Comparing growth of the freshwater rotifer *Brachionus calyciflorus* in four differently modified versions of Guillard's WC medium

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Master Thesis

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UNIVERSITY OF OSLO

2017

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http://www.duo.uio.no/

Print: Reprosentralen, University of Oslo

Acknowledgements

Completing the master level is a challenging task and for me it was even much more challenging because of my unusually difficult situation during my studies. It would have been absolutely impossible for me to do this without the amazing help and support from all the people who walked with me along this long path.

My deepest and most sincere gratitude goes to my main supervisor professor Tom Andersen for his brilliant guidance, constant generous care and unmatched patience. You are way more than my supervisor and being the least of your students is one of my greatest honors.

My deepest heartfelt appreciation goes to my co-supervisor professor Josefin Titelman for her excellent advice, ceaseless positive energy and so much motivation all along the project. I can never thank you enough.

Sincere thanks to my co-supervisor professor Ketil Hylland for all his help and advice especially in the earlier stages of the project.

I would like to express many heartfelt thanks to Per-Johan Færøvig for always being available for technical help and support and truly making the lab work a much more pleasant experience. Also many sincere thanks to Berit Kåsa for her kind help and support whenever I was in need.

A special thank you to my family in Iran and here in Norway for their care and support and for making it possible for me to pursue my studies in the master level.

I want to thank all my classmates, lab-mates, friends in the department, PhDs and postdocs in AQUA for being such great friends. Studying in UiO became an amazing and unforgettable experience for me. In particular, I want to thank Catharina Broch from my heart for spending so much time to answer my numerous questions even though you had so much to do. Special thanks to Jan-Erik, Raul, Thomas and Daniel for all your help and support. My special thanks goes to Jan David Heuschele for all your generous help in the lab and your patience in answering my questions. A thousand heartfelt thanks to Silje Marie Kristiansen for being such an amazing friend and classmate, for your endless genuine care and constant emotional support and motivation. Many many thanks to Cathrine, Ellen, Andre Moan, Julie, Anne Karin, Malin, Inger, Espen and Sabrina for being great friends and awesome company.

Many heartfelt thanks to the administration in the department of biosciences especially to Lise Bøknes for her trust and understanding and offering precious advice so that I could focus on moving forward with my studies even during hard times.

I would like to thank AQUA, the department of biosciences, the faculty of Mathematics and Natural sciences and the University of Oslo for giving me this truly great opportunity.

Abstract

Rotifers and phytoplankton have been vastly used by scientists as model organisms in toxicological, eco-toxicological, ecological and other studies. In order to conduct experiments on interactions between animals and food organisms, it is necessary to develop a medium that adequately supports the growth of both algae and zooplankton without the need to alter the medium to accommodate either the algae or the animals. Several types of culture media have been developed to grow different types of organisms in particular phytoplankton and zooplankton. Little research has been carried out to compare the capacities and potentials of different types of media to rear these model organisms.

Brachionus calyciflorus and *Chlamydomonas reinhardtii* were chosen as a model system to compare the carrying capacity of four types of media for algae and rotifers. Both of these are widely used as test organisms or together as a model system in various studies. All four types of media were modified versions of Guillard's WC medium with #1 & #2 being based on tap-water and #3 & #4 based on Distilled water.

Phytoplankton biomass was assessed by using different techniques including in-vivo fluorescence and absorbance, in-vitro chlorophyll fluorescence, pigments concentration estimates using absorbance spectra data and algae cell count. Rotifers' growth was measured by counting and calculating the number individuals per milliliter.

In general, the algae grew reasonably well in all four media while rotifers only grew in the tap-water based media. Apart from some speculations, the definite reason for this significant difference in rotifer growth between tap-water based and distilled water based media is unknown and further research is needed to better explore and understand the features and capacities of different types of culture media.

1. Introduction

In every branch of biosciences, scientists have sought to choose organisms which best suit the purposes and the conditions of their experiments. Model organisms represent only a small fraction of the biodiversity that exists on Earth, although the research that has resulted from their study forms the core of biological knowledge (Hedges, 2002).

In toxicology and eco-evolutionary biology species of rotifers have been used by many scientists as model (test) organisms e.g. (Janssen, Rodrigo, & Persoone, 1993), (C.P. Charoy", 1995), (Benjamin L. Preston *, 1999), (Strojsova, Nedoma, Sed'a, & Vrba, 2008), (Alvarado-Flores, Rico-Martinez, Adabache-Ortiz, & Silva-Briano, 2015), etc. . Rotifers are very attractive test organisms for aquatic toxicity assessment for reasons of ecological relevance, biological characteristics and general practicability (Janssen et al., 1993). This great interest has been due to the central role of rotifers in freshwater planktonic communities, the ease and speed of making quantitative measurements of mortality and reproduction, their sensitivity to common pollutants, the commercial availability of cysts, and the existence of reliable, standardized protocols. They often play a key role in the dynamics of freshwater and coastal marine ecosystems(Snell & Janssen, 1995).

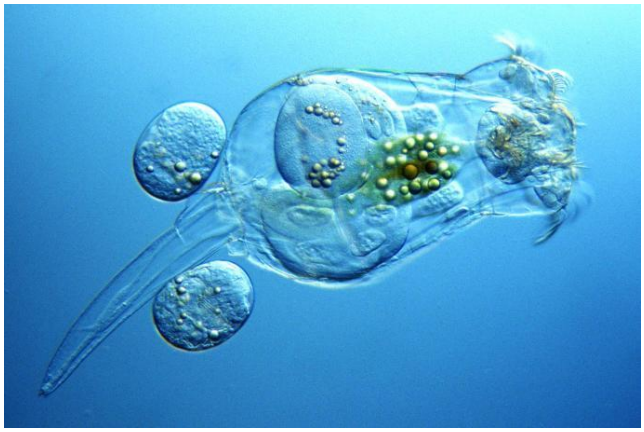
In order to conduct experiments on interactions between animals and food organisms, it is necessary to develop a medium that adequately supports the growth of both algae and zooplankton without the need to alter the medium to accommodate either the algae or the animals (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998). Many defined freshwater algal media have been designed (see (Stein, 1973)for early efforts), but four are in wide use: Guillard's WC (Guillard, 1975), Fraquil(Morel, J. C. Westall, & Chaplick, 1975)ASM (Carmichael & Gorham, 1974), and DYIII (Lehman, 1976) (Kilham et al., 1998). Despite the variety of the media used in different studies (e.g. EPA synthetic freshwater medium, Guillard's WC medium, COMBO medium, Z4 medium, SE medium, etc.) little research have been carried out to compare the capacity of the different media in supporting the growth of test organisms [Tom Andersen, personal communication].

This experiment was designed and conducted in an attempt to further explore the capacity of one type of these developed media (Guillard's WC) in growing algae and rotifers. The Four media types used in this study were modified versions of Guillard's WC medium and they were made using different types of water for comparison. The details about composition of the media are described in chapter 2 (see Table 2.1.).

A model system consisting of a freshwater rotifer (*Brachionus calyciflorus*) and a species of single celled green algae (*Chlamydomonas reinhardtii*) was chosen to test the carrying capacity of the media for each of the organisms (Fig.1.1.).

Various methods are in use for assessment of phytoplankton biomass including measuring chlorophyll fluorescence in-vivo and in-vitro and cell counts (Vollenweider, Talling, & Westlake, 1974) (Mayer, Cuhel, & Nyholm, 1997). It is also possible to assess the growth of phytoplankton by estimating pigments' concentrations from their absorbance data (Thrane et al., 2015). Each of these methods has its own advantages and disadvantages in terms of efficiency, investments and precision. I have employed these methods to assess the algae growth and tried to briefly describe my experience with each of them.

Rotifers of the genus *Brachionus*, especially *B. calyciflorus*, are particularly suited as test organisms for eco-toxicological studies because of their cosmopolitan distribution, rapid reproduction and short generation time, ease of culture, and the availability of resting eggs (Janssen et al., 1993). The green algae *Chlamydomonas reinhardtii* has been used in eco-toxicological studies together with the rotifer *B. calyciflorus* both as the main test organism (e.g. (Fischer, Roffler, & Eggen, 2012) and as food for the rotifer (Felpeto & Hairston, 2013)).



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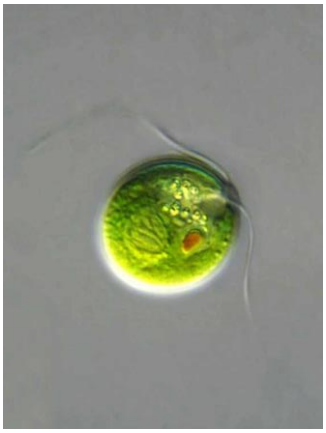

By:Magnhild Jenssen (NMBU)

Fig.1.1. *Brachionus calyciflorus* on the left and *Chlamydomonas reinhardtii* on the right.

Aims

1. To compare the carrying capacity of the four types of media for the phytoplankton *Chlamydomonas reinhardtii* and the freshwater rotifer *Brachionus calyciflorus*.
2. To compare the different methods of assessing growth in phytoplankton, particularly their precision and efficiency.
3. To observe the relationship between population density of rotifers (the amount of grazing) and the conversion rate of chlorophyll a to its degradation product (Pheophytin a).
4. To explore the fluctuations in the measured parameters in phytoplankton and their relationship with algae and rotifers' population dynamics.

2. Materials and Methods

The experiment

The experiment was designed to determine which one of the four growth media show the highest carrying capacity for phytoplankton (*Chlamydomonas reinhardtii*) and rotifers (*Brachionus calyciflorus*). Each treatment (media type) consisted of four replicates. I started by preparing the growth media. After that the stock cultures of experimental organisms were made ready and finally the experimental setup was started. The experiment lasted for 18 days.

Growth media

The four media were modified versions of Guillard's WC medium (Kilham et al., 1998). Media #1 and #2 were based on Oslo municipal tap water (TW) with #1 being aged. Medium #3 was based on distilled water (DW) and #4 on deionized distilled water (DDW using MilliQ®). The chemical composition of the 4 types is summarized in Table 2.1.

All glassware used in media preparation were first washed with non-phosphate washing agent and sterilized at 150˚C for 3 h. I first prepared stock and working solutions of nutrients, which were then added to the various water types when preparing the final media. Primary stock and vitamin solutions were made according to Guillard's WC medium (Kilham et al., 1998). The details of media preparation and chemical composition of all stock and working solutions are provided in Appendix 1 (Table A1). Distilled water was used in making all primary stock and working solutions.

Five liters of each medium (#1 to 4) were made using volumetric flasks (1000 mL and 2000mL). First the flask was filled with the corresponding water type up to 75% of the volume according to the protocol of (Kilham et al. 1998). One mL L^{-1} of every corresponding working solution was then added and finally 100µL L^{-1} of the vitamins solution was added to the medium. In the end, the flask was filled up with more water to reach the desired volume. For medium #1 five liters of Oslo municipal tap water was first left for aging for two weeks, while being aerated using an aquaria bubbling stone. Medium #1-4 were then stored in Erlenmeyer flasks at 19˚C until setup of the experiment. The flasks were covered with Aluminum foil to block light as an additional measure to keep the media sterile.

Table 2.1. Compounds used in making the 4 types of media. All values are in $mg.L^{-1}$ of the final medium. The two compounds Na2SiO39H2O and Na3VO4 were not used in making the media as they were not necessary for organisms used in this project.

Contents Base	Medium #1 Tap water (aged)	Medium #2 Tap water (direct)	Medium #3 Distilled water	Medium #4 DDW (MilliQ®)
Major elements				
K_2HPO_4	8.71	8.71	8.71	8.71
$NaNO_3$	85	85	85	85
$CaCl_2\ 2H_2O$	------	------	36.76	36.76
$MgSO_4\ 7H_2O$	------	------	36.97	36.97
$NaHCO_3$	------	------	12.6	12.6
H_3BO_3	------	------	24.0	24.0
Algal Trace Elements				
Na_2EDTA	4.36	4.36	4.36	4.36
$FeCl_3\ 6H_2O$	3.15	3.15	3.15	3.15
$MnCl_2\ 4H_2O$	0.18	0.18	0.18	0.18
$CuSO_4\ 5H_2O$	0.0025	0.0025	0.0025	0.0025
$ZnSO_4\ 7H_2O$	0.022	0.022	0.022	0.022
$CoCl_2\ 6H_2O$	0.01	0.01	0.01	0.01
$NaMoO_4\ 2H_2O$	0.0063	0.0063	0.0063	0.0063
Vitamins				
Thiamin HCl	0.1	0.1	0.1	0.1
Biotin	0.0005	0.0005	0.0005	0.0005
B_{12} Vitamin	0.0005	0.0005	0.0005	0.0005

Culturing of Experimental Organisms

The phytoplankton (*Chlamydomonas reinhardtii*) was used as food for the rotifers (*Brachionus calyciflorus*) in the experiment. Both phytoplankton and rotifer stock cultures were grown in medium #3 and kept under a 16/8 h light /darkness period at 19˚C. Light (~6700 lm) was provided with two 1200mm fluorescent tubes (L 36W/840. LUMILUX. Cool white Osram, Germany). The experiment was conducted under the same environmental conditions as culturing unless mentioned otherwise. Phytoplankton culturing was conducted in 50mL tissue culture flasks.

Dormant rotifer eggs (Pentair aquatic eco-systems, USA) were allowed to hatch in ~50mL of medium #3 in plastic Petri dishes (diameter: 82mm) for 24 h under constant light at 22˚C. Subsequently, rotifers were fed *C. reinhardtii* at ad libitum concentrations. The following day rotifers were transferred into a 400mL tissue-culture flask with fresh medium and ad libitum food. The culture flask was kept uncapped and at 19˚C under

16/8 h light/dark period. After 96 h the culture was split in two and diluted with fresh medium. This step was repeated such that rotifers to be used in the experiment came from 4 different flasks. Bottle-top 500mL 0.02 μm filters were used to sterilize the medium for the rotifer cultures.

Experimental setup

The experiment consisted of 4 treatments (Media #1-4); each with 4 replicates (Fig. 2.1). Sixteen 175 mL tissue-culture flasks were first filled with 150 mL of the corresponding media and inoculated with approximately 150000 *C. reinhardtii* cells to achieve a targeted start concentration of 1000 cells ml^{-1}, and kept at the experimental conditions until addition of rotifers. Cell concentrations were measured using a cell counter device (CASY Model TT Ser.No. TT-2CA-1075) (see Appendix 1, Fig.A4).

Rotifers used in the experiment were pipetted out individually from a mix of the cultures under a dissecting microscope. For each flask (hereafter called unit), thirty rotifers were isolated in Petri dishes containing 5ml distilled water (30 x 16). The experiment started when the aliquots of rotifers were carefully transferred to the experimental units (i.e. time=0).

Air-pumps, elastic tubing, plastic sterile valves, veterinary needles and rubber stoppers were used to flow air into every flask (Fig. 2.1). A separate air pump with a 0.2 μm air filter and a "distributor flask" were used to aerate the units of each treatment to avoid contamination between treatments. Each "distributor flask" was filled with distilled water (to compensate for evaporation).

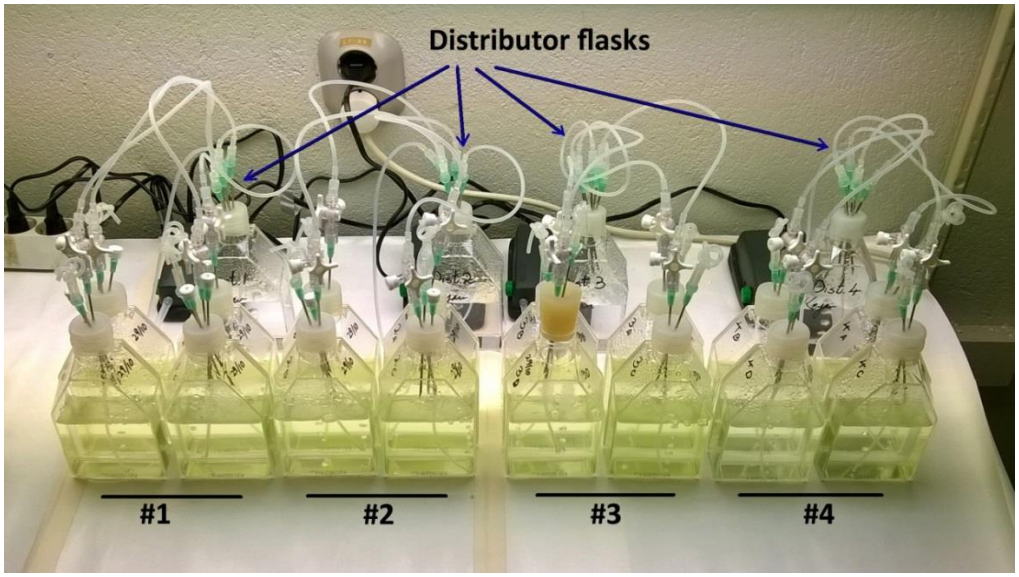

Fig.2.1. The experimental setup in the temperature controlled room (at time=0). The flasks are arranged in groups of four (replicates of each treatment) as marked in the picture. Each group is connected to a separate distributor flask which in turn is connected to an air pump.

Sampling and measurement

During the experiment, every 2 to 4 days (~45-90 h) samples were taken from each unit and replaced with fresh medium of corresponding type. Samples were then used to measure in-vivo fluorescence, absorbance, phytoplankton cell count and rotifers abundance. A portion of each sample was also transferred to a well-plate and frozen in -18°C to be used later for pigment extraction and analysis.

Prior to each sampling the flasks were moved to a laboratory. All sampling and refilling procedures were carried out under a fume hood to avoid contamination. Flask contents were first homogenized by gentle mixing. Using a digital measure, 50 g water samples were first taken. Subsamples of these 50g portions were then used for specific analyses (see below). Fifty grams of the corresponding fresh medium was then added to each flask after filtration through bottle-top 0.2 µm filters. The flasks were typically returned to the temperature-controlled room within 40-45 mins.

In vivo fluorescence and Absorbance

Five samples of 200 µL (as technical replicates) were sampled with a micropipette and placed in a well-plate (see Appendix 1, Fig A1.). Absorbance was then measured at wave lengths of 500 nm and 700 nm and fluorescence in the range of 460-680 nm in a plate reader device (Biotek Synergy Mx. See Appendix 1, Fig.A3).

In-vitro fluorescence and absorbance

Three samples of 200 µL (as technical replicates) were placed in another well-plate and immediately frozen at -18˚C. The samples were later freeze dried and pigments were extracted before subsequent measurement of chlorophyll fluorescence and absorbance.

After the last round of sampling, the first four well plates were dried using a vacuum freeze-drier device. The drying process took ~20 h. 2oo µL 96% ethanol was then added to each well. For each sample (each of the 16 units), one well with 200 µL 96% ethanol was included as a blank (see appendix 1, Fig A2. for plate layout). The well-plates were then capped, wrapped in aluminum foil to block light and left in a refrigerator at 4˚C for 4-6 h for extraction. Fluorescence in the range of 430-675 nm and absorbance in the range of visible light 400-700 nm was then read in the plate reader device (Biotek Synergy Mx). See Appendix 1 for further device specifications and protocols.

Phytoplankton abundance

Phytoplankton concentration was measured using 1 mL samples on the CASY cell counter device. The samples were diluted in the special solution provided for the device to reach 10 mL in volume prior to measurement. Towards the end of the experiment, when concentrations were high, the rate of dilution had to be increased as there is a limit for the maximum number of cells in a sample that can be measured by the device. Dilution is of course accounted for in the results.

Rotifer counts

Rotifers were counted under a dissecting microscope in samples ranging from 20 mL (without technical replicates) in the beginning of the experiment to samples of 1 mL (with 5 technical replicates) towards the end of the experiment. Sample volumes depended on the concentration of rotifers. Counts were converted to rotifers mL^{-1}.

Statistical analysis

Calculation of Chlorophyll A concentration using in-vitro fluorescence data

To calibrate the in-vitro fluorescence data, a series of solutions of Chlorophyll A with standard concentrations was made and the fluorescence was measured using the same device and protocol as for the samples:

```
   Conc.std          meas.Fl
Min.   : 0.0625   Min.   :  40.67
1st Qu.: 0.2500   1st Qu.: 149.67
Median : 1.0000   Median : 589.67
Mean   : 2.9931   Mean   :1325.26
3rd Qu.: 5.0000   3rd Qu.:2399.33
Max.   :10.0000   Max.   :3817.67
```

Fluorescence data from the standard series was plotted (Fig.2.2.) a linear model with a polynomial was fitted to describe the relationship between the concentration of Chlorophyll And the measured fluorescence:

```
lm(formula = Conc.std ~ poly(meas.Fl, 2), data = std.data)
```

The polynomial was added for the measured fluorescence variable to adjust for the non-linearity in the graph that is probably due to self-absorption of fluorescence light in higher concentrations. A line was added to the plot to visualize the fit of the model:

```
> Fl.pred <- seq(0, 4000, 10)
> Conc.pred <- predict(m2, newdata=list(meas.Fl=Fl.pred))
> lines(Conc.pred ~ Fl.pred, lwd=2
```

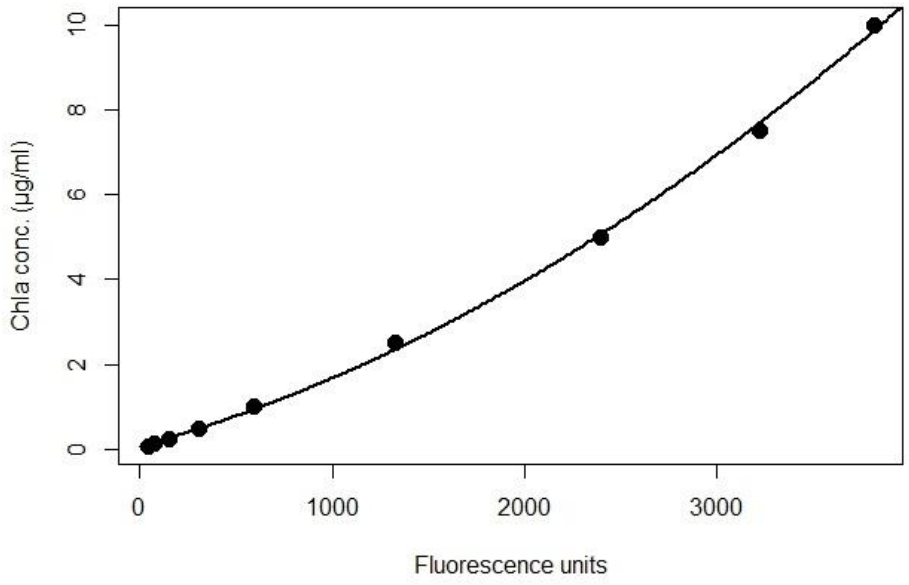

Fig.2.2. Chlorophyll A concentration by fluorescence from standard series data

The model was used to estimate the values of chlorophyll concentration for the samples (a new column was created in the existing data file containing these values):

```
> sample.data$Chl.conc <- predict(m2, newdata=sample.data)
> summary(sample.data)
      Days            Unit      Treatment       meas.Fl          Chl.conc
 Min.   : 3.80   1A     : 7   Min.   :1.00   Min.   :  85.0   Min.   :0.177
 1st Qu.: 6.40   1B     : 7   1st Qu.:1.75   1st Qu.: 806.5   1st Qu.:1.313
 Median :11.00   1C     : 7   Median :2.50   Median :1128.0   Median :1.933
 Mean   :11.19   1D     : 7   Mean   :2.50   Mean   :1158.3   Mean   :2.092
 3rd Qu.:16.30   2A     : 7   3rd Qu.:3.25   3rd Qu.:1539.5   3rd Qu.:2.829
 Max.   :18.10   2B     : 7   Max.   :4.00   Max.   :2446.0   Max.   :5.206
                 (Other):70
```

Pigment analysis from absorbance spectra data

In-vitro absorbance spectra data was used to estimate the concentration of different pigments in the samples. This was done using an improved version of the Gauss-Peak Spectra (GPS) method in which individual pigment spectra are presented as weighted sums of Gaussian functions. These functions are then used to model the absorbance spectra of phytoplankton pigment mixtures (Thrane et al., 2015). The background attenuation is also modeled as a linear combination of power functions of wavelength to be taken into account in deconvolution of the spectra (i.e. a spectrum to represent background attenuation is created using that model) (Thrane et al., 2015). By background spectrum we mean any light attenuation not attributable to the pigment absorption; that is mainly scattering by particles in the sample or absorption by non-algal components from the sample (Thrane et al., 2015). The spectra are reconstructed as weighted sums of pigment and background components (Thrane et al., 2015) . This improved version can calculate the concentration of 28 of the normally occurring pigments in natural phytoplankton communities (Thrane et al., 2015).

Since there is no pigment absorbance at 700 nm the amount of absorbance at this wavelength was subtracted from the data to obtain the actual pigment absorbance values at different wavelengths. This increased the signal to background ratio in the data that was going to be used in fitting the functions. After introducing the list of pigments of interest (Chlamydomonas pigments: Chlorophyll A, Chlorophyll b, Pheophytin a, Pheophytin b, β,β-Carotene, Lutein, 9'-cis-Neoxanthin, Violaxanthin) pigment weights were estimated using NNLS. The results of this estimation were plugged in the function "pigment.concentration" to calculate concentration of pigments. Fitted spectrum (pigments +background) was also calculated.

Two of the pigments (Pheophytin b and c.Neoxanthin respectively) whose concentration estimates had the least correlation with others were removed one by one from the list of pigments and each time the calculations were repeated with the new set of pigments. Figure.2.3. shows the correlation among calculated concentration values for different pigments.

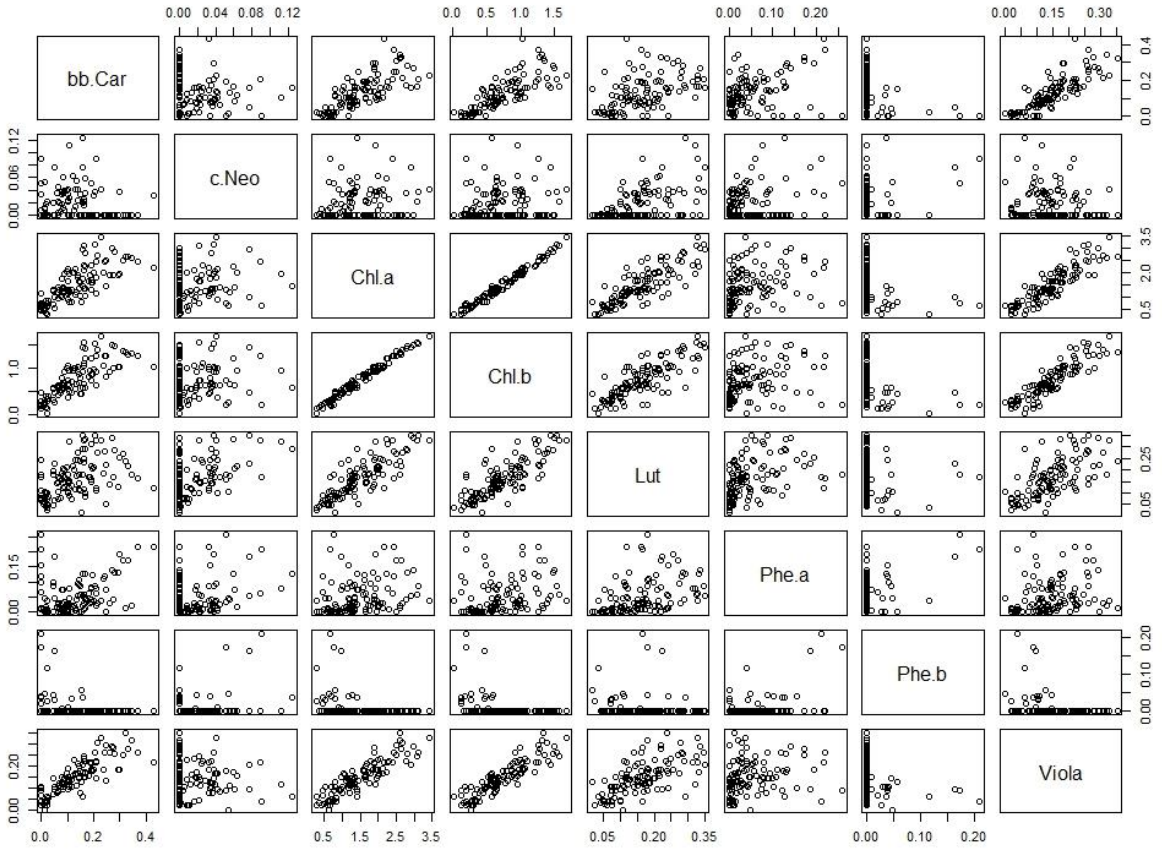

Figure.2.3. Correlation among calculated concentration values for different pigments. Note that calculated values for Pheophytin b and 9'-cis-Neoxanthin have the least amount of correlation to that of other pigments.

Mixed effects-modeling

Since the experiment involved taking sample from the same units over time, mixed effect modeling was used to avoid temporal pseudo-replication. In order to have proper estimates in the statistical analysis the assumptions of the theory need to be met, one of them being independence of errors. Error terms for samples that are taken from the same experimental units (flasks) do not qualify as independent. In such situations one needs to consider fixed effects and random effects.

In mixed-effects modeling in contrast to analysis of variance, sources of variation are categorized into fixed effects and random effects. Fixed effects are the factors that influence the mean of response and they have informative factor levels (such as treatment, sex, addition of a particular nutrient/stressor. In this study: treatment). Random effects influence the variance of response and they have rather non-informative factor levels (Unit, patch, subplot, blood type, Genotype. Here: unit).

Additionally, one will have higher number of degrees of freedom (thus higher precision) when using a mixed effect model to analyze the same dataset since instead of many regressions mixed effect models use all the data points to fit a line (curve in non-linear mixed effect models).

In this study non-linear mixed effects modeling was used to analyse in-vivo fluorescence data since we had non-linearity in most units. Library “nlme” was introduced to organize the data in the form of “grouped data” and to define non-linear mixed effect models. Fluorescence data from the last two days of sampling was not needed to fit nlme models since the data from the first 5 samplings already included an asymptote (see Fig.3.1.). Data from the last two days, therefor, was not used in that part of analysis.

3. Results

In-vivo fluorescence and absorbance

As it is apparent in Fig.3.1. there is an obvious difference between treatments based on tap water (#1, #2) and those based on distilled water (#3, #4). After day 14 of the experiment, the fluorescence values for treatments #1 and #2 declines dramatically whereas for treatments #3 and #4 this value reaches a plateau. This is more clearly seen in the plot with log-transformed values of in-vivo fluorescence (see Fig.3.2.).

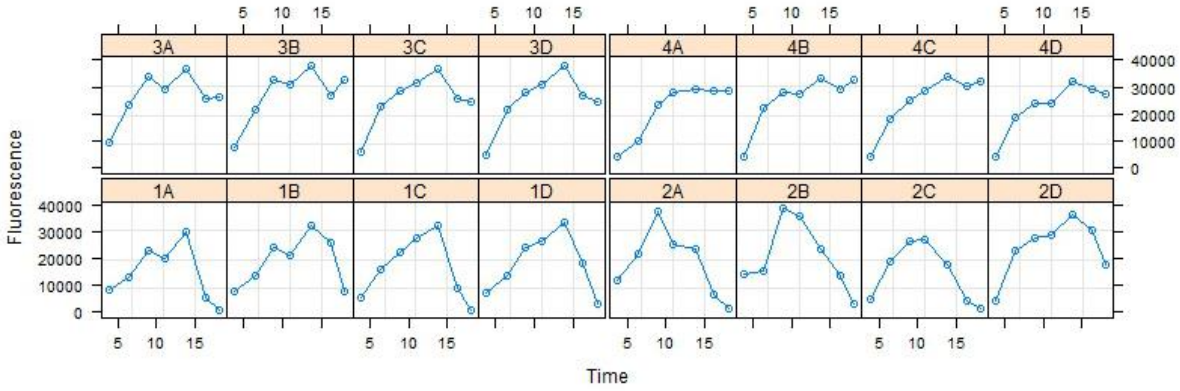

Fig 3.1. in-vivo fluorescence plotted against time for each unit

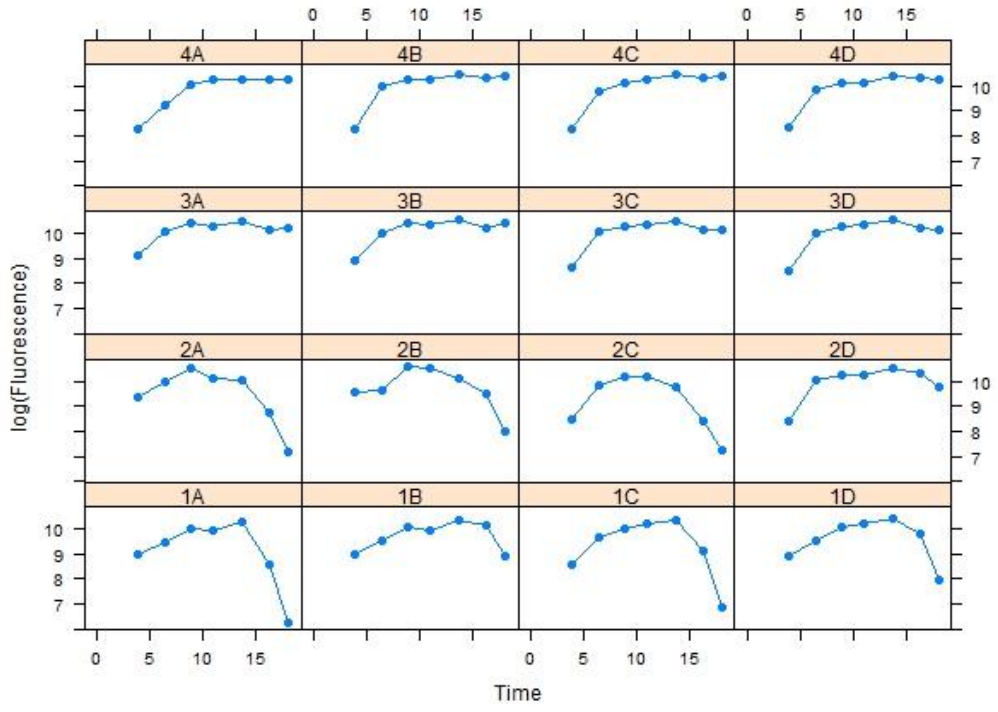

Fig.3.2. Log transformed values of in-vivo fluorescence plotted against time for each unit. Note the decline after day 14 in all units of treatments #1 and #2.

In-vivo absorbance in 500nm (pigment absorbance) and 700nm (no absorbance by pigments) are highly correlated (see Fig.3.3.). By subtracting the values of measured absorbance at 700nm (scattering) from those at 500nm the amount of light absorbed by pigments is obtained. These values are not very correlated with in-vivo fluorescence. (Fig.3.4.).

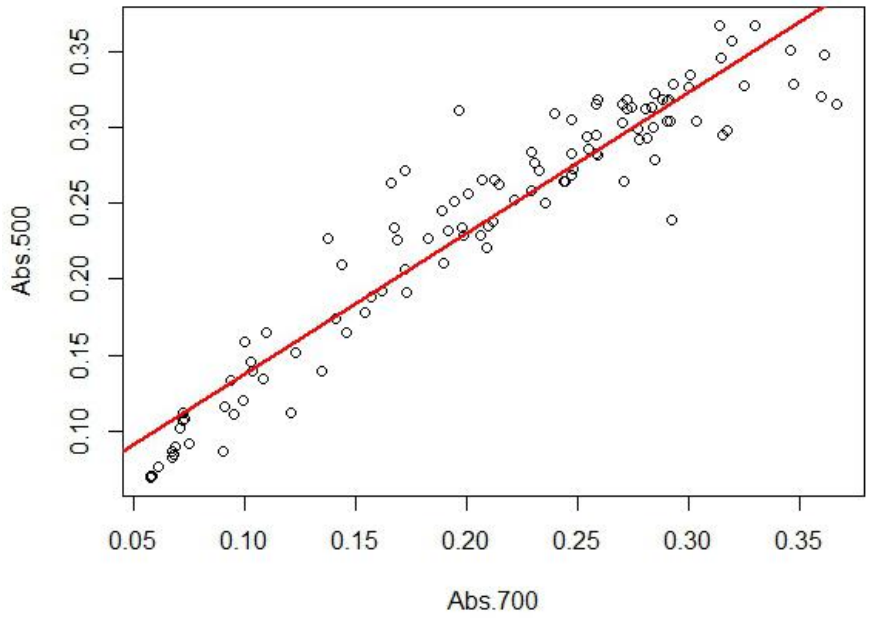

Fig.3.3. Correlation between measured absorbance at 500nm and 700 nm. (slope=0.928681, p= 2e-16)

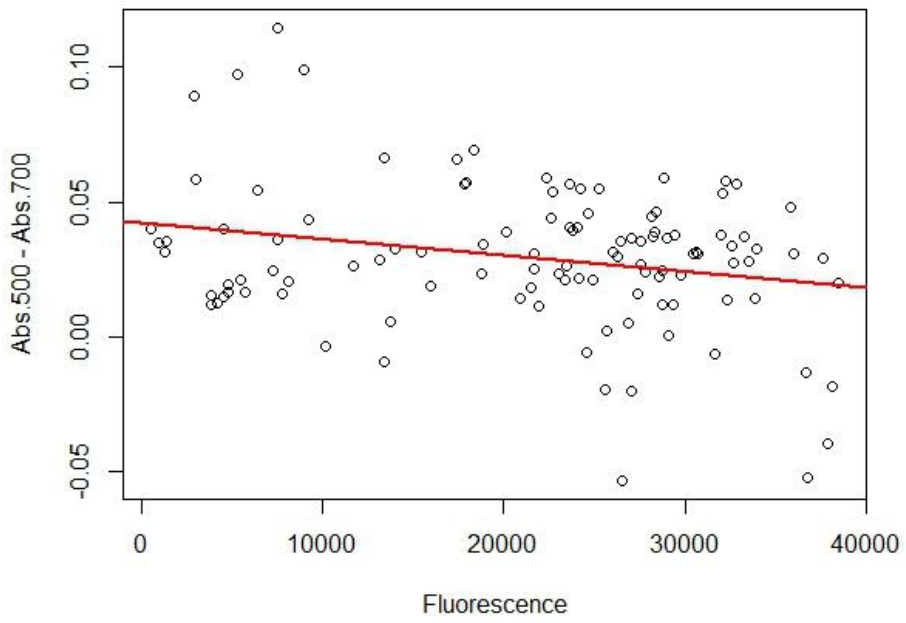

Fig.3.4. Correlation between pigment absorbance and in-vivo fluorescence. (Slope=-6.032e-07, standard error = 2.277e-07, Adjusted R-squared: 0.05141)

Absorbance spectra data

In the plot that is depicting all in-vitro absorbance spectra three main peaks are seen at around 430-440 nm and 460-470 nm and 660-670 nm (see Fig.3.5). The amount of absorbance between treatments varies significantly as absorbance values for treatments #3 and #4 were much higher than those of treatments #1 and #2 (see Fig.3.6.).

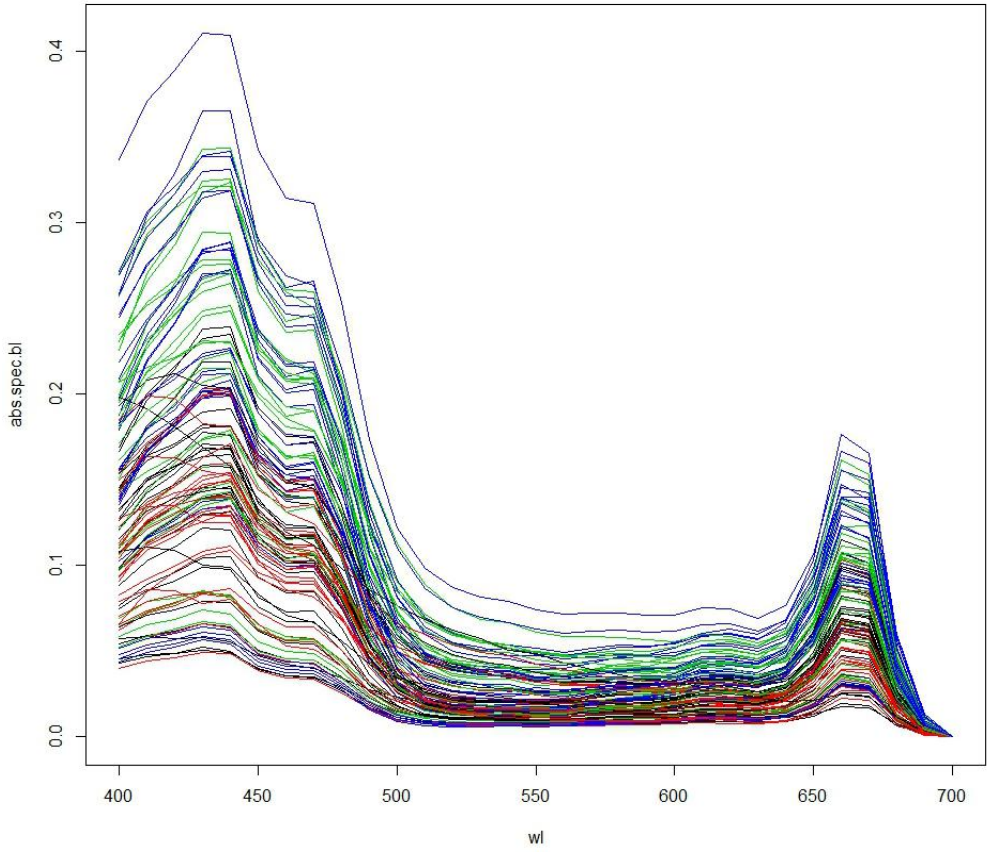

Fig.3.5. Absorbance spectra after subtraction of absorbance at 700nm.

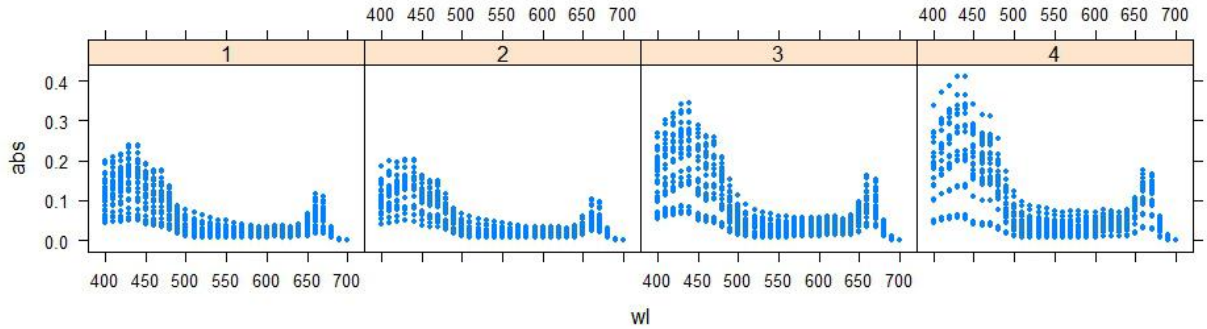

Fig.3.6. Absorbance values against wavelength grouped by treatment.

Pigment data

The average estimated concentrations of Chlorophyll A for treatments #1, #2, #3 and #4 were 1.1, 1.3, 1.6 and 2.1µgr/l respectively. It is apparent that the estimates were higher for treatments #3 and #4 than those of treatments #1 and #2 (see Fig.3.7.). The average estimated concentrations of Pheophytin a for treatments #1, #2, #3 and #4 were 0.005, 0.01, 0.05 and 0.035 µgr/l respectively. These estimates are also higher for treatments #3 and #4 than those of treatments #1 and #2. The highest variation is seen in treatment #4 (see Fig.3.8.).

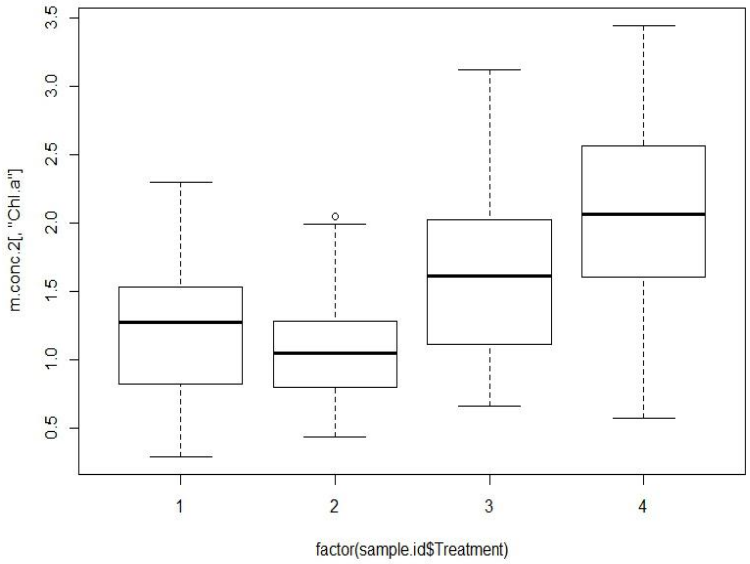

Fig.3.7. Chlorophyll A concentration estimates in different treatments

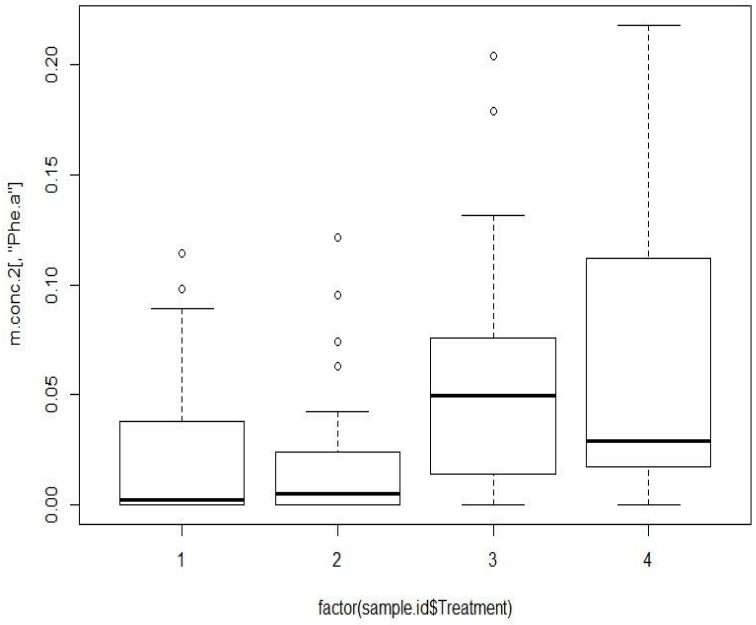

Fig.3.8. Pheophytin a concentration estimates in different treatments.

The values of concentration of Chlorophyll A estimated from deconvolution of absorbance spectra is highly correlated with those values calculated from in-vitro fluorescence measurements (see Fig.3.9.). Chlorophyll A concentration (estimated from absorbance spectra) is also fairly correlated with the concentration of Chlorophyll b and Lutein but not with the concentration of Pheophytin a (see Fig.3.10.)

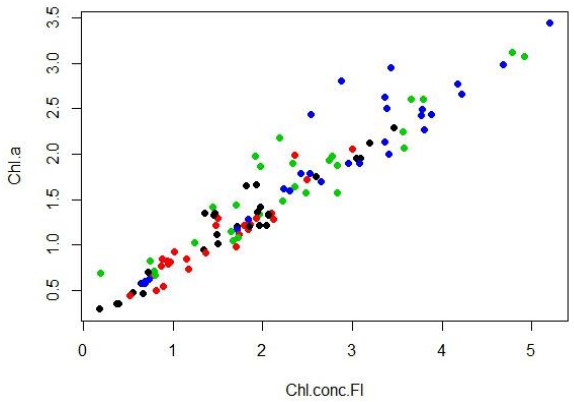

Fig.3.9. Correlation between concentration of Chlorophyll A (from absorbance spectra) and that of Chlorophyll A from in-vitro fluorescence.

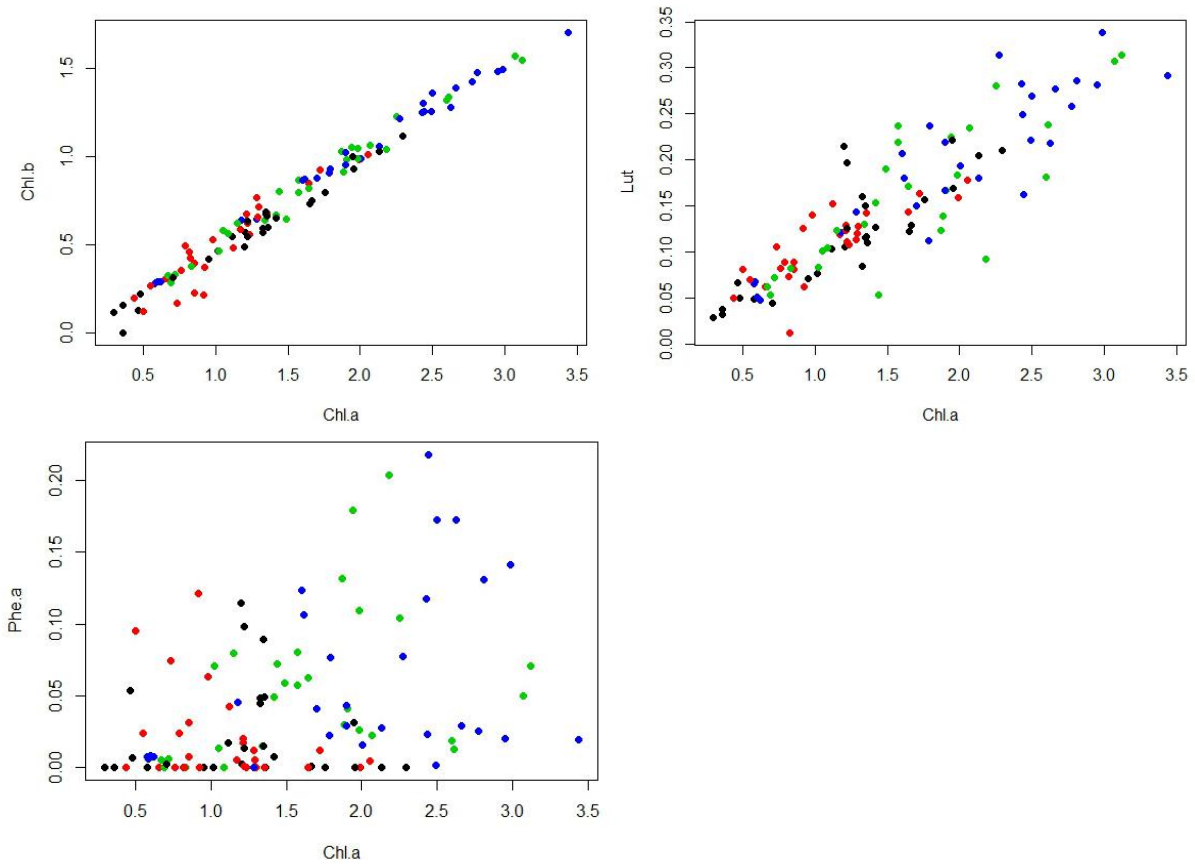

Fig.3.10. correlation between concentration of Chlorophyll A and three other pigments (Chl.b, Lut, Pheo.a) all estimated from absorbance spectra deconvolution.

Fig.3.11. shows that there was not much correlation between the concentration of Chlorophyll A and the values of algae cell count. The ratio of Pheophytin a concentration to that of Chlorophyll A was also calculated and its correlation with the values of rotifers' abundance was explored. Fig.3.12. visualizes this correlation.

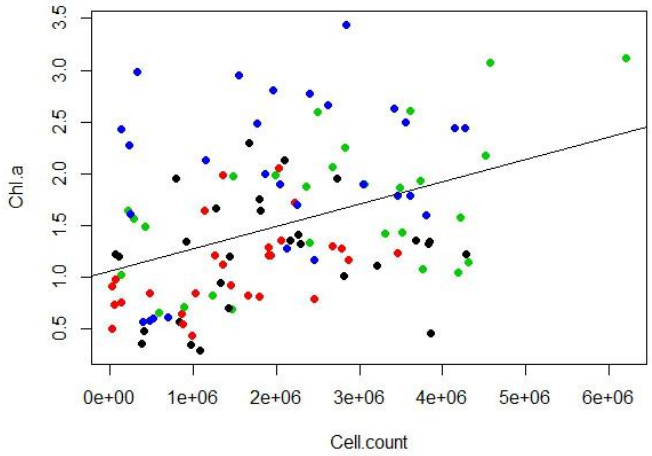

Fig.3.11. Chlorophyll A concentration from absorbance spectra vs. algae cell count values.

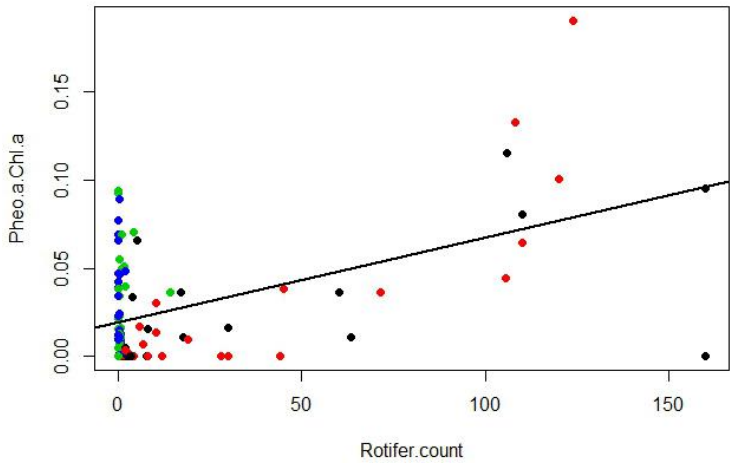

Fig.3.12. Correlation between Pheophytin a/Chlorophyll A ratio and number of rotifers per milliliter. Slope= 0.000481, standard error = 7.988e-05, Adjusted R-squared: 0.2413

Mixed effects analysis

In-vivo fluorescence

Fitting a non-linear model using "nlsList" delivered a list of parameter estimates (namely: Asym, Scal and xmid). The scatter plot matrix shows that these estimates are more or less correlated (Fig.3.13.). The function "nlme" was used to fit the model to observe the fixed effects and random effects. Results showed highly significant fixed effects and that random effects were highly correlated (more than 90%) (see appendix 2 (A2.1)).

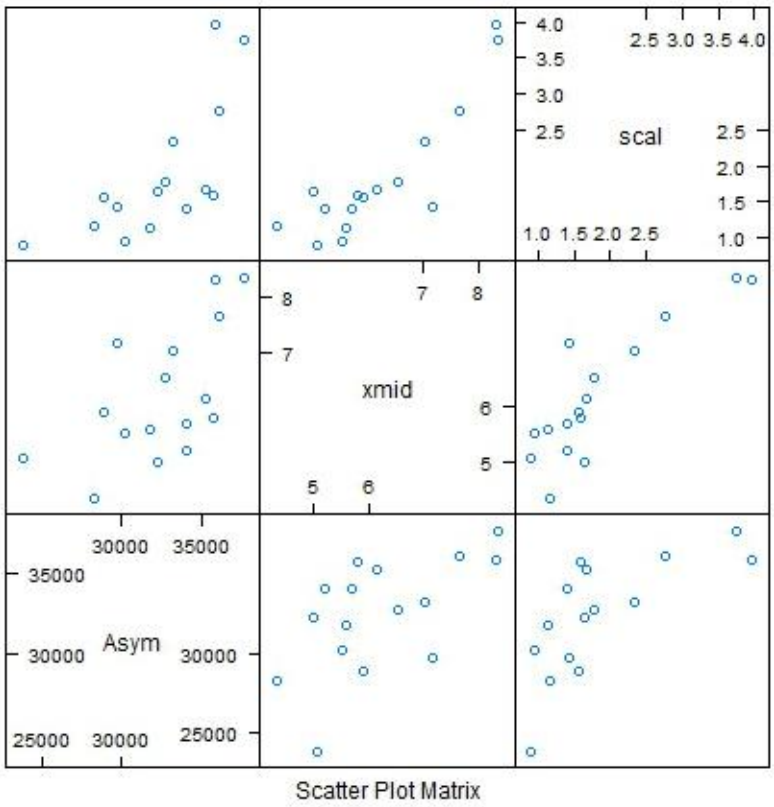

Fig.3.13. correlation between the parameter estimates of the non-linear model using nlsList.

Augmented predictions of the model suggested that treatments #1 and #3 respectively had the lowest and highest fluorescence values compared to what the model had predicted (see Fig.3.14.). Standardized residuals seem to be fairly normally distributed along the quantiles of standard normal (see Fig.3.15.).

Since the factor "Treatment" was assumed to be the fixed effect, the model was modified so that only the asymptote (i.e. carrying capacity) varied with treatment. This new model suggested that treatment #3 had the highest carrying capacity and that all treatments had the same maximal specific growth rate (i.e. μmax = $\frac{1}{\exp(scal)}$). The model delivers much less correlated estimates and it is preferred upon the first model according to the result of analysis of variance (see appendix 2 (A2.2)). Fig.3.16. shows augmented predictions for the model. Note that asymptote varies among treatments and is highest in treatment #3. The distribution of residuals for this model is also shown to be reasonably normal except for one extreme value (see Fig.3.17).

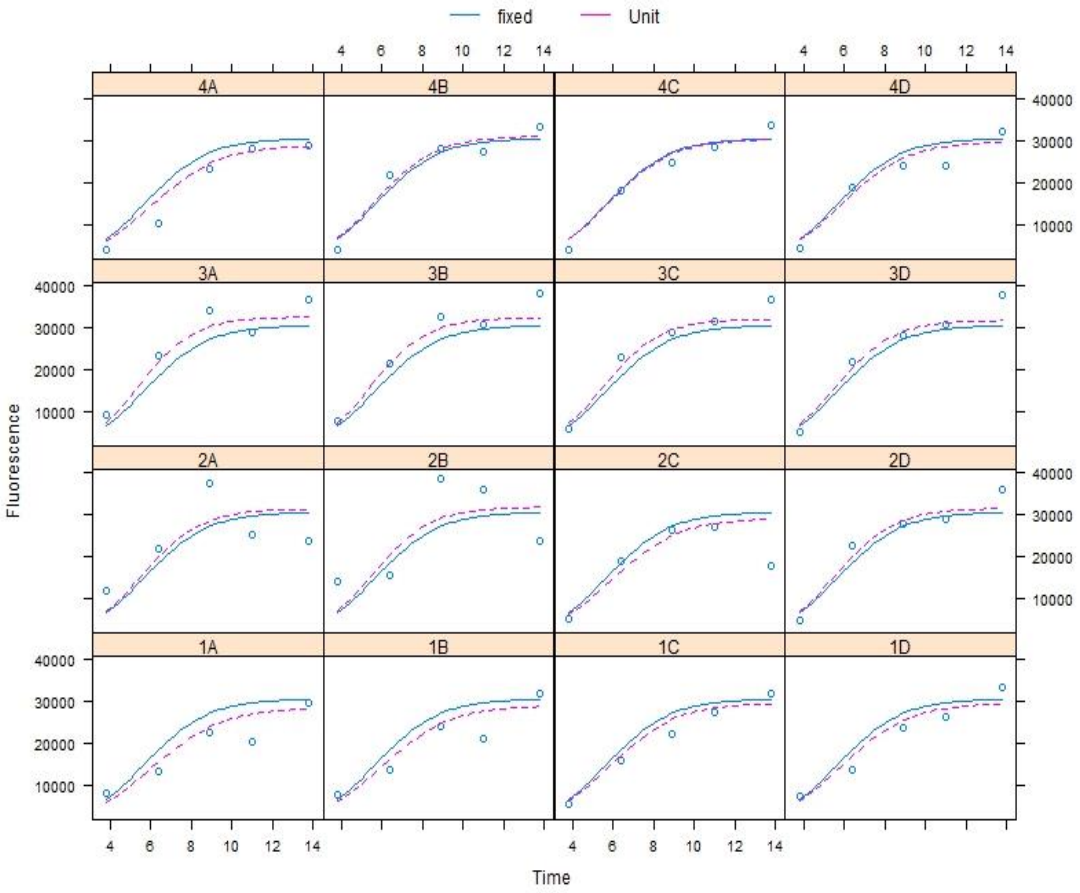

Fig.3.14. Augmented predictions plot comparing the fitted curve and the values of each unit to the fitted curve predicted by the model.

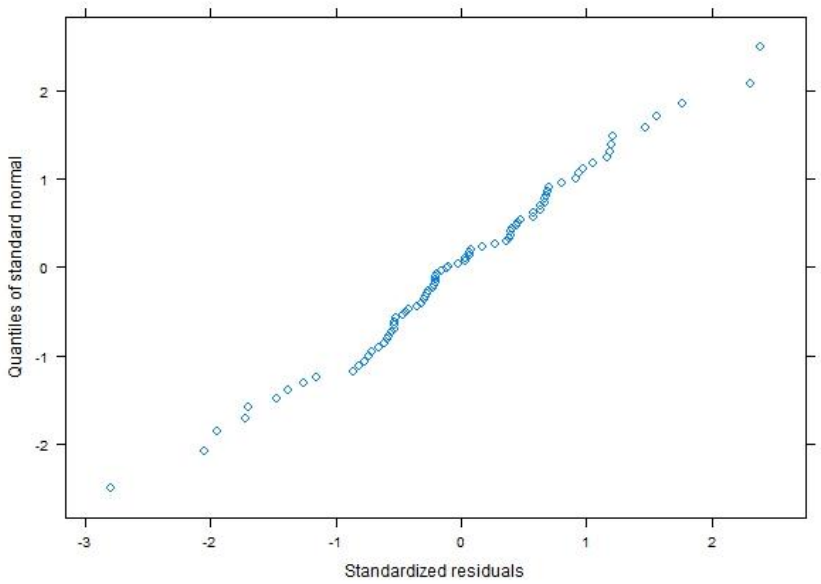

Fig.3.15. qqnorm plot shows the distribution of standardized residuals along the quantiles of standard normal.

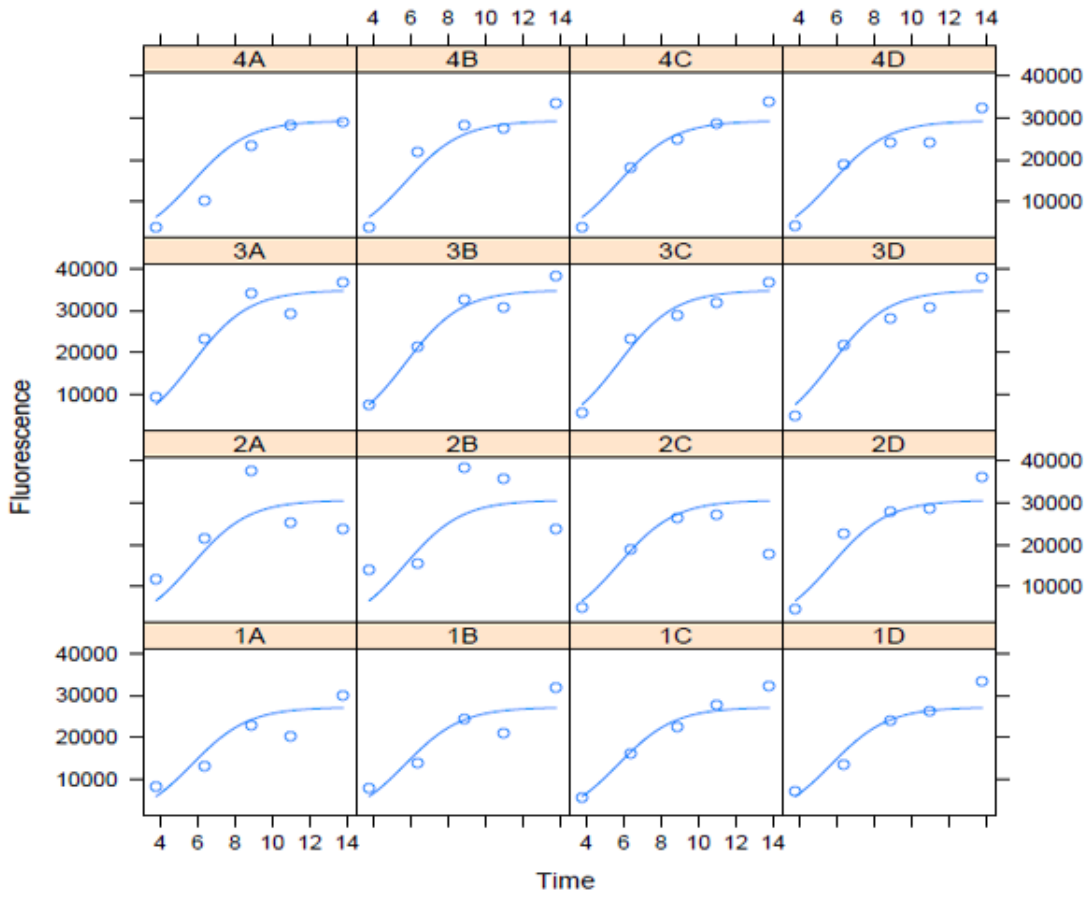

Fig.3.16. Augmented predictions presents the values of each unit and the curve fitted by the model. Note that the predicted asymptote is different for each treatment.

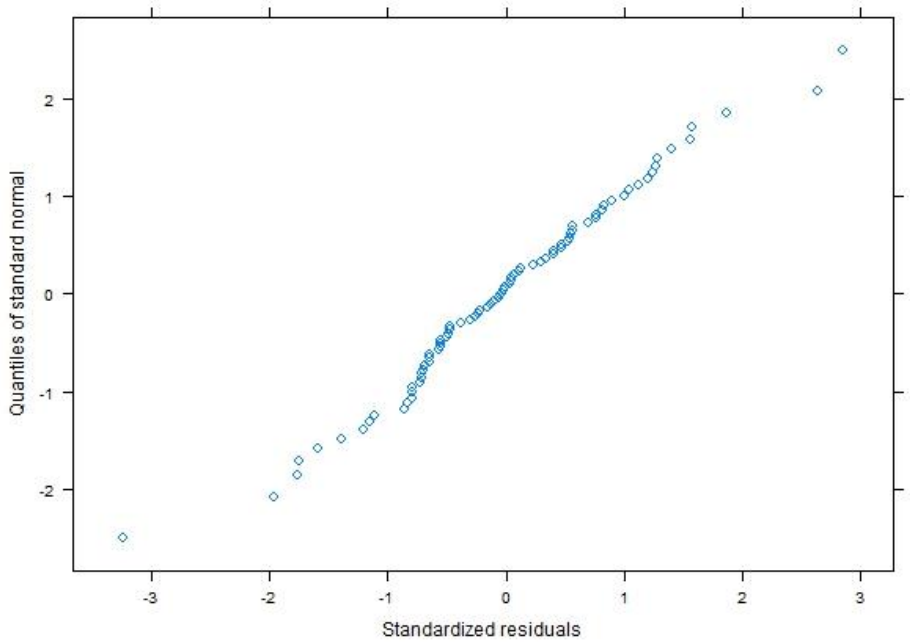

Fig.3.17. The distribution of standardized residuals remained reasonably normal for the modified model.

Algae cell count

Patterns in the population of algae cells were more or less similar between treatments. Maximum population density and the degree of fluctuation however varied considerably. Fig.3.18. shows the fluctuations of algae population along time in all four treatments. Range and degree of variation in the measured number of algae cells is also presented in Fig.3.19. It appears that treatment #3 had the highest capacity for algae growth among other treatments.

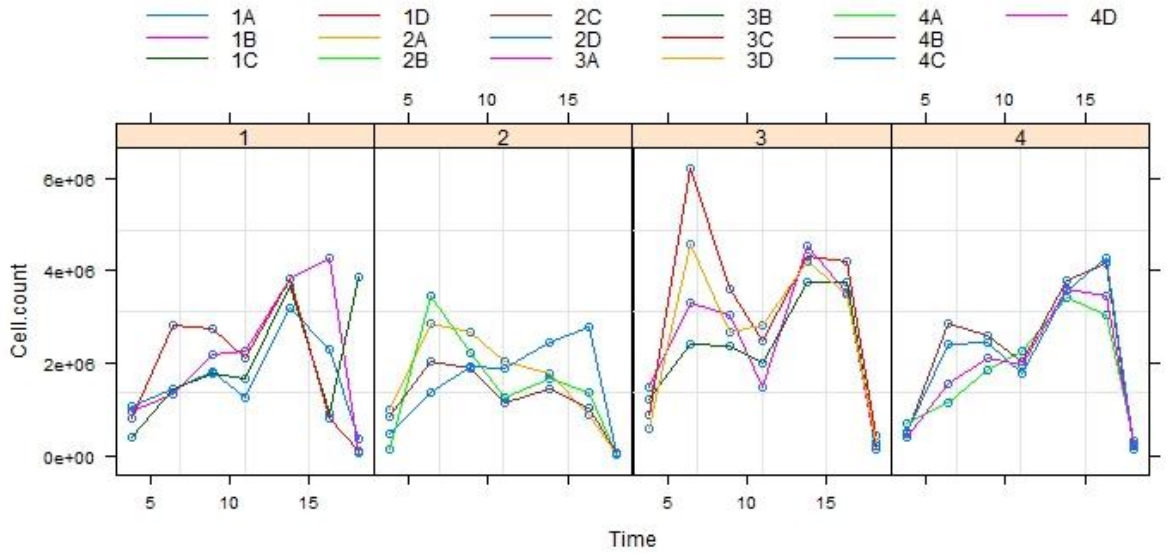

Fig.3.18. Variations of algae population in different treatments. (up to day 14 of the experiment)

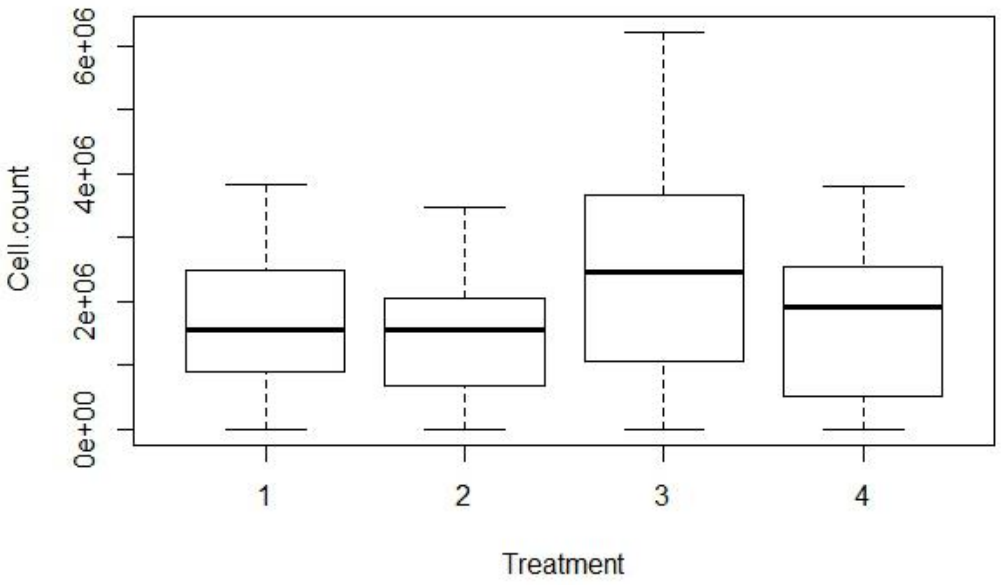

Fig.3.19. abundance and variation in the number of algae cells per milliliter

Rotifer counts

A dramatic difference was observed in the results of rotifer counts between treatments #1, #2 and treatments #3, #4. The number of rotifers kept increasing until the end of the experiment up to more than 150 rotifers per milliliter in the first two treatments while no such increase happened in treatments #3 and #4. Changes in abundance of rotifers and range and variation of this parameter are shown in Figures (3.20.) and (3.21.).

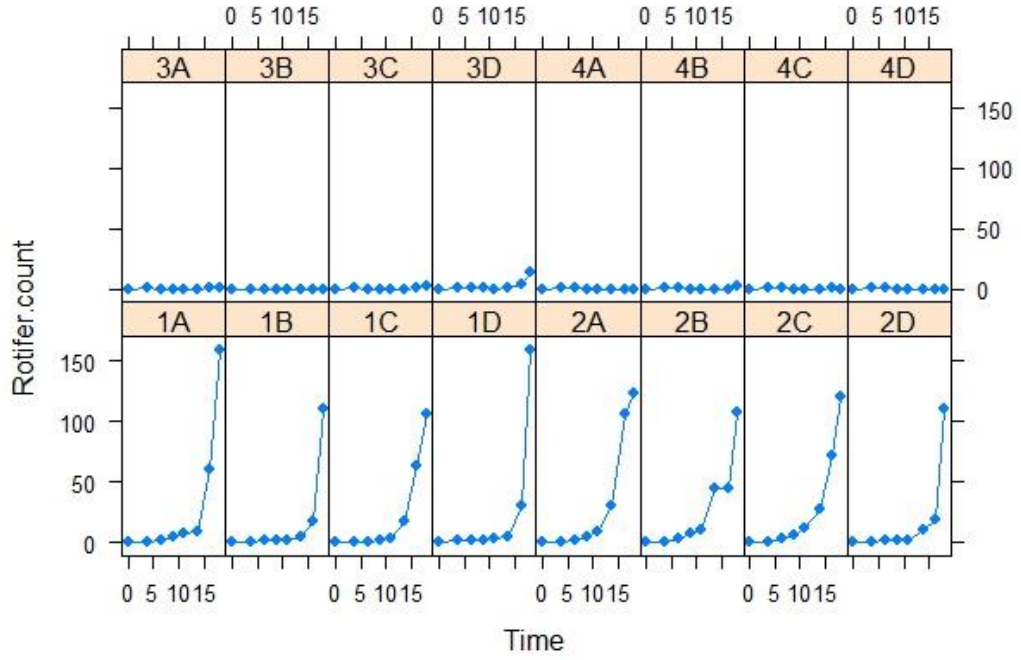

Fig.3.20. Changes in the number of rotifers presented for each unit

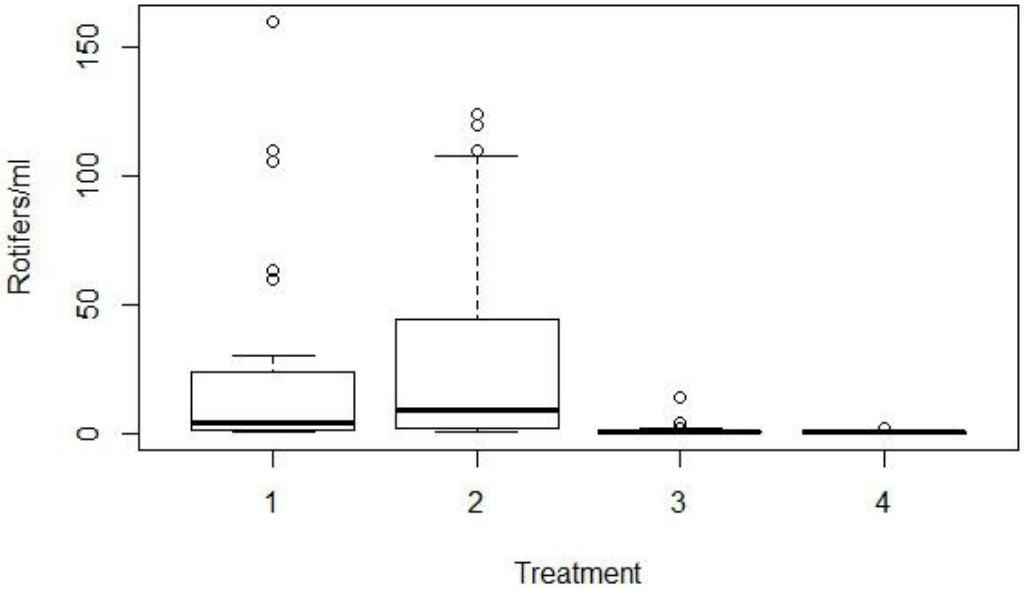

Fig.3.21.Comparison of the abundance and range among treatments

4. Discussion

Chlamydomonas growth assessment methods

In-vivo fluorescence

I used a few different methods to assess algal growth. The most efficient among those is measuring in-vivo fluorescence since it involves a rather simple and time saving procedure. The major advantage of using fluorescence is that the measurement is easy to make (Falkowski & Kiefer, 1985). However, despite some sound theoretical models describing variable fluorescence there are many environmental factors influencing fluorescence about which little is known (Falkowski & Kiefer, 1985). Thus I believe this method is especially suitable for comparing the growth of *Chlamydomonas* in different units/media, etc.

In-vitro fluorescence

In terms of precision however, it seems that measuring the fluorescence from extracted pigments is favored. Although this method is more time consuming and demanding, there are advantages to it compared to the "in-vivo" alternative: The extraction stops the electron transfer and other processes which interact with chlorophyll fluorescence when measured in vivo. As a result the response is stabilized and the sensitivity improved (Mayer et al., 1997). This method also provides the opportunity to calculate the concentration of Chlorophyll A in the samples based on measured fluorescence from standard pure stock solutions of the pigment.

Absorbance spectra analysis

"Gaussian Peaks" method (GPs) was used for estimating the concentration of individual pigments in the samples (Thrane et al., 2015) (Fig.3.7. & Fig.3.8.). While it has the same preparation requirements as for in-vitro fluorescence (freeze-drying and pigment extraction) it is also very time consuming to read the absorbance and extract the results data from the device. However, high degree of correlation between the concentrations of Chlorophyll A estimated using the GPs method and that of Chlorophyll A calculated from in-vitro fluorescence (Fig.3.9.) suggests that GPs is a rather reliable method for estimating Chlorophyll concentrations. Thrane et al. have compared their pigment concentrations estimated by GPs with concentrations obtained using high performance liquid chromatography (HPLC) and found that the deviance between observed and fitted spectra was generally very low; indicating that measured spectra could successfully be reconstructed as weighted sums of pigment and background components. In general, It is a fast, inexpensive, and high-throughput alternative for screening of pigment composition in samples of phytoplankton material (Thrane et al., 2015).

"Chlamydomonas" cell count

Counting the number of algae cells was another method for observing the growth of *Chlamydomonas*. While the number of cells per unit of volume can reveal the pattern of changes in the population it is neither the most precise nor the most efficient method of assessing the growth of *Chlamydomonas* among the above mentioned methods. This method requires some preparation and the measurement itself is a rather slow process. Conditions which are quite obscure may alter the relation between the division rate and the growth in size of the cells of the population (Ketchum & Redfield, 1949). The synthesis of new material proceeds with greater regularity than does the cycle of cell division. Because of this consideration, cell counts are not necessarily a precise index of the growth of the culture, and some of the irregularity in growth curves based on cell counts may be thus explained(Ketchum & Redfield, 1949).

Best type of media for growing *Brachionus calyciflorus*

Among the four types of media #1 and #2 clearly provide better growth conditions for the rotifers (*Brachionus calyciflorus*) than #3 and #4. Between treatments #1 and#2 however no significant distinction is observed (seeFig.4.1.).

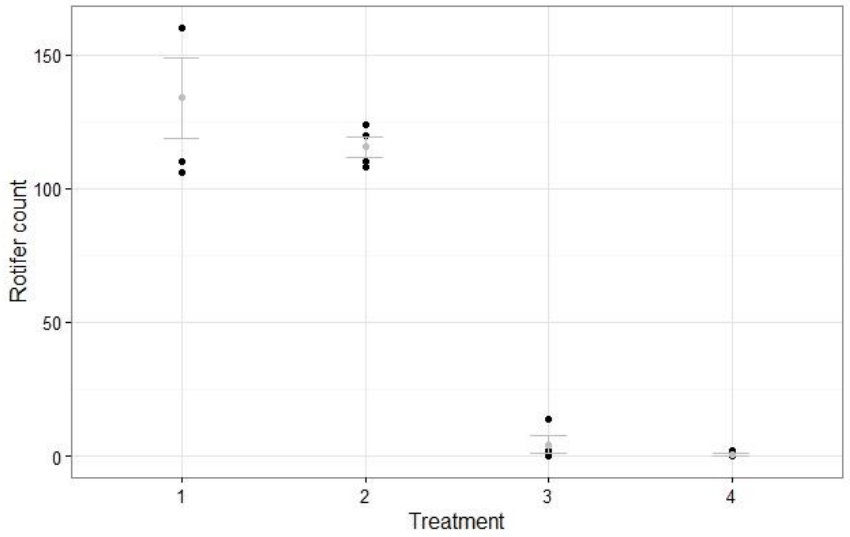

Fig.4.1. comparing the results of samplings on day 18.

Grazing by rotifers and chlorophyll degradation

Welschmeyer and Lorenzen 1985 assume that pheopigments detected in nature are produced mostly by the activity of herbivorous grazers. Owens and Falkowski (1982) have provided in vitro evidence for a cellular magnesium-releasing enzyme which catalyzes the conversion of chlorophyll a to Pheophytin A (Welschmeyer & Lorenzen,

1985). One may infer that in an environment with a growing population of rotifers, the rate of conversion of Chlorophyll A to Pheophytin A should be increasing and therefore, the ratio of Pheophytin A to Chlorophyll A concentration should vary in relation to the number of rotifers [Tom Andersen, personal communication]. Accordingly, in treatments #1 and#2 which show a high rate of reproduction (in contrast with treatments #3&#4, there was a positive correlation between the ratio of Pheophytin A / Chlorophyll A and the number of rotifers. (Fig.3.12.).

Explanation of the differences between treatments

This can be discussed in two parts: In the first 14 days of the beginning of the experiment the measured fluorescence and the number of algae cells are increasing. This increase is generally higher for treatments #3 and #4 which is more or less reflected in both fluorescence and cell count. Lower levels of Fluorescence and algal population density in treatments #1 and#2 can be explained by rotifer population growth in these treatments.

In the second part (after day14), the number of algae cells is generally dropping in almost all units but in treatments #3 and #4 the level of measured fluorescence does not decrease as much. That is because in treatments #1 and #2 there was a rapid growth in rotifer population and they were grazing on the remaining population of algae. In treatments #3 and#4, only the number of algae cells decreases since the number of rotifers is low in these treatments and the algae cells just die and their pigments are released into the water. Hence, we don't see a significant drop in the level of in-vivo fluorescence.

Conclusion

The experiment was designed and conducted to compare the carrying capacity for two model organisms (a freshwater rotifer and a single celled alga) in four media types. Two of the media were based on tap-water and the other two modified versions of Guillard's WC medium based on distilled water and deionized distilled water(MilliQ).

The algae grew in all the four media reasonably well while the rotifers only grew in the tap-water based media (#1 and #2). This could be due to presence of some micro-nutrients in the undefined tap-water base which are non-existent in the other two media. It also might be because of the fairly high amount of Borate in distilled water based media (#3 and #4). These however are only speculations and further research is needed to investigate the robustness and possible mechanisms behind the results obtained in this study.

References

Alvarado-Flores, J., Rico-Martinez, R., Adabache-Ortiz, A., & Silva-Briano, M. (2015). Morphological alterations in the freshwater rotifer Brachionus calyciflorus Pallas 1766 (Rotifera: Monogononta) caused by vinclozolin chronic exposure. *Ecotoxicology, 24*(4), 915-925. doi:10.1007/s10646-015-1434-8

Benjamin L. Preston *, G. C., Terry W. Snell. (1999). Effects of pentachlorophenol on predator avoidance behavior of the rotifer Brachionus calyciflorus. *Aquatic Toxicology, 44*, 201–212.

C.P. Charoy", C.R. Janssenb, G. Persooneb, P. Ckment". (1995). The swimming behaviour of Brachionus caZyczj2oru.s (rotifer) under toxic stress. I. The use of automated trajectometry for determining sublethal effects of chemicals. *Aquatic Toxicology*(32), 271-282.

Carmichael, W. W., & Gorham, P. R. (1974). IMPROVED METHOD FOR OBTAINING AXENIC CLONES OF PLANKTONIC BLUE-GREEN-ALGAE. *Journal of Phycology, 10*(2), 238-240.

Falkowski, P., & Kiefer, D. A. (1985). CHLOROPHYLL-A FLUORESCENCE IN PHYTOPLANKTON - RELATIONSHIP TO PHOTOSYNTHESIS AND BIOMASS. *Journal of Plankton Research, 7*(5), 715-731. doi:10.1093/plankt/7.5.715

Felpeto, A. B., & Hairston, N. G. (2013). Indirect bottom-up control of consumer-resource dynamics: Resource-driven algal quality alters grazer numerical response. *Limnology and Oceanography, 58*(3), 827-838. doi:10.4319/lo.2013.58.3.0827

Fischer, B. B., Roffler, S., & Eggen, R. I. L. (2012). Multiple stressor effects of predation by rotifers and herbicide pollution on different Chlamydomonas strains and potential impacts on population dynamics. *Environmental Toxicology and Chemistry, 31*(12), 2832-2840. doi:10.1002/etc.2010

Guillard, R. R. L. (1975). Culture of phytoplankton for feeding marine invertebrates. *InW. L. Smith &M. H. Chantey (eds), Culture of Marine Invertebrate Animals. Plenum Publishers, New York:*, 29–60.

Hedges, S. B. (2002). The origin and evolution of model organisms. *Nature Reviews Genetics, 3*(11), 838-849. doi:10.1038/nrg929

Janssen, C. R., Rodrigo, M. D. F., & Persoone, G. (1993). ECOTOXICOLOGICAL STUDIES WITH THE FRESH-WATER ROTIFER BRACHIONUS-CALYCIFLORUS .1. CONCEPTUAL-FRAMEWORK AND APPLICATIONS. *Hydrobiologia, 255*, 21-32. doi:10.1007/bf00025816

Ketchum, B. H., & Redfield, A. C. (1949). SOME PHYSICAL AND CHEMICAL CHARACTERISTICS OF ALGAE GROWTH IN MASS CULTURE. *Journal of Cellular and Comparative Physiology, 33*(3), 281-299. doi:10.1002/jcp.1030330303

Kilham, S. S., Kreeger, D. A., Lynn, S. G., Goulden, C. E., & Herrera, L. (1998). COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia, 377*, 147-159. doi:10.1023/a:1003231628456

Lehman, J. T. (1976). PHOTOSYNTHETIC CAPACITY AND LUXURY UPTAKE OF CARBON DURING PHOSPHATE LIMITATION IN PEDIASTRUM-DUPLEX (CHLOROPHYCEAE). *Journal of Phycology, 12*(2), 190-193. doi:10.1111/j.0022-3646.1976.00190.x

Mayer, P., Cuhel, R., & Nyholm, N. (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research, 31*(10), 2525-2531. doi:10.1016/s0043-1354(97)00084-5

Morel, F. M. M., J. C. Westall, & Chaplick, J. G. R. J. P. (1975). *Description of the algal growth media 'Aquil' and 'Fraquil'.* Retrieved from Water Quality Laboratory:

Snell, T. W., & Janssen, C. R. (1995). Rotifers in ecotoxicology: A review. *Hydrobiologia, 313*, 231-247. doi:10.1007/bf00025956

Stein, J. R. e. (1973). *Handbook of Phycological Methods: Culture Methods and Growth Measurements.* Cambridge University Press.

Strojsova, M., Nedoma, J., Sed'a, J., & Vrba, J. (2008). Diet quality impact on growth, reproduction and digestive activity in Brachionus calyciflorus. *Journal of Plankton Research, 30*(10), 1123-1131. doi:10.1093/plankt/fbn069

Thrane, J. E., Kyle, M., Striebel, M., Haande, S., Grung, M., Rohrlack, T., & Andersen, T. (2015). Spectrophotometric Analysis of Pigments: A Critical Assessment of a High-Throughput Method for Analysis of Algal Pigment Mixtures by Spectral Deconvolution. *PLOS ONE, 10*(9), 24. doi:10.1371/journal.pone.0137645

Vollenweider, R. A. e., Talling, J. F. e., & Westlake, D. F. e. (1974). *A manual on methods for measuring primary production in aquatic environments*: Blackwell Scientific Pub.

Welschmeyer, N. A., & Lorenzen, C. J. (1985). CHLOROPHYLL BUDGETS - ZOOPLANKTON GRAZING AND PHYTOPLANKTON GROWTH IN A TEMPERATE FJORD AND THE CENTRAL PACIFIC GYRES. *Limnology and Oceanography, 30*(1), 1-21.

Appendix 1

Table A1. Procedures for making primary stocks, working solutions and final media.

Solution	Compound	Amount added to 50 ml primary stock solution	Amount added to 100 ml working solution	Amount taken from primary stock solution: Primary stock solutions	Amount taken from working solution: Working solutions	Dissolved into 1L of corresponding base water (final volume)
Major elements	K_2HPO_4		0.871 gr	→	**1ml** →	→
	$NaNO_3$		8.5 gr	→	**1ml** →	
	$CaCl_2 2H_2O$		3.676 gr	→	**1ml** →	
	$MgSO_4 7H_2O$		3.697 gr	→	**1ml** →	
	$NaHCO_3$		1.26 gr	→	**1ml** →	
	H_3BO_3		2.4 gr	→	**1ml** →	
Algal Trace Elements (ATE)	Na_2EDTA		0.436 gr	→	→	**Final Medium**
	$FeCl_3 6H_2O$		0.315 gr	→	→	
	$MnCl_2 4H_2O$	9 gr →		100 µL →	→	
	$CuSO_4 5H_2O$	0.125 gr →		100 µL →	**1ml** →	
	$ZnSO_4 7H_2O$	1.1 gr →		100 µL →	→	
	$CoCl_2 6H_2O$	0.5 gr →		100 µL →	→	
	$NaMoO_4 2H_2O$	0.315 gr →		100 µL →	→	
VIM	Thiamin HCl		20 mg	→	**100 µL** →	

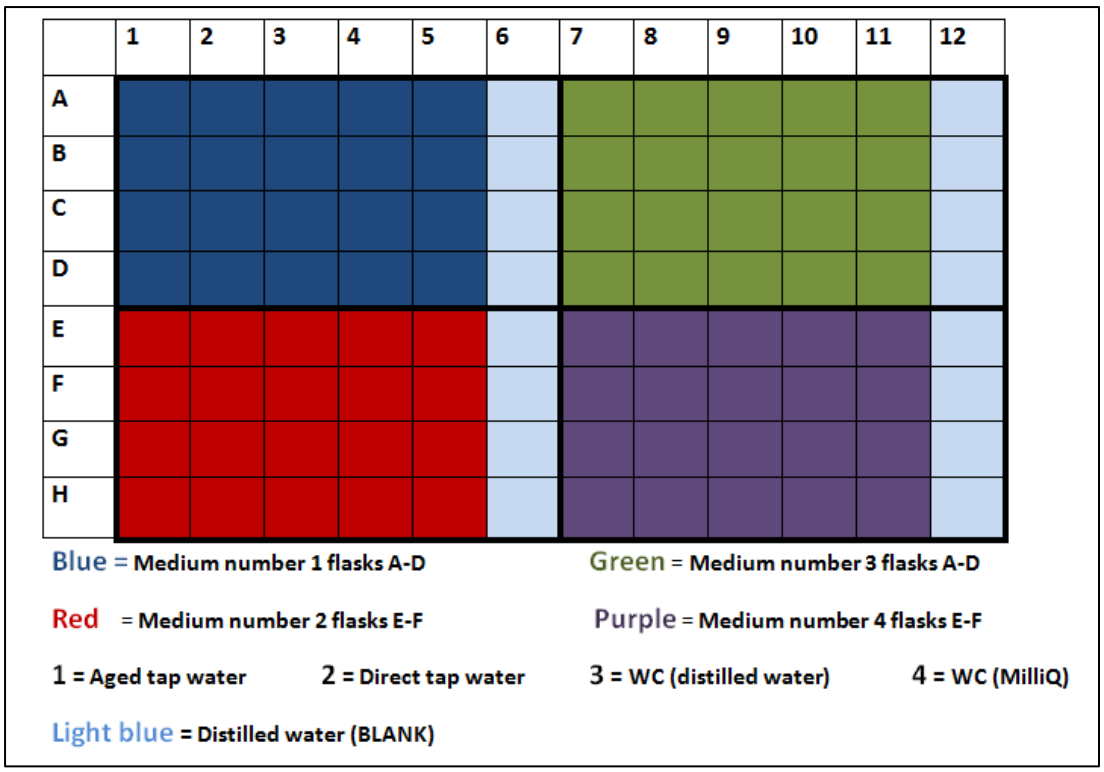

Fig.A1. Plate layout for in-vivo flourescence and absorbance.

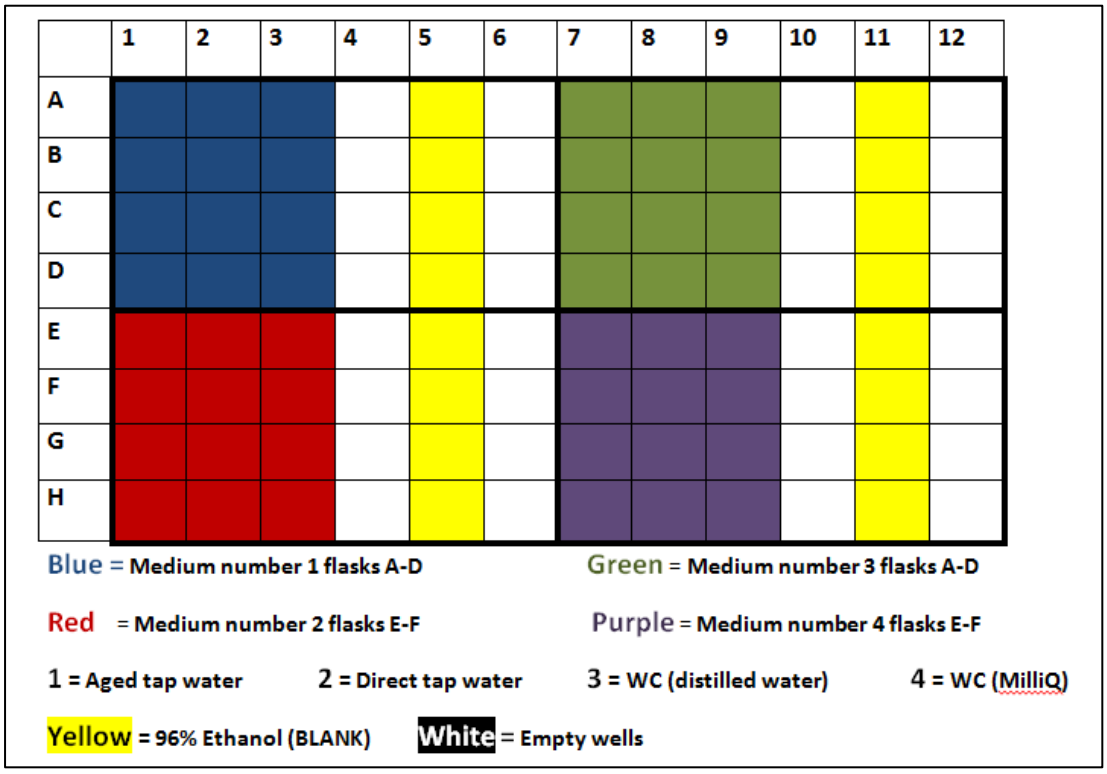

Fig A2. Plate layout for Chlorophyll fluorescence and absorbance

Fig.A3. The plate reader device (BioTek Synergy Mx)

Fig.A4. CASY cell counter device.

Specifications and protocols used for the plate reader device:

- **Biotek Synergy Mx | BioTek Instruments**.
  HIGHLAND PARK, BOX998
  WINOOSKI, VT 05404-998
  TEL. 802-655-4040 SN 219551
  MADE IN THE U.S.A.

- **Protocol for reading in-vivo absorbance and fluorescence:**
  Read absorbance in 500 nm
  Read absorbance in 700 nm
  Read fluorescence in the range of 460-680 nm
- **Protocol for reading Chlorophyll Absorbance and fluorescence:**
  Read absorbance in the range of 400-700 nm
  Read fluorescence in the range of 430-675 nm

Appendix 2

A2.1

```
> # Independent parameter estimates for each unit
> summary(m.0 <- nlsList(SSlogis, data = gd))
Call:
  Model: Fluorescence ~ SSlogis(Time, Asym, xmid, scal) | Unit 
   Data: gd

Coefficients:
   Asym
   Estimate Std. Error   t value    Pr(>|t|)
1A 35903.91  24461.387  1.467779 0.199249913
1B 37776.87  22228.142  1.699506 0.203199269
1C 33238.28   7547.537  4.403857 0.005588596
1D 36211.01  11102.274  3.261584 0.012858305
2A 28321.58   3071.680  9.220225 0.033832092
2B 32315.62   3926.986  8.229115 0.069895711
2C 23726.35   2888.674  8.213581 0.017966908
2D 31751.01   3222.869  9.851785 0.007777240
3A 34077.76   3526.666  9.662883 0.007644886
3B 35744.50   4105.909  8.705623 0.006679907
3C 34042.75   3679.235  9.252671 0.005195894
3D 35293.59   4518.591  7.810750 0.011004798
4A 29744.61   4647.343  6.400349 0.001619864
4B 30188.95   2987.211 10.106065 0.004195830
4C 32751.50   5076.780  6.451234 0.008014152
4D 28859.66   4121.572  7.002101 0.015923924
   xmid
   Estimate Std. Error  t value    Pr(>|t|)
1A 8.324282  6.3821903 1.304299 0.234976312
1B 8.359303  5.2991484 1.577480 0.225592946
1C 7.025531  1.6426973 4.276826 0.005922526
1D 7.646370  2.3246083 3.289315 0.012646489
2A 4.320513  0.8081840 5.345953 0.091648707
2B 4.992110  0.9431239 5.293165 0.147790362
2C 5.070161  0.9047496 5.603938 0.037448729
2D 5.580823  0.7198406 7.752860 0.012469197
3A 5.222722  0.7831843 6.668573 0.015852493
3B 5.795383  0.8375770 6.919224 0.010513153
3C 5.686438  0.7822300 7.269522 0.008377105
3D 6.132597  0.9152616 6.700376 0.014866712
4A 7.159199  0.9938609 7.203422 0.001279471
4B 5.529694  0.6930216 7.979108 0.006705452
4C 6.525783  1.0875555 6.000414 0.009246342
4D 5.914675  1.0281829 5.752551 0.023326557
   scal
    Estimate Std. Error  t value   Pr(>|t|)
1A 3.9745417  3.8660434 1.028064 0.31651194
1B 3.7621850  3.2329922 1.163685 0.32983691
1C 2.3242339  1.3810481 1.682949 0.03649120
1D 2.7702619  1.7359375 1.595830 0.05064145
2A 1.1550120  0.8764980 1.317758 0.52799932
2B 1.6503296  0.9875999 1.671051 0.54269524
2C 0.8819923  0.5884620 1.498809 0.31139705
2D 1.1372150  0.6062458 1.875832 0.16495348
3A 1.4000253  0.7229787 1.936468 0.15031829
3B 1.5773858  0.7818741 2.017442 0.10603220
```

```
3C 1.3928615  0.7102587 1.961062 0.09948301
3D 1.6711652  0.8397130 1.990162 0.13764123
4A 1.4295537  0.8251716 1.732432 0.02145247
4B 0.9485896  0.5299354 1.790010 0.11235655
4C 1.7803026  0.9637921 1.847185 0.08632087
4D 1.5553540  0.9498073 1.637547 0.20856783

Residual standard error: 4728.108 on 32 degrees of freedom
```

```
> # Parameter estimates assumed to be drawn from a normal distribution (=
random effects)
> summary(m.1 <- nlme(m.0)) # Highly correlated random effects, highly sig
nificant fixed effects
Nonlinear mixed-effects model fit by maximum likelihood
  Model: Fluorescence ~ SSlogis(Time, Asym, xmid, scal)
 Data: gd
       AIC      BIC    logLik
  1586.692 1610.512 -783.3459

Random effects:
 Formula: list(Asym ~ 1, xmid ~ 1, scal ~ 1)
 Level: Unit
 Structure: General positive-definite, Log-Cholesky parametrization
         StdDev       Corr
Asym     1747.6317270 Asym   xmid
xmid        0.2823084 -0.918
scal        0.1626633 -0.914  0.990
Residual 3948.5370584

Fixed effects: list(Asym ~ 1, xmid ~ 1, scal ~ 1)
         Value Std.Error DF  t-value p-value
Asym 30631.421  947.3446 62 32.33398       0
xmid     5.780    0.2114 62 27.33746       0
scal     1.523    0.1868 62  8.15556       0
 Correlation:
     Asym  xmid
xmid 0.342
scal 0.403 0.326

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.8028642 -0.5364691 -0.1106758  0.6317417  2.3944878

Number of Observations: 80
Number of Groups: 16
```

A2.2

```
> # Let only the carrying capacity (Asym) vary with Treatment (starting va
lues w. zeros for each treatment contrast)
> summary(m.2 <- update(m.1, fixed=list(Asym ~ Treatment, scal + xmid ~ 1)
, start=c(30000, 0, 0, 0, 5.8, 1.5)))
Nonlinear mixed-effects model fit by maximum likelihood
  Model: Fluorescence ~ SSlogis(Time, Asym, xmid, scal)
 Data: gd
      AIC      BIC    logLik
  1576.93 1607.896 -775.4649

Random effects:
 Formula: list(Asym ~ 1, xmid ~ 1, scal ~ 1)
 Level: Unit
 Structure: General positive-definite, Log-Cholesky parametrization
                  StdDev       Corr
Asym.(Intercept) 3.393605e-03 As.(I) xmid
xmid             8.031756e-05 0
scal             7.768814e-05 0      0
Residual         3.922063e+03

Fixed effects: list(Asym ~ Treatment, scal + xmid ~ 1)
                     Value Std.Error DF   t-value p-value
Asym.(Intercept) 27187.033 1276.1922 59 21.303244  0.0000
Asym.Treatment2   3478.581 1627.8883 59  2.136867  0.0368
Asym.Treatment3   7607.086 1633.7007 59  4.656352  0.0000
Asym.Treatment4   2112.821 1626.9165 59  1.298666  0.1991
scal                1.511    0.1872 59  8.070526  0.0000
xmid                5.731    0.2020 59 28.374200  0.0000
 Correlation:
                 As.(I) Asy.T2 Asy.T3 Asy.T4 scal
Asym.Treatment2 -0.618
Asym.Treatment3 -0.593  0.501
Asym.Treatment4 -0.626  0.501  0.500
scal             0.345  0.035  0.075  0.021
xmid             0.347  0.035  0.076  0.021  0.275

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.23938641 -0.64195889 -0.03949479  0.56354597  2.85290694

Number of Observations: 80
Number of Groups: 16
> # Parameter estimates much less correlated (-0.6 -> + 0.5)
> # Treatments 2/3 probably have higher carrying capacity than 1/4, with t
reatment 3 as highest
> # All media have the same maximal specific growth rate (μmax = 1 / exp(s
cal)) and starting population size (xmid)
> anova(m.1, m.2) # AIC/BIC in favour of m.2
    Model df      AIC      BIC    logLik   Test  L.Ratio p-value
m.1     1 10 1586.692 1610.512 -783.3459
m.2     2 13 1576.930 1607.896 -775.4649 1 vs 2 15.76192  0.0013
```