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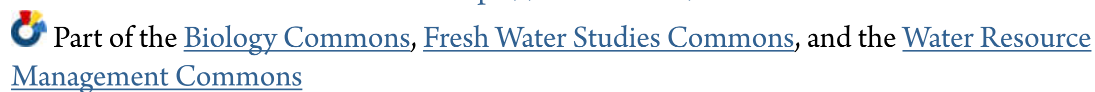
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PHYTOPLANKTON LIFE HISTORY EVENTS: RESTING STAGES AND PHYSIOLOGICAL CELL DEATH

by

Christine Kozik

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Masters of Science

in Biological Sciences

at

The University of Wisconsin-Milwaukee

August 2013

ABSTRACT

PHYTOPLANKTON LIFE HISTORY EVENTS: RESTING STAGES AND PHYSIOLOGICAL CELL DEATH

by

Christine Kozik

The University of Wisconsin-Milwaukee, 2013
Under the supervision of Professors John A. Berges and the late Craig D. Sandgren[‡]

Understanding and predicting changes in phytoplankton populations requires knowledge of key life history processes such as recruitment from benthic resting stages and losses due to sedimentation and cell death. Currently, these processes are poorly understood in freshwater systems. Phytoplankton resting stage and cell death life history events were separately examined in two freshwater systems in Wisconsin, four northern lakes and an urban pond. In the northern lakes, sedimentation and benthic recruitment were examined using sediment and recruitment traps that were sampled weekly over two summers. Sedimentation and benthic recruitment contributed little to changes in standing crop chl *a*, but rather vegetative growth, grazing pressure and mortality likely caused the observed changes in standing crop chl *a*. Food web structure affected sedimentation and recruitment for taxa that produce resting stages. The proportion of *Dinobryon* that encysted was greater in a planktivore-dominated lake compared to the lake that had

[‡] Deceased December 24, 2011

greater grazing pressure due to dominance of the food web by piscivores. Benthic recruitment of dinoflagellates was greater in the planktivorous versus the piscivorous system, suggesting grazer inhibition of benthic recruitment.

In a separate study, the occurrence of cell death was examined in an urban pond. Fluorescence microscopy was used to detect necrotic cells, as indicated by positive staining with SYTOX Green®, and apoptotic cells, as indicated by positive staining with FITC Annexin-V, during the mid-July to mid-November 2010 sampling period. Cell death occurred in a number of phytoplankton taxa. Abiotic stress likely caused death near the end of the sampling period, during temperate autumn. Biotic factors, such as viral or chytrid fungal infection, and allelopathy, may have contributed to cell death when populations were increasing or at their peaks. Finally, though death is implicated as a potentially important process to nutrient cycling in aquatic systems, it could not fully explain observed changes in total dissolved phosphorus in the urban pond.

To the loving and ever-patient Robert Polsen, whose support and thoughtfulness has helped me to reach this point sooner than would otherwise have been possible.

TABLE OF CONTENTS

GENERAL INTRODUCTION 1

LITERATURE REVIEW: PHYTOPLANKTON RESTING STAGES 6

LITERATURE REVIEW: PHYTOPLANKTON CELL DEATH 18

OBJECTIVES 25

PART I: INFLUENCE OF FOOD WEB CONFIGURATION ON PHYTOPLANKTON

RESTING STAGE ECOLOGY AND ITS EFFECTS ON POPULATION DYNAMICS

INTRODUCTION 26

MATERIALS AND METHODS 30

RESULTS 40

DISCUSSION 61

LITERATURE CITED 74

PART II: INCIDENCE OF CELL DEATH IN A EUTROPHIC URBAN PARK POND

INTRODUCTION 77

MATERIALS AND METHODS 82

RESULTS 88

DISCUSSION 101

LITERATURE CITED 112

GENERAL DISCUSSION 117

REFERENCES 122

APPENDIX: Viral and bacterial abundance (Hanson, 2010), and community zooplankton biomass (B. Angel, 2006 *unpublished data*) in Estabrook Park Pond (43°01'N 87°54'W, Milwaukee, WI, USA). 131

LIST OF FIGURES

Figure 1. Birth and loss processes that act on phytoplankton populations, and the generalized life history for cyst producing phytoplankton 4

Figure 2. Diagram of recruitment and sediment traps suspended along a transect line. 35

Figure 3. Epilimnetic standing crop chl *a* (µg chl *a* L^{-1}) for Paul, Peter, West Long, and East Long Lakes during the 1996 and 1997 sampling periods. 42

Figure 4. Relationships between observed standing crop chl *a* and that predicted from previous standing crop chl *a* with added recruitment and less sedimentation. 46

Figure 5. Changes in epilimnetic abundance of vegetative cells and the dynamics of vegetative cell sediment and recruitment fluxes (as cells d^{-1}) for *Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in the epilimnion of Peter (1996) and Paul (1997) Lakes. 50

Figure 6. Changes in the epilimnetic abundance of cysts and the dynamics of cyst sediment and benthic recruitment fluxes for *Anabaena* spp., *Cryptomonas* spp., Dinobryon spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. 52

Figure 7. Sedimentation rates of vegetative cells and cysts (cells m^{-2} d^{-1}) for *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. 54

Figure 8. Benthic recruitment rates of vegetative cells and cysts (cells m^{-2} d^{-1}) for *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) andPaul (1997) Lakes. 55

Figure 9. Percentage of the total epilimnetic standing crop (cysts and cells) represented by sedimented vegetative cells (black bars), sedimented cysts (light grey bars), benthic recruited vegetative cells (dark grey bars) and benthic recruited cysts (white bars) on each sampling date. 56

Figure 10. Seasonal trends of light, temperature, and oxygen at the surface of Estabrook Park Pond in 2010. 90

Figure 11. Trends in nutrients and particulate matter in Estabrook Park Pond, including data from previous work (1999, 2000, M. Murru, unpublished) and the present study (2010). 92

Figure 12. Trends in abundance and cell death for Chlorophycean taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® and FITC Annexin-V primarily during the latter half of the 2010 sampling period. 95

Figure 13. Trends in abundance and cell death for Chrysophycean taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V primarily near the end of the sampling period. 97

Figure 14. Trends in abundance and cell death for taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V both before and after a peak in abundance. 98

Figure 15. Trends in abundance and cell death for taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V during peaks in abundance. 99

LIST OF TABLES

Table 1. Characteristics of lakes in the Trophic Cascade Project used in Part I of the present study prior to initial 1993 nutrient enrichment. 33

Table 2. Ecosystem manipulations for the Trophic Cascade Project lakes used in Part I of the present study. 34

Table 3. Phytoplankton taxa counted in the samples from Peter (1996) and Paul (1997) Lakes. 39

Table 4. Results of regression analysis on the observed standing crop (measured as chl *a*) and that predicted from the previous standing crop plus benthic recruitment and less sedimentation for each of thermal strata of the lakes in Part I of the present study on resting stage ecology. 44

Table 5. Sedimentation (%S) and benthic recruitment (%R) as percentages of the epilimmetic abundance (both vegetative cells and cysts, on a daily basis) for *Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. 58

Table 6. Results from the regression analyses on the measured abundance and that predicted from the previous abundance plus benthic recruitment and less sedimentation for the taxonomic groupings counted in the epilimnion of Peter (1996) and Paul (1997) Lakes. 60

Table 7. The phytoplankton taxa that exhibited positive staining with SYTOX-Green® and FITC Annexin-V, and that were able to be identified under fluorescent microscopy and were counted in Part II of the present study on cell death. 87

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GENERAL INTRODUCTION

Historically phytoplankton taxa were thought to live indefinitely in the pelagos until either grazed by herbivores or sedimentation took them out of the photic zone (Franklin, Brussaard & Berges, 2006). Yet life history events such as resting stage formation and germination (Ishikawa & Taniguchi, 1996), and physiological cell death (Vardí *et al*., 2007) may also be important processes contributing to phytoplankton population dynamics. However, the significance of life history events to population dynamics is often overlooked because of cryptic and transient nature of life phytoplankton history transitions (Sandgren, 1988).

Many phytoplankton taxa alternate between benthic resting stages and pelagic vegetative existence as part of their life history (Figure 1; see also Sandgren, 1988; von Dassow & Montresor, 2011). Resting stages are physiologically and morphologically distinct from vegetative cells and are characterized by a reduced metabolic rate, the presence of ample storage products, and a resistant cell wall (Fryxell, 1983). Moreover, resting stage formation (*encystment*) and germination (*excystment*) often occur in response to environmental changes in temperature (e.g. Anderson & Rengefors, 2006), light (e.g. Adams & Duggan, 1999) and nutrient availability (e.g. Figueroa, Bravo & Garcés, 2005). In addition, excystment and encystment dynamics may be affected by the inhibitory effect of grazer exudates (suggested by Hansson, 1993; Rengefors, Karlsson & Hansson, 1998). Together

these characteristics suggest that resting stages function as a survival mechanism during periods unfavorable to pelagic growth (Starr, 1955; Livingstone & Jaworski, 1980; Coleman, 1983; Reynolds, 1988; McQuoid & Hobson, 1996).

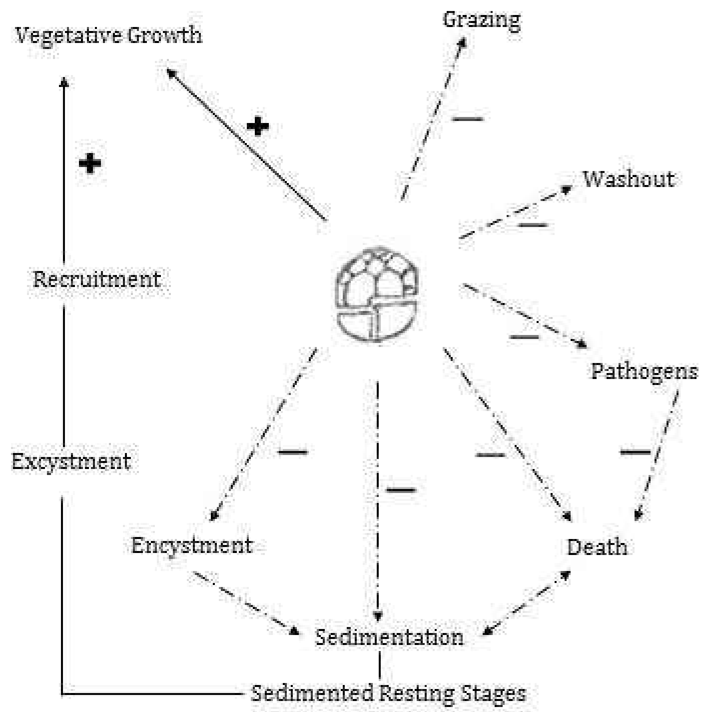
Phytoplankton may also undergo programmed cell death (PCD) in response to unfavorable environmental conditions. Laboratory studies have demonstrated that nutrient starvation (e.g. Berges & Falkowski, 1998), light limitation (e.g. Segovia & Berges, 2009), UV radiation (Jiménez *et al*., 2009), hyperosmotic stress (e.g. Ning *et al*., 2002) and heat shock (Jiménez *et al*., 2009) may each elicit a death response. Other factors that have potential to contribute to death and further population declines in the field include the presence of pathogens like algacidal bacteria (Kim *et al*., 1998), viruses (Brussaard, 2004) and chytrid fungi (Ibelings, 2004; Alster & Zohary, 2007), and also allelopathy (Legrand *et al*., 2003). Furthermore, the death response observed depends on the intensity, duration, and type of stress to which cells are exposed (Jiménez *et al*., 2009).

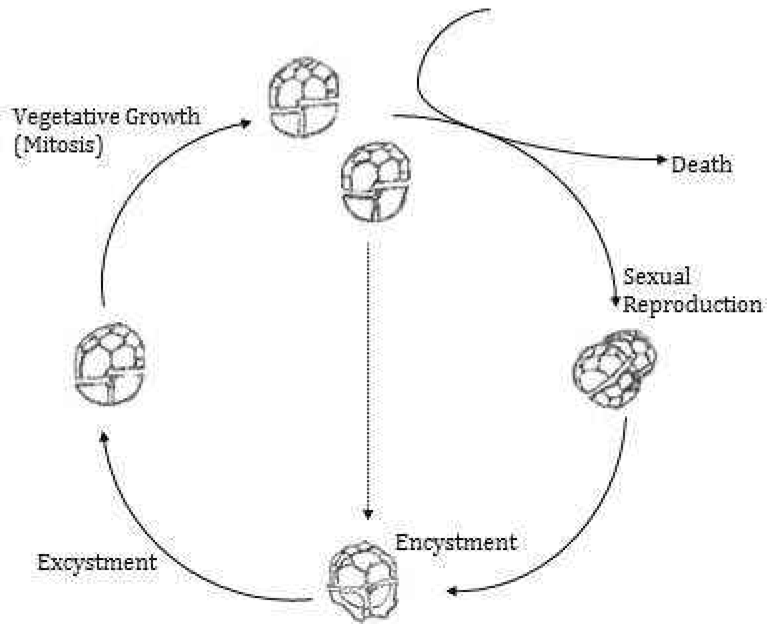
Necrosis and apoptosis are two types of cell death considered to be at opposite ends of the death response continuum (Jiménez *et al*., 2009). Necrosis is the form of death triggered when cells are exposed to a lethal level of stress causing irreparable damage (Henriquez *et al*., 2008), and is characterized by cellular swelling, membrane permeabilization, and organelle dysfunction (Bialik *et al*., 2010). Unlike necrosis, apoptosis is regarded as a form of programmed cell death (PCD) because events are metabolically regulated by the expression of specific death genes (Franklin *et al*., 2006; Bialik *et al*., 2010). The early stages of apoptosis are characterized by the translocation of phosphatidylserine from the inner plasma

membrane to the outer layer resulting in "blebbing" of the membrane (Segovia & Berges, 2009). Other morphological features of apoptosis that have been observed in phytoplankton are DNA condensation, margination, and fragmentation, followed by cell shrinkage, and membrane permeabilization associated with secondary necrosis (Franklin *et al*., 2006).

It is important to examine both the effects of phytoplankton resting stage dynamics and programmed cell death on population dynamics. Both resting stage formation and programmed cell death are controlled by environmental stress. Moreover, there is evidence that resting stage formation and apoptotic pathways may be linked (Vardí *et al*., 1999). Distinguishing the proportion of a population that undergoes resting stage transformation or programmed cell death, and also the conditions that trigger these life history transitions, will become important for future studies on biogeochemical cycling of nutrients in aquatic systems.

Figure 1. Birth and loss processes that act on phytoplankton populations, and the generalized life history for cyst producing phytoplankton (in this case a dinoflagellate). The top panel shows that pelagic phytoplankton populations may grow via vegetative growth (mitosis), and via excystment from benthic resting stages followed by recruitment into the pelagic environment. Grazing, washout, pathogens, encystment, sedimentation, and death may all contribute to losses from the pelagic population. The bottom panel shows the life history of a cyst-producing phytoplankton (in this case a dinoflagellate). Vegetative growth occurs by mitosis. Cells may die, undergo direct encystment, or sexual reproduction followed by encystment as the population ages and experiences stress. Excystment followed by vegetative growth under favorable conditions seeds subsequent pelagic populations.


Vegetative Growth
Grazing
Washout
Recruitment
Pathogens
Excystment
Encystment
Death
Sedimentation
Sedimented Resting Stages


Vegetative Growth
(Mitosis)
Death
Sexual
Reproduction
Excystment
Encystment

LITERATURE REVIEW: Phytoplankton resting stages

Morphological Characteristics and Function

Resting stages are physiologically distinct life history stages produced by many freshwater phytoplankton. Their physiological and morphological characteristics indicate that these stages primarily serve as a survival mechanism to over-winter or over-summer periods unfavorable to pelagic growth (Starr, 1955; Livingstone & Jaworski, 1980; Coleman, 1983; Reynolds, 1988; McQuoid & Hobson, 1996). Specialized resting stages are characterized by a reduced metabolic rate, the presence of ample storage products, and a resistant cell wall (cyanobacteria see Rippka & Herdman, 1985; Adams and Duggan 1999; dinoflagellates see Wall & Dale, 1968; Chapman, Dodge & Heany, 1982; Kokinos, 1994; chrysophytes see Sandgren, 1991 and refs. therein; chlorophytes see Starr, 1955; Coleman, 1983; Malmberg & Van Winkle-Swift, 2001; diatoms see McQuoid & Hobson, 1996). These characteristics allow specialized resting stages of some taxa to remain viable in the sediments for decades after production (Lund, 1954; Livingstone and Jaworski 1980; McQuoid, Godhe & Nordberg, 2002), though different types of resting stages vary in their ability to remain viable over prolonged periods as a consequence of morphological and physiological differences (Chapman *et al*., 1982; Agrawal, 2009).

In addition to general survival, resting stages are sometimes attributed greater importance and may be regarded as a perennation strategy (a strategy for year-to-year survival). A number of perennation strategies exist and more than one type may be exhibited within a taxon (Reynolds, 1988; Rengefors, Gustafsson & Stahl Delbanco, 2004). Perennation strategies of freshwater phytoplankton include:

the maintenance of year-round pelagic *refugia* populations; benthic vegetative cells; and benthic resting stages which include resting cells (akinetes) and specialized benthic resting stages generally referred to as cysts or spores. Determination of the significance of resting stages as a perennation strategy may be difficult because more than one perennation strategy may be exhibited within a taxon.

Resting Stage Formation: Regulation and Patterns of Encystment

The formation of resting stages couples pelagic phytoplankton to the benthic environment. Two encystment strategies may be distinguished based on the factors that initiate encystment. In one strategy, freshwater phytoplankton form resting stages in response to environmental cues that cause physiological stress (encystment under *exogenous* control) (Sandgren & Flanigan, 1986). These cues act as triggers or regulators of encystment rates and are often related to changes in nutrient concentration, light, and temperature (chlorophytes: Trainor, 1960; Cain & Trainor, 1976; Coleman, 1983; cyanobacteria: Wymann & Fay, 1986; Adams & Duggan, 1999; Hori *et al*., 2003; dinoflagellates: Chapman & Pfiester, 1995, and refs. therein; diatoms: McQuoid & Hobson, 1996, and refs. therein). Alternatively, *endogenous* induction of resting stage formation relies on a developmental mechanism to determine the timing of resting stage production. Endogenous cues may be related to the number of cellular divisions or extracellular hormone concentrations; it is a bet-hedging strategy that may be best suited for taxa that experience strong regulation of vegetative growth by environmental factors because reproduction and encystment would be cued before the population becomes sharply truncated. This strategy has been observed for the production of zygotic resting

stages in chrysophytes (reviewed in Sandgren, 1991), diatoms (McQuiod & Hobson, 1996; Jewson *et al*., 2008), and green flagellates (Kirk & Kirk, 1986). These strategies are important because the factors that regulate resting stage production may be indicative of the state of population growth and physiological state of taxa. As a result, patterns of resting stage production may be apparent relative to the population growth rate.

The timing of resting stage production relative to the vegetative growth rate may have important implications to community dominance patterns. The pattern of resting stage production relative to the vegetative dynamics of a population may be classified as *extrinsic* or *intrinsic* encystment. Populations in a stationary or declining growth phase often experience physiological stress. Encystment that tends to occur during stationary or declining growth is termed *extrinsic* encystment and is often under exogenous control (Sandgren, 1981). This type of encystment has been observed for cyanobacteria (Wymann & Fay, 1986; van Dok & Hart, 1996), dinoflagellates (Wall & Dale, 1968; Heaney & Talling, 1980; Zohary *et al*., 1998), and chrysophytes (Sandgren, 1981, Sandgren & Flannigan, 1986). Green algae may also exhibit extrinsic encystment due to likely reliance of coccoid forms on resting cells as benthic propagules and nutrient limitation triggering sexual production of resting cysts in flagellate taxa (Happey-Wood, 1988).

Alternatively, *intrinsic* resting stage formation occurs during periods of exponential growth and is typically associated with endogenous inducing factors. Intrinsic encystment has been observed for chrysophytes known to excrete mating hormones (Sandgren, 1981; Sandgren & Flannigan, 1986) and may be a potential

pattern for diatoms. Diatom resting spores are thought to be the result of sexual reproduction cued by an internal "sex-clock" (reviewed in Cherpurnov *et al*., 2004). Environmental cues are then believed to trigger sexual reproduction once cells have reached a critically small size as a result of successive mitotic size reductions. However, this idea is currently challenged as evidence of size maintenance in diatom populations is more closely examined (Hildebrand, Frigeri & Davis, 2007). Regardless, the take-home message is that the timing of encystment relative to the physiological state of a population may differentially affect water column abundance dynamics. Extrinsic encystment may contribute more rapidly to population decline than intrinsic encystment because extrinsic cyst formation occurs during a period of stagnant or declining growth during which the population often experiences physiological stress, in contrast to intrinsic encystment that occurs at the point of increasing population growth rates.

Resting Stage Germination and Recruitment: Regulation and Patterns of Excystment

Resting stages form a benthic seed bank from which germinating propagules recruit to the pelagic environment. Regulation of resting stage germination and recruitment is primarily under exogenous control with some taxa exhibiting a degree of endogenous regulation. Resting cysts of dinoflagellates have a well-documented maturation period that is required prior to germination, and after which, cysts may remain viable in a quiescent state until germination (e.g. see Anderson, 1998). Required dormancy periods for zygotic resting stages have also been reported for chlorophytes (see Agrawal, 2009), diatoms and chrysophytes (Sandgren, 1991 refs. therein). Maturation periods are likely important in

preventing resting stage germination during seasonally predictable periods unfavorable to vegetative growth and may promote synchronous recruitment. Germination of resting stages across taxa primarily occurs in response to light, temperature, and oxygen (Hansson, 1993, 1994; Chapman & Pfiester, 1995; Brunberg & Blomqvist, 2003; McQuoid & Hobson, 1995). In addition, grazer exudates have been shown to have an inhibitory effect on germination (Rengefors *et al*., 1998) and may play a role in regulation of recruitment (Hansson, 1993; Anderson & Rengefors, 2006).

Germinated resting stages enter the water column through the process of recruitment. Recruitment strategies are classified based on the synchronicity of propagules entering the water column. Phytoplankton recruitment may be synchronized and occur from mass numbers of benthic propagules or through continuous recruitment from a small number of benthic propagules. Synchronous recruitment has been observed in lab and field studies for cyanobacteria (Head, Jones & Bailey-Watts, 1999) and dinoflagellates (e.g. Anderson, 1998; Rengefors & Anderson, 1998; Anderson & Rengefors, 2006). Requisite maturation periods of resting stages may contribute to mass recruitment events as has been suggested for dinoflagellates. In addition, these studies have also revealed that taxa that exhibit pronounced and distinct germination optima for a given set of conditions tend to have a synchronous recruitment pattern. Wind-driven mixing events may also influence mass recruitment from benthic propagules and have been implicated as an important factor in the seasonal initiation of diatom blooms (Lund, 1955; Schelske, Carrick & Aldridge, 1995; McQuoid & Godhe, 2004). Also of note is that taxa with a

synchronous strategy often are mixing-tolerant R-strategists (diatoms) or slow-growing, stress-tolerant S-strategists (cyanobacteria, dinoflagellates).

Alternatively, a persistent recruitment strategy may be employed in which a few benthic propagules continually germinate and recruit to the water column. This strategy is likely representative of species with a more broad range of conditions over which benthic propagules germinate, and may be more adaptive for fast-growing opportunistic taxa (C-strategists) whose vegetative populations are strongly regulated by grazing (Reynolds, 1988). Chrysophytes are heavily grazed freshwater taxa and laboratory studies suggest that the persistent recruitment strategy may be employed (Sandgren, 1981, 1988). Continual, or persistent, recruitment has also been suggested for other taxa including dinoflagellates (Anderson & Rengefors, 2006; Blanco, Lewis & Aldridge, 2009). These strategies suggest that taxa that exhibit a mass recruitment strategy have a greater reliance on benthic propagules for pelagic establishment compared to taxa with a continuous recruitment strategy that would rely on rapid vegetative growth for pelagic establishment.

Resting Stage Sedimentation and Recruitment: Significance to Community Abundance Dynamics

Resting stage sedimentation and recruitment represent potentially important loss and gain processes that contribute to seasonal periodicity. Sedimentation of cells out of the photic zone is recognized as a loss process that may significantly contribute to pelagic population decline, especially for non-motile forms (e.g. Uhlmann, 1971; Reynolds, Morison & Butterwick, 1982). The relative

contribution of resting stage sedimentation to population decline may be a reflection of the significance of these stages as a perennation and survival mechanism (Sandgren, 1988). Recognition of the significance of resting stages as a potential perennation strategy has led to research aimed at determining the proportion of sedimented material comprised of specialized resting stages because these stages represent contributions to the benthic seed bank for subsequent recruitment. Quantification of resting stage sedimentation focused on toxic and bloom-forming marine dinoflagellates (e.g. Ishikawa & Taniguchi, 1996; Montressor, Zingone & Sarno, 1998; Morquecho & Lechuga-Devéze, 2004) and freshwater cyanobacteria (Fallon & Brock, 1980; Verspagen *et al*., 2005). At times, high rates of encystment have been implicated as a driving factor causing bloom collapse (Heaney, Chapman &Morison, 1983; suggested by Anderson, 1998; Wang, Qi & Yang, 2007), while in other systems encystment contributes little to population decline (e.g. Pollingher, 1988). Furthermore, mass encystment and resting stage sedimentation events in response to changes in the chemical and/or physical environment have been suggested as a factor contributing to phytoplankton succession in deep lakes (e.g. Crumpton & Wetzel, 1982; Reynolds, Wiseman & Clarke, 1984; Sommer, 1985) and marine systems (e.g. Anderson & Rengefors, 2006; Estrada *et al*., 2010).

Recruitment into the water column from benthic propagules may periodically account for a significant proportion of limnetic biomass and may also contribute to seasonal succession. Episodes of mass recruitment over a defined time period have been shown to contribute a significant proportion of biomass to

dinoflagellates (Heaney *et al*., 1983; Ishikawa & Taniguchi, 1996); cyanobacteria (Head *et al*., 1999; Brunberg & Blomqvist, 2003); and diatoms (McQuoid & Hobson, 1995; Jewson *et al*., 2008). These studies demonstrated that the pelagic biomass for some resting-stage-producing taxa could not have been accounted for by vegetative growth alone.

Patterns of community succession may also be influenced by the dependency of taxa on resting stages for perennation in addition to mandatory maturation periods of zygotic resting stages. Studies have shown that the timing of first appearance in the water column for cyst-forming taxa may be explained by resting stage maturation and required environmental conditions for germination (Rengefors & Anderson, 1998; Head *et al*., 1999; Anderson & Rengefors, 2006). Conversely, taxa that rely on *refugia* populations tend to have a more stochastic appearance in the water column associated with favorable vegetative conditions (Rengefors & Anderson, 1998; Head *et al*., 1999; Anderson & Rengefors, 2006). Though resting stages may be important for the initial establishment of pelagic populations, the relative contribution of recruited cells to the water column standing crop will depend on the upward flux of recruiting cells and the presence of *refugia* populations at the onset of favorable vegetative conditions (Head *et al*., 1999). Benthic stages may be important for initial establishment in the water column, but further recruitment may contribute little to the standing crop if *refugia* populations exist at the onset of favorable vegetative conditions. Studies on the sedimentation of and recruitment from benthic resting stages are important

because valuable quantitative information is provided on life history population dynamics that are useful for developing taxa-specific bloom-development models.

Incorporation of internal and external factors that regulate life history transitions is important for development of taxa specific population models. Internal factors are important because internal nutrient quotas may affect the regulation of resting stage formation and recruitment. One model that incorporates regulatory internal and external factors that control life history transitions predicts that the effect of excystment and recruitment on pelagic cell abundance to be greater during periods of pelagic population decline, and that cyst-forming taxa are less susceptible to extinction than non-cyst formers (Estrada *et al*., 2010). This model also predicts that cyst-forming taxa are more predictable in their timing of appearance in the community compared to non-cyst forming taxa (Estrada *et al*., 2010). The latter prediction is in accordance with the observed stochastic appearance of non-cyst forming taxa in marine (Rengefors & Anderson, 1998; Anderson & Rengefors, 2006) and freshwater studies (Head *et al*., 1999).

Seed Banks and Temporal and Spatial Variation in Recruitment

A number of factors must be considered when examining the effect of resting stages on community seasonal dominance patterns. In addition to the topics previously discussed, the spatial distribution of resting stages in lake sediments is another important factor that requires attention.

The accumulation of resting stages in the benthos is referred to as a “seed” or “propagule bank” and represents the potential pelagic inocula for resting stage-forming taxa. Lake mixing events disturb the sediment surface and aid recruitment

of phytoplankton from the benthos by inoculating the water column with propagules. The extent and frequency of these events affect the size of the effective seed bank and the potential significance of mixing events to recruitment. Sediment from the entire lake basin may contribute to pelagic seeding during whole-lake mixing events that typically occur in spring and autumn in temperate climates. Conversely, during periods of stratification or limited mixing, the physical and chemical gradients formed may act as a barrier to limnetic recruitment from deeper water and restrict the effective seed bank to sediments above the thermocline (Hansson, 1995; Brunberg & Blomqvist, 2003). If the stratification is weak, however, it may be possible for recruitment to occur from depths below the thermocline as suggested by Head *et al*. (1999). Recruitment from the littoral zone may also be enhanced during periods of stratification as a result of the greater effect of wind-driven mixing in the epilimnion (Rengefors *et al*., 2004) and likeliness of bioturbation (Stahl-Delbanco & Hansson, 2002) contributing to sediment resuspension events.

Lake bathymetry is also important when determining the size of the seed bank during periods of stratification. Basins with a more gradual slope may have a larger effective seed bank when strongly stratified than basins that are more steeply sloped due to greater sediment surface area when assuming the thermocline is the same depth. The frequency of mixing events is also affected by lake bathymetry. Shallow lakes with a large surface area tend to mix more readily (Schelske *et al*., 1995) than deep lakes with a small surface area that exhibit stronger stratification. As a result, pelagic establishment of phytoplankton may be more highly correlated

with mixing events in shallow lakes, than to environmental cues that trigger germination and recruitment into the water column through buoyancy regulation or self-propelled movement. Similarly, the pelagic community of a strongly stratified lake may become largely a reflection of excystment from resting stages in epilimnetic sediments due to more frequent mixing, bioturbation, and a strong thermocline that may act as a barrier to vertical migration from the hypolimnion.

All phytoplankton taxa with benthic stages have the potential to recruit during mixing events but successful recruitment from resting stages will depend on whether resting stages have germinated (or have the ability to germinate) and if the water column conditions are able to support vegetative growth. For example, zygotic resting stages that have not reached maturity at the time of a mixing event will not be able to successfully recruit. It may be argued, however, that “older” resting stages from previous years would also be able to inoculate the water column if the “younger” resting stage were immature at the time of mixing. While this may be the case, as has been suggested to have happened in a study by Heaney *et al*. (1983), viability has been shown to decrease as specialized resting stages age (Livingstone & Jaworski, 1980; McQuoid *et al*., 2002). As a result, the ability of “old” resting stages to inoculate the water column during a mixing event will depend on the time spent in the water column as well as water column conditions with respect to germination and vegetative growth. Turbulence during mixing events differentially affects phytoplankton growth rates as a consequence of effects on nutrient uptake and shear stress on rates of cell division (see Sullivan & Swift, 2003 and ref. therein). Large-scale mixing favors disturbance tolerant taxa like diatoms

(Reynolds *et al*., 1984) and contributes to the seasonal transition of phytoplankton in lakes (Song *et al*., 2010).

Unfortunately, in the expanse of research conducted on phytoplankton resting stage ecology, there have been few comparative studies that examine the significance of resting stages to pelagic phytoplankton abundance dynamics under different trophic conditions. The Trophic Cascade Hypothesis predicts that lakes with food-webs that are piscivore dominated will have a greater abundance of zooplankton (and less phytoplankton) compared to lakes that are planktivore dominated (Carpenter *et al*., 2001). The effect of grazer exudates on phytoplankton resting stage dynamics has been little researched but a few studies have observed an inhibitory effect of grazer exudates on dinoflagellate germination (Rengefors *et al*., 1998) and recruitment (Hansson, 1993), and increased encystment co-occurring with increased grazer abundance in the field (Anderson & Rengefors, 2006). In light of these studies, a glaring gap in knowledge exists in understanding the effects of food-web structure on phytoplankton resting stage dynamics. This knowledge is directly important to an applicable to models aiming to examine the effect of biotic perturbations to phytoplankton population dynamics, and to understanding potential consequences of lake management practices and the response of phytoplankton communities to environmental perturbations.

LITERATURE REVIEW: Physiological cell death

The occurrence of cell death is well studied and an important phenomenon in metazoan development (Lam, 2004; Ulukaya, Alcilan & Yilmaz, 2011; van Doorn, 2011), but development of an understanding of its occurrence among phytoplankton and significance to ecosystem processes is still in its infancy despite almost two decades of research. Historical equations describing birth and loss processes of phytoplankton did not include a term for cell death (Uhlmann, 1971). Equations developed later acknowledged *in-situ* cell death as a loss process (Reynolds, 1980) but it was deemed negligible compared to grazing and sedimentation losses. Recently, phytoplankton ecologists have begun to examine the occurrence of and factors associated with different types of cell death responses in laboratory and field settings by using fluorescent microscopy techniques.

Modes of Cell Death: Necrosis and Apoptotic PCD

Two modes of cell death that have been distinguished and measured in studies on cell death in phytoplankton are apoptosis and necrosis. Apoptosis is regarded as a form of programmed cell death (PCD) because events are regulated by the expression of specific death genes (Bialik *et al*., 2010). Conversely, necrosis has been classified as a death response that does not involve metabolic regulation, is devoid of new gene expression (Franklin *et al*., 2006), and is not considered a form of PCD by this definition. However, a number of recent studies on the mechanisms regulating cell death events in metazoans have revealed that necrosis and apoptosis are both under genetic control and both should be considered forms of PCD (as reviewed in: Henriquez *et al*., 2008; Bialik *et al*., 2010). Mechanistic connections in

the biochemical pathways of apoptosis with necrosis form part of the foundation for considering necrosis as an active response initiated by the cell (as reviewed in Henriquez *et al*., 2008; Bialik *et al*., 2010). Regardless of whether apoptosis and necrosis are both under genetic control, the events in the biochemical pathways result in different death morphologies that may subsequently affect intercellular communication and dissolved nutrient cycling in aquatic systems.

Studies on metazoans describe a number of biochemical and morphological characteristics that distinguish apoptosis and necrosis as different modes of cell death. Apoptosis is triggered by sub-lethal forms of stress and upon activation leads to the synthesis of new proteins involved in the death sequence (Proskuryakov, Konoplyannikov & Gabai, 2003). Cysteine aspartic proteases (caspases) are involved in the regulation of events in apoptosis (Vardí *et al*., 1999; Segovia *et al*., 2003; Jiménez *et al*., 2009) and may be activated by high levels of internal reactive oxygen species (ROS) (Vardí *et al*., 1999; Segovia & Berges, 2009). The early stages of apoptosis are characterized by the translocation of phosphatidylserine from the inner plasma membrane to the outer layer resulting in "blebbing" of the membrane (Segovia & Berges, 2009). Other morphological features of apoptosis that have been observed in phytoplankton are DNA condensation, margination, and fragmentation, followed by cell shrinkage, and membrane permeabilization associated with secondary necrosis (Franklin *et al*., 2006).

Necrosis represents a physiological state of dying in which the cell may not recover from damage and may occur independently or coupled with other forms of PCD. This form of death is triggered when cells are exposed to a lethal level of stress

causing irreparable damage (Henriquez *et al*., 2008; Bialik *et al*., 2010). Cellular swelling, membrane permeabilization, and organelle dysfunction are key morphological features that characterize necrotic cells (Bialik *et al*., 2010). Ultimately, the cell swells and lyses in a necrotic death response rather than cellular shrinkage and collapse into apoptotic bodies, as is the case for apoptosis. In metazoans apoptotic bodies are targeted for phagocytosis by the immune system, but in phytoplankton this does not occur and apoptosis is followed by necrosis. Furthermore, the physiological and biochemical changes associated with death pathways may affect the quantity and compositions of exudates secreted by cells into the environment and predispose neighboring cells to a particular death response (Vardí *et al*., 2007).

Methods of Cell Mortality Detection: Fluorescent probes SYTOX-Green and FITC Annexin-V

Fluorescent staining techniques have been used to determine modes of cell death in laboratory and field studies. Indicators of dead cells are called mortal stains because they are only able to penetrate compromised membranes (Lobban, Chapman & Kremer, 1988). SYTOX-Green® (Segovia and Berges 2009) and Evan's Blue (Lobban *et al*., 1988) are two types of mortal stains have been employed to indicate dead cells. SYTOX-Green® is a fluorescent nucleic acid probe that has been increasingly used in studies on phytoplankton death because of the potential to be used in conjunction with flow cytometry for rapid culture analysis, but traditional analysis with fluorescent microscopy is common (Peperzak & Brussaard, 2011). This stain provides an adequate method for detecting necrotic cells within a natural

assemblage because of the broad range of phytoplankton classes that are receptive to the probe, though differential sensitivity to the probe has been observed reportedly in Euglenophyceae (Peperzak & Brussaard, 2011). Using mortal stains alone does not determine the mode of cell death, but rather is simply an indicator of dead cells. Elucidation of the mode of death would require the use of a mortal stain in conjunction with other techniques that interact with biochemical or physiological features characteristic of a death pathway.

Loss of membrane symmetry is a useful feature for detecting apoptotic cell death. Loss of membrane symmetry occurs early in PCD for metazoans, prior to membrane permeabilization associated with secondary necrosis of the cell. FITC Annexin-V is a fluorescent probe used to detect the loss of membrane symmetry characteristic of apoptosis (Segovia & Berges, 2009). FITC Annexin-V binds to the phosphatidylserine residues found on the inner cell membrane. During apoptosis the phosphatidylserine is translocated from the inner membrane to the outer membrane. However, FITC Annexin-V may also enter cells with a compromised membrane and apoptotic and necrotic cells would be expected to stain positive. As a result, FITC Annexin-V must be used in conjunction with a mortal stain, like SYTOX-Green® so that the proportion of a population actually experiencing apoptosis can be determined by subtracting the proportion of SYTOX-Green® positive (dead) cells from the observed proportion of FITC Annexin-V positive cells.

Cell Death in Field Populations

The occurrence of cell death in phytoplankton has long been apparent in lab and field studies. Evidence of cell death lies in historical observations of "culture crashes" (e.g. Fogg, 1965) and un-balanced whole-lake budgets of birth and loss processes in which sedimentation and grazing cannot wholly account for population (see Franklin *et al*., 2006). Despite these observations, many studies continued to focus on factors and physiological processes necessary to maintain cell vitality and growth rather than the processes that led to cell mortality. Recognition of this gap in knowledge has lead to work examining the factors that trigger a death response in lab and field populations of phytoplankton, and how death events in a community contribute to observed dominance patterns.

A variety of abiotic stressors have been used to experimentally initiate apoptosis and necrosis in laboratory studies on phytoplankton. Examples include nutrient starvation (Berges & Falkowski, 1998; Vardí *et al*., 1999; Jiménez *et al*., 2009), light limitation (Segovia & Berges, 2009), UV radiation (Jiménez *et al*., 2009), hyperosmotic stress (Ning *et al*., 2002, Jiménez *et al*., 2009), and heat shock (Jiménez *et al*., 2009). A number of death pathways (including apoptosis and necrosis) may be possible within a species and the death response initiated depends on the intensity, duration, and type of the stress to which cells are exposed (Jiménez *et al*., 2009). Moreover, one study observed an increase in caspase production after lethal stress exposure regardless of the death morphology of the cell (Jiménez *et al*., 2009). This finding is consistent with studies that suggest interconnected

biochemical pathways are involved in determining the type of death response in metazoan cells (as reviewed in Henriquez *et al*., 2008; Bialik *et al*., 2010).

In phytoplankton, a link was suggested between a caspase-mediated death response and production of resting cysts in the dinoflagellate *Peridinium gatunenese* (Vardí *et al*., 1999). The study by Vardí *et al*. (1999) examined, at the end of a bloom season in Lake Kinneret, the relationship among dissolved CO_2 limitation, accumulation of intracellular ROS concentration, and cysteine protease activity in programmed cell death. Culture work in this study revealed that percent SYTOX-Green® positive and percent ROS positive cells were indirectly related to the concentration of dissolved CO_2. In addition, the results for the ROS portion of the culture study were corroborated in the field. Furthermore, it was found that use of E-64, a cysteine protease inhibitor, blocked accumulation of ROS in cells and reduced the number of SYTOX-Green® positive cells. E-64 inhibition was also associated with increased cyst formation in field samples of *P. gatunenese*. As a result, the data suggest a regulatory role of caspases in oxidative cell death and cyst formation for this species. Interestingly, sexual reproduction and subsequent resting cyst formation in the chlorophyte *Volvox carteri* has been shown to depend on the presence of ROS (Nedelcu, Marcu & Michod, 2004). The potential link between mechanisms controlling death responses and the production of resting stages is intriguing and warrants further investigation.

As evidence of cell death in the field mounts much of the recent research aims to determine the occurrence of *in-situ* phytoplankton death and the significance to population dynamics. For example, some studies have suggested that

death events can in some cases be responsible for bloom collapse (Vardí *et al*., 2007) and represent an important succession mechanism (Sigee *et al*., 2007). Sensitization of cells in a population to death as a result of oxidative stress and excreted sensitizing proteases (Vardí *et al*., 2007) represents one possible mechanism of bloom collapse and succession. Additionally sensitization may be responsible for an observed "death threshold" for a colonial cyanobacteria, in which a minimum proportion of cells were observed dead in colonies staining positively with a mortal stain (Sigee *et al*., 2007). The significance of death as a mechanism in bloom collapse and succession may also depend on a lake's nutrient status and a taxon's relative abundance in a planktonic community. The occurrence of cell death has been suggested to be a more significant loss process in oligotrophic than eutrophic lakes, and to have a greater effect on minor community components compared to dominants (Agustí *et al*., 2006). Continued work on the occurrence of PCD in phytoplankton is needed to disentangle the mechanisms responsible for mass death events versus "cryptic" death events that occur in minor community components or small proportions of community dominants. In addition, continued work is also needed to understand the relative significance of PCD to intra- and inter-cellular communication and nutrient cycling in aquatic systems.

OBJECTIVES

This research aims to explore the occurrence of life history transformations in phytoplankton communities. In Part I, the objective was to determine whether sedimentation and benthic recruitment could explain changes in phytoplankton standing crop chl *a*. In addition, the effect of food-web structure on sedimentation and benthic recruitment dynamics was also examined for taxa that produce resting stages. Specifically, I hypothesized that sedimentation due to cysts to be more important in explaining changes in taxa abundance than sedimentation due to vegetative cells in lakes that had higher grazing pressure (piscivorous dominated lakes) by large-bodied planktonic grazers (e.g. *Daphnia pulex*) because cysts would act as a way to escape herbivory. In addition, I hypothesized a lower rate of benthic recruitment from cysts in lakes with higher grazing pressure by large-bodied planktonic grazers due to suppression of germination by grazer exudates. In Part II of this research, the occurrence of physiological cell death in a eutrophic, urban, park pond was determined using the mortal stain SYTOX Green® to indicate necrosis and the stain FITC Annexin-V as an indicator of apoptosis. I expected dead and apoptotic cells to occur in a variety of phytoplankton taxa throughout the mid-July to mid-November sampling period. Both abiotic (light, temperature, oxygen, and nutrients) and biotic (pathogens) environmental parameters were expected to be potential causes of cell death in the field, and result in different patterns of cell death relative to time of year and cell abundance.

PART I: INFLUENCE OF FOOD WEB CONFIGURATION ON PHYTOPLANKTON RESTING STAGE ECOLOGY AND ITS EFFECTS ON POPULATION DYNAMICS

Introduction

Many phytoplankton taxa alternate between benthic resting stages and pelagic vegetative existence as part of their life history (Figure 1; see also Sandgren, 1988; von Dassow & Montresor, 2011). Resting stages are physiologically and morphologically distinct from vegetative cells and are characterized by a reduced metabolic rate, the presence of ample storage products, and a resistant cell wall (Fryxell, 1983). Moreover, resting stage formation (*encystment*) and germination (*excystment*) often occur in response to environmental changes in temperature (e.g. Anderson & Rengefors, 2006), light (e.g. Adams & Duggan, 1999) and nutrient availability (e.g. Figueroa, Bravo & Garcés, 2005). In addition, excystment and encystment dynamics may be affected by the inhibitory effect of grazer exudates (as suggested by Hansson, 1993; Rengefors, Karlsson & Hansson, 1998). It follows that formation of and germination from resting stages may differentially affect phytoplankton population dynamics in lakes differing in nutrient status and food web configuration.

Resting stages in the benthos form a "seed" or "propagule bank" and represents the potential pelagic inocula for resting stage-forming taxa. The depth of benthic recruitment relative to the thermocline must also be considered. During periods of stratification or limited mixing, the physical and chemical gradients

formed within the thermocline may act as a barrier to benthic recruitment to the epilimnion from deeper sediments, and restrict benthic recruitment to sediments above the thermocline (Hansson, 1995; Brunberg & Blomqvist, 2003). If stratification is weak, however, it may be possible for recruitment to occur from depths below the thermocline (Head, Jones & Bailey-Watts, 1999). Recruitment from the littoral zone may also be enhanced during periods of stratification as a result of the greater effect of wind-driven mixing (Rengefors, Gustafsson & Stahl-Delbanco, 2004) and likeliness of bioturbation (Stahl-Delbanco & Hansson, 2002) contributing to sediment resuspension events. Thus, I expect benthic recruitment from epilimnetic sediments to contribute more to changes in epilimnetic standing crop chl a, than benthic recruitment from sediments other thermal strata to the standing crop of that thermal strata.

Few studies have concurrently examined the effect of nutrient input and food web configuration on phytoplankton sedimentation and recruitment, and the significance to population dynamics (Hansson, 1996); none have specifically addressed the effects of these factors on encystment and excystment, and the potential significance of resulting resting stage sedimentation and benthic recruitment from resting stages to explaining phytoplankton population dynamics. Trophic cascade theory suggests that piscivore-dominated systems, with few planktivorous fishes, have more large-bodied zooplankton (e.g. *Daphnia*), and subsequently lower phytoplankton biomass compared to planktivore-dominated systems (Carpenter & Kitchell, 1993). Given the importance of abiotic factors, including nutrients, and biotic factors, like the presence of grazers, to controlling

encystment and excystment dynamics, it is possible that food web configuration may effect encystment and excystment dynamics of phytoplankton taxa in the field. Specifically, I hypothesized that sedimentation due to cysts to be more important in explaining changes in taxa abundance than sedimentation due to vegetative cells in lakes that had higher grazing pressure (piscivorous dominated lakes) by large-bodied planktonic grazers (e.g. *Daphnia pulex*). In addition, I hypothesized that lakes with higher grazing pressure by large-bodied planktonic grazers would have lower rates of benthic recruitment from cysts because the presence of exudates from large bodied grazers like *D. pulex* would inhibit phytoplankton cyst germination.

The goal of this study was to determine whether sedimentation and benthic recruitment was important to explaining changes in community standing crop (chl *a*) and, or abundance of resting stage producing taxa. First, I wanted to determine whether sedimentation and gains via benthic recruitment can explain changes in standing crop at the community level, using measurements of pelagic chl *a*, sedimented chl *a* and chl *a* that recruited from the benthos, in lakes that differed in nutrient status and food web structure. In addition, in order to determine the effect of food web structure on the significance of sedimentation and benthic recruitment to explaining changes in abundance of resting stage producing taxa, counts on Lugol's preserved samples were also performed. I expected sedimentation of cysts to contribute more to the decline in pelagic abundance than sedimentation of vegetative cells in lakes that had a higher grazing pressure. I also expected a lower

rate of benthic recruitment from cysts in lakes with higher grazing pressure by large bodied planktonic grazers (piscivore dominated lakes) compared to the rate of benthic recruitment from cysts in lakes with reduced grazing pressure (planktivorous lakes).

MATERIALS AND METHODS

Study Site and Sampling

Data examined were collected from four lakes in 1996 and 1997 at the University of Notre Dame Ecological Research Center (UNDERC) located in Land O' Lakes, WI, USA (89°32' W, 46°13' N) in conjunction with the NSF-funded Trophic Cascade Project (BSR9007196) (Carpenter *et al.*, 2001). These soft water, humic-stained forest lakes used in the project are of similar morphometry (Table 1; see Hansson, 1996 for bathymetric maps). Paul Lake was an unmanipulated lake with a piscivore-dominant food web, which served as a reference lake to three other manipulated lakes (Peter, East Long and West Long Lake) that were subject to food web reconfiguration and nutrient enrichment (Table 2). East Long and West Long Lakes are two basins of the same lake that were separated by a neoprene curtain during the Trophic Cascade Project and were treated as separate lakes (Carpenter *et al.*, 2001). Peter, East Long, and West Long Lakes were subject to nutrient enrichments, and Peter and East Long Lakes were made to be planktivore-dominant by removal of piscivores, while West Long Lake was made to be piscivore-dominant through addition of piscivores. East Long Lake became dystrophic in 1997 due to hydrological changes resulting from separation from the West Long Lake basin, and was not included in the present study.

A transect line that suspended the recruitment and sediment traps ran from the shore to the center of each of the lakes (Figure 2). Recruitment and sediment traps were suspended at fixed depths in each of the lakes. A Hydrolab DataSonde-3 (Hach Hydromet, Loveland, CO, USA) was used to record temperature, pH,

conductivity and oxygen profiles from the center of each lake at the time of trap-setting and collection. Light penetration was also measured using a LI-COR LI-193SA spherical quantum sensor attached to a LI-1000 data logger (LI-COR Biosciences, Lincoln, NE, USA). Funnel traps as described in Hansson (1996) with 300µm Nitex mesh over the opening were suspended large side down to collect algae recruiting from the benthos in samples bottles that were attached to the narrow end of the funnel. Prior to deployment, recruitment traps were filled with prescreened epilimnetic water. Recruitment traps without preservative were deployed alongside recruitment traps that contained saline, acid Lugol's iodine. Saline acid Lugol's iodine was used in order to increase the density and prevent leakage of the preservative from the sample bottles (C.D. Sandgren pers. comm.). Traps were deployed for one-week intervals. Upon sample collection, 100mL of the preserved recruitment trap samples were transferred to French square bottles for later microscopic enumeration. Unpreserved recruitment trap samples were used for chl *a* analysis. Recruitment trap samples for chl *a* analysis were combined dependent on position in the epilimnion, metalimnion and hypolimnion.

Sediment traps consisted of paired PVC-pipes (8 traps total, 6.4cm dia., 5 X 1 aspect ratio) attached to collection bottles, and were suspended from the transect line into the hypolimnion at the center of each lake. Prior to deployment, sediment traps were filled with prescreened epilimnetic water. One of the paired sediment traps also contained saline acid Lugol's iodine preservative.

Samples were collected weekly and were processed in a manner similar to recruitment traps for enumeration and chl *a* analysis. Whole-water samples from the center of the lake were also collected weekly from each thermal stratum at the time of trap setting for subsequent chl *a* analysis and counting.

Table 1. Characteristics of lakes in the Trophic Cascade Project prior to initial 1993 nutrient enrichment. Photic depth is depth of 1% irradiance. East and West Long Lakes are separate basins of the same lake. † Color is based on absorbance at 440nm. From Carpenter *et al*. (2001).

Characteristic	East Long Lake	Paul Lake	Peter Lake	West Long Lake
Area (ha)	2.31	1.74	2.67	3.39
Mean depth (m)	4.9	3.9	6	4.4
Max. depth (m)	14.4	12.8	19.9	17.7
Color†	9.5	1.6	1.5	3.9
Thermocline depth (m)	2.8	4.1	4.2	4.2
Photic depth (m)	2.6	5.7	6.3	5.1
Depth to anoxia (m)	4.5	6.5	7.9	5.1

Table 2. Ecosystem manipulations for the Trophic Cascade Project lakes in Land O' Lakes, WI (89°32' W, 46°13' N). Lakes were subjected to food-web manipulations in 1991 and to nutrient enrichment regimes during 1993-1997. Nutrient enrichments in 1996 and 1997 are reported in N:P by atoms, and were 34.4 and 31.5 respectively (Carpenter *et al*., 2001).

Lake Basin	**Nutrient Enrichment**	**Food-web configuration**
Paul (control)	-	Piscivore dominant. Largemouth bass, *Micropterus salmoides*.
Peter	+	Planktivore dominant. Golden shiners (*Notemigonis chrysoleucas*), fathead minnows (*Pimephalus promelas*), and red dace (*Phoxinus eos)*
West Long	+	Piscivore dominant. Largemouth bass *(Micropterus salmoides*), smallmouth bass (*Micropterus dolomieu*), and yellow perch (*Perca flavescens*). Perch died in 1992-1993, but lake remained piscivore dominant.
East Long	+	Intended planktivore dominant, but hydrologic changes caused low and variable fish populations and was excluded from the 1997 portion of this study.

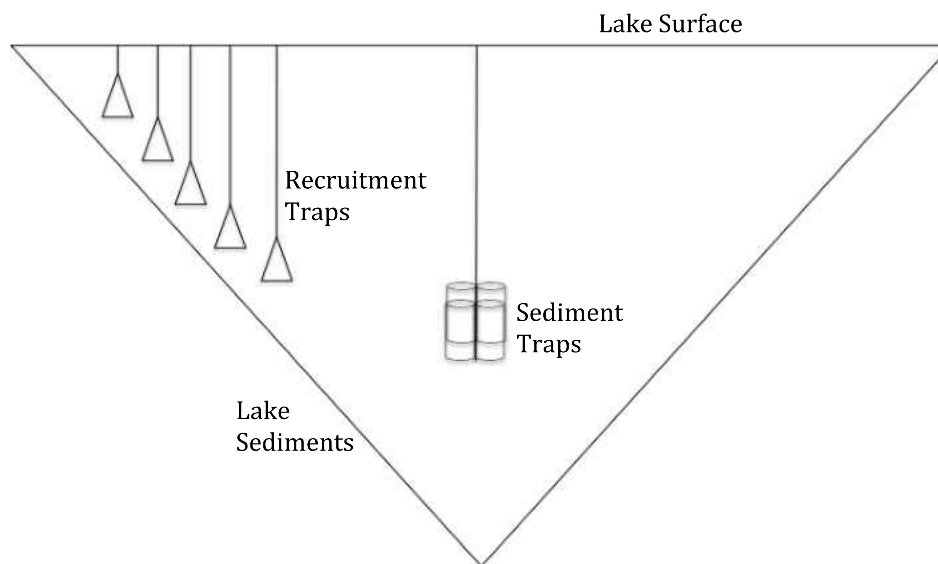


Figure 2. Diagram of recruitment and sediment traps suspended along a transect line. Inverted funnel traps attached to bottles were used to collect recruiting phytoplankton from the benthos and were suspended at fixed depths, 0.5m above the sediment surface (Hansson, 1996). Sediment traps were constructed from PVC pipes (6.4cm dia., 5 X 1 aspect ratio) attached to bottles and suspended in hypolimnion in the center of each lake. Each trap was deployed in pairs to collect separate samples for chl *a* analysis and enumeration.

Chlorophyll a Analysis

Chlorophyll *a* (chl *a*) was used to quantify community standing crop. It was measured immediately after collection of recruitment trap, sediment trap, and whole water samples. For recruitment and sediment traps, samples from the same thermal stratum were combined prior to chl *a* analysis. Whole water samples were taken from each thermal stratum on the date of recruitment/sediment trap sample collection and trap-setting. Replicate subsamples were filtered onto Gelman Supor 800 polycarbonate filters (0.8μm pore size), extracted in 90% acetone (buffered with 1g MgCO3 L^{-1}) for seven days at -10°C, and chl *a* measured using the fluorescence technique of Strickland and Parsons (1972).

Significance of Sedimentation and Benthic Recruitment to Standing Crop

Chl *a* data and phytoplankton cell count data were used to assess the significance of sedimentation and benthic recruitment to changes in community standing crop and taxa water column abundances respectively. All chl *a* values were corrected to represent the total amount of chl *a* recruiting into, sedimenting from, or present in a given thermal stratum on each sampling date. Additionally, recruitment and sediment trap total chl *a* values were corrected for deployment time in order to be comparable to measured water column chl *a*.

Next, predicted standing crop chl *a* values for sample dates were calculated as follows:

$$P_{(t+1)} = W_t + R_{(t+1)} - S_{(t+1)}$$

Where P is the predicted standing crop chl *a* value at sample time *t+1*, W is the water column chl *a* for a given thermal stratum at time *t*, R is the recruited chl *a* at

time *t+1* and S is the sedimented chl *a* at time *t+1*. Recruitment and sediment trap chl *a* values at time *t+1* were used because traps were deployed for one week, and values represent the daily total chl *a* recruited or sedimented between sampling periods. Regression analyses were then run on the predicted versus measured standing crop chl *a* values for each of the thermal strata in Paul, Peter, East Long and West Long Lakes that were sampled in 1996 and 1997. A Bonferroni correction was applied in order to decrease the chance of error at $\alpha=0.05$ and 21 tests, so values of $P<0.002$ were considered significant.

Phytoplankton Counts

Phytoplankton counts were used to measure the abundance of vegetative cells and cysts. Counts done on a subset of thermal strata from the experimental lakes sampled in 1996 and 1997 which had the lowest *P*-values for relationships between predicted and measured standing crop chl *a*, and which differed in food web structure. A subsample of the Lugol's-preserved recruitment, sediment, and whole water samples were settled in a 50mL column overnight into a 2mL Utermöhl counting chamber and counted with an Olympus IX70 inverted microscope. Settled samples that appeared too dense to count were diluted by a 1:1 or 2:1 factor with reverse osmosis water. Taxa were counted as "particles" such that colonies or solitary cells were counted as a single particle. Random fields, transects, and half-wells were used to count taxa with a goal of achieving 100 counts, or the number counted within 20 minutes per taxon (Venrick, 1978). In addition, vegetative cells and cysts of resting stage producing taxa were counted separately in recruitment and sediment traps, and vegetative cells or cysts were considered separately in later

calculations of, for example, rates of benthic recruitment and sedimentation. Individual species were also grouped into larger taxonomic categories because determination of resting stage identity to species was not possible in the present study. Taxa were identified with keys from Prescott (1962) and Dillard (2007).

To examine whether sedimentation or benthic recruitment could explain changes in pelagic abundances of resting stage producing taxa, counts focused on taxa that produce specialized resting stages. Regression analyses were conducted on measured versus predicted abundance for *Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates. Predicted abundances were calculated in the same manner as predicted chl *a*, and reflect total contributions of sedimented and recruited cysts and cells to the water column abundance. *P*-values <0.05 were considered to be significant. Recruited cysts were included in prediction calculations because germinating cysts differentially use storage products; starches are used before lipids, which may cause a change in buoyancy allowing turbulence independent recruitment of cysts into the water column (Binder & Anderson, 1990), and so their presence in recruitment traps may not have been the result of sediment disturbance. In addition, the percent of epilimnetic water column abundance due to resting stage sedimentation and benthic recruitment was also compared among taxa. Taxa counted in both study lakes are presented in Table 3.

Table 3. Phytoplankton taxa counted in the samples from Peter (1996) and Paul (1997) Lakes.

Species	Class
Dinobryon bavaricum †	Chrysophyceae
Dinobryon cylindricum†	Chrysophyceae
Dinobryon divergens †	Chrysophyceae
Dinobryon sertularia†	Chrysophyceae
Synura spp. †	Chrysophyceae
Dinobryon cyst †	Chrysophyceae
Cryptomonas erosa †	Cryptophyceae
Cryptomonas marssonii †	Cryptophyceae
Cryptomonas ovata †	Cryptophyceae
Cryptomonas reflexa†	Cryptophyceae
Cryptomonas cyst *	Cryptophyceae
Anabaena affins ‡	Cyanophyceae
Anabaena circinalis ‡	Cyanophyceae
Anabaena wisconsinense ‡	Cyanophyceae
Peridinium umbonatum †	Dinophyceae
Peridinium cinctum †	Dinophyceae
Peridinium wisconsinense †	Dinophyceae
Ceratium hirundinella †	Dinophyceae
Gymnodinium spp. †	Dinophyceae
Dinoflagellate cyst ‡	Dinophyceae

† Taxonomic reference Dillard (2007)
‡ Taxonomic reference Prescott (1962)
* *Cryptomonas* cysts were distinguished from chlorophyte cysts based on descriptions in the literature and sample observations of taxonomic composition.

RESULTS

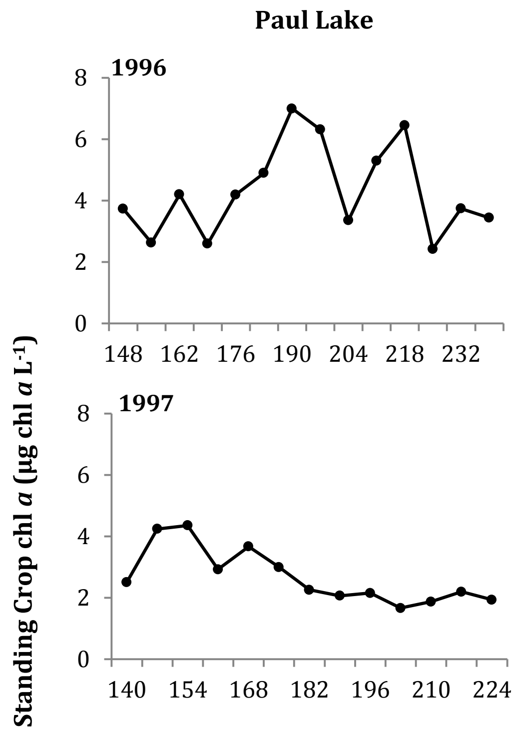
Contribution of Sedimentation and Benthic Recruitment to Standing Crop: Chlorophyll Analysis

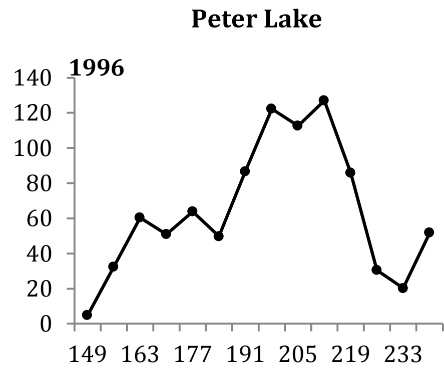
The epilimnetic chl *a* concentration of Peter, East Long and West Long Lakes were greater than the epilimnetic chl *a* concentration of Paul Lake during the 1996 and 1997 sampling period (Figure 3). Moreover the nutrient enriched lake with piscivores (West Long Lake) had a lower epilimnetic chl *a* concentration than the nutrient enriched lakes that lacked piscivores in both 1996 (Peter and East Long Lakes) and 1997 (Peter Lake) (Figure 3).

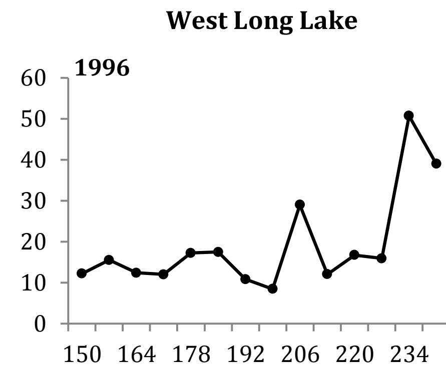
In general, predicted and measured standing crop chl *a* were poorly related; no significant relationships ($P>0.002$) were found (Table 4). The four lowest *P*-values for relationships between predicted and measured standing crop chl *a* were observed for Peter 1996 epilimnion ($P<0.006$), East Long 1996 metalimnion ($P<0.05$), Paul 1997 epilimnion ($P<0.01$), and Peter 1997 hypolimnion ($P<0.04$) (Figure 4, Table 4). Peter and East Long Lakes were eutrophied and lacked piscivores, and Paul Lake was not subject to nutrient additions and piscivores were present (Table 2). There were no obvious differences in the contribution of sedimented chl *a* and benthic recruitment of chl *a* to standing crop chl *a* among lakes that were manipulated and those which were not manipulated.

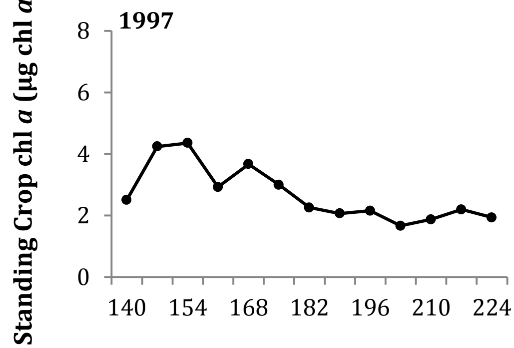
The measured standing crop chl *a* was often greater than the predicted standing crop chl *a* for lakes that had the lowest *P*-values (Figure 4, Table 4) for the regression on the predicted and measured chl *a* standing crop. This was particularly true for epilimnion of Peter and Paul Lakes in 1996 and1997 respectively (Figure 4). In the metalimnion of East Long in 1996 and the hypolimnion of Peter in 1997, the measured and predicted chl *a* standing crop were generally more closely matched (Figure 4).

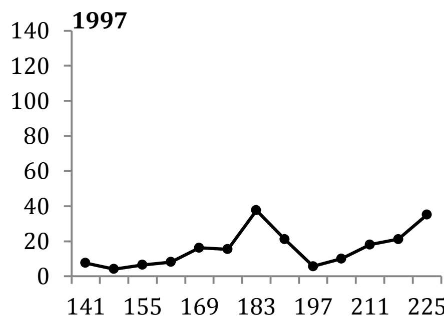
Figure 3. Epilimnetic standing crop chl *a* (μg chl *a* L^{-1}) for Paul, Peter, West Long, and East Long Lakes during the 1996 and 1997 sampling periods.

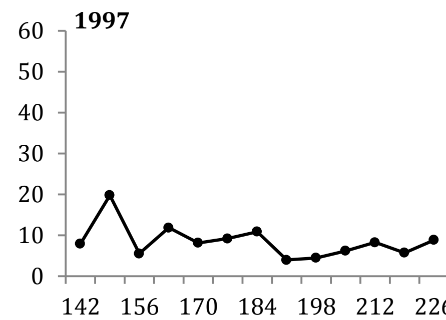

Paul Lake
1996
Standing Crop chl a (µg chl a L^{-1})
0
2
4
6
8
148 162 176 190 204 218 232


Peter Lake
1996
0
20
40
60
80
100
120
140
149 163 177 191 205 219 233


West Long Lake
1996
0
10
20
30
40
50
60
150 164 178 192 206 220 234


1997
0
2
4
6
8
140 154 168 182 196 210 224


1997
0
20
40
60
80
100
120
140
141 155 169 183 197 211 225


1997
0
10
20
30
40
50
60
142 156 170 184 198 212 226

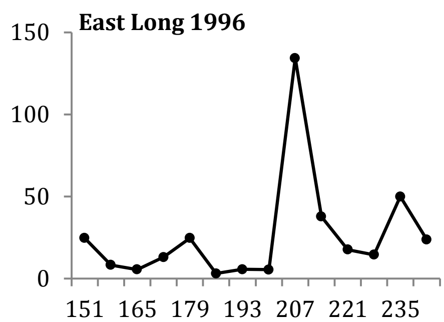
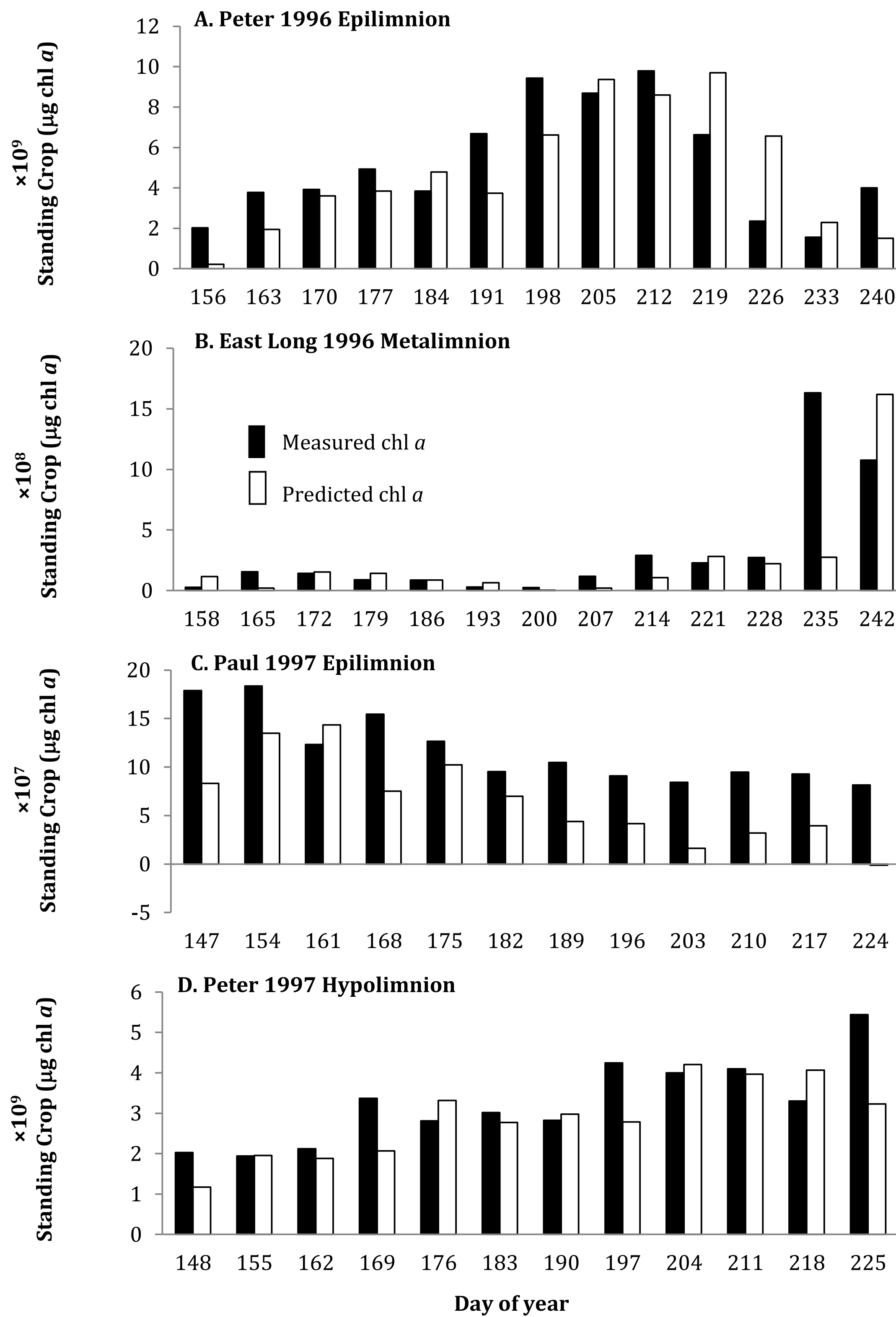

East Long 1996
0
50
100
150
151 165 179 193 207 221 235

Table 4. Results of regression analysis on the observed standing crop chl *a* and that predicted from the previous standing crop chl *a* plus benthic recruitment and less sedimentation for the thermal strata in Paul, Peter, East Long and West Long Lakes in 1996 and 1997. Asterisks (*) denote thermal strata for the lakes sampled in 1996 and 1997 with the lowest *P*-values for contributions of sedimented chl *a* and benthic recruitment of chl *a* to standing crop chl *a*.

Lake, thermal strata	Slope	R^2	*p*-value	
Paul 1996				
Epi	0.24	0.045	0.49	
Meta	-0.23	0.03	0.57	
Hypo	0.23	0.046	0.48	
Peter 1996				
Epi	0.8	0.52	0.005	*
Meta	0.33	0.12	0.25	
Hypo	0.5	0.24	0.092	
West Long 1996				
Epi	0.31	0.12	0.24	
Meta	0.17	0.02	0.64	
Hypo	0.21	0.037	0.53	
East Long 1996				
Epi	0.02	0.0004	0.95	
Meta	0.51	0.33	0.041	*
Hypo	0.17	0.028	0.59	
Paul 1997				
Epi	0.9	0.53	0.008	*
Meta	0.63	0.24	0.1	
Hypo	0.15	0.022	0.65	
Peter 1997				
Epi	0.4	0.25	0.095	
Meta	0.46	0.2	0.15	
Hypo	0.57	0.38	0.033	*
West Long 1997				
Epi	-0.16	0.026	0.62	
Meta	0.073	0.006	0.82	
Hypo	-0.17	0.029	0.6	

Figure 4. Relationships between measured standing crop chl *a* and that predicted from previous standing crop chl *a* with added benthic recruitment of chl *a* and less sedimented chl *a*. Present here are the measured (black bars) and predicted (open bars) standing crop chl *a* for the thermal strata of the lakes sampled in 1996 and 1997 that exhibited the lowest *P*-values for the contribution of sedimented chl *a* and benthic recruitment of chl *a* to standing crop chl *a*. A. Peter 1996 Epilimnion ($P<0.006$). B. East Long 1996 Metalimnion ($P<0.05$). C. Paul 1997 Epilimnion ($P<0.01$). D. Peter 1997 Hypolimnion ($P<0.04$). Values are extrapolated over the volume of the thermal stratum indicated for each lake. Note changes in Y-axis scales.



A. Peter 1996 Epilimnion
×10^9
Standing Crop (µg chl a)
12
10
8
6
4
2
0
156
163
170
177
184
191
198
205
212
219
226
233
240
B. East Long 1996 Metalimnion
×10^8
Standing Crop (µg chl a)
20
15
10
5
0
Measured chl a
Predicted chl a
158
165
172
179
186
193
200
207
214
221
228
235
242
C. Paul 1997 Epilimnion
×10^7
Standing Crop (µg chl a)
20
15
10
5
0
-5
147
154
161
168
175
182
189
196
203
210
217
224
D. Peter 1997 Hypolimnion
×10^9
Standing Crop (µg chl a)
6
5
4
3
2
1
0
148
155
162
169
176
183
190
197
204
211
218
225
Day of year

Taxa Sedimentation and Benthic Recruitment Dynamics

In both lakes, the epilimnetic abundance of vegetative cells reached a maximum during or before the first week of June (approximately day 156) for *Cryptomonas* spp., *Dinobryon* spp. and dinoflagellates (Figure 5). Only dinoflagellates in Paul Lake maintained a presence in the epilimnion after their initial peak and collapse (Figure 5). *Anabaena* spp. appeared later in the epilimnion of Peter and Paul Lakes on July 2nd (day 184) and June 22nd (day 174) respectively, and peaked around this time (Figure 5). Free akinetes (not attached to filaments) of *Anabaena* spp. were not observed in either lake, and vegetative colonies were the only life history stage that contributed to changes in epilimnetic abundance (Figures 5 & 9). In general, the patterns of sedimentation and benthic recruitment of vegetative cells, relative to standing crop, were often similar to the patterns of sedimentation and benthic recruitment observed for cysts (Figures 5 & 6). One exception is the sedimentation of cysts that co-occurred with peak epilimnetic standing crop prior to sedimentation of vegetative cells for *Dinobryon* spp. in Peter Lake (Figures 5 & 6).

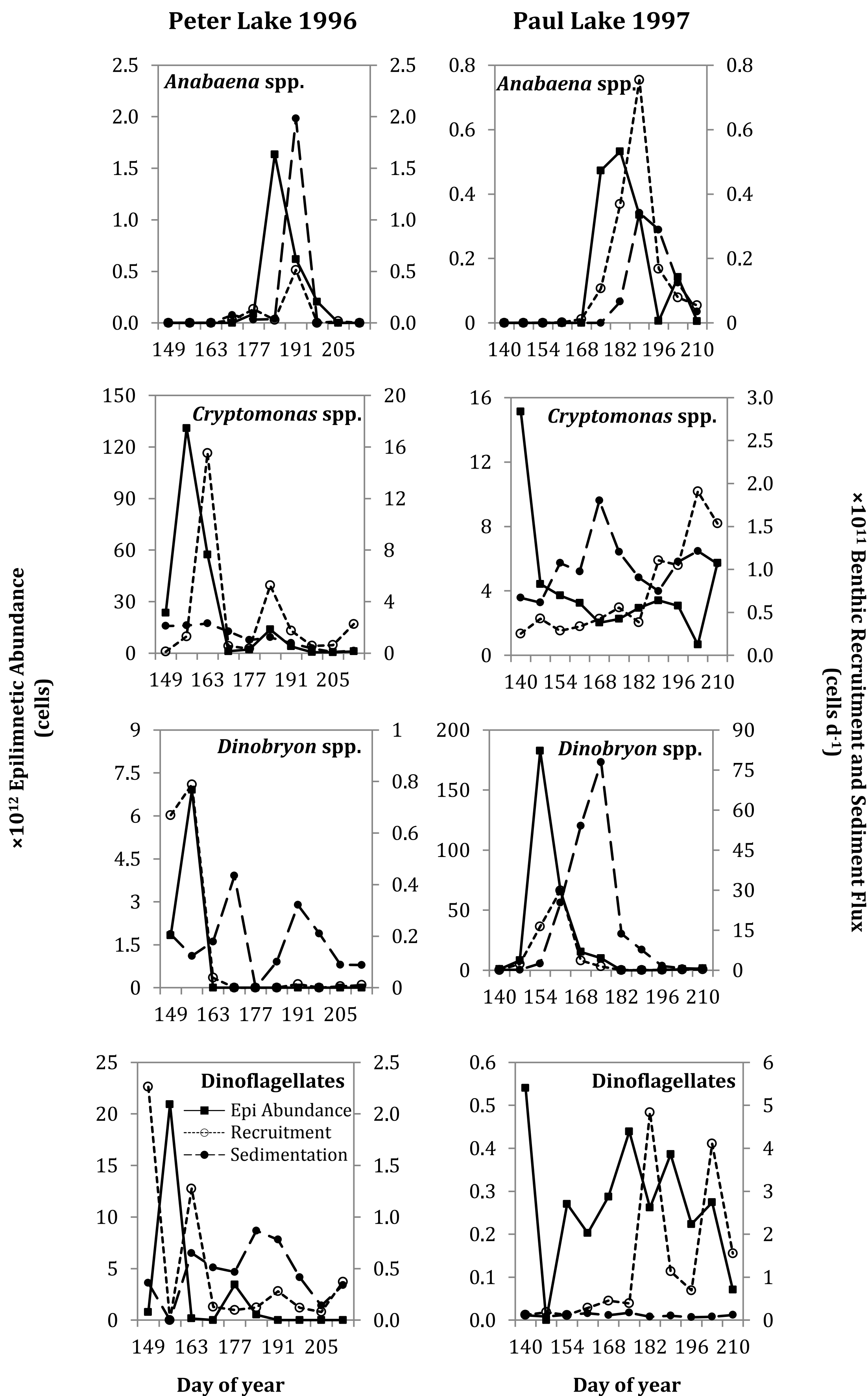
Total (vegetative cells and cysts) sedimentation and total benthic recruitment varied among taxa both when expressed as a percentage of epilimnetic abundance (Table 5, Figure 9), and when expressed as a rate (Figures 7 & 8). As a percentage of epilimnetic abundance, total sedimentation was often more than twice total benthic recruitment for *Dinobryon* spp. in both Peter (all three occurrences) and Paul Lakes (seven of 10 occurrences) (Table 5, Figure 9). *Cryptomonas* spp. followed the same pattern as *Dinobryon* spp. in Paul Lake (six of

11 occurrences), but not Peter Lake (four of 10 occurrences) (Table 5, Figure 9). In contrast, for dinoflagellates total benthic recruitment was often more than twice the percentage of total sedimentation in both Peter (three of five occurrences) and Paul Lakes (six of 11 occurrences) (Table 5, Figure 9).

In both Peter and Paul Lakes, most of the epilimnetic abundance sank as vegetative cells than as cysts for all taxa on most sampling occasions, when expressed as both a percentage of epilimnetic abundance (Figure 9) and as a rate (Figure 7). *Dinobryon* spp. in Peter lake were an exception where the percent epilimnetic abundance that sank as cysts was greater than thirty times the percent that sank as vegetative cells on two sampling occasions (days 149 & 156, Figure 9). The percent epilimnetic abundance due to sedimented cysts was also greater on five of 11 occasions for dinoflagellates in Paul Lake, but the greatest difference, occurring on May 26th (day 147), was only about three and a half times the percent due to sedimented vegetative cells (Figure 9). Losses due to cysts of *Cryptomonas* spp. (as a rate and percentage of epilimnetic abundance) on the first four sample dates in Peter Lake also represented approximately twice that of losses due to sedimented vegetative cells (Figures 7 & 9).

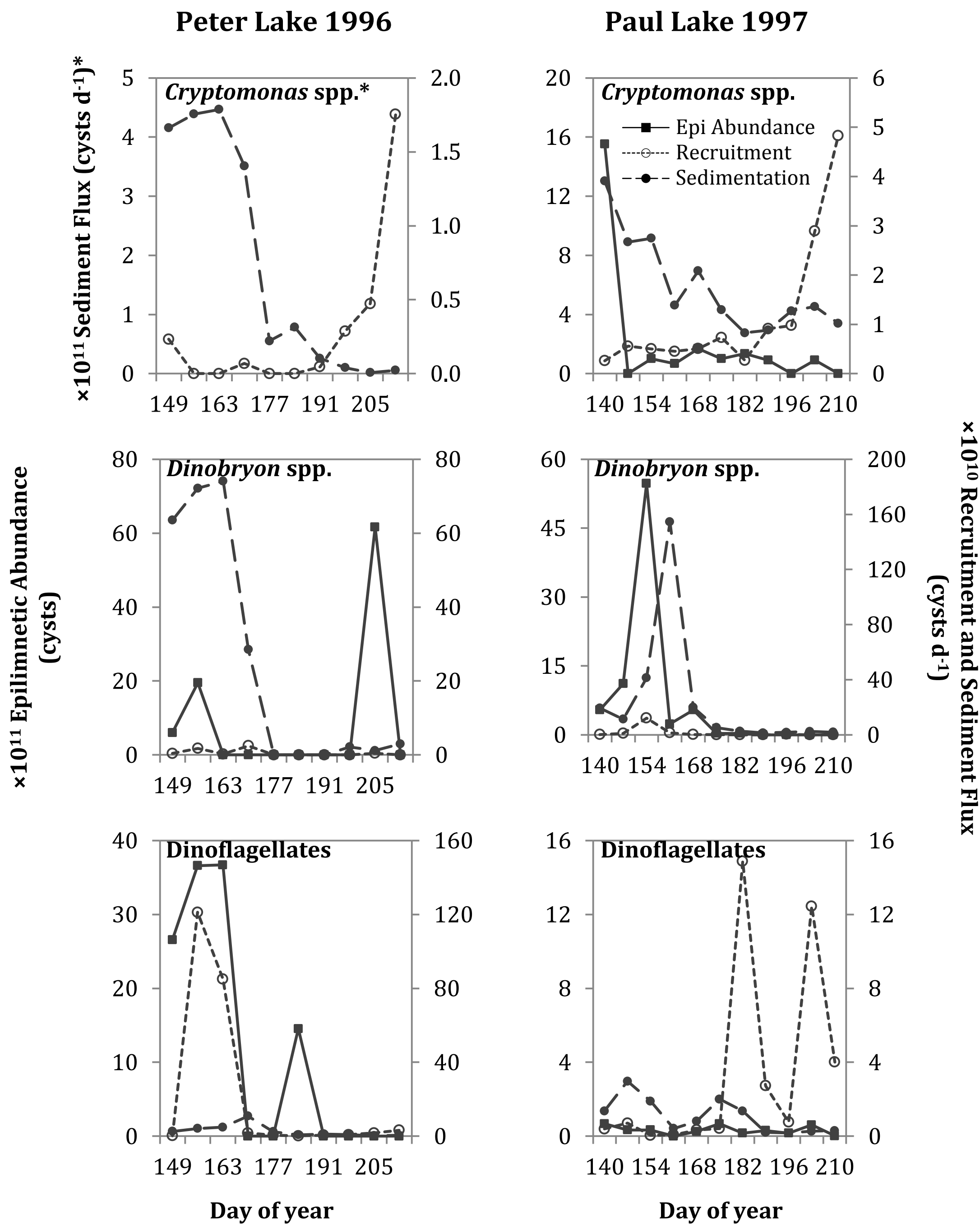
As was the case for sedimentation, benthic recruitment of vegetative cells was generally greater than that of cysts for all taxa on most sampling occasions in both Peter and Paul Lakes (Figures 8 & 9). Benthic recruitment of cysts exceeded that from vegetative cells on only two occasions, in both cases dinoflagellates, in Peter Lake (Figures 8 & 9).

Figure 5. Changes in the epilimnetic abundance of vegetative cells and the dynamics of vegetative cell sediment and benthic recruitment fluxes (cells d^{-1}) for *Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in the epilimnion of Peter (1996) and Paul (1997) Lakes. Solid lines with squares represent epilimnetic abundance of cells, long dashed lines with closed circles are the flux of cells to sediments, and short dashed lines with open circle are the flux of cells that recruited to epilimnetic water (cells d^{-1}). Integrated epilimnetic trap samples were used to calculate benthic recruitment flux; an average of the hypolimnetic sediment trap samples were used to calculate sediment flux. Epilimnetic abundances on the primary axes are calculated as the total number of cells and the sediment and recruitment fluxes on the secondary axes are calculated as the number of cells sedimented or recruited from the benthos per day. The x-axis labels are the corresponding day of year sample dates. Note different y-axes scales on figures.



Peter Lake 1996
Paul Lake 1997
Anabaena spp.
Cryptomonas spp.
Dinobryon spp.
Dinoflagellates
Epi Abundance
Recruitment
Sedimentation
×10^12 Epilimnetic Abundance (cells)
×10^11 Benthic Recruitment and Sediment Flux (cells d-1)
Day of year

Figure 6. Changes in the epilimnetic abundance of cysts and the dynamics of cyst sediment and benthic recruitment fluxes (cysts d^{-1}) for *Anabaena* spp., *Cryptomonas* spp., Dinobryon spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. Solid lines with squares represent epilimnetic abundance, long dashed lines with closed circles are flux of cysts to sediments, and short dashed lines with open circle are the recruitment flux of benthic cysts to epilimnetic waters. Integrated epilimnetic trap samples were used to calculate benthic recruitment; an average of the hypolimnetic sediment trap samples were used to calculate sedimentation. Epilimnetic abundances on the primary axes are calculated as the total number of cysts, and the sediment and benthic recruitment fluxes on the secondary axes are calculated as the number of cells sedimented or recruited per day. The x-axis labels are the corresponding year-day sample dates. Note different y-axes scales on figures. Also note that the sediment flux is on the primary axis for only *Cryptomonas* spp. in Peter Lake (1996).



Peter Lake 1996
Paul Lake 1997
Cryptomonas spp.*
Cryptomonas spp.
Epi Abundance
Recruitment
Sedimentation
Dinobryon spp.
Dinobryon spp.
Dinoflagellates
Dinoflagellates
×10¹¹ Sediment Flux (cysts d⁻¹)*
×10¹¹ Epilimnetic Abundance (cysts)
×10¹⁰ Recruitment and Sediment Flux (cysts d⁻¹)
Day of year
Day of year

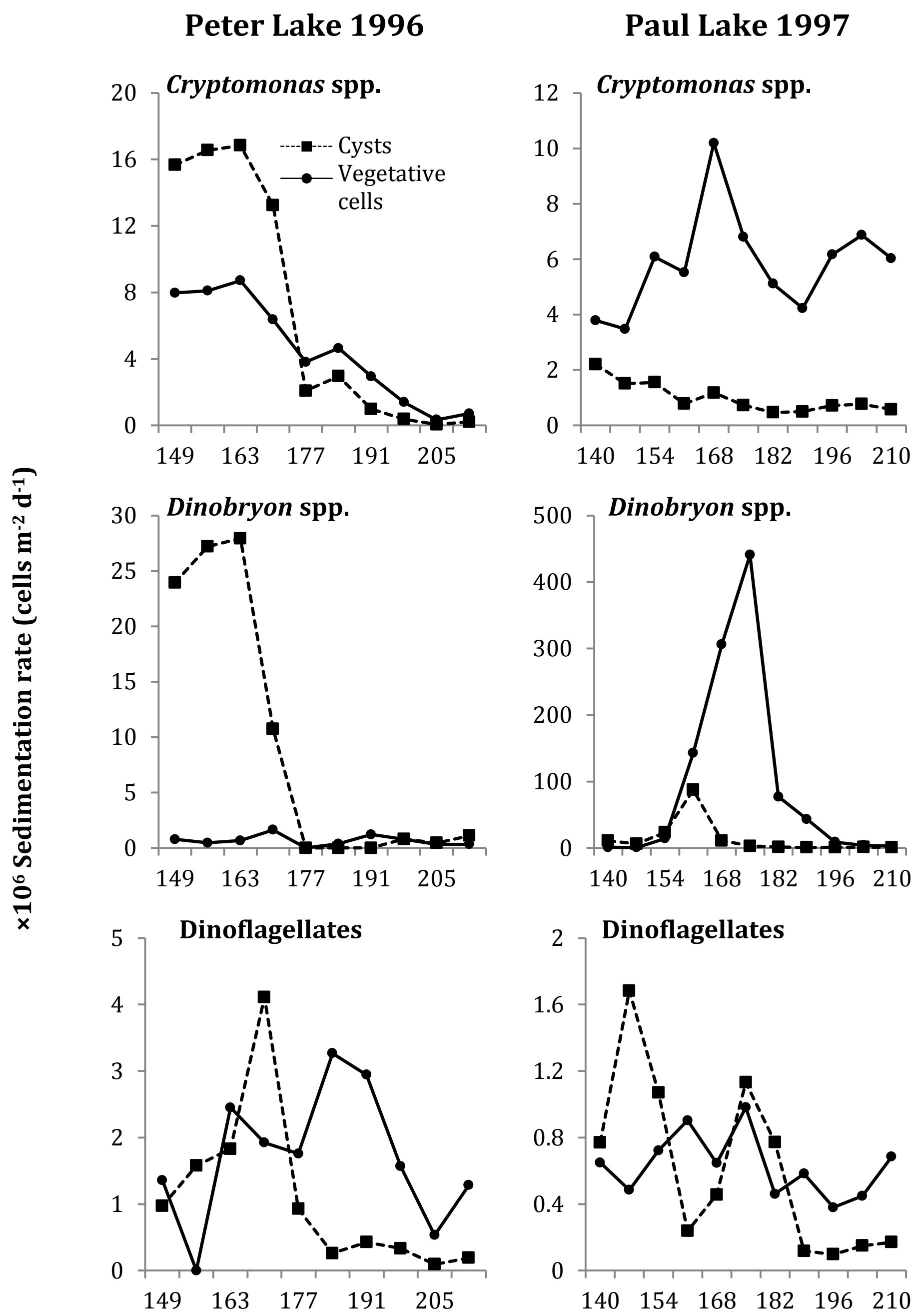


Figure 7. Sedimentation rates of vegetative cells and cysts (cells m^{-2} d^{-1}) for *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. Solid lines with circles represent the sedimentation rate of vegetative cells; dashed lines with squares represent the sedimentation rate of cysts. The unit cells is used to represent either vegetative cells or cysts. An average of the hypolimnetic sediment trap samples were used to calculate sedimentation. The x-axis labels are the corresponding year-day sample dates. Note changes in y-axes scales.

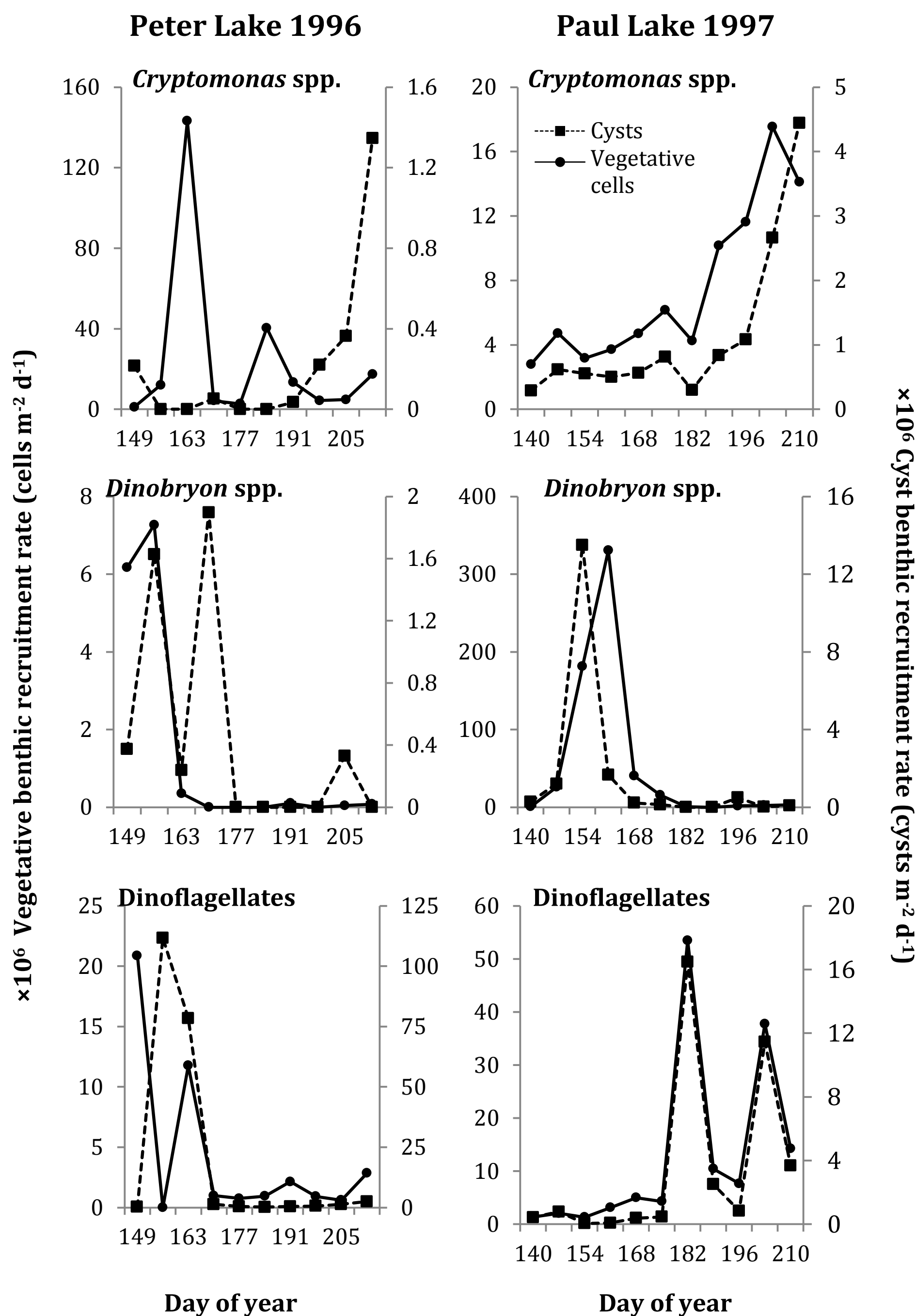
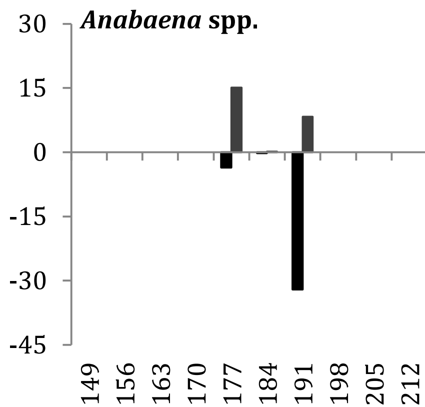


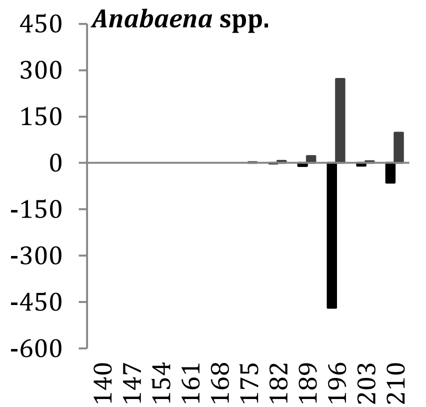
Figure 8. Benthic recruitment rates of vegetative cells and cysts (cells or cysts m^{-2} d^{-1}) for *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. Solid lines with circles represent the benthic recruitment rate of vegetative cells; dashed lines with squares represent the benthic recruitment rate of cysts. Integrated epilimnetic trap samples were used to calculate benthic recruitment. The x-axis labels are the corresponding year-day sample dates. Note changes in y-axes scales.

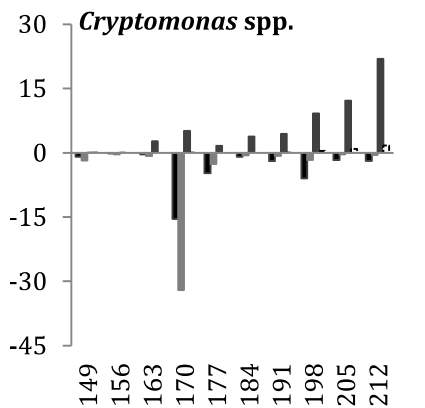
Figure 9. Percentage of the total (cysts and cells) epilimnetic abundance represented by sedimented vegetative cells (black bars), sedimented cysts (light grey bars), benthic recruited vegetative cells (dark grey bars) and benthic recruited cysts (white bars) on each sampling date. Values are calculated on a whole-lake basis. Integrated epilimnetic trap samples were used to calculate benthic recruitment; an average of the hypolimnetic sediment trap samples were used to calculate sedimentation. Note different scales on figures.

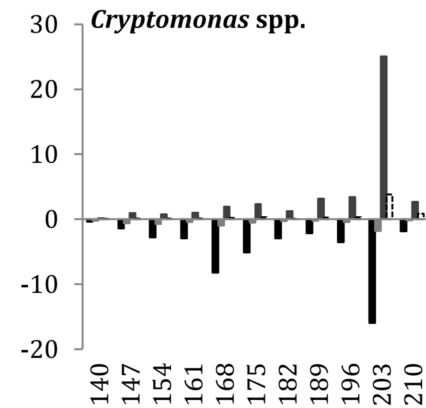
Peter Lake 1996 | **Paul Lake 1997**

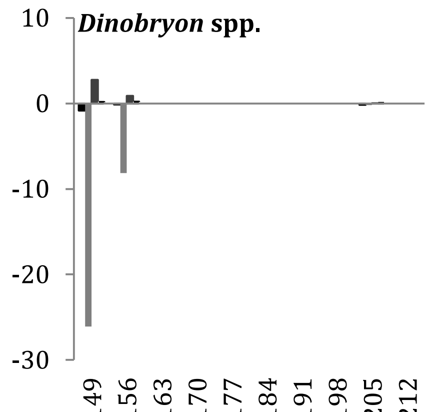
% Epilimnetic Abundance

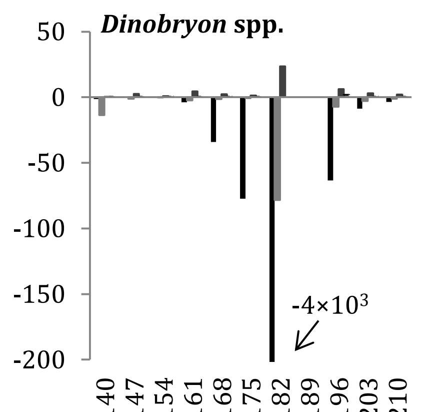


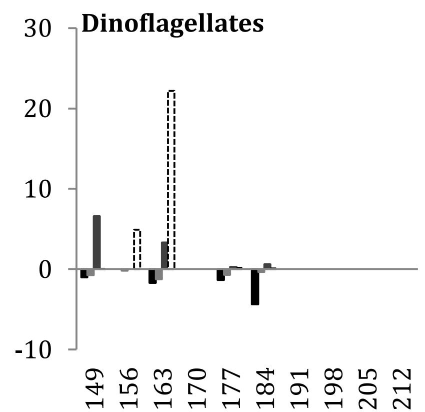












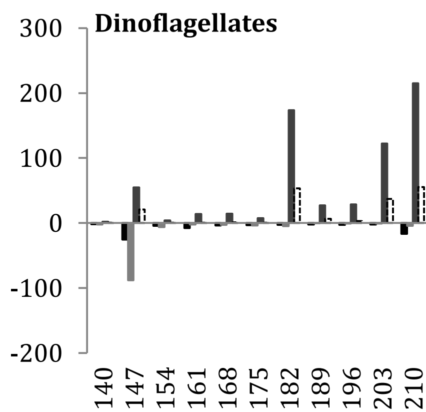


Table 5. Sedimentation (%S) and benthic recruitment (%R) as percentages of total (vegetative cells and cysts) epilimnetic cell abundance calculated on a daily basis for *Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., and dinoflagellates in Peter (1996) and Paul (1997) Lakes. Values in bold indicate the direction of the higher flux.

		Day of year 1996										
		149	156	163	170	177	184	191	198	205	212	
Anabaena	% S					3.6	**0.2**	**32**				
spp.	% R					**15**	0.18	8.3				
Cryptomonas	% S	**2.7**	**0.50**	1.2	**47**	**7.4**	1.5	2.6	7.6	2.1	2.4	
spp.	% R	0.060	0.1	**2.7**	5.1	1.7	**3.8**	**4.4**	**9.6**	**13**	**24**	
Dinobryon	% S	**27**	**8.3**							**0.3**		
spp.	% R	2.9	1.1							0.08		
Dinoflagellates	% S	1.8	0.2	3.0		**2.1**	**4.7**					
	% R	**6.6**	**4.9**	**25**		0.44	0.72					
		Day of year 1997										
		140	147	154	161	168	175	182	189	196	203	210
Anabaena	% S						0.0	1.2	10	**468**	**8.8**	64
spp.	% R						**2.3**	**6.9**	**23**	272	5.6	**98**
Cryptomonas	% S	**0.64**	**2.0**	**3.5**	**3.4**	**9.2**	**5.6**	**3.2**	2.4	**4.0**	18	2.0
spp.	% R	0.17	1.1	0.88	1.2	2.2	2.7	1.3	**3.4**	3.7	**29**	**3.5**
Dinobryon	% S	**15**	1.4	0.35	**6.2**	**35**	**78**	**4115**		**71**	**12**	**4.7**
spp.	% R	0.63	**2.6**	**0.94**	4.6	2.3	1.4	23		8.0	3.0	2.1
Dinoflagellates	% S	**4.1**	**113**	**10**	9.9	6.2	7.4	7.8	3.0	3.5	3.2	21
	% R	2.6	76	4.0	**14**	**16**	**8.5**	**227**	**34**	**32**	**160**	**270**

Contribution of Sedimentation and Benthic Recruitment to Standing Crop: Taxa Analysis

None of the taxa examined (*Anabaena* spp., *Cryptomonas* spp., *Dinobryon* spp., or dinoflagellates) had a significant relationship between the total epilimnetic abundance of cells and cysts, and that predicted from previous week's epilimnetic abundance plus total benthic recruitment minus total sedimentation (P>0.05 in all cases, Table 6).

Table 6. Results from the regression analyses on the measured total abundance (vegetative cells and cysts) and that predicted from the previous week's total abundance plus total benthic recruitment and less total sedimentation for the taxonomic groupings counted in the epilimnion of Peter (1996) and Paul (1997) Lakes.

	Slope	**R^2**	***P*-value**
Peter 1996 Epilimnion			
Anabaena spp.	0.21	0.055	0.55
Cryptomonas spp.	0.33	0.11	0.37
Dinobryon spp.	-0.11	0.015	0.76
Dinoflagellates	0.14	0.019	0.73
Paul 1997 Epilimnion			
Anabaena spp.	0.57	0.29	0.11
Cryptomonas spp.	0.75	0.051	0.53
Dinobryon spp.	0.23	0.053	0.52
Dinoflagellates	-0.4	0.036	0.6

DISCUSSION

Changes in Community Standing Crop

The results of the present study indicate that sedimentation and benthic recruitment were not sufficient to account for observed changes in standing crop chl *a* (Table 4) or taxa epilimnetic abundance (Table 6) in lakes of different nutrient status and food web structure. As a result, gains via vegetative growth and losses due to factors such as grazing were more important to changes in standing crop chl *a*. The observation that measured epilimnetic chl *a* was often greater than that predicted from the previous week's chl *a* plus benthic recruitment minus sedimentation support the idea that vegetative growth was more important to changes in standing crop chl *a* than recruitment (Figure 4). For example, chl *a* increased by a factor of 1.74 between day 184 and 191 in the epilimnion of Peter Lake in 1996 (Figure 4). This is equivalent to a daily growth rate of 0.079 and a doubling time of 8.77 days, similar to an *in situ* doubling time of 10.7 days calculated for *Peridinium limbatum* in a eutrophic, temperate bog (Graham *et al*., 2004). With this doubling time, 2.27×10^{9} µg chl *a*, of the 2.84×10^{9} µg chl *a* growth that occurred between July 2nd and July 9th (days 184 and 191), is accounted for by vegetative growth. In addition, 5.51×10^{7}µg chl *a* was recruited between these days, and an additional 9.96×10^{7} µg chl *a* was added due to vegetative growth of recruited cells. This gives a total of 6.26×10^{9} µg chl *a* due to vegetative growth and benthic recruitment. Growth of recruited cells was underestimated due to the fact that calculations were based on recruitment trap chl *a* averaged over the seven days between sampling period, and may account for the lower amount of chl *a* calculated

than what was actually measured in the water column on July 9th (day 191, 6.68×10^9 μg chl *a*, Figure 1). Additionally, it must be noted that we may expect that chl *a* results and taxonomic results, discussed separately, to diverge because the traps used for taxonomic analysis were preserved, while the traps for chl *a* analysis were not preserved. As a result, the potential for growth and excystment would be different between the traps. Regardless, the calculation demonstrates that vegetative growth of the standing crop chl *a* overwhelmingly accounts for the increase in biomass rather than benthic recruitment and growth of cells recruited from the benthos.

Sedimentation weakly contributed to changes of standing crop chl *a* in the present study, and may be the result of buoyancy regulation mechanisms characteristic of the dominant phytoplankton groups. Buoyancy regulation mechanisms that reduce sedimentation losses include mucilaginous coatings and the use of flagella (Reynolds *et* al., 1982). The dominant taxa in these lakes were flagellates and consisted of dinoflagellates, chlorophytes, chrysophytes, and cryptomonads (Carpenter *et al*., 2001). These groups often appeared in low abundances in the sediment traps, but may have been found in deep chl *a* peaks previously observed in these lakes (Carpenter & Kitchell, 1993). Non-motile phytoplankton are particularly affected by sedimentation losses and may significantly contribute to population decline of these forms during the stratified period (e.g. Uhlmann, 1971; Reynolds, Morison & Butterwick, 1982). Washout from these seepage lakes (Carpenter & Kitchell, 1993) was also unlikely to significantly contribute to phytoplankton losses in the present study, due to long residence times

ranging from 1285 to 1914 days in the experimental lakes (Cole & Pace, 1998 as referenced in Carpenter *et al*., 2001).

Grazing more likely explained differences in the standing crop chl *a* than sedimentation in the present study. Overall, lakes that were nutrient enriched (Peter, East Long and West Long) had a higher chl *a* concentration than the control lake (Paul), but enriched piscivorous lakes (West Long) had a lower chl *a* concentration compared to enriched planktivorous lakes (Peter and East) (Figure 3). Previous work on the lakes used in the present study found that food web manipulations resulted in a zooplankton assemblage dominated by large-bodied zooplankton (*Daphnia pulex*) in piscivorous West Long Lake in both 1996 and 1997, which were generally excluded from planktivorous Peter Lake in both years and from East Long Lake in 1997 (Carpenter *et al.*, 2001). It was also found that the areal chl *a* was related to mean grazer body length in the experimental lakes of the present study, and that the biomass of zooplankton in nutrient enriched lakes was greater than in the reference lake (Carpenter et al., 2001). Both increased mean body length and zooplankton biomass have, at times, accounted for increased clearance of phytoplankton (Cyr & Curtis, 1999). For example, a zooplankton community dominated by large cladocerans (e.g. *Daphnia* spp.) had a clearance rate between 0.15 and 0.29 mg algal biomass L^{-1} d^{-1} compared to results that indicated phytoplankton biomass may actually increase in communities dominated by small-bodied cladocerans such as *Bosmina* spp. (Cyr & Curtis, 1999). Together, these previous findings suggest that differences in standing crop between nutrient enriched planktivorous and piscivorous lakes were due to piscivorous fishes

preying upon planktivorous fishes, which enabled large-bodied zooplankton to thrive, graze upon the phytoplankton and, thereby, reduce increases in standing crop chl *a*.

Taxa Comparisons of Sedimentation and Benthic Recruitment

Contrary to my hypothesis, for most resting stage producing taxa, sinking due to cysts was greater than sinking due to vegetative cells in lakes with lower grazing pressure (planktivorous lakes) rather than in lakes with greater grazing pressure (piscivorous lakes) (Figure 9). Sinking cysts of *Dinobryon* spp. in planktivorous Peter Lake contributed more to epilimnetic losses than sinking cysts in piscivorous Paul Lake where a greater proportion of the population sank as vegetative cells (Figure 9). The overall pattern of *Dinobryon* spp. cyst production relative to epilimnetic abundance was consistent between the two lakes. In both Peter and Paul Lakes, resting stage production (Figure 6) coincided with periods of increasing epilimnetic abundance (Figure 5), as has been previously found for sexual cyst production in *D. cylindricum* (Sandgren, 1981). Encystment with increasing population is considered a bet-hedging strategy for survival because cysts are created before the population experiences limitation of resources necessary to produce cysts (Sandgren, 1988). In addition, production of sexually produced cysts depends on cell density and the ability to detect a mate (Sandgren, 1981). It is therefore reasonable to assume that sexual cysts are more likely to be produced as the population grows and reaches peak abundance. Sexually produced cysts are often not morphologically distinguishable from asexually produced cysts

in *Dinobryon* spp. (Sandgren, 1981). As a result, I could not determine whether cysts produced during increasing abundance were of a sexual nature.

These differences in the rate of cyst production for *Dinobryon* spp. between the two lakes could be the result of either difference in conversion of vegetative cells into asexually produced cysts, or as a result of differential success of sexual reproduction. Of the sinking material, the percentage of the epilimnetic cell abundance that sank as cysts (0.52%-78.4%) was generally lower than the percentage of sinking vegetative cells (0.13%-4037%) in Paul Lake, where as a greater percentage sank as cysts (0.19%-26.1%) than cells (0.14%-0.85%) in Peter Lake (Figure 9). If cysts were sexually produced, differences in cell density and the ability to find a mate and, or the presence of highly compatible mating types could also lead to differences in cyst production between the lakes (Sandgren, 1981). In addition, gametes may have been grazed at a greater rate in Paul Lake, and would also account for the lower observed percentage of sinking cysts versus cells when compared to Peter Lake. Clone crossing experiments, as were performed by Sandgren (1981), would be needed to determine compatibility of mating types within each of the mating systems as determined by the frequency of sexual cyst production. In addition, determination of grazing rates of mature *Dinobryon* colonies and sexual gametes by the dominant grazers in each system would also need to be determined to validate or refute the possibility of grazing inhibition of successful sexual reproduction.

Only dinoflagellates supported the hypothesis that recruitment from cysts would be suppressed in lakes with higher grazing pressure (piscivorous lakes).

Unlike other taxa, recruited cells and cysts from the benthos represented comparatively more of the dinoflagellate epilimnetic abundance than sedimented cells and cysts in both Peter and Paul Lakes (Table 5). In Peter and Paul Lakes, the rates of peak benthic recruitment of dinoflagellates in the form of vegetative cells (respectively 2.09 $\times 10^7$ and 5.35 $\times 10^7$ cells m^{-2} d^{-1}) and cysts (respectively 1.12 $\times 10^8$ and 1.65 $\times 10^7$ cells m^{-2} d^{-1}) (Figure 8) were greater than the rates of peak sedimentation of vegetative cells (respectively 3.27 $\times 10^6$ and 1.13 $\times 10^6$ cells m^{-2} d^{-1}) and cysts (respectively 4.11 $\times 10^6$ and 1.68 $\times 10^6$ cells m^{-2} d^{-1}) (Figure 7). A previous study by Hansson (1996) also found that recruitment exceeded sedimentation, when normalized to vegetative cell abundance, for two dinoflagellate taxa examined in Peter and Paul Lakes. For example, the percent of the vegetative abundance of *Peridinium pusillum* represented by recruited cells ranged from approximately 10%-55% and 20%-100%, in Peter and Paul Lakes respectively, compared to 1%-10% represented by sedimented cells in both lakes; normalized recruitment exceeded normalized sedimentation on all occasions (Hansson, 1996). This was also generally true for dinoflagellates in the present study (Figure 9). On three of five occasions, normalized benthic recruitment (0.44%-25%) exceeded sedimentation (0.2%-4.7%) in Peter Lake and on eight of 11 occasions in Paul Lake (2.6%-270% and 3.0%-113%, respectively) (Figure 9).

Vegetative growth alone could not have accounted for the increase in dinoflagellate epilimnetic abundance in Peter Lake between May 28th (day 149) and June 4th (day 156) (Figure 5). Given a doubling time of 10.7 days and a growth rate of 0.065 d^{-1} (Graham *et al.*, 2004) for *P. limbatum*, only 1.03 $\times 10^{12}$ cells of 20.9 $\times 10^{12}$

cells observed could be accounted for on June 4th (day 156). Benthic recruitment and growth of recruited cells and cysts from the benthos accounted for an additional 3.74×10^{11} and 1.41×10^{13} cells respectively in the epilimnion of Peter Lake. However, an additional 5.44×10^{12} cells were unaccounted for by epilimnetic vegetative growth and benthic recruitment. Benthic recruitment and migration into the epilimnion from other thermal strata may account for the discrepancy in the calculated and observed epilimnetic abundance of dinoflagellates in Paul Lake. It has been previously found by Hansson (1996) that a majority of the dinoflagellate *Gymnodinium* in Paul Lake is recruited below the thermocline, and though poorly represented in epilimnetic waters in that study, this demonstrates that phytoplankton species may have been recruited from specific depths not examined in the present study.

Despite low rates of dinoflagellate sedimentation (peak rates of sinking vegetative cells ranging from 1.13 to 3.27×10^{6} cells m^{-2} d^{-1}, and cysts from 4.11 to 1.68×10^{6} cells m^{-2} d^{-1}) (Figure 7) as compared to benthic recruitment (peak rates of benthic recruited vegetative cells ranging from 2.09 to 5.35×10^{7} cells m^{-2} d^{-1}, and cysts from 1.65×10^{7} to 1.12×10^{8} cells m^{-2} d^{-1}) (Figure 8), over time, sedimented cysts accumulate in lake sediments forming a seed bank that has the potential to inoculate pelagic populations. Resting cysts of dinoflagellates have a specific well-documented maturation period before germination may be cued by external factors (e.g. see Anderson, 1998). These requisite maturation periods may contribute to mass recruitment events (Rengefors & Anderson, 1998; Anderson & Rengefors, 2006). Mass recruitment episodes have been shown to contribute a significant

proportion of biomass to the standing crop of dinoflagellates in other systems (Heaney, Chapman & Morison, 1983; Ishikawa & Taniguchi, 1996), and may help explain periods with exceptionally high rates of benthic recruitment (Figure 8) in this study.

Interestingly, the peak rate of benthic recruitment of dinoflagellates from cysts was nearly seven times greater in Peter Lake (11.2×10^7 cells m^{-2} d^{-1}) than the peak rate of benthic recruitment from cysts in Paul Lake (16.5×10^6 cells m^{-2} d^{-1}). One possible explanation would be the use of the cyst stage as a mechanism to escape herbivory. The food web of Peter Lake was dominated by planktivores compared to piscivores in Paul Lake (Table 2). Differences in food web structure caused differences in the zooplankton assemblages between the lakes such that large-bodied cladoceran grazers (e.g. *D. pulex*) and copepods (e.g. *Cyclops varicans*) dominated Paul Lake, and were generally excluded from Peter Lake (Carpenter *et al*., 2001).

Previous work by Rengefors *et al.* (1998) demonstrated that zooplankton exudates of unknown composition could reduce the rate of dinoflagellate excystment in taxa that did not have a grazer-resistant growth form. This would include *Peridinium* and *Gymnodinium* spp. that dominated dinoflagellate taxa in Peter and Paul Lakes during the present study (Table 3). Additionally, another study on these same lakes suggested a possible role of grazer exudates on the regulation of recruitment by suppressing germination (Hansson, 1993). The work by Hansson (1993) revealed reduced rates of recruitment for two dinoflagellate taxa in the presence of grazers, compared to treatments with fewer grazers, in both

enclosure and whole-lake experiments. For example, *P. pusillum* recruited at a rate of nearly 2.0×10^6 cells m^{-2} d^{-1} in the presence of grazers, compared to approximately 8.5×10^6 cells m^{-2} d^{-1} in the absence of grazers (Hansson, 1993).

The differences in the rate of benthic recruitment from cysts in planktivorous Peter Lake compared to piscivorous Paul Lake may have been the result of metabolic effects of grazer exudates on cyst germination. Cysts differentially use storage products during germination, such that starch is metabolized prior to lipid metabolism (Binder & Anderson, 1990). This causes the ratio of starches to lipids to decrease within the cyst, and cysts become more buoyant as starches are metabolized. It has previously been demonstrated by Rengefors *et al*. (1998) that grazer exudates, of unknown composition, reduce the excystment rate of some dinoflagellate taxa. As a result, it is possible that grazer exudates inhibit germination, and therefore benthic recruitment, by affecting cyst metabolism during germination. Inhibition of starch metabolism within cysts would cause there to be fewer buoyant cysts, and would account for the lower rate of dinoflagellate cysts observed in recruitment traps in Paul Lake (1.65×10^7 cells m^{-2} d^{-1}) compared to Peter Lake (1.12×10^8 cells m^{-2} d^{-1}). Additional work is needed to determine the chemical nature of grazer exudates, and the consequences specific constituents have on phytoplankton metabolism during cyst germination.

Finally, mortality should also be considered to explain changes in taxa abundance that is not explained by sedimentation or grazing pressure. For example, dinoflagellates were not observed on June 11th (day 163) in Peter Lake following peak epilimnetic abundance of 20.9×10^{12} cells on June 4th (day 156) (Figure 5).

Between June 4th and June 11th (days 156 and 163), 3.84 $\times 10^{11}$ cells and cysts were lost due to sedimentation. Hansson (1996) determined clearance rates of different dinoflagellate taxa in Peter Lake. Given the greatest clearance rate, for *P. pusillum*, of 2.22 liters mg zooplankton^{-1} d^{-1} and 0.052 mg zooplankton L^{-1} in Paul Lake (Hansson, 1996), a total of 2.42 $\times 10^{12}$ cells were cleared from the epilimnion. As a result, sedimentation and grazing accounted for a total loss of 2.80 $\times 10^{12}$ cells, leaving 1.81 $\times 10^{13}$ cells lost by processes other than sedimentation and grazing. Cell death triggered by oxidative stress has been implicated as a driver of bloom collapse for *P. gatunense* in Lake Kinneret (Vardí *et al*., 1999). *P. gatunense* is also a cyst producing dinoflagellate, and a link between cell death and cyst formation, mediated by cysteine proteases, has been suggested to exist for this taxon (Vardí *et al*., 1999). Future studies need to examine both losses due to cyst formation and cell death simultaneously to explore the relationship of these loss processes in both laboratory and field settings.

Implications and Future Directions

Overall, this study suggests that while sedimentation and recruitment may not be responsible for explaining changes in standing crop at the community level, these processes may be important to taxa-specific abundance dynamics. In addition, the results of this study have important implications for lake management strategies. First, the results demonstrate that benthic recruitment and sedimentation weakly affect phytoplankton community dynamics for the studied lakes. Vegetative growth, rather than benthic recruitment, accounted for most of the observed increases in standing crop during the summer sampling period. The

importance of benthic recruitment to pelagic abundance may vary seasonally. For example, benthic recruitment may be more important during the temperate winter when temperature and irradiance are low and reduce vegetative growth rates. In addition, benthic recruitment from cysts is more significant to increases in standing crop during wind-driven mixing events (Lund, 1955; Schelske, Carrick & Aldridge, 1995; McQuoid & Godhe, 2004). However, the present study was conducted during the summer stratified period, possibly limiting benthic recruitment. In terms of management for possible algal blooms in these lakes, applications of algaecide may be best administered first during temperate spring mixing events to prevent mass establishment from suspended cysts, with a second dose during the summer stratified period if phytoplankton biomass becomes problematic.

Sedimentation was also weak during the present study, which may be because sedimentation during the stratified period primarily affects non-motile phytoplankton (e.g. Uhlmann, 1971; Reynolds *et al*., 1982). The community was dominated by flagellates that were capable of regulating buoyancy in the water column. Grazing is a loss process that likely controlled the community abundance of phytoplankton in the study lakes. As a result, lake modifications to increase piscivory may prevent apparent increases in phytoplankton biomass in systems dominated by edible (<35μm) flagellate phytoplankton and filter-feeding cladocerans.

In terms of key taxa, the present study demonstrated that the relative composition of vegetative cells and cysts composing sedimented and recruited material may vary both within and among taxa. However, it is not clear whether

these differences are necessarily a result of food web effects or nutrient enrichment because only one of the study lakes (Peter Lake) used for taxonomic analysis underwent nutrient enrichment (Table 2). Additional phytoplankton counts focusing on resting stage producing taxa need to be conducted for the other manipulated lakes to address the effect of food web structure on phytoplankton resting stage ecology. Work is also needed on the effect of grazer exudates on resting stage formation and germination at the taxonomic level to evaluate the effects of food web manipulations on phytoplankton resting stage ecology. These studies should focus on the effects of grazer exudates on changes in biochemical composition and metabolic activity of phytoplankton resting stages in order to determine whether there are physiological effects of the presence of grazers on germination. To date, no study has been found that analyzes the effect of grazer exudates on cyst metabolic activity.

For some taxa examined mortality may be an important loss process. A potential link between death and cyst formation pathways has been previously suggested for dinoflagellates under oxidative stress (Vardí *et al*., 1999), however additional work is needed to determine how much and what kinds of stress determine cell fate. A number of biological processes are regulated by reactive oxygen species (ROS) such as growth, the cell cycle and programmed cell death (PCD) (Miller *et al*., 2008). In phytoplankton ROS have been implicated as triggers in activation of death (Thamatrakoln *et al*., 2012) and sex genes (Nedelcu *et al*., 2004). Involvement in the activation of sex is significant because sex is a precursor to cyst formation in many freshwater phytoplankton taxa, but it must also be noted

that cysts may be formed asexually (Sandgren, 1988). The ability to recover from, or acclimate to, ROS accumulation and to continue vegetative growth depends on the age and health of cells, in addition to the type, intensity, and duration of stress exposure (Thamatrakoln *et al*., 2012). As a result, it is possible that initiation of sex and cyst formation are also adaptive responses to ROS-generating stress stimuli, and initiation may partially depend on the effectiveness of ROS scavenging mechanisms to avoid death. Experiments are needed that determine how the type, intensity, and duration of stress exposure affect up- and down- regulation of genes associated with ROS scavenging mechanisms as it pertains to death and cyst formation pathways. Understanding the linkages between death and cyst formation pathways will be important for modeling bloom behavior and developing ballast water management strategies for cyst forming taxa responsible for harmful algal blooms. These strategies will focus on application of lethal doses of stress that up-regulate death genes and silence genes necessary for cyst formation.

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PART II: PHYSIOLOGICAL CELL DEATH IN PHYTOPLANKTON

INTRODUCTION

Mortality in phytoplankton has not been treated consistently in the literature. Historical equations describing birth and loss processes of phytoplankton did not include a term for cell death (Uhlman, 1971). Equations developed later acknowledged *in-situ* cell death as a loss process (Reynolds, 1980) but it was deemed negligible compared to grazing and sedimentation losses. However recent studies have suggested that death events may at times be responsible for bloom collapse (Vardí *et al*., 2007), possibly through intercellular signaling mechanisms (Vardí *et al*., 2006), and represent an important succession mechanism (Sigee, Gallois & Dean, 2007). Moreover, cell death in the field may have important implications for biogeochemical cycling in aquatic systems.

Phytoplankton may also undergo programmed cell death (PCD) in response to unfavorable environmental conditions. Laboratory studies have demonstrated that nutrient starvation (e.g. Berges & Falkowski, 1998), light limitation (e.g. Segovia & Berges, 2009), UV radiation (Jiménez *et al*., 2009), hyperosmotic stress (e.g. Ning *et al*., 2002) and heat shock (Jiménez *et al*., 2009) may each elicit a death response. Other factors that have potential to contribute to losses due to death and further population declines in the field include the presence of pathogens like algacidal bacteria (Kim *et al*., 1998), viruses (Brussaard, 2004) and chytrid fungi (Ibelings *et al*., 2004), and also allelopathy (Legrand *et al*., 2003). Furthermore, the death

response observed depends on the intensity, duration, and type of stress to which cells are exposed (Jiménez *et al*., 2009).

Necrosis and apoptosis are two types of cell death considered to be at opposite ends of the death response continuum (Jiménez *et al*., 2009). Necrosis is the form of death triggered when cells are exposed to a lethal level of stress causing irreparable damage (Henriquez *et al*., 2008), and is characterized by cellular swelling, membrane permeabilization, and organelle dysfunction (Bialik *et al*., 2010). Unlike necrosis, apoptosis is regarded as a form of programmed cell death (PCD) because events are metabolically regulated by the expression of specific death genes (Franklin, Brussaard & Berges, 2006; Bialik *et al*., 2010). The early stages of apoptosis are characterized by the translocation of phosphatidylserine from the inner plasma membrane to the outer layer resulting in "blebbing" of the membrane (Segovia & Berges, 2009). Other morphological features of apoptosis that have been observed in phytoplankton are DNA condensation, margination, and fragmentation, followed by cell shrinkage, and membrane permeabilization associated with secondary necrosis (Franklin *et al*., 2006).

Fluorescence staining techniques have been used to determine modes of cell death in laboratory and field studies. Indicators of necrotic (dead) cells are called mortal stains because they are only able to penetrate compromised membranes (Lobban, Chapman & Kremer, 1988). SYTOX-Green® (Segovia & Berges, 2009) and Evan's Blue (Lobban *et al*., 1988) are two types of mortal stains have been employed to indicate necrotic cells. SYTOX-Green® is a fluorescent nucleic acid probe that has been increasingly used in studies on phytoplankton death because of the potential

to be used in conjunction with flow cytometry for rapid culture analysis, but traditional analysis with fluorescence microscopy is common (Peperzak & Brussaard, 2011). This stain provides an adequate method for detecting necrotic cells within a natural assemblage because of the broad range of phytoplankton classes that are receptive to the probe, though differential sensitivity to the probe has been observed namely for Euglenophyceae (Peperzak & Brussaard, 2011). Using mortal stains alone does not determine the mode of cell death, but rather is simply an indicator of necrotic cells. Elucidation of the mode of death would require the use of a mortal stain in conjunction with other techniques that interact with biochemical or physiological features characteristic of a death pathway.

Loss of membrane symmetry is a useful feature for detecting apoptotic cell death and occurs during the early stages of metazoan apoptosis (van Doorn, 2011), prior to membrane permeabilization associated with secondary necrosis of the cell (Franklin *et al*., 2006). FITC Annexin-V is a fluorescent probe used to detect the early loss of membrane symmetry characteristic of apoptosis (Segovia & Berges, 2009). FITC Annexin-V binds to the phosphatidylserine of the inner cell membrane. During apoptosis the phosphatidylserine is translocated from the inner membrane to the outer membrane. However, FITC Annexin-V may also enter cells with a compromised membrane and apoptotic and necrotic cells would be expected to stain positive. As a result, FITC Annexin-V must be used in conjunction with a mortal stain, like SYTOX-Green® so that the proportion of a population actually experiencing apoptosis can be determined by subtracting the proportion of SYTOX-Green® positive cells from the observed proportion of FITC Annexin-V positive cells.

Field studies on cell death in populations of phytoplankton have yielded a number of interesting hypotheses. For example, Sigee *et al*. (2007) suggested the possibility of a "death threshold" for colonial cyanobacteria exhibiting positive staining with SYTOX-Green®. Agustí *et al*. (2006) postulated differential significance of cell death as a loss process among oligotrophic versus eutrophic lakes, and also dominant versus minor community components.

This study aims to examine the incidence of phytoplankton cell death in a eutrophic, urban, park pond using the fluorescent stains SYTOX-Green® and FITC Annexin-V. I was particularly interested in detecting the occurrence of apoptosis using FITC Annexin-V because this method has not been tried much at all in the field, primarily being used in laboratory studies (Segovia & Berges, 2009). I expected dead and apoptotic cells to occur in a variety of phytoplankton taxa throughout the sampling period because previous work demonstrated a wide variety of phytoplankton taxa are receptive to SYTOX Green® (Peperzak & Brussaard, 2011). Because both abiotic and biotic factors may be responsible for phytoplankton cell death, I expected different patterns of cell death to be observed relative to time of year and cell abundance. I expected abiotic factors to be responsible for death events when cell abundance was low, indicating unfavorable environmental conditions for growth. Environmental parameters of light, temperature and oxygen were measured to determine if these factors could be abiotic explanations of cell death events. Pathogens are an example of a biotic factor that may be responsible for phytoplankton cell death. Because pathogen transmission increases with increasing host density (Thingstad & Lignell, 1997), I

expected cell death due to biotic factors to occur during periods of increasing or peak phytoplankton cell abundance. Virus abundance data from previous studies (Appendix) in Estabrook Park Pond (Hanson, 2010), and observations of chytrid fungi, were used to help explain cell death that occurred during increasing or peak cell abundance. Finally, because phytoplankton cell death has been suggested to be important to nutrient cycling in aquatic systems (e.g. Bidle & Falkowski, 2004), I wanted to determine if cell death could account for observed increases in total dissolved phosphorus.

METHODS

Study Site and Sampling

Estabrook Park Pond WI, USA (43°01'N 87°54'W) is a eutrophic urban park pond that has been the site of limnological studies and classroom research at UW-Milwaukee for decades. Sampling was conducted weekly from July-November 2010. A YSI 600XL Sonde (YSI Inc., Yellow Springs, CO, USA) was calibrated prior to recording water surface temperature, specific conductance, oxygen, and pH on each sampling occasion. Regression analyses were run on temperature and oxygen against the day of year to determine whether significant ($P<0.05$) changes occurred from the July to November. Sub-surface water samples were collected with a 1L Swing Sampler (Nasco, Fort Atkinson, WI, USA) and combined from eight locations around the perimeter of the lake. Combined samples were transported to the lab and sub-sampled for cell death staining and nutrient analyses, and 100mL of the combined sample was preserved with acidified Lugol's iodine for microscopic enumeration.

Light Data

Light data were obtained from the 2010 climate data provided by the National Weather Service Forecast Office in Milwaukee, WI (Mitchell Field). Total surface irradiance (mol photons m^{-2}) between sampling time points and daily total surface irradiance (mol photons m^{-2}) for the day prior to sampling were calculated.

Regression analyses were run on the total surface irradiance and daily total surface irradiance against day of year to determine whether significant seasonal changes occurred (P<0.05).

Detection of Cell Death in a Community of Phytoplankton

A portion of the collected whole-water sample was screened over a 153µm Nitex mesh to remove large animals before a known volume was concentrated over a 10µm Nitex mesh using the methods and an apparatus similar to that described by Reavie, Cangelosi & Allinger (2010). The concentrated particles were re-suspended with filtered lake water to a known final volume and this concentrated sample was used for detection of necrotic and apoptotic cells using the epifluorescent stains SYTOX-Green® and FITC Annexin V respectively. The Lugol's preserved whole water sample was used to enumerate the actual abundances of positively stained phytoplankton taxa previously enumerated and identified under fluorescent microscopy.

SYTOX-Green® Staining SYTOX-Green® (Invitrogen S7020) is a mortal stain that enters cells with compromised membranes, binding to fragmented DNA, and is indicative of necrotic cells. 10µL of SYTOX-Green® working stock was added to 1mL of the concentrated phytoplankton sample (0.5µM final SYTOX-Green® concentration) and incubated in darkness for 15 minutes. Stained samples were then filtered onto a Nucleopore® membrane (Nuclepore® Corporation, Pleasanton, CA, USA, product number 110611, 25mm diameter, 2.0µm pore size) prior to a gentle wash with filtered lake water. Before the Nuclepore® membrane was exposed to air 2-3 drops of gluteraldehyde were added to the lake water to preserve

the stained cells on the membrane. The Nuclepore® membrane was then transferred to a microscope slide dotted with 1-2 drops of Resolve™ low-fluorescence microscope immersion oil (Stephens Scientific, Denville, NJ, USA product number M6005-1L) in order to "clear" the slide for subsequent microscopy. The preserved and cleared slide was stored in a dark container at 4°C until the stained cells could be enumerated and identified (within 1 week of preparation). Immediately prior to epifluorescence microscopy, 30µL of a freshly made antifade mounting solution (50% glycol, 50% phosphate buffer solution pH 7.5, 0.01% p-phenylenediamine) was added to the membrane in order to prolong fluorescence. An Olympus BX41 (BX41TF) epifluorescent microscope with a FITC filter set (Chroma 41001: HQ480/40x band-pass excitation, and Q505 long-pass dichroic mirror and HQ535/50m band-pass emissions) was used to observe positively stained cells (green light emission). All identifiable taxa (Table 7) on the prepared slide in three to four transects at 400x magnification were counted and the proportion of SYTOX-Green® positive counts calculated. Attempts were made to resolve taxa to species level using keys (Prescott, 1962; Dillard, 2007).

FITC Annexin-V Staining FITC Annexin-V (Invitrogen A13199) is a stain used to indicate apoptotic cell death by binding to phosphatidylserine (PS) that is normally on the inner surface of the plasmalamellae, but is translocated to the outer surface during the early stages of apoptosis (Berges & Falkowski, 2004). Two-mL of the phytoplankton sample concentrate were centrifuged at 7500rpm for 5 minutes and the pellet was resuspended in 100µL of Hepes buffer solution. 5µL of the FITC Annexin-V reagent was then added and the sample was allowed to incubate

in darkness for 15 minutes. The stained sample was then diluted to 1mL with Hepes buffer and was filtered onto a Nuclepore® membrane (product number 110611, 25mm diameter, 2.0μm pore size), washed, preserved, stored, and counted in the same manner as in the SYTOX-Green® preparation; fluorescent microscopy also utilized the same FITC filter set. The proportion of cells staining with FITC Annexin-V was corrected for false positives since the stain also enters cells with compromised membranes and stains the inner membrane. Thus the true proportion of FITC Annexin-V positive counts were equal to the observed proportion of FITC Annexin-V positive counts less the proportion SYTOX-Green® positive counts.

Lugol's Abundance Counts Counts were performed on samples preserved in acidified Lugol's iodine and taxa abundances determined. Counts focused on taxa that were identifiable in the stain counts and aimed to achieve counts of 100 per taxon or better than those obtained on slides prepared with stained samples (Venrick, 1978). Preserved samples were settled in an Utermöhl chamber for approximately 24 hours before counting. A combination of random fields, transects, and half-wells were used to obtain counts and taxa abundances were subsequently calculated. Keys were used to identify taxa (Prescott, 1962; Dillard, 2007).

Nutrient Analysis

Phosphorus, nitrogen, and carbon were measured and used to calculate molar C:N:P values as a measure of community level stress. Analytical triplicates of total and dissolved phosphorus were measured using the persulfate oxidation method (Menzel & Corwin, 1965). Results were read using an LKB Biochrom

Ultrospec® II spectrophotometer (LKB Biochrom Ltd, Cambridge, U.K., model number 4050) and subsequently used with equations from a standard absorption curve to calculate total and dissolved phosphorus. Particulate phosphorus was determined by subtraction of the dissolved fraction from the total, and this value was used in the determination of molar C:N:P values. A regression analysis was run on total phosphorus measured in this study and that measured in a previous study to determine whether seasonal changes in total phosphorus were typical in this lake, indicated by a significant correlation ($P<0.05$). In order to determine whether cell death accounted for changes in total dissolved phosphorus (TDP), I calculated the potential total TDP released from SYTOX-Green® and FITC Annexin-V positive cells on the sampling day where a peak in TDP occurred. Internal phosphorus quotas from Smith & Kalff (1982), and a 21% conversion of internal phosphorus to biologically useable forms upon viral lysis (Gobler *et al*., 1997) were used in calculations. Finally, to determine elemental carbon and nitrogen, a known volume of the combined water sample was filtered onto pre-ashed (460°C for 8h) Whatman® GF/F glass microfiber filters (Whatman Inc., Clifton, NJ, USA, 47mm diameter) before analysis with a Thermo Electron Flash EA 1112 series CNS analyzer (Thermo Scientific, Rodano, Milan, Italy), and values used to calculate molar C:N:P ratios.

Table 7. The phytoplankton taxa that exhibited positive staining with SYTOX-Green® and FITC Annexin-V, and that were able to be identified under fluorescent microscopy and were counted in the present study.

Taxa	**Class**
Ankistrodesmus spiralis	Chlorophyceae
Franceia Droescheri	Chlorophyceae
Kirchnieriella obesa	Chlorophyceae
Pediastrum boryanum	Chlorophyceae
Pediastrum duplex	Chlorophyceae
Pediastrum simplex	Chlorophyceae
Scenedesmus quadricauda	Chlorophyceae
Staurastrum chaetoceras	Chlorophyceae
Staurastrum polymorpha	Chlorophyceae
Tetraedon minimum	Chlorophyceae
Dinobryon sertularia	Chrysophyceae
Mallomonas caudata	Chrysophyceae
Mallomonas tonsurata c.f.	Chrysophyceae
Peridinium spp.	Dinophyceae
Synedra spp.	Bacillariophyceae
Microcystis aeruginosa	Cyanophyceae
Euglena spp.	Euglenophyceae

RESULTS

Environmental Conditions: Light, Temperature, Oxygen, and Nutrients

Light, temperature, and oxygen all significantly changed during the sampling period ($P<0.05$ for each). Temperature and both the total surface irradiance and daily total irradiance decreased throughout the sampling period (Figure 10). Oxygen concentration increased from day 195 (July 13th) until day 292 (Oct. 18th), after which point oxygen concentration began to decrease (Figure 10).

Total phosphorus (TP) was significantly correlated ($P<0.05$) with TP measured from July to October 1999 in a previous study on Estabrook Park Pond. The 1999 study measured TP from May 4th (day 124) through November 9th (day 313) during which time TP peaked in mid-July (day 201) followed by a steady decline until the end of the 1999 sampling period (Figure 11). This trend corresponded well with measured TP of the present study which peaked on July 13th (day 195), and was followed by a steady decline (Figure 11). Total dissolved phosphorus (TDP) was highest on July 13th (day 195) reaching 1.7 µM, but remained at ~0.7 µM throughout the rest of the sampling period, except on Oct. 25th (day 299) when the concentration spiked to nearly 1.4 µM (Figure 11). Cell death at the end of the sampling period coincided with a peak in the concentration of total dissolved phosphorus on Oct. 25th (day 299) reaching 1.36 µM from 0.78 µM on Oct. 18th (day 292, Figure 11). Simultaneously, about 162 cells mL^{-1} of the identifiable taxa stained positively with SYTOX-Green® and FITC Annexin-V on Oct. 25th (day 299). Previous studies determined the minimum internal quota of freshwater phytoplankton on average ranged from approximately 10 to 100 $\times 10^{-9}$ µmol P cell^{-1} (Smith & Kalff,

1982), and a 21% conversion of internal phosphorus to biologically useable forms upon viral lysis (Gobler *et al*., 1997). Assuming the upper limit for the internal P quota of 100×10^{-9} µmol P $cell^{-1}$ and a 21% conversion, 3.4×10^{-3} µM dissolved phosphorus would have been released on Oct. 25th (day 299).

Figure 10. Seasonal trends of light, temperature, and oxygen at the surface of Estabrook Park Pond in 2010. The total surface irradiance represents the sum of the irradiance between sampling dates (typically 7 days), while the daily total surface irradiance (mol photons m^{-2}) is the total for the day prior to the sampling date. Irradiance was obtained from the National Weather Service Forecast Office in Milwaukee, WI (Mitchell Field), and temperature and oxygen values were determined *in situ* with a YSI 600XL sonde.

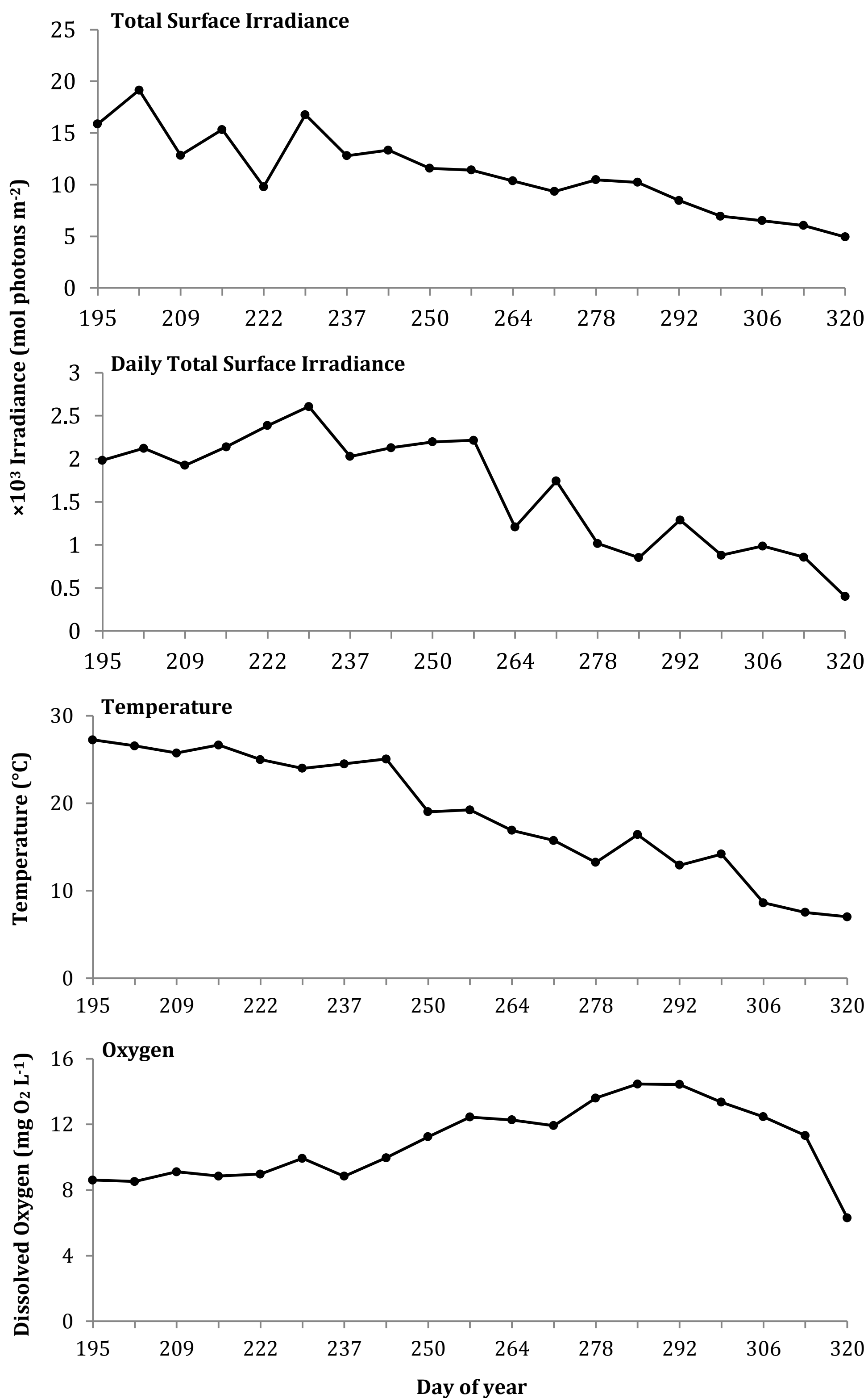
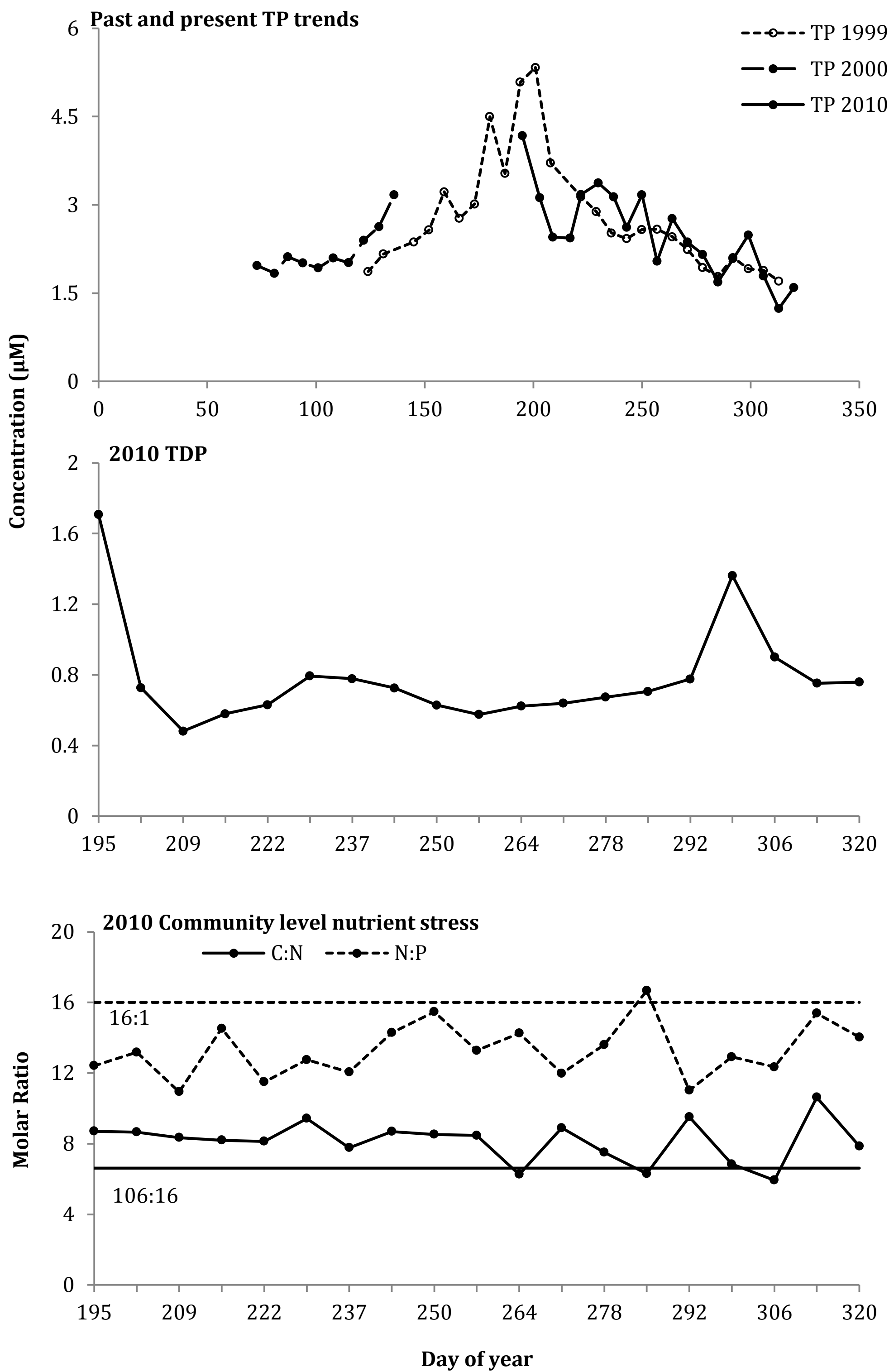

Total Surface Irradiance
Daily Total Surface Irradiance
×10³ Irradiance (mol photons m⁻²)
Temperature
Temperature (°C)
Oxygen
Dissolved Oxygen (mg O₂ L⁻¹)
Day of year
195
209
222
237
250
264
278
292
306
320

Figure 11. Trends in nutrients and particulate matter in Estabrook Park Pond, including data from previous work (1999, 2000, M. Murru, unpublished) and the present study (2010). The first panel represents total phosphorus (TP) trends in 1999, 2000, and 2010. The second panel represents trends in total dissolved phosphorus (TDP) during the 2010 sampling period. The third panel represents particulate C:N (solid line with circles) and N:P (dashed line with circles) molar ratios; the horizontal lines represent the Redfield ratio typical of nutrient sufficient cells. Phosphorus determination was accomplished by the persulfate oxidation method (Menzel & Corwin, 1965). Determination of carbon and nitrogen for calculations of molar C:N and N:P ratios was accomplished with a Thermo Electron Flash EA 1112 series NCS analyzer. Note differences in y-axes scales.



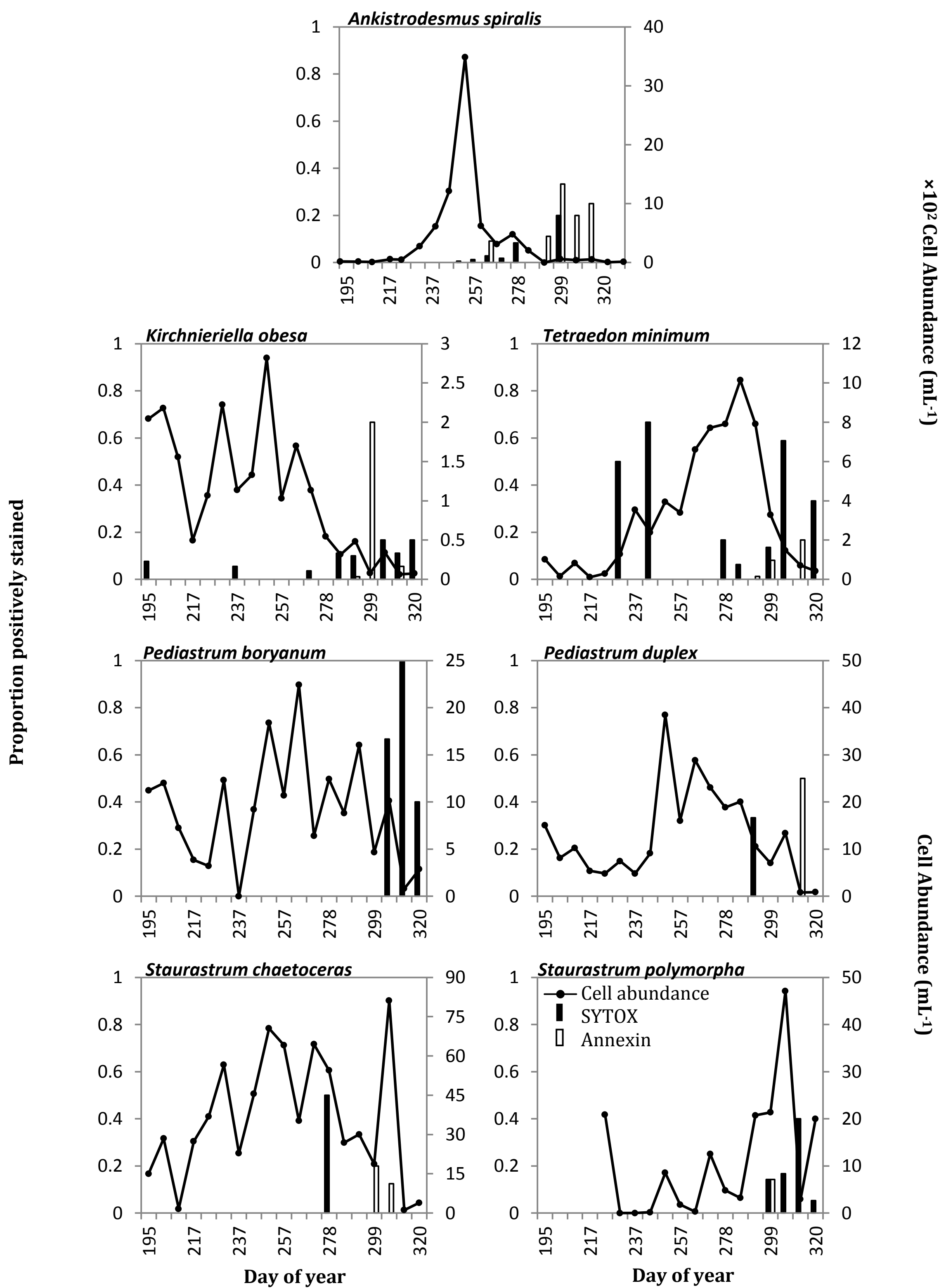
Past and present TP trends
TP 1999
TP 2000
TP 2010
Concentration (μM)
2010 TDP
2010 Community level nutrient stress
C:N
N:P
16:1
106:16
Molar Ratio
Day of year

Phytoplankton Cell Death

The proportion of dead cells (i.e. positively stained with either SYTOX-Green® or FITC Annexin-V) was typically less than 0.6 for all taxa examined (Figures 12-15), though in a few cases (one occasion for *Pediastrum Boryanum* and twice for *Microcystis aeruginosa*) all cells counted stained with SYTOX-Green® (Figures 12 & 15). Samples that stained positively with FITC Annexin-V were generally associated with or followed by samples that stained positively with SYTOX-Green® (Figures 12-15).

Staining data presented in Figures 12-15 are grouped by the staining patterns observed. Overall, there was little consistency in staining patterns within the classes of taxa examined (Table 7, Figures 12-15), but three main patterns emerged: Type (1) SYTOX-Green® or FITC Annexin-V positive cells near the end of the sampling period (i.e. after day 278 [Oct. 4], typified by *Ankistrodesmus spiralis* [Chlorophyceae] and *Staurastrum polymorpha* [Chlorophyceae]) (Figures 12 & 13); Type (2) SYTOX-Green® or FITC Annexin-V positive cells both before and after a peak in abundance (e.g. *Tetraedon minimum* [Chlorophyceae]) (Figure 14); or Type (3) SYTOX-Green® or FITC Annexin-V positive cells coincident with a peak in abundance (Figure 15), typified by Euglena spp. (Euglenophyceae) and *Staurastrum polymorpha*. In general, positive staining that occurred during peak abundance was followed by a decrease in abundance (Figure 15).

Figure 12. Cell abundance and cell death for Chlorophycean taxa in Estabrook Park Pond that positively stained with SYTOX-Green® and FITC Annexin-V primarily during mid-September to mid-November in 2010. Symbols represent total abundance (based on settled counts). Bars represent the proportion of cells/colonies that positively stained with SYTOX-Green® (black bars) and FITC Annexin-V (white bars), in independent stained samples, indicating dead and apoptotic cells respectively. Note differences in scaling of the secondary y-axes.



Ankistrodesmus spiralis
Kirchnieriella obesa
Tetraedon minimum
Pediastrum boryanum
Pediastrum duplex
Staurastrum chaetoceras
Staurastrum polymorpha
Cell abundance
SYTOX
Annexin
Proportion positively stained
×10² Cell Abundance (mL⁻¹)
Cell Abundance (mL⁻¹)
Day of year

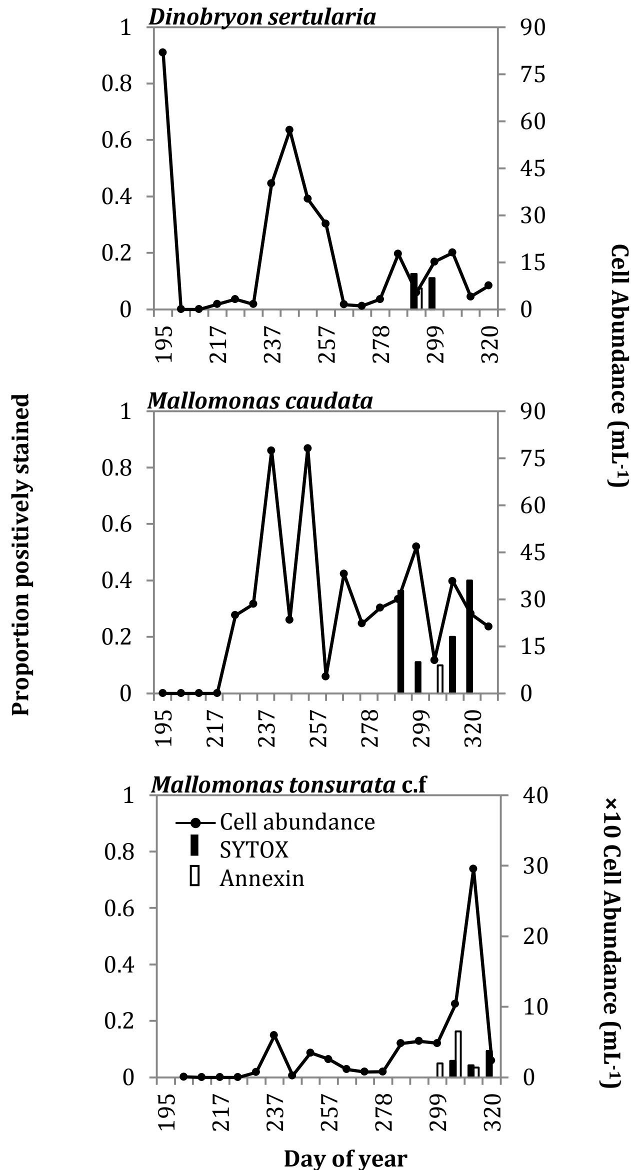


Figure 13. Cell abundance and cell death for Chrysophycean taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V primarily during mid-September to mid-November 2010. Symbols represent cell abundance (based on settled counts). Bars represent the proportion of cells/colonies that positively stained with SYTOX-Green® (black bars) and Annexin-V (white bars), in independent stained counts, indicating dead or apoptotic cells respectively. Note differences in scaling of the secondary y-axes.

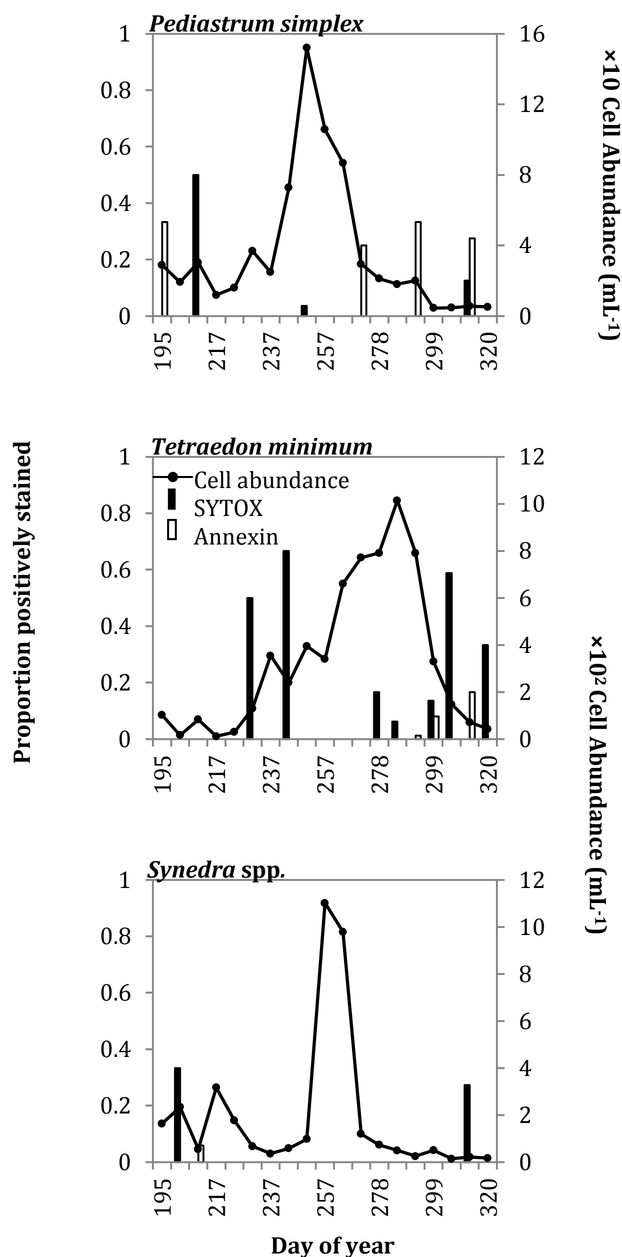
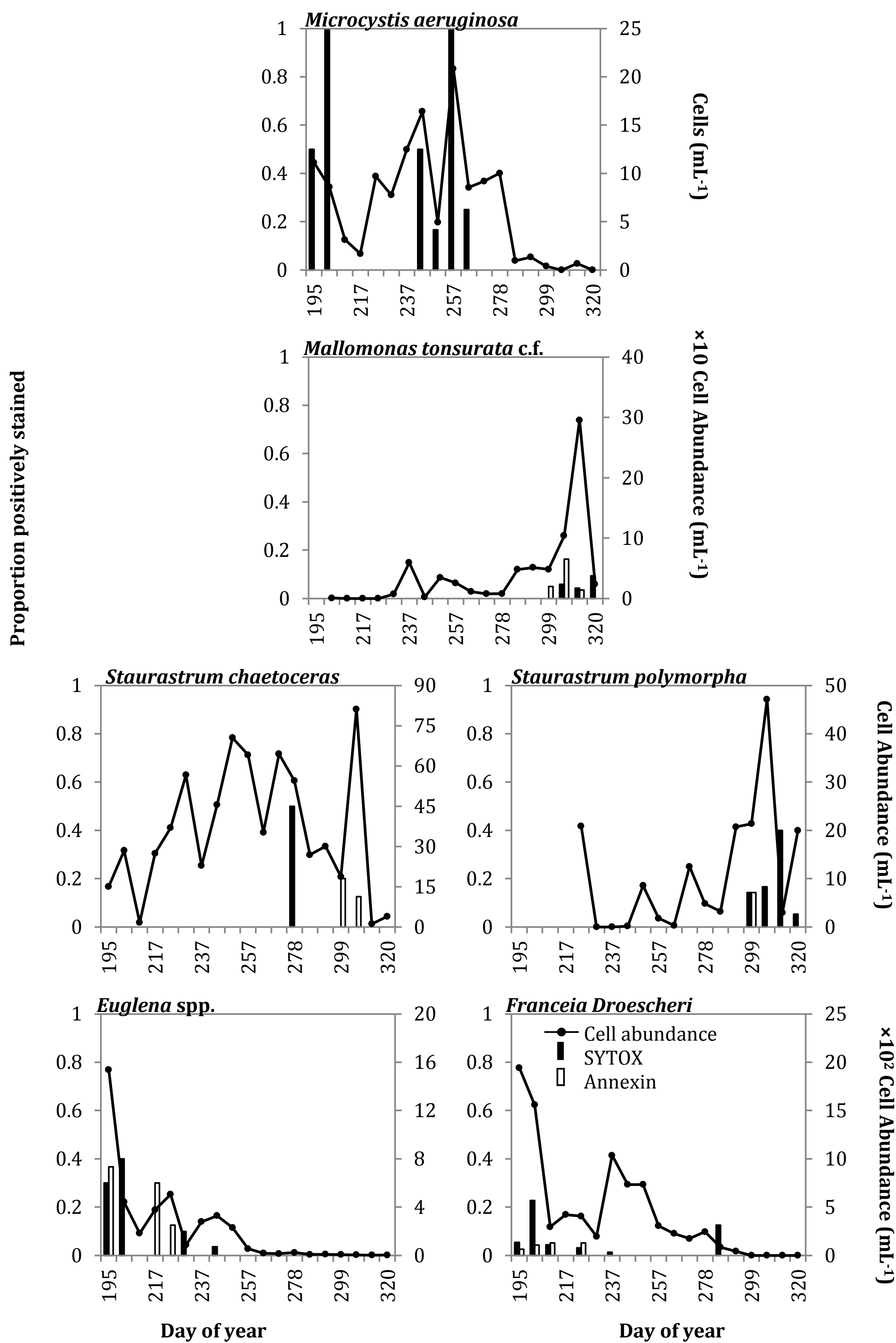


Figure 14. Cell abundance and cell death for taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V both before and after a peak in cell abundance. Symbols represent cell abundance (based on settled counts). Bars represent the proportion of cells/colonies that positively stained with SYTOX-Green® (black bars) and Annexin-V (white bars), in independent counts, indicating dead and apoptotic cells respectively. Note differences in scaling of the secondary y-axes.

Figure 15. Cell abundance and cell death for taxa in Estabrook Park Pond in 2010 that positively stained with SYTOX-Green® or Annexin-V during peaks in abundance. Symbols represent cell abundance (based on settled counts). Bars represent the proportion of cells/colonies that positively stained with SYTOX-Green® (black bars) and Annexin-V (white bars), in independent stained counts, indicating dead and apoptotic cells respectively. Note differences in scaling of the secondary y-axes.



Microcystis aeruginosa
Cells (mL-1)
Mallomonas tonsurata c.f.
×10 Cell Abundance (mL-1)
Proportion positively stained
Staurastrum chaetoceras
Staurastrum polymorpha
Cell Abundance (mL-1)
Euglena spp.
Franceia Droescheri
Cell abundance
SYTOX
Annexin
×10² Cell Abundance (mL-1)
Day of year
Day of year

DISCUSSION

SYTOX-Green® is a mortal stain used as an indicator of cell death. The use of SYTOX-Green® as an indicator of death has been well validated (Veldhuis, Kraay & Timmermans, 2001), but concerns have been expressed that there is staining of live cells or failure to stain dead cells, depending on species (Tang & Dobbs, 2007). Moreover, affinity for staining may vary among phytoplankton groups, for example rhodophytes and euglenophytes stain less readily stained with SYTOX-Green® than other groups of phytoplankton (Peperzak & Brussaard, 2011). In some studies, increasing the concentration of SYTOX-Green® (Binet & Stauber, 2006) and, or the incubation time were adequate to achieve better staining (Zetsche & Meysman, 2012). In the present study, a positive control was attempted by preserving samples overnight with 0.1% gluteraldehyde prior to staining with SYTOX-Green® and analysis with a flow cytometer; no evidence of unstained cells was detected (Kozik & Berges, *unpublished data*). It is not clear what a good negative control would be, but variation in staining frequency from zero to high numbers of stained cells within taxa over the course of the study period suggest the method yields some reliable results. Other techniques that involve enzymatic cellular digestion of samples have also been used as a means of assessing cell viability in field samples (Agustí & Sánchez, 2002). Since cellular digestion detects the presence of living cells rather than dead cells, it would be interesting to compare results of these techniques side by side across species.

FITC Annexin-V was used as an indicator of cells undergoing apoptotic-like PCD. Unlike SYTOX-Green®, FITC Annexin-V has not been tried much in the field. In

addition, no clear control exists for FITC Annexin-V, but positive staining has been observed in other studies along with additional evidence corroborating apoptotic-like cell death, such as increased caspase activity in both *Dunaliella tertiolecta* (Chlorophyceae) (Segovia & Berges, 2009) and *Thalassiosira pseudonana* (Diatomaceae) (Thamatrakoln *et al*., 2012).

Other studies examining the frequency of cell mortality in natural populations have yielded estimates similar to those obtained in the present study. The proportion of positively stained cells with SYTOX-Green® in this study generally ranged from ~5% to 60%, and from ~5% to 30% for FITC Annexin-V (Figures 12-15). For example, the percent SYTOX-Green® positive cells ranged from 6.3% to 67% for *Tetraedon minimum*. This is comparable to results from other field studies. In Roshtherne Mere, a eutrophic, temperate lake, 17%-50% of *Microcystis flos-aquae* (Cyanobacteria) were dead (Sigee *et al*., 2007), and ~ 65% of the *Peridinium gatunense* (Dinophyceae) population was dead near the end of a bloom in Lake Kinneret, Israel, compared to approximately 5% dead at the start of the bloom period (Vardí *et al*., 1999). A natural coral bleaching event recorded a maximum of 22% dead symbiotic dinoflagellate cells using SYTOX-Green® (Franklin *et al*., 2004). One taxa in the present study demonstrated 100% staining (*M. aeruginosa*, Figure 15) on a few occasions. This was likely the result of there being a low number of cells or colonies on the counted filter, and perhaps if more cell or colonies had been counted, the percent stained would have been less than 100%.

As expected, a number of different phytoplankton taxa positively stained with SYTOX-Green® and FITC Annexin-V (Figures 12-15). In addition, differences in

the observed staining patterns suggest different factors may have been responsible for causing phytoplankton cell death in an urban, eutrophic park pond from mid-July to mid-November of 2010. In this study it was found that the pattern of cell death relative to time of year and, or abundance was not consistent within classes (Figures 12-15). For example *Ankistrodesmus spiralis*, *Pediastrum simplex* (Chlorophyceae), and *Franceia Droescheri* (Chlorophyceae) are all chlorophytes. However, *A. spiralis* exhibited positive staining with SYTOX-Green® and FITC Annexin-V primarily toward the end of the sampling period (type one staining, Figure 12), *P. simplex* exhibited staining both before and after a peak in abundance (type two staining, Figure 14), and *F. Droescheri* positively stained earlier during the sampling period during peaks in abundance (type three staining, Figure 15). Both abiotic and biotic factors likely contributed to differences in mortality observed among the taxa examined. It has been demonstrated that a single species may exhibit different death morphotypes in response to different environmental stimuli (Jiménez *et al*., 2009), so it is likely that different stressors are responsible for different death events in the present study.

Cell Death and Abiotic Factors

If apoptotic-like PCD is the result of successive exposure to sub-lethal levels of stress (Proskuryakov, Konoplyannikov & Gabai, 2003), due to rapidly changing conditions, we would anticipate it to be more prevalent during temperate fall, or spring, when light and temperature are more variable. It was predicted that abiotic factors were more likely responsible for cell death observed at the end of the sampling period because sampling was conducted during temperate fall, when

temperature and irradiance decrease, and vegetative growth conditions become less favorable. In the present study, an interaction between light and temperature may have resulted in the increased incidence of apoptotic-like PCD in the latter half of the sampling period for observed staining patterns one (Figures 12 & 13) and two (Figure 14). However, staining at the end of the sampling period did not always coincide with low cell abundance (Figure 12-14) as was predicted. Most staining occurred in autumn after approximately Oct. 4th (day 278) when daily total irradiance averaged approximately 897 mol photon m^{-2}, and temperatures averaged below 12°C (Figure 10).

Low temperatures reduce metabolic processes by slowing down enzymatic reactions (Davison, 1991). The reduction in the ability at low temperatures to use energy derived from light reactions in enzymatic reactions, such as C fixation, may lead to photoinhibition under light saturated conditions (Davison, 1991), and result in the generation of reactive oxygen species (ROS) (Skjånes, Rebours & Lindblad, 2013). Accumulation of ROS within the cell has been observed along with other hallmarks of apoptotic-like death in phytoplankton such as cytoplasmic shrinkage (Vardí *et al*., 1999), DNA fragmentation and loss of membrane asymmetry (Segovia & Berges, 2009). However, production versus irradiance curves were not determined for the taxa in the present study to determine light saturation at different temperatures, nor were ROS concentrations measured within cells. Laboratory experiments that examine the occurrence of PCD under changing temperature and light conditions are needed in order to determine whether changing temperature and light conditions that are typical of temperate fall could

explain apoptotic-like PCD observed in the latter half of the sampling period of this study (Figures 12-14).

Previous studies have implied that phytoplankton cell death may be important to nutrient cycling in aquatic ecosystems (e.g. Bidle & Falkowski, 2004; Strzepek *et al*., 2005; Evans & Brussaard, 2012), yet few studies have been conducted that assess the biologically usable constituents of cell lysates released from apoptotic phytoplankton cells (e.g. Gobler *et al*., 1997). The results of this study do not support the importance of phytoplankton cell death to phosphorus cycling in aquatic systems. It was determined that only 3.4×10^{-3} µM of the 0.58 µM increase in dissolved phosphorus would have been released from dead and dying cells when TDP peaked on Oct. 25th (day 299), assuming the upper limit for the internal P quota of 100×10^{-9} µmol P cell^{-1} (Smith & Kalff, 1978) and a 21% conversion of particulate phosphorus to the dissolved fraction (Gobler *et al*., 1997). Even a 100% conversion (1.6×10^{-2} µM) to dissolved phosphorus would not account for the increase observed on Oct. 25th (day 299). Though bacterial conversion of particulate to dissolved phosphorus occurs at a slower rate, on the order of days to weeks, (Gobler *et al*., 1997), a 100% conversion of released phosphorus during the three weeks prior to and occurring on day 299 (6.4×10^{-2} µM) would also not account for the 0.58 µM increase in TDP.

The disconnection between cell death and the observed increase in TDP on Oct. 25th (day 299) may be due underestimation of dead or dying cells and, or differences in staining affinity. One reason dead or dying cells were underestimated was because a number of flagellate taxa were unidentifiable and were not counted

on the filters viewed under fluorescence microscopy, and therefore could not be included in calculations. In addition, the duration of apoptosis in animal cells has been reported to occur in as little as 2 or 3 hours to as long as 48 hours depending on the type of cell (Kanduc *et al*., 2002). So it is possible that apoptotic-like PCD occurred after sampling, but before being stained with SYTOX-Green® and FITC Annexin-V. Affinity for staining with SYTOX-Green® and FITC Annexin-V may have also varied among taxa, for example poor staining of euglenophytes (Peperzak & Brussaard, 2011), so dead and dying cells of certain taxa may have gone undetected. This may have been circumvented had staining protocols been optimized for dominant taxa by adjusting the incubation time and concentration of stains as community assemblages changed throughout the study. Another explanation for the disconnection between TDP released and the increased observed on Oct. 25th (day 299) is that phosphorus is rapidly recycled in freshwater systems, and may have been utilized by other cells upon release. Lastly, rainfall on Oct. 23rd and 24th (days 297 and 298) totaled 2.64cm, and surface run-off may have also accounted for the increase in TPD observed on Oct. 25th (day 299).

Live-dead assessment for all members of a phytoplankton community is needed to quantify the contribution of phytoplankton cell death to nutrient cycling. Flow cytometric methods would be ideal for this type of assessment due to rapid sample analysis. Flow cytometric analysis of natural phytoplankton communities stained with SYTOX-Green® and FITC Annexin-V was able to easily distinguish positively stained cells from unstained cells by setting a fluorescence threshold for defining positive staining cells, and also by gating to restrict staining events into size

fractions (Kozik & Berges, *unpublished*). The fraction of the phytoplankton community that is determined to be dead or dying can then be used to determine the contribution of phytoplankton death to nutrient cycling in aquatic systems. However, more experiments are needed that quantify the release of dissolved and particulate fractions of nutrients from a variety dead and dying phytoplankton taxa in order to improve quantification of the contribution of death events to nutrient cycling in aquatic systems.

Cell Death and Biotic Factors

Biotic factors, including viral and fungal pathogens, may have accounted for phytoplankton death when abundance was increasing or at its peak (Figure 15). Pathogens increase with increasing host populations (Thingstad & Lignell, 1997) because transmission is dependent upon host density (Brussaard, 2004). Viruses are one algal pathogen that has been studied in detail (Gachon *et al*., 2010). The highest viral abundances observed in Estabrook pond were not associated with bacterial abundance (Hanson, 2010, Appendix-Figure 1). Rather, greatest viral abundance, from about Aug. 7th through November 5th (days 220 to 310), overlapped with peak chl *a* concentration on Aug. 7th (day 220) (Appendix-Figure 1). In addition, viral abundance remains high following the decline in chl *a* which suggests that high viral abundance may be the result of cell lysis and release of viral particles. Other studies have demonstrated that viral infection induces symptoms of PCD in phytoplankton (e.g. Segovia & Berges, 2005; Bidle *et al*., 2007), and is a potential mechanism of bloom collapse (Bratbak, Egge & Heldal, 1993). This is similar to the observation in the present study that the abundance of some taxa, for

example *Euglena* spp. (Figure 15), declined following apoptotic-like PCD at peak abundance.

Chytrid fungi were also observed infecting cells of some chlorophyte taxa (e.g. *Staurastrum* spp.) in the present study, however the incidence of infection was not recorded. Abundance of chytrid fungi has been shown to increase with increasing host populations, and infection may occur on both healthy and senescent cells (reviewed in Ibelings *et al.*, 2004). Infection by chytrid fungi has been observed to produce a hypersensitivity response in phytoplankton after zoospore attachment, causing the generation of ROS in the host cells (Ibelings *et al.*, 2004). Moreover, infection by chytrids have been suggested as the cause of bloom collapse in Lake Biwa, Japan (Kagami *et al.*, 2006), and was attributed as the cause of cell death in *Anabaena* colonies in a eutrophic lake (Sigee *et al.*, 2007). It is possible that in some instances chytrids may have accounted for recorded necrotic or apoptotic-like death. In general, the mechanism and prevalence of chytrid induced cell death warrants further investigation.

Grazers have been observed to produce changes in phytoplankton life history via their exudates (Rengefors, Karlsson & Hansson, 1998), so it is plausible that exudates may also induce a death response. Work by Rengefors *et al.* (1998) demonstrated that grazer exudates, of unknown composition, were able to induce encystment of the dinoflagellate *Peridinium aciculiferum*. In addition, work by Vardí *et al.* (1999) revealed that death and cyst formation pathways are linked in *P. gatunense* via control by cysteine proteases. As a result, it is possible that phytoplankton may exhibit a hypersensitivity response when in the presence of

great enough concentrations of grazer exudates, and under the appropriate (stressful) environmental conditions. Grazer abundance and exudates were not tracked in the present study, but the influence of grazer exudates on life history events, death and cyst formation, should be examined in future studies.

Allelopathic interactions may also explain death events in the field. A variety of chemical compounds have been identified in allelopathic interactions, such as aldehydes, which are produced by a variety of marine and freshwater phytoplankton (Ribalet *et al*., 2007). A number of phytoplankton taxa, including chlorophytes, produce allelopathic chemicals that result in inhibition of photosystem II and death in other algae and cyanobacteria (see Table 1 in Legrand *et al*., 2003). In the present study *Euglena* spp. exhibited increased necrosis and apoptotic-like PCD (Figure 15) as the abundance of chlorophyte taxa, for example *A. spiralis* (Figure 12) and *T. minimum* (Figure14), increased. However, determining the effect of allelopathic interactions in the field is difficult and was not attempted in the present study (Maestrini & Bonin, 1981). Laboratory studies would need to be conducted to establish whether filtrates from field isolates inhibit growth and, or instigate death in other taxa (e.g. Suikkanen, Fistarol & Granéli, 2004). It would also need to be established that competition, or grazing and, or sedimentation, does not account for the succession pattern observed. Since it is difficult to remove allelopathic interactions from competition experiments, it may be necessary to identify and knockout the genes involved in allelochemical production prior to performing competition experiments under different environmental regimes.

Future Directions

This study demonstrated that cell death occurs in a wide array of phytoplankton taxa in the field, and may occur when cell abundances are high or low. This suggests that different environmental factors may have been responsible for eliciting cell death in an urban, eutrophic park pond throughout the study period. It was also found the cell death could not explain changes in TDP, perhaps due to the rapid recycling of phosphorus in freshwater systems. However, the significance of various abiotic and biotic factors that triggers cell death to population dynamics, succession, and nutrient cycling remains to be examined in the field.

Abiotic factors that trigger cell death has largely been the focus of laboratory (e.g. Jiménez *et al*., 2009; Segovia & Berges, 2009) and field examinations (e.g. Vardí *et al*., 1999) compared to biotic causes of death due to viruses and chytrids. When biotic factors have been considered, viral mediated cell death has largely been the focus of study (e.g. Vardí *et al*., 2009; Evans & Brussaard, 2012). Additional studies are needed that examine the mechanisms and importance of other biotic factors, like allelopathy, chytrid infection, and presence of grazer exudates, to cell death in both the laboratory and field. Laboratory studies are needed that examine the significance of allelopathic chemicals to eliciting death and causing a decline in abundance separate from abundance dynamics caused by resource competition. Continued work on chytrid fungal infection triggering cell death is also needed. Specifically, studies should focus on the environmental and genetic conditions that elicit a hypersensitivity death response during chytrid infection. In addition, the

possibility of grazer exudates as a potential death response trigger remains to be examined in the laboratory. Concurrent with studies on cell death, other life history responses, like cyst formation, should also be examined as alternative responses affecting community dynamics and ecological memory.

Finally, to quantify the importance of death events to nutrient cycling in aquatic systems, laboratory studies are also needed that quantify the bioavailability of nutrients released from phytoplankton cells upon lysis. These studies should be combined with experiments on the recycling rate by bacteria of particulate to dissolved fractions if we are to understand both the short and long term consequences of phytoplankton cell death to biogeochemical cycling.

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GENERAL DISCUSSION

The primary goal of this thesis was to examine phytoplankton life history transformations in the field, in particular, resting stage ecology and cell death. It was found that while sedimentation and recruitment may not be responsible for explaining changes in standing crop at the community level, these processes may be important to taxa-specific abundance dynamics. It was also found that cell death occurs in a wide variety of phytoplankton taxa in the field, which may be the result of different abiotic and biotic factors, and that these death events do not contribute to changes in TDP in the field.

Two possible outcomes of a cell exposed to stress are the formation of resting stages (Sandgren, 1988) and cell death (Jiménez *et al*., 2009), and a mechanistic link between these processes has previously been proposed (Vardí *et al*., 1999). In the present study, resting stage ecology and cell death were examined at separate times and in separate systems. Although it was found that resting stages might not account for significant ($P<0.002$) changes in standing stock in lakes of differing food web structure (Figure 4), differences in the contribution of resting stages to sedimentation losses and gains via recruitment were observed among taxa (Figure 9, Table 5). In addition, dinoflagellate taxa exhibited a greater peak recruitment rate (Figure 8) in Peter Lake compared to Paul Lake where herbivory was stronger (Carpenter *et al*., 2001). The second half of the study examined the prevalence of cell death in a eutrophic, urban, park pond. In this half of the study, it was observed that different stress stimuli likely operate at different times, relative to population abundance and time of year, to induce cell death in the field.

Phytoplankton cysts were rarely observed during abundance counts in the second half of the study, but were prevalent in the first half of the study. This begs the question of what determines whether a cell forms a cyst or dies when faced with stress.

In plants, reactive oxygen species (ROS) have been established as being toxic yet also important signal transducers involved in gene regulation, and due to toxicity, intracellular levels are tightly controlled by ROS scavenging networks, such as antioxidants (reviewed in Miller, Shulaev & Mittler, 2008). A number of biological processes are regulated by ROS such as growth, the cell cycle and PCD (Miller *et al*., 2008). In phytoplankton ROS have been implicated as triggers involved in activation of death (Thamatrakoln *et al*., 2012) and sex genes (Nedelcu *et al*., 2004). Involvement in the activation of sex is significant because sex is a precursor to cyst formation in many freshwater phytoplankton taxa; but it must also be noted that cysts may be formed asexually (Sandgren, 1988). The ability to recover, or acclimate, to ROS accumulation, and to continue vegetative growth depends on the age and health of cells, in addition to the type, intensity, and duration of stress exposure (Thamatrakoln *et al*., 2012). As a result, it is possible that initiation of sex and cyst formation are also adaptive responses to ROS-generating stress stimuli, and initiation of sex and, or cyst formation may partially depend on the function of ROS scavenging mechanisms to avoid death.

Additional work is needed to determine the identity of compounds responsible for orchestrating cellular events determining cell fate, and deducing the interplay of ROS and ROS scavenging networks during PCD or cyst formation.

Cysteine proteases have duel functions and are involved in orchestrating the events of apoptotic-like PCD in phytoplankton (e.g. Segovia & Berges, 2005; Bidle *et al*., 2007), in addition to having housekeeping functions (Thamatrakoln *et al*., 2012). The specific response of cysteine proteases to ROS stress is dose-dependent (Thamatrakoln *et al*., 2012). Sublethal levels of ROS activate housekeeping functions of cysteine caspases whereas lethal levels trigger mediation of apoptotic-like death (Thamatrakoln *et al*., 2012). This is seemingly counterintuitive to studies that state apoptotic-like death is caused by sublethal levels of stress (e.g. Jiménez *et al*., 2009), however these studies often do not measure stress in terms of ROS, but rather variables like light intensity which influence ROS levels within a cell (Skjånes, Rebours & Lindblad, 2013). Future studies will benefit from standardizing measures of stress in terms of internal ROS concentration, so that comparisons between studies are easier to make.

Genes for the production of other compounds involved in inducing PCD, such as sphingolipids and subsequent ceramide production (Vardí *et al*., 2009), have also been found to be up-regulated during ROS stress and expression of PCD related genes (Thamatrakoln *et al*., 2012). However, it is not known whether, and under what conditions, genes associated with cyst production may also be up-regulated. Determination of genes associated with cyst formation is a necessary step for the study of cell acclimation to stress. Next, the internal and external conditions under which cyst formation genes are up-regulated must be determined. In addition, it will be necessary to measure ROS, the activity of ROS scavenging mechanisms, and the presence of cysteine proteases and other compounds expressed in association

with PCD in future studies on phytoplankton cell death and cyst formation, so that links between cell fates may be discovered.

Finally, additional work is needed to examine the occurrence, expression, and consequences of expression of anti-death genes. Previous work has identified genes whose expression would inhibit apoptotic-like death (Thamatrakoln *et al*., 2012), but relationships to cyst formation have not been examined. It is possible that some anti-death genes promote cyst formation by inhibiting expression of death genes, or altering the translation of death genes into products used for cyst formation. For example, application of a cysteine protease inhibitor has been demonstrated to reduce ROS production and cause cyst production in a dinoflagellate (Vardí *et al*., 1999). Cellular constituents that may act as cysteine protease inhibitors, and the genes that encode them, need to be identified so that the pathway linking cell death and cyst formation can be more thoroughly studied.

Continued work on the mechanistic connections linking cell death and cyst formation has important implications for developing ballast water sterilization techniques and models of biogeochemical cycling. It will become important to determine of the proportion of cells that undergo cell death or cyst formation under a given set of environmental conditions. This will be important for both ballast sterilization and models discerning the contribution of phytoplankton lysis to biogeochemical cycling because the type (Jiménez *et al*., 2009) and dose (Thamatrakoln *et al*., 2012) of the stressor may affect the proportion of dead or cyst-forming cells. In order for ballast sterilization to be effective, the proportion of

cells undergoing death must be maximized and the proportion forming cysts minimized.

In terms of biogeochemical cycling, determination of cell lysis versus cyst production is important because cells that lyse act as a source of nutrients, whereas cysts act as a sink. Moreover, cysts forming over deep water in the oceans are less likely to germinate and repopulate surface waters, thus acting as a more permanent nutrient sink. This will be especially important for modeling the role of coccolithophorids as acting as either a source or sink for carbon in our oceans. In addition, models on biogeochemical cycling must also consider the age of the bloom because stationary phase cultures have demonstrated elevated death when exposed to oxidative stress compared to exponentially growing cultures (Thamatrakoln *et al*., 2012). As a result, the age of the bloom may also be influential with regards to the proportion of cells capable of forming cysts and requires examination. Overall, the investigation of the causes and cellular mechanisms controlling life history transitions can no longer be considered in isolation, but rather must be considered in tandem if we are to more accurately assess biological responses in a dynamic world.

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APPENDIX

Viral and bacterial abundance (Hanson, 2010), and community zooplankton biomass (B. Angel, *unpublished data*) in Estabrook Park Pond (43°01’N 87°54’W, Milwaukee, WI, USA).

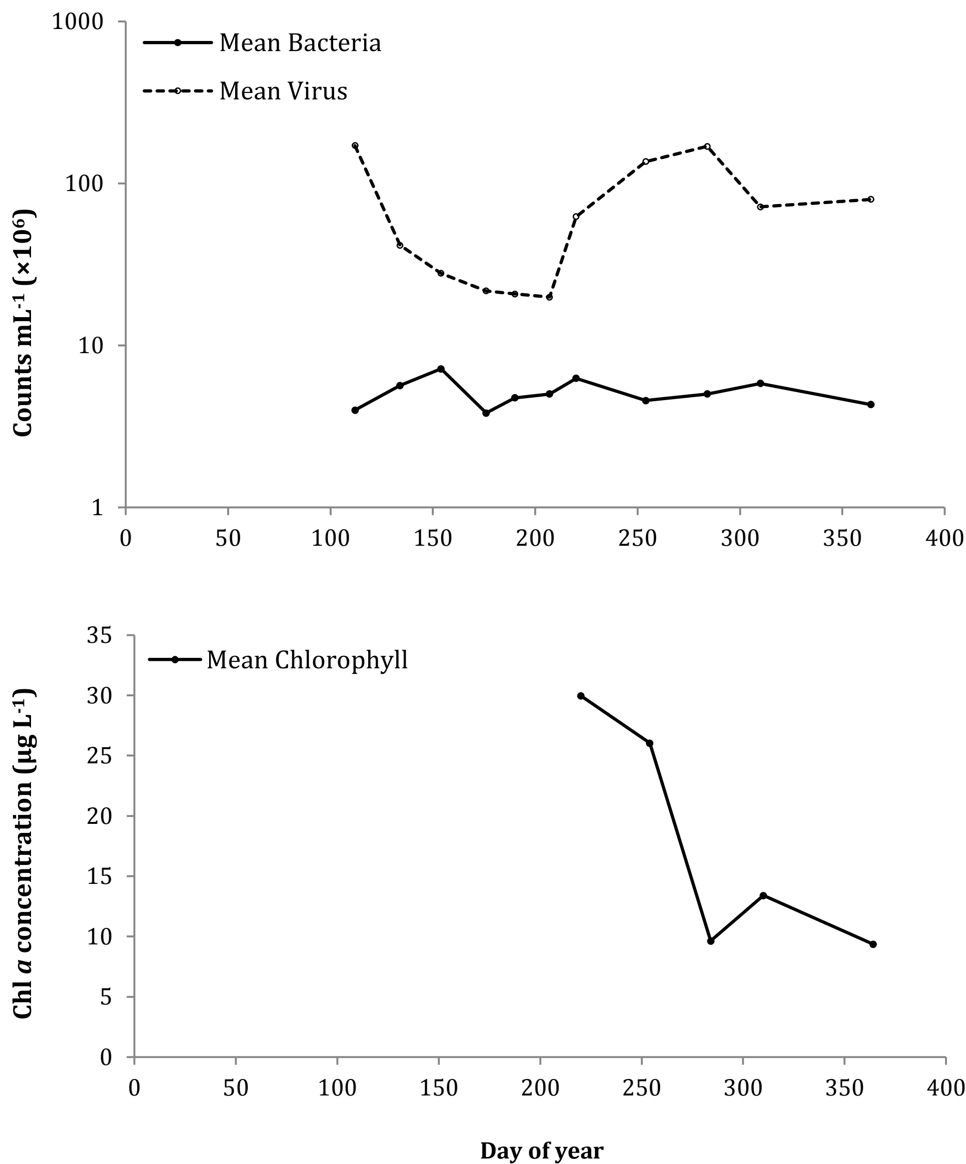

Figure 1, Appendix. Mean bacteria and virus abundance, and mean chl *a* concentration (µg L^{-1}) in Estabrook Park Pond from April 21st through December 29th, 2008. The top panel is mean bacteria and virus abundance. Mean bacteria abundance is the solid black line with solid markers; mean virus abundance is the dashed line with open markers. The bottom panel is mean chl *a* concentration. X-axis sample dates are presented as the 2008 day of year.

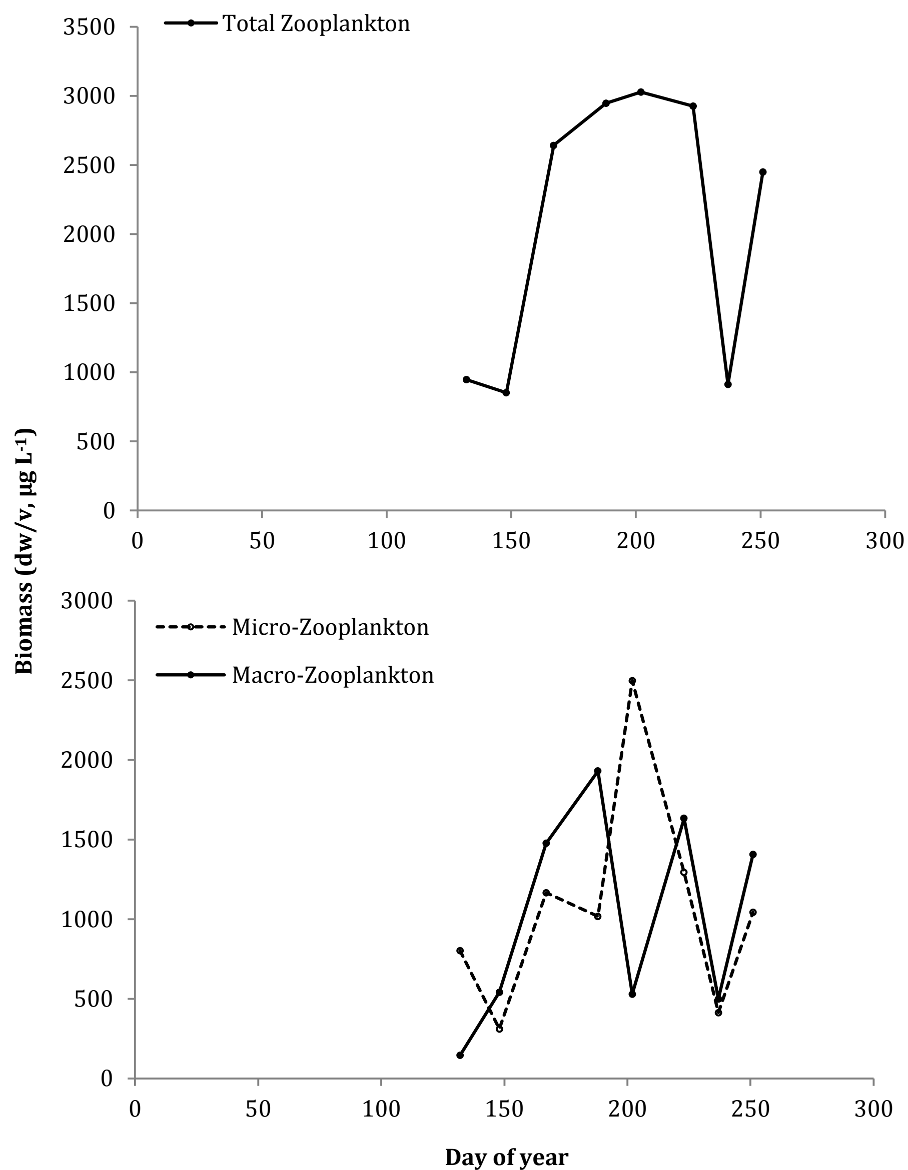

Figure 2, Appendix. Total zooplankton biomass (dw/v, µg L^{-1}), and macro- and micro- zooplankton biomass in Estabrook Park Pond from May 12th through September 8th, 2006. X-axis sample dates are presented as the 2006 day of year. Unpublished data are courtesy of B. Angel.

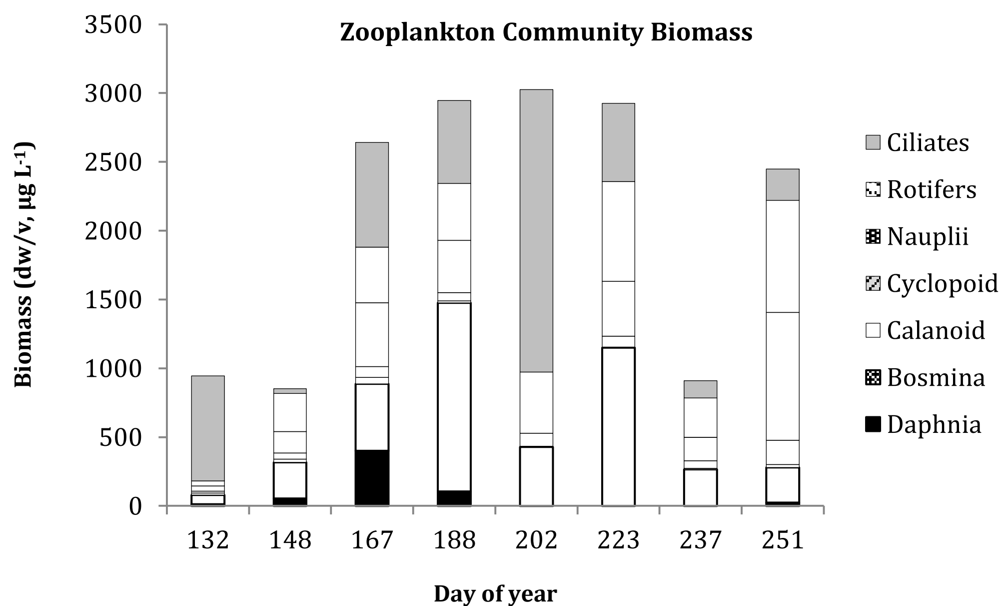

Figure 3, Appendix. Zooplankton community biomass (dw/v, µg L^{-1}) of Estabrook Park Pond from May 12th through September 8th, 2006. X-axis sample dates are presented as the 2006 day of year. Unpublished data are courtesy of B. Angel.