

Appendix 1. Phytoplankton Bioassay

Methodology

A priori it was proposed that phosphorus was the limiting nutrient as the nitrate concentrations in the inflowing groundwater were 5.46-6.47 mg L⁻¹. However, bioassays were conducted to assess whether phosphorus or nitrogen was the limiting nutrient and to estimate the pelagic phytoplankton growth rate at different nutrient concentrations. Incubation experiments were performed in August 2014 during an 11 day period, using water collected from Ewens Ponds at 100 µmol m⁻² s⁻¹ (cool white, fluorescent lamps) with a light-dark cycle of 12 h:12 h, and temperature of 20°C. As the phytoplankton concentration was close to zero a pure culture of green algae, *Ankistrodesmus falcatus*, was used as inoculum. The initial cell density of all treatments was adjusted to approximately 1000 cells mL⁻¹.

In order to reduce the effect of nutrients accumulated in the cultured cells, algae were maintained in nitrate- and phosphate-free BG 11 media for five days before the experiment to deplete the intracellular store. Four nutrient treatments were used: no nitrate or phosphate added (control); Phosphorus addition comprising 100 µmol L⁻¹ of K₂HPO₄; nitrogen addition as 1000 µmol L⁻¹ NaNO₃; both K₂HPO₄ and NaNO₃ added at the aforementioned concentrations (N+P). Additionally, algae growth was examined using seven levels of PO₄³⁻P addition (0.1, 0.2, 0.5, 1, 2, 5, 10 µmol L⁻¹), all with nitrogen addition as 1000 µmol L⁻¹NO₃⁻. Nutrients other than phosphorus and nitrogen for phytoplankton growth during the bioassay were provided by adding stock solutions following the formula of BG-11 media (Stanier et al., 1971).

Chlorophyll-a was measured spectrophotometrically (spectrophotometer: Libra S22 Biochrom, Cambridge, UK) from hot ethanol extracts of GF/C filtered samples. Cell counting was undertaken with an OLYMPUS BX40F4 optical microscope (Olympus, Tokyo, Japan) following standard procedures (American Public Health Association, 2005).

Phytoplankton growth rates were calculated on chlorophyll-*a* concentration (μ_{Chla}) and cell numbers (μ_{cell}) using the following equation $\mu = \ln(X_t/X_0)/t$ where X_t is final chlorophyll-*a* concentration or cell number, X_0 is initial Chl-*a* or cell number, and t is the duration of incubation.

Nonlinear regression was used to fit growth rates with phosphate concentration following growth kinetics by Monod (1950). Statistics were performed using SPSS 19.0 (IBM, Armonk, NY, USA), and values were logarithmically transformed to meet the requirements for parametric tests when necessary. ANOVA was used to test for differences in data between cultures with variable nutrient supply patterns. Nonlinear fitting parameters were determined using OriginPro 9.0 (OriginLab, Northampton, MA, USA).

Results

Nutrient addition to water samples significantly increased the phytoplankton cultures chlorophyll-*a* (Chl-*a*) content and cell number with respect to the control ($p < 0.01$). The highest Chl-*a* and cell concentrations were obtained in treatments with excess phosphorus and nitrogen (P+N). The total Chl-*a* developed at the end of the treatment was significantly higher when adding phosphorus than nitrogen. The increase in cell number obtained with addition of P alone was the same as that obtained by adding both P and N, showing that P was the controlling factor for growth (Figure A1.1).

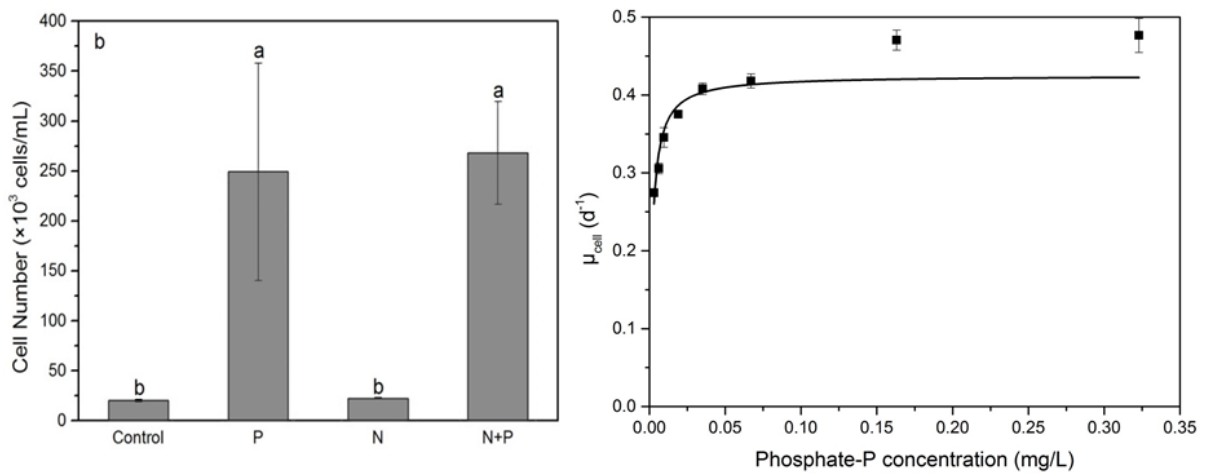


Figure A1.1 On the left, cell number at the end of the incubation experiments in samples with excess of phosphorus (P), nitrogen (N) and both (P+N). Bars are standard deviations, columns labelled with different letters are significantly different. On the right, estimated growth rates at different phosphate concentrations.

The bioassays confirmed the initial hypothesis that phosphorus was the limiting nutrient in Ewens Ponds. Thus, one of the main factors controlling phytoplankton development is closely associated with phosphorus increase while nitrogen was present at concentrations that were excess to demand.

The second bioassay experiment allowed the estimation of phytoplankton growth rates at different P concentrations in the presence of excess nitrogen. Algae growth rate increased consistently until the P concentration reached about 0.035 mg L^{-1} (Figure A1). Maximum growth rates were respectively 0.3 d^{-1} and 0.43 d^{-1} when accounting for biomass change as chlorophyll-*a* or cell number. Half-saturation constants were respectively $0.016 \text{ mg P L}^{-1}$ for chlorophyll-*a* and $0.019 \text{ mg P L}^{-1}$ for cell number.

References

American Public Health Association. 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, *American Water Works Association*, Water Environment Federation.

Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). *Bacteriological Reviews* 35: 171-205.

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