

Cranfield University

Centre for Water Sciences

PhD

2009

Daniel Murray

The potential of barley straw as an algal and cyanobacterial growth
control

Supervisors: Prof Simon A. Parsons and Dr Bruce Jefferson

June 2009

ABSTRACT

Algal and cyanobacterial¹ blooms are increasingly becoming a problem to water utilities in the UK and around the world. Conditions such as increasing eutrophication and poor management of water bodies are consequently leading to increases in the number and severity of algal bloom events. These blooms present serious issues to both utilities and the environment; problems include deoxygenation of water bodies leading to fish kill and rapid algal growth leads to overgrowing of other plant species in the water, while blooms of toxic cyanobacteria can lead to the closure of reservoirs and a number of algae can lead to pump and filter blockages in treatment works. The problems created by algal and cyanobacterial blooms are becoming increasingly expensive to offset and while some technologies such as dissolved air flotation exist to control them at treatment level, there are few effective options to tackle blooms where control would be most effective, at the source. What methods there are for source control are often unreliable or almost as bad for the environment as the blooms themselves such as, in the use of chemical pesticides.

Barley straw has been shown to have the potential to be an effective control of algal and cyanobacterial blooms at the reservoir level, being able to inhibit a variety of species at practical straw concentrations, but little is known about how it functions or how its 'effect' is developed. This study identified specific areas which when investigated provided insight into these gaps in current knowledge. An analysis of the chemicals proposed as released by decaying barley straw has shown that they can control algal blooms at concentrations similar to what has been detected in the field. Investigations into the decay of barley straw has shown that barley straw decays in water in a way consistent with species that breakdown the lignin section of the straw potentially releasing the proposed chemicals. This finding was supported by the effect being consistent when field rotted straw, fresh straw rotted in the lab, and straw cultured in the lab with species specifically adapted to breaking down the lignin section of the straw were compared. Attempts at pre-treating the straw have shown that microbiological activity is important in developing the algistatic effect, which could lead to the possibility of keeping straw 'ready for use' thus removing the current need of several months wait before barley becomes effective. These findings have also provided a definitive route by which barley straw develops its effect, namely, microbiological decay of the lignin fraction of the straw leading to the production of phenol chemical release.

Acknowledgements

The work contained in this thesis was made possible through the sponsorship of Anglian Water, Northumbrian Water, Thames Water and Yorkshire water without whose contribution this thesis would not have been possible. I like to particularly thank my supervisors Prof Simon Parsons and Dr Bruce Jefferson for giving me the opportunity to do my PhD on a subject I love, and for all the help with the corrections and planning.

I'd like to thank everyone at Water Sciences for making my experience there so good. I'd particularly like to thank Lewis, Diane and Rita and Rukhsana for keeping me motivated and for all the help and advice with planning the work. Dave and Tom I'd also like to thank for all the fun and "having a goosy" at my chemostats whenever I was away.

Special thanks to all my family for always being around when I needed them and for putting up with me while I was writing up, and to Jan for all the proof reading and help with my spelling!

And for those who helped me start but weren't here to see me finish.

Dan

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Chapter 1

Introduction

1 Introduction

1.1 Project background

Blooms of fresh water algae, whether in water bodies or in water treatment works, can pose serious problems to the environment as well as considerable challenges in terms of mitigation, clean up and repair, or replacement of equipment such as filters and pumps. These blooms are caused when conditions come together, including sunlight, warm temperatures and eutrophication of the water body, allowing unrestricted algal growth (Mason, 2002). Eutrophication is caused by excess nutrients such as phosphorus entering the water system, often from external sources such as agricultural or industrial runoff (Mason, 2002). The excess of nutrients means there are no limitations to slow bloom formation and algae rapidly overgrow everything else in the system at a rate where even predation can no longer make an impact on their population. The factors causing algal blooms are difficult to control or plan for since, in some years, there will be blooms and in others there will not and bloom conditions will not develop. The causes of eutrophication, for example, are diverse; it can stem from multiple sources and this makes mitigation complex, expensive and sometimes even impossible (Mason, 2002; Pretty *et al.*, 2003).

Because of the difficulties in controlling the causes of algal blooms, the focus of many water utilities has generally been on preventing the blooms from growing or mitigating the downstream impacts. The impact of an algal bloom in a source water can affect a range of areas including: blocking of filtration systems and pumps which results in high costs through repair and replacement (Pretty *et al.*, 2003), problems with taste and odour (Pretty and Ward, 2001), and range though to serious environmental issues including: de-oxygenation of water bodies which results in fish kill, the smothering of macrophytes and, of particular concern, the release of toxins associated with cyanobacteria blooms (Jones and Orr., 1993). As cyanobacteria such as the blue-green algae *Microcystis aeruginosa* die, their cells lyse and release potent toxins into their surroundings, thereby producing substances like the cyanotoxin microcystin LR (Jones and Orr, 1993). These toxins are potent at very low

concentrations and can lead to sickness or the death of anything that drinks the water, including livestock and birds. These toxins can also pose risks to human populations; thus entire reservoirs can be rendered out of action when cyanobacterial blooms occur. As algal blooms tend to occur during dry months when water demand is highest, closing entire reservoirs can pose considerable problems. The World Health Organisation (WHO) has recently produced specific guidelines relating to microcystin LR and considers concentrations of above 1 ug L^{-1} as unsafe in its 2006 water quality guidelines (WHO, 2006). Concentrations in blooms are often many orders higher than this and concentrations as high as 1800 ug L^{-1} have been reported (Jones and Orr, 2003). This problem can only be expected to get worse as eutrophication continues to increase. The cost associated with algal blooms in general is already considerable and is estimated to be in excess of £150 million y^{-1} in the UK alone, with £19 million y^{-1} of this related to cleaning up toxins and other algal decomposition products (Pretty *et al.*, 2003).

The tools currently available to water utilities to prevent or mitigate algal blooms, while various, are often of limited use and effect, because they are either too costly to have in place constantly, or have drawbacks to their use, or cannot be applied on the larger scale required in a reservoir in order to make them practical long-term solutions (Barrett, 1994; Caffrey and Monahan, 1999). Various avenues have been explored in the quest to deal with algae and have included biological, chemical and mechanical means. Biological methods such as manipulation of a system's food web to increase grazer populations are expensive, time consuming, cannot be guaranteed and can be easily off set by factors such as eutrophication (Olin *et al.*, 2006). Chemical control methods, such as copper sulphate, can be highly effective at controlling algal blooms (McKnight *et al.*, 1983). The use of pesticides, though, is limited due to their serious side effects, like causing cell lysis, and many chemical controls are non-specific and kill off many other organisms in the system including higher plants. These plants provide competition for light and nutrients but are slower growing than algae and their removal means that future blooms have one less factor to offset their growth. Also, certain pesticides are known to bioaccumulate in higher organisms over time and this can lead to more damage to the local environment than the blooms themselves and can even pose a risk to human populations (Barrett, 1994). Mechanical methods such as artificial shading can be effective in water treatment works but generally have

proven largely unsuccessful at controlling algal populations at a larger scale; methods such as surface or benthic filtering often fail to remove all the algae, therefore only delay the problem at the cost of massive disturbance to the system (Ceccherelli and Piazzini 2005) and alternatives such as dredging the sediments to remove nutrients are even more damaging to the ecosystem.

The shortfalls in the currently available methods of controlling algal growth have meant that there has been a search in recent years for practical alternatives, especially those that act at the source level as it is preferred to prevent blooms becoming a problem in the first place. Processes such as dissolved air flotation can be effective in controlling algae once they reach the water treatment works (Henderson *et al.*, 2008) but they do not tackle the problem at its source. Reedbeds have been proposed as a possible solution to controlling algal growth at the reservoir as they perform a number of actions that could help to offset their growth, including: providing shading and helping to reduce phosphate levels. Overall though, to date, they appear to have only limited use as a standalone process, being unable to compensate fully for problems such as eutrophication (Garbett, 2005). Perhaps the most promising of the emerging processes to deal with algal growth, barley straw is also one of the least understood. Several studies over the last 2 decades have attested to its ability to significantly inhibit a wide range of algal species (Everall and Lees, 1996; Everall and Lees, 1997; Barrett *et al.*, 1999) and the conditions for this appear to be simple to maintain, namely well oxygenated water and neutral pH (Barrett, 1994), conditions common in many reservoirs. This makes barley straw extremely practical to use, and potentially low cost, low maintenance and requiring little input once applied (Everall and Lees, 1996; Everall and Lees, 1997).

In the numerous studies undertaken to assess the potential of barley straw to date, it has been suggested that, when it is submerged in water for a period of at least 3 months, a process occurs that results in the inhibition of algae. Previous studies have discounted factors such as the straw acting as a nutrient sink or providing shelter for enough grazers to offset the growth increase under bloom conditions (Welch *et al.*, 1990) as the source of the effect. What many studies have concluded is that the effect of barley straw is most likely to be chemical in nature (Welch *et al.*, 1990; Everall and Lees, 1996; Everall and Lees, 1997; Waybright *et al.*, 2009), and that these chemicals

are released from the barley straw into the surrounding waters as it decays. The fact that decay appears to be necessary has led many to the conclusion that microbiological activity is somehow important (Newman and Barrett, 1993), and others, based on evidence obtained in other areas such as rotting wood studies, have suggested that lignin may be the source of the chemicals (Ball *et al.*, 2001). But in summary, beyond the knowledge that barley straw can inhibit algal growth, it also has the potential to be a very useful and cost effective tool and the idea that the effect of barley straw is chemical in nature, very little is known until now about how this effect comes about, what is the source and even if the chemicals suggested can inhibit algal growth - all vital areas of knowledge if barley straw is to be optimised or even used. Barley straw is currently not a standard method of algal control due to the lack of knowledge about its use, though it has been used by Severn Trent Water and trials have been carried out on sites owned by Thames Water and Yorkshire Water. Barley straw has been tested successfully under a number of field conditions in the UK and Ireland including reservoirs (Everall and Lees 1996) and canals (Caffrey and Monahan, 1999) and it has also been studied further afield in other parts of Europe and the US (Ferrier *et al.*, 2005, Gulati and Donk, 2002). Outside industry, barley straw is commonly used on a small scale in the UK to control algal growth in ponds with a variety of products being available from many garden suppliers.

1.2 Motivation and project development

When considering the need for a new and effective tool to combat algal blooms and the current lack of knowledge surrounding barley straw, a consortium of water utilities - Anglian Water, Northumbrian Water, Thames Water and Yorkshire water - identified that there was scope to carry out research into the nature of the effect of barley straw and its usefulness as an algal control method. The work presented in this thesis is the result of that research. The project initially examined whether the chemicals suggested as a product of decaying barley straw could in fact inhibit algal growth and do this at the concentrations suggested. Later investigations were designed to study the processes involved in the evolution of the effect of barley straw and whether this process and its effect can be improved. Particular emphasis was placed on exploring the role of microbiological decay by fungi, and the differences between the decay of fresh and rotted barley straw, observing what happens to the straw

structure during this decay. All this data has been used to provide information on how barley straw functions and recommendations regarding its usefulness to industry.

1.3 Aims and Objectives

The purpose of this thesis is to develop an understanding of the process leading to the algistatic effect of barley straw (barley straw's effect has been described as algistatic, i.e. rather than killing the algae outright like a pesticide (an algicidal effect) it prevents rapid recruitment in the population, thus a viable, albeit reduced population remains). The areas of understanding which lack exploration include: the role of microbiology, the nature of possible chemical release, and whether any released chemicals can in fact inhibit algal growth. Knowledge is also to be increased with regards to optimising the use of barley straw by reducing the delay factor for it to work and guaranteeing its effectiveness. In order to meet these aims the following areas were focused on:

- (1) Do chemicals associated with decaying barley straw inhibit algal growth at amounts that could be produced?
- (2) Is decay necessary, can the potential of barley straw be artificially removed, and are there differences between rotted and fresh straw?
- (3) Is the decay of straw consistent with fungal activity?
- (4) Is there potential for chemical production due to lignin breakdown during decay?
- (5) Can white rot fungi be used to improve the activity of barley straw and is their action consistent with naturally active straw?

In order to meet these aims, the experimental work carried out over this thesis focused on the following areas:

- (1) Chemicals detected in the presence of decaying barley straw were assessed at the range they were found in previous studies to see if algae could be significantly inhibited at those levels.
- (2) Fresh and rotted barley straw were treated in the laboratory to see if the inhibitory portion of the straw could be removed and used separately from the straw to inhibit algal growth. Differences between the performance of rotted and fresh straw would provide insight into the nature and production of the naturally occurring inhibitors.
- (3) Long-term studies were set up in the laboratory using continuous cultures to investigate the role of decay, using fresh and rotted barley straw, and barley straw pre-cultured with lignin-degrading white rot fungi. The effect of all three straws was monitored to see if they would all produce an effect, to assess the time required for all to produce that effect and to see if the scale of effect was similar over all straw forms.
- (4) Fresh straw decayed in the laboratory using continuous cultures was assessed to see if its decay was consistent in a way that could lead to the production of chemicals. This included quantification of fungal biomass and lignin degradation within the straw over time.

1.4 Thesis plan

All work in this thesis was written by the 1st author Daniel Murray and edited by Prof Simon Parsons and Dr Bruce Jefferson (supervisors). All experimental work was undertaken by the 1st author. The initial chapter of this thesis consists of a literature review (Chapter 2), over the course of this chapter the problems associated with algae blooms are explained as are the problems in dealing with them. Current methods of controlling algal blooms are outlined and their shortcomings, and why barley straw is being considered as an alternative. The work to date looking at the inhibitory potential of barley straw is covered and areas such as species affected, conditions required and theories of how inhibition may work are explained and where possible discounted.

Chapters 4, 5 and 6 contain the technical and experimental sections. Chapter 4 investigates the inhibitory potential of chemicals associated with decaying straw. Analysis of the literature was used to select 13 chemicals and these were assessed to see if they could inhibit algal growth at the concentrations reported to be associated with straw. Their effectiveness would show if the theory that chemicals generated by decaying straw are responsible for barley straw's effect and the nature of the effective chemicals would provide insight into their possible source. This chapter is presented as a paper, *Inhibition of three algal species using chemicals released from barley straw*, Murray, D, Parsons, S, A and B, Jefferson, and has been accepted for publication in the Journal; *Environmental Technology*.

Chapter 5 consists of an investigation into the nature of the algal inhibitors produced by rotting barley straw and if that potential can be removed on demand by artificial means. Fresh and rotted straw were assessed during this study and their inhibitory abilities compared to each other. Three treatments of straw included: an extract of straw prepared by autoclaving in water and then filtering out the straw, then the second straw extract was autoclaved without water, and finally the straw was autoclaved in water, then the supernatant was filtered off and the remaining straw assessed. The autoclave was used as it provided a potential method of degrading the lignin fraction of the straw and releasing any bound chemicals.

Chapter 6 involved the analysis of the decay process and its importance in generating the inhibitory effect of barley straw. This study was carried out using a controlled environment so that the decay process was the only factor and it was done by using continuous cultures maintaining a steady state algal population. Three forms of barley straw were compared during this study: fresh straw, rotted straw and the third barley straw form, which was straw seeded with lignin-degrading white rot fungi, these being considered the group most likely to speed up the process but not the scale of barely straws effect. All three straws had their overall effectiveness compared. Consistent effect across the 3 straw types encouraged the second part of this chapter which was a more detailed investigation into the decay of fresh straw. This investigation consisted of acid digestible fibre analysis and ergosterol analysis. These were used to assess the presence of fungi, the effect that degradation had on the lignin content of the straw and whether the degradation of fresh straw naturally was

consistent with white rot fungi being present especially during the period when the straw was effective. Assessment was also done on whether enough lignin was degraded to account for the concentrations of chemicals required to inhibit algal growth. This chapter is presented as a paper, *The impact of conditioning of barley straw on the inhibition of Scenedesmus SP.* Murray, D, Parsons, S, A and B, Jefferson and has been submitted to the journal *Water Research*.

Chapter 7 gives the final discussion and conclusions of the thesis and contains a summary of the findings from all the experimental chapters and how they relate to the literature. It also outlines the process, which has been determined by this thesis that leads to the inhibitory effect of barley straw and makes recommendations for its use, possible optimisation and suggestions for work to be undertaken in the future.

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Chapter 2

Literature review

The causes and problems associated with algal blooms, why barley straw may be a possible solution and how it might work.

2 The causes and problems associated with algal blooms, why barley straw may be a possible solution and how it might work - a literature review.

2.1 Introduction

Algae are a natural and vital part of aquatic ecosystems, being the primary producers of the food chain (Carpenter *et al.*, 1987). Under normal conditions such as clean unpolluted, uneutrophicated water and usual weather conditions, they pose little problem to the environment or water treatment works (Mason, 2002). However, under eutrophic conditions, algae populations can quickly reach an exponential rate of growth (Mason, 2002) often leading to the formation of an algal bloom. Usual algal populations range from 5-15,000 cells ml⁻¹, for spring blooms and populations can reach 50,000 cells ml⁻¹, with summer blooms reaching up to 100,000 cells ml⁻¹ and severe blooms reaching populations of over 1,000,000 cells ml⁻¹ (Bauer *et al.*, 1998; Albay and Akcaalan, 2003; Hoeger *et al.*, 2004). The impact of algal blooms is well reported and includes:

- (i) The de-oxygenation of water systems leading to macrophyte, invertebrate and fish kill. This can be disruptive to fisheries and aquaculture.
- (ii) The overwhelming of water filter systems by algae which results in blockages and damage to water treatment equipment. (Shumway, 1990; Pretty, 2002; Pretty *et al.*, 2003).
- (iii) The limiting of light penetration into benthic regions, affecting the growth of charophytes and rooted macrophytes. (Middleboe and Markager, 1997; Mason, 2002;).
- (iv) The development of aesthetic changes such as affecting the taste and odour of water as well as the colour. (Pretty and Ward, 2001; Pretty *et al.*, 2003).
- (v) In the case of a number of blue green species such as *Microcystis aeruginosa*, cell lysis can release potent toxins, such as microcystin LR into water (WHO, 2006).

In recognition of this particular toxicity hazard, the World Health Organisation (WHO) has recommended a maximum of $1 \mu\text{g l}^{-1}$ in its drinking water quality guidelines (WHO 2006). Under bloom conditions, the concentration of this toxin has been measured at levels as high as $1800 \mu\text{g l}^{-1}$ (Jones and Orr 1993) which would render entire water systems hazardous to other animals including humans and lead to the closure of water bodies (Jones and Orr, 1993; Chorus *et al.*, 2000; Carmichael, 2001; Mankiewicz *et al.*, 2003).

Pretty (2003) analysed these issues and approximated the costs of blooms to be between £75-114.3 million a year for England and Wales. Treating water affected by algal toxins and decomposition products for potable use was alone responsible for over £19 million of the costs. The development of policy response costs - i.e. costs incurred responding to eutrophication - was responsible for an additional £54.8 million on top of the original £114.3 million.

Current methods of controlling algal blooms in freshwater bodies range from mechanical to biological and chemical systems. The main types of algal controls include: the application of ferric salts to remove phosphorus (Surampalli *et al.*, 1995; Kang *et al.*, 2003;), mechanical controls include raking surface waters to remove filamentous algae (Barret, 1994) and biological methods such as food web manipulation (Mason, 2002; Turker, 2003; Olin *et al.*, 2006). Unfortunately, mechanical and biological manipulations have drawbacks. Biological manipulation requires much time, constant management and control of lake use (Olin *et al.*, 2006) and factors such as background turbidity (Scheffer *et al.* 1997, 1998) and the level of eutrophication can offset its effectiveness. Also, the presence of protected species in the ecosystem would possibly rule out the use of bio-manipulation. Mechanical removal such as dredging to remove nutrients (Jeppesen *et al.*, 1999; Zarull *et al.* 2002), and filtering surface waters and scraping filamentous algae off the surface (Ceccherelli and Piazzzi 2005) can never fully remove all the algae or nutrients. Instead, mechanical methods merely tend to offset the problem for a short period of time (Ceccherelli and Piazzzi, 2005). Such massive upheaval often leads to damage of the less hardy species present including macrophytes and benthic filter feeders. These species recover slowly and, when present, provide shading and grazing pressure, thereby reducing algae mass, and their removal and the ability of algae to recover

quickly leads to an eventual increase in the frequency of blooms and the scale at which they occur.

Chemical methods of controlling algal blooms for example, atrazine (Pratt *et al.*, 1997), CuSO₄ (Mcknight *et al.*, 1983; Van Hullebusch *et al.*, 2003), Terbutryn (Gibson and Barrett, 1989) and Diquat (Peterson *et al.*, 1997) have proven highly effective at controlling algae blooms at relatively small doses (Peterson *et al.*, 1997; Van Hullebusch *et al.*, 2002^b). Whilst effective, many of these chemicals have been subsequently proven to cause more harm than good, being either non-specific, so harming a range of species which are often higher plants and other competitors (Barrett, 1994) or through bioaccumulation or biomagnification into higher organisms (Murray-Gulde *et al.*, 2002; Van Hullebusch *et al.*, 2002^b), or persistence in the environment (Birmingham and Coleman 1983; Pratt *et al.*, 1997; Van Hullebusch *et al.*, 2003). Table 2.1 summarises the commonly used methods of controlling algae in freshwater and in treatment works.

Table 2.1 Common methods used to control algal growth in source waters and at treatment works.

Source water	Mechanism	Description of method	Success	Example References
Bio-manipulation	Biological control	The Ecosystem itself is modified to be used as a tool in management. By changing the species composition it is hoped that a new stable system will be created that is self sustaining. This can include: the addition of new plant species, predator species, pathogens or even removing selected key species from the system such as removing top predators thereby allowing grazer populations to increase. Often a combination of all is used.	Has been shown to be effective at reducing nutrient availability, increasing grazing pressure, reducing turbidity, encouraging macrophyte growth. All are useful at reducing algae problems and reducing/offsetting eutrophication. Not every water body can be modified (protected species) and manipulation is not always successful. Timescale means this requires years to carry out and constant monitoring afterwards and can be very expensive. Potentially, in the long-term, a self-managing and sustainable method.	Olin, M., Rask, M., Ruuhijarvi, J., Keskitalo, J., Horppila, R., Tallberg, P., Taponen, T., Lehtovaara, G., Sammalkorpi, I., (2006)
Copper sulphate	Chemical control	CuSO ₄ is dosed directly into the water body, usually via a sprayer attached to the back of a boat. Once dissolved into the water it is a potent algae toxin causing cell death and lysies.	Has been used as a standard method of algae control in some parts of the world for decades. Very effective at low doses but it does persist in sediments, move out of the target area and damage non-problem species and there is evidence that it bioaccumulates in higher species.	Van Hullebusch, E., Chatenet, P., Deluchat, V., Chazal, P, M., Froissard, D., Botineau, M., Ghestem, A., Baudu, M (2003)

Pesticides	Chemical control	Pesticides are dispersed into the water usually by spraying from a boat. Once in the water, the chemicals rapidly kill off the algae population and can persist in the water body for several weeks, preventing further growth. The algae then take time to re-grow to previous populations.	Pesticides such as Diquat and Artazine are effective inhibitors of algae growth. Their use is limited e.g. aquaculture, as they can persist in water for weeks, and affect higher organisms such as amphibians. Many are non-selective and can affect higher plant growth as well as algae.	Erturk, H., Walker, P. N., (2003)
Pre-oxidation	Chemical control	Oxidising chemicals such as Ozone kill algae biomass and prevent further growth. Dead cell matter can then be filtered out and the water moved on into the treatment works.	Can be a very effective treatment when used as in conjunction with other techniques i.e. filtration. Ozonation is the more effective as it does not only cause lyses of cells but also breaks down extracellular organic matter (EOM). Chlorination also causes cell lyses increasing the concentration of EOM in the water.	Babel, S., Takizawa, S., (2000)
Surface mixing	Hydrological	The water body is actively mixed e.g. using pumps, air or rotors. This prevents algae from stabilising near the surface and thus reduces their access to light and hence, their growth	Effective at reducing cyanobacterial levels. Mixing prevents sinking of greens and brings up nutrients from depth, stimulating diatoms, eventually leading to higher biomass. Useful as an instant control when bloom occurs.	Visser, P. M., Ibelings, B. W., Van der Veer, B., Koedood, J., Mur, L. R., (1996)

Sedimentation	Hydrological control	Coagulants are added to the water causing the algae to agglomerate and sink.	Can be appropriate but DAF is usually more effective as algae are buoyant.	Ma, J., Lei, G., Fang, J. (2007)
Surface / Benthic raking	Mechanical control	The benthos or the surface of the water is scraped with rakes or nets. This can be done manually or mechanically. The scraping can remove large stands of algae especially filamentous, large or colonial algae bound within excreted EOM.	Initially successful. But raking in all cases leaves fragments of algae behind which rapidly re-grow to previous levels. Can also damage higher plants and large grazers making problems worse	Gulati, R, D., Donk, E (2002)
Filtering	Mechanical control	Surface waters are actively pumped from the water source through fine filters where the algae are removed. Once filtered the water can be returned to the original water source, thus reducing the algae density, or be further processed.	Can be very effective at removal of algae (e.g. > 90%) but requires space and equipment. Also not all algae are removed due to factors such as size. The filters themselves if exposed to the elements may themselves become the site of algae blooms. But can be a very effective method especially at treatment scale.	Naghavi, B., Malone, R, F., (1986)

Shading	Mechanical control	The water body is artificially shaded by stretching material across it such as plastic sheeting. The material shades the water body reducing the amount of light available for photosynthesis, thereby reducing algae growth.	Best suited to within water treatment works as covering large sections of reservoirs affects other species such as higher plants and benthic organisms and species which use light as a guide for breeding. It can also disrupt the food chain and prevent recreational use. Does not limit influxes of algae from outside.	Linang, Y., Chen, X, -C., Kong, H, -N., Wu, D, -Y., Huang, Y, -Y. (2007)
Dissolved air flotation	Mechanical control	Dissolved air floatation and coagulants are used to cause agglomeration of the algae biomass.	Proven method of removing algae in water treatment systems, with removal in excess of 90%. Not all algae are significantly affected and not suitable at reservoir scale.	Henderson, R., Parsons, S. A., Jefferson, B. (2008)
Ferric salts	Nutrient limitation	Ferric salts are dosed into the water usually from a sprayer attached to the back of a boat. Once in the water Phosphorous binds to the ferric and sinks out of the system into sediments, reducing availability to algae for growth	Marginal and temporary but cheap to use. Drops in total P and chlorophyll levels; however without total management of system, phosphorus returned to near original levels within 3 months. <i>cCyanobacteria</i> not affected as can withstand very large fluctuations in nutrient levels.	Gulati, R. D., Donk, E., (2002)

Dredging sediments	Nutrient limitation	Sediments act as a nutrient store releasing their load throughout the year and when disturbed. By dredging surface sediments, this store can potentially remove a large nutrient source for algae later on. Furthermore, wintering populations of algae such as <i>M. aeruginosa</i> can be reduced.	Can reduce cChlorophyll a concentration. Appears to have little effect on <i>cCyanobacteria</i> . New sediment layers have same release properties as old. Also may damage any macrophyte species increasing problem. May be effective if combined with input control (e.g. catchment management) and ferric dosing before dredging. Expensive	Van der Does, J., Verstraelen, P., Boers, p., van Roestel, J., Roijackers, R., Moser, G. (1992).
Alum	Nutrient limitation	Alum is dosed in to the water usually from a sprayer attached to the back of a boat. Once in the water P binds to the alum and becomes unavailable eventually sinking out of the system into sediments, reducing availability for algae for growth.	Initially effective but, without control of sediment release and external inputs, algae levels rapidly returned to pre-treatment levels e.g. within 3 years.	Funk, W, H., Gibbons, H, L., Bailey, G, C., Mawson, S., Gibbons, M. (1982)
Reed beds	Nutrient limitation + light limitation	Floating reed beds provide shading, thereby reducing algae growth. They provide a shelter for algae grazers and could also act as a nutrient uptake. Inlet reed beds act as filters removing incoming nutrients and reducing the food available for algae growth.	Floating beds have been shown to reduce phosphate levels in lakes and reed beds also function well. Though as a standalone technique it appears to have limited effect compared to chemical dosing.	Garbett, P. (2005)

This PhD is investigating one of the alternative methods of controlling algal growth in large freshwater systems such as reservoirs, with the use of barley straw.

2.2 Why barley straw?

The potential for barley straw to inhibit the growth of other plants and deter pest microorganisms and animal has been known since 1960 and a number of studies have shown barley straw to possess superior adaptations when compared to other plants, this allows it to successfully defend against a variety of species e.g. wheat (Borner, 1960; Reed and Jensen, 1989), pathogens (Stoessl and Unwin, 1970), and even predators (Zuniga *et al.*, 1988). These powerful defences have been attributed to chemicals produced by barley straw rather than to anything structural and Borner (1960) reported the production of phenolics by barley straw and the ability to retard the growth of wheat and rye roots at phenolic concentrations as low as 10 mg l⁻¹. Two alkaloids, ramine and horadenine, for example, are both known to play potent parts in barley straw's defences (Overland 1966; Swain 1977).

Barley straw residues are also known to be phytotoxic to other plants (Reed and Jensen 1989). Furthermore, barley straw appears able to use the same chemical release pathways it uses to compete with other plants to defend itself against problems such as fungal attack. Studies such as that by Stoessl and Unwin (1970) have shown that hordatines produced by barley straw are able to prevent the germination of fungi at concentrations as low as 10⁻⁵M. Barley straw is also known to produce chemicals that are effective at deterring predators such as greenbug (Juneja and Gholson, 1976), where the defence was shown to be due to the free benzyl alcohols contained in the structure of barley straw. Compounds found in barley straw including hydroxamic acids and gramine have been shown to deter grain aphids (Zuniga *et al.*, 1988; Barria *et al.*, 1992).

The structure of barley is also known to be different from other straws in that it possesses a higher than normal lignin content (Love *et al.*, 1998; Goh and Tuta, 2004) and Lima *et al.* (2007) points out that lignin is a source of phenolic material and it is well known that phenolic compounds are inhibitors of plant growth and may be particularly effective on algae (Barrett, 1994; Everall and Lees 1996, 1997). Overall,

it appears that barley straw's effectiveness over other plant types is due to its ability to successfully inhibit and out-compete other plants, as it contains within its structures a wide variety of chemicals including phenolics (Lima *et al.*, 1997), alkaloids (Swain, 1977), alcohols (Juneja and Gholson, 1976) and hydroxamic acids (Barria *et al.*, 1992) which are also likely to affect its interaction with algae.

2.3 Barley straw for algae control

The use of barley straw for controlling algal growth started in the late 1970s and early 1980s when farmers reported that the accidental addition of rotted straw to a lake reduced the growth of filamentous algae (Welch *et al.*, 1990). Winfield (1985) proposed the first mechanism for this observation: that bacterium immobilised on the straw, out-competed algae for nutrients such as dissolved phosphate. Laboratory tests supporting this mechanism showed that barley straw had a capacity of 5 µg P per g straw per hour. Street (1978) reported observations on adding straw to a gravel pit, they found that the straw increased the invertebrate concentration fivefold; the invertebrates consumed the algae which reduced their numbers. In other early trials including Champ and Caffrey (1994), they placed 120 loose bales of straw in a 12 ha eutrophic lake in the south of Ireland and reported improvements in transparency and evidence of improved macrophyte plant growth.

Welch *et al.* (1990) investigated the use of rotted straw on the growth of *Cladophora glomara* and *Spirogyra* sp. in a series of canal locks. They dosed the canals (2 x 100m x 10m) 10 times over 2 years with 20 x 20 kg bales of barley straw and reported a significant reduction in the growth of *Cladophora* over 2 consecutive years. The authors identified a number of potential mechanisms including nutrient limitation, pesticide release from the straw and increased invertebrate grazing, all of which they were able to rule out. They proposed that the delay in the inhibitory effect observed was likely to be due to the biological decomposition of the straw releasing inhibitory chemicals. However, they did question the concentration required and estimated, from their observations, that a decay of 5 kg of straw a month would yield a maximum of 37 µg L⁻¹ of compound. Ridge and Barrett (1992) revisited the canal site and again showed inhibitory effects of the straw even a year after its application. Barrett (1994) later tested the hypothesis of pesticide release from straw by comparing barley straw

with organically grown barley straw and confirmed that pesticide residues are not involved in the anti-algal activity of the straw.

Gibson *et al.* (1990) and Pillinger *et al.* (1992) continued the investigation initiated by Welch using more controlled laboratory experiments. Gibson *et al.* (1990) investigated eight different algae including the filamentous species *Cladophora glomerata*, *Klebsormidium rivulare*, *Oedogonium* sp, *Spirogyra* sp, *Stigeoclonium tenue*, *Ulothrix trentonense* and the unicellular species *Chlorella vulgaris* and *Selenastrum capricornutum*. They tested barley straw and hay at concentrations of 100 kg m^{-3} and 40 kg m^{-3} , (approximately ten times higher than that used by Welch *et al.*, 1990) and found reductions of between 60 and 77% in the presence of rotted straw. They reported that fresh straw showed an inhibitory but not lethal effect after being rotted for 2 months, increasing to 63% after 6 months. Removing the straw led to the algae counts recovering to original levels. Similar findings were also reported by Newton and Barrett (1993), who demonstrated that autoclaving the straw removed any inhibitory effect, again supporting a biologically enhanced release of chemical compounds, but they were unable to suggest if this was due to the straw or the microbes growing on it.

Newman and Barrett (1993) investigated the control of *M. aeruginosain* an aerated tank with rotted straw for 85 days. A 94% inhibition of growth was reported for the combination of straw and liquor collected after the autoclaving of rotted straw at a dose of 2.57 g m^{-3} .

Barrett *et al.* (1999) reported positive results from a long term trial of barley straw in a water supply reservoir near Aberdeen. The reservoir had historical problems associated with a spring bloom of diatoms ($>40,000 \text{ cells ml}^{-1}$) and a summer bloom of *Anabaena* sp. The result of these blooms had led to the reservoir being taken out of service on a number of occasions. Barley straw was applied 2 to 3 times per year to this reservoir between March 1993 and February 1998 at doses of between 28 g m^{-3} and 6 g m^{-3} and algae cell numbers were found to be significantly reduced when compared to the 1991 and 1992 data. The population dynamics were similar but levels of diatoms and, more markedly, *cyanobacteria* were found to be significantly reduced. For example, in August 1992 the mean monthly cell counts were 10,500

cells ml⁻¹ which reduced to an average of 1580 cells ml⁻¹ over the next 6 years. Barrett *et al.* (1999) calculated the mean daily dose by dividing the straw dose over 3 years by time and gave a value of 0.04 g/d/m³ for effective application.

Caffrey and Monahan (1999) reported the use of barley straw to control the growth of the filamentous alga *Cladophora glomerata* in a canal system. They assessed the impact of barley straw over 4 years (1990 – 1994) in a 1 km stretch of canal against an upstream untreated 1 km stretch of canal, then performance was quantified by measuring the amount of algae biomass harvested (measured as g dry weight m⁻²). Barley straw was dosed at 10 g m⁻³ regularly over the 4 year period and the results were impressive with significant reduction in algae recorded in the first year of application followed by 2 years of no filamentous algae formation in the control stretch of the canal. By 1994, algae levels had been reduced to such an extent that the aquatic rooted macrophyte *Myriophyllum verticillatum* had established itself in the treated reservoir, possibly due to the removal of the carpet forming *Cladophora* which allowed enough light for it to grow. The macrophyte appeared unaffected by the presence of barley straw.

Moor (1975) reported large numbers of *Asellus aquaticus* in and beneath the straw mattress, which is interesting since *Asellus* is known to eat algae as part of its diet. It only makes up a small amount, 14 % (Moor 1975), so whilst feeding would have very little effect on the environment's algae population, *Asellus* is also known to consume decaying plant matter as well as the fungi and bacteria living on them. This would support the conclusions of Welch *et al.* (1990) who proposed that the time delay in the effectiveness of barley straw was due to the time needed for an appropriate microbiological community to build up.

Terlizzi *et al.* (2002) reported the first evaluation of barley straw on dinoflagellates in salt water. The barley straw was prepared as described by Gibson *et al.* (1990) where 360g of chopped barley straw was rotted for sixty days in an aerated 18 litre bucket. The tests were undertaken at doses ranging from 7.2 to 14.5 kg straw per m⁻³ of culture and incubated for 3 weeks, and performance was measured by comparing cell counts against an un-dosed control. The results were mixed; some species were inhibited (*Gyrodinium galatheanum*, *Gymnodinium sanguineum*, *Heterocapsa*

triquetra and *Heterocapsa pygmaea*) and some were stimulated (*Gyrodinium instriatum*, *Prorocentrum minimum* and *P. micans*). The best results were found for *Heterocapsa triquetra* and *Heterocapsa pygmaea* for which algae counts were reduced by 55 and 80% respectively compared to the control. Later, the same group (Ferrier *et al.*, 2005) evaluated 12 species of freshwater algae and here the selected dose was 7.2kg straw per m⁻³. The test ran for 2 weeks and performance was measured in terms of chlorophyll a concentrations. Growth of *M. aeruginosa* and *Dinobryon* sp. was significantly inhibited by barley straw, which echoes the inhibition reported by Newman and Barrett (1993) and Martin and Ridge (1999). *Synura petersenii* was completely removed indicating that barley straw was in this case acting as an algicide. In comparison, growth of the species *Selenastrum capricornutum*, *Spirogyra* sp., *Oscillatoria lutea* var. *contorta*, and *Navicula* sp. was stimulated.

Barrett (1994) proposed three mechanisms for the anti-algae effect: (i) that a natural component is released from straw aided by the decomposition of the cell structure, (ii) that a natural component of the straw is converted into an active molecule by either a microbial or chemical reaction or, (iii) that an antibiotic is generated by microorganisms in / on the straw. Barrett (1994), when reviewing the works of Welch and Pillinger, suggested that phenolics in the straw were oxidised under alkaline pH and well-aerated conditions to form quinines, and many of the studies where barley straw has been successfully applied have been under these conditions (Everall and Lees, 1996, 1997; Caffrey and Monahan, 1999). Several studies report the presence of various phenolic chemicals released from rotting barley straw (Ridge and Pillinger, 1996; Everall and Lees, 1996, 1997) and other plant types (Pillinger *et al.*, 1995, Park *et al.*, 2006).

Whilst these compounds may be active in their own right, a number of authors have suggested an alternative path by which barley straw may function. It has been proposed that, under the right conditions, the chemicals released during the decay of straw could also form humic acids (Nakamuro *et al.*, 1990; Amador *et al.*, 1991; Yavmetdinov *et al.*, 2003). These could break down further in the presence of sunlight, possibly leading to the production of hydrogen peroxide. Nakamuro *et al.* (1990) suggested that under ideal laboratory conditions, where lab-generated ozone was directly added to the sample at a rate of 75 mg min⁻¹, 100 mg l⁻¹ of humic acid

yielded 1 mg l⁻¹ H₂O₂. Yavmetdinov *et al.* (2003) suggests that under natural conditions 25 g l⁻¹ straw would produce 500 mg of humic acid over 45 days. This would mean, following Nakamuro *et al.* (1990) findings, that under ideal conditions, 25g l⁻¹ barley straw in the field could potentially produce up to 111 µg day⁻¹ of H₂O₂. Peterson *et al.* (1995) looked into the use of H₂O₂ to control algal growth and suggested that in the case of *Aphanizomenon flos-aquae*, 800 µg L⁻¹ of H₂O₂ would be needed to affect nitrogen fixation. So, when taking into account the findings of Nakamuro *et al.* (1990) and Yavmetdinov *et al.* (2003), concentrations of around 180 g l⁻¹ barley straw under ideal conditions would be required to generate sufficient levels of H₂O₂. Such a concentration of straw is far higher than has been commonly and successfully used to control algae (Table 2.2). Furthermore, when considered with the short life time of H₂O₂ (Peterson 1995), it would mean H₂O₂ is unlikely to even inhibit algae growth let alone achieve the scale and efficiency that has been reported.

Pillinger *et al.* (1992) investigated the findings of Welch *et al.* (1990) and Gibson *et al.* (1990) to question whether the presence of fungi contributed to the algistatic effect of barley straw and whether, in particular, the fungi itself had any algaestatic properties. They tested 29 different fungi types isolated from straw and found that only *P. dispersa* and *Z. leucotricha* were able to affect the growth of *M. aeruginosa* and *C. vulgaris*. There was some evidence that *F. tricinctum* inhibited the growth of *C. vulgaris* although this was inconclusive. So, whilst it appears that the presence of fungi is important in the action of barley straw, it is not the presence of fungi alone that is inhibitory.

Pillinger *et al.* (1995) investigated the link between lignin and the algistatic properties by studying the inhibitory effects of brown and white rotted woods (sycamore, elm, birch and hazel). Brown rotted materials are those which have been decayed by brown rot fungi. These specialised feeders selectively remove the cellulose from plant material eventually leaving a brown powder which is enriched in lignin and almost devoid of cellulose (Kirck and Alder, 1970). This is possible via the use of complex enzyme systems revolving around glucanases and cellulases (Valaskova and Baldrin 2006; Frankland *et al.*, 1982). White rot fungi functions in the opposite manner, in that only they are able to efficiently digest the lignin material within the plant. This is done via their use of specialised enzyme systems dependent on manganese peroxidase,

lignin peroxidase and laccase (Granit *et al.*, 2007). The end product here is a white fibrous material containing almost no lignin and leaving only the cellulose and fibre portions of the plant.

The algaestatic performance of the wood samples was tested using a standard algae toxicity test, using *C. vulgaris* and *M. aeruginosa* as the test species. Pillinger *et al.* (1995) showed that brown rotted wood was more inhibitory than white rotted wood at doses of 2, 5 and 10 g L⁻¹. Pyrolysis-GCMS was used to characterise the wood samples, finding mostly compounds originating from polysaccharides from white rot wood whilst a number of phenolic compounds were identified from brown rotted wood. These were thought to be methoxy phenols including 2,6 dimethoxyphenol. The substitution of methoxy to dimethoxy groups appeared to be a characteristic of brown rotted wood. The most effective sample - brown rotted elm - had the highest ratio of syringyl lignin to guaiacyl lignin (calculated at 1.8 compared to all other wood types that were all below 0.5). Barley straw is also a good source of lignin which makes up roughly 10-15% of its mass (Rowland and Roberts, 1994). This is not as high as in woody plants; for example, 29.1% of *Calluna vulgaris* is lignin (Rowland and Roberts, 1994), or in some leaf litters such as spruce, which has been estimated at up to 29 % (Taylor *et al.*, 1989; Rowland and Roberts, 1994). However, barley straw has a higher lignin content than many other terrestrial plants and it will certainly be higher than almost all commonly found aquatic plants (Hume *et al.*, 2002).

Phenolic compounds such as p-coumaric and ferulic acids are known to be inhibitory towards *C. vulgaris* (Dedonder and Van Sumere, 1971) and *M. aeruginosa* (Newman and Barrett, 1993). For example, Schrader *et al.* (1998) reported a 5 day, lowest observed effective concentration (LOEC) of 1641.6 mg l⁻¹ for p-coumaric acid on *Oscillatoria cf. Chalybea*, and ferulic acid was found to be highly effective on *Oscillatoria* with a 5 day LOEC of just 1941.8 µg l⁻¹. Lignin in barley straw contains polyphenolic compounds and Pillinger *et al.* (1994) suggested that this lignin would, under basic and well-aerated conditions, be a major source of oxidisable phenolic material. To test this hypothesis they investigated the algaestatic properties of tannins released from barley straw and those from oak leaves. The barley straw (15 g in 15 litres) was aerated for 3 months prior to use to allow them to assess the impact of aeration as oxidised phenols are generally more toxic than non-oxidised phenols.

They showed that algae growth was inhibited by quinone compounds especially under aerated conditions. For example, 1,2 naphthoquinone and 9,10 phthalanthrone quinone had IC_{50} values of 40 and 25 $\mu\text{g l}^{-1}$ respectively. Other authors have found similar properties for tannins (Hussein-Ayoub and Yankov, 1985; Ridge and Pillinger, 1996; Ridge *et al.*, 1999; De Nicola, 2004) and similar effects have been reported for leaf litter in water. This is all in line with early findings from the likes of Fitzgerald *et al.* (1952) who found that 2,3-dichloronaphthoquinone killed *M. aeruginosa* as a dose of 2 $\mu\text{g l}^{-1}$.

Everall and Lees (1996) reported the results of a field study of barley straw application (50 g m^{-3}) in a water supply reservoir in Derbyshire, UK. They assessed the impact of straw on algal counts and water quality, and also undertook a 'semi-quantitative' environmental assessment and found no deterioration in biological quality downstream of the barley straw. There was no change in phosphorus or nitrate levels in the reservoir but the application of barley straw led to a decrease in algae numbers from 55,000 cells ml^{-1} to less than 5,000 in the reservoir when compared to previous years. Water samples were collected for analysis by GCMS both upstream and downstream of the straw and these showed there was a release of a wide range of chemical compounds including 2, 6 dimethoxyphenol and 3 methylbutanoic acid from the straw. This supports the mechanism proposed by Pillinger *et al.* (1994) and Barrett (1994) and the study was reproduced by Everall and Lees (1997) in another reservoir which had suffered severe blooms of *Oscillatoria tenuis*. There, barley straw was applied at a dose of 25 g m^{-3} and again there were significant reductions in the algae concentrations from 100,000 cells ml^{-1} down to 10,000 cells ml^{-1} in the dosed reservoir and 12 days later in a lower reservoir. GCMS was used to identify compounds released by the straw and the presence of phenolic compounds such as 2 methoxyphenol and 2, 6 dimethoxyphenol. Key conditions were reported as aeration, alkaline pH and close proximity of the straw to the algae bloom. Yavmetdinov *et al.* (2003) suggested that, if up to 2.36 % of the mass of straw decayed, this could be converted to humic-like substances (HLS). Within 45 days this would mean that 25 g l^{-1} of straw could yield 500 mg of humic substances. A similar amount was suggested by Welch *et al.* (1990) and this could suggest that the levels of chemicals proffered by Everall and Lees (1996, 1997) were realistic barley straw doses (Gibson *et al.*, 1990; Newman and Barrett, 1994).

Ridge *et al.* (1999) compared the performance of barley straw and deciduous leaf litter in controlling algae growth. They tested *C. vulgaris*, *M. aeruginosa*, *Cladophora glomerata* and *Asterionella formosa* at a dose of 5.5 kg m³ in a well-aerated pool over a 2.5 year period. The results showed an inhibitory effect over the initial 90 day period for both barley straw and leaf litter and linked the effect to the presence of fine particulate material. The authors proposed the inhibitory effect was due to the release of oxidised tannins during microbial decay and suggested using natural dams to capture leaf litter on the inlet to lakes as a way of engineering the effect or the planting of deciduous trees close to the lakes.

To date, several trials using barley straw to inhibit algal growth have been carried out in the field, including those mentioned above by Newman and Barrett (1993) and Everall and Lees (1996, 1997), along with laboratory trials including Gibson *et al.*, (1990) and Ferrier *et al.*, (2005). These tests have looked at several different algal species ranging from green algae such as *C. vulgaris* to cyanobacterial species including *M. aeruginosa* through to diatoms such as *Navicula* sp. The impact of straw on these species is summarised below in Table 2.2.

From Table 2.2, it can be seen that barley straw has been tested successfully on a variety of species from all the major taxa including Chlorophyta and Cyanophyta, and common laboratory species such as *C. vulgaris* (Pillinger *et al.*, 1995), and species of major concern, including *Anabaena* spp (Barrett *et al.*, 1999) and *M. aeruginosa* (Ridge *et al.*, 1999). It can also be seen that barley has been tested in a variety of concentrations both as straw and liquor. It is also clear that barley has been tested in both field and laboratory conditions with varying degrees of success: for example, 25 % inhibition has been reported in the case of *Anabaena* sp (Barrett *et al.*, 1999) through to inhibitions as high as 95 % for *M. aeruginosa*.

Table 2.2 Summary of the algae species barley straw has been tested on. Details of straw used, species, response to straw and conditions.

Species	Division	Problems caused by species	Straw details	Response of species to barley straw	Conditions	Reference
<i>Anabaeea</i> Sp	Cyanophyta	Toxic. Taste + odour. Filter-clogging	21 tonnes (3x7 over 25000m ³) Dec 1.6t (50 nets of 32kg). 1.9tonnes 10m/20kg. Sausage = > 28g m ³ barley straw.	Once straw introduced algae reduced to 25% for rest of Exp. 0.04g/d/m ³ . no effect on annual species subsection, just numbers.	Field. Reservoir.March 1993/Dec 1993 then 1994-7 for sausages	Barrett, P. R .F., Littlejohn, J. W., Cunrow, J. (1999)
<i>Asterionella</i> Sp	Chrysophyta	Odour/ taste. Filter Clogging	21 tonnes (3x7 over 25000m ³) Dec 1.6t (50 nets of 32kg). 1.9tonnes 10m/20kg. Sausage= >28g m ³ barely straw	Before Exp frequent blooms of 40000+ cells ml-1. Once straw introduced reduced to 25% for rest of Exp.	Field. Reservoir.March 1993/Dec 1993 then 1994-7 for sausages	Barrett, P. R. F. Littlejohn, J. W. Cunrow, J. (1999)
<i>Chlorella pyrenoidosa</i>	Chlorophyta	Can lower DO. Filter-clogging	fungi spp. 4 weeks	Not believed fungi responsible. May play role in action or role of breaking straw down. Noted that p.dispersa (constant) and z.leucotricha (temporary) create zones of inhibition.	Lab. 4 Weeks	Pillinger, J. M., Cooper. J. A., Ridge, I. and Barrett, P,R F. (1992)
<i>Chlorella vulgaris</i>	Chlorophyta	Can lower DO. Filter-clogging	2,5 or 10 g ⁻¹ sycamore, 1,2 or 4g ⁻¹ Elm	Significant control (<0.001). Increased inhibition at lower concentrations by autoclaving in medium first.	Lab. 3-4 days	Pillinger, J,M., Gilmour, I., Ridge, I., (1995)
<i>Chlorella vulgaris</i>	Chlorophyta	Can lower DO. Filter-clogging	Range up to 1 ml. 40/25ugl for quinines	Tannins and oak leaves in oxidised conditions = Inhibitory. As did quinones 50% inhibition.	Lab. 3-4 days	Pillinger, J. M., Cooper, J. A and Ridge, I. (1994)

<i>Chlorella vulgaris</i>	Chlorophyta	Can lower DO. Filter-clogging mat forming	40 g wet wt. Rotted then autoclaved no effect.	<0.001. Significant reduction in ug l ⁻¹ Chlorophyll a	Lab. 6months/ 3 days. Till significant growth in controls.	Gibson, M. T., Welch, I. M., Barrett, P. R. F., Ridge, I., (1990)
<i>Chlorella vulgaris</i>	Chlorophyta	Can lower DO. Filter-clogging	Leaves.5.5 cm ³ . Liquor and ground + liquor.	Both rotted leaves and liquor effective for 80-90 days up to 80%. 364-456 days 95% inhibition. Then reduction in effectiveness but significant inhibition for rest of exp. filtered liquor effective till day 509.	Lab + simulated field 2.5 years.	Ridge, I., Walters, J. and Street, M. (1999)
<i>Cladophera glomerata</i>	Chlorophyta	Can lower DO. Filter-clogging mat forming	Bales over sites (20bales 10 bales then 50kg) every start-2 months-14 months.	Removal of straw from locks lead to re-establishment of algae bloom. Where straw kept algae remained strongly inhibited.	Field (canal) 14 months.	Ridge, I., Barrett, P. R. F.(1992)
<i>Cladophera glomerata</i>	Chlorophyta	Can lower DO. Filter-clogging mat forming	4 years 20x20kg Over 3 locks topped up 10 times.	No effect 1 year. No algae growth in treated locks in following years + areas below straw filled locks.<0.001 diff from up stream.	Field. 15.10.86 -5-4-89	Welch, I. M., Barrett, P.R.F. Gibson M. T and Ridge, I. (1990)
<i>Cladophera glomerata</i>	Chlorophyta	Can lower DO. Filter-clogging mat forming	40g wet wt	<0.001. Significant reduction in ug l ⁻¹ Chlorophyll a.	Till significant growth in controls.	Gibson, M. T., Welch, I. M., Barrett, P. R. F and Ridge, I. (1990)
<i>Cladophera glomerata</i>	Chlorophyta	Can lower DO. Filter-clogging	Leaves	Inhibited by up to 65.4%	Field. Ponds 1993-8/94.	Ridge, I., Walters, J and Street, M. (1999)

<i>Cladophera glomerata</i> (mostly)	Chlorophyta		Straw in loose bags at 50m intervals. = 10g m ³	By August 1991 no algae present. Control section = 113m m-2. Aug 1991-Aug 1993. No filamentous algae. Nov 62g m-2 (occurred as replacement straw not added. When added in Nov = no effect.). Allowed establishment of macrophyte <i>Myriophyllum verticillatum</i> (25% cover).	Field. Canal.Oct 1990-1994.	Caffery, J and Monahan, C (1999)
<i>Dinobyran</i> sp	Chrysophyta	Taste + odour	both(liquor +liquor and straw	Showed significant inhibition for sterile liquor (<0.05) inhibition for straw + liquor seen but not significant.	Lab. Two weeks	Ferrier, M. D. <i>et al</i> (2005)
<i>Gymnodinium sanguineum</i>	Dinoflagellate		(Estuarine salt water). 9 ml unfiltered barley straw extract. +7 ml inoculums	Inhibition <0.05	Lab.three weeks. Tested once a week.	Terlizzi, D. E., Ferrier, M. D., Armbruster, E. A and Anlauf, A. K. (2002)
<i>Gyrodinium galatheanum</i>	Dinoflagellate		(Estuarine salt water). 9ml (7.25 g l ⁻¹) unfiltered barley straw extracts. +7ml inoculums	Two strains tested growth of 9.5%(Chesapeake bay) and 13.5%(Hyrock farm) at two weeks for samples then both decreased in week three - 27.6% and -35%. Bay sample repeated with double amount of straw I.e. 14.5 g l ⁻¹ . Resulted in significant inhibition <0.05.	Lab.three weeks. Tested once a week.	Terlizzi, D. E., Ferrier, M. D., Armbruster, E. A and Anlauf, A. K. (2002)
<i>Heterocapsa pygmaea</i>	Dinoflagellate		(Estuarine salt water). 9ml (7.25 g l ⁻¹) unfiltered barley straw extracts. +7ml inoculums	80% reduction. Very effective on <i>Heterocapsa</i> group.	Lab.three weeks. Tested once a week.	Terlizzi, D. E., Ferrier, M. D., Armbruster, E. A and Anlauf, A. K. (2002)

<i>Heterocapsa triquetra</i>	Dinoflagellate		(Estuarine salt water). 9ml (7.25 g l ⁻¹) unfiltered barley straw extract. +7 ml inoculum.	55% reduction	Lab.three weeks. Tested once a week.	Terlizzi, D. E., Ferrier, M. D., Armbruster, E. A and Anlauf, A. K. (2002)
<i>Kelbsormidium rivulare</i>	Chlorophyta		100 g wet wt.	<0.01. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls	Gibson, M.T., Welch,I. M., Barrett,P. R. F and Ridge, I. (1990)
Lake Study	All	NA	2.5 tonnes across reservoir = 50g m ³	Significant reduction in chlorophyll a. 80-140 ug-1 before addition 0-40 after. Significant population change. i.e.before = Aphanizomenon, Anabaena, Pandorina and Cryptomonas. After =Large but few Volvox and Sphaerocystis.	Field .8 april-3 oct 1994	Everall, N.C and Lees. D.R (1996)
Lake Study. Esp. <i>Ocellatoria tenuis</i>	All	NA	3.5 tonnes = 25gm-3	Initial reduction in average monthly phytoplankton count (<0.0001) Chl a (<0.05) and cyanobacterial dominance (<0.0001).	12 April 1995. 3 month	Everall, N.C. and Lees, D.R. (1997).
<i>Melosira, Gloenkenia, Stephanodiscus, Synedra, Tabellaria, Pediastrum, Staurastrum, Chlorella Spp</i>	Various	Odour/ taste. Filter Clogging	Initially 22 tonnes (3x7 bales over 25000m3). Additional Dec 1.6t (50 nets of 32kg). 1994 study 1.9tonnes in 10m/20kg sausage = 6 - 28 g m ³	Once straw introduced algae reduced to 25% for rest of Exp. 0.04 g d ⁻¹ m ³ .	Field. Reservoir. March 1993/Dec 1993 then 1994-7 for sausages	Barrett, P. R. F., Littlejohn, J.W. and Cunrow, J. (1999)
<i>Microcystis aeruginosa</i>	Cyanophyta	Toxic. Taste +odour probs	Both(liquor +liquor and straw.	Biomass significantly lower than control <0.01.	Lab. Two weeks	Ferrier, M. D. <i>et</i> <i>al.</i> ,(2005)
<i>Microcystis aeruginosa</i>	Cyanophyta	Toxic. Taste+odour probs	2,5,10 g-1 sycamore, 1,2,4 g-1 Elm	Significant difference (<0.001) for all woods.	Lab. 3-4 days	Pillinger, J. M., Gilmour, I. and Ridge, I. (1995)

<i>Microcystis aeruginosa</i>	Cyanophyta	Toxic. Taste+odour probs	Range up to 1 ml. 40/25 ug l ⁻¹ for quinones	Points towards oxidised phenolic compounds. Quinones 1000x more effective than ferulic and p-courmaric acids. 2,3 dichloronaphthoquinone 2ug ⁻¹ complete kill microcystis..	Lab.3-4 days. Straw 3 months before use.	Pillinger, J. M., Cooper, J. A. and Ridge, I. (1994)
<i>Microcystis aeruginosa</i>	Cyanophyta	Toxic. Taste+odour probs	Straw added 22may 1991. sampled 78-92 days after after addition. Straw = 2.57gm m-3	95% inhibition with 2.57gm-3 dwt..	Lab. 72h	Newman, J. R. and Barrett, P. R. F. (1993).
<i>Microcystis aeruginosa</i>	Cyanophyta		2.5-5 gm-3	95% inhibition.	See Newman. J.R. (1993)	Barrett, P. R. F. (1994).
<i>Microcystis aeruginosa</i>	Cyanophyta		Leaves.5.5cm3/ Liquor and ground + liquor.	50% inhibition. Fluctuations over exp ie. tannins-lag phase-lignin.	Lab +simulated field 2.5 years	Ridge, I., Walters. J. and Street. M. (1999)
<i>Oedogonium Sp</i>			100 g wet wt.	<0.001. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls.	Gibson, M. T., Welch, I. M., Barrett, P. R. F. and Ridge, I. (1990)
<i>Saprolegnia diclina</i>	Fungi	Fish disease	Liquor / straw at 1g l ⁻¹ dry wt.	Growth suppressed in presence of straw. Lasted 6 months. Inhibition occurs in light or dark. Effective at 10g l-1 -1000g l-1.	Lab. Seven days. Looking at mycelium.	Cooper, A. J, Pillinger, J. M. and Ridge, I. (1997)
<i>Saprolegnia ferax</i>	Fungi	Fish disease	Liquor / straw at 1g l ⁻¹ dry wt.	So significant inhibition till 10-11 days.	Lab. Seven days. Looking at mycelium	Cooper, A. J., Pillinger, J. M. and Ridge, I. (1997)
<i>Saprolegnia parasitica</i>	Fungi	Fish disease	Liquor / straw at 1g l ⁻¹ dry wt.	Growth suppressed in presence of straw + Liquor. Lasted 6 months.	Lab. Seven days. Looking at mycelium.	Cooper. A.J. Pillinger. J.M. Ridge I. (1997)
<i>Selenastrum capricornutum</i>	Chlorophyta		40 g wet wt	<0.001. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls.	Gibson, M. T., Welch, I. M., Barrett, P. R. F. and Ridge, I. (1990)

<i>Spirogyra</i> Sp	Chlorophyta	Mat-Forming, Filter-clogging.	Both(liquor +liquor and straw.	Increased growth in presence of unfiltered (<0.05) Inhibition in presence of sterile liquor (<0.01).	Lab. Two weeks	Ferrier, M. D. <i>et al</i> , (2005)
<i>Spirogyra</i> Sp	Chlorophyta		100 g wet wt.	<0.001. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls	Gibson, M. T., Welch, I. M., Barrett,P. R. F. and Ridge, I. (1990)
<i>Stigeoclonium tenue</i>			100 g wet wt.	<0.05. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls	Gibson, M. T., Welch, I. M., Barrett, P. R. F. and Ridge, I. (1990)
<i>Synura Petersenii</i>	Chrysophyta	Taste + odour	Both (liquor +liquor and straw.	Complete inhibition <0.01. For both sterile liquor and liquor and straw. Chlorophyll a undetectable at end of two week period.	Lab. Two weeks	Ferrier, M. D. <i>et al</i> ,(2005)
<i>Ulothrix trentonense</i>	Chlorophyta		100 g wet wt.	<0.001. Significant reduction in ug l-1 chlorophyll a.	Till significant growth in controls	Gibson, M. T., Welch, I. M., Barrett,P. R. F. and Ridge, I. (1990)

Barley straw has been effectively tested at a range of concentrations, from 1 kg m^{-3} (Newman and Barrett, 1993) through to $25 - 50 \text{ kg m}^{-3}$ (Everall and Lees, 1996). Everall and Lees (1996) suggested that lower amounts may be effective. Barley straw is never really applied in a controlled, kg m^{-3} measure but it is instead added to the water in bales near to areas of good flow and close to where the algae blooms occur. Laboratory studies such as those carried out by Gibson *et al.*, (1990) used lower amounts of straw at dose around 4 kg m^{-3} . All the trials to date have used fairly similar conditions (Gibson *et al.*, 1990; Welch *et al.*, 1990; Newman and Barrett, 1993; Everall and Lees 1996, 1997). The straw has been anchored near the surface in the flow of water to allow aeration, or directly aerated, and exposure to sunlight and generally alkaline water conditions are other constants. Some salt water species have also been tested (Terlizzi *et al.*, 2002) but again the water is fairly similar. The other constant finding from these studies is that barley straw needs 3+ months to become effective.

This 3 month delay makes barley straw unsuitable for rapid application and a number of studies have attempted to bypass the decay period in an attempt to circumnavigate this problem (Gibson *et al.*, 1990). Attempts have been made to directly remove the inhibitory fraction of barley straw in the form of an extract - such as studies by Grover *et al.* (2007) and Waybright *et al.* (2009) - and these studies strongly support the hypothesis that it is the release of chemicals that leads to the inhibitory effect, by producing extracts from which any straw particles and microorganisms have been filtered out so that only chemical factors remain. Waybright *et al.* (2009) showed how *M. aeruginosa* was successfully inhibited by several different extracts produced from decayed barley straw. Chemical analysis of extracts obtained using filtration and fractionation showed that the inhibitory effect of barley straw was most likely derived from liberated phenol chemicals of a size between 1000-3000 Da. Other studies including Ferrier *et al.* (2005), Terlizzi *et al.* (2002) and Ball *et al.* (2001) have shown that an extract prepared by filtering waters in which decaying barley straw was present in concentrations of around 7 g l^{-1} can inhibit the growth of several species including; *Synura peterseni*, *Dinobryon* sp, *M. aeruginosa* and *Spirogyra* sp, *Heterocapsa triquetra*, *Heterocapsa pygmaea*, *Gymnodinium sanguineum* and *Gyrodinium galatheanum*, *Microcystis* sp and *Scenedesmus* sp. To date, most of these extracts have used rotted barley straw in order to obtain an effective extract. There is

some evidence that the extract is stable after it is produced (Ferrier *et al.*, 2005) which means that there could be potential for it to be stored and used as required later. Until now, the extracts prepared from straw have still relied on time to decay the straw to its active phase. The recent Waybright *et al.* (2009) study, with its finding that phenolic chemicals are most likely responsible for its effect, has opened up a new possibility. Now that phenolic chemicals have been identified as the group responsible for inhibition, research can be undertaken into studying which phenolics are effective and analysing methods of removing and storing the active fraction. This gives the possibility of using the active chemicals directly when required, or bypassing the need to wait for the straw to decay, and storing a prepared extract indefinitely to apply as required. All of which would greatly increase the flexibility of barley straw and potentially guarantee its effectiveness and efficiency.

2.4 Conclusions

The mechanism by which barley straw inhibits algal growth is not fully understood and whilst several hypotheses have been proposed, the most viable explanation focuses on microbiological decay assisting in the release of inhibitory compounds (Barrett 1994; Pillinger *et al.*, 1993; Everall and Lees, 1996, 1997; Love *et al.*, 1998; Witka-Jezewska *et al.*, 2003; Goh *et al.*, 2004; Ferrier *et al.*, 2005).

The most likely explanation for the source of this release seems to be fungal decay. In particular, White rot fungi function in a way that degrades lignin (Granit *et al.*, 2007) and it may be phenolic by-products from this decay that inhibit algal growth. Pillinger (1992) and Witka-Jezewska *et al.* (2003) have both shown that a variety of fungi are associated with rotting barley straw and Pillinger *et al.* (1995) found free lignin material associated with the waters around decaying straw. Phenolics are already known to be able to inhibit the growth of algae (Newman and Barrett, 1993; Schrader *et al.*, 1998) and several toxic phenols have already been found with decaying barley straw (Everall and Lees, 1996, 1997). Recently, chemical analysis suggested that phenolic chemicals were the sole group responsible for the effectiveness of an extract prepared from decaying barley straw (Waybright *et al.*, 2009).

To date, it is this hypothesis that appears most likely to be the source of barley straw's inhibitory effect, although more work still needs to be carried out to investigate the role of decay and, under controlled conditions, to explore further the other possible methods of inhibition. Use of extracts (Ball *et al.*, 2001; Ferrier *et al.*, 2005; Waybright *et al.*, 2009) may prove ideal for water utilities by providing a more readily available and responsive option for controlling algae.

2.5 References

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Chapter 3

Materials and methods – Growth media recipes

3 Growth media

3.1 Jaworski's Medium – Algae

Jaworski's medium was used in the growing of all algae in chapters 4, 5 and 6. The medium was prepared according to the CCAP recipe (Culture Collection of Algae and Protozoa, Argyll, UK) below. All stock chemicals were purchased from ACROS Chemicals, Geel, Belgium.

Table 3.1 Jaworski's medium stock solutions

Stock solution	Chemical	Amount (g l ⁻¹)
1	Ca(NO ₃) ₂ . 4H ₂ O	20
2	KH ₂ PO ₄	12.4
3	MgSO ₄ .7H ₂ O	50
4	NaHCO ₃	15.9
5	EDTAFeNa	25
	EDTANa ₂	2.25
6	H ₃ BO ₃	2.48
	MnCl ₂ .4H ₂ O	1.39
	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	1
7	Cyanocobalamin	0.04
	Thiamine HCl	0.04
	Biotin	0.04
8	NaNO ₃	80
9	Na ₂ HPO ₄ .12H ₂ O	36

Stock solutions were prepared by dissolving the chemicals required for each stock in 1 l deionized (DI) water in a volumetric flask. Once dissolved, all stocks apart from (7) were transferred to clean acid-washed glass bottles and sterilised by autoclaving (121 °C, 15 psi, 15 mins) (PriorClave, London, UK). Due to the heat sensitivity of the chemicals, stock (7) required filter sterilising instead of autoclaving. This was carried out under aseptic conditions in a laminar flow cabinet (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK) using a 1 l Corning disposable filter unit in pore size 0.22 µm . All stocks were stored in a fridge at 5 °C. Medium was prepared as it was

required by adding 1 ml l⁻¹ of each stock to make the amount of medium demanded at that point. For example, if 0.5 l of medium was needed, 0.5 ml of each stock would be added to 0.5 L of DI water. All stocks apart from (7) were added and the media was then autoclaved (121 °C, 15 psi, 15 min's) (PriorClave, London, UK) and allowed to cool. Once cool, stock (7) was added under aseptic conditions.

Malt extract peptone agar – Fungi

All fungal species were maintained in pure cultures on malt extract peptone agar. Agar was prepared according to the recipe shown below (Table 3.2). All chemicals were purchased from ACROS (Geel, Belgium):

Table 3.2. Malt extract peptone agar recipe

Stock	Chemical	Amount (g l ⁻¹)
1	Malt extracts	30
	Soya peptone	3
	Agar	15

The medium was prepared by mixing the media ingredients in 1 L of DI water then autoclaving (121 °C, 15 psi, 15 min's) (PriorClave, London, UK) in a sealed bottle. Once autoclaved, the agar media was allowed to cool to 50 °C in a temperature controlled water bath (Grant Y28; Grant Instruments, Cambridge, UK). Having cooled, the media was poured into petri dishes straight away ((pre-sterilised, Fisher Scientific, Loughborough, UK); this had to be done to prevent the agar setting in the bottle. All this was carried out under aseptic conditions inside a laminar flow cabinet (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK). The plates were allowed to set, then were sealed with parafilm and stored at 5 °C until needed.

Chapter 4

Inhibition of Three Algae Species Using Chemicals Released From Barley Straw

Submitted to: *Environmental Technology*

4 INHIBITION OF THREE ALGAE SPECIES USING CHEMICALS RELEASED FROM BARLEY STRAW.

D. Murray, B. Jefferson and S. A. Parsons*

Centre for Water Sciences, Cranfield University, Cranfield, Bedfordshire, MK43
OAL, UK

4.1 Abstract

Algae blooms are a significant problem in the UK and in particular in water sources that supply potable water treatment works. A wide range of methods to control algae have been tested and whilst many are effective they all have disadvantages. The use of barley straw to control algae growth in reservoirs is one option that is gaining popularity but little is known about its mode of action. One suggested mechanism is that as the straw is broken down algastatic chemicals such as phenolics are released. Here we have used an algae inhibition test to evaluate the effect of chemicals reported to be released from straw on three common algae species: *Chlorella vulgaris*, *Microcystis aeruginosa* and *Scenedesmus subspicatus*. It was shown that of the chemicals assessed, many produced an algastatic effect on the growth of the three common algal species tested with 2-phenyl-phenol being the most effective whilst P-Cresol and benzaldehyde were shown to be effective at concentrations similar to those that have been reported downstream of rotted straw (EC_{50} of 85 mg l^{-1} and EC_{50} of 28 mg l^{-1} respectively) (EC_{50} , concentration required to effectively reduce the population by 50 %). *Scenedesmus subspicatus* proved much more resistant to the chemicals tested than the other species.

Keywords: algae bloom; barley straw; *Chlorella vulgaris*; *Microcystis aeruginosa*; *Scenedesmus subspicatus*

4.2 Introduction

Algae blooms are a significant problem in the UK and in particular in water sources that supply potable water treatment works (Henderson *et al.*, 2008). The algae can produce aesthetic changes to a water body, such as colour or taste and odour issues, they can lead to the overwhelming of filtration processes (Greene and Hayes, 1981) and certain species have been shown to release toxic metabolites which can cause health problems (Hutson *et al.*, 1987). Pretty (2003) analysed the impact of algae blooms and approximated the costs to be between £75m – £114m a year for England and Wales.

Options for controlling algal blooms range from simple mechanical processes such as dredging or raking surface waters to remove filamentous algae (Barret, 1994) through to more complex biological processes and chemical processes. Whilst effective at first, mechanical processes only tend to offset the problem for a short period of time and can damage less hardy species such as macrophytes and benthic filter feeders (Ceccherelli and Piazzzi, 2005). Biological options such as food web manipulation look interesting at first glance but have considerable drawbacks. Biological manipulation requires time, constant management and control of lake use (Olin *et al.*, 2006) and factors such as background turbidity (Van Den Berg *et al.*, 1998) and level of eutrophication can offset the effectiveness.

Chemical control options include the dosing of metal salts or clays to control phosphorus levels and hence control algae growth (Kang *et al.*, 2003; Surampalli *et al.*, 1995) through to dosing chemicals such as atrazine, copper sulphate or diquat that are toxic to the algae at relatively low doses (Pratt *et al.*, 1997; Peterson *et al.*, 1997; Van Hullebusch *et al.*, 2003). Whilst attractive due to their efficacy, many of these chemicals have been subsequently proven to cause more harm than good, such as by being non-specific. Thus, they harm a range of species that are often higher plants and other competitors (Barrett, 1994) either through bioaccumulation or biomagnification into higher organisms (Murray-Gulde *et al.*, 2002; Van Hullebusch *et al.*, 2003) or through persistence in the environment (Birmingham and Coleman 1983; Pratt *et al.* 1997; Van Hullebusch *et al.*, 2003).

One option that is gaining popularity in the UK is the use of barley straw, where bales of the straw are suspended in surface water reservoirs. This is not a new option and work in the 1970s suggested that barley straw may be a potential solution to controlling algal growth at reservoir level (Street, 1978). The activity of barley straw is usually described as being algistatic rather than algicidal and a range of mechanisms have been proposed to explain its success ranging from adsorption of phosphorus (Winfield *et al.*, 1985) through to the direct or indirect release of toxic or inhibitory compounds (Barrett 1994; Ridge and Pillinger, 1996).

However, although most of the mechanisms have been ruled out by laboratory research, the most viable explanation revolves around microbiological decay assisting in the release of inhibitory compounds (Pillinger *et al.*, 1993; Barrett 1994; Everall and Lees 1996; Love *et al.*, 1998; Witka-Jezewska, 2003; Goh and Tuta, 2004, Ferrier *et al.*, 2005). It was the research of Everall and Lees (1996), who reported the results of a field study of barley straw application (50 g m^{-3}) in a water supply reservoir in Derbyshire, UK, that has come closest to proving this mechanism. They assessed the impact of straw on algae count and water quality and showed how the application of the barley straw led to a decrease in algae numbers in the reservoir from 55000 cells ml^{-1} to less than 5000 cells ml^{-1} when compared to previous years. Water samples were collected and chemical analysis upstream and downstream of the straw showed the release of 38 identified compounds including 2, 6 dimethoxyphenol and 3 methylbutanoic acid plus 30 unidentified compounds (Table 4.1). The study was reproduced (Everall and Lees, 1997) in another reservoir which had suffered severe blooms of *Oscillatoria tenuis*. For this trial, barley straw was applied at a dose of 25 g m^{-3} and there was a significant reduction in algae concentrations from 100000 cells ml^{-1} down to 10000 cells ml^{-1} in the dosed reservoir and 12 days later this was replicated in a lower reservoir. Substances identified as released by the straw included phenolic compounds such as 2 methoxyphenol and 2, 6 dimethoxyphenol.

Table 4.1 Chemicals found in waters associated with rotting barely straw ($\mu\text{g l}^{-1}$) and from methylated and unmethylated extracts from barley rotting in water ($\mu\text{g kg}^{-1}$).

COMPOUNDS FROM ROTTING BARLEY STRAW			
Compound	Max concentration $\mu\text{g l}^{-1}$	Compound	Max concentration $\mu\text{g l}^{-1}$
9 Eicosene ¹	1000	3 Methyl-3-buten-2-one ¹	10
Hydrocinnamic acid ¹	1000	3 Pentan-2-ol ²	10
Hexadecanoic acid ¹	1000	9 Hexadecanoic acid ²	10
1 [4 Hydroxy-3,5 dimethoxyphenyl] ethanone ²	100	9 Octadecanoic acid ¹	10
1 Phenyl-ethanone ²	100	Acetic acid ²	10
2, 6 Dimethoxy-4-phenol ²	100	Benzaldehyde ²	10
2 Methybutanoic acid ¹	100	Benzene ²	10
3 Phenyl-2-propenoic acid ²	100	Butanoic acid ¹	10
4 Methyl phenol ²	100	Dodecanoic acid ¹	10
Benzene acetic acid ²	100	2, 3 Dimethyl-3-buten-2ol ²	1
Benzene acetonitrile ¹	100	2 Ethylhexyl ester of 3-[4-methoxy phenyl]-2-propanoic acid ¹	1
Benzoic acid ^{1, 2}	100	2 Heptadecanone ²	1
Carboxylic acid ¹	100	2 Methyl-1, 3-dioxolone ²	1
Ethenylbenzene ²	100	2 Methyl-2-propenoic acid ester ²	1
Heptanoic acid ¹	100	2 Pentadecanone ²	1
Hexanoic acid ¹	100	4 [1, 1-Dimethylethyl] benzoic acid ¹	1
Octadecanoic acid ¹	100	Dimethyl disulphide ²	1
Octadecanol ¹	100	Dimethyl trisulphide ²	1
Phenol ²	10	Docosanoic acid ²	1
[1, 1-Dimethylethyl]-4-methoxyphenol ²	10	Eicosanoic acid ²	1
[1, 1-Biphenyl]-2-ol ¹	10	Pentanoic acid ²	1
1, 2 Dimethylbenzene ²	10	Tetradecanoic acid ¹	1
2 Methoxy-4-methyl phenol ²	10	Geosmin ²	0.1
3 Ethyl-2, 2-dimethyloxinane ²	10	MCPA ¹	0.1
3 Methylbutanoic acid ²	10	MCPP ¹	0.1
3 Methyl-3-buten-2ol ²	10		

COMPOUNDS FROM METHYLATED & UNMETHYLATED EXTRACT			
Compound	Max concentration $\mu\text{g kg}^{-1}$	Compound	Max concentration $\mu\text{g kg}^{-1}$
Hexanoic acid ²	1000	2,6 Dimethoxy-4-[2 propenyl] phenol ²	1000
Octanoic acid ²	1000	5,6,7,7A-Tetrahydro-4, 4, 7A-trimethyl-2 [4H] benzofuranone ²	1000
Nonanoic acid ²	1000	1,1,4,4 Tetramethyl-2, 6-bis[methylene] cyclohexone ²	1000

Decanoic acid ²	1000	1 Hexacosene ²	1000
Dodecanoic acid ²	1000	Acetic Acid ²	100
Tetradecanoic acid ²	1000	3 Methylbutanoic acid ²	100
Hexadecanoic acid ²	1000	2 Methylbutanoic acid ²	100
1 Methylnaphthalene ²	1000	Heptanoic acid ²	100
2 [1-1-Dimethylethyl]phenol ²	1000	[1,1-Dimethylethyl]-4-methoxyphenol ²	100
2,3 Dihydrobenzofuron ²	1000		

Paper noted where chemical or maximum chemical concentration identified ¹Everall and Lees (1996), ²Everall and Lees (1997).

This paper looks at the chemicals shown to be released from barley straw and tests their impact on three common algae species: *C. vulgaris*, *M. aeruginosa* and *S. subspicatus*. The inhibition of algae growth was evaluated using the OECD 201 (Organisation of Economic Cooperation and Development, Paris, France) algal growth inhibition test which is an accepted method for screening chemicals in this way (Eisentrager *et al.*, 2003; Pavlic *et al.*, 2006).

4.3 Material and Methods

4.3.1 Algae Inhibition Test

4.3.1.1 Preparation and maintenance of algae

Pure cultures of *Chlorella vulgaris* 211/11b, *Microcystis aeruginosa* 1450/3 and *Scenedesmus subspicatus* 276/20 were obtained from the CCAP (Culture Collection of Algae and Protozoa, Argyll, UK) and kept in exponential growth in CCAP recipe *Jaworski's Medium* (J. Media) with weekly sub-culturing. All glassware, measuring cylinders and cotton wool bungs (Fisher Scientific, Loughborough, UK) used in culturing were acid -washed and autoclaved at 121 °C, 15 psi, for 15 minutes (PriorClave, London, UK) and allowed to cool to room temperature before use. All pipettes used were variable volume pipettes (Fisher Scientific, Loughborough, UK) and all tips were disposable 1ml Fisher brand pipette tips. All culturing was carried out under standard aseptic conditions, via the use of a laminar flow cabinet (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK). Culturing was performed by measuring 100 ml of new media into a 250 ml Erlenmeyer flask; to this 5 ml of pure algal culture was added and the flask was then stoppered with a sterile cotton

wool bung and placed on to a *Bibby Stuart* SSL1 orbital shaker (Barloworld Scientific, Staffordshire, UK) at 110 rpm at room temperature (18-20 °C). Growth was carried out under the constant light of 2x 15w Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK).

The chemicals were obtained from the following sources: 2, 6 dimethoxy phenol, 2-methylbuteric acid, acetophenone, benzaldehyde, bezenzyl cyanide, benzoic acid, heptanoic acid, hexanoic acid, hydrocinnimac acid, p-cresol, 2 Phenyl-phenol, trans cinimmamic acid and copper sulphate Fisher Scientific – ACROS chemicals (Geel, Belgium). 3-Methylbutryic acid, Sigma-Aldrich Co (Dorset, UK)

4.3.1.2 Assessment of chemical toxicity

The chemicals were assessed using the OECD (Organisation of Economic Cooperation and Development) (Paris, France) 201 “*Algal toxicity test*” method using *Chlorella vulgaris* 211/11b, *Microcystis aeruginosa* 1450/3 and, *Scenedesmus subspicatus* 276/20 as the test species (CCAP, Argyll, UK). The OECD 201 test was selected as it is carried out using cell counts. This is beneficial when testing for inhibition as unviable cells can be ignored. It also offers benefits over population estimation via chlorophyll analysis as chemicals such as copper sulphate can cause cell lyses, meaning there is a possibility for liberated chlorophyll to skew test data. The tests were carried out in acid-washed, autoclaved (121 °C, 15 psi, 15 min’s) (PriorClave, London, UK) 250 ml Erlenmeyer flasks with sterile cotton wool stoppers containing 100 ml of J. Media to which a starting concentration of 10000 cells ml⁻¹ was added. Starting cell concentrations were calculated by use of a haemocytometer (Fisher Scientific, Loughborough, UK), where samples from pure cultures of the algae were added to the counting chamber by use of a 1 ml Fisher brand, variable volume pipette and sterilised 1ml disposable tips (sterilisation done via autoclaving). All 9 squares on both grids of the haemocytometer were counted. This meant that the total cells counted in each grid could be x10000 to give the cells ml⁻¹ in the starting culture, and this was carried out 3 times, whenever a dilution was prepared, to ensure accuracy. The culture could then be diluted with fresh media to give a concentration

of 1000000 cells ml⁻¹; and 1 ml could then be added to the 100 ml sample to give 10000 cells ml⁻¹.

The effectiveness of each chemical was tested at 5 different concentrations: 10, 100, 200, 500 and 1000 µg l⁻¹. Samples were prepared from 10000 µg l⁻¹ stock solutions and dilutions made for each chemical in clean sterile glassware. 1, 0.5, 0.2, 0.1 and 0.01 ml could then be pipetted into the samples to give the desired chemical starting concentration. These concentrations had been selected to reflect the concentrations at which the chemicals had been reported as being found in the literature studied (Everall and Lees 1996; Everall and Lees, 1997). Each experiment was conducted in triplicate and 6 controls were used for comparison. The cultures were grown on a *Bibby Stuart* SSL1 (Barloworld Scientific, Staffordshire, UK) orbital shaker under two 15w Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK). Cell counts were carried out every 24 h for 72 h using a haemocytometer, counting the entire main 9 squares on both sides and repeating 6 times for each flask to reduce error.

4.3.1.3 Data analysis

The cell count data were analysed according to the OECD 201 test's recommended methods. Firstly, cell count data were plotted against time using the average of the 6 samples from the 3 repeats for each chemical, giving a total of 18 cell counts for each chemical concentration, each day; this was compared to the average of the 36 cell counts obtained for the 6 controls, each day. 95 % Confidence intervals were used as an assessment of reproducibility. This could be used to show the effect of each concentration on growth over time (Figs 4.1, 4.2). The % inhibition of each chemical concentration on each species was assessed initially by calculating the differences in the area under the plotted cell counts (Equation 1), then by calculating the inhibition of cell growth by each test substance concentration data (Equation 2) to produce growth inhibition curves such as in fig 4.3:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \quad (1)$$

A = area

N_0 = nominal number of cells/ml at time t_0

N_1 = measured number of cells/ml at t_1

N_n = measured number of cells/ml at time t_n

t_1 = time of first measurement after beginning of test

t_n = time of nth measurement after beginning of test

(2)

$$I_a = \frac{A_c - A_t}{A_c} \times 100$$

I_a = % inhibition of growth curve by test concentration

A_c = Area under the control growth curve

A_t = Area under test substance concentration growth curve

The statistical significance of any effect could then be assessed by using a single factor ANOVA to compare the data from each concentration to the control. Chemical effectiveness was then compared to the effectiveness of CuSO_4 , a known algicide.

4.4 Results and discussion

4.4.1 Chemical selection

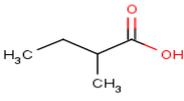
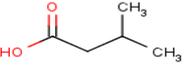
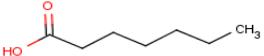
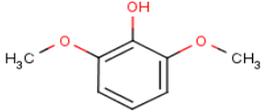
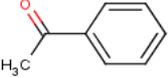
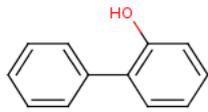
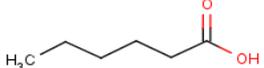
It was not possible to test all compounds, so the initial list of 39 chemicals identified by Everall and Lees (1996) was screened to find chemicals that were soluble, found at reasonable concentration ($>10 \mu\text{g l}^{-1}$), and contained either phenolic groups or had previous evidence of algae toxicity. A number of the chemicals were selected as they are phenolic compounds including 2, 6 dimethoxyphenol, 2, 6 dimethoxy-4-phenol, p-cresol and 2-phenylphenol. For example p-cresol is a soluble, known algal toxin which has previously been reported to be formed by microbial degradation (Hwang and Maloney, 1996). Other compounds such as heptanoic acid (Fukuda *et al.*, 2004), hexanoic acid (Elliott *et al.*, 1978; Armstrong and Armstrong, 2002), benzoic acid (Lu and Metcalf, 1975), and hydrocinnamic acid (Dedonder and Van Sumere 1971), were selected as they have been shown previously to be toxic to algae or higher plants. Fuuda *et al.* (2004) showed that a 0.1 M suspension was able to reduce the viability of

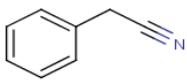
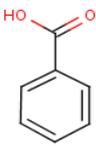
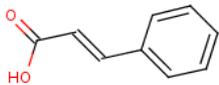
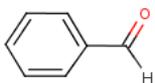
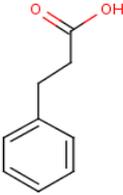
crab grass by 30 % compared to the control and Armstrong and Armstrong (2002) were able to significantly reduce the growth of various organelles in the plant *Phragmites* with concentrations of between 0.56 and 1.4 mM. Lee and Chen (2008) have shown that various forms of benzoic acid can be very effective at inhibiting algal growth, despite the lack of detailed algal toxicity data on them in the literature. For benzoic acid itself they reported that a NOEC of 4.81 mg l⁻¹. 40 phenolic compounds were tested for toxic effects on *Chlorella* by Dedonder and Van Sumere (1971). They found that chemicals such as p-nitrophenol, 2, 4-dihydroxybenzaldehyde and hydrocinnamic acid were able to produce inhibitions of 5 × 10⁻⁵M at concentrations under a few milligrams l⁻¹. Hydrocinnamic acid has also been shown to be a potent allopathic chemical on higher plants with concentrations of less than 200 ppm being able to reduce the root and shoot biomass of *Schizachyrium scoparium* by almost 80% (Williamson *et al.*, 1992).

The final compounds selected for testing were decided upon by simple elimination. This was based on existing toxicity knowledge, such as that summarised in table 4.1: concentration associated with barley straw, solubility, and whether the chemical was a phenol or not. As it can often be seen in the literature outlined above, phenolic chemicals are often among the more effective chemicals suggested to come from barley straw. The chemicals selected for analysis are described in table 4.2 where their common names have been used for ease of reference (as in all other proceeding tables and figures). Copper sulphate was also chosen as a bench mark by which to test the chosen compounds.

Table 4.2 Description of structure and basic chemical properties of compounds selected for assessment. (# Chemicals common names have been used for ease of reference). **Table also gives a summary of why chemicals were selected for assessment.**

Chemical [#]	Structure	pKa	Log p	Water Solubility (mg/l)	Reason for selection
P-Cresol		10.26	1.94	2.15E+04	Soluble, phenolic Known algal toxin, Can be produced by microbiological degradation

2-Methylbutanoic acid		4.87	1.18	4.50E+04	Soluble, phenol Known toxic chemical. Related to 3-methylbutanoic acid known algal toxin Kompany-Zareh (2008)
3-Methylbutanoic acid		4.77	1.16	4.07E+04	Soluble, phenol, naturally occurs in plants, can be toxic at low concentrations Kompany-Zareh (2008)
Heptanoic acid		4.8	2.42	2.82E+03	Soluble. Phenol, Phytotoxin.
2,6 Dimethoxyphenol		9.98	1.15	1.72E+04	Soluble, Phenol, reported toxic to cyanobacteria, Pillinger <i>et al.</i> , 1994
Acetophenone		21.55	1.58	6.13E+03	Soluble, Ketone, effects on some aquatic organisms
2-Phenylphenol		9.97	3.09	7.00E+02	Phenol, widely used as an agricultural fungicide
Hexanoic acid		4.879	1.92	1.03E+04	Soluble, Known phytotoxin

Benzyl cyanide		1.56		1.00E+02	Soluble, Known Phytotoxic compound
Benzoic acid		4.19	1.87	3.40E+03	Soluble, Known to inhibit algal growth (Lu and Metcalf, 1975)
Trans-cinnamic acid		4.44	2.13	5.46E+02	Soluble, Phenol
Benzaldehyde		0.96	1.48	6.57E+03	Aldehyde, Known to be toxic to algae.
Hydrocinnamic acid		4.66	1.84	5.90E+03	Known to effect plant protein production. known toxicity to algae .Mulavoa and Rappaport (1973)
Copper sulphate		5.64		2.31E+05	Reference compound

4.4.2 Inhibition testing

Results from the growth inhibition tests for *C. vulgaris* and *M. Aeruginosa* are shown below (Figures 4.1, 4.2, 4.3 and Table 4.3). Figures 4.1 and 4.2 show the effect of time and concentration of each chemical tested. The data was used to generate growth inhibition curves (Figure 4.3), which show the inhibitory effect of 3 of the most effective chemicals on the 3 algal species. The growth curves were used to estimate the EC₅₀ for all tested chemicals: for *C. vulgaris* and *M. aeruginosa*, and the 2 best chemicals plus copper sulphate for *Scenedesmus subspicatus* (Table 4.3). It should be noted that due to lab conditions and the differences between the species tested. There was a small variation in growth rates observed in the controls, though this was deemed to not have a significant impact on the overall study.

Table 4.3 The ability of 13 chemicals associated with decaying barley straw, plus copper sulphate, to affect the growth of *C. vulgaris*, *M. Aeruginosa* and *S. subspicatus*. Data for EC₅₀ values are displayed in µg l⁻¹. Only CuSO₄, 2 phenyl phenol and benzaldehyde tested with *Scenedesmus*.

Chemical	EC ₅₀ (µg l ⁻¹)		
	<i>Chlorella</i>	<i>Microcystis</i>	<i>Scenedesmus</i>
CuSO ₄	22	10	20
2 Phenyl phenol	10	150	200
Benzaldehyde	28	100	300
3-Methylbuteric acid	85	500	
P-Cresol	110	150	
2-Methylbuteric acid	110	300	
Benzoic acid	140	250	
Heptanoic acid	150	650	
Benzyl cyanide	190	No effect	
Trans-cinnamic acid	270	No effect	
Acetophenone	1000	No effect	
2,6 Dimethoxy phenol	>1500	No effect	
Hexanoic acid	No effect	1000	
Hydrocinnamic acid	No effect	No effect	

If we consider the control data we can see that over the 72 hour test, the algae cell count increased fairly consistently from 10000 cell ml⁻¹ up to 1000000 cell ml⁻¹. EC₅₀ values were calculated for each compound (Table 4.3) and it was clear that the majority of the chemicals tested, apart from hexanoic and hydrocinimmamic acids, produced an inhibitory effect on *C. vulgaris* (Table 4.3). 2, 6 dimethoxyphenol also had a limited effect with the EC₅₀ found to be 1500 µg l⁻¹ which was significantly greater than the concentration in which it was reported to be found in the field (10 µg l⁻¹). A number of chemicals produced an inhibitory effect but the EC₅₀ values were well in excess of the levels reported to be released from barley straw. For example, trans-cinimmamic acid and acetophenone were shown to have EC₅₀ values (for *C. vulgaris*) of 270 and 1000 µg l⁻¹ whilst reported to be found at concentrations closer to 100 µg l⁻¹ associated with barley in the field (Everall and Lees 1996; Everall and Lees, 1997) (Table 4.3). P-Cresol was shown to be effective (EC₅₀ of 85 µg l⁻¹) at concentrations similar to those reported, as was benzaldehyde (EC₅₀ of 28 µg l⁻¹). Of all the chemicals tested, 2 phenyl-phenol was the most effective and would also be active at the concentrations reported by Everall and Lees (1996) (Figure 4.3, Table 4.3).

The data appear to show that, even at high end concentrations, the majority of the chemicals tested only ever produced an algalstatic effect, fitting in with the effects observed in previous studies (Welch *et al.*, 1990; Pillinger *et al.*, 1994; Pillinger *et al.*, 1995; Everall and Lees 1996; Everall and Lees, 1997). There are three possible exceptions to this: 2 phenyl phenol, which appeared to become algacidal to *C. vulgaris* at concentrations of over 100 µg l⁻¹, and 3-methylbuteric acid and benzene cyanide that also appeared to have algicidal effects at higher concentrations when tested with *C. vulgaris*. Copper sulphate, as expected, showed strong toxicity for all three species (Figure 4.3) and algicidal effects were observed for all three species at over 100 µg l⁻¹.

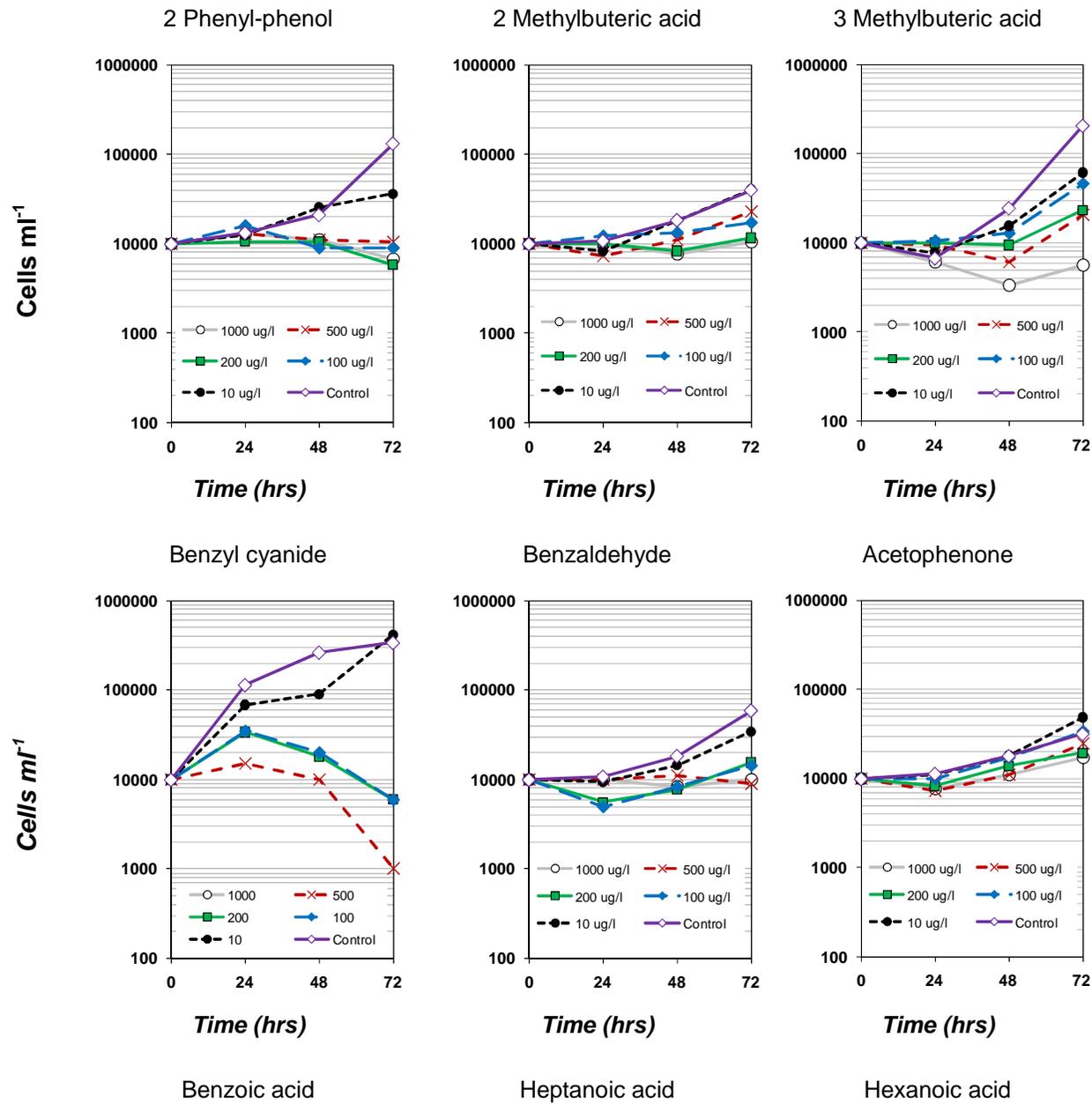
M. aeruginosa was significantly more resistant to the chemicals released from straw than *C. vulgaris* (Table 4.3) - which is surprising as many of the laboratory studies of barley straw have shown that it is more effective against *M. aeruginosa* than *C. vulgaris* (Newman and Barrett, 1993; Martin and Ridge, 1999; Ferrier *et al.*, 2005;

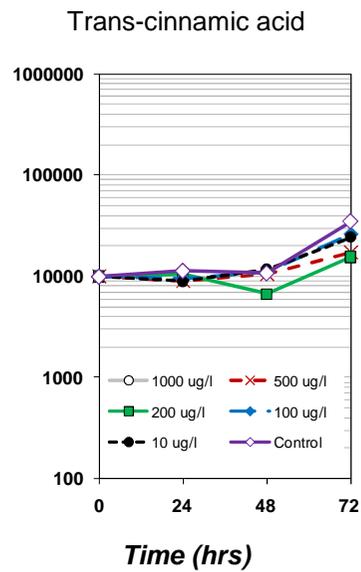
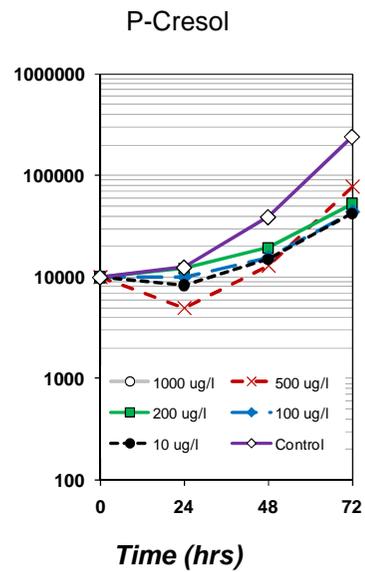
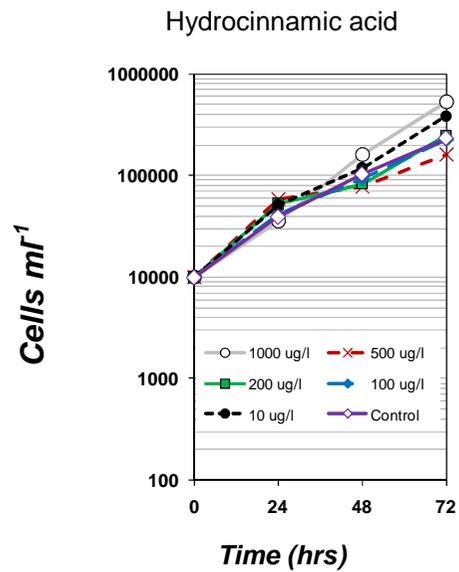
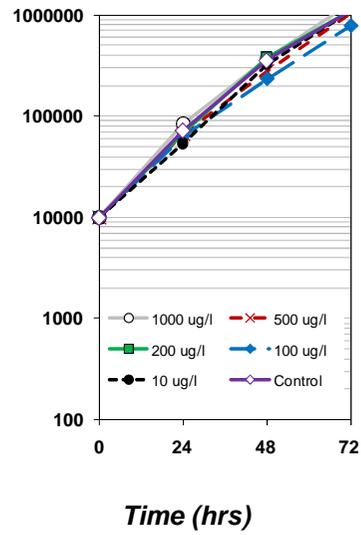
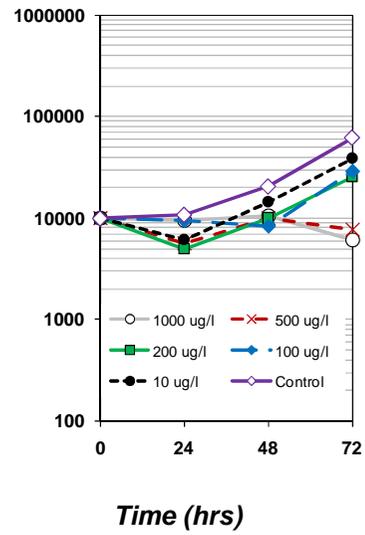
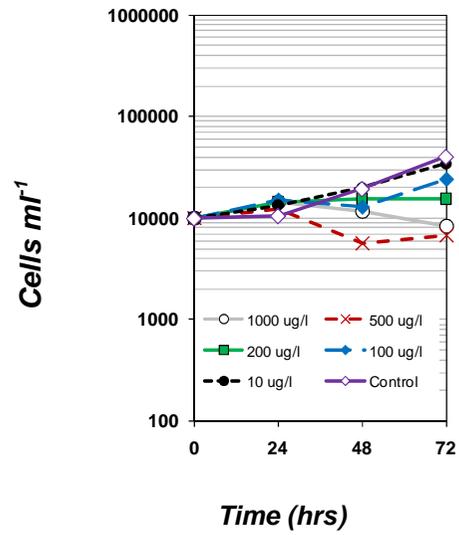
Waybright *et al.*, 2009). Of the chemicals tested, benzyl cyanide, trans cinimamic acid, acetophenone, 2, 6 dimethoxyphenol and hydrocinimamic acid all had no effect on the growth of *M. aeruginosa* at any of the concentrations under examination.

Previously, work going back over 50 years has highlighted the anti-algal properties of certain organic chemicals. For example, Fitzgerald *et al.* (1952) showed that 2, 3-dichloronaphthoquinone acted as an algaecide for *M. aeruginosa* at a dose of $2 \mu\text{g L}^{-1}$. Schrader *et al.* (1998) reported a 5 day, lowest observed effective concentration (LOEC) of $1641 \mu\text{g L}^{-1}$ for P-coumaric acid on *Oscillatoria cf. chalybea*, whilst ferulic acid was found to be effective on *Oscillatoria* with a 5 day LOEC of $1941 \mu\text{g l}^{-1}$.

Phenolic chemicals have been particularly effective (Tang and Waiss, 1978; Pillinger *et al.*, 1994). The lignin in barley straw is known to contain polyphenolic compounds (Pillinger *et al.*, 1994). It has been suggested that lignin under both basic and well-aerated conditions is a potential source of oxidisable phenolic material. Pillinger *et al.* (1994) showed that the growth of *M. aeruginosa* was inhibited by quinone compounds, especially under aerated conditions. For example, 1, 2 naphthoquinone and 9, 10-phanantherene quinone had reported IC_{50} values of 40 and $25 \mu\text{g L}^{-1}$ respectively. Other authors have found similar properties for tannins released from leaf litter in water (Hussein-Ayoub and Yankov, 1985; Ridge and Pillinger, 1996; Ridge *et al.*, 1999; De Nicola *et al.*, 2004).

The two most inhibitory chemicals, 2 phenyl phenol and benzaldehyde were tested on a third species of algae, the hardy green alga *S. subspicatus*, for comparison to the other species (4.3). Previous reports have shown that this species was not inhibited at all by barley straw (Martin and Ridge, 1999) but *S. subspicatus* is a significant issue in the UK (Henderson *et al.*, 2008). *S. subspicatus* proved much more resistant to 2 phenyl phenol and Benzaldehyde with EC_{50} values of 200 and $300 \mu\text{g l}^{-1}$ respectively. Copper sulphate remained constant with an EC_{50} of $20 \mu\text{g l}^{-1}$.





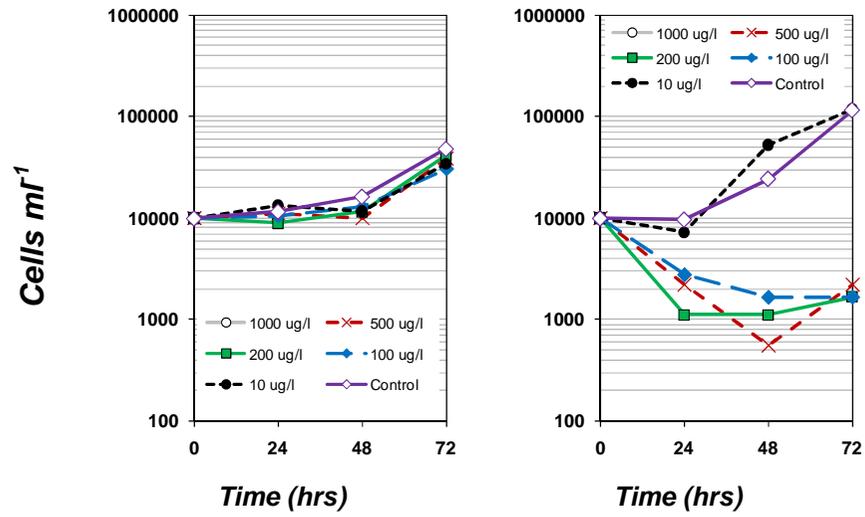
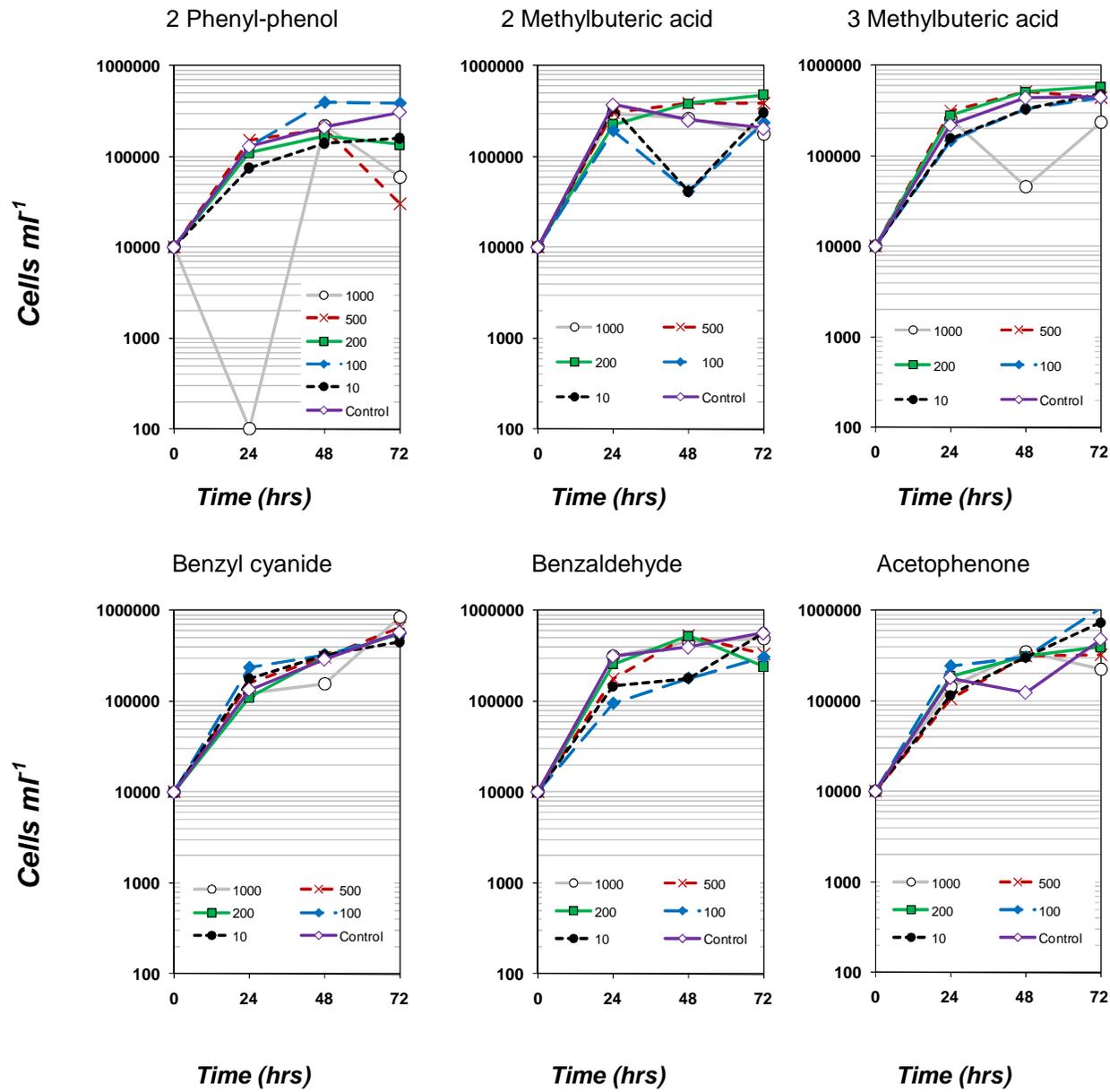
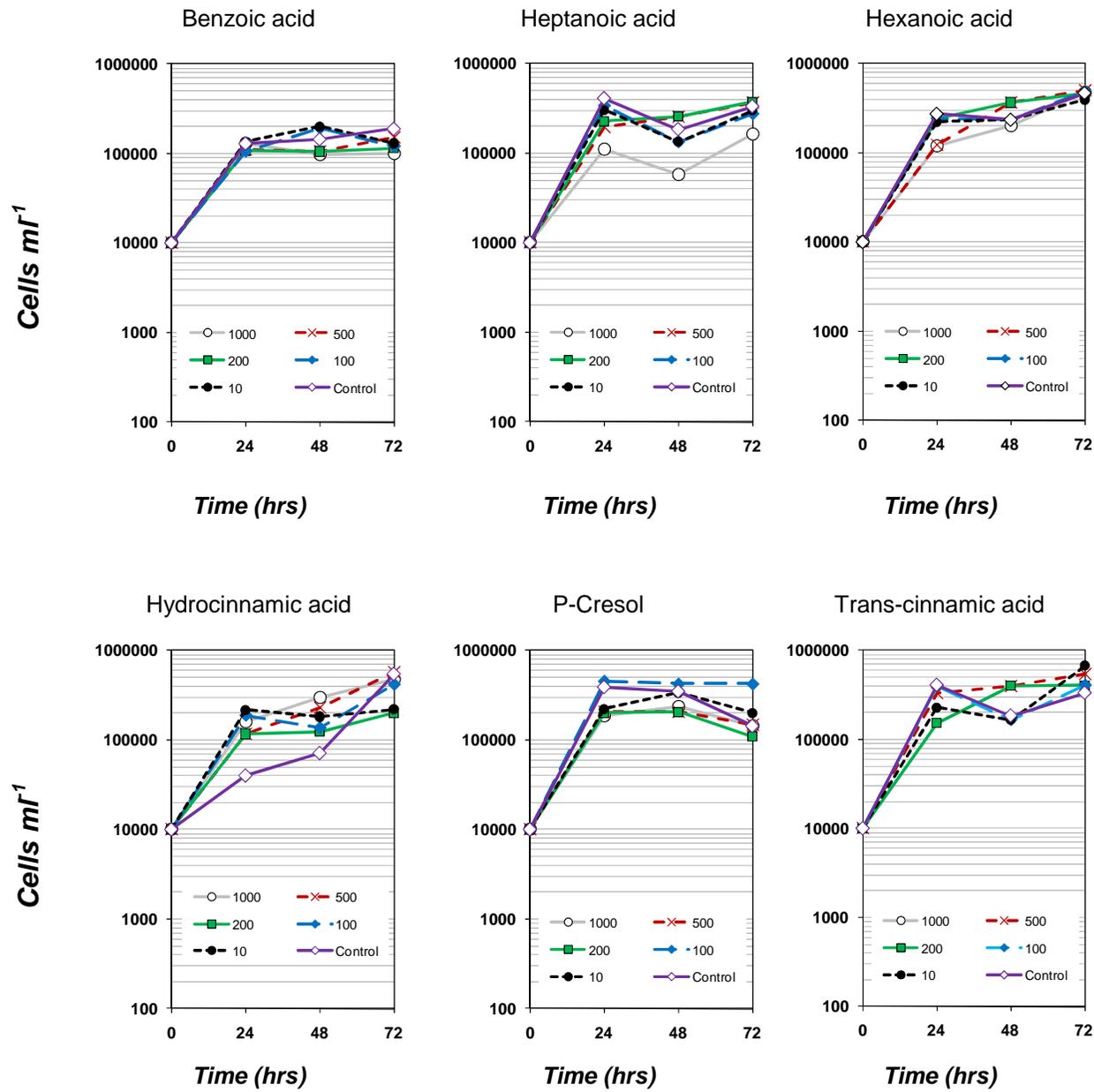


Figure 4.1. Effects of 13 tested chemicals on the growth of *C. vulgaris* over 72 h. Chemicals tested at a range of concentrations from 10 - 1000 $\mu\text{g l}^{-1}$





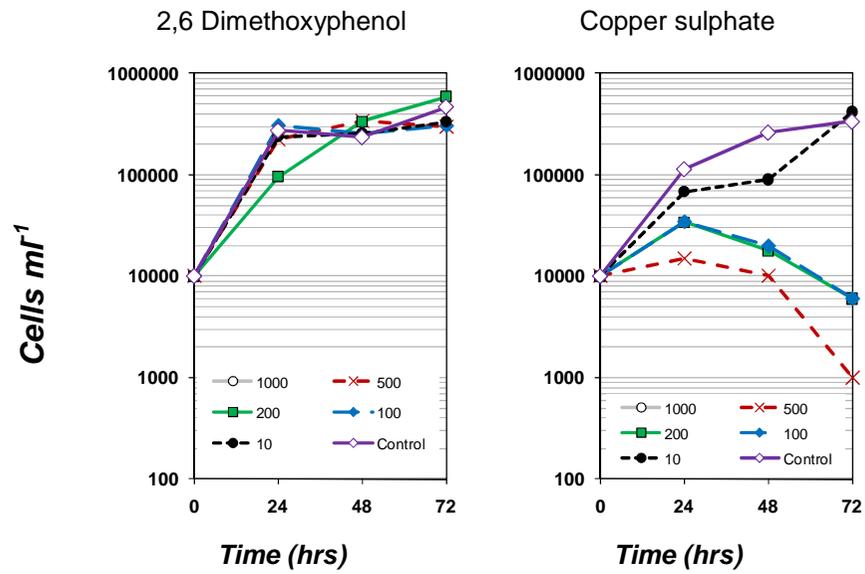


Figure 4.2. Effects of 13 tested chemicals on the growth of *M. aeruginosa* over 72 h. Chemicals tested at a range of concentrations from 10 - 1000 $\mu\text{g l}^{-1}$

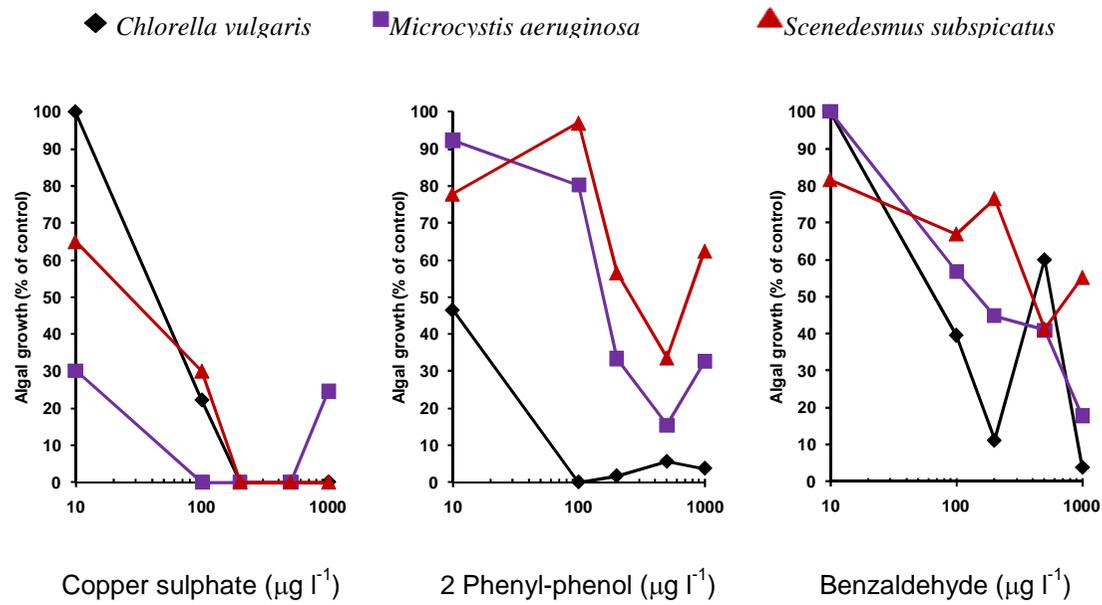


Figure 4.3. Growth responses of *C. vulgaris*, *M. aeruginosa* and *S. subspicatus* to varying concentrations of Copper sulphate and two selected barley straw compounds.

4.5 Conclusions

The aim of this trial was to see if the chemicals associated with decaying barley straw could be responsible for its inhibitory effect. It has shown that, of the chemicals assessed, many produced an algastatic effect on the growth of the 3 algal common species tested. This finding coincides with the inhibitory effect of barley straw that has been reported to date (Gibson *et al.*, 1990; Newman and Barrett, 1993; Ridge *et al.*, 1999). Furthermore, the effectiveness of the chemicals, especially phenolics, supports the suggestion that the effect of barley straw is derived from the release of phenolic chemicals produced during the breakdown of lignin (Barrett, 1994; Pillinger *et al.*, 1994). How, when and why this release occurs is the focus of our current research activity.

4.6 Acknowledgements

The authors would like to acknowledge the financial support for this project from Anglian Water, Northumbrian Water, Thames Water and Yorkshire Water.

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Chapter 5

Assessing the effects of fresh and rotting barley straw extract on the growth of three algal species.

5 Assessing the effects of fresh and rotting barley straw extract on the growth of three algal species.

D. Murray, B. Jefferson and S. A. Parsons*

Centre for Water Sciences, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

5.1 Introduction

The use of barley straw to control algae growth in reservoirs and other water bodies has been reported since the 1970s (Street, 1978) and there are a significant number of robust studies which show its effectiveness in laboratory and field studies (examples include; Welch *et al.*, 1990; Barrett, 1994; Ridge and Pillinger, 1996 and the recent series of papers by Ferrier *et al.*, 2005). There is a common theme in many of these papers in that the effectiveness of the straw is dependent on it being rotted (Welch *et al.*, 1990; Pillinger *et al.*, 1994; Cooper *et al.*, 1997). Studies have reported that barley straw requires at least 3 months before it becomes effective even under ideal conditions (Gibson *et al.*, 1990; Pillinger *et al.*, 1994; Terlizzi *et al.*, 2002) and may take up to 6 months before its maximum effectiveness can be seen (Gibson *et al.*, 1990). This observation has been linked to a mechanism whereby barley straw's mode of action is related to the microbiological decay of the lignin portion of the straw (Pillinger *et al.*, 1994). Lignin is difficult to break down and requires specific slow-growing species such as white rot fungi which take several months to develop (Granit *et al.*, 2007). The importance of microbiological decay has also been shown in the study carried out by Gibson *et al.* (1990) where it was demonstrated that sterilising rotting barley straw disrupted its ability to inhibit algal growth. The ultimate product of this activity is proposed to be the liberation of inhibitory chemicals bound within the barley straw which are most likely phenolic in nature (Waybright *et al.*, 2009).

Water utilities are considering barley straw as a passive control solution for algae in water supply reservoirs but the uncertainty about when barley straw will become active is a major concern. The current solution is to have barley straw in position all year round as the precise moment when the algae will bloom and become a problem is not always clear (Henderson *et al.*, 2008). Many of the other algae control options that the water utility has available lack the preventative nature of barley straw and act either on the bloom once it has begun to develop or by removing the algae further

downstream (Henderson *et al.*, 2008; Babel *et al.*, 2000). Hence, we are interested in whether or not we can manipulate barley straw to allow us better control of its efficacy.

Several papers have investigated the use of an extract from barley straw, and have met with varying success (Ball *et al.*, 2001; Grover *et al.*, 2007) (Table 5.1). Ball *et al.* (2001) produced an extract by chopping up either fresh or rotted barley straw and boiling it. The resulting material was then filtered to obtain a liquid extract. They tested whether the extract could inhibit the growth of *Microcystis aeruginosa* and *Scenedesmus* sp and found that, while the fresh straw extract had little effect, the rotted straw extract was highly effective at the equivalent of 100 g m⁻³ of decaying straw for *Microcystis*. Terlizzi *et al.* (2002) in a study that investigated the inhibition of 11 saltwater algal species also tested a rotted straw extract, although they found varying success. Roughly half the species were significantly inhibited by the extract at concentrations equivalent to 7250 g m⁻³ of rotting barley straw (~80 %, Table 5.1). The extract was particularly effective against *Heterocapsa triquetra* and *Heterocapsa pygmaea*. Ferrier *et al.* (2005) reproduced the study on 12 fresh water algal species although in this case the extract was sterile filtered before use. Half of the species tested showed no reaction to the extract (equivalent to 7200 g m⁻³ rotted barley straw) (Table 5.1) but several species including *Synura peterseni*, *Dinobryon* sp, *M. aeruginosa* were significantly (>90%) inhibited. While it is not yet fully understood why some species are more susceptible than others, there are general indications, such as that *Scenedesmus* sp and *Chlorella* sp are generally known as hardy algal species (Baos *et al.*, 2002) and that, whilst they have been affected by barley straw, they are generally more resistant (Ball *et al.*, 2001). Cyanobacteria such as *Microcystis* sp have been found to be particularly susceptible to barley straw (Ferrier *et al.*, 2005; Newman and Barrett, 1993). Since cyanobacteria are simpler organisms than algae and lack some of their refinements such as internal organelles, it may be that their simpler structure makes it harder for them to mitigate the effects of barley straw (Prokhotskaya and Stienberg, 2007).

Grover *et al.* (2007) carried out a study into the effectiveness of a commercially available barley straw extract in which they had detected the presence of phenolic chemicals. Phenolic chemicals have been suggested previously as the source of barley straw's inhibition (Everall and Lees, 1996). Unfortunately, the extract in this case was unable to inhibit the growth of *Prymnesium parvum* (Roelke *et al.*, 2006), even at concentrations of 750 g m⁻³ of extract, far higher than the recommended dose of 15 g of extract m⁻³. The paper itself points out that *Prymnesium paryum* is already known as being resistant to the effects of barley straw. Waybright *et al.* (2009) recently published a detailed study looking at chemical and physically isolated extracts (Table 5.1). These

include a variety of fresh and rotted straw extracts and their performance when assessed against *Microcystis aeruginosa*. The most effective extract was prepared by filtering through a YM-3 (molecular weight cut off 3000 Da) Amicon ultra filtration cell and produced a significant inhibition of *Microcystis aeruginosa* at 1000 g m⁻³. A basic extract prepared by filtering the waters surrounding barley straw to remove particulate material also significantly inhibited the growth of *Microcystis aeruginosa* growth at an extract concentration equivalent to 1720 g m⁻³ of barley straw. While most of the extracts are effective, many of the papers to date where whole rotting straw has been used to control algal growth have reported successful results at far lower concentrations such as the approx 50 g m⁻³ used by Everall and Lees (1996) or the 28 g m⁻³ used by Barrett *et al.* (1999).

At first, it would appear that the concentrations of straw used in the production of extracts such as: the 1.24 – 3.03 kg m⁻³ used by Waybright *et al.* (2009), or the 7.25 kg m⁻³ used in the study undertaken by Terlizzi *et al.* (2002), are far higher than the straw concentrations used in field trials with actual straw. Field trials have often quoted very small straw concentrations as being able to affect algal growth, Everall and Lees (1997), for example, in their reservoir trial quote an effective concentration of just 25 g m⁻³. Barrett *et al.* (1999) in their reservoir trials quote an effective dose of between 6 and 28 g m⁻³. When the actual conditions of the application are considered, this reported dose is not entirely accurate. For instance in the Barrett *et al.*, (1999) study, initially 2.3 tonnes of straw were anchored at 3 locations in the reservoir and later 50 nets each with 32 kg were applied. In the case of the Everall and Lees study (1997), 3.5 tonnes of straw was broken up and applied to 6 locations in the reservoir. From this data it becomes clear that, while the straw concentrations they both report in g m⁻³, may be correct from a global point of view, such as the 250,000 m⁻³ site used by Barrett *et al.* (1999), the straw itself is clearly concentrated at specific locations. Without accurate knowledge of the dimensions of the bales used, it is impossible to calculate the actual g m⁻³ in these areas but is it obviously far higher than then g m⁻³ quoted. The local concentrations are, therefore, more likely to be closer to the kg m⁻³ used in the extract studies than is at first apparent. Overall, actual barley straw has been tested at a range of concentrations and conditions in the field and lab from the lows of 5 g m⁻³ used by Barrett (1994) to inhibit *Microcystis* or the 25 - 50 g m⁻³ quoted by Everall and Lees (1996, 1997) through to the concentrations as high as 40 kg m⁻³ (Gibson *et al.*, 1990), though, as discussed, the lower concentrations may be misleading global values or not very repeatable, as was noted by Ridge and Pillinger (1999) who found concentrations of at least 0.5 - 1 kg m⁻³ were required. The concentrations at which extracts have been prepared and used fit into this range but have generally been used at concentrations far higher than quoted in field trials. Although as previously pointed

out, this may be misleading and the 0.5 – 1 kg m⁻³ mentioned by Ridge and Pillinger (1999) or figures such as the – >3 kg m⁻³ used by Waybright *et al.* (2009) may not be accurate, whilst concentrations as low as 0.1 kg m⁻³ have been reported. It would appear that, generally, the effectiveness of extracts prepared from rotted straw are not dissimilar from the amounts of physical straw used, though in general it appears that barley straw and its extract are effective at a range of chemical concentrations.

Key findings to date have included the identification of phenolic compounds associated with decaying straw, their ability to inhibit algal growth (Everall and Lees, 1996, 1997; Murray *et al.*, 2009; Waybright *et al.*, 2009) and that the active fraction of an extract was made up of phenolic compounds of a size range of 1000-3000 Da (Waybright *et al.*, 2009). These findings are of considerable importance as they confirm what has, until now, been the most popular hypothesis: that liberated phenolic chemicals are responsible for the inhibitory effect of barley straw (Everall and Lees, 1996). The reservoir studies reported by Everall and Lees (1996, 1997) identified a range of chemicals associated with rotting barley straw that had the potential to inhibit algal growth such as: 2 phenylphenol and P-Cresol (MW 108 and 170 respectively). The chemicals had a lower molecular weight than that suggested by the Waybright *et al.* (2009) study but both are likely to be indicative of the type of compounds released. Although previous research has been unable to pinpoint the exact process by which these chemicals are evolved, the Waybright *et al.* (2009) and Everall and Lees (1996, 1997) studies both identified two peaks of inhibition: an initial short release most likely attributed to soluble material such as hemicelluloses and some types of lignin, and the later, more prolonged release which they suggest is most likely attributable to microbiological decay of the lignin substructure of the straw (Caffery and Monahan, 1999).

Here, we have undertaken a series of experiments to test the proposed hypothesis that barley straw can yield algistatic compounds. Extracts were collected by autoclaving and filtering to remove any particulate material. Autoclaving was used in an attempt to extract the maximum amount of removable material from the fresh and rotted samples, to make sure all microbiological activity was stopped, and to keep the process the same over all straw types for better comparison. We were also interested in seeing if the combined high temperature and pressure conditions in the autoclave would liberate compounds straight away rather than waiting several days or weeks for them to be liberated.

Whilst many of the compounds found associated with decaying barley straw are soluble and have boiling points below 100 °C (well below the temperature range in an autoclave - for example, P-cresol has a boiling point of 35.5 °C), they are still bound within the lignin-cellulose matrix of the barley straw (Rogalinski *et al.*, 2008), thus preventing their release. Lignin makes up 10-15% of barley straw (Ball *et al.* 2001; Rowland and Roberts 1994) and it is known that this matrix is very stable, water resistant and difficult to degrade (Rogalinski *et al.*, 2008). This combination makes it resistant to simple heating as this is not enough in itself to break the bonds of lignin. Many industries that seek to liberate lignin, either for chemical production or in the pulp and paper industry, try to break down lignified material using processes involving a combination of heat, pressure and steam or very hot water, the very environment offered by an autoclave (Demirbas, 2005; Rogalinski *et al.*, 2008). This should provide an environment where it is possible to break down the lignin and liberate the bound chemicals. It has already been shown that many of the chemicals associated with decaying barley straw, when free, are soluble at low temperatures and can inhibit algal growth (Chapter 3), but under natural conditions cannot readily be released from the straw. It is hoped that the use of the autoclave will potentially allow us to bypass the rotting phase. If an extract could be prepared directly from fresh straw, it would go some way to proving that chemical inhibitors are contained in the barley straw and could be responsible for the inhibition of algal growth. It is hoped that there will be differences between fresh and rotted straw, as this may give clues to the type of process and chemicals involved. It is hoped that, as in the Welch *et al.* (1990) study, autoclaving rotting straw will reduce its effectiveness. This could show that there is a difference between the early stage, soluble chemicals detected by Everall and Lees (1996, 1997) and Waybright *et al.* (2009) and the chemicals released later on as the straw decays. It may be that the soluble content of the straw can be removed directly from fresh barley straw by autoclaving, such as has been done previously with other lignified material (Demirbas, 2005; Rogalinski *et al.*, 2008). While chemicals produced naturally later on, when barley straw is most effective, are more fragile and broken down by autoclaving or as Everall and Lees (1996, 1997) point out, are more volatile, and therefore possibly boiled off, such as P-cresol with its low bp of 35.5 °C. It is hoped that two points will be proven in this study; i) that barley straw on its own is not inherently effective so that a process is required to produce an inhibition. ii) That two distinct phases of chemical release can be attested to: namely, soluble, removable lignin and, at a later stage, lignin degradation products.

If a portion of the inhibitory effect of barley straw could be extracted and used on demand, this would be greatly beneficial to water utilities. This paper looked at the efficacy of the straw and the

extracts, and tested their impact on three common algae species: *Chlorella vulgaris*, *Microcystis aeruginosa* and *Scenedesmus subspicatus*. The inhibition of algae growth was evaluated using the Organisation of Economic Cooperation and Development (OECD Paris, France) 201 algal growth inhibition test which is an accepted method for studying algal inhibition.

Table 5.1 Overview of previous studies using an extract prepared from rotted barley straw

Author	Species	Extract	Suggested nature of extract	Effect on algae	Findings
Waybright, T, J <i>et al.</i> , (2009)	<i>Microcystis aeruginosa</i>	<p>i) 50.3g dry straw in 5L D.I. water for 154 days at room temp, filtered before use.</p> <p>ii) Straw extracted with 1:1 dichloromethane/methanol, straw then soaked in aerated water at room temp for 37 days then filtered.</p> <p>iii) as (ii) but soaked overnight then homogenised and left for 12h before filtering</p> <p>(iv) straw extracted using ethanol, frozen over night then centrifuged the supernatant was then removed and tested</p> <p>(v) as (iv) except the precipitate rather than supernatant was tested.</p> <p>(vi) Supernatant passed through polyamide.</p> <p>(vii) samples where passed through a YM-1, YM-3 or YM-10 filter</p> <p>(viii) Peaks isolated using ELSD where tested</p>	<p>A)MWCO gives an indication of size of active fraction of extract of 1000-3000 Da</p> <p>B) Removal of effect by Polyamide suggested chemicals such as phenols responsible for effect</p> <p>C) Precipitate not effective, soluble compounds responsible for effect</p>	<p>(i) Algistatic at 1720 mg l⁻¹</p> <p>(ii) Algistatic at 2400 mg l⁻¹</p> <p>(iii) Algistatic at 3030 mg l⁻¹</p> <p>(iv) All showed similar properties to their crude initial extract</p> <p>(V) Produced no effect</p> <p>(vi) No effect</p> <p>(vii) YM-1 filtrate 1,240 mg l⁻¹, YM-3 and YM-10 1000 mg l⁻¹. YM-1 not as effective as crude extract or supernatant</p> <p>(viii) ELPA serial dilution concluded that concentration of 11.17 mg l⁻¹ matched initial crude extracts</p>	<p>1) Aqueous samples retain algal static activity.</p> <p>Precipitates do not</p> <p>2) Phenolic chemicals are most likely involved.</p> <p>3) Tannins most likely not involved as tests show extract functions after hydrolysis.</p> <p>4) 2 inhibition peaks</p> <p>Suggests two processes involved in inhibition. A) Initial peak due to release of soluble material. B) Later release may be due to microbiological metabolism</p> <p>5) Microbial species themselves not source of effect i.e. release of antibiotics. Rather their activity may lead to the release of inhibitory compounds</p>

Grover, J, P <i>et al.</i> , (2007)	<i>Prymnesium parvum</i>	Premade extract obtained from “Ecological laboratories” and tested at : (i) 0.015 ml l ⁻¹ (Low dose) (ii) 0.75 ml l ⁻¹ (High dose)	Analysis of extract showed presence of phenolic chemicals using thin-layer chromatography	(A) Low dose (0.015 ml l ⁻¹) appeared to significantly stimulate exponential growth by 10% at 30°C and cellular chlorophyll content was 2 X the control. At 10 and 20°C no significant effect. (B) At 10°C low dose (0.015 ml l ⁻¹) treated samples developed a carbon requirement 40% lower than the control.	1) The high and low concentrations tested produced no significant inhibition of exponential growth or endpoint. Low dose produced some significant stimulation of growth. 2) <i>P.parvum</i> known already to be resistant to barley straw. 3) Phenolic chemicals were detected in the extract.
Ferrier <i>et al.</i> , (2005)	<i>Ulothrix fimbriata</i> , <i>Scenedesmus quadricauda</i> , <i>Selenastrum capricornutum</i> , <i>Spirogyra</i> sp, <i>Chlorella vulgaris</i> , <i>Microcystis aeruginosa</i> , <i>Oscillatoria lutea var. contorta</i> , <i>Anabaena flos-aquae</i> , <i>Navicula</i> sp, <i>Synedra</i> sp, <i>Synura petersenii</i> , <i>Dinobryon</i> sp	360g straw left in 18L aerated, reverse osmosis water for 60 days. Liquid then sterile filtered (0.22µm). Filtrate used in study (Equiv 7.2g l ⁻¹)	No analysis of extract composition carried out. Extract sterile filtered so no non-soluble matter	(A) <i>Synura peterseni</i> showed and algacidal effect with 100% removal (B) <i>Dinobryon</i> sp, <i>Microcystis aeruginosa</i> (around 90%) and <i>Spirogyra</i> sp (around 40%) where all significantly inhibited. (C) <i>Navicula</i> sp and <i>Selenastrum capricornutum</i> where significantly stimulated with the samples chlorophyll content rising from around 100 to 700 ug-l ⁻¹ . (D) <i>Oscillatoria lutea</i> var, <i>Ulothrix fimbriata</i> , <i>Chlorella vulgaris</i> ,	1) Study showed effect is not always inhibitory. Some species are infect stimulated (i.e. <i>Navicula</i> sp) or killed (i.e. <i>Synura peterseni</i>) 2) Effectiveness after filtering suggests chemical action. 3) Showed flagellated species can also be affected 4) Showed that extract remains stable for at least 2 weeks once produced

Terlizzi, D, E <i>et al.</i> , (2002)	(salt water) <i>Gyrodinium galatheanum</i> , <i>Gymnodinium sanguineum</i> , <i>Heterocapsa triquetra</i> , <i>Heterocapsa pygmaea</i> , <i>Gyrodinium instriatum</i> , <i>Prorocentrum minimum</i> , <i>Prorocentrum micans</i> , <i>Gyrodinium estuariale</i> , <i>Gyrodinium uncatenum</i> , <i>Ceratium furca</i> , <i>Peridinium sp</i>	(i) Rotted straw was prepared by leaving 360g straw in 18 l aerated, reverse osmosis water for between 60 days and 6 months. An unfiltered extract used in test at an equivalent of 7.25 g l ⁻¹ . (ii) <i>Gyrodinium galatheanum</i> also tested with a higher concentration equivalent to 14.5 g l ⁻¹	No assessment of the content of the extract was undertaken	<i>Anabaena flos-aquae</i> , <i>Scenedesmus quadricauda</i> , all showed no significant reaction to the extract.	(A) <i>Heterocapsa triquetra</i> , <i>Heterocapsa pygmaea</i> significantly inhibited (55% and 80% respectively) (B) <i>Gymnodinium sanguineum</i> , inhibited in similar fashion to <i>H. Triquetra</i> and <i>H. pygmaea</i> though not significant (p < 0.05) (C) <i>Gyrodinium galatheanum</i> stimulated at week 1 inhibited at week 2 for normal extract. Significant inhibition with double strength extract with a population drop from 10x10 ⁴ to 3x10 ⁴ cells ml ⁻¹ (D) <i>Prorocentrum minimum</i> , <i>Prorocentrum micans</i> , <i>Gyrodinium instriatum</i> , significant stimulation (38%, 80% and 75% increase respectively). (E) <i>Gyrodinium estuariale</i> , <i>Peridinium sp</i> , non significant stimulation (21% and 90% increases respectively) (F) <i>Gyrodinium uncatenum</i> , <i>Ceratium furca</i> . No response to barley straw (data not shown)	1) Effect not always found to be inhibitory in fact several species were found to be significantly stimulated by barley straw extract 2) First evidence extract can inhibit some saltwater species
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Ball, A, S <i>et al.</i> , (2001)	<i>Microcystis sp</i> , <i>Scenedesmus sp</i>	<p>2.5 g of straw was blended to a grist then boiled for 2.5 h in 250 ml water, cooled then filtered (Whatman GF/C) then readjusted to 250ml. Fresh and rotted straw tested. Extract was tested at % extract of total volume.</p> <p>(i) 1% V/V (ii) 0.5% V/V (iii) 0.1% V/V (iv) 0.05% V/V (V) 0.01% V/V (vi) 0.05% V/V (vii) 0.001% V/V (<i>Scenedesmus</i> only)</p>	Production of extract due to microbiological activity.	<p>(A) <i>Microcystis sp</i> was significantly inhibited by all concentrations of rotted straw extract with chlorophyll a concentrations generally being 10 x lower than those of the control concentration of 3170 $\mu\text{g l}^{-1}$. A small stimulation in growth was seen in fresh straw extract samples with an increase in the chlorophyll concentration to 4700 $\mu\text{g l}^{-1}$ compared to the control concentration of 3170 $\mu\text{g l}^{-1}$.</p> <p>(B) <i>Scenedesmus sp</i> was inhibited by rotting barley straw extract down to an extract concentration of 0.01% V/V, where a drop in chlorophyll a concentration from 2500 $\mu\text{g l}^{-1}$ to around 250 $\mu\text{g l}^{-1}$ was observed.</p> <ol style="list-style-type: none"> 1) Both species significantly inhibited by rotting straw extract 2) Fresh extract had a small stimulating effect 3) Results appear to confirm the necessity of microbiological decay in order for straw to become inhibitory 4) Extract stable for at least 28 days 5) Wheat straw is effective, reported that it has a lower lignin content
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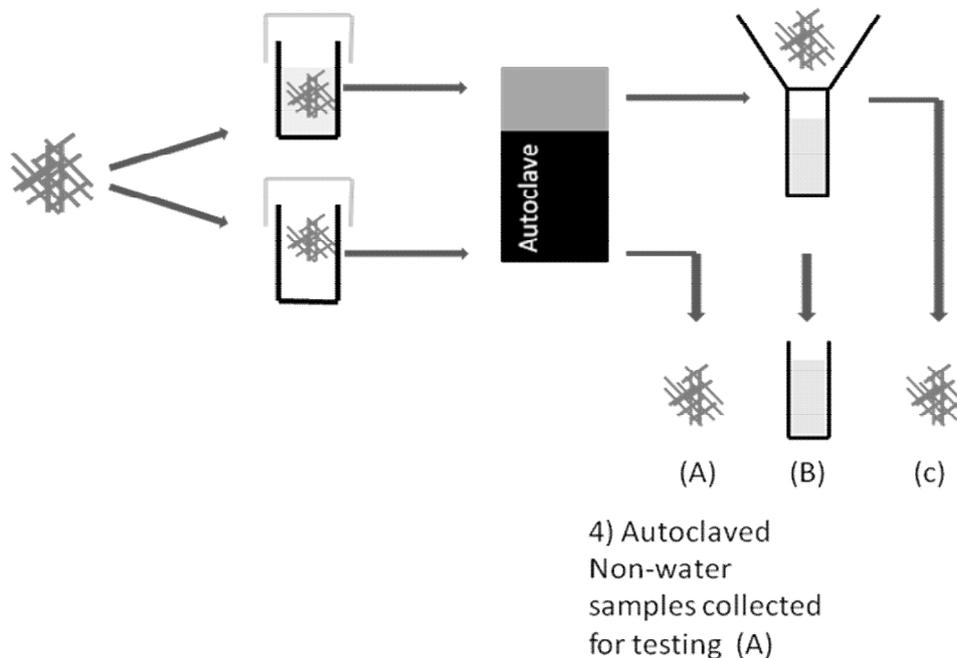
5.2 Materials and Methods

5.2.1 Straw preparation

Barley straw was assessed in two forms; fresh organic straw sourced from a local pet shop and rotted straw collected from a reservoir site in the Yorkshire Water region, 4 months after its initial application. The rotted straw was stored in a sterile plastic bag to prevent it drying out during transport back to the laboratory. Here it was sorted by hand to separate the straw from any organisms and detritus. Both the fresh and rotted straw were stored in the dark inside a temperature – controlled, cold room until used. The fresh and rotted straw was pre-treated in 3 ways as outlined below (Figure 5.1, Table 5.2).

- 1) A hot aqueous extraction was performed by autoclaving 15 g (dry weight) fresh or rotted barley straw in 1L DI water at 121 °C, 15 psi for 15 minutes then filtering through 0.7mm Whatman GF/C. Paper.
- 2) Sterile samples of both straw types were prepared by placing fresh and rotted straw samples (15 g dry weight) in 1 litre glass jars and covering with foil and then autoclaved at 121 °C, 15 psi for 15minutes.
- 3) Fresh and rotted straw samples were autoclaved in water and filtered as for the extraction, after which the straw was kept and rinsed thoroughly with deionised water.

- 1) 15 g fresh / rotted straw prepared
- 2) Straw sealed in glass jar using foil. 1x jar contains just straw. 1x jar has 1 L D.I water added
- 3) All straw samples autoclaved at 121 °C, 50 atm for 15 minutes
- 5) Autoclaved straw in water filtered. Filtrate collected for testing (B). Separated straw collected for testing (C)



2) Straw placed in glass jar and covered using foil. 1x jar contains just straw. 1x jar has 1 l D. I. Water added

Figure 5.1. Overview of straw sample preparation

Table 5.2 Overview of rotted and fresh straw samples and references

Straw type	Sample explanation		
	Sample 1	Sample 2	Sample 3
Fresh (FS)	Description	Filtrate from fresh straw autoclaved in DI water	Fresh straw remaining after autoclaving and filtration
	Sample label	Fresh straw extract (FSE)	Residual fresh straw (FSR)
Rotted (RS)	Sample 1	Sample 2	Sample 3

Description	Autoclaved rotted straw - No D.I water	Filtrate from rotted straw autoclaved in D.I water	Straw remaining after autoclaving and filtration
Sample label	Autoclaved rotted straw (RSA)	Rotted straw extract (RSE)	Residual rotted straw (RSR)

5.2.2 Preparation and maintenance of algae

Pure cultures of *Chlorella vulgaris* 211/11b, *Microcystis aeruginosa* 1450/3 and *Scenedesmus subspicatus* 276/20 were obtained from the Culture Collection of Algae and Protozoa (CCAP Argyll, UK) and kept in exponential growth in CCAP recipe *Jaworski's Medium* (J. Media) with weekly sub-culturing. All glassware, measuring cylinders and cotton wool bungs (Fisher Scientific, Loughborough, UK) used in culturing were acid-washed and autoclaved (121 °C, 15 psi, for 15 minutes) (PriorClave, London, UK) and allowed to cool to room temperature before use. All pipettes used were variable volume pipettes and all tips were disposable 1 ml Fisher brand pipette tips and all inoculations was carried out under standard aseptic conditions, via the use of a laminar flow cabinet (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK). Culturing was carried out by measuring 100 ml of new media into a 250 ml Erlenmeyer flask and to this 5 ml of pure algal culture was added. The flask was then stoppered with a sterile cotton wool bung and placed onto a *Bibby Stuart* SSL1 orbital shaker (Barloworld Scientific, Staffordshire, UK) at 110 rpm at room temperature (18-20 °C). Growth was carried under the constant light of 2x 15w Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK)

5.2.3 Assessment

The straw and extract samples were assessed using the Organisation of Economic Cooperation and Development (OECD, Paris, France) 201 “Algal toxicity test” method using *C. vulgaris* 211/11b, *M. aeruginosa* 1450/3 and, *S. Subspicatus* 276/20 as the test species (CCAP, Argyll, UK). The OECD 201 test was selected as it is carried out using cell counts. This is beneficial when testing for inhibition as unviable cells can be ignored. It also offers benefits over population estimation via chlorophyll analysis as chemicals to cause cell lysis are reduced, meaning that otherwise there

would be a possibility for liberated chlorophyll to skew test data and, since the exact nature of barley straws effect on the extract composition is unknown, this was deemed an appropriate route to take. The tests were carried out in acid-washed, autoclaved (121 °C, 15 psi, 15 min's) (PriorClave, London, UK) 250 ml Erlenmeyer flasks with sterile cotton wool stoppers containing 100 ml of *J. Media* to which a starting concentration of 10000 cells ml⁻¹ were added. Starting cell concentrations were calculated by use of a haemocytometer (Fisher Scientific, Loughborough, UK), where samples from pure cultures of the algae were added to the counting chamber by use of a 1 ml Fisher brand, variable volume pipette and sterilised 1ml disposable tips (Sterilisation took place via autoclaving). All 9 squares on both grids of the haemocytometer were counted, and this meant that the total cells counted in each grid was multiplied by 10000 to give the cells ml⁻¹ in the starting culture; this was carried out 3 times whenever a dilution was prepared to ensure accuracy. The culture could then be diluted with fresh media to give a concentration of 1000000 cells ml⁻¹; 1 ml could then be added to the 100 ml sample to give 10000 cells ml⁻¹.

The effectiveness of the straw samples was tested at 5 different concentrations 15, 5, 1, 0.5 and 0.1g l⁻¹, and extract samples were tested at concentrations the equivalent of 15, 5, 1, 0.5 and 0.1 g of straw per litre of water. These were prepared by adding the desired amount of straw or extract to the algal cultures under aseptic conditions inside a laminar flow cabinet (LABCAIRE, SC-R laminar flow cabinet, Somerset, UK). These concentrations were selected to reflect the range at which barley straw and its extract has been tested under field and laboratory conditions and also a large dose was tested beyond what would be practical (Everall and Lees 1996, 1997; Ball *et al.*, 2001; Ferrier *et al.*, 2005). The important factor taken into account was the amount of barley straw biomass that has been reported as being convertible into phenolic compounds (Welch *et al.*, 1990; Yavmetdinov *et al.*, 2008). This was done to ensure that the straw mass was enough to potentially yield chemicals at the concentrations that were reported in previous studies such as Everall and Lees; (1996, 1997) lake studies in Derbyshire, and the concentrations that were reported in the previous chapter as able to inhibit algal growth. Each experiment was conducted in triplicate and 6 controls were used for comparison. The cultures were grown on a *Bibby Stuart* SSL1 (Barloworld Scientific, Staffordshire, UK) orbital shaker under 2x 15w Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK). Growth was assessed via

cell counts which were carried out every 24 h for 72 h using a haemocytometer, counting the entire main 9 squares on both sides and repeating 6 times for each flask to reduce error.

5.2.4 Data analysis

The cell count data was analysed according to the OECD 201 tests recommended methods (OECD, Paris, France). Firstly, cell count data were plotted against time using the average of the 6 samples from the 3 repeats for each chemical, giving a total of 18 cell counts for each chemical concentration, each day; this was compared to the average of the 36 cell counts obtained for the 6 controls, each day. These data were used to calculate % inhibition caused by the straw and extract samples on each species by measuring the differences in the area under plotted cell counts (Equation 1), then by calculating the inhibition of cell growth by each test substance concentration data (Equation 2) to produce growth inhibition histograms seen in Figures 5.2 and 5.3:

(1)

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

A = area

N_0 = nominal number of cells/ml at time t_0

N_1 = measured number of cells/ml at t_1

N_n = measured number of cells/ml at time t_n

t_1 = time of first measurement after beginning of test

t_n = time of nth measurement after beginning of test

(2)

$$I_a = \frac{A_c - A_t}{A_c} \times 100$$

I_a = % inhibition of growth curve by test concentration

A_c = area under the control growth curve

A_t = area under test substance concentration growth curve

5.3 Results

Fresh straw had little effect on *C. vulgaris* or *M. aeruginosa* with around 5 - 15 % inhibition aside from the 15 kg m⁻³ dose where an inhibition of around 40 % (Figure 5.2) was observed for both species. No EC₅₀ for fresh straw (Table 5.3) was attainable within the range tested. FSE was more effective on both species and in particularly *M. aeruginosa* where a very significant effect was observed with almost complete inhibition being seen at concentrations of 500 g m⁻³ and over. No significant effect on growth was observed up to a dose of 1 kg m⁻³ (Figure 5.2) and the inhibition rose to approx 90 % (Figure 5.2) for all the higher concentrations. For *C. vulgaris* a 40% inhibition was achieved at 1 kg m⁻³ dose and 80 % at 15 kg m⁻³ (Figure 5.2).

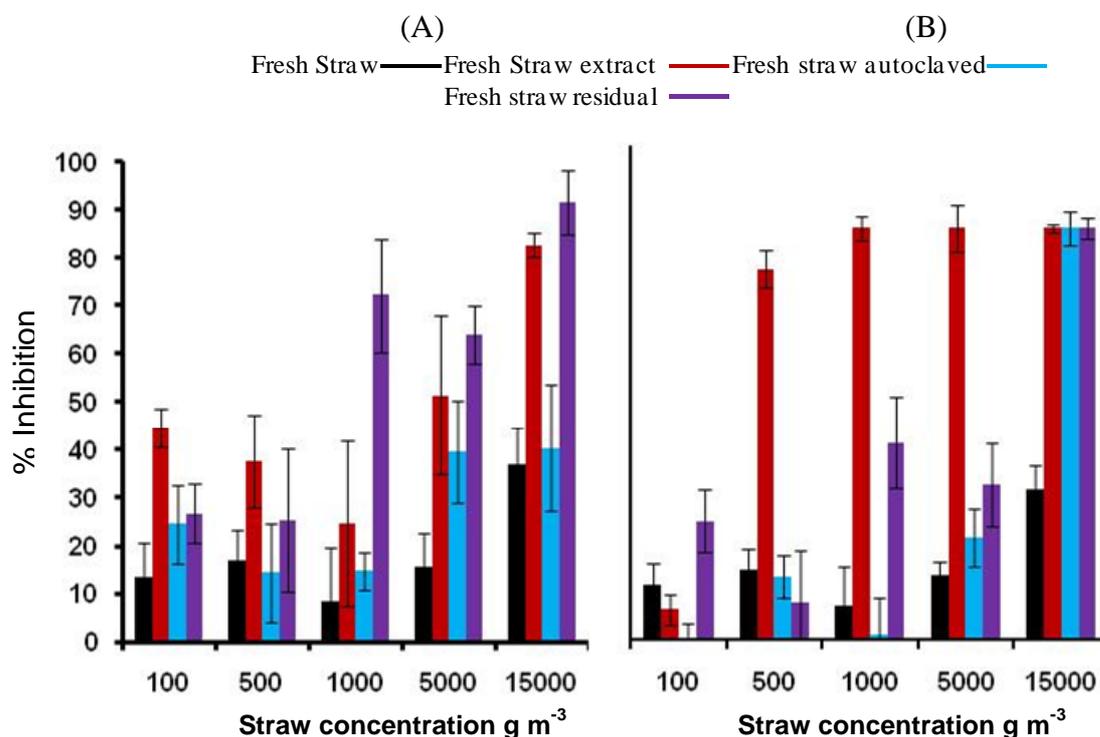


Figure 5.2. The effects of fresh barley straw and 3 pre-treatments on the growth of *C. vulgaris* (A) and *M. aeruginosa* (B). In the Text straw samples are abbreviated as; Fresh straw (FS), Fresh straw extract (FSE), Fresh straw autoclaved (FSA), Fresh straw residual (FSR). Each straw concentration was tested at 100, 500, 1000, 5000 and 15,000 g m⁻³. Data is shown as % inhibition of growth compared to the control.

FSR and FSA also affected algal growth; FSR in particular was effective on *C. vulgaris* with a 20 % inhibition being observed at 0.5 kg m^{-3} , increasing to 70% at 1 kg m^{-3} (Figure 5.2). FS and FSA at higher concentrations took up considerable space in the flask and this may have reduced access to light and further reduced algal growth. This could explain the sudden increase in inhibition for FS, FSR and FSA at the highest concentrations where there was generally a rise in inhibition. This is also true for *M. aeruginosa* which usually grows more efficiently at lower light intensities (Klein and Cronquist, 1967). This would explain the high EC_{50} observed for the 3 straw types (FS, FSA, FSR) (Table 5.3) as nothing would happen until high enough straw concentrations were used to reduce light enough to result in inhibition. It is clear that in the case of FS (where the EC_{50} is low), inhibitory chemicals can be extracted which have a definite effect on algal growth, reflected in FSE being the most effective of all the FS treatments and one of the 2 best tested overall.

FSE was also tested on a third species of alga *S. subspicatus*. *Scenedesmus* species are generally known to be more resistant to barley straw than *Microcystis* and *Chlorella* (Ball *et al.*, 2001; Ferrier *et al.*, 2005). In this case, *M. aeruginosa*, as in other studies, was the most affected, with an EC_{50} of $> 0.2 \text{ kg m}^{-3}$ (Table 5.3) a similar level to that found by Ball *et al.* (2001). In this case, the EC_{50} for *Chlorella* and *S. subspicatus* appeared similar at around 1 kg m^{-3} (Table 5.4), which is more susceptible than has been found in other studies such as that by Ferrier *et al.*, (2005). While the effectiveness of *M. aeruginosa* was found to be similar to the literature, the method of preparation in this case was different, so it may be that in this case the mechanism of inhibition was not the same.

RS, RSE and RSA were also tested alongside FS (Figure 5.4). Several studies have shown that *M. aeruginosa* is susceptible to the effects of RS and other decaying plant matter (Ferrier *et al.*, 2005; Martin and Ridge 1999; Pillinger *et al.*, 1995). In this study it appeared less so. Martin and Ridge (1999) found 0.07 kg m^{-3} RS was able to inhibit *M. aeruginosa* by 50 % whilst here, at a similar concentration (0.1 kg m^{-3}), inhibition was only ~10 % (Figure 5.3). Inhibition was seen to rise to 25 - 30% by 0.5 kg m^{-3} (Figure 5.3) and an EC_{50} was calculated as 0.75 kg m^{-3} (Table 5.3). 100 % inhibition was achieved at 15 kg m^{-3} although, at the highest concentration, light access may have been playing a role. In contrast to the literature, where *M.*

aeruginosa is usually more susceptible than *C. vulgaris* (Ferrier *et al.*, 2005) to RS, this study found *C. vulgaris* more susceptible to RS at lower concentrations with 40 %, 65 % and 85 % inhibition at 0.1, 0.5 and 1 kg m⁻³ respectively (Figure 5.3) and an EC₅₀ of 0.17 kg m⁻³ (Table 5.3) compared to *M. aeruginosa* EC₅₀ of 0.75 kg m⁻³. RSE had limited effect on *M. aeruginosa* and *C. vulgaris*, with an inhibition of approximately 15 – 20 % (Figure 5.3) at all concentrations, and no estimable EC₅₀ (Table 5.3) for *M. aeruginosa*. Furthermore, inhibition may be lower when variation is taken into account, a conclusion which fits with findings by Welch *et al.* (1990) who discovered that autoclaving effective rotting barley straw reduced its ability to inhibit algal growth. RS was judged the most effective of the RS samples and, alongside FSE, was one of the two most effective samples. Therefore, it was also assessed against *Scenedesmus* and again was found the most resistant of the 3 species, requiring an EC₅₀ of at least 1 kg m⁻³ (Table 5.3).

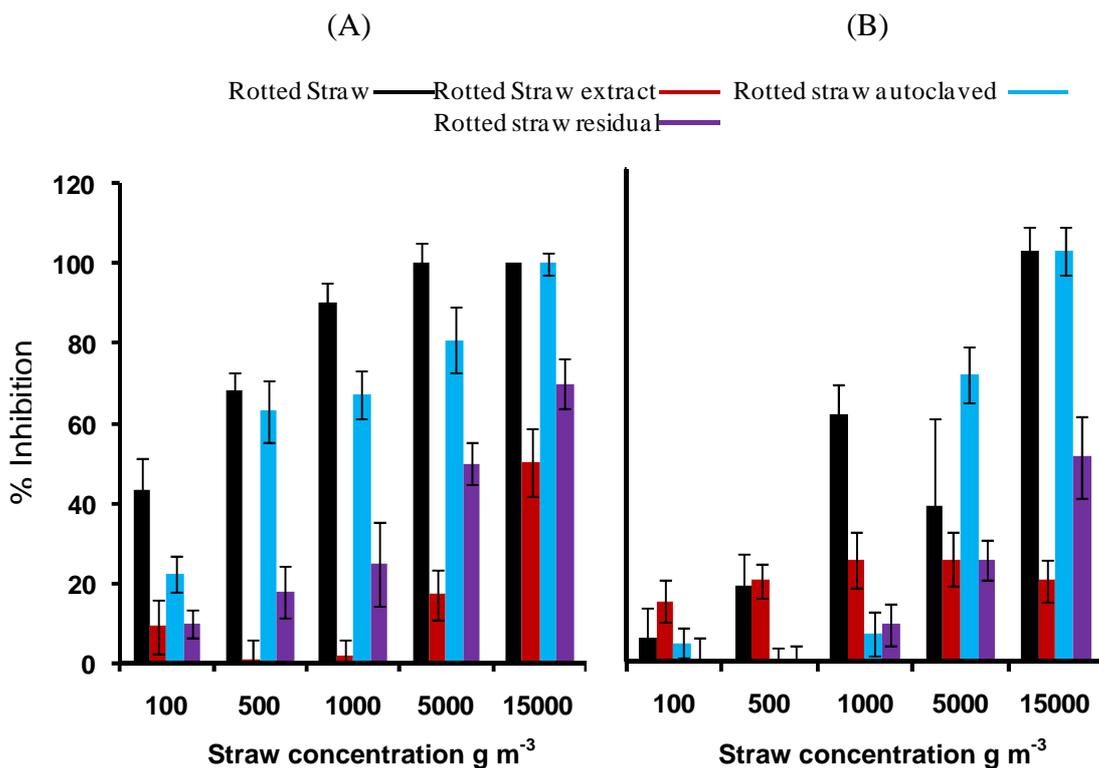


Figure 5.3. The effects of rotted barley straw and 3 pre-treatments on the growth of *C. vulgaris* (A) and *M. aeruginosa* (B). In the Text straw samples are abbreviated as; Rotted straw (RS), Rotted straw extract (RSE), Rotted straw autoclaved (RSA), Rotted straw residual (RSR). Each straw concentration was tested at 100, 500, 1000, 5000 and 15,000 g m⁻³. Data is shown as % inhibition of growth compared to the control.

Despite the limited effect RSE had on *M. aeruginosa*, it was still more effective than when tested on *C. vulgaris*, where there was no significant inhibition until 5 kg m⁻³ at

which point growth was reduced by 25 %, and 50 % at 15 kg m⁻³ (Figure 5.3). RSA produced significant inhibition of *C. vulgaris*, with 25 % inhibition being seen at 0.1 kg m⁻³ rising to 70 % at 1 kg m⁻³ and complete prevention of algal growth at 15 kg m⁻³ and an EC₅₀ of 0.3 kg m⁻³ (Figure 5.3, table 5.3). *M. aeruginosa* was also significantly inhibited but not until concentrations of 1 kg m⁻³ and over, with inhibitions of 10 %, 70 % and total inhibition at 1, 5 and 15 kg m⁻³ respectively (Figure 5.3). RSR produced a similar level of effect to what was seen with RSA, but was generally seen to be less inhibitory to algal growth. As with RSA, *C. vulgaris* was the more inhibited, 10 % at 0.1 kg m⁻³, compared to the 25 % seen at 0.1 kg m⁻³ RSA. The inhibition produced by RSR vs *C. vulgaris* rose to 25 % at 1 kg m⁻³ and produced a maximum inhibition of 70 % at 15 kg m⁻³ (Figure 5.3). RSR vs *M. aeruginosa*, as in the case for RSA, saw no inhibition of growth until 1 kg m⁻³ where growth was reduced by 10 %, a similar inhibition to that observed for RSA at this concentration. The level of inhibition rose after this point eventually reaching 50 % inhibition at the highest concentration (Figure 5.3).

Both RSR and RSA appeared largely ineffective, which was reflected in the low levels of inhibition observed until concentrations of over 1 kg m⁻³ and the high EC₅₀ requirements at 4 and 15 kg m⁻³ (Table 5.3) for *C. vulgaris* and *M. aeruginosa* respectively. It was noted that RSR and RSA degraded considerably after autoclaving, leading to considerable clouding of the water at the higher concentrations, and this may have resulted in a large reduction in the levels of light available and could be responsible for the large rise in inhibition seen at 5 and 15 kg m⁻³ (Figure 5.3). *M. aeruginosa* is already known to be more adapted to low light conditions (Klein and Cronquist, 1967) and it may be that this adaptation is why *M. aeruginosa* appears less affected by RSR and RSA.

From a comparison of RS and FS and their treatments, it is clear that there is a difference in the sources of inhibition in FS and RS. FS on its own appears inert, producing very little inhibition except at the highest amounts of straw, which required processing to become effective. This is in contrast to RS which was able to inhibit algal growth with no further processing. Also giving very contrasting results were the extracts produced from the two barley straw types, where RSE had little impact on any of the species tested whilst FSE was effective especially on *M. aeruginosa*. The

fact that treating RS appears to disrupt its effectiveness yet treating FS renders it more active suggests that the cause of the inhibition is different. While FS appears to contain extractable chemicals that can survive the autoclave process, the inhibitory factor of RS seemingly cannot. This may be due to the chemicals associated with RS and hence still likely to be in RSE, such as P-Cresol, being ‘boiled off’ or denatured by the process. The fact that two different types of inhibition have been observed concurs with previous findings, where two different waves of chemical release have been seen in the straw (Waybright *et al.*, 2009): an initial release of soluble chemicals and a later release of chemicals as barley straw underwent decay. This reduction of effectiveness when RS was autoclaved has been previously reported by Welch *et al.* (1990). *M. aeruginosa* in this study was found to be the most susceptible species overall to barley straw, a finding which is consistent with previous studies (Ball *et al.*, 2001; Ferrier *et al.* 2005; Newman and Barrett, 1993).

Table 5.3 EC₅₀ (g m⁻³) values for the fresh and rotted barley straw forms and extracts tested on *C. vulgaris*, *M. aeruginosa* and *S. subspicatus*. * The 2 most effective were also tested on *Scenedesmus*.

Sample	EC ₅₀ (g m ⁻³)		
	<i>Chlorella</i>	<i>Microcystis</i>	<i>Scenedesmus</i> *
Fresh straw (FS)	>15000	>15000	
Autoclaved fresh straw (FSA)	>15000	7000	
Fresh straw extract (FSE)	5000	190	100-1000
Extracted fresh straw (FSR)	700	8000	
Rotted straw (RS)	170	750	100-1000
Autoclaved rotted straw (RSA)	300	3000	
Rotted straw extract (RSE)	15000	>15000	
Extracted rotted straw (RSR)	4000	15000	

5.4 Discussion

Two separate questions were addressed in this study. Firstly, a comparison was made of the inhibitory properties of fresh and rotted straw, and secondly, whether inhibition

could be effected by treating the straw or producing extracts via autoclaving was investigated. Several studies carried out previously have shown the potential of extracts prepared from rotting barley straw to inhibit a range of algal growth (Terlizzi *et al.*, 2002; Ferrier *et al.*, 2005; Waybright *et al.*, 2009) at concentrations as low as 1.24 kg m^{-3} and one study found that *M. aeruginosa* could be significantly inhibited at extract concentrations as low as 0.1 kg m^{-3} (Ball *et al.*, 2001). All these extracts have been based on rotted straw and filtering to obtain the extract, but this study is the first successful attempt to produce an extract directly from fresh barley straw without the need for rotting. The previous study by Ball *et al.* (2001) boiled rotted and fresh straw in an attempt to yield an extract and found that, while rotted straw was effective, fresh straw had no effect. The extract produced from fresh barley straw in this study was found to be successful at inhibiting algal growth, and, as in the literature where other extracts have been used (Ball *et al.*, 2001), *M. aeruginosa* was the most susceptible species with an EC_{50} of 0.19 kg m^{-3} . Previous studies have concluded that the source of inhibition is the degradation of lignin as the barley decays (Everall and Lees 1996; Welch *et al.*, 1990). Lignin, as has been discussed, is known to be hard to break down and is resistant to heating (Rogalinski *et al.*, 2008), which is why the simple boiling employed by Ball *et al.* (2001) was unable to produce an effective extract. In order to break down lignified material, most processes use a combination of pressure, heat and steam or hot water (Demirbas, 2005), producing an environment similar to that offered by an autoclave hence its use in this study, and it would appear that in the case of fresh straw an effective chemical extract can be obtained. This may be of considerable use to water utilities as a method of offsetting algal blooms. However, more research would need to be done into the nature of the chemicals present and, from this present study, the high requirement of *C. vulgaris* suggests that investigations into which species are vulnerable would also need to be carried out.

An important finding from this study is that an extract cannot be prepared from rotting straw by autoclaving, as very little inhibition was seen until concentrations over 5 kg m^{-3} , and this is in stark contrast to previous studies like those of Ball *et al.* (2001) and Waybright *et al.* (2009) who found significant inhibition produced by extracts at concentrations of $< 1.5 \text{ kg m}^{-3}$. The difference in this case was in the preparation of the straw. Everall and Lees (1996, 1997) found several phenolic chemicals associated with decaying barley such as P-Cresol and Waybright *et al.* (2009) also suggested

similar conclusions. It is possible that autoclaving, in this case, boils off or denatures the active parts of the extract, rendering it inert. Welch *et al.* (1990) have already reached similar conclusions when they found that autoclaving rotted straw reduced its effectiveness - which has also been found in this study. In contrast to RSE, FSE was one of the two best treatments found in this study, since it was able to inhibit all three algal species, for which outcome differences in the lignin present in fresh and rotted straw may account. It is likely that, by the time lignin becomes naturally effective, the easily soluble fraction that can be directly extracted from fresh straw has been removed. This may not happen all at once but rather slowly over the initial weeks. Indeed, both Everall and Lees (1996, 1997) and Waybright *et al.* (2009) found two waves of chemical release: an initial short-lived release and the longer prolonged release of the effective chemicals after several months. It may be that autoclaving extracts all the initial chemicals at once, and at that concentration they are able to inhibit algal growth. Whereas, by the time straw is naturally effective, all these compounds have been removed and so are no longer there to be extracted. The fact that autoclaving rotted straw resulted in it breaking up suggests that matter has been lost by the time it becomes effective, and the extractable compounds may be contained within this fraction. The source of the inhibitory chemicals has often be attributed to degrading lignin which leads to the liberation of phenolic chemicals (Pillinger, 1993; Barrett, 1994; Pillinger *et al.*, 1994, 1996) such as those detected in the studies by Everall and Lees, (1996, 1997) and Waybright *et al.* (2009). Research carried out into when the degradation of lignin yields phenolic compounds (Garrottel *et al.*, 1999; Demirbas, 2005; Rogalinski *et al.*, 2008) has found that the more processed lignified material becomes, the more difficult it becomes to liberate phenolic material. It may be that this is the case with rotted straw, that the loss of material as it decays in water helps to make it even more stable, meaning it can no longer be simply extracted, and a more complex process must occur in nature at this point to allow the liberation of the detected phenolics. This is why many stress the importance of the rotting process (Barrett, 1994) as the more complex means available to microorganisms would allow the further breakdown of and liberation of material past the point at which the autoclave fails. Some evidence of this has already been produced by Pillinger *et al.* (1992) where fungi, the main decaying group of lignified material, were found to be associated with rotting straw and, in a later study (Pillinger

et al., 1995), where it was shown that wood rotted by specialised lignin - degrading fungi was more inhibitory to algae.

The outcome of this study has been to present evidence that chemicals that are inhibitory to algal growth can be directly extracted from fresh barley straw but that this process cannot be repeated with rotted straw in its naturally inhibitory phase. This suggests that two different groups of chemicals are involved in the inhibition demonstrated in this study: soluble chemicals that can be extracted from fresh straw but which are no longer present later on as the straw decays, and the products of later stage decay which are either too fragile to survive or are boiled off by autoclaving and require a more complex process in nature, one which is not yet fully understood, in order to be liberated. While the extract from fresh straw may not be the same as the chemical groups produced by the rotting of straw, it does offer the possibility of at least releasing some of the potential of barley straw on demand, though more work must first be carried out into the nature and safety of the chemicals produced in this fashion and whether the species affected are as broad in range as those reported for rotted barley straw.

5.5 References

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Chapter 6

The impact of conditioning of barley straw on the inhibition of *Scenedesmus* Sp

Submitted to: *Water Research*

6 The impact of conditioning of barley straw on the inhibition of *Scenedesmus sp.*

Daniel Murray^a (d.murray@cranfield.ac.uk), Simon A. Parsons^a

(s.a.parsons@cranfield.ac.uk) and Bruce Jefferson^{a,*} (b.jefferson@cranfield.ac.uk)

^aCentre for Water Science, Cranfield University, Bedfordshire, MK43 0AL, UK

*Corresponding author. Tel.: +44 (0)1234 754813. Fax: +44 (0) 1234 751671

6.1 Abstract: The current paper investigates the role of conditioning on the efficacy of barley straw to inhibit the alga *Scenedesmus*. Fresh, pre-rotted and white rot fungi augmented straw was tested in a series of continuous culture experiments over the course of 15 weeks. All three systems were seen to be effective at inhibiting the algae with differences observed in terms of the lag time before inhibition occurred and the rate of algae decline. Lag times of 8, 4 and 1 week were recorded for the fresh, rotted and fungi treated straws respectively with the maximum inhibition rate of >70000 cells.weeks⁻¹ observed for the fungi pre-treated system. Overall, the results indicate that pre-treatment is a viable method to enable barley straw to be used in a more reactive manner. It is postulated that during pre-treatment no alternative source of nitrogen is available, enhancing the lignin breakdown pathway which causes the release of the inhibitory substances.

Key Words: Barley straw, Algal control, *Scenedesmus*

6.2 INTRODUCTION

Algae are photosynthetic, aquatic plants that are ubiquitous in surface waters. Whilst at low concentrations algae do not pose a significant problem, during periods of seasonal algal bloom surface waters can become very polluted, disrupting recreational use and significantly impairing drinking water production. Reported bloom concentrations in the UK are as high as 2000000 cells.ml⁻¹, occur between February and November, typically lasting a few weeks to several months, formed by species including: *Microcystis*, *Aphanizomenon*, *Asterionella*, *Melosira*, *Anabena*, *Cyclotella*, *Pediastrum*, *Coelastrum* and *Scenedesmus* (Henderson *et al.*, 2008a). The impact of such blooms in regards to water production is seen both in terms of increased operating costs through reduced filter run times or increased coagulant demand, as well as reduction in product water quality in terms of colour, taste and the formation of disinfection byproducts (Henderson *et al.*, 2008a). Whilst surface water treatment plants can largely cope with the blooms through proper control of the coagulation process (Henderson *et al.*, 2008b), source control remains a key strategy to preserve costs and resources. Included in such source control techniques are bubble curtains, chemical dosing, ultrasound and barley straw (Purcell *et al.*, 2008). Barley straw has been used in the UK since the 1970s in a range of application scales including household ponds, canals, streams and potable water reservoirs. Reports on the use of barley straw indicate it is highly effective, consistent and produces no associated damage to desired species such as invertebrates and fish (Everall and Lees, 1997; Barrett *et al.*, 1999). Barley straw is normally applied as loosely bundled bails at key location in the reservoir at dose rates from 5 g m⁻³ (Barrett, 1994) up to 40 kg m⁻³ (Gibson, 1990) depending on whether localised or overall averaged concentrations are reported. In laboratory trials, where extract from rotted barley straw was applied, effective inhibition occurred at equivalent straw concentrations of around 1-2 kg m⁻³ (Waybright *et al.*, 2009). Fresh straw has been shown to be ineffective and so addition of the bails is recommended several months before the blooms are likely to occur as the straw is principally effective before or during exponential growth phases and must be degraded.

To date, most work carried out to understand the application of barley straw in inhibiting algal growth has focused on demonstrating the efficacy of the technology.

This has led to the hypothesis that chemicals produced during decay of the straw are responsible for inhibition (Everall and Lees, 1996). Further work has identified these chemicals to be phenolic in nature (Everall and Lees, 1996, 1997; Waybright *et al.*, 2009) and derived from lignin as the plant material decays rather than directly from the microbial species responsible for the decay (Pillinger, 1993; Barrett, 1994; Pillinger *et al.*, 1994). Lignin is a tough insoluble matrix that is very difficult to degrade resulting in the need for the development of white rot fungi communities within the straw matrix in order for the phenolic compounds to be liberated (Garrotel, *et al.*, 1999; Demirbas, 2005; Rogalinski *et al.*, 2008).

To date, little work has focused on the role of decay and whether it can be enhanced to provide a more controlled response within water treatment applications. The current study complements the previous work and attempts to understand the role of pre-treatment with white rot fungi by conducting trials using continuous cultures over time scales appropriate to the applications in question. As part of the trial a pre-treatment with 3 common white rot fungal species: *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* has been conducted to establish the potential for an engineered enhancement to the use of barley straw.

6.3 MATERIALS AND METHODS

6.3.1 Algae preparation

Cultures of pure *Scenedesmus* sp was chosen due to its robustness and ease of growth in laboratory conditions. Isolation was carried out initially during a trial run of the continuous cultures using samples collected from Yorkshire Water's Scout Dyke reservoir. Once *Scenedesmus* was identified, 10 sterile falcon test tubes were filled under aseptic conditions with 9 ml of *Jaworski's Medium* (J media) using clean 10 ml variable volume pipettes and sterile tips. A serial dilution was then undertaken to isolate the species following standard methods. 4 such serial dilutions were prepared under aseptic conditions inside a laminar flow cabinet (LABCAIRE, SC-R laminar flow cabinet, Somerset, UK). Once prepared, the falcon tubes were sealed and left on a *Bibby Stuart* SSL1 orbital shaker (Barloworld Scientific, Staffordshire, UK) under 2x 15w Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK). After

2 weeks the cultures were inspected microscopically and pure cultures of *Scenedesmus* that had been isolated were then combined and used to seed batch cultures.

Batch cultures were produced by adding 5 ml of the pure *Scenedesmus* culture to 100 ml J. media in a sterile 250 ml Erlenmyer flask under aseptic conditions inside a laminar flow cabinet (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK). *Scenedesmus* was added via 1 ml variable volume pipette. Once complete the flask was then sealed with a cotton wool bung. All glass wear, bungs and pipette tips were sterilised via autoclaving (121 °C, 15 psi, 15 minutes) (PriorClave, London, UK). Cultures were grown on a *Bibby Stuart SSL1* orbital shaker (Barloworld Scientific, Staffordshire, UK), at 110 rpm at room temperature (18-20 °C), and this was carried out under the constant light of two 15W Hagen Sun-glo tube lights (Maidenhead Aquatics, Woburn Sands, UK). Cultures were maintained by weekly sub-culturing until required. This was done by transferring 5 ml culture to 100 ml fresh J media following the same method as above. Cultures were inspected microscopically every 2 weeks to ensure purity.

6.3.2 Fungi preparation

3 species of white rot fungi; *Phanerochaete chrysosporium* DSMZ 1556, *Pleurotus ostreatus* DSMZ 1833, *Trametes versicolor* DSMZ 11269 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All three species were maintained in pure cultures on malt extract peptone agar. The 3 species were selected as they have been shown to be particularly good producers of the lignin and complex phenol degrading laccase enzyme (Hou *et al.*, 2003).

Fungi were grown by transferring 1 cm² section of the culture with an ethanol washed, flame sterilised, scalpel. The process was under aseptic conditions inside a laminar flow cabinet and the plates outside were wiped with ethanol before and after use (LABCAIRE, SC-R Laminar flow cabinet, Somerset, UK). The plate was then sealed with parafilm and kept inside a sealed environment chamber (Sanyo MLR-450H, Sanyo, Japan) at 20 °C, 100 % humidity and in the dark. Fungi were re-cultured until

required, which was typically every month or when the plate was completely covered with growth. Re-culturing was carried out using a fresh clean agar plate inside the laminar flow cabinet under aseptic conditions.

6.3.3 Continuous culture preparation

The *Scenedesmus* cultures were used to seed the continuous cultures (100 l) (Maidenhead, UK). Prior to use the continuous cultures were thoroughly cleaned with ethanol and then filled with dechlorinated tap water, sealed with cling film to prevent invasion by outside organisms, and continuous cultures were allowed to stand for 2 days before use. The continuous cultures were kept in a temperature control room at 20 ± 2 °C, light was also controlled by equipping all tanks with 2 x 20 W Hagen Aqua-glo aquarium lights (Maidenhead Aquatics, Woburn Sands, UK) equipped with a light timer (B&Q, UK) set to a 16h light 8h dark cycle. Maintenance of the cultures was carried out based on the principle of a continuous culture, where the cultures in the tanks were changed at a rate of 10% a day, using dechlorinated tap water via a peristaltic pump (Watson-Marlow Bredel Pumps, UK). Water was dechlorinated by allowing it to stand for at least 48 hours before use and it was constantly filtered through an external Fluval 305 cartridge filter (Maidenhead Aquatics, Woburn Sands, UK) and checked via titration using standard methods. No media addition was used as preliminary work showed that the algae could grow consistently with just tap water. The tanks were kept mixed with (*AquaClear Powerhead 50*, Maidenhead Aquatics, Woburn Sands, UK) aerating aquarium pumps. The algal cultures were allowed 1 month to settle and acclimatise and then blank data was collected for 3 weeks before straw was added to the tanks. In total 2 tanks were prepared for each of the straw forms to be tested: fresh, rotted and fungal pre-treated and 4 further tanks were kept as controls. The continuous cultures used for the analysis of fungal activity (acid digestible fibre 6.3.7, ergosterol 6.3.6) was prepared and maintained exactly the same as the fresh straw continuous cultures. The straw was prepared and samples taken according to the methods below.

6.3.4 Straw preparation

Three sources of barley straw were used in this test:

- (i) Fresh organic straw which was obtained from a local pet shop.
- (ii) 4 month old straw (rotted straw) obtained from a surface water reservoir, in the Yorkshire region.
- (iii) Fungal treated fresh straw.

Fungal treated straw was prepared by taking 25 g of fresh straw, which was placed into a 1.5 l clean, acid-washed beaker sealed with tin foil and autoclaved (121 °C, 15 psi, 15 mins) (PriorClave, London, UK). Once autoclaved the straw was stored in an ethanol-cleaned, sealed environment chamber (Sanyo MLR-450H, Sanyo, Japan) at 100% humidity and in the dark, overnight to cool. The cooled straw was then carefully opened under aseptic conditions inside a laminar flow cabinet (LABCAIRE, SC-R laminar flow cabinet, Somerset, UK), when 3 x 1 cm² squares of *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* were placed throughout the straw. 1 square of each species was placed at the bottom, middle and top of the straw, fungal samples were removed by cutting out a 1cm² section of the agar plate with an ethanol-washed, flame-sterilised scalpel, sterilised this way before each use. Once the fungi were added the straw was resealed and placed in the environment chamber (Sanyo MLR-450H, Sanyo, Japan) where it was kept in the dark at 20°C and 100% humidity for 2 months until the fungi had thoroughly spread throughout the straw.

Once obtained (or adequately colonised by fungi), 2 x 25 g bundles for each of the three straw types was prepared inside a laminar flow cabinet (LABCAIRE, SC-R laminar flow cabinet, Somerset, UK). Each tank received 1 x 25 g bundle of straw which was suspended from the top of the tank by plastic string (Maidenhead Aquatics, Woburn Sands, UK) to keep the straw at the surface of the water. The bundles were prepared by carefully weighing out 25 g (dry weight) of each straw type, twice, inside a laminar flow cabinet under aseptic conditions. The bundles were tied up using

sterile plastic string, which was used as it is inert and would not affect the environment of the tank. As soon as each 25 g bundle was ready; it was immediately transferred to an individual tank. Operating conditions were designed to reflect previous studies and field trials (Everall and Lees, 1996; Welch *et al.*, 1990) which have been carried out to coincide with bloom periods. This included a summer daylight cycle - in this case set to 16 h day 8 h night and 20 °C – and the dilution was calibrated in a trial run prior to the test to ensure that a dilution was used which would allow the algae to maintain a constant population level.

6.3.5 Sampling

Water samples were collected weekly from the main body of each tank for the duration of the test. Samples were taken via an acid-washed, sterilised (via autoclaving at 121°C for 15 mins) 50 ml syringe and an attached 20 cm length of Nalgene tubing. All water samples were analysed using standard methods including dissolved oxygen and temperature (OxyGuard Handy Beta, OxyGuard International, Birkerød, Denmark), pH (Jenway 3540 pH meter (Jenway, Essex, UK). Alkalinity was measured using a standard titration (Pohland and Bloodgood, 1963), using a 100 ml sample in a clean acid-washed beaker, a few drops of bromocresol green indicator was then added to the sample, which was then titrated with 0.1 M H₂SO₄ until a colour change was observed. Dissolved organic carbon (DOC) was measured using a Shimadzu TOC-5000A TOC (Shimadzu, Milton Keynes, UK). Cell counts were taken using a Spectramax plus 384 96 plate reader (Molecular Devices, Sunnyvale, USA), reading at 687 nm using a prepared standard curve and regularly checked against haemocytometer counts to keep counts within 10% of each other.

6.3.6 Acid Digestible Fibre

Acid digestible fibre analysis was carried out on order to assess the effect of fungal activity on the decay of the lignin, cellulose and fibre content of the straw. By doing this insight was provided in to nature of the fungal activity and weather that activity was consistent with white rot fungi. Samples were collected from fresh straw at 0, 1, 3, 5 and 9 months for acid digestible fibre analysis (ADF). The lignin, cellulose and fibre content of the straw samples were analysed according to the acid-detergent

method described by Rowland and Roberts (1994). This involves simmering 0.7 g (W1) of a straw sample in a 100 ml cetyltrimethyl ammonium bromide (CTAB) solution ((1ml CTAB dissolved in 99ml D.I. water)(ACROS Chemicals, Geel, Belgium) inside a 250 ml Erlenmeyer flask. The top the flask was plugged with an inverted 50 ml volumetric flask to prevent evaporation. Samples were simmered at a temprature just short of boiling (Approx 90 °C), for 1 hour and this was followed by immediate transfer of the straw to a no.2 porosity sinter (Fisher Scientific, Loughborough, UK) (W2). The straw was then rinsed with 50 ml aliquots of boiling water followed by acetone (Fisher Scientific, Loughborough, UK) until no more colour was removed; filtering was through the sinter under vacuum. Once rinsed, the samples were dried at 105 °C for 2 h, and then weighed once cooled (W3). Glass measuring cylinders and clean, acid washed, glass bottles were used for the preparation of all chemicals. Once weighed, the sinter was halffilled with cooled (15 °C) 72% H₂SO₄ (Fisher Scientific, Loughborough, UK) and stirred with a glass rod. The acid was allowed to drain away under gravity and continuously topped up with fresh acid for 3 h. After 3 h the acid was filtered off under vacuum and the sample rinsed with boiling water followed by acetone until colour was no longer removed. The sinter was then dried at 105 °C for 2 h and weighed (W4) followed by 2 h at 550 °C and then weighed again (W5).

The weights were then used to calculate the following:

$$\% \text{ Acid digestible fibre} = (W3 - W2) \times 100 / W1$$

$$\% \text{ Lignin} = (W4 - W5) \times 100 / W1 \quad \%$$

$$\text{Cellulose} = (W3 - W4) \times 100 / W1$$

6.3.7 Ergosterol

Fungal population was quantified using ergosterol analysis according to the rapid ultrasonication method described by Ruzicka *et al*, (1995). Ergosterol is a cell wall constituent almost exclusive to fungi, because of this and its relative simplicity to extract, yields can be directly related to fungal biomass. Samples in this study were taken at 3, 6 and 9 months from both the liquid and straw phases. Ergosterol was quantified by taking the filtrates from samples from either the bulk or within the straw

matrix through a 0.7 micron filter paper and adding it to a 50 ml polyethylene tube. A duplicate was used for each sample; pre-spiked using a variable volume pipette with 100 µg of ergosterol (Sigma-Aldrich Co, Dorset, UK) in a 1 ml n-hexane-propan-2-ol (98: 2) solution (Hexene and isopropanol, Acros Chemicals, Geel, Belgium). After 15 min, a 10 ml mixture of methanol and ethanol (Fisher Scientific, Loughborough, UK) (4:1) was added to all samples, which were then cooled to 4 °C for two hours. After 2 h, 20 ml n-hexane-propan-2-ol was added to the samples, or 19 ml in the case of the spiked samples. Samples were ultrasonicated at 150 W for 200 s using a VirTis - Virsonic 600 ultrasound probe (VirTis, NY, USA), whilst kept on ice. The samples were then allowed to settle for 30 s and the top 2 ml (top layer) was transferred to a microfuge tube and centrifuged for 10 min at 10,000 rpm. The top 1.5 ml was then used to calculate the ergosterol concentration via HPLC. All pipettes used were cleaned with ethanol before use and all tips were new fisher disposable tips autoclaved before use (autoclaving at 121 °C, 15 psi for 15 mins) (PriorClave, London, UK).

HPLC analysis of the samples was carried out in order to quantify the concentrations of ergosterol present. Ergosterol quantification was carried out using a Shimadzu SCL- 10A HPLC (Shimadzu, Milton Keynes, UK) using a Licrosorb Si 60 (10 µm) column (Phenomex, Macclesfield, UK) following a 4 x 3.0 mm guard cartridge (Phenomex, Macclesfield, UK). The columns were flushed with n-hexane-propan-2-ol at 1.5 ml min⁻¹. For sample analysis 100 µl was injected at 1.5 ml min⁻¹ and absorbance measured at 282 nm.

6.4 RESULTS

Reduction in the concentration of *Scenedesmus* due to the application of barley straw was observed to follow a three stage profile characterised by: (1) a lag phase where no inhibition took place, (2) a declining phase where the concentration of algae decreased over time and (3) a steady state phase where a new stable concentration of algae existed (Figure 6.1). The initial lag phase lasted for 4 and 8 weeks for the rotted and fresh straw respectively indicating the importance of pre-treatment. The observation is consistent with previous finding which have indicated that up to 12 weeks are required before addition of fresh barley straw is seen to be effective suggesting that

key transformations must occur within the straw before it becomes effective at inhibiting algal growth (Gibson *et al.*,1990; Pillinger *et al.*,1994; Terlizzi *et al.*,2002).

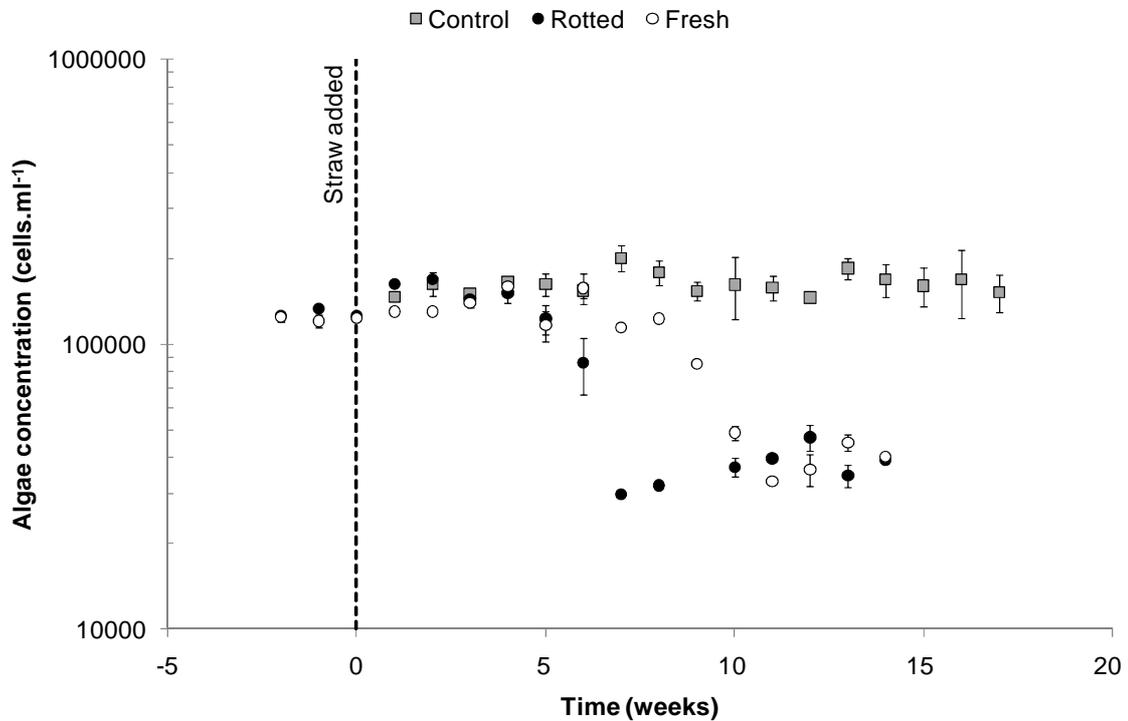


Figure 6.1 Effect of fresh and rotted straw on the growth of *Scenedesmus*. Error shown as 95% confidence interval

In the case of fresh straw, the population decreased from an average of 130000-150000 cells.ml⁻¹ during the lag phase to 85831±34320 cells.ml⁻¹ in week 9 and further to 48901±23673 cells.ml⁻¹ by week 10 (Figure 6.1). In comparison, in the case of rotted straw the algal population decreased from a lag phase concentration of 140000 cells.ml⁻¹ to 85831±19418 cells.ml⁻¹ in week 6 and 29761±713 cells.ml⁻¹ by week 7. Consequently, the new steady state population sizes were established 3 and 4 weeks after lag phase indicating that pre-treatment influenced the rate of cell decline. The declines rates of *Scenedesmus* sp during this phase were approximately 30000 and 40000 cells.week⁻¹ for the fresh and rotted straw respectively (Figure 6.1). Analysing the data in terms of a first order kinetic rate yields a rate constant of 0.45 weeks⁻¹ and 0.52 weeks⁻¹ for the fresh and rotted straw respectively. Reported growth rates of *Scenedesmus* sp in controlled conditions for maximum growth are around 1000000 cells.week⁻¹ with a rate constant of around 0.5-1.5 d⁻¹ indicating that suppression of new growth was the controlling factor in the successful use of barley

straw (Mohammed and Markert, 2006). The average algae concentration during the inhibited steady state phase varied between 31966-45121 cells.ml⁻¹ for the rotted straw and 33124-47182 cells.ml⁻¹ for fresh straw exhibiting no significant difference based on an ANOVA test at a 95% confidence level. Overall, this demonstrates that the application of barley straw at a dose of 0.25 kg.m⁻³ produced significant but not complete inhibition of the algae which was independent of pre-treatment. Comparison with previous investigations confirm the applied dose rate used in the current study to be appropriate when applied as either extract (Ball *et al.*, 2001) or whole straw (Murray *et al.*, 2009). In contrast, doses of an extract equivalent to 7.2 kg.m⁻³ in laboratory trials, have been reported to be ineffective at controlling the growth of *Scenedesmus* but effective against *Synura* and *Microcystis* which were significantly inhibited within a few weeks (Ferrier *et al.*, 2005). Comparing the finding between the current study and those previously reported such as the studies by Ball *et al.*, 2001 and Ferrier *et al.*, 2005, suggest that the kinetics of algal inhibition by barley straw is species specific. Such a finding is not unexpected as the characteristics of different algae are known to vary widely in terms of their physiology (Andersen, 2005) and the organic matter that surrounds the algae surface (Henderson *et al.*, 2008c). As the inhibition mechanism for barley straw requires the adsorption of the toxin onto the algal surface, both the inherent toxicity of the cell and the transfer of the toxin through the algal organic matter (AOM) layer will be important. Consequently, appropriateness of barley straw in inhibiting different algal species is not expected to follow traditional biological classification based on differences in pigmentation and cell complexity arising as a result of evolution (Bellinger, 1992). Instead, species from all phyla are expected to be susceptible. Parallels can be made with other forms of treatment where effectiveness mirrors specific chemical and physical properties rather than biological classification. For instance, the use of low energy ultrasound has been shown to be effective only for filamentous algae irrespective of phyla (Purcell *et al.*, 2008). Similarly, optimum conditions for coagulation and clarification of algae have been shown to relate to the charge density of the algae and AOM as well as shape factors that cross all phyla (Henderson *et al.*, 2008). Consequently, barley straw appears to fit this pattern such that generalisation cannot be made regarding susceptibility based on biological classification.

Further investigation into the role of pre-treatment confirmed its importance during the trial with white rot fungi pre-treated straw. The lag phase was observed to last only 1 week, 3 weeks shorter than that observed with pre-rotted straw. The impact of the straw was very quick with the cell population decreasing from 123504 ± 1586 cells.ml⁻¹ before the addition of straw to 63219 ± 5373 cells.ml⁻¹ within the first week representing a minimum decline rate of 70000 cells.week⁻¹ and a first order rate constant of 0.67 weeks⁻¹ (Figure 6.2). A second reduction to 26875 ± 2000 cells.ml⁻¹ was observed after 11 weeks which then returned to the previous steady state value. The control concentration increased during the equivalent time period suggesting the effect was not due to the environmental conditions over the three week period. The steady state value varied between 63219 and 78750 cells.ml⁻¹ which is higher than those experienced previously with the fresh and rotted straw although the applied dose rate was the same. However, all three systems produced significant levels of reduction with an overall average inhibition level of 75, 75 and 60 % for the fresh, rotten and fungi pre-treated straw respectively. The levels reported confirm previous studies which found 75% inhibition in a field trial (Barrett *et al.*, 1999) and higher than the >50% reported for a field trial of mixed algal species (Everall and Lees, 1996). Similarity is also observed concerning the duration of the inhibition which was seen to continue up to the end of the experiments during the current investigation, a maximum of 13 weeks in the case of the fungi pre-treated straw.

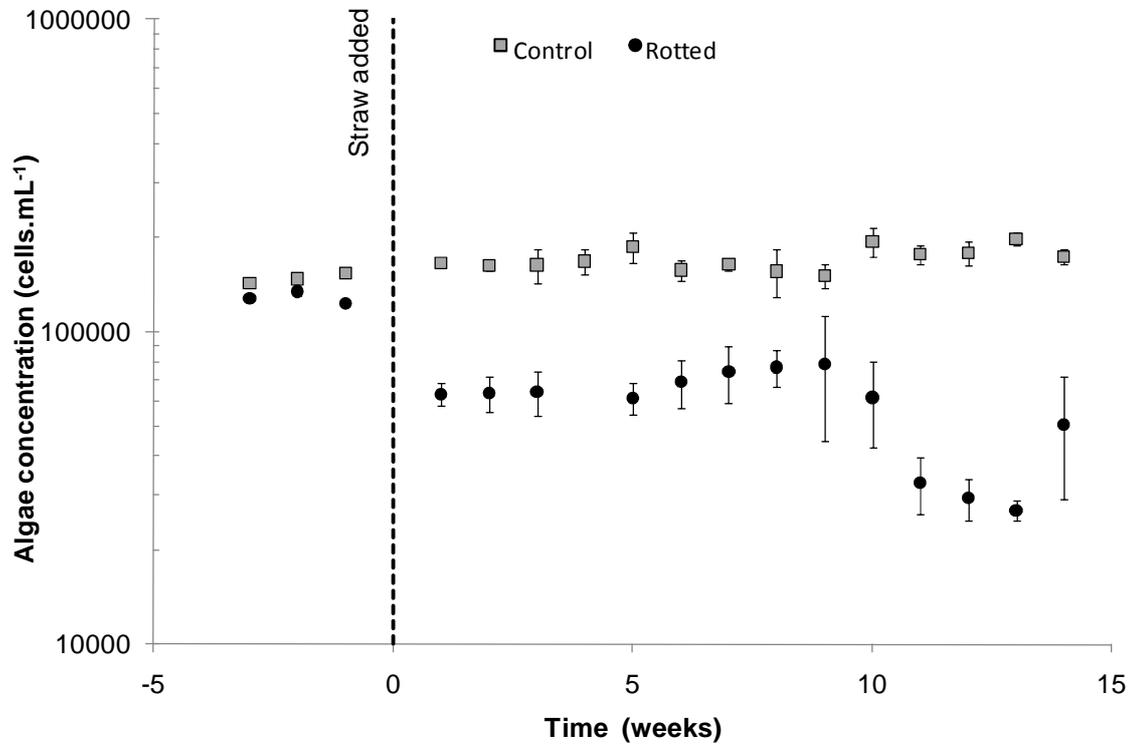


Figure 6.2. Effects of fungal-treated straw on the growth of *Scenedesmus*. Growth effect is shown as the total cells ml⁻¹. Error shown as 95% confidence interval.

No significant differences were observed between the controls and straw continuous cultures during all trials. In the first trial pH and temperature, alkalinity and DOC remained constant at 7.5 – 8 and 20 °C ± 2, 45 ppm and 4-6 mg.l⁻¹ respectively. This compared to equivalent values of 7.5 – 8 and 20 °C ± 2, 50 ppm and 6 mg l⁻¹ during the second trial. The only major difference was observed DO which was 6 and 9 mg.l⁻¹ for the first and second trial, although both were above any potential oxygen limitation effects.

6.5 Analysis of straw characteristics during degradation

Analysis of the degradation of straw through ADF and ergosterol analysis revealed that the cellulose component was preferentially consumed (Figure 6.3, 6.4). To illustrate, initial composition of the straw was 0.4, 0.1 and 0.5 in terms of the mass

fraction of cellulose, lignin and fibre respectively. As the trial progressed, the cellulose content decreased to a mass fraction of 0.25 by month 3 and ultimately become non-detectable after month 5 (Figure 6.3, 6.4). The mass fraction associated to fibre remained effectively constant across the trial such that the lignin mass fraction increased commensurately with the decrease in cellulose reaching a maximum value of around 0.5 by month 9. The initial preferential reduction in cellulose has previously been reported with a decrease in mass fraction from 0.4 to 0.15 in the initial 28 days (Ball *et al.*, 2001) which compares to the decrease from 0.4 to 0.24 in the current case. Similar results have also been reported during investigation of the degradation of cellulose-lignin systems in agricultural contexts (Wessén and Berg, 1986). Exact mechanisms are unclear due to the complexity of the system but lignin degradation is thought to occur in order for the fungi to access nitrogen rather than for obtaining metabolic energy (Schlegel, 1997). The preferential degradation of cellulose within the system reflects the need for the lignin to be solubilised by complex non-specific enzymes such as lignin peroxidase and laccase (Thiruchelvam and Ramsay, 2007) which require H₂O₂ to work effectively (Schlegel, 1997). The H₂O₂ is thought to be produced as a by-product of the cellulose degradation pathway and hence cellulose degradation is an important precursor to lignin degradation (Schlegel, 1997).

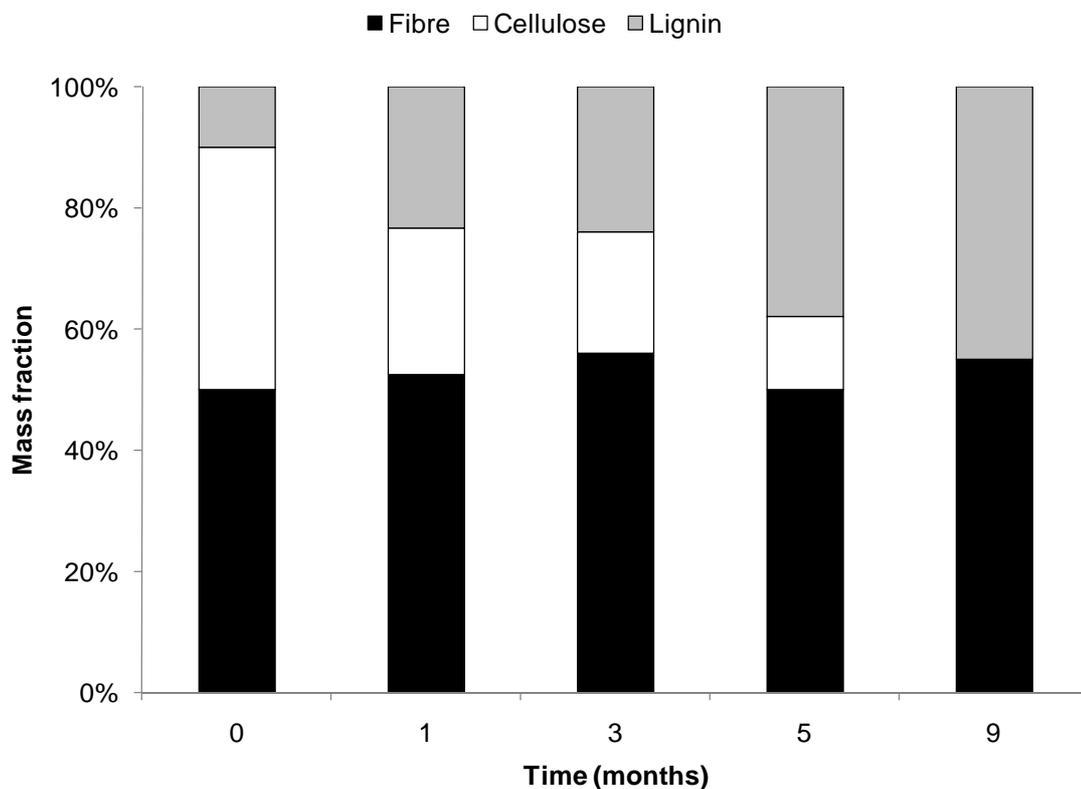


Figure 6.3: Composition of straw during fungal pre treatment following initial content of fresh barely straw through to 9 months decay..

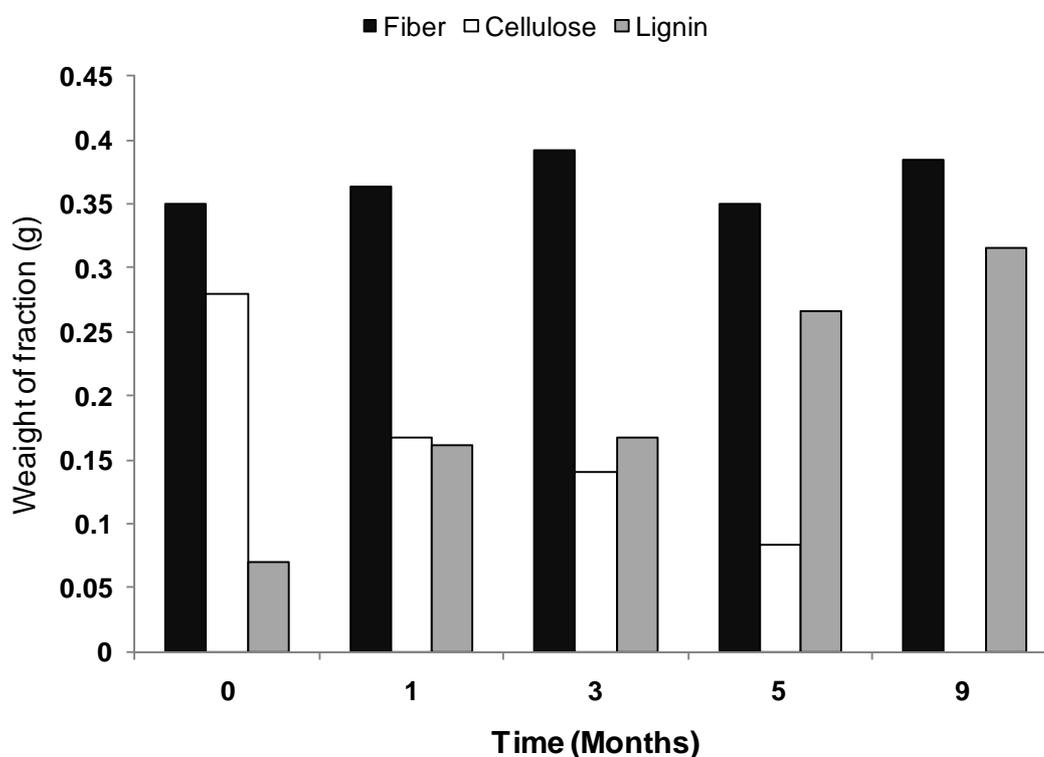


Figure 6.4: Absolute concentrations of the fibre, lignin and cellulose fractions from rotting barely straw samples (0.7g). Taken at 0, 1, 3, 5 and 9 months.

Fungal biomass increased from less than 0.01 mg.ml^{-1} before the start of the experiment to 0.26 mg.ml^{-1} after 9 months in terms ergosterol analysis (Figure 6.5). The concentration profile is indicative of exponential growth indicating that the fungal community was not substrate-limited over the course of the trial. Conversion of the data into a first order rate expression yields a rate constant of 0.38 month^{-1} which compares to reported growth rates for fungi in other situations of $0.2\text{-}2 \text{ d}^{-1}$ indicating that fungal growth is relatively slow in the current environment. Similar findings have been reported using ergosterol analysis on the fungi population grown on wheat straw (Robertson *et al.*, 2008) where exponential growth was observed in terms of an initial lag phase of 21 days and increase in fungal mass of 300% after 84 days. This compares to 550% higher over the same time period in the current case reflecting differences in the choice of substrate and species. In both cases, significant fungal growth coincides with a significant decrease in the cellulose mass fraction and the

onset of lignin degradation is consistent with the reported mechanism by which barley straw inhibits algal growth (Barrett, 1994). The maximum concentration of $260 \mu\text{g}_{\text{ergosterol}}\cdot\text{g}^{-1}$ detected in the current study is considerably lower than the $4055 \mu\text{g}_{\text{ergosterol}}\cdot\text{g}^{-1}$ reported after 35 days under enriched conditions for *P. chrysosporium* but is similar to those reported when using wood block at $421 \mu\text{g}_{\text{ergosterol}}\cdot\text{g}^{-1}$ (Niemenmaa *et al.*, 2008) demonstrating that fungal growth will be restricted in barley straw, generating the reported lag phase and the prolonged operation.

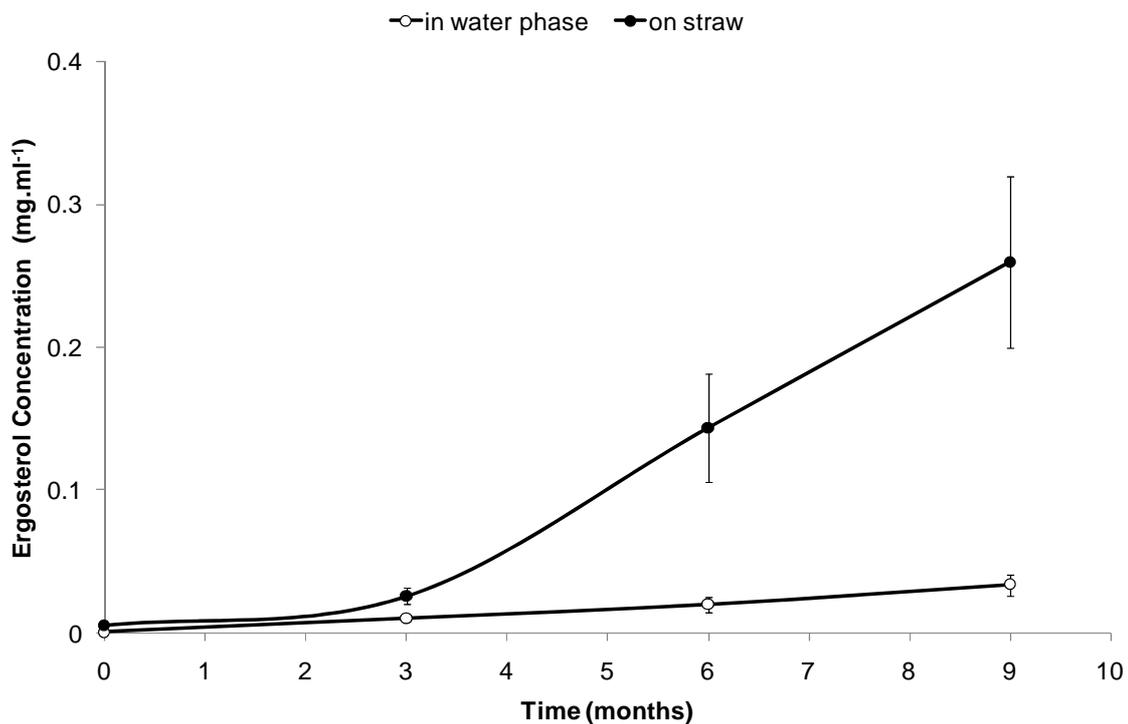


Figure 6.5 Fungal biomass development during straw degradation.

6.6 Discussion

In the current study all three straw systems were effective at inhibition of *Scenedesmus subspicatus* and generated an approximately similar level of inhibition. The key difference was observed in terms of the kinetics of both the onset and rate of inhibition (Table 6.1). The results presented here support postulated theories associated with the mechanisms of inhibition due to barley straw, centred around the release of phenolic compounds through the degradation of lignin (Pillinger, 1993; Barrett, 1994; Waybridge *et al.*, 2008). Previous work has evidenced the pathways of both phenolic compound release during straw degradation (Everall and Lees, 1996,

1997) as well as the inhibiting effect of phenolic compounds (Murray *et al.*, 2009). Explanation of the differences observed in the lag phase for the 3 systems are consistent with typical biological systems where a population needs to be developed and follows an exponential growth cycle such that duration of the lag phase relates to the initial population size. However, explanation for the differences in the rate of inhibition is less clear, although it is likely to mirror environmental conditions. In cases where the straw is degraded in the reservoir, alternative sources of nitrogen will be available, especially in locations that are likely to bloom. Given that lignin degradation relates to a response to nutrient limitation during secondary metabolism (Kirk and Farrell, 1987), the presence of alternative sources of nitrogen is likely to influence fungal activity. A parallel can be made to studies on fungi growth on copper-treated wood where nutrient supplementation has been shown to greatly enhance fungal activity and kinetics (Humar and Pohleven, 2005). Consequently, controlled pre-treatment by fungal augmentation in a reactor provides the ideal conditions to maximise the activity of the fungi in terms of lignin degradation and hence release of the phenolic toxins required to inhibit algal growth.

Table 6.1: Comparison of effect of treatment on performance.

Straw treatment	Lag time (weeks)	Rate of algae decline (cells.week ⁻¹)	Inhibition level (%)
Fresh	8	30000	75
Rotted	4	40000	75
Fungal pre treatment	1	>70000	65

The practical significance of the results relates to the responsiveness of the solution. Current approaches require long-term planning to ensure that the straw is inhibitory during the exponential growth phase of the bloom. Pre-treatment effectively renders the solution more responsive and as such can be applied in situations where blooms are unexpected in terms of location or time. The need to establish fungal populations remains the critical step and, whilst pre-treatment reduces the time required, the net benefits of developing pre-treatment reactors remains uncertain. Further work is

required to optimise the pre-treatment stage and to minimise the duration of pre-treatment as well as to maximise the inhibitory impacts of adding the straw.

6.7 Conclusions

The principle of utilising an engineered pre-treatment to enhance the impact of barley straw has been demonstrated. The enhancement is seen in terms of both a reduction in the lag time and the rate of inhibition. The consequence of this is that pre-treatment enables barley straw to be utilised in a more responsive way to manage unpredicted algal bloom outbreaks.

6.8 Acknowledgements

The authors would like to acknowledge the financial support for this project from Anglian Water, Northumbrian Water, Thames Water and Yorkshire Water.

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Chapter 7

Final Discussion

7. Final discussion

7.1 Introduction

Algae presently pose a considerable problem to industry and in managing water bodies, blooms of algae in eutrophicated water bodies can rapidly to an overwhelmed filtration systems and damage to water treatment processes (Shumway 1990; Pretty 2002; Pretty *et al.*, 2003) and can pose serious problems for the environment including limitation of light to the benthos and deoxygenation of the water body (Middleboe and Markager, 1997; Mason 2002). The worst cases are blooms of *cyanobacteria* (blue-green algae); these blooms lead not only to taste and odour problems but the lysis of the cells as they die releases potent toxins that can lead to the closure of reservoirs and even treatment works (Pretty and Ward 2001; Mankiewicz *et al.*, 2003; WHO, 2006). Traditional approaches to controlling algal blooms have revolved around various chemical and mechanical means, though all have considerable drawbacks; for instance pesticides such as diquat and copper sulphate are effective inhibitors of algal growth but are not target specific, this means that large parts of the ecosystem can be affected and higher organisms killed (Erturk and Walker, 2003; Van Hullebusch *et al.*, 2003). Mechanical methods such as surface raking and filtering often fail to remove the entire bloom so at best only delay the problem (Gulati and Donk, 2002) and further downstream methods such as pre-oxidation are effective when used with other methods but are costly, do not solve the source of the problem and can cause cell lysis meaning further costs are required to remove any released toxins. The costs associated with dealing with algal problems are considerable being estimated to account for at least £ 150 million year⁻¹ (Pretty *et al.*, 2003).

Barley straw has been put forward as a potential solution to this problem since early studies by Street (1978) and the presence of barley straw decaying in water has been shown to inhibit a wide range of algal species (Chapter 1, Table 1) including the problem blue-green algae *Microcystis* sp (Newman and Barrett, 1993) and the filter clogging green alga *Cladophora glomerata* (Welch *et al.*, 1990). Work on barley straw to date has focused mostly on showing it has an effect, whether this is in the field or the lab (Newman and Berrett, 1993; Barrett *et al.*, 1999). While this approach

has helped to show the scale of effect and range of species susceptible, it has not allowed an adequate investigation into the process leading to the production of inhibition. Some studies have shown that the end product responsible for inhibition is most likely liberated phenolic chemicals (Everall and Lees, 1996, 1997; Waybright *et al.*, 2009) but how this comes about during decay has been to date only theory. This study, alongside showing that rotting barley straw is effective, has shown that the types of chemicals suggested as the possible inhibitors can inhibit algal growth and has for the first time thrown light on the process that leads to inhibition. With this new understanding possible means to improve and use the properties of barley straw more efficiently are also recommended.

The proposed process by which barley straw's affect is produced is outlined below (Figure 7.1), each section of which will be outlined and discussed in order to present an overview of the results and their significance.

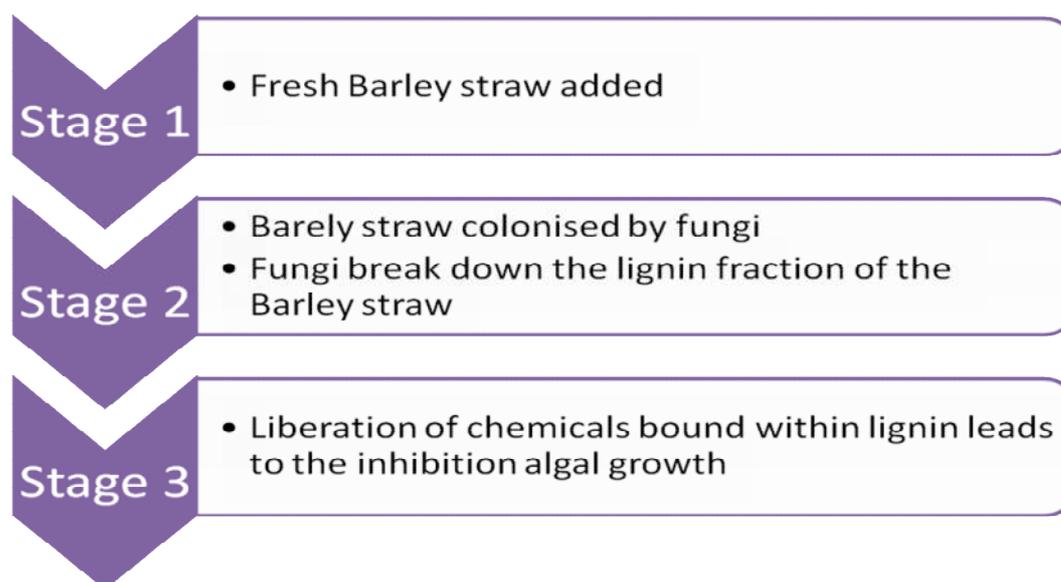


Figure 7.1 Outline of the process which leads to the production of algalstatic compounds from barley straw in water.

7.2 Stage 1 - Effects and potential of fresh straw

In order for barley straw to inhibit algal growth it has been suggested that decay is first required (Barrett, 1994, Ferrier *et al.*, 2005) the work carried out during this study using continuous cultures and attempting to pre-treat the straw has borne this

out as both have shown that fresh straw alone cannot inhibit algal growth. This lack of effect is because most of the inhibitory fraction of the straw is bound within the insoluble fraction of the lignified portion of the plant and requires a process such as decay to liberate it (Everall and Lees 1996, 1997; Rogalinski *et al.*, 2008). Previous studies have attempted to circumvent this problem by preparing an extract from barley straw (Ball *et al.*, 2001; Ferrier *et al.*, 2005; Waybright *et al.*, 2009). While these extracts were able to inhibit algal growth at concentrations as low as 100 g m^{-3} (Ball *et al.*, 2001) they were all still based on rotted straw; the attempts at using fresh straw, such as by Ball *et al.*, (2001), again like using whole fresh straw failed to inhibit algal growth. While previous attempts to use fresh straw have proved ineffective, it is known that when fresh straw is first immersed in water there is a brief chemical release (Everall and Lees, 1996, 1997) and it has been shown that, given enough energy, at least a portion of lignified plant material can be broken down and solubilised (Demirbas, 2005; Rogalinski *et al.*, 2008). Because of this it was decided there was still scope to explore the potential use of fresh straw to inhibit algal growth, as work had yet to be carried out on fresh straw with the aim of attempting to solubilise the lignified material and allowing all the naturally soluble material present early on to be applied in one dose.

It has been shown in this study that an effective extract can be obtained from fresh barley straw when an environment conducive to breaking down lignin is supplied; in this case the combination of heat, steam and pressure offered by an autoclave was used, and the extract obtained was able to inhibit all algae species tested including *Microcystis* with an EC_{50} of 190 g m^{-3} being found. It was interesting that rotted straw, even though able to inhibit algal growth, cannot be extracted in this way; in fact similarly to the findings of Welch *et al.* (1990) autoclaving the straw disrupted its effect. This suggests a difference in the chemicals involved in the fresh extract and when straw becomes naturally active, since, as mentioned previously, it has been shown that there is some natural chemical release when barley is first added to water (Waybright *et al.*, 2009) and lignin cannot be completely broken down by autoclaving (Garrott *et al.*, 1999; Demirbas, 2005; Rogalinski *et al.*, 2008). It is likely that the extract prepared by autoclaving removed all of the easily soluble material in one go rather than over a period of days / weeks and at the increased concentration offered by this an effective extract can be produced. By the time straw becomes naturally active

all the easily removable fractions will be gone so would no longer be present for extraction; instead the chemicals that are produced during this time are known to be phenolic in nature and derived from lignin (Everall and Lees 1996, 1997; Waybright *et al.*, 2009) and some of these chemicals such as P-Cresol have low boiling points and Waybright *et al.* (2009) suggests some may also be quite complex. This means a heat intensive process such as autoclaving would boil off or denature any of the chemicals being produced when straw is naturally active so, combined with the removal of the stable early chemicals, this would explain why rotted straw cannot be extracted via autoclaving. Instead a different process is required in order to liberate the inhibitory chemicals bound within the lignin of the barley, and only microbiological decay by fungi in nature can do this. This study has shown it may be possible to liberate at least a portion of the effective part of the barley straw without the wait for decay, by using artificial means. With further study this may be of use to utilities as an alternative method to control algal growth, although without decay or artificial treatment fresh barley straw shows no ability to inhibit algal growth.

7.3 Stage 2 - The importance of lignin and the role of fungi

Since fresh barley straw on its own has been shown in this study to have no inhibitory effect, a process must be required to render it effective. Previous studies have reached the conclusion that the production of barley straw's effect is related to the decay process, as it is only when straw has been rotted for a period of months that an effect is induced (Barrett *et al.*, 1999; Welch *et al.*, 1990). This study has shown that in the initial months after straw has been added to water only the cellulose fraction of the straw is degraded and during this time no effect is seen, it is only once significant fungal growth and lignin degradation was observed that inhibition was seen. This study has provided evidence for the first time that fungal decay of the lignin section of the straw is important in developing inhibition in barley straw, and this has supported previous studies such as Pillinger *et al.* (1994, 1995) where it was theorised that lignin degradation in barley straw could be the source of inhibition. Lignin itself is a complex insoluble matrix that is resistant to degradation requiring specialised species in nature to break it down (Pillinger *et al.*, 1995) and as explained lignin is a known source of phenolic material (Demirbas, 2005; Rogalinski *et al.*, 2008). In addition phenolic chemicals have been found in extracts produced from rotting straw

(Waybright *et al.*, 2009) and in the waters around decaying barley straw (Pillinger *et al.*, 1995) and furthermore it is known that barley straw has a higher than average lignin content (Hume, 2002).

Since this study has shown that actively degrading straw is inhibitory to algal growth and that this occurred in relation to both fungal activity and lignin decay, it would be wise to test fungal species which are able to break lignin down. In nature this is carried out by a specially adapted fungal group, the white rot fungi. With this in mind it was decided to pre-culture fresh straw with 3 species of white rot fungi, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*. It was found that when the pre-cultured straw was applied, algal growth was inhibited almost immediately, but that the scale of effect was comparable to field and lab rotted straws that were also tested. With the evidence that fresh straw's decay was consistent with fungal degradation this study can conclude for the first time that there is a direct link between microbiological decay of the straw, specifically by lignin degrading fungi and barley straw's inhibitory effect. This knowledge could be of great use to utilities as an understanding of the process that leads to barley straw becoming active can help to ensure success when it is applied and in future perhaps enhance the process. There is also opportunity to improve the usefulness of the straw, because as the fungi are fairly low maintenance, there is potential to keep straw with active communities of degrading fungi on standby without expending much effort or energy. This would mean that rather than applying the straw then having to wait several months for an effect, active straw could be applied and an effect seen almost instantly. Such an ability would provide great flexibility in dealing with algal events and would mean that straw could be only applied when needed rather than keeping it in "just in case".

7.4 Stage 3 –Liberated chemicals inhibit algal growth

Before this study it had been suggested that the compounds released during decay of barley straw were responsible for algal inhibition (Pillinger *et al.*, 1994, Everall and Lees, 1996, 1997). During the course of this study, attempts at pre-treating the straw and later on the use of white rot fungi, with the specific aim of breaking down lignin to produce chemicals, both showed that released chemicals were responsible for inhibiting algal growth. The chemicals responsible for inhibition have been suggested to be phenolic chemicals derived from the straw as lignin is broken down. As

discussed, lignin is already a known source of phenolic chemicals (Lima *et al.*, 2007) and these chemicals are known as able to inhibit algal growth (Newman and Barrett, 1993, Schrader *et al.*, 1998). Importantly, previous studies have shown liberated lignin in the waters around decaying barley straw (Pillinger *et al.*, 1994) and analysis carried out by Waybright *et al.* (2009) concluded that phenolic chemicals were responsible for decaying barley straw's inhibition, and attempts at pre-treatment over the course this study also suggested chemical production was responsible for inhibition. Everall and Lees (1996, 1997) in their 2 studies were able to not only detect phenolic chemicals, but to actually point out individual compounds. Whilst all these studies are important in showing that chemicals can and are produced by decaying barley straw, they haven't to date provided information on the effectiveness of the chemicals themselves.

Because of this it was decided to test several of the chemicals detected by the Everall and Lees (1996, 1997) studies, as their effectiveness would be useful in showing that chemicals produced by decaying straw can inhibit algal growth. The effectiveness of phenolic chemicals would be particularly useful as it would give support to the findings of Waybright *et al.* (2009). Finally if any individual chemical were particularly effective it may allow identification of the "main ingredient" which could, after further development, be directly applied to prevent algal growth. After an initial study of the pre-existing literature 13 chemicals were chosen to be assessed. Selection was based on existing data such as, chemical toxicity, solubility and if the chemical was a phenol or not. Chemicals chosen for assessment included p-cresol, 2 phenyl phenol and benzoic acid (a full list of chemicals is provided in chapter 3). Encouraging results were obtained from the study as many of the chemicals were found to have at least a mild inhibitory effect on algal growth such as the EC₅₀'s of 110 µg l⁻¹ for both p-cresol and 2-methylbuteric acid vs. *Chlorella* and 150 and 300 µg l⁻¹ respectively vs. *Microcystis*. Several of the chemicals proved to be highly effective at inhibiting algal growth, such as 2 phenyl phenol having an EC₅₀ of just 10 µg l⁻¹ for *Chlorella*, even lower than what was obtained for CuSO₄ (22 µg l⁻¹). Individually the chemicals found associated with decaying barley straw have been shown to be able to significantly inhibit algal growth at concentrations noted by Everall and Lees (1996, 1997) over the course of their 2 studies. When the factors such as cumulative effect of all the chemicals together, the possibility for synergism

and the fact that the chemicals may be present in higher concentrations than measured due to their volatility (Everall and lees 1996, 1997) it is clear that chemicals liberated from barley straw could easily be responsible for the inhibition seen. The particular effectiveness of phenolic chemicals seen in this study also supports the findings of Waybright *et al.* (2009) who suggested that they were the main chemical group responsible, and the evidence already provided in this study, and outlined above, has shown that the process leading to barley straw becoming effective is one which directly leads to the liberation of chemical compounds contained within the straw, giving an overall conclusion that chemicals liberated during barley straw's decay are responsible for algal inhibition.

7.5 Conclusions & recommendations

This study has provided evidence for the first time outlining the chain of events that leads to the production of barley straw's inhibitory effect. Once added to water, barley straw is colonised by white rot fungi, these species are able to degrade the lignin section of the straw liberating the large variety of phenolic chemicals bound within, and these chemicals are released into the surrounding waters and inhibit the growth of the surrounding algae. During the course of showing this, this study has been able to show that the recommended conditions for producing barley straw's effect are correct (Waybright *et al.*, 2009) and if they can be maintained, barley straw can be made active faster (Chapter 4). Importantly, now that lignin-degrading fungi have been shown to be responsible for generating the effect of barley straw there is a possibility to maintain straw in readiness to be deployed where blooms arise, this trial having shown that straw pre-cultured with white rot fungi is active almost on contact with algae so none of the previous waiting for several months would be required. This knowledge of how the inhibition is produced also provides a possibility for guaranteeing barley straw's effect; this will be of great help to utilities when planning their algal control programs. Now that white rot fungi's importance has been noted it may be possible to improve the scale of effect by screening different species to see if they can function more efficiently / effectively under the conditions barley straw becomes effective. Use of better species alongside preculturing will allow vast improvements in optimising the process and deployment of barley straw.

This study has show there is potential to directly use the chemicals associated with barley straw. Some of the chemicals can be obtained by artificial means such as by extraction as in chapter 4 or by applying individually (chapter 3) once identified by chemicals analysis (Everall and Lees 1996, 1997). Using the chemical potential of straw in these ways may provide a way of rapidly offsetting algal blooms, but is not ultimately recommended at this time as 1) it is not known what the long term effect of adding massive doses of the chemicals in one application may be; 2) The full potential of barley straw is not used in these ways as only an individual or group of chemicals is applied, this loss may lead to a reduction in the range of affected species or an increase in the speed of resistance development; 3) The fringe benefits of using whole straw are lost, such as low maintenance required, the reported positive effects of barley straw on the ecosystem, such as promotion of a more diverse community (Everall and Lees, 1997) and the “green credentials” of using such as method. However, there may be situations where a chemical deployment is more appropriate, such as to offset blooms that arise in works, meaning the potential of chemical applications should not be overlooked, and identification of the most effective chemicals may lead to benefits in further understanding and optimising barley straws effect. These short-comings are not present when using barley straw itself and now that the production of its effect can brought on sooner the previous issues of time delay are no longer a problem. In light of this, this study recommends that larger scale studies be carried out using fungal pre-cultured straw, which if successful could be maintained centrally and deployed wherever algal blooms begin to form.

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