A Biofilter Process for Phytoplankton Removal prior to Potable Water Treatment: a Field and Laboratory Study

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Abstract

Phytoplankton blooms compromise the quality of freshwater ecosystems and the efficient processing of water by treatment works worldwide. This research aims to determine whether in-situ filamentous biofiltration processes mediated by living roots and synthetic filters as media can reduce or remove the phytoplankton loading (micro-algae and cyanobacteria) prior to a potable water treatment works intake.

The underlying biofiltration mechanisms were investigated using field and laboratory studies. A novel macroscale biofilter with three plant species, named the "Living-Filter", installed in Farmoor II reservoir, UK, was surveyed weekly for physicochemical and biological variables under continuous flow conditions during 17 weeks. The efficiency of a mesoscale biofilter using the aquatic plant *Phalaris arundinacea* and synthetic filters, was tested with *Microcystis aeruginosa* under continuous flow conditions and in batch experiments. The 'simultaneous allelochemical method' was developed for quantifying allelochemicals from *Phalaris* in aqueous samples. Microscale studies were used to investigate biofilter allelochemical release in response to environmental stressors and *Microcystis* growth inhibition in filtered and unfiltered aqueous root exudate.

Results demonstrate that the removal of phytoplankton biomass by physical mechanisms has a removal efficiency of ≤45% in the "Living-Filter" (filamentous biofilter plus synthetic fabric) and that the removal of *Microcystis* biomass using only biofilters was 25%. Chemical mechanisms that reduce *Microcystis* cell numbers are mediated by allelochemicals released from biofilter roots. Root exudate treatments on *Microcystis* revealed that *Microcystis* growth is inhibited by allelochemicals, not by nutrient competition, and that protists and invertebrates play a role in removing *Microcystis*.

Filamentous biofilters can remove phytoplankton biomass by physical, chemical and biological mechanisms. Biofilters and synthetic filters in combination improve removal efficiency. Application of macroscale biofilters prior to potable water treatment works benefits the ecosystem. Plant properties, biofilter size to surface water ratio, and retention time must be considered to maximise the benefits of biofiltration processes.

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Table of Contents

List of Tables

List of Figures

Glossary

Algae (here micro-algae): eukaryotic photosynthetic micro-organisms where the pigments for photosynthesis are contained in a special structure, the chloroplast

Algastatic: is a generic term used to describe a substance that inhibits grow of phytoplankton (cyanobacteria and micro-algae) cells, the effect could be reversed if the agent is removed. It is the specific term for a substance that inhibits grow of algae/microalgae

Algicide/algicidal: is a generic term used to describe a substance that damage irreversibly phytoplankton cells. It is the specific term of a substance that damage irreversibly algae/microalgae.

Allelochemicals: secondary metabolites that are not essential for the survival or development of the organism that produces them and can inhibit or stimulate another organism.

Allelopathy: defines the inhibitory (suppressant) or stimulatory effect produced by one species on another species mediated by the release of secondary metabolites (chemicals).

AWB: artificial water bodies

Biomass: total mass of living organisms per unit of surface or volume

Biocide: a substance that damage irreversibly living cells or organisms

Biostatic: a substance that inhibits grow or reproduction of living cells or organisms

Chemical bridging: neutralization of the natural electric charge of suspended particles by the addition of a coagulant for binding particles to form aggregates facilitating removal

Chlorophyll-*a*: a pigment of photosynthetic organisms. It is use as a surrogate of phytoplankton biomass.

CWs: constructed wetlands

Cyanobacteria (or blue-green algae): prokaryotic photosynthetic organisms which lack of chloroplasts, so the pigments (for photosynthesis) are found in the cytoplasm

Cyanocide/cyanocidal: is the specific term of a substance that damage irreversibly cyanobacteria cells

Cyanostatic: is the specific term of a substance that inhibits grow of cyanobacteria cells, the effect could be reversed if the agent is removed

DO: dissolved oxygen

DOC: dissolved organic carbon

DOM: dissolved organic matter

DBPs: disinfection by-products

Eukaryotic: organisms where the genetic material (DNA) of the cell is organized in a structure (the nuclei).

Freshwater systems: describes lakes and reservoirs in an ecological context.

FTWs: floating treatment wetland(s)

GAC: granular activated carbon

HPLC: high performance liquid chromatography

HLR: hydraulic loading rate

HRT: hydraulic retention time

O.D.: optical density

Periphyton: micro-organisms that live attached to a surface

Photosynthesis: the process where large organic compounds are produced essentially from carbon dioxide and water using light as the energy source

Phytoplankton: includes micro-algae and cyanobacteria. It defines unicellular organisms that drift with water currents and carry out photosynthesis

POC: particular organic carbon

Prokaryotic: cells without nucleus, the genetic material is dispersed in the cell and also lack of other cellular organelles (structures)

Raw water: refers to chemically untreated surface water

RE: removal efficiency

RGF: rapid gravity filter or rapid gravity filtration

Root extract: product obtained after cutting and maceration of roots

Root exudate: substances released by plants to the environment

Filter run time: length (time) of filter performance measured in hours.

SSF(s): slow sand filter(s) or slow sand filtration

Surface reservoir or reservoir: is a man-made or impounded water source for potable water treatment works.

Surface water: in this thesis includes streams, rivers, ponds, lakes and reservoirs

Thames Water: Thames Water Utilities Limited

TOC: total organic carbon

WTWs: water treatment works or potable water treatment works

WWTWs: waste water treatment works

Zooplankton: protists and invertebrates that drift with water currents

Chapter 1. Introduction and background

1.1 Context and justification

The quality and quantity of water in surface reservoirs is of paramount importance to the water supply industry, ensuring that the treatment of water will produce safe drinking water and that water demand is met (Brooks *et al.*, 2016). Water has become a scarce resource in many regions of the world due to overpopulation, climate change and excess of macro-nutrients, primarily phosphorus and nitrogen (Heathwaite, 2010). The excess of nutrients that reduces aquatic biodiversity and promotes algal growth with adverse effects to the balance of food webs in aquatic systems is termed eutrophication (Lürling and De Senerpont Domis, 2013).

Eutrophication is arguably the primary water issue at the global scale (Lürling *et al.*, 2016a). A symptom of eutrophication is the presence of phytoplankton blooms, but particularly cyanobacteria blooms (Reynolds *et al.,* 2012; Ger *et al.*, 2014). A further symptom observed in shallow lakes and reservoirs (<5 m max. depth) is a loss of vegetation, caused by the shading effect of phytoplankton blooms, increased periphyton on plants, or both. This loss of vegetation and provision of refuge leads to a loss of zooplankton and grazing due to an increase in zooplankton predation by planktivorous fish. Moreover, a decrease in vegetation implies a decrease of phytoplankton suppressants released by submerged vegetation (Phillips *et al.*, 1978; Scheffer *et al.*, 1993).

Cyanobacteria blooms in surface reservoirs are a global issue threatening water quality, with significant economic, ecological and public health consequences (O'Neil *et al.,* 2012; Ibelings *et al*., 2016; Pearl *et al*., 2016). Cyanotoxins are known to be hepatotoxic, causing gastrointestinal and skin illnesses and if ingested can be fatal to humans, livestock and wildlife (Carmichael and Boyer, 2016). An established cyanobacteria bloom can be defined as a concentration of 100,000 cells mL^{-1} or ~ 50 µg L^{-1} chlorophyll-a with potentially an average

content of 20 µg L^{-1} cyanotoxins in recreational water (Chorus and Bartram, 1999; Falconer, 2005). Chlorophyll-a is also frequently used as an indicator of phytoplankton biomass (Bowes *et al.,* 2012). The World Health Organization guideline for microcystin concentration in drinking water is 1 µg L^{-1} (Chorus and Bartram, 1999), although these are only provisional guidelines for total microcystins-LR (a type of cyanotoxin) (WHO, 2011).

The presence of phytoplankton blooms in surface waters is associated with operational challenges in potable water treatment works (WTWs) (Henderson *et al.,* 2008; Jarvis *et al.,* 2008) and these can be due to an increased number of suspended cells in the water or the production of secondary metabolites such as cyanotoxins, geosmin or 2-methyl-isoborneol (MIB) from cyanobacterial blooms (Carmichael, 2001; Gurbuz *et al.,* 2009; Peter *et al.,* 2009). Cyanotoxins are cyclic heptapeptides that affect phosphorylation in cellular processes with more than 90 variants described. The most commonly detected cyanotoxins worldwide are microcystins (Falconer, 2007; Newcombe, 2009). Geosmin and MIB are volatile organic compounds which are the source of customer complaints due to earthy and mouldy tastes and odours in final drinking water (Jarvis *et al.,* 2008). Both the cells and their polysaccharide exudates are associated with disruption of the treatment coagulation process, as a result of inhibiting the adhesion of colloidal particles to form stable floc blankets, and hence increasing the chemical dose demand (Sun *et al.,* 2013). Filter blocking, because of the phytoplankton cells, leads to a decrease in filtration running times which reduces water production rates; as a result the increase in the frequency of filter backwashing shortens their lifespan (Bauer *et al*., 1998; Ribau and Joao, 2006).

Recent literature also highlights the role of phytoplankton in the formation of disinfection-by-products (DBPs) which carry unwanted health risks for human populations (Tomlinson *et al.,* 2016). However, the pool of dissolved organic matter (DOM) in aquatic systems is dynamic, varied and complex in their reactivities (Yates *et al*., 2016). The hydrology of natural systems, e.g. uplands and lowlands will influence the composition and

delivery of natural organic matter (Johnes, 2007; Yates *et al*., 2016). Disinfection-by-products formation occurs when organic matter (allochthonous or autochthonous) react with chlorine, chloramine and ozone which are used as disinfectants in the treatment of water. Allochthonous organic matter can derive from waste water effluents and the breakdown of vegetation from external catchment sources, the latter known as natural organic matter (NOM). Autochthonous organic matter is derived from algae and is also known as algal organic matter (AOM) (Tomlinson *et al.*, 2016). The discrimination of DOM origin and fractions has been possible by spectrophotometric analysis measuring absorption in the visible, UV-A and UV-B wavelength ranges (Spencer *et al*., 2007). Recent advances in the spectrophotometer technology made possible to determine variations in wavelength of chromophoric DOM *in situ* and real time (Spencer *et al*., 2007; Yates *et al*., 2016). Algal-derived dissolved organic matter which is rich in hydrophilic content (carboxylic acids, polyuronic acids, aminoacids, peptides and carbohydrates), reacts with chlorine, and the preferred disinfectant in WTWs (Tomlinson *et al*., 2016).The risks of DBPs in potable water can be potentially heightened by the increased frequency in cyanobacteria blooms; hence, the removal of DBPs precursors is the preferred way of reducing the risks (Bond *et al.,* 2011).

One way of dealing with phytoplankton loading for the potable water industry is by increasing the chemical dose in WTWs and testing new chemicals that reduce the formation of residual by-products e.g. ferrates (Sharma *et al.,* 2015). However, the intensive chemical use of sulphuric acid, potassium permanganate, aluminium and ferric salts to prepare the water for coagulation and flocculation processes; added to the use of chemicals for disinfection that involves ozone, chlorine, ammonia and sulphate; will generate treatment residuals: sludge, brines and toxic waste. All these can add to the problems of contamination and salting of surface water (Shannon *et al.,* 2008), and increase the area of land needed for disposal of sludge.

An alternative way of dealing with phytoplankton loading in WTWs is through the trialling and implementation of innovative technology, such as Mecana Pile Cloth media filter (Mecana, 2016), a filtration process which uses numerous micro fibres arranged at depth. This concept is currently being tested by water companies in the UK (Tupper *et al*., 2016). The raw water flows into the filter tank and passes through the filter cloth; whilst the solids retained by the cloth increase the hydraulic resistance, the head loss also increases and this triggers an automated filter cleaning cycle without interrupting the filtration process.

In the light of the above points, WTWs are under huge pressure to deal effectively with phytoplankton loading. However, the increase in associated processes required to deal with these issues requires more land, capital and operational investment. This consequently increases the cost of the final treated water (Purcell *et al.,* 2013). Moreover, the financial implications and land limitations associated with increasing the number of processes affect developed and developing countries alike, although the challenge is aggravated in developing countries where the infrastructure is also limited (Shannon *et al.,* 2008; Purcell *et al.,* 2013).

The Water Supply Innovation team in Thames Water Utilities Ltd. (Thames Water) in an attempt to move away from the traditional approach taken by the water supply industry, increasing the number of processes in WTWs, designed the "Living-Filter" in collaboration with AquaticEngineering, UK. Thames Water is the largest water supply and sewerage company in the UK serving 15 million people, and their vision for creating the Living-Filter was to implement an eco-technological process in a surface reservoir avoiding the extra use of land for new treatments. The Living-Filter is an ecologically engineered, field-scale, in-reservoir floating treatment wetland (FTW) installed in Farmoor II reservoir, with the aim of pre-treating the raw water flowing into the WTWs (Figure 1.1). A reduction of phytoplankton load means a reduction of chemical dosing in the WTWs; as a consequence, less sludge is generated from the treatment that would need to be disposed of via landfill, and potentially the Living-Filter will reduce operational expenditure.

Farmoor II Reservoir

Area:	1 km^2
Depth:	11 m
Volume:	9.3 x 10^6 m^3
Outflow:	45 - 95 x 10^3 m^3 d^{-1}

Living-Filter plant-bed

Area:	210 m^2
Depth:	1 m ~ 2m (root growth)
Vol.:	210 m^3
Flow:	10–20 m^3 h^{-1} m^{-2} (HLR)

Figure 1.1 Aerial image of Farmoor reservoir (I and II), showing the location of the River Thames intake, Living-Filter and outlet (or intake) to the WTWs. Farmoor II reservoir and Living-Filter morphometric data (Google Maps, 2013).

The mechanisms expected to operate within the pre-treatment Living-Filter biofiltration process are based on a) filtration mechanisms on a filamentous biological medium, such as plant roots, and b) ecological mechanisms. Filtration is a process for the removal of particles from a suspension by passage of the suspension through a porous medium (Crittenden *et al.,* 2012). Filtration on a filamentous medium entails several mechanisms that can be broadly grouped into transport and attachment mechanisms, both based on filtration theory that comprises a system of mass conservation and kinematic relations. The ecological mechanism is based on the trophic cascade hypothesis and the concept of biomanipulation (Shapiro *et al.,* 1984; Carpenter *et al.,* 1985; 1987). These mechanisms operate in biofilter treatment processes in WTWs such as slow sand filters (SSF), rapid gravity filters (RGF) and granular activated carbon (GAC), but with the difference that the media are granular instead of filamentous (Magic-Knezev and Van Der Joit, 2004; Nakamoto, 2014).

While biofilter treatment processes with granular media are known to contribute to phytoplankton removal in WTWs, the use and contribution of biofilters with filamentous media as a pre-treatment process for phytoplankton removal have not been investigated before. This research tests the Living-Filter as an in-reservoir, field-scale and biofilter pre-treatment process; it also uses laboratory-scale biofilters with filamentous media to investigate and identify the underlying mechanisms that could contribute to phytoplankton removal. The research builds on a range of physicochemical and biological relations at the macro, meso and micro scales. Finally, it seeks to improve future designs of field-scale biofilters for phytoplankton removal prior to WTWs.

1.2. Eutrophication: a major driver for the establishment of phytoplankton blooms

The global scientific community accepts that anthropogenic eutrophication associated with an increasing global human population has stimulated the occurrence of phytoplankton blooms. Some of these can be harmful, particularly cyanobacteria blooms with some species

producing toxins known as cyanotoxins (O'Neil *et al.*, 2012). Indeed, in the presence of macronutrient enrichment of phosphorus and nitrogen, the phytoplankton community shifts to a cyanobacteria-dominated community.

Surface waters are among the most sensitive of all ecosystems to changes in flow and temperature in the UK (Watts *et al.*, 2015; Whitehead *et al.*, 2009; Bussi *et al.*, 2016) and worldwide (Paerl and Paul, 2012; Whitehead *et al.*, 2016). In the UK, a number of studies have predicted the impact that climate change will have on river flows and the effecs of rising temperatures on rivers and lakes (Bowes *et al.*, 2009; Whitehead *et al.*, 2009). The predictions are that flooding will increase nutrient transfer from catchments to rivers, and that river flows with reduced velocity and longer residence times, will create conditions that are strongly related to large phytoplankton blooms (Bowes *et al.*, 2009; Watts *et al.*, 2015; Paerl *et al.*, 2016). Higher temperatures, in conjunction with the transfer of nutrients from the catchment to rivers, lakes and reservoirs, will accelerate metabolic processes associated with the release of nutrients from the sediment; mediated by microorganisms; this, in turn, will lead to an increase in the severity of the blooms (Paerl *et al.*, 2011; Elliot, 2012; Whitehead and Crossman., 2012; Arnell *et al.*, 2015; Watts *et al.*, 2015).

1.2.1 Farmoor I and II reservoirs: external nutrient loading from the River Thames

Farmoor I and Farmoor II reservoirs were built in 1967 and 1976 respectively, and they supply water to Swinford and Farmoor WTWs. Together these WTWs provide potable water to Oxford as well as other parts of Oxfordshire, and Berkshire. The demand for water production at each WTWs is 45-90 x $10^3 m^3 d^{-1}$, with an average of around 65 x $10^3 m^3 d^{-1}$. High water demand is expected during the summer, when chlorophyll-a is also at its highest concentration, complicating the treatment and potentially reducing production outputs. These are man-made reservoirs, built in locations where no body of water existed before, and which are denominated artificial water bodies (AWB) that need to achieve good ecological potential

(GEP) by 2015 under the European Water Framework Directive (Directive 200/60/EC), instead of good ecological status required for rivers and lakes (HM-AWB, 2015). However, there is no clarity regarding the exact meaning of GEP for AWB (Hering *et al*., 2010). These surface reservoirs are riverbank reservoirs with the bulk of water sourced only from the River Thames.

The River Thames is located in the south of England, with a total length of 354 km, a catchment of around 9948 km², and is highly urbanised, including among other major cities: Swindon, Oxford, Reading and London (Octavianti, 2014; Whitehead *et al.*, 2015; Bussi *et al*., 2016). The WTWs and waste water treatment works (WWTWs) in the River Thames catchment are managed by Thames Water. The River Thames supplies water to 14 million people and receives effluents from 63 WWTWs (Kinniburgh and Barnett, 2010), of which 24 are located in the River catchment between Somerford and Swinford (Octavianti, 2014). The river intake for Farmoor I and II drinking water reservoirs is in the section of river with the lowest slope (0. 019%), which is located between Newbridge and Swinford (Whitehead *et al.*, 2015).

Phosphorus (P) loading to the River Thames in the section between Somerford and Farmoor can be from sewage effluents (point source pollution), or from catchment run-off from arable crops (diffuse sources) (Kinniburgh and Barnett 2010; Heathwaite 2010; Whitehead *et al.*, 2015). The Urban Waste Water Treatment Directive (UWWTD) was introduced in the European Union in 1991 to reduce nutrient loading from sewage effluents, and reductions in soluble reactive phosphorus concentrations have been achieved since then (Bowes *et al.*, 2012). Currently, the maximum P concentration from tertiary effluents is 0.2 mg L^{-1}. However, nutrient mobilization from catchments and how these interactions take place with water bodies remains a subject of investigation (Haygarth *et al.*, 2005; Neal and Heathwaite, 2005; Burt *et al.*, 2011; Musolff *et al*., 2016; Yates *et al*., 2016). For example in a meta-analysis study of 110 catchments carried out in Germany, Musolff *et al*., (2016) found

that in catchments with decrease NO_3^- levels (<6 mg L^{-1}) was linked to an increase of soluble Fe^{+2}, thus mobilizing previously adsorbed compounds such as P and dissolved organic carbon (DOC). Consequently, P and DOC levels increased from riparian waterlogged soils to streams, rivers and to receiving drinking water reservoirs. Implications are that decrease in nitrate concentrations might raise other water quality concerns that affect negatively treatment options and costs (Musolff *et al*., 2016). Nevertheless, understanding the delivery of phosphorus and organic matter to streams from contributing sources in their catchments is a complex problem; and considering that also involves understanding temporal, hydrological and geographical variability (Yates *et al*., 2016).

Nitrogen (N) fractions found in aquatic systems include oxidised and reduced inorganic N (nitrate, ammonium, ammonia and dissolved inorganic nitrogen) and also organic N (urea, amino acids, proteins, nucleic acids). Inorganic forms are dominant in eutrophic waters, while organic forms are common in meso- and oligotrophic waters (Durand *et al.,* 2011). Nitrogen can reach freshwater ecosystems by several pathways: atmospheric deposition onto the catchment or directly onto the water body; leaching from naturally rich N soils from erosion, or from the catchment, direct intrusion from sewage effluents (Durand *et al.,* 2011); and groundwater which is a flow pathway for the infiltrated nitrogen from polluted soils into surface water (Rivett *et al.,* 2008). Since 1991, a maximum acceptable nitrate (NO_3^-) concentration of 50 mg L^{-1} (11.3 mg NO_3-N L^{-1}) was established for all water bodies (WHO, 2011; DWI, 2016). However, the pattern in the UK for NO_3^- concentrations indicate that there is a very slow decline since the mid 1980's (Howden and Burt, 2008; Burt *et al.,* 2011). Despite the numerous UK policies and legislation since the Control of Pollution Act (1974) and European Directives e.g. Drinking Water Directive (EU 80/778), nitrogen in groundwater is still a risk for public health (Howden *et al*., 2009; Worral, 2009; Burt *et al*., 2011).

1.2.2 Internal loading in lakes and reservoirs

Internal nutrient loading is responsible for the limited success of many restoration attempts on freshwater systems after reducing external loading (Cooke *et al.*, 2005). Phosphorus binding or mobility results from physical, chemical and biological processes, including temperature, redox conditions, dissolved oxygen, pH and bacteria re-mineralization (Wetzel, 2001; Meis *et al.,* 2012; Dittrich, 2013). Phosphorus fractions found in the sediment of lakes and reservoirs include soluble and loosely adsorbed orthophosphate (PO_4^{3-}), redox-sensitive iron (Fe)-bound P, and P bound to hydrated oxides of aluminium (Al^{+3}) and non-reducible Fe (surface-bound), calcium (Ca)-bound P (apatite-P), and organic P (Dittrich *et al.,* 2011; Meis *et al.*, 2012).

Average temperature increases due to climate change, impact eutrophic lakes and reservoirs by promoting stratification in the water column. Stratification separates the water column in three defined layers. The epilimnion is the upper layer of the water column that is well mixed, well illuminated and warmer than the other two layers. The thermocline or metalimnion is the layer where the water temperature changes more rapidly and is also well mixed. The hypolimnion is the bottom layer where there is no mixing, an absence of light, low dissolved oxygen levels, low redox potential and colder temperature (Wetzel, 2001).

Photosynthesis and respiration are the major biological processes affecting pH by changing the amount of CO_2 in the water (Figure 1.2. The consumption of CO_2 during photosynthesis to produce carbohydrates and oxygen, alters the carbon (C) equilibrium, the H^+ uptake increases the pH (equation 1.1) (Brönmark and Hansson, 2005) whilst respiration contributes to CO_2 in the water (equation 1.2) (Stumm and Morgan, 2012).

Figure 1.2 shows in the pH-carbon dioxide-bicarbonate system (eq. 1.1) that when CO_2 is taken up by photosynthesis, free H^+ ions are associated with bicarbonate and carbonate ions, leading to fewer free H^+ and thereby a higher pH.

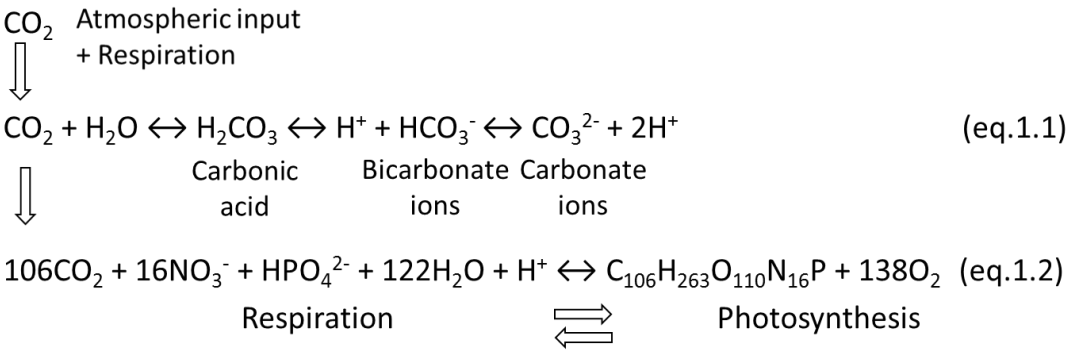


Figure 1.2 Equilibrium processes of the pH-carbon dioxide-bicarbonate system; and photosynthesis and respiration processes (modified from Brönmark and Hansson, 2005; and Stumm and Morgan, 2012).

Warmer temperatures favour cyanobacteria blooms that dominate the epilimnion. The blooms induce daily changes in the water pH that respond to changes in dissolved carbon dioxide concentrations during photosynthesis (diurnal changes) and respiration (nocturnal changes) processes. Moreover, pH ≥8 promotes desorption of P (PO_4^{3-}) from Al^{+3} and Fe^{+3} metal oxides (Perkins and Underwood, 2001; Lukkari *et al.,* 2007). The processes in the hypolimnion, accentuate microbial activity in the proximity of the sediment-water interface (Bostrom, 1988; Foley *et al.,* 2012; Carey *et al.,* 2012) where redox-sensitive Fe-P bindings under decreased redox potential conditions will promote the release of PO_4^{3-} by reducing Fe^{+3} to Fe^{+2} complexes. These processes can be mediated by chemolitotrophic bacteria or by direct reduction of iron (Fe^{+3}) complexes.

1.3 Phytoplankton blooms

Phytoplankton in freshwater comprise a large group of photosynthesising organisms that include micro-algae and cyanobacteria. Micro-algae are eukaryotic organisms (with a nucleus) which include the taxonomical classes Chlorophyceae, known as green algae, Bacillaryophyceae (diatoms), Chrysophyceae (golden algae) and Pyrrophyceae (John *et al.,* 2011). Cyanobacteria are prokaryotic organisms also known as blue-green algae. Common micro-algae blooms are those formed by diatoms and occasionally by golden algae in

oligotrophic waters. However, the term ‘algal blooms’ in surface water is attributed to cyanobacteria blooms and among these, *Microcystis* spp. is the most frequent species to form blooms (Matthijs *et al.,* 2016). Bloom-forming cyanobacteria are gas-vacuolated with self-regulating buoyancy enabling them to seasonally dominate the phytoplankton of eutrophic lakes and reservoirs by outcompeting micro-algae for light and nutrients (Walsby, 1975; 1992 Leigh *et al.,* 2010; Carey *et al.,* 2012; Visser *et al.,* 2016). Many cyanobacteria species such as *Anabaena* spp. and *Aphanizomenon* spp. are diazothropic, so they can fix atmospheric nitrogen (N_2) into ammonia via a nitrogenase enzyme (Durand *et al.,* 2011).

Micro-algae and cyanobacteria produce external polysaccharides (EPS) which have been shown to protect them against harmful environmental changes, predators such as zooplankton grazers, to facilitate adhesion to surfaces, or to aid buoyancy (Whitton and Potts, 2000; Pereira *et al.,* 2009; Harel *et al.,* 2012). The EPS can also change the rheological properties of the water, increasing the viscosity, and consequently reducing eddies (Jenkinson and Sun, 2011).

A summary of abiotic and biotic triggers leading to cyanobacteria blooms in the air-water interface, water column and water-sediment interface taking place in a lake or reservoir can be seen in Figure 1.3, with kind permission from Paerl *et al.,* (2011) and Alan Joyner (Institute of Marine Sciences, UNC).

1.3.1 Phytoplankton blooms and ‘limiting nutrients’ in lakes and reservoirs

The limiting nutrient concept (Liebig’s Law of the Minimum) refers to the element that is least available relative to the needs of the organism, and is related to the idea that nutrient deficiency at any given time in a photosynthetic organism can be traced to a single element (Lewis *et al.,* 2011).

Phosphorus is considered to be the limiting nutrient in lakes and reservoirs (Wetzel 2001). The strong relationship between total phosphorus (TP) and chlorophyll-*a* (Chla)

concentrations (Cardoso *et al.*, 2007; Phillips *et al.*, 2008; Spears *et al.*, 2013), and on TP and cyanobacteria levels (Carvahlo *et al.*, 2011) is well established, but the relationship between total nitrogen (TN) and Chla is very weak (Lewis *et al.*, 2011). However, Phillips *et al.*, (2008) found that in shallow polyhumic lakes with high alkalinity the relationship between TN and Chla was a better predictor than TP and Chla, suggesting N as a limiting factor.

Although the role of nitrogen as a limiting nutrient is well established for estuaries and coastal environments, its role is still being documented in freshwater ecosystems (Conley *et al.*, 2009; Howarth *et al*., 2011; Paerl and Paul 2012). Nitrate (NO_3^-) is the predominant form of nitrogen in eutrophic waters and in the sediment-water interface it can be an oxidant for P cycles, releasing dissolved PO_4^{3-} and dissolved Fe^{+2}, by reducing Fe^{+3} (Hemond and Lin 2010).

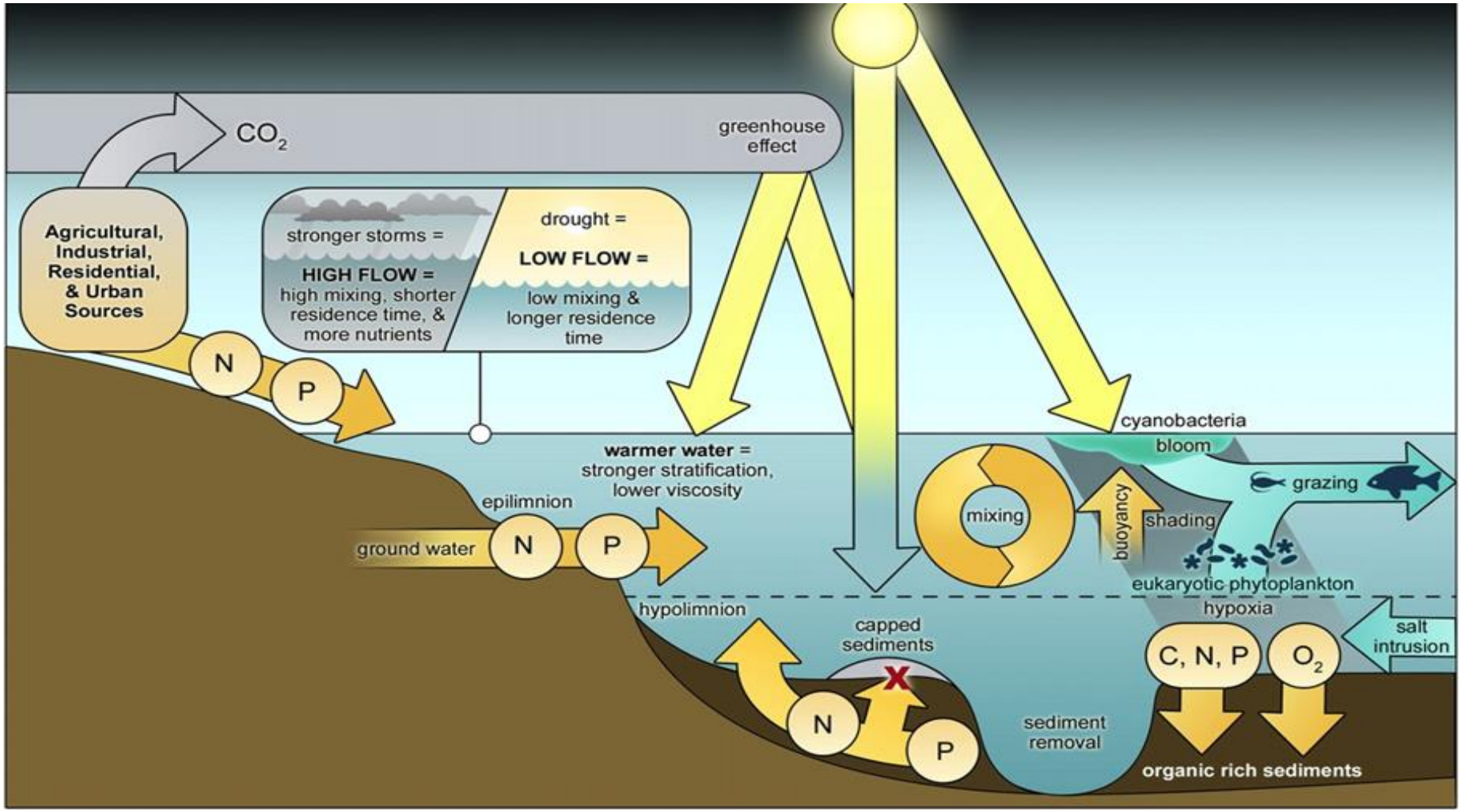


Figure 1.3 Schematic of the environmental processes that control cyanobacterial blooms, including anthropogenic and climate change impacts on aquatic systems. Licence provided by Elsevier and Copyright Clearance Center, number 3887730642248.

Nevertheless, the idea that nitrogen is a co-limiting nutrient is still largely debated for freshwater systems (Durand *et al.*, 2011). Nitrogen is considered a co-limiting nutrient in eutrophic freshwaters during the summer when the earlier algal blooms have exhausted the dissolved N in the water column, and delivery from diffuse catchment sources is at its minimum (Durand *et al.*, 2011). However, it is argued that, because many cyanobacteria species are diazothropic, nitrogen in freshwater ecosystems is not a limiting factor (Paerl and Paul 2012). Indeed cyanobacteria blooms very frequently consist of dominant non-N_2-fixing cyanobacteria species, such as *Microcystis* spp. accompanied by much less abundant N_2-fixing cyanobacteria species such as *Aphanizomenon* spp. or *Anabaena* spp. Moreover, the average increase in surface water temperatures associated with climate change has an important role in the metabolism and internal recycling of phosphorus and nitrogen (Carey *et al.*, 2012).

1.4 Management of phytoplankton blooms

Restoration refers to the management activities that aim to return freshwater systems to a previous balanced ecological state by applying remediation measures. Remediation in surface waters includes measures applied to control a source of pollution. Phosphorus reduction has been the main driver for lakes and reservoir restoration programmes to meet the requirements of the EC Water Framework Directive (WFD; Directive 2000/60/EC) (Phillips *et al.*, 2008; May *et al.*, 2009, Carvahlo *et al.*, 2013). A number of in-lake remediation techniques have been applied over the past five decades to manage cyanobacteria blooms and these measures include physical, chemical and bio-ecological techniques as illustrated in the following examples.

1.4.1 Physical techniques

Physical techniques such as artificial mixing systems involve bubble-plume systems for using air or oxygen. With these systems the advantage that the buoyancy of cyanobacteria

cells have over non-buoyant or non-motile phytoplankton to access optimum light conditions is reduced, thus affecting the amount of photosynthetic carbon fixation and subsequently reducing growth (Huisman *et al.,* 2004 Visser *et al.,* 1996, 2016). Air-based systems such as bubble-plume curtains are used in Europe, for example, in lake Nieuwe-Mere in the Netherlands (Visser *et al.,* 2016); and surface reservoirs in the South-West of England, UK (Castro-Castellon, 2013). This method is effective as long as the velocity of the bubbles is faster than the cells reaching the surface to keep the cells always entrained in the water column. Thermal stratification is lost, but temperature in the hypolimnion is increased (Visser *et al.,* 2016). Jetting systems are mainly used for mixing purposes leading to disrupt the thermal stratification and increase oxygenation in the water body (Castro-Castellon, 2013). Jetting systems are installed in Farmoor reservoirs but were not operational during the period of the study. Oxygen-based systems are used in the USA, for example, in Spring Hollow reservoir. The oxygenation of the sediment-water interface prevents the release of phosphorus and maintains the phytoplankton cells entrained in the water column (Gantzer *et al.,* 2009a). However, due to the increase in oxygen uptake some metabolic processes such as denitrification are accelerated (Gantzer *et al.,* 2009b; Bryant *et al.,* 2011a).

Another physical technique is ultrasound that relies on collapsing the cyanobacteria gas vesicles or damaging the integrity of their photosynthetic component and making them more susceptible to cavitation. The assumption is that cyanobacteria gas vacuoles collapse as a result of the ultrasound resonance (Rajasekhar *et al.,* 2012). However, the diameter of the gas vacuoles (40-60 nm) and the length (~600 nm) (Walsby, 1994 Dunton and Walsby, 2005) will collapse when ultrasound is used at high intensity (≥800 kHz) in the laboratory in small volumes, killing cyanobacteria. At lower intensities has no lethal effect with some filament breakage and cell de-clumping (Lürling *et al*., 2016b). It is rarely used in surface water remediation, except for research or trial purposes. Trials carried out in the field have shown no effects on cyanobacteria when compared to controls (Lürling *et al*., 2016b), but also

intracellular cyanotoxins might be released, particularly for the susceptible filamentous cyanobacteria species (Rajasekhar *et al.,* 2012). Although sonication could degrade cyanotoxins (640 kHz for 2-5 min), it is only needed for 10 seconds at frequencies as low as 19-20 kHz to cause 90% zooplankton (>100 µm) mortality (Rajasekhar *et al.*, 2012; Purcell *et al.,* 2013; Lürling *et al*., 2016b).

1.4.2 Chemical techniques

Chemical measures entail the addition of metals such as ferric and aluminium salts that act as flocculants and precipitate cyanobacterial blooms as well as binding P (Cooke *et al.,* 2005; Jancula and Marsalek, 2011; Lürling *et al.,* 2016b). Copper is an effective short term algicide, but is now banned from many countries (including UK) because of its toxicity to zooplankton (Hannson *et al.,* 1994; Matthijs *et al*., 2016) as well as resistance to the algicide by the cyanobacteria, *Microcystis* spp. (Garcia-Vilada *et al.,* 2004).

Natural compounds (known as allelochemicals) extracted and synthesized from plants are also considered in the management of cyanobacteria blooms. Allelopathy is defined as the inhibitory (suppressant) or stimulatory effect produced by one species on another species mediated by the release of secondary metabolites, named allelochemicals, that are not essential for the survival or development of the organism that produces them (Gross, 2003). Bundles of barley straw have been used in surface waters for algal control in the UK since 1980s, and Welch *et al*. (1990) used barley bundles in the Chesterfield canal against *Chladophora* spp. (a green algae). Barrett *et al*. (1999) used 50 g m^{-3} in a surface water reservoir in Scotland, although results were not immediate, successful cyanobacteria control was achieved in the long term (up to three seasons later). The activity of barley straw seems to be algastatic rather than cyanostatic or cyanocidal, with no deleterious effect reported on other aquatic life (Ferrier *et al.,* 2005, Iredale *et al*., 2012; Matthijs *et al*., 2016).

Bioassays of allelochemicals such as indolic alkaloids from extracts of *Phalaris arundinacea* (canary grass) and phenolic compounds from decaying *Hordeum vulgare* (barley straw) have been tested against cyanobacteria cultures of *Anabaena* spp., *Microcystis* spp and micro-algae (*Chlorella* spp. and *Scenedesmus* spp.). The mode of action of indolic alkaloids on these species is by blocking the transfer of electrons in the photosystem II in the cyanobacteria cells (Li *et al.,* 2005; Hong and Hu, 2007; Murray *et al.,* 2010). However, the use of extracted allelochemicals for inhibiting algal growth are limited to laboratory experiments (Jancula and Marsalek, 2011; Huang *et al*., 2016).

The use of herbicides such as diuron is effective for managing blooms at very low concentrations (1-5 µg L^{-1}) and used in aquaculture at >100 µg L^{-1} concentrations but these herbicides are very toxic for other aquatic organisms (Jancula and Marsalek, 2011; Matthijs *et al*., 2016). Herbicides cannot be used in surface reservoirs, and in the UK their presence in drinking water is regulated by legislation from the Drinking Water Inspectorate at maximum levels of 100 ng L^{-1} for combined pesticides (DWI, 2016).

Geo-engineering refers to activities that interfere with biogeochemical processes using materials aimed at controlling eutrophication or cyanobacterial blooms by inactivating P, such as Phoslock™, a lanthanum-bentonite clay (Spears *et al.,* 2013) or Sinobent®, an iron, magnesium, calcium and nitrate modified bentonite (Lürling *et al.,* 2016a). Lanthanum-bentonite clay prevents nutrient release from sediments by binding phosphorus, particularly soluble reactive phosphorus, to the metal-clay (Spears *et al*. 2013; Lürling *et al.,* 2013b). The addition of nitrate to the modified bentonite Sinobent® can increase the redox potential in the sediment and thus lower the internal P loading (Goldyn *et al.,* 2014; Lürling *et al.,* 2016a).

1.4.3 Bio-ecological measures

Bio-ecological measures include barley bundles and biomanipulation. Barley hay bundles inhibit phytoplankton by allelopathic mechanisms, where phytoplankton cells are

damaged by the release of phenolic compounds which is mediated by microbial biodegradation (Murray *et al.,* 2010; Jancula and Marsalek, 2011). Biomanipulation is a management tool which intends to restore a freshwater body to a "clear water state" from a "turbid water state" by lessening phytoplankton biomass and/or nutrient enrichment by manipulating submerged vegetation and fish population (Phillips *et al.,* 2005; Sondergaard *et al.,* 2007; Jeppensen *et al.,* 2007).

The concept of biomanipulation is derived from the trophic cascade hypothesis (top-down: bottom-up model) and the holistic food web model (Hansson *et al.,* 1999). The trophic cascade hypothesis states that nutrients set the potential productivity of freshwater systems and that deviation from this potential are due to food web interactions (Carpenter *et al.,* 1985). Bottom-up mechanisms refer to sediment macronutrient reduction and top-down mechanisms refer to balancing the food web by increasing zooplankton communities for grazing on phytoplankton. Zooplankton are favoured by promoting vegetation cover or manipulating fish populations (Phillips *et al.,* 2005; Jeppensen *et al.,* 2007; Reynolds *et al.,* 2012; Fan *et al.,* 2013; Lürling and De Senerpont Domis, 2013). However, Schou *et al.,* (2009) found contradictory results when testing the hypothesis that macrophyte growth provided refuge for zooplankton against fish fry in two (a clear and a turbid) shallow lakes in Denmark. In their research they used a system of submerged cages with upright plastic plants imitating Ivy. The plastic plants were inside artificial plant beds (APB) and were tested in a clear and a turbid lake during the same period of time. They found there were diel migratory zooplankton patterns and species behaviour differences in the two lakes, where zooplankton sought nocturnal refuge, but the fish densities in the APB were twice as high in the turbid lake than in the clear lake.

Vegetation cover of submerged macrophytes is required for the long-term stability of clear water conditions in shallow lakes and reservoirs and should be part of any remediation program (Hilt *et al.,* 2006; Lauridsen *et al.,* 2015). Macrophytes are defined as the vast group

of emergent, submerged and floating plants which include a number of taxonomic vascular families (e.g. Cyperaceae) and macro-algae (e.g. Charophyceae). It is believed that one of the contributions by macrophytes to the clear-state of freshwaters is mediated by the release of allelochemicals which inhibit phytoplankton growth (Phillips *et al.,* 1978; Hilt and Gross, 2008). Other contributions are resource competition (nutrients and light) and the provision for zooplankton refuge (Phillips *et al*., 2016). Therefore, the inhibition of phytoplankton in the presence of macrophyte is a subject that is much debated because some authors believe the separation between resource competition and chemical inhibition might not be possible (Inderjit and Moral, 1997). Another major issue is that an understanding of the mode of action of these inhibitor mechanisms (light, nutrients or allelochemicals) in natural situations is difficult to achieve (Hilt *et al.,* 2006; Gross *et al.,* 2007; Hilt and Gross, 2008). However, in nature an increased macrophyte biomass excludes an increased phytoplankton biomass and *vice versa* (Phillips *et al*., 2016).

Constructed wetlands (CWs) and floating treatment wetlands (FTWs) are ecologically engineered systems, designed using ecological principles (i.e. bottom-up mechanisms) and applied to remediating the deleterious effects caused by anthropogenic activities (Mitsch, 2012). Plants are supported in an artificial structure where the roots are embedded in a substrate (sand, soil or gravel) or artificial biological matrix (BioHaven®) in an enriched media (e.g. peat soil) and the direction of the inflowing water can be horizontal or vertical, surface or subsurface (Kadlec, 2009; Chen, 2011). These systems have been mainly used to treat tertiary sewage effluent, stormwater and domestic grey waters (Vymazal, 2013a); less frequently for industrial waters (e.g. sugar, dairy, meat and winery) (Vymazal, 2014), and a few designs have been used to treat surface waters, mostly in rivers to prevent eutrophication (Ozkundakci *et al*., 2010) or to remediate eutrophication (Tanner and Headley, 2011; Zhao *et al*., 2012). The performance of these planted systems when compared to unplanted systems has generally resulted in a much higher uptake of N or forms of N (e.g. NH_3), P and total

suspended solids. However, there are differences in performance depending on the plant species used (Vymazal, 2013b). Removal processes of N and P load from the inflow are generally carried out by microorganisms. The success of removal processes varies depending on the type of substrate (soil or biofilm carriers) that is used to which newly formed biofilm communities will adhere but also on plant nutrient uptake (Ballantine and Tanner, 2010; Zhu *et al.,* 2011; Wang *et al.,* 2012). Ozkundakci *et al.,* (2010) had partial success using a combination of measures to reduce P levels in surface water in a river in New Zealand. Constructed wetlands (CW) were used to manage non-point source pollution from the catchment in combination with improved farming practices and chemical application for internal loading control. However, despite the implementation of this measure aiming to remove P, the role of the CW was not evaluated no the impact of changing farming practices in the catchment. Zhao *et al.* (2012) used nine floating plant species with a biofilm enriched matrix in a FTW. Although they claimed that removal of nitrogen, phosphorus, suspended solids and chlorophyll-*a* was better during the summer-autumn than in the spring-winter season, their study was carried out in a subtropical monsoon region, where there is no change of seasons, during a period of five months (June - November 2008).

Despite the range of innovative practices used to control and manage eutrophication and the establishment of cyanobacterial blooms, the success of remediation and restoration measures is limited. An example of successful restoration is the work carried out in the Norfolk Broads, UK, based on biomanipulation concepts (Moss *et al.,* 1996). The limited success of controlling phytoplankton blooms is a result of a) inappropriate selection of the restoration measure or misinterpretation of the results, based on weak scientific foundations regarding the problem and the freshwater system to restore (Hilt *et al.,* 2006); b) water authority managers' attitudes to seeking immediate solutions to the problem which impede a science based evaluation of the applied restoration measure (Reynolds *et al.,* 2012; Spears *et*

al., 2015; Lürling *et al.*, 2016b); c) resilience of the system to restoration (Gross *et al*., 2007; Spears *et al*., 2015).

A deeper understanding of the underlying mechanisms of these restoration measures continues to be needed to maximise the opportunities of success in the long term, more so, in the light of uncertainties and risk due to climate change that is exacerbating cyanobacterial blooms. Nevertheless, bio-ecological measures are the most popular because of their lower impact on the environment compared to physical or chemical measures (Moss, 2007; Lu *et al.*, 2014). Although this research does not attempt to restore a surface water reservoir using the Living-Filter, one aspect of the research is to focus on testing whether phytoplankton can be removed from the water flowing into the WTW. That said, physicochemical and biological mechanisms from the trophic cascade hypothesis and biomanipulation concepts are expected to operate in the Living-Filter.

1.5 Ecological filtration

1.5.1 Floating treatment wetlands in surface waters

Floating treatment wetlands systems (FTW) are a hybrid between natural floating wetlands and CWs. Natural floating wetlands are a marsh of vascular vegetation on a mat of rich organic matter (roots, pit and detritus) that floats over a layer of water (De Stefani *et al.*, 2011). Floating treatment wetlands are characterized by emergent macrophytes growing in a nutrient enriched buoyant mat (e.g. soil, peat or biofilm as Biohaven®) that has roots reaching beneath the water for the uptake of nutrients, biofilm attachment and entrapment of suspended solids. The shade provided by the structure may also benefit algal management (Tanner and Headley, 2011). Because water level fluctuations do not affect their performance, FTWs can be used in running waters (Revitt *et al*., 2001; De Stefani *et al.*, 2011; Zhao *et al*., 2012). The removal efficiency of pollutants depends primarily on the hydraulic and hydrological conditions, as well as the pollutant loading and vegetation type (Kadlec, 2009; Chen, 2011).

The first FTW installed in a surface reservoir was in Upper Shustoke, UK, in 2001 for P removal and cyanobacteria bloom management. The reed-bed (*Phragmites australis*) structure (490 m^2) was installed in a shallow freshwater reservoir with (92 x $10^3 m^3$) volume capacity and the FTW almost cover the width of the reservoir. The reeds were fully grown by 2003. The most notable result was the reduction in orthophosphate and limited algal growth (Garbett, 2005). However, due to the fact that barley straw was used at the same time, the results cannot be attributed to the FTW alone (Li *et al.*, 2010). There were a few issues that hampered the success of the installation. First, the monitoring started three years after installation, because the plants were too small and they needed time to grow and develop. Secondly, the sampling points were too far away from the structure, and thirdly, the system was monospecific. Despite this, Garbett's (2005) installation opened new possibilities for using FTW in surface reservoirs for WTWs, and provided impetus for the Living-Filter tested at Farmoor II reservoir.

Vincens (2007) used a FTW mesocosm originally developed in raw water tanks at Kempton Park, a Thames Water site, and then carried out tests at a waste water lagoon in Wide Bay Water Corporation (WBWC), Queensland, Australia. The aim was to investigate the potential of reed-beds for removing algae from waste water. The mesocosm was a raft of 4 m^2 with the reeds growing hydroponically in a coconut matrix. The study focussed on the ecology of the roots of the reeds (*Phragmites australis* and *Phalaris arundinacea*). The research also described a number of attached organisms (peryphyton) to the roots, and concluded that potentially the best reed to use for the purpose of removing algae was *Phalaris* because its morphology was denser than *Phragmites*. Although this system was used in the treatment of waste water, the species information emerging from the research could have influenced the selection of plants by Thames Water for the Living-Filter at Farmoor II reservoir.

A floating reed-bed installation the size of a football pitch was deployed in New Zealand in shallow Lake Rotuehu (Headley and Tanner, 2012), with the aim to trap suspended solids from agricultural and livestock run-off. Most of these FTW systems are installed in shallow freshwater systems and sewage effluent ponds, and they also have in common a substrate of enriched media (Ballantine and Tanner, 2010; Tanner and Headley, 2011; Wang, 2012).

There are two mechanisms that have been demonstrated to operate in FTWs in eutrophic freshwater systems; one is rhizofiltration, with the uptake of nutrients (particularly of nitrogen and phosphorus) in the plant biomass. The other is the metabolic activity of the nutrients carried out by the biofilm adhered to the plants' roots (Li *et al*., 2010). However, some aspects are still understudied, for example the role of algae (phytoplankton) which is studied only as another organism of the microbial community (biofilms) that remove or metabolize nutrients (Chen, 2011). No other research on FTW systems has attempted to examine the removal of phytoplankton from inflowing water to a WTW.

1.5.2 The Living-Filter: a floating treatment wetland process for pre-treatment of potable water.

The Living-Filter is the second FTW installed in a surface reservoir in the UK, and therefore applications of FTWs in surface reservoirs are scarce (Garbett, 2005; Li *et al.*, 2010; Wang, 2012). This is the first attempt at studying the functionality of a FTW for the reduction or removal of phytoplankton biomass at a reservoir WTW intake. Moreover, there are a number of unique engineering features in the design that make it the first of its kind. The Living-Filter novel design features are:

a) The distribution of the three plant species that cover the 178 m^2 of the Living-Filter's floating plant-bed, as shown in Figure 1.3-A. The plant-bed is a modular galvanized caged structure, with a platform area of 210 m^2 (21 m Width x 10m Length), of which 32 m^2 is

wooden decking to access the sampling sites. The structure is 20 m away from the WTW intake (Figure 1.3-B).

b) Two lateral submerged impermeable curtains are parallel and attached to the wall of the abstraction tower. The curtains run downwards from the surface water level to the sediment bed (10 m H x 65 m W) (Figure 1.3-A and 1.3-B). The curtains are folded and anchored with chains to create a fixed barrier, thus preventing mixing from water that has not flowed through the roots of the plant-bed.

c) Two baffles between the plant-bed and the WTW intake. The first runs two metres downwards from the surface; the second baffle runs from two metres below the water surface to the sediment bed (Figure 1.3-B).

The water flows into the Living-Filter at the plant-bed, passes through the roots and the non-woven plastic matrix, and then passes over the second baffle and under the first baffle to arrive at the WTW intake (Figure 1.3-A and 1.3-B). The Living-Filter can be classified as a horizontal, subsurface flow, floating emergent treatment wetland (HSSF-FTW) (Kadlec, 2009; Chen, 2011). A key feature of the Living-Filter, shared with other types of HSSF-FTW, is that the flow and treatment occur primarily within the root-zone of the plants in a hydroponic context. However, the Living-Filter differs from other FTW in that there is no gravel, soil, granular media or nutrient enriched substrate (Headley and Tanner, 2012).

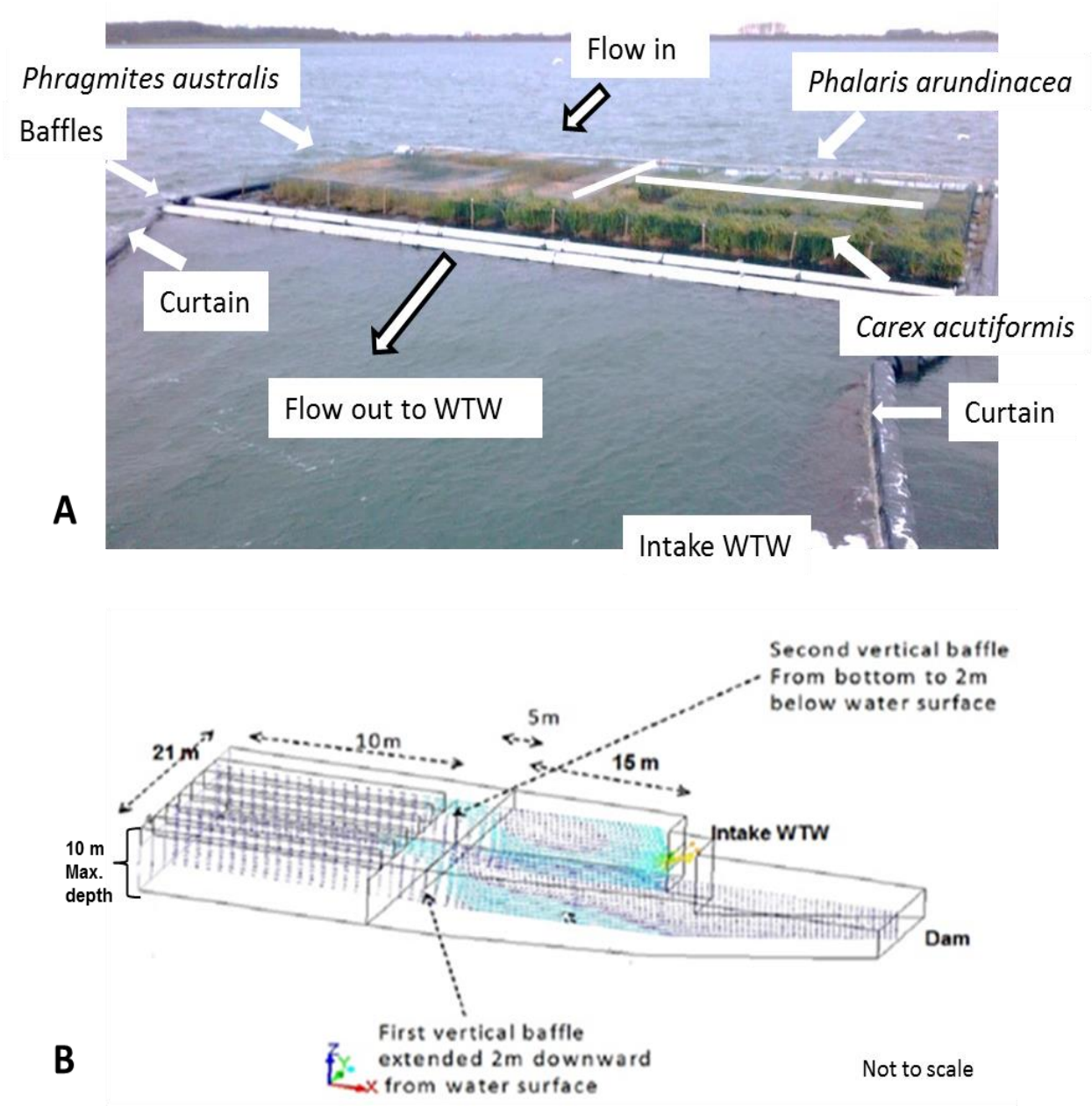


Figure 1.4 A- Living-Filter six months after installation in winter 2012-2013 showing: the plant-bed and plant distribution, direction of flow, and the position of the curtains and baffles. B- Schematic side view of the Living-Filter with dimensions of the plant-bed (21 m W x 10 m L), the distance between baffles (5 m) and the distance between the 1st baffle to the WTW intake (15 m). (Not to scale).

1.5.3 Biofilter treatment processes in water treatment works

Biofilter treatment processes are a well-established process in WTWs, where biofiltration takes place on granular media, such as sand, silica, anthracite or granular carbon,

used as single media or in combination for slow sand filter (SSF), rapid gravity filter (RGF) and granular activated carbon (GAC) processes (Magic-Knezev and Van Der Joit, 2004). Filtration with SSF is generally used in the initial treatment of the raw water, the water is filtered, either directly or after a period of settlement at slow flow rates (0.2 – 0.6 m h^{-1}), onto the granular filter media. Raw water treated with acid, aluminium or ferric salts or ozone can be filtered onto SSF, RGF and GAC (Patterson and Jefferson, 2006). Phytoplankton removal in the SSF is mediated by the organisms from the living top layer of the SSF, the so-called "Schmudzdeke", rich in organisms such as protists, phytoplankton, algae, invertebrates and small fish, and because of this, SSFs are considered by some as an ecological filter (Nakamoto, 2014). Furthermore, this latter author proposes changing the name from SSF to ecological purification system, because he considers that there is no mechanical filtration only an ecological process in line with a food chain or food web model from the trophic cascade hypothesis of Carpenter *et al.,* (1985). The removal of pollutants and contaminants is mediated by a biodegration mechanism carried out by the microorganisms constituting the biofilm on the granular media (Magic-Knezev and Van Der Joit, 2004; Simpson, 2008). Biofilms in RGF and GAC systems benefit from backwashing without chlorine, where the GAC becomes biologically active carbon (BAC); it thus outperforms the inert media because it simultaneously biodegrades and adsorbs organics (Persson *et al.,* 2005).

1.5.4 Biofilters with filamentous media: biofiltration and rhizofiltration

Biofilters based on plant rhizofiltration with a high-surface-area and operating at the bench-scale have been hydroponically developed and limited to the absorption of nutrient or metal ions from waste waters (Dushenkov *et al.*, 1995; Salt *et al*., 1995; Raskin and Enley, 2000). Marchand *et al.* (2014) combined plants and biofilms for metallic ion removal using planted and unplanted Bio-rack™ to increase the uptake rate of copper. Kurzbaum *et al.* (2012) separated the role of the roots and the associated biofilm for the removal of pollutants

in rhizofiltration processes in a hydroponic system, whilst Weiss *et al.* (2014) used recirculating metal-rich flows to investigate the effects of water flux through the roots of hydroponically grown plants on metallic ion removal.

1.6 Rationale for the study

Research into the removal processes of phytoplankton cells or suspended particles in a FTW is little reported. Perhaps the complexity of the subject, the need to work with whole plants in the field (Doran, 2009), and the difficulties imposed by field-scale research in limnological environments hampers the elucidation of conclusive cause and effect relationships. That said, the observations from field data provide valuable ecological information to generate hypotheses to be tested at the bench-scale (Benton *et al.,* 2007).

Initially, testing of the field-scale Living-Filter as a biofilter system for phytoplankton removal was carried out during the summer of 2013 (July to October). A number of questions were generated from this macroscale study in order to identify underlying physico-chemical and biological mechanisms that could remove phytoplankton biomass. *Microcystis aeruginosa* was used as a model organism in laboratory-scale continuous flow and batch experiments using biofilters with the hydroponically supported roots of *Phalaris arundinacea*, one of the three species constituting the plant-bed of the Living-Filter (*Phragmites australis*, *Phalaris arundinacea* and *Carex acutiformis*). The mesoscale research generated new questions that in order to be answered needed the development of a trace-organic chemistry method. Thus the microscale relations between the *Phalaris arundinacea* roots (biofilters) and the rhizosphere, as well as between the rhizosphere and *Microcystis aeruginosa* cells, were investigated together and independently.

The context for the study of the Living-Filter is illustrated in Figure 1.4.

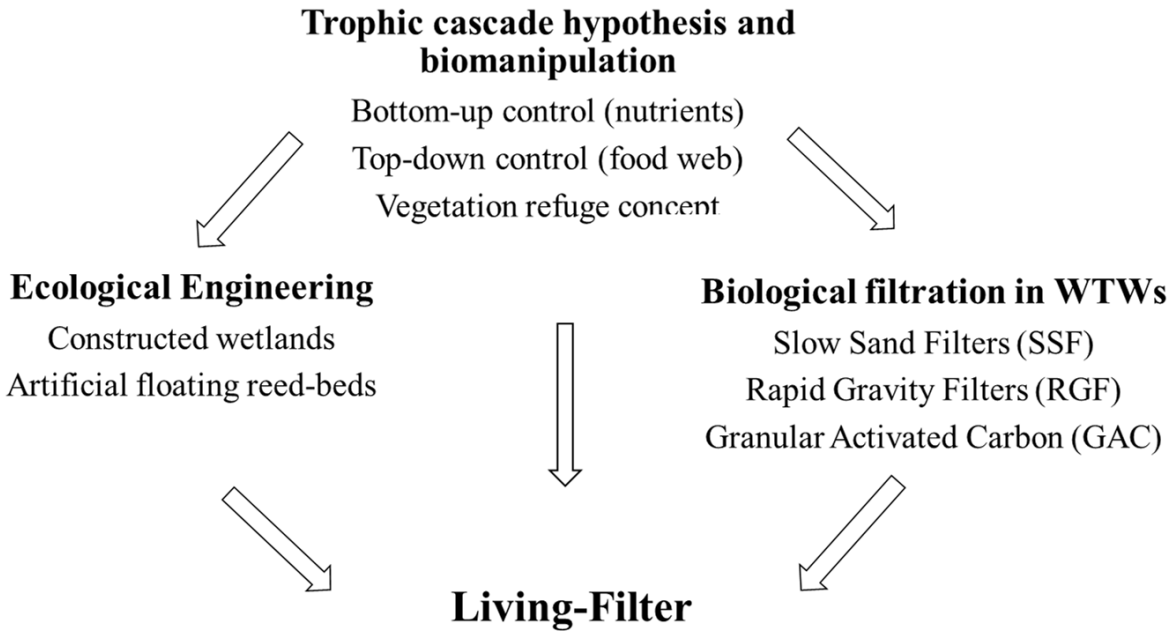


Figure 1.5 Schematic illustration of the conceptual framework of the Living-Filter.

1.7 Aims of the current research:

1. To explore if the field-scale filamentous biofilter known as the "Living-Filter" can be used as a pre-treatment process for the reduction or removal of phytoplankton loading onto a potable water treatment works.

2. To investigate the underlying physico-chemical and biological mechanisms of the biofilter process in laboratory scale filamentous biofilters and synthetic filters for the removal of phytoplankton in continuous flow and batch experiments.

3. To make recommendations for the specification of future field-scale filamentous biofilter pre-treatment processes designed to maximise the advantages of eco-technology for phytoplankton removal.

To meet the aims of the research two overarching questions were posed:

1. Can the Living-Filter be used as an in-reservoir pre-treatment filtration process for the reduction of phytoplankton biomass onto potable WTWs?
2. Can the underlying physico-chemical and biological mechanisms that might operate in biofilters be identified in macro-, meso- and microscale studies?

1.8 Thesis structure

This thesis is divided into seven chapters in order to address the research questions and to meet the aims. **Chapter 2** is descriptive and gathers information from environmental surveys on the field-scale biofilter, the "Living-Filter", as a pre-treatment process for the removal of phytoplankton and tests how effective the system under the given conditions.

Chapter 3 is a bench-scale biofilter experiment using self-grown *Phalaris arundinacea* roots for investigating physical mechanisms, such as the role of flow and root density of *Microcystis aeruginosa* removal, a species of phytoplankton, to test the concept of the "Living-Filter".

Chapter 4 responds to the observations and questions generated from the results in Chapter 3 by suggesting that chemical mechanisms (nutrient competition or allelochemicals) were also involved in the removal of *Microcystis aeruginosa* by the roots of the biofilter plants. The determination of allelochemicals in aqueous root extracts and exudate was paramount, and for this purpose a specific and quantitative analytical trace organic method was developed and optimized. It was applied to the experiments in chapters 5 and 6.

Chapter 5 presents the findings of allelochemical presence in the roots of the Living-Filter plants and in biofilters. Laboratory experimental work was carried out to test the response of released allelochemicals to environmental stressors.

Chapter 6 determines the minimum concentration of commercial allelochemicals necessary for inhibitory effects, and tests the response of *Microcystis aeruginosa* to root exudates over time.

Chapter 7 presents recommendations for the future field-scale design of floating biofilters based on the results obtained in the foregoing experimental chapters and compared to similar systems described in the literature.

Chapter 8 presents a synthesis of the thesis, the conclusions from this research and future research.

Chapter 2. Comparison of physicochemical and biological variables upstream and downstream of the Living-Filter

This chapter focuses on the macroscale study of the Living-Filter which comprises surveys carried out over 17 weeks.

2.1 Introduction

Thames Water Utilities Ltd., UK (TW) identified a seasonal (July - November) increase in the coagulant dose demand (poly-aluminium chloride 10%) in the water treatment works (WTW) associated with an increase in chlorophyll-*a* (as a measure of phytoplankton biomass) levels in Farmoor II reservoir, Oxfordshire, UK. The coagulant is added during the coagulation process to neutralize the particles (e.g. phytoplankton cells). A negative charge facilitates the formation of micro-flocs (aggregation of neutral particles) which during flocculation form stable flocs that are removed during the dissolved air flotation process.

The physical filtration process expected to take place in the Living-Filter could be mediated by three mechanisms: screening or straining, sedimentation and adsorption. In screening the larger particles will be retained at the front of the filter because they are separated on the surface of the media due to their large size. Sedimentation is the process whereby particles are retained by deposition in the porous interspace and adsorption is mediated by forces and charges on the particles. The particles, however, might be resuspended if shear forces (e.g. strong flow velocities) remove them from the attached surfaces. Tanner and Headley (2011) showed how the removal of copper ions, phosphorus and fine suspended particles (<0.4 μm) from synthetic storm water occurred via the biofilm which adhered to the roots of a floating wetland mesocosm in a batch experiment. They also showed that direct rhizofiltration (i.e. direct uptake of macro and micronutrients by the roots) of copper and P could have contributed to the removal processes. Scholz (2006) pointed out that various factors affect the biofiltration efficiency in ecological engineering designs, such as: organic

biomass, organic loading rate, hydraulic loading rate, depth of media, packing density of the media, media size and the total surface area of the media available.

The Living-Filter main characteristics that make it unique are: the plant-bed combined with curtains and baffles. The plant-bed is a floating emergent treatment wetland (FTW) growing in a hydroponic context and in absence of other enriched nutrient media apart from the natural nutrient from the reservoir and a hollow supporting plastic fabric. The submersed curtains and baffles create a waterway so that the particles in the inflow are removed when passing through the roots prior to the WTW. There is no loss of momentum because of the low drag of the inflow passing through the high porosity of the roots and the plastic fabric, as pointed out by Aberle and Jarvela (2013) in a high porosity media. However, the increase in flow velocity would force the roots to bend due to the natural flexibility of the plant species roots potentially decreasing their role as a filtration media (Aberle and Jarvela, 2013; Shi *et al*., 2013).

The Living-Filter uses macrophytes that belong to two commonly selected families of plants: Poaceae and Cyperaceae (Vymazal, 2012). *Phragmites australis* (Cav.) Trin. ex Steud (common reed) and *Phalaris arundinaceae* L. (reed canary grass) are from the Poacea family; and *Carex acutiformis* Ehrh. (lesser pond sedge) belongs to the Cyperaceae family (hereafter *Phragmites*, *Phalaris* and *Carex).* The chosen species have been used in other FTWs, and a key characteristic is that are very adaptive to harsh environmental conditions (Vymazal and Krőpfelová, 2005). Other common species used are *Typha latifolia* (cattail), *Juncus effusus* (soft rush), *Iris pseudacorus* (yellow flag), other *Carex* spp., *Glyceria maxima* (sweet mannagrass), *Canna indica* (Indian shot, canna), and *Chrysopogon zizanioides* (vetiver grass), but a broad range of plants are suitable for these purposes (Chen, 2011).

Phragmites seasonally regulates the mobilization of nutrients with two periods of rhizome biomass loss. One is between April-May when carbohydrates are mobilized to form the new shoots. The other is during winter with 30% biomass loss due to rhizome mortality

(Granrli *et al*., 1992). Nutrient uptake of nitrogen (N) is seasonal, decreasing during spring/early summer and increasing during summer/autumn mobilization to the roots, but no similar pattern is observed for phosphorus (P) or potassium (K). This suggests that the uptake of P and K is directly from the substrate (Granrli *et al*., 1992; Lawnizack *et al*., 2010). Comparative studies between *Phragmites* and *Carex* show that *Phragmites* develops a greater biomass above ground but *Carex* has a greater P uptake (Lawnizack *et al*., 2010).

Phalaris is a perennial grass, highly adaptable and competitive. The growing season starts as soon as the daily mean temperature is above 5°C and the inflorescence forms at the end of June/beginning of July (Sahramaa and Jauhiainen, 2003). The species has a percentage of aerenchyma and gas spaces in the roots that make it well adapted to waterlogged conditions (Miller and Zedler, 2003). *Phalaris* is used in phytoremediation (removal of nutrients and pollutants) and is favoured due to its multiple ways of reproduction (seeds, tillers and rhizomes) (Lavergne and Molofsky, 2004). *Phalaris* is used to restore abandoned mine pits for carbon extraction because their capacity to store carbon is greater than other invasive plants (Zhou *et al*., 2011).

Carex is a highly productive species, generally found growing in eutrophic fens, and the uptake of N rates in this species is greater than in other *Carex* species (Aerts and Caluwe, 1997). The aerenchyma adaptation found in *Phalaris* is similarly seen in *Carex* (Granrli and Weisner, 1992). *Carex* nutrient cycling is related to its high productivity with very low decomposition rates, contrary to what is expected for a species with high productivity in eutrophic environments (Van Der Werf *et al*., 1988).

Promoting vegetation coverage to increase zooplankton populations with the assumption that the increase in zooplankton will intensify grazing on phytoplankton is one of the objectives of biomanipulation (Muylaert *et al*., 2010). Another objective is to increase zooplankton by reducing zooplanktivory (fish fry), known as top-down mechanisms in biomanipulation (Ger *et al*., 2014; 2016). Farmoor II reservoir lacks natural vegetation, and

therefore it is expected that the roots of the plant-bed of the Living-Filter will provide some refuge to the existing zooplankton community, consequently contributing towards the reduction of phytoplankton biomass at the WTW intake. The roots and synthetic fabric of the plant-bed could also play a role in reducing phytoplankton biomass through the physical filtration processes of entrapment and deposition of suspended particles (i.e. phytoplankton cells), as well as through metabolic bio-chemical processes from the plants and biofilm attached to the roots and synthetic fabric.

The retention of suspended particles by biofilms or adhesion to biofilms is expected to introduce changes in the hydrodynamics of the Living-Filter, and the adsorption of chemicals influences biochemical and energy flux processes in the system (Faulwetter *et al.,* 2009; Stout and Nüsslein, 2010; Battin *et al.,* 2016). The production of polysaccharide exudates (EPS) by phytoplankton protects them against harmful environmental changes (e.g. desiccation) (Potts and Whitton, 2005; Pereira *et al.,* 2009). It could also make them inedible to zooplankton grazers and this is likely to be the result of co-evolutionary dynamics (Ger *et al.*, 2016). The EPS can be divided into released soluble polysaccharide (RPS) to the surrounding media, and EPS associated with the cells that form the sheaths, capsules or mucilage surrounding the cells (Pereira *et al.,* 2009; Kehr and Dittman, 2015). Cyanobacterial EPS varies in density and composition, but contains uronic acids and sulphate groups (a unique feature from Prokaryotes shared with EPS from Archaea and Eukaryotes) that give a negative charge and sticky behaviour to the overall macromolecule (Micheletti *et al.,* 2008; Di Pippo *et al*., 2013; Liu *et al*., 2014; Svan and Eriksen, 2015). These groups also facilitate the adhesion to a solid substrate, preventing them from being washed away from their natural habitat by water flow (Scott *et al.*, 1996; De Philippis *et al.*, 2005). Some of the chemical characteristics of the cyanobacterial RPS (high molecular masses and viscosity) can modify the rheological properties of water, acting as thickening agents (Jenkinson, 1986; Pereira *et al.,* 2009). *Microcystis* sp. can produce extracellular fibres of polysaccharide origin (Harel *et al*., 2012).

This may increase the bulk viscosity and reduce eddies (Jenkinson and Sun, 2010), thus intervening in the diffusion of nutrients from the cells in division, the regulation of the vertical migration of colonies and the exchange of resources within and between populations (Sukenik *et al*., 2002; Harel *et al*., 2012). Chemical removal processes are the focus of most studies on biofilm and hydrodynamic environments, whilst very few studies are on particle retention processes (Stout and Nusslein, 2010; Battin *et al.*, 2016). The production of polysaccharide fibres and the increased viscosity might increase the adhesion of cells to the biofilm and the biofilm attachment on the roots, the plastic fabric or the metal cage structure of the Living-Filter.

This chapter aims to answer the following research questions:

1) Are there physico-chemical and biological spatial changes in the water upstream and downstream of the Living-Filter?

2) Are there physico-chemical and biological spatial changes within the plant-bed itself?

3) Is there any removal of chlorophyll-*a* by the Living-Filter during the period of study?

4) Will the Living-Filter provide refuge to zooplankton?

2.2 Materials and Methods

To facilitate the surveys and monitoring of the Living-Filter, three floating walkways were built. The plant-bed structure consists of seven modules made with a galvanized steel mesh sheet labelled from A to G (Figure 2.1-A) with dimensions (1.2 m Height x 21 m Width x 10 m Length). The diameter of the mesh in the sheets is 7.6 cm Lx L. Each module (3 m W) has three cages and is separated from other modules by a baffle that runs downwards 2 m from the water surface. Each cage is 1.2 m H x 1m W x 3.3 m L and contains 10 m^2 unfolded area of plastic fabric (Figure 2.1-B). The plants grown in 10 cm thick coconut coir mats were less than a year old when first placed on top of the cage structure (Figure 2.1-B) in July 2012 with some plants tied to the structure. The mats were lost within a year, but the plants had

already rooted. The plant-covered area was estimated for each cage in order to calculate the plant coverage of the entire plant-bed structure. The initial plant coverage was 85% (179 m^2 from 210m^2), with the remaining 15% (31 m^2) taken up by wooden decking on the plant-bed.

The Living-Filter sampling sites can be seen in Figure 2.1-A. Stage 1 (S1) is the area outside the boundaries of the Living-Filter and stage 2 (S2) is within the plant-bed structure. Inside the Living-Filter enclosure is stage 3 (S3) which is immediately downstream of the plant-bed structure and stage 4 (S4) which is 10 m away from the WTW intake. Stages 1, 3 and 4 are depth sampling sites (in the water column) where samples are taken at three depths each 1, 4 or 5 (depending on the thermocline) and 8 m (Figure 2.1-C). In S2, from left to right, modules A to D have primarily *Phragmites* and modules E to G have *Phalaris* and *Carex* Hence, *Phragmites* covers 50% of the total plant-bed, Phalaris 23% and *Carex* 27% as shown in Figure 2.1-A. There is only one sampling depth (1m) for each sampling site S2 in A to G (Figure 2.1-A).

2.2.1. Sampling design and collection

To investigate changes in the physico-chemical and biological variables, 17 weekly surveys (July to October 2013) were carried out at 16 sampling sites (located in stages 1 to 4). The biological variables monitored were zooplankton (identification and biomass) and chlorophyll-*a* (a surrogate measure of phytoplankton biomass). The scope of sampling at S1, S3 and S4 was to allow investigation of the changes in water quality upstream and downstream of the plant-bed of the Living-Filter. The scope of sampling within the plant-bed (S2) was to explore differences in water quality between the sampling sites (A to G). Differences in physico-chemical and biological variables between all stages 1, 2, 3, and 4 were investigated via samples taken at 1 m depth, where the average value of the variable for the sites A to G in S2 was compared with S1, S3 and S4 at 1 m depth.

Depth samples were taken at positions based on historical stratification data that were confirmed with data taken during the current survey. Hence, 1 m corresponds to the epilimnion (0-3 m), 4 or 5 m corresponds to the metalimnion and 8-11 m corresponds to the hypolimnion. The thermocline was generally weak with temperature differences of 1°C - 1.5°C in the water column from the top (0 m) to the bottom (11 m) of the reservoir.

Dissolved oxygen (DO) and temperature (^{o}C) (T) depth profiles were taken with a Hach Intellical LDO rugged sensor from 23rd August to 14th October 2013. Samples for physico-chemical and biological analyses were collected with a Van Dorn sampler (~ 2 L capacity), between 09:00 and 13:00 hours, each survey last about two hours. Samples were pooled and transferred to a plastic container, of similar shape to a jerrycan, with volume approximately 5.5 L. The Van Dorn sampler was deployed at least three times per each depth measurement at stages 1, 3 and 4.

Samples from Stage 2 (sites A, B, C, D, E, F and G) were taken with a Phil Sampler, which is a device operated by a pole manufactured in stainless steel and unplasticized polyvinyl chloride with a removable bottle (500 ml) at the end of it (Duncan & Associates, UK). The bottle was refilled 11 times for each sampling site to fill up the 5.5 L containers. To facilitate sampling within the roots and fabric-filled cages (Figure 2-B), a square of 20 cm L was cut off from the top face of the galvanized cage and a piece of pipe (15 cm diameter x 50 – 70 cm length) was inserted to create a permanent sampling point. The samples were taken once the bottle passed through the pipe and reached the bottom (1 m depth) of the galvanized stainless steel structure. All samples were kept in shade conditions until taken for chemical and chlorophyll-*a* analysis to Spencer House, the Thames Water Utilities analytical laboratory which is a United Kingdom Accredited Service (UKAS) laboratory.

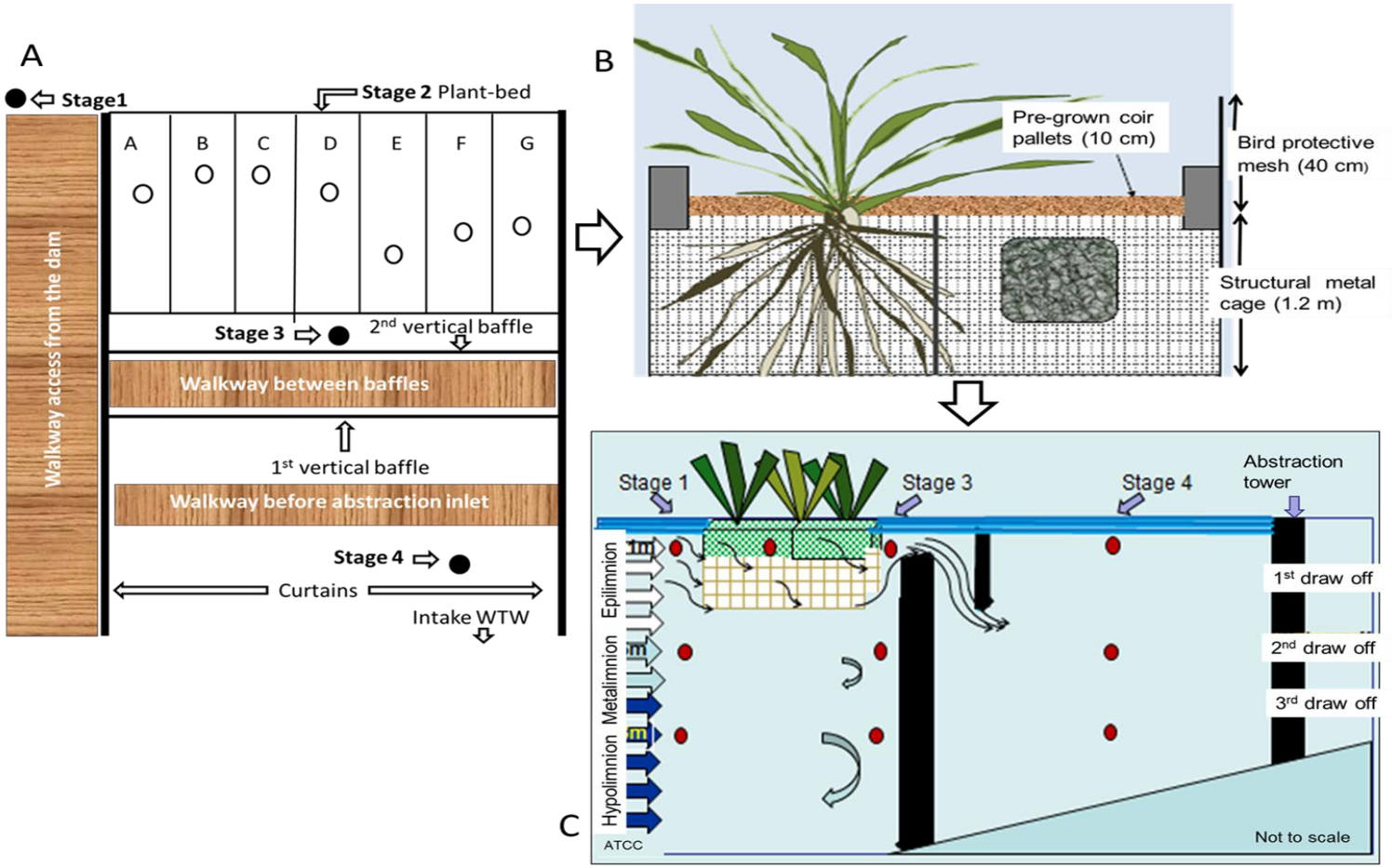


Figure 2.1 A- Schematic aerial view of the Living-Filter showing the position of the walkways, plant-bed structure, curtains, baffles and stages 1-4. Stages 1, 3 and 4 sampling sites are shown by dark circles. Stage 2 comprises seven modules (labelled A to G) and the seven sampling sites are shown by clear circles. B- Schematic cross-section of the plant's position and roots in the galvanized cage structure (1.2 m H) (AquaticEngineering, UK). C- Schematic cross-section of the floating plant-bed in relation to the baffles and depth sampling sites for stages 1, 3 and 4. (Not to scale)

2.2.2 Physico-chemical and chlorophyll-*a* variables measured

Physico-chemical and metabolic variables that are related with the quality of the water were measured *in situ* and in the laboratory. The physico-chemical variables measured *in situ* were DO and T; and laboratory analyses were for pH, chloride (Cl^{-}), suspended solids (SS) and alkalinity (Alk). Microbial activity was prevented or minimized by maintaining the samples in shade conditions during the survey and by having them refrigerated within less than three hours of collection, hence preventing changes in the pH and other variables. A number of metals and their soluble forms which are uptaken as micronutrients by phytoplankton were measured in the laboratory including: iron (Fe^{+3}), manganese (Mn^{+3}), aluminium (Al^{+3}), soluble Fe (sFe^{+2}), soluble Mn (sMn^{+2}) and soluble Al (sAl^{+2}). High concentration levels of these variables are relevant to the water supply industry, particularly the soluble forms of iron and manganese that are more difficult to remove during treatment. Nitrogen is one of the three macronutrients that supports phytoplankton productivity in freshwater. In the water supply and wastewater industry nitrogen fractions are measured as total oxidized nitrogen (TON) which is the sum of nitrate and nitrite (NO_2^{-}); and dissolved inorganic nitrogen (DIN) is measured as the sum of the concentrations of nitrate and ammonium (NH_4^{+}). Nitrate is not measured in the water supply and wastewater industry routinely. Phosphorus (P) and silica (Si) are the other two macronutrients supporting phytoplankton productivity. These macronutrients were measured as total phosphorus (TP) and soluble reactive phosphorus (SRP), and silica dioxide SiO_2^{-} is referred to as Si. A number of biological and chemical metabolic variables were measured: biological oxygen demand measured at five days (BOD5) and chemical (COD) oxygen demand, particulate organic carbon (POC), total organic carbon (TOC) and dissolved organic carbon (DOC). Chlorophyll-*a* (Chla) was used as a measure of the phytoplankton biomass. Moderately polluted surface waters have BOD5 values $\geq 3.5 \leq 5$ mg L^{-1} and COD $\geq 25 \leq 100$ mg L^{-1} (European Environment Agency, 2014). A brief description of the methods is presented in Appendix A.

2.2.4. Chlorophyll-*a* loading and removal efficiency

To investigate whether filtration physical processes for the removal of Chla took place within the Living-Filter, the Chla loading and removal efficiency was calculated. Thames Water provided the recorded daily hydrological flow data of the water to be treated at the WTW during the period of study which was used to estimate the weekly Chla loadings. The range of hydrological flows for daily water demand range was 45 x10^3 m^3d^{-1} 90 x10^3 m^3 d^{-1} (average of 65 x10^3 m^3d^{-1}).

2.2.4.1 Chlorophyll-a loading

Calculations for Chla loading on S2 were made by using Equations 2.1:

Chla loading = (g m^{-3} w^{-1}) (eq. 2.1)

where Chla loading is grams of Chla per cubic meter per unit time, $t = (t_2 - t_1)$ in this case week (w) one is subtracted from week two ($w_2 - w_1$), so that time refers to Chla g m^{-3} w, and V is the volume of the plant-bed cage (V=210 m^3).

2.2.4.2 Chlorophyll-a removal efficiency

The removal efficiency as a percentage (RE %) was calculated based on Chla concentration and the volume of the inflow water to the works, Equation (2.2), where C_i and C_f (mg L^{-1}) are the initial and final concentrations of Chla; C_i is upstream of the plant-bed and C_f is Stage 3 or Stage 4:

$$RE\% = \frac{(C_i - C_f)}{C_i} \times 100 \quad \text{(eq. 2.2)}$$

2.2.5 Biological organisms in the Living-Filter system

2.2.5.1 Plants and root development

To assess the aerial shoot and root development of *Phragmites*, *Phalaris* and *Carex*, three individuals of each plant species were randomly chosen 18 months after installation. The length of the aerial shoot from the base to the highest point and the root length at the longest distance from the root apex to the base of the aerial shoot were measured.

2.2.5.2 Periphyton qualitative observations

Qualitative observations with a Go-Pro underwater camera were made of the galvanized stainless steel cage structure of the Living-Filter plant-bed structure on only one occasion due to health and safety regulations (a minimum of two people on the walkway) and logistic circumstances in place (extending the surveys for another hour having a knock on effect on sample preparation and transport to the Spencer House laboratory in Reading). A photographic, video and a collected sample were taken from the internal face of the curtain on September 2013.

2.2.5.3 Phytoplankton identification and biomass

A subsample of 100 ml from the 5.5 L containers was transferred to dark 125 ml bottles and fixed with 1 ml Lugol's iodine 1%. The method followed for phytoplankton enumeration and identification was membrane filtration concentration, as described by the Standing Committee of Analysts (1990). This method was validated against the Uttermöhl method (1935) using an inverted microscope (x400) (laboratory Dr H. Townley, Begbroke Science Park, University of Oxford). To validate the method, aliquots of 1 ml were taken from pure cultures of *Anabaena* spp., *Aphanizomenon* spp., *Microcystis* spp., *Chlamydomonas* spp., *Chlorella* spp. *Closterium* spp., *Pediastrum* spp. and *Cyclotella* spp., obtained from Dan Fagan at the School of Biological Sciences, University of Bristol.

For fixed samples, a known volume was filtered through a pre-damp (deionized water) 47 mm Millipore nitrate cellulose membrane of 0.45 μm pore size (Millipore, UK). A vacuum pump was applied at a low pressure differential of 0.3 atm, but stopped just before the water had drained completely to avoid damaging the morphology of the cells, and was dried at 20^{o}C (in an incubator or at room temperature) for 10 minutes. The membrane was mounted on a slide with immersion oil. Cells, filaments and colonies of phytoplankton were counted in at least 30-50 fields (depending on the load) under the microscope at x400 magnification. The mean of the cell count from 10 filaments of different lengths was obtained. For some small

colonial cyanobacteria, cell counts were carried out directly (*Microcystis* spp.) and the final colony counts were the mean of five colonies. In the case of large *Microcystis* spp. colonies (≥ 200 μm), a quadrant of the colony was counted and then multiplied by four. The same method was applied for colonial Bacillaryophyta or Chlorophyta. The number of total phytoplankton observed was converted to total number of cells per ml^{-1}, Equations. 2.3 and 2.4:

$$\text{Total algae} = (CF \times /N_i)/ (N_f \times V_f) \quad \text{(eq. 2.3)}$$

$$\text{Conversion factor} = A_f/A_m \quad \text{(eq. 2.4)}$$

where (CF) is the conversion factor; (Nf) is the number of individuals counted; Vf is the volume of sample filtered. A_f is the area of the filter and A_m is the area of view under the microscope. The software Opticount was used for the counting process (Hepperle, 2004).

2.2.5.4 Zooplankton identification, quantification and biomass

To identify and estimate zooplankton abundance and biomass, a subsample of 1L from the 5.5 L containers was filtered through a 47 mm Millipore nitrate cellulose membrane of 5 μm pore size (Millipore, UK), to enable the collection of small rotifers. A vacuum pump was applied at a low pressure differential of 0.3 atm, but stopped just before the water had drained completely to avoid damaging their morphology. The membrane was removed and placed inside a 50 ml Falcon tube. The retained residual was rinsed with 1-2 ml (depending on the loading on the membrane) of ultrapure water (UPW) and 0.5 – 1ml of carbonated water added drop by drop as a buffer. Ethanol 74% was then added in a 1:3 ratio (1 part UPW + carbonated water and 3 parts of ethanol). A subsample of 1 ml was obtained with a calibrated automatic volumetric pipette with a wide mouth tip and placed onto a Sedgwick-Rafter chamber (20 mm W x 1 mm D x 50 mm L). All organisms were counted under a Leica Wild 3M stereoscope microscope using x16 ocular for larger organisms. The x40 ocular is used for counting small zooplankton and to take body measurements for biomass estimation. Zooplankton abundance was calculated as the number of organisms per m^3. Initially it was calculated as n, the number of organisms per L, using Equation (2.5), where N = number of organisms counted per ml; V_s

= Volume of the sample in ml (after re-suspension with UPW and alcohol); and V_f = Volume of reservoir water filtered in litres.

$$n = NV_s/V_f \quad \text{(eq. 2.5)}$$

n is multiplied by 1000 to obtain the number of organisms per m^3 (Wetzel and Likens, 2000). The zooplankton (*Daphnia* spp., *Cyclops* spp. and *Diaptomus* spp.) biomass was calculated using the dry weight of 35-50 individuals of each taxon, as described by McCauley (1984).

*2.2.*6 Statistical analyses

Data management and exploratory statistical analysis were undertaken using Excel (Microsoft © 2010), Minitab v. 17 and SPSS v. 22 software. Parametric tests were used if data met the required assumptions of normal distribution, equal variances and independent data otherwise non-parametric tests were used. Data were transformed to meet the parametric assumptions of a normal distribution (the data are distributed around the mean); homoscedasticity (equal variances) and independence of the data (where one data point does not influence the outcome of another). Normality tests were carried out with the Anderson-Darling test, and the Levene's test was used to test for equality of the variances (Dytham, 2011). If assumptions could not be met, non-parametric tests were used. To work with these tests the data are ranked and the tests are performed on the ranks instead of the actual data. Non-parametric tests also make fewer assumptions about the type of data or their distribution on which they can be used (Fowler *et al*., 2009).

Interval plots with a 95% confidence interval for the mean were selected for the presentation of the means and standard error, because these are the most useful measure of the dispersion of a distribution and are preferred to compare several sets of observations (Dytham, 2011).

To compare the average and test for significant differences of matched pairs of the physico-chemical and biological variables between pairs of the sampling sites: upstream the plant-bed (S1), within the plant-bed (S2), downstream immediately after S2 (S3) and downstream before the abstraction intake (S4) the following tests were carried out. Paired t-test was used for the variables that met the parametric assumptions and Wilcoxon-sign-test if these were non-parametric. The test statistic of Wilcoxon is *T*, and it quantifies both the direction and magnitude of all the changes in a set of matched pairs. The smaller value of the sums of ranks between the matched pairs is the test statistic *T*, and the probability of distribution was checked against the relevant statistical tables (Fowler *et al.*, 2009). To compare more than two sampling sites and determine the pairs that were different, the Friedman's test for repeated measures was used. For this test the sampling week was taken as the repeat level factor and significant findings (α=0.05) were followed up with the Wilcoxon sign-rank test and application of a Bonferroni correction.

The hypothesis null for these tests is that there is no difference between the pairs. The effect size was calculated to estimate the magnitude of the effect that was studied. The effect size is a standardized measure of the significance of a statistical test that takes into account the size of the observations and can be compared with other studies. The criterion of interpretation of the effect size is: small effect (<0.3), medium effect (>0.3 and <0.5), large effect (>0.5) (Nakagawa and Cuthill, 2007; Field, 2009).

2.3. Results

2.3.1. Exploring physico-chemical and chlorophyll-*a* variables in the Living-Filter system

Physico-chemical and Chla variables were measured and analysed upstream and downstream of the plant-bed and within the plant-bed to investigate possible downstream

changes of these variables caused by the Living-Filter, and to observe changes between the sites within the plant-bed which may be influenced by the different species of plant.

The results of DO and T measurements taken at each depth (mean±SE) for S1, S3 and S4 from 23rd August to 14th October are shown in Figure 2.2. The results show that DO decreased with depth at all stages, although there was a considerable decrease (> 0.5 mg L^{-1}) at specific depths. Mean DO levels dropped from the surface (0 metres) to five metres depth 0.7 mg L^{-1} in S1 and 1 mg L^{-1} in S3; but in S4, DO levels dropped 0.5 mg L^{-1} from the surface to five metres, and 1.2 mg L^{-1} to seven metres. It is important to note that although the mean DO levels are similar at the surface, in S3 and S4 the range of DO levels are more variable than at S1. The DO levels in S1 at nine metres range is 7.7 – 7.9 mg L^{-1} compared to the range 7.2 – 8.2 mg L^{-1} for S3 and S4. The mean temperatures were similar between (0-3 m) depths; however, the mean temperatures from 4-9 m depths appear to be slightly higher (0.2 ° C) in S4.

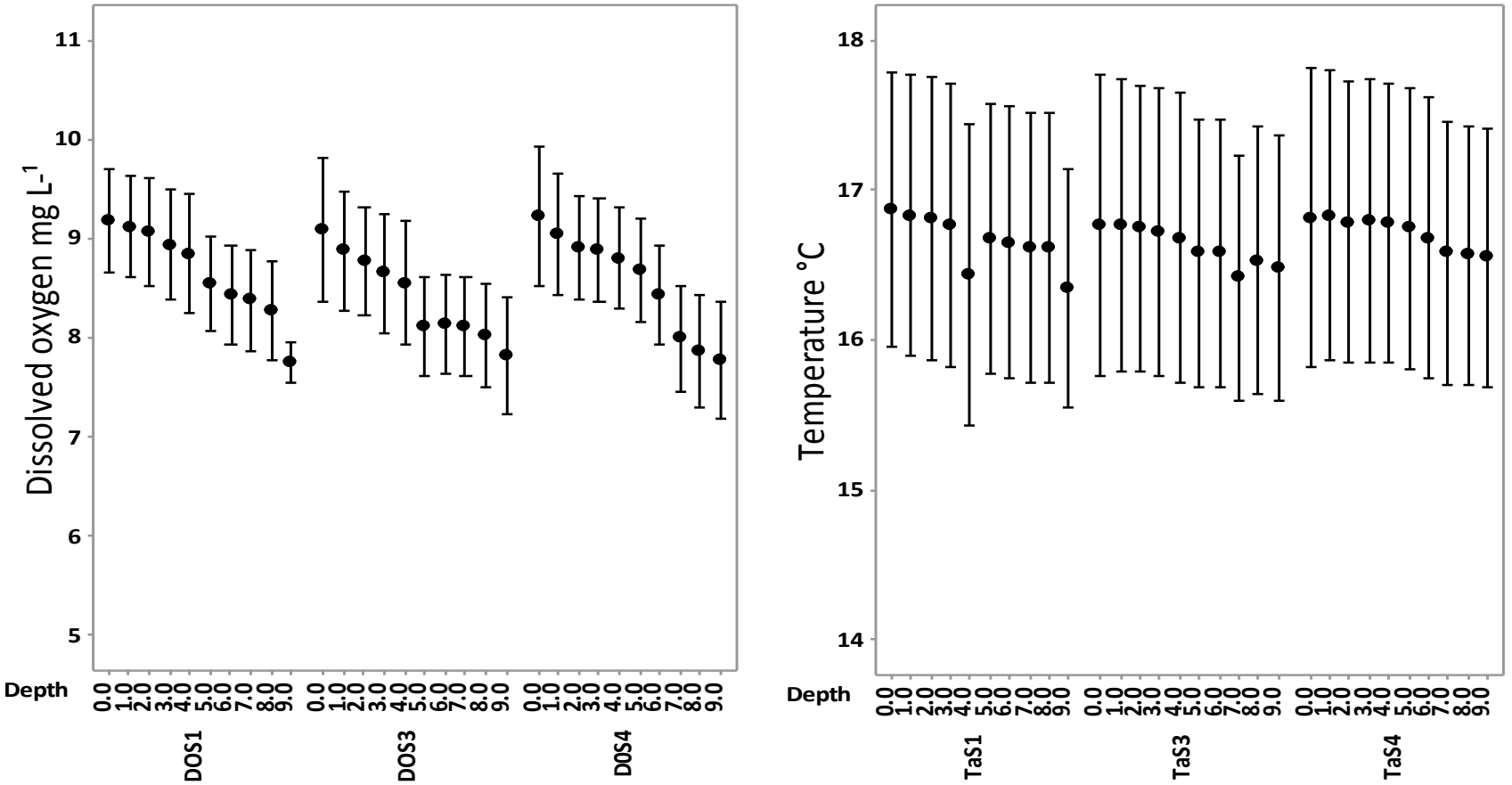


Figure 2.2 Interval bars (mean ±SE) for dissolved oxygen (DO) (mg L^{-1}) and temperature (T) (°C) versus depth profiles (Depth) of the water column at stages S1, S3 and S4 from 23rd August to 14th October 2013 (*n*=60).

Figure 2.3 shows plots for mean±SE for selected variables (Fe^{+3}, Mn^{+4}, sFe^{+2}, sMn^{+2}, SS, pH, COD and BOD) for S1, S2, S3 and S4 and significant variation in the mean values upstream and downstream of the Living-Filter, and also within the plant-bed. The mean of Mn^{+4} and Fe^{+3} for S1, S3 and S4 are smaller than most sites in S2, whilst the mean for sMn^{+2} in S1 is higher than the means of the other stages. Suspended solids, BOD and COD means are highest at site S2E where *Carex acutiformis* grows.

Figure 2.4 shows plots for mean±SE for DIN, NO_2^-, NH_4^+, TP, TOC, DOC, POC and Chla. This figure shows higher means for TP, POC and Chla in the plant-bed (S2), compared to S1, S3 and S4. For S2, S2E is higher than for any other site. The mean of NO_2^- in S1 is considerably higher than any of the other sites, whilst the mean of $NO_2^-$and mean NH_4^+concentration in S2E and S2F are the lowest.

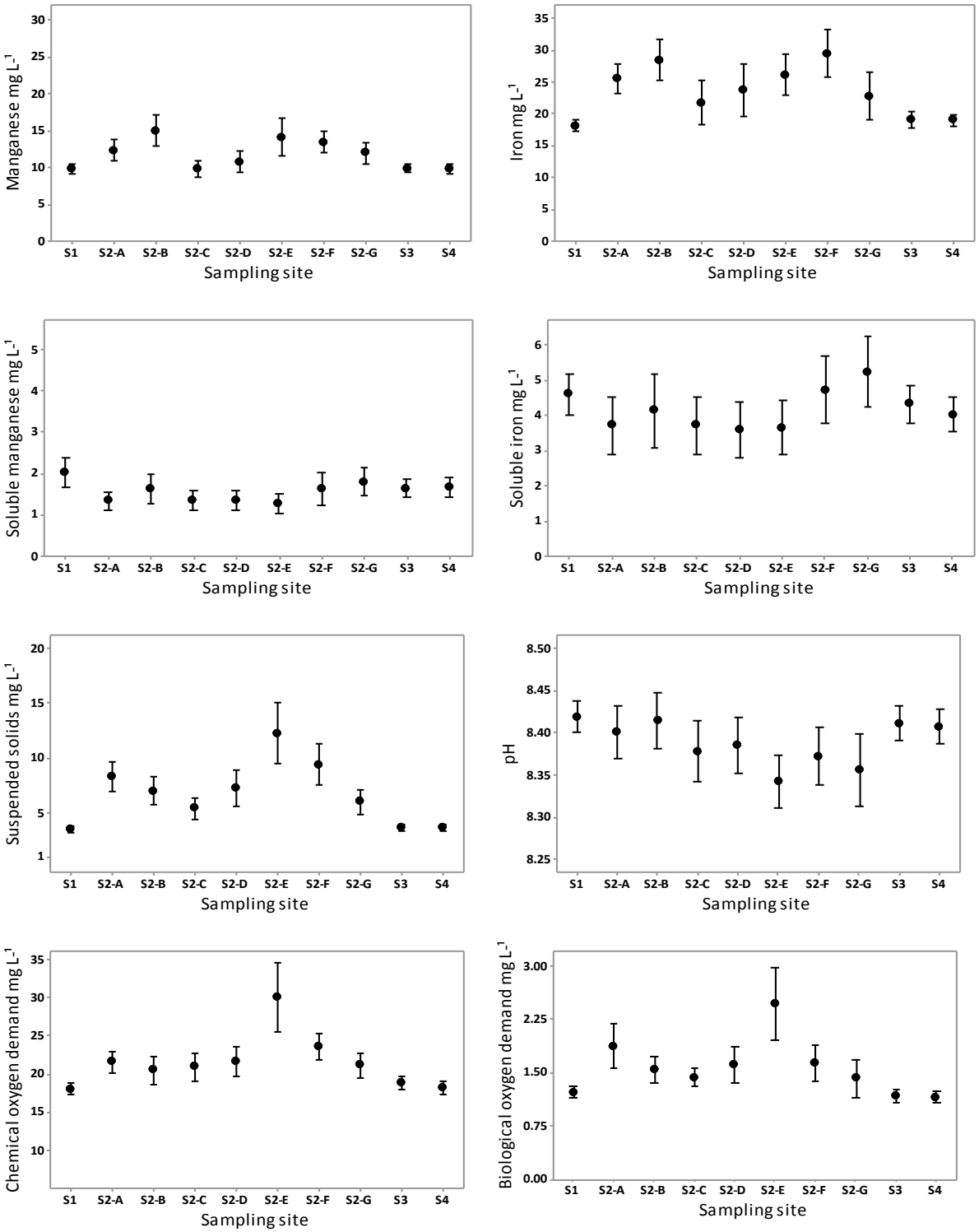


Figure 2.3 Interval bars (mean ±SE) for manganese, iron, soluble manganese, soluble iron, suspended solids, pH, chemical and biological oxygen demand. Stages S1, S3 and S4 include all sampling sites (min. *n*=46; max. *n*=51). The interval bars are for each site at S2 (A, B, C, D, E, F, G) (min. *n*=15; max. *n*=17).

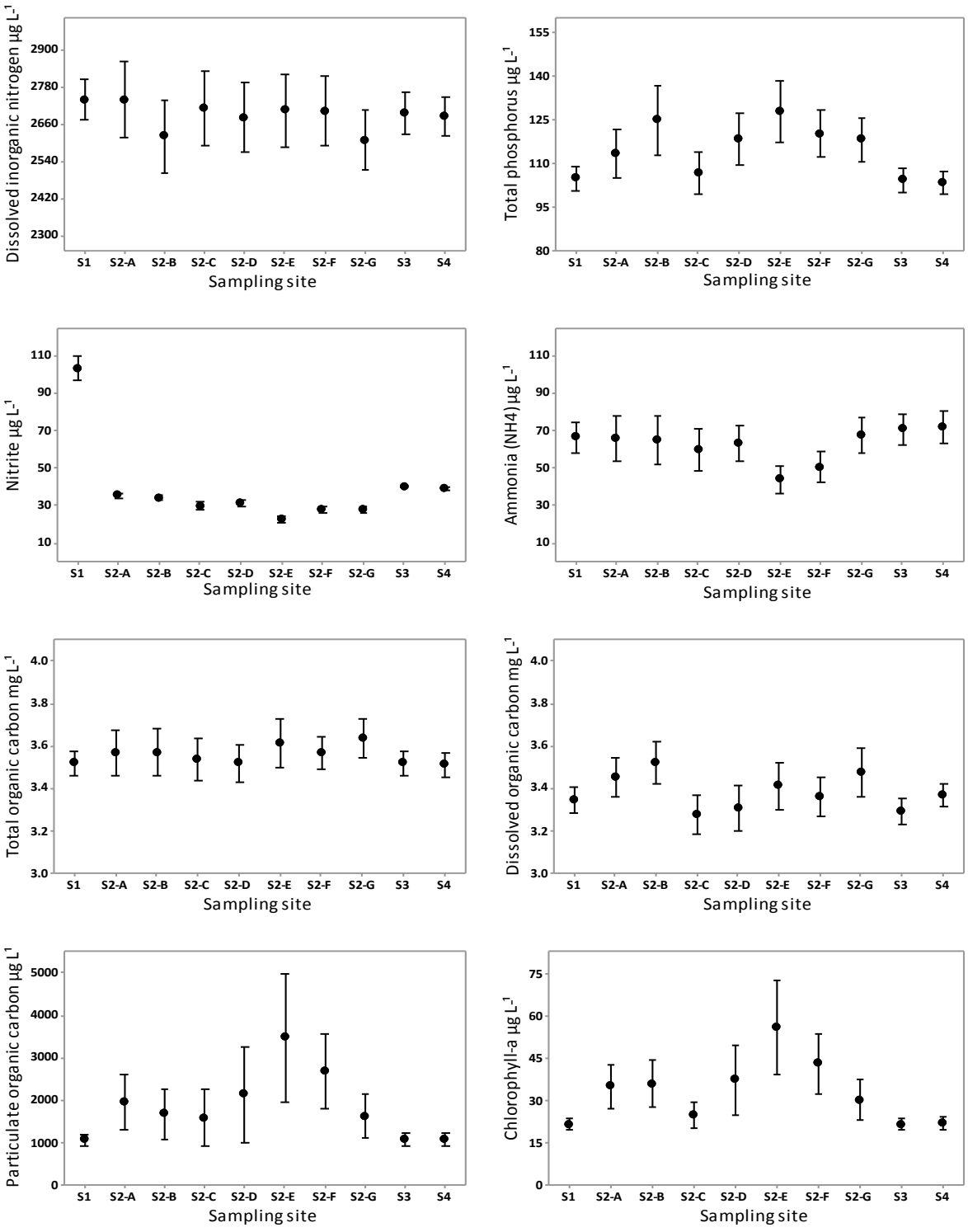


Figure 2.4 Interval bars (mean ±SE) for dissolved inorganic nitrogen, total phosphorus, nitrite, ammonium, total, dissolved and particulate organic carbon and chlorophyll-*a*. Sampling sites S1, S3 and S4 include all depths (min. *n*=46; max. *n*=51). The interval bars are for each site at S2 (min. *n*=15; max. *n*=17).

Table 2.1 summarizes the significant results for the tests of paired differences and repeated measures with the effect size. Dissolved oxygen was significantly different between

S1, S3 and S4 (data from all depths) with S1>S3 and S1>S4. At 1 m depth, there were significant differences for DO between S1, S2, S3 and S4. There was a reduction in DO in S2 compared to S1 and S4, the magnitude of the effect size of these results was large (> 0.5). The Living-Filter DO mean values are lower (spatially) within the plant-bed compared to the water column, regardless of whether samples are from upstream or downstream. Significant temperature differences between S1, S3 and S4 showed that downstream (S3) the temperature was reduced compared to upstream although with a small effect size (<0.3). Downstream temperature was cooler at S3 compared to the downstream temperature at S4, with a medium effect size (0.39)

Significant differences upstream and downstream of the plant-bed were found between S1-S3 for sMn^{+2}, where S1 scores were higher than S3. Also significant were the differences between S1, S3 and S4 for the variables DIN and NO_2^- where S1 was higher than S3 and S4. Ammonium was higher at S4 than S1. The t-test and Wilcoxon sign-rank test revealed a significant increase in Fe^{+3}, Mn^{+4}, SS, BOD, COD, TP, POC and Chla within the plant-bed (S2) compared to S1, S3 and S4 with a large effect size (>0.5); whilst pH was significantly lower in S2, also with a large effect size.

Significant differences between the sites A, B, C, D, E, F and G in the plant-bed were found for DO (Table 2.2). Site E showed the lowest DO with a large effect size. Manganese was higher in site C (the site closest to the open water within the plant-bed) than A, B and F, all with a medium to large (0.45 and 0.5) effect size. The effect size was 0.45 for the differences in pH, with a low pH for site E compared to sites A, C and F. The SS in site C was lower when compared to A, B and F with medium to high effect. Nitrite was higher at sites A, B and C than D, E, F and G; site E was the lowest for NO_2^-. Site E had higher significant POC than the other sites, with a large size effect for most of these results. The differences between the sites seem to be related to either the location of the sampling site and/or the species of plant surrounding the sampling site.

Table 2.1 Comparison of physico-chemical and biotic variables between Living-Filter sites: upstream and downstream of the plant-bed and within the plant-bed (average of sites A to G). The results from the test used (Matched-pair *t*-test, Friedman's and Wilcoxon sign-rank test), level of significance, degrees of freedom, number of samples and effect size are included.

Living-Filter System	Site / Paired differences	Measured Variable	Paired *t* test	Friedman's χ	Wilcoxon's sign-rank *T*	*z*	*p*-value	Significance *p*	*df*	*n*	Effect size *r*
Up & down stream	Stages S1-S3-S4	DO		49.45			0.000	<0.05	2	60	
Depths (0-9m)	S1-S3				3	-5.75	0.000	<0.017		60	0.52
	S1-S4				15	-2.53	0.005	<0.017		60	0.23
	Stages S1-S3-S4	Temp.		21.98			0.000	<0.05	2	60	
	S1-S3				11	-2.69	0.003	<0.017		60	0.25
	*S3-S4				4	-4.24	0.000	<0.017		60	0.39
Depth (1m)	All stages										
	S1-S2	DO	3.96				0.011	<0.05	5	6	0.87
	S2-S4		-3.38				0.020	<0.05	5	6	0.83
	S1-S2	Fe^{+3}	-4.28				0.001	<0.05	16	17	0.73
	S2-S3		4.13				0.001	<0.05	16	17	0.71
	S2-S4		5.14				0.000	<0.05	16	17	0.79
	S1-S2	Mn^{+4}	-4.15				0.001	<0.05	16	17	0.72
	S2-S3		4.6				0.000	<0.05	16	17	0.65
	S3-S4		4.87				0.000	<0.05	16	17	0.58
	S1-S3	sMn^{+2}	2.05				0.046	<0.05	47	48	0.29
	S1-S3	DIN	3.13				0.002	<0.05	49	50	0.41
	S1-S4		4.66				0.000	<0.05	49	50	0.55
	S1-S3	NO_2^-	9.74				0.000	<0.05	48	49	0.81
	S1-S4		9.66				0.000	<0.05	48	49	0.81
	S1-S4	NH_4^+	-2.74				0.008	<0.05	49	50	0.36

(Continue)

Living-Filter System	Site / Paired differences	Measured Variable	Paired *t* test	Friedman's χ	Wilcoxon's sign-rank *T*	Wilcoxon's sign-rank *z*	Significance *p*-value	Significance *p*	Significance *df*	Significance *n*	Effect size *r*
	S1-S2	SS	-3.57				0.003	<0.05	16	17	0.67
	S2-S3		4.07				0.001	<0.05	16	17	0.71
	S2-S4		4.01				0.001	<0.05	16	17	0.70
	S1-S2	pH	2.72				0.015	<0.05	16	17	0.56
	S2-S3		-2.69				0.016	<0.05	16	17	0.56
	S2-S4		-2.54				0.023	<0.05	15	16	0.55
	S1-S2	BOD	-2.83				0.013	<0.05	15	16	0.59
	S2-S3		3.22				0.006	<0.05	15	16	0.64
	S2-S4		4.32				0.001	<0.05	15	16	0.74
	*S1-S2	COD			2	-3.48	0.000	<0.007	16	17	0.60
	S2-S3				4	-2.82	0.002	<0.007	16	17	0.48
	*S1-S2	TP			3	-3.34	0.000	<0.007	16	17	0.57
	S2-S3				3	-3.34	0.000	<0.007	16	17	0.57
	S2-S4				2	-3.48	0.000	<0.007		17	0.60
	*S1-S2	POC			0	-3.62	0.000	<0.007		17	0.62
	S2-S3				1	-3.57	0.000	<0.007		17	0.61
	S2-S4				1	-3.64	0.000	<0.007		17	0.62
	*S1-S2	Chla			4	-2.64	0.003	<0.007		17	0.45
	S2-S3				3	-2.91	0.001	<0.007		17	0.50
	S2-S4				4	-2.68	0.003	<0.007		17	0.46

<0.007 and <0.017 are one-tailed level values of significance after the Bonferroni correction. *the test was based on the negative ranks

Table 2.2 Comparison of physico-chemical variables between sites sampled at the plant-bed of the Living-Filter. The results from the test used (Matched-pair *t*-test, Friedman's and Wilcoxon sign-rank test), level of significance, degrees of freedom, number of samples and effect size are included.

Site	Measured	Paired *t*	Friedman's	Wilcoxon's sign-rank			Significance			Effect size
Paired differences	**Variable**	**test**	χ	***T***	***z***	***p*-value**	***p***	***df***	***n***	***r***
Stage 2	DO									
A-E		3.4				0.019	<0.05	5	6	0.84
B-E		3.51				0.017	<0.05	5	6	0.84
C-E		2.95				0.032	<0.05	5	6	0.80
D-E		3.69				0.014	<0.05	5	6	0.47
E-F		-5.32				0.003	<0.05	5	6	0.61
E-G		-3.45				0.018	<0.05	5	6	0.44
Stage 2	Mn^{+4}		15.26			0.018	<0.05	6	16	
A-C				2	-2.53	0.005	<0.007		16	0.45
B-C				2	-2.99	0.001	<0.007		16	0.53
*C-F				2	-2.52	0.004	<0.007		16	0.45
Stage 2	pH		14.22				<0.05	6	16	0.00
A-E				4	-2.96	0.001	<0.007		16	0.52
C-E				5	-2.64	0.003	<0.007		16	0.47
C-G				3	-2.69	0.003	<0.007		16	0.48
*E-F				3	-2.53	0.005	<0.007		16	0.45
Stage 2	SS		22.54				<0.05	6	14	
A-C				3	-2.64	0.003	<0.007		16	0.47
B-C				3	-2.74	0.005	<0.007		15	0.50
*C-E				3	-2.89	0.001	<0.007		16	0.51
*C-F				2	-2.61	0.003	<0.007		16	0.43

(Continue)

Site	Measured	Paired *t*	Friedman's	Wilcoxon's sign-rank			Significance			Effect size
Paired differences	**Variable**	**test**	χ	***T***	***z***	***p*-value**	***p***	***df***	***n***	***r***
*D-E				3	-2.84	0.001	<0.007		16	0.50
E-G				4	-2.59	0.004	<0.007		17	0.44
Stage 2	NO_2^-		34.31				<0.05	6	15	
A-D				2	-2.7	0.002	<0.007		17	0.46
A-E				0	-3.52	0	<0.007		16	0.52
A-F				2	-3.39	0	<0.007		17	0.58
A-G				0	-3.4	0	<0.007		16	0.60
B-E				2	-3.01	0.001	<0.007		15	0.55
B-F				4	-2.51	0.005	<0.007		16	0.44
B-G				3	-2.76	0.002	<0.007		16	0.49
C-E				3	-2.5	0.005	<0.007		15	0.46
D-E				2	-3.34	0	<0.007		17	0.57
E-F				4	-2.68	0.005	<0.007			
Stage 2	POC		29.89				<0.05	6	16	
*A-E				3	-2.96	0.001	<0.007		17	0.51
*B-E				4	-2.38	0.008	<0.007		16	0.42
*C-E				2	-3.15	0	<0.007		16	0.56
*C-F				2	-3.1	0	<0.007		16	0.55
*C-G				4	-2.48	0.005	<0.007		16	0.44
*D-E				2	-3.01	0.001	<0.007		17	0.52
E-F				3	-2.72	0.002	<0.007		17	0.47
E-G				2	-2.89	0.001	<0.007		16	0.51
Stage 2	SRP		16.51			0.011	<0.05	6	15	
B-D				4	-2.39	0.007	<0.007		15	0.44
B-E				4	-2.79	0.002	<0.007		15	0.51

<0.007 and <0.017 are one-tailed level values of significance after the Bonferroni correction. *the test was based on the negative

2.3.2 Chlorophyll-*a* loading and removal efficiency

2.3.2.1 Chlorophyll-a loading

The loading of Chla (g m^{-3} w^{-1}), that was measured as the mass accumulated per unit volume per unit time (week), followed the seasonal trend in Chla productivity in the reservoir. High Chla loads were seen from 19th August (week 8) until 23rd September (week 13) year, with a maximum load in week nine (Figure 2.5).

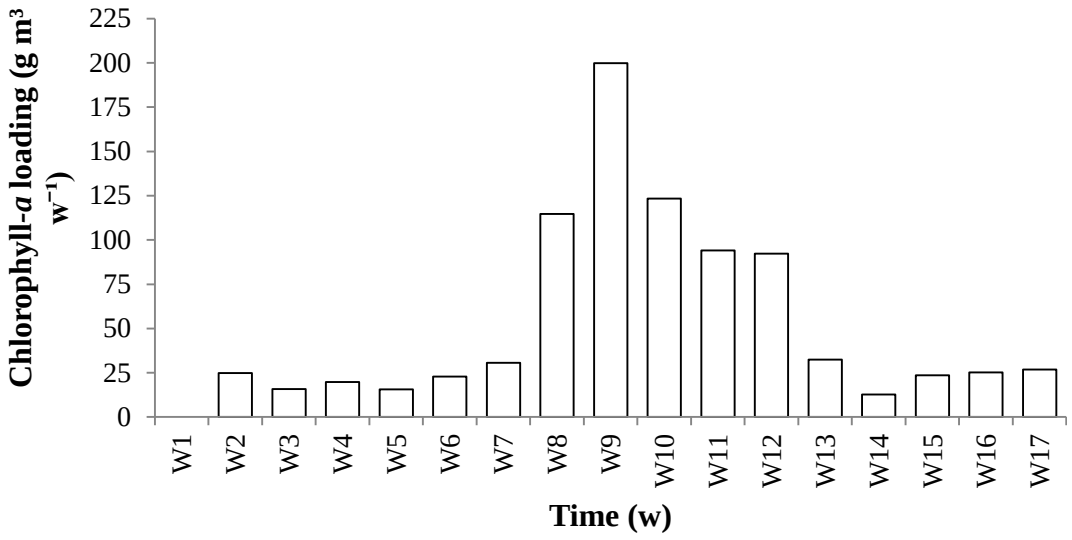


Figure 2.5 Weekly (w1-w17) chlorophyll-*a* loading onto the plant-bed of the Living-Filter from 2nd July 2013 (w1) to 31st October 2013 (w17).

2.3.2.2 Chlorophyll-a removal efficiency

Measured values for the removal efficiency (RE%) of Chla approached 45% between S1 and S3 and approached 40% between S1 and S4, during the first seven weeks of the survey. After this period, some removal was observed between 20-10% on only five occasions. There were three large negative RE% or releases (≥ 90%) of Chla biomass, meaning that Chla was higher downstream than upstream. In the context of a filtration process, similar events take place when there is a breakthrough in the filter so there is no longer retention, or the filtered particles become unbound so are released. The *releases* were

observed in weeks seven and 13 between S1 and S4, and also in week 13 between S1-S3 (Figure 2.6). The first large *release* took place at the end of August/early September, which was the period when the first cyanobacteria bloom was observed. The other two *releases* were observed six weeks later at the end of the summer.

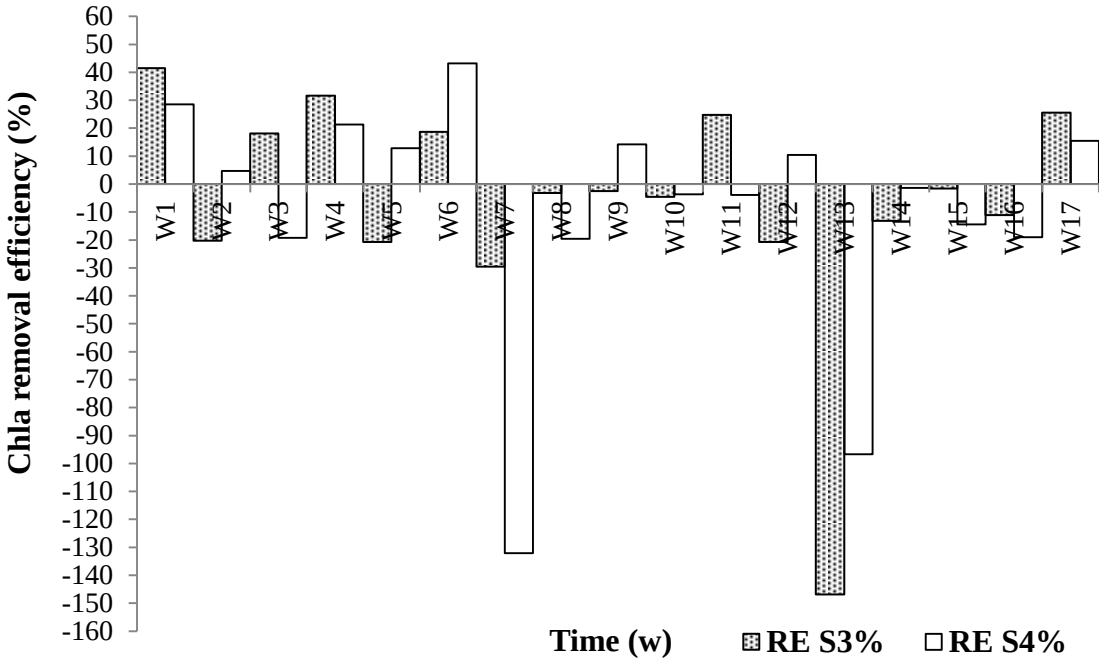


Figure 2.6 Weekly (w1-w17) chlorophyll-*a* removal efficiency (RE%) at Stage 3 (S3) and Stage 4 (S4) from 2nd July to 31st October 2013.

2.3.3. Biological variables

2.3.3.1 Assessment of the plants and root development

Three individual plants from each of the three species were sampled. The size range of aerial shoots was 0.50-0.70 m when first positioned on the cages in July 2012. During the survey period, *Phragmites* showed the tallest aerial shoots with the shortest roots. *Carex* roots were the longest; and *Phalaris* roots showed the greatest density and most abundant fine roots (Table 2.3).

Table 2.3 Shoot height and root length (mean±SE) Showing dimensions of the plant species sampled (n=3) from the Living-Filter plant-bed in 2013 and 2014.

Plant species	Aerial plant height (m)		Root length (m)
	2013	2014	2014
	mean±SE	mean±SE	mean±SE
Phragmites	1.66 ±0.24	2.08±0.26	0.15±0.08
Phalaris	1.53±0.12	1.1±0.36	1.5±0.15
Carex	1.1±0.23	1.4±0.18	1.74±0.12

The plant species coverage (m^2) on the plant-bed is shown for 2012 and 2013 in Table 2.4.The figures showed a reduction in plant coverage for 2013, with the side of the structure facing the open water devoid of vegetation.

Table 2.4 Plant species coverage (m^2) on the plant-bed structure for years 2012 and 2013.

	Year 2012	Year 2013
	m^2	m^2
Plant coverage	179	115
Phragmites	90	45
Phalaris	41	26
Carex	48	44

2.3.3.2 Periphyton qualitative observations

Hydra spp. was observed to colonize the metal cage structure of the Living-Filter plant-bed, and a freshwater sponge colonized the curtains and baffles.

2.3.3.3 Phytoplankton qualitative observations

A total of 27 phytoplankton genera have been identified (Table 2.5). The most abundant genera appeared to be the colonial *Microcystis* spp., identified as *Microcystis aeruginosa* and filamentous cyanobacteria *Aphanizomenon* spp., green algae *Cosmarium* spp., identified as *Cosmarium bicaudatum*, followed in abundance by *Sphaerocystis* spp. and *Oocystis* spp.

Diatoms were less frequent but dominated by *Aulacoseria* spp. identified as *Aulacoseria* granulata.

Table 2.5 Phytoplankton genera grouped by Phyla identified in all stages in the water column (S1, S3 and S4) and in the plant-bed (S2).

Phytoplankton			
Phyla	**Genera**	**Phyla**	**Genera**
Cyanobacteria	*Anabaena* spp.	Bacyllaryophyta	*Aulacoseria* spp.
	Aphanizomenon spp.		*Asterionella spp.*
	Microcystis spp.		*Cocconeis* spp.
	Woronichinia spp.		*Cymbella* spp.
Chlorophyta	*Chlorella* spp.		*Fragilaria* spp.
	Closterium spp.		*Gomphonema* spp.
	Coelastrum spp.		*Gyrosigma* spp.
	Cosmarium spp.		*Navicula* spp.
	Desmodesmus spp		*Nitzschia* spp.
	Dictyospherium spp.		*Stephanodiscus* spp.
	Monoraphidium spp.	Chrysophyta	*Cryptomonas* spp
	Oocystis spp.		*Ochroomonas* spp.
	Pediastrum spp.	Dynophyta	*Ceratium* spp.
	Sphaerocystis spp.		*Peridinium* spp.
	Staurastrum spp.		

2.3.3.4 Zooplankton composition, abundance and biomass

A total of 22 taxa of zooplankton and aquatic organisms were identified. The juvenile phases (Nauplii) of *Cyclops* spp. and *Diaptomus* spp. were included in the analysis of the zooplankton composition (Table 2.6).

The relative abundance of the identified taxa was compared between stages S1, S3, S4 and S2. *Daphnia* spp. was the most abundant species at S1, S3 and S4 and *Cyclops* spp. was most abundant at S2 (Figure 2.7); the organisms that correspond to the numbered taxa in Figure 2.8 can be found in Table 2.6.

Table 2.6 Zooplankton taxa (genera, order or phyla) and other aquatic organism are ranked according to their frequency of occurrence the samples from the water column (Stage 1, 3 and 4) and in the plant-bed (S2).

S1, S3 and S4	Rank	Plant-bed (S2)	Rank
Daphnia spp.	1	*Cyclops* spp.	1
Keratella spp.	2	*Daphnia* spp.	2
Nauplii small	3	*Keratella* spp.	2
Cyclops spp.	4	*Chydorus* spp.	2
Diaptomus spp.	5	*Nauplii small*	3
Asplanchna spp.	6	*Diaptomus* spp.	4
Nauplii large	7	*Nauplii large*	4
Polyarthra spp.	8	*Nais* spp.	4
Oligochaeta	9	*Asplanchna spp.*	5
Brachionus spp.	10	*Canthocamptus*	6
Mite	11	Ostracoda	7.5
Canthocamptus	12	*Polyarthra* spp.	9
Mosquito larvae	13	Mite	10
Bosmina spp.	14	Mosquito larvae	11
Chydorus spp.	15	*Gammarus* spp.	12
Bryozoa	16	*Briozoa*	13
Ostracoda	17	*Bosmina* spp.	13.5
Nematode	18	*Brachionus* spp.	15.5
Chrionomid	19	Chrionomid	17
	20	Mollusca	18.5
	21	*Limnae* spp.	19

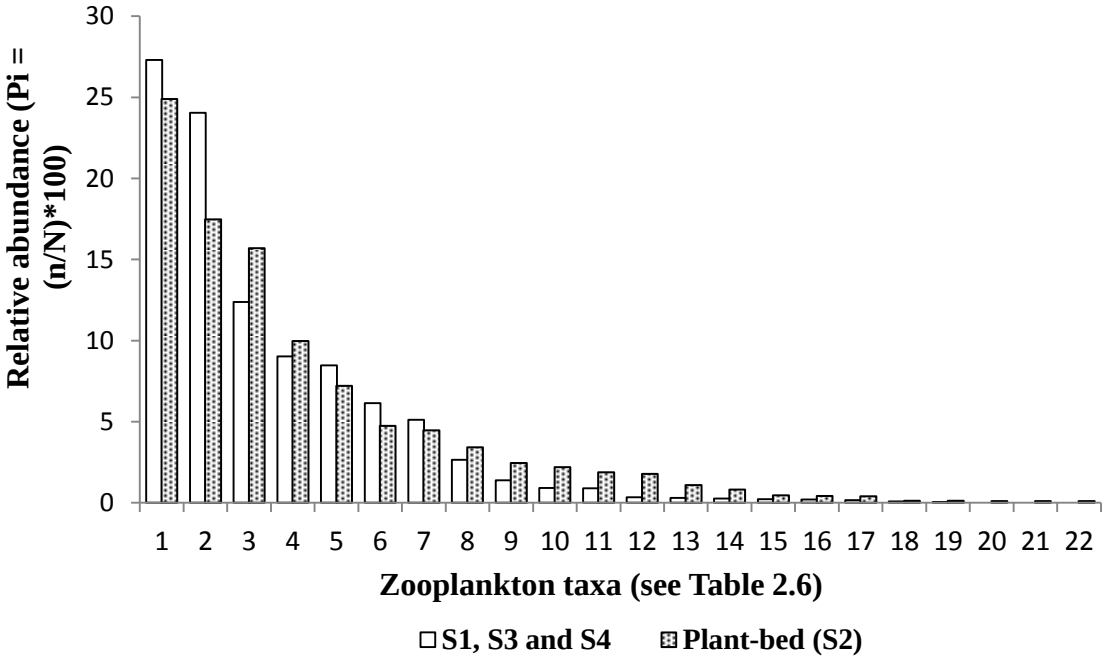


Figure 2.7 Relative abundance (%) of zooplankton and pelagic aquatic organisms, Pi=n/N*100, by zooplankton taxa (see Table 2.6).

Dispersion of the zooplankton taxa (Relative abundance %) at 1, 5 and 8 m depths from stages S1, S3 and S4 (Figures 2.8, 2.9 and 2.10) is shown in accordance with Table 2.6.

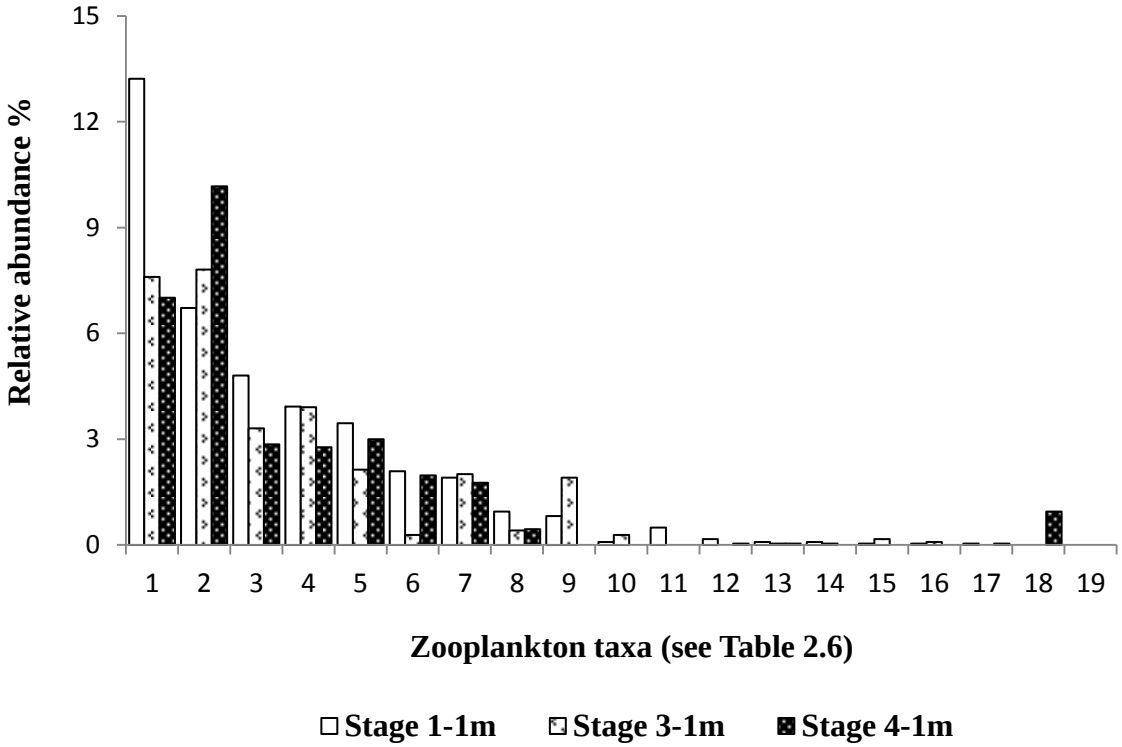


Figure 2.8 Dispersion of the zooplankton taxa (%) at 1m depth at S1, S3 and S4.

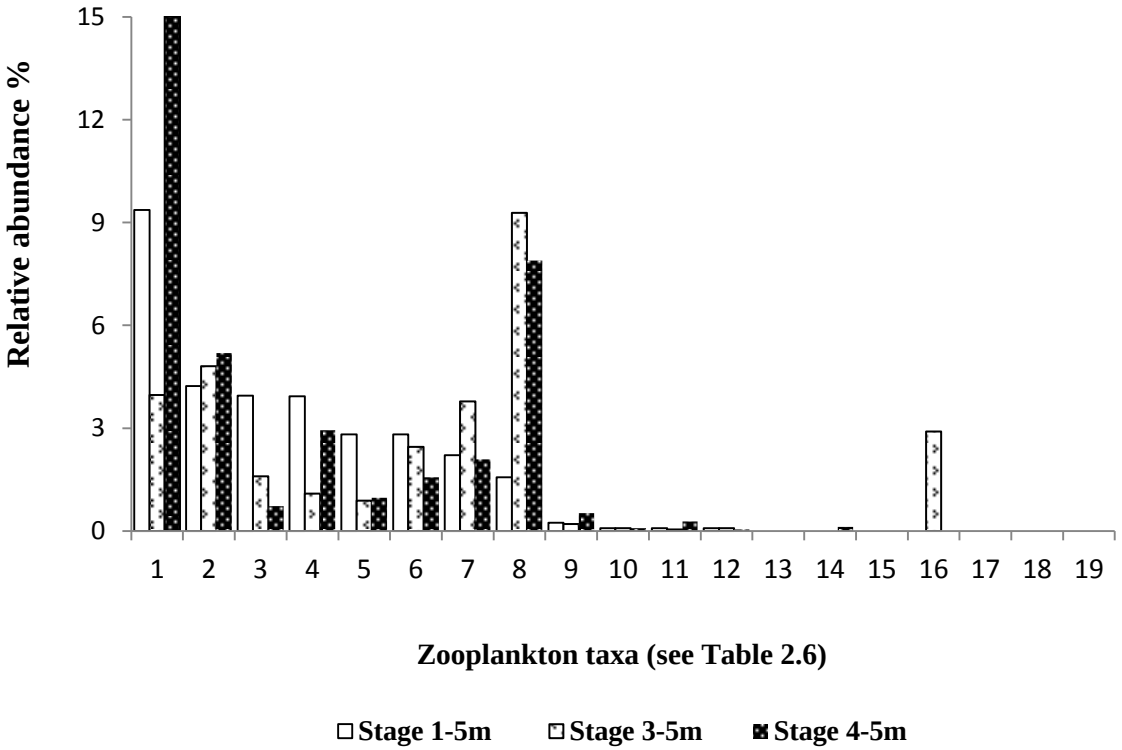


Figure 2.9 Dispersion of the zooplankton taxa (%) at 5m depth at S1, S3 and S4.

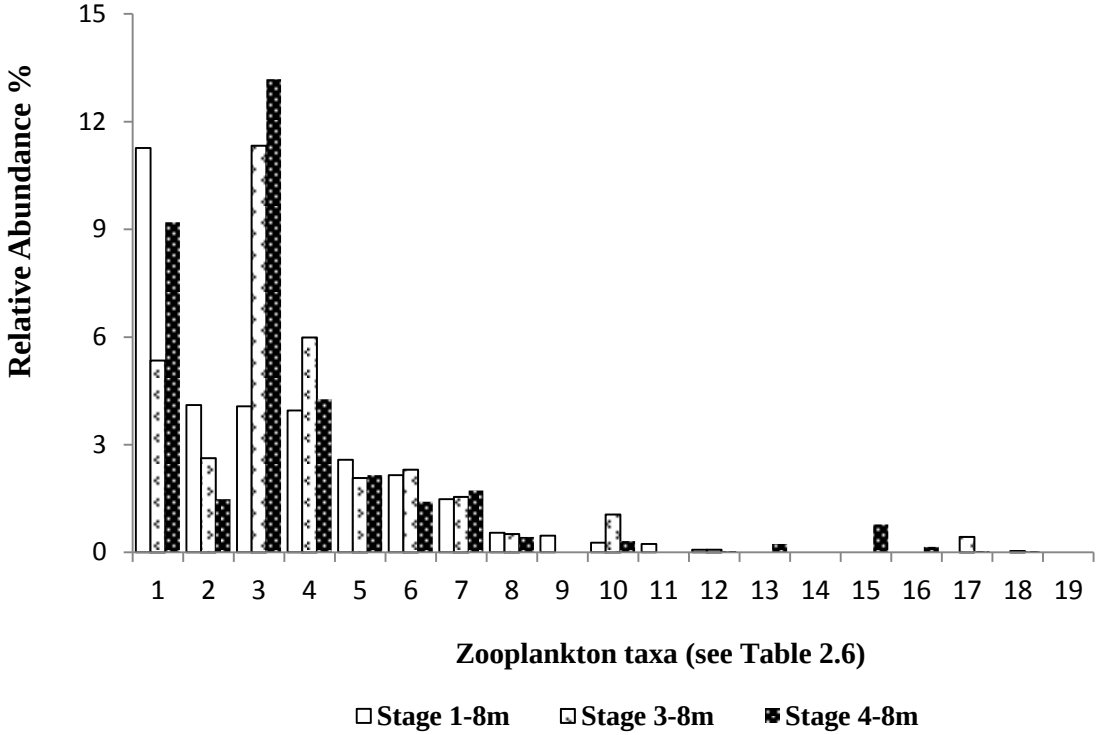


Figure 2.10 Dispersion of the zooplankton taxa (%) at 8m depth at S1, S3 and S4

Daphnia spp. and *Cyclops* spp. were significantly more abundant at S1, with a medium effect size for the differences S1-S3 (0.36) and a small effect size for S1-S4 (0.25) (Table 2.7). Statistically significant differences were found with the Friedman's test between the sampling sites of S2 for *Daphnia* spp., *Keratella* spp. and *Canthocamptus* spp. The paired differences of sites E-F were significant with a large effect size. No significant differences were found between other pairs with the Wilcoxon sign-test (Table 2.7).

Table 2.7 Comparison of zooplankton abundance between Living-Filter sites: upstream and downstream of the plant-bed and within the plant-bed. Results for Friedman's and Wilcoxon sign-rank test, level of significance, degrees of freedom, number of samples and effect size are included.

Living-Filter	Site / Paired differences	Measured Variable	Friedman's	Wilcoxon's sign-rank			Significance			Effect size
			χ	*T*	*z*	*p*-value	*p*	*df*	*n*	*r*
Up & down stream	Stages S1-S3-S4	*Daphnia* spp.	26.16			0.000	<0.05	2	45	
Depths (0-9m)	S1-S3			5	-3.37	0.000	<0.017		45	0.36
	S1-S4			9	-2.33	0.009	<0.017		45	0.25
	Stages S1-S3-S4	*Cyclops* spp.	19.22			0.000	<0.05	44	45	
	S1-S3			7	-3.56	0.000	<0.017		45	0.37
	S1-S4			9	-2.96	0.001	<0.017		45	0.31
Plant-bed	Stage 2	*Daphnia* spp.	12.99			0.043	<0.05	6	14	
	E-F			0	-2.81	0.001	<0.007	6	14	0.54
	Stage 2	*Keratella* spp.	15.12			0.019	<0.05	6	14	
	Stage 2	*Canthocamptus* spp.	15.66			0.016	<0.05	6	14	

A decline in zooplankton numbers can be seen following a seasonal trend. Drastic fluctuations are observed at S1, S3 and S4 (w6) and (w13-14) (Figure 2.11).

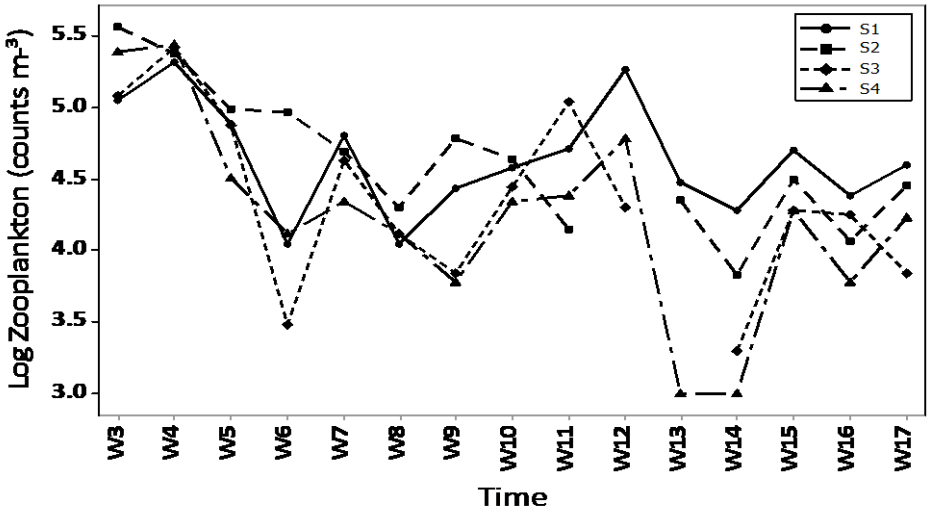


Figure 2.11 Time series of logarithm counts of zooplankton per cubic metre (m^{-3}) for 15 weeks at 1 m depth from 22nd July to 31st October 2013. S1=continuous line, S2=dashed line, S3=dotted line and S4=dashed-dotted line.

No significant relationships were found between the zooplankton biomass (*Daphnia* spp, *Cyclops* spp. and *Diaptomus* spp.) and Chla biomass in Figure 2.12.

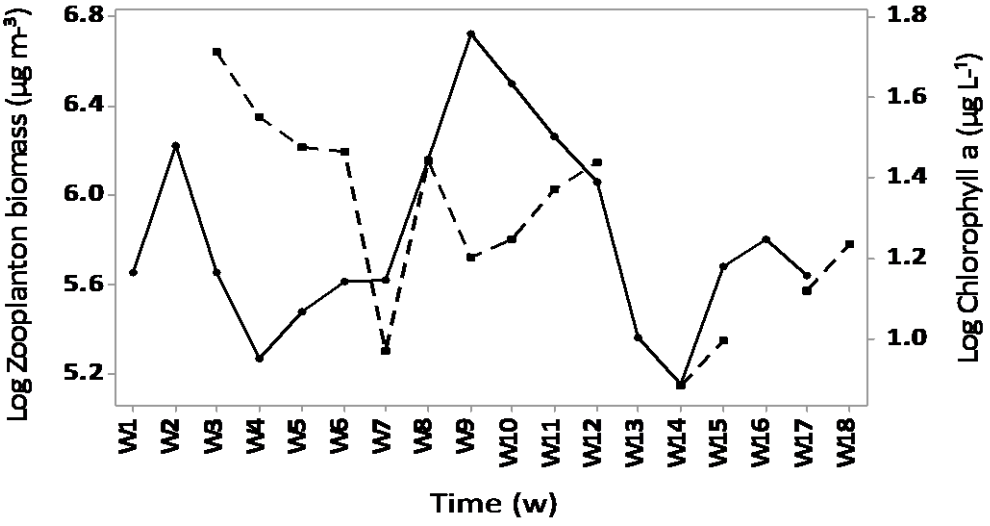


Figure 2.12 Time series of the logarithm for average zooplankton biomass (dashed line) (µg m^{-3}) and of chlorophyll-*a* (continuous line) (µg L^{-1}) at S3.

2.4. Discussion

Surveys of the water column were undertaken to identify physico-chemical changes induced by the Living-Filter upstream (S1) and downstream (S3 and S4) and in the plant-bed (S2). Although statistically significant differences were found for some of the measured variables, the raw water quality supplied to the WTW was not adversely affected by the Living-Filter installation during the period of study.

2.4.1 Were there physico-chemical and chlorophyll-*a* water quality differences upstream and downstream of the plant-bed of the Living-Filter?

The Living-Filter may have induced physico-chemical changes for some variables between the stages as significant differences were found in DO, temperature, pH, sMn, DIN, NO_2^- and NH_4^+. The differences in DO, higher in S1 (all depths) may be related to the demand for oxygen by biochemical processes in S2. No hypoxia was seen in any of the stages, even at S4 which showed the lowest DO concentration (7.2 mg L^{-1}). The lower temperatures in S3 are likely to be a consequence of the shading effect on the water column by the plant-bed (Table 2.1). Furthermore, at 1m depth it was found that S2 differed from the other stages by a significant decrease in DO and an increased COD and BOD but were <25 mg L^{-1} and (< 3 mg L^{-1}) respectively (Table 2.1). This provides further evidence that biochemical processes are taking place: either biochemical activity originating from the roots themselves, the biofilms attached to the roots and fabric, or the organisms within the rhizosphere, or a combination of all these. As found by Kyambadde *et al*. (2005) and Osem *et al*. (2007), these activities could be related to the uptake of nitrogen and the denitrification/nitrification processes. In this study, DIN, NO_2^- and sMn are significantly higher upstream of the plant-bed. The increase in NH_4^+ in S4 might be related to biochemical processes enhanced by the increased temperature in S4. This increase in temperature should be monitored further and addressed in future designs to prevent promoting conditions for phytoplankton growth. The treatment of the raw water

supplied to Farmoor and Swinford WTWs was not affected by the Living-Filter installation during the period of study.

The significant increase in SS, TP, POC and Chla in S2 (Table 2.1), with particularly high means at site E and F for Chla and POC (Figure 2.5), strongly suggest that the plant-bed has a role in trapping particulates. These results indicate that the increase in particulates may be due to phytoplankton cells, as Chla is also increased. Furthermore, the increase in TP could be related to the retention of cyanobacteria cells, which store polyphosphate granules in their cells and also play a role in the cycling of nitrogen and phosphorus in water bodies (Carey et al., 2012; Cottingham *et al*., 2015).

2.4.2 Were there physico-chemical and chlorophyll-*a* differences within the plant-bed itself?

Surveys within the plant-bed (S2) were undertaken to investigate whether physico-chemical differences could relate to the coverage of the plant species.

Physico-chemical differences were found between sites within S2. Site E is markedly different from the other sites (Figures 2.4 and 2.5), with significant low DO, pH, NO_2^- and high SS, POC and Chla. This site is the furthest sampling point away from where the inflowing water first enters the plant-bed, and is surrounded by *Carex* plants (Figure 2.2-A). However, *Carex* roots are the longest and form dense roots which could further explain the higher SS and POC retention. A high retention in SS and POC, accompanied by a high BOD and COD, therefore means that microbial activity and biodegration processes are operative and greater than for other sites. These results are also supported by the fact that site F, with *Phalaris* and *Carex* in the vicinity, showed similar mean values to site E for SS, POC and Chla. *Phalaris* has long and dense roots with a "bushy" morphology. By contrast, site C (which is the closest point to the entering inflowing water) showed the lowest (statistically significant) levels of Mn, SS, POC and Chla, whilst sites A and B showed higher significant

differences for NO_2^- and SRP. These sites (A, B and C) are surrounded by *Phragmites*, which is known for its high removal of nitrogen and phosphorus as highlighted by Vymazal and Kropfelova (2005).

Carex showed good growth performance on the Living-Filter with well-developed plants above the water (Table 2.3). *Carex* and *Phalaris* covered about 50% of the plant-bed in 2012, however 8.3% of *Carex* and 36.5% of *Phalaris* were lost in 2013 (Table 2.4). Initially *Phragmites* covered 50% of the plant-bed, but 50% of the plants were lost in 2013 (Table 2.4). An explanation might be related to the position of the lost plants on the plant-bed platform. Most of the lost plants were placed on the periphery of the structure, being directly exposed to environmental conditions such as wind and wave action. However, there might other explanations as *Phragmites* also showed a well-developed aerial shoot length and rhizome, but a root length of less than 20 cm which suggest that nutrient concentrations/availability or flow velocity could have been an issue (Chen, 2017). Moreover, *Phragmites* plants lose about 70% in root biomass over winter, which regrows during Spring/Summer, providing little consistent density/surface area for particle trapping or biofilm attachment. Therefore, future designs should include protection on the windward periphery of the plant-bed which is exposed to the wave action damaging the plants or planting the most robust and resilient species in the periphery.

Nevertheless, the plants growth on the Living-Filter indicates that the influx of nutrients in the reservoir water is sufficient to meet their nutritional requirements. The annual TP in eutrophic Farmoor II reservoir is $\geqslant 80\ \mu g\ L^{-1}$ but $\leq 200\ \mu g\ L^{-1}$. Growth performance is one of the requirements for phytoremediation when using plants in eutrophic environments (Ansari *et al.*, 2014). *Phalaris* and *Phragmites* aerial shoot length in their first and second season in the Living-Filter installation are within those reported for these plants from constructed wetlands in their second or third growing season, or even longer for *Phragmites* (Vymazal and Kropfelova, 2005). Thus, indicating a rapid acclimation to the conditions in the surface water

reservoir. However, the good growth performance and nutrient removal of *Phragmites* is overshadowed by its poor root development, making it unsuitable for Living-Filter designs. It is worth remembering that as well as roots, the plant-bed cage also contained a synthetic plastic filter which might have also contributed to mechanical filtration. The purpose of the Living-Filter design is to remove suspended particles and phytoplankton cells, prior to potable WTWs, therefore suitable root development and morphology should be a requirement in plant selection for future designs.

2.4.3 Was there any removal of chlorophyll-*a* by the Living-Filter?

To investigate whether physical filtration processes for the removal of Chla (as a surrogate of phytoplankton biomass) take place within the Living-Filter, Chla loading and removal efficiency (RE%) were estimated.

Results for the Chla RE% ≤45% were very promising in the first seven weeks of the survey coincided with low loadings (Figure 2.6). The Chla loading doubled between weeks 8 and 12, which could have overloaded the plant-bed leading to the two large *releases* and suggests there a six-week filtration cycles (Figure 2.7). The first Chla *release* was seen at stage 4 (S4), downstream the plant-bed and before the abstraction intakes, in late August/early September. The second Chla *release* was seen at stage 3 (S3), immediately after the plant-bed, and S4. However, two cycles (monitored for a season) is not sufficient to confirm if these observations are related to physical filtration processes.

Following the filtration theory, as the phytoplankton biomass accumulates, the efficacy of removal is initially enhanced in the narrower pores and the size of the macro-pores between the roots/filaments of the fabric is reduced as more material is trapped causing the flow to short-circuit along the less resistant root systems (Knowles *et al*., 2011). Furthermore, the strength of attachment of the trapped and adhered particles will also depend on the shear forces of the inflowing water; high flow velocities are likely to cause greater shear stress and

the trapped and adhered particles will become detached. The effect of flow velocity could be more important at increasing shear stress than the role it could have on the deformation of the roots, because the flow velocity (10-20 m h^{-1}) was roughly the same during the period of study including the period of high efficiency removal. The release at S3 and S4 in week 13 might be explained by filtration processes, because it occurred after the high Chla loading (Figure 2.5) on the plant-bed (S2). However, it does not explain the *release* at S4 in week 8.

The *releases* at S4 in weeks 8 and 13 might have another explanation. Cyanobacteria blooms are most frequent mid-Summer/early autumn (Visser *et al*., 1996, 2016) and dormant cyanobacteria cells in the sediment which can migrate vertically from the sediment to the surface with the right environmental conditions increasing chlorophyll-*a*. The area of the body of water downstream the plant-bed to the abstraction tower is not covered (Figure 2.1-C), therefore an increase in Chla in the water body could be measured at S3 and S4, but particularly at S4, because S3 is immediately after the plant-bed. A way to overcome this situation in future field-scale biofilter designs could be covering this area so that removal efficiency could be attributed to filtration performance. However, the design and materials, to cover this area have to be approved in the UK by the DWI as water quality cannot be compromised.

The Living-Filter partially fulfilled its aim by showing removal of phytoplankton biomass (measured as chlorophyll-*a* for almost half of the period of study, but these results lead to further questions: 1) Are the flow rate and flow velocity too high? The Living-Filter flow rate is ~86 m^3m^{-2} h^{-1} with a 10-20 m h^{-1} flow velocity compared to a slow sand filter (SSFs) flow rates of 0.2-0.6 m^3 $h^{-1}m^{-2}$. Slow sand filtration rates enables mechanical, biological and ecological filtration mechanisms. A high flow rate (3 – 30 m^3 m^{-2} h^{-1}) used in rapid gravity filters (RGFs) enables mechanical and biofiltration mechanisms (Bar-Zeev *et al*, 2012), where ecological mechanisms might are also be operating. A major difference between SSFs and RGFs when used in the first stages of potable WTWs is that untreated filtered water

is loaded onto SSFs, whilst in RGFs the water has been chemically treated and particles size ≥ 1 μm removed. In the context of field-scale filamentous biofilters such as the Living-Filter, high flow velocities might be counteractive for filtration mechanisms, reducing removal efficiencies. Aberle and Jarvela (2013) pointed out that high flow velocities for herbaceous flexible vegetation in riparian flows will force the shoots to bend, thus equally in the Living-Filter high flow velocities might force the roots to bend thus reducing the surface area for retention of suspended particles. 2) Is the Living-Filter appropriate for the reservoir's depth? Similar floating emergent treatment wetlands are generally operated in water bodies less than 2-3 m depth. Although this is a novel prototype and a first attempt to use a FTWs in a deeper reservoir, efforts should be made to improve the design and implementation of similar eco-technological systems with applicability to a wide range of water bodies. The current design of the Living-Filter includes curtains and baffles which aid water flow through the system. Therefore the role of baffles should be reviewed in future Living-Filter designs if implemented in shallow waters. As the system matures, it is expected that the roots within their natural limits will grow deeper into the water column increasing the likelihood of physical filtration processes in deeper water bodies like Farmoor II reservoir. 3) Is the Living-Filter the right size for this reservoir based on the Chla load and the inflow of water to the WTW? This research will attempt to answer this question in Chapter 7. 3) Is the Living-Filter appropriate for the reservoir's depth? Similar floating emergent treatment wetlands are generally operated in water bodies less than 2-3 m depth. Although this is a novel prototype and a first attempt to use a FTWs in a deeper reservoir (~11 m deep), efforts should be made to improve the design and implementation of similar eco-technological systems with applicability to a wide range of water bodies. The current design of the Living-Filter includes curtains and baffles which aid water flow through the system. Therefore the role of baffles should be reviewed in future Living-Filter designs if implemented in shallow waters. As the system matures, it is expected that the roots within their natural limits will grow deeper into

the water column increasing the likelihood of physical filtration processes in deeper water bodies like Farmoor II reservoir.

2.4.4 Has the Living-Filter provided refuge for zooplankton?

Zooplankton samples from the water column and the plant-bed were analysed in an attempt to answer this question. *Daphnia*, a generalist Cladoceran organism, is the most abundant taxa in the water column, whilst *Cyclops,* a specialist Copepod (Ger *et al*., 2014), is the most abundant taxa within the plant-bed (Figure 2.7). Although statistically *Daphnia* was more abundant in site E than F on the plant-bed, results showed that *Daphnia* and *Cyclops* were significantly more abundant upstream or away from the plant-bed (S1) (Table 2.7), where there is no root coverage for refuge. Although these were unexpected results, other authors have found a similar pattern which is known as the "shore-avoidance" hypothesis (Hulsmann *et al*., 1999). Zooplankton avoids diurnal planktivory by migrating horizontally away from the macrophyte rich shores, although other researchers have also found the opposite pattern (Moss, 1991). Moreover, Schou *et al*. (2009) using floating plastic plants in cages demonstrated differences in the diurnal horizontal migratory pattern for *Daphnia* and *Cyclops* in a eutrophic lake. They found higher abundances of *Daphnia* in the open water whilst *Cyclops* was in the pelagic zone, but *Cyclops* aggregated at night within the cages. The Living-Filter is installed in a eutrophic reservoir and the results might have been influenced by the time of day of the sampling (9:00-13:00). However, the trend abundances of the zooplankton community from each stage (S1, S2, S3 and S4) showed the most stable community abundance was within the plant-bed (S2), despite the downward temporal trend observed for each stage as the surveys approached autumn (Figure 2.10). Zooplankton communities able to graze on phytoplankton and cyanobacteria include four major groups: rhizopods, ciliates, heterotrophic flagellates, rotifers and crustacean (Gerphagnon *et al*., 2015). Of these, rotifers and crustaceans were identified in this study (Table 2.6) and their

vertical distributions are presented in Figures 2.9, 2.10 and 2.11. These initial results suggest that the Living-Filter might be providing refuge for the zooplankton. However, further analysis on zooplankton spatial distributions with surveys of their migratory patterns will be necessary to further support these observations.

Chapter 3. Removal of *Microcystis* cells in a continuous flow mesocosm under two flow conditions.

This chapter covers the operational system of an experimental mesocosm that was used to investigate the removal of a phytoplankton species by filamentous biofilters composed of living plants and synthetic material under continuous flow.

3.1 Introduction

The Living-Filter, a floating treatment wetland (FTW) is used as a pre-treatment filtration process in a UK surface water reservoir to remove phytoplankton loading onto a potable water treatment works (WTW). Results from the field-scale study presented in Chapter 2, section 2.3.2.2, showed a ≤45% chlorophyll-*a* removal efficiency (RE %) (as phytoplankton biomass) only in the first seven weeks of the study (Castro-Castellon *et al*., 2016).

The daily water demand is on average 67.5 x 10^3 m^3d^{-1} in Farmoor and Swinford WTWs. The following are the minimum required processes in WTWs which are implemented at Farmoor and Swinford WTW. Once the water enters the WTW, it is acidified to a pH between 7.0 – 7.3 to optimize the chemical bridging between the coagulant (polymers of aluminium or ferric salts) neutralizing the charge of the suspended particles (generally negatively charged) in the abstracted water (Ratnayaka *et al*., 2009). By a slow mixing process, flocculation enhances the attachment mechanisms between the particles and the coagulant to form a stable floc of aggregated particles. Dissolved air flotation (DAF) follows flocculation, where the flocs are "air-lifted" by the injection of pressurized (4-8 bar) air bubbles of micrometre size (30-80 µm). The bubbles attach themselves to the flocs and by reducing the floc density there is a separation of the floating floc-blanket from the clarified water (Parsons and Jefferson, 2006). The floc-blanket is removed as the clarified water flows to the bed filtration process which aims to remove particles of size 0.1 – 50 µm, including clays and silts, micro-organisms and precipitates of organisms and metal ions. The technology for the bed filtration

process includes slow or rapid gravity sand filters. The particles removed are smaller than the granular media bed (500 – 2000 µm) and the media used can be sand, anthracite, activated carbon or a combination of these media. The removal takes place by diffusion, interception, sedimentation and attachment (Parsons and Jefferson, 2006). Another filtration process takes place by adsorption to remove chemical compounds (e.g. pesticides, geosmin, etc.) using granular media of activated carbon, and the clarified water is then disinfected with chlorine and the pH adjusted (Ratnayaka *et al*., 2009). The biofiltration process in slow sand filters, rapid gravity filters and granular activated carbon is well-established in the water treatment works (Magic-Knezev and Van Der Kooij, 2004; Bar-Zeev *et al*., 2012) where the removal of pollutants and contaminants are mediated by biodegradation mechanism carried out by the microorganisms constituting the biofilm on the granular media (Simpson, 2008).

The Living-Filter comprises two reeds (*Phragmites australis* and *Phalaris arundinacea*) and the sedge *Carex acutiformis* growing hydroponically and rooted in a non-nutrient rich plastic matrix that provides a filamentous media for filtration processes. Therefore, it is expected that physical entrapment of suspended particles and biofilm growth development takes place on the Living-Filter roots, synthetic plastic fabric, metal cages, curtains and baffles of the installation under continuous flow conditions. During biofiltration processes, the biofilm will remove suspended particles and colloids from the water (liquid phase) as they pass through the solid phase (a layer of porous material). The roots and synthetic plastic fabric of the Living-Filter form the layers of porous material. Compared to granular media in bed filtration processes, the diameter of the roots in the Living-Filter is far less uniform, but initially the synthetic fabric filaments are more uniform, and both roots and synthetic fabric have a higher porosity (~90-95%) than the granular media. Therefore, it is expected that plants with a higher root density, particularly a fine root density, will reduce the porosity and thus increase the efficiency of the filtration process.

Tanner and Headley (2011) showed the treatment capabilities of a floating wetland mesocosm (0.7 m^3) batch experiment for the removal of copper ions, phosphorus and fine suspended particles (<0.4 μm) from synthetic storm water in New Zealand. Although they used an enriched media with biofilm carriers, they found the removal of all three variables were significantly higher in the presence of living roots compared to plastic roots despite having similar surface areas. These results suggest that the biofilm developed on the roots of living plants may have benefited in their development from the production of organic exudate or were more effective at the absorption of nutrients. These results also suggest that direct rhizofiltration (i.e. direct uptake of copper/phosphorus) could have contributed to the removal processes.

Biofilters at the bench-scale based on plant rhizofiltration with a high surface area have been hydroponically developed and limited to absorbing nutrient or metal ions from waste waters (Dushenkov *et al.*, 1995; Enley and Raskin, 2000). Marchand *et al.* (2014) combined plants and biofilms for metallic ion removal using planted and unplanted Bio-rack™ to increase the uptake rate of copper. Kurzbaum *et al.* (2014) separated the role of the roots and the associated biofilm for the removal of pollutants in rhizofiltration processes in a hydroponic system, whilst Weiss *et al.* (2014), used recirculating metal-rich flows to investigate the effect of water flux through the roots of hydroponically grown plants on metallic ion removal.

Phytoplankton defines unicellular organisms that drift with the currents and carry out oxygenic photosynthesis whilst living within the illuminated layer of all aquatic systems (Marañon *et al.*, 2009). The removal mechanisms of suspended particles or phytoplankton which include cyanobacteria and micro-algae, by interception and deposition filtration processes using the roots of living plants or synthetic filters in a FTW have not been systematically studied. Doran (2009) pointed out that the complexity and difficulty posed by

working with whole plants in the field do not provide clear cause and effect relationships. That said, observations from field monitoring provide valuable ecological data to generate hypotheses to be tested at the bench-scale (Benton *et al.,* 2007).

During the field-scale study, the dominant group of phytoplankton in the reservoir were the cyanobacteria *Microcystis* spp. (the most abundant) and *Aphanizomenon* spp.; and two microalgae, the diatom *Aulacoseria* spp. and the green algae *Cosmarium* spp. from the groups Bacillariophyta and Chlorophyte respectively. The occurrence of *Microcystis* blooms in drinking water reservoirs is a serious challenge which arises worldwide, and the limno-ecological and reservoir management measures applied for their control are widely published in the scientific literature (Ger *et al.,* 2014). The event of phytoplankton blooms implies the presence of an excessive number of cells (suspended particles); furthermore *Microcystis* blooms tend to contribute to a rise in pH of the water, because of the consumption of CO_2 during photosynthesis (Maberly, 1996). As a result, the acid dose requirement in the WTW must be increased to optimise the coagulation process. Moreover, under predictions of future climate change with increasing atmospheric CO_2 and temperature, Sandrini *et al*. (2016) found that *Microcystis* can adapt genetically to this scenario impacting surface waters and their treatment. As the suspended particles must be chemically bound by the coagulant, the polymers of aluminium or ferric dose demand are increased to form the stable flocs for the separation of the particles from the water.

Therefore, the concept of the Living-Filter as an in-reservoir biofiltration system for phytoplankton reduction by biofilters in a novel, continuous flow mesocosm experiment was tested at two flow velocities. To carry out the experiment, hydroponic cultures of *Phalaris arundinacea* and cultures of two cyanobacteria and two micro-algae were carried out. The species selected were *Microcystis aeruginosa* Kützing. emend. Elenkin (1924) (CCAP 1450/3); *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault (1886) (CCAP 1401/3);

Aulacoseria granulata (Ehrenberg) Simonsen (1979) (CCAP 1002/1) and *Cosmarium bioculatum* Brébisson ex Ralfs (1848) (CCAP 612/17). Each species will be tested independently.

In this work, filtration units of hydroponically developed *Phalaris arundinacea* (biofilters) and synthetic filters of plastic material are used in a novel set-up under continuous flow conditions to study *Microcystis* spp. cell removal by deposition throughout the filter media (depth filtration). The aim of this study is to gain a fundamental understanding of the interactions of *Microcystis* cells with synthetic filters and biofilters comprised of plant roots, and in each case determine the mechanisms of *Microcystis* removal by the filter. The outcome of this work will provide insights for improving future designs of a field scale Living-Filter.

The following research questions were posed for the work presented in this chapter:

1) Are there differences in the cell removal efficiency between biofilters and the plastic filters at high flows (54±2ml min^{-1})?

2) Are there differences in the cell removal efficiency between biofilters and the synthetic filters at low flows (1.2±0.3ml min^{-1})?

3) What other mechanisms might be operating in the biofilters for the removal of *Microcystis* cells?

3.2 Materials and methods

3.2.1 Operational system

An experimental mesocosm was set up to investigate whether hydroponically developed roots and/or synthetic plastic filters can be used at the field-scale for removing *Microcystis aeruginosa* (hereafter referred to as *Microcystis*) from inflowing raw water prior to a potable WTWs intake. The mesocosm system consisted of triplicate filter units with three different types of filter media: one biofilter (i.e. *Phalaris arundinacea* roots) (hereafter refered to as *Phalaris*) and two synthetic monofilament filters (i.e. a plastic three dimensional mesh). In

addition, control units with no filter media were included. *Microcystis* was cultured in photobioreactors and later mixed with tap water to constitute the synthesized laboratory feed, which was pumped to the filter and control units. A schematic of the mesocosm design is displayed in Figure 3.1. All units were covered at the top; the units with the biofilters were also covered where there were no plants, so that all units received the same light intensity. Triplicates of the units (controls, biofilters and plastic fabric) were placed randomly in groups of three on the laboratory bench.

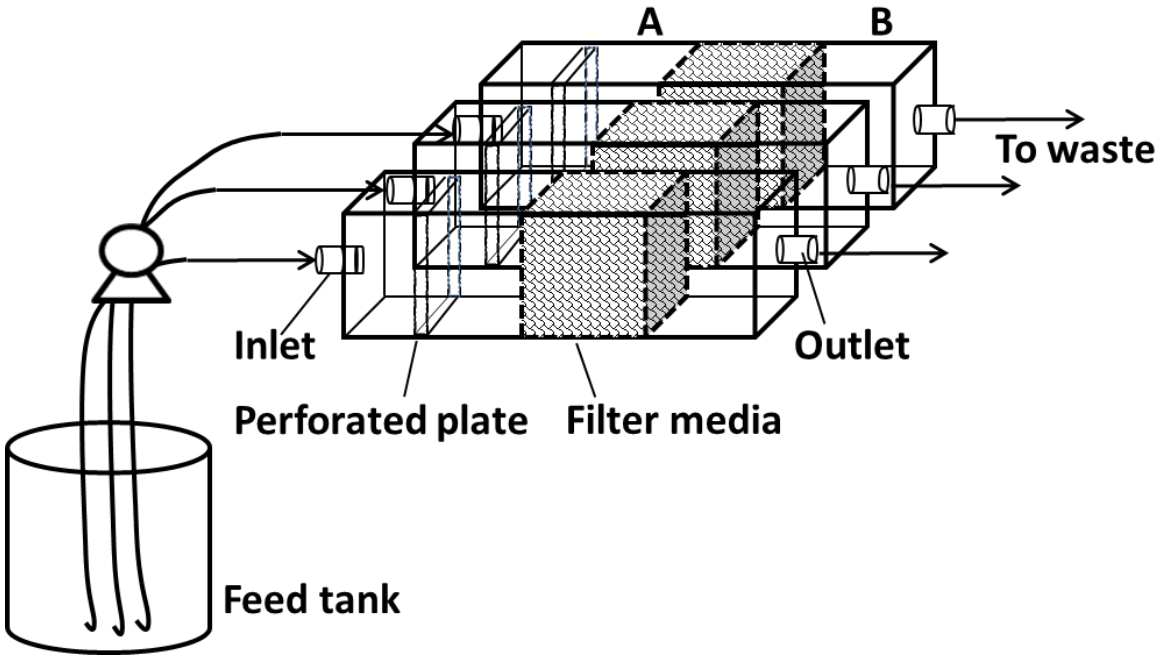


Figure 3.1 Schematic of the mesocosm set up showing only three horizontal filtration units. Furthest unit showing the sampling points, where: A=upstream and B=downstream of the filter; nearest unit showing inlet and outlet of the units, the perforated plate and the position of the filter media.

The filtration units (0.32 m L x 0.104 m W x 0.11 m H) were designed in collaboration with the company Tuan Ta Ltd., London, UK, and made of sheets of acrylic transparent material with an internal perforated plate placed at 0.05 m from the inlet. The units were operated in continuous flow mode, with water mixed by air-bubbling and supplied from a 120

L high density polyethylene tank, via nine individual lines of Marprene long-life flexible tubing (1.6 x1.6 mm). The inflow was controlled by nine pump cassettes in three peristaltic pumps (Watson-Marlow Series 500 x2 and a Series 325).

3.2.2 Hydraulic configuration of the filtration units

Two different flow velocities were chosen for running the experiments. A high flow rate (54±3.0 $cm^3\ min^{-1}$) with a filtration velocity of ~ 0.29 m h^{-1} was chosen to resemble the range of filtration velocities applicable for slow sand filters (typically run at 0.2 - 0.6 m h^{-1}). This is because slow sand filtration is an effective ecological process in potable water treatment works (Campos *et al*., 2002; Nakamoto, 2014). A low flow-rate (1.3±0.2 $cm^3\ min^{-1}$) and filtration rate (1.3±0.2 $cm^2\ min^{-1}$) was chosen to investigate whether deposition processes could take place in these filters. The hydraulic properties of the system are summarized in Table 3.1.

Table 3.1 Hydraulic properties of the filtration units design for two flow conditions

Experimental flow	***Q*** **($cm^3\ h^{-1}$)**	**Area (cm^2)**	**Volume (cm^3)**	***V*=*Q*/*A*** **(cm h^{-1})**	**HRT= Vol/*Q***
Low	60	10	33	0.5	55h 47min
High	33	10	33	29	55min

HRT: hydraulic retention time

3.2.3 Photobioreactors for growing *Microcystis*

Four phytoplankton species, two cyanobacteria, a diatom and a green algae were obtained from the Culture Collection of Algae and Protozoa (CCAP), UK. The species selected, *Microcystis, Aphanizomenon flos-aquae, Aulacoseria granulata* and *Cosmarium bioculatum,* were cultured in the stock solution medium supplied by CCAP: the two cyanobacteria species in BG11 medium, *Aulacoseria granulata* in diatom medium, and *Cosmarium bicaudata* in 3N-BBM+V medium However, two months into the research, and due to the different

culturing requirements of these species, and the logistics and limitations of laboratory space even before culturing the species at a larger scale to run the experiments, it was decided to carry out the experiments with only the most dominant species, *Microcystis*.

Aseptic techniques and materials were used throughout the experiment. Four 150 cm^3 sterile flasks, each with 50 cm^3 media, were spiked with 1cm^3 of the *Microcystis* stock culture and kept on the bench under fluorescent laboratory lights providing photosynthetic active radiation (PAR) of 10 µmol photons m^{-2} s^{-1} (Imai *et al.,* 2008). The flasks were shaken manually every three to four days and tested weekly to check the cultures were not contaminated with bacteria or fungi prior to adding 10 cm^3 of fresh media, maintaining *Microcystis* at the exponential growth phase. An aliquot of 5 cm^3 of the *Microcystis* suspension was used as inoculum to grow larger quantities in photobioreactors. The photobioreactors consisted of 1 L Duran bottles, placed in an open water bath with a thermostat maintaining the temperature at 22±1ºC. The bottle mouth was closed with a sterile foam plug covered with aluminium foil. To the initial volume of 300 cm^3, 200-250 cm^3 of BG11 was added weekly up to a volume of 1 litre. Air was bubbled into the solutions at a rate of 0.1 – 0.4 L min^{-1}, with three periods of 4 hours on/off, 12:12 hours on/off. An additional fluorescent lamp provided 35 µmol photons m^{-2} s^{-1} to the closest positioned photobioreactors and a minimum of 10 µmol photons m^{-2} s^{-1} to the furthest, and the photoreactors were rotated weekly. The growth was monitored by measuring changes in optical density (O.D) at 750 nm in a spectrophotometer (Shimadzu 1800 UV-VIS) (Dagnino *et al.*, 2006).

3.2.4 *Microcystis* cells: particles in suspension

Microcystis cells are spherical particles with a mean diameter of 3.2 µm ± 0.8µm (n = 30), as observed under a light microscope (Eclipse E-200 Nikkon, UK). Cells that were in division, representing approximately 20% of the cell population, were also included in the average size.

Once the system was operational, an increase in the concentration of *Microcystis* cells was expected upstream of the filter media as the units are pre-filled with clean tap water. A concentration change factor was estimated to ensure the cells were not accumulated before the perforated plate (with 64 holes of 0.036 cm diameter) inserted five centimetres after the inlet in the acrylic units (Figure 3.1). The particle concentration in continuous flow mode can be expressed as a dimensionless parameter, which represents the ratio of the particle concentration upstream of the filter media relative to the concentration at the feed inflow to the filtration unit, following Equation (3.1),

$$\text{Inlet concentration change factor (ICF)} = C_a/C_i \qquad \text{(eq. 3.1)}$$

Where, ICF is the inlet concentration change factor, C_a is the concentration of cells on the upstream side of the filter at sampling point A (A in Figure 3.1) and C_i is the concentration of cells in the inflow upstream of the filter unit at the inlet (Inlet in Figure 3.1). This parameter will indicate the filtration mode of the filter media (US-EPA, 2005). Hence, ICF =1 indicates the cell concentration is the same at the inlet and before the filter media; ICF $\geq$1 indicates the cells are accumulating at the surface of the filter media or that there is some backflow; ICF $\leq$1 indicates the cells are passing through the filter or that there is no accumulation of cells at the inlet.

3.2.5 *Microcystis* cell count

Counts of *Microcystis* cell were estimated manually and by flow cytometry using the following methods.

3.2.5.1 Microcystis manual cell count: haemocytometer and optical density calibration measurements

Aliquots of 1 ml from *Microcystis* cultures and samples from each of the acrylic units of the mesocosm (biofilters, synthetic fabric 1 and controls) were transferred into 1.6 safe-lock microcentrifuge tubes (ThermoSientific, Fisher, UK). All samples were preserved with 10 µl

Lugol's solution 1% (Cole-Palmer, UK), and an aliquot of 10 µl of the preserved sample was loaded onto each chamber of the improved Neubauer haemocytometer (AC 2000 standard two chambers 1/400 Hawksley, UK). Manual counting of *Microcystis* cells was performed in triplicates at x400 magnification in the centre square of an inverted microscope (Motic AE 2000, UK) following Imai *et al.* (2008).

A calibration curve was created between *Microcystis* cell counts from the cultures and their optical density (O.D.) measured at 620 nm (Vezie *et al.*, 2002) in a Shimadzu 1800-UV spectrophotometer. The minimum absorbance value accepted for the calibration were those at or above 0.06 O.D. at 620 nm, readings below this figure do not follow a straight line. The resulting linear calibration Equation (3.2) was

$$Y=731218x + 26888 \quad \text{(eq. 3.2)}$$

Where, Y= number of cells and x= the absorbance at 620 nm, and a constant of 26888.

To ensure measurable changes in the reactors a minimum O.D. at 620 nm value was set up at 0.3 (~2.5 x 10^{-6} cells ml^{-1}) in six litres of *Microcystis* to allow for a final dilution in the feed tank of 1:10. The experiments were carried out using cultures with O.D. 0.605 ±0.005 (~4.5 x 10^{-6} cells ml^{-1}) standard error (SE). The O.D. after dilution in the feed was 0.061 ±0.004 SE (1.5 x 10^{-6}).

3.2.5.2 Microcystis cell counting by flow cytometry

The large number of samples generated and the poor count discrimination of low O.D. readings (<0.005) led to the use of flow cytometry for subsequent cell counts using a BD Accuri C6 instrument (BD Biosciences, UK) at the Centre for Ecology and Hydrology (CEH), Wallingford, UK. Flow cytometry is a rapid technology that allows the analysis of millions of single-cells from aqueous samples in 1-5 minutes. It measures the scattered light and the fluorescence emitted by the cells when passing one by one through a zone of intense

illumination (laser beam) in a fast flow media (Ormerod, 2009; Adan *et al*., 2016). *Microcystis* are single celled organisms that emit fluorescence when excited with light and are ideal for flow cytometry. Parameters of measurement were selected according to the method developed by Dr Daniel Read (CEH) for identifying and counting cyanobacteria and microalgae based on their intrinsic fluorescent pigment properties (Read *et al.*, 2014) and using a low sample volume (0.5 ml) from a loaded 96-deep well plate (ThermoScientific, UK). The data were analysed using the BD Accuri C6 software.

The fluidic system in the instrument is designed so that the suspended cells in the sample are delivered one by one to a specific point with the illuminating beam. The velocity of the samples loaded into the channel was set at 35 µl min^{-1}. The instrument measures the light scattered by the cells at right angles to the laser beams (called side scatter, SS) and light scattered in a forward direction (forward scatter, FS). The size and shape of the cells affects the forward scatter whilst small structures (internal or external) of the cells affect more the SS. The forward scatter threshold was set up to 15000 events, and therefore some debris and instrument noise will be ignored. The instrument is equipped with blue (488 nm) and red (638 nm) excitation lasers and four emission filters (Table 3.2). The data were displayed using density dots for two parameters using a bivariate histogram, or cytogram (C), where the dot density of a particular population of cells forms a specific shape called a region (Dubelaar and Jonker, 2000). A region can be drawn using a fluorescence parameter to define the population of interest (called a signature), or a region can be used to limit the cells that are drawn on a light scatterplot (called a gate).

Table 3.2 Naturally occurring fluorescent pigments in phytoplankton and their detection on the BD Accuri C6 (BD, 2015).

Pigments	Excitation (nm)	Emission (nm)	C6 Detector (filter)
Chlorophyll *a*, *b*	488	>640 nm	FL3 (670 LP)
Phycoerythrin	488	575 nm	FL2 (585 ±20)
Phycocyanin	640	650 nm	FL4 (675 ±12.5)
Allophycocyanin	640	646 nm	FL4 (675 ±12.5)

A manual gate was drawn around the cell population on a plot of chlorophyll-*a* fluorescence (FL3; 488 nm excitation, 640 nm emission) versus phycocyanin fluorescence (FL4; 640 nm excitation, 650 nm emission) and was used to discriminate and count the cells against volume calibrated fluidics. The combination of these filters provides an accurate signature of the population of *Microcystis* cells as shown in Figure 3.2.

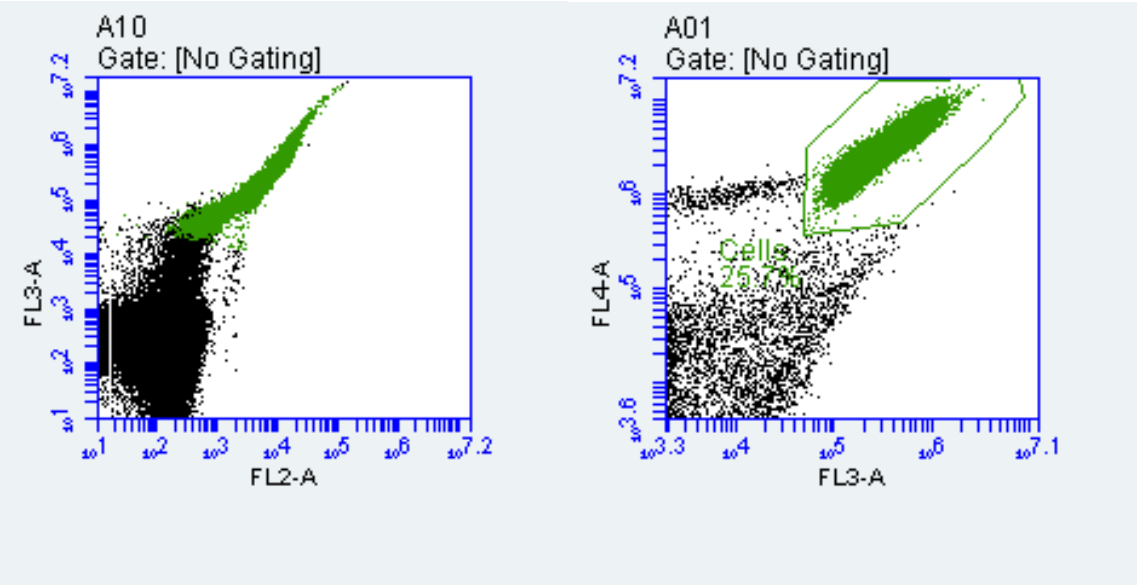


Figure 3.2 Flow cytometry plots showing the gating of *Microcystis* counts (coloured green); with Filters 2 and 3 (left), and with Filters 3 and 4 (right).

3.2.6 Fluid flow of particles (*Microcystis*) through biological and synthetic filamentous filter media

Three filamentous filter media were compared: the living roots of hydroponically grown *Phalaris arundinacea* and two synthetic monofilament fabrics with different packing densities (fabric 1 and fabric 2). The packing density for the filter media was estimated from Equation (3.3):

Packing density= solid volume occupied by roots or fabric/ total volume (eq. 3.3)

where the total volume was measured in a 1 L measuring cylinder, filled-up to 500 ml. Stainless steel cages (of total volume 10 cm^3) were made to contain the biofilters and synthetic media, and empty cages were placed in the control units. The wire diameter was 1.1 mm, with an aperture width of 14 mm.

3.2.7 Production of hairy roots to maximize filtration processes in biofilters

Production of hairy roots can be achieved by hydroponically-grown cultures in solutions with low concentrations of macronutrients (Shanks and Morgan, 1999; Shane *et al*., 2005). To maximize the production of hairy roots, and hence the filtration process, the plantlets were cultured hydroponically in Hoagland's solution at 50% concentration. A 100% Hoagland's solution was prepared following Taiz *et al.* (2015) (Table 3.3). The Hoagland solution is a nutrient solution widely used for hydroponic plant growth (Hoagland and Arnold, 1950).

The selection of the Hoagland's solution concentration was made after carrying out a treatment experiment consisting of five solutions, of which Hoagland's solution at 100% concentration was taken as a control. The treatment included two diluted solutions of the Hoagland's macronutrients solution (at 50% and 25%) and two solutions lacking in KNO3 and another in KH_2PO_4 (no- N and no-P) respectively.

Table 3.3 Hoagland's solution modified from Arnos (1979) in Taiz *et al*. (2015).

Compound concentration	Molecular weight (g mol^{-1})	Concentration stock solution (g L^{-1})	Volume of stock per litre of sol. (ml)
Macronutrients			
1M KNO3	101.1	101.1	5.0
1M $Ca(NO_3).9H_2O$	236.16	236.16	5.0
1M KH_2PO_4	136.06	136.06	1.0
1M $MgSO_4.7H_2O$	246.48	246.48	2.0
0.064M Fe-EDDHA	435.2	15.0	1.5
Micronutrients (all prepared in a 1 L solution)			2.0
0.025M KCl	74.55	1.864	
0.0125M H_3BO_3	61.83	0.773	
0.001M $MnSO_4.H_2O$	169.01	0.169	
0.001M $ZnSO_4.7H_2O$	287.54	0.288	
2.5 x 10^{-4} M $CuSO.5H_2O$	249.68	0.062	
2.5 x 10^{-4} M H_2MoO_4	161.97	0.040	

Approximately 300 *Phalaris* seeds were purchased from British Wildflower Plants, Norfolk, for all experiments. Single seeds were placed in 25 coconut coir pellets. The roots of three week old plantlets were carefully cleaned free of residual coconut fibres by hand and rinsed with tap water. The plantlets were rolled in strips (0.03 m L x 0.01 m W x 0.005 m D) of foam and transferred to 15 cm^3 Falcon™ tubes with the respective solutions. The tubes were covered individually with aluminium foil to prevent light from promoting algal growth in the solution (Figure 3.3). Seeds of *Carex acutiformis* were also obtained and the same procedure described for growing *Phalaris* seeds was followed but did not germinate despite a total of four attempts with from two suppliers.

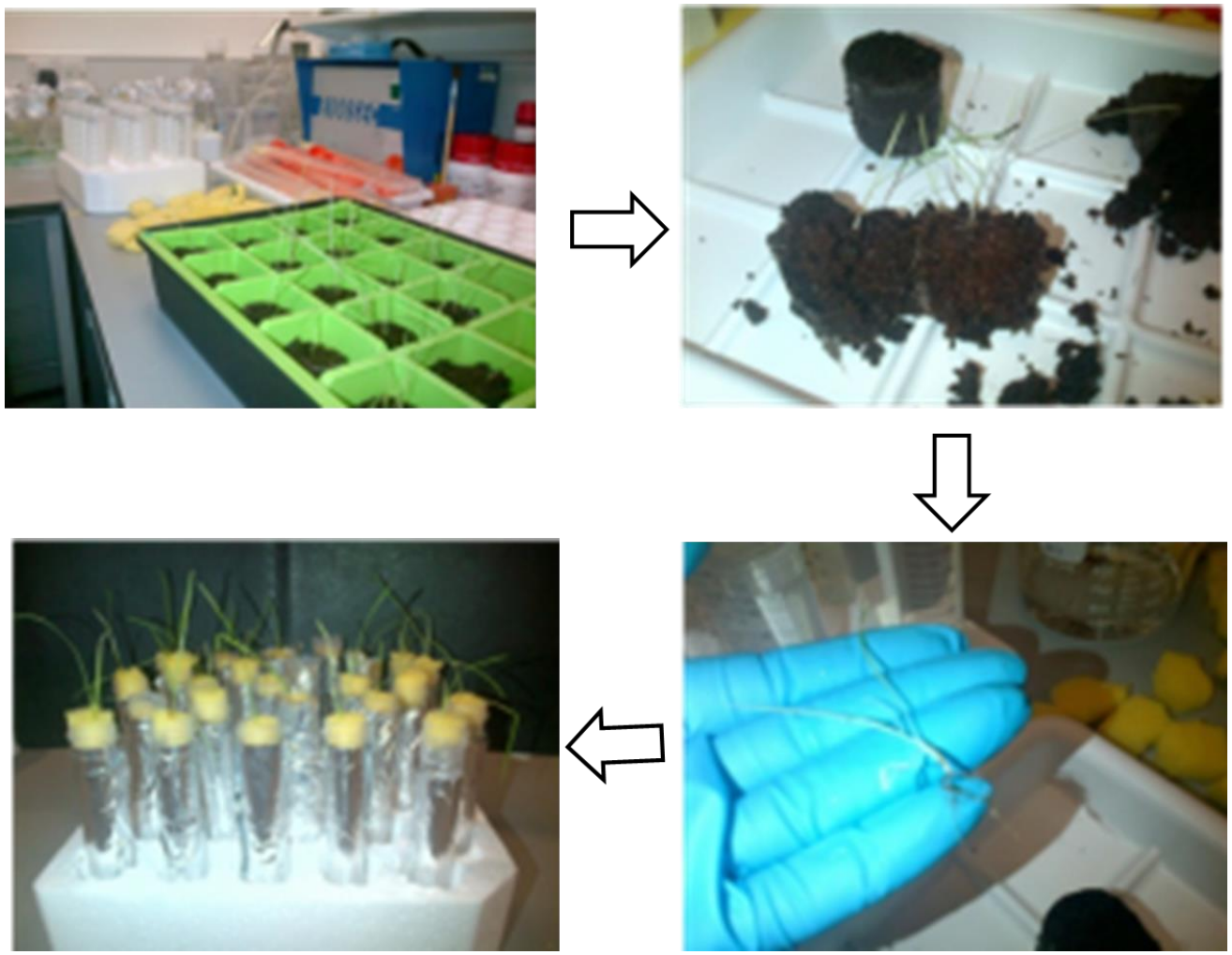


Figure 3.3 Schematic process for the development of *Phalaris arundinacea* hairy roots. From left to right: three week old plantlets in coconut coir; preparation of plantlets for hydroponic culture; cleaned root plantlets; plantlets in their respective treatments at day 0 of the experiment.

To select the appropriate concentration of Hoagland's solution to ensure healthy plant growth and the highest possible production of hairy roots, several criteria were taken into account: plant survival, length of roots, length of shoots, leaf colouration (green/yellow) and density of hairy roots which was estimated by visual inspection of the roots in relation to the filled-space in the tubes. Three weeks after the plants were first placed in the greenhouse, a semi-quantitative scale for assessing plant survival and development was used (Abundant=5, Common=4, Frequent=3, Occasional=2, Rare=1, absent=0), and plant viability was scored one (=1) for each live plant (Table 3.4).

The highest scoring treatment was that with Hoagland's solution at 50% concentration, although survival was highest with the most diluted concentration which also had the greatest root density. It appears that the lack of nitrogen is crucial for shoot and root development compared to the lack of phosphorus. Kuzbaum *et al.* (2012) also used a 50% Hoagland's concentration in their phytoremediation experiments. Once the treatment was selected, a further 75 seeds were grown following the procedure described, with the difference being that 50 cm^3 Falcon™ tubes were used instead of 15 cm^3 to provide greater volume for root development.

Table 3.4 Semi quantitative scoring for the selection of Hoagland's concentration

Phalaris arundinacea	**Hoagland's Solution**				
	100%	**50%**	**25%**	**No-N**	**No-P**
Day 0	5	5	5	5	5
Day 7 - Survival	3	4	5	4	4
Day 14 - Survival	3	4	5	3	3
Shoot development	5	4	3	2	3
Root development	5	4	1	1	2
Hair development	2	3	4	1	4
Total Score	23	24	23	16	21

3.2.8 Biofilters: hydroponic growth of *Phalaris arundinacea*

Hydroponic culture of whole plants is one of several experimental approaches available for phytoremediation and biotechnology research (Conn *et al.*, 2013). It is the third most complex experimental system, the first being the culture of whole plants in potted soil under greenhouse cultivation and the second being the cultivation of whole plants in the field (Doran, 2009).

Seeds of *Phalaris* (British Wildflower Plants, Norfolk, UK) were cultivated in sterile coconut coir pellets. After two weeks, the roots of plantlets were rinsed with tap water, rolled up individually in strips of foam and transferred to 50 cm^3 Falcon™ tubes. A hollow plastic straw was inserted into the tubes to facilitate gas interchange and the supply of the Hoagland's solution, and they were refilled with the 50% Hoagland's solution every three/four days. The plants were placed in a growing shed (1.5 m H x 1 m W x 1 m D) equipped with two blue 125 Watt compact fluorescent lamps (CFL) and a Sun Mate Grow CFL reflector. Only one of the lamps was on while the plantlets were eight weeks old, then two lamps were used beyond eight weeks. The photoperiod was adjusted to a 10:14 hr light/darkness cycle (Conn *et al.*, 2013). The photosynthetic active radiation (PAR) of 40 µmol photons m^{-2} s^{-1} (range 30-45 µmol photons m^{-2} s^{-1}) (Lindig-Cisneros and Zedler, 2001) was measured with a terrestrial quantum sensor LI-190SA and light meter LI-250A (Li-Cor International, Ltd., UK). The PAR was 100 µmol photons m^{-2} s^{-1} (range 80-120 µmol photons m^{-2} s^{-1}). The tubes were covered individually with aluminium foil to prevent light damage to the roots.

After 10 weeks, the roots were 8-10 cm length for 48 plants, and sets of 16 plants were prepared to create the biofilters. To do this, the roots were carefully passed through the mesh (1 cm) of the stainless steel cages with the shoots on top of the cage, in a similar way to the plants in the Living-Filter. The biofilters were first transferred to 2.1 L tanks, and when the plants were 18-20 weeks' old the biofilters were ready for the mesocosm filtration experiment and transferred to the acrylic units (Figure 3.4).

Figure 3.4. Left to right: (a) *Phalaris* two week old plants, b) biofilters with the 8-10 week old plants (16) (c) biofilters in 2.1 L containers; (d) Biofilter root density at 18 – 20 weeks just before starting the trial and experiments.

At the end of the mesocosm experiment, the root bio-volume was estimated by calculating the volume to the closest geometrical shape, in this case a cylinder. The roots were cut separating them from the shoots and were wrapped in a sheet of paper. The length of the measured paper corresponds to the length of the roots and the diameter is that of the "cylinder" (Faulwetter *et al.*, 2009).

3.2.9 Synthetic filter media: two types of monofilament non-woven plastic material

The synthetic filtration media consisted of two types of monofilament non-woven plastic material of different packing densities. These synthetic media were tested to compare their filtration removal efficiencies for *Microcystis* with that of the biofilters. The synthetic media is made up of plastic monofilament layers bonded by heat to create a sheet of fabric. Differences in flexibility that exist between fabric 1 and fabric 2 are based on the diameter of the filaments and the number of filament layers that create the sheet of fabric. Fabric 1 was used in the Living-Filter as a supporting material in the Living-Filter. The filament diameter of fabric 1 and fabric 2 is 0.5 and 0.03 mm, respectively. Fabric 1 is a two-layered filament sheet of 0.8 mm thickness and fabric 2 is a multi-layered filament sheet of 1.8 mm thickness. Six stainless steel cubic cages (10 cm^3) were made to contain the fabrics, which were cut in squares of 10 cm^2 (10 cm x 10 cm). Because of the difference in thickness of the multi-layered filament sheets, the number of squares or pieces used to fill the cages varied. Hence, 15 pieces from fabric 1 and six from fabric 2 were used for three cages. These pieces were placed one after another until they were tightly filling the cages. The pieces were placed in the cages with the largest surface area facing the direction of the flow. New cages were made and fabric cut for each flow experiment.

3.2.10 Removal efficiency of filtration units

Removal efficiency (RE%) was calculated for every paired inflow-outflow sample taken from the filtration units. This parameter is calculated with the assumption of a similar inlet and outlet flow rate, and is shown in Equation (3.4):

$$RE\ \% = \frac{(C_0 - C)}{C_0} \times 100 \qquad \text{(eq.3.4)}$$

where: C_0 is the concentration of *Microcystis* cells at the inlet and C is at the outlet. The units were cells cm^{-3} measured by flow cytometry (see 3.2.5.2).

3.2.11 Testing the concept of *Microcystis* removal by biofilter roots.

Mesocosm experiments were carried out at two continuous flow conditions to test for *Microcystis* cell removal by the hydroponic roots of living plants. The design at high flow-rate (54±3.0 $cm^3\ min^{-1}$) included biofilters and the two types of synthetic fabric (fabric1 and fabric2). Fabric 2 with the higher packing density was used as a positive control, and units of empty cages were used as negative controls. The design at low flow rate (1.3±0.2 $cm^3\ min^{-1}$) included biofilters, one type of synthetic fabric (fabric 1) and negative controls. The two flow conditions and three filter media experiments were each run in triplicates.

Prior to starting each of the experiments (high flow rate and low flow-rate), the filtration units filled with de-chlorinated tap water were left to stabilize for 2-3 hours (high flow rate) and 48h (low flow rate). The outflow was set to 54±3.0 $cm^3\ min^{-1}$ and 1.3±0.2 $cm^3\ min^{-1}$ for high and low flow rates, respectively. The replicates of high flow rate experiments ran for five hours. Replicates of the low flow experiment were run for five, seven and eleven days, which corresponded to two, three and approximately five hydraulic retention times. An LED lamp was inside the feed tank for the 11 day run of experiments to prevent cell death.

Samples for the high flow experiment were taken every 55 minutes at the sampling points shown in Figure 3.1, including the feed tank (x3); inlet (x1), sampling point A and B (x1).

Daily sampling (aliquots of 1 cm^3) was carried out for the low flow rate experiment from the feed tank (x3); inlet (x1) and sampling points A (x1), B (x3) (depths 1, 5 and 9 cm) for each filtration unit.

3.2.12 Data processing and statistical analysis

Derived variables (ratio, percentages and rates) and exploratory statistical analysis were undertaken using Excel (Microsoft © 2010) and SPSS v. 22.0 (IBM Graduate Advanced pack statistic software, UK). Data transformation, statistical tests explanation and criterion for interpreting the effect size was explained in section 2.2.6 in chapter 2.The non-parametric Friedman's test was used for repeated measures. The repeat level was the measure before-after the filters which is suitable because of the number of *Microcystis* cells ml^{-1} data. If the results were significant the Wilcoxon sign-rank test was used and a Bonferroni correction applied. The test statistic of Wilcoxon is *T*, and it quantifies both the direction and magnitude of all the changes in a set of matched pairs. The smaller value of the sums of ranks between the matched pairs is the test statistic *T*, and the probability of distribution was checked against the relevant statistical tables.

3.2.13 Exploring chemical mechanisms: allelopathy and nutrient competition

Observations from the behaviour of *Microcystis* cells during the low flow rate experiments needed to be investigated to identify whether allelopathy was an underlying chemical mechanism behind the removal of cells in the biofilters (Hilt and Gross, 2008; Rojo *et al.*, 2013). Two integrated samples of root exudate (from three biofilters) were screened and analysed using gas chromatography coupled with mass spectrometry (GC-MS) to identify potential allelochemical compounds. This analysis was performed at Wessex Water Scientific Centre, Bath, UK; a United Kingdom Accredited Systems laboratory for carrying out chemical and biological testing analysis in aqueous samples.

To confirm that the observed pattern in the biofilters of "lack of colour" and reduction in *Microcystis* cell number during the runs of low flow rate experiments was due to allelopathy, other chemical mechanisms such as a change in pH needed to be excluded. These variables and the temperature were measured in two biofilter and control units and carried out for five days. The pH and temperature were measured with a Hach Intellical pH probe and HQ40D meter reader. Biofilters and controls were filled with 75% Hoagland's' solution to ensure a nutrient rich media for the plants. These were compared to biofilters and controls containing just tap water. Equal volumes (300 cm^3) with a concentration $O.D._{.}$ = 0.3 at 620 nm, of *Microcystis* culture were added to each biofilter (final dilution 1:10). All units were covered with a lid so the light penetration was the same.

3.3 Results

3.3.1 *Microcystis* cell removal during high flow-rate experiments

The packing densities of the synthetic filters with fibrous media were 0.03 for fabric 1 and 0.07 for fabric 2. The estimated packing density of the biofilters was 0.05. The inlet concentration factor of biofilters and fabric 2 was ≤1, suggesting that the system operates in deposition filtration mode for both types of fibrous media (Figure 3.5-A). The biofilter RE% of *Microcystis* cells is presented in Figure 3.5-B.

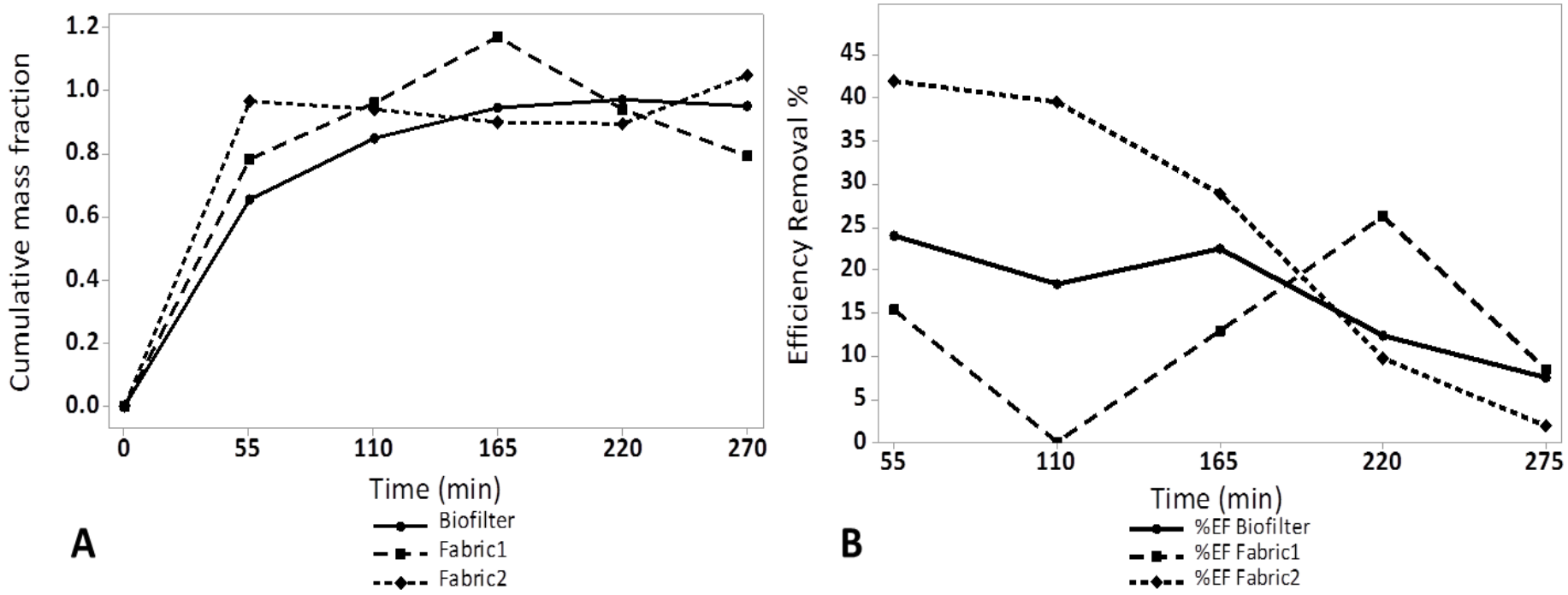


Figure 3.5-A. Inlet concentration change factor: biofilters (solid line); fabric 1 (dashed line) and fabric 2 (dotted line) as a function of time (min). 3.5-B Removal efficiency (RE%) of *Microcystis* cells by the fibrous media in relation to hydraulic retention time (min).

The results show that all units, i.e. the biofilter and fabric 2 (higher packing density) were effective in removing *Microcystis*, which was confirmed by lower cell numbers in the outflow compared to the inflow. Wilcoxon-Sign Test T=8, z=-4.34, p<0.5 with a medium effect r=-0.47 for biofilter and T=5, z=-0.46, p<0.5, also with a medium effect (r=-0.47) for fabric 2.

3.3.2 *Microcystis* cell removal during low flow-rate experiments

The results displayed in Figure 3.6-A-B are from the biofilter experiments run for five hydraulic retention time (5 HRT). Six filtration units were used for each pair of biofilters and controls, biofilters and matrix with the feed volume (100 L). The biofilter ICF was >1 at 4 HRT before dropping rapidly (Figure 3.6-A). The ICF results could have been affected by the increase in cells in the tank over periods of 48-72 hours as shown in the growth model. The results displayed for fabric 1 are from the run of 3 HRT (Figure 3.6-B). The biofilters showed RE% 40- 55% within the first three days and thereafter, dropping to RE% 10-20%; after three days the RE% for fabric 1 was 8-20% (Figure 3.6-B).

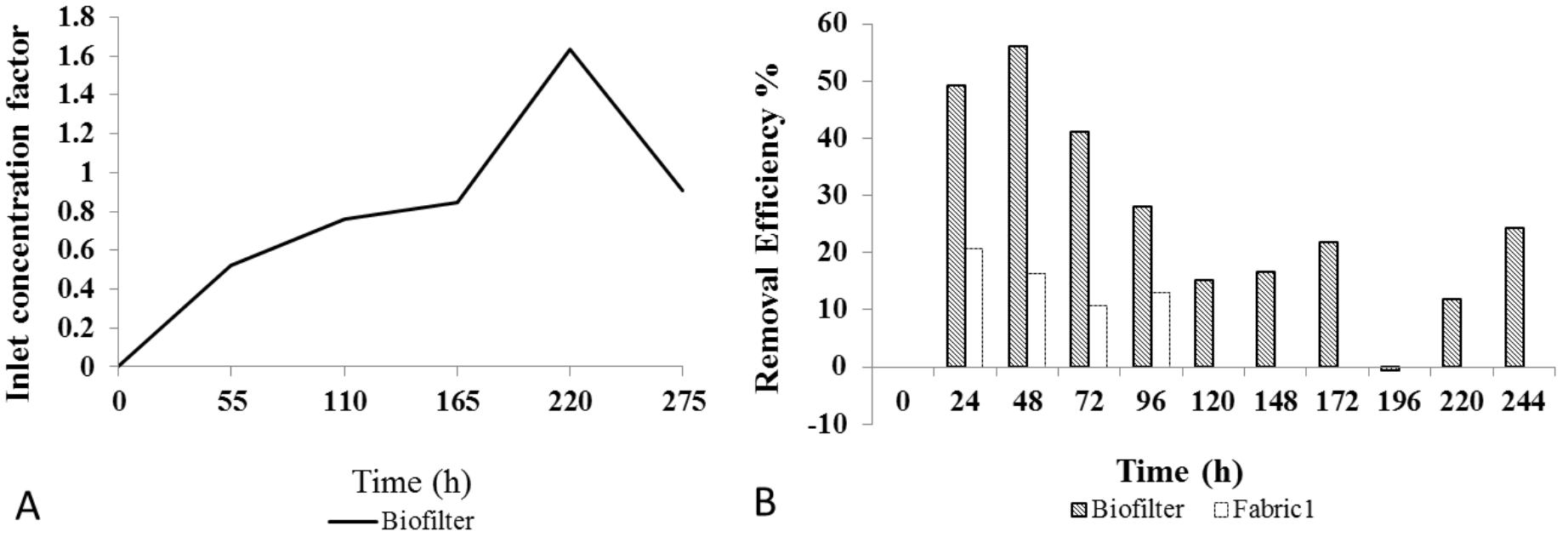


Figure 3.6-A. Inlet concentration change factor for the biofilters (solid line as a function of time in hours equivalent to hydraulic retention time). 3.6-B Removal efficiency (%) of *Microcystis* cells by the biofilter and fabric 1 in relation to time (hours).

Microcystis cell counts were increasing in the feed tank, and therefore the data were fitted to a growth curve (Figure 3.7). Results from the ANOVA showed that the data fit well to this model (r^2=0.96, adj. r^2=0.91; $F_{(1,8)}$=82.7, p=<0.05) (ln$Y$=(12.5+(0.007*t)).

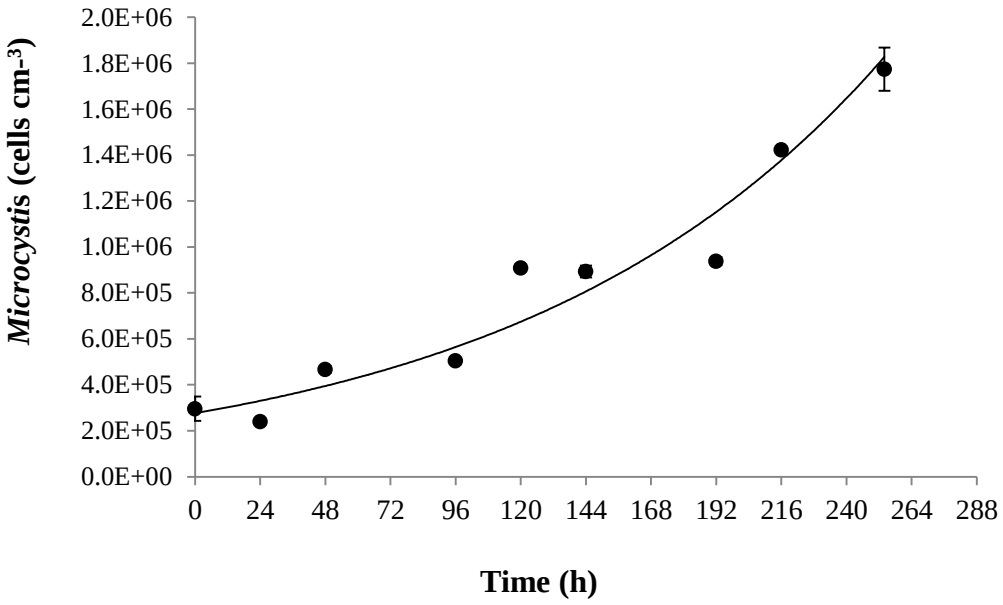


Figure 3.7 Growth curve fitted to *Microcystis* (cell counts ml^{-1}) (mean±SE) at intervals of 24 hours (h) (Time). Time is the predictor with a unit time of 24 hours; the dependent variable is the natural logarithm of *Microcystis* (mean±SE). (ln$Y$ = b_0 + (b_1 * t).

Visual differences in the colouration of the algae were observed between the biofilters, the fabric units, and the controls. Results are shown for the biofilters and the controls in Figure 3.8-A-B. The biofilters show a total loss of green colour, which is a visual indicator of the absence of *Microcystis* cells. On the other hand, the fabric units and controls show green colouration. The same qualitative pattern was observed in each unit of the biofilters, fabric units and controls, and for all replicates of the experiments at low flow-rate (run at 2 HRT and 3 HRT (x2) where O.D and a calibration curve were used for quantification of *Microcystis* cells. Cell numbers (by flow cytometry) increased four and a half times in the controls and fabric 1 (low packing density) compared to the biofilters. It was found that the number of cells

from the feed tank was not constant, with an increase in cell numbers every 48-72 hours (results not shown). However, the number of cells in the biofilters remained at $1x10^6 \pm 2x10^5$ ml^{-1} from day five onwards (Figure 3.8-B).

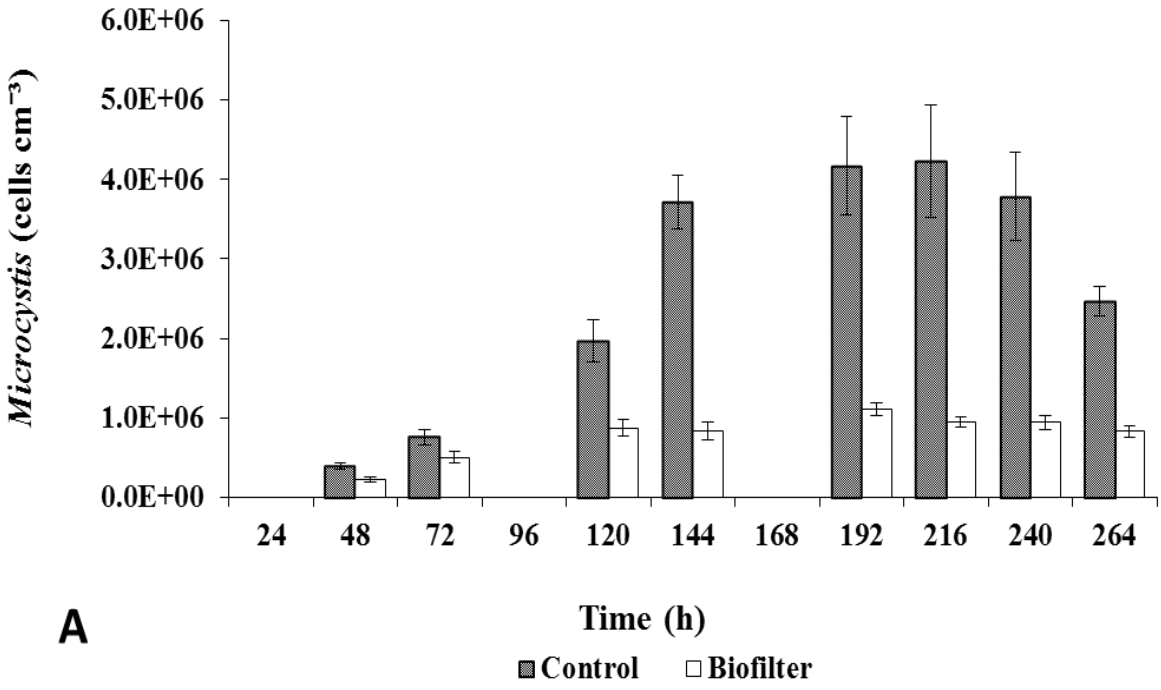
Significant differences in cell numbers were found between the inflow and outflow in the biofilters, with numbers being higher in the inflow than in the outflow (Wilcoxon sign-test T=5, z=-5.164, p<0.05, r=-0.53). No significant differences in the number of *Microcystis* cells were found in the controls.

3.3.3 Exploring chemical mechanisms: allelopathy

Allelochemicals (i.e. phenols) and other secondary metabolites (i.e. cholesterol) were found in the screened samples. Table 3.5 shows the identified compounds with the gas chromatography- mass spectrometry analysis (GC-MS) screening from the root exudate samples.

Table 3.5 Results of the GC-MS screening analysis

Chemical identified	Concentration (ng L^{-1})
Stigmasterol	660
Phenols	620
Salicylates	440
Cholesterol	250
β-cyclocitral	220



A

B.i

B.ii

Figure 3.8-A. Quantitative changes in average concentration *Microcystis* (cells ml^{-1}±SE) for a mesocosm experiment run for 11 days (5 HRT). Controls (dark), biofilters (clear) (n=3). 3.8-B. Qualitative green colouration changes in the control units (B.i) indicating the presence of *Microcystis* cells, and loss of colouration in the biofilter units (B.ii). Units were covered during experiment and uncovered for the picture.

3.4. Discussion

Microcystis is one of the most widespread bloom-forming cyanobacteria found globally in surface reservoirs, and there has been an increasing interest in eco-biological systems for its removal. The purpose of this study was to investigate whether *Microcystis* cells could be removed by the roots of *Phalaris* in biofilters, under continuous flow conditions. A further investigation established the possible interactions between inflowing *Microcystis* cells with biofilters and synthetic filters.

3.4.1 Were there differences in the cell removal efficiency between *Phalaris* roots and the plastic filters at high flows (54±2 ml min^{-1})?

The results from this experimental mesocosm demonstrate removal of suspended particles by filamentous biofilters and synthetic plastic fabric under continuous flow conditions. *Microcystis* cell removal by physical processes in filamentous biofilters has not been reported before.

Microcystis cells (as suspended particles) in relation to both filamentous media (natural and synthetic plastic) are transported within the fluid media without accumulation either before the perforated plate or the filter media (ICF=1) (Figure 3.5-A). Differences in the inlet concentration change factor at different retention times demonstrated that there is less hindrance to the cells passing through fabric 1, reaching 1 at 55 mins than for biofilters, where the inlet concentration factor reaches 1 at 220 mins.

Filamentous biofilters show a modest performance at removing phytoplankton cells (25%) by filtration processes under the high flow treatment. By comparison the removal efficiency of the plastic filters (fabric 2) was 15% higher than the biofilters (40%). These results are contradictory to the findings of Tanner and Headley (2011) where a greater reduction (34-42%) of suspended particulate load was found from natural roots in FTWs compared to artificial floating plastic roots (26.8%), or unplanted floating mats (17-21%). By

contrast, their mesocosm did not consider flows as a variable and the FTWs (0.36 m^2) used consisted of a floating mat with enriched soil. They contest that FTWs provide additional advantages, such as a source for biofilm attachment, changes in physico-chemical conditions, or release of bioactive compounds. Whilst Tanner and Headley (2011) did not mention the potential role that the release of bioactive compounds (e.g. allelochemicals) from FTWs could have in aquatic environments, Nakai *et al*., (2008) suggested the potential applicability of FTW to control cyanobacteria.

The field-scale Living-Filter RE% (≤45%) and mesocosm experiment findings showed that filamentous media (natural roots and synthetic) in aquatic environments under continuous flow conditions can remove phytoplankton biomass or *Microcystis* cells. The findings suggest that there might be an additive effect and that by combining biofilters and synthetic plastic filters their removal efficiency would increase and should be considered in future designs.

3.4.2 Were there differences in the cell removal efficiency between *Phalaris* roots and the synthetic filters at low flows (1.2±0.3 ml min^{-1})?

During low flow rates, the biofilters showed removal efficiencies twice as high as those observed under high flow rate conditions suggesting, that the slow flow velocity might affect the removal process in a positive way. These results are interesting, because despite injecting an increased number of cells from the feed tank over periods of 48-72 hours which followed a sigmoidal growth curve, this increase in cells was not observed in the biofilters, only in the controls and fabric. A growth model fitted to the cell abundance data in the feed tank explained 91% of the variance of the data (Figure 3.7). This growth could also have affected results for the ICF (Figure 3.6-A), as an increasing cell concentration would directly affect the ratios. The resulting increase in cell numbers was seen in the control and fabric units, but not in the biofilters. Hence, the number of cells in the controls, ≥ 3.0 x10^6 cells ml^{-1}, was at least

twice as high as in the biofilters, where they did not increase above 1.2 x10^6 cells ml^{-1} (Figure 3.8-A).

The most striking results found during the low flow experiment were the loss of colouration (bleaching) (Figure 3.8-B). "Bleaching" or chlorosis is defined in the literature as the change in pigmentation from blue-green to yellow-green to yellow (or orange) (Collier and Grossman, 1992; Baier *et al.,* 2014) in non-N_2 fixing cyanobacteria (like *Microcystis*) under nitrogen starvation conditions. Chlorosis is a process generally observed after 72 hours of nitrogen starvation in cultures of cyanobacteria model organisms, such as *Synechocystis* and *Synechococcus* (Krasikov *et al.,* 2012). However, there is no reference in the literature to the chlorotic process as a complete lack of colouration (as clear as tap water) as was observed in the biofilters. The chlorotic process of *Microcystis* in the biofilters is observed after 72 hours, and at 96 hours there is a total absence of colouration. Moreover, these cells were re-cultured but no growth was observed. Dagnino *et al.* (2006) described a chlorotic process in nutrient-depleted *Microcystis* cultures. After 3-5 days, they showed lack of colouration, but from blue-green to very pale blue-green and decreasing cell density. They explained this as a result of intercellular signalling present in the medium of the nutrient-starved *Microcystis*. Intercellular signalling is a chemically mediated process between microorganisms (e.g. cyanobacteria and algae/organisms of higher taxa) also known as allelopathy (Gross *et al.,* 2003). Allelopathy is the direct or indirect, harmful or beneficial effect of an organism, generally a plant, on another organism (plant, animal, fungi or bacteria) by the release of chemical compounds (allelochemicals) into the environment (Leao *et al*., 2009). Reduction in cell numbers was also seen, measured and compared between the biofilters and the controls. These results suggest that either chemical or biological mechanisms occur in the biofilters. Chemical mechanisms are: nutrient competition between the roots in the biofilters and *Microcystis* cells, or allelopathy with inhibiting/biocide effects (or both) where the allelochemicals induced chlorosis in the *Microcystis* cells. It is important to point out that the

Microcystis cells were continuously injected to all the units in increasing concentrations, but the only units with loss of colouration and reduction in cell numbers were the biofilters.

3.4.3 Were there other mechanisms involved in the removal of *Microcystis* cell numbers from the biofilters?

These preliminary results suggest that the chlorotic effect could be triggered by allelopathy and the reduction in *Microcystis* cell numbers in the biofilters could be triggered by allelopathy, nutrient competition or biological mechanisms. Allelochemical compounds with biostatic/biocidal effects (Laue *et al.*, 2014) were identified from aqueous root exudate in the screening analysis at nanogram concentrations (Table 3.5). These concentrations mimic natural aquatic environments, where the allelochemicals released by macrophytes are diluted in the surrounding water (Hilt and Gross, 2008; Rojo *et al.*, 2013), with suppressive effect on phytoplankton growth rates (Korner and Nicklisch, 2002; Mulderij *et al.*, 2007; Chang *et al.*, 2012). By contrast, laboratory experiments are generally carried out at low volumes (microliters), with *Microcystis* cells at concentrations of $1x10^5$-$1x10^6$ and in the presence of milligram concentrations of allelochemical compounds. These allelochemical compounds are either extracted from the roots (after maceration with methanol 70%, evaporation and resuspension in methanol) or supplied commercially to demonstrate their biocidal effect (Lürling and Beckman, 2013; Lürling and Van Oosterhout, 2014).

Preliminary results suggest for the first time that root exudates allelochemicals released from the roots of *Phalaris* in an aqueous environment reduce *Microcystis* cell numbers through a chemical mechanism in low flow conditions. However, it is possible that nutrient competition as another mechanism might be operative as discussed by Inderjit and Del Moral (1997) or more recently by Lürling *et al*. (2006). In order to further investigate this mechanism in aquatic systems, quantification of allelochemicals from aqueous root exudate samples at low concentrations is necessary.

Chapter 4. A simultaneous method for multiple allelochemical detection, identification and quantification using high performance liquid chromatography

This chapter focuses on the development of a simultaneous method for determining multiple allelochemicals in aqueous samples from root extract and exudate using high performance liquid chromatography with an ultraviolet diode array detector (HPLC-UV-DAD) and solid phase extraction (SPE).

4.1 Introduction

The results of the reduction in *Microcystis aeruginosa* (hereafter *Microcystis*) cell numbers ml^{-1} (*Microcystis* growth) in the biofilters during the low flow experiments, accompanied by the "bleaching" of the cells and the screening confirmation of allelochemical presence by gas chromatography mass spectrometry (GC-MS) suggests that a chemical mechanism is operative.

Interactions between macrophytes and phytoplankton in aquatic environments are described via two main mechanisms: resource competition and allelopathy (Inderjit and Del Moral, 1997; Gross *et al*., 2007) and the separation of their individual contributions still remains difficult and continues to be the subject of ongoing debate and investigation. Resource competition in aquatic environments refers to competition for abiotic factors (e.g. light) and biotic factors. The former includes macronutrients (e.g. nitrogen and phosphorus) and micronutrients (e.g. cobalt and zinc) (Vanderstukken *et al*., 2011; 2014). Unlike resource competition, allelopathic effects depend on the addition of a chemical compound, named allelochemicals, to the environment, and can be regarded as a form of interference competition (Begon *et al.*, 2006). Such interference occurs among a variety of organisms, from higher plants to macro- and microalgae, bacteria and viruses (Van Donk *et al*., 2002;

Legrand *et al*., 2003; Ger *et al*., 2016). Therefore a first step to investigate allelopathic interactions is to identify and quantify the existing allelochemicals. Allelochemicals can be termed algastatic/ algicide or cyanostatic/ cyanocidal. However, the term algastatic or algicide is used for phytoplankton in general.

A methodological approach proposed by Willis (1985) for allelopathic research consists of six requirements: 1) a clear pattern of inhibition between two species; 2) identification of allelochemical production by one of the two species; 3) allelochemical release from the plant into the environment or surroundings; 4) allelochemical transportation and/or accumulation in the environment or surroundings; 5) uptake by the target species, and 6) that the observed pattern of inhibition cannot be explained solely by physical factors or other biotic factors, especially competition and herbivory. Despite his proposed methodology, Willis (1985) argues that meeting these six points does not prove that allelopathy is operative, only that it offers the most reasonable explanation of the observed pattern. A pattern of inhibition was observed in this research as presented in section 3.3.2 in chapter 3, under continuous low flow conditions.

The scientific literature gives account of identified allelochemicals in cereals, but many of these allelochemicals can be broken down into derivative compounds of four major groups: phenolic compounds, alkaloids, quinones and hydroxamic acids (Sanchez-Moreira *et al.* 2003). Phenolic compounds are naturally present in plants, containing at least one aromatic ring with hydroxyl and other radicals; this group includes flavonoids e.g. naringin and tannins e.g. tannic acid. Alkaloids are a very diverse group that share a nitrogen atom with a heterocyclic ring and a common origin from aminoacids and bases (Guirimand *et al*., 2010). This group include indole alkaloids e.g. gramine and 4-nitroindol-5-carboxaldehyde. Quinones are also a large group that derive from conversions of polycyclic aromatic compounds that form very reactive bioactive compounds e.g. 9, 10-anthraquinones (Nikai *et al.* 2008; Bahrs *et al*., 2013). Hydroxamic acids are weaker hydrophilic organic compounds

than the structurally related carboxylic acids (Kakkar, 2013). Other compounds that have allelopathic activity are fatty acids, such as polyunsaturated fatty acids e.g. linoleic acid and stigmasterol (Bertin *et al*., 2003)

A major difficulty for the isolation of bioactive compounds is that they are often produced either in the laboratory or the field below detection limits. These are generally ≤100 ng ml^{-1} and detection with a high performance liquid chromatography with ultraviolet diode array detector (HPLC-UV-DAD) can be challenging. Detection of such low concentrations are possible when more sophisticated systems are coupled with mass spectrometry (MS), or tandem MS/MS, GCMS or GCMS/MS, both with ion-quadruple detectors (Li *et al*., 2011). Production of highly active compounds at such low concentrations is a more energy-efficient strategy for the species producing the bioactive compound. Furthermore, in aquatic environments, the distances between cells are critical, because chemical information is transmitted by diffusion and advective laminar flow (Wolfe, 2000) for which allelopathic compounds with a small molecular weight are favoured because of their faster diffusion. Added to this are the distances between cells, which are important as the allelochemicals must reach the target species in order to have an effect. Furthermore, the concentration of the allelochemicals will be diluted as the distance travelled between the producer plant organs and the target cells is increased (Nan *et al.*, 2008; Bauer *et al*., 2009). The dilution of these compounds in such environments is a major problem for measuring concentrations (Rojo *et al*., 2013).

The plants used in the Living-Filter: *Phragmites* and *Phalaris* (also in the biofilters) belong to the Poaceae family; and *Carex* to the Cyperaceae family. Moreover, many plants used in FTW frequently include at least one genera of the Poaceae family. This family includes cereals used for human and animal consumption, for example, barley, rice, corn and wheat among others (Brezinova and Vymazal 2014; Kellogg 2015). *Hordeum vulgare* L. (barley straw) is one of the cereals most studied in remediation, such as the management of

cyanobacteria blooms because of the presence of phenolic allelochemicals released with decomposing barley with known algicidal activity (Murray *et al*. 2010; Bahrs *et al.* 2013).

Ostrem (1987) was the first to identify nine alkaloids in *Phalaris* spp. which include hordenine, gramine, methyl tryptamines, and carbolines. *Phalaris* seeds contain highly saturated fats including: linoleic acid, oleic acid, palmitic acid and linolenic acid (Abdel-Aal *et al.*, 2011; Li *et al.*, 2011). Other lipophilic compounds that can be found in *Phalaris* are tocopherols, carotenoids, sterols, and lecithin (Slavin and Yu 2012).

Following the methodology of Willis (1985), and taking into account the information on allelochemicals in cereals, the results and observations on the reduction of *Microcystis* cell numbers reported in chapter 3, provides further direction to the research. This chapter will investigate the production, identification and quantification of allelochemicals in the roots of the Living-Filter plants and biofilters, as wells as in the biofilter root exudate.

Several analytical methods have been used to identify allelochemicals, such as; the use of high performance liquid chromatography (HPLC) with an ultraviolet (UV)/ diode-array detector (DAD) coupled to either mass-spectrometry (MS) or the electrospray ion trap mass spectrometry technique (HPLC-DAD-ESI-MS/MS), or gas chromatography (GC-MS/MS) (Barrajon-Catalan *et al*., 2011; Nakai *et al*., 2008, 2012; Gaujac 2012; Skhryl *et al*., 2016). These methods generally are developed to target a particular group of allelochemicals, for example phenolic acids (Li *et al*., 2011), anthraquinones (Shkryl *et al*., 2016), alkaloids (Zhou *et al*., 2006) and less frequently developed to identify several groups (Mizukami *et al.*, 2007). For the latter, the method developed used the allelochemical bands from a thin layer chromatography (TLC), and then the allelochemicals were confirmed by HPTLC and later validated using HPLC-MS. This is a very laborious and costly procedure for identifying allelochemicals from different chemical groups. By contrast, methods developed to use a single HPLC-UV-DAD run for multiple identification and separation of various

allelochemicals include those of Mizukami *et al.* (2007) for catechins, gallic acid, strictinin, and purine alkaloids in green tea, however, these are all alkaloids. Slavin and Yu (2012) developed another method for tocopherol, carotenes and phytosterols (e.g. stigmasterol) from soya beans. The main advantage of these methods is that they allow the analysis of a sufficient number of samples to enable understanding of the variability in allelochemical levels associated with plant genetics and growth conditions (Slavin and Yu, 2012)

In this research, the need to develop a method for the detection, identification and quantification of allelochemicals or potential allelochemicals in aqueous samples was paramount. This came after realizing the high costs of GC-MS analysis for the investigation of allelochemicals for a large number of aqueous samples, and the absence of a simultaneous method (i.e. involving multiple allelochemicals) for this purpose, either by GC-MS or HPLC, in the organic chemistry laboratories in Oxford. Although allelochemicals can be produced and released into the environment by most plant organs, studies on the detection, identification and quantification of allelochemicals or potential allelochemicals in aqueous samples from root-wet extracts from *Phragmites, Phalaris or Carex* and root exudate from *Phalaris* have not previously been described in the literature. Moreover, the development of this new method is critical for the progression of this research as will be seen in subsequent chapters. The method will be referred to the 'simultaneous allelochemical method' or 'simultaneous method' in this thesis.

The research question posed for this chapter is: Can the simultaneous allelochemical method detect identify and quantify multiple allelochemicals in aqueous samples?

4.2 Materials and methods

In this section, the analytical methodology for the development of the simultaneous method for detection, identification and quantification of allelochemicals in root wet-extracts and exudates using (SPE) and HPLC-UV-DAD is summarised, with a detailed description

where is indicated in Appendix A. The steps to purify and concentrate aqueous samples with organic content by the SPE process and to be used for analysis in the HPLC are given, together with the steps for method validation, precision and accuracy. Finally, the procedure for processing the root biomass to obtain aqueous extracts, and root exudates, to be purified and concentrated by SPE, is presented. The sample processing procedure and the application of the simultaneous method will be used in subsequent chapters (chapters 5 and 6).

4.2.1 Allelochemicals selection and preparation

The selection of nine chemicals used as reference in the method is based on the GC-MS screening analysis results from the root exudate samples (section 3.3.3.1, chapter 3), on published literature for *Phalaris* and Poaceae, and on their known algastatic/algicidal or cyanostatic/cyanocidal activity against cyanobacteria and micro-algae (Hong *et al*., 2009; Shao *et al*., 2013; Xiao *et al*., 2014).The allelochemicals purchased from Sigma-Aldrich were: anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringin, stigmasterol, tannic acid and 4-indole-5-carboxaldehyde. The molecular weights and purity of the allelochemicals are shown in Table 4.1 and the chemical structural formulae can be seen in Appendix C.

Stock solutions for each allelochemical were determined by weighting 10 mg of each compound using a digital four point calibrated balance (ATILON, Acculab-Santorious group, UK) in a weighting boat and transferring this into a 100 ml volumetric flask (Fisher) with methanol (quality for pesticide residue analysis, 99.9%, Fluka – Sigma-Aldrich, UK) to obtain a final concentration of 100 mg L^{-1}. These stock solutions were used to prepare the final concentrations in the experiments, and were also used in the method development for identification and quantification of allelochemicals in aqueous and root material and to determine the percentage inhibition on *Microcystis* growth in chapter 5 (Figure 4.1).

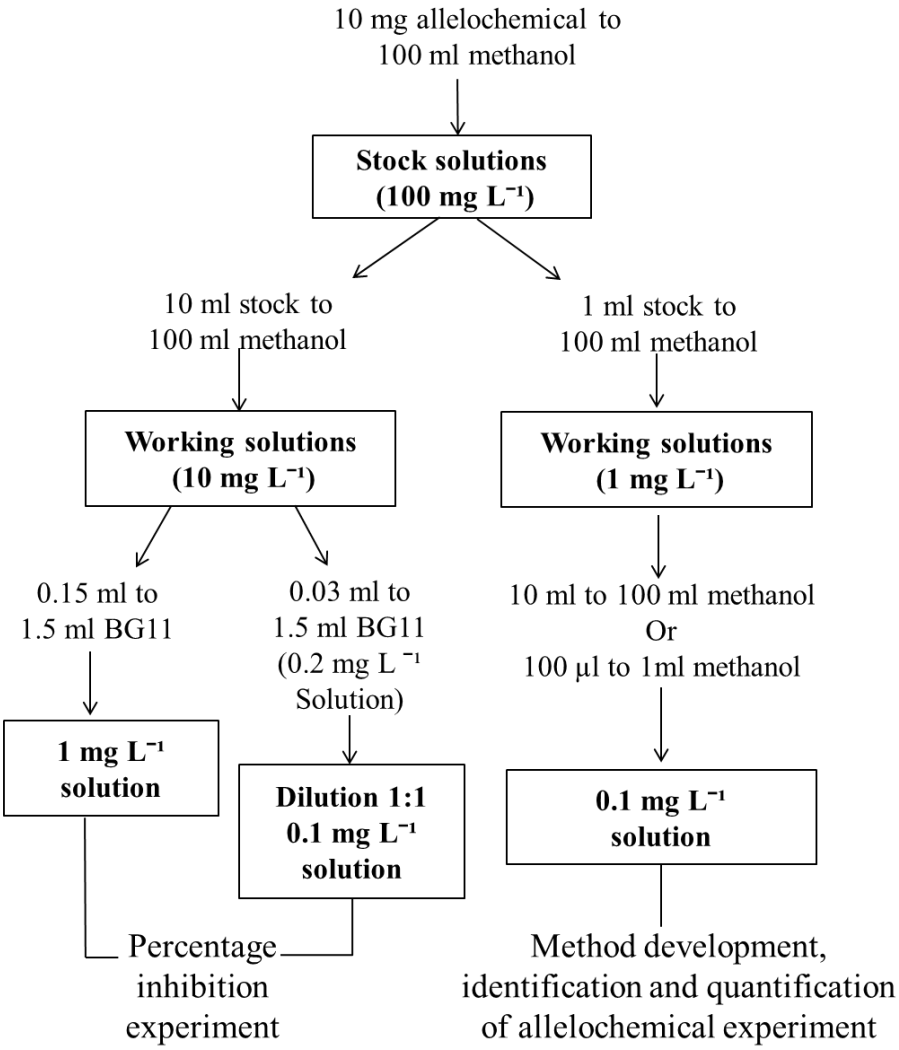


Figure 4.1 Flow chart illustrating the preparation of allelochemical solutions: a) percentage concentration for inhibition of *Microcystis* growth, and b) method developed for allelochemical identification and quantification in aqueous and root material.

Table 4.1 Summary of the allelochemicals purchased from Sigma-Aldrich, UK. A summary of their synonyms, type of chemical, empirical formulae, molecular weight and purity.

Compound	Synonym	Type	Empirical formulae	Molecular weight ($g\ mol^{-1}$)	Purity
Anthraquinone	9,10-dihydro-9-10-diketoanthracene	Quinone	$C_{14}H_8O_2$	208.21	Analytical standard
Gallic acid	3,4,5-Trihydroxybenzoic acid	Phenol	$(HO)_3C_6H_2CO_2H$	170.12	97.5-102%
Gramine	3-(Dimethylaminomethyl) indole	Alkaloid	$C_{11}H_{14}N_2$	174.24	≥99%
Hordenine	4-(2-Dimethylaminoethyl)	Alkaloid	$C_{10}H_{15}NO$	165.23	≥97.5%
Linoleic acid	*cis*-9,*cis*-12-Octadecadienoic acid	Fatty acid	$CH_3(CH_2)_4\ CH{=}CHCH_2\ CH{=}CH(CH_2)_7CO_2H$	280.45	≥98.5%
Naringin	4′,5,7-Trihydroxyflavanone 7-rhamnoglucoside	Polyphenol	$C_{27}H_{32}O_{14}$	580.53	≥95% (HPLC)
Stigmasterol	3β-Hydroxy-24-ethyl-5,22-cholestadiene,	Sterol	$C_{29}H_{48}O$	412.69	Analytical standard
Tannic acid	Gallotannin	Polyphenol	$C_{76}H_{52}O_{46}$	1701.20	Analytical standard
4,5-Nitroindol-carboxaldehyde	3-Formyl-4-nitroindole	Alkaloid	$C_9H_6N_2O_3$	190.16	≥97%

4.2.2 Standards, mixed-standards and non-extracted standards preparation

The working solutions (*hereafter* standards) of the nine analytes (anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringin, tannic acid, 4-5 indole-aldehyde and stigmasterol) were prepared, using methanol as the solvent, to a final concentration of 1 mg L^{-1}. Also, nine individual analytes were prepared to a final volume of 100 ml (1:100) and three mixture standard solutions. From this point onwards in this chapter the term standard is applied to the mixed solutions of analytes. The term 'analytes' refers to allelochemicals that are not in a mixed standard.

A mixed standard solution (mix-A) included anthraquinone, gallic acid, gramine, hordenine, naringin, 4-nitroindol-5carboxaldehyde and stigmasterol (6:100). A mixed solution (mix-B) of two standards was prepared with stigmasterol and linoleic acid (2:100) and another mix solution (mix-C) with all of them (9:100). The initial spectrum for individual standards was obtained by measuring the absorbance of 1 ml of the standards in a quartz cuvette in the Shimadzu UV-1800 spectrophotometer with a wave-length spectrum from 190 to 400 nm. The internal standard used in the method was naringin (Goufo *et al*., 2014) (Appendix C).

4.2.3 Purification and concentration of analytes by solid phase extraction procedure

This process is aimed at purifying and concentrating the investigated compounds that would have been otherwise undetected by HPLC-UV-DAD as they might be present at low concentration in the samples. It is also used to determine the recovery of the analytes by the SPE procedure. Thus, a mixed-standard sample is extracted by SPE and analysed by HPLC-UV-DAD. The results of the analytes are compared to that of non-extracted standards.

The SPE was carried out using columns of 3 ml volume capacity containing 100 mg of non-polar polymeric polystyrene divynilbenzene (PS-DVB) particles (Isolute® 101, Biotage). These columns were attached to a 12 port Visiprep manifold (Supelco™ – Sigma-Aldrich). Each port is provided with valves for flow control and PTFE needles. The vacuum pressure in

the Visiprep chamber is control by the valve and manometer. The process is aided in some steps by a vacuum pump. The SPE involved five steps: column conditioning, sample adsorption, elution, drying and resolution of eluent, which are fully detailed in Appendix B-2.

4.2.4 Instrumental determination, quality assurance and quality control

The Agilent 1200 LC series system used consisted of a binary pump, auto-sampler, column oven and UV-DAD detector. A HPLC Agilent column (C18, 150 mm x 5 mm) was used to achieve separation with an aqueous / organic mobile phase gradient elution consisting of acidified (0.1% acetic acid) DI Water and acidified (0.1% acetic acid) acetonitrile, with starting conditions of 5% acetonitrile to 95% water (5:95). Solvent conditions were tested prior to accepting the method. The column was protected by a 20 mm x 3.9 mm guard column of the same packing material. The binary pump was used with two solvent reservoirs to deliver the appropriate eluent mix. Column temperature was maintained at 40°C. Following injection at 5% acetonitrile: 95% acidified water (5:95), isocratic conditions were held for 10 min, followed by a 5 min linear gradient to 95% acidified acetonitrile: 5% acidified water (95:5), held for 5 min, then a subsequent 5 min linear gradient to return to initial conditions (5:95). Total run time was 45 min. The UV absorbance detector collected data at three wavelengths 205, 250 and 280 nm. Peak identity was validated through the use of retention times of external non-extracted and extracted standards with their respective spectrums. All samples were kept at 5°C at all times. The HPLC operating system used was the Agilent ChemStation software.

4.2.5 Peak validation

Peak identity was validated through the use of retention times of the extracted and non-extracted standards and spectrum characteristics (Mizukami *et al*., 2007). The spectra of the peak standards in the Agilent 1200 LC series were compared with those obtained in the Shimadzu UV-1800 spectrophotometer.

4.2.6 Calibration, sensitivity and recovery

Standard calibration curves were constructed with the non-extracted mix standards (A, B and C) with three concentrations (100, 500 and 900 µg L^{-1}) to a final volume of 1 ml; each was injected in triplicate. The internal standard present in the samples (at a concentration of 100 µg L^{-1}) was used to verify the concentrations of these dilutions (Mizukami *et al*., 2007). These dilutions were not extracted by SPE serving as a reference and quality control for extracted standards by SPE (Slavin and Yu, 2012). The extracted mixed-standards had a final concentration of 0.9 mg L^{-1} spiked with 0.1 mg L^{-1} of the internal standard. The peak area was plotted against the concentration of prepared non-extracted standards and concentrations were all calculated with the ChemStation software.

The sensitivity of the method was determined by the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ are based on the peak-to-peak noise of the baseline near the analyte peak obtained by the analysis of the internal standard solution (Villagrasa *et al*., 2009), as shown in equations 4.2 and 4.3 respectively:

LOD (mg L^{-1}) = (3.3 x standard deviation of the analyte concentration) / slope of the calibration curve of the analyte (eq. 4.2)

LOQ (mg L^{-1}) = (10 x standard deviation of the analyte concentration)/ slope of the calibration curve of the analyte (eq. 4.3)

The recovery of the extraction method was determined through the use of three sets of spiked samples with the internal standard, performed in three independent measurements and calculated as in equation 4.4:

% Recovery =

(Extracted mixed-standard mg L^{-1} / Non-Extracted mix-standard mg L^{-1}) x 100

(eq. 4.4)

4.2.7 Precision and accuracy of the method

The precision and accuracy of the analytical method is based on the intra-day and inter-day variability of the non-extracted mix standard (Mizukami *et al*., 2007; Villagrasa *et al*., 2009). The precision is assessed by injecting the non-extracted standard up to nine times in the same day. The accuracy is assessed by performing inter-day analyses of the non-extracted standard solutions, injected in triplicate on at least two separate sequential days. The triplicate analyses were tested as single data points to calculate the relative standard deviation expressed in percentage (RSD%) between days for each compound.

The RSD% was calculated by dividing the standard deviation of the analyte in a sample by the average of the analyte in the sample, and the result was multiplied by 100.

4.2.8 Quantitative analysis and calculations using external and internal standard procedures

These two methods are carried out with the ChemStation software provided by the HPLC-UV-DAD, but the procedure is presented here as it is used for semi-quantitative analysis. The magnitude of response to the analytes in the samples is quantified using an external standard analysis (ESTD) with four point multi-level calibration, which includes three concentrations and a point at the origin. The multi-level calibration can be used to confirm linearity of the calibration range. The regression analysis equation obtained from the calibration curve describes the best-fit of the line through the data points (eq. 4.5)

$$y = mx + c \qquad \text{(eq. 4.5)}$$

where: x = peak area; *m*= the slope of the regression line; *c*=the intercept of the regression line with the y-axis; .*y*= the concentration of an analyte in a sample.

This method allows the quantitative determination of analyte concentrations within the range of the external standard concentrations and semi-qualitatively for peaks out of the range of calibration by extrapolation..

The internal standard (ISTD) analysis adds a known concentration of a compound to be used as a normalizing factor, being added to the blank, non-extracted and extracted standards and samples; it compensates for losses during sample preparation and solid phase extraction. The ratio between the maximum ISTD area in the same set of samples (batch), and the peak area of the internal standard for a sample is obtained. This ratio is the ISTD factor and is applied to all the peak areas in a particular sample. For example, if the batch contains 10 samples, there will be 10 internal standard factors. Therefore all peak areas are normalized in relation to the internal standard. This method allows for the normalization of the peak areas of all standards and samples.

4.2.9 Sample preparation for aqueous root extract and exudate

To investigate allelochemicals in aqueous samples from root extracts from *Phragmites*, *Phalaris* and *Carex* and root exudate obtained from the biofilters containing, laboratory grown *Phalaris* (section 3.4.2.1), the procedure for sample processing is as follows (Figure 4.2).The roots are cleaned, disinfected with 1% sodium hypochlorite (NaOH) solution, and finally rinsed and dried with a paper towel. The roots were then weighed on a digital balance (AND F3000t A&D Company Limited, UK) and their volume estimated by water displacement in a measuring cylinder of 1 L capacity. Then the roots are cut coarsely (e.g. with a blender) and 300 ml of 30% methanol in DI water is added. The samples are filtered and centrifuged until there is a clear separation of root material and the supernatant. A 100 ml volume of the supernatant is concentrated by SPE. All samples were eluted from the columns with 1 ml of 1% formic acid in methanol; the extracts were air dried (0.8 L min^{-1}) through a Visidry (Supelco – Sigma-Aldrich) and the dried residue was resuspended with 1 ml 10% acetonitrile: 0.1 % acetic acid in DI water.

Root exudate samples of 100 ml taken from each biofilter and were concentrated by solid phase extraction (SPE) using a Visiprep (Supelco™ – Sigma-Aldrich).

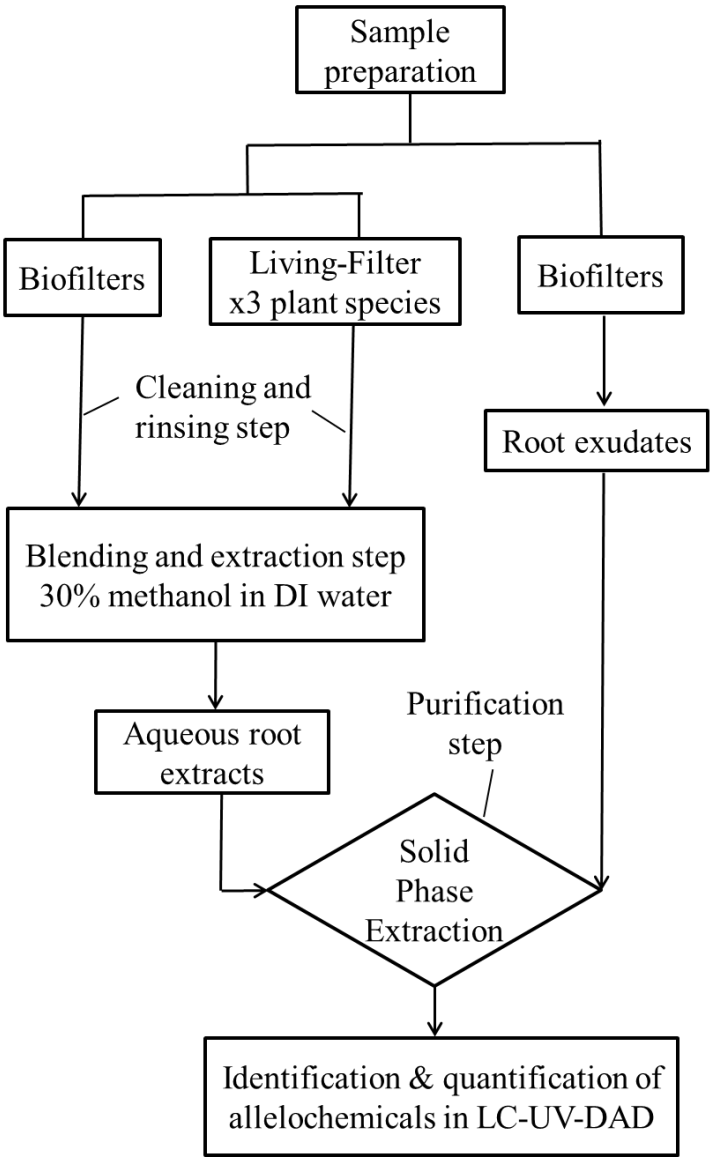


Figure 4.2 Diagram of the sample preparation procedure for the identification and quantification of allelochemicals in root exudate and root extract.

A maximum of 10 samples was extracted at a time. A blank and a mix-standard were always extracted alongside the samples to complete a batch of 12 samples for SPE. The blank and mixed-standards were prepared with de-chlorinated tap water with some granules of sodium thiosulphate to prevent the potential suppression of organic chemical compounds (Sigma-Allelopathy, UK). The blank was spiked with the internal standard to a final concentration of 100 µg L^{-1}. The mixed-standards were prepared in 100 ml flasks with 1 ml of the mixed-standard solution (1:100). The internal standard is added to all samples

immediately before the samples pass through the cartridges. All samples in the batch were processed at the same time.

4.3 Results

4.3.1 Simultaneous allelochemical method

Analysis was carried out using an Agilent 1200 Series LC-UV-DAD system at the laboratories of the Scientific Centre, Wessex Water Ltd. The data obtained were processed and analysed using Agilent ChemStation software.

Several attempts (up to 15) were made: using different solvents (methanol and acetonitrile); using gradient concentrations of these solvents (percentage of the organic solvent and ultra-pure water); applying solvent acidification or using unacidified solvent, varying the volume of the injection (50 µl and 100 µl), varying the elution time for each sample (30 min to 55 min) and varying the selection of the range of wavelength.. The quality assurance and quality control of the instrumental method was evaluated by measuring sensitivity, selectivity and precision (repeatability and reproducibility) (Villagrasa *et al*., 2009).

4.3.2 Peak validation

Results from the method developed to identify allelochemicals by HPLC-UV-DAD are shown in Table 4. 2. The table shows the analysis of the non-extracted standards with their respective retention times. As seen on Table 4.2, six of the nine analytes in the mix standard were detected. The undetected analytes were: the phytosterol stigmasterol; the fatty acid linoleic acid; and the phenolic acid tannic acid.

The spectrum of the peaks corresponded to those obtained individually in the UV-1800 and double checked with their spectrum in the HPLC-UV-DAD from the stock solutions and from the non-extracted and extracted mixed-standards.

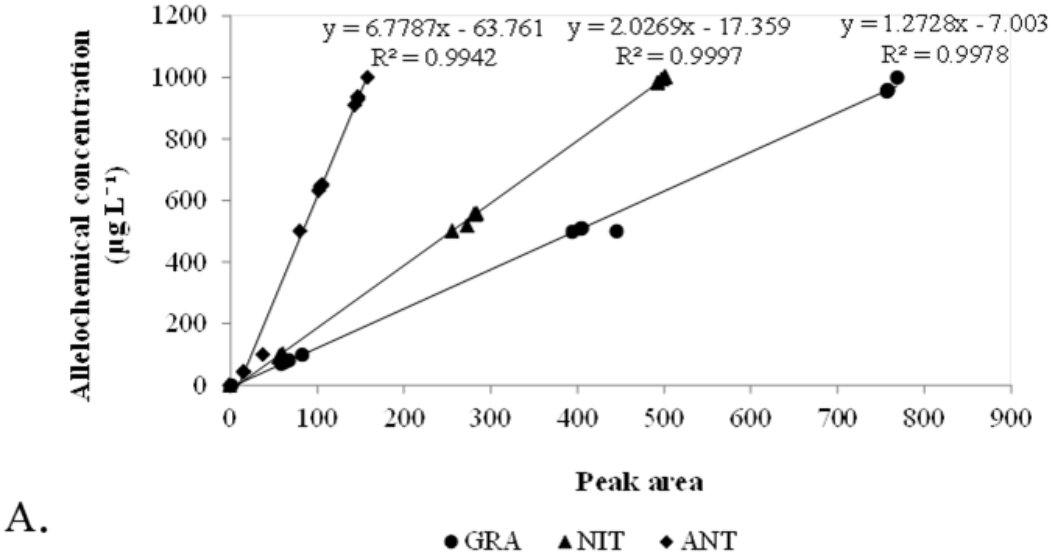
Table 4.2 Chemical compounds and their retention time (RT) in minutes and seconds analysed from the non-extracted standard (Nx) at the three tested concentrations (High= 0.9 mg L^{-1}; Mid=0.5 mg L^{-1}; Low=0.1 mg L^{-1}).

Non-extracted standard	Nx High (0.9 mg L^{-1}) mean±SD (*n*=3)	Nx Mid (0.5 mg L^{-1}) mean±SD (*n*=3)	Nx Low (0.1 mg L^{-1}) mean±SD (*n*=3)
Hordenine	6.946±0.001	6.947±0.001	N/A
Gallic acid	7.740±0.020	N/A	N/A
Gramine	14.941±0.005	14.976±0.002	15.050±0.003
Naringin	17.144±0.055	17.139±0.016	17.15±0.05
4-Nitroindol-5-carboxaldehyde	19.720±0.003	19.717±0.001	19.718±0.003
Anthraquinone	25.193±0.010	25.176±0.001	25.176±0.005

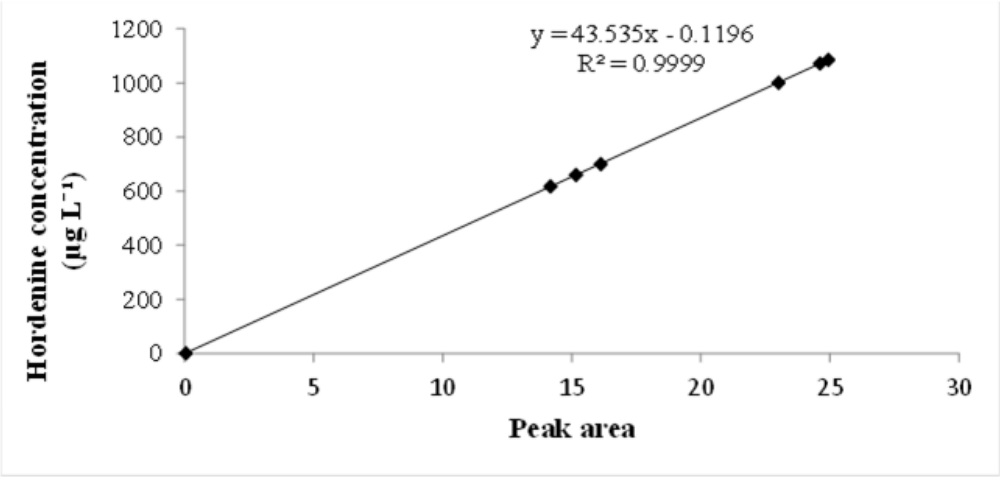
N/A= not available

4.3.3 Calibration, sensitivity

A four-point calibration curve of the peak concentration response in milliampere units was created for gramine, anthraquinone and 4-nitroindol-5-carboxaldehyde (Figure 4.3-A). A three-point calibration was created for hordenine (Figure 4.3-B) and a two-point calibration for gallic acid (not shown) and the internal standard naringin (Figure 4.3-C).



A.



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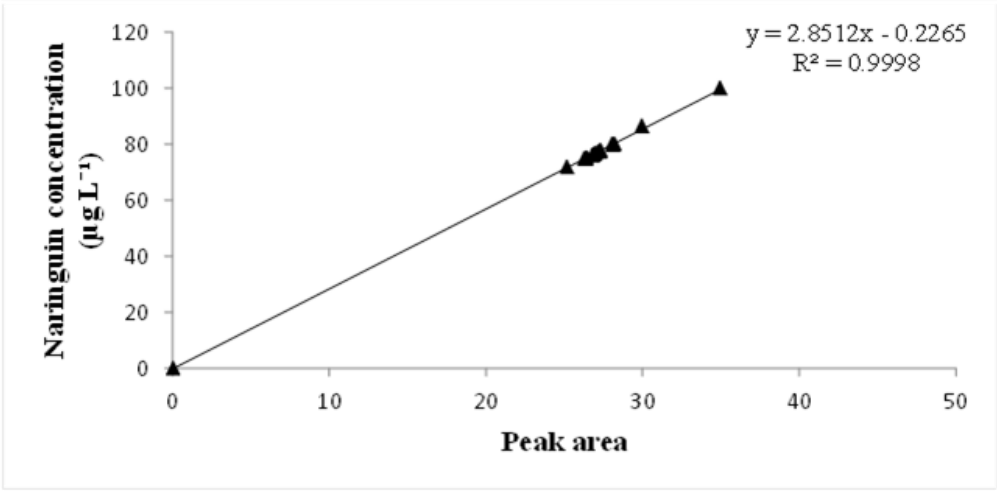

C.

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Figure 4.3-A. Calibration curve of analytes: gramine (GRA), 4-nitroindol-5-carboxaldehyde (NIT) and anthraquinone (ANT). 4.3-B. Calibration curve of hordenine standard. 4.3-C. Calibration curve of naringin, internal standard.

The limits of detection (LOD) and limits of quantification (LOQ) are shown in Table 4.3. Despite the low limits of detection and quantification calculated for hordenine, a peak for this analyte could not be detected with the low non-extracted mix-A standard (100 µg L^{-1}), nor with the mix-A extracted standard. This might be due to a suppression effect of the peak detection by other analytes present in the mixture at the low concentration range.

Table 4.3 Limits of detection and limits of quantification for the selected analytes used in the method.

Analyte from mix-A non-extracted standard solution	Limit of Detection (µg L^{-1})	Limit of Quantification (µg L^{-1})
Hordenine	3.63	11.01
Gramine	13.46	40.78
Naringin	9.11	38.86
4-Nitroindol-5-carboxaldehyde	14.83	44.93
Anthraquinone	29.20	88.48

Chromatograms of these standards at three concentrations are shown in Figure 4.4. Hordenine was not detected at the low concentration (100 µg L^{-1}). Gallic acid was only detected at the mid (500 µg L^{-1}).

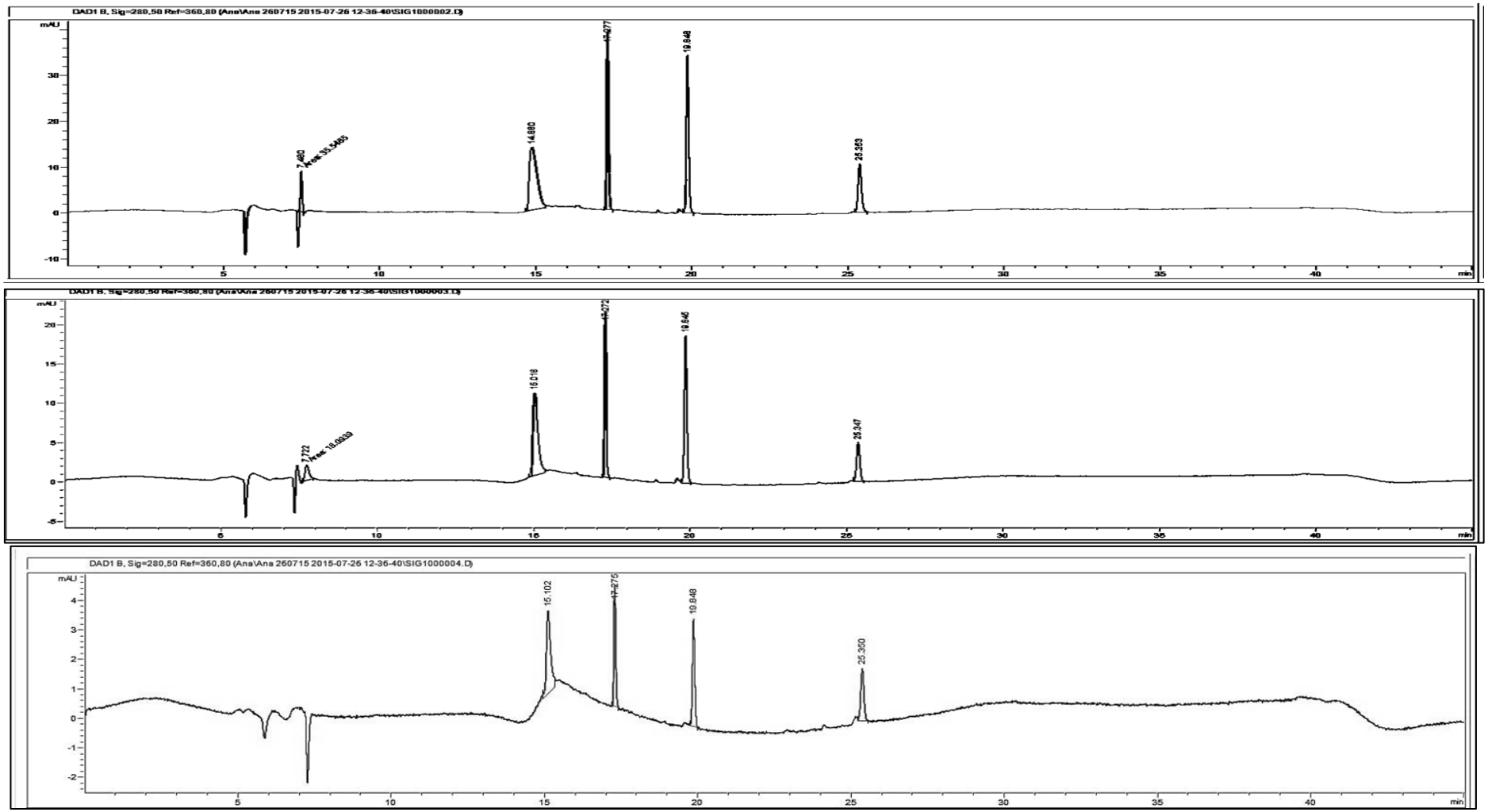

Figure 4.4 Chromatograms of mix-A non-extracted standards at three concentrations: Top= 0.9 mg L^{-1}, Middle: 0.5 mg L^{-1} and Bottom: 0.1 mg L^{-1}, where X-axis is retention time in minutes and Y-axis is milliampere units.

The recovery of the SPE procedure was carried out on normalized concentrations for the non-extracted and extracted mix-A standard solution. The recovery (%) was analysed for the triplicates of three independent samples extracted in three consecutive days (n=9) and run in the HPLC-UV-DAD at different times in the same day. The results in Table 4.4 show that gallic acid and hordenine were not detected in the extracted mix-A standard solution. The observed recovery (48.3%) for gramine is slightly less than the minimum required (50%) for the recovery of an extracted standard. Despite these results, the recovery of this analyte was taken into account for the quantitative analysis. This decision was made because gramine standard deviation and RSD were small suggesting that the recovery can be improved by addressing issues at the SPE, and are not due to the method itself. This is shown for the recovery results of the analytes with the non-extracted mix-A standard solution (Table 4.5). Overall results for % RSD are less than 5%, meeting the accuracy and precision parameters for method validation.

Table 4.4 Percentage of recovery of analytes in the mix-A extracted standard solution by SPE. Samples were from three independent extractions run at different times in the same day (n=9).

Analytes from the mix-A extracted standard solution	Recovery (%)	Standard deviation (SD)	Relative standard deviation (%)
Gallic acid	N/A	N/A	N/A
Hordenine	N/A	N/A	N/A
Gramine	48.3	1.1	2.2
Naringin	90.1	4.1	4.6
4-Nitroindol-5-carboxaldehyde	101.2	1.0	1.0
Anthraquinone	108.9	1.0	0.9

4.3.4 Precision and accuracy of the method

The precision and accuracy of the method was carried out on normalized concentrations of the non-extracted mix-A standard. The precision for the analytes: hordenine, gramine, naringin, 4-nitroindol-5-carboxaldehyde and anthraquinone was checked by analysing %RSD of triplicate results (*n*=3). The results for RSD were hordenine: 4.33%; gramine: 0.01%, naringin: 4.71%; 4-nitroindol-5-carboxaldehyde: 1.02% and anthraquinone: 1.51 %. All these results were ≤5% RSD, which are within the maximum acceptable precision criteria (Sivakumar *et al.*, 2007; Villagrasa *et al*., 2009).

The accuracy was calculated with the normalized concentrations of the analytes in the non-extracted mix-A standard solution, taking into account the target percentage concentrations (100, 50 and 10%) which corresponded to the 900, 500 and 100 μg L^{-1} concentrations.

Results from Table 4.5 show that naringin had the lowest recovery for the method but it is >80%. The current method meets the assumption of the RSD being less than 15%, although the ideal accuracy in a method validation is such that the RSD would be less than 2% (Villagrasa *et al*., 2009).

Table 4.5 Data used to determine the accuracy of the method for the selected analytes using non-extracted mix-A standard solution, (n=9 measurements, except for hordenine n=6).

Analyte in the non-extracted mix solution	Recovery (%)	Standard deviation (SD)	Relative Standard Deviation (%)
Hordenine	103.8	7.3	7.1
Gramine	104	7.7	7.4
Naringin	82.5	5	6
4-Nitroindol-5-Carboxaldehyde	106.8	4.1	3.8
Anthraquinone	100.1	14.4	14.4

4.4 Discussion

This discussion answers the question: Could the simultaneous allelochemical method detect, identify and quantify multiple allelochemicals in aqueous samples?

The developed simultaneous method is a tool that enables meeting the second requirement proposed by Willis (1985), which is the identification of allelochemical production by one of the two species suspected of producing allelochemicals. The first requirement was met in the previous chapter, with the observation of a clear pattern of inhibition.

Analysis was performed using a four point calibration with mixed solutions prepared at concentrations of 900, 500 and 100 µg L^{-1}. Some authors recommend calibrations to be made with six or seven data points, including a point through the origin, and these could be useful particularly for investigative purposes. Relevant root extracts and exudates were purified and concentrated by SPE procedure to produce usable samples in the instrument.

The simultaneous method addressed the needs for an analytical method for the investigation and determination of allelochemicals in aqueous samples from root wet-extracts and root exudates. The method enabled the multiple detection, identification and quantification of hordenine, gallic acid, gramine, naringin, 4-nitroindol-5-carboxaldehyde and anthraquinone out of nine selected allelochemicals. Single detection, identification and quantification using this method were also possible for five allelochemicals determined simultaneously, as well as gallic acid. Gallic acid only had one point calibration at the highest concentration, therefore it was not considered as being sufficiently calibrated for quantitative analysis.

Hordenine was not detected after the first three months of having run the method; however, these could not be investigated within the time frame of this research and logistics of using the instrument but further research should reinstate hordenine and gallic acid. The

allelochemicals tannic acid, linoleic acid and stigmasterol could not be detected using the described method. A possible explanation is that tannic acid easily breaks down into other phenolic derivatives with short run-times leaving the column before the change of gradient composition in the first few minutes. Linoleic acid and stigmasterol are lipophilic, and may require a different solvent gradient composition or are retained longer in the column requiring longer runs. However, numerous attempts were made before six allelochemicals were detected in a single run, and despite testing with a 55 minutes run-time, the lipophilic compounds were not detected. Despite the limitations of the developed simultaneous allelochemical quantification method with a single injection, the method is a very promising tool for quantifying allelochemicals from aqueous root exudate samples that could be applied for investigation of selected allelochemicals in surface waters.

One of the limitations of developing new methods or using an instrument with a limited library of chemical compounds is that many detected analytes remain unknown.

Chapter 5. Determination of allelochemicals in root biomass from the Living-Filter plants and in biofilter root exudate as a response to environmental stressors

This chapter investigates allelochemical content in the aqueous root extracts of *Phragmites*, *Phalaris* and *Carex* from the Living-Filter, and *Phalaris* from the biofilters, using the simultaneous multiple allelochemical method described in the previous chapter. The release of allelochemicals into the aqueous root exudate from the biofilters, in response to environmental stressors, will be evaluated.

5.1 Introduction

An aspect of aquatic allelopathy and allelopathy in general that remains elusive is to determine if or which abiotic and biotic factors or their interaction triggers the release of allelochemicals (Bauer *et al*., 2009). Legrand *et al*. (2003) suggested that the effects of depletion should be avoided when studying allelopathy because in light, nutrient or space-limited conditions the production of an allelopathic compound may be enhanced while the target may become more sensitive. Yet, nutrient-depleted conditions for both donor and target species could yield useful information if the effects of competition are well separated from those of allelopathy (Bauer *et al*., 2009), because allelopathic interactions that are not detectable due to adaption may become evident under physico-chemical stress (Vanderstukken *et al.*, 2011, 2014). However, many experiments report that in ideal light, temperature and nutrient enriched conditions the production of allelochemicals is increased (Svanys *et al*., 2014).

Floating treatment wetlands (FWT) frequently include at least one genera of the Poaceae family, such as *Phragmites* spp., *Phalaris* spp. and *Hordeum* spp. (Brezinova and Vymazal, 2014; Kellogg, 2015). *Hordeum vulgare* L. (barley straw) is perhaps the most studied Poaceae because of the presence of allelochemicals; in particular, phenolic

compounds are released from decomposing barley (Murray *et al*., 2010; Bahrs *et al.,* 2013). The Living-Filter uses *Phragmites*, *Phalaris* (Poaceae) and *Carex* (Cyperaceae), and moreover, *Phalaris* is the species that is used in the biofilters grown hydroponically under controlled laboratory conditions in chapter 3 (section 3.2.4.1).

Allelochemicals are produced in any organ of the plant which can be released to the environment from leaves, shoots, roots, flowers and pollen as exudates, leachates or volatile compounds (Dhaouadi *et al.*, 2015), although this does not mean that the organ that produces them, necessarily releases them (Sanchez-Moreiras *et al.,* 2003). The mechanisms of allelochemical action and their effects on other plants, animals and microorganisms are well studied in terrestrial environments (Cheema *et al*., 2012), with a focus on the Poaceae, because it includes the largest food crops supporting humanity. For example rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) (Chon and Kim, 2004; Sanchez-Moreiras *et al.*, 2004).

Investigating the allelochemical content in root extracts from *Phragmites*, *Phalaris* and *Carex* could inform the selection of species used in FTWs and enhance the root characteristics observed in the physical removal of phytoplankton (large root biomass and density). Quantifying allelochemical content in root extracts and in root exudates in relation to root biomass could serve as a tool for the estimation of plant/root density in future biofilter designs.

The outcome of this research will provide greater insight into the potential removal of *Microcystis* by bio-chemical mechanisms promoted by hydroponic roots; inform the plant coverage needed for effective biofilters; and, therefore, increase the efficiency and effectiveness of using biofilters for water treatment.

The aim of this chapter is to detect, identify and quantify allelochemicals or potential allelochemicals in aqueous root extracts of *Phragmites, Phalaris* and *Carex* from the Living-

Filter, and *Phalaris* from the biofilters, and to evaluate the allelochemical release response to environmental stressors (nutrient and *Microcystis*). The following specific research questions were investigated:

1) Are there interspecies differences in allelochemical composition from the Living Filter plants: *Phalaris*, *Phragmites* and *Carex?*

2) Are there differences in allelochemical composition in aqueous samples derived from root extracts of *Phalaris* grown in the field-scale (Living-Filter) versus that grown in the laboratory (biofilters)?

3) Are there differences in allelochemical composition in aqueous samples derived from root extract and root exudate from laboratory *Phalaris*?

4) Are there differences in the release of allelochemicals in response to different environmental stressors, and in particular differing nutrient and *Microcystis* treatments?

5.2 Materials and methods

The methods used in this chapter are those described in section 4.2, chapter 4. Roots of plants (from the Living-Filter and biofilters) were weighed and measured, and their aqueous samples and exudates were analysed for allelochemical detection, identification and quantification. A biofilter experiment was also carried out to test whether allelochemical release responds to environmental stressors and whether they can be measured.

5.2.1 Sample preparation for analysis of aqueous root extract and exudate

To investigate allelochemical content and potential variability in aqueous samples of root extract from *Phragmites*, *Phalaris* and *Carex* from the Living-Filter, and root extract and exudate from the laboratory biofilters, samples were prepared as in Figure 4.2. This figure summarizes the procedure explained in full in section 4.2.9, chapter 4.

5.2.2 Investigation of allelochemicals in biofilter root exudates as a response to *Microcystis* and nutrient environmental stressors

An experiment (designated Exp I) was carried out to investigate the rate of production of allelochemicals by biofilters under two nutrient conditions (tap water and Hoagland's 75%) and in the presence and absence of *Microcystis aeruginosa* over a period of five days (Figure 5.2).

Twelve jars of 2 L capacity and a diameter of 10 cm were used to hold each of 10 plants of *Phalaris arundinacea,* all three months of age. The biofilters were grown hydroponically as in section 3.2.8, but with the difference that they were kept under light conditions until the end of the five day experiment. The treatments were applied as in Figure 5.1.

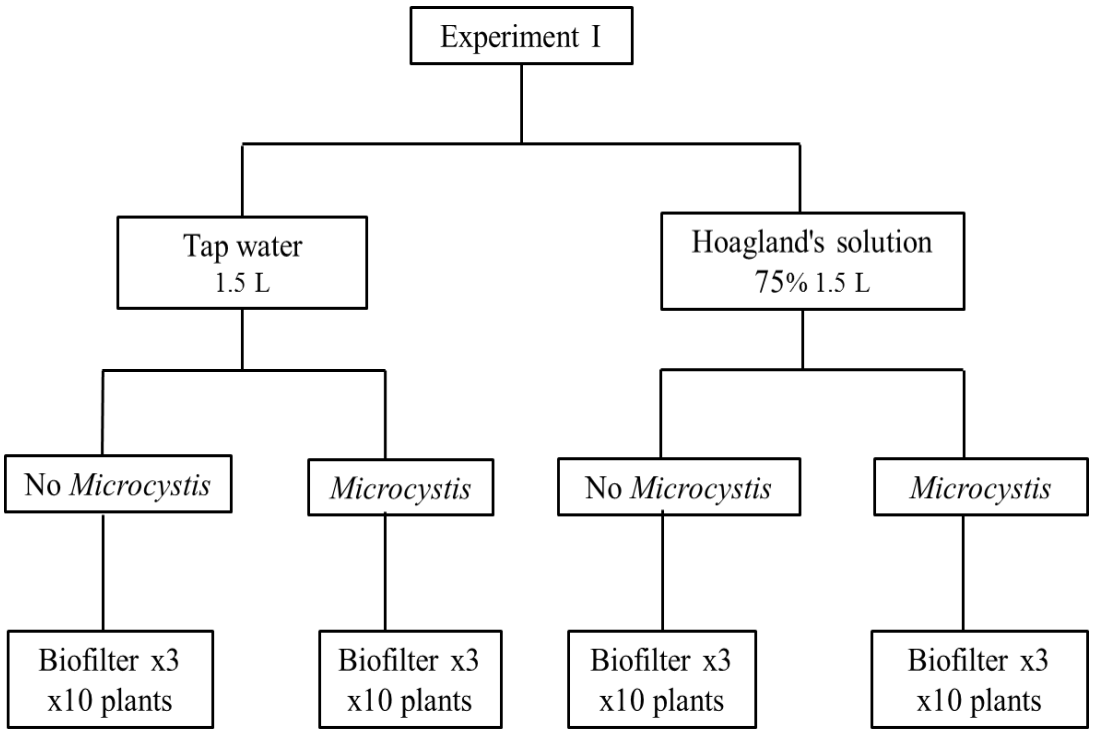

Figure 5.1 Experimental design to investigate the rate of allelochemical production under two nutrient conditions (tap water and Hoagland's 75%) and in the presence and absence of *Microcystis* over a period of five days.

5.2.3 Calculations for analyte concentrations in aqueous samples and root extracts

The concentration of analytes in the total volume of samples and root biomass was calculated as follows:

Concentration of analytes in the sample = Response of the analyte x M (or DF)

(eq. 5. 1)

Response of the analyte = the peak area; M= multiplier, to take into account sample weights; and DF = dilution factor or concentration factor, to take into account differences in the volumetric preparation of sample and standard solutions.

5.2.4 Data processing and statistical analysis

Exploratory and statistical analysis were undertaken using Excel (Microsoft © 2010), Minitab 17 and SPSS v. 22.0 (IBM Graduate Advanced pack statistic software, UK). Data were checked to see if parametric assumptions were met: a normal distribution, equal variances and independence of the data. Kendall's *tau*-test (*T*) was applied as the non-parametric correlation test to determine if there were associations, one variable depends on another variable, between weight of the biofilter roots (section 5.3.1) and the allelochemical concentration released. Interval plots with a 95% confidence interval for the mean were selected for the presentation of the allelochemical means (see section 2.2.6, chapter 2 for the test explanation). To determine the effect of two independent variables (treatment and *Microcystis*) on a dependent variable (living-root from biofilters) with five replicates, a two-way ANOVA with replication was applied to Exp. I. The treatment (tap water and Hoagland's) and the absence/presence of *Microcystis* were fixed factors (Sokal and Rohlf, 2003).

5.3 Results

5.3.1 Allelochemicals from root extract in aqueous samples: inter-species variability

The estimation of the identified allelochemical concentration (µg L^{-1}) from aqueous root extract for *Phragmites, Phalaris* and *Carex* is shown in Figure 5.2. This estimation is semi-quantitative because the concentration for the known allelochemicals was above the highest concentration for the non-extracted mix-A standard (900 µg L^{-1}) used in the calibration. Linearity could not be assumed.

Gramine was only found in *Phragmites* roots. The highest concentration was for naringin from *Phalaris*; and naringin was also the highest allelochemical concentration found in *Phragmites*. The highest concentration of an allelochemical in *Carex* was 4-nitroindol-5-carboxaldehyde.

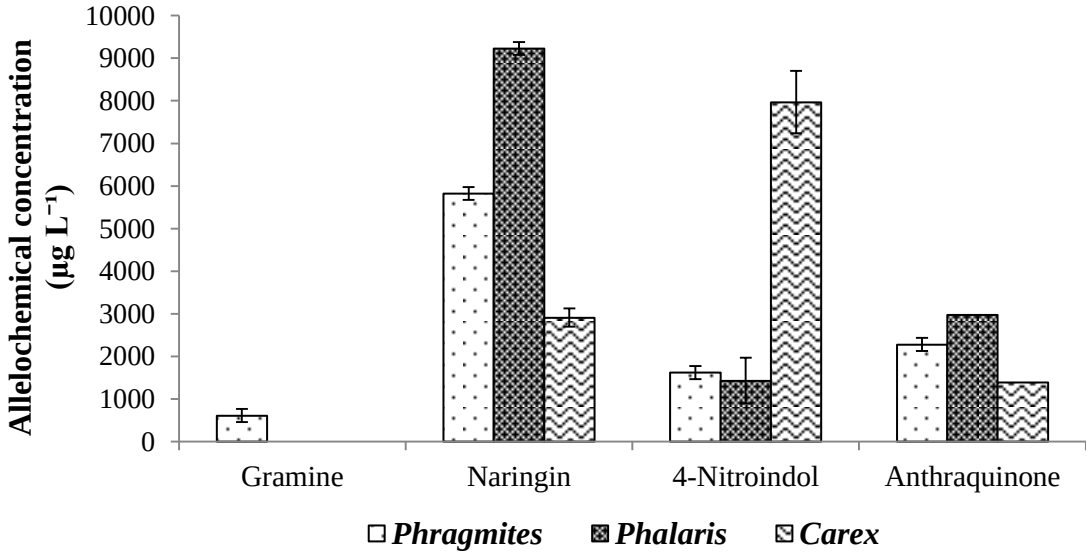

Figure 5.2 Allelochemical concentration (µg L^{-1}) from the aqueous root extracts of the species from the Living-Filter: *Phragmites* (n=6), *Phalaris* and *Carex* (n=9).

The estimation of the allelochemical concentration for root biomass (µg g^{-1}) from *Phragmites, Phalaris* and *Carex* and the biofilters is shown in Figure 5.3.

For root biomass the results are triplicates of three independent samples (n=9) for *Phalaris* and *Carex*, and from two independent samples for *Phragmites* (n=6). *Phalaris*

showed the highest concentration of naringin and anthraquinone, whilst *Carex* showed the highest content of 4-nitroindol-5-carboxaldehyde. The estimation for root biomass from the biofilters each with 16 plants, showed only the presence of gramine and naringin (n=3).

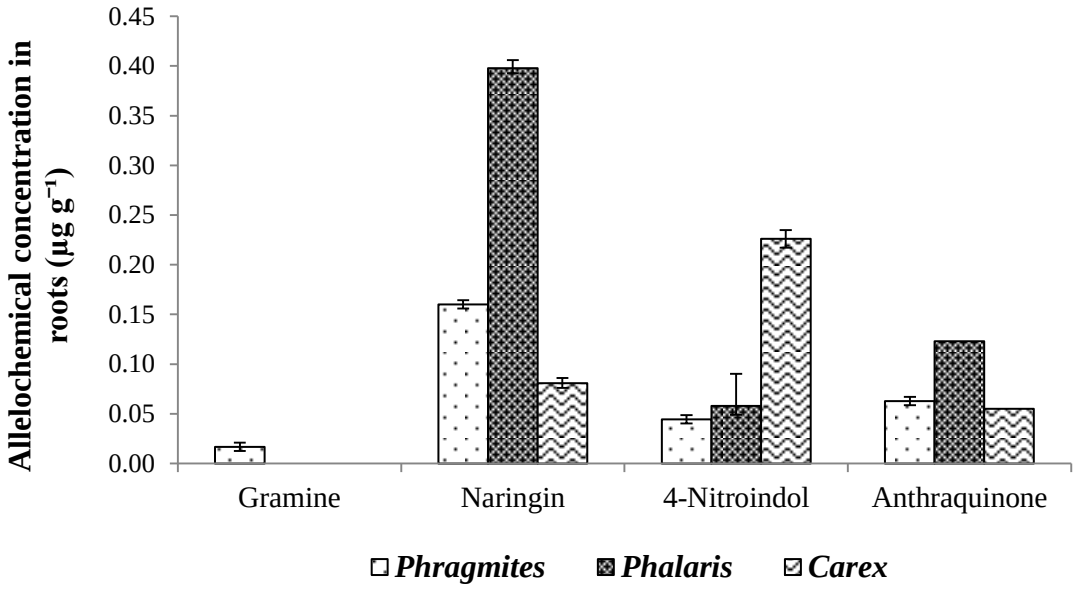

Figure 5.3 Allelochemical concentration of the root biomass (µg g^{-1}) from the three species in the Living-Filter: *Phragmites, Phalaris* and *Carex.*

It is important to note that chromatograms from standard mixed solution samples (both non-extracted and extracted) showed an almost flat baseline as shown in Figure 4.4, chapter 4. This is not the case for aqueous samples with organic material which are likely to show a variable baseline with peaks and valleys (Al Harun *et al.*, 2015). Furthermore, the calculation of the limits of detection (LOD) and quantification (LOQ) accounts for false increments in the areas of the peaks for any particular method.

Figure 5.4 shows a number of unidentified peaks for the three species, the peak areas differ, with some showing very large areas, suggesting large concentrations of these compounds. *Phragmites* shows 28 unidentified peaks, *Phalaris* 40 and *Carex* 35.

5.3.2 Allelochemicals from root extract in aqueous samples: intra-species variability

The detection, identification and quantification of allelochemicals in root extract samples from Living-Filter and laboratory *Phalaris* showed qualitative, quantitative and semi-quantitative differences. The chromatogram for *Phalaris* from the Living-Filter in Figure 5.4 and from the laboratory in Figure 5.5 (bottom) showed not only that there are differences in the concentration of the allelochemicals identified from the root extracts, but also differences in the number of unknown analytes. There were 40 unknown analytes in *Phalaris* from the Living-Filter, compared to 17 from the laboratory samples.

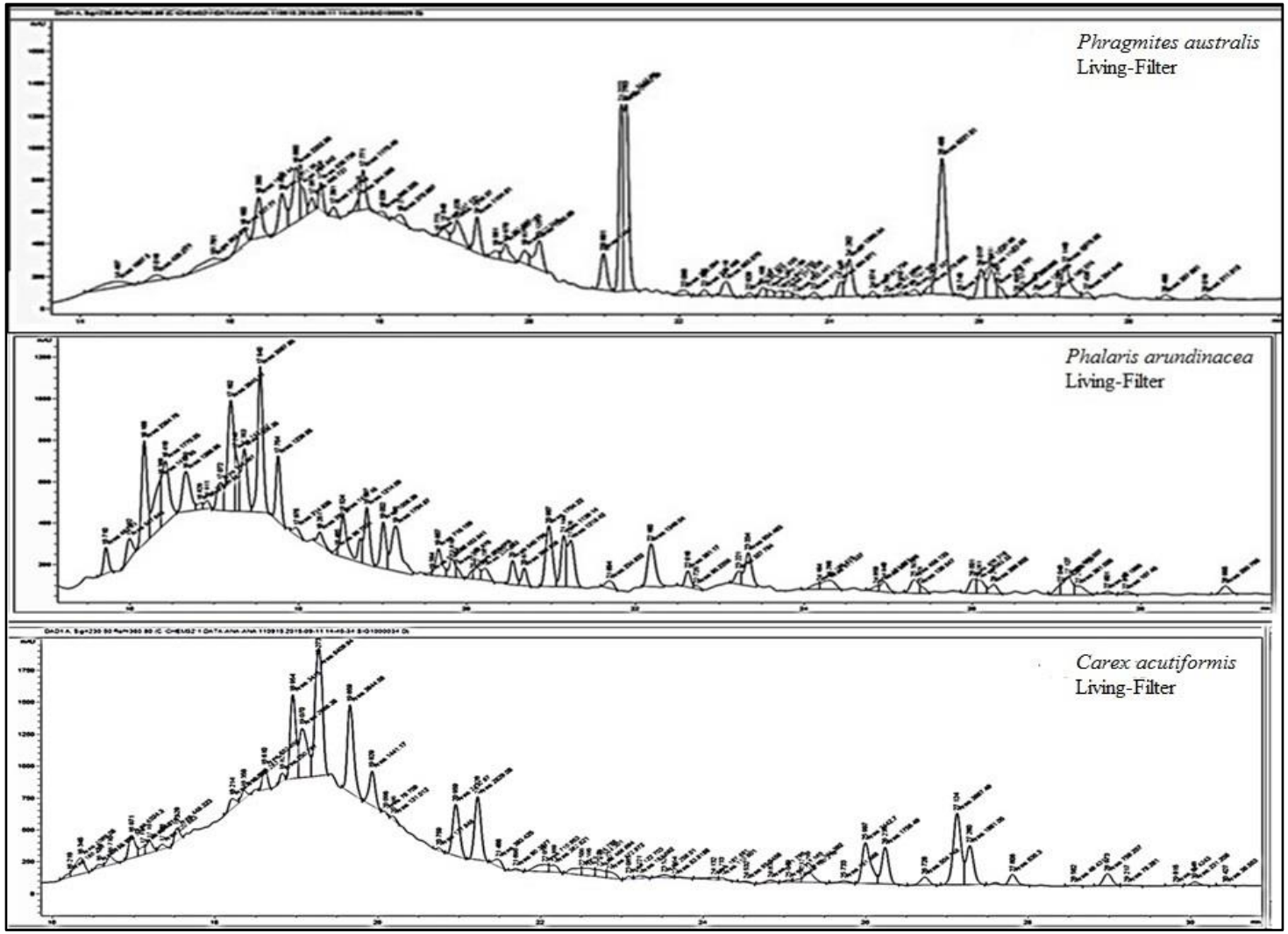

Figure 5.4 Chromatogram of the analytes from the three plant species in the Living-Filter: the *x*-axis shows the retention time in minutes and the *y*-axis shows the signal intensity of detected analytes in milliampere units.

5.3.3 Comparison between root exudate and root extracts from laboratory Phalaris

Detected peaks of potential allelochemicals were more numerous and also their areas were larger in the root extracts (with 20 peaks) from laboratory *Phalaris* (biofilters) than in the root exudate (8 peaks), as shown in Figure 5.5. The root exudate chromatogram corresponds to an experiment where the biofilters were exposed to the presence of *Microcystis*. All samples were measured in triplicates. Except for the identification and quantification of naringin and 4-nitroindol-5-carboxaldehyde, the other six peaks did not correspond to any of the analytes used in the standard solution. The concentration of naringin matched that of the internal standard. The allelochemical 4-nitroindol-5-carboxaldehyde was quantified with a concentration of 96.13±10.08 μg L^{-1} (mean±SE). By contrast, in the root extract from the biofilters three allelochemicals were identified as shown in Table 5.1. Gramine and naringin concentrations were higher than the high standard with concentrations of 3,447±1043 μg L^{-1} (mean±SE, *n*=3) and 4.688±1433 μg L^{-1} (mean±SE, *n*=3) respectively; and 4-nitroindol-5-carboxaldehyde was identified in only one of the three root extract biofilter samples (176.02 μg L^{-1}). The other 17 peaks detected in the root extract sample are unknown analytes that showed high responses; with peak size areas of, ≥ 900 μg L^{-1}.

Table 5.1 Allelochemicals that were identified and quantified in root exudates and root extracts (n=3).

Allelochemicals	Root exudate mean±SE μg L^{-1}	Root extract mean±SE μg L^{-1}
Gramine		3447±1043
Naringin (ISTD 100 μg L-1)	109.3±9.9	4688±1433
4-Nitroindol-5-carboxaldehyde	96.13±10.1	176±10.1

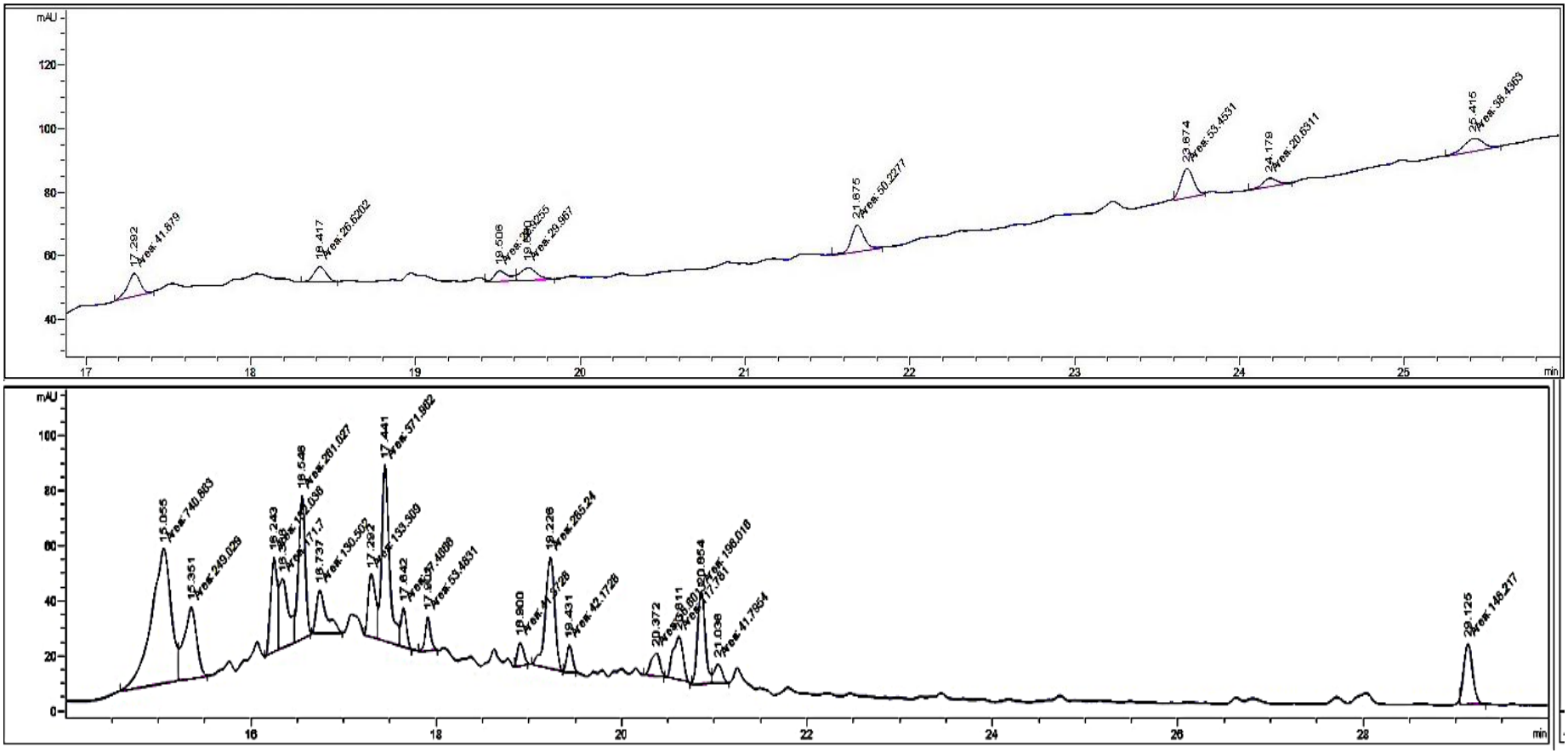

Figure 5.5 Chromatograms of a root exudate sample (top graph) and of a root extracted sample (bottom graph). These samples were from laboratory-grown *Phalaris*. The number of detected peaks for the exudate was eight and for the extract it was 20.

5.3.4 Allelochemicals in biofilter root exudate as a response to environmental stressors

A comparison of results investigating the allelochemical response (µg L^{-1}) of biofilters to different nutrient treatments (tap water and 75% Hoagland's solution) and *Microcystis* (presence or absence), as environmental stressors, is presented in Figure 5.6. The results showed that the overall response of the three quantified allelochemicals (mean±SE) in tap water and without *Microcystis* appears to be the lowest. The highest concentration (µg L^{-1}) released of an allelochemical was for 4-nitroindol-5-carboxaldehyde.

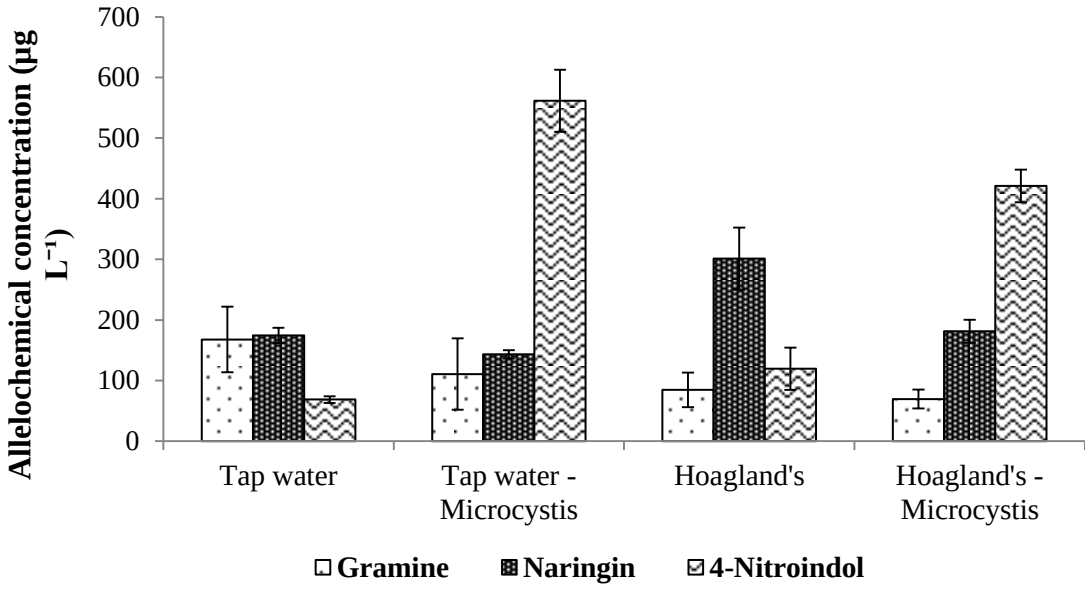

Figure 5.6 Comparison of allelochemical concentrations (µg L^{-1}) for gramine, naringin and 4-nitroindol-5-carboxaldehyde (mean±SE) released by the biofilters as a response to different treatments of nutrients (tap water and Hoagland's) and *Microcystis* (absence or presence).

Traces of anthraquinone were also found in tap water and Hoagland's in the absence of *Microcystis* (at or below LOD = 29.20 µg L^{-1}). Gallic acid was identified by retention time and chemical spectrum with a large area (suggesting a high concentration) in a few samples. But because it had only one point calibration it was not quantified. Two allelochemicals

gramine (all below LOD) and 4-nitroindol-5-carboxaldehyde with concentration of 107.0 ± 1.7 µg L^{-1} (mean±SE) were found in the samples from the *Microcystis* cultures.

Figure 5.7 shows that the mean concentrations of gramine (µg L^{-1}) (top-left plot) released by the biofilter roots into the aqueous media appears unaffected by the *Microcystis* treatment. It also shows that under Hoagland's treatment the production of gramine decreases. In contrast to gramine, the biofilter roots release higher concentrations of naringin under Hoagland's treatment and in the absence of *Microcystis* (top-right plot). The bottom-left plot shows that 4-nitroindol-5-carboxaldehyde is released at high concentrations when in the presence of *Microcystis* in both nutrient conditions.

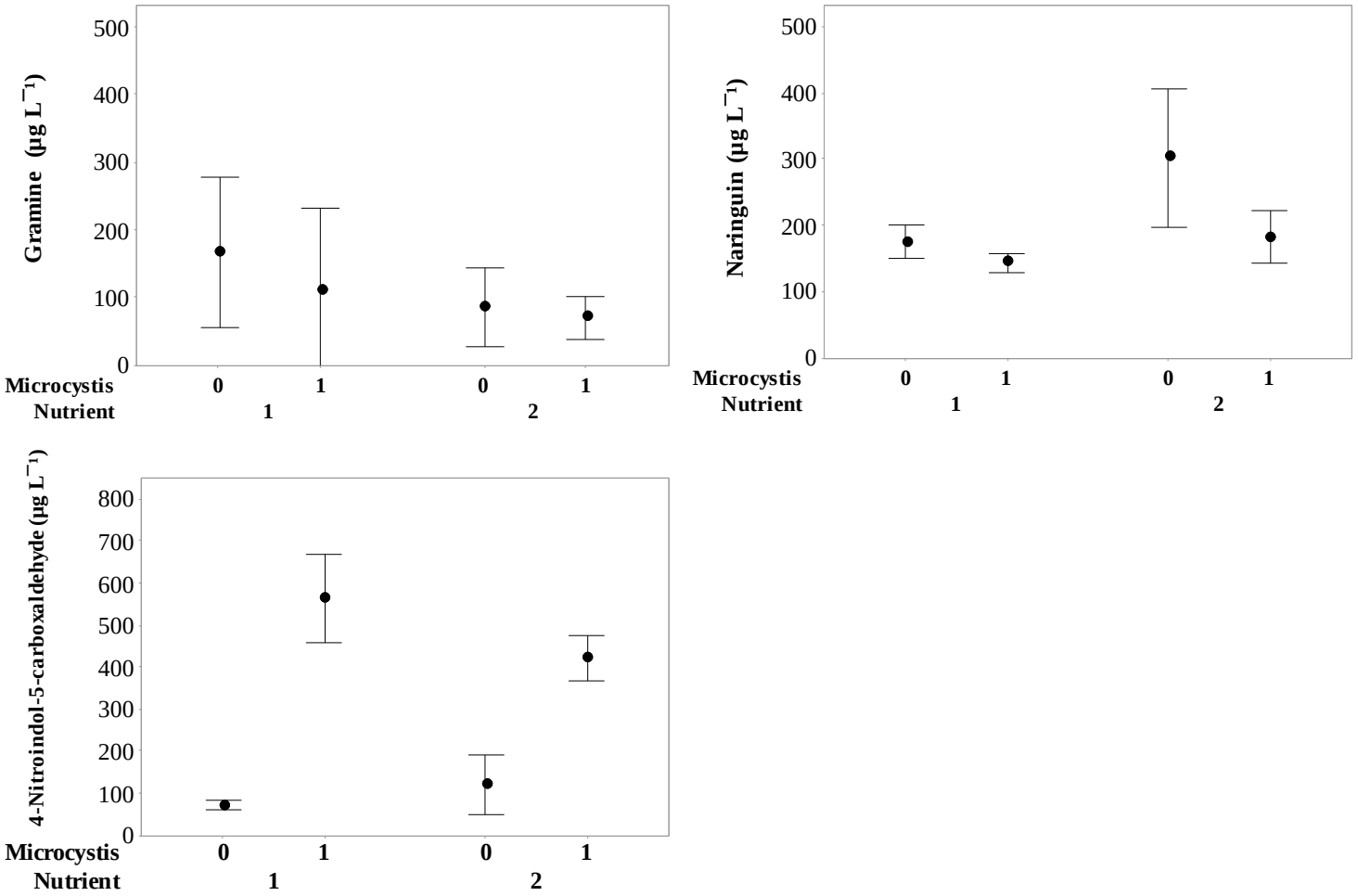

Figure 5.7 Interval plots of gramine (top left), naringin (top right) and 4-nitroindol-5-carboxaldehyde (top left) (µg L^{-1}) confidence means 95% for treatment conditions: *Microcystis* (absence = 0, presence =1) and nutrient (tap water =1, Hoagland's' 75% = 2). (n=18 for each data set).

The allelochemical concentration data from the root exudates were transformed to fit the assumptions of a normal distribution and equal variances. The allelochemical concentration mean±SE for each treatment is presented in Appendix D.

To determine statistically significant differences between the treatments and their interactions, the data were analysed with a two-way ANOVA with replicates. The difference in mean concentration of the quantified allelochemicals in response to environmental stressors, where n=18 in each case, was only statistically significant for the production of 4-nitroindol-5-carboxaldehyde ($F_{3,32} = 39.54$, $p<0.05$). The significant effect of the increased production of 4-nitro-5-carboxaldehyde was in response to the presence of *Microcystis*.

The association between allelochemical concentration in the root exudate and the root biomass from biofilters (n=12) showed no statistically significant correlations (Figure 5.8). The actual figures can be found in Appendix E.

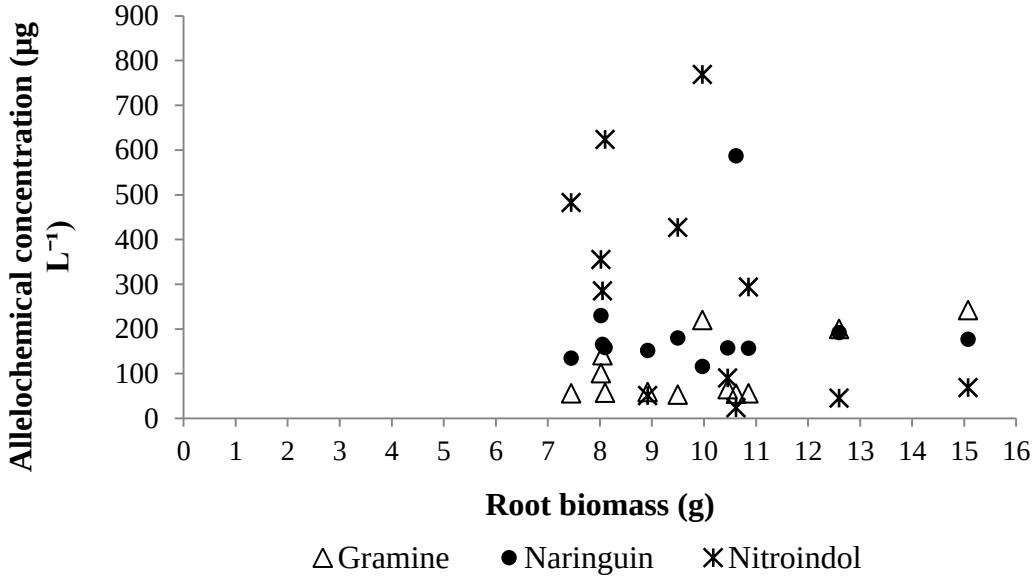

Figure 5.8 Relation between biofilter root biomass (g) and released allelochemical concentration (µg L^{-1}) in the root exudate.

5.4 Discussion

5.4.1 Were there inter-species differences in allelochemical composition from the Living-Filter plants: *Phalaris, Phragmites* and *Carex*?

Interspecies variability is shown qualitatively and quantitatively in this study. On the one hand, the quantitative estimation of the identified allelochemicals gramine, naringin, 4-nitroindol and anthraquinone in *Phalaris, Phragmites* and *Carex* showed the concentration of these allelochemicals varies among species (Figures 5.2 and 5.3). On the other hand, the array of peaks of chemical compounds displayed in the chromatogram (Figure 5.4) from the aqueous root extract of the three species illustrates the interspecies variability. These compounds might be potential allelochemicals that might have cyanocide/cyanostatic or algicide/algastatic effect.

However, many results for the identified allelochemicals with the simultaneous method were semi-quantitative due to being greater than the calibration range. This was the case for most aqueous samples from root extracts. Possible ways of avoiding semi-quantitative results would be either diluting the samples, increasing the concentration of the high standard above 900 µg L^{-1} or adding more data point concentrations above 900 µg L^{-1} for the calibration (section 4.2.6). However, dilution of the samples is laborious and time consuming, making the whole process less efficient, as dilutions would have to be tailor made for the samples. Increasing the number of data point(s) for the calibration seems the most sensible option for higher standard concentrations above 900 µg L^{-1} (for example adding one point at 2000 µg L^{-1}), as this will ensure linearity is maintained at higher concentrations and thus facilitate quantification.

In this work, a large number of peaks with considerably large areas, possibly with high concentration of the analyte, were detected in all samples, and more so for samples from root extracts; however, these peaks remain as unknowns. In this case, coupling the HPLC-UV-

DAD system with an MS or MS/MS system could improve the identification of unknown analytes with the method, because identification by MS or MS/MS is based on the unique mass signatures of the analytes. Also, with MS coupled systems analytes could be detected with concentrations a magnitude lower, as suggested by Villagrasa *et al*. (2009). For example the limit of detection concentration can be 20 ng L^{-1} (0.02 µg L^{-1}) compared to the simultaneous allelochemical method where the lowest limit of detection was 3.6 µg L^{-1} (Table 4.3). Nevertheless, knowing the variability of chemicals in these plants, having an efficient method to identify and measure the concentrations and establishing their allelopathic activity, it is a step closer to predicting actual concentrations of released allelochemicals involved in algicide/algastatic activity in aquatic environments.

The application could be used not only for selecting suitable plant species in a biofilter design prior to a potable water treatment, but for identifying the allelochemical content in the root extracts for FTWs. In this manner, the potential control by allelochemicals of cyanobacteria (or phytoplankton) loading onto a potable WTWs or cyanobacteria blooms in aquatic environments could become an effective measure.

5.4.2 Were there intra-species differences in allelochemical composition from field-scale (Living-Filter) *Phalaris* and that grown in the laboratory (biofilters)?

Intra-species variability in *Phalaris* was shown in the findings of this research from field and laboratory *Phalaris* using root extracts, where the total number of peaks (45) and the size of their areas were shown to be larger in *Phalaris* from the field-scale Living-Filter (Figure 5.4) compared to the laboratory samples (20 peaks) (Figure 5.5 – bottom graph). In samples of field *Phalaris* allelochemical compounds with known algastatic/algicidal effects (Laue *et al*., 2014) were detected, identified and quantified from the root extracts. The number of alkaloids and their concentration varies genetically in natural populations, but species or genetic variations of *Phalaris* without alkaloids have not yet been found (Ostrem, 1987).

Although it is likely that individual genetic variability determines the composition of synthetized analytes, some authors have highlighted the role that microorganisms from the periphyton or biofilm (the community of organisms attached to surfaces) and endophytes (the community of microorganisms living within the organic tissue) can play on the production and/or release of allelochemicals. Although the roots from *Phalaris* growing in the Living-Filter and in the laboratory were disinfected to ensure removal of the periphyton, the roots were not sterile and endophytes could still be present. Hence, growth conditions alone might not be playing a role, but it might determine which community of endophytic organisms is established in the root tissue. Kusari *et al*. (2012) pointed out that the endophytic process requires a physical encounter between the plant and the microorganism, which are generally fungi, to establish the conditions of association for what is called "balanced antagonism". This might explain the greater variability of chemical compounds for *Phalaris* from the Living-Filter compared to the laboratory. Furthermore, plant domestication in controlled environments is an option for the selection and production of plants for biofilters. Plant domestication consists of selecting and cultivating, plants that produce desired allelochemicals or secondary metabolites in sufficient quantities that they can be used as biocides. This is an extended practice worldwide that has shown renewed interest in the wake of microorganisms' resistance to antibiotics and antiparasitics (Martinez-Diaz *et al*., 2015).

5.4.3 Were there differences in allelochemical composition between root extract and root exudate in aqueous samples derived from laboratory *Phalaris*?

There were differences in allelochemicals derived from root extracts and those from root exudate from laboratory *Phalaris*. Two identified allelochemicals (anthraquinone and 4-nitroindol-carboxaldehyde) were quantified and semi-quantified from the root exudate, whilst three identified allelochemicals were semi-quantified from the root extracts (Table 5.1). Although the number of identified allelochemicals is small compared to the number of

unidentified chemicals, the number of allelochemicals decreases in the root exudates compared to the root extracts (Figure 5.5).

The reason for the lack of association between the concentration of released allelochemicals in the water and the concentration within the root biomass is not clear (Figure 5.8). It could be that after five days of being exposed to different treatments the allelochemicals released by the root biomass might have been broken down into subcomponents which were not measured or identified. These results, however, may be supported by Sanchez-Moreiras *et al*. (2003) who pointed out that although several organs in a plant can produce allelochemicals it does not mean that the producing organ also releases them. Hence, it might be that in this case, the stress signal is received by the roots, and then allelochemicals are mobilized within the plant from another organ, for example the leaves (Ostrem, 1987), and are later released by the roots. Another explanation could be that allelochemicals stored in the roots, are released in conjunction with those that are mobilized from other parts of the plant. This explanation is supported by Bertin *et al*. (2003) who found that all roots secrete large quantities of chemical compounds and root exudate is the most important source of allelochemical input into the terrestrial rhizosphere.

5.4.4 Were there allelochemicals in aqueous root exudate released as a response to environmental stressors?

The concentration for three identified and quantified allelochemicals (gramine, 4-nitroindol-5-carboxaldehyde and naringuin) in the root exudate from *Phalaris* biofilters were different depending on the exposure to the treatments (nutrient or *Microcystis* spp. cells) (Figure 5.6), and did not seem to relate to the root biomass (Figure 5.8). Although statistically significant differences were only found for the released concentrations of 4-nitroindol-5-carboxaldehyde in response to *Microcystis* presence (Figure 5.8) regardless of the nutrient media, it was found that overall the concentration of released allelochemicals was higher in

the enriched nutrient media (75% Hoagland's solution) than in tap water, in the presence or absence of *Microcystis*. These results are supported by Svanys *et al*. (2014) that enriched nutrient conditions might stimulate the production of allelochemicals and that there are specific sensitivities to certain types of biological stressor. However, the results are contrary to Legrand (2003) that allelochemicals are supressed in these conditions. This could explain the differences for allelochemical release from the biofilters in the presence or absence of *Microcystis*. For example, although naringin was released in both nutrient media and in the presence/absence of *Microcystis,* its concentration was higher in the enriched media only when *Microcystis* cells were absent. In contrast 4-nitroindol-5-carboxaldehyde was higher when in the presence of *Microcystis* (Figure 5.7). The sensitivities to biological stressors from the biofilter roots might respond to *Microcystis* produced metabolites, such as microcystin-LR (cyanotoxin) and other allelochemicals. These metabolites are considered contributors to the formation and/or maintenance of cyanobacterial blooms (Keating, 1977, 1978; Sivonen and Börner, 2008), either deterring and/or killing potential grazers (Gerphagnon *et al*., 2015; Ger *et al*., 2016), and inhibiting and/or killing micro-algae growth (Suikkanen *et al*., 2004).

In this research, naringin was used as an internal standard in the simultaneous allelochemical method to compensate for any differences in recovery following SPE (thus standardising and normalising the result data so that it could be equally comparable). Ideally the internal standard should have a similar chemical structure to the investigated analytes but should not be a frequently found analyte itself. Naringin, however, was frequently found in the investigated samples. Interestingly, Xie *et al*. (2014) found that naringin inhibits the hepato-pancreatic uptake of microcystin-LR in freshwater snails. Moreover, Takumi *et al*., (2015) demonstrated that the same allelochemical can attenuate the cytotoxicity of microcystins-LR in the hepatocytes of mammals. The production of naringin by the biofilters and plants studied here is an added benefit of using a biofilter process for removal/reduction of phytoplankton.

Overall, findings from this research are very important as the future installation of biofilters in eutrophic surface waters could stimulate, rather than hinder, the release of allelochemicals into the surrounding water. Further research was undertaken and presented in the next chapter to demonstrate the effectiveness of the released allelochemicals at inhibiting *Microcystis* growth but also to discriminate from other mechanisms that might be operative in the presence of biofilters and *Microcystis* in an aqueous environment.

Chapter 6. The effect of commercial allelochemicals and biofilter root exudates on the growth of *Microcystis aeruginosa*

This chapter investigates and quantifies the inhibition of *Microcystis* growth by commercial allelochemicals and by biofilter root exudates in aqueous environments in function of time.

6.1 Introduction

The restoration of water bodies using macrophytes to limit the growth of cyanobacteria, either by nutrient competition or by the release of allelochemicals with algastatic/algaecide effects is a subject of continuous investigation (Korner and Nicklisch, 2002; Mulderij *et al.*, 2007; Hilt and Gross, 2008; Chang *et al.*, 2012; Rojo *et al.*, 2013; Laue *et al.*, 2014; Matthijs *et al*., 2016). Approaches to the study of this subject include macrophyte interference experiments using crude extracts or purified compounds obtained from submerged macrophytes (Wium-Andersen *et al*., 1982; Della Greca *et al*., 1989; Erhard & Gross, 2006). Culture filtrates of submerged macrophytes suppressing phytoplankton growth were used by Korner and Nicklisch (2002) who suggested that suppressant compounds may be released in the surrounding water at concentrations that can affect phytoplankton productivity (Declerck *et al*., 2007; Mulderij *et al*., 2006) but these suppressants (allelochemicals) were not identified. The inhibitory effect of root exudates on a target organism was demonstrated by Chang *et al*. (2012) with an experiment where the root exudate from a batch of seeds that germinated on wet filter paper in petri dishes was in contact with a new batch of seeds that replaced the first batch. The germination of the new seeds was affected by the root exudates of the preceding seeds. However, aspects that remain problematic in the understanding of allelopathic interactions include the scale of investigation, field, experimental or a combination of both (Bauer *et al.*, 2009; Vanderstukken *et al.*, 2011, 2014) and the role of allelopathy in the dynamics of cyanobacteria blooms (Gerphagnon *et al*., 2015). Other

experiments have been performed under conditions where alternative control mechanisms could be excluded, such as nutrient competition (Mjelde and Faafeng, 1997; Vanderstukken *et al*., 2011) or zooplankton grazing (Van Donk and Van de Bund, 2002; Vanderstukken *et al*., 2014).

Other methods focus on the demonstration of the production of allelopathic compounds by an organism, and the estimation of the role and importance of allelopathic interactions in the field (Leflaive and Ten-Hage, 2007; Leflaive *et al*., 2009). Cross-culturing is a method used to identify allelopathic interactions between phytoplankton (micro-algae and cyanobacteria) and consists of culturing a target species in a medium enriched with filtrate from the culture of different micro-algae/cyanobacteria whose allelopathic activity is being investigated (Suikkannen *et al*., 2004). A number of experiments using cross-culturing with cyanobacteria filtrates, cyanobacteria cells and micro-algae species as targets have shown the allelopathic effect from cyanobacteria (Keating, 1977, 1978; Falch *et al*., 1995; Pushparaj *et al*., 1999). These authors considered that the observed allelopathic effect explains, in part, the success of, *Aphanizomenon flos-aquae*, *Anabaena* spp., and *Nodularia harveyana* in aquatic environments. However, in the field these effects might be hindered taking into account the mathematical model of Jonsson *et al*. (2009). These authors pointed out that whilst the cell to cell contact will facilitate the allelochemical to be delivered to the target cell even in a turbulent environment, the probability of allelochemicals reaching the target cell immediately decreases if the distance between the cells is greater than the square radius of the target cell. Hence, in Jonsson *et al* (2009) mathematical model, if the target cell is damaged in an initial encounter, later encounters are immediately reduced. They also proposed that allelopathy can be explained if cell numbers are sufficiently large, comparable to that of an established bloom and that the effect of allelochemicals is reduced if the number of cells is low (<5 µg L^{-1}).

Proof of the bioactivity of allelochemicals in aquatic environments, involving release from the donor and transport to the target cells might be difficult because active and passive

transport mechanisms are involved for both release and uptake of allelochemicals (Gross *et al*., 2012). Excretion is an active mechanism of allelochemicals release that requires specific organs for their storage. Exudation is a passive mechanism where allelochemicals are released by diffusion into the surrounding environment. Allelochemical uptake, is perhaps more difficult to explain as it appears to require specific channels (e.g. ATP- transporters) or passive transport across lipid membranes is also possible (Gross *et al*., 2012). These authors discussed that while actively transported compounds can be large (i.e. molecular weight > 1000 Da.), polar (hydrophilic) substances capable of passively crossing membranes should be lipophilic or at least amphiphilic (having both polar and non-polar molecular elements).

Allelochemicals affect photosynthesis and enzymatic processes (Bauer, 2009).They can inhibit the photosystem II (PSII) by interfering with electron transfer (Laue *et al.,* 2002), and can cause a higher redox midpoint potential for the non-heme iron group, located between the primary and the secondary quinine electron acceptors (denominated QA and QB in the PSII). The alkaline phosphatase enzymatic processes can be affected (Gross *et al.,* 2003), where their inhibition damages the whole organism, decreasing the dehydrogenase activity in *M. aeruginosa* and thus resulting in increased levels of reactive oxygen species (ROS) (Hong *et al.,* 2008). Consequently, the oxidative damage of cellular structures will lower activities of antioxidant enzymes, leading to metal ion leakage and changes in cellular integrity (Li and Hu, 2005).

However, these studies are based on the evidence of reduction of phytoplankton species but do not show the presence of allelochemicals, the allelochemical concentration or other biological mechanisms that might also be operative. Gerphagnon *et al.* (2015) reviewed the role of protozoan grazing (amoeba, ciliates and flagellates), including eukaryotic infection by fungi and prokaryotes (environmental bacteria and viruses) and the lytic effect on the phytoplankton cells showing the key role of these microorganisms in the regulation of phytoplankton blooms. The role of ciliates have been highlighted by Lishke *et al*. (2016) who

demonstrated that in the absence of large-bodied crustaceans populations (e.g. *Daphnia* and *Cyclops*), they dominate top-down grazing mechanisms in eutrophic lakes.

The standard methodology proposed by Willis (1985) for allelopathic research is plagued with studies that do not meet all requirements. The present research attempts to meet the six requirements described above: first, the pattern of inhibition of *Microcystis* in the presence of roots of *Phalaris* (biofilters) needs to be established (section 3.3.2); second, allelochemicals are produced by biofilters, specifically by the roots of *Phalaris* (section 4.3.2); and third that allelochemicals are released into and measured in the surrounding water (section 4.3.3). If the fluid media (water) carries or accumulates the allelochemicals to the target cells (*Microcystis*), then the fourth requirement is met. The fifth and the sixth requirements are expected to be met with the experiments carried out in this chapter, and were set up to: a) investigate the mode of action of the allelochemicals by evaluating the inhibition response of *Microcystis* growth to the exposure of nine known allelochemicals as a function of a range of concentrations; and b) to discriminate if the observed reduction in *Microcystis* growth is due to chemical mechanisms by allelochemicals, nutrient competition or biological mechanisms by investigating differences between the effects of root exudates and filtrates on the reduction of *Microcystis* growth as a function of time.

No other study has been found attempting to determine the allelopathic effect of root exudates and filtrates from biofilters in aqueous samples, nor the effects of *Phalaris* on *Microcystis.* The aim of this chapter is to determine whether identified allelochemicals are the chemical mechanism involved in the reduction of *Microcystis* cell numbers (ml^{-1}) and their effectiveness at inhibiting *Microcystis* growth. An overarching question is posed: Can the observed inhibition of *Microcystis* growth in the biofilter units be due to the effect of released allelochemicals from the roots of *Phalaris*, or due to biological microbial activity, or a combination of these?

The following specific research questions were asked:

1) Are there differences in *Microcystis* growth when exposed to three concentrations of nine selected allelochemicals (gallic acid, hordenine, gramine, naringuin, 4-nitroindol-5carboxadelhyde, anthraquinone, tannic acid, linoleic acid and stigmasterol)?

2) Are there differences in the allelochemical rate of production from biofilters as a function of time?

3) Are there differences in the effect of the unfiltered and filtered root exudate on *Microcystis* growth as a function of time?

4) Are there other mechanisms involved in the reduction of *Microcystis* growth?

6.2 Materials and Methods

To answer the research questions, two main experiments were carried out. The first experiment investigated the effect of commercially obtained allelochemicals on *Microcystis* growth at three concentrations and the percentage inhibition of allelochemicals on *Microcystis*. The second experiment investigated the effect of aqueous root exudate, potentially containing allelochemicals, on *Microcystis* growth.

6.2.1 *Microcystis* growth response to commercial allelochemicals: selection, preparation and percentage inhibition of allelochemical concentration

The aim was to determine the effects and percentage of inhibition of the selected allelochemicals: anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringuin, stigmasterol, tannic acid and 4-indole-5-carboxaldehyde and a mix of these compounds (Sigma-Aldrich, see section 4.2.1.). The range of allelochemical concentrations used on *Microcystis* growth was: 1000, 100 and 10 µg L^{-1}.

Although the ideal number of dose-tests for cyanobacteria growth response is six concentrations, it is recommended that a minimum of three concentrations be selected

(OECD, 2011). The response of *Microcystis* growth will be evaluated as a function of the exposure concentration and compared with replicates of a control with no allelochemicals.

Microcystis cells from a culture at the exponential growth phase were taken to carry out the experiments. Controls included *Microcystis* in BG11 and methanol media as a positive control and with BG11 media as a quality negative control. The concentration of *Microcystis* cells was measured as follows: One millilitre aliquots were transferred from the culture flasks into a spectrophotometer quartz cuvette (Sigma-Aldrich, UK). The optical density O.D was measured in a Shimadzu spectrophotometer at wavelengths 600, 610, 620 and 680 nm. Measurements were performed with 1:1 culture dilutions to reproduce the final dilution in the microtubes. From the microtubes, aliquots were taken for measurements in the 96-well plates. A new calibration curve was created with cell counts determined manually by haemocytometer using the method described in 3.2.5.1.

The experimental procedure was as follows: Two sets of 36 clear microcentrifuge tubes of 1.6 ml (Fisherbrand, UK) were autoclaved and dried in an oven at 40 °C (Genlab 100 litres, UK). Aliquots of 0.675 ml and 0.735 ml of both BG11 and *Microcystis* cells in BG11, were transferred for each set of microtubes (1000 $\mu g\ L^{-1}$, 100 $\mu g\ L^{-1}$). Two sets were prepared with a final concentration of 100 $\mu g\ L^{-1}$; one set was diluted to achieve a final concentration of 10 $\mu g\ L^{-1}$ (as in Figure 4.1). Each treatment set consisted of 10 standards (nine individual and the mixed-standard with nine allelochemicals) including the target concentrations, a positive and a negative control. All treatments and controls were carried out in triplicate.

Aliquots of 0.3 ml were taken from the microtubes and transferred into two columns of a 96-well plate (Thermo Scientific™ clear flat bottom), and their O.D was measured at a wavelength of 595 nm excitation with 615 nm emission in a Tecan Infinite F200 plate reader. This procedure was carried out at time zero (initial day), 72 hours (day 3) and 120 hours (day five), denoted respectively as *t0*, *t3* and *t5*. The OECD (2011) states 72 hours as the optimum

duration of the test, although this can be extended depending on the species, particularly for slow growing organisms. Under constant temperatures (here at 21°C) *Microcystis* is a slow growing cyanobacterium, therefore the study of growth response to allelochemicals was extended to 120 hours.

The inhibitory effect of the allelochemicals on the *Microcystis* growth was estimated by calculating the percentage of inhibition over the control, following Hong *et al*., (2009) as in equation (5.1):

$$\text{Inhibition (\%)} = ((C_i - Cf)/ C_i) *100 \quad \text{(eq. 6.1)}$$

where Cs is *Microcystis* cell numbers in the microtubes treated with each of the allelochemicals, and C_0 is the positive control.There was a total of six replicates per sample per treatment.

6.2.2 Experimental design

Experiment II (Exp. II) consisted of three biofilters labelled A, B and C (Figure 6.1). The biofilter units (of 3 L capacity) were filled with tap water and 1 L samples were taken from each biofilter unit (x3).after the biofilters were immersed in the water for 24, 72 and 120 hours (one, three and five days). The water was changed after each experimental day and the roots were rinsed thoroughly each time. The decision to select this number of days was based on the results from the low flow experiment, where the growth of *Microcystis* increased every 72 hours in the feed tank, and accumulated in the control units. However, in the biofilter units, the reduction of cells and lack of colouration was seen at 72 hours and evident at 120 hours (section 3.3.2). These results suggested that chemical activity compatible with allelochemical activity was observed from 72 hours onwards. Therefore, if such algastatic/algicidal activity was due to the release of allelochemicals by the roots in the water, it justifies the choice of time frame used in this experiment for measuring the allelochemicals from the root exudate.

Figure 6.1 presents the experimental design of Experiment II. The batch experiments were named One-Day, Three-Day and Five-Day experiment according to the number of days the biofilters were immersed in the water. Each 1 L sample was subdivided into two subsamples. One subsample was used for the estimation of the production rate by the biofilters (3x 100 ml each) and the other subsample was used to investigate the effect of root exudate on *Microcystis* cell number.

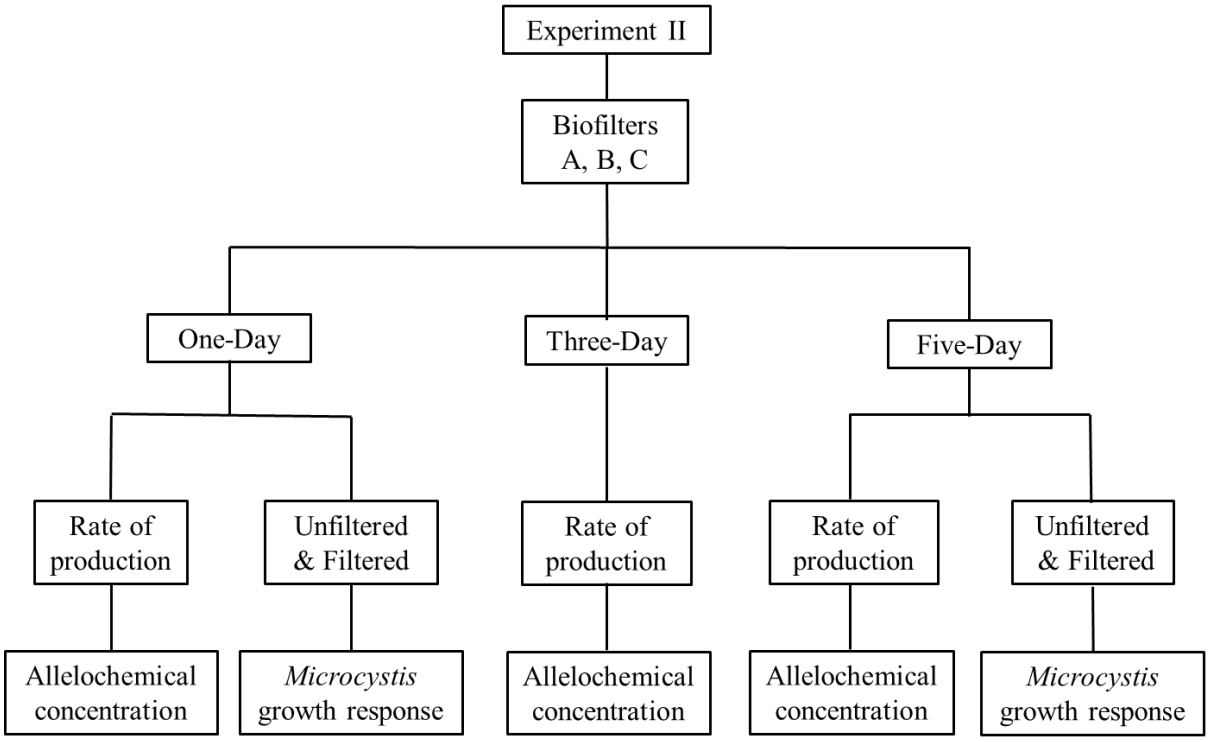

Figure 6.1 Schematic of the experimental design for the estimation of allelochemical production and *Microcystis* growth response to the unfiltered and filtered root exudate treatments.

6.2.2.1 Estimation of allelochemical production rate from biofilters

The estimation of the production rate of allelochemicals as shown in Figure 6.1 was carried out using three biofilters in tap water (each with 16 plants of *Phalaris* and produced as described in section 3.2.8) and in the absence of *Microcystis*. Three replicates of 100 ml (one per each biofilter) were extracted and analysed with the simultaneous method in the HPLC-UV-DAD as described in section 4.2. The length of the experiment was in total 216 hours

(nine days), calculated by adding 24, 72 and 120 hours of One-Day, Three-Day and Five-Day experiments.

6.2.2.2 Microcystis growth response to root exudate and filtered root exudate

The following experiments were carried out to investigate: a) if there are differences between the effects of root exudates and filtrates from the biofilter units on the reduction of *Microcystis* growth as a function of time; and. b) to ascertain if the observed reduction in *Microcystis* growth is due to allelochemicals or biological mechanisms.

Allelochemicals were expected to be present in the root exudate, as a result of the findings presented in sections 5.3.3 and 5.3.4. Triplicates from root exudate samples were filtered to separate allelochemicals from potential biological microorganisms (bacteria, fungi and protozoa) that could contribute to the reduction of *Microcystis* growth (measured as cell numbers). Two controls were used: a) using BG11 to determine the fitness of *Microcystis* cells and the response of *Microcystis* growth to ideal conditions, compared with the treatments; and b) using tap water to determine *Microcystis* growth response in the same media as the treatment.

The procedure for the experimental design was as follows. One litre samples from biofilters labelled A, B and C were taken from the One-Day experiment. Three 500 ml subsamples (A, B and C) were subdivided into two, each of 250 ml each. The 250 ml subsamples were dispensed in aliquots of 22.5 ml to a set of five 50 cm^3 Falcon™ tubes (Falcons). The Falcons were prefilled with 2.5 ml BG11 and 2.5 ml of *Microcystis* from a 250 ml culture flask to obtain a dilution (1:10) in the Falcons and an $O.D_{620\ nm}$ 0.605 ±0.005 (~4.5 x 10^{-5} cells cm^{-3}). The other 250 ml root exudate sample was dispensed, after filtration, in another set of five 50 cm^3 Falcons. Aliquots of 22.5 ml were filtered using a 0.2 µm pore size and 30 mm diameter filter (Fisherbrand, UK) per sample. All Falcons were prefilled with 2.5 ml BG11 and 2.5 ml cell culture at the exponential phase. The controls contained either 22.5 ml of tap water or 22.5 ml of BG11. All Falcons were exposed to a light cycle (12:12) at

photosynthetic active radiation (PAR) of ~10 µmol photons m^{-2} s^{-1} with a laboratory temperature 21-23 °C. Three sets of samples were taken: after thoroughly mixing the Falcon tubes at the start of the experiment (t_0), at three days (t_3), and at five days (t_5) (Table 5.1). Aliquots of 0.5 ml with a calibrated pipette were taken from the Falcon tubes and transferred to a 96-deep well plate (ThermoScientific, UK). Measurements were counts of *Microcystis* cells by flow cytometry (section 3.2.5.2 in chapter 3). The data were analysed using the BD Accuri C6 software. This procedure was repeated for the Five-Day experiment.

6.2.3 Filtered non-extracted standards preparation

To test if the filter used for sample filtration could interfere with allelochemical concentration that could lead to misinterpretation of the results, triplicates of the non-extracted mix-A standard (section 4.2.2, chapter 4) were filtered. These filtered samples were analysed in the same batch as the triplicates of the unfiltered non-extracted mix-A standard samples with the simultaneous method.

The reason for using non-extracted mixed-standard samples was to minimize the risk of introducing potential interferences that could confound the results if using another matrix and because there might be loss of some allelochemicals during the SPE process. The three filtered non-extracted standard samples were obtained by taking three 1 ml aliquots from the standard flask with 900 µg L^{-1} concentration. (chapter 4) using a sterile 1 ml syringe and passing them through Millipore 0.2 µm filters into test tubes. Then, to measure the allelochemical concentration after filtrations, these were analysed as described in section 4.2.2.

6.2.4 Microscopic observations of filtered and unfiltered root exudate samples

An aliquot of 1 ml was taken at 120 hours from the unfiltered (x5) and filtered Falcons from Day-One and Five-Day experiment and dispensed in a test tube. The samples were

mounted and covered with glass slides, then were observed under a light microscope (Eclipse E-200 Nikkon, UK).

6.2.5 Data processing and statistical analysis

Exploratory and statistical analyses were undertaken using Excel (Microsoft © 2010) and SPSS v. 22.0 (IBM Graduate Advanced pack statistic software, UK). Data were checked to meet the parametric assumptions: a normal distribution and equal variances (section 2.2.6, chapter 2). Because some of the data were repeated measurements, the sample was measured three times; no checks for independence were carried out. Thus, because measurements from an experiment taken on day 5 (*t5*) cannot be independent from a measurement taken on day 3 (*t3*) the repeated measures analysis of the variance (RM-ANOVA) test is used for this situation and this is why it was chosen to analyse the data (Dytham, 2011).

In a RM-ANOVA some measurements are dependent or influenced by the previous measurement, therefore the Maulchy's test of sphericity is carried out to make sure that the variation within the dependent measurements is similar to the independent variables or between treatment levels of the design. Sphericity is measured by the differences between pairs of scores computed for each tested element and the variance for each set of differences is calculated (Field, 2009).The assumption of sphericity is a condition that needs to be met in repeated-measures designs. If the test statistic is significant ($p< 0.05$), it indicates that there are significant differences between the conditions, or non-significant when $p>0.05$. The Bonferroni correction was applied to the standard errors.

A repeated measures analysis of variance (RM-ANOVA), with three levels, mixed model with replication was applied to the data. The measured dependent variable is *Microcys*tis growth (cell counts) and the independent factors were: biofilter units, treatment and time. The design had three levels: 1) The biofilter with three biofilter units being tested (a=3); 2) Two types of treatment (b=2); unfiltered exudate and the filtrate of the exudate

sample, each with five replicates (c=5) per unit; and 3) Time was the repeated fixed factor in this design (d=3) with the time scale selected based on the *Microcystis* growth cycle (72 hours). Hence, measurements were carried out as follows: t_0= initial day corresponding to the day when *Microcystis* was inoculated to each replicate; t_3=72 hours after inoculation; t_5=120 hours after inoculation. Each replicate (n=5 per treatment) was measured once at the specified times (n=3 for each replicate, constituting the repeated measurements.

To determine if differences in the response of *Microcystis* growth to the treatment were due to allelochemicals, the response was measured and evaluated as the average of *Microcystis* cell growth at 72 hours in comparison to the average cell numbers on the initial day. To determine if the response differences to the treatment were due to biological microorganisms, the response was measured and evaluated as the average of *Microcystis* cell growth at 120 hours in comparison to the average cell numbers on the initial day when the root exudate was filtered. The filter (0.2 µm pore diameter) removes bacteria and protozoa from the root exudate.

Parametric assumptions (normal distribution and equal variances) could not be met for the Five-Day experiment, despite the data being archsinh-transformed to meet the assumptions. The archsinh transformation is a suitable transformation when there are zeros in samples of count data (Fowler *et al*., 2013). The non-parametric Friedman's test was used to analyse the data from the Five-Day experiment. This test used two factor levels (treatment and the biofilter units) with a repeated measure (time), and there was more than one sample (n=5 replicates). To carry out the test, SPSS was used following Dytham (2011). Kruskal-Wallis test, another non-parametric test, was used to test for differences between the groups of time (t1, t3 and t5) for unfiltered and filtered root exudate samples from biofilters A, B and C.

6.3 Results

6.3.1 Effect of the commercially obtained allelochemicals on *Microcystis* growth and percentage of inhibition

The results from *Microcystis* (growth %) in the presence of 1000 µg L^{-1} and 100 µg L^{-1} of anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringuin, stigmasterol. tannic acid, 4-nitroindol-5-carboxaldehyde and the mix of the eight compounds are shown. There was no inhibition of *Microcystis* growth at 10 µg L^{-1}. The optical density ($O.D_{620nm}$) of *Microcystis* cells in the microtubes and plates was 0.103; this figure corresponds to a cell concentration of approximately 35,000 cells ml^{-1}. Results for the treatment of *Microcystis* cells with allelochemical concentrations of 1000 µg L^{-1} are shown in Figure 6.2.

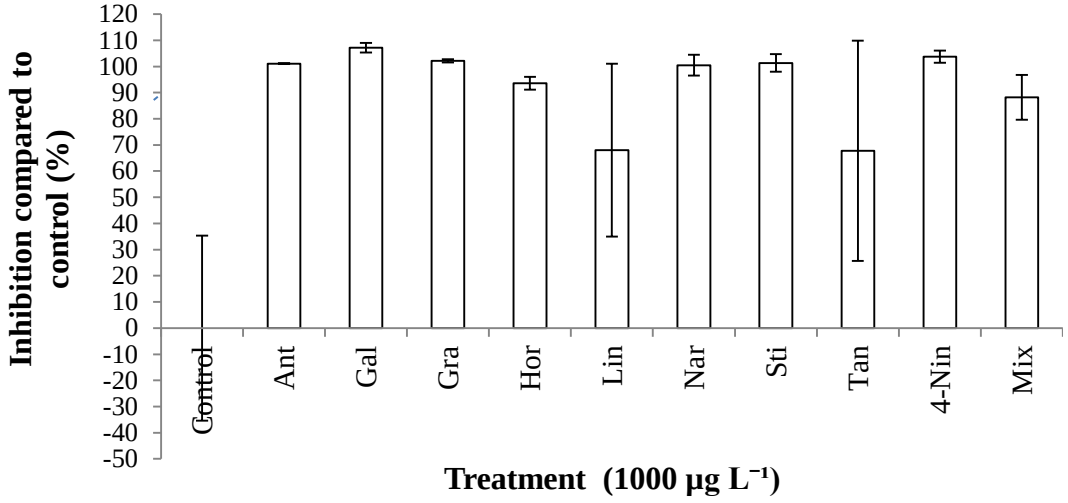

Figure 6.2 Percentage inhibition of *Microcystis* by commercial allelochemicals (mean±SD) with a concentration of 1000 µg L^{-1} compared to a control of *Microcystis* (mean±SD) (*n*=6).

The percentage of inhibition of *Microcystis* growth by seven of the nine compounds was above 92% compared to the controls (Figure 6.3). The standard deviations were small for all compounds, including the mix-A allelochemical standard. The mean percentage of inhibition of the mix-A allelochemical standard was 87%, and Linoleic and Tannic acid was 68% but the response of *Microcystis* to these two compounds showed large standard deviations.

Results for the treatment of *Microcystis* cells with allelochemical concentrations of 100 µg L^{-1} can be seen in Figure 6.3.

Here the mean percentage of inhibition of *Microcystis* growth by eight compounds and the mix was above 50% compared to the controls. Gallic acid and hordenine mean percentage of inhibition were the largest (above 80%); however, anthraquinone stimulated *Microcystis* growth by up to 70% compare to the controls.

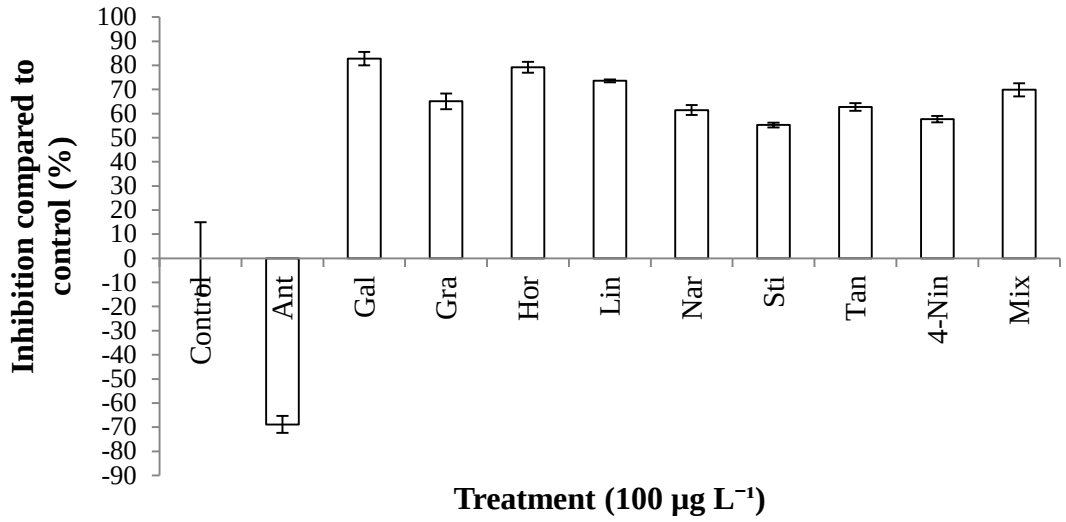

Figure 6.3 Percentage inhibition of *Microcystis* by commercial allelochemicals (mean±SD) with a concentration of 100 µg L^{-1}compared to a *Microcystis* control (mean±SD) (n=6).

6.3.2 Rate of production of allelochemicals in aqueous samples derived from root exudate

The estimation of the rate of production carried out with biofilters immersed in tap water and in the absence of *Microcystis* cells from One-Day, Three-Day and Five-Day experiment (Figure 6.1).

The peaks of the analytes were identified as anthraquinone, naringuin and 4-nitroindol-5-carboxaldehyde. Naringuin concentrations corresponded to the concentration of the internal standard (section 4.2.2). Anthraquinone and 4-nitroindol-5-carboxaldehyde showed a decrease

in their concentration over time (Figure 6.4). The results from the experiments were semi-quantitative for anthraquinone for two reasons. First, the results One-Day (24 hrr) showed concentrations higher than the highest non-extracted standard used in the calibration. Secondly, the results were at or below 80 µg L^{-1} for Three-Day (72 hr) experiment, and for the Five-Day (120 hr) experiment were below the Limit of Quantification (LOQ). Results for 4-nitroindol-5-carboxaldehyde were semi-quantitative for Five-Day, as they were below LOQ. The LOQ and limit of detection can be found in Table 4.4, section 4.3.3.

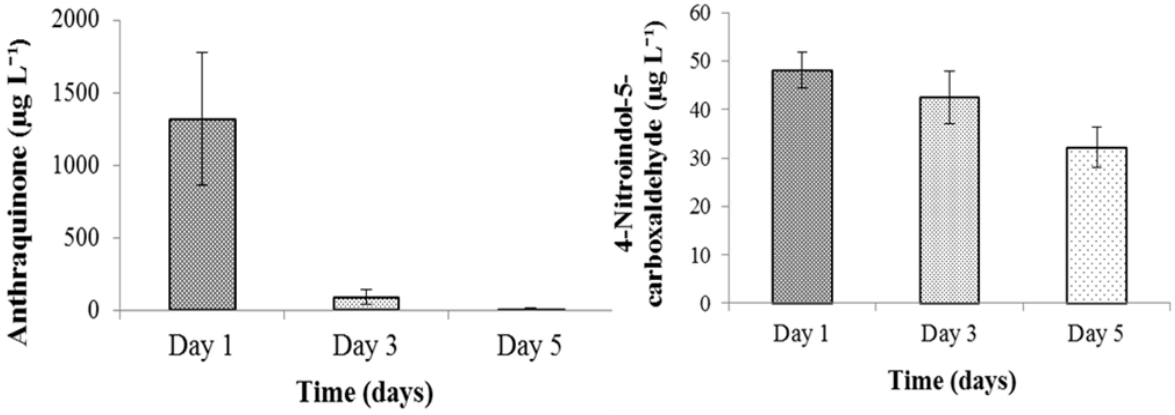

Figure 6.4 Decrease in the concentration (µg L^{-1}) of anthraquinone and 4-nitroindol-5-carboxaldehyde in the root exudates from biofilters (n=3) with time (24 hr, 72 hr and 120 hr).

At the end of the experiment the roots of the biofilters were weighted (g) as described in section 4.2.9, (chapter 4), results were as follows: biofilter A = 44.63 g, B= 39.6 g and C= 92.6 g.

6.3.3 Determining the effect of root exudate on *Microcystis* growth

A number of batch experiments were carried out to determine if chemical or biological mechanisms were involved in the phenomena of reduced *Microcystis* cell numbers and lack of colouration from the biofilters that was observed during the low flow experiments (section 3.3.2 in chapter 3). Three specific research questions were investigated in this section. The first was to determine if there were differences in the effect of the unfiltered and filtered root

exudate on the growth of *Microcystis* (cells ml^{-1}). The second addressed whether the differences observed in biofilters, root exudate and filtrate and their interactions changed as a function of time. The third was to determine differences in the root exudate from the three biofilters A, B and C.

Results for testing the allelochemical concentration after filtration in non-extracted mix-A standards are shown in Table 6.1. The results showed that there were no statistically significant differences between the filtered and unfiltered non-extracted mix-A standard for gramine, naringuin and 4-nitroindol-5-carboxaldehyde. There were statistically significant differences for anthraquinone (t=-30.9, p<0.05), and the concentration of anthraquinone was less in the unfiltered sample (779.3±65.9) compared to (1238.7±15.5) filtered samples.

Table 6.1 Allelochemical concentration (µg L^{-1}) (mean±SD) from unfiltered and filtered non-extracted (900 µg $L^{-1.}$) mix-A standard (n=3). Paired *t*-test, *t* (statistic test), *df* (degrees of freedom) and *p*-value.

Allelochemicals		Non-extracted standard (at 900 µg L^{-1})		Paired *t*-test		
		Unfiltered mean±SD	Filtered mean±SD	*t*	*df*	*p*
Gramine	µg L^{-1}	931.3±34.8	921.7±44.4	0.26	2	0.814
Naringuin (ISTD 100 µg L-1)	µg L^{-1}	99.95±4.73	93.3±3.9	3.46	2	0.074
4-Nitroindol-5-carboxaldehyde	µg L^{-1}	896.2±25.2	896.5±11.6	0.73	2	0.54
Anthraquinone	µg L^{-1}	**779.3±65.9**	**1238.7±15.5**	-30.9	2	0.001

α = 0.95, p =<0.05

As seen in Figures 6.5 and 6.6, there were large differences in the number of *Microcystis* cells from the treatments (unfiltered and filtered) compared to both controls,: BG11 and tap water. The control in BG11 showed the response of *Microcystis* growth in ideal conditions, and the control in tap water demonstrated the growth response in the same media

that the treatments were carried out in. These figures also show that after 72 hours of inoculation, *Microcystis* growth decreases in the controls and the treatments for the One-Day experiment (Figure 6.5), whilst in the Five-Day experiment the decrease at 72 hours takes place only in the controls. In the Five-Day experiment, the standard error at 72 hours is large for the unfiltered treatment while for the filtered treatment the number of cells increases more than the number of cells in both controls (Figure 6.6).

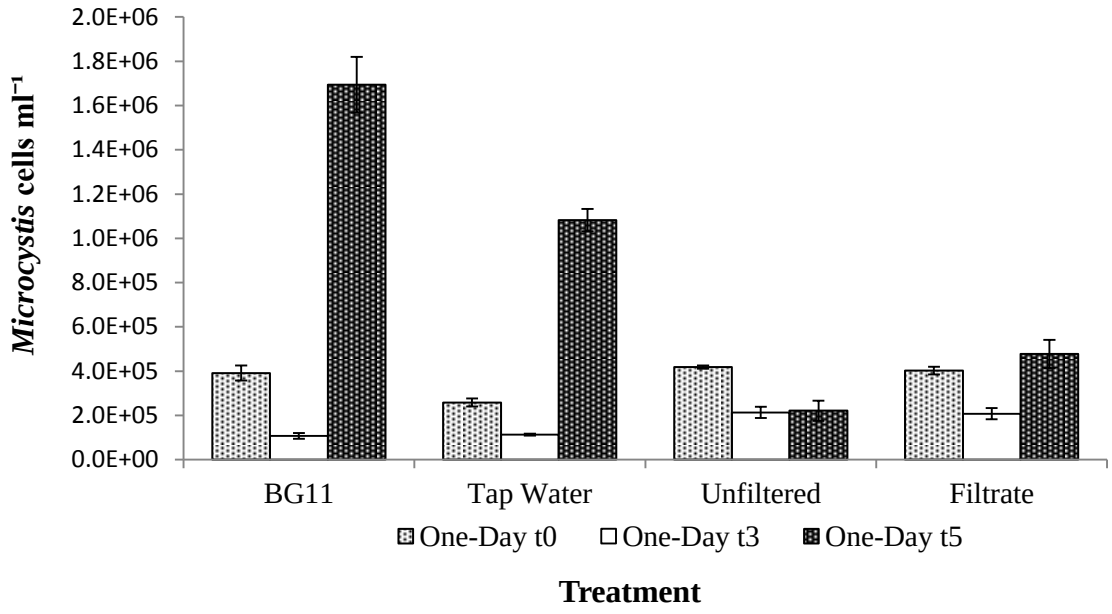

Figure 6.5 *Microcystis* growth (cells ml^{-1}) (mean±SE) for the controls (BG11 and tap water) and the response to the One-Day root exudate exposure with treatment (unfiltered and filtered) (*n*=15) on the initial day (*t0*), three (*t3)* and five days (*t5*).

The Falcon tubes were as clear as tap water at 120 hours for the unfiltered One-Day and Five-Day experiment with no signs of green colouration. These results will be further analysed to determine the significance of the root exudate treatments (unfiltered and filtered) in the One-Day experiment by repeated measures ANOVA and the non-parametric repeated measures Friedman's test for the Five-Day experiment.

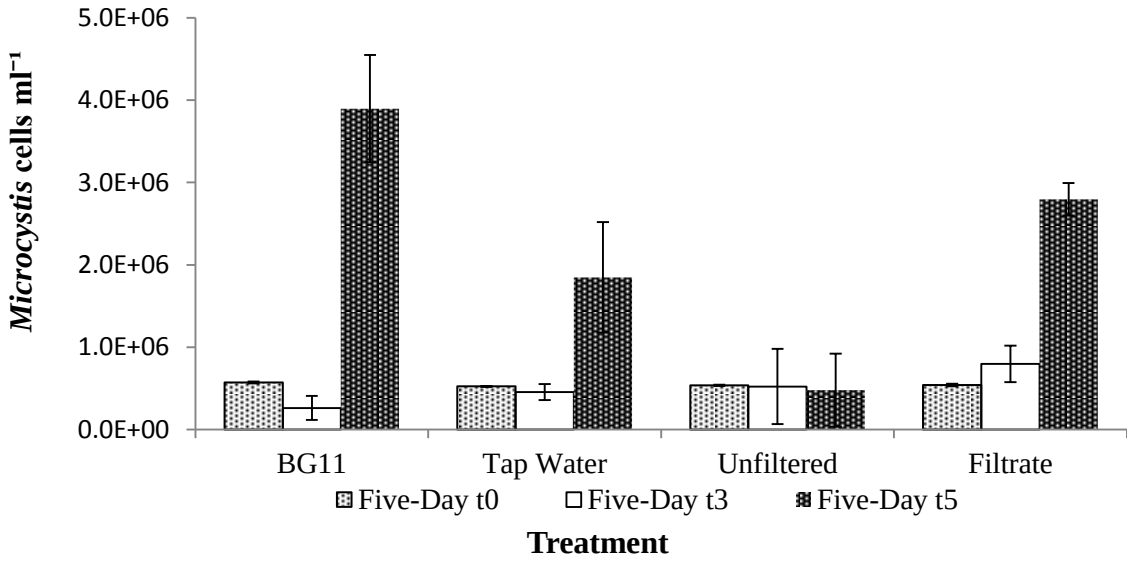

Figure 6.6 *Microcystis* growth (cells ml^{-1}) (mean±SE) for the controls (BG11 and tap water) and the response to the Five-Day root exudate exposure with treatment (unfiltered and filtered) (*n*=15) on the initial day (*t0*), three (*t3)* and five days (*t5*).

6.3.3.1 One-Day root exudate treatment experiment on Microcystis growth

To determine differences between the One-Day root exudate treatments, changes over time and their interactions, the data were analysed with a RM-ANOVA. The data fit the assumptions of a normal distribution and equal variances. The result the Maulchy's test of sphericity was non-significant (p>0.05) for the main effect biofilter (p=0.084) nor was it significant for the interaction biofilter*treatment (p=0.75), hence meeting the assumptions.

The RM-ANOVA table (Table 6.2) showed that biofilter (root exudate source) and the interaction biofilter*time were non-significant (p>0.05). However, significant differences (p<0.05) were found for the interaction biofilter*treatment. These results indicate that there are differences in the effect of the treatment (unfiltered and the filtered) on *Microcystis* growth response, and that there is no difference between the three biofilters, the source of the root exudate samples.

Table 6.2 Results from the repeated measures ANOVA (*F* and *p*-values) showing the effects of biofilters, unfiltered and filtered root exudate treatment and their interaction as a function of time (*t0, t3* and *t5)* on *Microcystis* growth (cells ml^{-1}).

Source of variation	SS	*df*	MS	*F*	*p*-value
Biofilters	$8.23*10^{8}$	2	$4.12*10^{8}$	0.35	0.709
Biofilters*Time	$4.69*10^{10}$	4	$1.17*10^{10}$	0.994	0.430
Error (biofilters)	$2.8*10^{11}$	24	$1.17*10^{10}$		
Treatment	$1.37*10^{11}$	1	$1.37*10^{11}$	11.58	**0.005**
Treatment*Time (interaction)	$3.59*10^{11}$	2	$1.79*10^{11}$	15.19	**0.001**
Error (treatment)	$1.42*10^{11}$	12	$1.18*10^{10}$		
Biofilters*Treatment	$2.81*10^{11}$	2	$1.40*10^{11}$	10.98	**0.001**
Biofilters*Treatment*Time (interaction)	$5.17*10^{11}$	4	$1.28*10^{10}$	10.13	**0.001**
Error (Treatment*Time)	$3.07*10^{11}$	24	$1.28*10^{10}$		

SS= Sum of squares; *df*= degrees of freedom; *MS*=mean square, *F*= F distribution

Microcystis growth response (cells ml^{-1}) to treatment and time was significant ($p<0.05$). This significance indicated *Microcystis* growth decreased with the unfiltered treatment compared to the filtered treatment. *Microcystis* growth also decreased significantly at time (*t3*) (72 hours) compared to the initial day (*t0*) of inoculation. The results of the effect of the interaction between treatment and time were significant ($p<0.05$), with the greatest positive effect on the growth of *Microcystis* observed at five days (*t5*) with the filtered treatment (Figure 6.7).

There was a significant interaction effect between: time, biofilter and the type of treatment (Table 6.2). To break down their interaction, RM-ANOVA was performed comparing time at 72 and 120 hours with the initial day of inoculum as a control. Because no significant differences were found between the biofilters, biofilter A was taken as the control at t_0. *Microcystis* growth response to the interaction effect was greatest between biofilter A and biofilter C, compared to filtered and unfiltered treatment, as a function of time, $F_{(2,}$

$_{12)}$=18.60, p=0.000 (p<0.05). There was also a significant difference between biofilter B and biofilter C with $F_{(2, 12)}$=9.342, p=0.000 (p<0.05) (Figure 6.8).

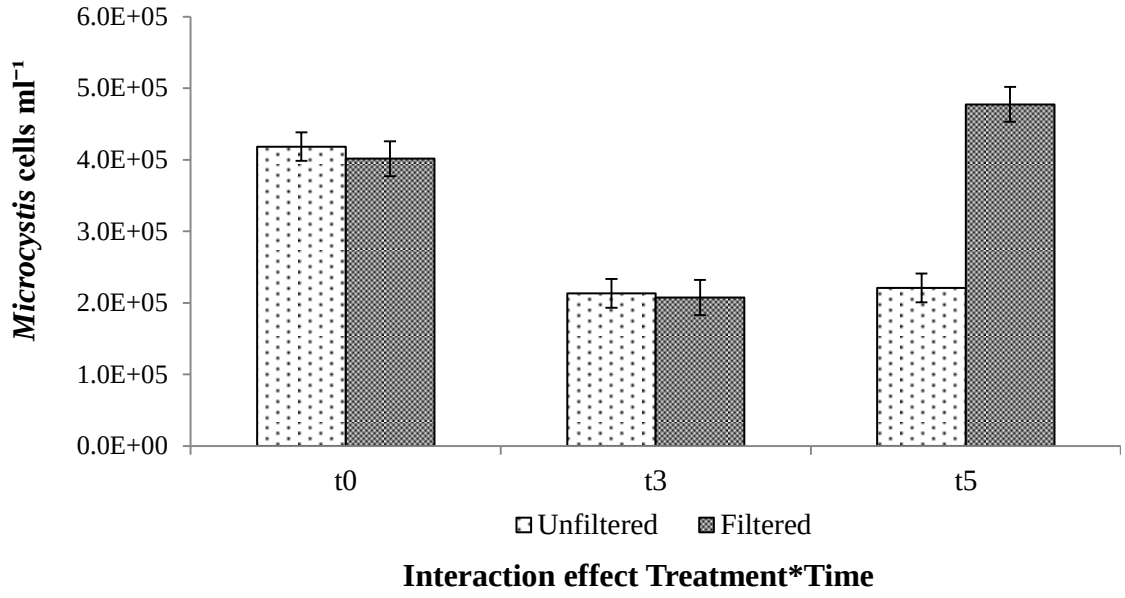

Figure 6.7 The mean (±SE) of *Microcystis* growth (cells ml^{-1}) in response to unfiltered and filtered treatment of the aqueous root exudate (*n*=15) as a function of time (days = t*0*, t*3* and t*5*).

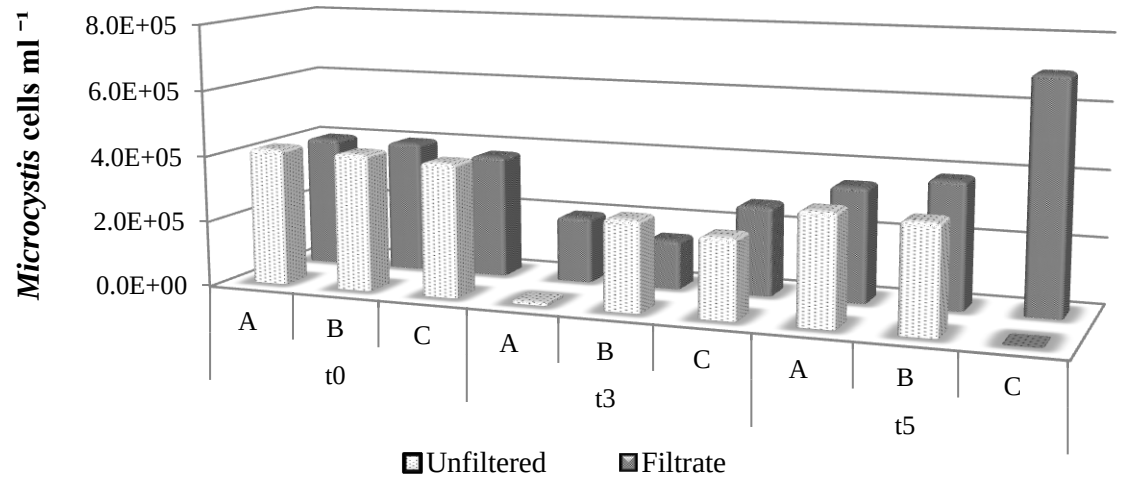

Figure 6.8 The mean (±SE) of *Microcystis* growth (cells ml^{-1}) in response to a factorial combination of the biofilters as the source of the root exudate treatment (unfiltered and filtered) (*n*=15) as a function of time (days = *t0*, *t3* and *t5*).

6.3.3 2 Five-Day experiment

Results for the Five-Day experiment with the non-parametric Friedman's test showed there were significant differences between the treatments (unfiltered and filtered) from biofilter C (X^2= 46.7,*df*=5, p<0.05, n=10). The Kruskal Wallis test allowed for testing paired differences of the medians. Significant differences (p<0.05) were found for the variable Time (*t1* and *t3*) for unfiltered and filtered samples from biofilter B and C (n=10). Some data were missing for biofilter A, the data from (*t5*) could not be used in the test (Table 6.3).

Table 6.3 Unfiltered and filtered results from biofilters A, B and C with grouping variable Time (*t1* and *t3*) using Kruskal Wallis test. The test statistic (X^2), degrees of freedom (*df*) and *p*-value are given.

	Biofilters - Treatment					
	A-Unfiltered	**B-Unfiltered**	**C-Unfiltered**	**A-Filtered**	**B-Filtered**	**C-Filtered**
(X^2)	2.455	6.818	6.902	6.860	4.811	5.771
df	1	1	1	1	1	1
p-value.	0.117	0.009	0.009	0.009	0.028	0.016

6.3.4 Microscopic observations of unfiltered and filtered root exudate samples

A number of protozoans and invertebrates were observed from unfiltered samples but no microorganisms from the filtered samples were observed apart from *Microcystis* cells. Protozoans present were heliozoans, ciliates and zooflagellates. Invertebrates present were rotifers. Although some micro-algae (green algae) were also observed during the light microscopic analysis, the quantitative analysis by flow cytometry of the root exudates showed only one population of cells counted that corresponded to a population of *Microcystis*.

6.4 Discussion

Unfiltered and filtered root exudates were used to evaluate their effect on *Microcystis* growth. Allelochemical compounds with algastatic/algaecide effects on *Microcystis* growth were detected, identified and semi/quantified from the root exudate.

6.4.1 What is the biological effect of commercially obtained allelochemicals on *Microcystis*?

Findings on the effective algastatic/algaecide effect of the allelochemicals on *Microcystis* growth showed this species was strongly suppressed at the highest concentration (1000 μg L^{-1}) by all allelochemicals whilst the minimum effective percentage inhibition found was at 100 μg L^{-1}.

The percentage of inhibition at the highest concentration was ≥87% for most allelochemicals (gallic acid, gramine, hordenine, naringuin, stigmasterol, 4-nitroindol-5-carboxaldehyde) including the mix of allelochemicals, except for linoleic and tannic acids. Although the inhibition response (68%) of *Microcystis* growth to linoleic and tannic acids was not the lowest, the fact the response of *Microcystis* growth was very similar at the 1000 and 100 μg L^{-1} concentration treatment is difficult to explain (Figure 6.2 and 6.3). The standard deviations for these two allelochemicals was large in comparison to the others tested. A possible explanation might be that linoleic acid and tannic acid are fatty acids causing cell aggregation at the highest concentration; hence some cells escape the direct contact with the allelochemical. However, the most interesting result was that the minimum effective percentage inhibition was ≥55% for almost all allelochemicals at 100 μg L^{-1}, including the mix of allelochemicals, with the exception of anthraquinone (Figure 6.3). The percentage inhibition of stigmasterol and 4-nitroindol-5-carboxaldehyde (55%) was the lowest for the inhibition of *Microcystis* growth.

One unanticipated finding was the synergistic effect (68%) of anthraquinone (100 μL^{-1}) on *Microcystis* growth (Figure 6.3). This synergistic effect at concentrations below a toxic threshold of a tested substance on a living organism (e.g. micro-algae, cyanobacteria, plant and zooplankton) is an effect known as hormesis (Forbes, 2000, Cerbin *et al*, 2010; OECD 2011). Hormesis might be involved in the evolutionary process of strain selection as discussed by Forbes (2000). The hormetic effect has been demonstrated on *Microcystis* growth when exposed to, among other substances, the allelochemical ethyl-2-methylacetoacetate isolated from *Phragmites communis* (Hong *et al.*, 2008); pesticides such as pentachlorophenol (De Morais *et al.*, 2014), and the antibiotic amoxicillin (Liu *et al*., 2016). However, the stimulatory effect of anthraquinone on *Microcystis* growth has not been described in the literature before.

The starting concentration of *Microcystis* cell ($3.5x10^4$ ml^{-1}) was within the recommended starting concentrations by the OECD (2011), which is an order of magnitude lower than that used in other studies, where concentrations of $1x10^5$-$1x10^6$ ml^{-1} are used in microliters volumes (Lürling *et al.*, 2013). The allelochemical concentrations used in other experiments are generally ≥1 mg L^{-1} from dried root extracts or commercial allelochemicals to demonstrate their algastatic/algaecide effect (Lürling and Beekman, 2010). This research was carried out using 1.5 ml as the final sample volume, which is an order of magnitude higher than other laboratory experiments where the final volume is in the order of microlitres.

Further work should include testing these allelochemicals with concentrations ranging between 10 and 1000 $\mu g\ L^{-1}$. The ideal number of six concentrations for testing minimum percentage inhibitory concentrations of allelochemicals should be met, to identify the minimum inhibitory concentration for anthraquinone and determine the lowest threshold with hormetic effect for the other allelochemicals.

The extrapolation of the effective minimum inhibitory concentration of released allelochemicals from biofilters or floating treatment wetlands to water bodies can be used to estimate their effective diluted concentrations in those water bodies and their potential for managing *Microcystis*, other cyanobacteria and micro-algae. Also, this research found that the concentration of the released allelochemicals through the roots was high enough to be effective as an algicide/algastatic (Figure 5.7 and 5.8).

6.4.2 Are there differences in the allelochemical rate of production from biofilters aqueous samples as a function of time?

The findings from the estimation of the rate of production experiment indicated there was a decrease over time in the release of the detected and identified allelochemicals, but time affected the two identified allelochemicals differently. Anthraquinone degraded drastically (from about (1300±270 µg L^{-1} to <LOD) (Figure 6.4) from 24 hours to 120 hours whilst 4-nitroindol-5-carboxaldehyde was more stable despite having relatively low concentrations. However, these results were obtained from an experiment carried out in the absence of a possible trigger such as *Microcystis* and also subject to the fact that the biofilter media was tap water. In a previous experiment (section 5.3.4, chapter 5) it was demonstrated that allelochemicals released into the surrounding media respond to stressors such as nutrient media and the absence and/or presence of *Microcystis*. Although anthraquinone was not detected in those experiments, it is important to mention that allelochemicals (in section 5.3.4) were measured at 120 hours, and only traces of anthraquinone were found.

6.4.3 Are there differences in the effect of the unfiltered and the filtered aqueous root exudate on *Microcystis* growth as a function of time?

These experiments were carried out exposing only *Microcystis* only to root exudate (in the absence of biofilters); and therefore, it can be hypothesised that the chemical mechanism observed for the suppression of *Microcystis* growth is likely to be due to allelochemicals and

not to nutrient competition between the roots and *Microcystis*. Although potential nutrient competition between other micro-organisms (bacteria and fungi) cannot be completely overruled in the unfiltered samples, their competition with *Microcystis* might be negligible because *Microcystis* is an oxygenic photosynthetic organism that transforms light energy into chemical energy (Beardall *et al*., 2009; Raven, 2009).

These experiments were carried out with aqueous root exudate samples without being extracted to test their activity on *Microcystis* cells. These are the first experiments testing the allelopathic effect of aqueous root exudate on *Microcystis* cells. Although, Nakai *et al.* (2008) also used aqueous root exudate for testing against *Microcystis* cells, the way their experiment was conducted is different from the one performed in this study but also questionable for the following reasons. First, their aqueous root exudate was extracted and concentrated in methanol (no percentage was given), therefore inhibition of *Microcystis* by methanol is likely. Second, bioassay inhibition results were reported for a 25 day experiment which seem to be a long time compared to inhibition experiments reported in the literature (Lürling *et al*., 2006; Jancula and Marsalek, 2011; Lürling and Van Oosterhout, 2013). Third, there was no quantification of the allelochemicals detected despite using GCMS.

The findings for the treatment of unfiltered and filtered root exudate for the One-Day experiment indicated there was an inhibitory effect on *Microcystis* growth after the cells were exposed for 120 hours to the treatment, compared to the controls (Figure 6.5). However, when comparing the effect of unfiltered and filtered treatments, there were significant differences, with an increase in the *Microcystis* growth response to the filtered treatment at 120 hours in the One-Day and Five-Day experiment (Figure 6.7).

These findings strongly support the hypothesis that the reduction of *Microcystis* cells is not due to nutrient competition, because there were no living roots physically involved in this experiment, also there was a supply of BG11 media for *Microcystis* cells, so that the cells would survive. Adding to this, is the fact that where inhibition was shown, the Falcons appear

clear with no signs of green, suggesting oxidative processes which are a characteristic of the effect of allelochemicals on cyanobacteria cells, including *Microcystis* cells.

The evidence is provided by the fact the aqueous root exudate was only added once and was left in contact with *Microcystis* cells for 120 hours. As demonstrated in this research the concentration of anthraquinone at 24 hours is high (1300 ± 270 μg L^{-1}) and is shown to be a strong inhibitor of *Microcystis* growth at ≥1000 μg L^{-1} (Figure 6.2). Despite this the allelochemicals showed degradation at 120 hours (Figure 6.4), so it is likely that the photosystem II of the cells has already been damaged within the first 72 and/or 96 hours. Therefore if low concentrations were present at 120 hours because of degradation, no hormetic effect (Figure 6.3) could have taken place because the first contact with the allelochemical was at high concentrations causing possibly irreversible damage to the cells (Shao *et al*., 2013; Laue *et al*., 2014)

The claim that the allelopathic effect of the single addition of aqueous root exudate to the *Microcystis* cells can cause a lasting effect is supported by the work of Suikkanen *et al*., (2004) who found that the responses of micro-algae were not significantly different, irrespective of the number of filtrate applications from the cultures of cyanobacteria. It might be that 4-nitroindol-5-carboxaldehyde could inhibit *Microcystis* growth at concentrations >10 μg L^{-1} but <100 μg L^{-1}, but this was not demonstrated here. Also other unidentified chemical compounds that could be allelochemicals, may be inhibiting *Microcystis* growth.

Furthermore, the inhibitory effect on *Microcystis* growth observed at 120 hours as well as the loss of colouration were also observed in the units with biofilters during the low flow experiments (3.3.2). The first observation of a reduction of *Microcystis* cell numbers was between 72 and 96 hours, being most evident at 120 hours in the low flow experiment.

6.4.4 Were there biological mechanisms involved in the inhibition of *Microcystis* growth?

The results show that allelochemicals are released from the roots, but there seems to be a biological mechanism that contributes to the further reduction of *Microcystis* growth. This statement is supported by the fact that anthraquinone was above 1000 μg L^{-1}, and no significant differences were found among the biofilters A, B and C in the One-Day experiment. Therefore, all root exudate samples of the three biofilters had a similar effect on suppressing *Microcystis* growth. By contrast, anthraquinone concentration was 30 ±0.2 μg L^{-1} when the Five-Day experiment started, and the hormetic effect, could explain the slight increase in *Microcystis* cells at 72 hours. Despite these results not being statistically significant, the means did differ (Figure 6.6.). The hormetic effect might explain the further increase at 120 hours in the filtered treatment. However, it cannot explain why such an increase of *Microcystis* growth was not observed in the unfiltered samples at 72 and 120 hours, taking into account that there is no difference between unfiltered and filtered allelochemical concentration (Table 6.1). These results strongly suggest there may be another mechanism involved in the unfiltered samples.

The previous observations are supported by the significant differences found for the interaction between the biofilter and treatment in the Day-One experiment that showed differences between A and C, and B and C biofilters. Similar results of significant differences were found between biofilter C compared to biofilters A and B in the Five-Day experiment. A possible explanation could be related to its larger biomass (C=92.6 g compared to A=44.63 g and B=39.6 g). Therefore, it is possible that in biofilter C, potentially larger protozoan and invertebrates (e.g. rotifers), bacterial and fungal biomass was supported by a larger root biomass. Although the reduction of *Microcystis* growth cannot be solely attributed to protozoa present in the root exudate, their role as a biological mechanism on *Microcystis* reduction is further supported by microscopic observations and the identification of protozoa and invertebrates in the root exudate samples.

Chapter 7. Characteristics and recommendations to improve future field-scale biofilters

This chapter focuses on the requirements for the design of an optimized field-scale floating biofilter based on the Living-Filter structural design as a prototype, take into account the main findings from the research presented in previous chapters: plant selection, hydraulic rate; Farmoor II reservoir and WTWs local conditions.

7.1 Introduction

In this research, filamentous biofilter processes have shown their capabilities for removing phytoplankton cells in macro-, meso- and microscale studies. The outcome of the removal efficiency of phytoplankton biomass by the Living-Filter was presented in chapter 2, and further questions that arose from the survey were briefly discussed (section 2.4.3). The concept of the Living-Filter for physical filtration was tested in chapter 3. Chemical and biological mechanisms were subsequently investigated and identified in meso- and microscale studies (chapters 3, 5 and 6). Recommendations for future field-scale biofilters include key features of the Living-Filter, such as the modular plant-bed structure, curtains and baffles (section 1.5.2, chapter 1), thereby maintaining its dual role by: firstly, removing phytoplankton biomass from inflowing water prior to potable WTWs, and secondly, reducing phytoplankton biomass in surface waters by top-down mechanisms. However, designing future macroscale biofilters ideally should also consider the limnology of the water body and the capacity of the water treatment works.

Field-scale biofilters should follow the design parameters for floating treatment wetlands (FTWs) or constructed wetlands (CWs) with horizontal subsurface flow (HSSF) (Rousseau *et al*., 2004). Horizontal subsurface flow design is defined as the horizontally flow of raw water (or pre-treated wastewater) through a treatment media (gravel, sand, peat, roots and rhizomes). Although the direction of flow in floating field-scale biofilter designs such as

the Living-Filter is horizontal, there is not a designated inlet or outlet as in the HSSF-FTWs or HSSF-CWs; the side of the biofilter where inflowing water first enters the plant-bed constitutes the inlet. The other end of the biofilter where the water leaves the plant-bed facing the abstraction tower is the outlet (Figures 1.3 and 2.1). The water flows through roots, rhizomes and a hollow plastic matrix.

According to Chin (2012), the FTW designs are either technology or performance-based. The first uses regulatory prescribed ratio of loading rates to FTWs size, while the second uses targeted outflow concentrations or removal rates as the basis for determining the FTW size. Hydrology is one of the most important variables in FTW design that will influence the physicochemical and biological conditions (Chin, 2012). A number of hydrological design models have been developed for treatment wetlands. Rousseau *et al*. (2004) discussed the most frequently used and pointed out their advantages, limitations and uncertainties. However, the efficiency of the biofiltration process, as mentioned by Scholz (2006) will depend on organic biomass, organic and hydraulic loading rate, depth, size and packing density of the media, as well as the total surface area of the media available. Thus, creating complex systems for modelling is increasingly difficult and the system variable is generally reduced to the most relevant variables that might offer closer predictions than in more complex models (Rousseau *et al*. 2004).

Thus, based primarily on the information gained in this study, the improved design should emphasize the requirements needed for promoting an increase in root density for entrapment of phytoplankton cells, an increase in concentration of allelochemicals, and an increase in contact time between allelochemicals and the phytoplankton. To promote top-down mechanisms in the restoration in eutrophic lakes and reservoirs the design should incorporate guidelines for determining the size of the vegetation cover that best promotes zooplankton populations (Hilt *et al*., 2006). The description, function and requirements presented in Figure 7.1 will be discussed in the body of this chapter.

Ideal field-scale floating biofilter system

	Description	Functions and Requirements
Plant-bed and caged structure	• Aquatic plant species • Biodiversity: with at least three native plant species • Adequate plat-bed size to reservoir ratio • Consider a suitable synthetic plastic fabric in the modular cage • Adequate protection/baffles on the periphery of the structure	• Robustness to extreme weather conditions (wind, insolation, wave action) • Bio-resilience to disease and extreme events • Filtration mechanisms can be improved by incorporating a suitable synthetic fabric
Roots	• Long roots (≥ 1 m) • High root density • Hairiness • High production of allelochemicals • Hydroponic growth	• Physical filter • Nutrient intake/storage • Chemical production/ storage/release
Rhizosphere	• Adequate flow rates • Provision of microhabitats for zooplankton refuge and reproduction • Supporting structure for biofilms • Gas interchange	• Increase retention and contact time • Increase grazing rates • Balanced gas interchange • Little accumulation of detritus (ideal)

Figure 7.1 Summary of an ideal field-scale biofilter system: description, minimum functionality and requirements.

The research questions to be addressed in this chapter are:

1) Can a plant-bed size be determined to promote biofiltration processes prior to potable WTWs and to reduce phytoplankton biomass?

2) Is there an optimum retention time to enhance physicochemical and biological mechanisms for the removal or reduction of phytoplankton biomass?

7.2 Recommendations for field-scale biofilter designs in surface water

7.2.1 Water quality variables

Treatment wetland designs (CWs or FTW) focus on reducing the following water quality variables: total suspended solids, total phosphorus, total nitrogen, biological and chemical oxygen demand (Rousseau *et al*., 2004) which can be used to determine macroscale biofilter performance in surface waters. Chlorophyll-*a* can be included as a variable for biofilter performance because is used to determine the water quality of freshwater systems (European Environment Agency, 2014). Farmoor II reservoir water quality variables were measured during the macroscale study (July and October of 2013) (section 2.2.2, chapter 2). The mean±SE, median, minimum and maximum figures of these variables are shown in Table 7.1 These data show the levels of these variables in Farmoor II reservoir.

Table 7.1 Farmoor II reservoir water quality variables (mean±SE, median, minimum and maximum values): total suspended solids, total phosphorus, dissolved inorganic nitrogen, biological chemical oxygen demand, and chlorophyll-*a* (mg L^{-1}) (n=17).

Variables	Mean±SE	Median	Min	Max
Suspended solids (mg L^{-1})	3.46±0.28	3	0.4	8
Total phosphorus (mg L^{-1})	0.10±0.004	0.09	0.1	0.16
Dissolved inorganic nitrogen (mg L^{-1})	2.73±0.06	2.63	2.66	3.68
Biological oxygen demand (mg L^{-1})	1.21±0.08	1.16	1.05	2.9
Chemical oxygen demand (mg L^{-1})	17.98±0.76	16.7	10	30.8
Chlorophyll-*a* (µg L^{-1})	21.59±2.24	13.96	5.86	58.7

7.2.1.1 Phytoplankton loading and correlated variables.

The surface water productivity will determine the phytoplankton loading for a field-scale biofilter design for phytoplankton removal. Therefore variables such as chlorophyll-*a* (a surrogate of phytoplankton biomass) and total suspended solids (SS) can be selected to determine the loading. For example, during the macroscale study, it was found that there was a good correlation between chlorophyll-*a* and suspended solids, with a coefficient of determination, r^2=0.78, p< 0.01, n=67 (number of samples) (Figure 7.2). These results suggest that either variable could be used to calculate the organic loading (phytoplankton biomass/suspended particles) for the biofilter design.

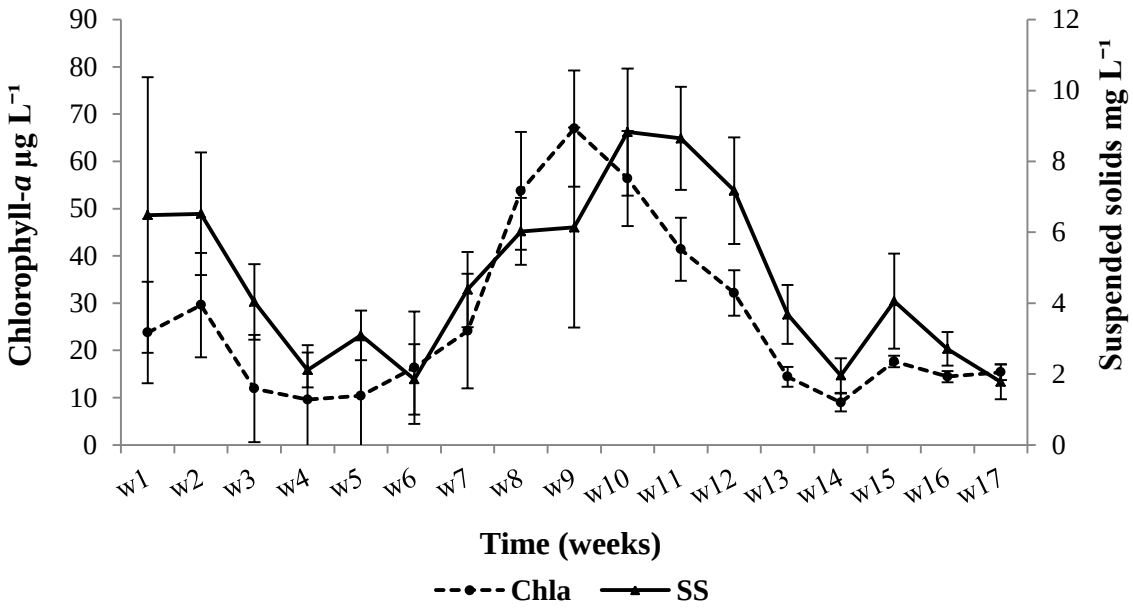

Figure 7.2 Trends in chlorophyll-*a* concentration (Chla µg L^{-1}) and total suspended solids (SS mg L^{-1}) (mean±SE), *n*= 4 (stages 1, 2, 3 and 4 as in section 2.1) from measurements taken during the survey period (July-October 2013) over 17 weeks (Time).

The biomass of *Microcystis* (as cell biovolume) from mesoscale experiments was estimated in order to calculate the loading onto the biofilters and synthetic filters. The biovolume of *Microcystis* cells counted by flow cytometry (section 3.2.5.2, chapter 3) was estimated following the method of Hillbrand *et al*. (1999). The geometrical shape for

Microcystis spherical cells corresponds to the volume of a sphere (volume= (4/3)(π*r³)). The cell diameter was measured using a lens with a calibrated graticule under a light microscope. The average cultured *Microcystis* cell diameter was 3.2±0.8 µm and the biovolume estimate was 17.16 µm³, whilst (Reynolds *et al*., 1980) estimated that *Microcystis* colonies in surface water can measure 40-1000 µm and the total volume of the colony might contain between 130-28,000 cells. Therefore the average of cell counts in a colony will provide an estimate of the colony biovolume. The relationship between *Microcystis* cells (counts ml^{-1}) and biomass (mg L^{-1}) is shown in Figure 7.3.

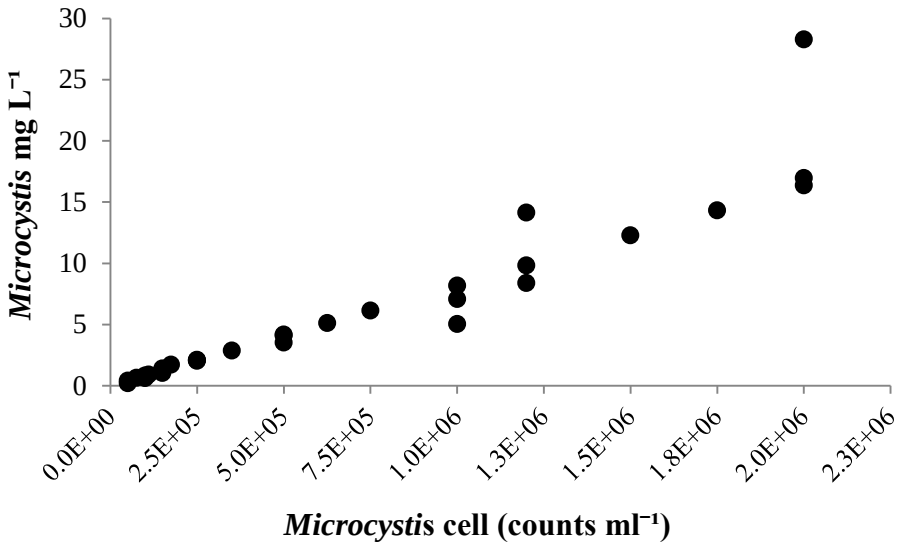

Figure 7.3 Association of *Microcystis* cell counts ml^{-1} and corresponding biomass (mg L^{-1}).

7.2.2 Hydraulic load and retention time

In a review of constructed wetland (CW) designs with HSSF, Wu *et al*. (2015) found that a greater hydraulic loading rate promotes fast flow through the media but reduce contact time, and hence reduce system performance. But longer hydraulic rates (<0.5 m d^{-1}) favour the establishment of microbial communities improving the system performance. This optimal hydraulic rate is 10 times lower, 0.02 m h^{-1} than for slow sand filters (SSF) where the hydraulic rate is 0.2 - 0.6 m h^{-1}, but the hydraulic differences can be justified by the higher

organic loading for CWs than for SSF. Therefore, field-scale biofilters need to have hydraulic rates similar to those of SSF. It was mentioned in chapter 2 that the hydraulic loading rate for the Living-Filter system was too high (at 10-20 m h^{-1}) compared to that of SSFs. Furthermore, the Living-Filter retention time, which is currently at 5-20 minutes is constrained by the water demand from the WTWs (average 65 x 10^3 m^3 d^{-1}), but should be increased in future designs. Moreover, Aberle and Jarvela (2013) have pointed out that high flow velocities for herbaceous flexible vegetation in riverine flows will force the shoots to bend. High flow velocities in the Living-Filter might force the roots to bend thus reducing the surface area for retention of suspended particles.

However, results from the allelochemical inhibition growth activity on *Microcystis* cells showed that after 72 hours (three days) there is a reduction in the number of cells (chapter 6). Therefore, and taking into account the above points, an ideal biofilter system for phytoplankton removal should have a retention time of at least three days. These results are in agreement with the findings of Wu *et al*. (2015) that a contact time between 2-5 days improves CW-HSSF microbial activity performance. Although it is worth bearing in mind that in their study the retention time recommendations for CWs are for treating tertiary waters or sewage effluents with higher organic/particle loadings than surface waters. The proposed contact time of at least three days for effective chemical removal (mediated by allelochemicals) might appear unrealistic for potable WTWs with large demands for water treatment, but the retention time could be shortened depending on the location, size of the plant-bed, as well as plant coverage, selection and demonstrably high production rates of allelochemicals into the water. Further discussion follows in section 7.2.4.

7.2.3 Plant selection versus artificial filamentous media

Plant selection and retention time are two fundamental variables for macroscale biofilter design for phytoplankton cells removal (Figure 7.1). Although plant selection has been

thoroughly discussed in previous chapters, particularly in chapter 5, a brief review of the main characteristics for selection is given, using new results extracted from previous data. These characteristics are described as follows:

- Root density: provides the media for physical filtration, thus the greater the density, the more efficient removal by filtration.
- Root allelochemical production/release: this is the chemical mechanism that was dominant for phytoplankton removal in laboratory biofilters. Thus, high allelochemical release is likely to maintain effective algicide/cyanocide concentrations preventing hormesis.
- Rhizosphere: provision of microhabitats and nutrient-rich exudate in the root zone will increase the abundance of biological organisms and enhance grazing.

7.2.3.1 Root density and morphology

Plant selection should be species-rich and include native macrophytes to enhance the local flora and invertebrate/pollinator communities. It should be managed to prevent colonisation of alien species and should generally promote species diversity. This research has shown the differences in root morphology, density and allelochemical content among the plant species sampled from the Living-Filter. These species, *Phragmites australis, Phalaris arundinacea* and *Carex acutiformis* live in natural waterlogged environments rooted in nutrient-rich sediments; or when used in CWs or FTWs are supplied with nutrient-rich wastewaters rooted in a type of media that substitutes for the sediment. In the Living-Filter the plants had to adapt to the hydroponic environment with local lower nutrient content (Table 7.1), and higher flow velocity conditions (> 5 cm s^{-1}). Chen (2017) found that macrophytes modify their shoot and root morphology when exposed to high flow velocities in streams and channels by developing dense roots to prevent being uprooted, and to facilitate the uptake of nutrients which can be quickly washed out. This author found that the shoot height to root length ratio (> 1.0) increases with greater flow velocities. The shoot height to root length ratio

estimated for the Living-Filter species two years after installation in 2014 is shown in Table 7.2, data for this table were taken from Table 2.3.

Of note is, the lowest shoot height to root length ratio of *Phragmites australis* which is accompanied by abundant rhizomes, and very short (0.15m±0.08) and sparse root morphology. These findings suggest that this species might have struggled to adapt to the local conditions at Farmoor II reservoir explaining why this species had the highest loss of plants (50%) on the plant-bed, a year after installation in 2013 (Table 2.2).

Table 7.2 Estimation of shoot height to root length ratio measured from *Phragmites australis*, *Phalaris arundinacea* and *Carex acutiformis* two years after installation (2014).

Plant species	Shoot Height to root length ratio
Phragmites australis	0.07
Phalaris arundinacea	1.36
Carex acutiformis	1.24

On the other hand, the shoot height to root length ratios of *Phalaris arundinacea* and *Carex acutiformis* with highly dense root formation suggest that these species would have developed advantageous adaptation mechanisms to local nutrient and flow conditions. However, only 4.1% of *Carex acutiformis* plants and a much 37% of *Phalaris arundinacea* were lost a year after installation. These differences could be attributed to other environmental factors, such as bird grazing which was observed during the study period and which may have weakened some of the new plants. Anecdotally, two months after installation, these plants were heavily grazed by migratory geese and a gabion was erected to protect the plants (Figure 2.1-B). Multispecies selection for the plant-bed will also provide greater resilience to adverse weather conditions and resistance to diseases, thus ensuring a long-term performance.

7.2.3.2 Roots and allelochemical production/release

Although no clear correlation was found between the quantity of allelochemicals produced and the root biomass, it has been shown that by selecting plants known to produce allelochemicals, release of allelochemicals into the surrounding water will take place. Rates of allelochemical production/release from macroscale biofilters roots in response to environmental stressors are expected and can be quantified, as it was found for the laboratory biofilters (chapter 5, sections 5.3.4 and 5.4.4). However, this is an area that requires further research in the field in order to produce improved field-based models of this relationship. Although some work has been carried out by Hilt *et al*. (2008), their studies are limited to the macrophyte *Myriophyllum* spp. in natural habitat.

The estimated daily change in allelochemical rate showed decreasing concentration levels of two identified allelochemicals. These measurements were made from aqueous root exudate samples in the absence of living roots. The daily change rate for anthraquinone was > 100% and 4.5% for 4-nitroindol-5-carboxaldehyde (Figure 6.4). Although further research is needed to estimate changing rates for other identified allelochemicals, this thesis has demonstrated that there is production of allelochemicals from living roots into the aqueous exudate environment. This observation contradicts Lürling *et al*. (2016*b*) who claim that there is no proof of allelochemical exudation from living plants into the surrounding water.

7.2.3.3 Rhizosphere

The removal efficiency (RE%) of *Microcystis* cells also depends on the grazing/algastatic/algicide activities of other biological organisms (protozoa, other phytoplankton species, bacteria and viruses) (Gerphagnon *et al*., 2014). The removal efficiency (RE%) of *Microcystis* cells from unfiltered and filtered aqueous root exudate was estimated using data from chapter 6 (section 6.3.3). The RE% was significantly different depending on the duration of the two experiments and the treatment used (section 6.3.3). The One-day experiment was carried out using a 24 hour old aqueous root exudate for five days;

and the Five-Day experiment was carried out using a 120 hour old aqueous root exudate for five days. The aqueous root exudate of the One-Day experiment was five days old in total and for the Five-Day experiment was 10 days old. Results are shown in Table 7.3.

There is only a difference of 10% RE of *Microcystis* cells between the two experiments for the unfiltered treatment, suggesting that some allelochemicals are still bioactive despite a potential decrease concentration after 10 days. There is a decrease of 23% RE between the unfiltered (83% RE) and filtered treatment (60% RE) for the One-Day experiment quantifying the role that biological organisms have in removing *Microcystis* cells. The absence of biological organisms, degraded allelochemicals, but also the potential hormetic effect of anthraquinone or other unidentified allelochemicals or compounds might explain the results for the filtered treatment for the Five-Day experiment, with the resultant large increase of *Microcystis* (-94%)

Table 7.3 *Microcystis* cells removal efficiency (RE%) for unfiltered and filtered aqueous root exudate for the One-Day and Five-Day experiments.

	Removal efficiency (%) at *t5*	
	One-Day experiment	**Five-Day experiment**
Aqueous root exudate	**5 days old**	**10 days old**
Unfiltered	83%	73%
Filtered	60%	-94%

Biological organisms are better supported by nutrient-rich exudates from biofilters (Svanys *et al*., 2014) than from artificial systems; biofilters can also contribute to a greater microbial biodiversity compared to artificial systems. Multispecies plant selection can mitigate the potential adverse effect that some allelochemicals might have on microbial communities (Puigagut *et al*., 2012). These authors found that in mesocosm experiment, ciliate abundance appeared to be affected by *Phalaris arundinacea* but not to *Typha latifolia* and *Phragmites australis*, although not statistical differences were found.

7.2.4 Determining a plant-bed size for a floating biofilter

The design and size of treatment wetlands is either technology-based or performance-based. Technology-based design uses regulatory prescribed loading rates to determine the size of the wetland whilst performance-based design uses targeted outflow concentrations, effluent loads, and/or removal rates (Chin, 2012). The total area of Farmoor II reservoir actually covered by the Living-Filter is 0.02% which is quite small for removing phytoplankton by chemical and/or biological mechanisms, and when taking into account the size of the reservoir (1 km^2), the volume (9.3 x 10^6 m^3) and the Living-Filter daily hydraulic loading.

For example, Hilt *et al*. (2008) found that for successful top-down mechanisms to operate, the percentage of vegetation coverage to the area of surface water varies from 3-100%, that successful chlorophyll-*a* and turbidity reductions are reported for 5-50% coverage; and that some improvement can be seen with a coverage of 1-2% if other measures are in place (Hilt *et al*., 2006; 2008). Therefore, based on vegetation coverage a field-scale biofilter system would need to be at least 50-100 times larger than the present Living-Filter (210 m^2) to fulfil the minimum ideal of a 1-2% coverage with an area of approximately 10,000-20,000 m^2.A macroscale biofilter of such size would be impractical in surface water reservoirs.

However, the recommendations for an effective CW-HSSF given by Wu *et al*. (2015) is at least 2,500 m^2 which is 10 times larger than the present Living-Filter, but with the advantage of being about 10 times smaller than the minimum size found by Hilt *et al*. (2008). Another aspect that must be taken into account in the design is the length to width ratio, which is recommended to be 3:1 or 5:1 by Sholz (2006) and 2:1-3:1 by Wu *et al*. (2015). The Living-Filter length to width ratio is 1:2.

Based on Hilt *et al*. (2006; 2008) the minimum size for a field-scale biofilter in a reservoir of 1 km^2 should be 180 m L x 60 m W (10,800 m^2), and following the recommendations of Wu *et al*. (2015), should be 80 m L x 25 m W with a plant-bed area of 2,000 m^2 (Figure 7.4). Although field-scale floating biofilters being used a pre-treatment

process should be placed by as close as possible of the inlets to the WTWs (Figure7.4), considerations of implementing another field-scale floating biofilter by the intake from the river to the reservoir (Figure 1.1) may be recommended. Risk assessments and consultation with the DWI need to be made for any implementation in surface water reservoirs. Because of DWI regulations and operational water abstraction management, field-scale biofilters should be installed at a distance from the inlet to the works (Figures 2.1-A and C; and 7.4). The uncovered area from the plant-bed of the biofilter to the WTWs inlet in Farmoor II reservoir is one and half times larger than the area of the plant-bed. Seasonally dormant cyanobacteria cells can migrate vertically in the water column which would have implications for the evaluation of the performance of future biofilters in the short-term (<3 years), as discussed in section 2.4.3.

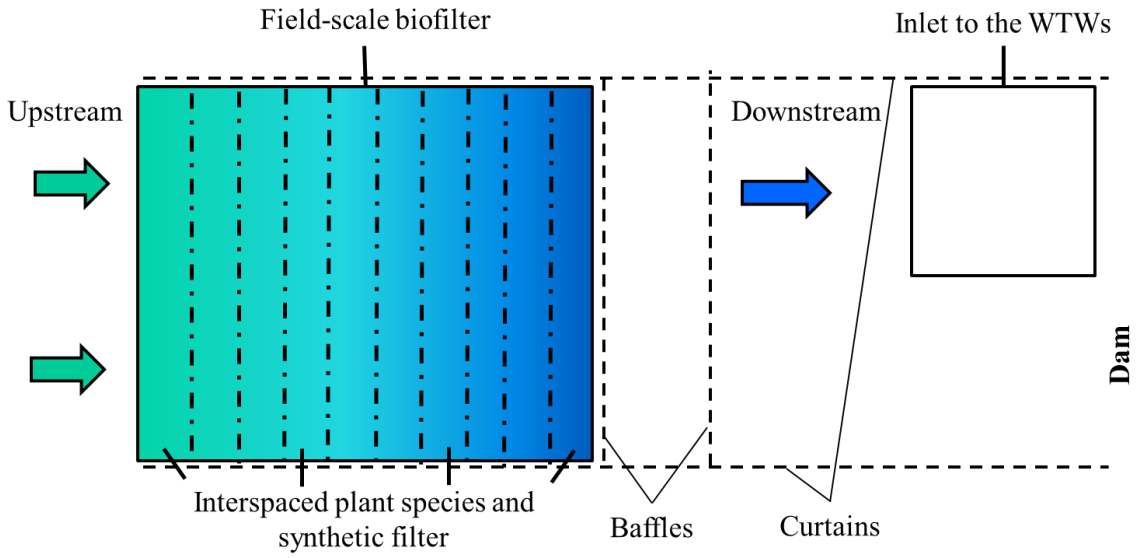

Figure 7.4 Aerial view of an ideal floating biofilter system for a 1km^2.

The plant-bed depth should be ≥1.5 m, which includes the 1 m cage structure depth and a minimum 1.5 m root length (Table 2.3) to give a plant-bed filtration volume of 3,375 m^3. Thus, if the average demand is 65 x 10^3 m^3 d^{-1}, the retention time is 1h 15 min. The retention time might increase slightly taking into account the porosity of the roots (1h 40 min) and the synthetic filter (fabric 2) (1 h 24 min) (Table 7.4). Nevertheless, it is clear that for such a high water demand the retention time for a macroscale biofilter design following the minimum

requirements of Wu (2015) is still too short. It seems unlikely that the effects of chemical and biological mechanisms for phytoplankton removal onto a WTW will be observed in the short term. However, observations from the Living-Filter surveys showed that zooplankton abundance was more stable within the plant-bed than in the water column despite the short retention time of the system (5-20 min). Thus, the increase in zooplankton abundance and stability of zooplankton communities would potentially mean a more effective phytoplankton grazing zooplankton community. In light of the above, the output of the WTWs has also to be considered before implementing a macroscale biofilter as a pre-treatment process in a surface reservoir.

Table 7.4 Summary of dimensions: biofilter roots and synthetic filters

Measurements	**Synthetic filters**		**Biofilters**
	Fabric 1	**Fabric 2**	**Roots (mean±SE)**
Diameter (mm)	0.5	0.3	1.42±0.23
Layer sheet thickness (mm)	0.8	1.8	N/A
Surface of filter mm^2	12	10.8	-
Porosity (%)	0.95	0.90	0.79
Density (in cages) (cm^3)	580	830	793±77
Weight (g)	-	-	
Root biomass (g)	-	-	63.6±8.1

The use of floating biofilters can provide improvements in water quality in deep surface waters such as reservoirs where it is not possible or undesirable, to plant vegetation, as well as in shallow waters to avoid having to plant in the substrate. It is clear that floating biofilters are suited to surface waters such as artificial water bodies or heavily modified water bodies, and could be used as a temporary measure for long-term restoration projects. Finally, plant-bed size for phytoplankton removal prior to potable WTWs depends on the local characteristics of the suspended solids loading (as a surrogate for phytoplankton biomass) and on the water demand. Further consideration in their implementation involves stakeholders who might be

opposed to large field-scale biofilter systems if these interfere with recreational activities (e.g. sailing or angling).

7.3 Implementation and operation of floating biofilters

The Living-Filter is the first macroscale biofilter with accompanying system of curtains and baffles to be used in a drinking water reservoir, and this is the first study carried out to investigate its performance across a range of scales. The Living-Filter structure is robust and withstood adverse weather conditions during its deployment. The Living-Filter system was commissioned for three years, and after decommissioning in 2015 the curtains and baffles were removed. The plant-bed was floated from the abstraction area in the North West of the reservoir to its current position in the South of the reservoir (Figure 1.1) where provides ecosystem services as a habitat for wildlife (birds and fish nursery). It is likely the Living-filter will remain in the reservoir at least until the summer of 2017 when it will have completed a five year period.

Operational management of CWs to treat secondary or tertiary wastewater effluents are faced with long-term (≥5 years) clogging of the system (Pedescoll *et al*., 2011). However, evidence shows that clogging depend primarily on the suspended solid loading into the system. For example, Chezeranc *et al*. (2009) found that after five years with a daily suspended solid loading of 3.2 g, their CWs systems were clogging. Whilst Pedescoll *et al*. (2011) reports that clogging of CWs was evident with daily suspended solid loading of 0.6 – 10 g for 3 – 4 years. High suspended solid concentration (4 - 10 mg L^{-1}) was seen in Farmoor II reservoir only in late summer (weeks 10 - 13) as shown in Figure 7.2; and were associated with the seasonal pattern of phytoplankton bloom. However, the magnitude of the suspended solids levels in the reservoir is lower than the levels seen in wastewater effluents. Therefore clogging of floating biofilters in surface reservoirs might take place at even longer time scale than those for CWs (≥5 years). Therefore planning to commission future macroscale biofilters

for at least 5-10 years is conceivable with an annual assessment and management programme, similarly to any other water industry assets.

7.4 Field-scale biofilter risks and management

Greenhouse gas emissions, carbon dioxide (CO_2), methane (CH_4), nitrous gas (N_20) are by-products generated by anaerobic processes from constructed wetlands (CWs) used primarily for wastewater purification (Carballeira *et al*., 2017). Mander *et al*. (2014) carried out an extensive literature survey on greenhouse gas emissions and CWs, with their analysis comprising 158 papers spanning 19 years (1994 -2013). They found that some types of CWs, e.g. free flow constructed wetlands (FCWs) and (HSSF CWs) generate high CH_4 emissions, yet the CH_4 emissions were one to two magnitudes lower than that found in conventional wastewater treatment works (WWTW). The emissions can increase if peatland is used as media, where there is already organic matter combined with anaerobic conditions.

A lot less attention have been given to the production of gas emissions from floating treatment wetlands (FTWs), natural wetlands (Audet *et al*., 2013), freshwater systems (Tangen *et al*., 2016) and oceans (Hamdan and Wicklam, 2016). Audet *et al*. 2013 found that riparian wetland restoration did not increase CH_4 emissions with levels similar to those found in natural wetlands; and that the restoration balanced the CH_4 fluxes in the system. By contrast, N_2O emissions were highly variable, both spatially and seasonally, and appeared to be related to N content in the top soil and restoration had no significant effect on N_2O emissions. It is unlikely that a fully functional macroscale floating biofilter with high retention capacity and low flow rate could increase CH_4 and N_20 to levels higher than those produced by natural wetlands, CWs or conventional WWTWs because there is no an enriched substrate media (e.g. soil, gravel or pit) and the roots are floating over the sediment bed. However, floating biofilter systems could rarely emit greenhouse gasses associated with severe phytoplankton blooms, established for several weeks in the reservoir and combined with high environmental temperatures, low dissolved oxygen in the water body (< 5 mg L^{-1})

(European Environment Agency, 2014) and very low flow velocities. Nevertheless; these emission levels would be below the levels from natural wetlands, CWs or conventional WWTWs but further empirical research is needed in this area.

To prevent the emission of greenhouse gasses, as well as the deterioration of the raw water quality to be treated in a potable water treatment works, the implementation of management measures is essential for any future designs and installation of macroscale floating biofilters. Management measures include a harvesting plan for the plants in the floating plant-bed to prevent accumulation of plant detritus in the sediment; and an aeration system to alleviate low oxygen conditions within the plant-bed (< 5 mg L^{-1}) (European Environment Agency, 2014).

Aeration systems (i.e. perforated pipes with compressed air) operated intermittently can be used to prevent a decrease in oxygen consumption, and a potential increase in biological and chemical oxygen demand. Air systems used in CWs have been reported to increase total nitrogen removal and ammonium efficiency removal (Fan *et al*., 2013; Wu *et al*., 2015). Wu *et al*., (2015) discussed in their review that the operation of these systems might interfere with of the settling of solids in CWs. Ouellet-Plomoundon *et al*. (2006) and Chazarenc *et al.* (2009) tested the role of aerated planted and unplanted mesocosms in HSSF-CWs. Their results suggested that aeration systems increased aerobic degradation processes, thus reducing pollutants and solids accumulation in unplanted CWs. Aeration was particularly important during the summer and winter months. These results also highlighted the importance of planted systems versus unplanted artificial media systems (Ouellet-Plomoundon *et al*., 2006).

In the context of macroscale floating biofilters in surface water used for potable water treatment, the loading of suspended solids, nitrogen and phosphorus, biological and chemical oxygen demand is lower than in CWs, but aeration systems might provide some benefits. On the one hand, aeration systems can interfere with the deposition of phytoplankton cells on the roots; on the other hand, aeration systems can contribute to the vertical diffusion of

allelochemicals which in turn might have positive and negative effects. The positive effect is that allelochemicals and phytoplankton might be homogenously distributed facilitating the contact between allelochemicals and target cells at inhibitory concentrations. The negative effect is that allelochemical concentrations can be rapidly mixed and diluted in the body of water by the mechanical action of the bubbling system, and their inhibitory effect on the target cells would, therefore, be lost. Nevertheless, the implementation of aeration systems should be in line with significant deterioration of water quality variables, such as an increase in total phosphorus, total nitrogen, ammonium, biological and chemical oxygen demand downstream the plant-bed. Moreover, allelochemical diffusion from the roots into the water of body is a subject that will need further research.

7.5 Final recommendations

It is important to acknowledge that some processes might operate in different ways at different scales, particularly in full-scaled systems that operate in a heterogeneous environment with interacting multispecies and processes prevailing at laboratory scales do not necessarily prevail at field scales. Furthermore, observations from the macroscale Living-Filter study in the natural environment showed the temporal and spatial variability of the system that could not be replicated at laboratory scale experiments. Scaling-up or scaling-down systems should also observe geometric, flow and fluid similarities. There were logistic limitations in this study that prevented achieving geometric and flow similarities between the mesocosm and the Living-Filter. Nevertheless, even if these similarities would have been met, the uncertainties derived from the interaction between biofilter multispecies with multispecies of phytoplankton would need to be studied further. Laboratory scale experiments certainly simplify and limit heterogeneity. Having said that, there is no doubt that laboratory-scale studies in this research have shown the existing local physicochemical and biological processes between the selected plant and cyanobacteria species providing foundation for further research. Otherwise local information on these processes would have been lost in the

context of field-scale observations but these, ideally, will need to be tested in full-scaled systems.

Finally, the installation of floating biofilters aims to facilitate treatment and reducing the use of chemicals, hence, it aims at increasing the life-span of traditional physical filter media, not to replace physical filter bed structures and filter media. Installing additional physical filter beds is not an easy option in an established water treatment work because of the capital investment and land required for the new infrastructure that would be necessary. By comparison, one advantage is that the design and implementation of an *in-reservoir* floating biofilter does not require land. Regarding the question of cost it should be noted that the cost of installing additional traditional filter systems in a water treatment work that requires new infrastructure, are massive and far outweigh the cost of setting up a floating field-scale biofilter.

Chapter 8. Synthesis and Future Research

8.1 Introduction

This chapter is a synthesis of the findings that attempts to answer the aims of this research project. The purpose of this research has been to investigate whether biofiltration processes mediated by living-roots as filamentous media could remove phytoplankton biomass and thus to justify the use of field-scale floating biofilters as a processing step for surface water prior to potable water treatment. Eutrophic surface reservoirs are affected by seasonal phytoplankton blooms, particularly cyanobacteria blooms which disrupt several processes during the treatment of raw waters for the production of potable water. In addition, cyanotoxins represent a health risk for humans when present in surface and treated waters, and for wildlife when present in surface waters. The current research thus responds to the needs of the water industry by investigating measures to alleviate excessive phytoplankton loading into potable WTWs, the latter arising as a result of phytoplankton blooms, particularly cyanobacteria in surface waters. This research has also shown the potential benefits of introducing a floating natural vegetation system to facilitate balanced aquatic food-webs in man-made surface reservoirs. Such a system could ultimately translate into improvement of the quality of the water body.

The specific aims of the research were as follows. Firstly, to test if the proposed field-scale floating filamentous biofilter, referred to here as the "Living-Filter", could be used as a pre-treatment process for the reduction or removal of phytoplankton loading onto a potable water treatment works. Secondly, to investigate the underlying physico-chemical and biological mechanisms of the biofilter process in laboratory scale filamentous biofilters and synthetic filters, in which these filters are employed for the removal of a cyanobacteria in continuous flow and batch experiments. Thirdly, to make recommendations for the design of future field-scale filamentous biofilter pre-treatment processes to maximise the advantages of

this eco-technology for phytoplankton removal. The adopted research methodology has addressed the aims of the research by integrating field-scale surveys with experimental work into spatial and temporal macro-, meso- and microscale studies. An overview of the research methodology and research design is shown in Figure 8.1.

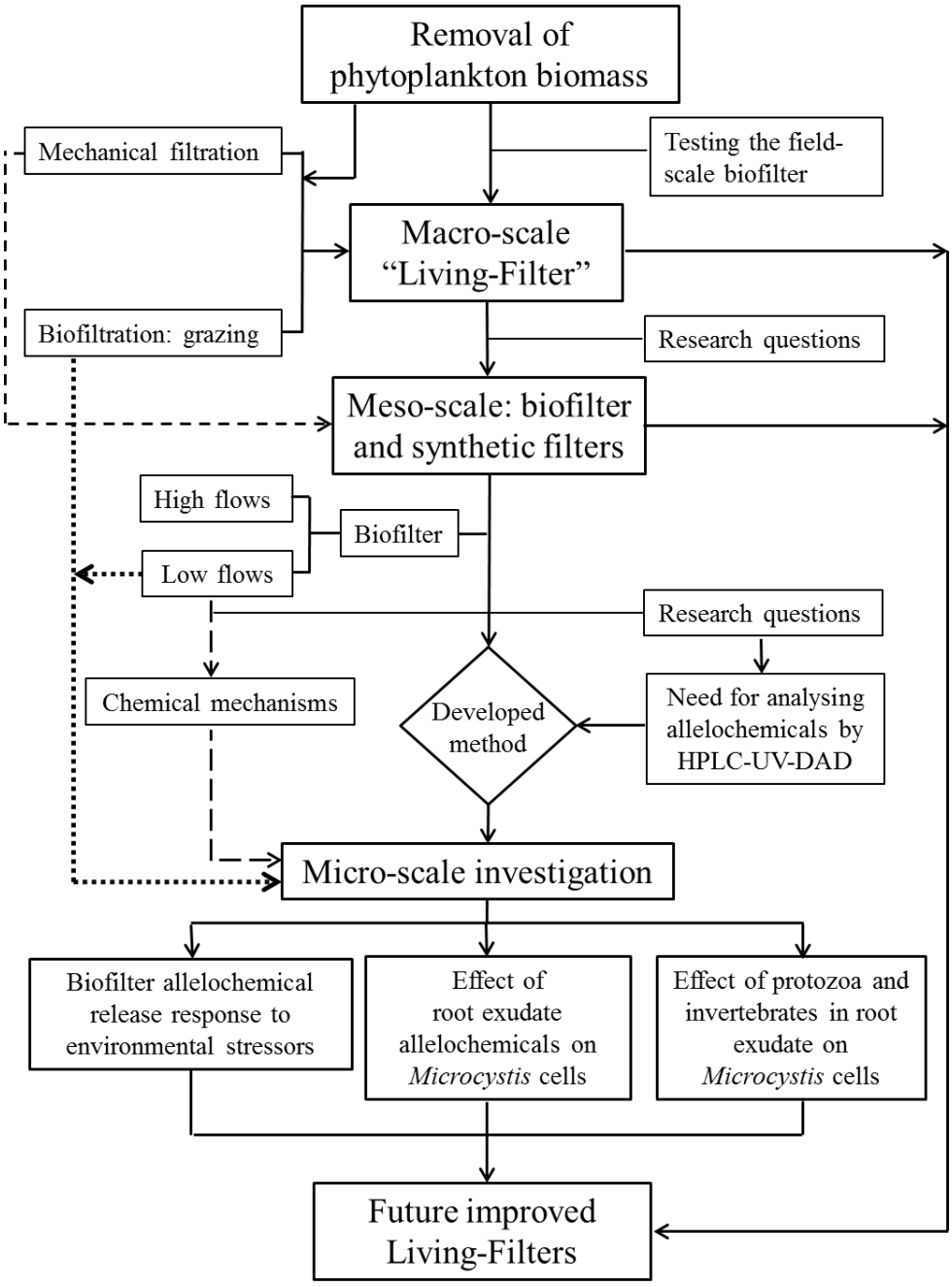

Figure 8.1 Schematic summary of the research approach for investigating "A biofiltration process for phytoplankton removal prior to potable water treatment: a field and laboratory study".

8.2 Research questions posed to meet the aims.

To meet the aims of the research, two overarching questions were posed:

1. Can the Living-Filter be used as an in-reservoir pre-treatment filtration process for the reduction of phytoplankton biomass onto potable WTWs?

2. Can the underlying physico-chemical and biological mechanisms that might operate in biofilters be identified from macro-, meso- and microscale studies?

The research findings were presented in six separate chapters. Questions generated from the research in line with the overarching questions were explored, investigated and answered for each chapter. These were summarized and discussed broadly in their respective chapters. A brief account of the research questions is presented here.

8.2.1 Chapter 2: Comparing physico-chemical and biological variables upstream and downstream of the Living-Filter.

This chapter focused on the macroscale studies with surveys and descriptive work. Four specific research questions were addressed:

1) Are there physico-chemical and biological changes upstream and downstream water of the plant-bed?

Findings showed that there were physicochemical and biological differences between upstream and downstream of the Living-Filter, with important raw water quality differences for potable water treatment. For example, soluble manganese, dissolved inorganic nitrogen and nitrite decreased downstream, although ammonium was increased at the potable WTWs intake. Within the plant-bed, total suspended solids, total phosphorus, particulate organic carbon, biological oxygen demand, chemical oxygen demand and chlorophyll-*a* increased whilst dissolved oxygen and temperature decreased when compared to upstream and downstream of the Living-Filter plant-bed. These results were explained by an increased in metabolic activity, whilst the temperature decrease is related to the plant-bed shading effect.

These findings supported the postulate that both mechanical filtration, i.e. particulate retention, and chemical mechanisms were operating in the Living-Filter plant-bed with no deleterious effect on the water to be treated in the WTWs.

2) Are there physico-chemical, biological and spatial changes within the plant-bed itself?

Physico-chemical water quality differences were found between the plant-bed sites. These findings seemed to be related to the plant species surrounding the respective sampling points. It was found that the roots of the plants differed in their morphological characteristics and density. The most effective species were *Phalaris arundinacea* and *Carex acutiformis*; *Phalaris* because of its root development and *Carex* because of its root development and robustness. The synthetic fabric in the plant-bed cages might have also contributed to the mechanical filtration. Nevertheless, these findings further support the postulate that mechanical filtration is an operating mechanism in the Living-Filter plant bed, and highlight the fact that the morphological characteristics of the plant species roots, and possibly the synthetic fabric, are relevant for the selection of the plants considered in future designs.

3) Was there any removal of chlorophyll-a by the Living-Filter during the period of study?

There was a removal of chlorophyll-*a* (as a surrogate of phytoplankton biomass) particularly during the first seven weeks of the study during the early summer, with encouraging figures up to 45% removal efficiency. However, in the following eight weeks there was chlorophyll-*a* removal on two occasions only (late summer). The removal efficiency figures do not suggest a finite filtration capacity of the Living-Filter. The efficiency removal may vary depending on the seasonal lake and reservoir phytoplankton productivity, therefore high chlorophyll-*a* loading during the spring and late summer might reduce the removal efficiency. The removal efficiency also depends on hydraulic loading rates and the size of the biofilter. Although these results led to further questions that were presented in the

chapter's discussion, mechanical filtration is again supported as an underlying mechanism. However, a weakness in the research is that these results are derived from only one particular Living-Filter; therefore, further research is needed to demonstrate more conclusively that mechanical filtration operates in general in biofilters with filamentous media.

4) Will the Living-Filter provide refuge to zooplankton?

A greater abundance of the large crustaceans *Daphnia* and *Cyclops* was found upstream of the Living-Filter than downstream and might be explained by the "shore avoidance" hypothesis. Results showed that the most stable zooplankton community abundance was within the plant-bed of the Living-Filter, suggesting that by being less exposed to external fluctuations (i.e. predation) and the plant roots providing favourable environmental conditions for food productions, there were indeed resources for larger populations of zooplankton.

8.2.2 Chapter 3: Removal of *Microcystis* cells in a continuous flow mesocosm under two flow conditions.

This chapter focused on determining if biofilters (roots of *Phalaris*) could remove *Microcystis* by mechanical filtration under two flow conditions. It also compared the removal efficiency of biofilters using two synthetic filters each with a different packing density in a continuous flow mesoscale set up. Three research questions were addressed:

1) Are there differences in the cell removal efficiency between biofilters and the synthetic filters at high flows (54±2ml min^{-1})?

There are differences in the removal efficiency between biofilters and synthetic filters. The biofilter removal efficiency was 25% and for synthetic filters it was 45% for fabric 1 and 15% for fabric 2. These differences were related to the packing density of the filamentous media. Despite the modest 25% biofilter removal efficiency, this appears to be the first published study that has demonstrated that biofilters with filamentous media can remove *Microcystis* cells by mechanical filtration. Furthermore the removal efficiency could be

improved if the biofilters are combined with synthetic filters, and arranged in a systematic manner than in the present Living-Filter, e.g. vertically in the cage structure.

2) Are there differences in the cell removal efficiency between biofilters and the synthetic filters at low flows (1.2±0.3ml min^{-1})?

There are differences in the removal of *Microcystis* cells by biofilters and synthetic filters at low flows, despite the continuous cell injection from the tank the cell numbers in the biofilters were about a trifold lower than in the controls and synthetic filters. There were two very interesting results. Firstly, biofilter removal efficiency was 50% over and above controls; secondly, the biofilter water was as clear as tap water compared to the green colouration of the synthetic filters and controls. These findings suggest that there are further mechanisms other than solely mechanical filtration operating in the biofilters, which prompted further investigations.

3) What other mechanisms are operating in the biofilters compared to synthetic filters?

Preliminary investigations suggested that chemical mechanisms, such as nutrient competition and/or allelopathy, or biological mechanisms, were taking place. Further, the lack of colouration in the biofilter units suggested that there was an oxidative process, probably due to an allelochemical effect, acting as a chemical mechanism. A screening analysis from aqueous root exudate samples revealed the presence of allelochemicals that could inhibit *Microcystis*. These findings prompted further investigations that revealed biological mechanisms are important contributors, but allelochemical mechanisms seem to be dominant.

8.2.3 Chapter 4: A simultaneous method for multiple allelochemical detection, identification and quantification using high performance liquid chromatography.

The research work presented in this chapter was derived from the biofilter observations made under low flow conditions and the need to have an in-house method for analysing a

large number of samples. The development of this method represented a progress in analytical research, and a research question was posed:

Can the simultaneous method detect, identify and quantify multiple allelochemicals in aqueous samples from root extracts and exudates?

The method successfully detected and quantified multiple phenolic and alkaloid compounds in aqueous samples from root extracts and root exudates. Although the method can be improved to incorporate a larger range of quantifiable concentrations, it successfully achieved its purpose with the novelty of quantifying phenolic and alkaloid compounds.

8.2.4 Chapter 5: Determination of allelochemicals in root biomass from the Living-Filter plants and in biofilter root exudate as a response to environmental stressors.

This chapter focused on descriptive, analytical and experimental macro-, meso- and microscale studies for the determination of allelochemicals in root biomass from the Living-Filter plants and in biofilter root extracts and exudate. The simultaneous method was applied for this research. Microscale studies were focused on the response of the biofilter to environmental stressors, nutrient rich media and *Microcystis* cells, by measuring allelochemical concentrations in the aqueous root exudate.

1) ***Are there interspecies differences in allelochemical composition between the following Living Filter plants: Phalaris, Phragmites and Carex?***

There was interspecies variability among the Living-Filter plant-bed species in allelochemical composition and concentration. Limitations in the range of quantification provided only semi-quantitative results in many cases; however, this issue could be addressed by incorporating more points of calibration in the method or using a coupled detection system with mass spectrometry. The study provided insights into plant selection criteria in future biofilter designs related to whether the allelochemicals produced by the plants roots are algicide/algastatic or if they are only cyanocide/cyanostatic.

2) Are there differences in allelochemical composition in aqueous samples derived from root extract from Phalaris grown in the field-scale Living-Filter versus that grown in the laboratory biofilters?

Intra-species differences were found in the composition and the concentration of allelochemicals for *Phalaris* between the field and the laboratory. Although genetic variability of the individuals undoubtedly plays a role, the environmental component of growth conditions might be decisive in expressing or suppressing some genes. Moreover, the exposure to different environmental conditions might determine the endophytic community established in the root tissue, which plays an important role in the production of allelochemicals. Laboratory studies are reliable for the set of measured variables, but provide a limited scope for the natural conditions and might reduce the variability of the response to changing natural conditions. Therefore, the plant domestication for the production of specific allelochemicals with algicide/algastatic and/or cyanocide/cyanostatic activity for future biofilters seems to be an area worthy of further exploration in seeking to reduce phytoplankton biomass.

3) Are there differences in allelochemical composition between aqueous samples derived from root extract versus derived from root exudate for laboratory Phalaris?

Findings showed that there were fewer allelochemicals released into the aqueous root exudate than the ones produced and/or stored in the root tissue. However, these results might be explained by a number of factors. First, allelochemicals might have been released as a response to environmental conditions; second, allelochemicals might have degraded quickly in the aqueous root exudate; third, allelochemicals were released at concentrations lower than the detection limits of the developed method.

4) Are there differences in the response of allelochemicals to different nutrient and Microcystis treatments as environmental stressors?

The concentration of the allelochemicals released into the water is higher in the enriched media than that in tap water, and the findings regarding the response to a biological stressor suggested that their release might be species specific. No associations were found between the root biomass and the concentration of allelochemicals in the water. All the same, installation of floating biofilters in eutrophic surface waters has the potential to stimulate the production of allelochemicals, contributing to phytoplankton reduction in inflowing waters and phytoplankton bloom control.

8.2.5 Chapter 6: The effect of commercial allelochemicals and biofilter root exudates on the growth of *Microcystis aeruginosa*

This chapter focused on experimental microscale studies. The growth inhibition of *Microcystis* was determined for commercial allelochemicals and root exudates. Furthermore, chemical and biological mechanisms were identified in the process of the reduction of *Microcystis* cell numbers.

1) Are there differences in Microcystis growth when exposed to concentrations of commercial allelochemicals?

Microcystis growth was inhibited at ≥100 μg L^{-1} concentrations of the tested commercial allelochemicals, except for gramine, which showed a hormetic effect on *Microcystis* at 100 μg L^{-1}. Further experiments should be carried out with a greater range of concentrations between >10 and <100 μg L^{-1} to determine if a hormetic effect from other allelochemicals exists and with other phytoplankton species. Although allelochemicals entering the water treatment works are likely to be removed by physico-chemical processes, further research should look into the potential challenges that allelochemical concentrations ≥ 100 μg L^{-1} could pose to potable water treatment works.

2) Are there differences in the allelochemical concentration produced from biofilters as a function of time?

There was a decrease in the concentration of allelochemicals over time. These findings are significant, as low allelochemical concentrations might have a hormetic effect on phytoplankton species. The latter would suggest that a minimum threshold should be achieved to maintain removal effectiveness, but this is an area that needs further research, as mentioned above.

3) Are there differences in the effect of unfiltered and filtered root exudate on Microcystis growth as a function of time?

Findings showed there was a decrease in *Microcystis* growth when exposed to unfiltered and filtered root exudate treatments as compared to the controls (BG11 and tap water). These findings are very important in aquatic ecology, as they demonstrate that allelopathy is the acting chemical mechanism for phytoplankton removal and not nutrient competition. This is because these experiments use root exudate itself in the absence of roots.

4) Are there other mechanisms involved in the reduction of Microcystis growth?

Differences between the two types of root exudate, unfiltered and filtered, on *Microcystis* cells numbers revealed that biological mechanisms also contributed to the removal and/or reduction of *Microcystis* cells numbers. In fact, after microscopic observation, findings suggested that protozoa and invertebrates (not Crustacean) significantly decreased *Microcystis* cell numbers in the presence of allelochemicals.

8.2.6 Chapter 7: Recommendations for future macroscale biofilter designs

In this chapter, the hypothetical design of an ideal Living-Filter is discussed based on both the findings from this research and the scientific literature.

1) Can a plant-bed size be determined for both a biofilter process prior to potable WTWs and as a measure for reducing phytoplankton biomass?

The selection of plant-bed size can be determined for both purposes: as a biofilter process and as a limnological restoration measure, which requires at least 1% of the area of

the surface water. The limitations are socio-recreational in that the biofilter might interfere with other activities in the surface waters.

2) Is there a suitable flow velocity to optimize physicochemical and biological mechanisms for the removal or reduction of phytoplankton biomass?

Yes, there are suitable flow velocities to optimize biofilters for phytoplankton removal that need to be carefully studied before design and taking into account the points highlighted in this research. However, this is a major constraint for large WTWs, because the water demand for the feed to the treatment works will determine the hydraulic loading rate. High hydraulic rates with associated high flow velocities are counteractive to the short retention time required for chemical and (probably) biological mechanisms for removal and/or reduction of phytoplankton biomass; in short, the two demands are conflicting.

8.3 Final conclusions

Filamentous biofiltration processes prior to potable water treatment works can remove or reduce phytoplankton biomass/loading onto potable WTWs. Therefore, biofilters can lessen disruption at the WTWs and mitigate the risks to human health associated with the phytoplankton blooms, but could also promote balanced aquatic food webs in surface waters. Floating pre-treatment biofiltration processes have not been implemented before in surface waters for the removal of phytoplankton.

This research project demonstrated the dual role that macroscale biofilters combined with a synthetic filter can play. One role is that the trapping of suspended particles (phytoplankton cells) from continuous flow and another is that during the period of study zooplankton grazers (crustaceans) maintained more stable population numbers within the field plant-bed than in the water column. The research also showed the role that "other grazers" (protozoa and non-crustacean invertebrates) could have in reducing phytoplankton cell numbers. Mechanical filtration was demonstrated as one of the mechanisms responsible for the removal of phytoplankton cells and *Microcystis* from continuous flow systems during

macro- and mesoscale studies. Although biological filtration mediated by zooplankton was inferred from the macroscale study, the microscale study findings showed the role that "other grazers" have at reducing *Microcystis*, and support the claim that biological filtration also takes place. A combination of mechanical and biological filtration provided by biofilters not only strongly supports the purpose of biofilters as a pre-treatment process of raw water for the removal of phytoplankton, but it would benefit the whole aquatic ecosystem by providing a refuge for zooplankton when floating macroscale biofilters are installed.

The current research from meso- and microscale studies also demonstrated that chemical mechanisms contributed to the removal of *Microcystis* cells. Allelopathy instead of nutrient competition was the chemical mechanism identified from biofilters of *Phalaris arundinacea*, a species widely used in ecological engineering designs, to remove and/or inhibit the growth of this species of phytoplankton. Generally, studies of floating treatment wetlands focus their investigations on the removal of nutrients, metals or other contaminants. Efforts to improve vegetation coverage of the sediment bed, as part of biomanipulation measures, are addressed to promote bottom-up and top-down mechanisms for the control of phytoplankton in shallow surface waters. Although the interaction of allelochemicals and phytoplankton has been suggested in the field, with some investigations carried out in parallel in the laboratory, most are bioassays with dried and resuspended extracted material from plants in the laboratory. Previous investigations have either supported resource competition or the allelochemical effect as a mechanism that inhibits phytoplankton, but they have failed to identify and measure allelochemicals from the aqueous plant environment.

This research is the first of its kind that has followed the requirements of Wills (1985) to study allelopathy and provides a holistic qualitative and quantitative demonstration of allelopathic interactions between two species. This statement is supported by the investigations and demonstrations of the initial observations of the potential allelopathic pattern, the development of a method for identification and quantification of allelochemicals

from wet-root extracts in the studied plants, allelochemical biodegradation as a function of time, and testing of the allelochemical effect from aqueous root exudates on a species of phytoplankton. However, this research has also demonstrated that under flow conditions the contact time between the cells and the allelochemicals released into the water has to be long enough to affect the cells. Moreover, the 96 hours' time of exposure of the cells to the allelochemicals confirmed the allelochemical inhibition effect observed at 72 hours. Because of this, the design aspects of floating biofilters have to be carefully chosen to balance the contact time with the required flow at the WTWs. This can be achieved or could be improved with an adequate ratio of the biofilter to surface reservoir size. Chemical mechanisms are an added benefit of biofilters in surface waters that supplement the physical and biological filtration benefits.

This research has shown the benefits, short term (mechanical and biological filtration) and long term (chemical and biological mechanisms) of field-scale installations like the Living-Filter. The dual benefits would be appealing not only to water industry managers but also to limnologists/environmental practitioners. To the water industry managers, it offers a biofiltration pre-treatment process but also improves the raw water quality. To the limnologists/environmental practitioners seeking the improvement of surface water quality, it is another remediation measure that could withstand uncertainties of climatic change, such as flooding. Hopefully, others will follow the lead of this relevant and valuable research on the subject of water supply and quality. However, as this has been the first step in the implementation of systems like this, there are also limitations.

The conclusions extracted from meso- and microscale laboratory experiments are reliable as well as the observations from the macroscale Living-Filter study. However, due to the limitations of the scale (meso and micro) of the laboratory experiments, the research topics addressed should be further analysed in full-scale systems.

Limitations in the study were that instead of installing one field-scale Living-Filter, three should ideally have been installed but also carrying out surveys for three years during the summer as initially planned. However, the ideal imposes a larger capital and human resources demand for its accomplishment. Nevertheless, if surveys would have been carried out over a three year period a larger data set within temporal scale could have proved if the physico-chemical and biological changes observed *in situ* were attributable to the Living-Filter. The method developed could be improved; due to logistic limitations in this work, only one species of phytoplankton was tested. Only one plant species, *Phalaris arundinacea*, could logistically be grown during this study, further studies should include other species, particularly species of the locally occurring macrophyte community composition ensuring biodiversity and the study of other allelochemicals from root exudates that might be equally effective against *Microcystis* to the ones study here. Although the target species tested is the most frequent nuisance bloom-forming cyanobacteria, there are other species of interest that should be studied in a similar way to broaden our understanding of the effects that allelochemicals exert on these species in aqueous environments.

Finally, this research answered the following questions:

1. Can phytoplankton cells be removed using *in situ* biofilter, prior to abstraction and conventional water treatment, to reduce the burden on physical filters in the WTW?

   Yes, they can be removed by *in situ* biofilters.

2. Do biofilters work by simple physical retention in filamentous (roots with root hairs) media?

   No, there is a combination of physical, chemical and biological mechanisms of which chemical is the dominant mechanism followed by biological mechanisms then physical mechanisms.

3. Is the biofilter physical effect enhanced with the addition of synthetic filter media to the *in situ* system?

Yes, it is.

4. Is there an additional biological effect (here referred to as top-down control) resulting from allelopathic suppression of the phytoplankton cells by allelochemicals exuded by the biofilter plants?

   Yes, there is.

5. Is the efficacy of allelopathic suppression a function of hydrological flow rates through the wetland or retention (and therefore exposure) time to the biofilter media?

   It is function of the hydrological retention time

8.4 Future research

Further research is needed to gain a deeper understanding of the chemical and biological mechanisms for phytoplankton removal:

1. Research into biochemical mechanisms are needed to deepen the knowledge of biodegradation processes at macro- and mesoscale levels, and to better understand the process of phytoplankton entrapment in a field-scale biofilter.
2. Understand the effect and duration of allelochemicals in aqueous environments on various phytoplankton species at macro- , meso and microscale levels.
3. Further refine the simultaneous allelochemical method developed for this research as it is a fundamental tool for allelochemical research.
4. Investigating plant selection for the production of allelochemicals taking into account their genetic plasticity, the environmental conditions and the endophyte community needed to maximize the production of desired allelochemicals. This could kick-start a plant domestication industry for macroscale biofilters used to improve water quality and the control of phytoplankton blooms.
5. Research on the interspecific differences in biofilter efficacy in terms of the target species (cyanobacteria and micro-algae) using root exudates from *Phalaris arundinacea, Phragmites australis and Carex acutiformis*.

6. Investigating plant selection considering locally occurring macrophytes to ensure biodiversity and root allelochemical production that might be equally or perhaps more effective against *Microcystis*, other cyanobacteria species or micro-algae.
7. Investigating interspecific differences in the health of the plants and their ability to withstand wind-stress in nutrient enriched waters as part of the biofilter system.
8. Investigating the impacts of nitrogen and phosphorus enrichment on root to shoot ratios in a comparative study among *Phalaris arundinacea*, *Phragmites australis* and *Carex acutiformis*, to establish how nutrient levels in a water body might affect the development of the plants in floating biofilters.
9. Investigating how effective will be the allelopathic processes of a mixed community of macrophytes and the epiphytic (biofilm) community that will grow on the root system of a floating biofilter on the phytoplankton community in a natural environment.
10. Research on the role that macroscale floating biofilters or wetlands might have on gaseous emissions of CH_4 and N_2O increased from trapped, biodegrading phytoplankton biomass in fully effective (high retention capacity, low flow rate, field-scaled) systems.
11. Evaluation of the unintended impacts of fully effective macroscale floating biofilters on climate change, relative to the gaseous nitrogen and carbon emissions from conventional filters within the water treatment works.

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Appendices

Appendix A. Procedure and quality assurance of analysis

These methods were developed in Spencer House, Thames Water Utilities laboratory, Reading, UK. The brief methods summary presented here are transcripts facilitated by the team leaders from the metal, Patrycja Kolodziej, and general chemistry, Jackie Brookling, laboratories; overseeing by Graham Coen, laboratory manager. The summary focuses on essential analytical procedure and quality assurance, details were omitted to maintain confidentiality

The methods included are for the analysis of particulate and soluble metals (aluminium, iron and manganese), nutrients (nitrogen, phosphorus and silica), total suspended solids, biological and chemical oxygen demand, particulate, total and dissolved organic carbon; and chlorophyll-*a*.

The analysis of samples for particulate metals is as follows: 125 ml of sample is digested with 1% nitric acid and is left for at least 11 hours in a 75 °C Oven Binder. Aliquots of 10 ml are transferred to test tubes in the autosampler of the instrument which is an inductively couple plasma (ICP). Calibration is set at three points which is different for each of the metals analysed. Hence, aluminium is set at 50, 250 and 500 mg L^{-1}; iron is set at 50, 500 and 2000 mg L^{-1}; and manganese is set at 10, 50 and 100 mg L^{-1}. The analytical quality control (AQC) standards are a mixed of the metals to analysed comprising the concentration of the prescribed concentration values: aluminium (200 mg L^{-1}), iron (200 mg L^{-1}) and manganese (50 mg L^{-1}). The analysis of samples for soluble metals is as follows: 10 ml of sample is filtered in a 0.45 µm filter using a syringe. The filtrate is digested with 10% nitric acid. All the reagents used are from Metlab, UK. The standards used for the AQCs are from Metlab and Inorganic Ventures, UK because two AQCs from two suppliers are used in each batch for quality assurance. The batch size is set to run with 90 samples.

Total oxidised nitrogen is determined as follows: an aliquot of 6 µl of sample is mixed with three reagents: 0.2 M sodium hydroxide, copper hydrazine and sulphanilamide/N-1-naphthylethylenediamine dihydrochloride. Nitrate is reduced to nitrite by hydrazine under alkaline conditions. The nitrite is then treated with sulphanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions to form pink azo-dye which is measured at 520 nm. The equipment used is Thermo Scientific Aquakem 600 with 0.1 mg L^{-1} limit of detection for potable matrix and with a calibration range 0-20 mg L^{-1} with 7 point calibration. The batch size is arbitrarily set to a maximum of 120 samples but the equipment is designed to run continuously so could run larger numbers of samples.

Silica content is determined as follows: an aliquot of 120 µl of sample is used with three reagents: ammonium molybdate, oxalic acid and ascorbic acid. The method is based on the formation of a silicomolybdate complex which is reduced by ascorbic acid to "molybdenum blue" measured at 700 nm in the Thermo Scientific Aquakem 600. The limit of detection is 0.1 mg L^{-1} with a six point calibration at a set range 0-30 mg L^{-1}. The batch size is arbitrarily set to a maximum of 95 samples but the equipment is designed to run continuously so could run larger numbers of samples.

Biological oxygen demand (BOD) is determined as follows: 200 ml of sample is diluted using synthetic river water comprising of deionised water spiked with a typical final effluent and the following chemicals: ferric chloride, calcium chloride, magnesium sulphate and phosphate buffer solution. For total BOD the only pre-treatment is the addition of allyl-thiourea at the time of analysis to inhibit the effect of nitrifying bacteria. The analysis takes five days to complete (5 day incubation at 20 degrees C in the dark). The limit of detection is 1.9 mg L^{-1}. The WTW Oxi 7310 meter and WTW StirrOx G probes are used to measure the dissolved oxygen on day one and day five of analysis. A Peerless BOD robot is used to fill the bottles with the river water and move the probes along to take the readings. The BOD is then calculated using the dilution factor and the difference in oxygen levels from start to end of

incubation. The instrument is calibrated daily in water-vapour saturated air. This gives a slope reading which the set limits, then the oxygen is check in air saturated water which is compared to a table of expected oxygen levels at the specified temperature and pressure provided by the supplier. The batch size is arbitrarily set to a maximum of around 150 samples but the equipment is designed to run up to 450 samples.

Chemical oxygen demand (COD) is determined as follows: 2 ml of sample is added to Dr Lange LCK114 cuvettes with a range 10-1000 mg L^{-1}. These cuvettes contain potassium dichromate, sulphuric acid, silver catalyst and mercuric sulphate (used to eliminate chloride interference). Samples are oxidised by refluxing in a sealed tube with potassium dichromate and sulphuric acid at 148 degrees C for 2 hours. Residual dichromate is determined photometrically at 605 nm. For total COD there is no pre-treatment, but for filtered COD, the samples are passed through a 0.45 µm filter before being added to the tube; and for settled COD, the sample is settled for 1 hour before adding to the tube. Flocculated COD the sample is flocculated and then filtered prior to adding to the tube. The analysis time is 2 hours. The limit of detection is 10 mg L^{-1}. The instrument used is Dr Lange DR3900 photometer with heating blocks for digestion. The response of the photometer is checked daily using tubes of known response at 690 nm. The batch size is arbitrarily set to a maximum of around 120 samples but the photometer can read continuously.

Total organic carbon is determined by measuring the carbon dioxide released by chemical oxidation of the organic carbon in the sample. A 20 ml sample is treated with phosphoric acid (5%) and purged with nitrogen to remove any inorganic carbon present. The sample, maintained at 85°C, is then treated with sodium persulphate solution (100 g L^{-1}) where any organic carbon present is oxidised to carbon dioxide. When the oxidation process is complete the sample is purged with a flow of nitrogen and the carbon dioxide evolved detected by a nondispersive infrared detector. The resulting mass of carbon dioxide is proportional to the mass of organic carbon in the sample. The analysis is based on the use of

the Analytical Carbon Analyser, Model 1030. The method uses a six point calibration at a range set 1-6 mg L^{-1}C. The minimum detection level is 0.1 mg L^{-1}

Dissolved organic carbon is determined as follows: a 0.45 µm polypropylene filter is washed with 60 ±10 ml of deionised water using a 20 ml disposable syringe. A 20 ml sample is taken with the syringe and passed through the washed filter. Then analysed as for TOC, results are reported in mg L^{-1}.

The frequency of analytical quality control (AQC) samples for the above analysis is set at a frequency of one per 19 samples. Each batch also uses two blanks and two samples spiked with a known concentration of the analyte. Results are sent electronically from the analytical instrument via the laboratory information management systems (LIMS) to the software system Sample Manager and are reported in mg L^{-1}.

The analysis of samples for suspended solids is as follows: pre-filtered glass fibre filter (GF/C) with 100 ml deionized water are dried in an oven for at least four hours. The filters are weighted in a calibrated four digit balance. A sample of 200 ml is filtered through the weighted filter, and then the filters are dried in an oven for at least four hours. Then the filters are weight again.

Particulate organic carbon (POC) is determined as follows: a sample of500 ml is filtered onto a 47mm GF/C heat-treated filter pad and submitted to chemical oxidation in a sulphuric acid – potassium dichromate complex which uses silver sulphate as a catalyst at a temperature of 80ºC ± 10ºC for one hour. Carbon bonds within the POC are broken and subsequently re-bond with oxygen provided by the potassium dichromate to form carbon dioxide. The potassium dichromate unused by the oxidation process is calculated by titration against ferrous ammonium sulphate. The titration end-point is determined by a colour change in the presence of a redox indicator (1,10-phenanthroline ferrous sulphate) through : yellow, green, blue and finally red. With the initial volume of potassium dichromate and the volume of FAS titrated known it is possible to calculate the volume of potassium dichromate (and therefore

oxygen) utilised in the reaction and from this the particulate organic carbon. Although the result is expressed in terms of carbon in µg L^{-1}, the result is extrapolated from the oxygen consumption of the oxidation reaction

Chlorophyll-*a* is determined as follows: 25 ml of sample is placed in the BBE algae analyser which uses fluorescence technology to measure the level of chlorophyll *a* present and provide a result in µg L^{-1}. Although no sample preparation is required the sample should be homogenised prior to analysis. The BBE analyses measures the chlorophyll-*a* level *in vitro*. The fluorometric method was validated against the previously used spectro-photometric method and an imprecision maximum value of 6.77%. A conversion factor of 1.6 is employed in the calculation to determine chlorophyll-*a* concentration.

Appendix B. Procedure for the multiple allelochemical method development

The method was developed in the Scientific Centre, Wessex Water, Saltford, Bath, UK, with assistance from Gary Hunt (ChemStation software and calibration), Alexander Jennings (HPLC preparation and solvent gradient tests) and overseen by Toni Hall.

Standards, mixed-standards and non-extracted standards preparation

The preparation of non-extracted standards for calibrating standards is as follows. Three sets of test tubes (13 mm x 10 mm, rimless Fisherbrand, Fisher Scientific, UK) were labelled as high (0.9 mg L^{-1}), medium (0.5 mg L^{-1}) and low (0.1 mg L^{-1}).The following was added to each tube; 70 µl of DI water, the standard mix (900, 500 and 100 µl) to the respective tubes, the internal standard (100 µl) to each tube and methanol (1000, 1400 and 1800 µl) to the respective tubes. These solutions were dried down in a Turbovap® LV (Biotage, UK) automated evaporation system for 15 min at 15 psi and 25°C carefully until almost dry. The DI water helps to retain the compounds and prevents loss of the compounds through complete evaporation of solvents. Then, the residue in the tubes is re-suspended with 1 ml of a solution made up with 10% acetonitrile (for pesticide quality analysis, 99.9%, Fluka- Sigma-Aldrich,

UK) in DI water. This is vortexed and pipetted using 150 mm glass Pasteur pipettes (Volac, Fisher Scientific, UK) into 2 ml screw-cap amber vials (2ml short thread vial, amber glass, label with filling lines and 1mm thick, red PTFE/white silicone, 9mm screw blue cap from Kinesis, UK).

Instrumental determination, quality assurance and quality control

The analytical work on the instrument employed (Agilent 1200 LC series system) was carried out in the Wessex Water Scientific Centre. Several attempts (up to 15) were made by using different solvents (methanol and acetonitrile), using gradient concentrations of these solvents (percentage of the organic solvent and ultra-pure water), applying solvent acidification or using unacidified solvent, varying volume of the injection (50 µl and 100 µl), varying the elution time for each sample (30 min to 55 min) and varying the selection of the range of wavelength. The gradient elution that allowed the detection, resolution and quantification of most standards was obtained after 15 different attempts with different combinations of the proportion of the mobile phases and the run time per sample. The quality assurance and quality control of the instrumental method was evaluated by measuring sensitivity, selectivity and precision (repeatability and reproducibility) (Villagrasa *et al*., 2009).

An Agilent 1200 LC series system was used in the method development process, consisting of a binary pump, auto-sampler, column oven and UV-DAD detector. A HPLC Agilent column (C18, 150 mm x 5 mm) was used to achieve separation with an aqueous / organic mobile phase gradient elution consisting of acidified (0.1% acetic acid) DI Water and acidified (0.1% acetic acid) acetonitrile, with starting conditions of 5% acetonitrile to 95% water (5:95). Solvent conditions were tested prior to accepting the method. The column was protected by a 20 mm x 3.9 mm guard column of the same packing material. The binary pump was used with two solvent reservoirs to deliver the appropriate eluent mix. Column

temperature was maintained at 40°C. Following injection at 5% acetonitrile: 95% acidified water (5:95), isocratic conditions were held for 10 min, followed by a 5 min linear gradient to 95% acidified acetonitrile: 5% acidified water (95:5), held for 5 min, then a subsequent 5 min linear gradient to return to initial conditions (5:95). Total run time was 45 min. The UV absorbance detector collected data at three wavelengths 205, 250 and 280 nm. Peak identity was validated through the use of retention times of external non-extracted and extracted standards with their respective spectrums. All samples were kept at 5°C at all times. The HPLC operating system used was the Agilent ChemStation software.

Peak validation

Peak identity was validated through the use of retention times of the extracted and non-extracted standards and spectrum characteristics (Mizukami *et al*., 2007). The spectra of the peak standards in the Agilent 1200 LC series were compared with those obtained in the Shimadzu UV-1800 spectrophotometer. The peaks with the same retention times in the samples as those in the standards of the Agilent system were cross-checked to verify if their spectrum was the same as those of the standards in the Shimadzu system.

Calibration, sensitivity and recovery

Standard calibration curves were constructed with the non-extracted mix standards (A, B and C) with three concentrations (1, 0.5 and 0.1 mg L^{-1}) to a final volume of 1 ml; each was injected in triplicate. The internal standard present in the samples (at a concentration of 0.1 mg L^{-1}) was used to verify the concentrations of these dilutions (Mizukami *et al*., 2007). These dilutions were not extracted by solid phase extraction (SPE) serving as a reference and quality control for the procedure with the corresponding mix extracted standard by SPE (Slavin and Yu, 2012). The extracted mix standard by SPE had a final concentration of 0.9 mg L^{-1} spiked with 0.1 mg L^{-1} of the internal standard. Peak area was plotted against the

concentration of prepared non-extracted standards and concentrations were all calculated with the ChemStation software.

The sensitivity of the method was determined by the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ are based on the peak-to-peak noise of the baseline near the analyte peak obtained by the analysis of the internal standard solution (Villagrasa *et al*., 2009) as in equations 4.2 and 4.3 respectively:

LOD (mg L^{-1}) = (3.3 x standard deviation of the analyte concentration) / slope of the calibration curve of the analyte (eq. 4.2)

LOQ (mg L^{-1}) = (10 x standard deviation of the analyte concentration)/ slope of the calibration curve of the analyte (eq. 4.3)

The recovery of the extraction method was determined through the use of three sets of spiked samples with the internal standard, performed in three independent measurements and calculated as in equation 4.4:

% Recovery =

(Extracted mix standard mg L^{-1} / Non-Extracted mix standard mg L^{-1}) x 100 (eq. 4.4)

Precision (repeatability) and accuracy of the method

The precision and accuracy of the method was determined following Mizukami *et al*. (2007) and Villagrasa *et al*. (2009). The precision of the analytical method is based on the repeatability (refers to the intra-day variation) and the accuracy is based on the reproducibility of the analytical results, which are respectively assessed by performing intra-day and inter-day analyses of the standard solutions.

The repeatability of the analytical method was assessed by performing HPLC-UV-DAD analysis of the mix-A non-extracted standard solution nine times on the same day. The relative standard deviation, expressed in percentage (RSD%), was calculated by dividing the

standard deviation of the analyte in a sample by the average of the analyte in the sample, and the result was multiplied by 100.

The accuracy of the results was verified by the reproducibility (inter-day variation) of the analytical results by performing HPLC-UV analysis of the mix-A non-extracted standard, injected in triplicate on at least two separate sequential days. The triplicate analyses were tested as single data points to calculate RSD% between days for each compound.

Purification and concentration of analytes by solid phase extraction procedure

This process is aimed at purifying and concentrating the investigated compounds which might be present at low concentration in the samples before being analysed in the HPLC-UV-DAD instrument. It is also carried out to determine the recovery of standards in an extracted solution of mix-standards compared to that of non-extracted standards and to estimate the concentration of allelochemicals present in the samples.

The SPE was carried out using columns of 3 ml volume capacity containing 100 mg of non-polar polymeric polystyrene divynilbenzene (PS-DVB) particles (Isolute® 101, Biotage). These columns were attached to a 12 port Visiprep manifold (Supelco™ – Sigma-Aldrich). Each port is provided with valves for flow control and PTFE needles. The vacuum pressure in the Visiprep chamber is control by the provided valve and manometer. The process is aided in some steps by a vacuum pump. The SPE involved five steps: column conditioning, sample adsorption, elution, drying and resolution of eluent.

1. Column conditioning. To increase purification and remove interferences from the PS-DVB phase, the labelled columns were flushed with methanol and then, to remove solvent excess, the columns were rinsed with distilled water (DI) (MilliPore Milli-Q system). These reagents flow through the column by gravity; the sample containers of 75 ml capacity (named ‘reservoirs’) are attached to the columns.

2. The sample adsorption step consisted of loading 100 ml of sample, addition of the internal standard and concentration of the desired standards. Half of the sample volume (50 ml) was loaded into the reservoir and all samples are spiked with the same concentration of internal standard (100 µg L^{-1}). The remaining sample volume is then loaded. A low vacuum pressure (0.6 kPa) is applied to pull the samples through the columns. Once the reservoirs are empty, the vacuum is released and the reservoirs are detached. To improve the purity of the extract, the columns are washed with 3 ml of a 30% methanol in DI water solution using a vacuum pressure up to 0.6 kPa. Then the columns are allowed to dry by drawing through air at a maximum vacuum pressure (2 kPa) for five minutes. The vacuum is released and labelled test tubes (Pyrex® glass test tubes without rim, medium wall) of 3 ml capacity are placed in the rack inside the manifold chamber and directly below their corresponding columns ready to receive the eluted concentrated extract.

3. Elution of analytes. This step involves the use of an organic solvent with a few percent of an organic acid to improve the recovery of acidic analytes. Here, 1 ml of 0.1% formic acid (LC-MS grade, 98%, Fluka, Sigma-Aldrich) in methanol was used as solvent eluent. To minimize the analyte-solvent volume, half of the solvent eluent volume is allowed to be soaked into the column for a couple of minutes, then collected in the test tubes by gravity flow. This step is repeated with the remaining solvent eluent, but after the eluent has drained by gravity flow, a low vacuum pressure is applied to gain as much of the remaining eluent as possible.

4. The drying step consists of the evaporation of the solvent eluent with a constant air flow (0.8 L min^{-1}) passing through the evaporation device (Visidry, Supelco – Sigma-Aldrich, UK). This device is attached to the Visiprep chamber. Evaporation of the solvent eluent is accelerated by placing the rack tubes in a water bath system with a temperature oscillating between 30-40°C. Most allelochemicals are generally destroyed at temperatures above 100°C. The samples should not be allowed to dry completely.

5. The resolution of the dried eluted analyte involves the addition of a solvent that closely matches the composition and concentration of the mobile phase solutions in the Liquid Chromatograph instrument. This solution consisted of 1 ml of 10% acetonitrile (Fisher pesticide residue grade, Sigma-Aldrich, UK) in DI water with 0.1 % of acetic acid (*Trace*Select® Ultra, for ultratrace analysis, ≥99.0%, Fluka, Sigma-Aldrich, UK). Half of the solution is loaded into the test tubes and homogenized using a Vortex-Genie 2 (Scientific Industries, UK) for a few seconds and finally transferred to 2 ml amber vials with caps. The final volume is 1ml.

Quantitative analysis and calculations using external and internal standard procedures

The magnitude of response to the analytes in the samples is quantified using an external standard analysis (ESTD) with four point multi-level calibration, which includes three concentrations and a point at the origin. The multi-level calibration can be used to confirm linearity of the calibration range. The regression analysis equation obtained from the calibration curve describes the best-fit of the line through the data points (eq. 4.5)

$$y = mx + c \qquad \text{(eq. 4.5)}$$

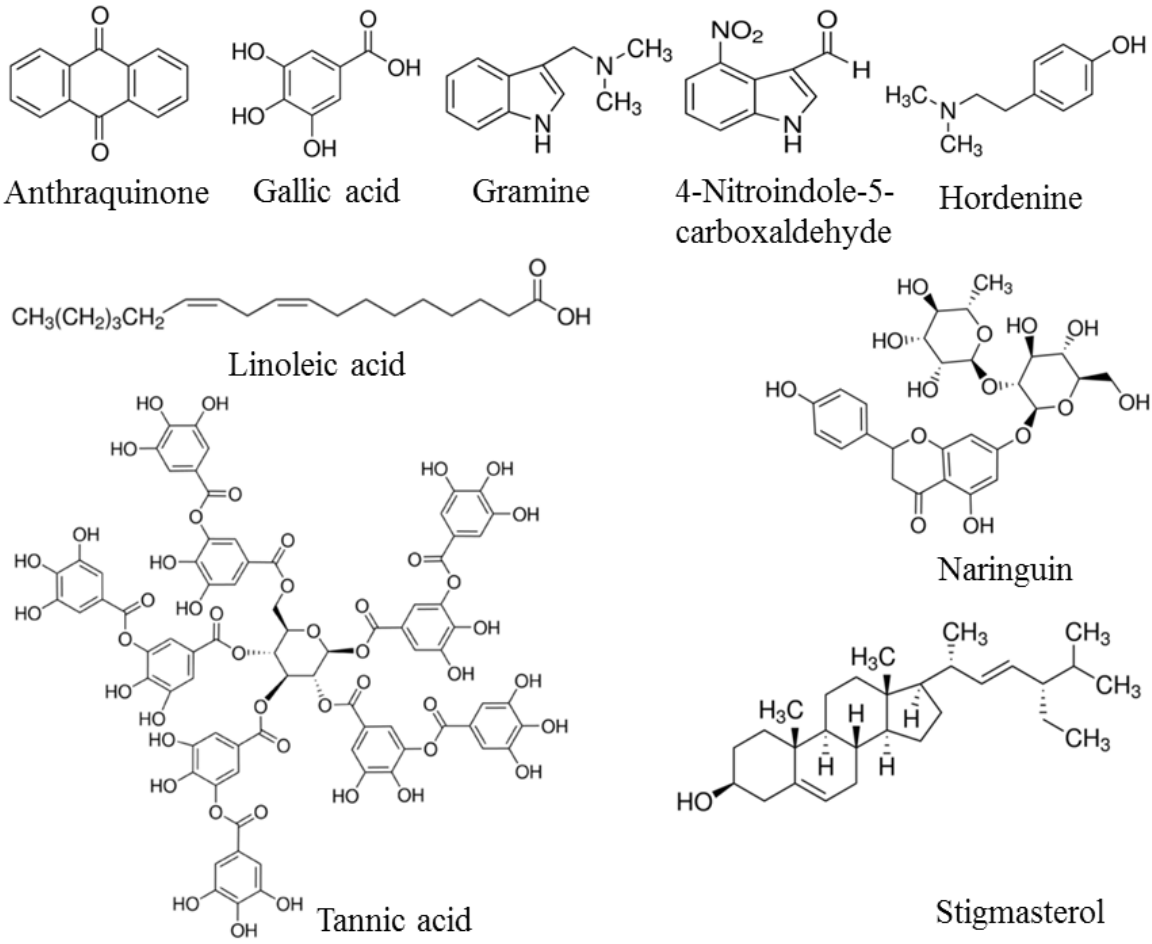
where: x = peak area; *m*= the slope of the regression line; *c*=the intercept of the regression line with the y-axis; .*y*= the concentration of an analyte in a sample is calculated using equation 4. 6:

This method allows the quantitative determination of analyte concentrations within the range of the external standard concentrations and semi-qualitatively for peaks out of the range of calibration by extrapolating the regression analysis.

The internal standard analysis (ISTD) adds a known concentration of a compound to be used as a normalizing factor, being added to the blank, non-extracted and extracted standards and samples; it compensates for losses during sample preparation and solid phase extraction. The ratio between the maximum internal standard area in the same set of samples (batch)

which have been extracted under the same conditions and the peak area of the internal standard for a sample is obtained. This ratio is the internal standard factor and is applied to all the peak areas in a particular sample. So, if the batch contains 10 samples, there will be 10 internal standard factors. Therefore all peak areas are normalized in relation to the internal standard.

Appendix C. Chemical structural formula of the selected allelochemicals.



Appendix D. Concentration of the allelochemicals gramine, naringuin and 4-nitroindol-5-carboxaldehyde (mean±SE) produced by biofilters for four treatments (n=18) and *Microcystis* (n=6).

Treatment	Gramine µg L^{-1} mean±SE	Naringuin µg L^{-1} mean±SE	4-Nitroindol-5-carboxaldehyde µg L^{-1} mean±SE
Tap Water	167.7±54.1	174.4±12.8	68.68±5.7
Tap Water & *Microcystis*	110.8±59.0	143.4±6.9	561.5±51.4
Hoagland's solution	84.8±28.5	301.2±51.1	119.5±34.9
Hoagland's'& *Microcystis*	69.4±15.6	181.1±19.06	420.9±26.9
Microcystis culture	< LOD*	-	107.0±1.7

*LOD= Limit of detection

Appendix E. Root weight (g) and volume (cm^3) from biofilter samples (x16 plants) and Living-Filter samples (x3)

Root Samples	Sample	Weight (g)	Weight (g) mean±SD	Biovolume (cm^3)
Biofilters (x3) each of *Phalaris* (x16) **plants**	1.	44.63	N/A	N/A
	2.	39.6		
	3.	92.6		
Living-Filter: *Phalaris* (x3)	1.	7.0	7.47±0.57	N/A
	2.	7.3		
	3	8.1		
Living-Filter: *Carex* (x3)	1.	10.4	10.70±0.30	N/A
	2.	11.0		
	3.	10.7		
Living-Filter: *Phragmites* (x3)	1.	11.6	10.90±0.70	N/A
	2.	10.2		
	3.	10.9		
Biofilters x4 sets of *Phalaris* (x10) **plants**	1.	N/A	12.7±1.33	16.7±2.08
	2.		9.6±0.81	11.3±3.06
	3.		9.2±0.75	10±2
(mean±SD) (n=3 biofilters)	4.		8.3±0.61	9.7±0.58