Investigating the use of variable fluorescence methods to detect phytoplankton nutrient deficiency

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Variable fluorescence of chlorophyll a (Fv/Fm), measured by pulse amplitude modulated (PAM) fluorometers, is an attractive target for phytoplankton-related water quality management. Lowered Fv/Fm is believed to reflect the magnitude of nutrient sufficiency or deficiency in phytoplankton. This rapid and specific metric is relevant to Lake Erie, which often experiences problematic Cyanobacteria blooms. It is unknown whether PAMs reliably measure phytoplankton nutrient status or if different PAMs provide comparable results. Water samples collected from Lake Erie and two Lake Ontario sites in July and September 2011 were analysed using alkaline phosphatase assay (APA), P-debt, and N-debt to quantify phytoplankton nutrient status and with three different PAM models (PhytoPAM, WaterPAM and DivingPAM) to determine Fv/Fm. The Lake Ontario, Lake Erie East and Central Basin sites were all N- and P-deficient in July, but only the East and Central Basin and one Lake Ontario site were P-deficient in September. The West Basin sites were P-deficient in July and one West Basin site and a river site were N-deficient in September. Between-instrument Fv/Fm comparisons did not show the expected 1:1 relationship. Fv/Fm from the PhytoPAM and WaterPAM were well-correlated with each other but not with nutrient deficiency. DivingPAM Fv/Fm did not correlate with the other PAM models, but correlated with P-deficiency. Spectral PAM fluorometers (PhytoPAM) can potentially resolve Fv/Fm down to phytoplankton group by additionally measuring accessory pigment fluorescence. The nutrient-induced fluorescent transient (NIFT) is the observation that Fv/Fm drops immediately and recovers when the limiting nutrient is reintroduced to nutrient-starved phytoplankton. A controlled laboratory experiment was conducted on a 2x2 factorial mixture design of P-deficient and P-sufficient *Asterionella formosa* and *Microcystis aeruginosa* cultures. Patterns consistent with published reports of NIFT were observed for P-deficient *M. aeruginosa* in mixtures; the pattern for *A. formosa* was less clear. This thesis showed

that Fv/Fm by itself was not a reliable metric of N or P deficiency and care must be taken when interpreting results obtained by different PAM fluorometers. NIFT analysis using spectral PAM fluorometers may be able to discriminate P-deficiency in *M. aeruginosa*, and possibly other Cyanobacteria, in mixed communities.

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Chapter 1

General Introduction

Phytoplankton is an important component of, and a base for, the lacustrine food web. Understanding the forces that shape their diversity, abundance and biomass is necessary in order for managers to make informed decisions and because not all lake systems are alike in this regard. Large lakes, such as the Laurentian Great Lakes have distinctive differences compared to smaller lakes, including their large volume and comparatively long residence time (Kalff 2002), ability to capture and retain heat (Fee et al. 1996), and internal currents that are governed by coriolis motion (Fee et al. 1996). Unlike flowing waters or smaller lakes, the nutrient regimes in these lakes will be different. As a result of these and other large lake features, their phytoplankton communities will be influenced in ways that may differ compared to communities in smaller lakes.

1.1 Lake Erie

Lake Erie is the smallest of the Laurentian Great Lakes by volume (Schertzer 1999) and is subdivided into three main basins: West, Central and East. The West Basin is the smallest and shallowest of the three basins, and receives a combined water discharge of approximately 5400 m^3s^{-1} from the Detroit and Maumee Rivers (Rasul et al. 1999) and all the nutrient loading associated with them (Agricultural Nutrients and Water Quality Working Group). The West Basin has been the site of large late summer/early fall *Microcystis* spp. blooms in recent years that represent a major challenge to environmental management. The Central and East Basins are larger and deeper, and receive a total water discharge less than $300m^3s^{-1}$ from their tributaries (Rasul et al. 1999). They are classified as meso-oligotrophic but there is persistent annual occurrence of hypolimnetic hypoxia in the Central Basin that is

exacerbated by increased phytoplankton productivity (Charlton et al. 1999, Munawar and Munawar 1999) and presents another management challenge.

As recently as the late 1960's, Lake Erie was considered a "dead lake" due to high algal biomass, low hypolimnetic oxygen and episodic fish kills (Charlton et al. 1999). The nitrogen-fixing cyanobacterium *Aphanazomenon* was at times the dominant phytoplankton group along with other filamentous nitrogen-fixing Cyanobacteria. In the 1970's, following the bi-national Great Lakes Water Quality Agreement (GLWQA), phosphorus (P)-loading reductions to the lake were implemented (Charlton et al. 1999). P-loading targets were met, dissolved oxygen levels increased (Munawar and Munawar 1999) and *Aphanizomenon* was no longer a dominant taxon. However, the accidental introduction and subsequent spread of *Dreissena polymorpha* and *D. rostriformis bugensis* (zebra and quagga mussels, respectively) has been linked to changes in internal cycling of nutrients, including a re-directing of nutrients toward the nearshore areas (Hecky et al. 2004). Concurrently, concentrations of certain nitrogen species, notably nitrate (NO_3^-) have been increasing in the lake (Charlton and Milne 2004, North et al. 2007), which should have the effect of making phosphorus relatively more scarce, and thus affecting a shift in phytoplankton community structure to ones dominated by those that require relatively little P compared to N (Harris 1986). Problematic *Microcystis* blooms emerged following P loading reductions under the guidelines of the GLWQA and the near-elimination of other Cyanobacterial blooms. There is evidence that P-loading has actually increased in recent years, however, and this may be contributing to *Microcystis*' dominance. As such, there is a push by agencies monitoring water quality to understand the nutrient dynamics of Lake Erie in order to set guidelines and develop possible remediation strategies.

1.2 Phytoplankton

Phytoplankton are photosynthetic microorganisms suspended in the water column that have arisen from multiple phyletic lineages; some groups are prokyarotic, such as the Cyanobacteria, while others are eukarotic, such as the Chlorophyta and Bacillariophyta (Harris 1986). All phytoplankton require nutrients and light to grow optimally, and of the required nutrients, nitrogen (N) and phosphorus (P) are required in relatively large amounts and can commonly be limiting. In freshwaters, it is generally accepted that P is the nutrient that ultimately limits total phytoplankton biomass and affects community composition (e.g. Schindler 1977), and variations in the severity of P limitation can influence phytoplankton community composition. The famous whole-lake enrichment studies in Lake 226 in the Experimental Lakes Area (Schindler 1971) provide powerful evidence of this. It is especially important to note that potentially problematic bloom-forming Cyanobacteria have been correlated with increased P concentrations in natural freshwater systems (Teubner and Dokulil 2002, Conroy et al. 2005).

Phytoplankton is an important base to the aquatic food web, and their abundance and composition can affect the composition of the food web and the stability of its linkages (Harris 1986). Invertebrates consume phytoplankton, are in turn consumed by larger invertebrate and vertebrate predators, which are themselves eaten by top predaceous fish (Carpenter et al. 1985). However, not all phytoplankton are nutritionally equal. Many zooplankton are constrained by their size or feeding mechanisms to a certain size class of phytoplankton (Harris 1986), as are many zoobenthic invertebrates (Harris 1986), including dreissenid mussels (Sprung and Rose 1988). Thus, phytoplankton that are individually too large, or that form large filaments or colonies, are less likely to be consumed (Fulton and Paerl 1987 and references therein). Some Cyanobaceria, including some *Anabaena* and *Planktothrix* species, also produce toxins (Sivonen and Jones 1999), which might also exclude them from zooplankton predation (Harris 1986). However, the main contemporary concern for Lake Erie water quality management is *Microcystis*

aeruginosa, of which some strains produce the hepatotoxin microcystin in varying concentrations per cell (Rinta-Kanto et al. 2009). A bloom of this particular taxon can be inedible for a number of reasons. This may include feeding exclusion by size (Vanni 1987) or avoidance of toxins (Fulton and Paerl 1987 and references therein). Even non-toxin producing blooms may still negatively impact the aquatic system. At cessation of a bloom, large-scale die-off of the cells results in their sinking to and decomposing on the lake bottom. Such processes consume oxygen, leading to a hypoxic or even anoxic hypolimnion, often the site of summer cold-water fish refugia; this may result in large-scale fish kills (Charlton et al. 1999).

1.3 Nutrients

Phytoplankton require many nutrients in varying concentrations, in addition to photosynthetically active radiation (PAR) to function. However, nitrogen and phosphorus are the major nutrients of management interest, and so only the relevant species of these two nutrients, in the context of eutrophication management in Lake Erie, are discussed here. There are many other nutrients that are important for optimal phytoplankton growth, but are not central to this thesis, as these are not nutrients that are most likely to become limiting to phytoplankton first. Nitrogen is an important component of proteins, forming an integral part of the N-C-C amino acid backbone (Falkowski and Raven 2007). Proteins are required for proper cell and organism functioning, and thus, relatively large amounts of this nutrient are required to meet this physiological need. Phosphorus is the major constituent of nucleic acids (i.e. RNA, DNA, and ribosomes) in cells (Falkowski and Raven 2007). Sterner and Elser (2002) determined that the major pool of phosphorus in cells is in the ribosomes, and thus, as with nitrogen, relatively large amounts of phosphorus are needed by cells.

Nitrogen (N) can take the aqueous form of nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_4^+), or gaseous atmospheric di-nitrogen (N_2) (Kalff 2002). It can be introduced into the system through the atmosphere, terrestrial and riverine sources, or through nitrogen cycling within the lake (Kalff 2002).

However, only N-fixing species of Cyanobacteria, are capable of transforming gaseous N_2 into NO_3^- and ultimately into NH_4^+, through specialized biochemical pathways (Kalff 2002). *Microcystis*, conversely, is not an N-fixer, and thus its dominance cannot be attributed to this particular physiological feature. NH_4^+ is the easiest form of combined nitrogen to assimilate, as it is already in the reduced form necessary for incorporation into amino acids and proteins (Falkowski and Raven 2007). NO_2^- is a relatively rare species of N in most oxic surface waters, and will not be commented on. NO_3^- is by far the most common form of dissolved inorganic combined N in freshwater systems, and in Lake Erie, relatively high concentrations of this species are found (Charlton and Milne 2004, North et al. 2007). However, despite its wide availability, its uptake may be constrained by the shortage of iron (Fe) (e.g. Twiss et al. 2005; North et al. 2007 and references therein) and light (Leonardos and Geider 2004). The iron and reductant (i.e. NADPH) generated by photosynthesis are both required within the cell to reduce NO_3^- to NH_4^+ and allow for biosynthesis of N-containing molecules (Falkowski and Raven 2007). Nitrogen deficiency, while not generally perceived as the limiting factor for phytoplankton biomass from a management perspective, is known to occur quite commonly and certainly has potential to influence short term phytoplankton dynamics and composition (e.g. Sterner 2008). There is evidence for phytoplankton N-deficiency in Lake Erie, despite its typically high nitrate concentrations (during spring and summer, North et al. 2007, Rattan et al. 2012).

Phosphorus (P) commonly can be found in inorganic dissolved phosphorus (PO_4^-), or bound in either organic (i.e. phospholipids) or inorganic (i.e. insoluble phosphate-iron flocs) compounds (Kalff 2002). Unlike N, phosphorus does not have a gaseous phase, so introduction to aquatic systems must ultimately be of terrestrial (external) or within-lake (internal) origin (Kalff 2002). Inorganic dissolved P is also known as soluble reactive phosphorus (SRP), and is the easiest form of P for uptake and assimilation for phytoplankton (Harris 1986). P may also occur in an insoluble form (e.g. flocs or apatite phosphorus species), which make it unavailable for use by phytoplankton (Kalff 2002). However,

dissolved organic phosphorus (DOP) wherein the PO_4^- group is covalently attached to another molecule, represents another important species of P (Nalewajko and Lean 1980, Harris 1986), such as a lipid chain (phospholipids). In order for the P to be of use to the phytoplankton cell, the PO_4 group must be cleaved from the rest of the molecule (Beardall et al. 2001b). Possibly because it lacks an atmospheric source, and because there are fewer inorganic chemical species that phytoplankton can utilize, P is the nutrient most limiting to phytoplankton growth. Most temperate freshwater systems are understood to be limited primarily by P (Sterner 2008), rather than N, although there are some exceptions to this generalization (e.g. Elser et al. 2009).

Phytoplankton can experience nutrient deficiency as either nutrient starvation or nutrient limitation. Nutrient starvation is the abrupt cessation of nutrient provision to the phytoplankton, such that the growth rate is uncoupled from the cells' metabolism (Parkhill et al. 2001, Wood et al. 2005). Between generations, the cellular quotas will differ (MacIntyre and Cullen 2005). This is often the case when the nutrient supply becomes unavailable, such as with the onset of stratification or at the termination of an algal bloom. Nutrient limitation, conversely, refers to phytoplankton growth of modified rate but with cellular quotas eventually remaining the same between generations in proportion to the provision of the limiting nutrient (MacIntyre and Cullen 2005). In nature, nutrient deficiency is often somewhere between starvation and limitation (Arrigo 2005).

There are many chemical tools available to determine nutrient concentrations in natural waters, which may be used to infer whether phytoplankton might be in need of nutrients or not. A variety of assays can be used to estimate phytoplankton physiological need for these nutrients. A few methods relevant to the studies presented in this thesis, as well as their advantages and disadvantages, are discussed briefly, but this is not a comprehensive list. The ambient concentrations of the various inorganic N species and the dissolved and suspended P species can be measured using well-known fluorescent (e.g. Holmes et al. 1999) and spectrophotometric (e.g. Murphy and Riley 1962) methods.

Lake Erie TP has previously been measured at concentrations of 0.27-0.57μM (Guildford et al. 2005), which falls within the range considered to be oligotrophic to mesotrophic (Charlton et al. 1999) and may be a low enough concentration to limit the growth of some phytoplankton with high P-requirements (e.g. Tilman et al. 1982, Harris 1986). However, ambient concentration is not necessarily the most informative parameter. Soluble reactive phosphorus (SRP) is sometimes over-estimated, especially in oligotrophic water (Harris 1986). This may be as a result of improper sampling handling and storage, including whether the samples are frozen (Jarvie et al. 2002) or because dissolved organic phosphorus (DOP) is inevitably part of the SRP measurement (Tarapchak et al. 1982, Harris 1986). Available phosphorus may actually be at more limiting concentrations than previously believed, although phytoplankton can use much lower levels of PO_4 than previously thought (Hudson et al. 2000). NO_3^- could also occur in high concentrations, but would be otherwise unavailable to phytoplankton if iron (Fe) is unavailable (e.g. Twiss et al. 2005, North et al. 2007). Another parameter of interest is nutrient ratios. The molecular ratio of C:N:P of approximately 106:16:1 has been found in seawater and was thought to reflect the general ratios of these elements in phytoplankton (Redfield et al. 1963, Sterner et al. 2008, Arrigo 2005). However, this ratio is variable and can differ between different systems because different phytoplankton may require N and P in ratios that deviate from this accepted ratio and from another phytoplankton group (Guildford and Heckey 2000, Arrigo 2005, Finkel et al. 2008, Sterner et al. 2008).

Physiology-based methods, that use process measurements (e.g. rates of enzyme catalysis) to infer condition, can be an improvement from these broad nutrient-requirement paradigms. Nutrient debts quantify physiological need based on the assumption that nutrient-deficient phytoplankton will engage in an increased rate (luxury) of uptake of the growth-limiting nutrient when it is provided. The greater the uptake rate for a given time period, the greater the physiological need for the nutrient of interest (Goldman and Gilbert 1983; Parslow et al. 1984; Cochlan and Harrison 1991; Beardall et al. 2001b). This can be done for both N and P deficiency. However, this sort of assay may be confounded by "bottle

effects" due to long (e.g. ≥24h) incubations (Beardall et al. 2001b). Like nutrient concentration measurements, they may be affected if zooplankton are not removed from the samples prior to incubation, as these organisms represent a nutrient recycling pathway that cannot be characterized concurrently (Jarvie et al. 2002) and also contain nutrients that could affect estimates of particulate concentrations. Thus, short-term incubations are best; however, with this method, a short incubation may not yield enough information to determine uptake rates of limiting nutrients. Another method nutrient deficiency can be characterized is through the use of inducible enzymes related to nutrient utilization, such as alkaline phosphatase (AP) for P-deficiency. AP cleaves the phosphate group from organic molecules that contain phosphate mono-ester bonds (Gonzalez-Gil et al. 1998), although Cyanobacteria and other taxa may also express phosphomonoesterases and phosphodiesterases (Whitton et al. 1991). Common alkaline phosphatase assays (APA) quantify deficiency based on the rate of PO_4 cleavage from a substrate molecule that becomes highly fluorescent when hydrolyzed (Huang et al. 1992, Gonzalez-Gil et al. 1998). APA provides a quicker measure than nutrient debts, typically taking <2h. However, heterotrophic bacteria also produce AP, and the protocol requires incubation at a physiologically unrealistic temperature (35°C). Enzymes such as Glutamate Synthetase (GS) (Everest et al. 1986) or Nitrate Reductase (Wynne and Berman 1990; Beardall et al. 2001b; Flynn et al. 2010) can be used for determining N-deficiency, but because N-deficiency could be relieved by either NO_3^- or NH_4^+, this particular deficiency is less easy to quantify. Bioreporters have been devised to assay expression of enzymes including AP and GS in selected Cyanobacterial strains, and can provide a quick and sensitive bioassay of nutrient availability or deficiency to the test organism (Gonzalez-Gil et al. 1998, Everest et al. 1986).

There are many other methods that can be used, though they are not discussed here in the interest of brevity and immediate relevance to this project. None of these methods, however, can act as the sole standard for assessing nutrient limitation. All are subject to uncertainties of interpretation in natural community studies. Concurrent and multiple measures are always advisable. This increases the logistical

and analytical demands, however, and may not be a viable strategy for workers in the field, especially if a large numbers of samples are to be taken, as is typical for spatial surveys in large lakes. It is known that photosynthetic activity and chlorophyll fluorescence can change in response to nutrient deficiency and can be assayed very rapidly and inexpensively. This is the basis of the proposal for the use of variable chlorophyll fluorescence as a means to detect nutrient deficiency in natural phytoplankton communities.

1.4 Photosynthesis

Phytoplankton are able to utilize photosynthetically active radiation (PAR), electromagnetic radiation between ~400-700nm. All photosynthetic organisms contain chlorophyll a (chl a), which has two absorption peaks around 430nm and 680nm respectively (Taiz and Zeiger 2006). Other taxonomically-specific accessory pigments have absorption peaks between the absorption peaks for chl a. Cyanobacterial phycobiliproteins extend the absorption range into the 700nm range, past the absorption maximum of chl a. These accessory pigments act to harvest and channel light at wavelengths to chl a, which is embedded in the reaction centres of the two photosystems (Johnson et al. 2011, Roy et al. 2011). This increases the amount of all PAR that can be utilized by phytoplankton. Indeed, Bidigare et al (1992) found that 60-90% of all phytoplankton-intercepted PAR was absorbed through accessory pigments (Johnson et al. 2011, Roy et al. 2011). Accessory pigments become important components for channeling light to the electron transport chain and for photochemistry.

In the current context, photosynthesis will be considered synonymous to photochemistry: the capture of photons starting in Photosystem II (PSII), the subsequent transfer of electrons through the electron transport chain (ETC) and Photosystem I (PSI), ultimately creating the photosynthetic yields of oxygen (O2) and carbon products of the general form n(CH_2O) (Falkowski and Raven 2007). Briefly, the important pigment molecule associated with photosynthesis and fluorescence is the chlorophyll a (chl a) molecule associated with PSII, termed P_{680}, as this is its peak absorption wavelength (Falkowski and

Raven 2007). Incoming light of all wavelengths enters the chloroplast, and through a variety of de-excitation pathways (e.g. accessory pigments, interactions with other molecules), is absorbed by P_{680}, which results in a charge separation in this molecule. That is, the photon energy elevates an electron in the porphyrin ring of chl a to a higher energy level. De-excitation results in an electron transfer to the next component in the electron transport chain, the electron acceptor, Q_A. Oxidized P_{680} cannot remain in this state, and strips electrons from the PSII D1 protein, which in turn strips electrons from other components further upstream of PSII, ultimately oxidizing water, forming O_2 and returning P_{680} to its ground state.

QA cannot remain reduced indefinitely, and passes the electron to the plastoquinone pool and the cytochrome b_6/f complex to PSI. PSI also contains a chl a molecule with a maximum absorbance in the far red, and thus is termed P_{700}. Excitation at 700nm yields a second charge separation of P_{700}, and the electron is donated to $NADP^+$. Transfer of electrons down the chain produces an H^+ gradient, and when combined with the $NADP^+$, produced the reductant NADPH. The H^+ gradient allows ATP Synthetase to produce ATP, a source of energy in the cell. These two products are then used to fix carbon dioxide into carbon products in the Calvin-Bessham-Benson Cycle (the "dark" reactions) (Falkowski and Raven 2007).

1.5 Variable Fluorescence

Light entering the chloroplast may have a variety of fates, but photochemistry, heat and fluorescence are the only products of incoming light energy dissipation in the commonly accepted model for interpretation of variable fluorescence (Krauss and Weiss 1991). The probabilities of these three fates are thus equal 1 (that is: $\Psi_P+\Psi_D+\Psi_F=1$) and for all ranges of fluorescence, Ψ_F is proportional to Ψ_D and inversely proportional to Ψ_P (Parkhill et al. 2001, Schreiber 2004). Thus, the more likely incoming light energy is to yield carbon and/or oxygen products, the less likely it is that it will contribute to

fluorescence products, and vice versa. This model predicts that the quantum yield of photochemistry (Φp) is equal to the maximum quantum yield, Fv/Fm, the difference between the maximum fluorescence (Fm) and the minimum fluorescence (Fo) normalized to the maximum fluorescence of a dark-adapted sample. Φp is also equal to the rate-constants for photochemistry (k_p), heat (k_d) and fluorescence (k_f) (i.e. $\Phi p = Fv/Fm = (Fm-Fo)/Fm = (k_p/(k_p+k_d+k_f))$ (Falkowski and Raven 2007).

Fo is the amount of fluorescence given off when all the reaction centres of all PSII complexes are completely "open" (in a reduced state), the quenchers (Q) are oxidized and able to receive the electron generated by the charge separation of the reaction centre from incoming light energy; therefore very little is re-emitted as fluorescence, as the energy is successfully quenched and Ψp is almost equal to 1 (Parkhill et al. 2001, Falkowski and Raven 2007). Fluorescence rises progressively (with intermediate values of Ψp and Ψ_f) with increasing light intensity up to a maximum Fm, when all reaction centres are "closed" (oxidized state), Q is reduced and is unable to transfer the energy (as electrons) down the rest of the electron transport chain (Falkowski and Raven 2007). Thus, $\Psi p=0$ and $\Phi p=0$, and the energy is released in the form of maximal fluorescence (Parkhill et al. 2001).

Variable fluorescence may reflect taxonomic differences between groups (Suggett et al. 2009), as well as light history and possibly even general phytoplankton health. To date, Fv/Fm has been used to assess UV damage and recovery to phytoplankton (e.g. Harrison and Smith 2009) as well as to probe nutrient status (e.g. Parkhill et al. 2001, Beardall et al. 2001a, Beardall et al. 2001b, Holland et al. 2005, Kruskopf and Flynn 2006, among others). This characteristic of Fv/Fm may confound results, however. The difference in Fv/Fm between taxonomic groups is especially obvious when comparing Cyanobacteria (prokaryotes) with the other eukaryotic phytoplankton (e.g. Chlorophytes, diatoms, etc.). Cyanobacteria do not contain organelles, and so their photosystems and other ETC components are associated with the thylakoid membrane (Campbell et al. 1998). PSI in Cyanobacteria also receives proportionately more incoming photons than in eukaryotic cells (Campbell et al. 1998) and phycobiliproteins fluoresce in the

same range of wavelengths as chl a, which may artificially inflate values of Fo (Cambell et al. 1998). Both these factors can make Fv/Fm appear as though it is surprisingly low compared to a eukaryotic sample of similar nutrient status. When Fv/Fm is taken following dark adaption, chlororespiration and a relatively large P_{700} fluorescence signal, possibly due to a state transition to state 2, where excess light energy is channeled toward PSI, may further lower the fluorescence associated with chl a in PSII (Gutu and Kehoe 2012). The overall effect is what appears to be a lower fluorescence from PSII-associated chl a.

The type of fluorescence measuring system can have an effect on the measurements of the quantum yield of PSII. Fast repetition rate fluorometry (FRRF) is a single turnover (ST) approach. FRRF uses very short and intense flashes of light to progressively oxidize the reaction centre of PSII and reduce only QA once (Suggett et al. 2003). Pulse amplitude modulated (PAM) fluorometry is a multiple turnover (MT) approach which creates multiple charge separations and a corresponding estimate of Fm (Suggett et al. 2003). As a result, Q_A, Q_B, and plastoquinone become fully reduced (Suggett et al. 2003). Because PAM also reduces plastoquinone to plastoquinol, it tends to measure a higher level of Fm than single turnover instruments like the FRRF. This is because plastoquinone can act as an additional fluorescence quencher, but plastoquinol cannot (Suggett et al. 2003). Because Fm is higher, Fv/Fm also tends to be higher in MT instruments compared to ST instruments (Ralph and Gademann 2005). Thus, workers are aware that results derived on ST instruments are not directly comparable with results derived on MT instruments. However, to date, comparisons solely between MT instruments (or ST instruments) have not yet been done. It might stand to reason that instruments that use slight variants of the same protocol would yield similar results; however, this can at present only be assumed. This thesis makes some of the first comparisons between three pulse amplitude modulated (PAM) fluorometers, which all use a MT protocol.

1.6 Photosynthesis, Variable Fluorescence and Nutrients

Physiological lack of nitrogen and phosphorus have similar effects on photosynthesis efficiency, but these deficiencies can, and usually do, target different aspects of the photosynthesis systems. For the better-characterized N deficiency, the number of functional reaction centres decreases (Kolber et al. 1988), but their absorption cross section increases in N-deficient cells. This "energetically isolates" the antennas from the rest of photochemistry (Falkowski and Raven 2007) by decreasing the efficiency of energy transfer (Kolber et al. 1988, Falkowski and Kolber 1995). Related to this, N deficiency is correlated with the preferential repression of chloroplast proteins (Plumley and Schmidt 1989, Beardall et al. 2001b), favours a shift to PSI excitation, a faster rate of quinone reduction (Geider et al. 1998), and an increase in xanthophyll cycle pigments (Geider et al. 1998), which are used for the dissipation of energy as heat (Govindjee 2004, Schreiber 2004).

The major form of P in cells is in nucleic acids, predominantly in ribosomes (Sterner and Elser 2002). P-limitation is associated with declines in the light harvesting complex of PSII, D1 protein, and the Rubisco large subunit (Geider et al. 1998), as well as decreases in terminal electron acceptors, and therefore a reduced electron transport rate and an increase in non-photochemical quenching (Wykoff et al. 1998). It has been observed, however, that cells may not show changes in their photosynthetic ability at the onset of P-starvation (Geider et al. 1998) because of their large intracellular P reserves (Whkoff et al. 1998).

As such, it might be possible to determine nutrient deficiency on a non-perturbed water sample just by using variable fluorescence measures, like Fv/Fm (e.g. Kolber et al. 1988, Falkowski and Kolber 1995). However, it appears that Fv/Fm is more sensitive to sudden changes ("pulsing") of N more so than the actual limitation of N (Parkhill et al. 2001). MacIntyre et al. (1997) found that when cultures of *Alexandrium tumerense* (Lebour) were fully acclimated to N-limitation, there was no change in Fv/Fm

compared to N-replete cultures (Parkhill et al. 2001, Kruskopf and Flynn 2006). The more chloroplast-specific molecular changes associated with N deficiency are not known for P deficiency, but there is still the expectation and some evidence that Fv/Fm can be depressed by P deficiency (e.g. Geider et al. 1998)

While some studies have found evidence of predictable changes (often decreases) to Fv/Fm in response to sub-optimal nutrient availability *in situ*, other studies have suggested that this may not always be the case (e.g. Parkhill et al. 2001; Kruskopff and Flynn 2006). Parkhill et al. (2001) have suggested that Fv/Fm might be able to detect acute nutrient deficiency or perturbation to nutrient status, but not chronic nutrient limitation. It appears that the specific type of deficiency can affect Fv/Fm. Nutrient perturbation appears to be the mechanism to detect nutrient deficiency (Parkhill et al. 2001, Beardall et al. 2001a). However, natural phytoplankton communities may be adapted to a certain nutrient regime at the time of sampling so that their growth is balanced (Shuter 1979; Eppley 1981; MacIntyre and Cullen 2005), and Fv/Fm may not appear depressed. Thus, Fv/Fm from nutrient-limited phytoplankton may not indicate nutrient deficiency, even though other nutrient status indicators suggest otherwise.

Parkhill et al. (2001) further suggest that perturbation treatments could increase the information obtainable from Fv/Fm. Beardall et al. (2001a, b) have developed a strategy to probe nutrient deficiency using this idea of perturbation: the nutrient induced fluorescent transient (NIFT). The NIFT response is based around the idea that carbon fixation, as well as nutrient uptake and assimilation, require both ATP and NADPH. Nutrient deficient phytoplankton, when the limiting nutrient is reintroduced, commonly engage in so-called luxury uptake (Duarte 1992). That is, they shuttle their energy (ATP) and reductant (NADPH) toward transport of the nutrient into the cell and incorporation into intercellular structures (e.g. proteins, nucleic acids, etc.). These ATP and NADPH molecules are then unavailable for carbon fixation, and photosynthetic C fixation yield drops temporarily (Turpin and Weger 1988, Beardall et al. 2001b). It has been previously observed that both photosynthetic yield (measured as O_2 and CO_2) and Chl a fluorescence both dropped in response to restoration of limiting N (Turpin and Weger 1988) and

fluorescence transients have also been observed in response to addition of P to P-limited phytoplankton (e.g. Petrou et al. 2008, Roberts et al. 2008). The physiological mechanisms for transient effects on Fv/Fm are not yet as well elucidated as a means to track photosynthetic yield compared to C-fixation, but Fv/Fm can be measured quickly so NIFTs might be a viable way to assess phytoplankton nutrient status with fairly short-term assays (≤1h). If spectral fluorescence of taxon-specific pigments can be overlaid on this variable fluorescence response, this could provide a great deal of information on group-specific nutrient status. This could prove to be a very useful tool and technique especially in understanding the proximate nutrient mechanisms surrounding blooms of *Microcystis aeruginosa*, which are currently affecting Lake Erie.

Finally, variable fluorescence gives an indication of phytoplankton health. More specifically, it gives an indication of PSII health, since its D1 protein is sensitive to damage (Falkowski and Raven 2007). Currently, variable fluorescence only gives information at the community level. There are a few issues inherent in this however. One is the influence of phytoplankton physiology. Some phytoplankton like Cyanobacteria, even when fully "healthy", typically only produce Fv/Fm ≤0.4, especially if they contain many phycobiliproteins (Campbell et al. 1998). Eukaryotes rarely exhibit a "healthy" Fv/Fm below ~0.55, and typically are expected to show Fv/Fm in the range of ≥0.65 (Falkowski and Raven 2007). It is then unknown whether the relative composition of the community will skew the Fv/Fm upwards or downwards.

1.7 Relevance of Variable Fluorescence to Water Quality Monitoring

Nutrients can affect photosynthesis; photosynthesis and variable fluorescence are related. It could be expected that variable fluorescence might reflect changes in phytoplankton nutrient status. The implications for water quality monitoring, especially for the purposes of understanding the dynamics of potentially problematic phytoplankton blooms, are huge. Variable fluorescence presents an attractive

option for monitoring water quality because of the rapidity with which information can be obtained. This has implications for finer spatial and temporal measurements, which can only help water quality managers make better informed decisions. Variable fluorescence is also specific to phytoplankton. Unlike other chemical methods and assays, chl a is most likely only to be attributable to phytoplankton in pelagic samples. Nutrient ratios might be skewed by detritus or zooplankton in the same sample and alkaline phosphatase is also expressed in heterotrophic bacteria (Jones 1972). Debts, especially P-debts, might be affected by the presence of bacteria since these are believed to be more competitive for SRP than phytoplankton (Tambi et al. 2009). In general, phytoplankton are most likely to experience chronic nutrient limitation, and not acute nutrient starvation, but starvation or some other perturbation is most likely reflected in a change in Fv/Fm. However, phytoplankton bloom cessation may be one of the most ecologically-relevant instances of sudden nutrient depletion and may be a situation for nutrient starvation, where phytoplankton growth rates are no longer in proportion to the nutrient supply (i.e unbalanced growth, Harris 1986, McIntyre and Cullen 2005); in essence, the phytoplankton "hit a wall". This dichotomy between what is most likely to be encountered in natural systems (limitation) and what is needed to detect nutrient deficiency (abrupt changes to nutrient status; starvation), might limit the usefulness of variable fluorescence in detecting nutrient deficiency *in situ*. However, these instruments could provide useful information for the purposes of understanding the nutrient mediators in bloom dynamics. Previous studies on Lake Erie and a few of the other Laurentian Great Lakes have provided some evidence that unperturbed phytoplankton Fv/Fm measured by PAM does reflect patterns of deficiency inferred from independent measurements (Rattan et al. 2012).

Fv/Fm might not be the most sensitive parameter, but the speed with which it can be measured makes it an attractive parameter to investigate and develop for water quality monitoring. However, Fv/Fm alone can only give an indication of the community chl a fluorescence, and thus, only provides information about phytoplankton health at the community level. To fully understand the potential

nutrient mechanisms behind bloom formation and cessation and understand the relationship between variable fluorescence signatures and bloom success, more information needs to be provided to investigate the phytoplankton group-specific variable fluorescence responses.

Spectral fluorescence, as found on the FluoroProbe (bbe) is capable of estimating phytoplankton community composition and relative abundance based on group-specific pigments. The PhytoPAM (Walz) is one of the instruments capable of measuring both spectral and variable fluorescence, and thus, bridges the gap in understanding group-specific variable fluorescence and may be useful in investigating group-specific phytoplankton nutrient status ("health"). However, this instrument has not yet been tested to see if it is sensitive enough to make these discriminations or even if the discriminations are correct; this is where this Master's work becomes relevant.

1.8 Scope of Thesis

Variable fluorescence is reflective of phytoplankton photosynthetic potential, which can be influenced by nutrient status of the phytoplankton, which in turn can reflect nutrient concentrations and nutrient loading in natural water bodies. This project fits within the mandate to understand nutrient controls on phytoplankton, but more specifically, to develop monitoring tools that may be used in the field to quickly and accurately collect pertinent data. The first major objective of the thesis is to determine whether comparisons with independent measures of P and N deficiency in Lake Erie phytoplankton support previous indications that Fv/Fm can serve as a nutrient deficiency indicator, and to assess the possibility that patterns observed in Fv/Fm may differ among fluorometers that all use PAM methodology but implement it in somewhat differing ways. Chapter two presents the results of field studies conducted in Lake Erie with three Walz PAMs (Water, Phyto, and Diving PAM) in 2011 that address this objective. The second major objective, and the subject of chapter three, is to explore the ability of PhytoPAM to correctly discriminate taxonomic composition and taxon-specific P status in

defined mixtures of laboratory-grown phytoplankton cultures. I hypothesized that either Fv/Fm itself or patterns of NIFT response would reveal responses to P deficiency, and that the PhytoPAM would continue to correctly identify taxa when mixed under P replete or deficient conditions. Chapter four provides a synthesis and summary of the thesis findings and outlook on directions for further study.

Chapter 2

Nutrient status of Lake Erie phytoplankton and its assessment by Pulse Amplitude Modulated (PAM) Fluorometry

Variable fluorescence of chlorophyll a (Fv/Fm) has been proposed as a metric of nutrient deficiency in phytoplankton and can be measured by a variety of Pulse Amplitude Modulated (PAM) fluorometers. This study tested the relationship of Fv/Fm to nutrient deficiency of natural phytoplankton communities, and the correspondence among results obtained with different PAMs (DivingPAM, WaterPAM, and PhytoPAM), using samples collected from Lake Erie and Lake Ontario in July and September 2011. Nutrient deficiency was measured as nitrogen (N)-debt, phosphorus (P)-debt, and alkaline phosphatase activity (APA). Lake Erie samples exhibited varying degrees of N- and P-deficiency, as well as sufficiency, in both months, with less deficiency in west basin in September than at most other places and times. Between-instrument correlations of Fv/Fm did not follow the expected 1:1 relationship, although WaterPAM and PhytoPAM correlated well with each other. DivingPAM Fv/Fm was the most different of the three and did not correlate positively with the others. DivingPAM Fv/Fm showed negative correlation with measures of P-deficiency; this is consistent with proposed effects of nutrient deficiency on Fv/Fm, but measurements with the other PAMs gave either no correlation or positive correlations. Workers must be aware that measurements between PAMs are not automatically comparable. Caution must also be exercised in interpreting Fv/Fm in the context of judging phytoplankton nutrient status.

2.1 Introduction

Lake Erie is considered, overall, a phosphorus (P)-limited lake, as phytoplankton biomass decreased following P-loading control measures under the guidelines of the Great Lakes Water Quality Agreement (GLWQA) (Charlton et al. 1999). This follows the single nutrient paradigm that P is the nutrient that will limit phytoplankton growth before any other nutrients (e.g. Schindler 1971, 1977). In response to decreased P-loading, problematic Cyanobacteria of the genus *Aphanizomenon* decreased, water clarity improved, and ambient concentrations of phosphorus, nitrate, and ammonium were within

acceptable limits (Charlton et al. 1999). While it is generally accepted that Lake Erie is a P-limited lake, there is some evidence that phytoplankton may experience nitrogen (N)-deficiency at times (Guildford et al. 2005, North et al. 2007, Rattan et al. 2012). This may be especially important as ammonium levels in Lake Erie have been at very low levels (Dove 2009), and nitrate, while available, may become unavailable if there is iron limitation, of which some evidence has been found (North et al. 2007). Multiple nutrient limitation on phytoplankton can reasonably be expected in Lake Erie, and has been observed in other systems (Sterner 2008, Elser et al. 2009). These temporal and spatial differences can influence patterns of phytoplankton occurrence, while not contradicting the P-limitation paradigm (Schindler 2012).

Because of its importance and impacts on higher orders of the aquatic food web and for the purposes of water quality monitoring, phytoplankton nutrient status needs to be monitored. Monitoring also gives an indication of short-term shifts in type and magnitude of nutrient deficiency, which in turn is likely to be indicative of short-term limitation patterns. There are a variety of analytical methods available (Table 2.1); however, they are often laborious, both in sample collection and analysis (Guildford et al. 2005, Rattan et al. 2012). This makes it difficult to sample on fine spatial and temporal time scales, thus making it more difficult to detect patterns of nutrient limitation. However, variable fluorescence has been proposed as a method that might be useful in detecting phytoplankton nutrient status (Genty et al. 1989, Geider et al. 1993, Kolber and Falkowski 1993, Babin et al. 1996(b), Parkhill et al. 2001). Variable fluorescence (Fv/Fm) is the difference between maximum fluorescence (Fm) and minimal fluorescence (Fo) normalized to the Fm (Fv/Fm= (Fm-Fo)/Fm). Its value is influenced by phytoplankton taxonomic composition (Suggett et al. 2009) and recent light history, including both exposure to ultraviolet (UV) radiation (e.g. Harrison and Smith 2009) or even varying intensities of photosynthetically active radiation (PAR) (Vassiliev et al. 1994).

Fv/Fm is related to photosynthesis. N and P deficiency have been demonstrated to affect photosynthesis (Falkowski and Raven 2007 and references therein). Thus, it is also expected that variable fluorescence would reflect this. Some examples are provided to illustrate the effects of N and P deficiency on PSII, and consequently, may also affect variable fluorescence. Briefly, N-deficiency is more directly associated with PSII disruption, as reduced N-nitrogen (in the form NH_4^+) is an essential component of all proteins, of which the D1 protein of PSII is made. The effects of N-deficiency vary, from decreases in functional reaction centres (Kolber et al. 1988) that lead to a decrease in energy transfer efficiency down the chloroplast transport chain (Kolber et al. 1988, Falkowski and Kolber 1995) to repression of chloroplast proteins (Plumley and Schmidt 1989, Beardall et al. 2001b). N-deficiency may also cause increased non-photochemical quenching by heat dissipation instead of photochemical or fluorescent mechanisms (Govindjee 2004, Schreiber 2004). These mechanisms include state transitions, increased rates of quinone reduction in the plastoquinone pool, and increases in xanthophyll pigments (Geider et al. 1998). P-deficiency may not be as obvious as N-deficiency, since the cellular components that would be most affected would be the nucleic acids, particularly ribosomes, which comprises the major pool of P (Sterner and Elser 2002). This may influence onset of unbalanced growth (i.e. P starvation), since it is possible for phytoplankton to make use of this cellular reserve to maintain intracellular function (Wykoff et al. 1998). However, P-deficiency has been previously associated with decreases in LHCII, decrease in D1, and decreases in the Rubsico large subunit (Geider et al. 1998), which has implications for carbon fixation (photochemistry). There may also be a decreases in substances at the end of the chloroplast electron transport chain (e.g. ATP, NADPH, etc.), which has the implications of a decreased electron transport rate, resulting in lower rates of photochemistry and increased rates of non-photosynthetic quenching (Wykoff et al. 1998); that is, energy is dissipated as heat. However, the evidence for the facility with which Fv/Fm might reflect nutrient status is mixed. Some workers have found good relationships between Fv/Fm and nutrient deficiency (e.g. Cleveland and Perry

1987, Kolber et al. 1988, Falkwoski et al. 1989, Geider et al. 1998), while others have not (e.g. Kruskopf and Flynn 2006, Suggett et al. 2009).

Rattan et al. (2012) found surprisingly robust relationships between variable fluorescence and nutrient deficiency in Lake Erie. Given the mixed evidence for the use of variable fluorescence to detect nutrient deficiency, Rattan et al. (2012) suggested further study, which this study attempted to fulfill. There is evidence that the type of instrument and the way variable fluorescence is induced can result in different measures of Fv/Fm (e.g. Ralph and Gademann 2005). Single-turnover (ST) instruments often yield lower Fv/Fm than do multiple-turnover (MT) instruments (Ralph and Gademan 2005). However, there do not appear to be any published comparisons between various MT instruments, particularly the pulse amplitude modulated (PAM) fluorometers. There are slight differences in saturation pulse intensity and wavelength, as well as slight differences in excitation peak of the saturation pulse (Table 2.2). There are two currently used for open-water samples (the WaterPAM and PhytoPAM), and another meant for benthic samples (DivingPAM). The differences of optical configuration(s) might influence measurement sensitivity and the outputs. This could help explain why the DivingPAM has yielded some very low Fv/Fm values for nutrient deficient phytoplankton in Lake Erie (Rattan et al. 2012) while other PAM fluorometers have not detected similarly low values in other nutrient-limited lakes (Harrison and Smith 2012).

This paper investigates the relationship between variable fluorescence and nutrient status in Lake Erie phytoplankton. Specifically, the objectives are as follows: (1) quantify the nutrient status of Lake Erie phytoplankton in all three (West, Central and East) basins in July and September 2011, as well some sites in Lake Ontario, hypothesizing that both N and P deficiency would occur; (2) determine the variability of Fv/Fm determinations between different PAM instruments, hypothesizing that instrument-specific differences may occur; (3) test the hypothesis that Fv/Fm would be negatively correlated with the severity of N and P deficiency, but that correlations would differ among PAM instruments.

2.2 Methods

2.2.1 Study Area and Sample Design

Lake Erie is the smallest of the Laurentian Great Lakes by volume and is segmented into three distinct basins: East, Central and West. The East Basin (EB) is the deepest of the three basins, with a maximum depth of 60m. The Central Basin (CB) is the largest basin by area, with a maximum depth of approximately 20m. Both these basins are considered oligotrophic (Charlton et al. 1999), with the EB usually containing lower nutrient concentrations of nitrogen and phosphorus than the CB. The West Basin (WB) is the smallest and shallowest basin, with a maximum depth of approximately 10m. The WB receives major inputs from the Detroit and Maumee Rivers. It is often characterized as mesotrophic, as these three tributaries are important sources of nutrient loading to this basin. Lake Erie is a temperate dimictic lake, and the EB and CB stratify in the summer and may inversely stratify in the winter. The WB does not usually stably stratify in the summer. The boundary area between the West and Central Basin has been termed the West Central Basin (WCB); it is an important transitional zone between these two basins, as it often contains interesting phytoplankton and nutrient profiles (Leon et al. 2005; Rattan et al. 2012).

Samples were taken from stations in the West, Central, and East Basins of Lake Erie and from two sites in Lake Ontario, one central station in Hamilton Harbour (43.289 N, 79.833 W) and another offshore from Grimbsy, Ontario (43.268 N, 79.621 W) during two cruises on the CCGS *Limnos* on July 11-16 and on September 6-9 and 12-14, 2011 (Fig 2.1, Table 2.3). Samples were obtained at a depth of 1m using a 10L niskin sampler. At station 880 (CB), samples were also collected 2m above lake-bottom. Samples were collected around the clock; some samples were taken during the day, and others taken at night depending on the ship's sailing schedule. These samples were collected into 20L carboys that had

been well rinsed with sample water prior to being filled with the sample water. Further samples for nutrient status assays were collected from these carboys. Care was taken to mix well the water in them, in case certain phytoplankton either sank or floated. These subsamples were taken within 30 minutes of collection with the Niskin water sampler.

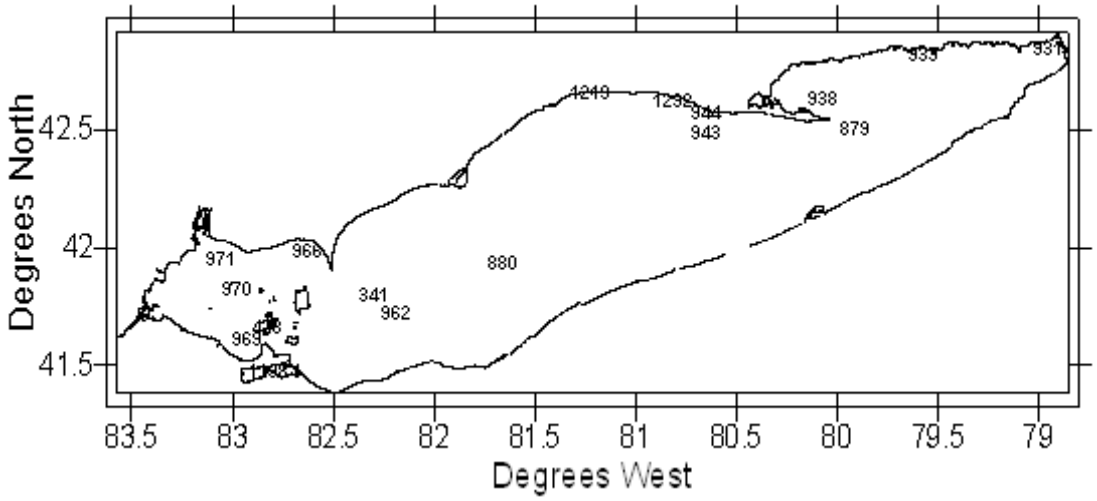

Figure 2.1 Map of Lake Erie sampling stations in July and September 2011

2.2.2 Nutrient Status Indicator Assays and Chlorophyll a (chl a) analysis

Alkaline phosphatase samples and 0.2µm filtrate were analyzed immediately to determine particulate alkaline phosphatase activity (APA), that is, the APA of intact phytoplankton, and not of dissolved alkaline phosphatase in the water as a result of cell lysis or planktivory. If samples could not be analyzed immediately, they were stored in the dark at 4°C and analyzed as soon as possible. The APA assay followed the methods of Healey and Hendzel (1979) and used 100µM 3-O-methylfluorescein phosphate (3-OMFP) as the substrate. Samples were incubated at 35°C for the duration of this analysis, as this is the temperature at which the sufficient-deficient thresholds were determined. Alkaline

phosphatase is an enzyme that is usually only induced when phytoplankton experience a shortage of soluble inorganic phosphorus (i.e. PO_4^-). A TD-700 fluorometer with a near-UV excitation lamp was used to measure the standardized fluorescence of 3-OMF. Chlorophyll *a* (chl *a*)-normalized hydrolysis rate of 3-OMFP to 3-OMF for each particulate (whole water minus 0.2µm filtrate) sample was calculated and the values derived gave information about the community's P-status (Table 2.1).

Table 2.1 Summary table of the various nutrient deficiency indicators and their ranges. Nutrient status indicator values are presented as in North et al. (2007) and Fv/Fm values as indicated in Falkowski and Raven 2007.

	Not deficient	Moderately deficient	Severely deficient	Deficient
N-debt (µmol NH_4^+µg $chla^{-1}$)	<0.15			>0.15
P-debt (µmol PO_4^- µg $chla^{-1}$)	<0.075			>0.075
APA (µmol 3-OMFµg $chla^{-1}h^{-1}$)	<0.003	0.003-0.005	>0.005	
Fv/Fm – eukaryotic phytoplankton	≥0.55			<0.40

N- and P-debt assays were conducted using the methods detailed in North et al. (2007), with NH_4^+ and PO_4^- as the N and P amendments, respectively, added to unfiltered water samples. Briefly, ~5µM (final concentration) of the nutrient amendment was added to filtrate, that had been passed through a 0.2µm polycarbonate filter in the case of N-debt and through a GF/F filter in the case of P-debt, and either analyzed immediately, or stored at 4°C until analysis (usually <3h). 40mL whole-water samples were amended in triplicate and incubated in the dark at ambient lake temperature for approximately 24h. Following incubation, N-debt samples were filtered through 0.2µm polycarbonate filters (AMD) and P-debt samples were filtered through GF/F (Whatman – 0.7µm) filters. While N-debt samples were analyzed immediately, filtered P-debt samples were stored at 4°C until analysis following the cruise. Samples were not frozen, as there have been suggestions that this changes the soluble reactive phosphorus (SRP) content of the sample (Jarvie et al. 2002).

Filtered N-debt samples were analyzed as soon as possible using the fluorescence method for ammonium described in Holmes et al. (1999). Before amending any of the samples with the reagent, any visibly coloured samples were read on the fluorometer to check for background fluorescence. Following incubation as described in Holmes et al. (1999), samples were read on a TD-700 fluorometer used to measure 3-OMF fluorescence for APA, as the wavelength of the excitation lamp also excites OPA fluorescence. SRP concentrations were measured spectrophotometrically, using the molybdate blue methods of Murphy and Riley (1962). Samples were filtered into 40mL acid-washed (10% HCl) screw-top falcon tubes, the cap sealed to the tube with parafilm to prevent contamination of the samples, and stored in the dark at 4°C until the end of the cruise. These samples were all analyzed against known standards of 0-250 $\mu gPO_4^{-}L^{-1}$ immediately upon return to U. Waterloo. Standards were run once at the beginning of analysis and once again at the end of the analysis. The standard curve slopes did not change significantly between the beginning and end of analysis, which never exceeded the six hours. The colour remains stable up to six hours, so the analysis time did not impact the accuracy of measurement. The difference between N or P concentration pre-incubation and post-incubation were normalized to chl a. Finally, because incubations were not all 24h long, these values were time-normalized.

Chlorophyll *a* was collected on 47mm GF/F (Whatman) filters by gentle filtration (<10mm Hg) and stored in the dark at -20°C until fluorometric analysis using the methods of Strickland and Parsons (1972). Briefly, chl a was passively extracted in 90% acetone at -20°C for 18-24h and its fluorescence was measured on a TD-10 fluorometer before and after acidification with 6N HCl. Chlorophyll concentration was calculated using the equation given in Strickland and Parsons (1972), with Fd and T values obtained from previous chlorophyll a and fluorometer calibrations in this lab.

2.2.3 Variable Fluorescence Measurements

Three pulse amplitude modulated (PAM) fluorometers were used: WaterPAM, DivingPAM, and PhytoPAM. Sample preparation for the WaterPAM and PhytoPAM was identical. 15mL of well-mixed sample water and 15mL of 0.2µm filtrate were dark adapted at ambient lake temperature in the on-board incubators for approximately 30 minutes prior to measurement in quartz glass cuvettes. For all instruments, preliminary measurements were done, according to manufacturer's instructions, to define the measuring and saturation light settings that would fully saturate fluorescence while maximizing Fv/Fm.

Table 2.2 Summary table of PAM differences

	DivingPAM	WaterPAM	PhytoPAM
Wavelength of saturation pulse maximum (nm)	Blue enriched white light (broad spectrum)	660	655
Saturation intensity (µmol PARm^{-2}s^{-1})	≤18,000	≤4000	≤4000
Wavelength(s) of measuring light maximum (nm)	650	650	470 520 645 650 665
Measuring light cutoff filter (nm)	670	695	695
Detection cutoff filter (nm)	>700	>710	>710

The DivingPAM uses a fibre optic probe for measurements and lacks the sensitivity necessary to make measurements at most natural concentrations so samples were filtered under low pressure (<10mm Hg) and concentrated onto a 25mm diameter GF/F (Whatman) filter as in Rattan et al. (2012). The volume required to achieve this was variable, depending on the biomass of phytoplankton at any given station. Briefly, well-mixed sample water was filtered until filtration rate slowed down to the point that water was just barely passing through the filter. The volume required from rivers and the West Basin was

usually 50 to 100mL, while the volume required in the Central and East Basins often exceeded 400mL. Filters were left damp, and stored in tight-fitting Petri dishes (to prevent desiccation) for the 30 minute dark incubation at ambient lake temperature.

While the three PAM instruments use the same multiple turnover protocol to close the reaction centres of photosystem II, they use different light sources and spectral filters to provide measuring and saturation pulse light, and spectral response of detectors also varies (Table 2.2). While differences in wavelength maxima and filter cutoffs are not necessarily large, the excitation and emission spectra may have maximal absorption in the regions concerned so differences could be important. The measuring light for the PhytoPAM is unique, as there are four diodes (Table 2.2) to excite spectral fluorescence of specific pigment groups (e.g. chl *b*, xanthophylls or phycobiliproteins) additional to chlorophyll *a*. The other major difference is the use by Diving PAM of broadband white light for saturation, while the other PAMs use red light.

For the PhytoPAM and WaterPAM, lake water filtrate was used to correct for background fluorescence from dissolved substances. For DivingPAM, a clean GF/F filter drenched with 0.2 μm filtrate was used as a blank. Samples in cuvettes were not stirred, as measurement times were short compared to the time it would take for the phytoplankton would either sink (e.g. diatoms) or float (e.g. Cyanobacteria). Gain was adjusted using whole water so that the measuring light intensity was set as low as possible to avoid inducing photosynthesis, but where fluorescence was detected between 300-600 fluorescence units. When the fluorescence reading (F_t) stabilized, Fv/Fm was measured, with inspection of the fluorescence dynamics to ensure that a credible maximum was observed.

2.2.4 Statistical Analyses

The data collected were used to attempt to answer the questions posed in the objectives. All analyses were conducted using R (version 2.13.1) unless otherwise stated. A correspondence analysis (CA) (function CCA in the vegan library) was used to visualize the relationship between Lake Erie stations, PAM outputs and N- and P- sufficiency and deficiency. For this particular analysis, the nutrient status indicators (N-debt, P-debt, and APA) were coded into binary values of sufficiency (1) and deficiency (0). The within-instrument replicability measurements were analysed using correlation analysis, standard error of the regression, and the average coefficient of variation. Between-instrument agreement for the 645nm diode, WaterPAM and DivingPAM was analysed using model II major axis linear regression (function lmodel2), average coefficient of variation (CV), principal components analysis (PCA) on a covariance matrix, and one-way ANOVA. A covariance matrix was chosen for PCA because only Fv/Fm data was used, and there was consistency in the data, two assumptions that are satisfied for this type of analysis (Legendre and Legendre 1998). One-way ANOVA was used because the sample size was just large enough and the assumption of normality was not violated (Sokal and Rohlf 2012). The specific diodes on the PhytoPAM were compared to each other, also using the average coefficient of variation. The CV allowed for comparisons between unequal sample numbers (Sokal and Rohlf 2012), in order to make comparisons between instruments and within instruments. Finally, agreement between the PAM outputs and the nutrient status indicators was visualized using scatterplots and analysed using Kendall associations (function Kendall) on all data.

2.3 Results

2.3.1 Nutrient Status of Phytoplankton

In July, all stations except the sole station in the West Basin of Lake Erie had indicator values above the conventional threshold values for deficiency, and could be considered to be both N- and P-deficient (Table 2.3a). In contrast, in September, most sites were not N-deficient, while most stations in the East and Central Basins, and some sites in the West Basin and at river mouths, were P-deficient (Table 2.3b). Even the eutrophic Hamilton Harbour site displayed P deficiency in July.

Table 2.3 Summary table of Lake Erie sample Fv/Fm. nutrient assay results, and keys to canonical analysis and principal components analysis points for (a) July and (b) September. Bolded values under N-debt, P-debt and APA indicate deficiency. Asterisks indicate severe P-deficiency in APA.

a.

	PhytoPAM	WaterPAM	DivingPAM	N-debt	P-debt	APA	CA site number	PCA site number
	Fv/Fm (mean)	Fv/Fm	Fv/Fm	(µmol NH_4^+ µg $chla^{-1}$)	(µmol PO_4^- µg $chla^{-1}$)	(µmol 3-OMF µg $chla^{-1}h^{-1}$)		
Lake Ontario								
1001	0.57	0.66	0.61	**0.1**	**0.096**	**0.0235***	1	1
3	0.53	0.65	0.18	**1**	**0.335**	**0.0461***	2	2
East Basin								
931	0.6	0.67	0.25	n/a	n/a	n/a	n/a	3
933	0.6	0.64	0.18	**1.58**	**0.395**	**0.0286***	3	4
879	0.47	0.55	0.14	**1.46**	**0.387**	**0.0468***	4	5
West-Central Basin								
341	0.51	0.65	0.45	**0.43**	**0.257**	**0.0372***	5	6
West Basin								
969	0.5	0.63	0.36	0.1	**0.107**	**0.0172***	6	7

b.

	PhytoPAM	WaterPAM	DivingPAM	N-debt	P-debt	APA	CA site number	PCA site number
	Fv/Fm (mean)	Fv/Fm	Fv/Fm	(µmol NH_4^+ µg $chla^{-1}$)	(µmol PO_4^- µg $chla^{-1}$)	(µmol 3-OMF µg $chla^{-1}h^{-1}$)		
Lake Ontario								
1001	0.45	0.564	0.415	0.06	0.035	**0.0116***	7	8
3	0.57	0.595	0.238	-0.08	**0.201**	**0.0364***	8	9
East Basin								
938	0.51	0.603	0.394	-0.04	**0.113**	**0.1827***	9	10
944	0.54	0.595	0.379	0.09	**0.261**	**0.0662***	10	11
943	0.57	0.611	0.329	0.01	**0.095**	**0.0417***	11	12
Central Basin								
880 (1m)	0.52	0.702	0.418	-0.14	**0.123**	**0.0376***	12	13
880 (Bottom minus 2m)	0.58	0.685	0.424	-0.07	**0.083**	**0.0463***	13	14
West-Central Basin								
962	0.48	0.548	0.383	-0.07	**0.135**	**0.0232***	18	19
341	0.53	0.571	0.401	-0.08	**0.145**	**0.0185***	17	18
West Basin								
971	0.54	0.58	0.298	-0.14	**0.171**	**0.0041**	14	15
970	0.49	0.403	0.302	-0.08	-0.001	0.0024	15	16
969	0.57	0.67	0.372	0.03	**0.14**	**0.0071***	16	17
478	0.36	0.5	0.455	**0.23**	0.059	0.0016	19	20
1163	0.51	0.496	0.452	-0.02	0.001	0.0004	24	25

	PhytoPAM	WaterPAM	DivingPAM	N-debt	P-debt	APA	CA site number	PCA site number
	Fv/Fm (mean)	Fv/Fm	Fv/Fm	(µmol NH_4^+ µg $chla^{-1}$)	(µmol PO_4^- µg $chla^{-1}$)	(µmol 3-OMF µg $chla^{-1}h^{-1}$)		
River Mouths								
1292	0.54	0.588	0.346	**1.41**	**0.1**	**0.0475***	20	21
1219A	0.54	0.603	0.588	-0.09	0.037	**0.0048**	21	22
1219B	0.52	0.585	0.564	-0.13	-0.028	**0.0061***	22	23
1219C	0.51	0.616	0.529	0.13	**0.096**	**0.0083***	23	24
1198B	0.5	0.454	0.452	0.05	0.004	0.0006	25	26

Correspondence analysis on bivariate (sufficient = “1” and deficient = “0”) nutrient status indicators yielded a good separation of sites between N- and P- sufficiency and deficiency (Fig. 2.2), explaining approximately 91% of the variation in the first two axes. All sites above the horizontal axis were P-sufficient and below were all P-deficient by one or both P-status indicators. The sites closer to the axis were P-deficient according to APA, but P-sufficient according to P-debt. All sites to the right of the vertical axis were N-sufficient, and to the left were N-deficient.

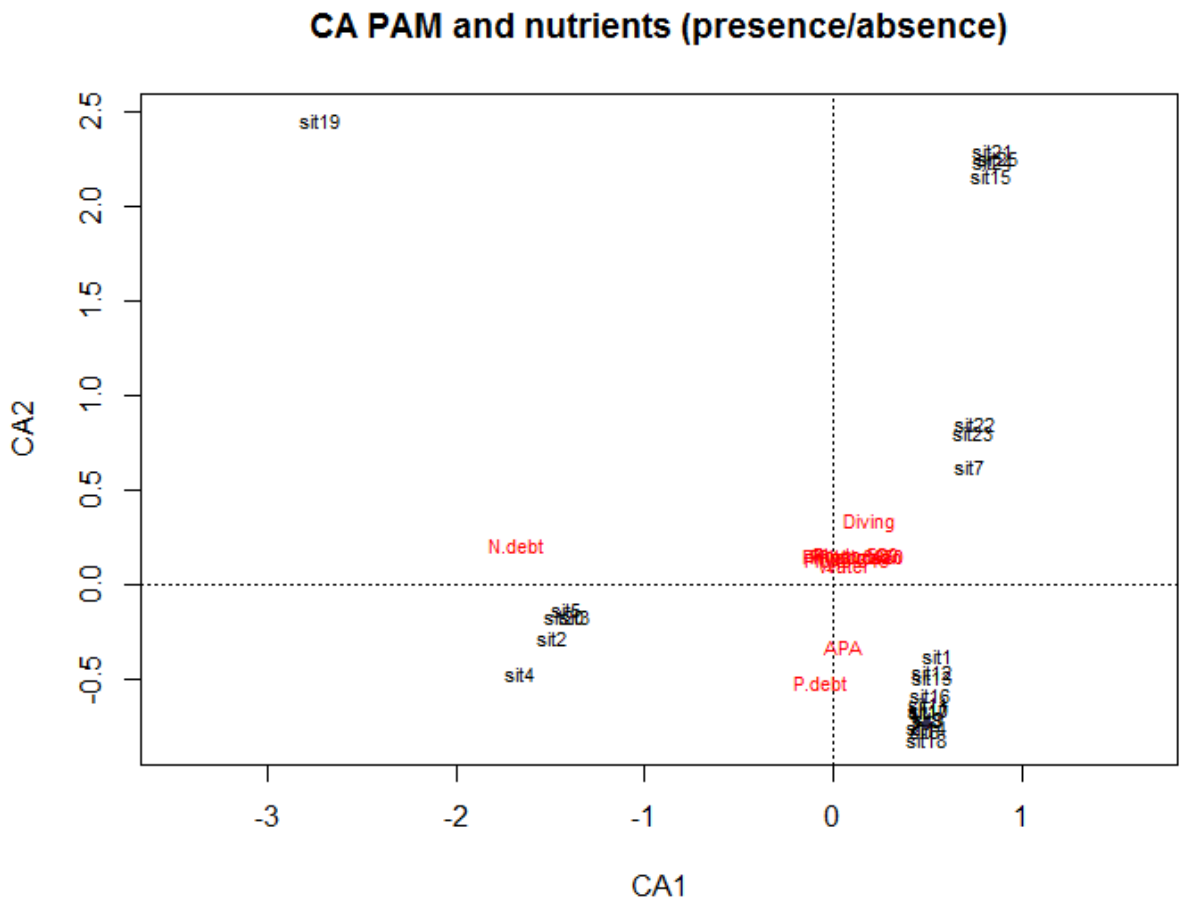

Figure 2.2 Correspondence Analysis of PAMs and nutrient deficiency and sufficiency

2.3.2 Replicability and between-instrument variations of Fv/Fm

Overall, the three PAM instruments had moderate to low variability (Table 2.4) for replicate measurements. The DivingPAM exhibited the largest range of variability in measurements, with a coefficient of variation (CV=sd/mean) between 0.3-24.2% while WaterPAM and the PhytoPAM had smaller and similar ranges of CV. The median CV was highest for PhytoPAM and similar between the DivingPAM and WaterPAM (Table 2.4).

Table 2.4 Coefficient of variation (%) for within-instrument variability on field samples

Instrument	CV min	CV max	Median CV
DivingPAM	0.3	24.2	2.6
WaterPAM	1.6	14.4	2.9
PhytoPAM mean yield	2.6	16.4	4.1
470 diode	1.5	14.9	4.1
520 diode	0. 5	19.3	4.0
645 diode	0. 7	15.9	7.2
665 diode	1.1	17.1	7.5

The 645nm diode channel on the PhytoPAM has a measuring wavelength similar to that of the WaterPAM and DivingPAM (650nm) and might be expected to provide similar values for Fv/Fm. However, based on model II major axis regression, Fv/Fm measured by the three instruments did not have 1:1 relationships with each other (Fig. 2.3, Table 2.5). The 95% confidence intervals (grey lines) of the regression slopes did not include one and the intervals around the regression (red) lines did not overlap with the 1:1 (green) line. WaterPAM had a regression line in the same direction and nearly parallel to the 1:1 line when compared to the 645 nm channel of the Phyto PAM (Fig 2.3) but the Water PAM values were significantly elevated. The DivingPAM, when compared against the 645nm channel and against the WaterPAM outputs (Fig 2.3) had a negative slope, a regression line clearly different from the 1:1 line, and a very poor R^2. There was essentially no correlation between

Diving PAM results and those from the other PAMs. The PhytoPAM gives Fv/Fm measures for the four diodes (470, 520, 645, 665nm) and a calculated "mean yield" (Table 2.4). The 520nm diode exhibited the largest range of variation (CV) for replicate measurements, but the 645nm and 665nm channels had the largest median CV of all the diodes.

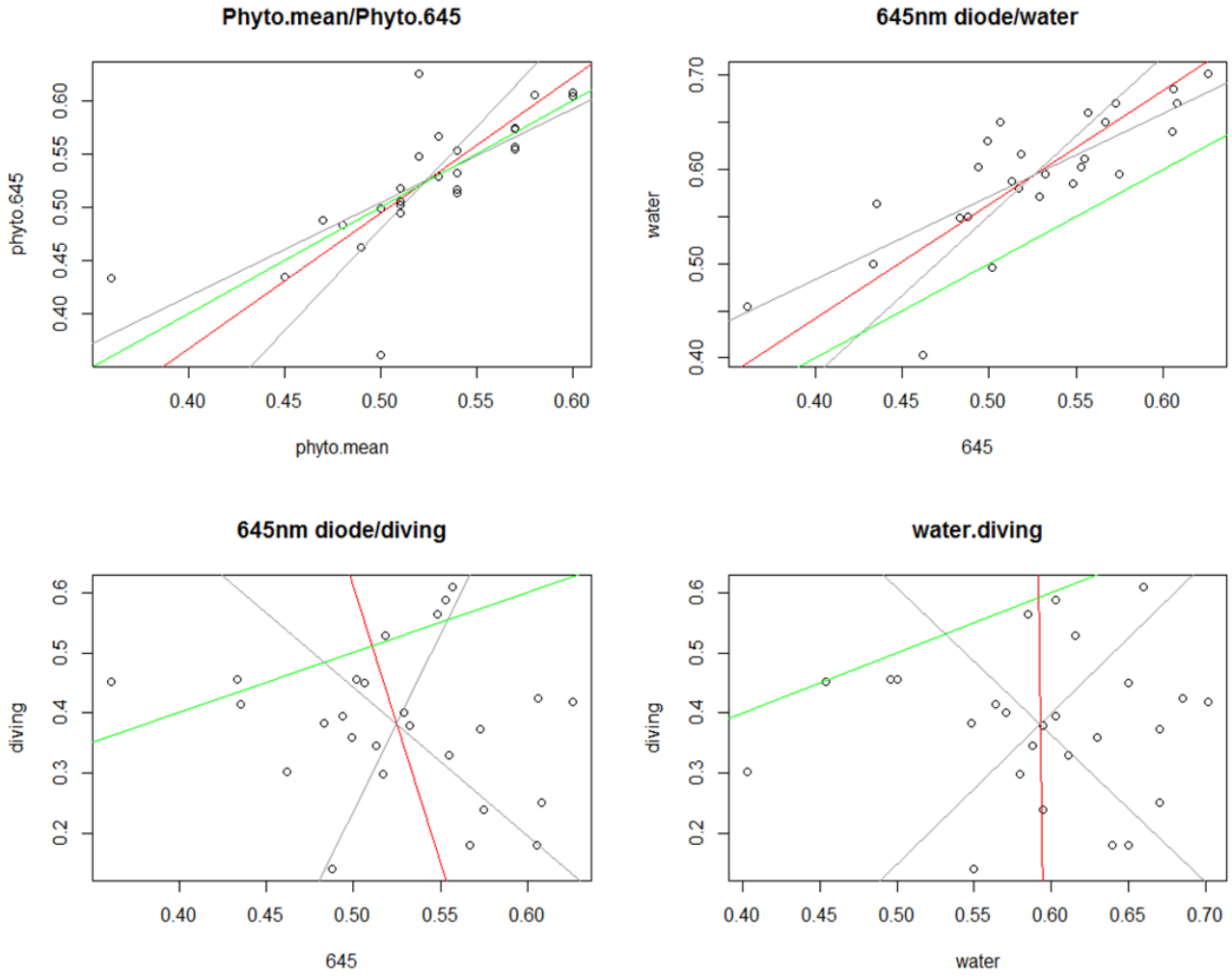

Figure 2.3 Model II major axis regression between various PAMs. Green line represents the hypothetical 1:1 relationship expected between PAMs, red line is the line of the regression, and grey lines represent 95% confidence interval around the regression.

Table 2.5 Comparison of slopes from outputs in Fig. 2.3

	R^2 (p-value)	Slope	t-test against hypothetical slope of 1 (p-value)
PhytoPAM mean yield vs. PhytoPAM 645nm diode	0.521 (3.16×10^{-5})	1.29	0.1371 (0.8921)
PhytoPAM 645nm diode vs WaterPAM	0.616 (2.09×10^{-6})	1.21	-8.1369 (1.722×10^{-8})
PhytoPAM 645nm diode vs. DivingPAM	0.0287 (0.4077)	-8.93	4.9381 (4.374×10^{-5})
WaterPAM vs. DivingPAM	0.000145 (0.953)	-94.61	7.6044 (5.848×10^{-8})

PCA provided an additional view of the correspondence among instruments, explaining 79% of the variation of Fv/Fm as measured by the different PAMs on the first two axes (Fig. 2.4). The WaterPAM and the PhytoPAM vectors, including those for all the PhytoPAM diodes, had similar directions and lengths, indicating a high degree of similarity in responses among stations. The DivingPAM vector was distinctly different, almost at right angles to the others and consistent with the lack of correlations in the regression analyses (Fig. 2.3). The DivingPAM vector was mainly aligned with the river sites (21, 22, 23), and sites with high biomass. Vectors for the other PAMs pointed towards a small group of stations (12, 13, 16) sampled in September but there was no obvious unifying factor among those sites, in terms of Fv/Fm or environmental features.

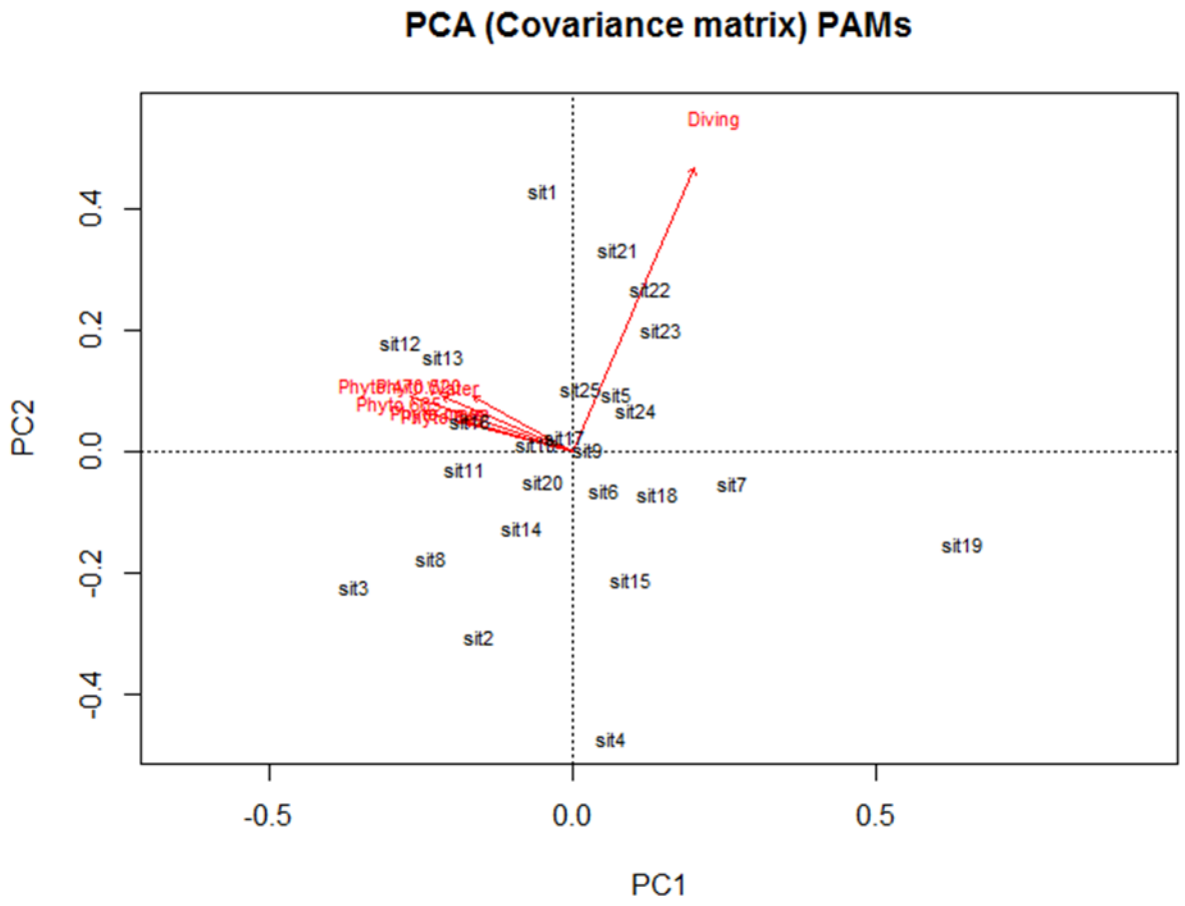

Figure 2.4 Principal components analysis of PAMs

One-way ANOVA further characterized differences among the PAM instruments (Fig. 2.5, Table 2.6). The DivingPAM produced a significantly lower mean Fv/Fm than the other instruments. The mean and diode-specific Fv/Fm by PhytoPAM was all similar to each other. Mean Fv/Fm by WaterPAM was higher than by other instruments, although its lower confidence interval overlapped with some of the upper confidence intervals of the PhytoPAM diode channels. A post-hoc Tukey HSD test (Table 2.6) indicated that the WaterPAM outputs were statistically different from

PhytoPAM mean yield, 520, and 645nm channels. None of the PhytoPAM channel means were statistically different from one another.

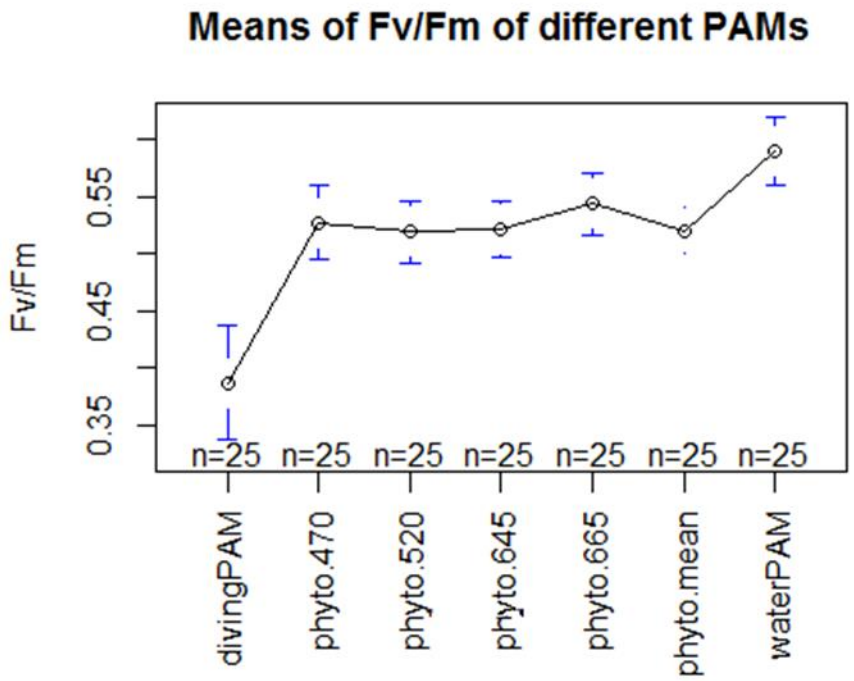

Figure 2.5 One-way ANOVA of PAM outputs. Means (symbols) and 95% confidence intervals (whiskers) for Fv/Fm measured by different PAMs (and by different diodes of PhytoPAM) on Lake Erie samples.

Table 2.6 Tukey HSD Post-Hoc adjusted p-values for ANOVA in Fig. 2.5. Bolded values highlight $p<0.05$, signifying statistically different means for Fv/Fm of one PAM or diode channel with another PAM or diode channel.

	Phyto.mean	Phyto.470	Phyto.520	Phyto.645	Phyto.665	WaterPAM
Phyto.mean						
Phyto.470	0.9999					
Phyto.520	1.0000	0.9998				
Phyto.645	1.0000	1.0000	1.0000			
Phyto.665	0.9320	0.9876	0.9166	0.9454		
WaterPAM	**0.0247**	0.0607	**0.0212**	**0.0287**	0.3318	
DivingPAM	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**

2.3.3 Relationship between PAM Fv/Fm and nutrient status indicators

Using Kendall's tau (τ) (Table 2.7) an association between chl a and Fv/Fm was apparent. The PhytoPAM 645nm channel and mean Fv/Fm, as well as the Water PAM Fv/Fm, were negatively associated with chl a, with $p<0.05$ for the 645 diode and $p<0.10$ for the others. DivingPAM Fv/Fm was, by contrast, positively associated with chl a with $p < 0.05$. The Diving PAM was the only instrument to give the expected negative correlation between Fv/Fm and nutrient status. The correlation with P debt had $p<0.05$, while the correlation with APA had $p>0.05$ for the complete data set but, with the exclusion of one apparent outlier point (see below), $p<0.05$. Diving PAM Fv/Fm did not have significant correlation with N debt. The other PAMs (and diodes) gave, surprisingly, Fv/Fm values that had mostly positive associations with nutrient status indicators, i.e. higher values when deficiency was stronger. This tendency was clearly significant ($p<0.05$) only for Water PAM Fv/Fm vs APA, but was borderline significant ($p<0.10$) for some other comparisons (Water PAM vs P debt).

P-debt and APA showed a significant ($p<0.05$) positive association among the stations sampled. N-debt had a positive and borderline significant ($p<0.10$) correlation with P-debt but less association with APA. Stations with higher chl a appeared to be less P deficient, as chl a had a strongly significant negative correlation with both APA and P debt. There was also a negative, but weaker and non-significant, correlation between chl a and N debt.

Table 2.7 Kendall association of all sites. Bolded values indicate $p<0.05$ and italicized values indicate $p<0.10$.

	Phyto.mean	Phyto.645	Water	Diving	Chl a	N.debt	P.debt	APA
Phyto.mean	**1.000**							
Phyto.645	**0.715**	**1.000**						
Water	**0.445**	**0.620**	**1.000**					
Diving	-0.101	-0.060	0.010	**1.000**				
Chl a	*-0.277*	**-0.337**	*-0.282*	**0.419**	**1.000**			
N.debt	-0.073	-0.101	0.078	-0.142	-0.179	**1.000**		
P.debt	0.180	0.217	*0.262*	**-0.452**	**-0.428**	*0.266*	**1.000**	
APA	0.218	*0.273*	**0.345**	*-0.268*	*-0.578*	0.215	**0.397**	**1.000**

Scatter plots of Diving PAM Fv/Fm against nutrient status indicators suggested that the significant negative correlation with P debt was evident in the data for each month, though the July data were few in number (Fig. 2.6 a). The lack of significant Kendall correlation with APA may have been due to one observation with very high APA (Fig. 2.6 b); with that point removed there was a significant ($p=0.036$) negative correlation. The relatively few observations with high N debt (>1, Fig. 2.6 c) also had P debt and APA above the P deficiency threshold; there was little evidence of systematic variations with N debt among the other points. Scatter plots of Fv/Fm against nutrient status indicators for the other fluorometers (not shown) did not provide any evidence that correlation analysis failed to detect negative correlations due to outliers, non-linearities, or threshold-type behaviour that might confuse the Kendall test. Observations in both months spanned most of the observed range in Fv/Fm and nutrient status indicators and failed to suggest negative associations between Fv/Fm by either PhytoPAM or WaterPAM and nutrient status.

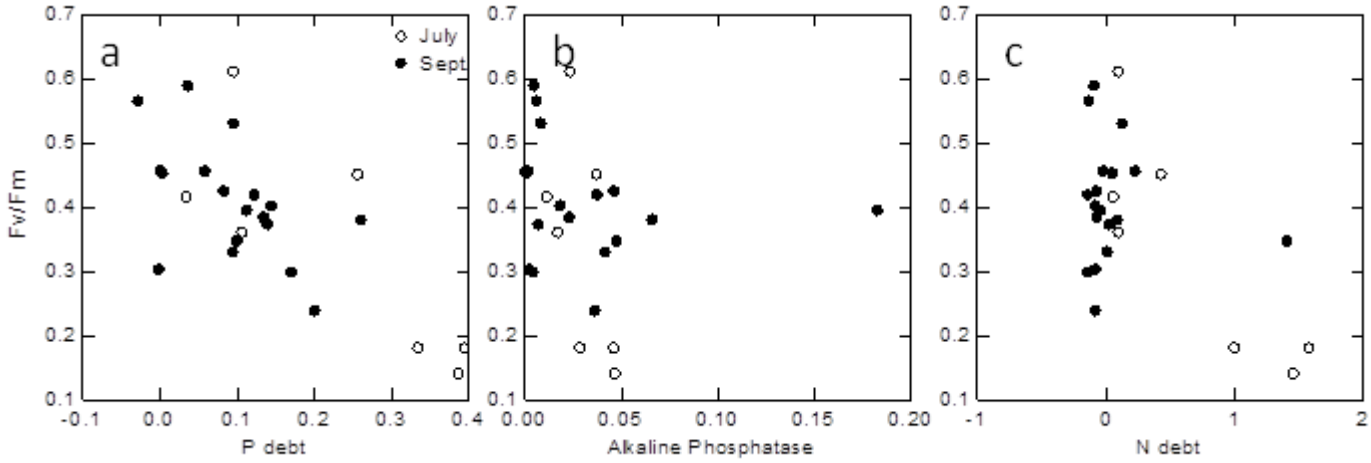

Figure 2.6 Scatterplots of DivingPAM Fv/Fm compared to nutrient-status assay results

2.4 Discussion

2.4.1 Lake Erie Phytoplankton Nutrient Status

In general, phytoplankton in Lake Erie (i.e.in the Central and Eastern Basins) are expected to show P-deficiency because Lake Erie is considered a P-limited lake. In Lake Erie's West Basin, however, *Microcystis* blooms appear to have become a regular occurrence in late summer in recent years, suggesting that P limitation may have been eased. Collecting data on phytoplankton nutrient status may contribute to a successive series of data "snapshots", which can be combined to infer longer term nutrient status trends. Previous work to characterise Lake Erie's phytoplankton nutrient status over a period of ~15 years has been published recently by Guildford et al. (2005) (all basins in 1997), North et al. (2007) (East Basin 2001-2003) and Rattan et al. (2012) (West and West-Central Basins 2005). This study is, in part, a follow up on Lake Erie's phytoplankton nutrient status (all basins and two western Lake Ontario stations), as these samples were collected in 2011. The broad patterns of phytoplankton nutrient deficiency found in this study were comparable to those found from 1997 to 2005 for mid-summer and early autumn. As in previous studies, there was evidence of P-deficiency in all basins. However, P deficiency was less prevalent in September than July, with many west basin stations having indicators below the threshold; Rattan et al. (2012) likewise found less P deficiency in September than July of 2005, though they sampled somewhat later in the month. There was evidence of N-deficiency in July but much less in September, consistent with Guildford et al. (2005), North et al. (2007), and Rattan et al. (2012), who all found N deficiency to be more common in spring and early summer than in late summer. Moon and Carrick (2007) did not see evidence of N-deficiency using bioassay experiments in the Central Basin during summer. However, their bioassays were testing more for ultimate community biomass limitation rather than for signs of

physiological stress (APA) or demand (debts) so cannot be directly compared (North et al. 2007). Like previous studies, the present results suggested that N deficiency generally became less common in the transition between summer and fall, suggesting a continued primacy of P as the limiting factor in algal growth during the late summer season. There was, however, evidence of N-deficiency in one West Basin site (station 478) and a river site in September, whereas Guildford et al. (2005) and Rattan et al. (2012) found none in September. Together with the relatively limited incidence of P deficiency in the West Basin in September, this could reflect the influence of P-loading from the anthropogenically-impacted Maumee River. Increased loading of bioavailable P from the Maumee in recent years (Agricultural Nutrients and Water Quality Working Group) may be helping to promote the late summer Cyanobacteria blooms that have become common in the West Basin. The largest *Microcystis aeruginosa* bloom documented to-date originated in the West basin in the late summer of 2011 (Bridgeman et al. 2013), starting about the time of the September survey.

No single measure of nutrient status is perfect; each assay suffers some sort of drawback, whether related to practical concerns (e.g. analysis time) or more theoretical concerns over interpretation (e.g. origin of APA activity in samples). Community-level sufficiency or deficiency may not be a good representation of individual phytoplankton group nutrient status (Arrigo 2005), especially in a highly heterogeneous assemblage. The thresholds for inferring deficiency may not always be entirely appropriate for use with natural populations, as they were often determined on only very few taxa (e.g. APA, Healey and Hendzel 1979), or performed under unnatural conditions (e.g. the 35°C incubation required for APA, dark incubation for debts). Using multiple assay types can help increase confidence in results. In the present case, APA and P debt gave similar, though certainly not identical, results for the distribution of P deficiency, as shown in the correspondence and correlation analyses. This might simply have been a reflection of the longer timescales necessary for a phytoplankton cell to down-regulate AP compared to the timescale necessary to satisfy its P-

deficiency through luxury uptake (e.g. Litchfield and Nguyen 2008). Agreement among P (APA, P-debt, and POC:PP ratios) and N (N-debt and POC:PN ratios) status indicators in Lake Erie was also reported by Rattan et al. (2012). The patterns of nutrient deficiency and sufficiency found in the present study are therefore likely to be meaningful, but a more extensive study with greater temporal and spatial resolution would be needed to draw conclusions about how N or P supply may have played a role in the cyanobacterial bloom of late summer 2011. The current study was designed more to evaluate the potential of variable fluorescence as an indicator that would allow the kind of spatial and temporal intensity needed to assess nutrient controls on phytoplankton in large lakes.

2.4.2 Within and among instrument variations of Fv/Fm

It is important to characterize the range of variability that might be expected from each PAM instrument, so that apparent inconsistencies among instruments can be assessed better. In this study, the CV for replicate measurements was usually $\leq$20%, considerably less than the variability between fluorometers and much less than the overall range of Fv/Fm observed. The disagreement between instruments appears to derive from genuine inter-instrument differences.

The PAM instruments examined here clearly did not give identical results for Fv/Fm. The causes may lie in the slight differences of optical configuration, differences in the sample preparation protocol, and interactions of both factors with the variable taxonomic composition and physiology of natural communities. Principle Components Analysis (PCA), one-way ANOVA and comparisons of coefficient of variation (CV) ranges all showed that the DivingPAM provided Fv/Fm estimates very different from, and uncorrelated with, those from WaterPAM or PhytoPAM. DivingPAM Fv/Fm did not correspond to either PhytoPAM or WaterPAM Fv/Fm. A possible reason for its differences may lie in the way samples must be concentrated on a filter to be measured. The delivery of saturation pulses and measurement of fluorescence may be affected by the filter and by interactions with

phytoplankton cells, detritus, and the filter itself that scatter or refract the measuring light and the fluorescence. The use of blanks might have partially corrected for scattering from the filter but cannot account for scattering geometry if the two filters may not have been exactly the same, and cannot account for detritus accompanying the samples. There also remains the possibility that the act of filtration to concentrate the phytoplankton onto a filter actually lysed or otherwise damaged some cells (e.g. Goldman and Dennet 1985, Fahnenstiel et al. 1994, Taylor 2010), decreasing what would have been the phytoplankton fluorescence signal and increasing the detrital fraction. There were comparably more flagellates, which are more fragile and easily damaged, in Lake Erie than diatoms or Cyanobacteria in the summer of 2011 (S. Watson, *personal communication*) so this may have served to influence the DivingPAM Fv/Fm. Fv might be affected as a result, as Fm might decrease and/or Fo might increase. This may explain in part why the DivingPAM Fv/Fm values were systematically lower than the values given by the WaterPAM and PhytoPAM. Filter blanks saturated with filtered lake water would not properly control for the possible accumulation of detritus and/or damaged cells arising during the sample filtration.

The sample filtration process needed for Diving PAM is the most obvious source of potential differences between instruments, but the differences between the other two PAMs show that it is not the only factor involved. Sample preparation for Water PAM and PhytoPAM was identical, indicating that differences between those instruments, and possibly some of their differences with DivingPAM, may be related to differences in optical arrangements or excitation protocols. One of the biggest differences is the use of white light for saturation by DivingPAM, but red light (of slightly differing peak wavelength) by the other PAMs. There was no clear evidence from the fluorescence saturation kinetics that we failed to attain a good estimate of Fm with any instrument so this particular difference may not be important, but it is conceivable that rapid induction of quenching by high-intensity white light may contribute to the systematically low Fv/Fm estimates by Diving PAM. The

more subtle differences of saturation irradiance wavelength between WaterPAM and PhytoPAM may seem less likely to produce differences in Fv/Fm, but some idea of the potential might be given by comparisons among the Phyto PAM diodes. While the differences between diodes pertain to measuring and not saturation light, they can help indicate how inter-instrument differences may arise from differences in spectral light-harvesting and energy transfer in natural samples of phytoplankton.

The PhytoPAM 645nm channel was chosen as a point of comparison between the PhytoPAM and both DivingPAM and WaterPAM because it was most similar to the 650 nm measuring light peak emission used by the other instruments. The present results indicated, however, that there was no significant difference in average Fv/Fm among any of the PhytoPAM diodes. This suggests that measuring light, and perhaps saturation light wavelength, was not a strong determinant of the measured Fv/Fm for the samples encountered in the present study. However, the degree of difference from Water PAM did differ among diodes, and the 665 nm diode gave Fv/Fm that was not significantly lower than from Water PAM. The relatively small differences in Fv/Fm between WaterPAM and PhytoPAM may therefore derive partly from differences in measuring and, perhaps, saturation light. The large differences with Diving PAM are not likely to be explained by such differences in measuring or saturation light wavelengths.

The lower Fv/Fm from PhytoPAM, compared to WaterPAM, may alternatively or additionally be due to differences in excitation protocol. With Water PAM (as with Diving PAM) Fo is determined with repeated flashes of weak measuring light, followed by the saturation flash and determination of Fm. For good Fv/Fm estimates, it is important that measuring light flashes do not significantly reduce PSII. In practice, care must be taken to optimize the flash frequency and intensity. The sequence is similar with PhytoPAM except that the measuring light involves successive application of flashes of light of different wavelengths from the different diodes. Optimizing the measuring light settings requires sufficient intensity and/or frequency of flashes that

Fo can be determined even for the diode(s) providing the least fluorescence response. PhytoPAM measures spectral fluorescence of pigments deemed characteristic of certain phytoplankton groups and certain groupings of spectral fluorescence are used to infer the community composition of the phytoplankton sample (Jakob et al. 2005). For example, the PhytoPAM associates high fluorescence in the 520nm diode and low fluorescence in the 470nm diode to be indicative of diatoms (Walz manual). However, many of these "group-specific" pigments are also accessory pigments in certain phytoplankton groups and may help to channel incoming photon energy to the reaction centre of PSII (Jeffrey et al. 2011, Roy et al. 2011). Thus, the photosystem may receive slightly more than the optimum minimum light to probe for Fo (i.e. cause a reduction of the acceptor side of PSII), resulting in chl a fluorescence that is somewhere between Fo and Fm (Schreiber 2004, Falkowski and Raven 2007). If the reducing effect is not dissipated in the interval such that the donor side of PSII is re-oxidized between successive diode flashes, Fo will be overestimated and Fv/Fm consequently underestimated.

Differences in optical configurations and excitation protocol may interact with variations of community composition to diminish the strength of inter-instrument correlations and possibly contribute to systematic disagreement. The particular arrangement of light harvesting and accessory pigments, configuration of light harvesting complexes (LHCs), and proximity of PSII to other components of the electron transport chain in the chloroplast varies among algal groups. Cyanobacteria, notably, do not have organelles. The photosynthetic electron transport chain is associated with thylakoids that are continuous with the plasma membrane (Falkowski and Raven 2007). Further, Cyanobacteria Fv/Fm may be artificially depressed following dark adaption as a result of overlapping phycobiliprotein fluorescence contaminating the chl a Fo signal, which tends to increase Fo and decrease Fv/Fm (Campbell et al. 1998). Small differences in the wavelengths of light allowed to reach the detector due to differences in the long-pass filter cutoff may also influence the

amount of phycobilin fluorescence, contributing to the fluorescence measured as Fo. These and many other factors may contribute to the lower Fv/Fm typical of Cyanobacteria and to community-level variations of Fv/Fm dependent more on taxonomic composition than nutrient status (Suggett et al. 2009). Differing sensitivity of different PAM instruments to different taxonomic components of the community, particularly Cyanobacteria, could thus account for some differences in measured Fv/Fm values. The limited degree of difference among the results from the different PhytoPAM diodes suggests this was not a dominant effect in our study, but it may have helped weaken correlations among instruments.

2.4.3 Variable Fluorescence to diagnose nutrient status

Not all phytoplankton taxa, or even individual cells, would be expected to have the exact same magnitude of nutrient deficiency or sufficiency. This may result in different community-level variable fluorescence responses, which may partially explain the variability associated with measures of Fv/Fm on natural samples. Rattan et al. (2012) used a Diving PAM to demonstrate strong negative correlations between Fv/Fm and P status (P-debt and APA) and N status (N-debt) in Lake Erie phytoplankton samples. In the present study, Diving PAM was the only instrument to show signs of such correlations. The correlation was strongest with P debt, but was also significant with APA if one suspected outlier was removed from analysis. Estimates of Fv/Fm by Diving PAM were not correlated significantly with N debt, and estimates from the other instruments were either not correlated, or were positively correlated, with P and N status indicators. While the present results do provide some confirmation of the findings of Rattan et al. (2012), they raise serious questions about the basis for the reported correlations between Fv/Fm and nutrient status. They also raise doubts about the applicability of Fv/Fm as a measure of nutrient deficiency in lake phytoplankton using PAM fluorometry. Results obtained here with WaterPAM and PhytoPAM were consistent with recent

findings that Fv/Fm is not characteristically or severely diminished in phytoplankton communities of P limited lakes (Harrison and Smith 2012), possibly because the phytoplankton are adapted to the limitation (Parkhill et al. 2001).

The DivingPAM Fv/Fm values were positively associated with high-biomass sites in the principal components analysis (PCA) and correspondence analysis (CA), and in particular the high biomass of river mouth and West Basin sites. The Fv/Fm values were directly correlated with Chl a concentrations among sites, while P debt and APA were negatively correlated. A parsimonious explanation may be that phytoplankton at the less P-deficient, high biomass, sites are less prone to damage when filtering samples for presentation to the Diving PAM, or that interference by detritus concentrated or generated in the procedure has less effect where Chl a biomass (and thus signal strength) is higher. The high biomass may have been due to an abundance of N-fixing Cyanobacteria, which are associated with a low N:P ratio (Kalff 2002), but without concurrent preserved samples to compare against these data, this is only speculative. This line of explanation may be supported by the observation that N debt did not correlate with Chl a among samples and Fv/Fm was, in turn, not correlated with N debt. In this view, Fv/Fm by Diving PAM was not measuring physiological differences related to P status so much as changes in phytoplankton biomass (and perhaps composition) associated with variations of P supply.

While N-debt did not associate significantly with any of the PAM instruments at $\alpha=0.05$, there was also a relatively small number of clearly N-deficient sites. Kendall association was chosen specifically to minimize the effects of a highly heterogeneous dataset (Kendall 1938), but it is possible that the extreme rarity of N-deficient sites (N-debt > 0.15μmolNμg $chla^{-1}$) still impacted the analysis, leading to the weak non-significant relationship. Visualization with scatterplots suggested that there might be some sort of non-linear relationship between the DivingPAM outputs and N-debt but it could not be established with the present data. A larger dataset reflecting more instances of N-

deficiency might clarify a relationship. Studies in marine systems with prevalent N limitation have, however, also failed to document strong and consistent depression of Fv/Fm that could be reliably traced to N deficiency (Olaizola et al. 1996).

Kendall associations tend to give more moderate relationships than either Pearson's r or Spearman's ρ. However, it seems likely that the absence of negative correlations between Fv/Fm measured by either WaterPAM or PhytoPAM and nutrient status is also a reflection of the number of influences on variable fluorescence and the physiological adaptability of phytoplankton in responding to environmental stressors. Additional influences include taxonomic differences (e.g. Suggett et al. 2009), and prior light exposure by UV (Harrison and Smith 2009) and PAR (Vassiliev et al. 1994, Sugget et al. 2009). It has been noted by other workers that phytoplankton adapted to a low-nutrient environment do not necessarily display evidence of impaired Fv/Fm (e.g. Parkhill et al. 2001; Kruspkoff and Flynn 2006) unless driven into relatively extreme, non-steady state, deficiency (Parkhill et al. 2001, Beardall et al. 2001b). It has been argued that the natural environment may pose multiple stresses that limit the ability of phytoplankton to maintain high Fv/Fm in the face of nutrient limitation (Rattan et al. 2012), even in approximately steady state situations. However, the present results are more consistent with evidence that, if measured with instruments specifically designed for work with natural communities at typically low Chl a concentrations (i.e. Water PAM and PhytoPAM, or fast repetition rates fluorometers), phytoplankton do tend to maintain Fv/Fm at a high level even when independent measures show that nutrient deficiency is strong (e.g. Harrison and Smith 2012).

The Diving PAM, and some other fiber optic PAMs used previously on phytoplankton (e.g. Marwood et al. 2000), were not originally designed for use on such low-concentration suspensions. It may not be a surprise that it seems to give anomalously low Fv/Fm values, although it has not previously been obvious that the values were out of the expected range (Marwood et al. 2000). The

evidence here that Water PAM and PhytoPAM also differ in their estimates (though much less dramatically) shows the need for caution in assessing and comparing results obtained by different models of PAM. Not explored here is the possibility that even different individual instruments of the same model may differ in their results, as recently shown for one type of spectral fluorometer (Twiss 2011). Pending further study, inter-calibration is advisable whenever an investigation involves use of data from multiple instruments.

Chapter 3

Use of spectral and variable fluorescence nutrient-induced fluorescence transients (NIFTs) to detect P-deficiency between phytoplankton groups

Nutrient-induced fluorescent transients (NIFTs), a pattern of Chl a fluorescence dynamics observable in nutrient-deficient phytoplankton when the limiting nutrient is added, may be a useful diagnostic to detect nutrient deficiency in phytoplankton. In this study the NIFT method was evaluated using the variable fluorescence ratio (Fv/Fm) as the observed fluorescence property and spectral fluorescence to discriminate the response of algal pigment groups. The pulse amplitude modulated fluorometer PhytoPAM was used to test the hypothesis that the cyanobacterium *Microcystis aeruginosa* (Kutz.em.Elenkin) and the diatom *Asterionella formosa* (Hass.) could be reliably discriminated and would show Fv/Fm dynamics (NIFT) diagnostic of P deficiency in mixtures and monocultures. In mixtures, the two taxa were correctly discriminated and a replicable pattern of Fv/Fm suppression followed by stimulation within 1h was observed in P-deficient *M. aeruginosa* when spiked with P. The pattern was not observed in controls, samples spiked with a non-limiting nutrient (N), or P-sufficient cultures. P-deficient *A. formosa*, by contrast, did not display dynamics of Fv/Fm that were specific to addition of P. The dynamics of Fv/Fm in both taxa were different in monocultures than in mixtures, possibly reflecting differences in severity of P deficiency. The results suggested that NIFT of spectrally-resolved Fv/Fm may provide a basis for inferring cyanobacterial, or at least *M. aeruginosa*, P status in mixed samples, but additional work with cultures of carefully-controlled condition is needed to identify the sources of variability in the observed kinetics.

3.1 Introduction

Water quality monitoring necessitates the development of consistently fast, accurate measuring tools. The use of variable fluorescence to track phytoplankton health has been one such avenue. Phytoplankton are a good candidate for water quality monitoring, as they are one of the bases of aquatic food webs (Kalff 2002), respond readily to external nutrient dynamics (Harris 1986), and are likely to be the only organisms in pelagic systems that contain chlorophyll a. Thus, phytoplankton variable fluorescence is a prime candidate to assess lake eutrophication and water

quality, as pertaining to the abundance, diversity and composition of potentially problematic phytoplankton. One such problematic taxon is the bloom-forming Cyanobacteria *Microcystis aeruginosa*, of which some strains produce the hepatotoxin microcystin (Rinta-Kanto et al. 2009). The development of spectral variable fluorescence measuring protocols, such as on the pulse amplitude modulated (PAM) fluorometer PhytoPAM, is an important contribution to understanding the nutrient aspect of *Microcystis* spp. bloom formation and cessation. This laboratory-based study makes some important first steps to developing monitoring strategies with this instrument.

There are physiological differences in nutrient requirements between broad phytoplankton taxa lines, however (Rhee 1978, Rhee and Gotham 1980, Tilman et al. 1982, Wynne and Rhee 1986). The Redfield ratio of 16:1 for nitrogen (N) and phosphorus (P) respectively is rarely encountered in freshwater phytoplankton (Arrigo 2005, Sterner et al. 2008). Furthermore, different groups vary in their intracellular ratios (Arrigo 2005). Thus, some groups will have an advantage depending on the current ambient nutrient availability. Cyanobacterial dominance correlates with high-P (eutrophic) conditions (Smith 1983, Watson et al. 1997, Niemisto et al. 2008) and they may require less P than chlorophytes (Millie et al. 2009 and references therein) and likely other groups as well. Diatoms are associated with the spring and fall blooms, which may reflect their higher nitrogen and silica requirements (Teubner and Dokulil 2002) so it would not be surprising to see them outcompeted by Cyanobacteria whose presence has been associated with a low N:P ratio (Watson et al 1997) and some of which can fix atmospheric dinitrogen, during summer stratification (Kalff 2002).

The current analytical methods for nutrient status, while informative, are often also time consuming and laborious. With the exception of a few specialized assays (e.g. fluorescence-labeled enzyme activity (FLEA) of alkaline phosphatase) (Gonzalez-Gil et al. 1998), all the phytoplankton nutrient status assays have resolution only to the community level. For the purposes of understanding Cyanobacterial blooms, which are of great concern to water quality in some parts of the Laurentian

Great Lakes (especially Lake Erie), this is not very informative for understanding the causal mechanisms behind these bloom formations, especially if nutrient (P) availability plays a significant role.

Variable fluorescence of chlorophyll a (chl a) has been proposed as a potentially useful tool for assessing phytoplankton nutrient status. It is the difference between maximal (Fm) and minimal (Fo) fluorescence normalized to the maximal fluorescence of a dark adapted sample (Falkowski and Raven 2007). Measurement at the community level (i.e. with non-spectral PAM fluorometers like WaterPAM, e.g. Harrison and Smith 2011) suffer the same limitation as most nutrient status assays in providing information only at the community level, and do not provide insight into the condition of a taxon of particular interest, such as *Microcystis*. Variable fluorescence changes have been tied to phytoplankton nutrient deficiency (e.g. Cleveland and Perry 1987, Kolber et al. 1988, Geider et al. 1998), but may be most informative if the system is perturbed by introducing the limiting nutrient back into the system (e.g. Parkhill et al. 2001, Beardall et al. 2001a, Holland et al. 2005, Petrou et al. 2008, Roberts et al. 2008). This is the basis for the "nutrient-induced fluorescent transient (NIFT)" (Beardall et al. 2001a, b). The NIFT is based on the idea that Fv/Fm is proportional to the quantum yield of photochemistry (Φ p) (Falkowski and Raven 2007). Φp can also be measured as amount of carbon fixed or oxygen produced (Falkowski and Raven 2007). However, nitrogen and phosphorus uptake and assimilation also require the energy (ATP) and reductant (NADPH), which is taken from the products of the chloroplast electron transport chain (Beardall et al. 2001b). Nutrient deficient cells have a higher initial rate of uptake of the limiting nutrient when it is provided compared to nutrient sufficient cells (Tilman and Kilham 1976, Kilham et al. 1977, Hameed et al. 1999), and so more of these ATP and NADPH molecules are expected to be shuttled away from carbon fixation and toward nutrient uptake and assimilation (Beardall et al. 2001b). Thus, Φp temporarily decreases, and Fv/Fm reflects this. However, nutrient re-uptake does slow down again, and Fv/Fm slowly rises.

This transient has been observed to occur on relatively short time scales (i.e. ≤1h), making it a useful assay for water quality measurements. There is now scope to extending this approach to the level of algal pigment groups using spectrally resolved variable fluorescence (Jakob et al. 2005).

However, the NIFT is a relatively new technique, and does not have the same body of data to support its use as do the traditional nutrient status assays. There are quite a few studies though (e.g. Moon and Carrick 2005, Sylvan et al. 2007) that have made use of bioassays. Simply, nutrients are added to a water sample and incubated for 24h. The difference in biomass measured as chl a is indicative of sufficiency or deficiency for the particular added nutrient (Lin and Schelske 1981, Moon and Carrick 2007). Specific to variable fluorescence, Fv/Fm is expected to be a larger value following the incubation (Wood and Oliver 1995, Beardall et al. 2001a, Sylvan et al. 2007), since low Fv/Fm has been observed to correspond to nutrient deficiency in phytoplankton in some studies (Cleveland and Perry 1987, Kolber et al. 1988, but see also Kruskopf and Flynn 2006, Harrison and Smith 2012 for evidence to the contrary). Concentrations immediately after inoculation and after the incubation time can give an indication of nutrient status through nutrient debts (Tilman and Kilham 1976, Kilham et al. 1977). There are some methodological issues surrounding this, including the problem of bottle effects, which can severely influence and even skew the results. The current study seeks to assess both the short-term responses one would expect to see under the NIFT response, as well as longer term responses that one would expect to see with a bioassay.

The PhytoPAM could represent an exciting step forward in the use of variable fluorescence to detect nutrient deficiency and attribute it to the broad phytoplankton groups its deconvolution protocol assigns as “blue”, “green”, and “brown”, which correspond generally to the Cyanobacteria, Chlorophyta, and Heterokontophyta, respectively (Walz manual). Four extra light emitting diodes (λ= 470, 520, 645, 665nm in addition to the 650nm measuring light) in the measuring head probe for taxon-specific accessory pigments (Jakob et al. 2005) and the deconvolution to assign PhytoPAM

groups are heavily influenced by the reference spectra (Walz manual). These raw data are analyzed with the PhytoWin software and the most likely Fv/Fm for each group is estimated by a linear unmixing algorithm (MacIntyre and Lawrenz 2011). However, the ability to attribute variable fluorescence to the correct group is only as good as its sorting algorithm (Jakob et al. 2005). A simplified model system of nutrient sufficient and deficient monocultures of *Microcystis aeruginosa* and *Asterionella formosa* (Fig. 3.2) was created to assess the NIFT technique on the PhytoPAM. The objectives were (1) to determine whether the PhytoPAM correctly discriminated the two phytoplankton groups in mixtures, and (2) showed a NIFT response specific to the nutrient status of the phytoplankton and addition of the limiting nutrient.

3.2 Materials and Methods

3.2.1 Culture Materials

Two uni-algal, but not axenic, cultures were used for this experiment: *Asterionella formosa* Hass. (CPCC 605) and *Microcystis aeruginosa* Kutz.em.Elenkin (CPCC 299). *Asterionella formosa* was maintained in full-nutrient Chu 10 medium, and *Microcystis aeruginosa* was maintained in full-nutrient BG-11 medium under ~50μmol PAR/m^2/s with a 14L:10D cycle at ~19°C. Once these cultures reached stationary phase as determined by previous students working with the same cultures, they were aseptically transferred in a laminar flow hood to new sterile media at a dilution of 1:10. Cultures required for experimentation were also transferred at this stage of growth.

3.2.2 Experimental Culture Procedures

The maintenance cultures were aspectically transferred into sterile medium and grown and maintained in sealed flasks separate from the experimental cultures, and were only unsealed for

transferring to prevent accidental inoculation with other algae from the air. The experimental cultures were also left sealed to prevent contamination from repeated openings. All cultures were gently swirled once a day to re-suspend them and redistribute nutrients. Previous work by others in the lab sampled the same cultures under the same conditions regularly to assess their biomass dynamics through exponential and stationary phase (*Asterionella formosa* – Milne 2011, Cater 2011; *Microcystis aeruginosa* – Reesor 2012, Holmes 2012). These assessments were done using *in vivo* chlorophyll *a* fluorescence estimates from the WaterPAM (Walz) fluorometer. When the chl *a* estimates began to level off, indicating entry into stationary phase due to P-deficiency (the only parameter that differed between high- and low-P cultures – see Appendix), the culture was either transferred or used in the experiments. Their results informed the timing of onset of P-deficiency in this setup to streamline the experimental process.

The experimental cultures were grown in two phases. Phase 1 was the "preconditioning" growth phase, and Phase 2 was the "experimental" growth phase. Full-strength media contained ~50μM-phosphorus (P), in the form PO_4^-, for the Chu10 medium and ~170μM-P for the BG-11 medium. The preconditioning phase was marked by media that contained moderately low, but not immediately growth-limiting, concentrations of phosphorus. In the case of Chu10, the medium contained ~1μM PO_4^- and in the case of BG-11, the medium contained ~3.4μM PO_4^-. This corresponds to a phosphorus concentration that is ~2% the original media concentrations. The cultures are able to grow in these media, but they became limited and reached stationary phase in their growth much sooner than in full strength medium.

Phase 2, the experimental phase, began when the Phase 1 preconditioning cultures became P-deficient. Preconditioned cultures were then transferred and scaled up, in triplicate, to either 1%-P (low-P) or 100%-P (high-P) medium. The final volume was ~500mL for each replicate (total volume ~1.5L). When the Low-P cultures went into stationary phase, both low- and high-P cultures were

checked for APA. Once confirmation of P-deficiency in the low-P cultures but not high-P cultures was obtained using the alkaline phosphatase assay (APA), the experimental procedure (detailed below) was implemented.

3.2.3 Nutrient Status Assays

Alkaline phosphatase (AP) is an inducible cell-surface enzyme that is typically only seen in large numbers in P-starved phytoplankton, although there are many other types of inducible enzymes (e.g. Whitton et al. 1991), which may be associated with extra- and/or intra-cellular phosphatase activity (e.g. White 2009). The alkaline phosphatase assay (APA; Healey and Hendzel 1979) was used for these experiments. Briefly, the low-P cultures were expected to become P-deficient much sooner than high-P cultures because of their much lower concentration of inorganic phosphorus (PO_4). As a result, these cultures were expected to induce AP in larger numbers in order to cleave the phosphate group off of organic molecules (e.g. pieces of phospholipid, nucleic acids, etc.) for their own growth. Cultures that contain excess amounts of inorganic PO_4 were not expected to induce this enzyme as it was not needed. The substrate used for APA was 3-O-methylfluoresceine phosphate (3-OMFP). Phytoplankton expressing high levels of AP would cleave the phosphate from 3-OMFP to yield the highly fluorescent product 3-O-methylfluoresceine (3-OMF). Concentration of liberated 3-OMF was calculated from a standard curve of known 3-OMF standard concentrations. The rate of particulate 3-OMFP to 3-OMF hydrolysis at 35°C was normalized to chl *a*. Rates ≥0.003μmol 3-OMF/μg chl *a*/h are indicative of deficiency and rates ≥0.005μmol 3-OMF/μg chl *a*/h indicates severe deficiency.

3.2.4 Experimental Procedure

The underlying theory for the experimental setup and protocol comes from the previously observed phenomena of a transient dip in fluorescence immediately following addition of a limiting nutrient, the nutrient-induced fluorescent transient (NIFT) (e.g. Turpin and Weger, 1988; Beardall et al. 2001b) (Fig. 3.1). The following method is novel because it assesses the short-term aspect of nutrient-addition perturbation (i.e. the NIFT response) on phytoplankton chl *a* variable fluorescence, as well as the long-term (24h) responses (e.g. bioassays). Previous studies have largely focused on one of either of these two responses to nutrient re-supply, but not both.

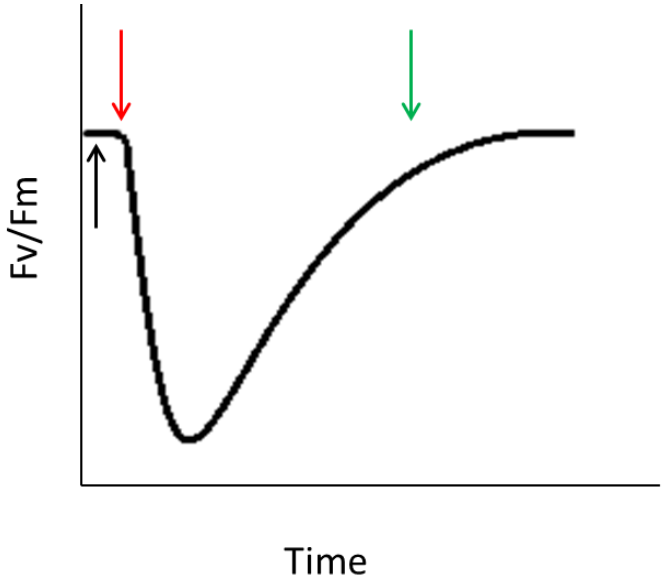

Figure 3.1 Typical nutrient induced fluorescent transient (NIFT) kinetic curve. Black arrow refers to Fv/Fm before amendment with the limiting nutrient. Red arrow indicates spiking with the limiting nutrient. Green arrow indicates recovery of Fv/Fm to (near) pre-amendment levels. Time is on the order of seconds to minutes.

Two pulse amplitude modulated (PAM) fluorometers were used in this study: the PhytoPAM and the WaterPAM. The PhytoPAM was used to assess if it can correctly distinguish which group,

"blues" (the Cyanobacterium *Microcystis aeruginosa*) or "browns" (the diatom *Asterionella formosa*), is P-deficient; this was the main objective of this experiment. However, characterizing the typical fluorescence response of each to nutrient re-supply in each of the two phytoplankton types was first needed. Therefore, the WaterPAM, which measures community response, was used on *M. aeruginosa* and *A. formosa* monocultures to determine this typical response.

3.2.4.1 Monoculture measurements

The same procedures were used for both *M. aeruginosa* and *A. formosa* monoculture readings on the WaterPAM and then again on the PhytoPAM. All procedures involving the WaterPAM or PhytoPAM were run in very dim light, too dark to stimulate photosynthesis, and therefore would not have influenced the variable fluorescence readings. The three replicates for the high-P cultures were mixed together, as were the replicates for the low-P cultures. Half of the volume (~750mL) from each nutrient treatment was reserved for single-culture readings on the PhytoPAM. From the remaining 750mL, 200mL aliquots were placed in acid-washed containers (3x200mL for low-P and 3x200mL for high-P) and dark adapted for 30 minutes. Previous work on these lab cultures has shown that there is high enough biomass in them to make any background fluorescence essentially negligible and produced values identical to values measured on distilled water at the low gain setting needed to measure their variable fluorescence. As a result, distilled water was sufficient to be used as a blank to correct the gain. After 30 minutes, each aliquot was thoroughly, but gently, mixed to re-suspend any phytoplankton that had settled, and run through the WaterPAM and Fv/Fm was measured.

Following this initial reading, one of the three replicates for each high- and low-P treatments were spiked with either Barnstead (ultrapure) water, 50µM (final concentration) NH_4^+, or 50µM (final concentration) PO_4^-. The water and the ammonium additions were controls: the water to ensure that

the act of liquid addition did not affect the NIFT, and the ammonium to ensure that addition of any non-limiting nutrient did not non-specifically induce a NIFT-like response. Each replicate was spiked and gently agitated to distribute the spike. Immediately after, a subsample was withdrawn for an Fv/Fm reading on the WaterPAM. The elapsed time between spike and reading was approximately 30s. The spikes were delivered in the following order: Barnstead control, N-control, and P-treatment to the low-P cultures and then to the high-P cultures. After this initial spike and read, the cultures were again subsampled and their Fv/Fm measured on the WaterPAM in the same order they were spiked and read initially. This continued for the first hour. After one hour, Fv/Fm was again recorded. This was replicated after all the treatments had been run through the first time. Fv/Fm was again measured twice for each treatment after 2h, 6h, 12h and 18-24h after spiking. PhytoPAM measurements used the same spiking and reading protocol, and were taken almost concurrently, with only a 3h delay. This was necessary, because it was not possible to do the high-frequency sampling in the first hour on the WaterPAM and PhytoPAM at the same time.

3.2.4.2 One-sample Re-suspensions

Because the mixed culture experiments required gentle filtration in order to re-suspend the two different cultures in a new medium, one-culture re-suspensions were necessary to ensure that neither the *M. aeruginosa* nor the *A. formosa* culture would react negatively to the process, thus skewing the two-culture NIFT experiment. One-culture re-suspensions were used so that any effect seen could be directly attributed to the particular alga. 200mL triplicate subsamples of low-P and then high-P culture were filtered under low vacuum onto 20μm polycarbonate filters in the case of *Asterionella formosa* and onto 5μm filters in the case of *Microcystis aeruginosa*. These conditions were chosen so that the culture medium could be drained off quickly with as little mechanical disruption to the cells as possible while retaining the majority of the culture on the filter. These filters

were then gently agitated in new, sterile, P-free medium of the same type (Chu10 for *A. formosa* and BG-11 for *M. aeruginosa*) to re-suspend the cells, and immediately dark adapted for 30 minutes then read at intervals up to 24h following re-suspension. Because of the very short time of dark adaption, P-deficiency could not have set in in the high-P cultures and the low-P cultures would not experience a NIFT response associated with competition between the uptake and assimilation pathways and photosynthetic dark reactions (Calvin cycle). Due to evidence from that Fv/Fm typically was affected within ~2h of these re-suspension experiments, the mixed culture experiments were modified so that the re-suspended culture could adapt to a new medium in dim light for a minimum of 1.5-2h before they were dark adapted for mixed culture NIFT experiments.

3.2.4.3 Mixed Cultures

Once it had been established that re-suspension would not have such a large effect on the variable fluorescence measurements in the context of the measurement experiments, *M. aeruginosa* and *A. formosa* cultures were mixed together in a 2x2 factorial design (Fig 3.2), re-suspended in appropriate P-free medium, and then the NIFT experiment conducted, using the protocols described above. That is, low-P *M. aeruginosa* (MaLP) cultures were mixed with 1) low-P *A. formosa* (AfLP) and 2) high-P *A. formosa*(AfHP) and that high-P *M. aeruginosa* (MaHP) cultures were mixed with 3) AfLP and 4) AfHP.

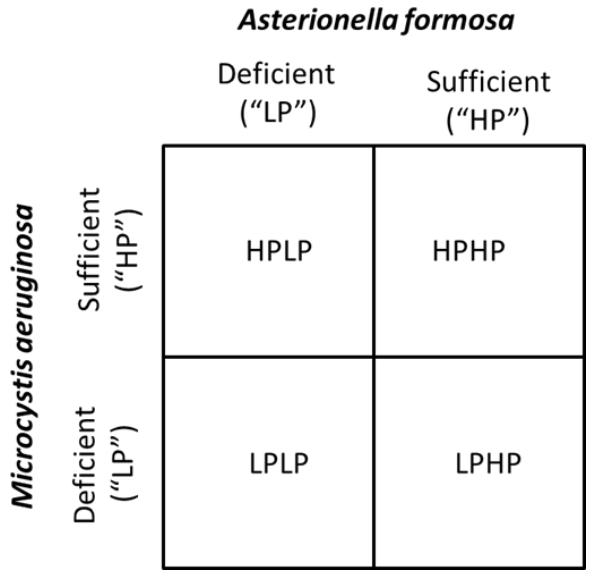

Figure 3.2 2x2 factorial design of mixture experiments. First two letters refer to *M. aeruginosa*'s P-status, and the second two letters refer to *A. formosa*'s P-status.

The specific method for determining re-suspension volumes are as described below. Each low-P and high-P culture was measured on the PhytoPAM where the mean gain was between 300 and 500 units and there was good separation between the four channels. Fluorescence (Ft) was noted for each culture. The high-P cultures had higher chl a fluorescence than the low-P cultures, and *Microcystis aeruginosa* tended to have higher biomass than *Asterionella formosa*. When the mean gain was not the same for the four cultures, a median gain value was selected. For example, if the gains for the four cultures were 5, 7, 8, and 9, then a "compromise" gain of 7 was chosen. Ft was then measured again for each of the cultures at this compromise baseline. The Ft values at this gain were then used to determine the approximate volume that must be filtered and re-suspended in 100mL of P-free BG-11 for *M. aeruginosa* and Chu10 for *A. formosa* to yield an approximate 50:50 fluorescence ratio. In all instances, the culture with the greater biomass was filtered, which operationally, was the high-P cultures.

To demonstrate this, the high-P culture of *M. aeruginosa* (MaHP) had an Ft value of 1485 at gain 5 and an Ft valued of ~2800 at gain 7. The low-P *A. formosa* (AfLP) culture had an Ft value of 1203 at gain 8 and an Ft value of 878 at gain 7. MaHP had approximately three-times the amount of Ft at the median gain 7. As such, the volume filtered (30mL) was approximately three-times less than the 100mL volume of the AfLP culture. This 30mL filtered sample was re-suspended in 100mL of P-free BG-11 medium and combined with 100mL of AfLP. Any extra P retained with the filtered cultures was diluted down to very low levels equal to or less than the low-P culture's P content. This was done to ensure that a pre-mature NIFT response would not occur.

3.2.5 Statistical Analysis

T-tests were conducted to determine if the Fv/Fm from the N- and P-amended was different from the Fv/Fm from the DIW control. This was done by subtracting the Fv/Fm values for all treatments at a given timepoint by the Fv/Fm of the control. Thus, the difference in N-treatment compared to control was expected to not be statistically differentiable from zero, but the P-treatment was expected to be statistically different from zero at timepoints that were chosen because there appeared to be significant differences when plots of these differences from control were assessed visually. Differences from the control were attributed to Fv/Fm changing as a response to nutrient amendment and needed to be formally tested to determine if the difference was statistically significant, and that the values actually fell outside the natural range of variability.

3.3 Results

Fv/Fm was measured prior to nutrient amendments for NIFT experiments and for both the *M. aeruginosa* and *A. formosa* cultures the P-deficient (LP) cultures showed a depressed Fv/Fm compared to the P-sufficient (HP) cultures (Table 3.1). The difference was slightly more pronounced between the LP and HP cultures in *M. aeruginosa*, however. The depressed (group-specific) Fv/Fm in the LP cultures was still apparent even in the mixtures with another LP or HP *M. aeruginosa* or *A. formosa* culture. However, the degree of depression for any of the cultures in the monoculture or mixture experiments differed. The difference in Fv/Fm values between the N-amendment or the P-amendment and the DIW control were plotted against time in the monoculture NIFT experiment (Fig. 3.3).

The points reflecting N-amendment were not expected to differ greatly from the y=0 line (meaning that the Fv/Fm of the N-amendment was the same as the DIW-amendment). Response to P-amendment was expected to take on the characteristic NIFT response characterized by workers such as Turpin and Weger (1987) and Beardall et al. (2001a) and as described in the materials and methods. For P-deficient *M. aeruginosa* (MaLP) measured on the WaterPAM, there was some evidence of depression of Fv/Fm following spiking with P, followed by a slow rise in Fv/Fm up to 64min after spiking. MaLP also appeared to respond to N-amendment by the end of the short-term NIFT measurements as well, but this effect was not the same as the P-amendment response. P-deficient *A. formosa* (AfLP) did not respond to P-amendment in the same way as MaLP. Instead of a depression in Fv/Fm immediately following spiking, there appeared to be a stimulatory effect of P addition. The MaHP and AfHP (P-sufficient) cultures did not show a very large difference from y=0 when amended with N or P, although the "pre" Fv/Fm values and values up to ~10-15 minutes following spiking were slightly elevated for both the N and P treatments. The PhytoPAM responses

were less defined than the WaterPAM responses. There was less evidence of the depression and stimulation of Fv/Fm in MaLP in response to the P-amendment, although the other measures, at least visually, appeared to be about the same. For AfLP read on the PhytoPAM, there was some evidence of stimulation following spiking, but the pattern was not the same as the pattern seen on the WaterPAM.

Table 3.1 "Pre" APA, chla, and Fv/Fm for P-deficient and P-sufficient (a) *Microcystis aeruginosa* and (b) *Asterionella formosa*. Bolded letters refer to the Fv/Fm of the particular culture in the mixture.

a. *Microcystis aeruginosa*

	Mean APA (n=3)		APA ratio LP:HP	Mean chl a (n=3)			Fv/Fm P-deficient (LP)		Fv/Fm P-sufficient (HP)	
	LP	**HP**		**LP**	**HP**	**PAM**	**mean**	**SD**	**mean**	**SD**
Monocultures	0.00395 (0.001381)	0.000206484 (4.00771E-05)	19.13	73.2 (26.5)	1187.1 (291.4)	WaterPAM (monocultures)	0.219	0.0180	0.520	0.0121
						PhytoPAM (monocultures)	0.260	0.0100	0.587	0.00577
July experiments	0.002683 (0.000339)	0.000606 (8.11E-05)	4.426	266.1 (25.3)	289.7 (32.8)	July - **LP**LP	0.439	0.00770	n/a	n/a
						July - **LP**HP	0.490	0.00628	n/a	n/a
September experiments	0.001794 (0.000198)	0.000343 (3.41E-05)	5.225	300.1 (34.1)	625.6 (32.8)	Sept - **LP**LP	0.455	0.00988	n/a	n/a
						Sept - **LP**HP	0.311	0.00544	n/a	n/a
						July - **HP**HP	n/a	n/a	0.535	0.00396
						July - **HP**LP	n/a	n/a	0.538	0.00602
						Sept - **HP**HP	n/a	n/a	0.524	0.00471
						Sept - **HP**LP	n/a	n/a	0.515	0.00501

b. ***Asterionella formosa***

	Mean APA (n=3)		APA ratio LP:HP	Mean chl a (n=3)			Fv/Fm P-deficient (LP)		Fv/Fm P-sufficient (HP)	
	LP	**HP**		**LP**	**HP**	**PAM**	**mean**	**SD**	**mean**	**SD**
Monocultures	0.051435 (0.006698)	0.001338 (1.08E-05)	38.44	67.9 (10.9)	193.9 (5.7)	WaterPAM (monocultures)	0.491	0.00361	0.552	0.00306
						PhytoPAM (monocultures)	0.370	0.0000	0.513	0.00577
July experiments	0.098057 (0.056985)	0.001286 (0.000267)	76.23	43.7 (23.1)	162.0 (41.6)	July - LP**LP**	0.385	0.0093	n/a	n/a
						July - HP**LP**	0.322	0.0117	n/a	n/a
September experiments	0.095244 (0.018341)	0.001216 (0.000101)	78.30	24.5 (4.8)	171.9 (9.0)	Sept - LP**LP**	0.333	0.00473	n/a	n/a
						Sept - HP**LP**	0.300	0.00879	n/a	n/a
						July - HP**HP**	n/a	n/a	0.505	0.00356
						July - LPHP	n/a	n/a	0.483	0.00739
						Sept - HP**HP**	n/a	n/a	0.500	0.00221
						Sept - LP**HP**	n/a	n/a	0.539	0.00550

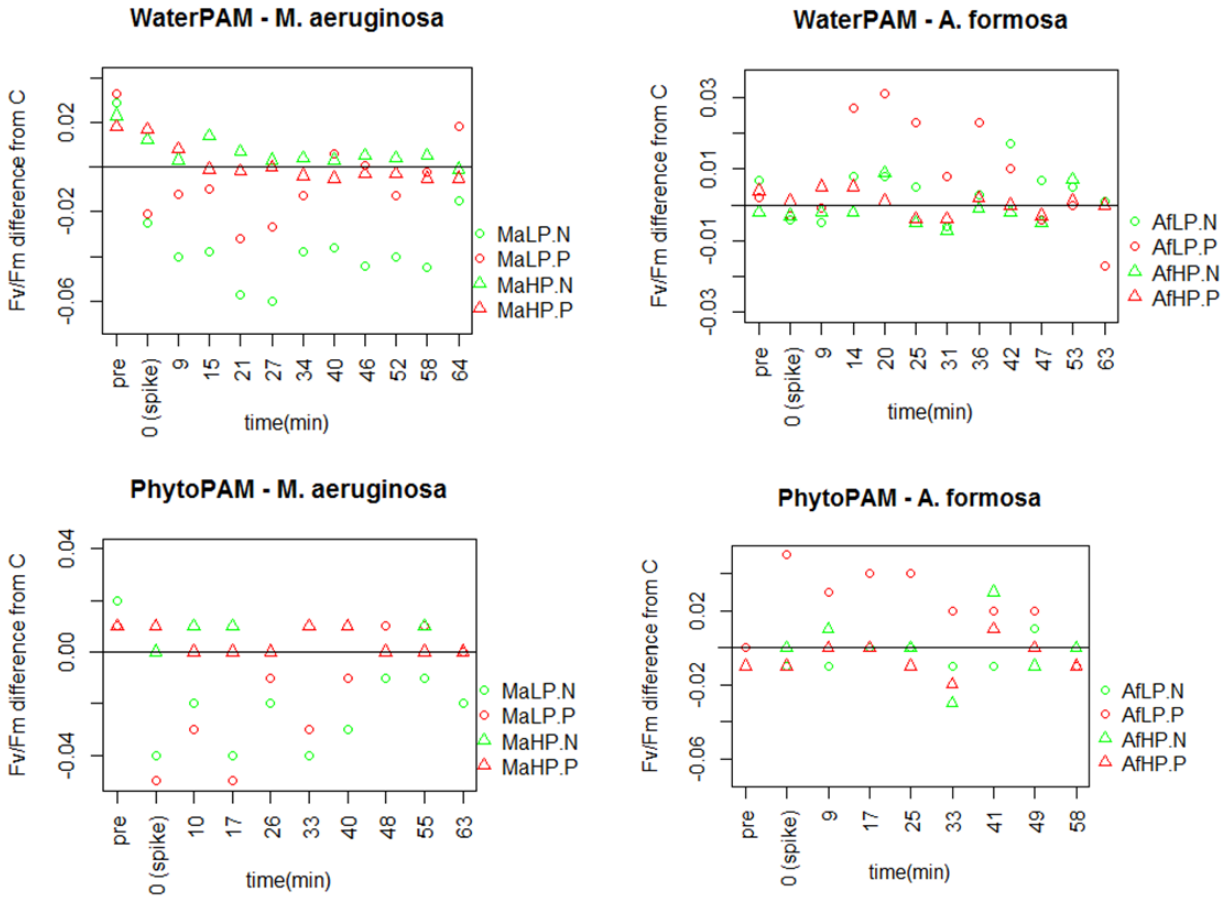

Figure 3.3 Difference in Fv/Fm from N- and P-amendments from control (y=0) (monocultures)

Fv/Fm was also measured on both instruments in the long term (Fig 3.4), but the PhytoPAM and WaterPAM outputs did not show very similar trends, nor did there appear to be any systematic differences between N- and P-amendment on the LP cultures compared to their HP counterparts. This was especially true for the P-treatments, although it appeared that N-treatments become stimulating later on. For AFLP amended with P (open red triangles), it seemed as though there was some stimulation by 24h following spiking, whereas all the other points were clustered on or near the y=0 line.

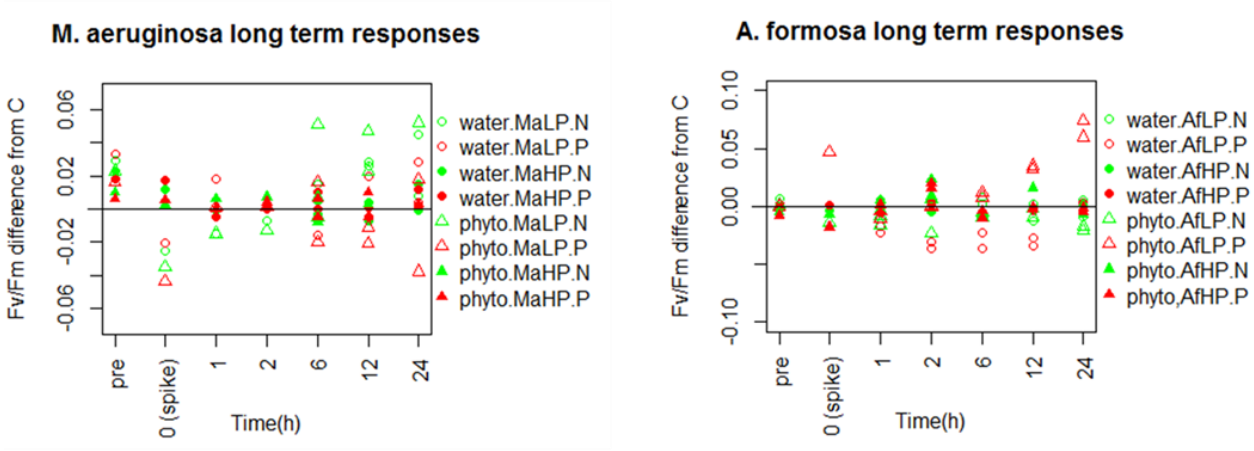

Figure 3.4 Differences in N- and P-amendment from control (y=0) in the long-term (monocultures)

Re-suspension seemed to affect Fv/Fm compared to control (un-re-suspended) values (Table 3.2), although the degree of change and the length of persistence in difference differed between cultures and their P-status. For MaLP, Fv/Fm initially differed between the re-suspension and the controls; however, by ~8h following re-suspension, the differences were fairly negligible. For AfLP, Fv/Fm was quite similar to start between the re-suspension and control, and did not change very much over the course of ~8.5h. MaHP yielded fairly similar Fv/Fm values between re-suspension and controls up to ~30-45min following re-suspension, although they did become more disparate with time. AfHP re-suspension and control values were fairly disparate to begin with, but the Fv/Fm values became more similar by ~8h.

Table 3.2 Comparisons of mean (n=3) un-re-suspended controls and re-suspensions. Standard deviations shown in brackets.

Time from first reading (h)	M. aeruginosa LP		A. formosa LP		M. aeruginosa HP		A. formosa HP	
	Control	**Re-suspension**	**Control**	**Re-suspension**	**Control**	**Re-suspension**	**Control**	**Re-suspension**
0	0.531(0.00208)	0.481(0.0212)	0.499(0.00208)	0.489(0.00551)	0.542(0.00416)	0.534(0.00557)	0.552(0.00252)	0.534(0.00436)
0.5-0.75	0.527(0.00289)	0.468(0.00919)	0.458(0.0609)	0.485(0.00300)	0.540(0.00346)	0.535(0.00611)	0.541(0.00493)	0.531(0.00306)
~8	0.457(0.00153)	0.473(0.0346)	0.487(0.00436)	0.485(0.00473)	0.419(0.00200)	0.386(0.01332)	0.521(0.00451)	0.524(0.00351)
20-24*	0.473(0.0255)	0.458(0.0148)	0.490(0.00473)	0.488(0.00872)	0.413(0.00231)	0.383(0.01250)	0.522(0.00208)	0.525(0.00200)

The differences of Fv/Fm from N-amendment or P-amendment and the control were also investigated in the short-term for the mixture experiments in July (Fig 3.5) and September (Fig 3.6). As with Fig. 3.2, it was expected that the HP cultures would not differ very much from the y=0 line, whereas the LP cultures amended with P would differ and the LP cultures amended with N would not. MaLP, regardless of whether it was mixed with AfLP or AfHP showed a depression in Fv/Fm following spiking with P and a rise to stimulation of Fv/Fm by ~0.75-1h following the spike. This trend was very clear, and rather different from the trend seen for MaLP amended with P in Fig. 3.3. The trend for AfLP was less clear, since it appeared as though both N and P were stimulating Fv/Fm. The HP cultures, as expected, did not deviate significantly from the y=0 line. There did appear to be a depression in the MaHP cultures treated with N. September experiment replicates reflected similar patterns. The only panel that reflected a clear NIFT-type of response was MaLP in response to P-amendment. Unlike the July replicate, the N-species used for this particular experiment was NO_3 instead of NH_4 and was used as the N-treatment for both *M. aeruginosa* and *A. formosa*. MaHP when treated with this form of N showed very little deviation from the y=0 line, and all treatments of *A. formonsa* (AfLP and AfHP) behaved in a similar manner regardless of the N-species.

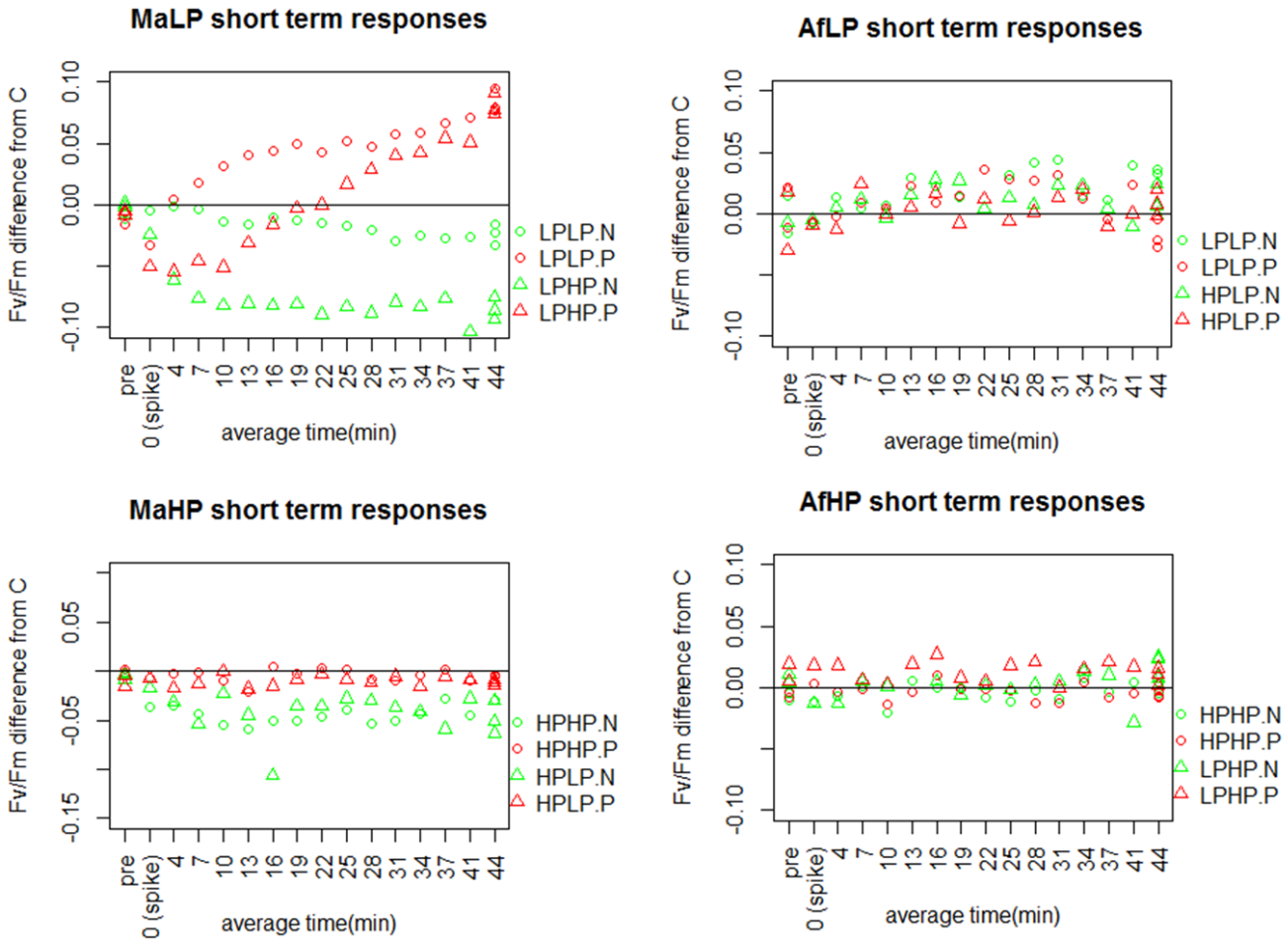

Figure 3.5 Fv/Fm differences between N or P treatments against control (y=0) in specific cultures from mixtures in July experiment replicate. First and last points are measures in triplicate.

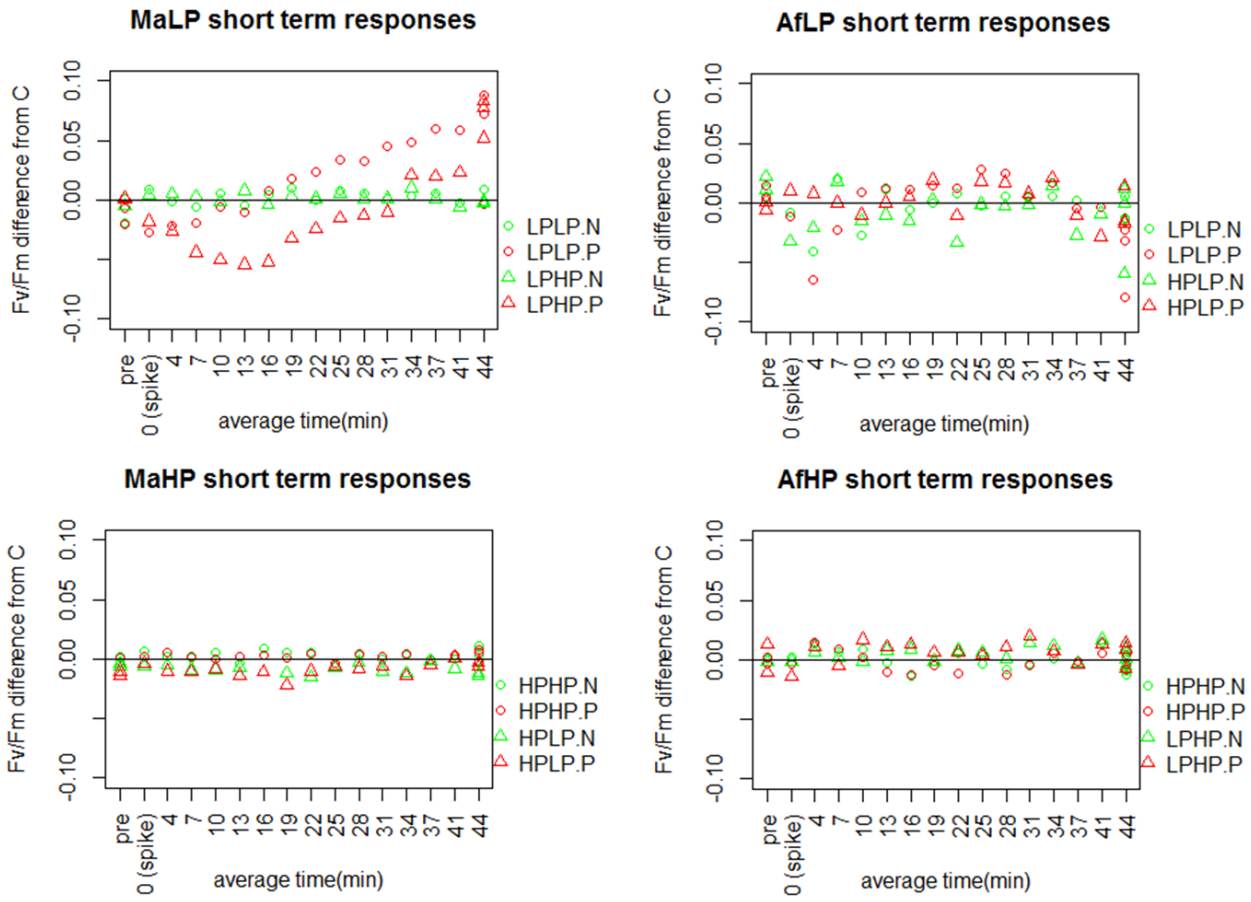

Figure 3.6 Fv/Fm differences between N or P treatments against control (y=0) in specific cultures from mixtures in September experiment replicate. First and last points are measures in triplicate.

T-tests were conducted on the mean of the third-last Fv/Fm points of each time-series measurements corresponding to ~30-40 minutes following nutrient amendments from Figures 3.5 and 3.6 (Table 3.3) because visual inspection suggested a significant effect of P-addition to MaLP. Fv/Fm of P-amended MaLP was significantly higher in the later stages than the control in both experiments and in the presence of either AfLP or AfHP. By contrast, Fv/Fm of MaHP was not significantly changed by P addition.

Table 3.3 T-test on difference of N and P treatment from control in (a) July and (b) September replicates. Means generated from the third-last points in each time-series measurement (e.g. the timepoints between ~35-40mins). Significant p-values ($p \leq 0.05$) are bolded.

a.

	mean	t-statistic	p-value
MaLP			
LPLP.N	-0.0260	-45.030	**0.000**
LPLP.P	0.0650	18.770	**0.003**
LPHP.N	-0.0873	-10.796	**0.008**
LPHP.P	0.0490	13.590	**0.005**
MaHP			
HPHP.N	-0.0390	-7.081	**0.019**
HPHP.P	-0.0040	-1.155	0.368
HPLP.N	-0.0427	-4.747	**0.042**
HPLP.P	-0.0100	-3.111	0.090
AfLP			
LPLP.N	0.0217	2.478	0.132
LPLP.P	0.0103	1.228	0.344
HPLP.N	0.0053	0.542	0.642
HPLP.P	0.0030	0.331	0.772
AfHP			
HPHP.N	0.0030	0.933	0.449
HPHP.P	-0.0030	-0.832	0.493
LPHP.N	-0.0017	-0.126	0.911
LPHP.P	0.0177	10.016	**0.010**

b.

	mean	t-statistic	p-value
MaLP			
LPLP.N	0.0020	0.961	0.438
LPLP.P	0.0557	14.481	**0.005**
LPHP.N	0.0017	0.360	0.753
LPHP.P	0.0213	24.190	**0.002**
MaHP			
HPHP.N	0.0010	1.000	0.423
HPHP.P	0.0020	1.309	0.321
HPLP.N	-0.0073	-2.524	0.128
HPLP.P	-0.0060	-1.377	0.303
AfLP			
LPLP.N	0.0017	0.640	0.588
LPLP.P	0.0027	0.372	0.746
HPLP.N	-0.0073	-0.618	0.600
HPLP.P	-0.0060	-0.418	0.717
AfHP			
HPHP.N	0.0047	0.992	0.426
HPHP.P	0.0030	1.500	0.272
LPHP.N	0.0087	1.442	0.286
LPHP.P	0.0053	1.071	0.396

The responses of the mixtures to nutrient amendment were also investigated over the long term up to 18-22h for both the July (Fig 3.7) and September (Fig 3.8) replicates. There was stimulation of MaLP Fv/Fm at 1h after P-amendment, but MaHP appeared to be stimulated in the long term as well. N-amendment with NH_4 in July resulted in a depressed MaLP Fv/Fm at 1h, but N-amendment with NO_3 in September did not. Both AfLP and AfHP Fv/Fm often overlapped at all timepoints regardless of amendment with P or N, even at 18-22h. There appeared to be some stimulation in Fv/Fm in response to P-amendment in the July replicate of AfLP culture mixed with the MaLP culture.

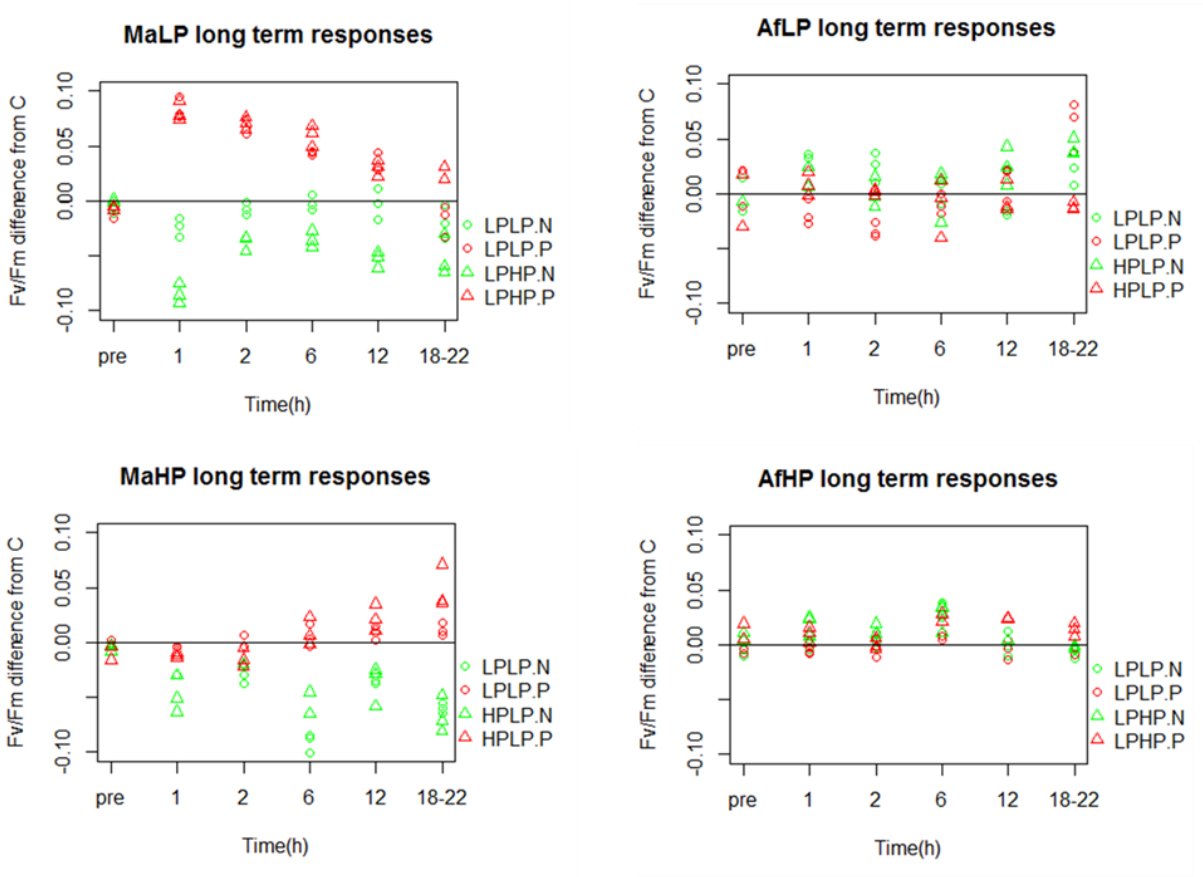

Figure 3.7 Fv/Fm differences of N and P treatments from control on mixtures in the long term for the July experiment replicate. Measurements were taken in triplicate.

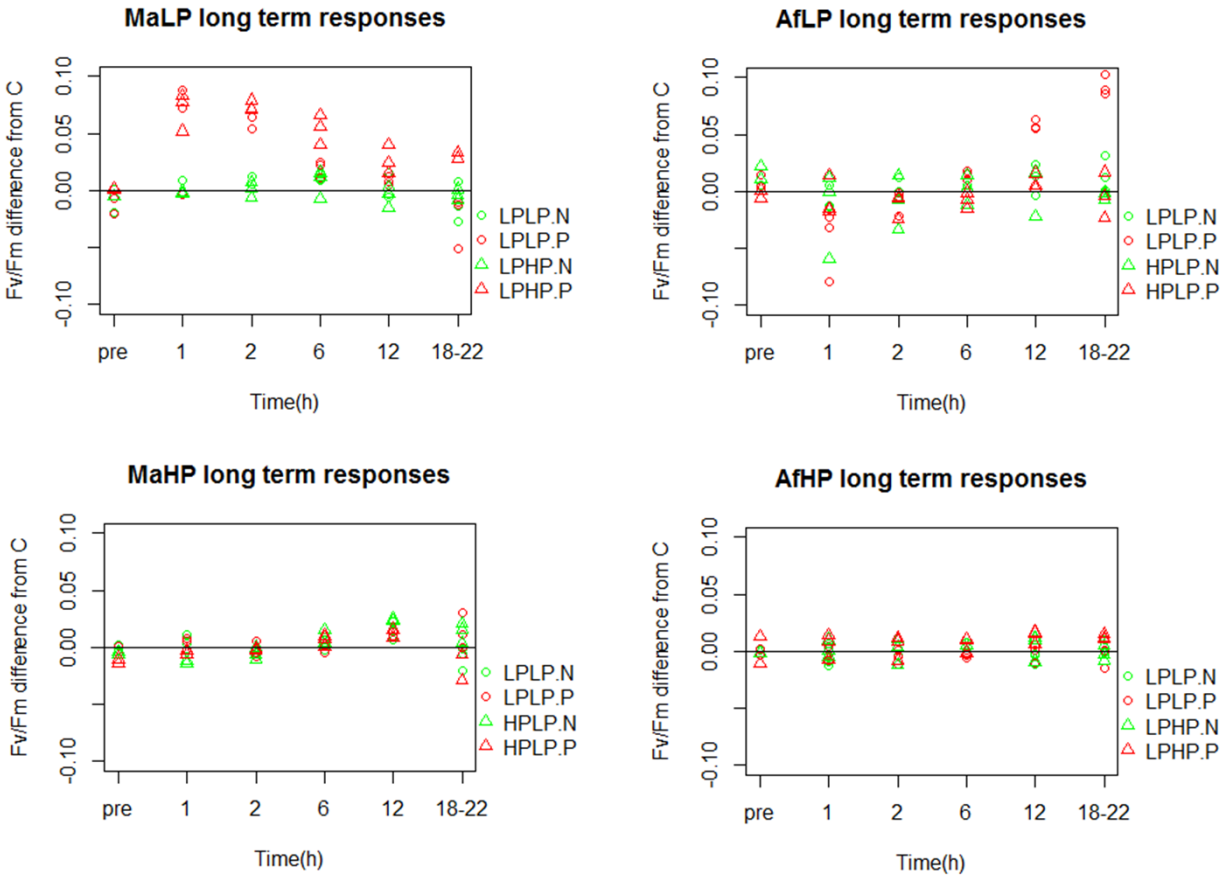

Figure 3.8 Fv/Fm differences of N and P treatments from control on mixtures in the long term for the September experiment replicate. Measures were taken in triplicate.

T-tests were again performed on the Fv/Fm differences of the N- and P-amendments compared to the control Fv/Fm for the measurements on both *M. aeruginosa* and *A. formosa* taken at 1h and at 18-22h after spiking (Table 3.4). Both the MaLP and MaHP cultures were significantly different from the control at 1h in the July replicate, but neither the AfLP nor AfHP were. At 18-22h following the spike, AfLP, except in the LPLP mixture, showed a significant difference from y=0 for both N- and P-amendments. However, AfHP in the HPLP mixture did show a significant difference from zero at this time, but did not show a difference in response to any other amendments. *M.*

aeruginosa in the LPHP and HPLP mixtures also showed a significant difference from y=0 at this time as well, but this difference was not seen in the LPLP and HPHP mixtures. The patterns in September were similar, though there were a few minor differences between these experiments and the July experiments. At 1h, MaLP was significantly different from control in the LPLP and LPHP mixtures when treated with P, but only the MaLP in the LPHP was significantly different. MaHP in the HPLP mixtures showed significant difference from control at 1h after spiking, but this pattern was not seen for MaHP in the HPHP mixture. AfLP and AfHP cultures in any of the mixtures did not differ significantly from the control. At 18-22h after spiking, only MaLP in the LPHP mixture showed a significant difference from control of all the cultures and mixtures. At the same timepoint, AfLP in the LPLP mixture and AfHP showed significant stimulations in Fv/Fm in response to P-amendment.

Table 3.4 T-tests at 1h and 18-22h following nutrient amendment consistent with differences from y=0 in figures 3.7 and 3.8 on (a) July and (b) September experiment replicates. Significant results ($p \leq 0.05$) are bolded.

a.

	T=1h			T=18-22h		
MaLP	**mean**	**t-statistic**	**p-value**	**mean**	**t-statistic**	**p-value**
LPLP.N	-0.0240	-4.8653	**0.0397**	-0.0193	-2.2308	0.1554
LPLP.P	0.0837	14.6887	**0.0046**	-0.0170	-2.0769	0.1734
LPHP.N	-0.0847	-16.1616	**0.0038**	-0.0513	-4.7503	**0.0416**
LPHP.P	0.0807	15.3981	**0.0042**	0.0237	6.4545	**0.0232**
MaHP						
HPHP.N	-0.029	-88	**0.000129**	-0.0597	-22.9186	**0.0019**
HPHP.P	-0.0043	-13.0000	**0.0059**	0.0117	3.5537	0.0709
HPLP.N	-0.0480	-4.9774	**0.0381**	-0.0667	-6.8239	**0.0208**
HPLP.P	-0.0117	-8.0296	**0.0152**	0.0483	4.2592	0.0510
AfLP						
LPLP.N	0.0313	9.5443	**0.0108**	0.0233	2.6923	0.1147
LPLP.P	-0.0180	-2.7034	0.1139	0.0627	4.7402	**0.0417**
HPLP.N	0.0097	1.2982	0.3238	0.0420	9.3142	**0.0113**
HPLP.P	0.0087	1.3628	0.3061	-0.0113	-5.1850	**0.0352**
AfHP						
HPHP.N	0.0007	0.2341	0.8367	-0.0063	-2.1377	0.1660
HPHP.P	-0.0057	-3.0533	0.0926	-0.0043	-1.8028	0.2132
LPHP.N	0.0187	3.4797	0.0736	-0.0023	-2.6458	0.1181
LPHP.P	0.0097	2.3600	0.1422	0.0140	4.0415	0.0561

b.

	T=1h			T=18-22h		
MaLP	**mean**	**t-statistic**	**p-value**	**mean**	**t-statistic**	**p-value**
LPLP.N	0.0057	1.7000	0.2312	-0.0110	-1.0769	0.3942
LPLP.P	0.0520	1.8324	0.2084	-0.0243	-1.8233	0.2098
LPHP.N	-0.0017	-2.5000	0.1296	-0.0037	-1.4084	0.2943
LPHP.P	0.0707	7.4443	**0.0176**	0.0297	17.8000	**0.0031**
MaHP						
HPHP.N	0.0043	1.2284	0.3442	-0.0090	-1.5828	0.2543
HPHP.P	0.0040	1.3093	0.3206	0.0137	1.5597	0.2592
HPLP.N	-0.0127	-19.0000	**0.0028**	0.0137	2.7094	0.1135
HPLP.P	-0.0040	-4.0000	0.0572	-0.0137	-1.7826	0.2166
AfLP						
LPLP.N	-0.0073	-1.1879	0.3568	0.0150	1.6531	0.2401
LPLP.P	-0.0447	-2.5726	0.1237	0.0927	17.6887	**0.0032**
HPLP.N	-0.0157	-0.7140	0.5493	-0.0033	-1.7961	0.2143
HPLP.P	-0.0060	-0.5990	0.6100	-0.0033	-0.2886	0.8001
AfHP						
HPHP.N	-0.0047	-0.7913	0.5117	0.0033	0.9853	0.4283
HPHP.P	-0.0027	-0.7559	0.5286	-0.0047	-0.9018	0.4623
LPHP.N	0.0020	0.4588	0.6914	-0.0020	-0.5283	0.6501
LPHP.P	0.0053	0.8421	0.4884	0.0127	10.5393	**0.0089**

3.4 Discussion

Previous work (e.g. Parkhill et al. 2001, Kruskopf and Flynn 2006), including the work presented in chapter 2 of this thesis, has provided evidence that variable fluorescence, Fv/Fm, is not a

consistent indicator of *in situ* phytoplankton nutrient status. However, phytoplankton fluorescence and photosynthetic yield measured as carbon fixation and oxygen evolution have been previously observed to decrease temporarily immediately following re-supply of a previously growth limiting-nutrient (Turpin and Weger 1988). This nutrient-induced fluorescent transient (NIFT) (Beardall et al. 2001a, b), may also be observed in time-course measures of Fv/Fm; this study provides some evidence of this effect.

The PhytoPAM represents an exciting step forward in variable fluorescence instruments, in that it is nearly-simultaneously able to detect the presence and relative intensity of group-characteristic accessory pigments in a sample (Jakob et al. 2005). This ability to assign variable fluorescence to different phytoplankton groups, but especially Cyanobacteria, means that the PhytoPAM, at least in theory, would be a useful tool in water quality monitoring. In freshwater, many of the bloom-forming taxa are Cyanobacteria (Downing et al. 2001). The recent resurgence of problem Cyanobacterial blooms in Lake Erie, with the largest *Microcystis* bloom occurring in fall 2011 (Bridgeman et al. 2013), highlights the potential use of the PhytoPAM in understanding the nutrient-related mechanisms behind the onset and cessation of these blooms. Largely for this reason, this study used *M. aeruginosa* to begin this investigation. Results from this set of experiments are not definitive, but suggested some potential for field applications of the NIFT techniques examined in this part of the thesis.

3.4.1 Monoculture Experiments

Monoculture NIFTs were intended to define the baseline Fv/Fm responses of each of the P-deficient and P-sufficient *M. aeruginosa* and *A. formosa* cultures when the nutrient treatments were added. These measurements should give information about one group's responses independent of any

influence from the fluorescence signal of the other group's response. The group of special interest was *M. aeruginosa* because of its importance in Lake Erie and other lakes.

The monoculture control experiment did not yield patterns wholly consistent with the patterns in the mixture experiments or from previous reported patterns of fluorescence dynamics (Beardall et al. 2001b, Holland et al. 2005), especially in the case of *M. aeruginosa*. AfLP showed a response atypical of the documented NIFT response. Instead of the transient suppression of Fv/Fm, there was instead an ephemeral stimulation in the short term (<1h) and there was no consistent evidence of stimulation as indicated by either instrument in the longer term. MaLP did not show any clear pattern of response to P-addition on either the short- or long-term compared to the response seen in the mixture culture experiments, which also contradicted previously-reported NIFT work. The Fv/Fm responses to introduction of the limiting nutrient did not demonstrate dynamics similar to previously published reports of NIFT patterns (Turpin and Weger 1988, Beardall et al. 2001b, Holland et al. 2004, Roberts et al. 2008, Petrou et al. 2008) and also differed from the results of the mixture experiments.

3.4.2 Mixed culture experiments

Both the P-deficient and P-sufficient cultures were treated with Barnstead (ultrapure) water, NH_4-N in July and NO_3-N in September, or PO_4-P. The DIW and N-amendments served as the controls. The reason for the change in N-species was as a result of inspecting the July mixture experiment data and observing that the addition of N seemed to be effecting a depression in Fv/Fm. Although the concentration of NH_4 was lower than what is considered toxic (50mM) to phytoplankton (Falkowski and Raven 2007), there was some concern that it was having a negative impact on the phytoplankton, which were grown with NO_3 as the primary N source. Previous studies have shown that N-deficient phytoplankton respond to resupply of N with fluorescence and

photosynthetic yield (O_2 produced or CO_2 fixed) following the prototypical NIFT pattern of a decrease immediately after amendment, with a slow rise in yield in the minutes following (e.g. Turpin and Weger 1988, Beardall et al. 2001a, Beardall et al. 2001b, Holland et al. 2005). The NH_4-N treatment in July showed a sustained depression following amendment, with no rise in Fv/Fm. This was especially apparent in the MaLP treatments amended with either N or P. While there was an Fv/Fm pattern consistent with the NIFT described in Beardall et al. (2001b) in response to P, there was only a sustained depression in Fv/Fm in response to N. The MaHP treatments also showed a sustained depression in Fv/Fm in response to NH_4-N but not to P, suggested that something about amending with a concentration of 50µM of NH_4 was detrimental to the phytoplankton in those treatments. When the N-amendment was changed to NO_3 for the second mixture experiment, the sustained Fv/Fm depression in relation to the N-amendment largely disappeared and N-treatment Fv/Fm did not significantly differ from the control Fv/Fm at any of the timepoints.

The NIFT kinetics in the mixture experiments for *M. aeruginosa* did not mimic the kinetics seen in the monoculutres. MaLP responded strongly and in a manner consistent with previous NIFT-type work when amended with P, by showing an initial short-term inhibition of Fv/Fm followed by stimulation within ~30min that lasted up to ~24h in response to the amendment. The kinetics of MaLP in response to P additions were different from responses to N addition, regardless of whether the N-species was NH_4 or NO_3, which is consistent with reports from workers such as Holland et al. (2005) for other taxa. The responses were consistent with the long-term stimulation seen by Rattan (2009) in Cyanobacteria-dominated, P-deficient natural communities.

A. formosa produced a pattern that was similar and somewhat consistent with the monocultures in terms of stimulation from the P-amendment, but there was no inhibition prior to the stimulation, contrary to the expected NIFT response. In addition, a similar response was seen for addition of the supposedly non-limiting nutrient N. There was also evidence of longer-term (e.g.

>1h) stimulation of Fv/Fm in AfLP in response to P, but this was mixture-dependent, and only seen in the *A. formosa* in the LPLP mixture, and not also in the HPLP mixture. Rattan et al. (2012) reported longer term stimulation of Fv/Fm in response to P, so it is possible that this response reflects recovery of nutrient sufficiency by amendment, but the results are inconclusive in the present experiments because of insufficient agreement with the monoculture controls.

3.4.3 Explanations for the differences between monocultures and mixed culture experiment outputs

The NIFT patterns replicated between the two mixture experiments on the PhytoPAM, which lends some support to the idea that the patterns were produced in response to the limiting nutrient (P) amendment, even if they did not necessarily correspond to the patterns observed on either the WaterPAM or the PhytoPAM with monocultures. It also seems unlikely that there was some instrument confusion about the two taxa. The PhytoPAM manual (Walz Manual) indicates that Cyanobacteria variable fluorescence is assigned mainly through the spectral fluorescence measured through the 645 and 665nm diodes, but not through the 470nm diode. Conversely, diatoms are generally assigned through the fluorescence from the 470 and 520nm diodes. These are the diodes that are targeted to deconvolute the raw signal and assign the group-specific Fv/Fm (Jakob et al. 2005). The reference spectra assigned to tell the instrument what is Cyanobacteria and what is a diatom in theses experiment also showed a distinct difference between the spectral fluorescence patterns of these two phytoplankton groups. Specifically, the spectral signature of the diatom reference (*Asterionella formosa*) was high fluorescence in the 520nm diode, moderate fluorescence in the 470 and 645nm diodes, and lower fluorescence in the 665nm diode. Conversely, the spectral signature for the Cyanobacteria reference (*Anabaena lemermanii*) had very low fluoresce in the 470nm diode, high fluorescence in the 645nm diode, and moderate fluorescence in the 520 and

665nm diode. A Cyanobacteria species (*Anabaena lemermanii*) other than *Microcystis* was purposely used to explore the utility of using a reference spectrum that was similar, but not exactly the same as the sample being read. This was done in part to mimic a similar field situation that is likely, where the reference species might not be the exact same as the species occurring and being measured at the field site. In these experiments, it appeared to work, but may not necessarily work in all cases, so workers must be aware of this when conducting their own readings on the PhytoPAM. This was also part of the rationale for studying *M. aeruginosa* and *A. formosa*, since it seemed unlikely, in theory, that the PhytoWin program could misattribute spectral fluorescence from one group to the other. Indeed, Fv/Fm was never spuriously measured in the "green" group, and the F_t for each of the "blue" and "brown" group was reflective of the intended 50:50 mixture proportions. Since there is very little overlap in the diodes that are used to assign Fv/Fm among groups, this also provides some evidence that the fluorescence patterns in the mixture experiments were representative of each taxon's response. *M. aeruginosa* and *A. formosa* also did not show strong long-term stimulation in the monoculture NIFTs, so it seems unlikely that the dynamics of Fv/Fm for *M. aeruginosa* in the mixture experiments was due to the deconvolution program misattributing the signals between the taxa.

For mixtures of a high-P taxon with a low-P taxon (i.e. the LPHP or HPLP mixtures), it is possible that there was P-contamination from the HP culture in the mixture, which may have accounted for some variation in the results. However, re-suspensions of the HP cultures was done to minimize such effects. In the event that some P contamination did occur, it should principally affect the apparent dynamics of the response. There were some differences in the dynamics in LPLP vs LPHP mixtures, but the main patterns were the same.

It is possible that another reason for the disparate results between the monoculture control and the mixture experiments lies in the degree of P-deficiency experienced by the cultures used in

each experiment and also in their chl a concentrations. The P-deficiency experienced by the P-starved *M. aeruginosa* and *A. formosa* cultures for the mixture experiments was similar between the July and September replicate experiments in terms of the ratio of alkaline phosphatase activity (APA) between the P-starved and P-sufficient cultures. However, the APA ratio between P-starved and P-sufficient cultures used for the monoculture experiment was much different, especially for *M. aeruginosa*. This was surprising, however, as they were grown and measured based on the standards from the previous experiments developed with Milne (2011) and Cater (2011) for *A. formosa* and with Reesor (2012) and Holmes (2012) for *M. aeruginosa*. The P-starved *M. aeruginosa* was more deficient in the monocultures than in the mixture experiments, as reflected in the greater APA value and lower Fv/Fm than those found in the mixture experiments. Perhaps the degree of deficiency was so great it impaired its ability to take up and assimilate the extra PO_4 when they were amended with the spike. In terms of the NIFT response, it might be that this response can become suppressed if the nutrient deficiency is sufficiently acute, or the culture has become semi-senescent (i.e. late stationary phase going into decline). Holland et al. (2005) observed loss of the NIFT response in extremely N-starved cultures, and this might have also been the case for the P-deficient *M. aeruginosa* used in the monoculture experiments. *A. formosa* APA and chl a concentrations did not vary as greatly between the LP monoculture and mixture experiments, which might partially explain why the apparent NIFT behaviour (or lack of it) was more similar between the two types of experiment for the diatom. Ideally, mixture experiments would have been done using the same cultures analyzed in the monoculture experiments but this was not logistically feasible. As a result, the sources of discrepancy between monoculture and mixture experiments are uncertain.

Results from previous work in this lab on P-deficient and P-sufficient *M. aeruginosa* (Reesor 2012, Holmes 2012) and *A. formosa* (Milne 2011, Cater 2011) cultures grown under identical conditions were used to inform the timings of the experimental cultures. The experiments for this

thesis were conducted under the assumption that the cultures grown under the same conditions and using the same protocols would experience the same growth dynamics and onset of deficiency in the P-deficient cultures as they did previously. Based on these previous data, *M. aeruginosa* became deficient within a week in low-P medium after pre-conditioning, and *A. formosa* became deficient within two weeks after pre-conditioning. Growth dynamics were tracked using *in vivo* fluorescence in the previous experiments done by these other students (see appendix). These dynamics informed them of onset of P-deficiency when the fluorescence appeared to plateau, suggesting growth rate was slowing down due to P-starvation. APA for the current experiments did not all exceed the conventional thresholds for P-deficiency as set out by Healey and Henzel (1979) (see appendix). However, Healey and Hendzel (1979)'s work was based on only a few taxa, none of which were *Microcystis* spp., and the degree of APA in the LP cultures was much greater compared to the HP cultures. It is also possible that *M. aeruginosa* uses other phosphatase enzymes (e.g. Whitton et al 1991, White 2009) that do not cleave 3-O-methylfluorescein phosphatase (3-OMFP) the same way alkaline phosphatase does, which could represent an underestimation of its true capabilities to hydrolyse PO_4 from organic molecules. It is reasonable to conclude that *Microcystis* in low P cultures was truly P deficient in this study, but the severity of the deficiency was not as fully characterized as it could be.

3.4.4 Conclusions and Future Directions

There is a possibility that group-specific NIFTS may be useful for detecting nutrient deficiency and discriminating differences among co-occurring phytoplankton groups, but these present results were not conclusive. More work is needed to characterize how the NIFT kinetics change with severity of P-deficiency, age of the culture, and imposition of steady state limitations vs. starvation conditions. Continuous cultures grown in chemostats would provide more physiologically

controlled material and may be a better model for natural phytoplankton communities where nutrient regeneration can help maintain more a steady state (limitation) and less of the extreme non steady state (starvation), conditions produced by typical batch cultures. Elucidation of a protocol that would give Cyanobacteria-, and perhaps specifically *Microcystis*-specific assays would be a very valuable tool to have for water quality managers, and so more of this type of work should be done.

Another limitation of this study is that it only assessed P-status, but not N-status. However, there is quite a bit of literature available tracking phytoplankton N-status against various measures of photosynthetic yield and fluorescence (e.g. Falkowski et al. 1989, Geider et al. 1993, Geider et al. 1998). Most freshwater systems are P-limited, but *Microcystis* has been observed in systems that experience N-limitation as well (Paerl et al. 2011); Lake Erie is one such example, and there is evidence for N and P co-limitation at times (e.g. Guildford et al. 2005, North et al. 2007, Rattan et al. 2012). Secondary limitation was also not assessed, although it is possible that it occurred, given the unique experimental setup that assessed short- and long-term Fv/Fm responses. This study only assessed P-starvation, but not P-limitation, so there is no way to know if the NIFT responses differ between these two forms of P-deficiency.

Future studies should focus on understanding the NIFT response in monocultures, and how the response changes with increasing P-deficiency to try and resolve the differences detected by the monoculture and mixture experiments in this thesis. Tests with different phytoplankton groups, such as the Chlorophyta, would also be important in further understanding how an artificial community's (i.e. a known mixture of phytoplankton cultures) composition influences group-specific Fv/Fm measurements. Natural phytoplankton communities are often heterogeneous, and it is important to know how well the PhytoPAM can reliably detect nutrient deficiency in these groups using the NIFT phenomenon.

Chapter 4

Synthesis and Summary

Among researchers, the use of variable fluorescence to detect or diagnose nutrient deficiency is a topic of much discussion and disagreement. Some workers have found evidence of nutrient deficiency when probing phytoplankton samples *in situ* for Fv/Fm. Others have found no evidence, while still others have only found evidence upon perturbation of phytoplankton samples. Further, many of these studies have been conducted on one-taxon "communities" like phytoplankton cultures or only measured the whole-community variable fluorescence. Whole-community Fv/Fm is a very coarse measurement, as it does not account for physiological and taxonomic differences between different phytoplankton groups.

This thesis attempted to clarify these issues, especially when the system is a mixed phytoplankton community, and test possible solutions that could be feasible for use in the field. Spectral variable fluorescence, as provided by instruments like the spectral fluorescence-capable pulse amplitude modulated (PAM) fluorometer, PhytoPAM, could represent a future for variable fluorescence technology as a useful water quality monitoring tool, given their relative ease-of-use and potential for shorter assay time. Coupling the spectral fluorescence that gives indications of phytoplankton group membership through accessory pigment composition and variable fluorescence, which can give some indication of nutrient status, especially when samples are perturbed, can give an indication of the types of phytoplankton groups that are nutrient deficient or sufficient. The WaterPAM and DivingPAM have also been used to assess phytoplankton variable fluorescence. However, there is no way to know whether or not any of the outputs from these instruments are

directly comparable, and thus, it is hard to draw conclusions from data gathered from different instruments.

Chapter two of this thesis set out to answer questions regarding interchangeability of different PAM fluorometers, and whether their outputs were statistically the same between the different instruments for the same sample. This chapter also sought to characterise Lake Erie's nutrient status, in terms of phytoplankton nitrogen (N) and phosphorus (P)-deficiency or sufficiency. Phytoplankton nutrient status in Lake Erie and two Lake Ontario sites showed that the phytoplankton were often P-deficient, especially in the Central and Eastern Basins, but also sometimes in the Western Basin. They also exhibited N-deficiency, and sometimes co-deficiency. Lake Erie was not permanently P-deficient, but showed spatial differences in phytoplankton nutrient status; this was especially true between the Western Basin, which showed P-sufficiency in September, and the Central and Eastern Basins, which showed consistent P-deficiency over both sampling trips. The classical P-control paradigm suggests that P is the nutrient that becomes limiting to phytoplankton first and thus shapes community composition and determines phytoplankton biomass. This is what is expected in P-limited lakes, as Lake Erie was believed to be following the P-loading controls under the Great Lakes Water Quality Agreement (GLWQA). However, evidence of N-deficiency and co-deficiency modifies the paradigm slightly. Different groups of phytoplankton have different N and P requirements, and a changing ratio of available N and P in the lake water medium has implications for community composition and diversity. A nutrient ratio heavily skewed toward either N or P will trend toward a less diverse community as one type of taxon becomes dominant, as in the cases of Cyanobacterial blooms. Analysis suggested that, on natural samples, agreement for P-debt was moderate and significant with DivingPAM. There were also moderate and non-significant relationships between the nutrient status indicators with PhytoPAM and a moderate significant relationship between APA and WaterPAM, and has been attributed to the importance of

phytoplankton phylogeny and previous light history on Fv/Fm. Thus, judicious use of PAM instruments in the field is needed, and awareness that outputs may be misleading when comparing samples read on different instruments, is necessary

P-limitation is believed to be the proximate controller that limits phytoplankton biomass, and especially Cyanobacteria, which are themselves correlated with higher concentrations of total P. It would be advantageous to know when Cyanobacteria are P-deficient, because they would seem unlikely to be in a position to form problem blooms. Being able to track on a fine scale where in Lake Erie *Microcystis* appears to be either P-deficient or P-sufficient would be advantageous, as this may help to correlate nutrient status with the position and extent of these blooms. In this way, it may be possible to see if the current P-loading management strategies are having a direct impact on controlling *Microcystis* biomass, and whether management strategies are working as well as expected. The PhytoPAM seems to be able to associate the Cyanobacteria-specific ("blue" channel) Fv/Fm with patterns consistent with the NIFT patterns indicative of nutrient deficiency. This could be a tool that can help understand this phenomenon. Being able to detect and determine phytoplankton nutrient status can provide information on short-term variability in phytoplankton nutrient status, which may be useful in detecting lake nutrient patterns if sampling is done consistently over the long term. Variable fluorescence seems to have only very limited usefulness on unperturbed samples. However, nutrient-induced fluorescent transient (NIFTs) may be a promising protocol to use to determine phytoplankton nutrient status for certain sampling applications and there have been some studies over the years providing evidence that it might be a replicable assay.

Chapter three sought to determine whether it is first possible to detect phosphorus deficiency using the nutrient-induced fluorescent transient (NIFT) response, and second, to correctly distinguish which one of two phytoplankton cultures in a roughly equal mixture (by fluorescence) was experiencing that deficiency. This second study also aimed to assess whether PhytoPAM is capable

of correctly attributing group-specific Fv/Fm, and if nutrient stress affects its ability to assign Fv/Fm to those groups. Numerous workers have observed that photosynthetic yield and fluorescence drop briefly in nutrient-deficient phytoplankton when the previously growth-limiting nutrient is reintroduced. This NIFT response was used to assess whether variable fluorescence could be used to detect nutrient deficiency in either *Microcystis aeruginosa* (Kutz.em.Elenkin) or *Asterionella formosa* (Hass.). PhytoPAM was chosen because of its purported ability to use spectral fluorescence to assign variable fluorescence signatures to broad phytoplankton groups. However, it was not known if these group-specific variable fluorescence responses are influenced by nutrient stress, in terms of both nutrient deficiency, and the NIFT response. The results from a 2x2 factorial design mixture experiment with *M. aeruginosa* and *A. formosa* suggested that PhytoPAM detects a NIFT response consistent with previously documented NIFT patterns in the P-deficient *M. aeruginosa* culture. *A. formosa* presented a more complex and variable response, so generalizations cannot be made from them. Interpretation of these results is complicated by a lack of agreement with the monoculture controls, and indeed, by a lack of convincing monoculture control results and alkaline phosphatase assay (APA) results. However, with this in mind, there is some evidence that use of NIFTs might be a useful metric for determining *Microcystis* nutrient status. PhytoPAM appears able to assign variable fluorescence to the correct phytoplankton groups in spite of their nutrient status, although more experiments are needed to fully differentiate the abilities of the spectral variable fluorescence (as measured on the PhytoPAM) to successfully attribute the correct nutrient status to different phytoplankton groups. This thesis made some important conclusions about spectral variable fluorescence and the ability to detect nutrient status between a cyanobacterium and a diatom, but better controls, and more and different taxa are needed to fully understand the capabilities and limitations of the instrument. Ways to automate the NIFT measurements also need to be explored to make this method truly feasible for high-frequency field sampling.

"Traditional" assays like nutrient debts and APA are still useful for understanding phytoplankton nutrient status, although they are limited in their resolution. Assays like spectral variable fluorescence NIFTs are meant to develop tools with a finer resolution, in terms of understanding group-specific nutrient-status. Other assays, such as the single-cell APA, fluorescence-labelled enzyme activity (FLEA), may be useful in understanding individual (colony) P-status; development of an analogous cell-specific N-status indicator is also of primary importance. These tools can help begin to discern the nutrient component of problem blooms of *Microcystis* in the Western Basin of Lake Erie, specifically in relation to the nutrient status of Cyanobacteria compared to other taxa during the major phases of a bloom such onset and cessation. Chronicling the nutrient status frequently in space and time between the different phytoplankton groups might give insights into the mechanism behind Cyanobacteria dominance during a bloom, especially in regards to whether nutrient availability plays a role, and how large this role is. NIFTs and spectral variable fluorescence may become useful in answering these questions, especially if automation is possible. This thesis work has provided evidence for correct differentiation of nutrient status between two different phytoplankton groups, and so provides some proof of concept for its use.

The studies presented in this thesis explored the use of variable fluorescence to detect nutrient deficiency, specifically P-deficiency, in phytoplankton. The first study helped put in context the low Fv/Fm results from a previous report using the DivingPAM and determined that PAM fluorometers, despite using the same excitation protocol and very similar excitation and measuring wavelengths, do not produce identical results for the same sample. The second study provided evidence that it may be possible to detect P-deficiency in *Microcystis aeruginosa*. PhytoPAM and NIFTs may become useful tools in understanding this taxon's potentially nutrient-mediated bloom dynamics in Lake Erie, and the results presented here provide a basis for further work on this topic.

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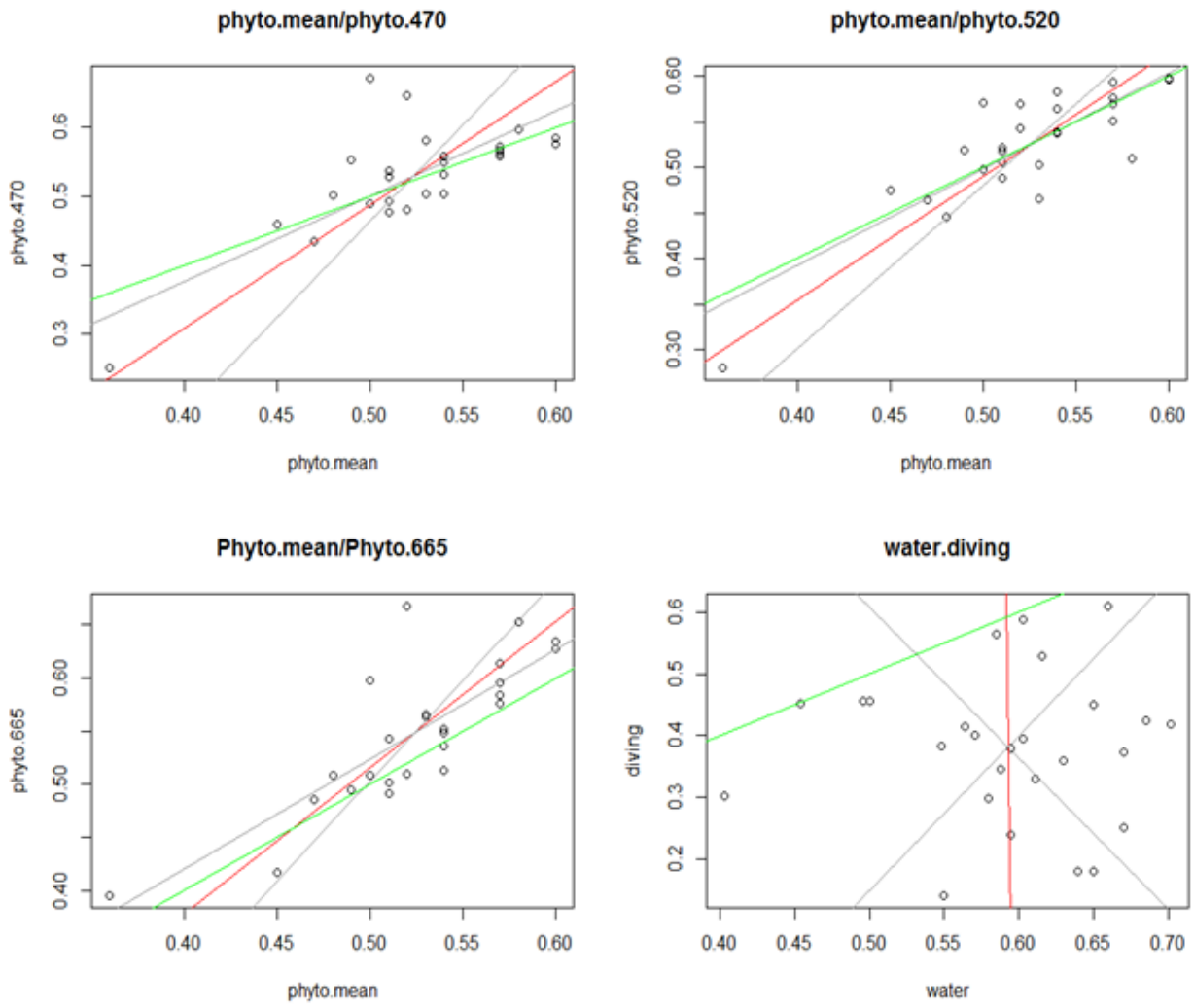
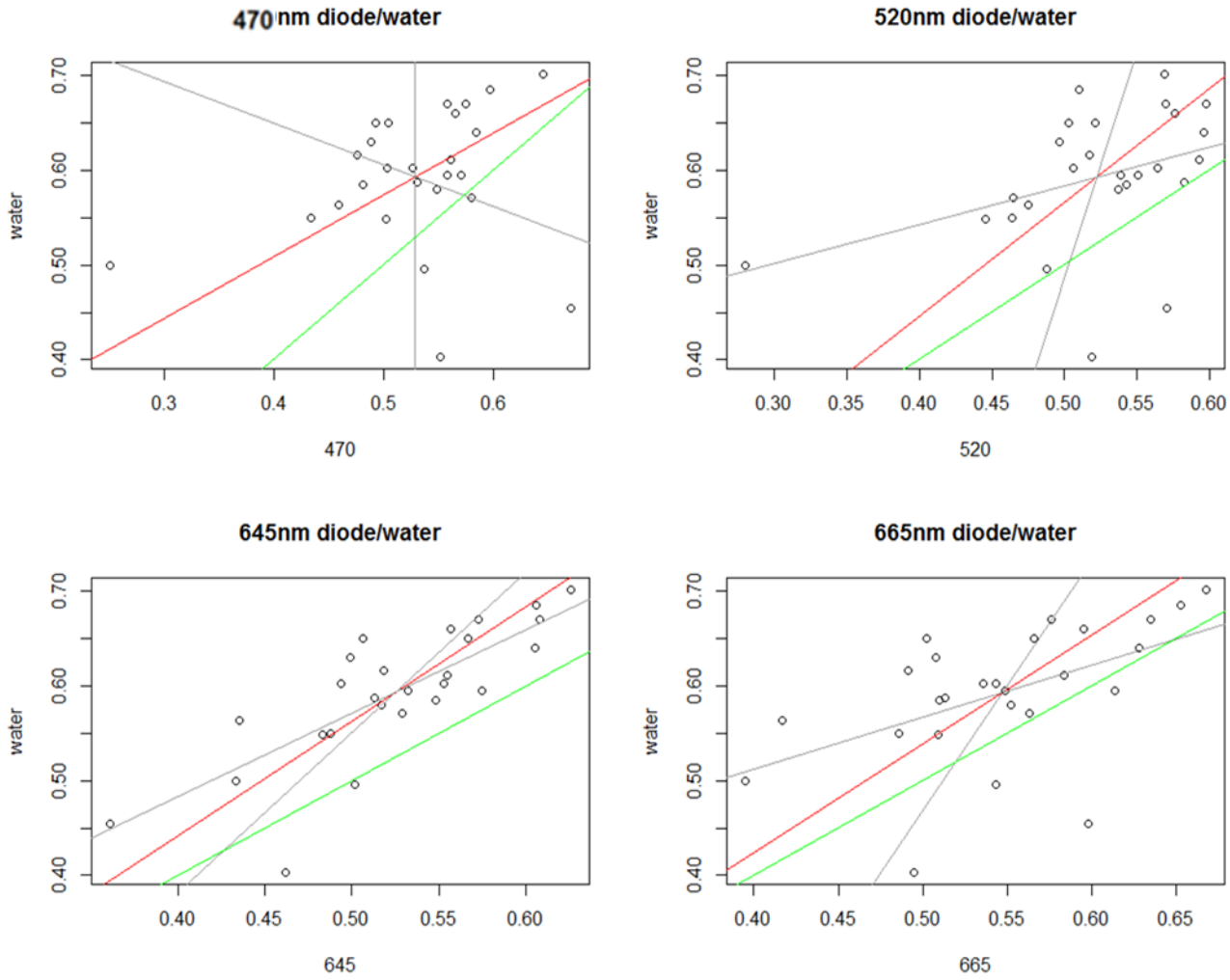
Appendix A

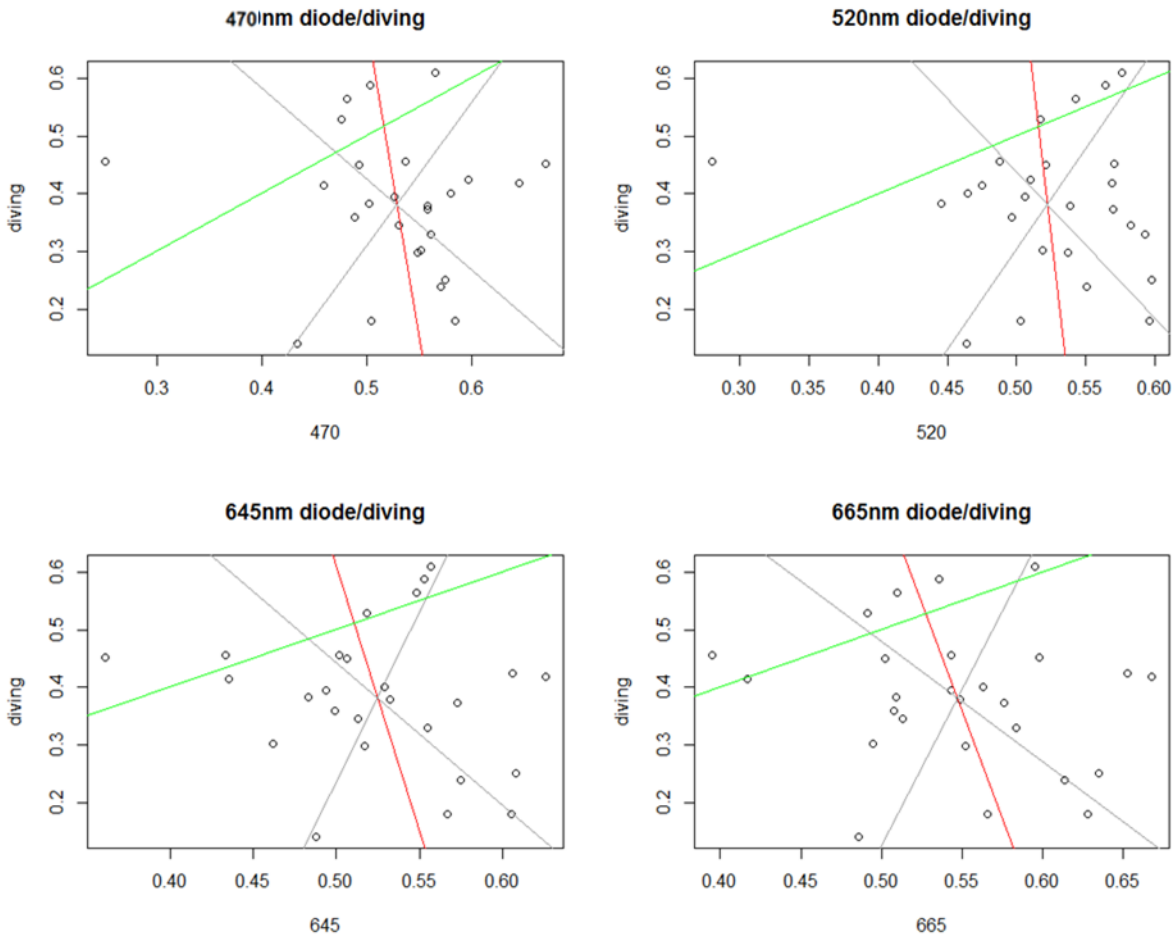
Supplementary figures and tables for Chapters 2 and 3



A2-1Model II major axis regression of the Phyto.mean channel outputs against other PhytoPAM diode channels and the WaterPAM regressed against the DivingPAM



A2-2 Model II major axis regression of the PhytoPAM diode channel outputs regressed against the WaterPAM outputs

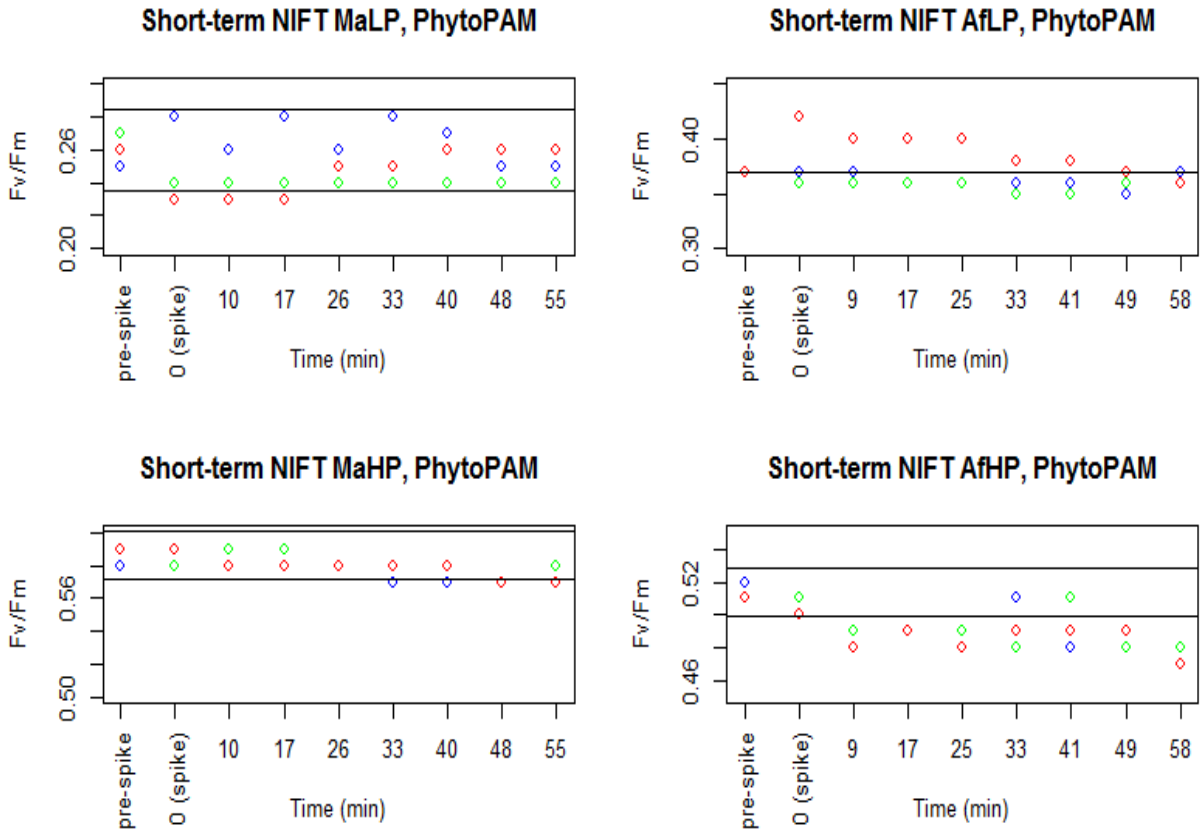


A2-3 Model II major axis regression of the PhytoPAM diode channel outputs regressed against the DivingPAM outputs

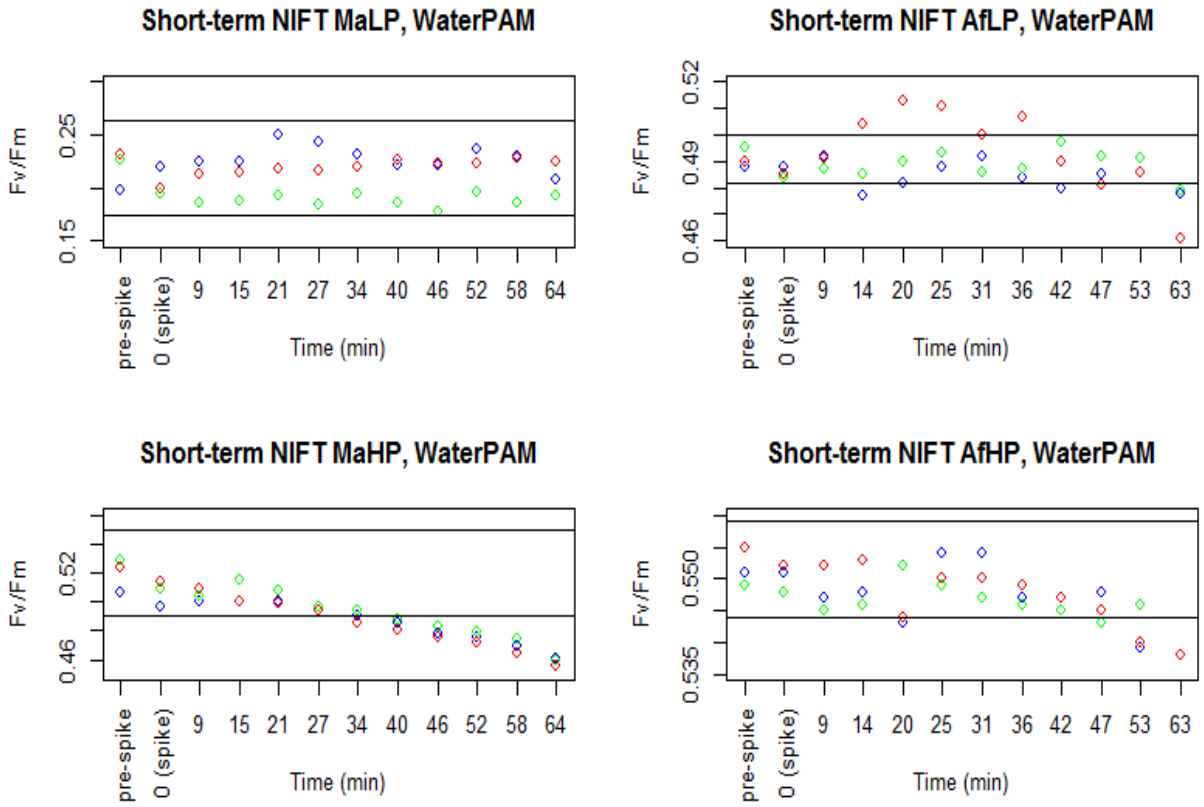
A2-4 Kendall correlation for sites exhibiting P-deficiency.

	Phyto.mean	Phyto.645	Water	Diving	Chl.a	N.debt	P.debt	APA
Phyto.mean	**1.000**							
Phyto.645	**0.681**	**1.000**						
Water	**0.430**	**0.638**	**1.000**					
Diving	-0.030	-0.043	0.077	**1.000**				
Chl.a	-0.179	-0.263	-0.173	**0.450**	**1.000**			
N.debt	0.005	-0.039	0.073	-0.189	-0.208	**1.000**		
P.debt	0.084	0.181	0.158	**-0.406**	**-0.377**	*0.280*	**1.000**	
APA	0.124	0.210	0.264	-0.177	**-0.568**	0.261	*0.305*	**1.000**

a.


Short-term NIFT MaLP, PhytoPAM
Fv/Fm
0.20
0.26
pre-spike
0 (spike)
10
17
26
33
40
48
55
Time (min)
Short-term NIFT AfLP, PhytoPAM
Fv/Fm
0.30
0.40
pre-spike
0 (spike)
9
17
25
33
41
49
58
Time (min)
Short-term NIFT MaHP, PhytoPAM
Fv/Fm
0.50
0.56
pre-spike
0 (spike)
10
17
26
33
40
48
55
Time (min)
Short-term NIFT AfHP, PhytoPAM
Fv/Fm
0.46
0.52
pre-spike
0 (spike)
9
17
25
33
41
49
58
Time (min)

b.

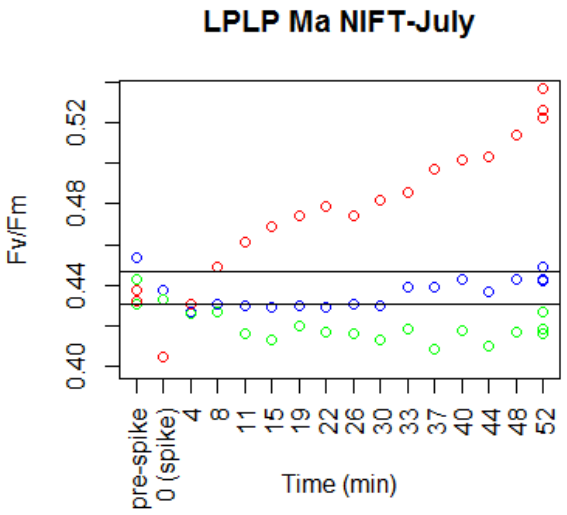


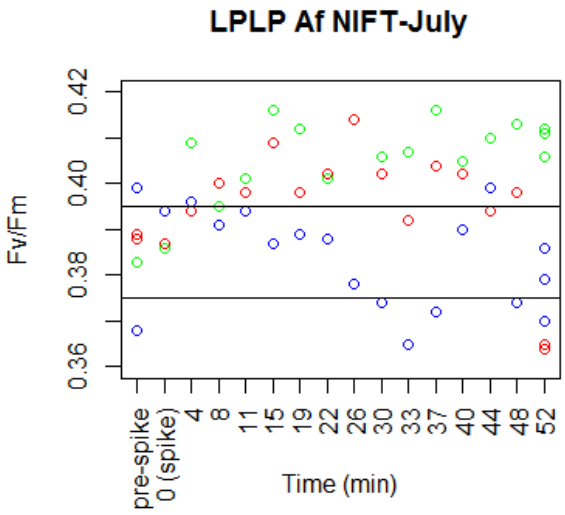
A3-1 Short-term NIFTS for monocultures measured on the (a) PhytoPAM and (b) WaterPAM. Blue points are DIW control, green points are N-amendment (second control) and red points are P-amendments.

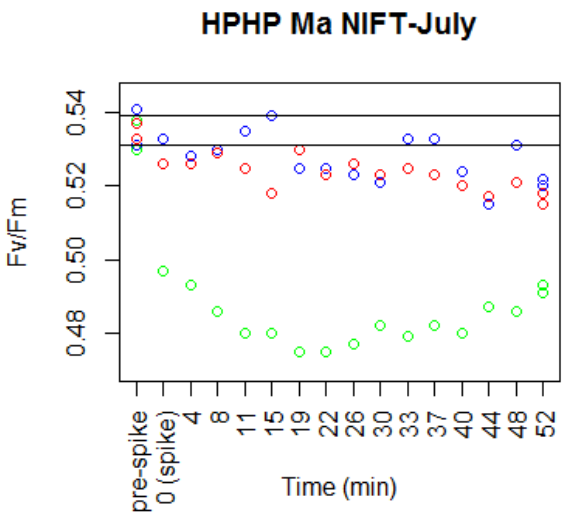
A3-2 Coefficients of variation (CV=SD/mean) of controls and their corresponding re-suspensions. (n=3 for each sample, true replicates – 3 different culture replicates tested)

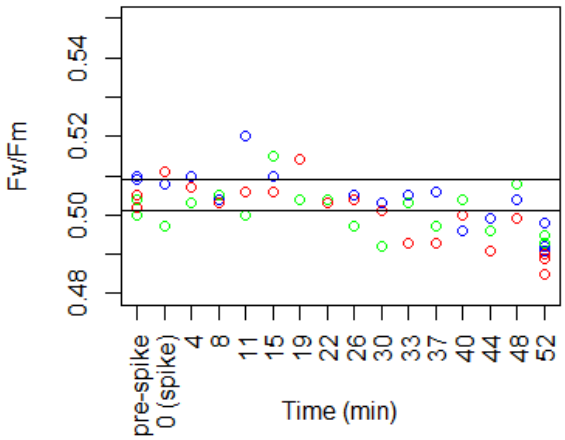
Time from first reading (h)	M. aeruginosa LP		A. formosa LP		M. aeruginosa HP		A. formosa HP	
	Control	Resuspension	Control	Resuspension	Control	Resuspension	Control	Resuspension
0	0.003923	0.044102	0.004174	0.011271	0.007686	0.010427	0.004556	0.008163
0.5-0.75	0.005474	0.019663	0.1329	0.006186	0.006415	0.011414	0.009124	0.005757
~8	0.00334	0.07333	0.008951	0.009751	0.004773	0.034469	0.008661	0.006698
20-24*	0.053818	0.032457	0.009638	0.017864	0.005587	0.032617	0.00399	0.00381

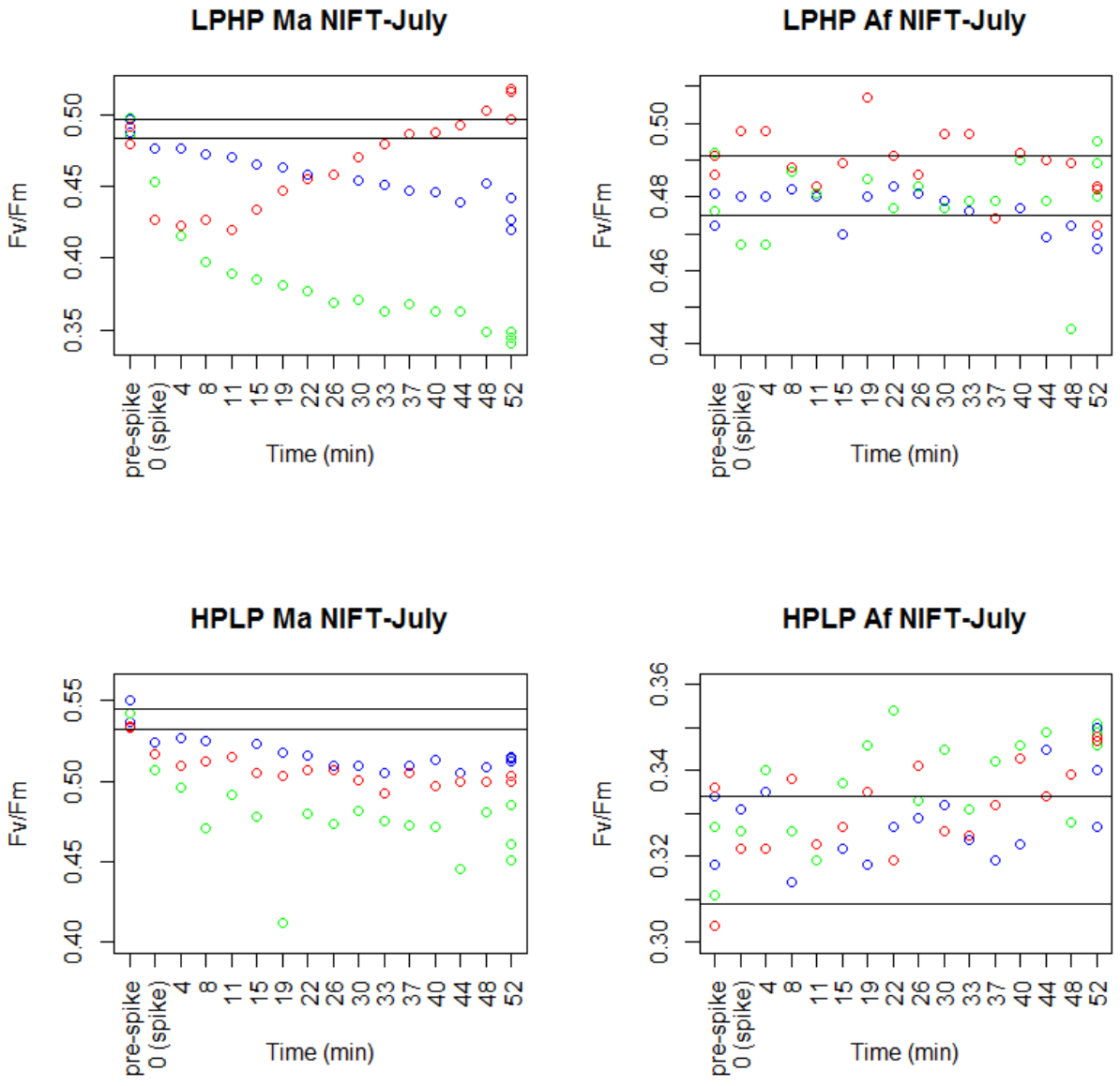
*~8.5h for A. formosa LP


LPLP Ma NIFT-July
Fv/Fm
0.40
0.44
0.48
0.52
pre-spike
0 (spike)
4
8
11
15
19
22
26
30
33
37
40
44
48
52
Time (min)

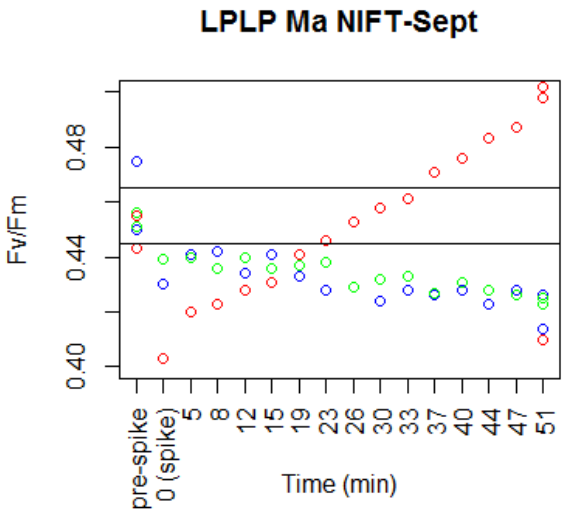

LPLP Af NIFT-July
Fv/Fm
0.36
0.38
0.40
0.42
pre-spike
0 (spike)
4
8
11
15
19
22
26
30
33
37
40
44
48
52
Time (min)

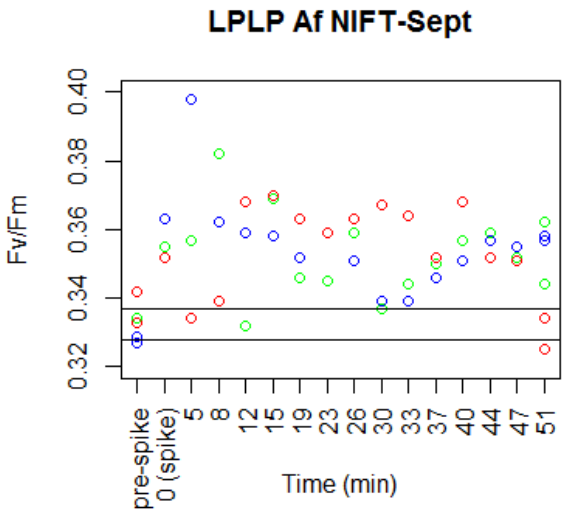

HPHP Ma NIFT-July
Fv/Fm
0.48
0.50
0.52
0.54
pre-spike
0 (spike)
4
8
11
15
19
22
26
30
33
37
40
44
48
52
Time (min)

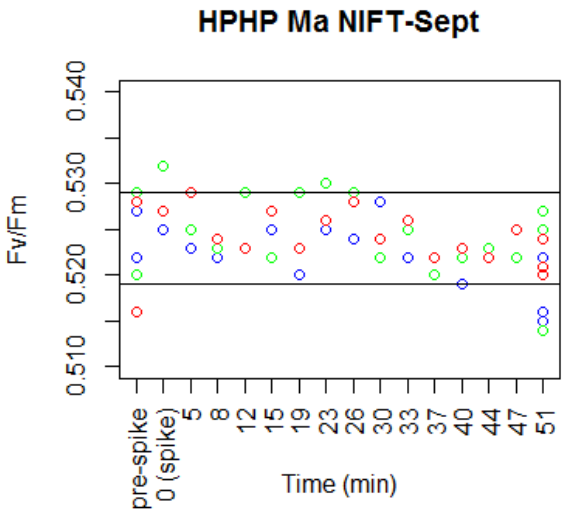

HPHP Af NIFT-July
Fv/Fm
0.48
0.50
0.52
0.54
pre-spike
0 (spike)
4
8
11
15
19
22
26
30
33
37
40
44
48
52
Time (min)

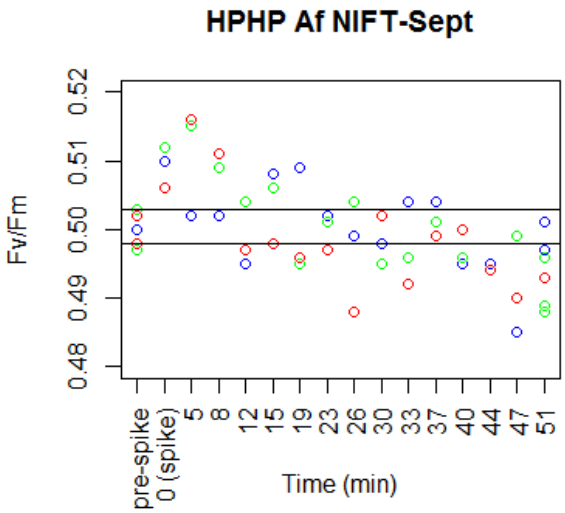


A3-3 Short-term NIFT responses of the individual LP or HP cultures in the different experimental manipulations for the July experiments (experiment replicate 1). Black lines denote the upper and lower 95% confidence intervals, blue points represent the DIW control treatment, green points indicate the N-treatment, and red points indicate the P-treatment.


LPLP Ma NIFT-Sept
Fv/Fm
0.40
0.44
0.48
pre-spike
0 (spike)
5
8
12
15
19
23
26
30
33
37
40
44
47
51
Time (min)


LPLP Af NIFT-Sept
Fv/Fm
0.32
0.34
0.36
0.38
0.40
pre-spike
0 (spike)
5
8
12
15
19
23
26
30
33
37
40
44
47
51
Time (min)


HPHP Ma NIFT-Sept
Fv/Fm
0.510
0.520
0.530
0.540
pre-spike
0 (spike)
5
8
12
15
19
23
26
30
33
37
40
44
47
51
Time (min)


HPHP Af NIFT-Sept
Fv/Fm
0.48
0.49
0.50
0.51
0.52
pre-spike
0 (spike)
5
8
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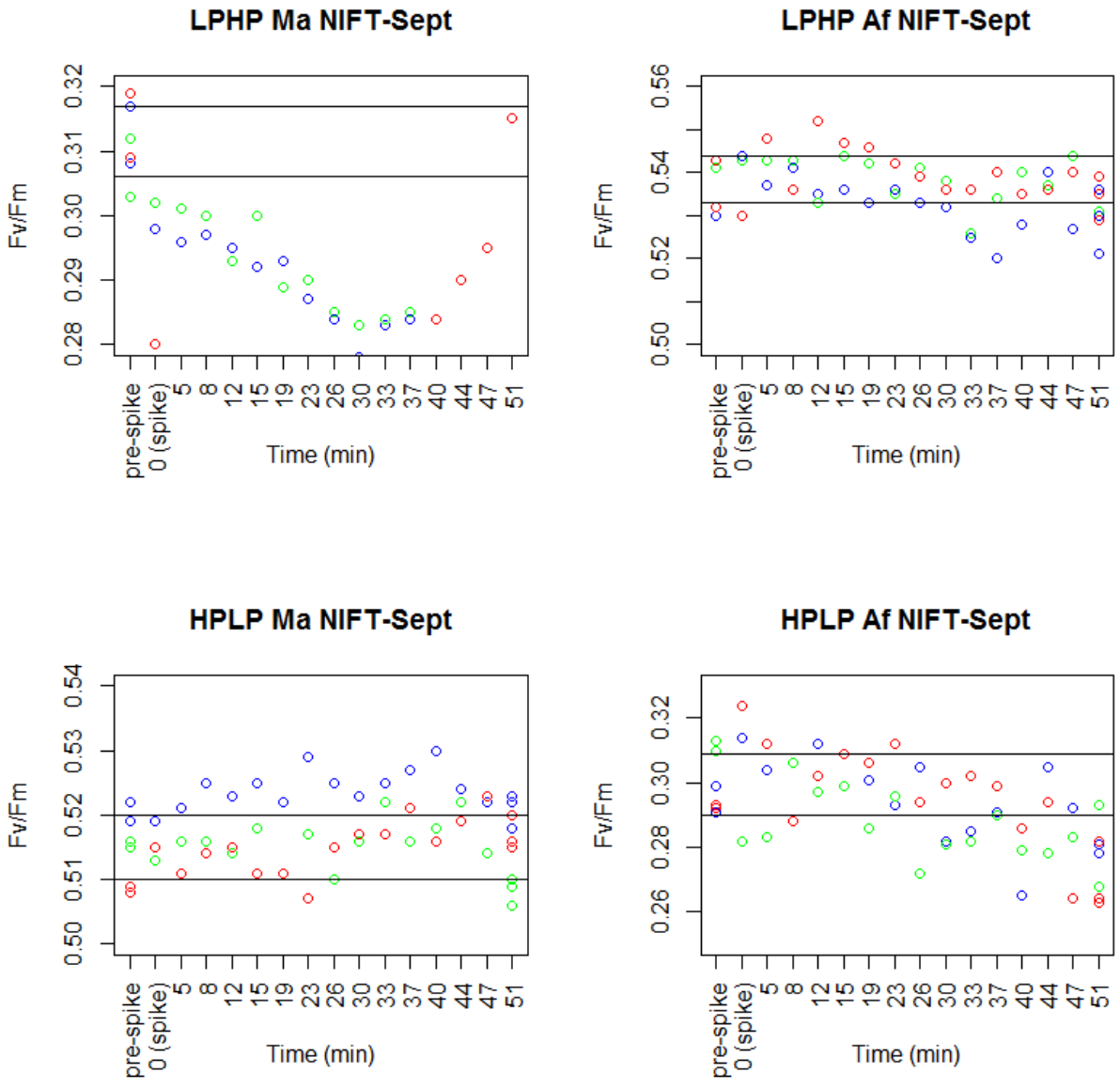
26
30
33
37
40
44
47
51
Time (min)



A3-4 Short-term NIFT responses for the September experiments (experiment replicate 2). All points and lines are the same as for Fig. A3-3.

A3-5 Mean values of Fv/Fm (SD in brackets) of long-term NIFT mixture experiments for (a) *Microcystis aeruginosa* and (b) *Asterionella formosa* in July and September.

a.

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July	LPLP			HPHP			LPHP			HPLP		
pre	0.439 (0.00770)			0.535 (0.00396)			0.490 (0.00628)			0.538 (0.00602)		
Time (h following spike)	C	N	P	C	N	P	C	N	P	C	N	P
1	0.445 (0.00379)	0.421 (0.00569)	0.528 (0.00777)	0.521 (0.00115)	0.492 (0.00115)	0.517 (0.00173)	0.430 (0.0112)	0.345 (0.00400)	0.510 (0.0116)	0.514 (0.00153)	0.466 (0.0175)	0.502 (0.00173)
2	0.440 (0.000577)	0.433 (0.00557)	0.507 (0.00721)	0.508 (0.00551)	0.480 (0.00404)	0.509 (0.00058)	0.404 (0.00666)	0.367 (0.00115)	0.475 (0.0118)	0.502 (0.00058)	0.481 (0.00100)	0.488 (0.00917)
6	0.425 (0.00200)	0.423 (0.00815)	0.469 (0.00289)	0.471 (0.00252)	0.380 (0.00666)	0.476 (0.01044)	0.345 (0.00289)	0.310 (0.00551)	0.404 (0.00702)	0.456 (0.00458)	0.404 (0.00737)	0.465 (0.0113)
12	0.394 (0.00361)	0.391 (0.0105)	0.428 (0.00625)	0.389 (0.00153)	0.356 (0.00436)	0.398 (0.00451)	0.306 (0.0153)	0.253 (0.0111)	0.336 (0.0101)	0.415 (0.00379)	0.378 (0.0165)	0.438 (0.00850)
18-22	0.376 (0.0191)	0.356 (0.00651)	0.359 (0.00503)	0.365 (0.00416)	0.305 (0.00557)	0.376 (0.00289)	0.265 (0.00577)	0.213 (0.0131)	0.288 (0.00058)	0.386 (0.0117)	0.320 (0.00551)	0.435 (0.00862)

Sept	LPLP			HPHP			LPHP			HPLP		
pre	0.455			0.524			0.311			0.515		
	(0.00988)			(0.00471)			(0.00544)			(0.00501)		
Time (h following spike)	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**
1	0.418	0.424	0.470	0.518	0.522	0.522	0.251	0.250	0.322	0.521	0.508	0.517
	(0.00693)	(0.00115)	(0.05200)	(0.00379)	(0.00700)	(0.00208)	(0.0104)	(0.00929)	(0.00608)	(0.00265)	(0.00208)	(0.00265)
2	0.408	0.416	0.466	0.505	0.506	0.503	0.221	0.222	0.295	0.511	0.505	0.509
	(0.00416)	(0.00436)	(0.00611)	(0.00513)	(0.00252)	(0.00577)	(0.00473)	(0.0110)	(0.00493)	(0.00153)	(0.00651)	(0.00100)
6	0.389	0.400	0.408	0.434	0.437	0.433	0.175	0.182	0.229	0.438	0.447	0.445
	(0.00231)	(0.00058)	(0.00964)	(0.00520)	(0.00173)	(0.00755)	(0.00917)	(0.00608)	(0.00400)	(0.00321)	(0.00874)	(0.00751)
12	0.368	0.370	0.376	0.360	0.375	0.371	0.156	0.151	0.182	0.321	0.345	0.335
	(0.00458)	(0.0141)	(0.00781)	(0.00436)	(0.00520)	(0.00252)	(0.0129)	(0.00379)	(0.00520)	(0.00458)	(0.00351)	(0.00306)
18-22	0.332	0.321	0.308	0.380	0.371	0.394	0.138	0.135	0.168	0.267	0.281	0.253
	(0.0191)	(0.0233)	(0.00404)	(0.0129)	(0.00321)	(0.00755)	(0.00702)	(0.0115)	(0.00458)	(0.00656)	(0.00551)	(0.00764)

b.

July	LPLP			HPHP			LPHP			HPLP		
pre	0.385 (0.00929)			0.505 (0.00356)			0.483 (0.00739)			0.322 (0.01173)		
Time (h following spike)	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**
1	0.378 (0.00802)	0.410 (0.00321)	0.360 (0.00723)	0.494 (0.00379)	0.494 (0.00115)	0.488 (0.00265)	0.469 (0.00306)	0.488 (0.00755)	0.479 (0.00608)	0.339 (0.01153)	0.349 (0.00252)	0.348 (0.00058)
2	0.379 (0.01155)	0.403 (0.00513)	0.345 (0.00854)	0.491 (0.00458)	0.495 (0.00306)	0.489 (0.00351)	0.471 (0.00321)	0.484 (0.00503)	0.471 (0.00351)	0.347 (0.00681)	0.348 (0.00896)	0.349 (0.00451)
6	0.377 (0.00700)	0.381 (0.00721)	0.367 (0.00231)	0.474 (0.00656)	0.504 (0.00173)	0.482 (0.00321)	0.449 (0.00551)	0.476 (0.00850)	0.475 (0.00265)	0.376 (0.0299)	0.377 (0.00586)	0.365 (0.00624)
12	0.381 (0.0117)	0.377 (0.0127)	0.392 (0.00985)	0.479 (0.00794)	0.479 (0.00379)	0.475 (0.00404)	0.347 (0.169)	0.458 (0.00351)	0.473 (0.0168)	0.388 (0.0156)	0.413 (0.00800)	0.384 (0.00115)
18-22	0.364 (0.0181)	0.387 (0.00379)	0.427 (0.00902)	0.480 (0.00306)	0.473 (0.00208)	0.475 (0.00115)	0.457 (0.00513)	0.454 (0.00451)	0.471 (0.00153)	0.412 (0.00513)	0.454 (0.00306)	0.400 (0.00808)

Sept	LPLP			HPHP			LPHP			HPLP		
pre	0.333			0.500			0.539			0.300		
	(0.00473)			(0.00221)			(0.00550)			(0.00879)		
Time (h following spike)	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**	**C**	**N**	**P**
1	0.357	0.350	0.313	0.496	0.491	0.493	0.529	0.531	0.534	0.276	0.260	0.270
	(0.00058)	(0.0104)	(0.02950)	(0.00611)	(0.00436)	(0.00000)	(0.00755)	(0.00000)	(0.00503)	(0.00681)	(0.0376)	(0.0107)
2	0.345	0.348	0.335	0.496	0.494	0.491	0.532	0.533	0.537	0.278	0.269	0.266
	(0.00416)	(0.00351)	(0.01002)	(0.00289)	(0.00100)	(0.00862)	(0.00569)	(0.0104)	(0.0101)	(0.0136)	(0.0148)	(0.0115)
6	0.337	0.341	0.352	0.491	0.492	0.487	0.533	0.538	0.540	0.290	0.292	0.283
	(0.00603)	(0.00700)	(0.00608)	(0.00153)	(0.00702)	(0.00058)	(0.00300)	(0.00300)	(0.00416)	(0.00379)	(0.00985)	(0.0104)
12	0.312	0.323	0.370	0.494	0.489	0.493	0.536	0.541	0.549	0.279	0.283	0.287
	(0.00458)	(0.0123)	(0.00200)	(0.00551)	(0.00721)	(0.00520)	(0.00513)	(0.00709)	(0.00231)	(0.0201)	(0.0191)	(0.0131)
18-22	0.319	0.334	0.411	0.499	0.503	0.495	0.539	0.537	0.551	0.307	0.304	0.304
	(0.0101)	(0.00681)	(0.00153)	(0.00404)	(0.00208)	(0.00651)	(0.00115)	(0.00569)	(0.00321)	(0.0160)	(0.0138)	(0.00400)

A3-6 *In vivo* fluorescence (F_t) monitoring of Phase II culture growth dynamics from previous experiments. Low-P cultures appear to enter stationary phase 5-6 days post-inoculation. Data from Reesor (2012).

Replicate 1	LP1	LP2	LP3	HP1	HP2	HP3
17 Jan 2012	296	298	302	301	286	263
19 Jan 2012	301	300	315	599	556	531
22 Jan 2012	317	338	336	612	571	592

Replicate 2	LP1	LP2	LP3	HP1	HP2	HP3
5 March 2012	334	336	328	310	340	368
6 March 2012	422	378	375	474	511	514
7 March 2012	410	394	390	700	478	746
8 March 2012	416	390	385	987	1058	1058
9 March 2012	412	422	426	1140	1235	1295

*low-P (~1μM PO_4) and high-P (~100μM PO_4) medium.

A3-7 *Microcystis aeruginosa* APA values. From Holmes (2012).

Previous Experiments	Mean APA (n=3)			Mean chl a (n=3)	
	LP	HP	APA ratio LP:HP	LP	HP
23 Jan 2012	0.006907	0.000607	11.4	108.9	379.1
	(0.001795)	(2.55E-05)		(26.9)	(28.5)
10 March 2012	0.005139	0.000643	7.99	63.3	375.1
	(0.003277)	(0.000109)		(32.2)	(72.4)