Cell death in individual freshwater phytoplankton species: relationships with population dynamics and environmental factors

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**ABSTRACT**
Understanding and predicting changes in phytoplankton populations requires knowledge of losses due not only to sedimentation and grazing, but also to intrinsic processes (here, collectively termed ‘cell death’). Cell death is poorly understood, especially in freshwater phytoplankton, but experiments in culture often suggest involvement of abiotic factors (e.g. temperature, light, nutrients). The occurrence of cell death was examined in a simple, natural environment: a small, well-mixed, temperate, urban pond during a period of phytoplankton growth, from mid-July to mid-November. Abundances of 18 phytoplankton taxa were measured weekly and fluorescence microscopy and staining was used to detect dead cells (using SYTOX which measures loss of membrane integrity) and cells undergoing cell death (using Annexin-V, which measures lipid inversions of membranes, an early signal of cell death). Dead and dying cells occurred in most phytoplankton taxa, but incidence and timing varied considerably, e.g. species like the chlorophyte *Ankistrodesmus spiralis* showed 20–30% of cells staining with SYTOX and Annexin in late autumn when the population was decreasing, while the dinoflagellate *Peridinium* sp. showed staining of up to 50% of cells with SYTOX throughout the period, and the cyanobacterium *Microcystis aeruginosa* occasionally showed staining of 100% of cells with SYTOX. Overall, there was some association between cell death staining and growth phase with 10–15% of the total community showing SYTOX and Annexin staining in late autumn, when most populations were declining. Cell death could not be correlated with thresholds or rapid changes in abiotic conditions (e.g. temperature, irradiance) or with indicators of nutrient limitation (e.g. N:P ratios). While abiotic factors have been clearly implicated in cell death within unialgal culture experiments, in natural freshwater ecosystems interactions between biotic factors, such as pathogens or allelopathy, may play greater roles in losses related to cell death and be distinct for different taxa.

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**Introduction**
Attempts to understand changes in phytoplankton populations have traditionally focused on the processes of growth, grazing and sedimentation; although models have sometimes included a term for ‘death’ which has been poorly defined and often deemed negligible compared with other losses (see Reynolds, 1984a). However, there is growing recognition that there can be internal cell processes involved in mortality (here termed ‘cell death’, see Franklin et al., 2006) and recent studies in marine and freshwaters have suggested that cell death can be a significant factor in population dynamics (e.g. Vardi et al., 1999; Agusti et al., 2006; Bidle & Falkowski, 2004).

Cell death has important ecological and biogeochemical implications. In fresh waters, cell death may at times be responsible for collapses of species that form blooms (Vardi et al., 2007), possibly through intercellular signalling mechanisms (Vardi et al., 2006), and may represent an important succession mechanism, with the potential for discrete environmental ‘thresholds’ to trigger death for different taxa (Sigee et al., 2007). Agusti et al. (2006) postulated more general differences in cell death in oligotrophic versus eutrophic lakes, and also among dominant versus rare phytoplankton species. In biogeochemical terms, cell death represents a rapid pathway from particulate to dissolved organic material (Berges & Falkowski, 1998) and a means of rapidly recycling nutrients, which may be especially important in nutrient-limited freshwater systems.

Causes and mechanisms of cell death in phytoplankton remain poorly understood. In some cases, cell death resembles programmed cell death (PCD) and can occur in response to unfavourable environmental conditions (see Berges & Choi, 2014). In particular, laboratory studies with unialgal cultures have demonstrated that nutrient starvation (e.g. Bidle & Bender, 2008), light limitation (e.g. Segovia et al., 2003), UV radiation and heat shock (e.g. Jiménez et al., 2009) and hyperosmotic stress (e.g. Ning et al., 2002) can elicit cell death. Other factors that potentially contribute to losses due to death in
field conditions include the presence of pathogens such as algaeidal bacteria (Kim et al., 1998), viruses (Brussaard, 2004) and chyrid fungi (Ibelings et al., 2004), as well as allelopathic interactions (Legrand et al., 2003).

Examining phytoplankton cell death in culture has been relatively straightforward (e.g. Berges & Falkowski, 1998; Bidle & Bender, 2008), but in the field, work has generally used community-level bulk assays based on strategies such as the release of intracellular enzymes (Brussaard et al., 1995) or staining or selective digestion of dead cells (Veldhuis et al., 2001; Agusti & Sanchez, 2002). Though careful application of these methods can provide some taxonomic resolution (e.g. with flow cytometry; Agusti et al., 2006), they are seldom applied at the species level. Alternatively, cell-specific staining has been used with some promise (Znachor et al., 2015). Resolving mortality at the species-level is important because it is clear that phytoplankton community dynamics are shaped by population dynamics (see Reynolds, 1984b). Since growth rates of species respond differently to different environmental factors such as light (e.g. Butterwick et al., 2005) and nutrients (e.g. Tilman, 1977) it seems likely that mortality will too. Thus, methods of examining species-specific mortality in the field are needed.

In order to develop an understanding of cell death beyond laboratory cultures, to examine the significance of phytoplankton cell death in natural ecosystems and determine how death processes vary among phytoplankton taxa in a community, studies need to focus on tractable environments where repeated measurement can be made and employ robust markers for diverse phytoplankton undergoing cell death. Small freshwater bodies provide good ecosystems in which to do this. Small lakes and ponds can be sampled frequently and comprehensively, yet support substantial phytoplankton biodiversity and have a global significance in terms of ecological and evolutionary processes that has been historically underappreciated (De Meester et al., 2005; Downing et al., 2006). In terms of selecting robust cell death markers there is, unfortunately, considerable confusion even in classification of cell death, making choices difficult (see Berges & Choi, 2014; Duprez et al., 2009; Galluzzi et al., 2009). To investigate dead and dying phytoplankton cells we employed robust fluorescence staining techniques which detect: (a) intact phytoplankton cells with membranes that are unable to exclude dyes (and are thus functionally ‘dead’; Veldhuis et al., 2001), and (b) phytoplankton cells that have membranes which show characteristics of one form of cell death, apoptosis (Berges & Choi, 2014). SYTOX-Green* is a fluorescent nucleic acid probe that stains dead cells (Peperzak & Brussaard, 2011). FITC-Annexin-V is a fluorescent probe that binds to phosphatidylserine (PS), which is exposed in the outer membrane of cells only during early apoptosis (e.g. Bidle & Bender, 2008). These two staining techniques have not, to our knowledge, been applied together in natural phytoplankton communities.

The present study aimed to explore the incidence and timing of phytoplankton cell death in a eutrophic urban pond by targeting a range of common taxa. SYTOX-Green* and FITC-Annexin-V were used with epifluorescence microscopy to count dead and dying cells in individual taxa over a spring to autumn time series. Abiotic environmental parameters that could potentially be associated with cell death were also examined. We aimed to test the feasibility of applying cell staining and microscopy to individual phytoplankton taxa in a natural community and to examine the following hypotheses:

1. Proportions of dead cells (SYTOX-stained cells) or cells with apoptotic characteristics (Annexin-stained cells) differ among different phytoplankton taxa during a summer–autumn period.
2. Occurrence of dead (SYTOX-stained) cells or cells with apoptotic characteristics (Annexin-stained) in a phytoplankton species are related to population growth, i.e. whether cell abundance is increasing, remaining stable or decreasing.
3. Occurrence of dead (SYTOX-stained) cells or cells with apoptotic characteristics (Annexin-stained) in a phytoplankton species are related to environmental conditions, specifically thresholds or rapid changes in irradiance, temperature or changes in nutrient limitation (indicated by changes in carbon, nitrogen or phosphorus ratios of cells).

Materials and methods

Study site and sampling

Estabrook Pond, Wisconsin, USA (43°01′N, 87°54′W) is a small (0.45 ha) urban pond that has been the site of limnological studies and classroom research at UW-Milwaukee for decades (see Table 1). The pond is shallow (mean depth 1.36 m), eutrophic with a tendency towards nitrogen limitation (Table 1) and is mixed frequently so that no stratification develops; this allows a relatively small-scale sampling programme to represent the phytoplankton dynamics in the pond. Sampling was conducted weekly July–November, 2010 (days of year 195–320). A YSI 600XL Sonde (YSI Inc., Yellow Springs, Colorado, USA) was used to record surface water temperature, specific conductance, dissolved oxygen and pH each week. Irradiance data for Milwaukee Airport (Mitchell Field, ~20 km distant) were obtained from
the National Weather Service Forecast Office in Milwaukee, Wisconsin (www.weather.gov/mkx/). Hourly integrated data were used to calculate total irradiance (µmol photons m\(^{-2}\)) for the week before sampling and for the 24 h prior to sampling. Subsurface water samples were collected from the shore with a 1-litre Swing Sampler (Nasco, Fort Atkinson, Wisconsin, USA) and water was combined from eight locations around the perimeter of the pond. Samples were placed in darkness in a cooler and transported to the laboratory where they were sub-sampled for cell staining and nutrient analyses within 20 min. Subsamples were preserved with acid Lugol’s iodine for enumeration of phytoplankton taxa.

**Phytoplankton analyses**

Immediately on return to the laboratory, a portion of the combined water sample was screened through 153 µm nylon mesh to remove large grazers before a known volume was concentrated with 10 µm nylon mesh using the methods described by Reavie et al. (2010). Samples were gently re-suspended with 0.2 µm-filtered pond water and the concentrated phytoplankton sample used for cell staining.

**SYTOX-Green® staining**

Ten µl of a 50 µM working stock of SYTOX-Green® (S7020 Invitrogen; Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was added to 1 ml of the concentrated phytoplankton sample (0.5 µM final SYTOX-Green® concentration) and incubated in darkness for 15 min. Stained samples were gently filtered onto 25 mm 2.0 µm membrane filters (Nuclepore® Corporation, Pleasanton, California, USA) and gently washed with filtered pond water containing 2–3 drops of concentrated glutaraldehyde (~1% w/v). This light preservation maintained the integrity of cells during counting but did not change proportions of stained cells for up to 48 h after slide preparation, thus extending the period in which slides could be counted, facilitating proper identification and enumeration. In addition, it was verified that heat-killing of samples (80°C for 5 min; Franklin et al., 2009) resulted in SYTOX staining of all individuals of species that could be examined using this technique. The membrane filter was transferred to a microscope slide dotted with 1–2 drops of Resolve™ low-fluorescence microscope immersion oil (Stephens Scientific, Denville, New Jersey, USA) in order to ‘clear’ the filter for microscopy. The prepared slide was stored in a dark container at 4°C until stained cells could be counted (within 2 d of preparation). Immediately prior to epifluorescence microscopy, 30 µl of a freshly made anti-fade solution (50% glycerol, 50% 100 mM phosphate buffer pH 7.5, and 0.01% (w/v) p-phenylenediamine; see Noble & Fuhrman, 1998) was added to the membrane to prolong fluorescence. Slides were viewed with an Olympus BX41TF epifluorescence microscope with FITC filter set (Chroma 41001: HQ480/40× band-pass excitation, and Q505 long-pass dichroic mirror and HQ535/50m band-pass emissions). All taxa that could be identified were counted in 3–4 transects at 400× magnification and the proportion of SYTOX-Green® stained cells recorded.

**FITC Annexin-V staining**

Subsamples of phytoplankton concentrate (2 ml) were centrifuged at 7500 g for 5 min and the pellet was re-suspended in 100 µl of binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 containing 140 mM NaCl and 2.5 mM CaCl\(_2\)). FITC-Annexin V reagent (A13199 Invitrogen; Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was added (5 µl, following the manufacturer’s protocol for microscopy), and the sample was incubated in darkness for 15 min. The stained sample was re-centrifuged, the supernatant discarded and the pellet re-suspended in 1 ml of binding buffer. This was filtered onto 25 mm, 2.0 µm membrane filter, washed, preserved, stored, visualized and counted as described above for SYTOX-Green® staining. Because the proportion of cells staining with FITC-Annexin-V also includes those cells with compromised membranes, in which the dye enters the cell and stains PS residues on the inside of the membrane, a correction must be performed (Vermesa et al., 1995). Typically, for mammalian cells, a red-fluorescing mortal stain (such as propidium iodide) would be used with green-fluorescing Annexin-V, allowing dual staining, but because phytoplankton contain red fluorescing chlorophylls, we were unable to find a stain suitable for dual staining. Thus, to correct for dead cells stained with Annexin, the proportion of SYTOX stained cells counted in parallel samples was subtracted from the proportion of Annexin stained cells to leave only those cells with PS residues on their outer membrane.
Lugol’s abundance counts

Total phytoplankton counts were performed on Lugol’s-preserved samples, settled in Utermöhl chambers for 24 h, with an Olympus IX-70 inverted microscope using 100× or 400× magnification. Phytoplankton were identified using regional keys (Prescott, 1962; Dillard, 2007) and counted (>100 cells or colonies per taxon) using random fields, transects, or full chambers, depending on the abundance of the taxon (Venrick, 1978).

For cell counts of preserved or stained samples, standard deviations were inferred from the actual number of cells counted, assuming a Poisson distribution (see Venrick, 1978). The validity of this approach was confirmed in preliminary experiments in which samples from the pond were analysed separately instead of being pooled and the standard deviations were calculated from multiple independent samples.

In order to identify periods of increase or decrease in cell abundance, natural logarithms of cell counts were plotted against day of the year and each plot was inspected for linear regions. Regions showing apparent linearity were analysed using linear regression and those with slopes significantly greater than zero (increase) or less than zero (decreases) \((p < 0.05)\) were noted.

Nutrient and particulate analyses

Analytical triplicates of total phosphorus (TP) and total dissolved phosphorus (TDP) were measured using persulfate oxidation (Menzel & Corwin, 1965) using whole water or GF/F filtrate, respectively. P concentrations were measured spectrophotometrically, using DL-glycerol-phosphate as a standard to correct for TDP digestion efficiency (typically > 85%). Particulate phosphorus (PP) was determined by subtraction of TDP from TP. To determine particulate elemental C and N, samples were filtered onto pre-ashed (460°C for 8 h) GF/F glass microfibre filters (13 mm diameter; Whatman Inc., Clifton, New Jersey, USA), dried and analysed with a Thermo Electron Flash EA 1112 series CNS analyser (Thermo Fisher Scientific, Waltham, Massachusetts), using acetalnilide as a standard. C and N content and particulate P were used to calculate molar C:N and N:P ratios.

Results

Environmental conditions: irradiance, temperature, oxygen and nutrients

Temperature and irradiance (measured on a daily or weekly basis), significantly decreased over the July–November sampling period \((p < 0.01, \text{linear regression analysis})\) (Fig. 1). pH did not vary consistently with time and was always between 7 and 8.3 \((p > 0.5, \text{data not shown})\). Dissolved oxygen concentration increased from day 195 (13 July) until day 292 (18 October), after which point oxygen concentration decreased \((\text{Fig. 1a, } p < 0.05, \text{linear regression analyses})\). Total phosphorus (TP) decreased significantly over the monitoring period \((\text{Fig. 2a, } p < 0.01, \text{linear regression analysis})\), while TDP was generally low at about 0.7 µM except for two peaks above 1 µM on day 195 (13 July) and day 299 (25 October) \((\text{Fig. 2a})\). TP data collected in 1999 agreed quite closely with the present study \((\text{Fig. 2a})\). C:N elemental ratios of particulate matter in the pond were always slightly above 106:16 (i.e. 6.6, the Redfield Ratio) and the N:P ratio was consistently close to or just below 16:1, but neither varied systematically over time \((\text{Fig. 2b, } p > 0.5 \text{ in both cases, linear regression analyses})\).

Phytoplankton population dynamics

Nineteen distinct phytoplankton taxa or groups of taxa monitored showed different population dynamics over the July–November sampling period (Fig. 3). Based on regression analyses of logarithmically transformed data (see Supplementary fig. 1), some taxa showed evidence of an increase, followed by a decline, in population over the period, e.g. Ankistrodesmus spiralis, Pediastrum simplex, Tetraedron minimum and Synedra sp. (Fig. 3a, f, j, p). Such a pattern was also seen in the abundance of the total
community (assessed from counts of preserved samples) (Fig. 3t), and, as well, in those species that could be successfully examined with the stains (SYTOX and Annexin) (Fig. 3t). Cells that could be examined using stains represented, on average, 54.2% (± 13.0) of total community abundance, but because they included, on average, larger cells, they probably represent a somewhat larger proportion of the total biomass. Another common pattern in abundance included a monotonic decrease in numbers, e.g. *Francea droscheri*, *Kirchneriella obesa*, *Peridinium* spp., *Euglena* spp. and Cryptomonads (Fig. 3b, c, n, q, r). Some species showed decreases in numbers only in the latter part of the time series, e.g. *Pediasstrum duplex*, *Scenedesmus quadricauda* and *Microcystis aeruginosa* (Fig. 3e, g, s), while other showed increases in abundance, e.g. *Staurastrum polymorphum*, *Mallomonas tonsurata* and *Aulacoseira* sp. (Fig. 3i, m, o). Still others showed no significant trend in abundance over the period, including *Pediasstrum boryanum*, *Staurastrum chae-toceras*, *Dinobryon sertularia* and *Mallomonas caudata* (Fig. 3d, h, k, l). By mid-November (day 320), counts in most taxa had declined to the minimum numbers observed.

**Phytoplankton cell death**

For most taxa, the percentage of cells which were positively stained with either SYTOX or Annexin was typically less than 60% (Fig. 3), however on one sampling date for *P. boryanum* and two dates for *M. aeruginosa*, 100% of cells counted were stained with SYTOX (Fig. 3d, s), indicating all cells were dead. All phytoplankton species enumerated showed positive staining with SYTOX at some point during the sampling period, but three taxa, *Aulacoseira* sp., *M. aeruginosa* and *P. boryanum*, showed no evidence of Annexin staining (i.e. indication of cells undergoing an apoptotic process), despite high proportions of dead cells stained with SYTOX (Fig. 3d, s). In other taxa, Annexin staining often coincided with, or was followed by, SYTOX staining. In only one case (*S. quadricauda*) did a taxon show a higher proportion of cells staining with Annexin than with SYTOX (Fig. 3g). SYTOX staining was found just before day 299 (October 26) in several taxa, e.g. *A. spiralis*, *K. obesa*, *S. polymorpha*, *T. minimum*, *D. terticularia* and *M. caudata* (Fig. 3a, c, i, j–l), coincident with an increase in TDP (Fig. 2a).

There were several additional taxa in which we observed SYTOX or Annexin staining of more than 5% of cells at one or more time points: the chlorophytes *Tetrastrum* sp., *Closteriopsis* sp., *Botryococcus* sp. and *Dictyosphaerium* sp.; the euglenoids *Phacus* spp. and *Trachelomonas* sp.; the haptophyte *Hymenomonas* sp.; the diatom *Fragilaria* sp.; and the cyanobacteria *Anabaena* sp. and *Merismopedia* sp. However, since each of these taxa occurred in less than one third of the total sampling points they were not suitable for inclusion in the analyses and we mention them only for completeness.

There were very few generalizations that could be made in terms of when dead and dying cells appeared with respect to increases and decreases in populations, and none of these was particularly consistent. For example, while some taxa tended to show staining when populations were declining and at low abundance, e.g. *A. spiralis*, *K. obesa*, *P. simplex*, *T. minimum* (Fig. 3a, c), others showed most staining when their populations were increasing and at relatively high numbers, e.g. *S. polymorpha*, *M. tonsurata* and *Aulacoseira* sp. (Fig. 3b, i, m, o). Examining the dead cells and those showing apoptotic characteristics at the community level, we note one relatively consistent trend: a tendency for dead and dying cells to occur in the autumn after day 270 (September 27) (Fig. 3t); such a pattern was seen even in taxa that didn’t show clear increases or decreases in the period.

**Fig. 2.** Seasonal trends in nutrient availability and particulate matter in water samples collected from Estabrook Pond during 2010. a) Phosphorus as total P and total dissolved P from 2010, also plotted with longer time series of TP from Estabrook Pond in 1999 (Murru and Sandgren, unpublished). b) Elemental ratios of C:N and N:P in particulate fraction of water samples collected. Redfield ratios typical for nutrient-sufficient cells are shown as horizontal lines.
Fig. 3. Seasonal patterns of cell abundance and cell death for 19 phytoplankton taxa measured in samples collected from Estabrook Pond for days of the year 195–320 (July–November) in 2010. Plots are labelled with taxon names within groups Green Algae (a–j), Chrysophytes (k), Synurophytes (l, m), Dinophyceae (Dinoflagellates) (n), Bacillariophyta (Diatoms) (o, p), Euglenoids (q), Cryptomonads (r) and Cyanobacteria (s), and in addition a plot of the sum of all species counted using Lugol’s preserved samples, and all species counted with SYTOX and Annex stains (t). Symbols represent total cell abundance per taxon based on settled counts. Bars show the percentage of cells staining with SYTOX-Green® (black bars) and FITC Annexin-V (grey bars), in independently stained samples collected on filters. Note differences in cell abundance scales among plots, and that percentage staining plots are given on one of two scales. For all data, error bars represent standard deviations, inferred from the actual number of cells counted to derive each abundance or percentage, assuming a Poisson distribution (see Materials and methods).
examined, e.g. *P. boryanum*, *D. sertularia*, *M. caudata* (Fig. 3d, k, l), suggesting the possibility of a link between an environmental parameter and cell death. However, there are no clear changes in environmental parameters that occur near or after day 270 (Figs 1, 2).

**Discussion**

Differences in patterns of abundance were observed in the 19 taxa monitored during this mid-summer to late autumn period in Estabrook Pond. This gave us the opportunity to associate the appearance of dead cells with both environmental parameters and phases of population growth. The data set provides a representative group of taxa in which to examine cell death in this ecosystem, though it is important to recognize that not all taxa present were enumerated because we wished to focus on species that we could readily and unambiguously identify close to species level when stained. Importantly, although populations of individual taxa were dynamic, with many demonstrating a pattern of increase and decline (similar to the total community) over the time course, most of the measured environmental parameters either varied little, or exhibited monotonic declines. Thus, there was little evidence that temperature, irradiance, pH or nutrients (as indicated from bulk composition of the phytoplankton community, C:N and N:P), were driving the dynamics of populations observed.

**Patterns of cell death**

Using SYTOX and Annexin staining, we found evidence for dead and dying cells in diverse phytoplankton taxa with varying patterns in freshwater field populations over a growth season. The present study represents, to our knowledge, the first application of both these stains in natural freshwater phytoplankton populations that also incorporates species-level resolution. The differences in proportions of stained cells, especially in relation to taxon abundance, supports our first hypothesis that phytoplankton taxa show variations in dead cells or cells with apoptotic characteristics (dying cells) over the sampling period. When positive SYTOX staining was observed, it was common to have 5–50% or more dead cells within that population. For example, cells of *T. minimum* showed 6–67% positive staining and 100% of *P. boryanum* stained positive with SYTOX on day 313 (8 November). This is comparable to ranges found from other field studies. For example, in another temperate eutrophic lake, Rosshtherne Mere, 17–50% of *Microcystis flos-aquae* (Cyanobacteria) were dead (Sigee et al., 2007). Using a cell digestion technique, Agustí et al. (2006) found 2–80% of phytoplankton cells dead in a set of Florida lakes, also noting a positive relationship between the proportion of live cells and the total chlorophyll *a* concentration, and that dominant taxa tended to show lower numbers of dead cells. In some cases our data also reflect this trend of more dominant taxa having fewer dead cells: incidence of 100% dead cells of *M. aeruginosa* and *P. boryanum* occurred when these taxa were at relatively low densities, whereas in very abundant taxa, e.g. *A. spiralis*, *F. droescheri* and *Euglena*, the maximum percentage of dead cells found was lower (20–40%). Rychtecký et al. (2014) also reported wide spatial and temporal variability in cell viability (using a derived index of membrane integrity based on SYTOX staining that does not allow direct comparison with the method used in the present study, but is presumably correlated with the percentage of living cells) among taxa in a eutrophic reservoir in the Czech Republic, but notably, little difference in that of the diatom *Asterionella formosa* in an April to October time series.

Problems with inconsistent staining with stains like SYTOX have led to concerns about applying such methods in the field (Tang & Dobbs, 2007; Gorokhova et al., 2012; Zetsche & Meysman, 2012; Rychtecký et al., 2014; see also MacIntyre & Cullen, 2016). In this study, we attempted to address these issues by counting only taxa we were confident we could resolve to species (or in some cases genus) under the microscope when stained. Also, to verify that staining of dead cells was complete, we used heat-killed field samples as positive controls to eliminate ‘false negatives’, i.e. dead cells that do not stain and so appear live (cf. Franklin et al., 2009). Although we have no true control for ‘false positives’ (i.e. living cells that stain as dead), the fact that all of our taxa show at least some time points without staining gives us confidence that we were able to distinguish meaningful differences with the stains. Another technique for assessing cell viability in field samples involves using flow cytometry and enzymatic digestion of dead cells to detect living cells by difference (Agustí & Sánchez, 2002). Preliminary results on Estabrook Pond samples show broad agreement between this method and SYTOX staining for the taxa we examined with microscopy that we can also identify using flow cytometry (Simmons & Berges, unpublished).

Annexin staining was seen in fewer samples and at lower percentages (2–30%) than SYTOX staining (Fig. 3). This makes some sense since it is possible for dead cells to persist for some time, while cells will be in the process of apoptotic cell death for only a short period (perhaps only 2–3 h, see Kanduc et al., 2002). The observation that there were taxa in which there was positive SYTOX staining but no Annexin staining (*P. boryanum*), and others where Annexin staining was more prevalent (*S. quadricauda*, *A. spiralis*) might mean that some species spend longer than others in the process of apoptosis, but it might also suggest that...
not all species stain equally well with Annexin. For example, the cyanobacterium *M. aeruginosa* showed no Annexin staining but readily stained with SYTOX. Phosphatidylserine residues are common to eukaryotic and prokaryotic membranes (Vance & Steenbergen, 2005), so there is no inherent membrane structure difference to account for differences in staining, and Annexin staining in cultures of the cyanobacterium *Aphanizomenon* has recently been reported (Dashkova et al., 2017), but the colonial habit of species like *M. aeruginosa* and the presence of extracellular polysaccharide (EPS) which holds colonies together (Li et al., 2013) may be complicating factors in staining efficiency. Unfortunately, it is unclear what might constitute a good positive control for Annexin staining. We have attempted to use classical ‘inducers’ of apoptosis in metazoans such as Actinomycin D, but with largely uninterpretable results (Berges, unpublished). Finally, at the time this project began, few alternative mortal stains existed with fluorescence in ranges suitable to allow dual-staining of phytoplankton to correct Annexin results for dead cell staining. However, a UV-exited dye, SYTOX-Blue (S34857 Thermo-Fisher Scientific, Waltham, Massachusetts, USA) is now available and could be employed in future work, simplifying measurements considerably.

**Cell death versus population dynamics and environmental conditions**

Our second hypothesis posited that dead or dying phytoplankton cells could be related to population dynamics, i.e. whether cell abundance is increasing, stable or decreasing. There was no general relationship for all taxa, yet in perhaps half of the cases dead and apoptotic cells occurred when populations were declining. Thus, the data offer limited support for this hypothesis. Znachor et al. (2015) used a SYTOX-based membrane integrity index to show that peaks of abundance due to growth in the diatom *Fragilaria crotonensis* coincided with low viability over a seasonal cycle in a temperate eutrophic reservoir. The Estabrook Pond dataset included only two diatoms (*Synedra* spp. and *Aulacoseira* sp.); *Synedra* spp. did not show this pattern, though there is some suggestion towards the end of the time series that increases in *Aulacoseira* sp. abundance may have been accompanied by appearance of dead cells. In contrast, *Peridinium gatunense* populations in Lake Kinneret showed about 5% staining at the start of the bloom (rapid growth) period, rising to ~65% dead at the end of the bloom period (Vardi et al., 1999). In Estabrook Pond, the dinoflagellate *Peridinium* spp. showed evidence of cell death throughout the time series with rates of dead cell staining (up to 55%) that were comparable to those found by Vardi et al. (1999) in Lake Kinneret (Fig. 3n).

Our third hypothesis proposed relationships between dead or apoptotic cells, species and environmental conditions, specifically, thresholds or rapid changes in irradiance and temperature or changes in nutrient limitation (indicated by changes in elemental ratios of cells). The decline in cell abundance in most taxa, and increase in dead cells in several taxa, during the late autumn period support the idea of some common environmental signal, possibly reduced light and/or temperature, limiting growth. However, the only clear trends during this period (irradiance and temperature) show gradual, not dramatic changes and the most staining occurred after day 278 (4 October) when average daily total irradiance was about 75 µmol photon m⁻², and temperature averaged 15°C, which would hardly be considered stressful conditions for temperate phytoplankton (see Butterwick et al., 2005). There is evidence that cell death can be associated with seasonal encystment in some species including dinoflagellates (see Vardi et al., 1999), but cysts were not obvious in samples at the time cell death was observed, making environmental signals less likely (although cysts may have sunk to the benthos and so have not been accurately sampled, and in addition some ‘pellicle cysts’ may not have been detected, see Bravo & Figueroa, 2014). Based on a lack of seasonal trends in C:N and N:P, it also seems unlikely that changing macronutrient availability in the pond provided a cue to increasing mortality after early October. In a temperate eutrophic reservoir system in the Czech Republic, loss of cell viability was correlated with nutrient effects (i.e. Si depletion) for the diatom *Fragilaria crotonensis* and light effects for the cyanobacterium *Aphanizomenon* (Rych捷k et al., 2014; Znachor et al., 2015). Dissolved silicate may become limiting in Estabrook Pond for some Si-demanding species, but it seems unlikely to be a seasonally limiting factor for most of the taxa measured (see Table 1). Overall, our last hypothesis linking cell death to environmental conditions cannot be supported but more complex interactions of abiotic factors may be influencing end-of-season mortality.

**Other factors in cell death**

Other factors, particularly biotic ones, may play roles in cell death in phytoplankton populations in Estabrook Pond. Viruses have received considerable recent attention in aquatic ecosystems and are linked not only with direct mortality of phytoplankton, but also with activation of internal cell death pathways (e.g. Bidle et al., 2007; Vardi et al., 2009; Evans & Brussaard, 2012). Concurrent work on viruses in Estabrook Pond does not show clear relationships between phytoplankton biomass (as chlorophyll a) and viral abundances, but it is probable that most viruses in Estabrook Pond
have bacterial rather than algal hosts (Hanson et al., 2017). Chytrid fungal infection of both healthy and senescent phytoplankton may occur, with zoospore attachment inducing a hypersensitive response in host cells (Belings et al., 2004). Although relatively few field data exist, infection by chytrids has been attributed to phytoplankton bloom collapse in Lake Biwa, Japan (Kagami et al., 2006, 2014) and cell death of Anabaena colonies in a eutrophic lake (Sige et al., 2007). In Estabrook Pond, chytrid infections of cells of some chlorophyte taxa, e.g. Staurastrum spp., were occasionally noted, but not quantified. Macro-grazers in the pond (dominated in recent years by small copepods; Simmons, unpublished) can certainly affect phytoplankton populations, though mechanisms by which grazing could generate ‘dead’ but still intact cells such as we counted in the present study are unknown. Allelopathic interactions among phytoplankton species may also explain death events in the field. A variety of chemical compounds, including aldehydes, have been associated with allelopathy in marine and freshwater phytoplankton (e.g. Legrand et al., 2003; Ribalet et al., 2007). This study presents many potential correlations among species that could be explored, but the statistical power of these is very low with a single year of data. Determining the effect of allelopathic interactions in the field is difficult (Maestrini & Bonin, 1981) and will thus rely on experimental approaches: for example, determining if filtrates from field isolates inhibit growth and/or instigate cell death in other taxa (Suikkanen et al., 2004).

This study has demonstrated that cell death occurs in a wide array of phytoplankton taxa in a freshwater ecosystem, and may occur when cell abundances are high or low. This suggests that different factors or combinations of abiotic and biotic factors can be responsible for cell death, possibly with distinct interacting factors for different taxa. Improving the power to determine cell mortality patterns from a time-series similar to that of the present study will require more intensive temporal sampling of a larger number of individual taxa. It is unlikely that this can be accomplished solely with microscopy, so flow cytometric methods for cell death detection (e.g. Bidle & Bender, 2008) and taxon identification (see Cellamare et al., 2010; Read et al., 2014) will be necessary.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Supplementary information**

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at https://doi.org/10.1080/09670262.2018.1563216

**Supplementary fig. 1** Seasonal patterns of cell abundance and cell death for 19 phytoplankton taxa measured in samples collected from Estabrook Pond for days of the year 195–320 (July–November) in 2010. Symbols represent total cell abundance per taxon based on settled counts, presented on logarithmic scales. Red lines indicate regions where linear regression analyses show statistically significant increases or decreases in abundance ($p < 0.05$). Other symbols are as for Fig. 3.

**Author contributions**

C. Kozik, C. D. Sandgren and J. A. Berges: original concept and planning of work; C. Kozik: majority of sampling and analyses; C. D. Sandgren, E. B. Young and J. A. Berges: supervision and assistance with fieldwork and analyses; C. Kozik, E. B. Young and J. A. Berges: drafting and editing of manuscript.

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