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# Characterisation of a major phytoplankton bloom in the River Thames (UK) using Flow Cytometry and High Performance Liquid Chromatography

H. L. Moorhouse[1, 2], D.S Read[1], S. McGowan[3], M. Wagner[1], C. Roberts[1], L.K. Armstrong[1], D.J.E. Nicholls[1], H.D. Wickham[1]. M.G Hutchins[1], and M.J. Bowes[1, *],

[1] Centre for Ecology & Hydrology, Wallingford, Oxon. OX10 8BB, United Kingdom

[2] Lancaster Environment Centre, Library Avenue, Lancaster University, Lancaster, LA1 4YQ

[3] School of Geography, University of Nottingham, University Park, Nottingham, NG7 2RD.

**Abstract**

Recent river studies have observed rapid phytoplankton dynamics, driven by diurnal cycling and short-term responses to storm events, highlighting the need to adopt new high-frequency characterisation methods to understand these complex ecological systems. This study utilised two such analytical methods; pigment analysis by high performance liquid chromatography (HPLC) and cell counting by flow cytometry (FCM), alongside traditional chlorophyll spectrophotometry and light microscopy screening, to characterise the major phytoplankton bloom of 2015 in the River Thames, UK. All analytical techniques observed a rapid increase in chlorophyll *a* concentration and cell abundances from March to early June, caused primarily by a diatom bloom. Light microscopy identified a shift from pennate to centric diatoms during this period. The initial diatom bloom coincided with increased HPLC peridinin concentrations, indicating the presence of dinoflagellates which were likely to be consuming the diatom population. The diatom bloom declined rapidly in early June, coinciding with a storm event. There were low chlorophyll *a* concentrations (by both HPLC and spectrophotometric methods) throughout July and August, implying low biomass and phytoplankton activity. However, FCM revealed high abundances of pico-chlorophytes and cyanobacteria through July and August, showing that phytoplankton communities remain active and abundant throughout the summer period. In combination, these techniques are able to simultaneously characterise a wider range of phytoplankton groups, with greater certainty, and provide improved understanding of phytoplankton functioning (e.g. production of UV inhibiting pigments by cyanobacteria in response to high light levels) and ecological status (through examination of pigment degradation products). Combined HPLC and FCM analyses offer rapid and cost-effective characterisation of phytoplankton communities at appropriate timescales. This will allow a more-targeted use of light microscopy to capture phytoplankton peaks or to investigate periods of rapid community succession. This will lead to greater system understanding of phytoplankton succession in response to biogeochemical drivers.

**Key words**

Algae; Cyanobacteria; Eutrophication; Water Quality; High-frequency monitoring; Photosynthetic pigments.

# 1 Introduction

Phytoplankton play a vital role within the aquatic ecosystem. In large rivers, they are often the principal autotrophs, supplying organic carbon to the system, oxygenating the water column and providing the base of aquatic food webs (Thorp and Delong, 1994; Wehr and Descy, 1998). However, under certain conditions (exacerbated by anthropogenic nutrient enrichment, river flow regulation, land-use change and food-web disturbance), excessive phytoplankton growth can occur (Bowes et al., 2012). These phytoplankton blooms can pose serious threats to ecosystem structure and function, water quality, and threaten drinking water supplies (Dodds et al., 2009). They also reduce sunlight penetration within the water column, resulting in the loss of macrophytes and the vital habitat that they provide for zooplankton, macroinvertebrates and fish. Blooms can be aesthetically unappealing, resulting in significant financial losses for the tourism industry and reduce the value of riverside properties (Pretty et al., 2003; Dodds et al., 2009). High densities of phytoplankton can also increase operating costs for water companies due to blocking of filters and toxin / taint production by cyanobacteria. When blooms terminate, there is often depletion in dissolved oxygen concentration as the algae are consumed by heterotrophs, and these oxygen sags can ultimately result in fish-kills (Hilton et al., 2006).

The plankton communities of marine, estuarine and lake environments have been extensively monitored, principally by chlorophyll analysis and algal identification/quantification by light microscopy. This has meant that the characteristics and drivers of phytoplankton succession are relatively well understood in these systems (Sommer et al., 2012). Over the last few decades, high performance liquid chromatography (HPLC) has been widely used to quantify pigments associated with phytoplankton groups (Barlow et al., 1993; Pinckney et al., 1998), which provide further information on phytoplankton biomass and community structure in estuarine and marine environments. In recent years, a new high-throughput technique, flow cytometry (FCM), has been employed to rapidly characterise and quantify marine and lake planktonic communities (Veldhuis and Kraay, 2004; Sarmento et al., 2006; Pomati et al., 2011). When used in combination, these techniques can provide a much more complete description of plankton dynamics, and are able to produce data at a much higher temporal resolution than traditional light microscopy alone.

In contrast, river phytoplankton succession has been relatively understudied in comparison to marine and lake environments (Reynolds and Descy, 1996). As a consequence, our knowledge of the causes of, and controls on river phytoplankton bloom development are not well understood (Reynolds, 1993). Previous studies of temperate rivers have shown how phytoplankton biomass can be affected by a combination of factors including hydrology (Reynolds and Descy, 1996; Hardenbicker et al., 2014), nutrient limitation (Tavernini et al., 2011; Wu et al., 2011), grazing (Basu and Pick, 1997; Twiss et al., 2010), light (Dokulil, 2014) and temperature (Desortova and Puncochar, 2011). Most river phytoplankton studies are based on infrequent (fortnightly to seasonal) chlorophyll analysis (to give an estimate of phytoplankton biomass), and/or identification and quantification by light microscopy to give some indication of phytoplankton succession.

Recent riverine studies have highlighted the rapid sub-daily changes in chlorophyll *a* concentration that can occur (Dubelaar et al., 2004; Bowes et al., 2016), highlighting the need for future river research to embrace new, high-throughput monitoring techniques such as HPLC and FCM to capture these short term phytoplankton dynamics and enable greater process understanding. However, to date, the number of riverine HPLC pigment studies are limited, and have been used to capture general changes in phytoplankton communities through the annual cycle (Descy and Metens, 1996; Dorigo et al., 2004) or along the river continuum (Dagg

et al., 2005; Descy et al., 2017). Application of FCM to rivers is extremely limited. Dubelaar et al. (2004) used FCM to characterise daily changes in the phytoplankton community over a two week period in the Oude Rijn River, Netherlands, highlighting the rapid dynamics that occur in riverine environments. FCM has also been used to quantify river bacterioplankton abundances (Paulse et al., 2007; Besmer et al., 2014; Read et al., 2015). Read et al. (2014) used FCM to enumerate ten phytoplankton groups (including diatoms, chlorophytes, cryptophytes and cyanobacteria) at 23 sites across the River Thames basin at weekly intervals, and these data have been utilised to model the algal dynamics and potential impact of climate change on phytoplankton community composition (Whitehead et al., 2015; Bussi et al., 2016).

This study aimed to characterise the phytoplankton community succession of the River Thames, southern England, at weekly interval through the spring to autumn period of 2015, using both traditional techniques (spectrophotometric chlorophyll *a* determination and light microscopy screening) alongside HPLC and FCM. To our knowledge, this is the first time these two instrumental techniques have been applied simultaneously to monitor a river phytoplankton bloom. The monitoring data was then assessed to identify strengths and potential weaknesses of these techniques, and to determine if greater understanding of phytoplankton dynamics can be achieved by combining these multiple approaches.

# 2 Methods

## 2.1 Study site

The River Thames is the longest river wholly in England (354 km), and the second longest in the UK, with a freshwater basin of 9948 $km^2$. It rises at Thames Head in Gloucestershire, and flows in an easterly direction through the relatively rural Cotswold Hills, then passing through a number of large towns and cities, such as Swindon, Oxford, Reading and Maidenhead, before flowing through the centre of London and into the North Sea (Fig 1). Approximately one fifth of the UK population (with a population density of 960 people $km^2$) reside in the catchment (Merrett, 2007). This study was conducted on the River Thames at the town of Wallingford, 134 km from the source. Previous studies along the River Thames have shown that chlorophyll maximum concentrations occur followed by depletions of soluble reactive phosphorus and silicon within the reach from Oxford to Wallingford (Lazar et al., 2012; Bowes et al., 2016) (Fig 1), making this productive site ideal to study phytoplankton dynamics, hence its choice for this study. Mean river flow at Wallingford is approximately 32 $m^3 s^{-1}$ with a base flow index of 0.64 (due to significant groundwater input from the Cretaceous Chalk and Oolitic Limestone geology) and mean annual rainfall of 715 mm (Marsh and Hannaford, 2008). The catchment area upstream of Wallingford is 4,213 $km^2$, with land use consisting of 45.1 % arable, 35.6 % grassland, 10.3 % woodland, and 7.3 % urban and semi-urban development (Fuller et al., 2002). The sewage treatment works (STW) population estimate upstream of Wallingford is approximately 1.03 million people (Bowes et al., 2014). The large number of STW, high population density and areas of relatively intensive agriculture result in the River Thames at Wallingford having elevated nutrient concentrations, with mean total phosphorus and soluble reactive phosphorus concentrations of 282 and 192 µg P $l^{-1}$ respectively, and nitrate concentrations of 6.7 mg $NO_3$-N $l^{-1}$ (from weekly measurements between Feb 2009 to April 2011) (Bowes et al., 2014).

**(Figure 1)**

## 2.2 Water quality analyses

Weekly water samples (8l) were taken from the River Thames at Wallingford between the 6th January and the 1st September 2015 as part of the Centre for Ecology & Hydrology's (CEH) Thames Initiative research platform (Bowes et al., 2014). Water samples (8 litres) were

collected from the euphotic zone of the river channel, and any visible plant / leaf material was removed from the bulk sample prior to processing, to minimise the contribution of macrophytes and allochthonous materials (such as leaves from riparian trees) to the pigment data. Sub-samples were filtered on-site (for subsequent nutrient analysis) through a 0.45 µm cellulose nitrate membrane (WNC; Whatman, Maidstone, UK), and all samples were stored in the dark at 4$^{o}$C, prior to analysis. Chlorophyll *a* concentrations were determined by filtering 1l of river water through a 1.2 µm membrane filter (GF/C grade; Whatman, Maidstone, UK) and then extracting the pigment in the residue with 90 % acetone overnight, and quantifying by spectrophotometry (Marker et al., 1980). Soluble reactive phosphorus and dissolved reactive silicon concentrations of the filtered subsamples were determined colorimetrically using the methods of Murphy and Riley (1962) and Mullin and Riley (1955) respectively, and nitrate – N concentration was determined by ion chromatography (Dionex DX500). Further details of the analytical methods are provided elsewhere (Bowes et al., 2012).

## 2.3 High Performance Liquid Chromatography

HPLC separates compounds according to their polarity and mass via the interaction of non-polar stationary (column of packing material) and polar mobile (solvents) phases. There are many different gradients and solvent combinations depending on the separation of the sample desired. In this instance, an adaption of Chen *et al*. (2001) was used as it separates the main chlorophyll and carotenoid pigments found in rivers. The weekly water samples (0.5 to 2l) were filtered through a 1.6 µm membrane (GF/A grade; Whatman, Maidstone UK) to collect phytoplankton, and the filters were then stored frozen (-80°C) and in the dark. The samples were extracted using acetone, methanol and HPLC grade water (80 : 15 : 5), then dried under $N_2$ gas before being re-dissolved in injection solution chemically similar to the mobile phase (70:25:5 = Acetone: IPR stock solution (1.875 g tetra butyl ammonium acetate, 19.25 g ammonium acetate, 250 ml de-ionised water): Methanol) (Chen et al., 2001; Leavitt and Hodgson, 2001). The HPLC system at the University of Nottingham comprised an Agilent 1200 series quaternary pump, autosampler, and photodiode array detector which detects UV and visible spectral data every 2 seconds. The eleven pigments quantified by this analytical method (with their related phytoplankton group) were chlorophyll a and β carotene (total algae), diadinoxanthin and diatoxanthin (diatoms), chlorophyll b and violaxanthin (chlorophytes) alloxanthin (cryptophytes), lutein and zeaxanthin (chlorophyte and cyanobacteria), sytonemin (cyanobacteria) and peridinin (dinoflagellates).

Each pigment used in the analysis was calibrated on the HPLC with phytoplankton pigment standards from DHI Denmark (c14.dhigroup.com), injecting a series of concentrations to allow the conversion of chromatogram peak areas to pigment molar weight per volume (McGowan, 2013). There was no commercial standard for scytonemin, and therefore we assumed that the absorption coefficient approximated to a common carotenoid lutein and used the calibration curve for this pigment. Therefore, the molar concentrations for scytonemin are estimates (as Figure 4c), but concentrations are directly comparable through time. The pigment standards provided reference spectra and retention times for comparison with samples to assist with pigment identification. To check for machine retention time drift which could lead to peak misidentifications, a non-quantitative extract of grass (containing lutein, chlorophyll a, chlorophyll b) was run at the start and end of each HPLC sequence. Samples were randomized within HPLC sequences to remove the possibility of machine drift systematically affecting the results. Phytoplankton group affinities were assigned following Leavitt and Hodgson (2001). The pigments lutein and zeaxanthin are presented together because they co-eluted.

## 2.4 Flow Cytometry

Phytoplankton samples for flow cytometry (ca. 20 ml sub-sample from the 8l bulk sample) were stored in 30 ml universal tubes at 4 °C before analysis within 24 hrs of collection. The flow cytometry data collection protocol is outlined in detail in Read et al. (2014). The method was used to identify ten major groups of phytoplankton based on phenotypic characteristics (size and pigmentation) using a Gallios flow cytometer (Beckman Coulter, High Wycombe, UK) equipped with blue (488 nm) and red (638 nm) solid state diode lasers. These groups were diatom / large chlorophytes (one combined group), nano- and pico-chlorophytes (three groups), cryptophytes (two groups) and cyanobacteria (four groups, including microcystis-like, synechococcus-like and phycoerythrin-rich cyanobacteria). Briefly, the analysis protocol uses a plot of yellow/green fluorescence (FL2 – 575 nm) against red fluorescence (FL4 – 695 nm) excited by the 488 nm laser to represent fluorescence from phycoerythrin versus chlorophyll. A second plot was used to identify and count cyanobacteria using red florescence (FL4 – 695 nm) excited by the 488 nm laser versus orange fluorescence (FL6 – 660 nm) excited by the 638 nm laser, representing chlorophyll versus the cyanobacterial pigment phycocyanin. Samples were spiked with a set volume of FlowCount (Beckman Coulter) counting beads, and run for five minutes per sample at a high flow rate. Data was processed using the software Kaluza Analysis v1.5a (Beckman Coulter, UK), and exported to a .csv file for plotting and interpretation.

## 2.5 Light microscopy screening

A 20 ml sub-sample from the 8 l bulk water sample was taken every fortnight between March-August 2015. On return to the laboratory, the sample was preserved with Lugol's iodine and kept in a dark, cold (4°C) environment. A 10ml subsample was allowed to settle in a sedimentation chamber and then multiple photographs were taken through an inverted microscope (Zeiss Axiovert 40CFL), to provide a visual record of community succession through the study period. These were used to confirm the presence of major taxonomical groups (pennate diatoms, centric diatoms, large chlorophytes, cryptophytes, dynoflagellates, and ciliates)

## 2.6 Statistical analyses

Statistical analyses were carried out in R (v.3.0.1) (R Core Team, 2013) using the package 'vegan' v2.0–10 (Oksanen et al., 2017). To determine the strength and significance of the correlation between community structures as captured by each of the two alternative metrics provided by FCM and pigment assemblage data, we carried out a Mantel correlation and test using vegan's 'mantel' function, with a significance test based on 9999 permutations. For this analyses, the characterization of community structure based on FCM was based on cell counts for diatoms, nano-chlorophyes, pico-chlorophytes, total cryptophytes, and total cyanobacteria. Accordingly, for the characterization of community structure based on pigment assemblage data, we used concentration data for six pigments associated with one or more of these specific groups of phytoplanktion, including alloxanthin (associated with cryptophytes), chlorophyll b, violaxanthin (both with chlorophytes), diadinoxanthin, diatoxanthin (both with diatoms), and lutein-zeaxanthin (chlorophytes and cyanobacteria). Mantel correlations were based on Bray-Curtis dissimilarity matrices for these two alternative characterizations of community structure, which were calculated using vegan's 'vegdist' function.

# 3 Results

## 3.1 Spectrophotometric chlorophyll *a* concentration

Spectrophotometric chlorophyll *a* concentrations from the weekly water samples showed that there was a single, large and prolonged algal bloom in 2015, with elevated chlorophyll *a* concentrations (greater than 15 µg $L^{-1}$) extending from the start of April until the end of June; a period of 13 weeks (Fig 2a). A bloom of such a long duration had not been observed in the middle reaches of the River Thames in the last 20 years (Kinniburgh and Barnett, 2010; Bowes et al., 2012). The average chlorophyll *a* concentration throughout this period was 118 µg $l^{-1}$, peaking at 186 µg $l^{-1}$ on 27th April and 236 on the 9th June. These 2015 peaks were relatively modest compared to chlorophyll maxima observed in previous years in the River Thames, which can have chlorophyll concentrations exceeding 350 µg $l^{-1}$. The chlorophyll maxima coincided with a reduction in dissolved reactive silicon concentrations (Fig 3).

## 3.2 Phytoplankton community succession by FCM and HPLC

The FCM and HPLC algal community matrices were well correlated (Mantel r2 = 0.67; P<0.001), suggesting that both techniques are detecting the major temporal changes to the algal assemblage. Concentrations of pigments representative of all algae (chlorophyll *a*, pheophytin *a* and β-carotene), diatoms (diadinoxanthin and diatoxanthin) and chlorophytes (chlorophyll *b*), and the FCM diatom and chlorophyte cell counts, all increased from the end of March, and throughout April (Fig 2b; d (individual pigment concentration and FCM group abundance time-series given in Supplementary Figs S1 and S2)). The majority of the increased biomass was due to increased numbers of diatoms / large chlorophytes (measured by FCM), which increased from 6,300 cells $ml^{-1}$ on 23rd March to 74,000 cells $ml^{-1}$ on 27th April (Fig 2b;3a). By the end of April, the diatom / large chlorophyte group contributed 56% of the total number of phytoplankton cells enumerated by FCM (Fig 2c). The increase in HPLC total pigment concentration was principally due to a 20-fold increase in chlorophyll *a* concentration, which occurred alongside an increase in diadinoxanthin, a pigment specifically associated with diatoms (Fig 3b). There were also rapid increases in pheophytin *a* and diatoxanthin, which are the degradation products of chlorophyll *a* and diadinoxanthin respectively, suggesting the presence of senescent material in the bloom. The close relationship between the ubiquitous pigment chlorophyll *a* to pigments found in diatoms suggests that this group of algae has driven these changes and there is minimal contamination from higher plant material. Dissolved reactive silicon concentrations rapidly decreased throughout this period (Fig 3a), from 3.89 Si mg $l^{-1}$ on the 2nd March to a potentially-limiting concentration of 0.50 mg Si $l^{-1}$ (Lund, 1950) on the 20th April (Fig 3a), due to diatoms utilizing dissolved silicon in the water column to produce frustules. The onset of this dissolved silicon depletion was approximately two weeks before the onset of the planktonic diatom bloom, indicating that benthic diatom growth was taking place in the upstream catchment as a precursor to the phytoplankton bloom, delivering Si-depleted waters downstream. The diatom bloom was sustained throughout April and May, reaching a FCM peak of 85,000 cells $ml^{-1}$ on 9th June. This coincided with the highest chlorophyll-*a* concentration (measured using both HPLC and spectrophotometry) observed throughout the study period (Fig 2c), and a major peak in diadinoxanthin concentration (Fig 3b), and a secondary dip in dissolved silicon concentration (Fig 3a). Nano-chlorophytes recorded by FCM also increased in number throughout this period, indicating that they comprised 20 to 25% of the total phytoplankton community population (Fig 2c).

Between the 9th and the 22nd June, there was a rapid decrease in both FCM phytoplankton cell numbers and pigment concentrations. This coincided with two concurrent dull days (with less than 1 hour sunshine per day) (Fig 4c) with associated rainfall and increased river flow, which has been demonstrated to be sufficient to terminate River Thames phytoplankton blooms in

previous years (Bowes et al., 2016). The bloom crash was predominantly due to a sharp reduction in the number of diatoms, with corresponding rapid decreases in chlorophyll *a*, pheophytin *a*, diadinoxanthin and diatoxanthin concentrations. Dissolved silicon concentrations also rapidly increased to background concentrations of >5 mg Si $l^{-1}$, indicating that diatom growth rate was minimal during this period. Very similar patterns of phytoplankton bloom development have been observed in both recent studies of the River Thames (Bowes et al., 2012; Read et al., 2014; Bowes et al., 2016), and a study of the River Thames at Reading in 1966 to 1968 (Lack, 1971).

**(Figure 2)**

**(Figure 3)**

FCM data showed a sudden increase in pico-chlorophytes and cyanobacteria from the 15$^{th}$ June, making up *ca.* 55% and 35% of the total phytoplankton cell count respectively throughout July (Fig 2b and c). Pico-chlorophyte (0.2 to 2.0µm diameter) numbers reached their peak of 145,000 cells $ml^{-1}$ in mid-August, equivalent to 75% of the total phytoplankton cell count. This coincided with an increase in the chlorophyte pigment violaxanthin, which peaked on the 10$^{th}$ August (Fig S1). However, by the end of June, HPLC pigment concentrations were generally low, and remained so until the end of the study period, indicating low phytoplankton biomass through July and August (Fig 2b; S1).

All four FCM cyanobacteria groups increased although the synechococcus-type group saw the greatest increases in cell abundance from the end of June to end of August (Fig 4a). This observation was supported by the HPLC data, which showed an increase in lutein-zeaxanthin pigment concentration (indicative of both chlorophytes and cyanobacteria) during the same period (Fig 4; S1). Lutein is also found in higher plants but its co-elution with zeaxanthin and relationship to FCM cyanobacteria counts suggest little contamination of samples with higher plant material. The concentration of scytonemin (a pigment specific to many strains of cyanobacteria, and produced to absorb ultraviolet light) peaked in early June (corresponding to the period of maximum sunshine hours) (Fig 4c), but was low during the late summer period. This may indicate that scytonemin concentration may be a response to high sunlight, rather than a specific indicator of cyanobacterial biomass.

**(Figure 4)**

### 3.3 Phytoplankton succession revealed by light microscopy screening

The light microscopy screening revealed a succession from sparse pennate diatom species to a dominance of centric diatoms and chlorophytes during the period of high chlorophyll *a* concentration from the middle of April to the middle of June (Fig 5). Cryptophyte numbers also increased towards the end of summer which follows the same trend of succession determined in the mid Thames at Reading in the 1960s (Lack, 1971). Further investigation of phytoplankton community succession and species shifts were not possible using this simple, rapid-screening method.

## 4 Discussion

### 4.1 Synthesis of analytical approaches

#### 4.1.1 Spring period (April to early June)

All analytical approaches identified the major diatom and chlorophyte bloom that occurred from April into early June (Fig 2). Dissolved reactive silicon concentration decreased from the

beginning of March (Fig 3), indicating rapid diatom growth in the benthic biofilms, but it was two to three weeks later that this was translated into rapid and sustained increases in phytoplankton biomass. The commencement of the phytoplankton bloom corresponded to a period of sunny conditions, low stable flows ($20 – 30\ m^3\ s^{-1}$) and river water temperatures rising above $9^oC$ (Fig 5). This water temperature has been identified in a previous study of the River Thames (from 2008 to 2013) as the key lower threshold temperature required for phytoplankton growth to occur (Bowes et al., 2016). This early bloom was predominantly due to a rapid increase in the abundance of diatoms. The light microscopy scanning technique was able to detect changes in diatom morphology through the early bloom period, shifting from pennate to centric forms. Higher river flow (compared to the summer and autumn period) could explain the prevalence of predominantly-benthic, pennate diatoms during this period, due to scouring and suspension of benthic pennate diatoms into the water column (Tekwani et al., 2013). This is further supported by the high concentrations of the diatom pigment diatoxanthin, which is a degradation product of diadinoxanthin and indicates the lower "health" of the algal cells resultant from sloughed, senescent benthic upstream production (Louda et al., 2002).

The decrease in total algae, diatoms and chlorophytes after reaching the first peak on the 27th April (detected by spectrophotometric chlorophyll, HPLC and FCM) corresponded to an increase in river flow and a decline in sunshine duration from the 3rd May, thus highlighting the effect of reduced water residence time on cell dilution and the potential for lower periphyton growth rates due to a lack of sunshine (Fig. 2; 3; 4). However, the subsequent low river flows, sunshine and increase in water temperature resulted in further diatom / chlorophyte bloom development, which suggests that precipitation did not remove a sufficient volume of cells for recruitment or dilute nutrient concentrations to prevent subsequent growth. The stable low flows coupled with rising water temperatures provided suitable conditions for rapid bloom recovery (Paerl and Huisman, 2008), peaking on the 9th June (Fig. 2, 3 and 4). Climate change will continue to mediate nutrient delivery and bloom-formation in the Thames and elsewhere (Wilby et al., 2006), thus it is important to consider how these factors interplay to enhance remediation success.

The timing of the two distinct peaks in phytoplankton biomass and diatom / nano-chlorophyte numbers (27th April and 9th June) was captured by spectrophotometric chlorophyll concentration and by HPLC and FCM data. The second peak on the 9th June corresponds to the highest spectrophotometric chlorophyll *a* concentration, FCM diatom concentration and the highest algal HPLC pigment concentrations (chlorophyll *a* and *b* , pheophyton *a*, and β carotene). The first peak corresponded to the maximum observed diadinoxanthin and diatoxanthin concentrations, showing that this first peak was primarily associated with diatom proliferation.

The first chlorophyll peak also coincided with the maximum HPLC peridinin concentration, which is a pigment specific to dinoflagellates. The increase in peridinin concentration began in the same week as the increase in the diatom pigments (Fig S1), indicating that either the dinoflagellate community responded to the same drivers as the diatoms (warm water temperatures, low flow and sunny conditions), or these species were primarily heterotrophic and increased according to an increase in diatom prey populations. If dinoflagellates were functioning mainly heterotrophically, this could explain the low concentration of the photosynthetic compound peridinin (Jeffrey et al., 1997) when light microscopy screening identified their continuing presence following the 9th June peak (Fig. 3; 5). Alternatively, the presence of heterotrophic ciliates detected by light microscopy images could explain the rapid decrease in peridinin from the 9th June and low abundance of dinoflagellates in the microscopy sample photographs (Fig. 3; 5). Some ciliates are known to consume dinoflagellates (Stoecker

et al., 1984) and prey as large as themselves (Kivi and Setälä, 1995), thus it is reasonable to deduce that the increase in predatory ciliates could account for the absence of dinoflagellates. These factors highlight the lower taxonomic strength of algal pigments to that of microscopy, but coupled together, its strength to detect important grazer-prey relationships and the autotrophic / heterotrophic balance, which have been understudied in the River Thames and rivers as a whole.

### 4.1.2 Summer period (July – August)

The proliferation of pico-chlorophytes and cyanobacteria throughout the July and August period, as quantified by FCM, was not detected by HPLC or spectrophotometric chlorophyll *a* analysis. This will, in part, be due to a proportion of the pico-chlorophytes being lost during the water sample filtering stages of both pigment methodologies, resulting in potential underestimations of chlorophyll concentrations by HPLC and spectrophotometry during this period. Pico-chlorophytes range from 0.2 - 2 µm in diameter, and the filtering stage for standard HPLC and spectrophotometric chlorophyll analysis is 1.6 and 1.2 µm pore size respectively. Therefore, for more accurate representation of the phytoplankton community, future pigment analyses should investigate using filter membranes with smaller pore-sizes that would retain pico-plankton. However, very low concentrations of total chlorophyll were also observed in the River Thames (ca. 10 km downstream of the Wallingford study site) during this period, using within-river fluorimeters (YSI EXO 2 probe), which were not similarly compromised by the sample filtration procedure. Between 6$^{th}$ July and 31$^{st}$ August, the average chlorophyll concentrations measured by spectrophotometry and the EXO probe were 11.9 and 12.2 µg l$^{-1}$. This confirms that the rapid decrease in chlorophyll *a* concentrations measured by HPLC and spectrophotometric methods reflected a real decrease in pigment concentration, rather than errors introduced by the methodology. These observations indicate that pico-chlorophytes and cyanobacteria were very numerate through the mid to late summer period (from the FCM data), but did not contribute significant biomass, as determined by the low pigment concentrations, and / or each cell contained lower quantities of pigment. This is an important observation that river phytoplankton communities remain active and abundant through the summer months, despite the low observed chlorophyll concentrations, and highlights the great potential of FCM for future river phytoplankton research.

This shift to smaller chlorophyte cell size has been observed previously, and attributed to a response to higher water temperatures (Daufresne et al., 2009), which is an important consideration for effective river management under future climate change. Seasonal phytoplankton succession in temperate eutrophic lakes follows a similar pattern to that of the River Thames, from spring diatom dominance through to growth in cyanobacterial groups in the late summer, although in lakes the spring crop often consists of fast-growing small centric diatoms and cryptophytes (Sommer et al., 1986), which are observed later in the River Thames. The residence time of the River Thames has been artificially increased in much of its catchment, from connection to canal networks (Bowes et al., 2012), and the building of extensive lock and weir systems, and has thus resulted in seasonal phytoplankton communities similar to those of temperate lakes. However, the presence of benthic pennate diatoms earlier in the spring is more typical of river systems which have experienced an increase in discharge and sloughing of the benthos, thus increasing the numbers of these species in the plankton (Lack, 1971).

**(Figure 5)**

## 4.2 Comparison of phytoplankton characterisation techniques

All analytical methods detected the main algal bloom period and indicated diatoms and chlorophytes were the dominant bloom-forming groups over the spring to early summer period. The FCM and HPLC pigment community distance matrices were strongly correlated, suggesting both methods had detected the same algal community changes over the eight month sampling period. However, as described previously, the peaks in FCM-derived diatom abundance differ to the peak concentrations of HPLC diatom pigments, suggesting that there are discrepancies in the nature of the data collected using these approaches (Fig. 2; 3). Explicitly, the earlier peak in diadinoxanthin concentration on 27$^{th}$ April could have been partly driven by increased dinoflagellate abundance, as indicated by the peak in peridinin concentration on the 27$^{th}$ April (Fig S1). Diadinoxanthin is not only associated with diatoms, but also occurs in dinoflagellates, prymnesiophytes and other algal groups (Leavitt and Hodgson, 2001). This would mean the second smaller peak of diadinoxanthin on the 9$^{th}$ June would likely correspond to a peak in diatom biomass, as suggested by the low peridinin concentration, the peak in FCM diatom cell abundance and a second smaller decline in dissolved reactive silicon at this time.

The chlorophytes are an important summer bloom-forming group in the River Thames as observed from past research (Lack, 1971) and the increase in chlorophyte pigments and microscope identification from the 26$^{th}$ May in this study (Fig. 3 and 5). However, the FCM nano-chlorophyte counts reach high numbers earlier in the spring from 30$^{th}$ of March to 27$^{th}$ of May at populations of >15000 cells ml$^{-1}$ (Fig. 2). Although key contributors to chlorophyll *a* (Rodríguez et al., 2006), it is difficult to determine the relative importance of the nano-chlorophytes to the total chlorophyll *a* pool using spectrophotometric and HPLC pigment analysis alone, especially in a period of high diatom abundance. The increase in HPLC and spectrophotometric chlorophyll *a* matched the increase in nano-chlorophytes and temperatures above 10°C from the 30$^{th}$ March, likely due to the warmer temperatures promoting the nutrient uptake capacity of these small taxa (Roy et al., 2011). Thus, FCM is a valuable method in identifying phytoplankton groups difficult to taxonomically identify by other methods.

However, there was inconsistency between the FCM and pigment-derived cryptophyte and cyanobacterial communities. These inconsistences could also indicate constraints of the FCM to differentiate between different groups with overlapping fluorescence and size classes. There are two main reasons why there is a discrepancy between FCM and HPLC. Firstly, the cyanobacterial pigment scytonemin peaked on the 1$^{st}$ June (Fig. 4c), compared to the higher values of the FCM cyanobacterial counts beginning 6$^{th}$ July (Fig. 4a), indicating that the colonial cyanobacterial which often have this pigment were not captured by FCM analysis. Scytonemin production is enhanced under high ultraviolet-A (UV-A) levels, increases in temperature and photo-oxidative conditions (Dillon et al., 2002). The colonial cyanobacterial pigment scytonemin is a UV-absorbing compound, hence its high values during the period from the 7$^{th}$ of April 2015 to the 29$^{th}$ of July 2015 when sunshine hours were at their greatest and water temperatures continued to rise (Fig 4). When the scytonemin concentration was normalised to the chlorophyll *a* concentration (to remove the influence of biomass), data shows there are peaks in scytonemin greater than chlorophyll *a* particularly on the 1$^{st}$ of June (Fig S3). This indicates greater scytonemin production compared to biomass, which is likely a result of UVR exposure. Thus, the HPLC data is likely capturing a change in functioning of these cyanobacterial groups, quantifying the production of this UV absorbing pigment as a response to bright sunshine, rather than quantifying the colonial cyanobacterial community biomass as a whole unlike the FCM data. The presence of this pigment not only provides insight into the changing light regimes of the River Thames but highlights the ability of cyanobacteria to outcompete other taxa who do not produce UV-screening compounds and are thus susceptible

to the damaging and inhibiting effects of UV-radiation on growth and photosynthesis (Rozema et al., 2002).

Colonial cyanobacteria were not captured by the FCM cyanobacteria counts which could be due to larger colonies not being detected by the FCM. For instance, *Microcystis* spp. can easily reach >20µm under nutrient-rich, high light and temperature conditions present in the Thames in summer (Reynolds et al., 1981), whilst the four cyanobacterial FCM-derived groups only count cells between 5-12µm or up to 20µm in the case of the *Microcystis*-like cyanobacteria group (Read et al., 2014) and could thus be missing the larger colonial forms.

Secondly, cryptophytes and cyanobacteria contain two phycobiliproteins: phycoerythyrin and phycocyanin used by FCM as a diagnostic of cyanobacteria and cryptophytes due to their fluorescence spectral signatures (Yentsch and Yentsch, 1979). Two groups of cryptophytes were identified by FCM with high phycoerythyrin fluorescence whose gated areas matched a single species *Cryptomonas curvata* during method calibration. A single diagnostic culture was also used for the *Synechococcus*- and *Microcystis*-like cyanobacteria (Read et al., 2014). This method requires further species calibration to appreciate the fluorescence characteristics of other species. Coupled with the highest value of 4.67 nmole $l^{-1}$ of the pigment alloxanthin (cryptophytes) on the 20th July (Supplementary Fig. 1) and the mismatch between the dates of the highest cryptophyte FCM counts (>20000 cells $ml^{-1}$) of the 8th to the 27th April, suggests these groupings may overlook the abundance of dominant cryptophyte taxa later in the year. The cell sizes of the FCM cryptophyte groupings are between 10-50 µm and could be capturing the larger colonial cyanobacteria mentioned above, whilst the high phycocyanin fluorescence of *Microcystis*-like cyanobacteria may be attributed to one of the FCM cryptophyte groups (Read et al., 2014). However, the light microscopy images indicated the presence of cryptophytes from the 27th April onwards although they were more abundant in the sample taken on the 20th July, matching the peak in the cryptophyte pigment alloxanthin (Fig 3; 5). Further calibration of the flow cytometry methodology with a wider range of pure cultures known to be present in the River Thames would increase confidence with phytoplankton group characterisation and increase insights into phytoplankton succession.

### 4.3 Do we get improved understanding of community structure / biomass by using multiple characterisation approaches?

This study has shown that monitoring of chlorophyll *a* by spectrophotometry (or use of within-river chlorophyll fluorescence monitors) provided a simple and effective indicator of river phytoplankton biomass, as the majority of the biomass consisted of chlorophyll *a*-containing diatoms and chlorophytes. FCM and HPLC give rapid quantification of cell abundance and an indication of biomass respectively, and when used together, provide a more coherent and insightful description of the phytoplankton community dynamics. The combination of these two techniques also infer changes in the amounts of pigment per cell, and how these change through the annual cycle in response to environmental drivers. In addition, the ability of HPLC to detect degradation products such as diatoxanthin and pheophytin *a* for diadinoxanthin and chlorophyll *a* respectively, provide added detail about the "health" of the algal community, and could be complemented by microscopy which can provide further detail into cell condition. However, whilst HPLC and FCM cannot specifically detect detrital matter which may include dead algal cells, FCM can also enumerate bacterioplankton whose community structure changes in relation to the nature of photosynthetically-derived carbon sources. This information is useful in providing further insights into autotrophic and heterotrophic production and river metabolism (Kirchman et al., 1991).

These complementary techniques also provide information on specific plankton groups not covered by the other. For instance, HPLC pigment analysis is able to estimate dinoflagellate biomass by measuring peridinin concentrations, whereas FCM is able to distinguish between four distinct cyanobacterial groups and different size classes of chlorophytes. The FCM methodology used in this study is unable to distinguish between diatoms and large chlorophytes, but when used alongside HPLC and dissolved silicon analysis in this study, provides the supporting evidence to indicate that the FCM diatoms / large chlorophyte group consists predominantly of diatoms in the River Thames.

Undertaking traditional light microscopy analysis is time-consuming, requiring sample digestion and slide preparation stages, followed by detailed identification (taking approximately 4 h per sample for the identification stage alone) which requires highly skilled and experienced technicians. This makes the technique costly and slow to produce results. In contrast, the HPLC methodology used in this study is able to prepare and analyse a batch of ca. 24 samples within 48 h. FCM requires minimal sample preparation, and could process up to 80 samples per day. However, light microscopy remains the most effective way to get data to species level, provides information on other important phytoplankton groups (such as crysophytes) and planktonic grazers (ciliates / zooplankton) that are not captured by either FCM or HPLC. If microscopy was to be used in combination with HPLC and FCM techniques, it can be targeted at critical times in the phytoplankton succession, based on rapid, high-throughput FCM and HPLC data (i.e. to capture pigment and cell abundance peaks; periods of rapid shifts in community structure; periods when new phytoplankton groups appear). This approach would make light microscopy much more cost effective and powerful as a technique, and would further enhance the high-frequency data produced by FCM and HPLC.

# 5 Conclusions

The use of FCM and HPLC pigment analysis in combination, offers rapid and cost-effective phytoplankton characterisation through estimates of biomass and cell counts of distinct algal groups. These techniques can be used to better target the use of algal identification by microscopy, and so could greatly reduce regulatory monitoring costs, and provide the higher resolution data that are required to improve our present system understanding.

Previous studies have already shown that rapid and complex dynamics of phytoplankton biomass and community structure can occur at a sub-daily timescales (Pomati et al., 2011; Bowes et al., 2016; Rode et al., 2016). To adequately capture these, and gain improved system understanding, it is vital that these dynamics are observed both in greater depth (in terms of characterising as many components of the plankton community as possible) and at higher temporal frequency. The development and application of techniques such as FCM and HPLC pigment analyses offer the opportunity to generate higher-frequency community assemblage data, due to the rapidity and ease of analysis, and the relatively low cost. As FCM and HPLC are both instrument-based techniques requiring minimal staff interactions to perform the analysis stage, they offer the exciting possibility of fully-automated, real-time monitoring of river phytoplankton communities at high frequency timescales. Such data, in combination with physical and chemical river monitoring at a similar frequency, would provide the knowledge-base required to understand the multiple-stressor controls on phytoplankton community succession required to improve management of river catchments to tackle eutrophication and future climate variability.

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Figure 3. Indicators of diatom bloom. (a) Diatom cell abundance per ml (enumerated by FCM) and accompanying dissolved silicon concentration decrease. (b) Concentrations of two main diatom-specific pigments, measured by HPLC.

Figure 4. (a) Cyanobacteria cell abundances of the four cyanobacterial groups identified by flow cytometry. (b) Cyanobacterial pigment concentrations by HPLC. (c) Scytonemin (a UV-absorbing cyanobacterial pigment) plotted against daily sunshine duration from the CEH meteorological station at Wallingford, measured using a Campbell-Stokes sunshine recorder.

Figure 5. (a) Representative light microscopy images of the phytoplankton community taken at the six time points through the Thames chlorophyll *a* bloom, 2015. pd = pennate diatoms; cd = centric diatoms; cr = cryptophytes; cl = chlorophytes; ci = ciliates, di = dinoflagellates. The time of sampling for each image is indicated in the upper graph, alongside water temperature and river flow.

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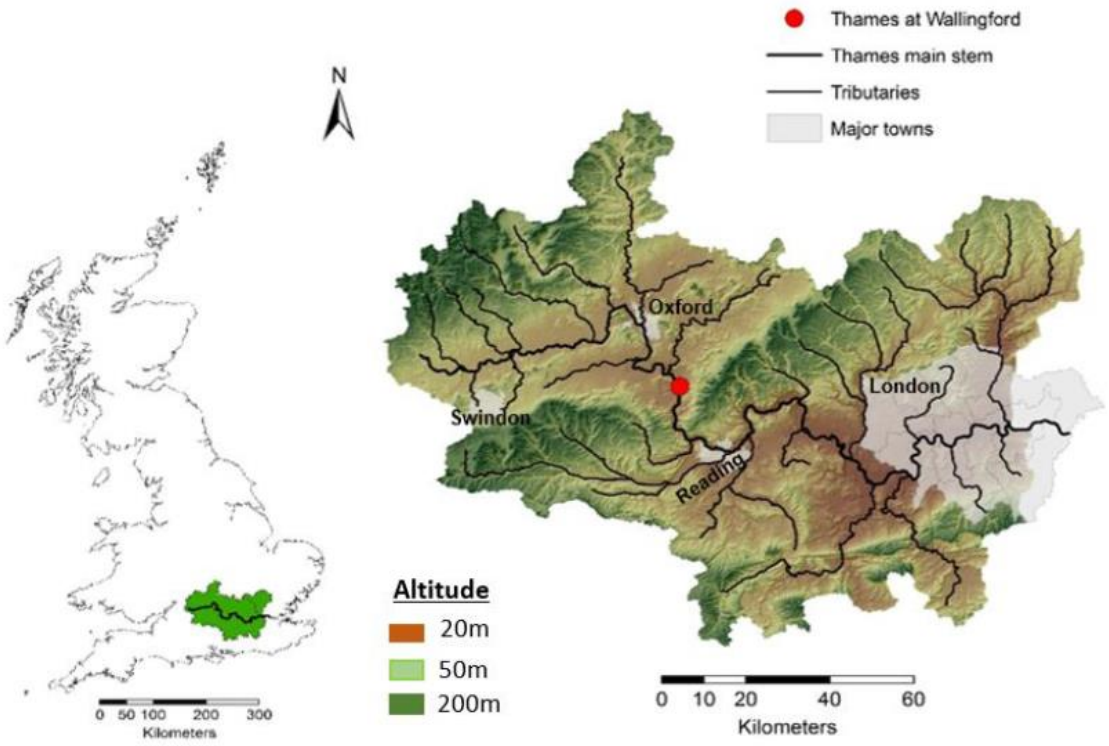
Thames at Wallingford
Thames main stem
Tributaries
Major towns
N
Oxford
London
Swindon
Reading
Altitude
20m
50m
200m
0 10 20 40 60
Kilometers
0 50 100 200 300
Kilometers

Fig 1.

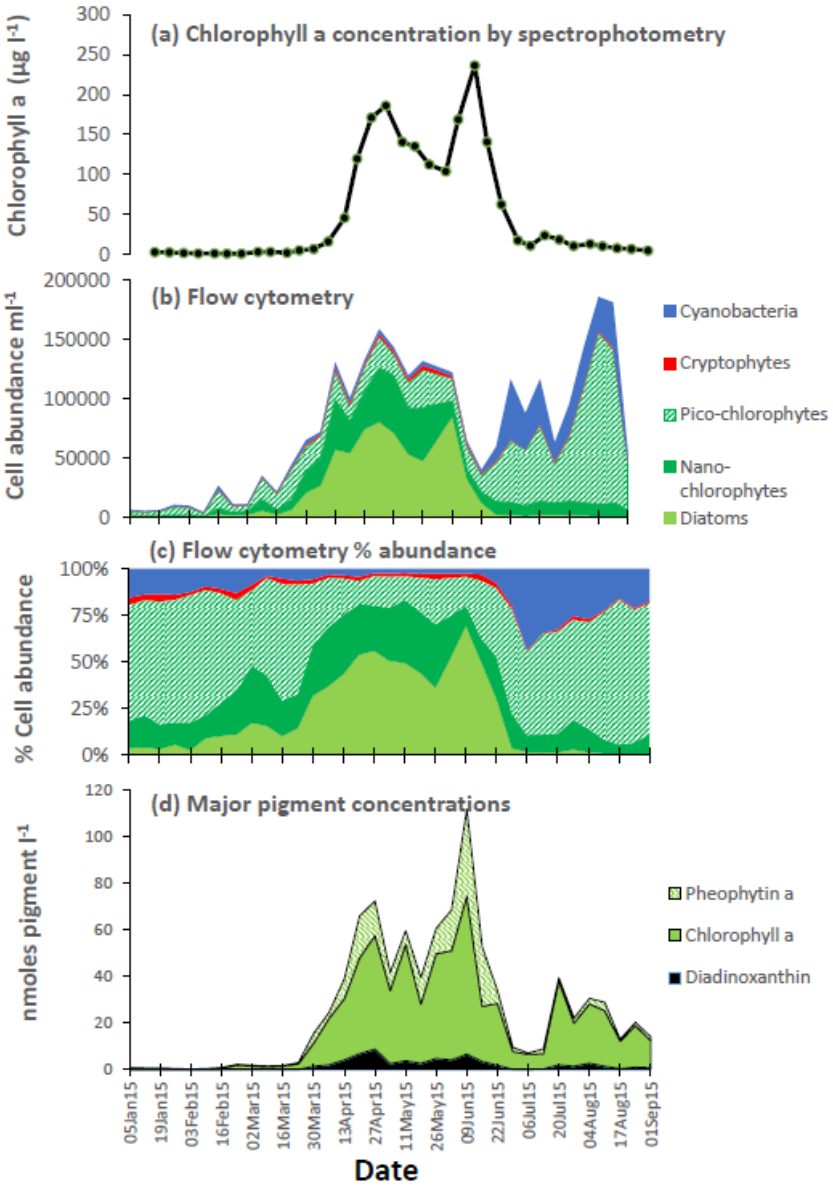
(a) Chlorophyll a concentration by spectrophotometry
Chlorophyll a (µg l-1)
300
250
200
150
100
50
0
(b) Flow cytometry
Cell abundance ml-1
200000
150000
100000
50000
0
Cyanobacteria
Cryptophytes
Pico-chlorophytes
Nano-chlorophytes
Diatoms
(c) Flow cytometry % abundance
% Cell abundance
100%
75%
50%
25%
0%
(d) Major pigment concentrations
nmoles pigment l-1
120
100
80
60
40
20
0
Pheophytin a
Chlorophyll a
Diadinoxanthin
05Jan15
19Jan15
03Feb15
16Feb15
02Mar15
16Mar15
30Mar15
13Apr15
27Apr15
11May15
26May15
09Jun15
22Jun15
06Jul15
20Jul15
04Aug15
17Aug15
01Sep15
Date

Fig 2.

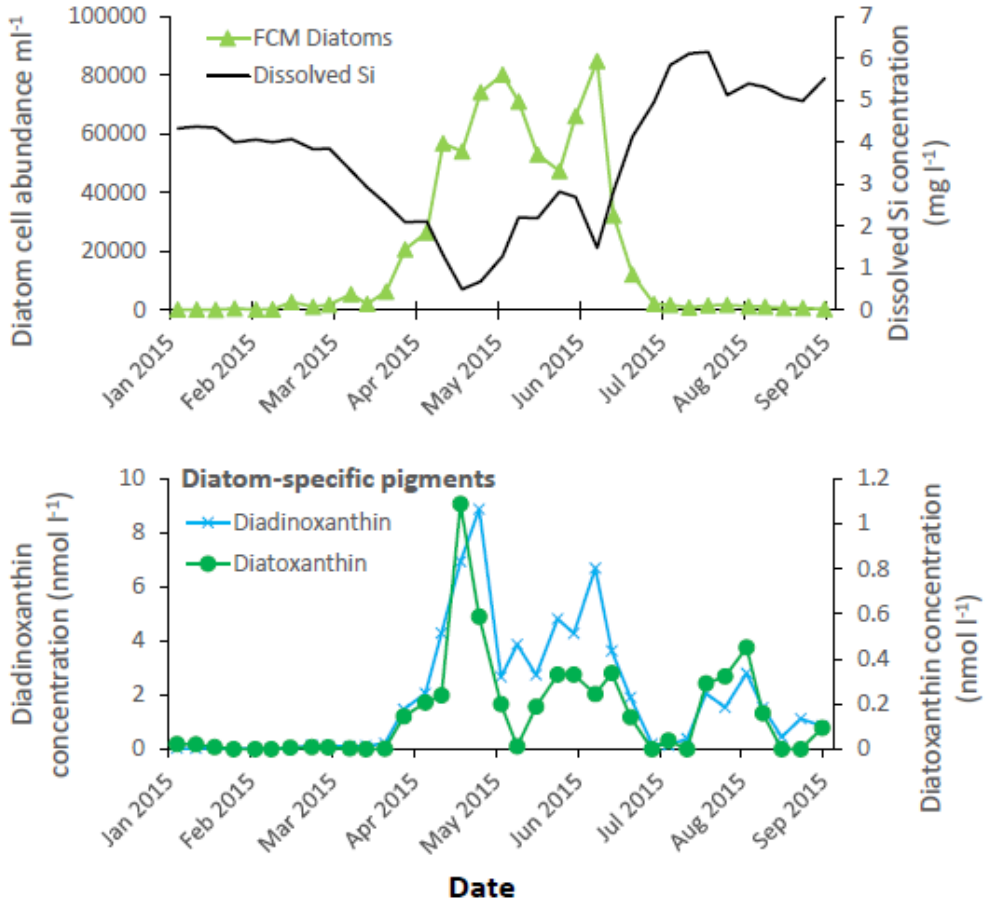
Diatom cell abundance ml-1
100000
80000
60000
40000
20000
0
FCM Diatoms
Dissolved Si
7
6
5
4
3
2
1
0
Dissolved Si concentration (mg l-1)
Jan 2015
Feb 2015
Mar 2015
Apr 2015
May 2015
Jun 2015
Jul 2015
Aug 2015
Sep 2015
Diatom-specific pigments
Diadinoxanthin concentration (nmol l-1)
10
8
6
4
2
0
Diadinoxanthin
Diatoxanthin
1.2
1
0.8
0.6
0.4
0.2
0
Diatoxanthin concentration (nmol l-1)
Date

Fig 3.

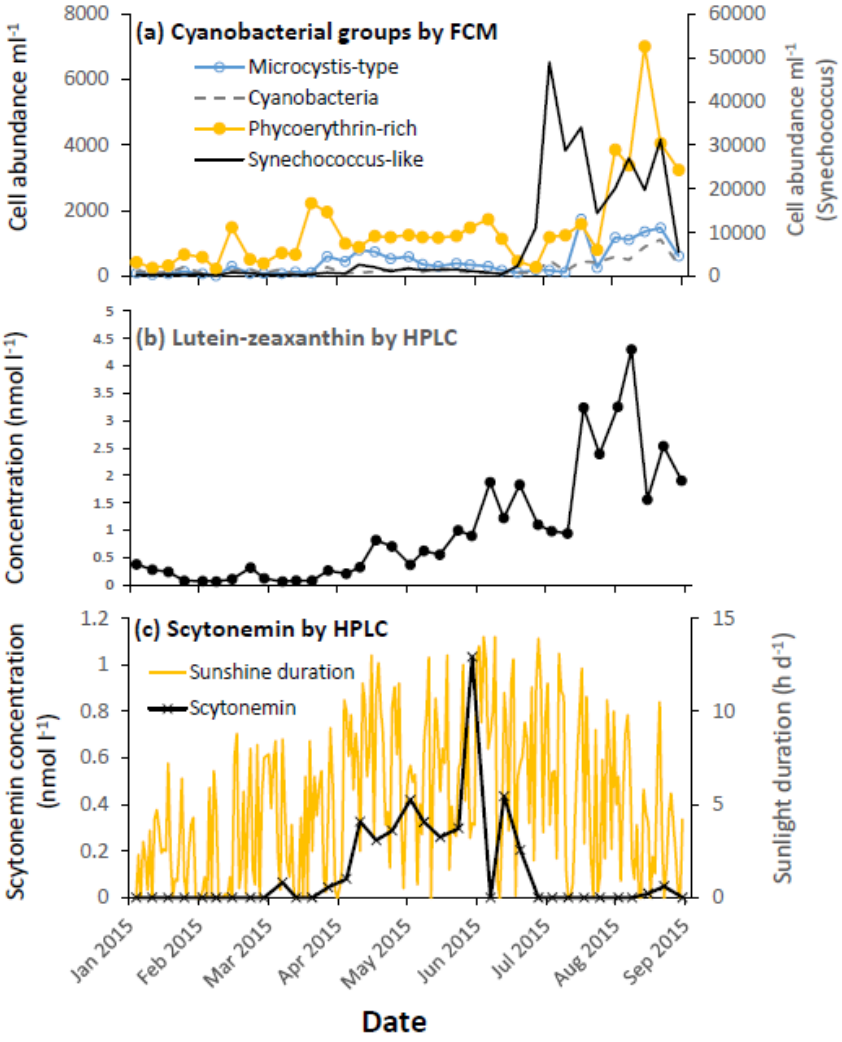
(a) Cyanobacterial groups by FCM
Microcystis-type
Cyanobacteria
Phycoerythrin-rich
Synechococcus-like
Cell abundance ml-1
Cell abundance ml-1 (Synechococcus)
(b) Lutein-zeaxanthin by HPLC
Concentration (nmol l-1)
(c) Scytonemin by HPLC
Sunshine duration
Scytonemin
Scytonemin concentration (nmol l-1)
Sunlight duration (h d-1)
Jan 2015
Feb 2015
Mar 2015
Apr 2015
May 2015
Jun 2015
Jul 2015
Aug 2015
Sep 2015
Date

Figure 4.

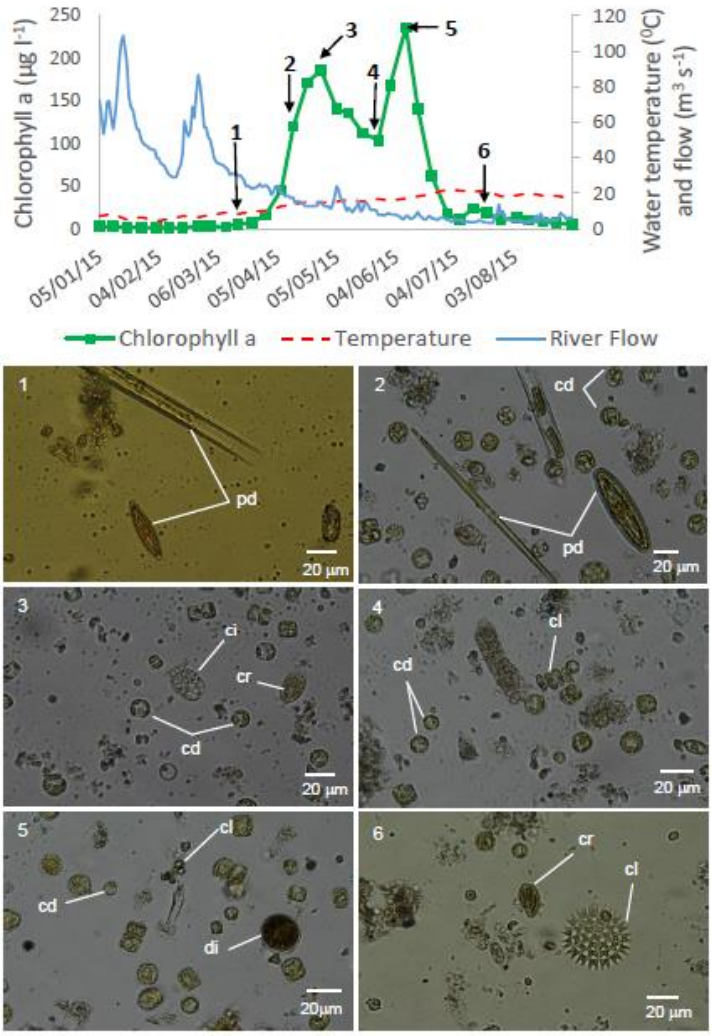
Chlorophyll a (µg l-1)
Water temperature (0C) and flow (m3 s-1)
250
200
150
100
50
0
120
100
80
60
40
20
0
1
2
3
4
5
6
05/01/15
04/02/15
06/03/15
05/04/15
05/05/15
04/06/15
04/07/15
03/08/15
Chlorophyll a
Temperature
River Flow
pd
cd
ci
cr
cl
di
20 µm
20µm

Fig 5.

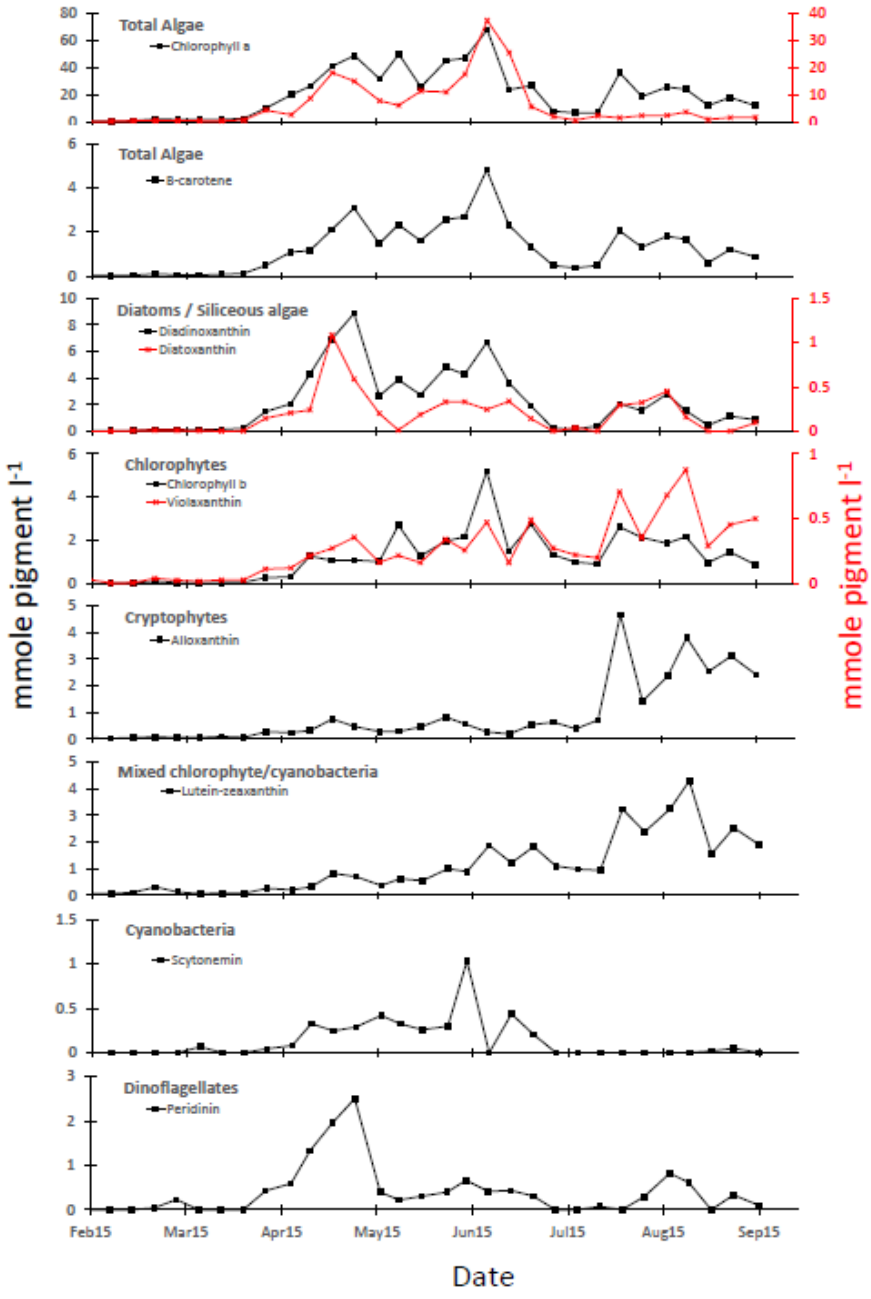
Total Algae
Chlorophyll a
Total Algae
B-carotene
Diatoms / Siliceous algae
Diadinoxanthin
Diatoxanthin
Chlorophytes
Chlorophyll b
Violaxanthin
Cryptophytes
Alloxanthin
Mixed chlorophyte/cyanobacteria
Lutein-zeaxanthin
Cyanobacteria
Scytonemin
Dinoflagellates
Peridinin
mmole pigment l-1
mmole pigment l-1
Feb15
Mar15
Apr15
May15
Jun15
Jul15
Aug15
Sep15
Date

Figure S1.

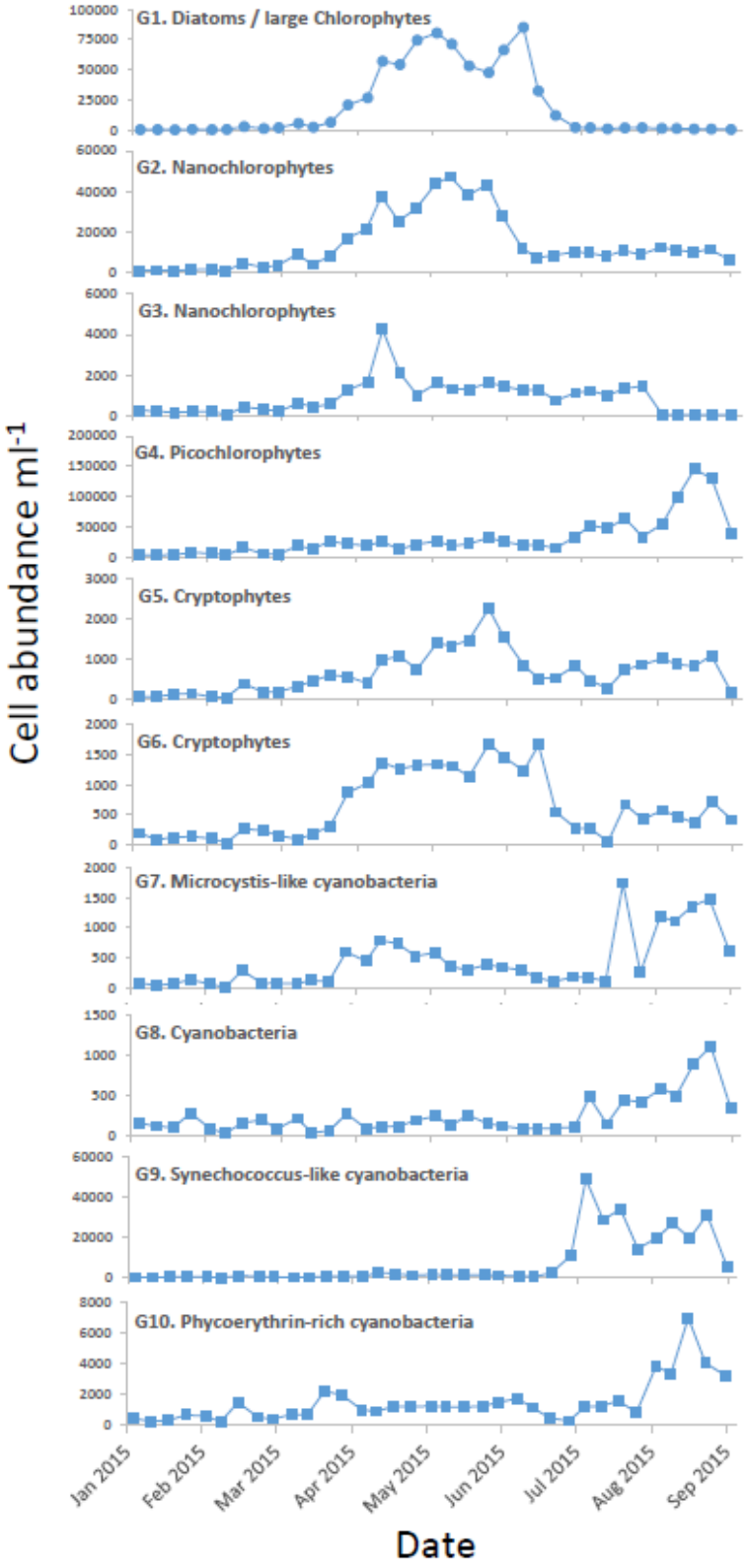
Cell abundance ml-1
G1. Diatoms / large Chlorophytes
G2. Nanochlorophytes
G3. Nanochlorophytes
G4. Picochlorophytes
G5. Cryptophytes
G6. Cryptophytes
G7. Microcystis-like cyanobacteria
G8. Cyanobacteria
G9. Synechococcus-like cyanobacteria
G10. Phycoerythrin-rich cyanobacteria
Jan 2015
Feb 2015
Mar 2015
Apr 2015
May 2015
Jun 2015
Jul 2015
Aug 2015
Sep 2015
Date

Figure S2.

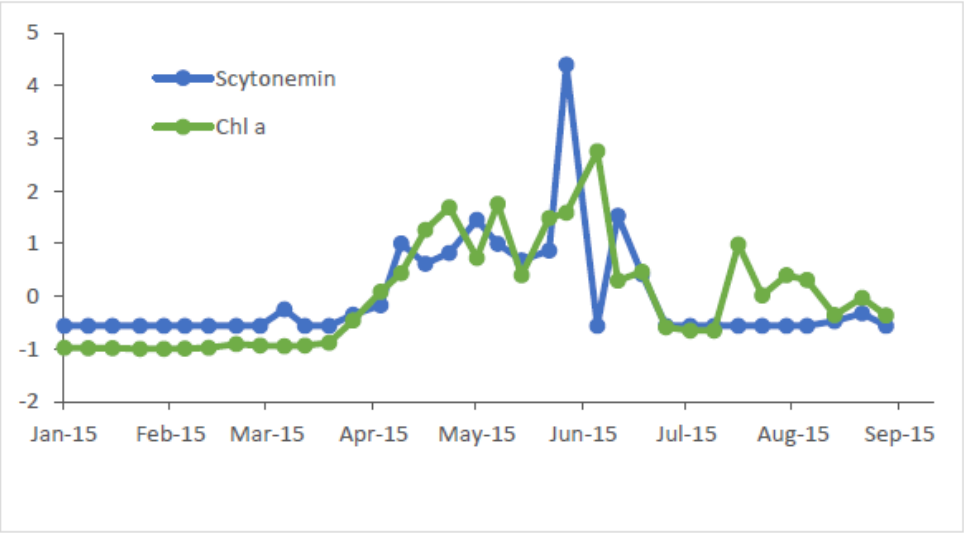
5
4
3
2
1
0
-1
-2
Scytonemin
Chl a
Jan-15
Feb-15
Mar-15
Apr-15
May-15
Jun-15
Jul-15
Aug-15
Sep-15

Fig S3.