Biodegradation of dissolved natural organic matter

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Abstract

There is currently under development a method for measuring biodegradability of dissolved natural organic matter (DNOM) at the Section of Environmental Sciences, Department of Chemistry, University of Oslo (UiO). The aim of this project is to find the optimum concentrations and ratios of nutrients that give the highest respiration rate of bacteria grazing on the DNOM. This is to ensure that only the quality of DNOM is the limiting factor for respiration. The respiration rates for concentrations 0.1, 1, 5, 10, and 20 mM P and ratios of 1, 2, 3, 4, 5, and 16N: 1P where measured and were found to not give any significantly different respiration rates. The most significant result was to find that there were very high standard divinations. This concludes that the concentration and ratio of nutrients do not play a critical factor in the measurement of the biodegradability of DNOM.

Preface

I would like to thank my co-supervisor Camille Crapart for helping me to get acquainted with the lab, to show me how all the equipment worked, to teach me how to execute a project like this and to teach me lots of chemistry along the way. My main supervisor Rolf D. Vogt for letting me do this project and finding a result in the endless row of numbers. Also, my office roommates Eline and Susanne for welcoming me into the group and answering all of my stupid newbie questions.

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Abbreviations and definitions

Abbreviation	Definition
BDOM	Bioavailable dissolved (natural) organic matter
DNOM	Dissolved natural organic matter
DOC	Dissolved organic carbon
HMW	High molecular weight
LMW	Low molecular weight
MW	Molecular weight
NIVA	Norsk institutt for vannforskning
RR	Respiration rates
SAR	Specific absorbance ratio
SDR	Sensor dish reader
sUVa	Specific UV absorbance
sViSa	Specific visual absorbance

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1. Introduction

1.1 Dissolved natural organic matter

Dissolved natural organic matter (DNOM) is a ubiquitous component of the environment. It plays an important role in controlling pollutant mobility, attenuating potentially damaging UV radiation (Findlay et al., 1986), heavy metal methylation and thus bioavailability of especially mercury. DNOM contains a large number of functional groups of weak organic acids. These allow it to regulate the pH and form strong metal-complexation with Fe, Al, and type B (heavy) metals, such as Hg and Pb (Rahman et al., 2010) and act as a transport medium for these metals. It can participate in redox reactions and since it is an organic compound it can also absorb and transport persistent organic micropollutants (POP) (Grannas et al., 2012). The DNOM also contains nutrients and act as an important source of food for heterotrophic aquatic organisms, fueling heterotrophic microbial-based food webs (Azam et al., 1983). DNOM plays therefore a key role in heterotrophic bacterial activities in freshwater watercourses, especially in dystrophic lakes. This indicates that DNOM generally plays a vital role in sustaining the aquatic ecosystem in the boreal domain, characterized by forests with organic-rich soils. This biogeochemical characteristic makes it important to have a method that can provide a sound measure of the biodegradability of DNOM. The measure of biodegradability of DNOM differs from measures of Bioavailable DNOM (BDOM). The biodegradability measures the rate in which the DNOM is biodegraded while the BDOM measures the amount of DNOM that is easily utilized for organisms.

DNOM is operationally defined as carbon-containing naturally derived organic compounds that can pass through a 0.45 μm filter. This limit is being historically linked to the microbiological standard for drinking waters (Nimptsch et al., 2014). The material is formed from the decay and oxidization of plant and microbial remains by biochemical and chemical reactions. Most of the DNOM in boreal surface waters are allochthonous, i.e. derived from the terrestrial environment. This allochthonous DNOM is an important food source for aquatic organisms. Heterotrophic bacteria can digest labile DNOM directly, as well as transform recalcitrant DNOM to more labile forms (Hessen and Tranvik, 1998).

Since microorganisms consume DNOM, they become food for other larger organisms. DNOM then becomes a part of a larger food-chain that sustains organisms on many trophic levels. As the organisms die, they decompose and the organic material slowly returns into DNOM, which then again becomes available for the microorganisms. This process of DNOM sustained organisms who in turn become DNOM as they die is referred to as the microbial loop(Azam et al., 1983).

1.2 Aim of study

A method for measuring biodegradability of DNOM is currently under development at the Section of Environmental Sciences, Department of Chemistry, University of Oslo (UiO). The biodegradability is measured by monitoring the bacterial response through consumption of oxygen over time in a concealed sample added inoculum in an incubator. The aim of this Undergraduate project is to test the effect of adding different concentrations and ratios of nutrients on the respiration rate (RR) of bacteria grazing on the DNOM. The goal is to optimize the sensitivity of the method while measuring the biodegradability of a material that is not very biodegradable at rather low concentration. The latter point is then to provide optimum conditions for biodegradation. This will also ensure that only the quality of DNOM is the limiting factor. There also needs to assess to what extent the respiration rate is related to other measures of biodegradability of DNOM, by studying the results of samples that have been determined using different methods. The hypothesis is that there are an optimal dose and composition of nutrients that ensure the best method of precision and accuracy.

2. Theory

2.1 Increase in dissolved natural organic matter in the Boreal domain

Levels of DNOM in surface waters are especially high in the boreal and sub-arctic areas. This pertains that DNOM imposes a significant physical and chemical impact on the surface waters in these regions. The DNOM found in northern terrestrial ecosystems,

especially in areas affected by permafrost, is of special interest in light of the climate change at northern latitudes. The large amounts of organic carbon in biomass, combined with relatively slow decomposition rates, provides a potentially large DNOM source. The permafrost will prevent DNOM to drain through the soil, and it will therefore be efficiently transported to surface waters (Wickland et al., 2007). This will change the ecological balance in these areas and may give unforeseen consequences. Therefore, knowing as much about the biodegradability of DNOM as possible is a prerequisite for sound and sustainable management of the fragile ecological balance of the boreal and sub-arctic areas.

2.2 Biodegradation

Biodegradation is the decomposition of material by biological means. In regard to DNOM, this pertains mainly to heterotrophic microorganisms. The biodegradability of DNOM is an important characteristic of the DNOM, as it provides a measure of the amount of food that is available as a source of nutrients and energy for heterotrophic aquatic organisms in a natural dynamic system.

2.3 Nutrient ratio

It is important to achieve an optimum environment for the bacterial growth in the method for measuring biodegradation of DNOM in order to achieve optimum sensitivity and so that the only factor limiting the biodegradation of DNOM by the bacteria during the incubation is the physicochemical characteristics of the DNOM. This ensures that the parameter reflects only the biodegradable character of the DNOM Access to available inorganic nutrient elements, such as nitrogen (N) and phosphorus (P), is particularly vital for bacteria to generate biomass (Correll, 1999, Elser et al., 2007) and life-sustaining energy. In oceans the molar ratio of Carbon: Nitrogen: Phosphorus in aquatic biomass is 106:16:1 (Marschner and Kalbitz, 2003). This is called the Redfield ratio after Alfred Redfield who was an oceanographer and who was the first to see this ratio connection. The ratio 16N:1P is the originally found ratio, but in 2014 there was a new study that reported a global median N:P ratio of 22:1 (Martiny et al., 2014). In a lack of any other data, it is assumed that the ratio of nutrients in bacteria in freshwater lakes is similar to

the ocean ratio (Cotner et al., 2010). This ratio is important because it is believed to be the molar ratio of nutrients (N:P = 16:1) that provides bacteria with the best living conditions. A large amount of organic matter is used to generate energy. The Redfield ratio of carbon to the other nutrients in biomass is thus not relevant in terms of optimum nutrient ratio in the food for the bacteria.

2.4 Aromaticity of the dissolved natural organic matter

There can often be observed a shoulder on the declining peak of the absorption spectra of DNOM in the UV range at 254 nm. This light attenuation is related to the conjugated double bonds from C=C and C=O who are characteristic for aromatic compounds and ubiquitous in DNOM. A strong overall correlation therefore exists between UV absorbance at 254 nm (Abs_{254}) and DNOM concentration in natural waters. Deviations from this correlation are due to the varying aromaticity of the DNOM. The specific UV absorbency index (sUVa) is a proxy for this degree of aromaticity. The sUVa is calculated by dividing the absorbance at 254 nm by the concentration of DOC in the sample. This index correlates well with the percent of the aromaticity of the DNOM sample (Weishaar et al., 2003). Longer chained conjugated systems are responsible for the absorbency in the visible region, which is explained by the bathochromic shift. That is a shift of absorbency towards longer wavelengths, redshift, induced by increasing length of conjugated systems. There can also be found a specific visual absorbance (sViSa) which serves as a proxy for the amount of higher molecular weight chromophores (Vogt and Gjessing, 2008). There are also two more ratios that are frequently used for DNOM characterization. One often-used ratio is the specific absorbency ratio (SAR) it is defined as the ratio of absorbency at 254 to 400nm, serving as a proxy for the relative contribution of lower to higher molecular weight chromophores (Vogt and Gjessing, 2008). Another is the E_4/E_6 ratio which is defined as the absorbency at 465 to 665nm and is found to decrease with increasing molecular weight, condensation, and aromaticity. Thus, serving as an index of humification. The bacteria grassing on DNOM can absorb light in the UV-spectrum. In the ViS-spectrum bacteria can scatter light.

3. Method and materials

3.1 Nutrients & Inoculum

Ammonium nitrate (NH_4NO_3) and potassium phosphate (K_2HPO_4+$3H_2O$) were used as a nitrate and phosphate source for the growth of bacteria in the inoculum as well as during the incubation. The ammonium (NH_4^+) is relatively unavailable for the bacteria to use compared to nitrate (NO_3^-). It is therefore not conceived to interfere with the targeted concentration of bioavailable reactive nitrogen. Potassium phosphate (K_2HPO_3 + $3H_2O$) is used as the phosphate source. Potassium (K^+) is an important nutrient ion on the other hand and may have an impact since it is a necessary compound for sustaining bacterial life, though in a much smaller amount then for nitrogen and phosphate.

3.1.1 Preparation of Inoculum

Inoculum is the bacteria culture added to the sample to start the biological activity. The raw water chosen was from the dystrophic lake Langtjern, where multiple biodegradation experiments have been done over many years, both by the Norwegian Institute of water research (NIVA) and UiO, so there is a good understanding of the water and bacteria there. It is also easily available as it is stored at the Department of Chemistry at UiO. The raw water was filtered through a 2.0 µm membrane filter to allow for bacteria to pass, while it removes predatory animals and microorganisms that would feed on the bacteria. 100 mL of the filtrate was transferred to a 250 mL Erlenmeyer flask. Then nutrients were added to assure that a sufficient amount of the bacteria grow exponentially. The nutrients added were nitrate (NO_3^-) and phosphate (PO_4^{3-}), using the salts K_2HPO_4+$3H_2O$ and NH_4NO_3. 1 mL of a 1 mM solution of PO_4^{3-} and NO_3^- were added to a final concentration of 0.1 mM in the Erlenmeyer flask. After adding the 1 mL of nutrients there were left 150 mL of headspace ensuring that the bacteria had enough oxygen. The inoculum was incubated at room temperature for two to five days on a shaking table at low shaking speed (50/min) to ensure the inoculum was properly saturated with oxygen. To avoid the growth of algae the solution was covered with aluminum foil to remove lighting.

There were initially made a few mistakes when preparing the nutrients for growing the bacteria in the inoculum. The first mistake was weighing out ten times too much of the nutrients, in the form of $K_2HPO_4+3H_2O$ and NH_4NO_3. This gave a final concentration of nutrients in the bacteria growth substrate of 1 mM N & P instead of the targeted 0.1 mM. The second time a mistake was done, it was to forget a middle step in preparing the nutrient solution. Instead of diluting, the nutrient salt was weight out and then added straight to the 100 mL solution of 0.2 µm filtrate containing the bacteria without zooplankton. This produced a final concentration of nutrients in the inoculum solution at 0.2 mM N & P. When these mistakes were discovered the experiments were repeated but with the correct concentration (0.1 mM N & P) of nutrients in the inoculum. This allowed for a comparison of the effect of the targeted and untargeted concentrations in the inoculum.

3.3 Calibration

The vials used in the incubator stand in a 24 well plate referred to as sets. Two sets can be measured in the incubator at a time. The four sets available were calibrated before the experiment where started. This was conducted in order to account for differences between the sets in regard to the standard calibration. The whole set was calibrated, not the individual wells. The calibration takes as a reference air-saturated water (containing 21 % O_2) and water depleted off O_2 (with ascorbic acid) at 25°C. Ascorbic acid is a mild reducing agent and gets degraded upon exposure to air converting the oxygen to water. Values measured are thus the references for a 21 % oxygen content and a 0 % oxygen content. Type 1 water, devoid of any DNOM, is used as a control.

3.4 Dissolved natural organic matter solution

The DNOM used in this experiment was prepared from a Reverse osmosis up-concentrated and freeze-dried DNOM isolate from Hellerudmyra, based on solutions of well-characterized DNOM isolates from the NOM-Typing project (Gjessing et al., 1999) sampled in October 1996. In the initial step of the isolation, the water sample was cation exchanged in which all cations were exchanged for sodium ions (Na^+) in order to avoid

clogging of the membrane by precipitating supersaturated salts. The DNOM used had a 33.7 % DOC content. As it is wanted to make a DNOM solution that is like what is found in nature, it was chosen to prepare a DOC solution with 20 mg C/L. To prepare that a 59.36 mg/L DNOM solution was made, 0.059 g of dried DNOM was measured out which was then dissolved with type 1 water in a 1 L volumetric flask.

3.5 Nutrient solution gradient

Nutrient solutions of phosphate and nitrate were prepared by adding phosphate and nitrate in different concentrations and ratios as listed in Table 1. The concentrations of phosphate were chosen based on results from previous experiments where the effect on the RR of concentrations of 10 mM P and 0.1 mM P had been measured (Håland, 2017, Holm, 2018). Therefore, the concentrations that were chosen to test was between 0.1 and 10 mM P, e.i. at 0.1, 1, 5, and 10 mM P, as well as one that was above this at 20 mM P. It was also important to try and find out where the nutrient solutions get toxic for the bacteria, therefore there was a wish to try higher concentrations and ratios than used earlier. The ratios were also chosen based on previous experiments where there had been used a 2N:1P ratio (Håland, 2017, Holm, 2018), both concluding that this was the best ratio between their experiments. Also looking at the original Redfield ratio to try higher ratios more similar to nature. The original choice was ratios of 1, 2, 5, 10 and 16N: 1P, but there was also needed to have blanks as references as well as a reference for the DNOM itself, the 'no nutrients' solution, containing only DNOM solution and inoculum. Meaning one of the ratios had to go. The choice ended on ratios of 1, 2, 5, and 16N: 1P, since 2N:1P is the ratio that has been tested before, 1N and 5N who are closer were chosen. The 16N was chosen as it is thought to be the natural ocean ratio. Meaning the 10N:1P was rejected.

Table 1. Concentrations of phosphate and their sets of N:P ratios, as well as the final concentration of phosphate (PO_4) and nitrate (NO_3) used during the incubation experiments.

0,1 mM P	**[PO_4]**	**[NO_3]**	**1 mM P**	**[PO_4]**	**[NO_3]**
1N:1P	0.1 mM P	0.1 mM N	***1N:1P***	1 mM P	1 mM N

2N:1P	0.1 mM P	0.2 mM N	***2N:1P***	1 mM P	2 mM N
			3N:1P	1 mM P	3 mM N
5N:1P	0.1 mM P	0.5 mM N	***4N:1P***	1 mM P	4 mM N
			5N:1P	1 mM P	5 mM N
16N:1P	0.1 mM P	1.6 mM N	***16N:1P***	1 mM P	16 mM N
5 mM P	**[PO_4]**	**[NO_3]**	**10 mM P**	**[PO_4]**	**[NO_3]**
1N:1P	5 mM P	5 mM N	***1N:1P***	10 mM P	10 mM N
2N:1P	5 mM P	10 mM N	***2N:1P***	10 mM P	20 mM N
			3N:1P	10 mM P	30 mM N
5N:1P	5 mM P	25 mM N	***4N:1P***	10 mM P	40 mM N
			5N:1P	10 mM P	50 mM N
16N:1P	5 mM P	80 mM N	***16N:1P***	10 mM P	160 mM N
20 mM P	**[PO_4]**	**[NO_3]**			
1N:1P	20 mM P	20 mM N			
2N:1P	20 mM P	40 mM N			
3N:1P	20 mM P	60 mM N			
4N:1P	20 mM P	80 mM N			
5N:1P	20 mM P	100 mM N			
16N:1P	20 mM P	320 mM N			

3.6 Final solution

The incubation sets were filled according to the following scheme: The first column was filled with ‘blanks’, i.e. type 1 water. The second column was filled with DNOM solution tough with inoculum, referred to as ‘no nutrients’. The four remaining columns were filled with DNOM solution, inoculum, and different nutrient solutions. Each row contains four vials giving every solution tested four replicates. In order to not affect the DNOM concentration significantly 25 mL of 20 mg C/L DOC solution was measured out and added 0.250 mL inoculum and 0.250 mL nutrient solution. To ensure no extra oxygen in a headspace or that oxygen makes its way into the sample about 5.1 mL of the solutions were filled in the 5 mL vials before they were sealed. The sets were put up as follows. Column 1 from A1-D1 contained Blanks, column 2 from A2-D2 contained ‘no nutrient' solution, column 3 from A3-D3 contained 1N:1P, column 4 from A4-D4 contained 2N:1P, column 5 from A5-D5 contained 5N:1P and column 6 from A6-D6 contained 16N:1P.

Except for concentrations 10 mM P and 20 mM P, where the order was turned having the blanks in A6-D6.

3.7 Instrumentation

The respiration rate was monitored by measuring the oxygen content using a PreSens SensorVial (SV-PSt5-4mL) with a SensorDish® Reader (SDR) Basic Set. The SensorVials used in the experiment are borosilicate glass vials, and screwcaps with a rubberized septum to seal the vials. To further ensure the seal Parafilm was wrapped around the screwcaps as well. A spot type sensor (PreSens Oxygen Sensor Spot SP-PSt5) is located at the bottom of each SensorVial. The sensors are made of hydrophobic silicon doped with rubidium and have a luminescent dye. The vials are placed in a 24-well plate, which is placed on top of the SDR. At every 15-second interval during the incubation, the SDR emits a flick of light that excites the dye. After excitation, the sensor phosphorescent light is detected by the SDR. Oxygen in the solution acts as a quencher for the phosphorescence and will reduce the luminescent lifetime. The partial pressure of oxygen in the sample thus determines the luminescent lifetime in the sensor. This luminescent lifetime is translated back to oxygen concentration by the software (PreSens SDR_v4.0.0), giving the concentration of oxygen in the sample.

3.8 Reactions in the incubator

The PreSens software calculates the oxygen concentration from the Stern-Volmer relationship, Equation 1.

$$\frac{I0}{I} = 1 + K_{sv} * [O_2]$$

Equation 1. Stern-Volmer relationship

Where I_0 denotes luminescent lifetime with no oxygen, I is the actual luminescent lifetime of the sample, Ksv denotes quenching rate and $[O_2]$ is the oxygen (quencher) concentration. K_{sv} is dependent on temperature. Examples of the time trends in O_2 concentration during the incubation are shown in Figure 1. The initial apparent increase in O_2 concentration is due to the effect on K_{sv} of the temperature increase in the sample upon entering the incubator. After reaching constant temperature the O_2 concentration

then tries to stabilize before starting the decomposition phase. The length of the following linear decline in O_2 concentration, during the decomposition phase, is depended on the amount of organic substrate and the available O_2, as well as other nutrients such as nitrate and phosphate. During the initial linear decomposition, one may assume that the amount of O_2 and nutrients is not a limiting factor. The rate of which the O_2 is consumed, i.e. the slope of the declining O_2 concentration, is then reflecting the biodegradability of the DNOM. The bacteria will often consume the most labile parts of the substrate first before they start to consume other more recalcitrant parts. At the end of the incubation, the decline in O_2 concentration decreases. This may be due to that there is no more DNOM to decompose, but it may also be due to lack of O_2 or nutrients.

3.9 Respiration rate

The respiration rate (RR), which is the slope of the decrease in O_2 concentration, it is a measure for the rate of biodegradation and thus the biodegradability of the DNOM. In each sample the RR was determined by calculating the reduction in oxygen concentration during its linear decrease, divided by the time period of the linear decrease (Amon and Benner, 1996), thereby determining the slope of the curve where oxygen decreases linearly. This was done in an R script (Appendix 8.2.2). Input to the program is the data from the PreSense monitoring of O_2 concentration during the incubation. The script graphs the oxygen consumption vs time and calculates the RR. The start of exponential consumption is defined as the point at which the measured values are more than 3x the standard deviation lower than the initial measurements. This ensures that the initial measurements, which may be skewed from initial temperature fluctuations are not included in the calculation.

3.10 Absorbance

The UV- and visual absorbance specters between 800-200 nm of the solutions were measured using a spectrophotometer (UV-1800 Shimadzu UV spectrophotometer) after the sets had been in the incubator for more than 150 hours. The spectrophotometer is background-corrected before the analysis using type 1 water. Under the analysis, there is kept a cuvette filled with type 1 water as a reference. The analysis starts at 800 nm

using a tungsten hydrogen lamp for UV-light, then at 341 nm the instrument automatically changes the lamp to a Deuterium lamp as it descends to 200 nm. Like mentioned in chapter 2.4 the bacteria in the samples absorb UV-light and scatter light in the ViS-light. Nitrate and phosphate also absorb light in the UV-spectrum. Therefore, the absorbance should be looked at in the ViS-spectrum. It is likely that the light will be scattered the same amount for the samples. The methods relevant for this experiment is therefore, sViSa and the E4/E6 ratio. sViSa would have been relevant if there had been done a measurement of the concentration of DOC after the incubation, there were not and therefore is the E3/E4 ratio the chosen method. E4/E6 ratio is defined as the absorbency at 465 and 665nm. It is found to decrease with increasing molecular weight, condensation, and aromaticity. This ratio thus serves as an index of humification.

4. Results and discussion

4.1 Concentration of Oxygen

The PreSens calculates the concentration of oxygen in μmol/L. These concentrations have been plotted against time during the incubation in order to provide an overview of the fluctuations that happen to the oxygen concentration in the sealed environments. Figure 1 shows the plots made by the PreSense software of O_2 in the inoculated sample with 10 mM Phosphate for the four replicates for the ratio 2N:1P. The red area is the slope used to calculate the concentration of oxygen. The blue is from the beginning of measuring to max value. The red area starts from the point in which value is below 3 x standard deviation of the blue area. What it shows is that for the first hours the oxygen concentration increases and then it slowly decreases. The increase is linked to the temperature rise from room temperature to a constant temperature of 25 ^{o}C as described in chapter 3.8. The following decrease is due to the bacteria's use of O_2 in their heterotrophic decomposition of DNOM.

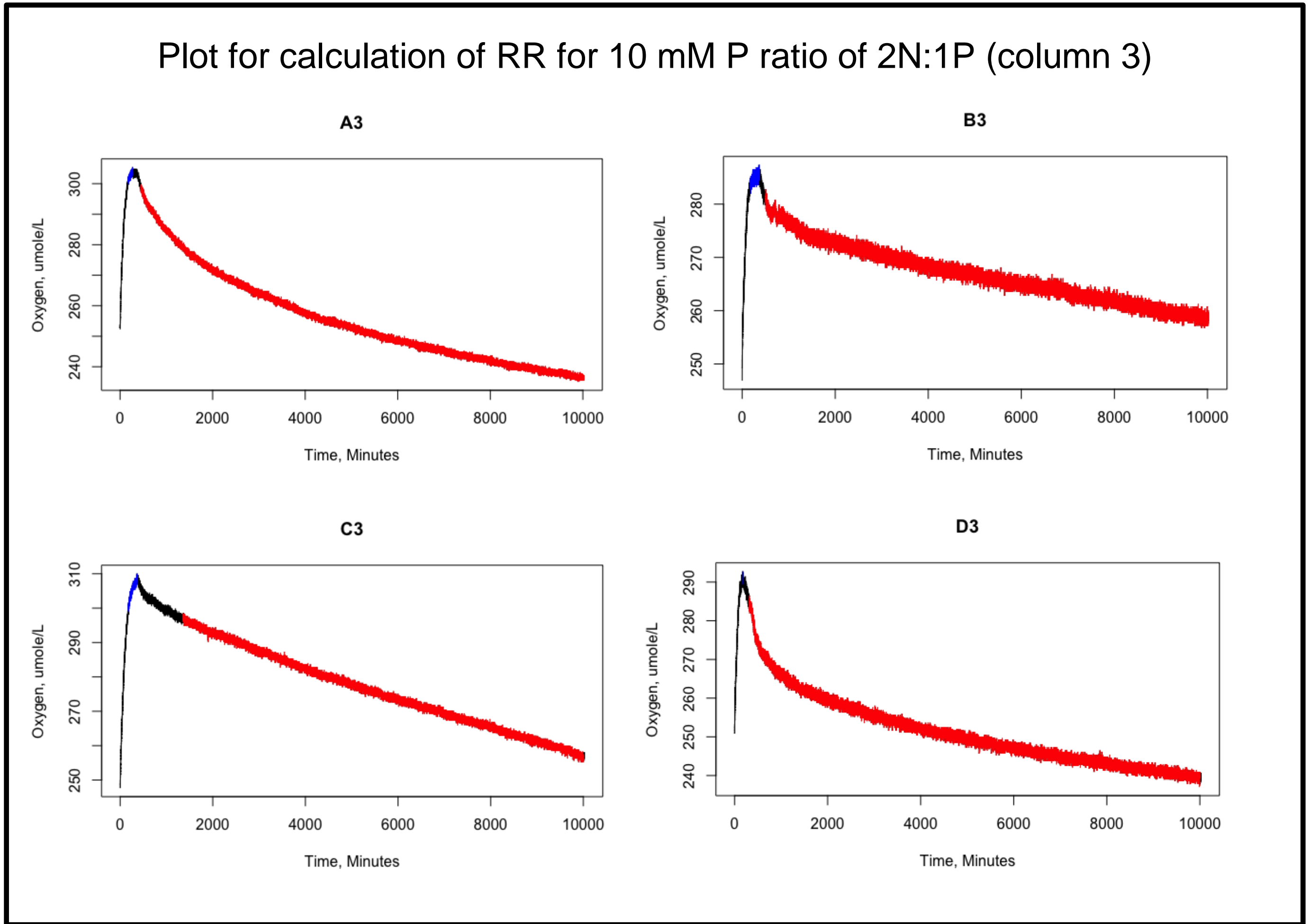

Figure 1. The decline in oxygen concentration for calculating the RR, for the concentration 10 mM P and ratio 2N:1P

4.2 Respiration rate

The respiration rate was calculated in an R studio script (Appendix 8.2.2). The program calculates the plot from Figure 1 of the concentration of oxygen and then finds the slope of the curve by dividing the change in concentration by time. The respiration rates (RR) were then calculated as the means of four replicates and presented in Table 2.

Table 2. Respiration rates for the means of concentration (0.1, 1, 5, 10, 20) mM P and ratios (1, 2, 3, 4, 5, 16)N:1P.

	0,1 mM P	1 mM P	5 mM P	10 mM P	20 mM P
Blanks	0.16 ± 0.03	0.21 ± 0.08	0.05 ± 0.01	0.12 ± 0.03	0.32 ± 0.06
No nutrient	0.4 ± 0.1	0.5 ± 0.2	0.31 ± 0.01	0.28 ± 0.06	0.3 ± 0.1

1N:1P	0.28 ± 0.06	0.38 ± 0.04	0.37 ± 0.07	0.32 ± 0.03	0.29 ± 0.09
2N:1P	0.36 ± 0.07	0.47 ± 0.06	0.33 ± 0.03	0.3 ± 0.1	0.6 ± 0.4
5N:1P	0.35 ± 0.06	0.5 ± 0.1	0.30 ± 0.03	0.36 ± 0.01	0.40 ± 0.06
16N:1P	0.34 ± 0.05	0.39 ± 0.08	0.25 ± 0.03	0.27 ± 0.07	0.11 ± 0.03

The respiration rates were then plotted against the ratio of nitrogen (1, 2, 3, 5, 16) for all the concentration of phosphorus (0.1, 1, 5, 10, 20) mM. Figure 2 is showing off both the means of respiration rates and the standard deviation.

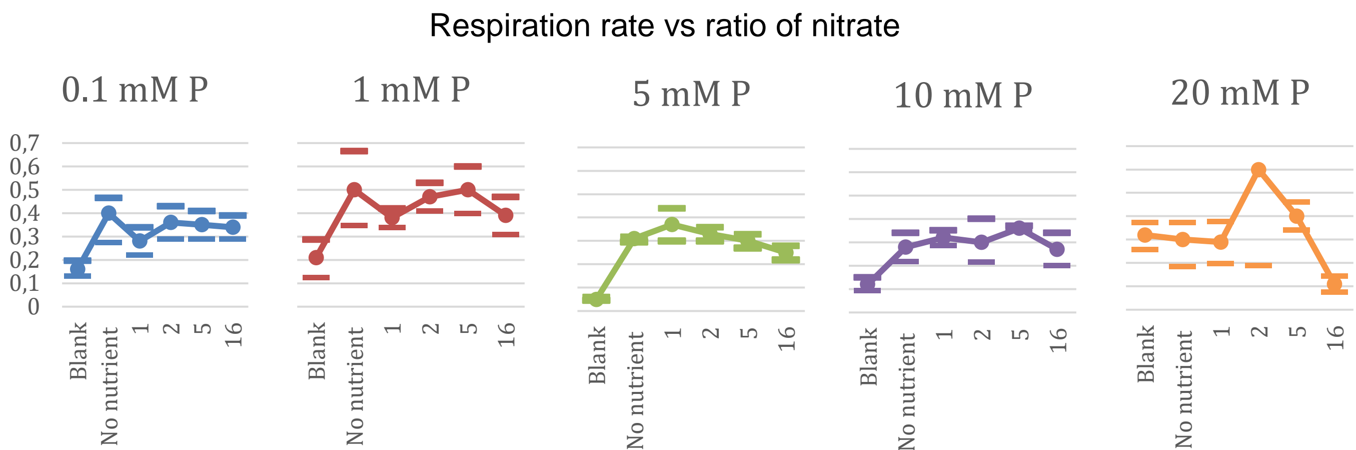

Figure 2. The respiration rates for the mean and standard deviation of concentrations 0.1, 1, 5, 10, and 20 mM P and ratios 1, 2, 5, 16N: 1P, blanks, and no nutrient solution.

For the concentrations that looked to generate the highest respiration rates, there was done a second run with ratios from 1N-5N:1P, as these ratios seemed to give higher respiration rates. The concentrations chosen were 1, 10, and 20 mM P. The respiration rates for the second run are listed in Table 3.

Table 3. Respiration rates for the concentration 1, 10, 20 mM P, blanks, and ratios 1-5N:1P

	1 mM P	10 mM P	20 mM P
Blanks	0.2 ± 0.1	0.15 ± 0.03	0.13 ± 0.02
1N:1P	0.32 ± 0.04	0.37 ± 0.04	0.38 ± 0.06
2N:1P	0.32 ± 0.05	0.27 ± 0.03	0.409 ± 0.002

3N:1P	0.27 ± 0.03	0.31 ± 0.02	0.5 ± 0.3
4N:1P	0.33 ± 0.04	0.30 ± 0.03	0.43 ± 0.06
5N:1P	0.28 ± 0.06	0.32 ± 0.05	0.37 ± 0.01

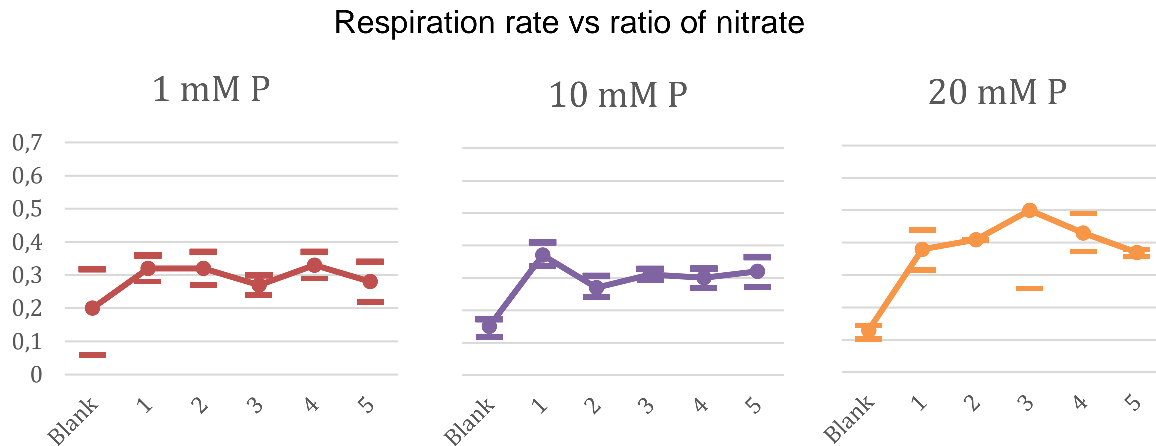

Figure 3. The respiration rates for the mean and standard deviation of concentrations 1, 10, and 20 mM P and ratios 1, 2, 3, 4, and 5N: 1P and blanks

The tested concentration of phosphorus was 0.1, 1, 5, 10, 20 mM and then the nitrogen was added in different concentrations depending on the targeted ratio to phosphorus (Table 1). The respiration rates show no clear response to neither the concentrations of phosphate nor the ratios of nitrate to phosphate. The standard deviations show large variations (Table 2, Figure 2). Moreover, the RR for the 'No nutrient' sample differs by almost a factor of 2. This is troublesome as these are sample replicates. Relative to the 'No nutrient' sample the addition of nutrients did not lead to any significant increase in RR, though there may be an indication of a reduced RR at high P and N:P ratios. The concentration 20 mM P and ratio 2N:1P has a high RR mean of 0.6 which would have been good had it not been for the standard divination of 0.4. Now with such a high standard deviation, there was done an outliner test (Grubbs -test).

$G_{exp} = \frac{suspect\ value - average}{standard\ divination}$ if $G_{exp} > G_{cri}$ then the suspect value can be rejected

For concentration 20 mM P and ratio 2N:1P the G_{exp} = 1.44 and the G_{cri} (p= 0.05) = 1.481 for n = 4 where n is number of replicates. This means that $G_{exp} < G_{cri}$ and that the

suspected value cannot be rejected. The big standard deviation must have an explanation as it is the exact same sample in all four replicates. The Grubbs-test was performed for all suspect values and is listed in appendix 8.3. The second run with ratios 1-5N shows different RR then for the same ratios and concentrations for the first run. They also have equally varying standard deviations. What also sets the tone for these RR is that there is seemingly no rational connection to where the RR is high and where it is low.

The rationale for the hypothesis was that there was an optimum concentration and ratio of nutrients was based on that other preceding studies (Håland, 2017, Holm, 2018, Martinez, 2017) have done similar experiments but used concentrations of 10 mM phosphorus and nitrate which is possibly so high they have become toxic for the bacteria. This toxicity is most likely why there is a decline at all phosphate concentrations from the ratio 5N:1P to 16N:1P. The toxicity is likely the reason for the concentration of 20 mM P and the ratio of 16N:1P has such a low RR of 0.11 ± 0.03.

4.3 Respiration rates with a higher concentration of N & P in the inoculum

There were made some mistakes in the preparation of the inoculum (Chapter 3.1.2) that were not discovered until after the sets were run on the incubator. Therefore, there is now also calculated respiration rates for the higher concentration of phosphate and nitrate in the inoculum for the concentration of 0.1, 1, 10, and 20 mM P. This means that the bacteria have had a higher nutrient solution in the growth phase.

Table 4. The Respiration rates for concentration 0.1- and 1-mM P, with the blanks, no nutrients solutions, ratios (1, 2, 5, 16) N:1P with the concentration 1 mM N & P in the inoculum. The respiration rates for concentration 10- and 20-mM P, with the blanks, no nutrients solutions, ratios (1, 2, 5, 16) N:1P with the concentration 0.2 mM N & P in the inoculum.

Concentration of N & P in the inoculum	*1 mM N & P in inoculum*		*0.2 mM N & P in inoculum*	
Concentration of P and ratio of N : P during the incubation	***0,1 mM P***	***1 mM P***	***10 mM P***	***20 mM P***
Blanks	0.17 ± 0.05	0.17 ± 0.03	0.098 ± 0.007	0.12 ± 0.03
No nutrients	0.41 ± 0.06	0.36 ± 0.03	0.3 ± 0.1	0.129 ± 0.005
1N:1P	0.5 ± 0.1	0.41 ± 0.09	0.4 ± 0.1	0.36 ± 0.03
2N:1P	0.4 ± 0.2	0.41 ± 0.02	0.51 ± 0.01	0.46 ± 0.05
5N:1P	0.28 ± 0.03	0.5 ± 0.1	0.32 ± 0.04	0.35 ± 0.02

16N:1P	0.28 ± 0.01	0.44 ± 0.08		0.4 ± 0.1	0.36 ± 0.03

What these RR show is by adding a higher concentration of nitrate and phosphate in the growth of bacteria in the inoculum has little or even positive effect on the RR. As these are generally higher RR but as varying in standard deviations as for the concentration of 0.1 mM nutrients in the inoculum.

4.4 Absorbance

The absorbance for 465 nm and 665 nm for the samples after incubation was found and divided to the ratios in Table 5. E_4/E_6 for the original DNOM solution was found to be 6.4.

Tabell 5. The E_4/E_6 ratio for concentrations 0.1, 1, 5, 10, 20 mM P, ratios 1, 2, 5, 16 N:1P, no nutrients and blanks.

E4/E6	**0.1**	**1**	**5**	**10**	**20**
Blank			0.6	3.4	1.0
No nutrients	5.8	3.4	5.6	5.8	8.1
1N:1P	3.8	3.4	5.0	6.3	8.7
2N:1P	3.3	3.6	5.0	6.2	9.3
5N:1P	3.4	2.7	4.6	6.9	8.0
16N:1P	3.6		5.3	7.8	9.0
Original DNOM	6.4				

The E_4/E_4 ratio can be shown visually (Figure 4) with the original DNOM solution as the black first column to compare with the solutions after incubation.

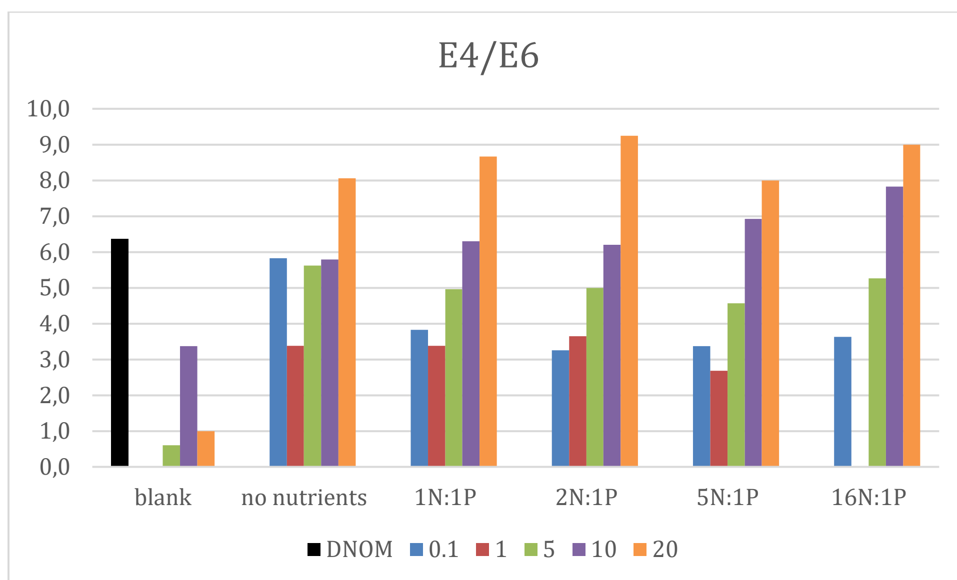

Figure 4. The E4/E6 ratio, with the original DNOM solutions ratio in black.

The absorbance for the ratios of nitrate is relatively equal, but the higher the concentration of phosphate the higher the E_4/E_6 ratio, except for the 0.1 and 1 mM P where 0.1 mM P has a higher ratio. By comparing the original DNOM solution to the samples after incubation there can be seen that the original DNOM solution has a ratio above the lowest concentrations but from concentration 10 mM P and ratio 5N:1P the samples have higher ratios. A decrease in E_4/E_6 ratio from the original DNOM solution to the samples after the incubation means that there is an increase of relative aromaticity and molecular weight (MW). Meaning that the bacteria have eaten the aliphatic LMW DNOM compounds and the ratio of HMW to LMW has shifted and in turn also leaving more aromatic compounds then aliphatic. This is what can be seen for the lower concentrations of phosphate, from 0.1 mM P to 10 mM P up to ratio 5N:1P. For the higher concentrations there seems the previously assumed toxicity is again confirmed, as there is a higher E_4/E_6 ratio then for the original DNOM solution.

5. Conclusion

This experiment shows that the concentration of phosphate and the ratio of nitrate is not a critical factor on the respiration rate. But there may still be some points to consider. The highest concentration of 20 mM P and a ratio of 16:1 seems to have poisoned the bacteria and should be avoided. Also, the lower ratios of N:P have higher variations and higher standard divinations. Therefore, it seems to have a ratio of N:P bigger than 5:1 can give more stable conditions, as they are less sensitive to the N:P ratio. Thus, giving the method of measuring the biodegradability a better accuracy, which is highly needed.

6. Future work

For future work, the most important part should be to achieve a lower standard deviations. The present method has too large uncertainties. Perhaps this is caused by signal variation in the wells and could somehow be accounted for by calibrating each well. Another potentially interesting issue is to look into adding more nutrients in the inoculum as the mentioned mistakes from Chapter 4.3, as this appears to give higher respiration rates, and not the poisoning effect that was feared.

7. References

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8. Appendix

8.1 Concentration of oxygen

The concentration of oxygen from the pre sens is a huge file for each set and have therefore been plotted against time (h).

8.1.1 Plot of the concentration of oxygen against time

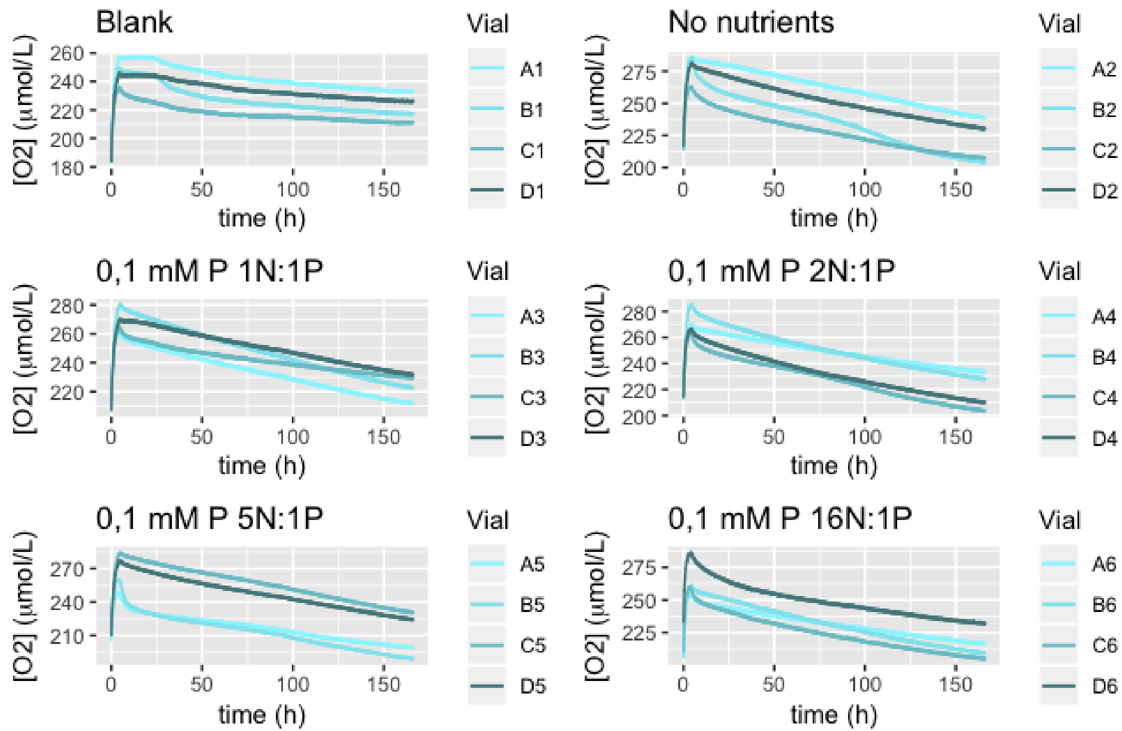

Figure 5 Concentration of oxygen for 0.1 mM P for ratios 1, 2, 5, 16 N, blank and no nutrient solution

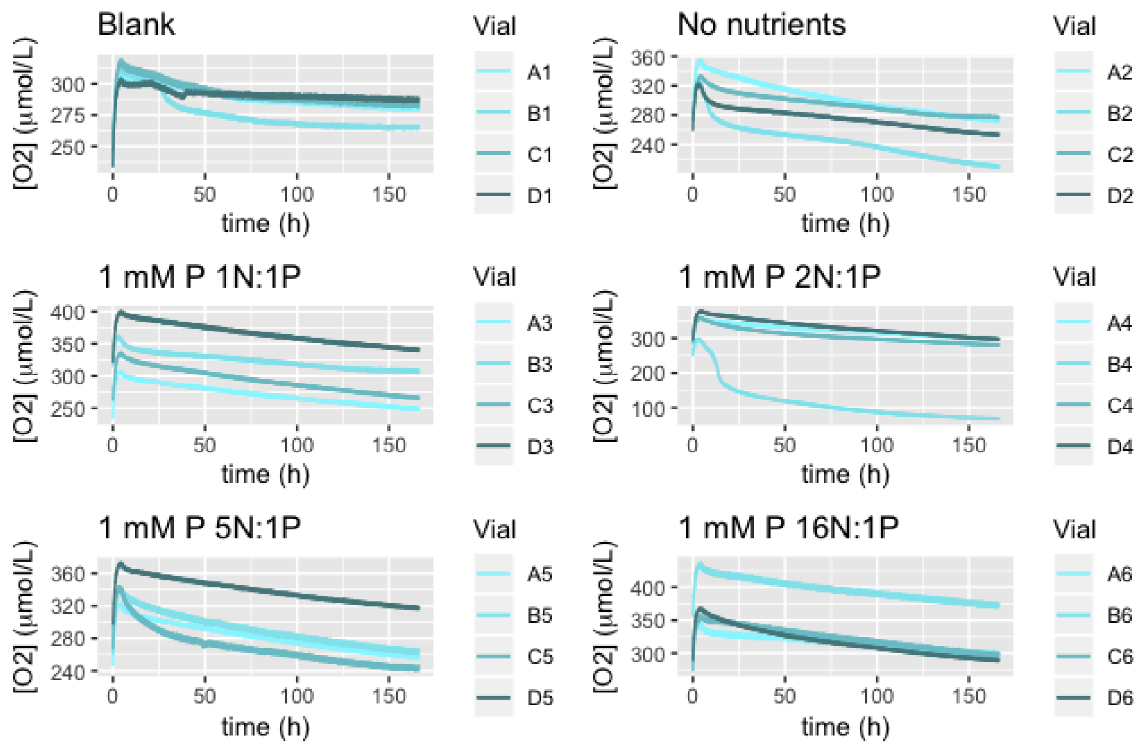

Figure 6. Concentration of oxygen for 1 mM P with ratios 1, 2, 5, 16 N, blank and no nutrient solution.

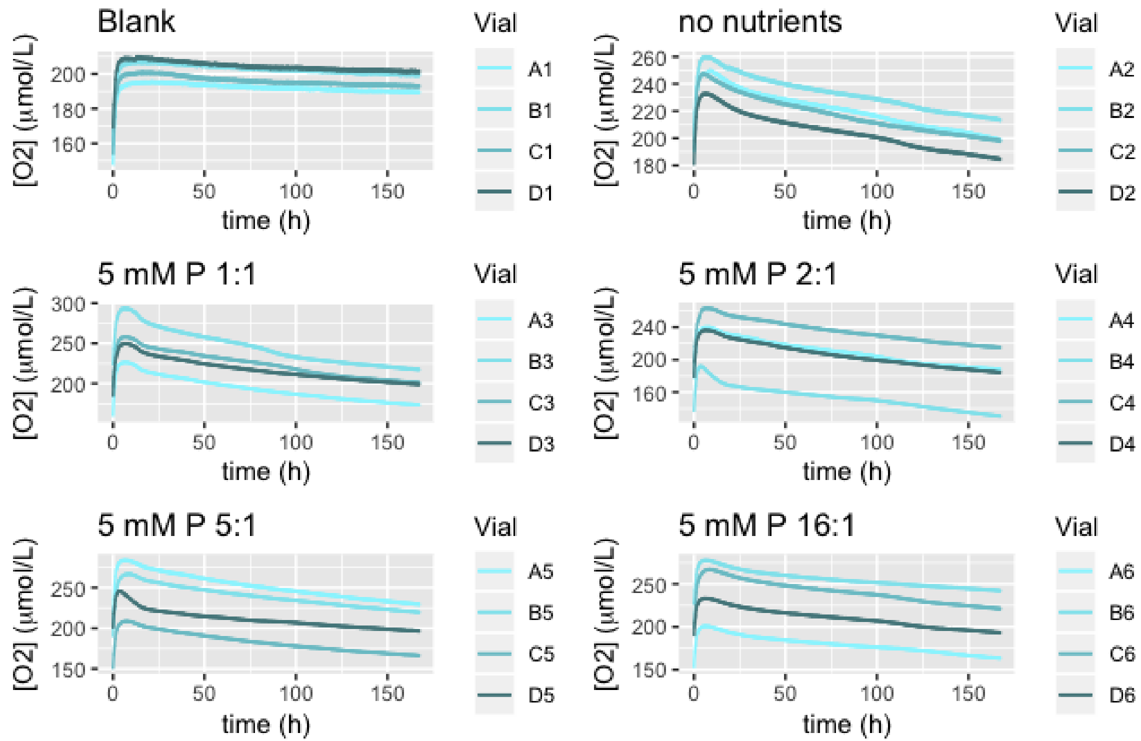

Figure 7. Concentration of oxygen for 5 mM P with ratios 1, 2, 5, 16 N, blank and no nutrient solution.

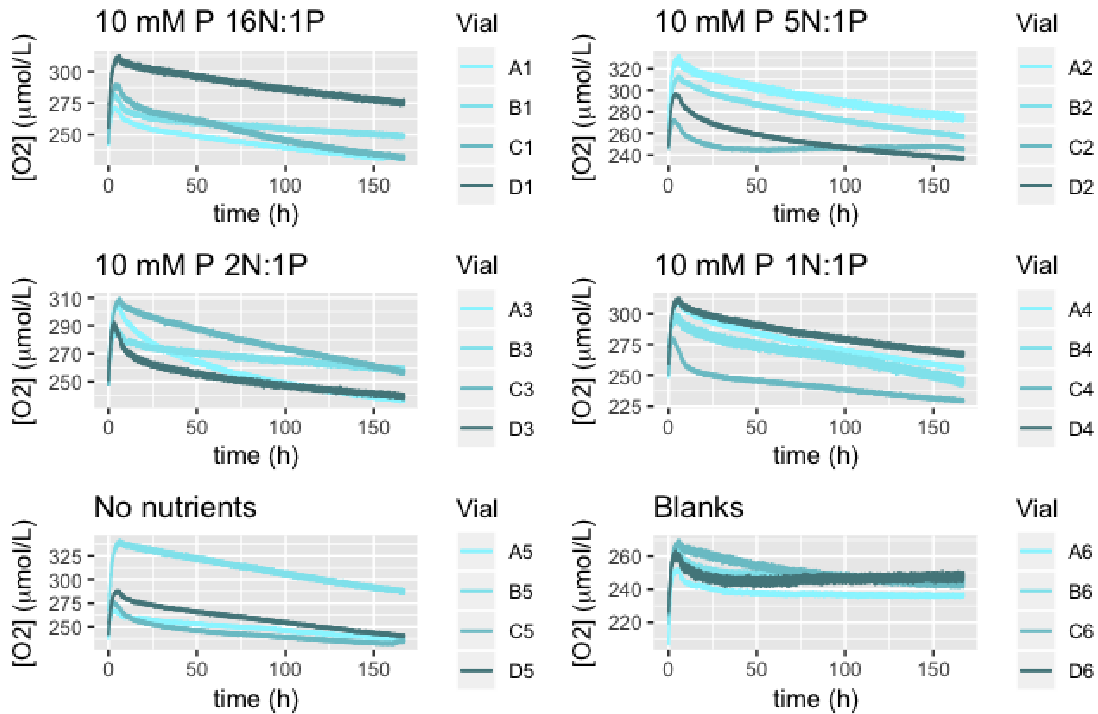

Figure 8. Concentration of oxygen for 10 mM P with ratios 1, 2, 5, 16 N, blank and no nutrient solution.

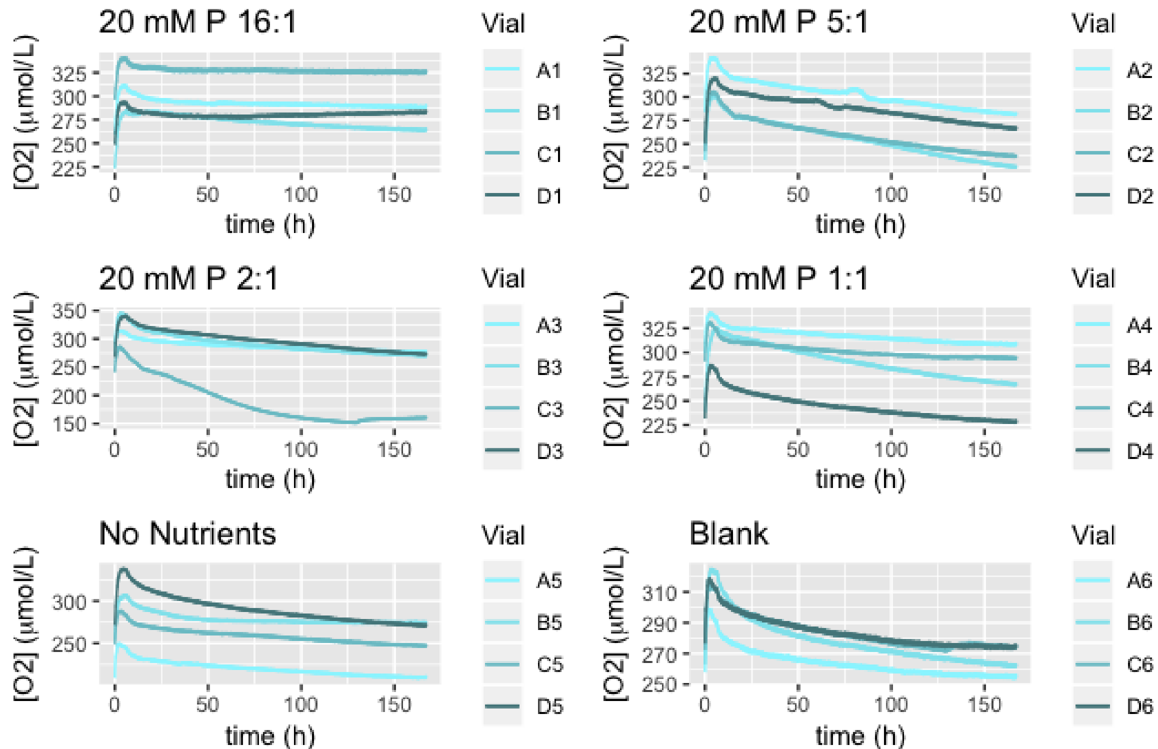

Figure 9. Concentration of oxygen for 20 mM P with ratios 1, 2, 5, 16 N, blank and no nutrient solution.

8.1.2 Plotting the concentration of oxygen

Plotting the concentration of oxygen was done using an R script, made by Camille

```
libraries <- c("dplyr","ggplot2","gridExtra","stringr")
lapply(libraries,library,character.only=TRUE)
#Load data to be plotted, in .csv format
nutrients <- read.csv2("5mM_510_25_3_Oxygen.csv")
vialplot <- function (data,line_number,plottitle){
  vials <- str_c(c("A","B","C","D"),line_number,sep="")
ggplot(data,aes(x=Time.Min./60))+geom_line(aes(y=data[,vials[1]],color=as.character(vi
als[1])))+
    geom_line(aes(y=data[,vials[2]],color=as.character(vials[2])))+
    geom_line(aes(y=data[,vials[3]],color=as.character(vials[3])))+
    geom_line(aes(y=data[,vials[4]],color=as.character(vials[4])))+
    labs(x="time (h)",y=expression(paste("[O2] (",mu,"mol/L)")),title=plottitle)+

scale_color_manual(name="Vial",values=c("cadetblue1","cadetblue2","cadetblue3","cad
etblue4"))
}
#applies the function vialplot to each line. 6 graphs are created
line1 <- vialplot(nutrients,1,"Blank")
```

```
line2 <- vialplot(nutrients,2,"no nutrients")
line3 <- vialplot(nutrients,3,"5 mM P 1:1")
line4 <- vialplot(nutrients,4,"5 mM P 2:1")
line5 <- vialplot(nutrients,5,"5 mM P 5:1")
line6 <- vialplot(nutrients,6,"5 mM P 16:1")
#creates an image with the 6 graphs above
grid.arrange(nrow=3,ncol=2,line1,line2,line3,line4,line5,line6)
###Creates a function that plots ALL the lines, one graph per line, with the same y scale.
Graphs are automatically saved in the working directory.
vialplot2 <- function (data,y_lim){
  for (line_number in 1:6){
    plottitle <- paste(deparse(substitute(data)),"_L",as.character(line_number),sep="")
#transforms data name and line_number in characters
    vialplot(data,line_number,plottitle)+ylim(y_lim)
    ggsave(paste(plottitle,".png",sep=""),width = 11, height =8)
  }
}
y_lim <- c(0,350)
vialplot2(nutrients,y_lim)
```

8.2 Respiration rate

8.2.1 Respiration rate average

The respiration rate as average, with standard deviation and relative standard deviation.

Table 5. The average respiration rate of concentrations (0.1, 1, 5, 10, 20) mM, ratios (1, 2, 5, 16) as well as blanks (type 1 water) and no added nutrients, with standard deviation.

	0,1 mM P	**1 mM P**	**5 mM P**	**10 mM P**	**20 mM P**
Blanks	0.16 ± 0.03	0.21 ± 0.08	0.05 ± 0.01	0.12 ± 0.03	0.32 ± 0.06
No nutrient	0.4 ± 0.1	0.5 ± 0.2	0.31 ± 0.01	0.28 ± 0.06	0.3 ± 0.1
1N:1P	0.28 ± 0.06	0.38 ± 0.04	0.37 ± 0.07	0.32 ± 0.03	0.29 ± 0.09
2N:1P	0.36 ± 0.07	0.47 ± 0.06	0.33 ± 0.03	0.3 ± 0.1	0.6 ± 0.4
5N:1P	0.35 ± 0.06	0.5 ± 0.1	0.30 ± 0.03	0.36 ± 0.01	0.40 ± 0.06
16N:1P	0.34 ± 0.05	0.39 ± 0.08	0.25 ± 0.03	0.27 ± 0.07	0.11 ± 0.03

Table 6. The average respiration rate of concentrations (1, 10, 20) mM, ratios (1, 2, 3, 4, 5) and blanks (type 1 water), with standard deviation.

	1 mM P	**10 mM P**	**20 mM P**

Blanks	0.2 ± 0.1	0.15 ± 0.03	0.13 ± 0.02
1N:1P	0.32 ± 0.04	0.37 ± 0.04	0.38 ± 0.06
2N:1P	0.32 ± 0.05	0.27 ± 0.03	0.409 ± 0.002
3N:1P	0.27 ± 0.03	0.31 ± 0.02	0.5 ± 0.3
4N:1P	0.33 ± 0.04	0.30 ± 0.03	0.43 ± 0.06
5N:1P	0.28 ± 0.06	0.32 ± 0.05	0.37 ± 0.01

Table 7. The average respiration rate of concentrations (0.1, 1, 10, 20) mM, ratios (1, 2, 5, 16) as well as blanks and no added nutrients, with standard deviation. With a higher concentration of N&P in the inoculum.

Concentration of N & P in the inoculum	1 mM N & P in inoculum		0.2 mM N & P in inoculum	
Concentration of P and ratio of N : P during the incubation	***0,1 mM P***	***1 mM P***	***10 mM P***	***20 mM P***
Blanks	0.17 ± 0.05	0.17 ± 0.03	0.098 ± 0.007	0.12 ± 0.03
No nutrients	0.41 ± 0.06	0.36 ± 0.03	0.3 ± 0.1	0.129 ± 0.005
1N:1P	0.5 ± 0.1	0.41 ± 0.09	0.4 ± 0.1	0.36 ± 0.03
2N:1P	0.4 ± 0.2	0.41 ± 0.02	0.51 ± 0.01	0.46 ± 0.05
5N:1P	0.28 ± 0.03	0.5 ± 0.1	0.32 ± 0.04	0.35 ± 0.02
16N:1P	0.28 ± 0.01	0.44 ± 0.08	0.4 ± 0.1	0.36 ± 0.03

8.2.2 Respiration rate calculation

The R script used to calculate the respiration rates (RR).

```
rm(list=ls())
graphics.off()

library("ggplot2")
library("readxl")
library("factoextra")
setwd("/maps")
raw_tbl <- read_excel("./name of document.xlsx", sheet = "sheet name")
summary(raw_tbl)
raw_tbl = data.frame(raw_tbl, check.names = TRUE) # new code
rownames(raw_tbl) = raw_tbl[,1]
raw_tbl <- raw_tbl[,-which(names(raw_tbl) %in% c("Date.Time"))] #new code
L = dim(raw_tbl)[1]
```

```
names(raw_tbl)[which(names(raw_tbl)=="T_internal...C.")] <- "TempC" # Change
column name to "TempC"
temp_cutoff = median(raw_tbl$TempC)- sd(raw_tbl$TempC[floor(L/2):L]) #new code
temp_cutoff_index = which(raw_tbl$TempC > temp_cutoff)[1]#new code
names = c()
plateau_index = c()
for (i in names(raw_tbl)) {
  if (!grepl("Time", i) && !grepl("TempC", i)) {
    plat = which(raw_tbl[,i] == max(raw_tbl[,i]))
    plat = plat[length(plat)]

    names= c(names, i)
    plateau_index= c(plateau_index, plat)
  }
}
indexes = data.frame(T_cut=rep(temp_cutoff_index, length(names)),
P_cut=plateau_index, S_cut=rep(NA, length(names)))
rownames(indexes) = names
for (j in names) {
  sel = raw_tbl[,j][indexes[j,"T_cut"]:indexes[j,"P_cut"]]
  slope_cut = which(raw_tbl[,j] >= mean(sel)-3*sd(sel))
  if (length(sel)>1) {
    indexes[j, "S_cut"] = slope_cut[length(slope_cut)]
  }

}
RR_names = c()
RR = c()
for (k in names) {
  #plotting + selection of cut-off point
  plot(raw_tbl[,"Time.Min."], raw_tbl[,k], type="l", main=k, ylab="Oxygen, ?mole/L",
xlab="Time, Minutes")
  plateau_idx =indexes[k,"T_cut"]:indexes[k,"P_cut"]
  lines(raw_tbl[plateau_idx,"Time.Min."], raw_tbl[plateau_idx, k], col=4)
    # calculating RR
  if (!grepl("Blank", k)) {
    print("Please click on the plot (just once):\n")
    loc = locator(1)
    if (raw_tbl[L,"Time.Min."] > floor(loc$x)) {
      End_cut = which(raw_tbl[,"Time.Min."]>floor(loc$x))[1]
    } else {
      End_cut = L
    }
    slope_idx = indexes[k,"S_cut"]:End_cut
```

```
    lines(raw_tbl[slope_idx,"Time.Min."], raw_tbl[slope_idx, k], col=2)
    RR_names = c(RR_names, k)
    #RR = c(RR,(raw_tbl[indexes[n,"P_cut"],n]-raw_tbl[L,n])/((raw_tbl[L,"Time/Min."]-
raw_tbl[indexes[n,"P_cut"],"Time/Min."])/60)) #old
    delta_oxygen = raw_tbl[indexes[k,"P_cut"],k]-raw_tbl[End_cut,k]
    delta_time = (raw_tbl[End_cut,"Time.Min."]-
raw_tbl[indexes[k,"P_cut"],"Time.Min."])/60
    # cat("delta oxygen:",delta_oxygen, "\n")
    # cat("delta time:",delta_time, "\n")
    RR = c(RR, delta_oxygen/delta_time)
  } else {
    slope_idx = indexes[k,"S_cut"]:L
    lines(raw_tbl[slope_idx,"Time.Min."], raw_tbl[slope_idx, k], col=2)
  }
}
RR = data.frame(RR=RR)
rownames(RR) = RR_names
#library("xlsx")
write.csv(x= RR, file= "./name of new document.txt")
```

8.3 Statistics

All RR has been calculated as average with standard deviation and relative standard deviation. Some samples have a suspiciously high standard deviation and have been tested with an outliner test.

Grubbs test: looking for outliners

$$G_{exp} = \frac{suspisius\ value - average}{standard\ divination}$$

G_{exp} > Gcri then the suspected value can be rejected
G_{cri} = 1.481 with n = 4 when n is number of replicates (p = 0.05)

8.3.1 Grubbs test for the first run of samples

Table 8. Respiration rates (RR) for all replicates with average, standard divination, relative standard divination, and G_{exp}.

	0.1 mM P				1 mM P			
	RR			G_{exp}	RR			G_{exp}
A1	0.17506679	Blank			0.18733718	Blank		
B1	0.20150008	X	0.16		0.30014238	X	0.21	

C1	0.15590159	S	0.03		0.23027345	S	0.08	
D1	0.12434845	RSD (%)	19.79		0.1075819	RSD (%)	39.06	
A2	0.3000949	No nutrients			0.52748387	No nutrients		
B2	0.51036299	X	0.4	B2: 1.47	0.71987558	X	0.5	B2: 1.34
C2	0.34965804	S	0.1		0.35135404	S	0.2	
D2	0.32069953	RSD (%)	25.8		0.42883535	RSD (%)	31.4	
A3	0.31054502	1N:1P			0.36600998	1N:1P		
B3	0.36023011	X	0.28		0.34305363	X	0.38	
C3	0.2191902	S	0.06		0.44447212	S	0.04	C3: 1.44
D3	0.24064421	RSD (%)	22.92		0.37271445	RSD (%)	11.48	
A4	***0.2258281***	2N:1P			0.39989091	2N:1P		
B4	0.35781371	X	0.36	A4: 1.51	***1.4187195***	X	0.47	B4: 1.5
C4	0.36819285	S	0,.07		0.50130372	S	0.06	
D4	0.35568326	RSD (%)	18.75		0.49840853	RSD (%)	12.37	
A5	0.30651985	5N:1P			0.4053832	5N:1P		
B5	0.44237144	X	0.35	B5: 1.47	0.487337	X	0.5	C5: 1.31
C5	0.33354247	S	0.06		0.61543197	S	0.1	
D5	0.33023108	RSD (%)	17.18		0.35399368	RSD (%)	24.5	
A6	***0.23859425***	16N:1P			0.29222395	16N:1P		
B6	0.32324478	X	0.34	A6: 1.48	0.41343685	X	0.39	
C6	0.34064371	S	0.05		0.37944344	S	0.08	
D6	0.34161497	RSD (%)	14.63		0.49377648	RSD (%)	21.15	
5 mM P					**10 mM P**			
	RR			G_{exp}	RR			G_{exp}
A1	0.045528343	Blank			0.269958844	16N:1P		
B1	0.054107155	X	0.05		0.210127514	X	0,27	C1: 1.40
C1	0.056093954	S	0.01		0.368905887	S	0,07	
D1	0.061472904	RSD (%)	12.20		0.36571151	RSD (%)	25,60	
A2	0.322441058	No nutrients			0.370413846	5N:1P		
B2	0.297001967	X	0.31		0.355524616	X	0,36	C2: -1.50
C2	0.306421772	S	0.01		***0.162856633***	S	0,01	
D2	0.304791827	RSD (%)	3.47		0.3650409	RSD(%)	2,07	
A3	0.331687312	1N:1P			0.425608377	2N:1P		
B3	0.478976712	X	0.37		0.18055346	X	0,3	A3: -1.34
C3	0.361110643	S	0.07		0.338777502	S	0,1	
D3	0.319669752	RSD (%)	19.54		0.319473626	RSD(%)	32,1	
A4	0.328251518	2N:1P			0.34821342	1N:1P		
B4	0.379086461	X	0.33	B4: 1.40	0.340152045	X	0,32	D4: -1.35
C4	0.302132069	S	0.03		0.315667482	S	0,03	
D4	0.32494577	RSD (%)	9.73		0.276765163	RSD(%)	10,02	
A5	0.342651395	5N:1P			0.194236748	No nutrients		
B5	0.297834077	X	0.30		0.339406503	X	0,28	A5: -1.38
C5	0.272209247	S	0.03		0.281136907	S	0,06	
D5	0.304655982	RSD (%)	9.57		0.302228144	RSD(%)	22,05	
A6	0.23467577	16N:1P			0.098644498	Blank		
B6	0.223782278	X	0.25		0.121650365	X	0,12	C6: 1.41

C6	0.294813874	S	0.03		0.161912225	S	0,03	
D6	0.254164982	RSD (%)	12.42		0.105926035	RSD(%)	23,16	

20 mM P				
	RR			G_{exp}
A1	0.144959313	16N:1P		
B1	0.132319282	X	0,11	
C1	0.100807625	S	0,03	
D1	0.066721263	RSD (%)	31,46	
A2	0.373384721	5N:1P		
B2	0.471135543	X	0,40	
C2	0.424318156	S	0,06	
D2	0.335508795	RSD(%)	14,76	
A3	0.235020635	2N:1P		
B3	0.467853013	X	0,6	C3: 1.44
C3	1.070635027	S	0,4	
D3	0.430288638	RSD(%)	65,6	
A4	0.196043061	1N:1P		
B4	0.369605245	X	0,29	
C4	0.22756152	S	0,09	
D4	0.363220758	RSD(%)	31,21	
A5	0.247905836	No nutrients		
B5	0.202000963	X	0,3	D5: 1.45
C5	0.253928609	S	0,1	
D5	0.417627127	RSD(%)	33,7	
A6	0.272751538	Blank		
B6	0.386503218	X	0,32	
C6	0.342030885	S	0,06	
D6	0.265601664	RSD(%)	18,28	

8.3.2 Grubbs test for the second run with ratios 1-5N

Table 9. Respiration rates (RR) for the second run of replicates with average, standard divination, relative standard divination, and Gexp.

	1 mM P				**10 mM P**			
	RR			G_{exp}	RR			G_{exp}
A1	0,37272055	Blank			0,143220099	Blank		
B1	0,14326503	X	0.2	A1:1.43	0,16832756	X	0.15	C1:-1.83
C1	0,16620585	S	0.1		0,107402543	S	0.03	
D1	0,07197007	RSD(%)	68.5		0,161834206	RSD(%)	18.84	
A2	0,29350769	1N:1P			0,347905861	1N:1P		
B2	0,28230889	X	0.32		0,379751268	X	0.37	C2: 1.35
C2	0,36478115	S	0.04		0,423236712	S	0.04	
D2	0,32459886	RSD(%)	11.68		0,343899083	RSD(%)	9.82	
A3	0,31622893	2N:1P			0,293019119	2N:1P		

B3	0,37571004	X	0.32		0,241323502	X	0.27	
C3	0,33602393	S	0.05		0,309208327	S	0.03	
D3	0,25277931	RSD(%)	16.02		0,251466566	RSD(%)	11.89	
A4	0,2285494	3N:1P			0,30128448	3N:1P		
B4	0,26708558	X	0.27		0,330506544	X	0.31	D4: 1.50
C4	0,29519094	S	0.03		0,301766396	S	0.02	
D4	0,26937207	RSD(%)	10.36		***1,012650754***	RSD(%)	5.38	
A5	0,29236053	4N:1P			0,326470307	4N:1P		
B5	0,38199334	X	0.33		0,322403673	X	0.30	
C5	0,33619768	S	0.04		0,26906869	S	0.03	
D5	0,3073899	RSD(%)	11.97		0,278576926	RSD(%)	5.38	
A6	0,20968327	5N:1P			0,330067403	5N:1P		
B6	0,34734123	X	0.28		0,297801968	X	0.32	
C6	0,29103843	S	0.06		0,26849215	S	0.05	
D6	0,25703415	RSD(%)	20.97		0,376981048	RSD(%)	14.60	

	20 mM P			
	RR			G_{exp}
A1	0,14698357	Blank		
B1	0,1003076	X	0.13	
C1	0,13552992	S	0.02	
D1	0,11718616	RSD(%)	16.43	
A2	0,45992226	1N:1P		
B2	0,39542313	X	0.38	
C2	0,32388101	S	0.06	
D2	0,33655415	RSD(%)	16.45	
A3	0,40774231	2N:1P		
B3	***0,3292841***	X	0.409	B3:-1,5
C3	0,41113295	S	0.002	
D3	0,40785153	RSD(%)	0.471	
A4	0,47670963	3N:1P		
B4	0,27808811	X	0.5	B4:1.41
C4	0,42037475	S	0.3	
D4	0,86370896	RSD(%)	49.1	
A5	0,38654201	4N:1P		
B5	0,3778762	X	0.43	
C5	0,49078157	S	0.06	
D5	0,45988273	RSD(%)	12.91	
A6	0,35843933	5N:1P		
B6	0,36915787	X	0.37	D6:1.49
C6	0,36963284	S	0.01	
D6	***0,4684312***	RSD(%)	1.73	

8.3.3 Grubbs test for inoculum with higher concentrations of N&P

Table 10. Respiration rates (RR) for replicates with a high concentration of N&P in the inoculum, with average, standard divination, relative standard divination, and Gexp.

	0.1 mM P (inoculum 1 mM nutrients)				**1 mM P (inoculum 1 mM nutrients)**			
	RR			G_{exp}	RR			G_{exp}
A1	0,151771385	Blank			***0,125154111***	Blank		
B1	0,111317894	X	0,17		0,192441539	X	0,19	A1: 1.482
C1	0,215219578	S	0,03		0,182061344	S	0,01	
D1	0,190961323	RSD (%)	17,95		0,182304317	RSD (%)	3,19	
A2	0,411804918	No nutrients			0,319201425	No nutrients		
B2	0,485138849	X	0,36		0,38487749	X	0,36	
C2	0,411737293	S	0,03		0,388594563	S	0,03	
D2	0,344310903	RSD (%)	8,77		0,362073666	RSD (%)	8,77	
A3	0,36168231	1N:1P			***0,543856911***	1N:1P		
B3	0,411956643	X	0,41		0,375895504	X	0,37	A3: 1.49
C3	0,676605728	S	0,09		0,354961531	S	0,01	
D3	0,56798038	RSD (%)	21,33		0,375889943	RSD (%)	3,28	
A4	0,669684683	2N:1P			0,410332817	2N:1P		
B4	0,408909144	X	0,41	A4:1.45	0,402336871	X	0,41	
C4	0,325771875	S	0,02		0,385587781	S	0,02	
D4	0,330499076	RSD (%)	3,88		0,423117832	RSD (%)	3,88	
A5	0,323645936	5N:1P			***1,188419068***	5N:1P		
B5	0,267094101	X	0,5	A5:1.41	0,372474522	X	0,7	A5: 1.43
C5	0,251248238	S	0,1		0,6333942	S	0,4	
D5	0,276808848	RSD (%)	27,8		0,446168743	RSD (%)	55,9	
A6	0,275802584	16N:1P			0,511700673	16N:1P		
B6	0,291738452	X	0,44		0,506125996	X	0,44	
C6	0,287716969	S	0,08		0,362472812	S	0,08	
D6	0,266979285	RSD (%)	18,81		0,370486066	RSD (%)	18,81	
	10 mM P (0.2 mM N&P in inoculum)				**20 mM P (0.2 mM N&P in inoculum)**			
	RR			G_{exp}	RR			G_{exp}
A1	0,10483932	Blank			0,158869	Blank		
B1	0,09113585	X	0.098		0,18646853	X	0.15	C1:1.491
C1	0,09942856	S	0.007		***0,68821779***	S	0.03	
D1	-	RSD (%)	7.009		0,11791356	RSD (%)	22.34	
A2	0,31721281	No nutrients			0,12836738	No nutrients		
B2	0,24440534	X	0.3	D2:1.41	0,13449367	X	0.097	C2:1.495
C2	0,21680961	S	0.1		***0,21913253***	S	0.005	
D2	0,49072527	RSD (%)	38.8		0,12529074	RSD (%)	4.828	
A3	0,29729735	1N:1P			0,34816998	1N:1P		
B3	0,3378573	X	0.4		0,33195351	X	0.36	D3: 1.41
C3	0,51720823	S	0.1		0,35511181	S	0.03	
D3	0,36696933	RSD (%)	25.3		0,40164855	RSD (%)	8.32	
A4	0,50880344	2N:1P			0,44206441	2N:1P		
B4	0,52114868	X	0.51	C4:-1.5	0,5349994	X	0.46	B5: 1.47
C4	***0,28564138***	S	0.01		0,44533789	S	0.05	

D4	0,51386286	RSD (%)	1.21		0,42357709	RSD (%)	10.82	
A5	0,35321421	5N:1P			0,32038529	5N:1P		
B5	0,33230516	X	0.32	D5:-1.42	0,3645443	X	0.35	A5: 1.44
C5	0,31953036	S	0.04		0,3594912	S	0.02	
D5	0,25584876	RSD (%)	13.31		0,37497354	RSD (%)	6.72	
A6	0,25012636	16N:1P			0,34087744	16N:1P		
B6	0,28564894	X	0.4		0,37414457	X	0.36	
C6	0,47478206	S	0.1		0,33747712	S	0.03	
D6	0,40263368	RSD (%)	29.4		0,39028128	RSD (%)	7.14	

8.4 Absorbance

The absorbance plotted against the wavelength to show the changes in absorbance. And calculated the ratio of E_4/E_6.

8.4.1 Absorbance for E_4/E_6

Table 11. Absorbance at 465 nm (E_4) and at 665 nm (E_6)

465	*0.1*	*1*	*5*	*10*	*20*
blank			0.75	13.5	1
no nutrients	33.5	31.25	29.5	42	32.25
1N:1P	37	38	32.25	47.25	39
2N:1P	40.75	41	32.5	46.5	37
5N:1P	39.333	43	34.25	46.75	38
16N:1P	39		30.25	45	38.25

665	0.1	1	5	10	20
blank			1.25	4	1
no nutrients	5.75	9.25	5.25	7.25	4
1N:1P	9.666667	11.25	6.5	7.5	4.5
2N:1P	12.5	11.25	6.5	7.5	4
5N:1P	11.666667	16	7.5	6.75	4.75
16N:1P	10.75		5.75	5.75	4.25

8.4.2 Absorbance plot 0.1 mM P

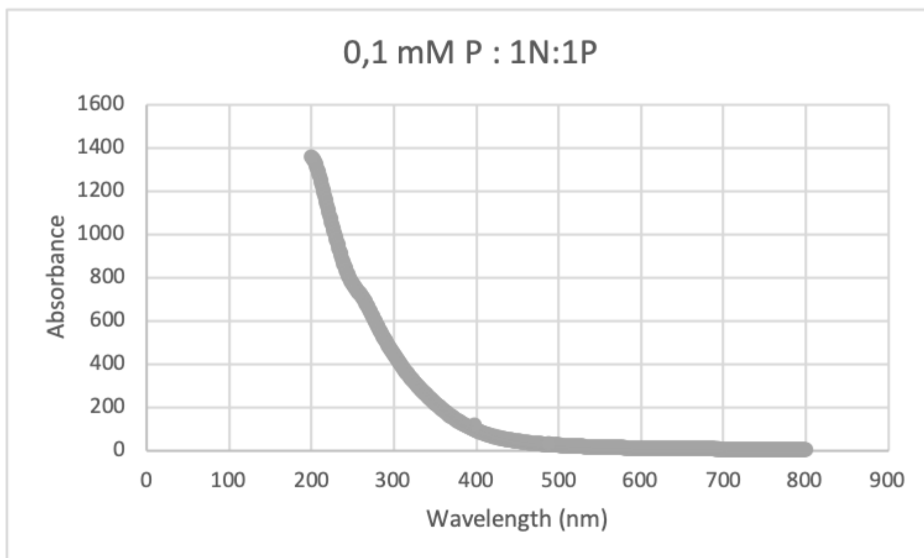

Figure 10. The absorbance for 0.1 mM P ratio 1N:1P, y-axis absorbance and x-axis wavelength (nm)

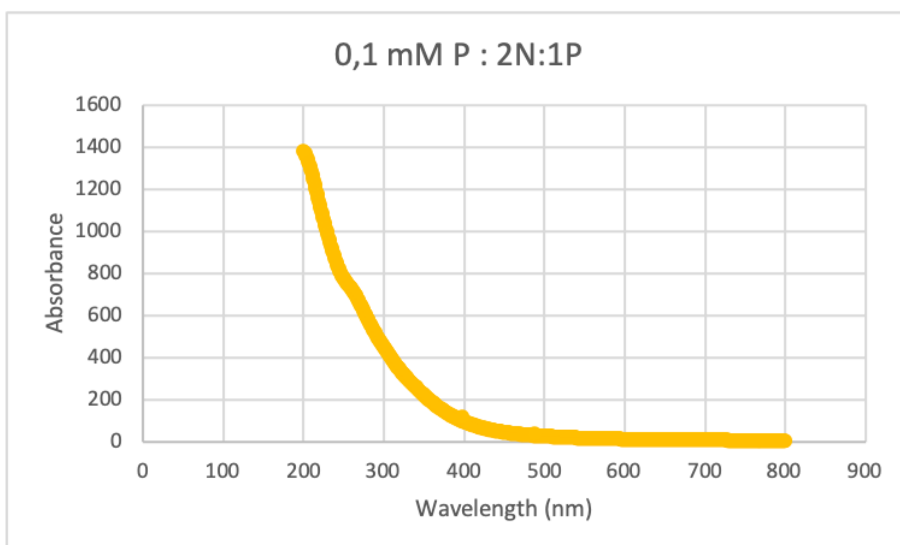

Figure 11. The absorbance for 0.1 mM P ratio 2N:1P, y-axis absorbance and x-axis wavelength (nm)

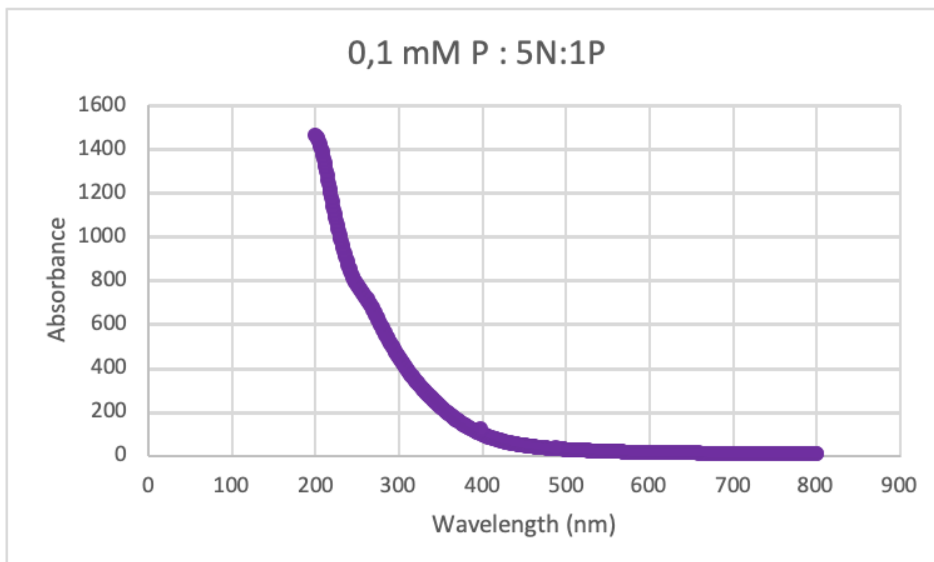

Figure 12. The absorbance for 0.1 mM P ratio 5N:1P, y-axis absorbance and x-axis wavelength (nm)

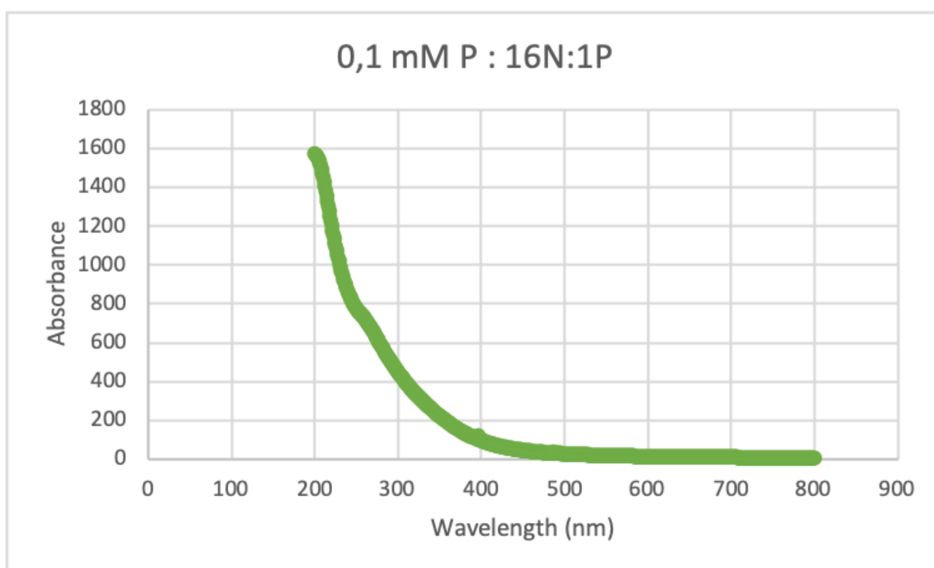

Figure 13. The absorbance for 0.1 mM P ratio 16N:1P, y-axis absorbance and x-axis wavelength (nm)

8.4.3 Absorbance plot 1 mM P

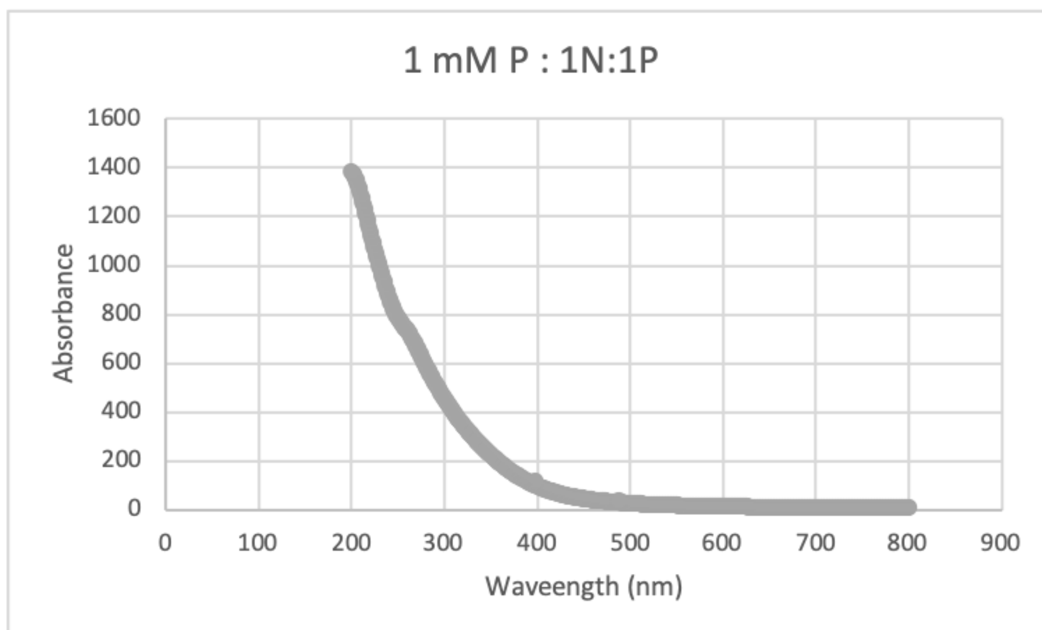

Figure 14. The absorbance for 1 mM P ratio 1N:1P, y-axis absorbance and x-axis wavelength (nm)

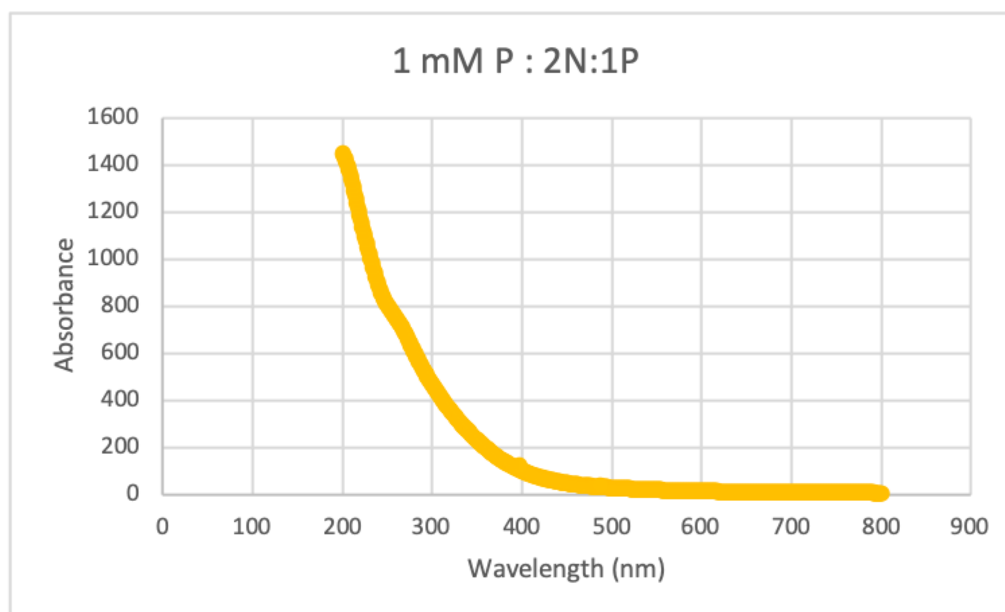

Figure 15. The absorbance for 1 mM P ratio 2N:1P, y-axis absorbance and x-axis wavelength (nm)

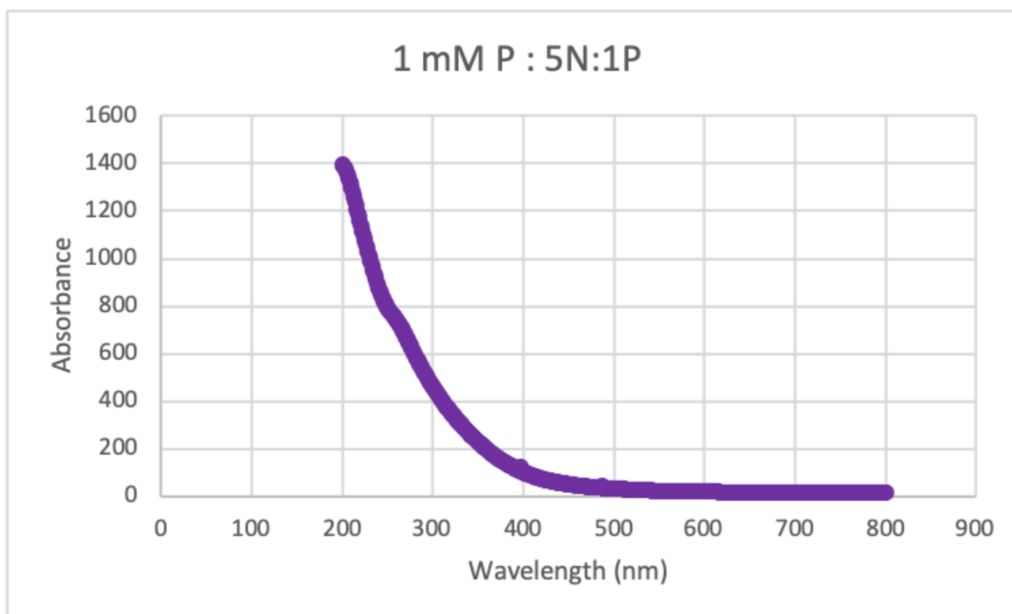

Figure 16. The absorbance for 1 mM P ratio 5N:1P, y-axis absorbance and x-axis wavelength (nm)

8.4.4 Absorbance plot 5 mM P

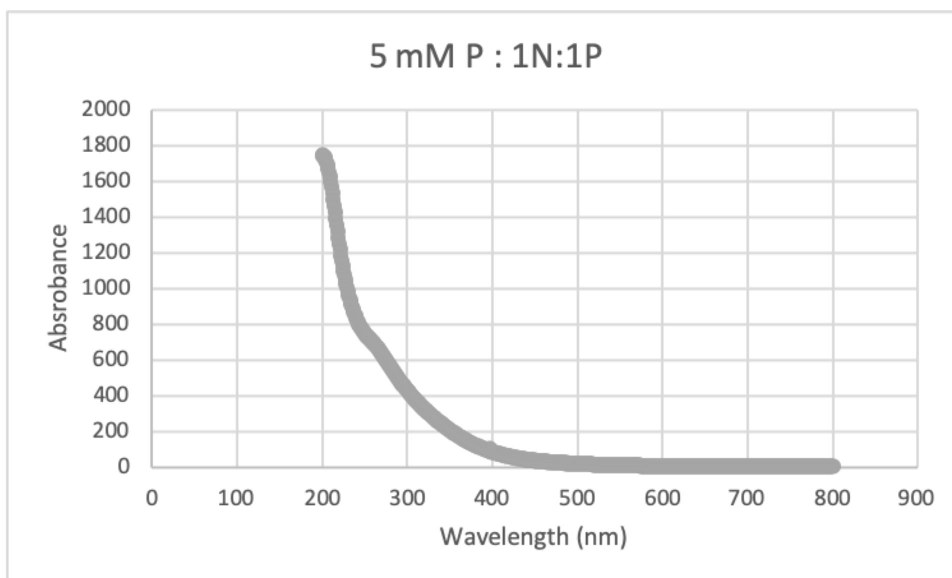

Figure 17. The absorbance for 5 mM P ratio 1N:1P, y-axis absorbance and x-axis wavelength (nm).

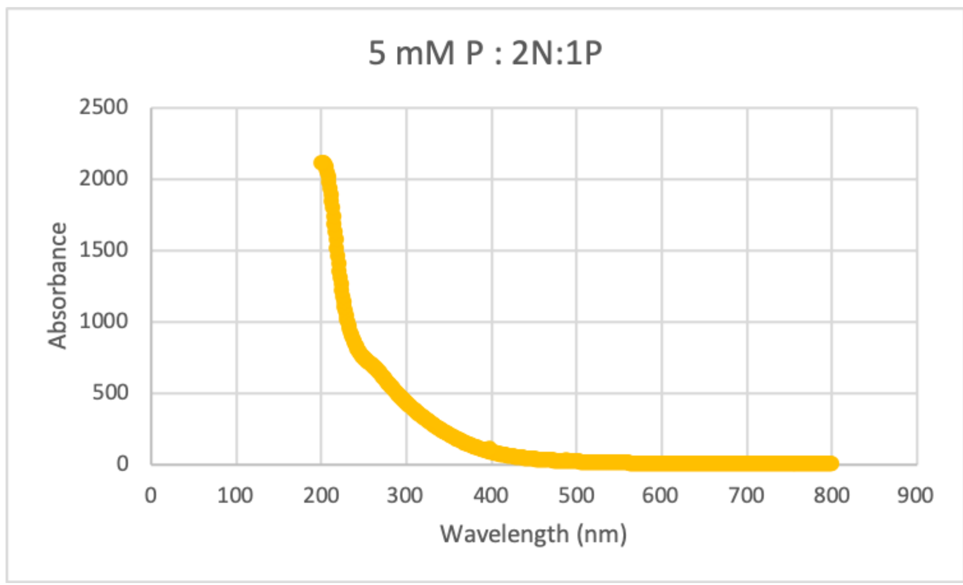

Figure 18. The absorbance for 5 mM P ratio 2N:1P, y-axis absorbance and x-axis wavelength (nm)

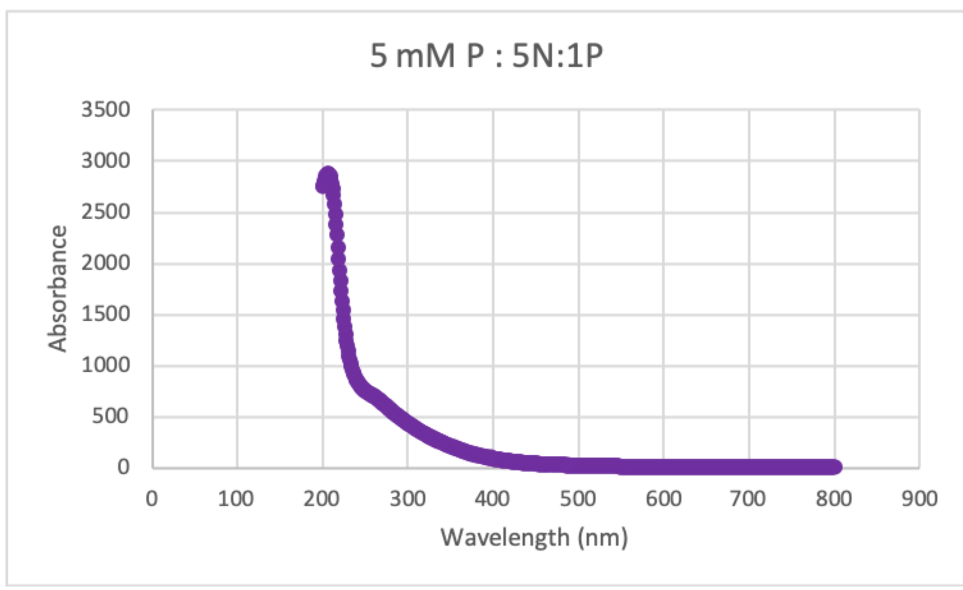

Figure 19. The absorbance for 5 mM P ratio 5N:1P, y-axis absorbance and x-axis wavelength (nm)

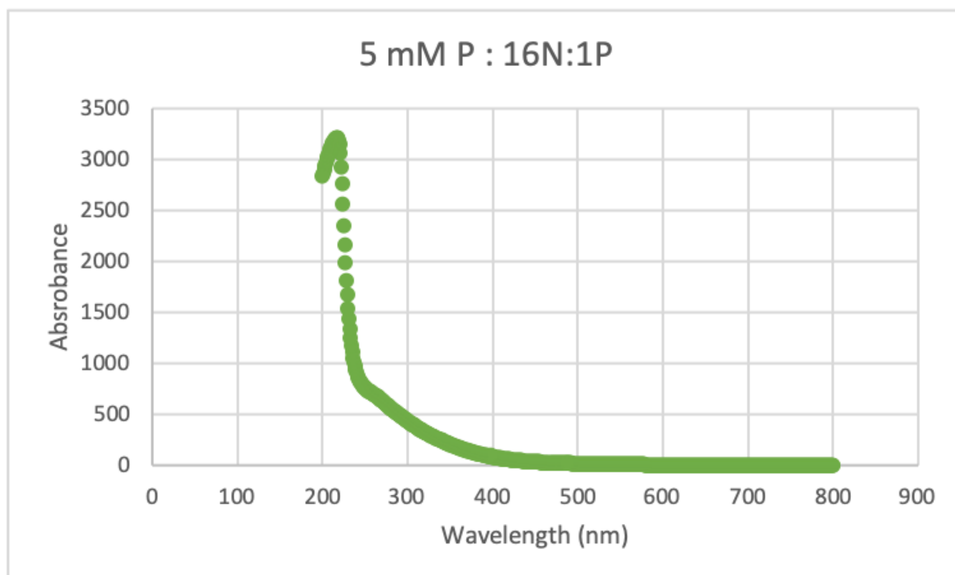

Figure 20. The absorbance for 5 mM P ratio 16N:1P, y-axis absorbance and x-axis wavelength (nm)

8.4.5 Absorbance plot 10 mM P

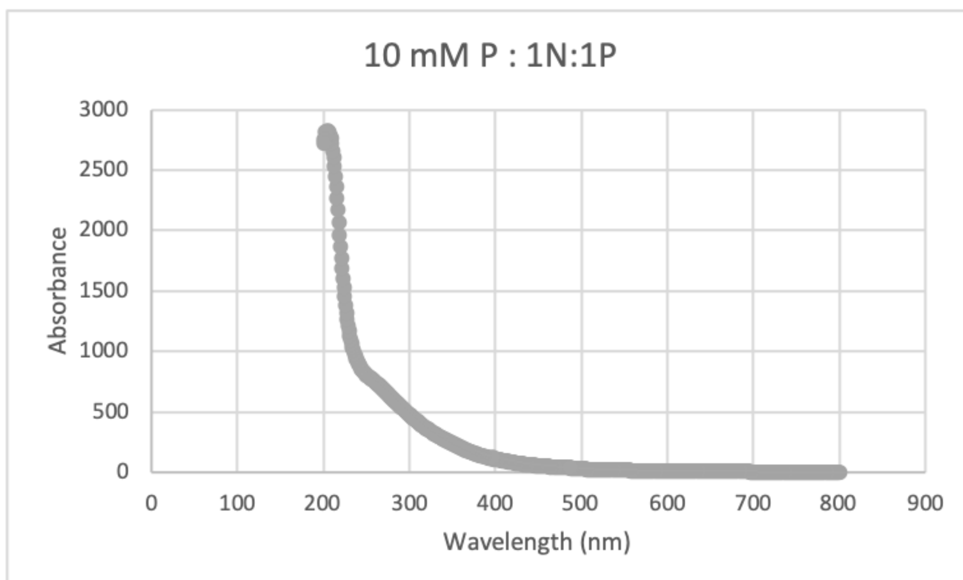

Figure 21. The absorbance for 10 mM P ratio 1N:1P, y-axis absorbance and x-axis wavelength (nm)

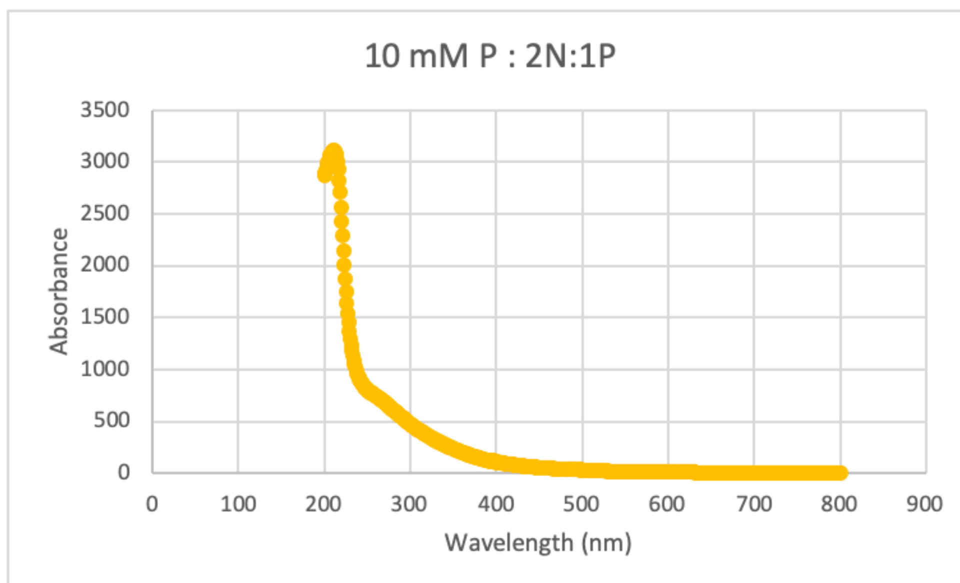

Figure 22. The absorbance for 10 mM P ratio 2N:1P, y-axis absorbance and x-axis wavelength (nm)

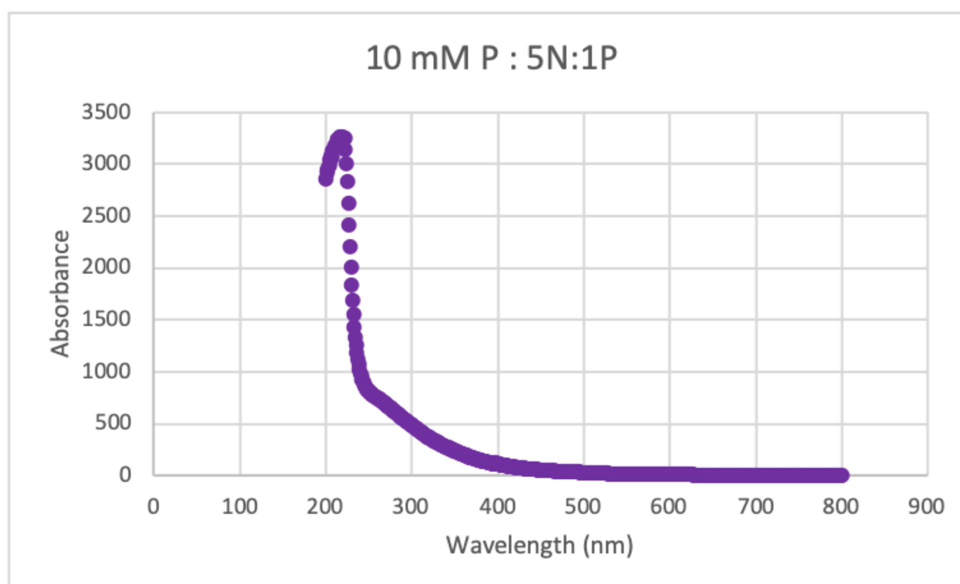

Figure 23. The absorbance for 10 mM P ratio 5N:1P, y-axis absorbance and x-axis wavelength (nm)

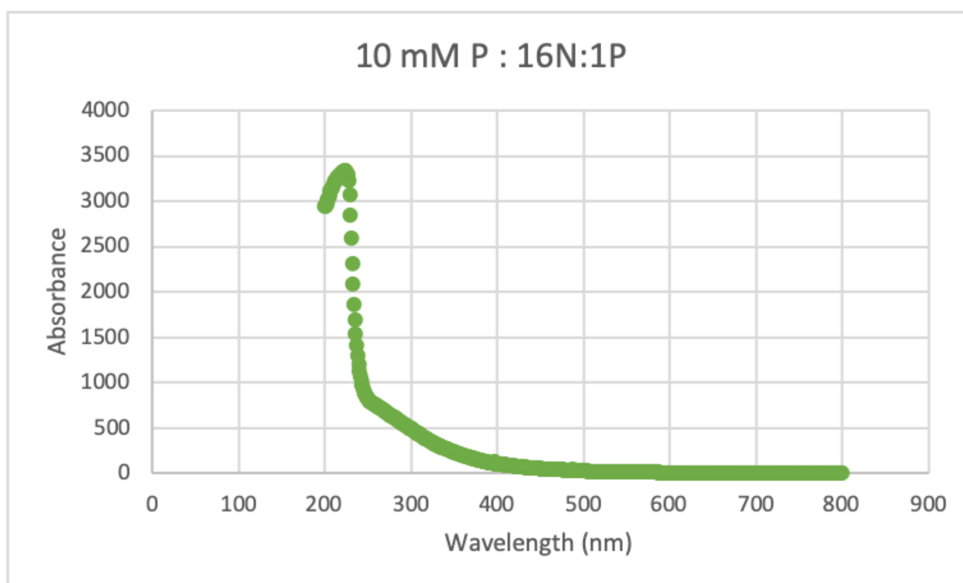

Figure 24. The absorbance for 10 mM P ratio 16N:1P, y-axis absorbance and x-axis wavelength (nm)

8.4.6 Absorbance plot 20 mM P

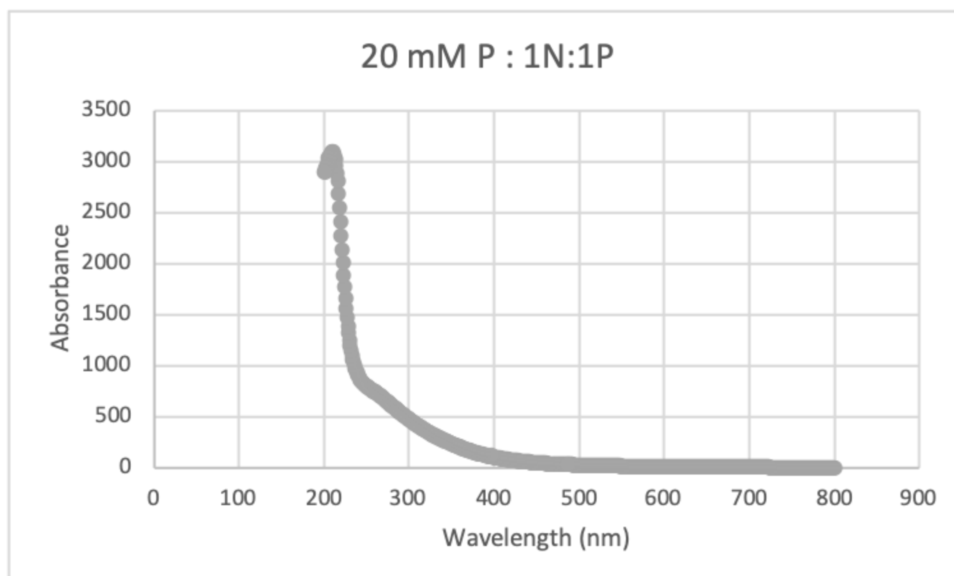

Figure 25. The absorbance for 20 mM P ratio 1N:1P, y-axis absorbance and x-axis wavelength (nm)

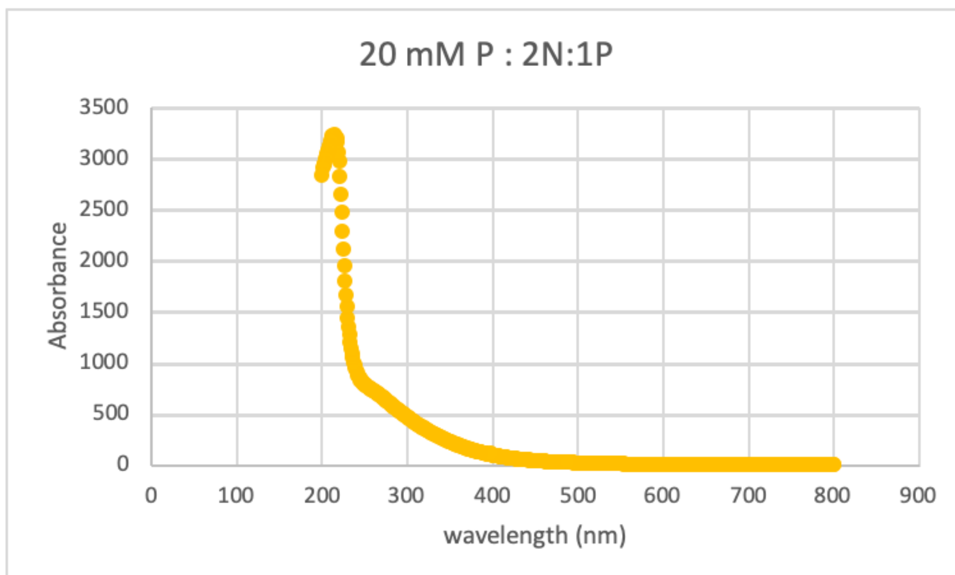

Figure 26. The absorbance for 20 mM P ratio 2N:1P, y-axis absorbance and x-axis wavelength (nm)

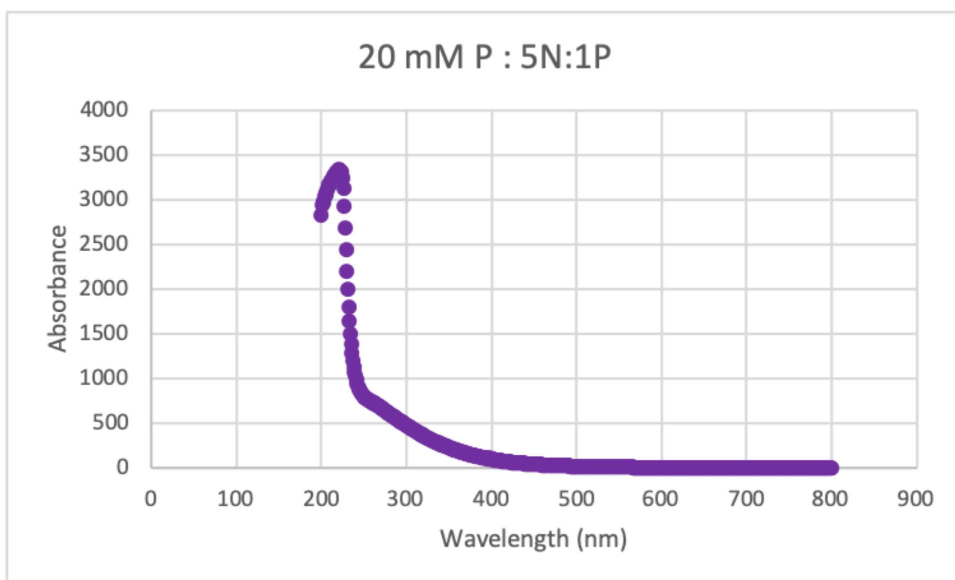

Figure 27. The absorbance for 20 mM P ratio 5N:1P, y-axis absorbance and x-axis wavelength (nm)

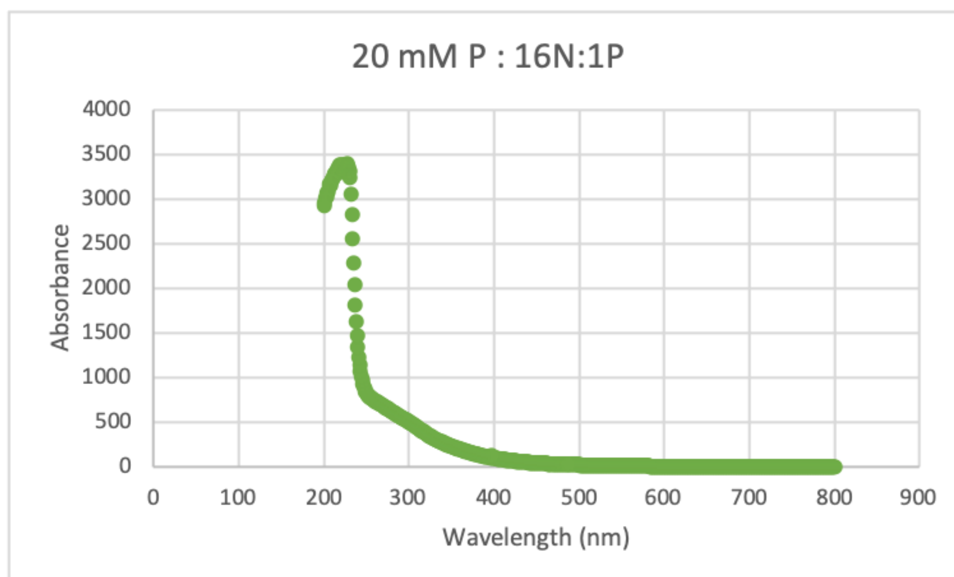

Figure 28. The absorbance for 20 mM P ratio 16N:1P, y-axis absorbance and x-axis wavelength (nm)