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Mini-project report

Identifying and Characterising Microalgal Strains that Overproduce Triacylglycerol as Potential Sources of Biodiesel

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26 March 2010

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Abstract

In support of the requirement to develop a sustainable alternative to conventional transport fuels, two species of microalgae were investigated to assess their suitability as third generation fuel crops. The algae were placed under NaCl stress and their lipid content measured at various time intervals and salinities. It was discovered that, overall, the yield of biomass decreased with increasing salinity, and the percentage of lipid to biomass increased with increasing salinity. The highest lipid to biomass yields were found in cultures aged approximately one week, which would provide a fast turnaround time for biofuel production on a commercial scale. Based on the data gathered so far, it would be difficult to say which species would be chosen in preference for biofuel production. Further work would be recommended to confirm the data, and to ascertain the concentrations of neutral and polar lipids produced by each species.

1. Introduction

Amid speculations that the peak of global oil production has already passed,[1] it is of great importance now to replace transport fuels with a sustainable alternative. Biodiesel from microalgae, a third generation fuel crop, is proving to be a promising alternative, [2-3] especially when compared to first and second generation crops. Microalgae are not in direct competition with food crops as first generation fuel crops are and, as the oil content of algae compares favourably with other energy crops, considerably less land would be required to produce an equal amount of biofuel.[2] A recent life cycle analysis [4] has shown the differences in land requirement to be less dramatic than originally thought; however, it is predicted that canola, for example, would need 2.0 ± 0.2 hectares (ha) to produce one functional unit of energy, compared to algae that would require 0.4 ± 0.05 ha. Similarly, switchgrass, a second generation fuel crop, would require over four times the amount land than algae to produce an equivalent amount of energy.

Algae can be encouraged to over-produce lipid under certain types of stress, commonly: nutrient deficiency in the form of nitrogen deprivation,[5] or when in high concentrations of sodium chloride. In this study, the concentration of NaCl was varied in order to trigger synthesis of glycerol, a compatible solute. With the purpose of maintaining cell volume and function, and to prevent Na^+ poisoning of cells and cell membranes, a mechanism that converts starch into the compatible solute comes into play when intracellular water is reduced due to high external salinities. [6] Glycerol production is a rapid process seen in actively growing cells and allows photosynthesis to recommence under non-ideal conditions. Another, slower, mechanism seen in cells that are no longer growing rapidly produces triacylglycerol (TAG) in response to growth inhibition. This mechanism will be exploited when using algae to produce biofuels, but further research is required to understand how TAG could be synthesised preferentially within the cell. The algae are harvested by filtration, flocculation or centrifugation [3-5] and the TAG extracted from the biomass, which can be utilised separately. The TAG is converted to methyl esters of fatty acids,[2] or biodiesel, via transesterification using an excess of methanol. The main weakness in this process is separating the algae from the growth medium; some research has been undertaken in the area of growing algae on solid surfaces [7-8], which would make harvesting far simpler. The solid can be lifted from the medium and the top layers of algae scraped away, to leave a root layer on the solid that can be returned the medium for further growth.

Commercially, algae can be grown in either raceway ponds or photobioreactors [2, 4] but experts are divided on which method to employ. Both utilise natural light to avoid lighting costs and each offer various pros and cons. Photobioreactors are tubular arrays of glass or plastic solar collectors, which are filled with the microalgal culture. These may be more useful for growing small amounts of concentrated algal culture, as the assembly is difficult to scale-up beyond a tube length of 80 m. This is due to a requirement to return the culture to a degassing zone to extract the oxygen produced in photosynthesis, in order to avoid photooxidative

damage to cells. Raceway ponds are closed-loop recirculation channels that are open to the atmosphere and very simple to construct. Production volumes from the ponds can be negatively affected by contamination with microorganisms that feed on the algae, as well as poor growth rates caused by dark zones at the bottom of the channels. However, the use of raceway ponds is perceived to be the least expensive option [5] and likely to be used in preference to the photobioreactor for algal production on a large scale.

Both methods of production require a source of CO_2 to enable photosynthesis and it has been proposed that industrially-recovered CO_2 is used.[5] Indeed, location adjacent to a coal-fired power station would provide a convenient source of CO_2 from flue gases, and co-firing [9] with algal biomass would be financially and environmentally advantageous. As production of third generation fuel crops has been associated with greenhouse gas emissions,[4] maximising the amount of energy extracted from the original culture would help algal biofuels in its claim of carbon neutrality. A similar idea to site algae production alongside an anaerobic digester [2, 5, 10] has been well-received – following lipid extraction, the remaining biomass is fed into the digestion vessel, from which methane and liquid digestate is collected.[11] Methane would provide heat and electricity for use in the production process; liquid digestate a suitable addition to the growth medium as a nutrient source and a good alternative to industrially produced fertilisers. A reliable and steady supply of a suitable growth medium is essential and seawater, in combination with a marine alga, is a convenient option.[12] Algae can also be used to remove phosphorous in wastewater [13] and it has been proposed that this mechanism be utilised as a novel alternative to the chemical treatment of waste stabilisation ponds, at the same time as relieving the freshwater burden of algal cultivation.

The aim of this project was to identify the conditions under which two species of algae produced the most lipid and to compare the results from each species. In doing so, various laboratory skills were acquired via several experiments: sub-culturing using aseptic technique, gravimetric lipid measurement, determination of chlorophyll content, the use of Nile Red-staining and fluorescence microscopy, and cell counting techniques. The two species of alga to be investigated were *Tetraselmis suecica* and *Dunaliella salina*. *T. suecica* is a unicellular, marine green flagellate with cell wall; some strains of *Tetraselmis* can grow in a salinity range of 8.6-34%.[14] It is known for its high vitamin E content [15]and could, therefore, be useful as a source of the vitamin for human and animal consumption. *Dunaliella salina* is also a unicellular flagellate, but has no cell wall. The species is halophilic and does not produce lipid as a response to nitrogen deficiency. All algae were grown in an incubation laboratory kept at a constant temperature of 25 °C, and with a light intensity of 80-120 $\mu mol\ m^{-2}\ sec^{-1}$.

2. Methods

Full methodology for the following procedures can be found in Appendix 6.1:

- Sub-culturing of Microalgae
- Determination of Chlorophyll Content of *T. suecica*
- Gravimetric Measurement of Lipid Content in Algal Strains
- Determination of Algal Cell Number
- Visualisation of Nile Red Fluorescence in Microalgal Cells using Fluorescence Microscopy

3. Results and Discussion

3.1 T. suecica

As *T. suecica* is a marine alga, it was anticipated that culture would grow well in 0.4 M NaCl medium and this was confirmed by the results seen in Appendix 6.2. The sample sub-cultured at 2.0 M NaCl was unsuccessful, which determined the salinity limit to be 1.5 M NaCl for this strain of Tetraselmis. Bacterial contamination was observed at 0.4 M NaCl in the flasks and in the Falcon tubes during lipid measurement, but the culture did not

appear to be adversely affected by this. The algae propagated more slowly with increasing salinity; this observation was illustrated by the chlorophyll results at 11 days, which show the chlorophyll content decreasing with increasing salinity (Table 1, below). This was further confirmed by the cell count, with a decrease of 5 x 10^6 cells mL^{-1} from 0.4 to 1.5 M NaCl culture. However, this trend was not consistent across the full age range when comparing samples cultured and tested for chlorophyll on different days; this may be due to a variation in the initial growth rate at different salinities. The lipid/chlorophyll ratio was calculated for use in determining the sample size for subsequent gravimetric lipid measurements, for a minimum target weight of 0.5 mg lipid per sample.

Sample Details		**Chl. Content (µg mL^{-1})**	**Biomass Content (µg mL^{-1})**	**Lipid Content (µg mL^{-1})**	**Cells mL^{-1}**	**Lipid / Chl. Ratio**	**Lipid / Biomass %**	**Lipid / Cell (pg)**
Medium NaCl (M)	**Age of Culture (Days)**							
1.5	61	8.860	336.7	121.7	n/d	**13.7**	**36.1**	**n/d**
1.5	21	1.831	103.8	40.0	n/d	**21.9**	**38.6**	**n/d**
1.5	11	3.439	113.3	48.3	4.00 x 10^6	**14.1**	**42.6**	**12.1**
1.0	11	4.303	198.3	66.7	6.36 x 10^6	**15.5**	**33.6**	**10.5**
0.4	11	5.538	220.0	66.7	9.02 x 10^6	**12.0**	**30.3**	**7.4**
1.5	9	2.250	80.0	65.0	n/d	**28.9**	**81.2**	**n/d**
1.5	7	n/d	53.7	32.5	n/d	**n/d**	**60.5**	**n/d**
1.0	7	1.775	62.5	61.2	n/d	**34.5**	**98.0**	**n/d**
0.4	8	1.710	127.5	81.2	n/d	**47.5**	**63.7**	**n/d**

Table 1. Combined mean results for *T. suecica* from gravimetric measurement of lipid content, determination of chlorophyll content, and cell counts. For full results, see Appendix 6.3 to 6.5.

As expected, the µg mL^{-1} of biomass in the cultures increased with age and the starting point was salinity-dependent, i.e. the mass was higher at lower salinities. The exception to this was the sample taken at 21 days, where the results for chlorophyll and biomass (µg mL^{-1}) appear out-of-trend. However, the lipid/biomass percentage was not out-of-trend; it may be possible that the culture was not as concentrated, but the cells within the culture were behaving normally. The lipid/biomass percentage from 1.5 M NaCl cultures, aged 11-61 days, was fairly constant with a mean value of 39.1%, decreasing slightly with age. This followed an initial peak of 60.5 and 81.2% at 7 and 9 days respectively. A trend towards increased lipid/biomass percentage with increasing salinity was observed in cultures aged 11 days. This trend can also be seen in the lipid/cell results, with 7.4, 10.5 and 12.1 pg of lipid per cell in 0.4, 1.0 and 1.5 M NaCl respectively. While the amount of lipid/cell provides information about the cell's productivity, the lipid content (µg mL^{-1}) may be more useful on a commercial level as the cell number is intrinsic within this value.

The lipid/biomass percentage was unexpectedly high in week-old (7-9 days) cultures at all salinities, with the result of 98% lipid/biomass for the 1.0 M NaCl culture assumed to be subject to a large degree of experimental error. However, the trend for high lipid/biomass values can also be seen in the results for *D. salina* (Table 2, below). These values may be explained by the compatible solute mechanism – in the very early days of propagation, the algal cell may be required to produce glycerol preferentially in order to gain the cell functions required for further growth or, indeed, survival. Once the cell has become accustomed to its surroundings, a more normal growth pattern can be established, which is observed here by the decreasing percentage of lipid/biomass over time. If this is the case, the ideal time to harvest the algae for biofuel production would be at 8 days from 0.4 M NaCl culture; if more biomass is desirable, and a longer lead-time tolerable, then harvesting at 61 days from 1.5 M NaCl would produce both high lipid and biomass yields.

3.2 Visualisation of Nile Red Fluorescence in T. suecica *using Fluorescence Microscopy*

Samples of algal culture at 0.4 M NaCl and 1.5 M NaCl were prepared using Nile Red stain. A greater number of complete algal cells were seen in the 0.4 M NaCl sample compared to the 1.5 M NaCl sample, where more cell breakage occurred due to the elevated salinity. Collection of lipid around the central cell mechanism was seen in both samples. For images, see Appendix 6.6.

3.3 D. salina

Sample Details		Chl. Content ($\mu g\ mL^{-1}$)	Biomass Content ($\mu g\ mL^{-1}$)	Lipid Content ($\mu g\ mL^{-1}$)	Cells mL^{-1}	Lipid / Chl. Ratio	Lipid / Biomass %	Lipid / Cell (pg)
Medium NaCl (M)	Age of Culture (Days)							
2.0	42	20.75	540.00	101.67	n/d	**4.90**	**18.83**	**n/d**
3.5	26	9.72	271.67	100.00	8.53×10^6	**10.29**	**36.81**	**11.72**
3.0	27	12.78	293.33	106.67	1.22×10^7	**8.35**	**36.36**	**8.73**
2.0	26	13.42	446.67	117.78	2.09×10^7	**8.78**	**26.37**	**5.64**
1.5	27	17.06	401.67	135.00	1.56×10^7	**7.91**	**33.61**	**8.65**
1.0	27	18.35	416.67	123.33	1.17×10^7	**6.72**	**29.60**	**10.51**
0.4	27	16.44	515.00	123.33	1.60×10^7	**7.50**	**23.95**	**7.69**
2.0	21	7.66	271.67	81.67	n/d	**10.67**	**30.06**	**n/d**
3.0	12	4.70	141.67	73.33	n/d	**15.59**	**51.76**	**n/d**
3.5	9	2.36	51.25	45.00	n/d	**19.03**	**87.80**	**n/d**
2.0	9	7.07	208.33	85.00	n/d	**12.02**	**40.80**	**n/d**
2.0	7	n/d	236.67	86.67	n/d	**n/d**	**36.62**	**n/d**
1.5	7	5.26	121.67	70.00	n/d	**13.30**	**57.53**	**n/d**
1.0	7	6.30	138.33	75.00	n/d	**11.90**	**54.22**	**n/d**
0.4	7	12.75	131.67	78.33	n/d	**6.15**	**59.49**	**n/d**

Table 2. Combined mean results for *D. salina* from gravimetric measurement of lipid content, determination of chlorophyll content, and cell counts.

As with *T. suecica*, the biomass of the cultures increased with age; the starting point was salinity-dependent, although this was not a linear relationship, as seen in *T. suecica*. It can be seen that the lipid/biomass content decreases with age, ranging from 36.6% to 18.8% in 2.0 M NaCl cultures from 7 to 42 days old, peaking at 9 days. A general trend towards increased lipid/biomass percentage with increasing salinity was observed in cultures aged 26-27 days, although this is again less linear than seen in *T. suecica*. The highest Lipid/Cell results were seen in 1.0 and 3.5 M NaCl salinity, containing 10.5 and 11.7 pg respectively. This relationship is non-linear, with a dip in the lipid/cell results at 2.0 M NaCl that may correspond to the salinity to which *D. salina* is most suited. As observed with *T. suecica*, the lipid/biomass content was high in week-old (7-9 days) cultures at all salinities. For further comment on *D. salina* results, see report of the same name and date by E.P.H. Hounslow.

4. Conclusions and Future Work

Based on the data gathered during this mini-project, it is difficult to say whether *T. suecica* or *D. salina* would be chosen for biofuel production. The results are, in some ways, incomparable; for example, *T. suecica* produces more lipid per cell than *D. salina*, but the values were not obtained from cultures of the same age. However, the cultures of *D. salina* contain more cells; therefore, the culture density may compensate for the lower lipid content per cell. The highest lipid per cell value obtained from *D. salina* is at a salinity of 3.5 M,

compared to *T. suecica* at 1.5 M NaCl. On a commercial scale, the difference in the cost of the NaCl may outweigh the revenue from the oil – a full life cycle analysis would be required to determine this. It may be more appropriate to compare the yield of lipid per volume of culture and, comparing the most productive cultures at around one week old, *D. salina* does perform better than *T. suecica*. However, this is achieved at a difference in salinity of 1.6 M NaCl (0.4 M vs. 2.0 M), which will again incur the financial burden of increased NaCl expenditure.

If the project were to continue for another six months, the experiments with week-old cultures should be repeated to confirm the high lipid/biomass percentages that were obtained. It would be beneficial to synchronise the sub-culturing of both species at all salinities, in order to have comparable results with respect to age. It may also be interesting to sub-culture in greater volumes, so that several samples may be taken at intervals from the same solution. This would provide more information on the behaviour of a single culture during its lifetime, although scale-up may have implications of light availability throughout the culture. Three determinations should be carried out when a sample is taken from the culture: chlorophyll content, lipid measurement and cell number. Knowing the weight of lipid per cell for each sample would be useful in determining the lipid activity of the cell at various ages; this information may be required commercially when deciding the optimum harvesting time. At present, there is no data on the type of lipid present in the cell – the measurements are *total lipids*, i.e. neutral and polar lipids are not distinguished. One species of alga may produce the valuable neutral lipid preferentially over polar lipid, and further research is needed in this area. A factor that could be investigated is the alga's response to movement.[7-8] *T. suecica* is a marine alga and may respond well to simulated wave surges, as can be achieved using a rocker-shaker. Another experiment may be to use a two-step culturing process – initially grow the alga at its preferred salinity, then transfer to a higher salinity to induce NaCl stress. This may result in an increased weight of lipid per mL, due to the incidence of more cells than would normally be found in a high salinity solution. Exactly how this process should be performed would need further research, however.

Research is currently ongoing in the many different areas of biofuel production from microalgae. Little is known about TAG formation [16] and which genes are involved in fatty acid synthesis in microalgae. As more is discovered about these mechanisms, it may be possible to engineer strains of algae with enhanced lipid production capabilities via bio-electromagnetic stimulation, [17] or the more conventional genetic and metabolic routes. Additionally, research is continuing in high throughput neutral lipid analysis,[18] which should make identification of overproducing strains quicker and easier. Biofuel from microalgae appears to be technically feasible, [19] if not currently financially so, but production within a biorefinery enhances the economic viability of the process. There is still some way to go before microalgal biodiesel becomes commercially available, but with the EU target of at least 10% biofuels in the transport sector by 2020 looming, and the threat of a rapid decline in conventional fuels, it is of utmost importance to advance this technology as quickly as possible for economic growth and stability.

5. References

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6. Appendices

6.1 Methodology

6.1.1 General Procedure for Sub-culturing of Microalgae

To a previously autoclaved conical flask containing Growth Medium (100 mL, Appendix 6.7), well-grown algal culture (5 mL) was transferred and the flask stoppered with a sponge bung; the flask was removed to the incubation laboratory for several days. All steps were completed aseptically. Flask sizes and volumes could be adjusted, providing the ratio of culture to medium remained at 5%. Several sub-cultures were prepared using salinities from 0.4 – 2.0 M NaCl Growth Medium. A full list of sub-cultures prepared can be seen in Appendix 6.2.

6.1.2 General Procedure for Determination of Chlorophyll Content of **T. suecica**

The following procedure was carried out in duplicate. To a 15 mL Falcon tube, algal culture (5 mL) was transferred aseptically and the sample centrifuged at 6000 rpm and 20 °C for 5 min. The supernatant fluid was discarded and the pellet re-suspended in H_2O (1 mL). (NB: Laboratory distilled water was used throughout.) The suspension was transferred to a Fast Prep tube (containing micro-beads) and beaten twice on Level 6 for 25 s. The sample was transferred back to the Falcon tube and acetone (4 mL) added; the sample was mixed and left to stand, out of direct sunlight, for a few minutes. The sample was centrifuged at 6000 rpm and 20 °C for 5 min, and the pellet colour checked – if the pellet was not completely white, the sample was mixed and centrifuged again. The optical density of the supernatant fluid of the samples was measured at 645 and 663 nm, and the µg chlorophyll mL^{-1} calculated using the following equation.

$$OD_{645} \times 202 = y$$

$$OD_{663} \times 80.2 = z$$

$$\frac{(y+z)}{2} = \mu g\ chlorophyll\ 5\ mL^{-1}$$

$$\frac{\mu g\ chlorophyll\ 5\ mL^{-1}}{5} = \mu g\ chlorophyll\ mL^{-1}$$

6.1.3 General Procedure for Gravimetric Measurement of Lipid Content in Algal Strains [12]

The following procedure was carried out in quadruplicate. To a 15 mL Falcon tube, algal culture (15 mL) was transferred and the sample centrifuged at 6000 rpm and 20 °C for 5 min. The supernatant fluid was discarded and the pellet re-suspended in H_20 (5 mL). The sample was centrifuged at 6000 rpm and 20 °C for 5 min and the supernatant fluid discarded; the washing step was performed twice. The pellet was re-suspended in H_2O (to a volume of 1 mL) and the sample transferred to a pre-weighed Eppendorf tube (fitted with a separate, pierced lid). The sample was frozen at -80 °C overnight (12-24 h) and then lyophilised for 48 h. The sample was weighed to estimate the weight of biomass. 2:1 v/v MeOH:$CHCl_3$ (500 µL) was added and the sample sonicated on ice for 1 min. The sample was centrifuged at 6000 rpm and 20 °C for 5 min and the volume of supernatant fluid estimated using a Gilson pipette; the pellet was discarded. MeOH, $CHCl_3$ and 1% $NaCl_{(aq)}$ were added to achieve a volume of 1.0 mL in the ratio 2:2:1. The sample was mixed, centrifuged at 6000 rpm and 20 °C for 2 min and the green, lower phase transferred to a pre-weighed Eppendorf tube. The solvent was allowed to evaporate in a fume cupboard overnight, or until dry, and the sample weighed to calculate the weight of lipid recovered.

6.1.4 General Procedure for Determination of Algal Cell Number

To an Eppendorf tube containing algal culture (900 µL), Gram's Iodine (100 µL) was added and the solution mixed. An aliquot (20 µL) of the stained algal culture was placed in the centre of the counting chamber of a Helber slide and the slide positioned on the microscope at x40 objective. The algal cells were counted in four large squares – one hundred small squares in total. The cell count was repeated five times for each sample at salinities 0.4, 1.0, and 1.5 M NaCl. Using the mean number of cells per small square, the number of cells in the original culture was calculated as follows:

$$Depth = 0.02\,mm;\ \ area\ of\ small\ square = {}^{1}/_{400}\ mm^2 = 2.5 \times 10^{-3} mm^2$$

$$\therefore volume\ of\ small\ square = 2.5 \times 10^{-3} \times 0.02 = 5.0 \times 10^{-5}\ mm^3 = 5.0 \times 10^{-8}\ cm^3 (\equiv mL)$$

$$Cell\ no.\ per\ small\ square \times \frac{1}{5 \times 10^{-8}} = cells\ mL^{-1}\ sample$$

$$\frac{Cells\ per\ mL\ sample}{9} \times 10 = cells\ mL^{-1}\ original\ culture$$

6.1.5 General Procedure for Visualisation of Nile Red Fluorescence in Microalgal Cells using Fluorescence Microscopy **[20-21]**

Nile Red (solid, 10 mg) was dissolved in Acetone (10 mL) to produce a 1 mg mL^{-1} stock solution. A dilute Nile Red solution (250 µg mL^{-1}) was then prepared using stock solution (2.5 mL) and Acetone (7.5 mL) and wrapped in aluminium foil. A dilute suspension of algal culture was prepared by determining the optical density at 720 nm and adjusting to 0.4 AU with fresh Growth Medium. The dilution was calculated using the following equation.

$$Conc_{stock} \times Vol_{stock} = Conc_{dil} \times Vol_{dil}$$

Diluted algal culture (3 mL) was transferred to a 15 mL Falcon tube and stained with dilute Nile Red solution (10 µL); the sample was mixed thoroughly and wrapped with aluminium foil. A slide of the stained sample was prepared and positioned on the fluorescence microscope using x20 and x100 (oil immersion) objectives.

6.2 Subcultures

Subculture		Original Culture		Comments / Observations
Date	**Medium NaCl (M)**	**Date**	**Medium NaCl (M)**	
19 Jan 10	1.5	19 Nov 09	1.5	3 flasks; grown well.
20 Jan 10	2.0	14 Jan 10	1.5	3 flasks; not grown – subcultures discarded.
26 Jan 10	0.4	19 Jan 10	1.5	1 flask; grown very well.
26 Jan 10	1.0	19 Jan 10	1.5	1 flask; grown well.
27 Jan 10	0.4	19 Jan 10	1.5	2 flasks; grown very well, sight yellow tinge.
27 Jan 10	1.0	19 Jan 10	1.5	2 flasks; grown well.
27 Jan 10	1.5	19 Jan 10	1.5	2 flasks; slow-growing.
11 Feb 10	0.4	27 Jan 10	1.0	1 flask; grown very well, sight yellow tinge.
11 Feb 10	1.0	27 Jan 10	1.0	1 flask; grown well.
11 Feb 10	1.5	27 Jan 10	1.0	1 flask; grown well.
22 Feb 10	0.4	11 Feb 10	0.4	1 flask; grown well.
22 Feb 10	1.0	11 Feb 10	1.0	1 flask; grown well.
22 Feb 10	1.5	11 Feb 10	1.5	1 flask; grown well.

6.3 Chlorophyll Content of T. suecica

Date	Sample Details		Sample Ref.	OD at 645 nm	OD at 663 nm	Chlorophyll Content (μg mL^{-1})
	Date	**Medium NaCl (M)**				
20 Jan 10	19 Nov 09	1.5	TS-A	0.238	0.539	9.130
			TS-B	0.220	0.517	8.590
28 Jan 10	19 Jan 10	1.5	TS-C	0.058	0.144	2.326
			TS-D	0.054	0.135	2.174
02 Feb 10	26 Jan 10	1.0	TS-E	0.040	0.094	1.562
			TS-F	0.052	0.117	1.989
03 Feb 10	26 Jan 10	0.4	TS-G	0.038	0.107	1.626
			TS-H	0.044	0.113	1.795
04 Feb 10	14 Jan 10	1.5	TS-I	0.039	0.137	1.887
			TS-J	0.041	0.118	1.775
22 Feb 10	11 Feb 10	0.4	TS-K	0.138	0.351	5.603
			TS-L	0.130	0.355	5.473
22 Feb 10	11 Feb 10	1.0	TS-M	0.095	0.285	4.205
			TS-N	0.100	0.297	4.402
22 Feb 10	11 Feb 10	1.5	TS-O	0.074	0.277	3.716
			TS-P	0.066	0.228	3.162

6.4 Lipid Content of T. suecica

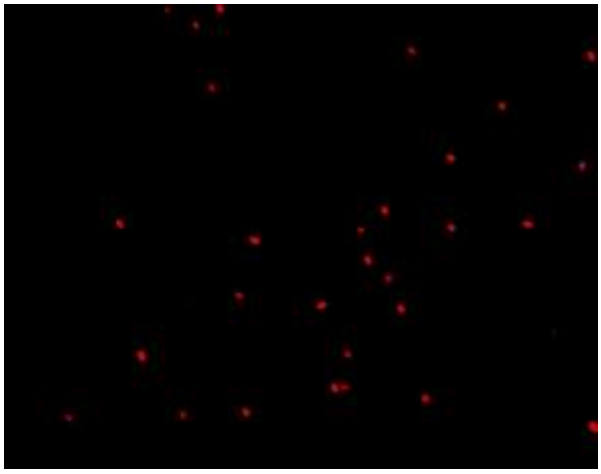
Date	Sample Details		Sample Ref.	Sample Volume (mL)	Weight of Biomass (mg)	Weight of Lipid (mg)	Weight of Lipid (μg mL^{-1})
	Date	Medium NaCl (M)					
20 Jan 10	19 Nov 09	1.5	TS-1	15	5.3	2.5	166.7
			TS-2	15	4.9	1.6	106.7
			TS-3	15	5.0	1.6	106.7
			TS-4	15	5.0	1.6	106.7
26 Jan 10	19 Jan 10	1.5	TS-5	20	1.2	1.0	50.0
			TS-6	20	1.2	0.8	40.0
			TS-7	20	1.0	0.4	20.0
			TS-8	20	0.9	0.4	20.0
28 Jan 10	19 Jan 10	1.5	TS-9	15	1.1	0.9	60.0
			TS-10	15	1.4	0.9	60.0
			TS-11	15	1.1	1.1	73.3
			TS-12	15	1.2	1.0	66.7
02 Feb 10	26 Jan 10	1.0	TS-13	20	1.2	1.2	60.0
			TS-14	20	1.3	1.0	50.0
			TS-15	20	1.3	1.4	70.0
			TS-16	20	1.2	1.3	65.0
03 Feb 10	26 Jan 10	0.4	TS-17	20	2.3	1.6	80.0
			TS-18	20	2.9	1.8	90.0
			TS-19	20	2.5	1.5	75.0
			TS-20	20	2.5	1.6	80.0
04 Feb 10	14 Jan 10	1.5	TS-21	20	2.3	0.8	40.0
			TS-22	20	1.8	0.8	40.0
			TS-23	20	2.4	0.8	40.0
			TS-24	20	1.8	1.7	85.0
22 Feb 10	11 Feb 10	0.4	TS-25	15	3.1	1.2	80.0
			TS-26	15	3.4	1.0	66.7
			TS-27	15	3.6	1.0	66.7
			TS-28	15	3.1	0.8	53.3
22 Feb 10	11 Feb 10	1.0	TS-29	15	2.2	1.0	66.7
			TS-30	15	3.4	1.0	66.7
			TS-31	15	3.4	0.9	60.0
			TS-32	15	2.9	1.1	73.3
22 Feb 10	11 Feb 10	1.5	TS-33	15	1.8	0.8	53.3
			TS-34	15	1.4	0.7	46.7
			TS-35	15	2.0	0.7	46.7
			TS-36	15	1.6	0.7	46.7

6.5 Cell Number of T. suecica

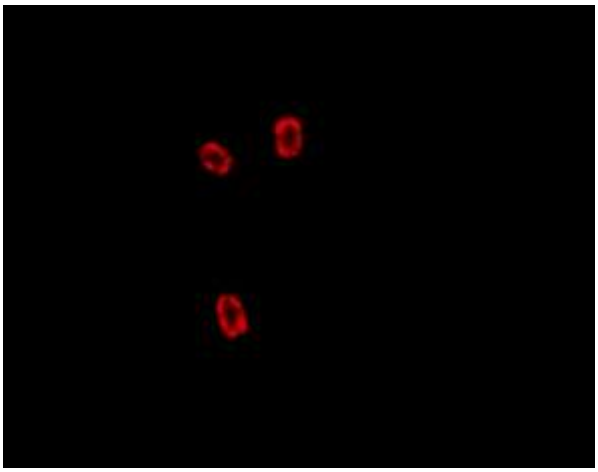
Sample Details		Slide No.	Cell No. per 100 Small Squares	Mean Cell No. per 100 Small Squares	Cells per mL of Original Culture
Date	**Medium NaCl (M)**				
11 Feb 10	0.4	1	37		
		2	27		
		3	56		
		4	40		
		5	43	40.6	9,0222,222
11 Feb 10	1.0	1	31		
		2	22		
		3	35		
		4	29		
		5	26	28.6	6,355,556
11 Feb 10	1.5	1	29		
		2	15		
		3	14		
		4	19		
		5	13	18.0	4,000,000

Note: Cells stained 22 Feb 10, counted 04 Mar 10.

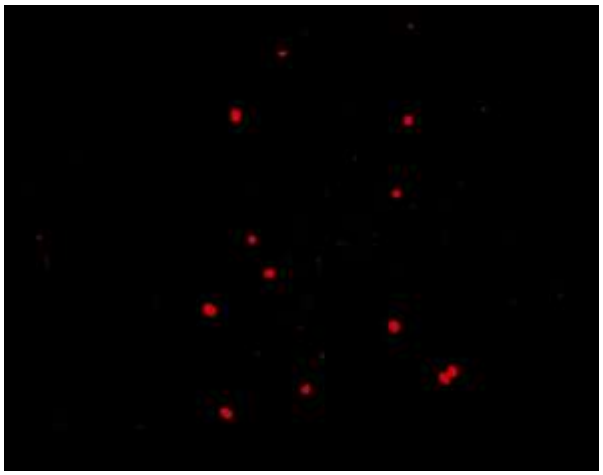
6.6 Visualisation of Nile Red Fluorescence in T. suecica *using Fluorescence Microscopy*



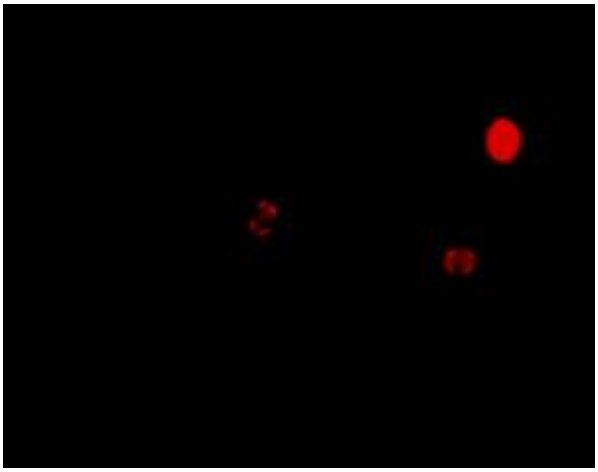
Culture: 22 Feb 10, 0.4 M NaCl, x20 objective.



Culture: 22 Feb 10, 0.4 M NaCl, x100 objective (oil immersion).



Culture: 22 Feb 10, 1.5 M NaCl, x20 objective.



Culture: 22 Feb 10, 1.5 M NaCl, x100 objective (oil immersion).

6.7 General Procedure for Preparation of Growth Medium

The following stock solutions were required for preparation of the Growth Medium:

Solution	**Compound (solid)**	**Weight (g)**	**Volume H_2O (mL)**
2.4 M $MgSO_4$	$MgSO_4.7H_2O$	133.1	225
2.0 M $MgCl_2$	$MgCl_2.6H_2O$	91.5	225
1.0 M $CaCl_2$	$CaCl_2.6H_2O$	33.1	225
4.0 M $NaNO_3$	$NaNO_3$	34.0	100
0.5 M Na_2SO_4	Na_2SO_4	63.9	900
0.1 M NaH_2PO_4	NaH_2PO_4	3.0	250
2.0 M KCl	KCl	74.6	500
1.0 M HEPES pH 7.6	$C_8H_{18}N_2O_4S$	59.6	250
1.5 mM FeEDTA pH 7.6	FeEDTA	0.0551	100
2.0 mM $CoCl_2$	$CoCl_2.6H_2O$	0.1071	225
0.2 mM $CuCl_2$	$CuCl_2.2H_2O$	0.0307	900

A solution of Trace Elements was prepared; the amounts below are for a volume of 400 mL H_2O.

Trace Element Concentration	**Compound**	**Amount**
185 mM H_3BO_3	H_3BO_3	4.576 g
7 mM $MnCl_2$	$MnCl_2.4H_2O$	0.5541 g
0.8 mM $ZnCl_2$	$ZnCl_2$	0.0436 g
0.02 mM $CoCl_2$	2.0 mM $CoCl_2$	4.0 mL
0.0002 mM $CuCl_2$	0.2 mM $CuCl_2$	0.4 mL

To a 1 L conical flask containing approximately 750 mL H_2O, an amount of NaCl was added to obtain the desired molarity (i.e. 87.66 g for a 1.5 M NaCl solution). The solution was stirred using a magnetic flea and the following stock solutions added:

Stock Solution	**Desired Concentration (mM)**	**Volume per 1 L (mL)**
2.0 M KCl	10	5
2.0 M $MgCl_2$	20	10
1.0 M $CaCl_2$	10	10
2.4 M $MgSO_4$	24	10
4.0 M $NaNO_3$	5	1.25
0.5 M Na_2SO_4	24	48
100 mM NaH_2PO_4	0.1	1
1.5 mM FeEDTA	0.0015	1
Trace Elements Solution	1 mL L^{-1}	1
1.0 M HEPES pH 7.6	20	20

The pH was checked and adjusted to pH 7.6 using NaOH or HCl, if necessary. The solution was then made up to 1 L with H_2O, and 1 g of $NaHCO_3$ per litre was added. The solution was then transferred into 1 L Duran bottles (to a volume of approximately 500 mL each) and autoclaved at 15 lb in^{-1} for 20 min, then allowed to cool.