneer | -21.07681, 148.63589 |
| Burdekin River | Burdekin | -19.64302, 147.39608 |
| Barratta Creek | Haughton | -19.70618, 147.14778 |
| Bluewater Creek | Black | -19.23952, 146.44566 |
| Herbert River | Herbert | -18.6316, 146.14013 |
| Tully River | Tully | -17.99361, 145.9411 |
| Bulgan Creek | Tully | -17.88245, 145.92297 |
| North Johnstone River | Johnstone | -17.54694, 145.93166 |
| Russell River | Mulgrave-Russell | -17.448658, 145.852609 |

**Supplementary Table S2** Herbicide sensitivity classification of benthic diatoms from selected literature

| ***Taxa*** | **Classification** | **Citation** |
|---|---|---|
| *Cocconeis placentula* | Tolerant | 1. Debenest et al. 2009 |
| *Achnanthidium catenatum* | Sensitive | 2. Larras et al. 2014a |
| *Achnanthidium minutissumum* | Tolerant | 3. Larras et al. 2013 |
| *Achnanthidium minutissumum* | Tolerant | 2. Larras et al. 2014a |
| *Achnanthidium minutissumum* | Tolerant | 4. Paule et al. 2015 |
| *Craticula accomoda* | Tolerant | 5. Larras et al. 2012 |
| *Cyclotella meneghiniana* | Sensitive | 5. Larras et al. 2012 |
| *Cyclotella meneghiniana* | Sensitive | 6. Larras et al. 2014b |
| *Diploneis oblongella* | Sensitive | 7. Ricart et al. 2009 |
| *Encyonema minutum* | Sensitive | 5. Larras et al. 2012 |
| *Encyonema minutum* | Sensitive | 3. Larras et al. 2013 |
| *Encyonema silesiacum* | Sensitive | 3. Larras et al. 2013 |
| *Eolimna minima* | Sensitive | 1. Debenest et al. 2009 |
| *Eolimna minima* | Tolerant | 5. Larras et al. 2012 |
| *Eolimna minima* | Tolerant | 8. Tiam et al. 2015 |
| *Fistulifera saprophila* | Tolerant | 6. Larras et al. 2014b |
| *Fragilaria capucina* var. *gracilis* | Tolerant | 9. Rotter et al. 2013 |
| *Fragilaria capucina* var. *vaucheriae* | Sensitive | 5. Larras et al. 2012 |
| *Fragilaria capucina* var. *vaucheriae* | Sensitive | 6. Larras et al. 2014b |
| *Fragilaria crotonensis* | Sensitive | 6. Larras et al. 2014b |
| *Gomphomena parvulum* | Tolerant | 5. Larras et al. 2012 |
| *Mayamaea fossalis* | Tolerant | 5. Larras et al. 2012 |
| *Melosira varians* | Tolerant | 1. Debenest et al. 2009 |
| *Navicula atomus* var. *permitis* | Tolerant | 7. Ricart et al. 2009 |
| *Navicula cryptotenella* | Tolerant | 7. Ricart et al. 2009 |
| *Navicula gregaria* | Sensitive | 7. Ricart et al. 2009 |
| *Navicula menisculus* | Tolerant | 7. Ricart et al. 2009 |
| *Nitzschia dissipata* | Tolerant | 1. Debenest et al. 2009 |
| *Nitzschia inconspicua* | Tolerant | 7. Ricart et al. 2009 |
| *Nitzschia palea* | Sensitive | 1. Debenest et al. 2009 |
| *Nitzschia palea* | Tolerant | 5. Larras et al. 2012 |
| *Nitzschia palea* | Tolerant | 9. Rotter et al. 2013 |
| *Nitzschia palea* | Tolerant | 6. Larras et al. 2014b |
| *Nitzschia palea* | Tolerant | 8. Tiam et al. 2015 |
| *Nitzschia paleacea* | Tolerant | 2. Larras et al. 2014a |
| *Planothidium lanceolatum* | Sensitive | 8. Tiam et al. 2015 |
| *Sellaphora minima* | Tolerant | 6. Larras et al. 2014b |
| *Tabellaria flocculosa* | Sensitive | 2. Larras et al. 2014a |
| *Ulnaria ulna* | Sensitive | 5. Larras et al. 2012 |
| *Ulnaria ulna* | Sensitive | 6. Larras et al. 2014b |

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**Supplementary Table S3** Herbicide sensitivity classification of benthic diatom taxa local to the study region; GBR catchment area, Australia.

| ***Taxa*** | **Classification** | **Paper** |
|---|---|---|
| *Achnanthidium minutissimum* | Tolerant | Wood et al. 2014 |
| *Achnanthidium minutissimum* | Tolerant | Wood et al. 2016a |
| *Adalfia* cf. *bryophila* | Tolerant | Wood et al. 2017 |
| *Adlafia* aff. *Bryophila* | Tolerant | Wood et al. 2016b |
| *Amphora* spp. | Tolerant | Wood et al. 2017 |
| *Cyclotella* sp. | Tolerant | Wood et al. 2017 |
| *Cymbella aspera* | Sensitive | Wood et al. 2017 |
| *Cymbella aspera* | Sensitive | Wood et al. 2016b |
| *Cymbella* sp. | Tolerant | Wood et al. 2017 |
| *Cymbella* sp. | Sensitive | Wood et al. 2016a |
| *Cymbella* sp. | Sensitive | Wood et al. 2014 |
| *Encyonema gracilis* | Sensitive | Wood et al. 2016a |
| *Epithemia adnata* | Tolerant | Wood et al. 2016b |
| *Epithemia* cf. *adanata* | Tolerant | Wood et al. 2017 |
| *Epithemia* cf. *cistula* | Sensitive | Wood et al. 2017 |
| *Epithemia cistula* | Sensitive | Wood et al. 2016b |
| *Eunotia* cf. *incisa* | Tolerant | Wood et al. 2016a |
| *Eunotia* cf. *minor* | Tolerant | Wood et al. 2017 |
| *Gomphonema clevei* | Sensitive | Wood et al. 2016b |
| *Gomphonema clevei* | Sensitive | Wood et al. 2017 |
| *Gomphonema gracile* | Sensitive | Wood et al. 2016b |
| *Gomphonema gracile* | Sensitive | Wood et al. 2016a |
| *Gomphonema gracile* | Sensitive | Wood et al. 2017 |
| *Gomphonema minutum* | Sensitive | Wood et al. 2016b |
| *Gomphonema minutum* | Sensitive | Wood et al. 2016a |
| *Gomphonema parvulum* | Sensitive | Wood et al. 2016a |
| *Gomphonema parvulum* | Sensitive | Wood et al. 2016b |
| *Gomphonema parvulum* | Tolerant | Wood et al. 2017 |
| *Gomphonema minutum* | Tolerant | Wood et al. 2017 |
| *Gomphonema truncatum* | Sensitive | Wood et al. 2016b |
| *Gomphonema truncatum* | Sensitive | Wood et al. 2017 |
| *Gyrosigma* sp. | Tolerant | Wood et al. 2017 |
| *Mayamaea atomus* | Tolerant | Wood et al. 2017 |
| *Mayamaea atomus* | Tolerant | Wood et al. 2016b |
| *Melosira varians* | Tolerant | Wood et al. 2017 |
| *Navicula* aff. *Rhynchocephala* | Tolerant | Wood et al. 2016b |
| *Navicula cryptocephala* | Tolerant | Wood et al. 2017 |
| *Navicula cryptocephala* | Tolerant | Wood et al. 2016b |
| *Navicula cryptotenella* | Tolerant | Wood et al. 2017 |
| *Navicula cryptotenella* | Tolerant | Wood et al. 2016b |
| *Navicula cryptotenella* | Tolerant | Wood et al. 2014 |
| *Navicula cryptotenella* | Tolerant | Wood et al. 2016a |

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|---|---|---|
| *Navicula schroeterii* | Tolerant | Wood et al. 2017 |
| *Navicula schroeterii* | Tolerant | Wood et al. 2016b |
| *Navicula subtillissima* | Tolerant | Wood et al. 2017 |
| *Navicula subtillissima* | Tolerant | Wood et al. 2016b |
| *Nitzschia paleaceae* | Tolerant | Wood et al. 2016b |
| *Nitzschia sigmoidia* | Tolerant | Wood et al. 2017 |
| *Pinnularia* sp. | Tolerant | Wood et al. 2017 |
| *Pinnularia viridis* | Tolerant | Wood et al. 2016b |
| *Pleurosira sp.* | Tolerant | Wood et al. 2017 |
| *Ulnaria ulna* | Sensitive | Wood et al. 2016b |
| *Ulnaria ulna* | Sensitive | Wood et al. 2016a |
| *Ulnaria ulna* | Sensitive | Wood et al. 2014 |
| *Ulnaria ulna* | Sensitive | Wood et al. 2017 |
| *Ulnaria ulna* | Sensitive | Wood et al. 2017 |

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**Supplementary Table S4** Complete species list of benthic diatoms collected during the field study from 16 rivers within the GBR catchment area and their SPEAR classification.

| ***Species*** | ***Genus*** | ***Order*** | **SPEAR** |
|---|---|---|---|
| *Achnanthes exigua* | *Achnanthes* | *Achnanthales* | 0 |
| *Achnanthes impexa* | *Achnanthes* | *Achnanthales* | 0 |
| *Achnanthes nodosa* | *Achnanthes* | *Achnanthales* | 0 |
| *Achnanthes oblongella* | *Achnanthes* | *Achnanthales* | 0 |
| *Achnanthidium minutissimum* | *Achnanthidium* | *Achnanthales* | 0 |
| *Achnanthidium minutissimum* var. *affine* | *Achnanthidium* | *Achnanthales* | 0 |
| *Achnanthidium sp.* | *Achnanthidium* | *Achnanthales* | 0 |
| *Actinocyclus normanii* | *Actinocyclus* | *Coscinodiscales* | - |
| *Amphora coffeaeformis* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora coffeaeformis* var. *borealis* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora delicatissima* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora holsatica* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora montana* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora pediculus* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora sp.* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora thumensis* | *Amphora* | *Thalassiophysales* | 0 |
| *Amphora veneta* | *Amphora* | *Thalassiophysales* | 0 |
| *Aulacoseira ambigua* | *Aulacoseira* | *Aulacoseirales* | - |
| *Aulacoseira crenulata* | *Aulacoseira* | *Aulacoseirales* | - |
| *Aulacoseira granulata* | *Aulacoseira* | *Aulacoseirales* | - |
| *Bacillaria paxillifer* | *Bacillaria* | *Bacillariales* | 0 |
| *Brachysira brachysira* | *Brachysira* | *Naviculales* | 0 |
| *Brachysira styriaca* | *Brachysira* | *Naviculales* | 0 |
| *Caloneis aerophila* | *Caloneis* | *Naviculales* | 0 |
| *Caloneis silicula* | *Caloneis* | *Naviculales* | 0 |
| *Cocconeis pediculus* | *Cocconeis* | *Achnanthales* | 0 |
| *Cocconeis placentula* | *Cocconeis* | *Achnanthales* | 0 |
| *Cocconeis placentula var. linearis* | *Cocconeis* | *Achnanthales* | 0 |
| *Cocconeis pseudothumensis* | *Cocconeis* | *Achnanthales* | 0 |
| *Craticula accomoda* | *Craticula* | *Naviculales* | 0 |
| *Craticula cuspidata* | *Craticula* | *Naviculales* | 0 |
| *Craticula halophila* | *Craticula* | *Naviculales* | 0 |
| *Craticula halophiloides* | *Craticula* | *Naviculales* | 0 |
| *Craticula molestiformis* | *Craticula* | *Naviculales* | 0 |
| *Craticula riparia* | *Craticula* | *Naviculales* | 0 |

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|---|---|---|---|
| *Ctenophora pulchella* | *Ctenophora* | *Fragilariales* | 1 |
| *Cyclostephanos dubius* | *Cyclostephanos* | *Thalassiosirales* | 0 |
| *Cyclostephanos tholiformis* | *Cyclostephanos* | *Thalassiosirales* | 0 |
| *Cyclotella atomus* | *Cyclotella* | *Thalassiosirales* | 0 |
| *Cyclotella comensis* | *Cyclotella* | *Thalassiosirales* | 0 |
| *Cyclotella meneghiniana* | *Cyclotella* | *Thalassiosirales* | 1 |
| *Cyclotella pseudostelligera* | *Cyclotella* | *Thalassiosirales* | 0 |
| *Cyclotella stelligera* | *Cyclotella* | *Thalassiosirales* | 0 |
| *Cymbella affinis* | *Cymbella* | *Cymbellales* | 1 |
| *Cymbella cistula* | *Cymbella* | *Cymbellales* | 1 |
| *Cymbella delicatula* | *Cymbella* | *Cymbellales* | 1 |
| *Cymbella laevis* | *Cymbella* | *Cymbellales* | 1 |
| *Cymbella tumida* | *Cymbella* | *Cymbellales* | 1 |
| *Diadesmis confervacea* | *Diadesmis* | *Naviculales* | 0 |
| *Diadesmis contenta* | *Diadesmis* | *Naviculales* | 0 |
| *Diploneis elliptica* | *Diploneis* | *Naviculales* | 1 |
| *Diploneis ovalis* | *Diploneis* | *Naviculales* | 1 |
| *Diploneis parma* | *Diploneis* | *Naviculales* | 1 |
| *Diploneis smithii* | *Diploneis* | *Naviculales* | 1 |
| *Diploneis subovalis* | *Diploneis* | *Naviculales* | 1 |
| *Encyonema gracilis* | *Encyonema* | *Cymbellales* | 1 |
| *Encyonema mesiana* | *Encyonema* | *Cymbellales* | 1 |
| *Encyonema minutum* | *Encyonema* | *Cymbellales* | 1 |
| *Encyonema silesiacum* | *Encyonema* | *Cymbellales* | 1 |
| *Encyonopsis cesatii* | *Encyonopsis* | *Cymbellales* | 1 |
| *Encyonopsis microcephala* | *Encyonopsis* | *Cymbellales* | 1 |
| *Encyonopsis perborealis* | *Encyonopsis* | *Cymbellales* | 1 |
| *Entomoneis alata* | *Entomoneis* | *Surirellales* | - |
| *Entomoneis costata* | *Entomoneis* | *Surirellales* | - |
| *Eolimna minima* | *Eolimna* | *Naviculales* | 0 |
| *Eolimna subminuscula* | *Eolimna* | *Naviculales* | 0 |
| *Epithemia adnata* | *Epithemia* | *Rhopalodiales* | 0 |
| *Epithemia sorex* | *Epithemia* | *Rhopalodiales* | 0 |
| *Eunotia arcus* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia bilunaris* var. *mucophila* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia exigua* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia faba* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia fallax* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia implicata* | *Eunotia* | *Eunotiales* | 0 |

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|---|---|---|---|
| *Eunotia incisa* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia minor* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia naeglii* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia muscicola* var. *tridentula* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia paludosa* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia pectinalis* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia praerupta* | *Eunotia* | *Eunotiales* | 0 |
| *Eunotia* sp. | *Eunotia* | *Eunotiales* | 0 |
| *Fallacia pygmaea* | *Fallacia* | *Naviculales* | 0 |
| *Fallacia tenera* | *Fallacia* | *Naviculales* | 0 |
| *Fragilaria capucina* var. *capucina* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria capucina* var. *gracilis* | *Fragilaria* | *Fragilariales* | 0 |
| *Fragilaria capucina* var. *perminuta* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria capucina* var. *rumpens* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria capucina* var. *vaucheriae* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria crotonensis* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria leptostriata* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria parasitica* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria* sp. | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilaria tenera* | *Fragilaria* | *Fragilariales* | 1 |
| *Fragilariforma virescens* | *Fragilariforma* | *Fragilariales* | 1 |
| *Frustulia rhomboides* | *Frustulia* | *Naviculales* | 0 |
| *Frustulia rhomboides var viridula* | *Frustulia* | *Naviculales* | 0 |
| *Frustulia vulgaris* | *Frustulia* | *Naviculales* | 0 |
| *Geissleria decussis* | *Geissleria* | *Naviculales* | 0 |
| *Gomphonema affine* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema angustum* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema angustum* var. *"subminutum"* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema bohemicum* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema clevei* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema gracile* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema micropus* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema minutum* | *Gomphonema* | *Cymbellales* | 0 |
| *Gomphonema olivaceum* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema parvulum* | *Gomphonema* | *Cymbellales* | 0 |
| *Gomphonema parvulum* var. *exillissimum* | *Gomphonema* | *Cymbellales* | 0 |
| *Gomphonema productum* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema pseudoaugar* | *Gomphonema* | *Cymbellales* | 1 |
| *Gomphonema pseudotenellum* | *Gomphonema* | *Cymbellales* | 1 |

| | | | |
|---|---|---|---|
| *Gomphonema truncatum* | *Gomphonema* | *Cymbellales* | 1 |
| *Gyrosigma attenuatum* | *Gyrosigma* | *Naviculales* | 0 |
| *Gyrosigma nodiferum* | *Gyrosigma* | *Naviculales* | 0 |
| *Gyrosigma parkerii* | *Gyrosigma* | *Naviculales* | 0 |
| *Gyrosigma spencerii* | *Gyrosigma* | *Naviculales* | 0 |
| *Hantzschia amphioxys* | *Hantzschia* | *Bacillariales* | 0 |
| *Hantzschia distinctepunctata* | *Hantzschia* | *Bacillariales* | 0 |
| *Haslea spicula* | *Haslea* | *Naviculales* | 0 |
| *Hippodonta capitata* | *Hippodonta* | *Naviculales* | 0 |
| *Karayevia clevei* | *Karayevia* | *Achnanthales* | 0 |
| *Karayevia laterostrata* | *Karayevia* | *Achnanthales* | 0 |
| *Kolbesia kolbei* | *Kolbesia* | *Achnanthales* | 0 |
| *Kolbesia ploenensis* | *Kolbesia* | *Achnanthales* | 0 |
| *Lemnicola hungarica* | *Luticola* | *Naviculales* | 0 |
| *Luticola goeppertiana* | *Luticola* | *Naviculales* | 0 |
| *Luticola mutica* | *Luticola* | *Naviculales* | 0 |
| *Mastogloia elliptica* | *Mastogloia* | *Mastogloiales* | - |
| *Mastogloia smithii* | *Mastogloia* | *Mastogloiales* | - |
| *Melosira* spp. | *Melosira* | *Melosirales* | 0 |
| *Melosira varians* | *Melosira* | *Melosirales* | 0 |
| *Meridion circulare* | *Meridion* | *Fragilariales* | 1 |
| *Navicella pusilla* | *Navicella* | *Cymbellales* | 1 |
| *Navicula aff subminiscula* | *Navicula* | *Naviculales* | 0 |
| *Navicula angusta* | *Navicula* | *Naviculales* | 0 |
| *Navicula bremensis* | *Navicula* | *Naviculales* | 0 |
| *Navicula capitatoradiata* | *Navicula* | *Naviculales* | 0 |
| *Navicula cari* | *Navicula* | *Naviculales* | 0 |
| *Navicula cincta* | *Navicula* | *Naviculales* | 0 |
| *Navicula constans* var. *symmetrica* | *Navicula* | *Naviculales* | 0 |
| *Navicula cryptocephala* | *Navicula* | *Naviculales* | 0 |
| *Navicula cryptotenella* | *Navicula* | *Naviculales* | 0 |
| *Navicula difficillima* | *Navicula* | *Naviculales* | 0 |
| *Navicula duerrenbergiana* | *Navicula* | *Naviculales* | 0 |
| *Navicula gottlandica* | *Navicula* | *Naviculales* | 0 |
| *Navicula gregaria* | *Navicula* | *Naviculales* | 1 |
| *Navicula heimansoides* | *Navicula* | *Naviculales* | 0 |
| *Navicula kotschyii* | *Navicula* | *Naviculales* | 0 |
| *Navicula lanceolata* | *Navicula* | *Naviculales* | 0 |
| *Navicula leptostriata* | *Navicula* | *Naviculales* | 0 |

| | | | |
|---|---|---|---|
| *Navicula libonensis* | *Navicula* | *Naviculales* | 0 |
| *Navicula menisculoides* | *Navicula* | *Naviculales* | 0 |
| *Navicula menisculus* | *Navicula* | *Naviculales* | 0 |
| *Navicula muraliformis* | *Navicula* | *Naviculales* | 0 |
| *Navicula notha* | *Navicula* | *Naviculales* | 0 |
| *Navicula peregrina* | *Navicula* | *Naviculales* | 0 |
| *Navicula phyllepta* | *Navicula* | *Naviculales* | 0 |
| *Navicula pseudokotschyii* | *Navicula* | *Naviculales* | 0 |
| *Navicula praeterita* | *Navicula* | *Naviculales* | 0 |
| *Navicula radiosa* | *Navicula* | *Naviculales* | 0 |
| *Navicula radiosafallax* | *Navicula* | *Naviculales* | 0 |
| *Navicula recens* | *Navicula* | *Naviculales* | 0 |
| *Navicula rhynchocephala* | *Navicula* | *Naviculales* | 0 |
| *Navicula schroeterii* | *Navicula* | *Naviculales* | 0 |
| *Navicula soehrensis* var. *muscicola* | *Navicula* | *Naviculales* | 0 |
| *Navicula splendicula* | *Navicula* | *Naviculales* | 0 |
| *Navicula subminiscula* | *Navicula* | *Naviculales* | 0 |
| *Navicula submuralis* | *Navicula* | *Naviculales* | 0 |
| *Navicula subrhynocephala* | *Navicula* | *Naviculales* | 0 |
| *Navicula tenelloides* | *Navicula* | *Naviculales* | 0 |
| *Navicula tripunctata* | *Navicula* | *Naviculales* | 0 |
| *Navicula trivialis* | *Navicula* | *Naviculales* | 0 |
| *Navicula vandamii* | *Navicula* | *Naviculales* | 0 |
| *Navicula veneta* | *Navicula* | *Naviculales* | 0 |
| *Navicula viridula* | *Navicula* | *Naviculales* | 0 |
| *Navicula viridula* var. *germanii* | *Navicula* | *Naviculales* | 0 |
| *Navicula viridula* var. *rostella* | *Navicula* | *Naviculales* | 0 |
| *Neidium affine* | *Neidium* | *Naviculales* | 0 |
| *Nitzschia accula* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia acicularis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia acidoclinata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia* aff. *Tropica* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia agnita* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia amphibia* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia angustatula* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia angustiforaminata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia braunii* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia capitellata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia clausii* | *Nitzschia* | *Bacillariales* | 0 |

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|---|---|---|---|
| *Nitzschia desertorum* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia dissipata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia diversa* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia dubia* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia elegantula* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia filiformis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia fonticola* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia fossilis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia frustulum* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia frustulum* var. *bulnhemiana* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia gessneri* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia graciliformis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia gracilis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia inconspicua* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia lacuum* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia liebetruthii* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia linearis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia linearis subtilis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia microcephala* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia obtusa* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia palea* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia palea var. thin variety* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia paleaceae* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia paleaformis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia perminuta* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia pumilla* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia pura* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia recta* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia reversa* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia sigma* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia sigmoidea* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia sinuata* var. *tabellaria* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia sociabilis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia solita* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia subacicularis* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia subcapitata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia umbonata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia valdecostata* | *Nitzschia* | *Bacillariales* | 0 |
| *Nitzschia valdestriata* | *Nitzschia* | *Bacillariales* | 0 |

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|---|---|---|---|
| *Nitzschia vermicularis* | *Nitzschia* | *Bacillariales* | 0 |
| *Opephora olsenii* | *Opephora* | *Fragilariales* | 1 |
| *Pinnularia appendiculata* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia borealis* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia braunii* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia gibba* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia intermedia* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia interrupta* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia legumen* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia microstauron* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia obtusa* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia similis* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia* spp. | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia subcapitata* | *Pinnularia* | *Naviculales* | 0 |
| *Pinnularia viridula* | *Pinnularia* | *Naviculales* | 0 |
| *Placoneis clementis* | *Navicula* | *Naviculales* | 0 |
| *Placoneis elginensis* | *Placoneis* | *Cymbellales* | 1 |
| *Planothidium delicatulum* | *Planothidium* | *Achnanthales* | 1 |
| *Planothidium frequentissimum* | *Planothidium* | *Achnanthales* | 1 |
| *Planothidium granum* | *Planothidium* | *Achnanthales* | 1 |
| *Planothidium lanceolatum* | *Planothidium* | *Achnanthales* | 1 |
| *Pleurosigma attenuatum* | *Pleurosigma* | *Naviculales* | 0 |
| *Pleurosigma elongatum* | *Pleurosigma* | *Naviculales* | 0 |
| *Psammothidium bioretii* | *Psammothidium* | *Achnanthales* | 0 |
| *Psammothidium saccula* | *Psammothidium* | *Achnanthales* | 0 |
| *Psammothidium scotica* | *Pseudostaurosira* | *Fragilariales* | 1 |
| *Pseudostaurosira brevistriata* | *Pseudostaurosira* | *Fragilariales* | 1 |
| *Pseudostaurosira zeillerii* | *Pseudostaurosira* | *Fragilariales* | 1 |
| *Reimeria sinuata* | *Reimeria* | *Cymbellales* | 1 |
| *Rhoicosphenia abbreviata* | *Rhoicosphenia* | *Cymbellales* | 1 |
| *Rhopalodia brebissonii* | *Rhopalodia* | *Rhopalodiales* | - |
| *Rhopalodia gibba* | *Rhopalodia* | *Rhopalodiales* | - |
| *Rhopalodia musculus* | *Rhopalodia* | *Rhopalodiales* | - |
| *Rossithidium linearis* | *Rossithidium* | *Achnanthales* | 0 |
| *Rossithidium pusilla* | *Rossithidium* | *Achnanthales* | 0 |
| *Sellaphora pupula* | *Sellaphora* | *Naviculales* | 0 |
| *Sellaphora seminulum* | *Sellaphora* | *Naviculales* | 0 |
| *Stauroneis anceps* | *Stauroneis* | *Naviculales* | 0 |
| *Stauroneis kreigerii* | *Stauroneis* | *Naviculales* | 0 |

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|---|---|---|---|
| *Stauroneis obtusa* | *Stauroneis* | *Naviculales* | 0 |
| *Staurophora wislouchii* | *Staurophora* | *Cymbellales* | 1 |
| *Staurosira construens forma venter* | *Staurosira* | *Fragilariales* | 1 |
| *Staurosira elliptica* | *Staurosira* | *Fragilariales* | 1 |
| *Staurosirella pinnata* | *Staurosirella* | *Fragilariales* | 1 |
| *Stenopterobia curvula* | *Stenopterobia* | *Surirellales* | - |
| *Surirella angusta* | *Surirella* | *Surirellales* | - |
| *Surirella biseriata* | *Surirella* | *Surirellales* | - |
| *Surirella brebissonii* | *Surirella* | *Surirellales* | - |
| *Surirella elegans* | *Surirella* | *Surirellales* | - |
| *Surirella linearis* | *Surirella* | *Surirellales* | - |
| *Surirella ovalis* | *Surirella* | *Surirellales* | - |
| *Surirella robusta* | *Surirella* | *Surirellales* | - |
| *Synedra acus* | *Synedra* | *Fragilariales* | 1 |
| *Synedra ulna* | *Synedra* | *Fragilariales* | 1 |
| *Tabularia fasciculata* | *Tabularia* | *Fragilariales* | 1 |
| *Tryblionella apiculata* | *Tryblionella* | *Bacillariales* | 0 |
| *Tryblionella calida* | *Tryblionella* | *Bacillariales* | 0 |
| *Tryblionella debilis* | *Tryblionella* | *Bacillariales* | 0 |
| *Tryblionella hungarica* | *Tryblionella* | *Bacillariales* | 0 |

Notes: - excluded do to lack of herbicide sensitivity data