Algal growth response and survival in a range of light and temperature conditions: implications for non-steady-state conditions in waste stabilisation ponds

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Abstract

Growth and physiological experiments were carried out using *Scenedesmus subspicatus* and *Chlorella vulgaris* as representative species typically found in waste stabilisation ponds. These experiments were designed to test the ability of the organisms to survive and grow under a range of different temperatures and light intensities that might occur in mid to high latitude regions. Growth was assessed using optical density and photosynthetic rate for a combination of temperatures of 5, 10, 15 and 20 ^{o}C at light intensities of 7.8, 15.7, 31.3, 47, 62.7 and 78.3 μmol m^{-2} sec^{-1}. *C. vulgaris* had a higher rate of growth and photosynthetic activity than *S. subspicatus* at low temperatures but had reached its maximum growth rate at 15 ^{o}C. *S. subspicatus* showed a higher growth rate than *C. vulgaris* at higher temperatures, and did not achieve its maximum growth rate over the range of temperatures studied. For both species light was not limiting to growth above 47 μmol m^{-2} sec^{-1}. Survival of the two species under dark conditions was tested at 4 ^{o}C and -20 ^{o}C using direct plating and growth tests. *C. vulgaris* was able to survive at 4 ^{o}C for a much longer period than *S. subspicatus* and a portion of the population was able survive -20 ^{o}C. The different responses of the two species to dark and cold conditions are indicative of the range that may occur across a wider population, and show why in practice some species may appear earlier and compete more effectively in early spring but then lose advantage as the temperature and light intensity increases into the summer.

Keywords

Algae, growth response, light, temperature, waste stabilisation ponds

INTRODUCTION

In temperate and continental regions of both the northern and southern hemisphere, waste stabilisation ponds (WSPs) are subject to seasonal variations in light and temperature: in extreme cases this may include ice cover for extended periods in winter. At higher latitudes reduced light intensity and changes in day length may also have a significant effect on pond performance. The current study examined the effect of these parameters on two algal species commonly encountered in WSPs, with a view to determining their influence on winter survival and spring recovery. All experiments were carried out under controlled conditions in laboratory batch culture.

MATERIALS AND METHODS

Cultures of *Scenedesmus subspicatus* CCAP 276/20 and *Chlorella vulgaris fo. viridis* CCAP 276/20 were obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology,

Ambleside, UK. Both species were cultured and maintained on Mineral Jaworski's Medium (JM) in which the available nutrient sources were bicarbonate (HCO_3^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and trace elements (CCAP, 2006). For growth related experiments the algal cells were first inoculated into sterile JM broth and allowed to grow for 1-2 days at 20 ^{o}C with constant illumination at 78.3 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at 100 rpm in a refrigerated temperature-controlled illuminated orbital incubator (Sanyo Gallenkamp). The algae were then sub-cultured into fresh JM medium and grown again under the same conditions to provide the inoculum for experimental flasks: this ensured that cultures were in good physiological condition at the start of each experiment. For the algal growth and photosynthetic rate determinations both species were then grown at four different temperatures (5, 10, 15, and 20 ^{o}C) and six light intensities (7.8, 15.7, 31.3, 47, 62.7 and 78.3 μmol m^{-2} sec^{-1}) in non nutrient-limited conditions ensuring that a period of exponential growth occurred. Growth was measured by optical density at 678 nm wavelength using a scanning Spectrophotometer (Cecil 3000 series) and cell counts were made using an improved Neubauer haemocytometer.

To determine photosynthesis rates 250 ml Erlenmeyer flasks used in the growth experiments were sealed with a silicon bung into which was inserted a gas sampling valve. Algal oxygen production was followed by sampling the headspace of the flasks and oxygen concentration was measured by gas chromatography (GC) (Varian, CP-3800) using a chrompack capillary column at 50 ^{o}C, manual gas injection through a gas loop injection port, and peak detection by thermal detector. Argon was used as the carrier gas at a flow rate of 6 ml min^{-1}. Standardisation of the GC used a commercial gas mixture SCOTTY II (mix 218) containing CO_2, CO, H_2, CH_4, O_2, and N_2 (Supelco Ltd, UK). Oxygen concentrations in the liquid phase at the start and end of each growth period were measured using a YSI 550A dissolved oxygen meter (YSI, Yellow Springs, USA). Chlorophyll was measured by acetone extraction using standard methods (APHA, 1996).

Nitrate and phosphate concentrations were measured after high speed centrifugation (Eppendorf 5417C, Hamburg) using a DX-500 ion chromatography system (Dionex, Sunnyvale, USA) equipped with electrochemical detector ED40 operating in a conductivity mode, anions column AS9-SC and anion self-regenerating suppressor ASRS-1. Carbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ solution) was used as an eluent at a flow rate of 2 ml min^{-1}.

The results were analysed by comparing specific growth rates (μ) and rates of reaction, with kinetic parameters derived using Monod or Michaelis-Menten equations.

For algal survival experiments the cultures were grown axenically at room temperature (20 $\pm$ 2 ^{o}C) and ambient light intensity until reaching a stationary phase of growth. They were then hardened for 48 hours by reducing the temperature to 8 ^{o}C at a constant illumination of 78.3 μmol m^{-2}s^{-1}. The biomass was allowed to settle, the culture medium was decanted and the algae resuspended in fresh JM medium. This suspension was subdivided into fifty 150 ml aliquots for each species and transferred into polypropylene bottles, half maintained at +4°C and the other half at –20 ^{o}C, in all cases in the dark and static. Biomass concentrations were 231.4 and 302.1 mg DW l^{-1} for *C. vulgaris* and *S. subspicatus* respectively. At 7-day intervals a sample was retrieved by taking one bottle for each species from each of the conditions used. The contents were warmed and a sample taken for determination of viability by three different methods. The first used a plating technique in which dilutions of 1/10, 1/100, 1/1000, 1/10 000 were surface spread onto solidified (15% agar) JM medium followed by incubation with continuous illumination for 7 days: the number of colonies was counted with each one assumed to represent a viable cell. The second method measured the growth response of a 100 ml aliquot of warmed sample which was placed in a 250 ml Erlenmeyer flask and incubated at 25 ^{o}C and 78.3 μmol m^{-2} s^{-1} of illumination for 24 hours. Changes in OD_{678}

were read every 8 hours. The third method measured the photosynthetic ^{14}C uptake, again using a 100 ml sample in Erlenmeyer flasks, with measurements taken at 8-hour intervals (Bartosh, 2005).

RESULTS AND DISCUSSION
Influence of light and temperature on specific growth rates
The aim of the work was to establish the specific growth rate for *S. subspicatus* and *C. vulgaris* at a matrix of different temperatures and light intensities. The actual specific growth rate was measured as the slope of the growth response curve during the exponential phase, and the value obtained for each combination of temperature and time is plotted as a single point in Figures 1 and 2. The maximum growth rate for each species for each temperature was assumed to be at a point when no increase in the specific growth rate was observed with increasing light intensity.

Both species approached their maximum growth rates at a light intensity of 47.0 µmol m^{-2} s^{-1}, irrespective of the temperature at which they were grown (Figure 1), although the values of maximum growth rate increased with increasing temperature. When the growth response of the two species is compared it can be seen that from 5 ^{o}C to 15 ^{o}C the maximum growth rate for *C. vulgaris* was higher than for *S. subspicatus* at all light intensities. At 20 ^{o}C, however, *S. subspicatus* showed a higher maximum growth rate than *C. vulgaris*. In fact the growth rate for *C. vulgaris* showed relatively little change between 15 ^{o}C and 20 ^{o}C whereas for *S. subspicatus* the rate of growth continued to increase.

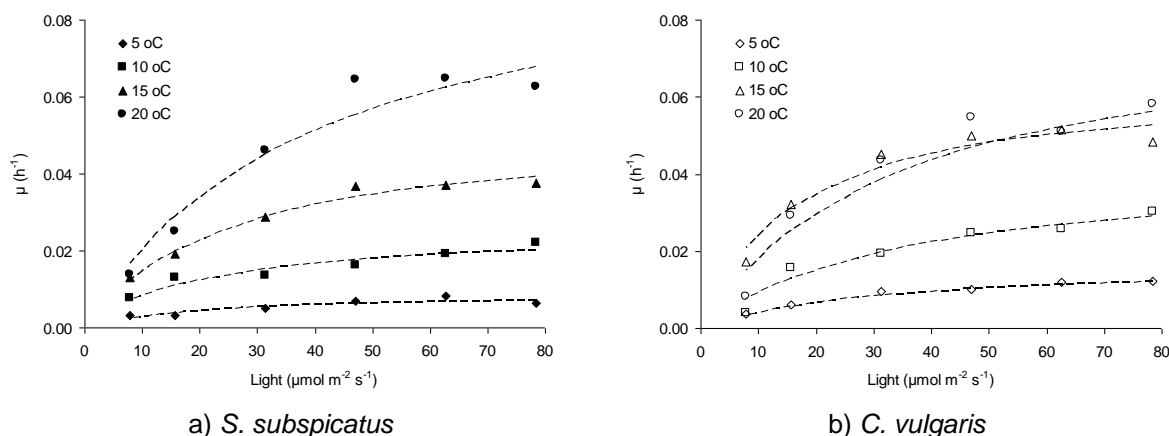
a) *S. subspicatus*   b) *C. vulgaris*

Figure 1 Growth rates of *S. subspicatus* and *C. vulgaris* as a function of light intensity at different temperatures. Dashed lines represent a Monod equation fitting to empirical results.

To provide a quantitative estimate of light limitation the experimentally obtained growth rates were fitted to the Monod equation. Values of μ_{max} for *S. subspicatus* appeared to be exponential in form (R^2=0.99), with empirical expression $\mu_{max} = 0.0046\ e^{0.1596\ T}$ where T is expressed in ^{o}C. The best fit of μ_{max} for *C. vulgaris* was linear (R^2=0.98), however, with empirical expression $\mu_{max} = 0.0042T$ where T is expressed in ^{o}C.

When the data are considered with respect to temperature (Figure 2) it can be seen that at light intensities up to 78.3 µmol m^{-2} s^{-1} growth rates for *C. vulgaris* were higher than those for *S. subspicatus* up to 15 ^{o}C. The growth rate for *S. subspicatus* increased with increasing light intensity up to 47.0 µmol m^{-2} s^{-1} at all temperatures, whereas for *C. vulgaris* the effect of increasing light intensity was smaller, with no appreciable effect at intensities greater than 31.3 µmol m^{-2} s^{-1}. At all light intensities tested for both species, growth rates showed a cut-off value at temperatures very close to 0 ^{o}C. This is of interest with respect to the start of the spring warm-up in cold climate

WSPs: in pilot-scale systems sub-ice water temperatures as low as -2.5 ^{o}C have been found and freezing at 0 ^{o}C has been prevented by the presence of salts, alginates and other impurities (Rspaev, 2005). Even when there is considerable light penetration, as is the case under clear ice, these two species would not grow until the water temperature rises above zero.
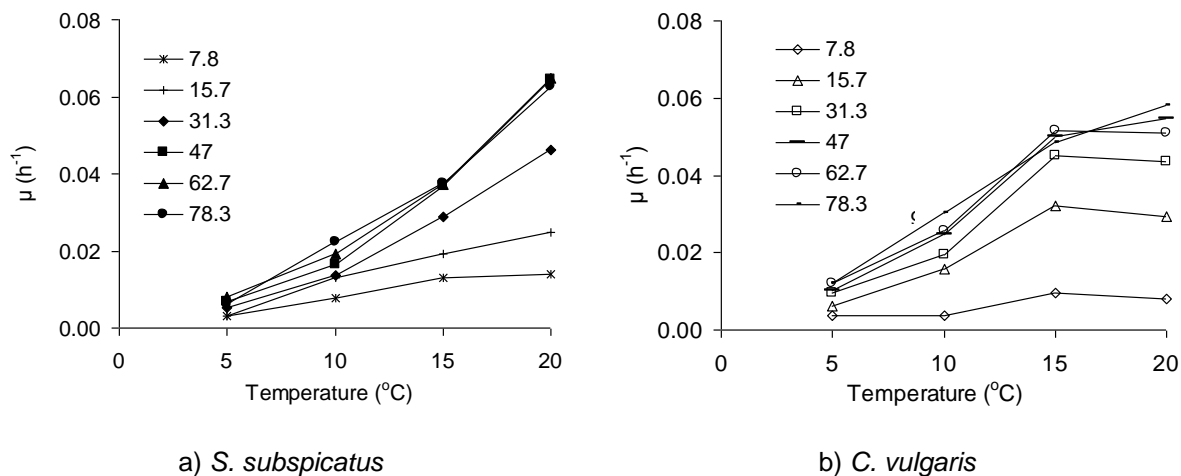

a) *S. subspicatus*   b) *C. vulgaris*

Figure 2 Growth rates of *S. subspicatus* and *C. vulgaris* as a function of temperature at different light intensities (μmol m^{-2} s^{-1}).

In summary, it appears that *C. vulgaris* is able to grow more vigorously than *S. subspicatus* at low temperatures and light intensities, but at temperatures above 15 ^{o}C the growth rate of *S. subspicatus* continues to rise while that of *C. vulgaris* shows no further increase. In practical terms this means that in the natural environment *C. vulgaris* may appear earlier and compete more effectively in early spring but may lose its advantage as the temperature and light intensity increases into the summer.

Photosynthesis. Rates of photosynthesis were determined for the same combinations of temperature and light intensity as for the growth rate determinations. The photosynthetic rate was determined during the exponential phase of growth, by measurement of oxygen in the headspace. Each value was plotted as a function of light intensity at each temperature: the results are shown in Figure 3.
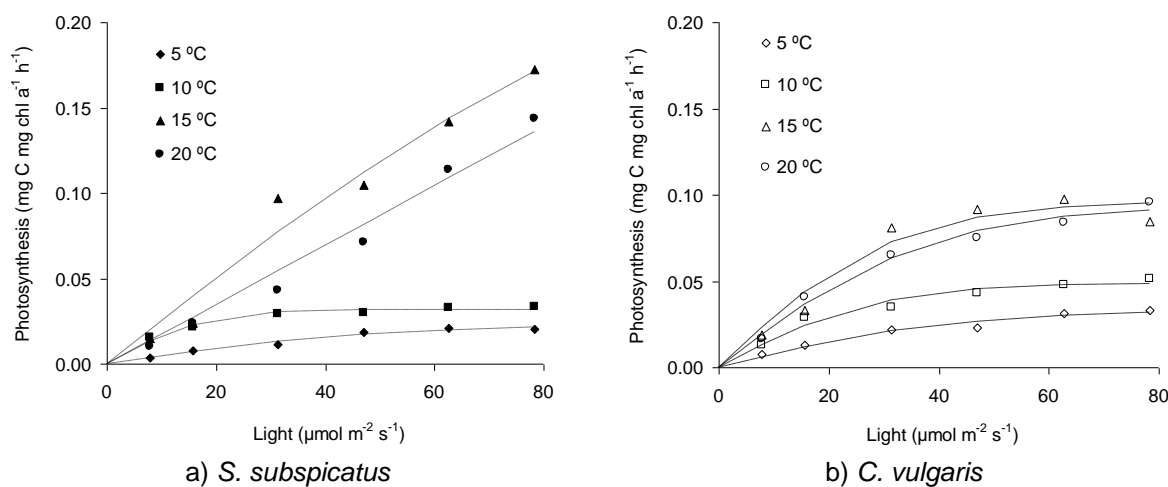

a) *S. subspicatus*   b) *C. vulgaris*

Figure 3 Photosynthetic rates of *S. subspicatus* and *C. vulgaris* as a function of light intensity at different temperatures. Dashed lines represent a Monod equation fitting to results

At 5 and 10 ^{o}C the photosynthetic rates of *C. vulgaris* cultures were clearly higher than *S. subspicatus* at all levels of irradiance. At 15 ^{o}C both species showed similar photosynthetic rates at lower light intensities, but *C. vulgaris* reached saturation at 47.0 μmol m^{-2} s^{-1} with an average rate of 0.091 mg C mg chl *a*$^{-1}$ h^{-1} whereas the rate for *S. subspicatus* cultures continued to rise and showed no saturation at the highest light intensity used. A photosynthetic rate of 0.173 mg C mg chl *a*$^{-1}$ h^{-1} was recorded for *S. subspicatus* at 78.3 μmol m^{-2} s^{-1}. A similar effect was seen at 20 ^{o}C except that the photosynthetic rates recorded for both species were lower than those measured at 15°C. These results are in agreement with Verity (1981), who showed that rate of photosynthesis varied markedly with growth temperature under light limiting as well as under light-saturated conditions.

Figure 4 shows photosynthetic rates plotted against temperature for different light intensities. While there is no well-defined cut-off value, it is clear that *C. vulgaris* is still active at 5 ^{o}C especially at the higher light intensities. Even though it is unlikely that significant growth would occur under ice with *C. vulgaris* it is possible that vegetative cells could still be photosynthetically active. Oxygenation under ice cover, as a result of photosynthesis, was observed by Mackenthun and McNabb (1961) in a WSP at Junction City, Wisconsin, with oxygen concentrations of up to 17 mg l^{-1}.
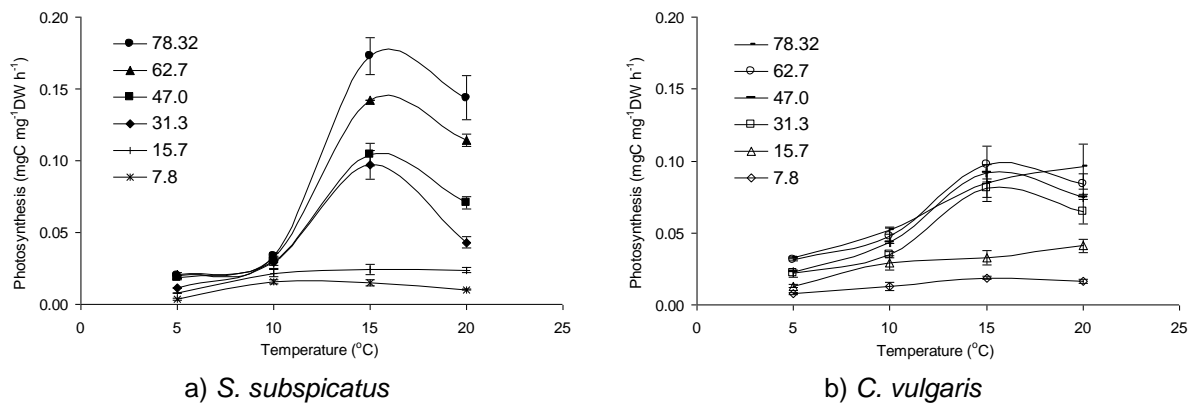

Figure 4 Photosynthetic rates of *S. subspicatus* and *C. vulgaris* as a function of temperature at different light intensities (μmol m^{-2} s^{-1}).

The results show the optimum temperature for photosynthesis in both species was around 15 ^{o}C. At temperatures below this a saturation value for photosynthetic activity was seen at 47.0 and 31.3 μmol m^{-2} s^{-1} for *C. vulgaris* and *S. subspicatus* respectively. At temperatures of 15 and 20 ^{o}C saturation was only apparent for *C. vulgaris*, and the results indicate that the optimum level of irradiance for *S. subspicatus* must be higher than 78.3 μmol m^{-2} s^{-1}. There was little difference between photosynthetic rates at 15 ^{o}C and 20 ^{o}C for *C. vulgaris*. These results again indicate that *C. vulgaris* is better adapted to growth at low temperature and light intensities, but may be out-competed by *S. subspicatus* in warmer brighter conditions.

Nutrient uptake. Rates of both nitrate and phosphate uptake were measured during exponential growth at the temperatures and light intensities tested. The uptake of nitrate by both species showed saturation kinetics at all four temperatures (Bartosh, 2005) and was also moderately influenced by light intensity. As the uptake of nitrate is an energy-dependent process (Brown and Johnson, 1977; Wood and Flynn, 1995), this result is expected since temperature and light affect both the energy production and utilisation efficiency of the cell. Analysis of variance showed that the rates of phosphate uptake for both species changed significantly in response to different temperatures or light intensities.

Survival of algae in darkness at low temperatures

Cultures of *S. subspicatus* and *C. vulgaris* were maintained stationary in complete darkness at +4 °C and -20 °C for up to 22 weeks. At 7-day intervals a sub-sample for each species at each temperature was tested for its viability by plate count, growth response (OD_{678}), and ^{14}C uptake. In addition to these physiological and growth tests any increase in dry weight or chlorophyll *a* content was recorded.

Plate counts. The results from plate counts are shown in Figure 5. The culture of *C. vulgaris* stored at +4 °C showed no major loss of viability, with an average survival rate over the experimental period of 74%. When stored at -20 °C there was an initial rapid loss of viability which then stabilised to give a survival rate of around 12% for the remainder of the experimental period. *S. subspicatus* cultures stored at +4 °C showed an average survival rate of 99% during the first 8 weeks of the experiment followed by a decrease that stabilised at about 12% in the final 4-5 weeks. When stored at -20 °C it was not possible to recovery any viable cells throughout the experimental period. In comparison, Dehning & Tizler (1989) measured the survival of *Scenedesmus acuminatus* for periods of 3 months in complete darkness at 7 °C and 22 °C. They found that 15-25% of cells survived, with better results at 7 °C. It was suggested that improved survival at 7 °C was due to lower metabolic activity. Cell viability as determined by cell numbers and dry weight decreased, and the volume of individual cells doubled. The techniques used differed slightly from that of the present study but the results confirm significant survival potential of this algal genera in the dark at low temperatures but above freezing.
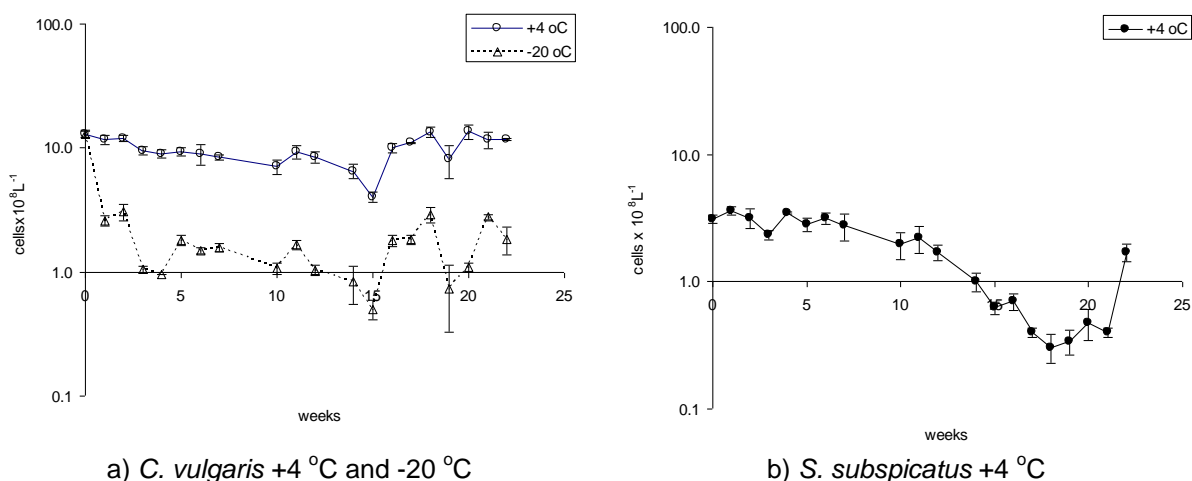

Figure 5 Viability of *C. vulgaris* (a) and *S. subspicatus* (b) cultures in number of cells x 10^8 l^{-1}.

Growth response curves. Figure 6a shows selected results for the revival of the *C. vulgaris* culture at +4 °C against those of the control (week 0). The rate of growth of the cultures maintained at low temperature is lower than in the control, reflecting the lower number of viable cells, but growth started without a lag and was at a constant rate over the 24 hour period. The effect at -20 °C was similar but showed a lag phase at the beginning of the revival. The results for *S. subspicatus* at +4 °C are shown in Figure 6b. These showed that the culture could be revived over the first 12 weeks but samples recovered after 14-22 weeks showed no increase in OD_{678} when incubated: in fact there was a decrease in turbidity which may indicate that cells were either lysing or their potential for light adsorption changed. Luder et al (2002) suggested that this effect may be due to degradation of the photosynthetic apparatus, when pigment from damaged cells is degraded in the medium. A similar but earlier decline in OD_{678} was seen in samples of *S. subspicatus* recovered from -20 °C, occurring from week 1 onwards.

Results for specific growth rate, dry weight and chlorophyll a content showed patterns of decline that were closely similar to those found from plate counting. On resuscitation from +4 ^{o}C, rates of photosynthetic carbon fixation fell to around 60% and 40% of initial values for *C. vulgaris* and *S. subspicatus* respectively. The rate for *C. vulgaris* fell to around 50% after exposure to -20 ^{o}C, while as expected *S. subspicatus* showed no photosynthetic carbon fixation.

The results from the three testing methods to assess survival potential suggest that *C. vulgaris* has a mechanism that allows it to survive for long periods in the cold and dark. It was also apparent that at least part of the population could survive freezing, although there was an initial high rate of kill. *S. subspicatus* on the other hand showed a very high percentage survival at low temperature and in the dark for short periods, but could not tolerate extended exposure without the cells, or a cellular component, breaking down. *S. subspicatus* showed no tolerance to freezing and the results from optical density measurements again indicated that there was cell or chloroplast damage.
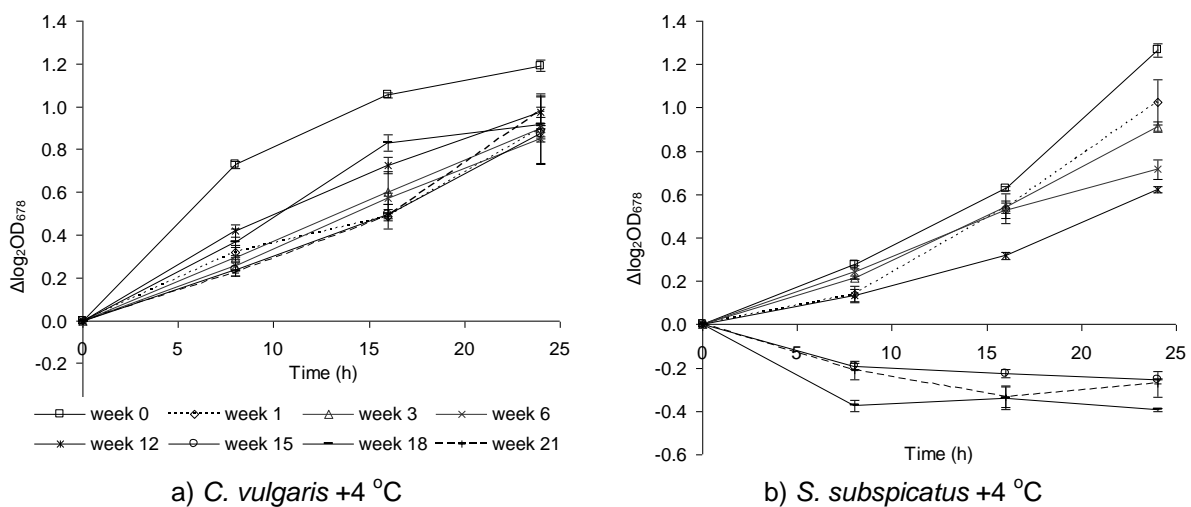

Figure 6 Growth response curves for group 1 *C. vulgaris* (a) and *S. subspicatus* (b) after selected periods.

It is well known that many members of the Chlorophyta are able to survive the effects of low temperature. For example, *Chlorella* sp. isolated from the Antarctic were used in repeated freezing and thawing experiments by Holm-Hansen (1963), suggesting that this species showed the highest resistance from eight tested. The increased resistance to damage by low temperatures displayed in species isolated from northern regions also confirms the influence of native environment on algal endurance. There is some evidence to suggest that the mechanisms for survival at low temperatures in unicellular algae may be similar to those in some higher plants. A study on *Chlorella ellipsoidea* (Hatano et al., 1976) showed on growth cessation in autumn that an accumulation of ATP and NADPH$_2$, due to a high chl **a** content, was necessary for the development of frost hardiness. The mechanisms for survival in the current experiments are unknown. Neither *Chlorella* nor Scenedesmus species are known to posses any special structures or reproductive adaptations and therefore the survival of both species on cooling probably reflects increased hardiness of the vegetative cells. The reasons why *S. subspicatus* could not tolerate extended storage at +4 ^{o}C in complete darkness was not investigated but could be due to the cells losing essential metabolites by diffusion: it is known that low temperatures increase the permeability of the membrane to solutes (Greiff 1960).

CONCLUSIONS
The results clearly show that of the two algal species tested, both of which are common inhabitants of WSP systems, *C. vulgaris* is much better adapted to withstand and grow under conditions of low

light intensity and temperature. Under higher light intensities and warmer conditions the rates of photosynthesis and growth achieved by *S. subspicatus* are substantially higher than the maximum rates observed for *C. vulgaris*. The implication of this finding is that in a mixed culture of the two species *C. vulgaris* may be out-compete *S. subspicatus* in winter conditions in temperate and cold climate regions, but itself be out-competed by *S. subspicatus* in the summer. Cells of *S. subspicatus* are damaged by even relatively short-term exposure to cold and dark and are completely disrupted by freezing, while *C. vulgaris* appears to have some adaptation mechanisms allowing survival of cold and dark conditions, and even of freezing in at least part of the population. In practical terms this means that in the natural environment *C. vulgaris* may appear earlier and compete more effectively in early spring but may lose its advantage as the temperatures and light intensity increases in the summer period.

The period of gradually falling temperatures in a WSP at the onset of winter provides a natural hardening process similar to that used to increase the resistance of cells to damage by freezing: the results showed that at least some species are likely to benefit from this. Likewise the growth response of different species to temperature increases in the spring may also be a deciding factor in which species can establish themselves. Of the two species studied *C. vulgaris* showed no significant lag at the beginning of the growth phase on revival from cold or frozen conditions whereas *S. subspicatus* showed some lag.

The different responses of the two species to dark and cold conditions are indicative of the range that may occur across a wider population, and have significant implications for over-winter survival and spring recovery. In particular this is likely to influence the population present from which spring blooms may form. A well-designed WSP is unlikely to freeze completely: there are potential survival niches in water close to 0 ^{o}C and within the frozen ice layer. The results indicated that survival in both is possible but is likely to show species differentiation. While light and temperature are particularly difficult environmental parameters to control, it is clear that species can survive in sufficient numbers to provide an inoculum for the spring bloom. It is also interesting to note that not only can algae survive at very low light intensities and temperatures: they can also photosynthesise and grow thus providing oxygen production in winter, albeit it at very low rates especially at high latitudes where daylight hours are limited.

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