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Nutrient reduction bioassays in the Waikato River

This report was commissioned by the Technical Leaders Group for
the Healthy Rivers Wai Ora Project

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Signed by:

Date: 11 May 2016

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Nutrient reduction bioassays in the Waikato River

*Prepared for Technical Leaders Group of the Waikato-Waipā Healthy
Rivers Waiora Project*

June 2015



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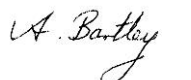

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Cover Photo: Dr Max Gibbs checking the bioassay incubations [Photo by John Quinn, NIWA]

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Executive summary

The Technical Leaders Group of the Waikato/Waipā Healthy River Waiora project asked NIWA to conduct a nutrient reduction bioassay on Waikato River water to determine whether reducing either N or P concentrations would cause a reduction in phytoplankton growth. The bioassay study was run in April 2015. The study also attempted to assess the nutrient limitation status of the phytoplankton in the river water with nutrient addition bioassays and tested the effect of pre-screening the river water through a 40µm mesh on the growth responses in the bioassays. Pre-screening is commonly carried out in such bioassays to remove zooplankton to try to reduce the variability in algal growth response due to zooplankton grazing.

High variability in the 40µm pre-screened water results led to these being discounted and only whole river water results were used to interpret the bioassay.

Due to unforeseen circumstances, the nutrient additions were lower than expected and interpretation of those results is based on a weight-of-evidence assessment rather than assessment of a direct response to the additions.

Within this limitation, the results showed that:

- the phytoplankton in the Waikato River at the time of sampling tended to be P-limited to growth
- the reduction of P and N nutrient concentrations by nominal 40% (actual reduction 21% for P) in the bioassay water resulted in a statistically significant reduction in growth relative to the control, $P=0.0025$ and $P=0.0135$, respectively, using a one-tail T-tests (95% confidence at $P<0.05$).

Further reductions in the N and P nutrient concentrations (nominal 70% and 39% actual, respectively) failed to reduce the growth further indicating that there may be constraints to the use of the nutrient reduction technique for this type of assessment.

1 Introduction

Two recent studies on phytoplankton biomass in the main stem of the Waikato River used nutrient (N and/or P) addition bioassay techniques to assess 1) the potential for nutrient limitation at four locations (below Lake Ohakuri, below Lake Karapiro, upstream of the Waipa River confluence at Ngaruawahia and at Rangiriri) (Gibbs et al. 2014a) and 2) factors such as retention time and thermal stratification in the hydro lakes (test case in Lake Karapiro) that may be influencing chlorophyll-*a* (Chl_a) concentrations (Gibbs et al. 2014b).

Peer review of these studies and subsequent discussion on potential nutrient limitation, produced general agreement that:

- In the river main stem, “Within the constraints of the studies, there were indications that phytoplankton growth was enhanced with the addition of N and P, and to a lesser extent, with P alone.” And “There were no apparent increases to N addition on its own. This suggests the potential for co-limitation of phytoplankton under these conditions.”
- In Lake Karapiro, chlorophyll-*a* concentration increased in response to added N, and this increase was not enhanced if P was also added.

While it was agreed that, under conditions where co-limitation occurred, addition of either N or P could stimulate phytoplankton growth, there was disagreement as to whether reductions in phytoplankton biomass would therefore be expected to result from reductions of N or P.

NIWA was asked conduct a further bioassay study to test the hypotheses that, if nutrient limitation bioassays indicate that phytoplankton are co-limited by N and P for growth:

1. addition of both N and P will stimulate growth, and
2. reduction of either N or P will reduce growth.

Because the original bioassay studies were conducted on water which had been pre-screened to remove zooplankton grazers larger than 40 µm (as is commonly done in bioassays to control the variability in results due to random inclusion of zooplankton grazers in treatments), a third hypothesis was added:

3. Removal of large zooplankton and phytoplankton >40 µm may affect growth responses.

If pre-screening reduces the phytoplankton biomass (as Chlorophyll-*a*) significantly it could bias the nutrient responses in bioassays because larger cell phytoplankton tend to have higher half-saturation coefficients for nutrients than smaller celled species, meaning that their growth is more sensitive to reductions in nutrient concentrations (Eppley and Thomas 1969; Halterman and Toetz 1984). Thus avoiding pre-screening is desirable if it does not result in problematic high variability in Chlorophyll-*a* results due to zooplankton grazing.

This report present the results of this nutrient reduction bioassay study.

2 Methods

The nutrient reduction bioassay study was conducted in mid-April using Waikato River water collected from beneath, but upstream of, the Bridge Street bridge within Hamilton City.

2.1 Experimental design

To test these hypotheses, Waikato River water from one site (Bridge Street ramp) were collected and incubated as per the earlier Waikato River study (Gibbs et al. 2014a), but with changes designed to resolve issues perceived in the earlier study. These are:

- The incubations were run for 24 hours only, not 5 days.
- The river water used was pre-screened at 40 μm to remove zooplankton.
- The incubations were repeated using whole (unscreened) water.
- Before the incubation began, nutrient concentrations were measured in the initial (T0) water of each treatment, i.e., controls, +N, +P, +N+P, -N and -P. Nutrient concentrations were also measured after the 24 hr incubation.
- Before incubation, chlorophyll *a* concentrations were measured in the control only for whole water and pre-screened <40 μm water to provide T0 values.
- For the nutrient depletion experiments the aim was to reduce the N and P by 40% and 70% corresponding to the reduction required for a 1 or 2 band shift in nutrient attribute state. This was achieved by dilution with nutrient depleted natural river water. (See technical details below).
- Because the -N and -P treatments required dilution of the specified nutrient, the concentration of the other non-treatment nutrient was balanced back to ambient concentration of that nutrient in the river water, using a standard solution of either N or P in deionised water.
- All incubations were done in triplicate.
- Chlorophyll *a* concentrations were measured in all incubations after the 24 hr incubation to provide T24 values.
- Zooplankton species and biomass were assessed on the Waikato River water at the time of sampling by passing 40 L of water through a 40 μm zooplankton net, as described in Gibbs et al. (2014a).
- The change in phytoplankton species composition was assessed on a single bottle each of raw river water and 40 μm screened river water that have been treated with +N+P.

2.2 Nutrient depleted water

Waikato River water was collected on 10 and 13 April 2015 with the latter comprising whole water and 40 μm pre-screened water (i.e., zooplankton larger than 40 μm removed). These samples were analysed for dissolved reactive phosphorus (DRP), ammoniacal nitrogen ($\text{NH}_4\text{-N}$) and nitrate nitrogen ($\text{NO}_3\text{-N}$) (Table 1).

Nutrient depletion required the removal of both N and P from the water without changing the ionic characteristics of the water i.e., alum or iron could not be used to remove DRP. The preferred option was to use biological stripping by adding a quantity of batch reactor pond algae or some macrophyte weed to a quantity of water then incubating the mixture for 63 h (over a weekend) under the artificial light system. Two different algal slurries were tested by adding 50 ml of each algal slurry into separate 20 L translucent containers of whole river water. The macrophyte weed used was Hornwort from the Waikato River (Lake Karapiro) with a handful of fresh fronds being added to whole river water in a clear 20 L Nalgene carboy. A 20 L sample of natural river water without amendment was also incubated beside the treatments as a reference. The samples were incubated at 18°C under artificial lights (Gibbs et al. 2014a) for 63 hours (weekend). Aliquots of these samples were filtered through Whatman GF/C glass fibre filters and analysed at the same time as the initial samples, before the nutrient depletion experiments were run (Table 1) to assess the efficacy of the nutrient depletion method and select the most appropriate dilution water to use.

Table 1: Concentrations (mg m⁻³) of DRP, NH₄-N, NO₃-N and Chla in water samples from the Waikato River on 10 and 13 April 2015. Nutrient depletion was achieved by incubating whole water with the macrophyte (Hornwort), treatment pond algae or just the natural algal assemblage in the river for 2 days under artificial lights at 18°C.

Date	Sample	DRP	NH ₄ -N	NO ₃ -N	Chla
10/04/2015	River water	19	34	191	
13/04/2015	Whole river water	18	25	171	5.0
13/04/2015	<40um river water	18	27	172	3.4
Incubations set up on 10/04/2015					
Analysed on	Treatment				
13/04/2015	+Hornwort fronds	6	3	1	
13/04/2015	+pond 1 algae	2	4	1	
13/04/2015	+pond 2 Algae	2	4	2	
13/04/2015	no additions	3	7	15	

The analytical results indicate that the two different pond algae incubations removed 89% of the DRP and 97% of NO₃-N while the Hornwort treatment removed 65% of the DRP and 97% of NO₃-N. The natural algal assemblage in the river water removed >80% of the DRP and >90 % of the NO₃-N without any additional treatment (Table 1).

Although the pond algae treatment removed the most DRP, the algae produced a strong green colour to the water and the water could not be used for the dilutions in the nutrient reduction incubations. Consequently, the Hornwort treated water was used for the dilutions.

2.3 Treatments

The incubation system was set up as described in Gibbs et al. (2014a) using three replicate 400 ml incubation jars each of control or treatment with 200 ml of sample, and a single bulk 2 L sample each of whole water and pre-screened water treated with +N+P. All incubation containers were stirred using gentle bubbling with filtered room air via an aquarium aerator pump (Figure 1). Nutrient bioassays were run as 24 hour growth assays on whole water and repeated on 40 µm pre-screened water (i.e., without large zooplankton). Nutrient additions of +P, +N and +N+P were compared with control water (no addition) to estimate the nutrient status of the phytoplankton in the river at the time of sampling.

Nutrient reductions were made by dilution of the whole or 40 μm pre-screened water with GF/C filtered nutrient depleted water to give reductions of either 40% or 70% of either N or P. When P reductions were made, the N concentration, which was also reduced, was balance back to the natural concentration by addition of the $\text{NO}_3\text{-N}$ stock solution. Similarly, when the N reductions were made, the P concentration was balanced back to the natural concentration by addition of the DRP stock solution. The nutrient dilutions were made by adding either 600 ml (40% reduction) or 300 ml (70% reduction) of the whole or 40 μm pre-screened water to a 1000 ml measuring cylinder and making the volume to 1000 ml with filtered nutrient depleted water. The required amount of balance nutrient was added before making up to volume to ensure good mixing. Then 200 ml of the final mixture was poured into each of the three incubation jars, ensuring that each replicate had identical starting chemistry. An aliquot of the mixture was retained for analysis to confirm the level of nutrient reduction.

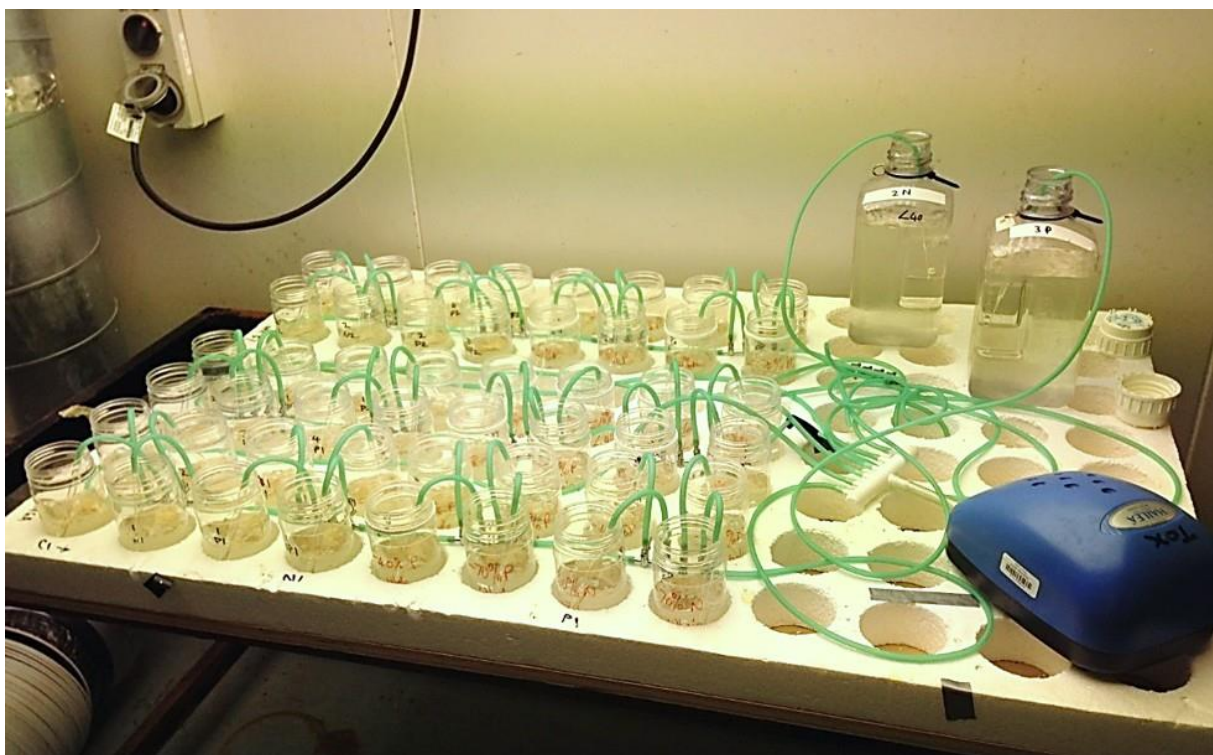


Figure 1: Incubation table showing the treatment incubation jar array, the two bulk samples and the aerator used.

Treatments were:

1. **+N:** an addition of sodium nitrate to a final concentration of plus 140 mg m^{-3} as nitrate-N ($\text{NO}_3\text{-N}$)
2. **+P:** an addition of potassium dihydrogen phosphate to a final concentration of plus 10 mg m^{-3} as dissolved reactive phosphorus (DRP)
3. **+N+P:** additions of both N and P to final concentrations of plus 140 mg m^{-3} $\text{NO}_3\text{-N}$ and plus 10 mg m^{-3} DRP
4. **CTRL:** no nutrient additions, to be used as the control
5. **-40%P:** P reduction with N balanced

6. **-70%P**: P reduction with N balanced
7. **-40%N**: N reduction with P balanced
8. **-70%N**: N reduction with P balanced
9. **whole vs pre-screened water**: All above treatments were repeated for both whole water and 40 µm pre-screened water, and
10. **+N+P bulk**: One each 2 L of whole or 40 µm pre-screened water with the +N+P nutrient additions. These samples were set up to examine any volumetric effects that might affect the 400 ml incubation jars.

Lighting consisted of a bank of 12 daylight fluorescent lights 0.5 m above the jars. The lights cycled on and off by timer for a 16 hour light and 8 hour dark cycle. Light levels were $\sim 170 \mu\text{Mol m}^{-2} \text{s}^{-1}$, which is equivalent to 20% of the average natural daily ambient photosynthetically available radiation (PAR) at the water surface of river where no riparian shading occurs.

2.4 Biology

The removal of large zooplankton in the pre-screening process could also remove large phytoplankton, thereby potentially affecting the growth of phytoplankton in the pre-screened water or allowing higher grazing pressure in the whole water. To understand the effect of the 40µm pre-screening on phytoplankton, phytoplankton cell counts and biovolume were measured on the river water before and after the pre-screening. The zooplankton biomass was also measured as per Gibbs et al. (2014a).

3 Results

3.1 Zooplankton

Zooplankton biomass were substantially lower at <10% of that measured in the previous Waikato River bioassay study in April (Table 2). This implies that zooplankton grazing pressure would not be an issue in the whole water bioassay incubations.

Table 2: Zooplankton biomass in April 2015 at Bridge Street compared with data from upstream and downstream sites in April 2014. (April 2014 data from Gibbs et al. 2014a).

Zooplankton species	Zooplankton biomass ($\mu\text{g L}^{-1}$)		
	April 2015	April 2014	April 2014
Site	Bridge Street	Karapiro	Ngaruawahia
Copepods	0.106	1.671	0.805
Cladocerans	0.019	0.579	0.772
Rotifers	0.022	3.681	1.260

3.2 Phytoplankton

There were reductions in cell counts of most phytoplankton species after the 40 μm pre-screening. These ranged from 10% up to 100% (Table 3). A few phytoplankton species cell counts remained unchanged indicating that these species were probably small cells. The average reduction of all species that reduced was 61.5%.

The greatest reduction in phytoplankton cell counts were for the diatom *Aulacoseira granulata* (50%), the Chlorophyte *Dictyosphaerium sp.* (32%) and cyanobacteria *Aphanocapsa sp.* (26%). While other species may have had greater percent reductions, their cell counts were much lower. Using just these results indicates that the average reduction in phytoplankton was more likely to be in the order 35%, which is in keeping with the measured 32% reduction in chlorophyll *a* concentrations in the pre-screened water (3.4 mg m^{-3}) compared with the whole water (5.0) (Table 1).

Table 3: Phytoplankton species composition list with cell counts for before and after 40 µm pre-screening. The Reduction column shows that most phytoplankton species cell counts decreased with the pre-screening but others were either not affected or appeared to increase.

Phyllum	Genus	Species	Whole cells/ml	Pre-screened cells/ml	Reduction %
Blue greens (Cyanobacteria)	Aphanizomenon	sp.	146	100	31.5
Blue greens (Cyanobacteria)	Aphanocapsa	sp.	447	331	25.9
Blue greens (Cyanobacteria)	Geitlerinema	sp.	6	24	
Blue greens (Cyanobacteria)	Microcystis	sp.	99	55	44.4
Blue greens (Cyanobacteria)	Phormidium	sp.	38	11	71.1
Blue greens (Cyanobacteria)	Planktothrix	sp.	8	0	100.0
Blue greens (Cyanobacteria)	Pseudanabaena	limnetica	9	14	
Blue greens (Cyanobacteria)	Pseudanabaena	sp.	4	0	100.0
Desmids (Zygnemophyceae)	Mougeotia	sp.	143	176	
Diatoms (Bacillariophyceae)	Attheya	sp.	11	11	
Diatoms (Bacillariophyceae)	Aulacoseira	granulata var. angustissima	1307	648	50.4
Diatoms (Bacillariophyceae)	Cyclotella	stelligera	44	22	50.0
Diatoms (Bacillariophyceae)	Synedra	sp.	2	1	58.3
Flagellates/Unicells	Flagellates/Unicells	<5um	834	736	11.8
Golden-brown algae (Chrysophyceae)	Synura	sp.	49	2	95.5
Green algae (Chlorophyta)	Coelastrum	cambricum	55	21	61.3
Green algae (Chlorophyta)	Crucigeniella	sp.	34	24	29.8
Green algae (Chlorophyta)	Dictyosphaerium	sp.	417	285	31.6
Green algae (Chlorophyta)	Micractinium	pusillum	6	0	100.0
Green algae (Chlorophyta)	Monoraphidium	sp.	11	11	
Green algae (Chlorophyta)	Pediastrum	sp.	7	2	77.8
Green algae (Chlorophyta)	Sphaerocystis	sp.	33	0	100.0
Green algae (Chlorophyta)	Westella	sp.	8	2	71.1
Small flagellates (Cryptophyceae)	Cryptomonas	sp.	22	22	

3.3 Nutrients

Nutrient data from the bioassay experiment at setup are presented in Table 4 and at the end of the incubation in Table 5.

The setup concentrations as measured showed that +N and +P additions increased the NO₃-N and DRP concentrations by about 12-14 %. However, while the N and P reductions showed that the NO₃-N concentrations were reduced by 40% and 70%, the DRP reductions were less than expected at 24% for the 40% and 67% for the nominal 70% in the <40µm pre-screened samples and 21% and 39% for the nominal 40% and 70% reductions, respectively, in the whole water (Table 4).

The less than expected DRP reductions stem from the use of nutrient depleted water (hornwort treatment), which still had about 6 mg m⁻³ DRP in it (Table 1).

After the 24 h incubation period, the NO₃-N concentrations showed small changes, whereas the DRP concentrations were reduced to near zero in all but the bulk containers (Table 5). It is noted that the NH₄-N concentrations increased in most incubation jars, implying some mineralisation of the dissolved organic N in the river water over the 24 h incubation period.

Table 4: Setup nutrient concentrations in the incubations. The percentage change in N and P are the measured values after setup. The expected changes are given with highlighting linking the nutrient reductions.

Sample	Set up nutrient concentrations (mg m ⁻³)					
	40µm pre-screened water			Change in N	P	Expected
	DRP	NH ₄ -N	NO ₃ -N	%	%	%
T0 Ctrl	13	17	176			
T0 +N	12	18	200	+14		
T0 +P	14	18	176		+14	
T0 +N+P	15	17	206	+22	+17	
T0 -40% P	10	10	120		-24	-40
T0 -70% P	4	7	81		-67	-70
T0 -40% N	10	11	107	-39		-40
T0 -70% N	11	5	53	-70		-70
T0 BULK +N+P	17	17	204	+35	+16	
Sample	Whole river water					
	40µm pre-screened water			Change in N	P	Expected
	DRP	NH ₄ -N	NO ₃ -N	%	%	%
T0 Ctrl	17	21	176			
T0 +N	16	22	200	+14		
T0 +P	19	20	175		+12	
T0 +N+P	19	25	207	+17	+18	
T0 -40% P	13	12	120		-21	-40
T0 -70% P	10	6	81		-39	-70
T0 -40% N	14	12	104	-41		-40
T0 -70% N	12	6	54	-70		-70
T0 BULK +N+P	18	20	190	+8	+8	

Table 5: Nutrient concentrations in initial (T0) water and in the T24 water after the 24 h incubation.

Sample	Nutrient concentration (mg m ⁻³)					
	40µm pre-screened water			Whole river water		
	DRP	NH ₄ -N	NO ₃ -N	DRP	NH ₄ -N	NO ₃ -N
T0 Ctrl	13	17	176	17	21	176
T24 Ctrl	0.3	38.3	185.3	0.6	56.7	181.0
T24 +N	0.6	30.3	217.0	2.0	36.7	209.7
T24 +P	1.0	7.3	178.0	2.0	41.2	185.0
T24 +N+P	0.6	4.3	205.7	1.0	2.0	206.3
T24 -40% P	0.6	37.0	130.7	1.5	24.2	116.0
T24 -70% P	0.6	37.0	75.7	1.3	55.3	76.3
T24 -40% N	0.0	28.0	111.7	1.5	54.7	113.0
T24 -70% N	0.3	29.6	56.6	1.5	73.0	60.3
T24 BULK +N+P	13	1	198	8	9	152

3.4 Bioassay

The chlorophyll *a* concentrations from the nutrient addition and reduction bioassay incubations were compared with the T0 and control concentrations to determine the growth response to the treatment. The chlorophyll *a* concentrations in the nutrient addition incubations were directly comparable with the T0 and control chlorophyll *a* concentrations, but the chlorophyll *a* concentrations in the nutrient reduction incubations required correction for the amount of dilution (% reduction) applied. To do this, a linear correction was applied by multiplying the chlorophyll *a* concentration measured by the total volume at set up (i.e., 1000 ml) divided by the volume of sample water used i.e., 600 ml for -40% or 300 ml for -70%.

The results are assessed as 'growth' if there was an increase in chlorophyll *a* concentration over the T0 concentration (Figure 2). In all cases the treatments and controls grew relative to the T0 value. This indicates that the phytoplankton were actively growing rather than stagnating or senescing.

The proportional increase in chlorophyll *a* over the T0 value in the 24 h incubation period ranged from 12% to 56% for the 40 μm pre-screened water tests and 26% to 94% for the whole water tests (Figure 3).

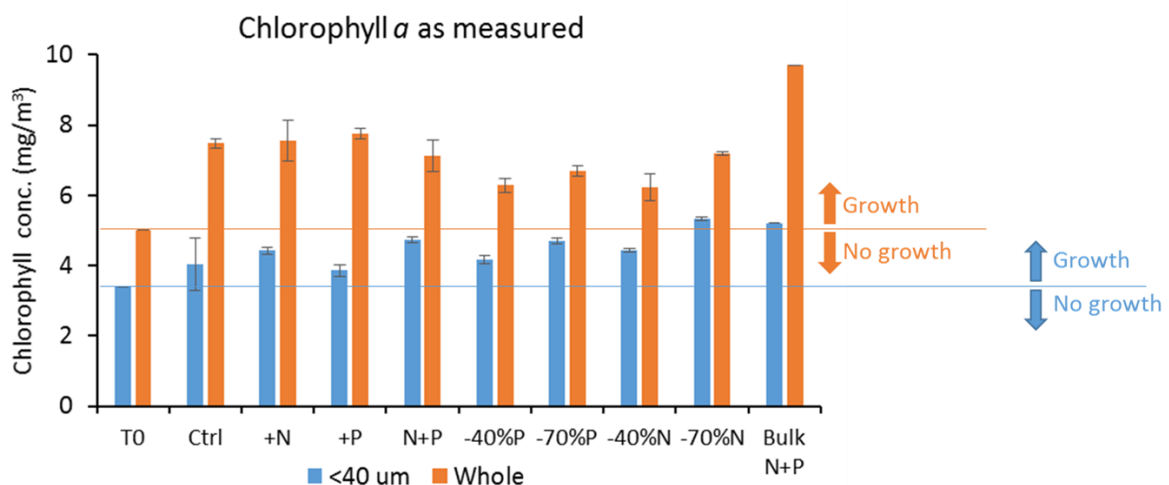


Figure 2: Comparison of mean chlorophyll *a* concentrations in the incubation jars in relation to N and P additions and nominal % reductions for pre-screened and whole water samples. Results are means of three samples. Error bars are \pm the standard deviation on the mean. Growth is an increase over the T0 value.

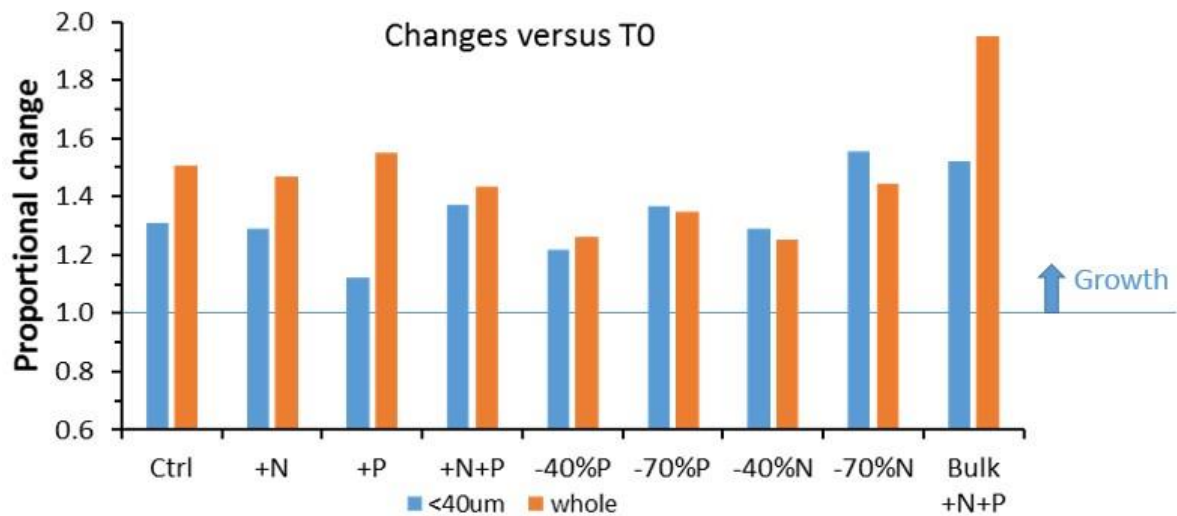


Figure 3: Proportional change over T0 in relation to N and P additions and nominal % reductions. Above the line indicates growth.

The proportional change in chlorophyll *a* relative to the control value in the 24 h incubation period ranged from +19% to -14% for the 40 μ m pre-screened water tests and +3% to -17% for the whole water tests (Figure 4).

Considering the whole water tests first, the relatively small nutrient additions in the +N, +P tests (+12-14% for each, Table 4) and +N+P tests (+17-22%) produced very little response. However, reducing P or reducing N by nominal 40% (actual 21%, Table 4) resulted in reduced growth by around 16-17%. This reduction in growth was only 11% for tests with P reduced by nominal 70% (actual 39%) and 4% for tests with N reduced by 70% (Figure 4).

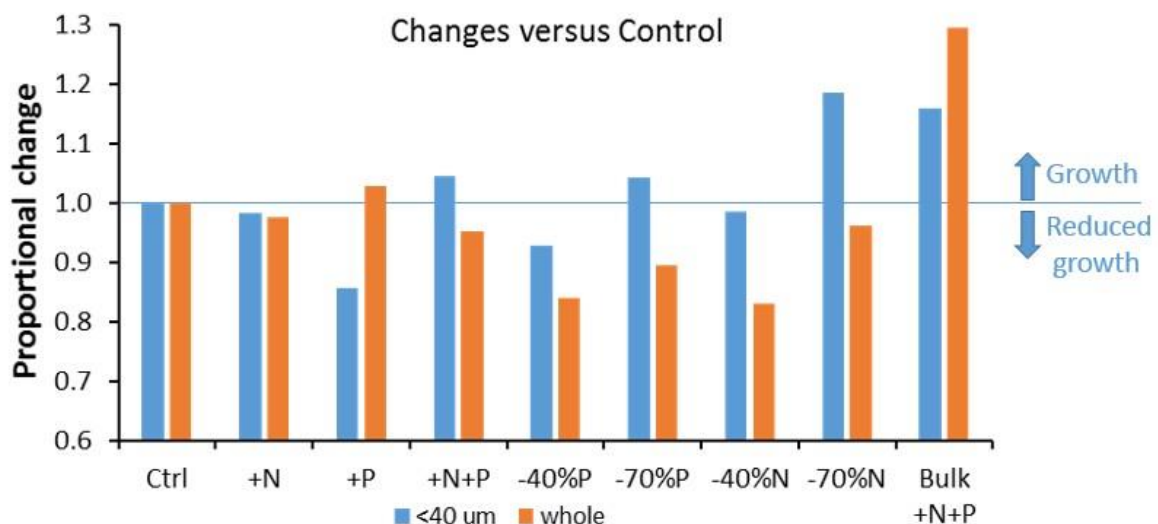


Figure 4: Proportional change over Control in relation to N and P additions and nominal % reductions. Values above the line indicate growth while values below the line indicate reduced growth.

In contrast, for the 40 µm pre-screened water tests, the nutrient addition tests +N, and +N+P produced very little response while there was reduced growth of 14% in the +P additions. Reducing P by 40% resulted in a 7% reduction in growth but there was little reduction in growth for the other P and N reductions. However, the -70% N reduction treatment appeared to produce a 19% increase in growth (Figure 4).

3.4.1 Sensitivity test

In order to answer the question, “Could the analytical method used ‘see’ a change in chlorophyll *a* given the levels of dilution used in the bioassay?”, the sensitivity of these results were tested by manipulating the analytical result, before correction for dilution, to produce a response change of 10%, i.e., a shift from no reduction to 10% reduction. At nominal 40% nutrient reduction, it would require an analytical error of 0.4 mg m⁻³ to produce a change in the result by 10%, and at nominal 70% nutrient reduction it would require an error 0.3 mg m⁻³ to change the result by 10%. The analytical method has a stated detection limit of 0.1 mg m⁻³ but can routinely discriminate to 0.01 mg m⁻³ chlorophyll *a*. This indicates that the method would be expected to ‘see’ levels of change produced in the bioassay of <3% and, therefore, the results (Figure 4) are likely to be real within the constraints of the method.

4 Summary and Discussion

The reasons for conducting this nutrient reduction bioassay were, as listed in the introduction and below, to test three hypotheses associated with the findings of nutrient limitation assays in two earlier reports (Gibbs et al. 2014a, b). These hypotheses have a pre-requisite condition of co-limitation by both N and P. Simply stated, under conditions where co-limitation occurs, addition of either N or P could stimulate phytoplankton growth. However, as to whether reductions in phytoplankton biomass would result from the reduction of N or P is uncertain. In this report the three hypotheses tested are:

1. addition of both N and P will stimulate growth, and
2. reduction of either N or P will reduce growth.
3. Removal of large zooplankton and phytoplankton >40 μm may affect growth responses.

While the intention was to add N and P to stimulate growth, the analysis after the incubations showed that the amount added was substantially less than expected. This is likely to have been an error during the preparation of the stock solution or a miscalculation of the spike needed to achieve the required nutrient addition. Unfortunately the error was not found until it was too late to repeat the experiment. Consequently, the interpretations of the results are based on the data obtained and often reflect the influence of the lower than expected nutrient additions.

4.1 Hypothesis 1

Addition of both N and P will stimulate growth:

- Although the phytoplankton were growing rapidly at the time of sampling, there was essentially no growth response by phytoplankton to the addition of N to the 40 μm pre-screened or whole river water. There was no indication that the phytoplankton had run out of N before the end of the incubation. This implies that, at the time of sampling, the phytoplankton in the river were not nitrogen limited for growth.
- There was a small growth response to the addition of P to the whole river water but there was a 14% reduction in chlorophyll *a* concentration to the addition of P to the 40 μm pre-screened water. This reduction appeared to be real with the standard deviation of the three replicates being 0.2 mg m⁻³ (Figure 2). There was an indication that the phytoplankton had run out of P before the end of the incubation because P concentrations were very low in all incubation jars (Table 5). The results are substantially different for the whole water and the 40 μm pre-screened water indicating that there may have been an effect of pre-screening the water (see hypothesis 3). With this in mind and within the constraints of the bioassay measurements, there was an indication of P limitation in the whole water but only at a level close to the sensitivity of the method.
- There was a varied response by phytoplankton to the addition of N + P to the 40 μm pre-screened and whole river water in the 400 ml incubation jars. The phytoplankton in the 40 μm pre-screened water had a small growth response, while those in the whole water appeared to have a slightly reduced growth. In contrast, the addition of N + P to the 2 L incubation jars produced a positive response from both the 40 μm pre-

screened and whole river water. While there was an indication that the phytoplankton in the 400 ml jars had run out of P before the end of the incubation, there was still measurable P in the 2 L incubation jars (Table 5), indicating that these results were more likely to be valid. If the tendency for P limitation was real, these results indicates the phytoplankton in the river at the time of sampling where most likely P limited to growth rather than being in a co-limitation state.

4.2 Hypothesis 2

Reduction of either N or P will reduce growth:

- While there was measureable growth in all treatments relative to the T0 samples, the whole water bioassays had less growth in the incubations with 40% or 70% nominal reductions in N or P than in the control. The reduced growth in the nominal 40% N or P reduction bioassays were substantial. These results demonstrate a clear and statistically significant reduction in biomass for a nominal 40% reduction of P ($P=0.0025$) and a 40% reduction of N ($P=0.0135$), using a one-tail T-tests (95% confidence at $P<0.05$). However, the expected further reduction in growth in the 70% nominal reduction in N or P did not occur. Instead a lesser reduction in growth effect occurred in the 70% nominal reduction incubations. This unexpected result may be an artefact of the incubation method.
- In the 40 μ m pre-screened water bioassay results were highly variable and inconsistent. There was less growth in the 40% nominal reduction in P bioassay compared with the controls. However, all other pre-screened treatments showed essentially no reduction in growth and there was more growth than in the control in the 70% nominal reduction in N incubation (Figure 4).

The reduction in growth relative to controls in the whole water for a 40% nominal reduction in N or P appears to support the hypothesis. Less reduction in growth for the 70% nominal reduction in N or P may be an artefact of the incubations that needs further investigation.

The reduction in growth in the 40 μ m pre-screened water for a 40% nominal reduction in P also supports the hypothesis. However, the highly variable results for the other treatments may have been an effect of pre-screening the water (see hypothesis 3).

4.3 Hypothesis 3

Removal of large zooplankton and phytoplankton >40 μ m may affect growth responses:

The analysis of the water shows that there was a low abundance of zooplankton at the time of sampling, which means that there was no need to remove large zooplankton to reduce grazing pressure. The results show that the pre-screening to remove zooplankton >40 μ m also removed about 50% of the large diatoms and an average of about 35% of all large phytoplankton, as indicated by the reduction in chlorophyll *a* concentrations between the initial whole and 40 μ m pre-screened water (Table 1). This level of modification of the phytoplankton assemblage in the river water may be the explanation for some of the variability in the 40 μ m pre-screened water results above. These observation support the hypothesis.

4.4 Interpretation of the nutrient addition and reduction results

Although the nutrient addition component of the experiment seems to be inconclusive due to the nutrient additions being substantially less than was intended, the results are still able to be interpreted in terms of chlorophyll responses to those nutrient additions. The logic is as follows:

- i) All the incubation jars showed an increase in chlorophyll *a* over the 24 hour incubation period. This shows that the phytoplankton were alive and growing.
- ii) Chlorophyll concentrations in the controls increased at a similar rate to those in the nutrient addition treatments, except for the bulk nutrient additions, which had higher chlorophyll concentrations at the end of the incubation period.
- iii) In all incubations except the bulk +N+P, the DRP concentrations had reduced to essentially zero. This indicates that the phytoplankton had run out of P in the 400 ml incubation jars. There was still DRP in the bulk +N+P incubation jars and the chlorophyll concentrations had increased above the control concentrations indicating that either N or P was stimulating growth.
- iv) The control chlorophyll concentrations were higher than those in the +N treatment but lower than those in the +P incubations. Although the differences are small, the implication is that +N did not stimulate growth while +P may have.

The interpretation of this information is that the phytoplankton in the river were short of P and that addition of P in the +N+P bulk incubation was the nutrient that stimulated the increase in the chlorophyll *a* concentration observed.

The conclusion from this is that the phytoplankton in the river at the time of sampling tended to be P limited.

It is evident that pre-screening of the river water had an effect on the bioassay. Consequently, because there was no need to remove large zooplankton grazers and the pre-screening reduced the phytoplankton biomass by about 35%, which potentially contributes to the high variability in the 40 μ m pre-screened water results, it would be reasonable to disregard the 40 μ m pre-screened water results and use only the, natural, whole water results to interpret this bioassay study.

To this end the proportional changes for each treatment relative to the control have been replotted without the 40 μ m pre-screened water results, for clarity (Figure 5). These show that the nominal 40% P reduction and 40% N reduction assays resulted in 16% and 17% reduction in chlorophyll *a*, respectively. This level of response is well above the sensitivity threshold of 3% (section 3.4.1) and the results are statistically significantly different from the control $P = 0.0025$ and $P = 0.0135$, respectively, using a one-tail T-tests (95% confidence at $P < 0.05$).

These results also show that at the nominal 70% N and P reduction there was no further reduction in growth, as indicated by chlorophyll *a* concentrations. This implies that there may be a limit to the amount of nutrient reduction that can be achieved with this type of experiment before the results become meaningless and an artefact of the experimental conditions.

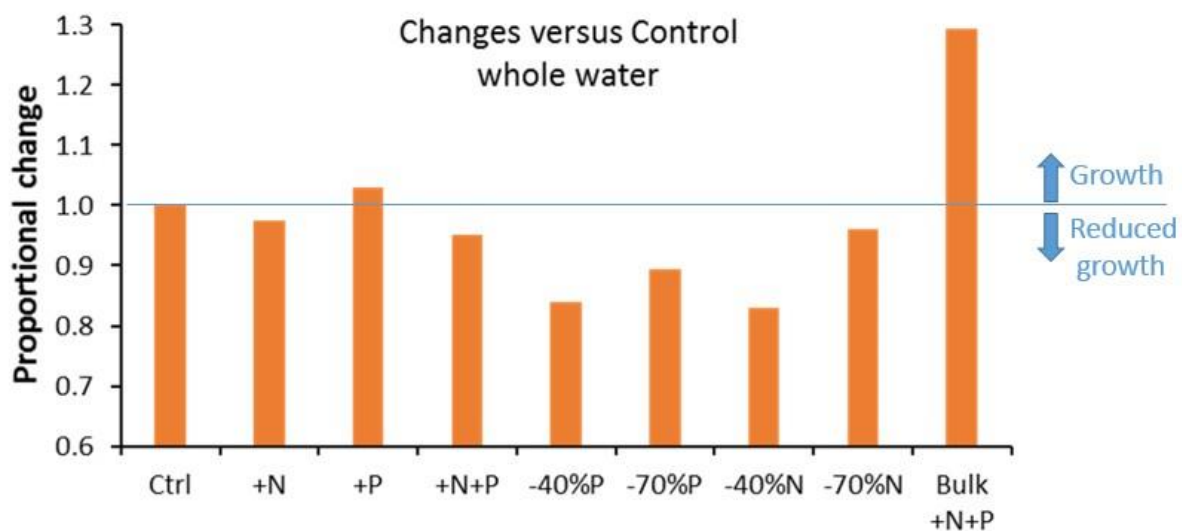


Figure 5: Proportional changes in chlorophyll a in the treatments relative to control for the whole water bioassays only. Analytical sensitivity on proportional change is 0.03.

5 Conclusions

The findings of this study indicate that:

- the phytoplankton in the Waikato River on 13 April 2015 tended to be P-limited for growth, and
- a reduction in P by 21% (nominal 40%) or N by 40% resulted in statistically significant reduced growth relative to controls, $P=0.0025$ and $P=0.0135$, respectively, using a one-tail T-tests (95% confidence at $P<0.05$).

6 Recommendations for future bioassays

The mesh size for pre-screening of water to remove large zooplankton should be large enough to pass most phytoplankton. More suitable mesh sizes are 100 to 130 μm . This is different from the 40 μm mesh size used to capture zooplankton.

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