Improved mass cultivation of the marine diatom *Chaetoceros calcitrans* for shellfish hatcheries

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Philosophy

In

Biotechnology

at Massey University, Palmerston North,

New Zealand

Simon John Charles Tannock

2006

Acknowledgements

This has been a great experience and one from which I shall walk away a more capable person. Top of the 'Thank you' list is my beloved Darlene, as she has supported, pushed, loved and listened to me as I have worked my way through this project. My sons, James and Andrew, have shown as much interest as you can expect from teenagers who have more important things on their minds than my research and I appreciate and love that they also listened to my overly detailed explanations to simple questions. Monique and Jasmine, thank you both for being the lovely supportive people you are.

My supervisors Dr Henry Kaspar and Prof. Yusuf Chisti have asked the right questions at the right time, but generally given me the free reign I was looking for. I very much appreciate Henry's open mind, open conversation and open door policy. The staff at the Glenhaven Hatchery are next on my thank you list, Nick King, who has the best grasp of how to make your toys do cool things remotely, Bridget Alexander and Nicky Roughton for allowing me to put dishes on the bench and not have to clean them myself. Dan M^{c}Call for asking lots of questions, which made me review and think more carefully about my processes. Achim Janke, the devil's advocate, always questioning and wanting big answers, thanks for the challenge. Daphne, for being a wonderful librarian. And to the rest of the staff who were happy to stop what they were doing to help lift and shift the monstrosity that is the pasteuriser into position, a couple more times than should have been necessary, thank you all. There are a huge number of people to thank at the Cawthron Institute. I won't provide a list of names as it really is so big. But thanks for the friendship, the help, the listening, the suggestions, the laughs, the games of pool, and the wonderful family feel that Cawthron has about it. That feeling isn't there so much now that Cawthron is getting so big, but I hope those that have been around for awhile will be able to keep that feeling alive.

John and Hilary Penno, from Technical Glass Products in Dunedin, BIG thanks. Not just for the help and exemplary service, but for the friendship and laughs. It is a great regret I haven't met you face to face. Keep sailing and laughing, you guys are great.

My six and a half years at Cawthron, two of them as a student, have been some of the most rewarding years I have had in a work environment. Any job is bearable if the people are good. I think I had the best of both worlds because, for the greater part, the job was challenging and the people have been fantastic. You know you are in a good place when the greater majority of your friends are your work colleagues. Or maybe it just means you need to get out more often?!?

So, thanks. Enjoy the thesis!

Simon T.

Tables of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1 Abstract

A medium for the optimal growth of *Chaetoceros calcitrans* in batch and continuous culture systems was developed. A method was developed for continuous culture of *C. calcitrans* that was free from detrimental infection by bacteria. The concentration of tested nutrients in the developed medium were sodium nitrate, 160 mg/L; sodium dihydrogen orthophosphate, 40 mg/L; and the molar Si:N ratio was 0.25 (99.9 mg/L sodium metasilicate). Isolated bacterial strains were shown to be detrimental to the growth of *C. calcitrans* in batch and continuous culture. Electrolytically treated water was suitable for the growth of *C. calcitrans*, but a subsequent flourish of bacterial growth at the late exponential phase reduced the quality of the algal cells and made the culture unsuitable for feeding to shellfish larvae. Heat treated water (95°C for ten and a half minutes) gave stable growth for the continuous culture of *C. calcitrans* in 38 L plastic bioreactor bags for at least 38 days. The superficial gas velocity in the culture bags was 0.09 L/min. Higher superficial gas velocities (e.g. 0.40 L/min were detrimental to *C. calcitrans*.

Chapter 2 Literature review

There has been limited investigation into the growth of the diatom *C. calcitrans*. There has been only one reasonably comprehensive study into the optimal conditions of this species (Krichnavaruk et al. 2005), but this was done at a temperature relevant to tropical climes (30°C) and in small (2.5 and 17 L) scale in batch and semi continuous cultures. A few other publications identify the effects of various nutrients on cell growth or composition. These results provide pointers to conditions which may lead to increased cell concentration or biomass. These studies were all performed with small scale axenic and non-axenic uni-algal cultures, filtered or autoclave sterilised medium and often at cell densities that would be considered to be very low from an aquaculture microalgae production perspective.

The following review is an outline of papers and results and includes information on other species of the genus *Chaetoceros* and other algae of aquacultural importance. Information is included from areas that are considered to be relevant to creating a better understanding of the conditions that might be optimal for producing *C. calcitrans*.

****

C. calcitrans (Figure 1) is a non-chain forming, non-motile, golden brown marine centric diatom of the Bacillariophyceae class (Laing, 1979). Its size ranges from 3 – 8 μm (excluding spines).

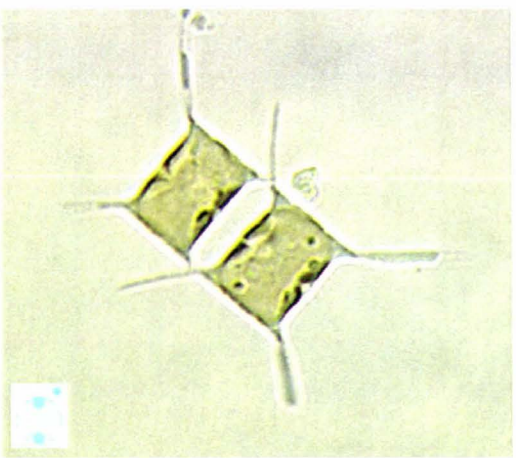


Figure 1- *Chaetoceros calcitrans*. Source: http://microscope.mbl.edu/scripts/microscope.php?func=imgDetail&imageID=12609.

C. calcitrans is a valuable aquaculture feed species because of its small size 30-50 μm^3 and a desirable fatty acid profile. It is relatively high in the fatty acids 14:0, 16:0, 16:1*n*7t, and 20:5*n*3. Polyunsaturated fats tend to be produced in early stationary phase

as compared to late-stationary phase in many other commonly used aquaculture species (Fernández-Reiriz et al. 1989). *C. calcitrans* tends to have higher fatty acid content (2.0 pg.cell-1) than other Bacillariophyceae diatoms (1.7 pg.cell-1) (Muller-Feuga *et al.*, 2003).

It is generally accepted that when growing *C. calcitrans*, like many other algae, the medium preparations of Guillard and Ryther (1962) (f series); Walne (1966); Laing (1979); Harrison et al. (1980), or a medium recipe similar to these will provide suitable growing conditions. In relation to the publications reviewed in this thesis the final method of medium preparation was often dependent on the experimenter and the nutrient being investigated e.g. nitrogen, silicate or iron.

2.1 Light

The effect of light intensity and temperature on *C. calcitrans* was investigated by Yanase & Imai (1968). Autoclaved medium was used and culture size was 60 mL. The cultures grown at 23 and 25°C had higher specific growth rates ($k= \log N_1 - \log N_0 / t_1 - t_0$) than other temperatures (17, 20, 27 and 29°C). At each temperature, the highest specific growth rate was found to be at 4,500 lux (60 $\mu mol.m^{-2}.s^{-1}$). Light levels of 8000 lux (110 $\mu mol.m^{-2}.s^{-1}$) and 12,500 lux (170 $\mu mol.m^{-2}.s^{-1}$) did not increase the growth rates at any temperature. The cultures at 500 lux (7 $\mu mol.m^{-2}.s^{-1}$) and 1500 lux (20 $\mu mol.m^{-2}.s^{-1}$) had significantly lower specific growth rates than the higher light cultures. Interestingly, the specific growth rate for *C. calcitrans* under these conditions (23°C and 4,500 lux (60 $\mu mol.m^{-2}.s^{-1}$) was 1.50 hr^{-1}, whereas for the other species tested (*Monochrysis lutheri, Platymonas sp.* and *Nitzschia closterium*) the specific growth rates ranged from 0.45 to 0.55 hr^{-1}. The optimum conditions found in this study are very similar to the recommended light levels of Laing (1979) (21°C and 4,750 - 5250 lux (64 – 71 $\mu mol.m^{-2}.s^{-1}$) for starter cultures (250 mL). For cultures of larger volume 2 – 20 L, Laing (1979) recommends an increase in illumination to 15,000 – 25,000 lux (200 – 335 $\mu mol.m^{-2}.s^{-1}$) which should allow harvest densities of 40,000 – 50,000 $cells.\mu L^{-1}$ in 2 L culture and 18,000 – 24,000 $cells.\mu L^{-1}$ in 20L culture. In cultures of 200 L, cell densities of 25,000 – 30,000 cells μL^{-1} at 15,000 lux are obtainable in an internal illumination bioreactor that maintains the culture in a sterile environment.

Comparing the intensity, and colour, of light for algal culture was investigated by Sánchez-Saavedra & Voltolina (1996). Cultures (in 250 mL Erlenmeyer flasks) received blue/indigo light (peak emission 440 nm), white light (peak emissions at 445 nm and 580 nm) or mixed light (blue/indigo light with a very low level (480 lux, 6.5 $\mu mol.m^{-2}.s^{-1}$ of white light) at two levels of irradiance. There was no significant difference in mean cell division rates between the low (7400 lux, 100 $\mu mol.m^{-2}.s^{-1}$) and high (29,600 lux, 400 $\mu mol.m^{-2}.s^{-1}$) levels of irradiance. Significant differences were present between light quality (colour) treatments. The *Chaetoceros sp.* and *Skeletonema costatum* cultures grew fastest in mixed (blue/indigo and low white) light than in blue/indigo light only. White light gave the slowest growth. White light and then mixed light gave the best

results for *Thalassiosira pseudonana*. Final cell numbers in *Chaetoceros sp.* were highest in the white light at any level of irradiance. Cell composition also varied between light colour and in some instances between irradiance levels. The mixed light at either irradiance resulted in the highest level of protein and the lowest level of carbohydrate. Harrison *et al.* (1990) found that in *C. calcitrans* cultures, low irradiance (14 or 44 $\mu mol.m^{-2}.s^{-1}$) significantly reduced the level of protein. The level of carbohydrate in *Chaetoceros sp.* was between 30 and 50% of the level produced by the other two algae tested. When *Chaetoceros sp.* was grown in mixed light the level of carbohydrate production was significantly lower than in blue/indigo light at the same high irradiance. Lipid levels of *Chaetoceros sp.* varied in mixed light, with low irradiance cultures producing significantly more lipid than high irradiance cultures (Sánchez-Saavedra & Voltolina, 1996). Harrison *et al.* (1990) commented on the work of Kowallik (1978), stating that light quality can also have an effect on the chemical composition of microalgae. Blue light generally increases protein content and red light increases cellular carbohydrates.

The length of the light path in the culture has an effect on growth. To compare the effect of light path length, cultures of *Chaetoceros muelleri* were grown in two flat plate bioreactors, with light path lengths (lpl) of 1 and 3 cm and constant photon flux density of 190 $\mu mol\ photon.m^{-2}.s^{-1}$. With equal initial inoculation densities, after 2 days the cell concentration in the 1 cm (lpl) bioreactor was low compared to the 3 cm (lpl) bioreactor. This difference suggests photoinhibition was occurring in the 1 cm (lpl) bioreactor. Increasing the initial inoculation concentration of the 1 cm (lpl) bioreactor by threefold created enough self-shading of the cells to allow an increase in growth. The 3 cm (lpl) bioreactor still outperformed the 1 cm (lpl) in total production and growth rate on a volume and areal basis (Göksan *et al*, 2003).

The age of the culture also had an effect on its composition (Sánchez-Saavedra & Voltolina, 1996). Regardless of light colour and irradiance level, cultures of *Chaetoceros sp.* and *S. costatum* in the early stationary phase had a tendency for higher protein and lipid content than during the exponential phase of growth (Sánchez-Saavedra & Voltolina, 1996). These results, regarding light profile and culture age indicate that the environment the algae is cultured in, and time of harvest, will have a considerable impact on the final or harvested biochemical composition of the culture.

2.2 Nutrients and Growth

The growth response of *C. calcitrans* to a range of initial silica concentrations was investigated by Laing (1985). The initial concentrations of silica were 350, 950 and 1400 $\mu g\text{-}at\ Si.L^{-1}$. N.B. The unit $\mu g\text{-}at.L^{-1}$ has generally been replaced by the unit $\mu mol.L^{-1}$, thus from this point on the latter unit shall be used. The medium was adjusted to 15‰ salinity. The increases in culture densities were proportionately related to the increase of Si concentration. Despite the large differences in cell densities between Si concentrations

there was no significant difference between the final biomass of the cultures (680 – 760 $mg.L^{-1}$). Although the biomass was similar across all three treatments there was proportionately more lipid and carbohydrate and less protein in the 350 $\mu mol.L^{-1}$ treatment than in the 1400 $\mu mol.L^{-1}$ treatment. All the Si had been absorbed by the cells in the 350 and 950 $\mu mol.L^{-1}$ media by the third day of culture. There was only a very small amount of Si left on the 4th and final day of culture in the 1400 $\mu mol.L^{-1}$ treatment. Although the results of this study illustrate the differences in growth characteristics over a range of Si concentrations, it does not identify the optimal initial concentration of silicate for batch cultures or the concentration of Si needed for growth to continue until some other nutrient, or light, becomes limiting. The experiments of Laing (1985) described above had Si:N molar ratios of between 0.15 and 0.59. Nutrient uptake generally follows the Michaelis-Menten equation, so nutrient uptake is dependent upon ambient concentration and each nutrient's kinetic parameters (Martin-Jézéquel *et al.* 2000; Kudo, 2003). The earlier work of Laing (1979) recommends a Si:N of 0.16 for aquacultural algae production of *C. calcitrans*.

Nutrient limitations cause the biochemical composition of algae to alter over time. This can have an effect on the nutritional value of algae when fed to shellfish larvae. Nitrogen is an essential nutrient, and the concentration and the form available in growth medium have a large effect on the level of growth (Harrison *et al.* 1990; Corzo *et al.* 2000; Lourenco *et al.* 2002; Lebeau and Robert, 2003). Harrison *et al.* (1990) investigated the effects of both nutrient and light limitation on three algae species commonly used in aquaculture, including *C. calcitrans*. Algae (all 3 spp.) starved of N for two days had a decreased percentage of protein and an increased percentage of carbohydrate compared to algae without nitrogen limitation. No difference in the lipid percentage was observed under these conditions. Under low irradiance conditions the amount of N per cell (an index of protein content) was lower than for cells grown at normal irradiance for *C. calcitrans*. Irradiance levels did not affect the N-content in the other species tested. Irradiance also has an effect on cell volume. Under low-light conditions the cells of *Thalassiosira pseudonana* were much smaller than of those grown under high-light conditions. This may have a significant effect upon animal growth if they are fed diets calculated by cell number.

In batch culture conditions, growth rates can be terminated quite abruptly by limitations of one of the macronutrients. The abruptness of the termination has a partial relationship to the ratio of the minimum to the maximum amount of nutrient per cell. The ranking from most abrupt to least abrupt termination of growth is Si > C > N > P. If ample nutrient is present growth slows when light becomes limiting. Thus if ample nutrient is supplied and pH is maintained within species specific tolerances the maximum biomass of a culture is dependent upon the amount of light the culture receives. Autoinhibition is one cause for exception to this general rule. Autoinhibition is when a metabolite of the cultured algae inhibits the growth of the culture as a whole. Semi-continuous and continuous culture systems could be a method of avoiding autoinhibition (Harrison *et al.* 1990).

Corzo *et al.* (2000) looked at *C. calcitrans* growth under N limitation and the production of transparent exo-polymer particles (TEP). They found that growth and biomass production were directly dependent on the initial nitrate concentration. TEP production was higher in N-limited cultures. Under N-limitation a large proportion of photosynthetically fixed C was channelled to TEP production. The TEP production influenced the rate of aggregation of particles. The ecological background to TEP production may be related to its gas bubble scavenging effect, which retards the rate at which cells sink and move out of the euphotic zone. TEP production is commonly associated with stationary and senescent phases of culture. *C. calcitrans* produces relatively large amounts of mucus-like material. Under N-limitation *Chaetoceros* spp. have shown increases in total carbohydrate; increased amounts of released free extracellular polysaccharides; and specifically, increases in the sugar-containing compounds on the cell surface. The amount of TEP production per unit of *C. calcitrans* biomass is inversely related to the level of N. N limitation increases the production of TEP. Therefore N limitation increases the stickiness of the *C. calcitrans* cells and aggregates may form (Corzo *et al.* 2000).

The biomass and biochemical profile of *C. calcitrans*, among others, was investigated by Fernandez-Reiriz *et al.* (1989). The culture conditions were; 15°C, 60 $\mu E.m^{-1}.s^{-1}$ (4440 lux), salinity 35‰ and aeration at 5 $L.min^{-1}$. The culture temperature, salinity and aeration rate used for this experiment were far from optimal for *C. calcitrans* and so these results could vary considerably if this experiment was repeated using optimal conditions. The culture was found to have a growth rate of 0.87 $divisions.day^{-1}$. The growth rate for *Pavlova lutheri* was 0.93 $divisions.day^{-1}$. This is at great variance to the results of Yanase & Imai (1968), where the specific growth rate of *C. calcitrans* was three times larger than that of *Monochrysis lutheri* (now generally referred to as *Pavlova lutheri*).

2.3 Bacteria

The beneficial and detrimental effect of the presence of bacteria in the medium algae grow in has been assessed by several researchers. Many researchers and hatcheries around the world have found that mass cultures of microalgae can be temperamental and susceptible to crashing as algae cell densities increase (Baker and Herson, 1978; Hirayama & Hirayama, 1993, 1996, 1997; Fukami *et al.* 1992, 1997; I.Laing, pers.comm.; C.Langdon, pers.comm.; S.Shotwell, pers.comm.). Baker and Herson (1978) found that *Thallasiosira pseudonana* growth was inhibited by the presence of the bacterium *Pseudomonas* T827/2B. They found that monocultures of *Pseudomonas* T827/2B did not grow in nutrient rich medium. However, in the presence of *Thallasiosira pseudonana* the bacterial growth was stimulated and the growth of the microalgae was inhibited in a way that has been termed 'indirect parasitism'. In this instance, one of the interacting populations releases a substance into the medium, resulting in the lysis of the second population of organisms (Fredrickson, 1977).

Following the lysis of the *T. pseudonana* the bacterium obtains its nutritional requirements (Baker and Herson, 1978).

The bacterial populations in semi mass cultures of diatoms were reported on by Hirayama & Hirayama (1993). These cultures were grown in open tanks with additional nutrients added. The results showed an initial increase in both bacterial and microalgae populations in the first two or three days. On the third or fourth day the microalgae population would reach a peak, whereas the bacterial population would reduce (not always significantly). Following this peak in the microalgae population the bacterial population would increase to greater levels than previously attained and the microalgae populations would significantly reduce. These researchers suggested that the two populations exhibited suppressive effects upon each other. Interestingly, no inocula were used for these experiments; the prevalent species at the time of the trial were simply allowed to grow. Within each trial the replicate cultures (2 or 3) had the same dominant species. Differences did occur between trials as to prevalent species and time of algae peak, but the general pattern of algae peak matching bacterial decline and then vice versa were the same across trials. This suggests that regardless of the algae and bacterial species that are prevalent during the year the interaction between the two populations is consistent (Hirayama & Hirayama, 1993).

The effects of 12 different bacterial strains upon the growth of *Chaetoceros gracilis* during coexistent culture was examined by Hirayama & Hirayama (1996). Nine of the bacterial strains had a significantly detrimental effect upon the growth rate of the *C. gracilis*. Two strains had no effect and one strain, *Flavobacterium* DN-10, had a significantly beneficial effect upon the growth rate of *C. gracilis* and did not alter the stability of the culture during the stationary growth phase. These same researchers also treated cultures of *Isochrysis galbana* and *Pavlova lutheri* with this beneficial bacterium *Flavobacterium* DN-10. The bacterium had no significant effect upon the growth rates of these two phytoflagellates, but the cell densities of the stationary growth phases were maintained for significantly longer periods than in the controls. *Flavobacterium* DN-10 was the dominant species (>45%) of bacteria in these cultures (Hirayama & Hirayama, 1997).

Fukami *et al.* (1997) described the work of Riquelme (1988) and how some species of algae do not grow in axenic cultures but will grow when bacteria are present. For example, it was found that *Asterionella glacialis* does not grow in axenic culture and that the bacterium *Pseudomonas* sp. 022 strain assisted the growth of *A. glacialis* by producing a glycoprotein that acted as a growth factor. This result, along with the others already noted here indicate that bacteria can play a part in the culture of algae and that the majority of bacterial strains tested have an inhibitive or detrimental effect on algal growth, but some strains are an essential part of stimulating the growth of microalgae.

2.4 Water Treatment for Algal Culture

Whilst value of some bacterial species in cultures is apparent, the majority of bacteria are, however, detrimental or have no effect. For these reasons, the reduction or destruction of all micro-organisms present in water to be used in aquaculture hatcheries and the like is of interest (Sako *et al.* 1988; Inouye *et al.* 1990; Frerichs, 1990; Liltved *et al.* 1995; Arimoto *et al.* 1996; Chang *et al.* 1998; Liltved & Cripps, 1999; Frerichs *et al.* 2000). There are several methods used to remove or reduce micro-organisms from water for algal culture. These methods are generally characterised as either chemical or physical methods. Chemical methods include disinfectants (sodium hypochlorite), electrolysis, and ozonation. Physical methods include filtration, heat and ultraviolet (UV) radiation (Arimoto *et al.* 1996; Frerichs *et al.* 2000).

The various methods of disinfection of water each have advantages and disadvantages; relating to cost, ease of preparation and how well algae grow in the treated water. The majority of research on the disinfection of water relates to the removal of viruses and bacteria to improve the husbandry of various aquaculture fish species or to make water potable. Several of the methods used have resulted in water that is either toxic to the organism destined to live in it (e.g. ozone (Wedemeyer, *et al.* 1979; White,1999)), formalin (Frerichs *et al.* 2000)) or still infected (electrolytes, pH (Frerichs *et al.* 2000)). Only those methods that may be relevant to the culture of *C. calcitrans* will be discussed further. These are heat, chlorination, electrolysis (sodium hypochlorite production in situ in sea water), UV and microfiltration.

Heating and cooling of water to remove various viral species has been investigated by several authors. Any temperature over 60°C was successful in inactivating most viruses; the degree of inactivation depending upon residence time at a specific temperature (MacLeod, 1965; Moats, 1971; Yura *et al.* 1993; Arimoto *et al.* 1996; Stabel *et* al. 1997; Chang *et al.* 1998; Frerichs *et al.* 2000; Kilsby *et al.* 2000). Samples held at 60°C for 30 minutes were found to be very low in viral activity (Arimoto *et al.* 1996). Frerichs *et al.* (2000) found that this same temperature and timeframe reduced viral activity by 100%. Samples held at 70°C for 5 and 30 minutes were found to have no viral activity at all (Chang *et al.* 1998). A method known as tyndalisation can be used to remove bacteria and viruses from culture medium. This method involves heating the medium to 60-70°C for 30 minutes and then allowing it to cool for 24 hours. Bacterial spores, unaffected by the heating process, may hatch after the initial heating and can be inactivated subsequently by repeating the heating cycle twice more (Gibson, pers.comm.).

Chlorination is a commonly used method of seawater disinfection in hatcheries (Janke, pers. comm. Langdon, pers. comm.). Chlorine appears to affect the cell's ability to synthesise protein and DNA, as well as disrupt the cell membrane's permeability (Haas and Engelbrecht, 1980). The factors that have an affect on the efficiency of the chlorination process are: the nature of the disinfectant (the kind of residual fraction);

concentration of the disinfectant; length of contact time with the disinfectant; temperature; the type and concentration of organisms; and the pH (White, 1999).

Following treatment of water with chlorine, the residual chlorine needs to be removed from the water so algae can be cultured. There are several methods available for the removal of chlorine. These include:

Activated Carbon – Not practical for algal culture as the treated water must be passed through the activated carbon filter and this creates opportunities for re-infection.

Sodium Thiosulphate ($Na_2S_2O_3$) - The most commonly used method in aquaculture. Sodium thiosulphate is not used in industrial processes to dechlorinate water as it was found to be lacking for a number of reasons. (1) – The reaction with chlorine was a stepwise one and required a longer time to run to completion. (2) – The reaction with chlorine is only stoichiometric at pH 2. (3) The degree of removal of chlorine is related to pH. Sodium thiosulphate is generally considered to be useful only in laboratory situations for chemical determinations.

Sodium Bisulphite ($NaHSO_3$) – This can be in powder or solution. It can be handled in stainless steel, PVC or fibreglass. The chlorine: sodium bisulphite mass ratio required is 0.685. For each part of chlorine removed, 1.38 parts of alkalinity as $CaCO_3$ will be consumed. When sodium bisulphite is mixed with acid it releases toxic gas (SO_2).

Sodium Metabisulphite ($Na_2S_2O_5$) – A powder that is highly soluble. The chlorine: sodium metabisulphite mass ratio is 0.746. For each part of chlorine removed, 1.38 parts of alkalinity as $CaCO_3$ will be consumed.

Sulphur Dioxide (SO_2) – A pungent gas that can be liquefied under pressure or at low temperatures, sulphur dioxide is not appropriate for algal culture systems.

(From White, 1999).

Disinfecting the water by using an electrolytic water treatment system is a relatively new technique that is not in wide use in aquaculture at this time. The system is akin to the chlorination process except that the hypochlorite is made in situ rather than being added to the water to be treated.

A Japanese manufacturer, Hoshizaki™ has designed and built a ROX™ water electrolyser. The system works as follows:

The ROX process is simple

Bacteria grows beneath the protein surface

ROX produces two kinds of water (Alkaline & Acidic)

Alkaline dissolves protein and oil

Acidic removes the bacteria

The process can be done in two easy steps

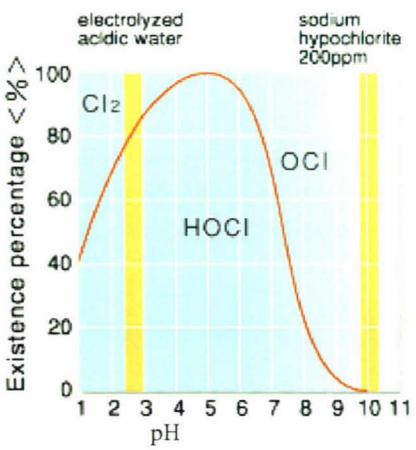
Step 1 Remove protein with Alkaline water

Step 2 Remove bacteria with Acidic water

Protein/Grease **Alkaline** **Acidic**

How does the ROX system work?

NaCl +	**H_2O** +	**electricity** =	**NaOH** +	**HClO**
(Salt)	(Water)		(Alkaline Water)	(Acidic Water)



pH=11.3	pH=2.7
Sodium Hydroxide	Hypochlorus Acid
-electrode	+electrode

(From Anon, http://www.hoshizaki.com/singapore, 2003)

Jorquera *et al.* (2003) attempted to grow *Isochrysis galbana* in water electrolytically treated (chlorinated) with the ROX system and subsequently dechlorinated with sodium thiosulphate. The growth was compared against cultures grown in autoclaved or UV-treated water. Growth of this microalga was significantly higher in electrolysed water than in either of the other treatments. Bacterial inactivation of the culture medium was compared between UV-irradiated water and varying levels of current (Amperes) used in the ROX electrolyser. The number of colony forming units (CFU) was found to be significantly higher in UV treated water than in water treated with 0.2 A of current. The numbers of CFUs remained at a similar level from 0.2 – 1.2 A. The difference between 1.2 and ≥1.3 A treatments was also significant. At intensities of 1.3 A and greater no CFUs were detected. Bacteriolysis was evident at current intensities of greater than 1.3 A. This total inactivation of bacteria not only occurred in raw seawater, but also in seawater that had had the bacterium *Vibrio anguillarum* inoculated into it at a concentration of 1.5×10^5 cells.mL^{-1} before the electrolysis treatment.

A less complex system of electrolysing seawater is the electrolytic cell used in saline swimming pool systems. Using similar technology as above, saline water (≥4‰) can produce enough sodium hypochlorite to keep algae, bacteria and viruses under control in a swimming pool environment (Hunter, T. pers.comm.).

Ultraviolet irradiation of water for the reduction of bacteria and viral pathogens is common in aquaculture (Liltved *et al.* 1995). Three pathogenic bacteria (*Vibrio anguillarum, V. salmonicida* and *Yersinia ruckeri*) were inoculated into water before UV irradiation treatment (2.7 mW.cm^{-2}). Inactivation of these bacteria was up to 99.999% (5 log). Because of water turbidity, the need for 1 µm filtration and the reasonably high level of system maintenance are disadvantages of the UV system and make it unsuitable for treating large volumes of water (Arimoto *et al.* 1996; Jorquera *et al.* 2003). These points are particularly relevant in regard to algal culture, as system reliability and consistent water quality are vital elements in algal production systems. For UV irradiation to be effective pre-filtration is necessary (Liltved & Cripps, 1999).

Laing (1979) used water that was filtered to 0.45 µm for cultures of *C. calcitrans* in 20 and 200 L volumes. Filtering to this level allowed for cultures to reach densities of 20,000 cells.µL^{-1}.d^{-1}. Although other researchers have used filtration for the preparation of medium, this has been done for very small volumes (<20 L). No research was found that directly compared filtration of large volumes of water with other methods of water sterilisation. Filtration was not assessed in further detail as a potential method of water treatment as the other methods described above were considered to be more suitable for batch and continuous culture water supply.

2.5 Photobioreactors

The type of water disinfection or sterilisation utilised or suitable for *C. calcitrans* may also have an impact on the type of photobioreactor design that can be utilised. The reverse is also true. Within the bounds of this review of optimal conditions for the microalga *C. calcitrans,* only the important points of design criteria shall be considered.

Photobioreactors are advantageous over open pond systems for high value species as the culture can be maintained as mono-specific. Because of the high level of control and monitoring of the growing conditions within a bioreactor e.g. O_2 accumulation, and pH control, greater levels of biomass productivity and sustainability are possible (Tredici, 1999).

The important factors to be considered in photobioreactor design are: surface-to-volume ratio; oxygen accumulation; mixing of culture; bioreactor orientation for light accumulation; temperature control; pH control; materials the system is made of (Tredici, 1999). The Glenhaven Aquaculture Centre Ltd (GACL), in Nelson, New Zealand is the research facility utilised by The Cawthron Institute. The Cawthron Institute initiated this research. At this facility algae culture occurs in a system that was purchased from Seasalter Shellfish Ltd (Whitstable, England). This system consists of 38 L polyethylene bags. They have an approximate diameter of 150 mm (depending on where along the length of the bag this measurement is taken) and are 2.2 m high. They are suspended on a frame beside banks (12 tubes/bank) of 'cool white' fluorescent lights. Air sparging is provided via an injection port at the base of the bag and medium is added at the top of the bag on a continuous basis. As medium is constantly supplied, algal culture is constantly harvested. Lighting is on a 24:0 L:D basis. The system is run chemo-statically i.e. the medium is supplied at a constant rate and the culture adjusts itself to the supply. The pH is maintained at approximately 8.0 with the injection of CO_2 gas into the air stream to alter pH.

2.6 Damage Caused by Bubbles

The starting point of this research is the experiences of culturing *C. calcitrans* at the GACL and the current system in use there. As well as the problems inherent in the water treatment system, the other major problem in this system is the occurrence of cell damage caused by gas sparging. Cultures of microalgae are sparged with air (plus an additional 1% CO_2 v:v) so as to ensure mass transfer (O_2 out, CO_2 in) and mixing within the culture medium for ensuring that cells are provided with nutrients, light and a homogenous growth environment (Joshi *et al.* 1996). Sparging in bubble columns similar to those used at GACL is an efficient method of gas transfer and culture mixing (Miyamoto *et al.*, 1988).

The unit of measurement for gas flow into columns such as photobioreactors is superficial gas velocity (U_t - m/sec). This is a measure of the flow rate (m^3/sec) divided by the cross-sectional area of the column the gas flows through (m^2). Krichnavaruk *et al.* (2005) reported that in an airlift photobioreactor (17 L culture volume) the highest cell densities were obtained with a U_t of 3.0 cm/sec. The cell densities they reported were 9000 cells/μL. These researchers did not use supplemental CO_2 to assist the culture with carbon assimilation and pH control. In this trial the researchers compared four superficial gas velocity rates (2, 3, 4 and 5 cm/sec). They found that initially each incremental increase in U_t improved the cell concentration over time. This observation applied to all except the 5 cm/sec treatment which did not grow well over the duration of the trial and finished with a cell concentration of 2500 cells/μL. This result would suggest that the high gas flow rate in the column they used caused damage to the cells in culture.

The deleterious effect associated with sparging is that bubbles can be very damaging to cells. Experiences with other forms of cell culture (animal and insect cells) have found similar problems with sparging or with cultures that are stirred at high rates, whereby the shear forces on cells reach a level where they are damaging (Tramper *et al.* 1986; Handa-Corrigan *et al.* 1989; Chalmers and Bavarian, 1991; Cherry and Hulle, 1992; Wang *et al.* 1994; Wu and Goosen, 1995; Joshi *et al.* 1996; Meier *et al.*1999; Kioukia *et al.*1996; Chisti, 2000; Csordas and Wang, 2004). The major reason for cell damage by sparging in animal and insect cell cultures is the lack of a cell wall (Zhang *et al.*1995; Joshi *et al.* 1996). That is not to say that cells such as microalgae are not also shear sensitive, but few studies have been done to characterize the shear sensitivity of these cells. Microalgae have a multi-layered cell wall which generally protects them from shear forces (Joshi *et al.* 1996). However microalgae cells have been damaged by gas sparging (Silva *et al.* 1987).

Bubbles have been found to be damaging because of the shear forces present on the gas-liquid interface (Kunas and Papoutsakis, 1990). When a bubble reaches the surface of the culture it rises above the water line where it forms a hemispherical cap. The thickness of this cap is typically 1-10 μm. The fluid within this cap drains downwards due to both

gravity and suction, generated by film curvature. The cap will thin until it ruptures (typically 0.1 μm), at which point the reducing pressure inside the bubble causes a flow of the liquid on the gas-liquid interface of the bubble into the region behind the bubble (Chalmers and Bavarian, 1991; Boulton-Stone and Blake, 1993; Wu and Goosen, 1995). It must be noted that this description of bubble rupture is only fully relevant to small ($d_b \leq 2$ mm) bubbles. Larger bubbles have reduced sphericity and it is less likely the receding film could initiate a flow underneath the bubble (Wu and Goosen, 1995). The typical velocities of this receding film liquid flow are within the range 1-50 $m.s^{-1}$ (Cherry and Hulle, 1992). Considering that any cell that is present within this bubble film before rupture was relatively stationary as it may have been adsorbed to the gas-liquid interface, the acceleration rate from 0 to > 1 m/s is considerable (Chalmers and Bavarian, 1991). These authors noted from the work of MacIntyre (1972) that the inner bubble surface (the bubble cavity wall) "would experience an acceleration approaching 10^6 g after the bubble burst." For a bubble with a film thickness of 2 μm, MacIntyre reported the film velocity to be 8 m/s. The toroidal ring (Figures 2 and 3) that forms as a bubble bursts may strike cells that are on a stationary part of the bubble film. The probability of this intense release of energy being sufficient to destroy cells is high (Chalmers and Bavarian, 1991). The numerical model of Boulton-Stone and Blake (1993) indicated that any bubble smaller than 2.5 mm in diameter is likely to have sufficient potential energy to damage cells as the energy dissipates during bubble rupture.

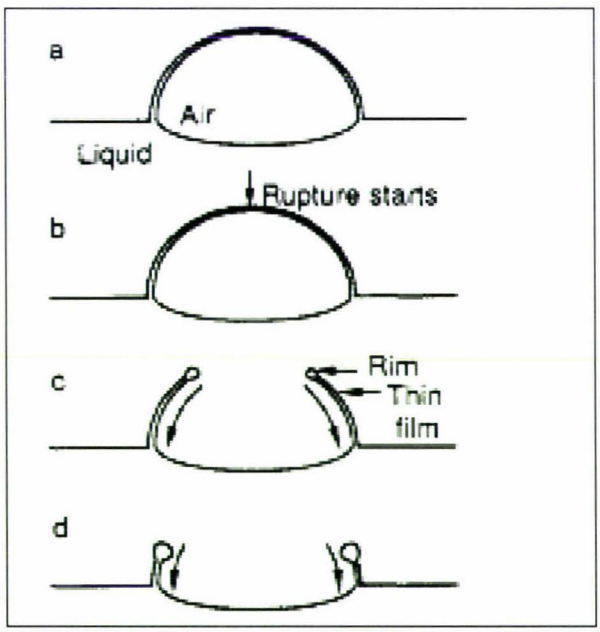


Figure 2 - Details of a bursting small bubble in water: (a) bubble at the surface, (b) spontaneous rupture, (c) retraction of film, and (d) growth of rim as film retracts further. Note the increasing toroidal ring, outlined further in Figure 3. (From Cherry and Hulle, 1992).

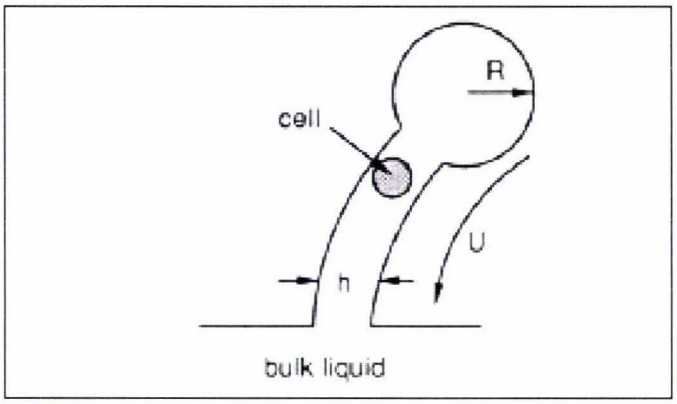


Figure 3 - Details of the toroidal ring of fluid in a bursting bubble. U= Culick's velocity and can be calculated to be between 3 and 8 m/s. (From Cherry and Hulle, 1992).

With such a body of research reporting on the damage to cells in culture from bubbling, and with the experiences of cell damage occurring in cultures of *C. calcitrans* in bags, it is necessary to address this problem. There are two methods of addressing the problem. One is to find an additive to the culture medium that affects a relevant part of the cell culture process; be it cell strength, water surface tension or cell hydrophobicity. The second would be to alter the way the mass transfer issues of cell culture are addressed. As it is commonly accepted that sparging is an effective way to ensure mass transfer (Merchuk, 1990; Merchuk *et al.* 1994; Contreras *et al.* 1999), other methods such as stirred reactors will not be considered. The other reason for this decision is that to consider stirred reactors would also require considerable alteration to the current photobioreactor design at the GACL, and this is not feasible. As such, altering bubble characteristics; either size, number, composition or the method the bubble has of entering and exiting the culture liquid are facets that can be considered as changeable. The third option is to combine aspects of these two methods for a suitable answer.

A commonly used additive that appears to meet the requirements of *C. calcitrans* culture is a surfactant known as Pluronic F-68 (aka PE6800). This is a non-ionic surfactant polyol; it is a block copolymer glycol of poly (oxyethylene) and poly (oxypropylene) (Papoutsakis, 1991; Chisti, 2000). The protective effect commonly attributed with Pluronic F-68 is the suppression of cell adsorption to the gas: liquid interface of bubbles (Handa-Corrigan *et al.* 1989; Chisti, 2000; Palomares *et al.* 2000) as well as by increasing the mean bursting membrane tension and the elastic area compressibility modulus (60% and 120% respectively in TB/C3 hybridoma cells)(Zhang *et al.* 1992). Additions of Pluronic F-68 to culture medium ranges from 0.01 – 0.3% w/v but generally the maximal level of protective effect is seen at around 0.05% w/v (Zhang *et al.* 1992; Chisti, 2000; Palomares *et al.* 2000). Ramirez and Mutharasan (1990) measured plasma membrane fluidity (PMF) to show that F-68 decreased the PMF, thus making the cell membranes more resistant to shear damage.

The addition of Pluronic F-68 showed a marked decrease in the hydrophobicity of the cell membrane in two types of insect cells. The adsorption process is probably the hydrophobic groups on the F-68 binding to the hydrophobic sites on the cell membrane. With the hydrophobic sites on the cell membrane now covered the cell-surface hydrophobicity reduces, or it becomes more hydrophilic. The efficacy of Pluronic F-68 is concentration dependent (Goldblum *et al.* 1990; Murhammer and Goochee, 1990; Wu *et al.* 1997). The protective effect upon cells is very rapid, as was described by Michaels and Papoutsakis (1991). Goldblum *et al.* (1990) cultured insect cells in medium, in the presence or absence of F-68. After the cell cultures had grown the F-68 free cells were spun down and re-suspended in medium that contained F-68. Five minutes after re-suspension the two cell lines were exposed to conditions that would normally cause cell damage due to shear stresses. There was no difference between the two cell lines; both had the same amount of protection from laminar shear stresses. This result agrees with that of other researches (Kunas and Papoutsakis, 1989; Michaels and Papoutsakis, 1991). To test the stability of the interaction between F-68 and the cell membrane, cells grown in the presence of F-68 were centrifuged and re-suspended in F-68 free medium. After a range of times in the new medium they were exposed to shear stresses. The protective effect of the F-68 continued to be observed for at least 130 minutes after re-suspension (Palomares *et al.* 2000).

The alternative to adding a cell protectant or similar additive to the culture medium is to alter the way sparging occurs in cell culture. Bubble size is certainly one factor known to have an effect on cell damage. The bubble diameter has an effect on the shape and its position at the air-liquid interface at the instance of rupture (Figure 4) (Wu and Goosen, 1995).

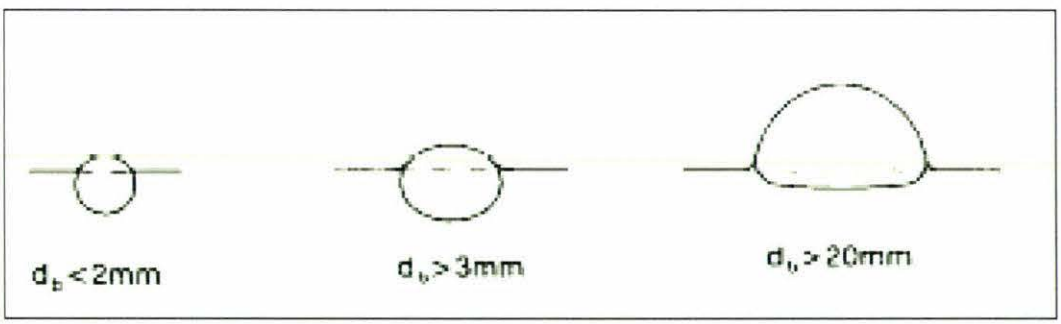


Figure 4 - The shape and position of different size bubbles at the air-liquid interface (Adapted from Wu and Goosen, 1995).

As can be seen from Figure 4 the bubbles with a large diameter (d_b >20mm) have an almost horizontal base that is not greatly depressed below the air:liquid interface. The small bubbles have a much greater excess pressure and can subsequently obtain a nearly hemispherical depression below the air: liquid interface (Wu and Goosen, 1995). When the bubble ruptures, as described above, the liquid mass of a spherical bubble moves to a central zone behind the bubble, where local turbulence is generated (Figure 5). This

turbulence would not normally be an issue if it occurred randomly within a culture. However, the base of the bubble is a region of cell aggregation because cells adsorb to rising bubbles and gather at the base of the rising bubbles (Chalmers and Bavarian, 1991; Bavarian *et al.* 1991).

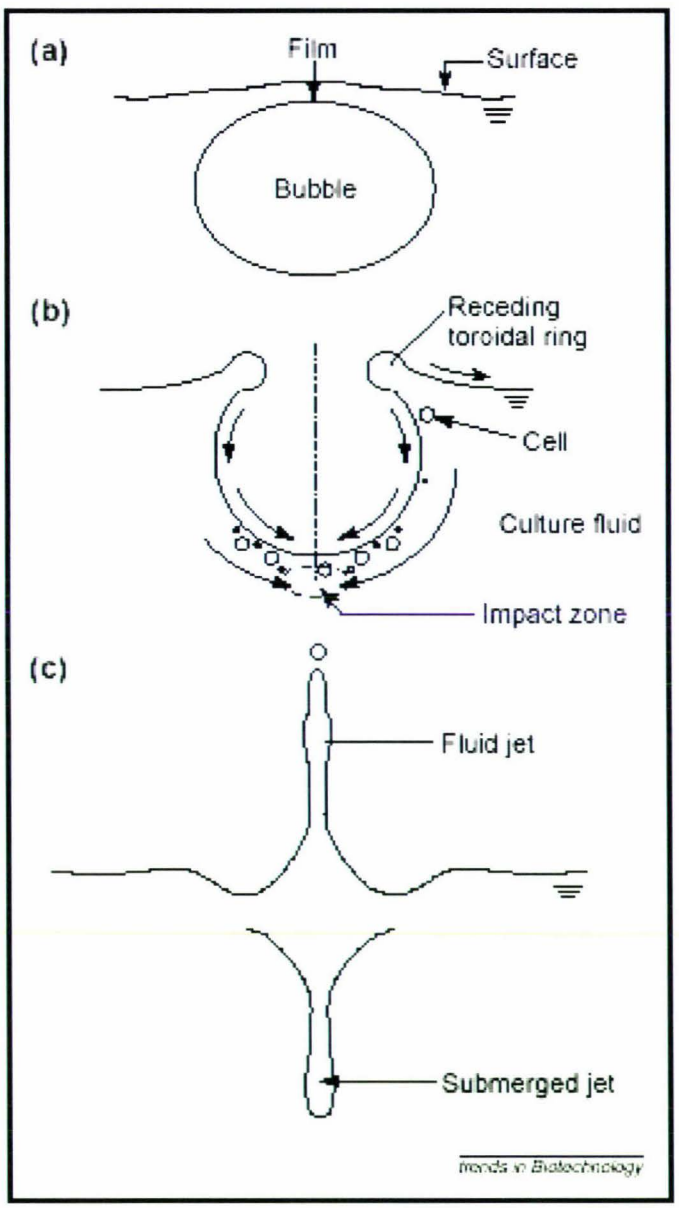


Figure 5 - Bubble rupture events. (a) The film between the bubble and the surface thins as the bubble rises. (b) A hole develops at the top of the bubble. The film forms a toroidal ring as it recedes back into the bulk liquid. Streams of water on the gas:liquid interface move quickly to the centre bottom of the bubble, this area becomes an impact zone of intense local turbulence. (c) jets of fluid above and below the impact zone are formed in a release of energy. (From Chisti, 2000).

This aggregation of cells at the base of bubbles (the impact zone) is a major factor in the level of damage caused by bursting of small bubbles (Chalmers and Bavarian, 1991; Bavarian *et al.* 1991). Along with the high energies already described in the receding films of bursting bubbles, it has been determined that small bubbles have the potential to be detrimental to cell cultures (Tramper *et al.* 1986; Handa-Corrigan *et al.* 1989; Chalmers and Bavarian, 1991; Bavarian *et al.* 1991; Cherry and Hulle, 1992; Boulton-Stone and Blake, 1993; Wang *et al.* 1994; Wu and Goosen, 1995; Joshi *et al.* 1996; Meier *et al.*1999; Kioukia *et al.*1996; Chisti, 2000; Csordas and Wang, 2004).

A seemingly simple answer to this problem is to ensure all bubbles are therefore large, i.e. $d_b > 4$ mm. The problem that arises from this conclusion is that one of the aims of gas sparging is mass transfer, and it is known that optimal mass transfer is obtained by the use of small bubbles due to their high surface area to volume ratio (Poulsen and Iversen, 1998).

Chapter 3 Materials and Methods

3.1 Assessment of conditions for producing optimal cell concentration in *Chaetoceros calcitrans*

3.1.1 Assessment of biomass production

The nutrient medium that was in use at the GACL was always considered to be Conway medium. Following the completion of the work to optimise the nutrient recipe for *C. calcitrans* the references that provide the medium recipes (Walne, 1966; Laing, 1979) were consulted to make comparisons. At this point an error was detected. The recipe that had been provided to the author as Conway medium had been copied erroneously. In the Conway medium recipe of Laing (1979) the constituents that make up Solution A are supposed to be added at a rate of 2 mL to 1 L of seawater. The recipe provided to the author upon initially starting work at GACL, and used in this work, indicated that solution A was to be added at a rate of 1.0 mL/L. Due to this inaccuracy, the trials (Sections 4.1.3, 4.1.4 and 4.1.5) have not compared the growth of *C. calcitrans* in Conway medium (the control) to enhanced medium. As there is this discrepancy, the medium used as the control throughout this work shall from hereon be referred to as Glenhaven medium.

The cell concentration and biomass of *C. calcitrans* as it is grown at the GACL needed to be established so any future experiments can be compared to the current method. The *C. calcitrans* was cultured in 1L round, flat-bottomed flasks (Figure 6). Five replicate flasks were cultured using 800 mL of autoclaved (15 min. 121°C) Glenhaven medium (Appendix 1) at 20°C.

Seawater salinity was set at 15‰ (parts per thousand salinity; Laing, 1985). Nutrient solution was as described by Walne (1966). The concentration of sodium metasilicate (not outlined by Walne) was set at 60 mg/L in the final medium. Aeration (0.2 µm minisart filtered) was provided at approximately 1 L/min with CO_2 supplementation (~1% by vol.). pH was set at 8.2 ± 0.2 and CO_2 flow was varied as required to manually maintain the required pH. The pH was measured using a Mettler Toledo Seven Easy pH meter and a Mettler Toledo InLab 421 Ag/AgCl pH probe.

Lighting was provided at 100 $\mu mol.m^{-2}.s^{-1}$ using Philips 'Cool-white' fluorescent lights. Each flask was housed in a white walled 'pigeon-hole' that was 200 mm wide and 300 mm high. The centre of the flask was 150 mm away from the front of the fluorescent tube. Each pigeon-hole compartment was exposed to 270 mm of fluorescent tube.

Samples were taken aseptically as required. The rubber bung for the 1 L flask (Figure 6 and Figure 7) was fitted with two L-shaped glass tubes (6 mm OD and 2 mm ID) for air

in and air out, respectively. The 'air in' fitting had a 150 mm long glass tube (dimension as above) connected to it via silicon hose (7.5 mm OD and 5.0 mm ID). This glass tube ensured that aeration into the flask occurred approximately 5 mm above the bottom of the flask. The L-shaped glass tubes and the 'air-in' and 'air-out' fittings were connected via silicon hosing (5.5 mm OD and 3 mm ID). The aseptic sampling system consisted of custom modified sample vials (Bonnet Equipment – 2 mL CLR BMV 10-425 THD). The sample vial had had the base cut off and was then heat annealed to round the cut edge. The septa were Bonnet Equipment 10 × 0.060 mm PTFE/SIL. Samples were obtained using custom-made 7 inch steel needles sourced from Phenomenex NZ (NLL-17.78/19BV-custom-made) and sterile B-D 5 mL syringes.

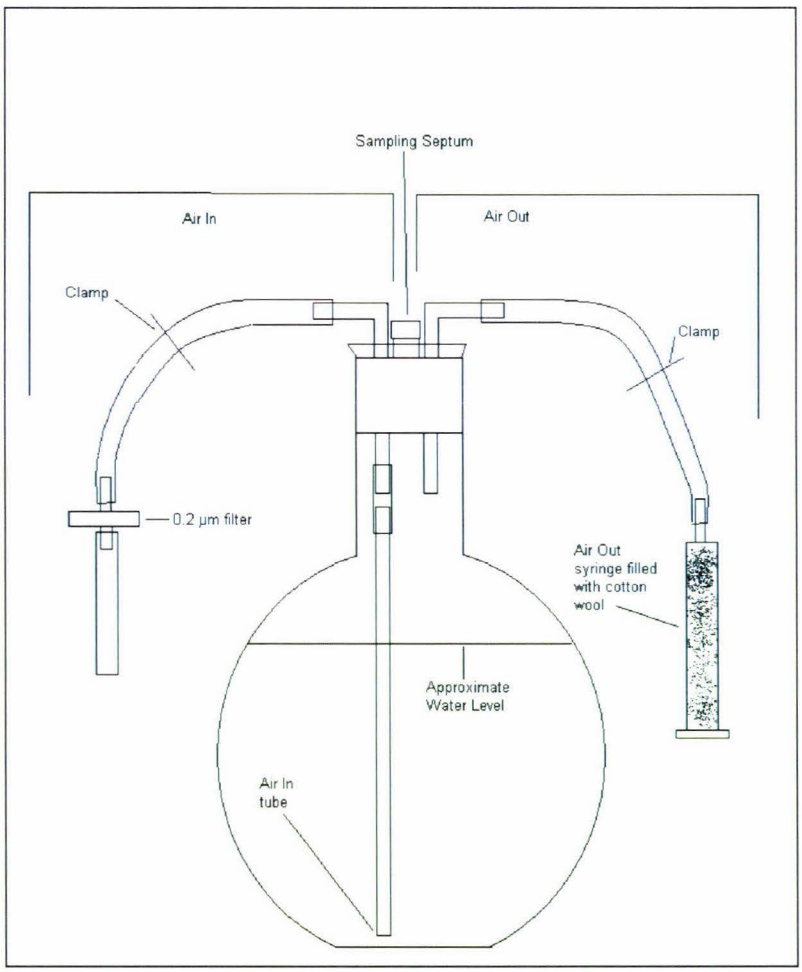


Figure 6 - Schematic drawing of the 1 L culture flask used for culture of *C. calcitrans* in all small scale experiments.

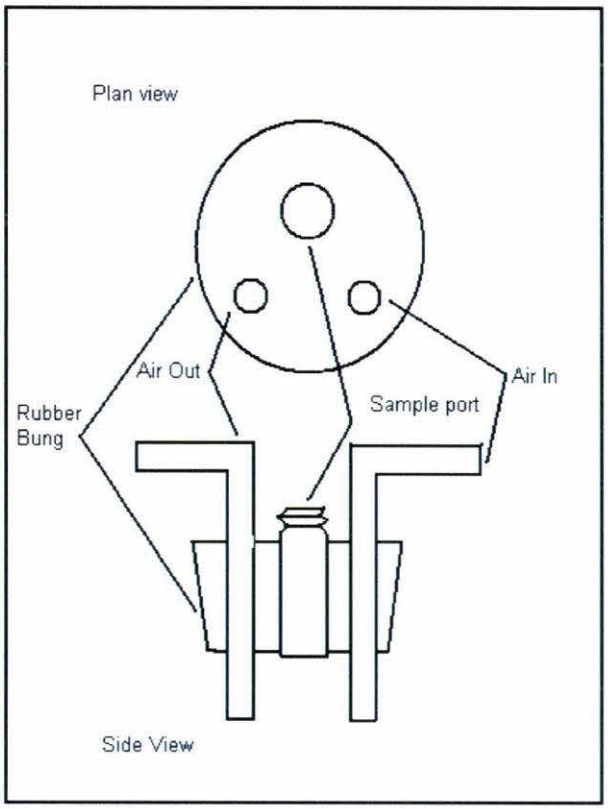


Figure 7 – Schematic drawing of the rubber bung and the air inlet, air outlet and sample port.

Each day a 3 mL sample was taken and held in a vial suitable for the spectrophotometer. These samples were read immediately after collection. The optical density was monitored over the growth period of the cultures using a Shimadzu UV-VIS – 160 spectrophotometer at 625 nm. In the instance where the absorbance reading of the culture was greater than 0.8 then the culture was diluted with medium that had been prepared in the same way as the culture medium and re-read. The reading was then corrected by multiplying it by the dilution factor.

In some sections of the results the specific growth rate (hr^{-1}) is presented. This value was calculated by plotting $\ln(N_1/N_0)$ vs time. The slope of the linear section of the plot was the maximum specific growth rate (Rasmussen *et al.* 1998).

3.1.1.1 The relationship between spectrophotometer absorbance readings and cell concentration

Results of cell growth are presented in this work as cell concentration in cells/μL. However, cell densities were most often measured using a spectrophotometer and the absorbance value was converted to cell concentration using a correction factor. To ensure that readings being taken on the spectrophotometer were reasonably representative

of the cell numbers present in cultures, samples across all treatments were randomly selected and monitored for both absorbance and actual cell number (counted on an Improved Neubauer double cell Haemocytometer). These samples were taken as the experiment progressed to ensure that both high and low cell numbers and absorbance levels were compared.

3.1.1.2 The biomass produced by *C. calcitrans* in batch culture

Before inoculation of the medium a sample was taken. Immediately after inoculation another sample was taken and read at 625nm using the spectrophotometer.

When the culture reached its maximum concentration the absorbance was recorded. A sample (100 ml or a similarly large precisely known volume) of the final culture was taken and filtered through a 0.45 μm (preweighed) membrane filter. The cake of algae was washed with a dilute salt solution (e.g. 0.15% wt/wt NaCl; 50 ml wash liquid) on a Buchner funnel. Then the filter paper and cake was quantitatively transferred to an aluminium foil square (preweighed) and kept in an oven overnight at 80° Celsius. The hot cake was then transferred to a desiccator at room temperature the next day. Once the cake had cooled, it was weighed accurately (3-decimal places). The weight of the filter paper and aluminium foil square was then subtracted to determine the exact weight of the biomass. This data was then used to calculate the exact dry weight of the biomass that was present in 100 ml of the culture.

3.1.2 The effect of salinity upon cell concentration of *C. calcitrans*

Flasks of medium were prepared and cultured as described in Section 3.1.1. The seawater's salinity was altered using dechlorinated freshwater before the additional medium ingredients were added. The medium was mixed in 5 L batches and then added in 800 mL aliquots to the flasks. *C. calcitrans* was cultured at six different salinities (12, 16, 20, 24, 28 and 32‰) (3 replicates). The treatments were inoculated with 50 mL of algae that was growing exponentially in medium of the appropriate salinity.

3.1.3 The effect upon cell concentration of *C. calcitrans* at different concentrations of sodium nitrate in the Glenhaven medium

The optimum levels of nutrients, for biomass production of *C. calcitrans* in an intensive algae culture system have not been established. Although the concentration of nitrogen in the medium was altered, dependent upon treatment, the molar ratio of Si:N was maintained at 0.36, the ratio in current use for culture of *C. calcitrans* at the Glenhaven Aquaculture Centre. See Table 1.

Weight of $NaNO_3$ (mg/L)	Weight of atomic N (mg/L)	Weight of atomic Si (mg/L)	Weight of $Na_2SiO_3.5H_2O$ (mg/L)
10.0	1.65	1.19	8.99
50.0	8.25	5.95	44.95
100.0	16.5	11.91	89.92
130.0	21.45	15.49	116.89
160.0	26.4	19.06	143.87
200.0	33.0	23.82	179.83

Table 1- Concentration of sodium nitrate and the corresponding concentration of sodium metasilicate required to maintain the molar Si:N molar ratio of 0.36.

The inocula for each treatment were grown in the corresponding medium of that treatment. Inoculum culture cell densities were counted on the day of inoculation and inocula volumes were calculated for each treatment so that each flask should have had a starting inoculum of 522 cells/μL. The inoculum for the 10 mg/L treatment only provided a starting cell concentration of 100 cells/μL because the inoculum itself was not dense enough to provide an inoculum of any greater density. Algal cultures were grown and monitored in the same manner as outlined previously in Section 3.1.1.

3.1.4 The effect upon cell concentration of *C. calcitrans* by altering the Si:N molar ratio in the Glenhaven medium

The molar ratio of Si:N in current use for the culture of *C. calcitrans* is 0.36 for cultures grown in 2.5 L flasks and 0.24 for cultures grown in 20 L carboys. The results of previous experiments (Section 4.1.3) on *C. calcitrans* showed the optimum concentration of $NaNO_3$ in the growth medium was 160 – 200 mg/L. Having set the $NaNO_3$ level at 160 mg/L the Si:N molar ratio was varied to identify the optimal ratio for *C. calcitrans*. The concentration of sodium metasilicate was adjusted to obtain the various ratios. See Table 2.

Si:N Ratio	Moles of Si	Actual weight of Sodium metasilicate (mg/L)
0.25	0.47	99.91
0.35	0.66	139.87
0.45	0.85	179.83
0.60	1.13	239.77
1.00	1.88	399.63

Table 2 – Si:N molar ratios and weight of sodium metasilicate required for a $NaNO_3$ concentration of 160 mg/L.

The inocula for each treatment were grown in the corresponding media of that treatment. Inoculum culture cell densities were counted on the day of inoculation and inocula volumes were calculated for each treatment so that each flask should have had a starting inoculum of 522 cells/µL. Algal cultures were grown and monitored in the same manner as outlined in Section 3.1.1.

3.1.5 The effect upon cell concentration of sodium dihydrogen orthophosphate concentration in the Glenhaven medium

Sodium dihydrogen orthophosphate is the chemical used to provide algae with the element phosphorus in the Glenhaven medium recipe. With the results of previous experiments (Sections 4.1.3 and 4.1.4) indicating the need for increasing the concentrations of sodium nitrate (160 mg/L) and sodium metasilicate (99.91 mg/L) to enhance cell production, so must the optimum concentration of sodium dihydrogen orthophosphate be identified. The current concentration of sodium dihydrogen orthophosphate in Glenhaven medium is 20 mg/L. The treatments for this experiment are to be 10, 20, 25, 30 and 40 mg/L. The concentrations of other nutrients (sodium nitrate, sodium metasilicate) are outlined in the results of previous experiments (Sections 4.1.3 and 4.1.4) or are standard to Glenhaven medium (Appendix 1).

The inoculum for each treatment was grown in the corresponding medium of that treatment. Inoculum culture cell densities were counted on the day of inoculation and inocula volumes were calculated for each treatment so that each flask should have had a starting inoculum of 522 cells/µL. Algal cultures were grown and monitored in the same manner as outlined in Section 3.1.1.

3.1.6 The comparative cell concentrations of *C. calcitrans* in two different nutrient recipes

The medium recipe known as Glenhaven medium has been investigated in previous experiments (Sections 3.1.3, 3.1.4 and 3.1.5) and has been shown not to produce optimal results in the microalga *C. calcitrans* (Sections 4.1.3, 4.1.4 and 4.1.5). The recipe developed from this previous work shall be referred to as CAW (as it was developed at the Cawthron Institute, Nelson, New Zealand). This investigation is to compare the performance of 200 mL starter cultures of *C. calcitrans* in Glenhaven medium (2 batches – one prepared by the author, and one prepared by the technicians at the GACL) and CAW (prepared by the author).

The culture flasks were 500 mL Schott conical flasks stoppered with Herenz MA steristoppers. A sampling port was created by inserting a Becton-Dickinson Precision Needle (16 G 1½) into the steristopper. The 7 inch sampling needle (Section 3.1.1) was

inserted into the larger needle so as to reduce possible contamination during sampling. See Figure 8. Over the top of the steristopper was folded a 300 mm × 300 mm square of aluminium foil. The foil assisted in reducing the opportunity for infection, as well as keeping the steristopper dry during autoclaving. This foil cap was removed to access the sampling port.

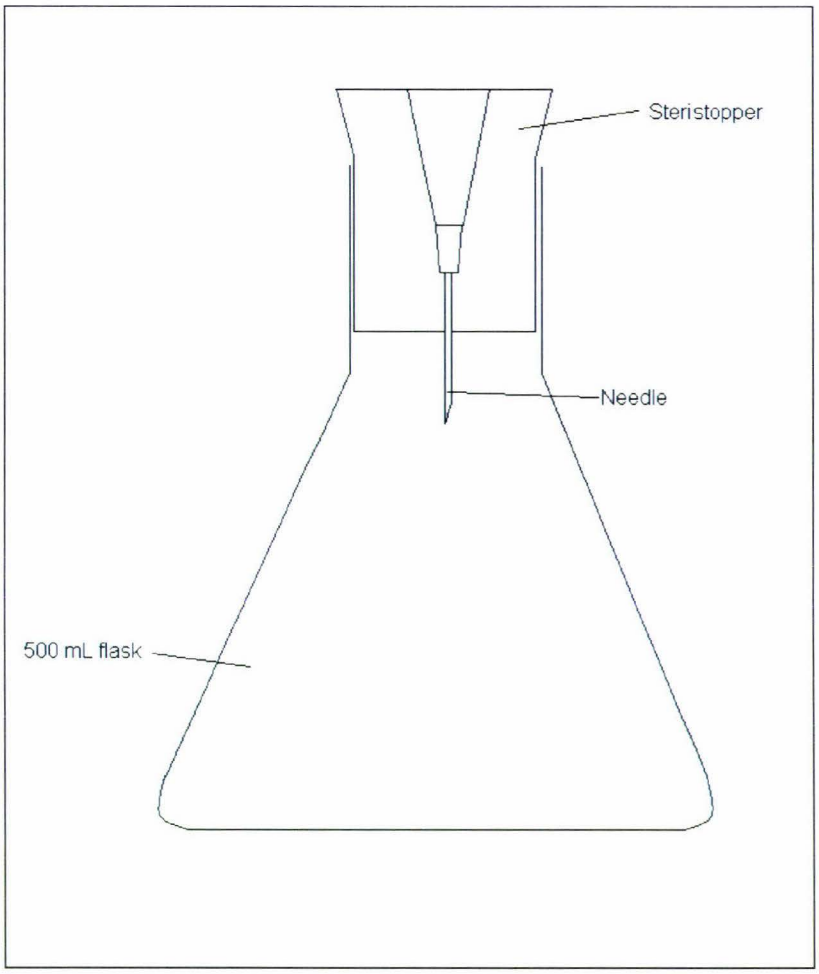


Figure 8 – Schematic drawing of the 500 mL culture flask with a 16-gauge needle as a sampling port.

The two recipes were also compared in aerated flasks as described in Section 3.1.1. The cultures were inoculated with exponentially growing *C. calcitrans* cells. The cells were monitored for cell concentration and at the end of the exponential phase 50 mL of each of the five replicates was taken and mixed together to form a 250 mL representative sample. The cell concentration of this sample was recorded. The two samples, one from each treatment were then filtered in the same way as described in Section 3.1.1.2.

3.1.7 The effect of Pluronic F-68 on *C. calcitrans* cell concentration

The culture of *C. calcitrans* in bags has been shown to be problematic on the basis of water quality (Sections 2.3 and 2.4) and one particular culture condition; that of damage by bubbles during aeration (Section 2.6). From the previous experiences of other researches working with cell cultures the issue of bubble damage has been found to be overcome by using additives to the culture medium that provide cell protection by one of several methods. In this section, one of these cell protectants is trialled to assess its suitability for use with *C. calcitrans*. The enhanced medium for *C. calcitrans*, established from previous experiments, now referred to as CAW medium was used for this experiment.

The CAW medium had various concentrations of the cell protecting chemical additive Pluronic F-68 added to it. These concentrations are outlined in Table 3.

Final percentage (w/v) of Pluronic F-68	Weight of Pluronic F-68 added to 5 L stock solution (g)
0.0	0.0
0.01	0.5
0.05	2.5
0.1	5.0
0.2	10.0

Table 3 - Concentration of Pluronic F-68 added to 5 L stock solutions of 15‰ seawater with CAW medium.

The inocula for each treatment were acclimated in the corresponding media of that treatment. Inoculum culture cell densities were counted on the day of inoculation and inocula volumes were calculated for each treatment so that each flask should have had a starting inoculum of 500 cells/µL. Algal cultures were grown and monitored in the same manner as outlined in Section 3.1.1.

3.1.7.1 The effect of Antifoam A in combination with Pluronic F-68 as additives to the culture of *C. calcitrans*

The experiment in Section 3.1.7 was repeated with the addition of Sigma Antifoam A concentrate (A6582) to reduce the deleterious effect the bubbles and froth that occurred as a consequence of adding Pluronic F-68 to the culture.

Meier *et al.* (1999) used Antifoam A in their culture work (100 ppm Antifoam A at 0.1% Pluronic F-68) and the same concentrations of application of Antifoam A were utilised for this experiment. The selected levels of Pluronic F-68 therefore dictated the amount of Antifoam A that was used so as to ensure a consistent ratio of Pluronic F-68:Antifoam A ratio across all treatments. See Table 4.

Pluronic F-68 % (w:v)	Volume of water in batch (L)	Pluronic F-68 weight (g)	Antifoam A volume (µL)
0.0	5	0.0	0
0.01	5	0.5	50
0.05	5	2.5	250
0.10	5	5	500

Table 4 – Concentrations of Pluronic F-68 to be tested and corresponding concentrations of Antifoam A.

The inocula for each treatment were grown in the corresponding media of that treatment. Inoculum culture cell densities were counted on the day of inoculation and inocula volumes were calculated for each treatment so that each flask should have had a starting inoculum of 522 cells/µL. The algal cultures were grown and monitored in the same manner as outlined in Section 3.1.1.

3.2 Identification of agars best suited to culture the bacteria present in non-axenic cultures of the microalga *C. calcitrans*, as well as treated and untreated sea water

To monitor the levels of bacterial infection in a *C. calcitrans* culture or in water treated to remove or reduce the level of bacterial infection it was necessary to culture samples of the treated waters, or the algal culture, on agar plates and calculate the bacterial levels from these results. The types of agar suited to culturing the bacteria common in these conditions were identified.

Five 1 mL samples were taken from water that had been treated through the Seasalter sea-water pasteuriser system. Also, a 3.0 L flask containing 2.5 L of water treated similarly was inoculated with *C. calcitrans* and allowed to grow for four days, at which time the culture 'crashed', possibly due to the bacteria in the water. Five 1 mL samples were taken from this 'crashed' culture, and five samples were taken from non-axenic 'production' cultures of *C. calcitrans* from the GACL.

The 1 mL samples (15 in total) were each added to a series of dilution tubes that contained 9 mL of ¼-strength Ringers solution. Each tube was vortex stirred to ensure thorough mixing and then 1 mL was added to the next dilution tube in series. The samples were diluted (1 mL: 9 mL) in series 6 times. A 0.1 mL aliquot of each dilution tube in the series was inoculated onto each of the 9 different agars to be tested. The inoculated aliquots were then smeared across the surface of the plate using standard sterile techniques to ensure the spread of any potential colony forming units (cfu). The inoculated plates (810 in total) were stacked in sample groups and cultured in a cabinet at 25°C for 72 hours, with inspections made at 24 and 48 hours.

Once the colonies had grown to sizes where a visual assessment of their appearance and form could be made, individual colonies were picked out and streaked onto an agar plate of the same type as the one upon which it had already grown. The streaking technique is standard to microbiology laboratories and is used to isolate individual colonies. At the time of picking out the colonies an estimate of the strain's density within the initial sample was made. This consists of counting how many other cfu's there are of similar appearance on the plate. This is an inexact but acceptable method.

All isolated colonies from the streaked plates were then used to re-inoculate all the types of plates being tested. From the results, it was established which agars were most supportive of the proliferation of bacterial cultures present in the water sources. The results of how well each isolated culture grew on the different plates were recorded. At this point the strain was re-plated on the agar it grew most prolifically on and a cfu from this plate was picked off and preserved for later use. The bacteria that were isolated from the initial plates were preserved using Protect Bacterial Preservers made by Technical

Service Consultants Ltd (UK). Manufacturer's instructions were followed. The preservers are suited for freezing bacterial isolates for any length of time.

The bacteria isolates were described in the following ways:

Original source;

The agar to which they are most suited to, and any other agars they grow on;

The cfu's (colony forming unit's) appearance, colour and shape on each agar;

Oxidase test - positive or negative;

Gram test - positive or negative;

Shape – rod or cocci;

Catalase test – positive or negative;

Motility test – motile or non-motile.

Within the scope of this work, identification of bacterial isolates to the genus and species level was considered unnecessary.

The agars to be used were:

Marine agar (MA) (BD - Difco™ Marine Agar)

Sheep blood agar (SBA)

Tryptone soy agar (TSA) (Oxoid CM131)

Tryptone soy agar (TSSW) (Oxoid CM131) made with sea water (15‰ salinity)

Vibrio chromogenic agar (VCA)

TCBS (Thiosulphate citrate bile sucrose) agar (TCBS)

Ryan's aeromonas agar (RAA) (Oxoid CM833 Aeromonas medium base)

MA + lysed *C. calcitrans* culture (MACc)

TSA + lysed *C. calcitrans* culture (TSACc)

Along with the first seven agars, the last two preparations containing lysed algae and medium from a healthy culture of *C. calcitrans* added to MA and TSA were used. The latter preparations were expected to be able to culture bacteria that may be somewhat specific to *C. calcitrans* and otherwise would not grow on any of the other agars (Gibson, pers. comm.).

All agars were made according to manufacturer's specifications or were purchased ready for use. The algae used for making the lysed preparation were lysed by exposure to

ultrasound (Ultrasonics, Inc.Sonicator™ Model W-220F). The power setting was at '7' and sonication was performed for 1 minute.

3.2.1 The effect of mono specific bacterial infections on the growth of axenic *C. calcitrans* in batch culture

The effect that a range of isolated bacteria had on axenic *C. calcitrans* cultures was assessed in an attempt to confirm the inference that the poor health of the *C. calcitrans* cultures grown in pasteurised water was a result of bacterial inhibition or stress. Nineteen of the bacteria that had been isolated from cultures of *C. calcitrans* grown in pasteurised water that had subsequently crashed were used for this assessment. As well as testing the isolated bacteria, axenic *C. calcitrans* cultures were also inoculated into flasks containing untreated seawater (15‰), pasteurised seawater (26‰) and autoclaved seawater without any bacterial isolate being added (control). All flasks that were to have mono specific bacterial broth inoculated into them were initially autoclaved at 121°C for 15 minutes as for the control.

Each bacterium had been isolated and frozen for preservation, as described in Section 3.2. A bead from the vial of each preserve was removed and added to either Marine broth or Tryptone soy broth made in sea water. The choice of broth depended on the isolate's growth performance on Marine agar and Tryptone soy agar. After 24 hours the broths were sampled to ascertain the bacterial density and 0.5 mL of broth was added to healthy, two day old *C. calcitrans* cultures growing in autoclaved seawater. The *C. calcitrans* cultures had been inoculated the previous day with 60 mL of exponentially growing axenic algae culture. The algae cultures were assessed qualitatively under the following parameters;

- foam or froth at the waterline of the flask.
- cell scum (cell aggregates) at the waterline.
- cell biomass collecting on the flask above the waterline.
- biofilm (cells adhering to the sides of the flask below the waterline).
- cells flocculating (dropping out of suspension).

The density of the infecting bacteria was measured at the start and finish of the culture cycle. The definition of 'finish' was deemed to be when the culture had clearly moved into a detrimentally affected condition.

3.3 Water treatment methods for the growth of *C. calcitrans* in batch and continuous culture

3.3.1 The electrolysis of 15‰ seawater

3.3.1.1 Testing the Monarch Pool Systems' seawater electrolysis device for the level of sodium hypochlorite

Seawater electrolysis has been established as a method of producing sodium hypochlorite in domestic swimming pools so as to kill bacteria and microalgae, and maintain the water quality in the swimming pool. A variation of this technology was trialled for the reduction of bacterial density so as to make it suitable for algal culture. Monarch Pool Systems (10-12 Kembla Way, Willetton WA 6155, Australia) produce a range of products for swimming pools. The Chloromatic ESR device is designed to work in pools with a NaCl salinity of 4‰ or greater. The system purchased for this work was modified slightly from the standard model. The controller that regulates how often the system operated was removed by the manufacturer, and replaced with a controller that varied the power output through the electrolysis cell. The power controller allowed the operator to vary the output in percent units. Maximum (100%) output = 20 Amperes and 50% output = 10 Amperes. See Figure 9.

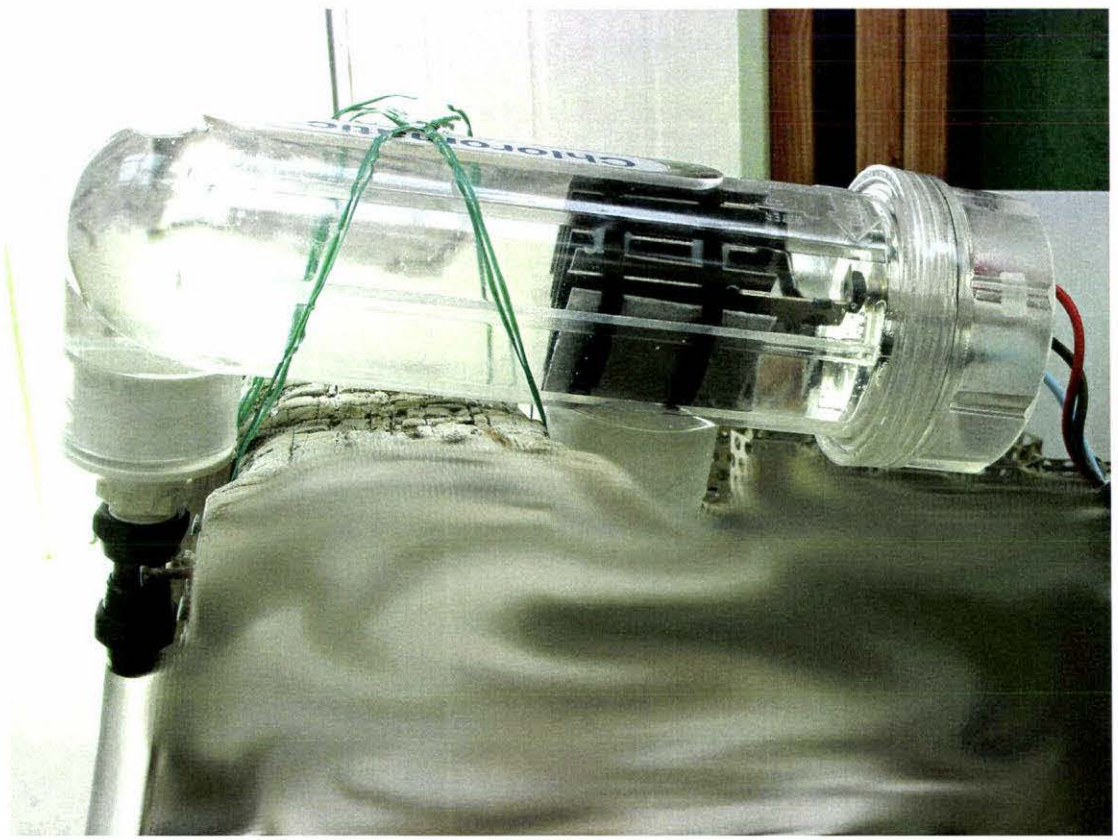


Figure 9 - The Monarch System's Chloromatic electrolytic cell. Water flows in over the electrolysis plates on the right and then out of the fitting on the left.

Following the manufacturer's operating instructions, the system was tested for levels of sodium hypochlorite production at various power levels, production of sodium hypochlorite at different water temperatures, and level of bacterial disinfection at different time intervals before chlorine neutralisation. In all instances, where different concentrations of hypochlorite were to be tested this was based upon power input levels of the electrolysis system and not set concentrations of the hypochlorite itself.

The level of sodium hypochlorite in samples was analysed on an ion-specific meter (Hanna Instrument USA, Model HI93734) as per the manufacturer's instructions. The protocol for dechlorination was taken from Jorquera *et al.* (2002) and a Microsoft Excel spreadsheet was written to allow for easy calculation of the correct level of sodium thiosulphate to add to chlorinated solutions to remove the chlorine. (See Appendix 2 for the MS Excel spreadsheet formulae). All sodium thiosulphate solutions added to samples had been autoclaved (121°C, 15 minutes) to ensure sterility.

As well as measuring the concentration of bacteria, a direct "Yes/No" test was performed to find if any culturable bacteria were present in treated samples. 20 mL samples of electrolysed and dechlorinated water were added to 20 mL sterile preparations of double strength Marine broth. The samples were incubated in the dark at room temperature for seven days on an oscillating table (200 cycles per minute). Any turbidity within the sample after this time was interpreted to be a "Yes" positive growth result, showing bacterial growth. Non turbid samples were allocated a "No" bacterial growth result. Control samples were autoclaved seawater.

Disinfection of water in different size vessels (1.0 and 20 L) was investigated to establish if disinfection was easily attainable in the vessels that were to be used for *C. calcitrans* culture during this part of the assessment. The system of algal culture developed in this work is meant to be for a commercial unit, thus a trial was performed to establish if 20 L Nalgene carboys containing electrolysed water, that had been prepared on any given day could be held for several days before de-chlorination, and still contain enough sodium hypochlorite to remain disinfected. Conversely, could an electrolysed carboy be prepared, dechlorinated, and held for several days and still be highly disinfected and culture *C. calcitrans*.

To test this situation, 12 carboys were filled to the very top with electrolysed water at 15‰ salinity seawater that had passed through the electrolysis unit running at 60% power (12 Amps and 30 L/min). A 40 L bin was filled with similarly treated water and the lids of the carboys were submerged in this water. All clamps and caps were opened and removed, respectively, from the lids to ensure that the chlorinated water would flush into the glass tubes and fittings of the carboy lid (Figure 10).

After 15 minutes the clamps were closed and caps replaced. The lids were then placed on the carboys. These were then left for 24 hours to ensure full disinfection.

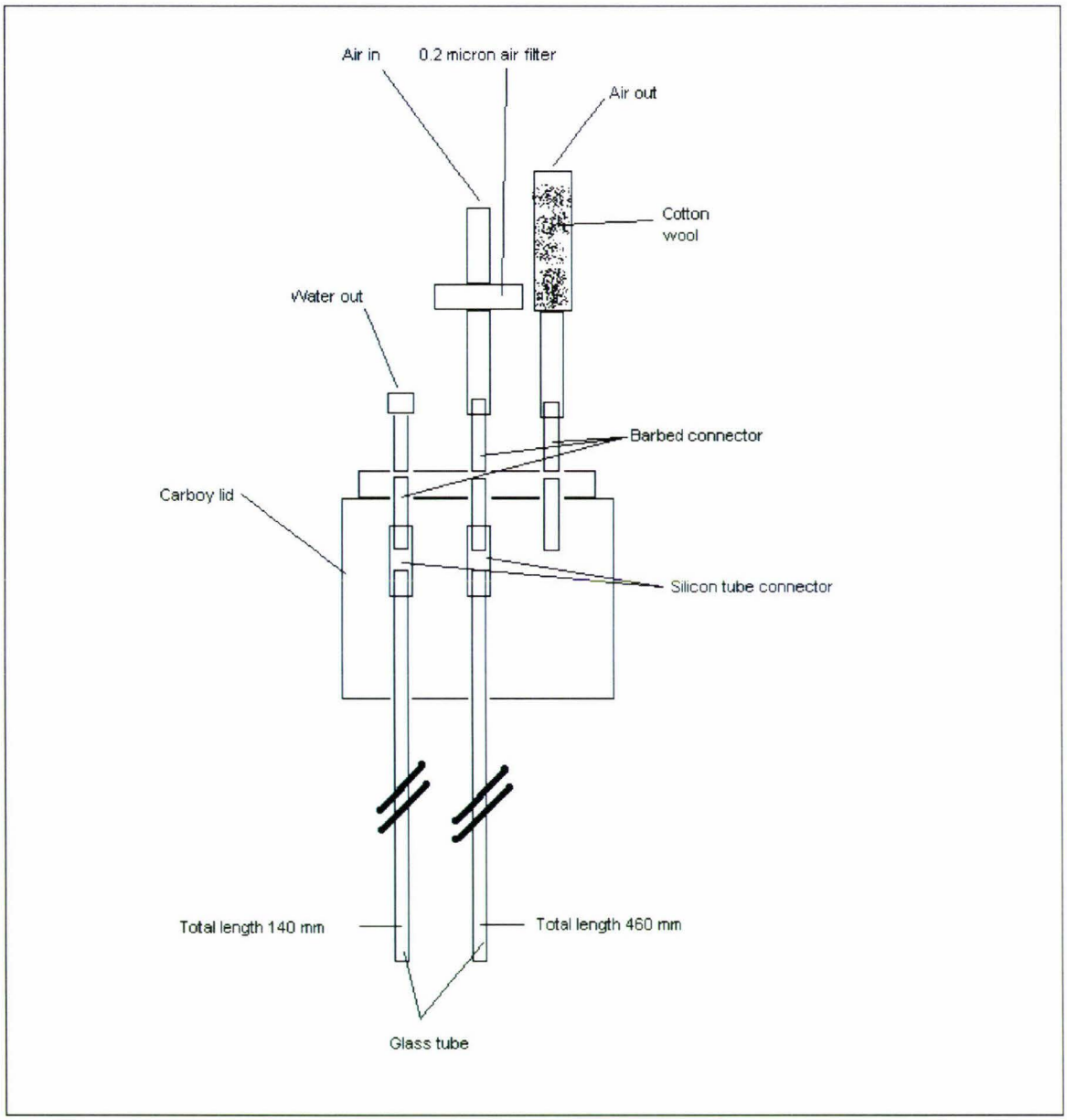


Figure 10 - Schematic drawing of the lid of a 20 L carboy.

After 24 hours, half the carboys were sampled for the level of total chlorine present. The remaining carboys were dechlorinated by attaching an airline to the 'air in' line of the lid. The 'water out' cap was removed and air was blown through the carboy. Water was forced from the carboy via positive pressure until the water level in the carboy reached the level of the 'water out' glass tube. The air line was clamped shut whilst a 10 mL

aliquot of autoclaved sodium thiosulphate was injected into the carboy through the 'water out' fitting. The 10 mL aliquot of sodium thiosulphate (20 g/L) was calculated to be sufficient to neutralise a maximum of 7 mg/L of total chlorine in 20 L. Any excess sodium thiosulphate was not considered to be detrimental to the culture of *C. calcitrans*. The cap was then replaced on the 'water out' fitting, and the 'air out' and 'air in' clamps were opened. The carboy was aerated through the 0.2 μm filter for one hour to ensure all the hypochlorite was removed.

After the dechlorination process the carboys (six in total) were sampled for the presence of bacteria. One millilitre samples were aseptically taken from each carboy and diluted in series following the methods in Section 3.2. All 0.1 mL aliquots were plated on Marine agar and cultured for 72 hours before colony forming units (cfu) were counted.

Five days after the first carboys were dechlorinated; the second six carboys were tested for total chlorine concentration and then dechlorinated. All twelve carboys were sampled and tested for presence of bacteria. Following this five carboys from each time regimen were then inoculated with *C. calcitrans* culture (~0.8 L of exponentially growing culture) and the growth was qualitatively monitored over the next seven days.

3.3.1.2 Cell concentration of *C. calcitrans* cultured in electrolysed water

The growth of *C. calcitrans* in 20 L carboys of electrolytically treated water was compared to the growth of *C. calcitrans* in similar carboys that had been disinfected by autoclaving. (The autoclaved carboys are referred to as disinfected and not sterilised, as the amount of time the carboys are treated in the autoclave with 20 L of water does not allow for the water temperature to reach 121°C as is normal in autoclaving. The average temperature reached in a 20 L carboy is 95°C. The bacterial spores that survive this process are generally not harmful to the *C. calcitrans* cultures.)

Following dechlorination (Section 3.3.1.1), the carboys were inoculated with *C. calcitrans* culture and have the nutrient solutions added. Two 100 mL Schott bottles per carboy were prepared prior to the carboy inoculation. These bottles contained the nutrients for the algal culture. The ingredients of the bottles are outlined in Table 5. These preparations were autoclaved for 15 minutes at 121°C.

Ingredient	Concentration of stock solution (g/L)	Bottle 1 Volume added (mL)	Bottle 2 Volume added (mL)
Sodium nitrate	160	20	
Sodium dihydrogen orthophosphate	40	20	
Nutrient mix	(see Appendix 1. Solution #1 minus the sodium nitrate)	20	
Vitamins	(see Appendix 1. Solution #3)	2	
Sodium metasilicate	60		33.3

Table 5– Recipe for nutrients for 20 L carboys.

The cell concentration of cultures of *C. calcitrans* cultured in autoclaved and electrolysed water in 20 L carboys was measured from the day of inoculation (Day 1) through to the day of harvest (Day 4), and the results were compared over the growth cycles. The cycles were not continued through to stationary and death phase as they were part of the algae production for shellfish breeding at the GACL, therefore they were utilised for feeding at the end of or near to the end of exponential growth.

3.3.1.3 The growth of bacteria in electrolysed water

From the results of Section 4.3.1.2, the culture of *C. calcitrans* with electrolysed water, it was hypothesised that water that had been electrolytically treated may promote bacterial growth if bacteria are present. This was tested by comparing bacterial growth in water (all 15‰ salinity) treated in different ways; untreated seawater (control), untreated seawater plus nutrients (control + nuts), autoclaved water with nutrients added, electrolysed water and electrolysed water with nutrients added. Five replicate 3.0 L flasks of each water treatment were prepared using the methods outlined in Section 3.3.1.1. In the treatments with nutrients added, this was at the standard concentration for a culture flask of that size (Appendix 1). One sample (40 mL) of each of the five replicate flasks was aseptically taken from the five treatments and added to a sterile 50 mL Falcon tube.

As the problem being investigated was bacteria that grew well in electrolysed water it was important to ensure that the bacterial inoculation used for this trial consisted of bacteria that grew well, and were acclimated to these conditions. This was accomplished

by preparing a culture of *C. calcitrans* in a 20 L carboy that had been disinfected using the electrolysis method. When the culture had reached the end of its exponential phase a 40 mL sample of the culture was taken and centrifuged at 2000 RPM (700 g) for 10 minutes at 4°C. The *C. calcitrans* cells formed a pellet in the centrifuge tube and the bacteria remaining in the supernatant were used as the inoculum. Samples were taken from the *C. calcitrans* culture before and after centrifugation and plated on Marine Agar (Section 3.2) to establish how many of the total bacteria were lost due to centrifugation.

Each replicate of each treatment was sampled prior to *C. calcitrans* supernatant inoculation to establish the background bacterial levels (Section 3.2). A 0.1 mL sample of supernatant was added to each replicate (40 mL). The replicates were then held in a rack in the dark at room temperature and stirred at 200 RPM on an oscillating table. One millilitre samples were aseptically taken from each treatment at 24 and 96 hours and diluted and plated on Marine agar using the methods in Section 3.2. Plates were cultured for 72 hours (25°C) before counting of cfu occurred.

3.3.1.4 The effect of activated carbon filtration upon the level of organic carbon in seawater

From the results of Section 4.3.1.3, the promotive effect of electrolysed seawater on the growth of bacteria was established. The level of organic carbon present in the water and the use of activated carbon filtration to remove organic carbon were investigated in an attempt to overcome this problem.

An activated carbon filter was constructed of 50 mm Iplex water pipe and Iplex fittings. The hose fittings for the filter were 20 mm Hansen pipe fittings. The 2.5 L of activated carbon granules (from Veolia Water Systems, Auckland, NZ) was retained in the pipe with a 100 mm plug of Dacron at both ends of the Iplex pipe. Seawater was passed through the filter at a rate of 8 L/hour to ensure a water/carbon contact time of at least 15 minutes (Figure 11). All water samples were measured for Non-Purgeable Organic Carbon (APHA 5310 B (modified) 20th ed. 1998) by Hills Laboratories, Hamilton, NZ.

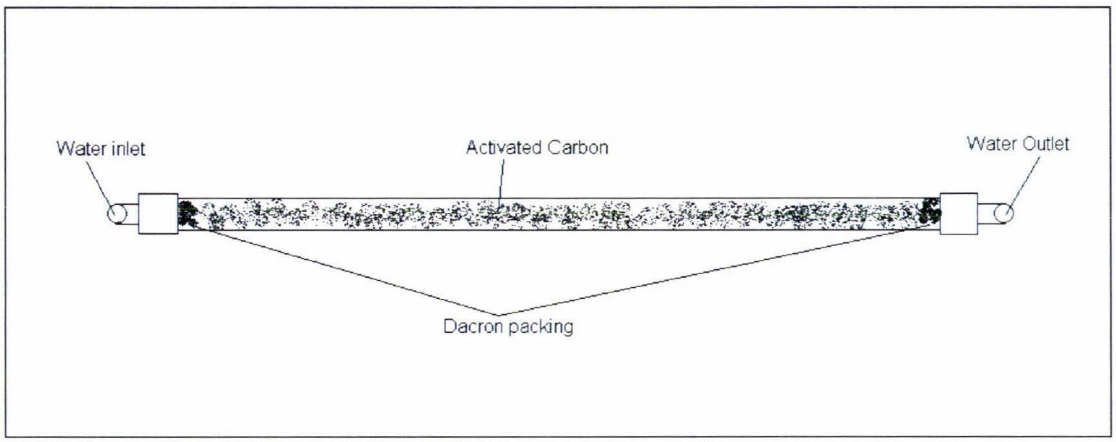


Figure 11 – Schematic drawing of the activated carbon filter built to remove organic carbon.

Results (Section 4.3.1.4) showed that the activated carbon filter removed at least 40% of the organic carbon from the seawater. A trial was performed to ascertain the organic carbon levels of water treated with electrolysis. Because of the order in which samples were taken, two separate batches of water had to be prepared, and samples were taken at various stages (Figure 12).

The treatments were as follows:

Sample A - Untreated 15‰ salinity sea water (Batch 1) (Control 1)

Sample B - 15‰ salinity sea water that was electrolysed and then treated with activated carbon (Batch 1)

Sample C - 15‰ salinity sea water that was electrolysed and then treated with activated carbon and then electrolysed again (Batch 1)

Sample D - Untreated 15‰ salinity sea water (Batch 2) (Control 2)

Sample E - 15‰ salinity sea water treated with activated carbon (Batch 2)

Sample F - 15‰ salinity sea water treated with activated carbon that was then electrolysed (Batch 2)

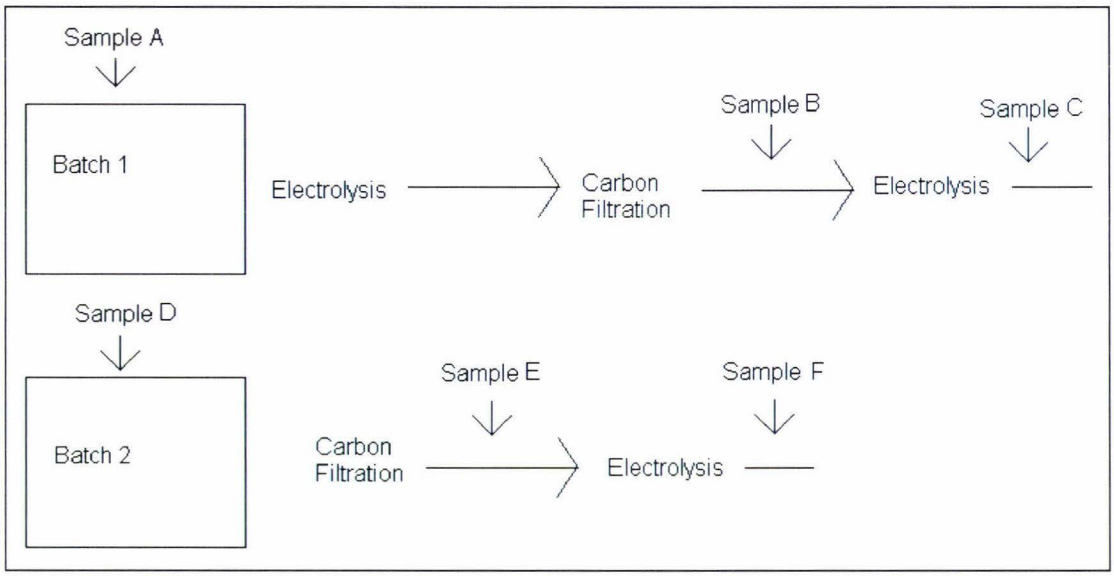


Figure 12 – Schematic drawing of the sample points in the water treatment process for the removal of organic carbon and water electrolysis experiment.

In parallel to this work to measure the organic carbon levels, a bacterial growth test was performed to find if any changes in organic carbon levels showed an equal or corresponding reduction in bacterial levels. This test followed the same methodology as the previous test (Section 3.3.1.3); aliquots were taken from sample points A, C and F and inoculated with bacteria obtained from *C. calcitrans* culture grown in electrolytically treated water.

3.3.2 Heat treating water for culturing *C. calcitrans*

The results of the previous investigations (Sections 4.3.1.1, 4.3.1.2, 4.3.1.3 and 4.3.1.4) into using electrolysed water have indicated considerable difficulties with the final water quality and the growth of bacteria in water treated with this method. For this reason it was decided to concentrate on a method of treating the seawater with heat to reduce bacterial spore survival. The Academic Press Dictionary of Science and Technology (1992) defines *Pasteurisation* as a "process in which a mild heat treatment is applied for a specific period of time to kill or inactivate disease causing organisms". In keeping with this definition, the heat treatment experiments in the remainder of this work will be in relation to developing a pasteurisation system for the treatment of seawater to allow the continuous culture of *C. calcitrans*.

3.3.2.1 The effect upon bacterial activity of heat treating seawater

Heating of water is known to reduce bacterial and viral activity (MacLeod, 1965; Moats, 1971; Yura *et al.* 1993; Arimoto *et al.* 1996; Stabel *et* al. 1997; Chang *et al.* 1998; Frerichs *et al.* 2000; Kilsby *et al.* 2000). In a trial similar to that of Kilsby *et al.* (2000) water was heat treated to assess inactivation over time.

Samples of 26‰ salinity water from the Seasalter pasteurising system that is currently in use at the GACL was sampled (30 mL) and added to a double strength Marine broth. The sample was taken from this system so as to ensure that bacteria tested in this trial are those that survive the current pasteurising system process of water treatment. This broth was grown for 24 hours at room temperature.

Ten treatments (5 replicates) were prepared with 10 mL of autoclaved 15‰ salinity seawater in 15 mL sterile Falcon tubes. 10 µL of the infected Marine broth (see above) was added to each tube before being sealed. Five tubes were put aside (no heat treatment) and the remaining tubes, held in a rack, were submerged to above the sample waterline in a hot water bath at 85°C. A separate Falcon tube with a standard -10 to 100°C thermometer inserted through the tube cap, with 10 mL of 15‰ salinity seawater was also held in the rack near the centre of the rack of tubes. When the water in this tube reached 85°C then the timer was started and dictated the removal of tubes. The tubes were removed at 5 minute intervals to obtain treatment times of 0, 5, 10 etc through to 45 minutes. As the 5 replicates of each treatment were removed they were placed in an ice bath to cool. Once all samples were heated and then cooled, 1 mL samples were taken of each replicate and diluted and plated on Marine agar using the methods in Section 3.2. Agar plates were cultured at 25°C for 96 hours before the cfu were counted.

3.3.2.2 Evaluation of flow dynamics in the Seasalter pasteuriser system used at the Glenhaven Aquaculture Centre Ltd

To ascertain the flow behaviour of the water in the present pasteuriser system, a heating kettle (60 L) was set up to have the same flow rate and air (bubble) rate as the unit being used. The air flow in the system was installed to mimic the bubbles of gas that normally leave the heat exchanger of the pasteuriser system and enter the kettle. These bubbles occur due to the degassing of the water as it heats up from ~17°C to ~80°C in the heat exchanger. The major difference between the model and the actual system was that the model was not heated, as no heat exchanger was available for use with the model. The water flow rate was established at 1.3 L/min and the air (bubble) rate was 50 ml/min.

A one millilitre dose of the fluorescent dye Rhodamine WT was added to the source tank (Time 0), and mixed quickly, to give a concentration of 473 parts per billion (ppb). Samples (30 mL) were taken every minute from Time 0 for 40 minutes.

The samples were measured for the concentration of dye on a Turner Designs (USA) 10AU Fluorometer. The Fluorometer had been set up to read Rhodamine WT dye at levels between 1 part per trillion (ppt) and 999 ppb.

3.3.2.3 Rhodamine WT dye concentration in a ten-chamber flow through system

The results in Section 4.3.2.2 indicated that the breakthrough time of the water in the heating kettle of the Seasalter pasteuriser system is unlikely to have been long enough to ensure the water was disinfected of bacteria and spores to a level suitable for the culture of *C. calcitrans*. As such, a system needed to be developed that would increase the breakthrough time of the water in the heating kettle, so that a greater proportion of bacteria and spores were inactivated during the heating process.

A multi-chamber flow through system with aeration as a form of agitation was investigated as a potential way of increasing the residence time of the water in a system. (NB. Unless otherwise stated, all preliminary experiments in this section of work were trialled with fresh water at room temperature.)

A series of ten buckets (10 L with 9.6 L working capacity) were set up so that water would flow from bucket 1 into bucket 2, bucket 2 into bucket 3 etc until the water flowed out of bucket 10 (Figure 13). The flow rate was 1.3 L/min. Aeration rate was not measured but was sufficient to ensure mixing in each bucket.

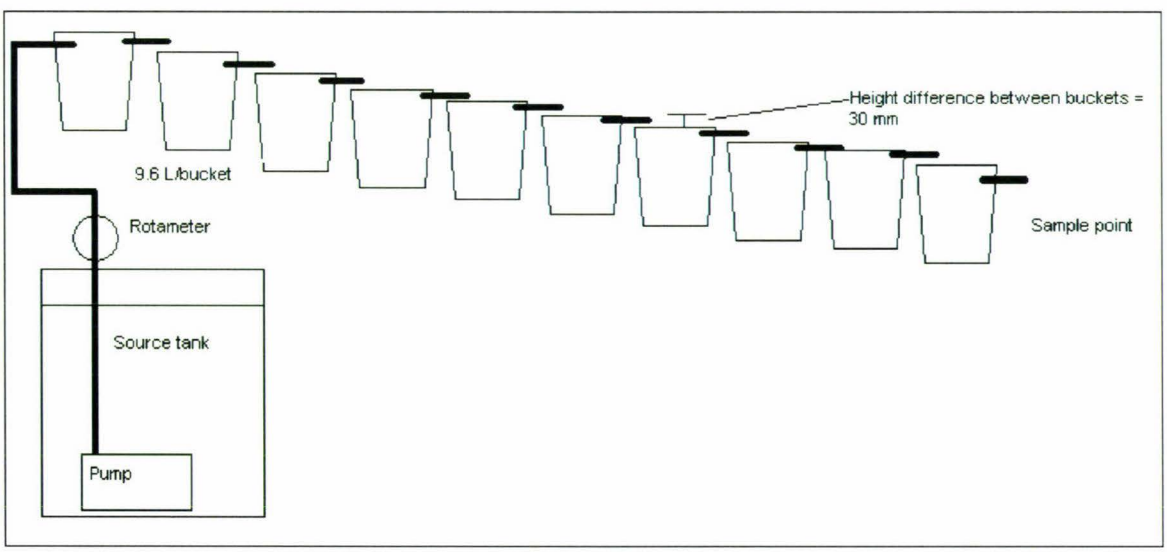


Figure 13 – Schematic drawing of the bucket layout for the multi chamber flow assessment.

As with the previous trial described above (Section 3.3.2.2), water was in a source tank and at Time 0 an aliquot of Rhodamine WT was added to establish an initial concentration of 593 ppb in trial 1 and 670 ppb in trial 2. As well as monitoring the concentration of Rhodamine WT in water flowing out of bucket 10 with each minute, the concentration of dye was monitored in each bucket by taking a sample (30 mL) from buckets 1-10 every ten minutes.

3.3.2.4 Breakthrough time and concentration of Rhodamine WT dye in water flowing through a 200 m long pipe

The results of the previous assessment (Section 4.3.2.3) of water in a multi-chamber system showed that a breakthrough time of 25-30 minutes was manageable. However, when the multi-chamber system was more fully considered from a design and operation perspective there were a number of issues that made it a less attractive option for continued consideration. Therefore, what was perceived to be a simpler method was considered.

A 200 metre long pipe (hose) constructed of medium density polyethylene (MDPE) with an internal diameter of 15 mm was stacked in a coil with each coil diameter between 0.5 and 0.7 m. The volume of the hose was calculated to be approximately 35 L. With a flow rate of 0.7 L/min the theoretical residence time is ~ 50 minutes.

i.e. Volume (π.r2.h; L)/Flowrate (L/min). Using similar methods as described in Section 3.3.2.2 to sample the dye concentration, Rhodamine WT, used as a dye tracer, was injected in-line and samples (30 mL) were taken from the pipe outlet, every 2 minutes for the first 20 minutes and the last 40 minutes and every minute in-between these times.

A second trial of this test was also completed. In this trial, every 50 m along the pipe a small disc was inserted, in line (Figure 14) that was intended to disrupt laminar flow in the pipe.

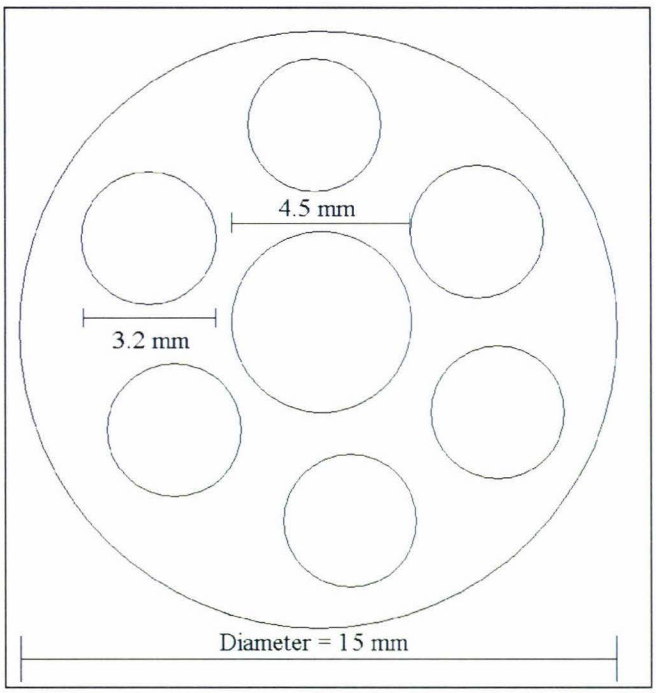


Figure 14 – Schematic drawing of the disc interrupter inserted into the 200 m pipe every 50 m.

3.3.2.5 Breakthrough time and concentration of Rhodamine WT dye in a prototype of a glass tube and silicone hose system

From the results of the last experiment (Section 3.2.4.1) with a 200 m pipe, a prototype design was proposed for testing. The prototype consisted of 66 Duran glass tubes at 1.5 m long (12 mm × 1.5 mm. The measurement of 1.5 mm relates to the wall thickness of the tube, therefore the internal diameter (I.D.) of the tube is 9.0 mm) that were connected with 190 mm long segments of silicone hosing (16mm × 3mm i.e.10 mm I.D) to give a continuous pipe length of approximately 108.2 m. (N.B. In the final system the silicon hose connectors were increased to 250 mm long. I.e. 190 mm of working length and 2×30 mm of connection length). The layout of the glass tubes consisted of a series of circles with increasing radii (see Table 6, Figures 15, 16 and17).

Radius of circle (mm)	Circumference (mm)	Number of tubes in the circle	Total number of tubes	Total length of tube in prototype (m)	Total length of tube in final system (m)
78.9	495.9	16	16	26	27
108.5	681.7	22	38	62	64
138.1	867.7	28	66	108	111
168.0	1055.6	30	100		169
197.3	1239.6	40	140		236
227.0	1426.28	46	186		314
256.5	1611.6	52	238		402

Table 6 – The specifications for the layout of the tubes in the tube pasteuriser system. This table contains details for the prototype, as well as the final tube pasteuriser system.

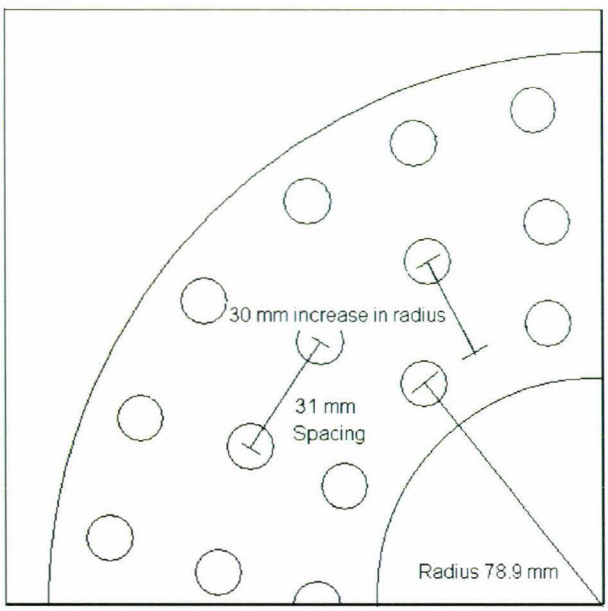


Figure 15 – Schematic drawing of a section of the layout of tubes for the tube system.

Figure 16 – The layout of the prototype tube system.

Figure 17 – The prototype tube system held in a temporary wooden frame.

In the case of the prototype glass tube and silicon hose system set up, the measurement of breakthrough time and Rhodamine WT dye concentration was performed slightly differently from previous experiments (Section 3.3.2.2). Firstly, instead of samples being taken every minute and then read individually through the Fluorometer, the Fluorometer was set up for continuous monitoring. This allowed the water from the tube system to

flow through the sampling cell and readings could occur as often as required. For this series of tests, sampling occurred every five seconds.

The second difference, from previous tests, was that the water run through the glass tube and silicon hose system was, cold for two trials and hot (~70 - 90°C) for two trials. A further change to the previous method was that a series of small clamps were trialled, instead of disc flow disrupters. The intention of these clamps was to change the profile of the silicon hoses from almost round to a very long ellipse (Figure 18). The final difference in this trial was that the dye was injected as one slug rather than by mixing into a source tank. This allowed both the appearance and dissipation of the dye to be recorded. The intention of this method was to be able to find the time required for the slowest moving dye to be washed from the system.

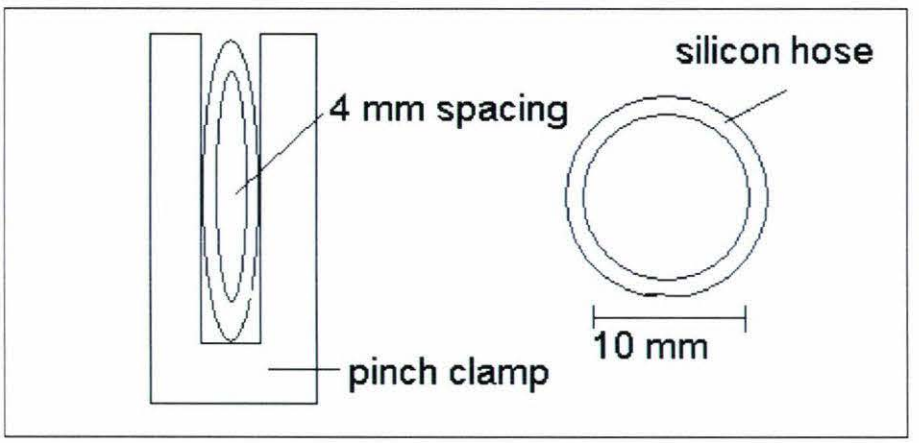


Figure 18 – A schematic diagram of the profile of the silicon hosing with and without a pinch clamp on it.

For the trials using hot water, the heating kettle, (described in Section 3.3.2.2) was used to heat the water before it was pumped through the tube system. The water temperature in the kettle and tube system did decrease as the test progressed, but the water in the tube system stayed over 70°C during the test. Once the water left the tube prototype it entered a short section of coiled glass tube that was submerged in an ice bath. The ice bath brought the water temperature down to ~25°C. This step was necessary as the Fluorometer was only able to withstand temperatures up to 50°C, and because Rhodamine WT gives lower fluorescence readings at higher temperatures.

3.3.2.6 Breakthrough time of Rhodamine WT dye in a full scale glass tube and silicon hose pasteuriser system

After assessing the results from the previous experiment (3.3.2.5), a design for a glass tube and silicon hose pasteurising system with a total length of tubing of 402 m was developed. The increase in the overall length of the tubing was to ensure that the desired breakthrough time of 25-30 minutes at 0.7 L/min would be obtainable.

Table 6 outlines the number of glass tubes and the total length of the system. A copper frame with the same tube layout as the prototype (Figures 15 and 19) was built to hold the tubes. This stand (Figure 19) was made from 2 mm thick copper plate and 25 mm diameter copper tubing for the legs and supports (built by the author). A custom built commercial hot water cylinder (HWC) was used for the water heater. The internal tank was copper; to ensure there were no red-ox reactions between different metals the tube stand was built from copper as well.

Figure 19 - The copper stand for holding the glass tubes in the water heater.

The glass tubes were installed into the stand in a sequential manner. Each glass tube was connected by a section of silicon hose (250 mm) to another glass tube three positions along in the same ring of holes. Once this circuit was filled, then the next circuit within the same ring was fitted. The most inner ring was filled first and then each subsequent ring after that (Figure 20).

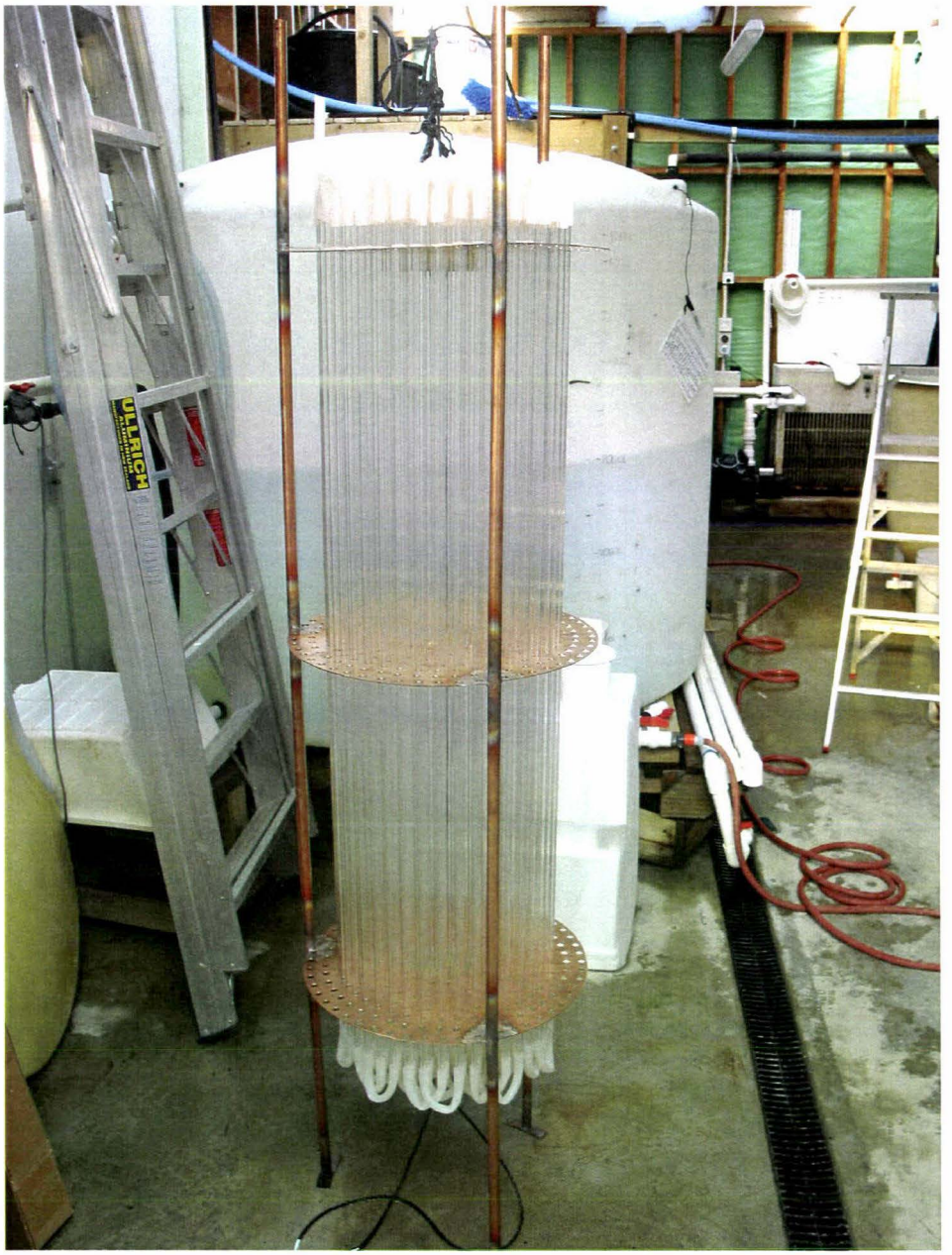


Figure 20 - The copper stand with ⅔ of the glass tubes fitted.

After the first 40 tubes a glass T-piece was fitted and the remainder of the system was divided into two parts. On one half of this system (99 tubes each half) a diaphragm pump operated by compressed air was fitted. The intention of this pump was to act as a booster pump to reduce the working pressure on the first pump. This pump failed due to the hot conditions (Section 4.3.2.6) and the system was re-plumbed so only one of the two 99 tube sections was used. This meant that overall the glass tube and silicon hose system consisted of 139 tubes and was 235 m long. To maintain the breakthrough time that the system was initially designed to have (30 minutes) the flowrate for the system (0.7 L/min) was reduced by half (0.35 L/min) in keeping with the reduction in length of the tube system.

Once the silicon hoses were slipped onto the glass tubes the system was fitted into the HWC. The necessary electrical and plumbing connections were made before the HWC and tube system were filled with water and heated to 95°C. Previous experience has shown that the glass and silicon tubing bond together after several days held at heat.

This method of fixing the silicon hose to the glass tube failed and a second attempt was made to bond the hose to the tubes. This method involved placing a bead of silicon sealant onto the glass tube and then pushing the hose onto the tube. An 80 mm length of copper wire (1 mm diameter) was then used as a twist tie around the union to further secure the hose to the tube (Figure 21).

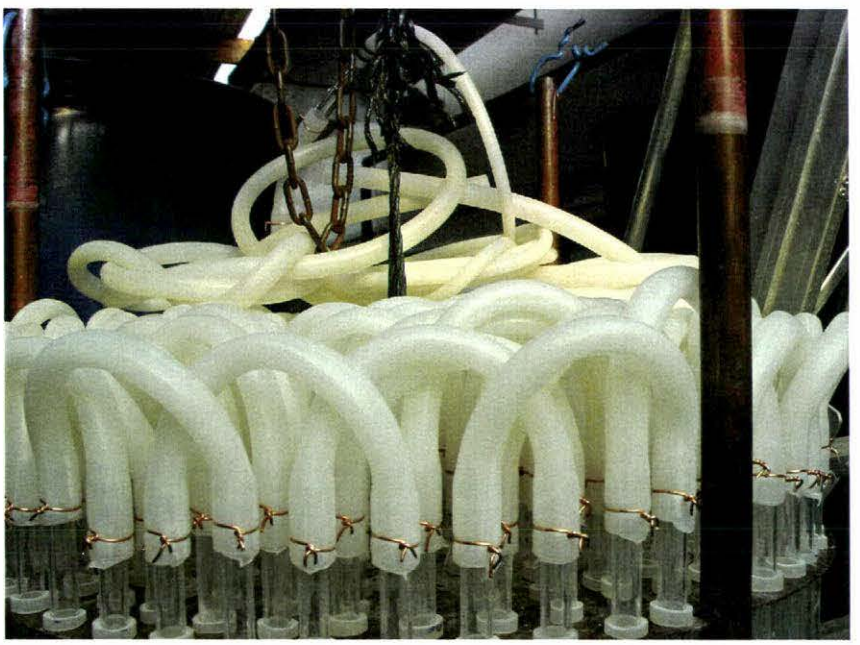


Figure 21 - The top of the glass tube system with all silicon hoses and copper wires fitted.

This method also failed. The final method trialled, successfully, was to make tubulations on the glass tubes with a proprietary two-part epoxy putty produced by Selleys called 'Knead It'. The tubulations were affixed by rolling out thin lengths of the putty and

putting them onto the glass tubes. The glass tube was then rolled against one's palm to bond the putty to the glass tube and create an even shape around the glass tube. Once the putty had cured onto the glass it was exceptionally hard. The silicon hose was then pushed over the glass tube and tubulation. As previously outlined, copper wire twist ties were used to further retain the hose onto the tube (Figure 22).

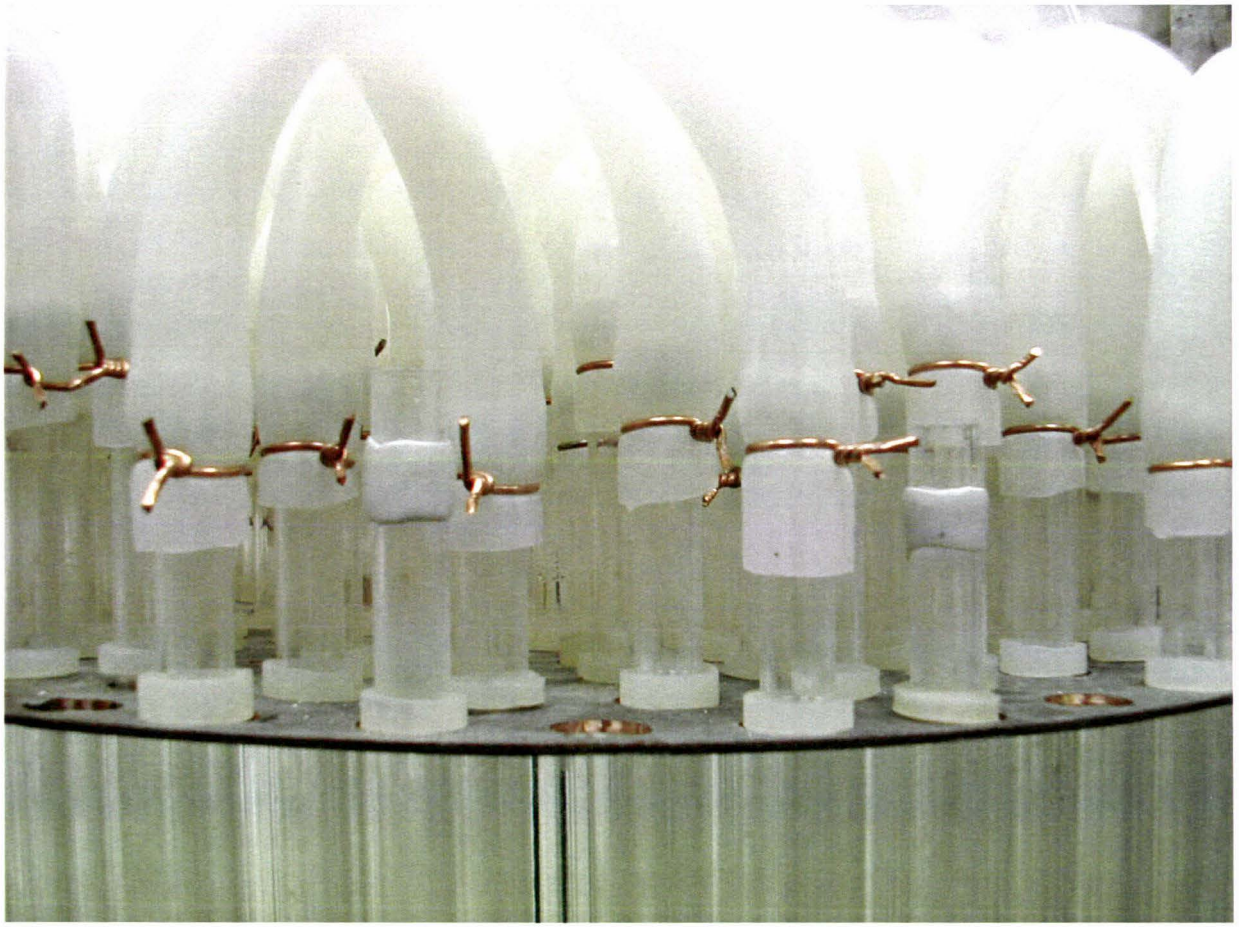


Figure 22 - The glass tube and silicon hose joints. The putty tubulations and copper wire connections are shown.

Once the glass tube and hose system was assembled, the whole frame was lifted up on a pulley and the HWC was placed underneath. The system was again lowered into the HWC (Figure 23 and Figure 24).

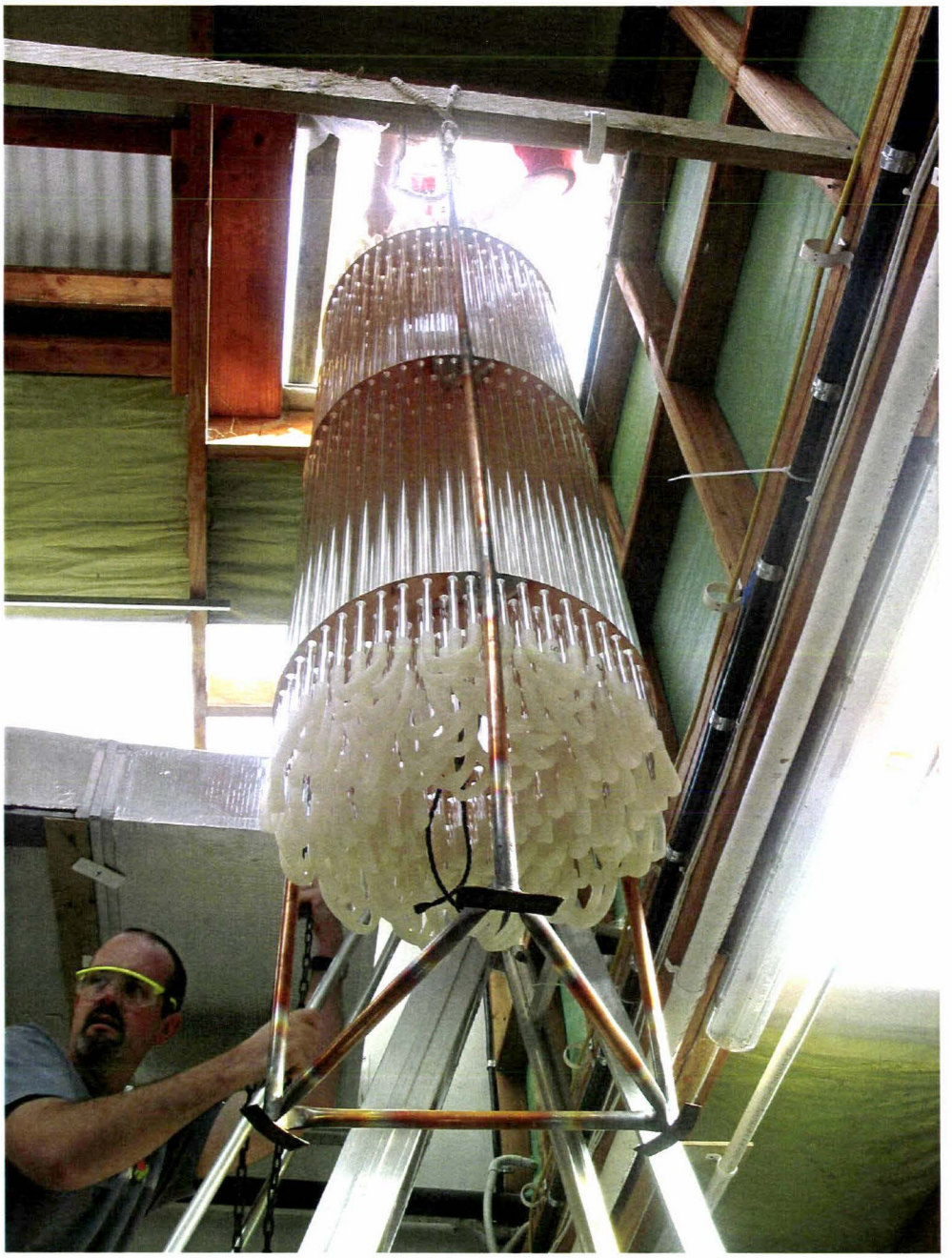


Figure 23 - The copper stand with the glass tube system lifted up so it can be lowered into the HWC. (The first trial).

Figure 24 - The copper stand system being lowered into the HWC.

The glass and tube system was fitted to the heat exchanger and all monitoring equipment, electrical power supplies and plumbing connections were fitted. Figure 25 is a schematic drawing of the system and Figure 26 is a photograph of the same.

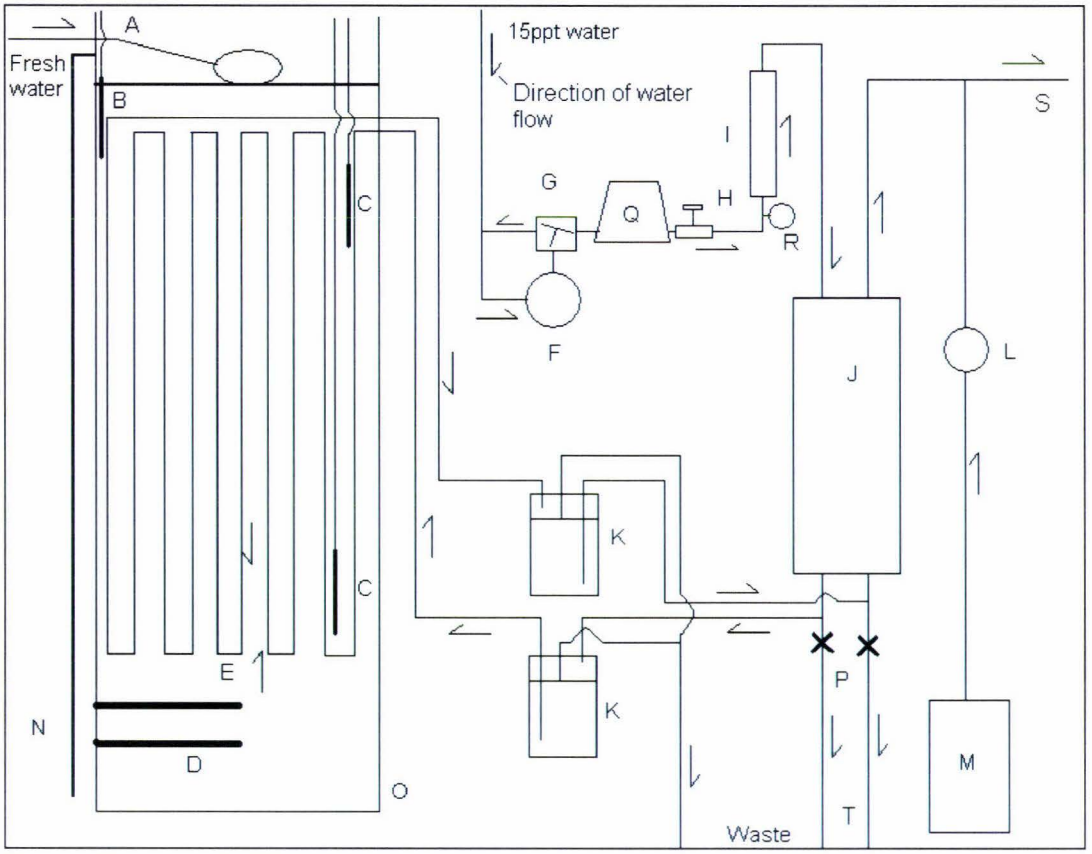


Figure 25 - A schematic drawing of the Tube Pasteuriser system.

Legend:
A – Ballcock. (¾" fitting) This ensures the water level in the heater is maintained.
B - Conductivity sensor (Eutech, EC-CONSEN46)
C - Temperature sensor (x2) (Intech Instruments, Pt100 RTD with teflon cable, RL-(TEFLON) S6.4-100-A2000)
D – 3 kW elements (x2)
E – 1.5m x 12x1.5mm glass tubes (128 in total)
F – Sea water pump – Wilden (Compressed air controlled)
Compressed Air control system (Including SMC –AFD20-02C-C; AW20K-02CH-C; AW2000)
G – Three way valve
H – Needle valve flow controller
I – Flow rotameter (0 – 2.0 L/min)
J – Heat Exchanger (31 plates (130x550mm plate) supplied by Seasalter Ltd, UK)
K - Degassing flask – Custom made unit designed by Simon Tannock, Nick King and John Penno, and built by John Penno of Technical Glass Products – Dunedin (Figures 27 and 28).
L – Nutrient pump (Milton Roy NSF Model P126-352TI)
M – Nutrient Carboy (Nalgene 20 L)
N – Overflow pipe in case of glass tube system or ballcock failure.
O - Hot Water Cylinder (Custom Built 710 L water heater. Built by Multi Machinery, Christchurch, NZ.)
P – Valve
Q – Wilden Dampener
R- Stainless steel pressure gauge (0 - 0.3 MPa)
S – Treated water (To algal culture bags in culture room)
T – Exit port for steam and waste water during start up and shut down.

Figure 26 - The whole unit assembled and installed.

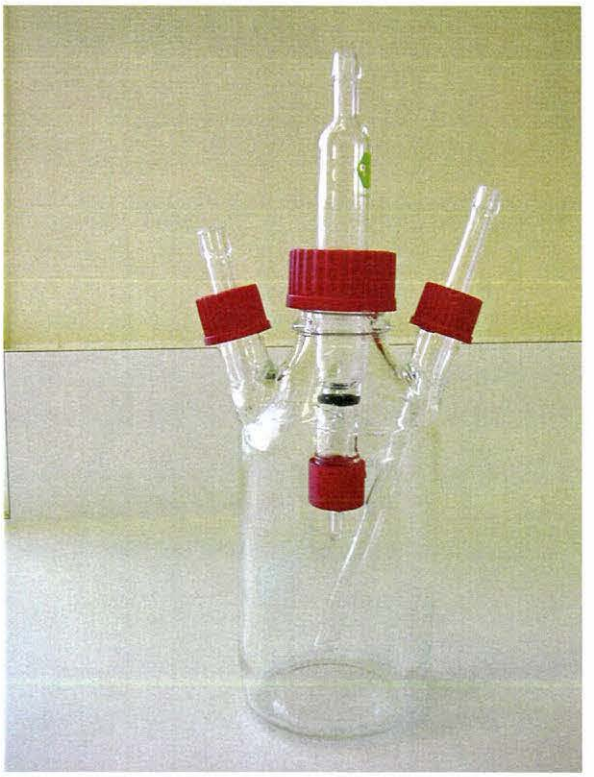


Figure 27 - The custom built degassing system.

Figure 28 - The disassembled fittings of the degassing system.

The entire system was initially run with freshwater not seawater in case of a system failure of the tube and silicon hose system in the HWC, as seawater in the copper cylinder would cause corrosion. The conductivity sensor was fitted to monitor the state of the fresh water in the heating cylinder. If there was a breech in the glass tube and silicon hosing system, the sea water would leak into the cylinder and the conductivity of the cylinder would increase and the monitoring system would set off an alarm.

The breakthrough and flushing times of dye in this system were measured in the same way as previously described in the prototype assessments in Section 3.3.2.5. Full instructions on the operation of the glass tube and silicon hose pasteuriser are outlined in Appendix 4.

3.3.2.7 The effect of heat treated water from the full scale glass tube and silicon hose pasteuriser system upon bacterial density

The heat exchanger and reticulation system had steam passed through it for a period of 15 minutes, from Point S in Figure 25 to the exit Point T of the heat exchanger. The temperature of the steam as it passed out of the heat exchanger and was discharged to waste was 97°C. The system was then closed off and was considered to be disinfected. The next step was to start pumping 15‰ salinity water through the system. Once the flow rate was established at 1.9 L/min and had been steady for 25 minutes a sample of the water was taken. The flow rate was then reduced to 1.7 L/min and left to stabilise again for 25 minutes before sampling. This procedure was repeated through to the flow rate 0.4 L/min. As well as the samples taken from the pasteuriser system, the following samples were taken: untreated seawater (15‰); untreated seawater (26‰); water treated with the Seasalter pasteuriser (26‰). All water samples were then assessed for the level of bacterial infection using the same method described in Section 3.2.

3.3.2.8 Cell concentration of batch cultured *C. calcitrans* grown in water treated in the full scale glass tube and silicon hose pasteuriser system

Two and a half litres of sea water (15‰) was added to 3.0 L conical flasks from the pasteuriser at a flow rate of 0.3 L/min. The flasks had previously had the CAW medium enrichments added to them and been autoclaved. As a control, 2.5 L of seawater (15‰) was autoclaved and was added to 3.0 L flasks as above. Once all flasks were filled with water they were inoculated with *C. calcitrans* from a culture in exponential phase.

Following the problems with the culture of the algae in this trial (Section 4.3.2.8) a change from the original plan for this experiment was adopted. One of the culture flasks (C1) from the pasteurised water treatment had 0.5 L of its algal culture aseptically

removed, and then 0.5 L of exponentially growing *C. calcitrans* culture from the autoclaved water treatment (control) was added. To a second flask (C2) from the pasteurised water treatment, after aseptically removing 0.5 L, exponentially growing culture of *Chaetoceros muelleri* was added. The source of the *C. muelleri* was a healthy bag growing continuously in the Seasalter pasteuriser system. The growth of these two flasks was monitored over the following days.

The results of this test (Section 4.3.2.8) above gave concern that something had occurred in the water making it unsuitable for algal culture. In a repetition of this trial, and in an attempt to reduce the unknown effect, water was treated at 75°C, instead of 95°C. Also, flasks were filled at a range of flow rates 0.35 -1.9 L/min in an attempt to establish a point where algal growth might occur. Water samples were taken as described in this and Section 3.3.2.7 and then inoculated with *C. calcitrans* growing exponentially in autoclaved medium. The water treatments were sampled for bacterial density before the algal inocula were added.

From the results of the trial above (Section 4.3.2.8), the same trial was repeated with the water being treated at 95°C and the range of flow rates was from 0.9 to 1.9 L/min. The density of bacteria in the water treatments was again measured.

3.3.2.9 Cell concentration of *C. calcitrans* in autoclaved water in the presence of the proprietary product "Selleys Knead It" putty

The glass tubes in the system had all had tubulations added to them in an attempt to reduce the chance of the silicon hose slipping off the ends of the tubes (Section 3.3.2.6). The putty used is a two part epoxy and contains sulphide and phenol products. Because of the large number of tubes and putty involved in this system, flasks were prepared that contained beads of the putty (5 mm diameter) and were autoclaved as normal along with the standard CAW nutrient addition. Treatments consisted of 0 beads (control), 1, 2 or 3 beads. Cell densities were counted on a daily basis using a haemocytometer.

3.3.2.10 Continuous culture of *C. calcitrans* in bags with water treated in the full scale glass tube and silicon hose pasteuriser

The most appropriate flow rate of the pasteuriser system had been assessed to be 1.3 L/min (Section 4.3.2.8). The required flow rate into bags was estimated to be 16 mL/min/bag. This relates to a total flow rate of 0.26 L/min for the 16 bags being set up. This left 1.04 L/min that would go to waste. It was necessary to design reticulation that ensured that the correct amount of water, at the correct head pressure, reached the bags, whilst the excess treated water was run to waste. Because the nutrients are added to the sea water after it has been treated in the pasteuriser the nutrient addition port was installed in the reticulation at a point after the waste water was removed (Figure 29).

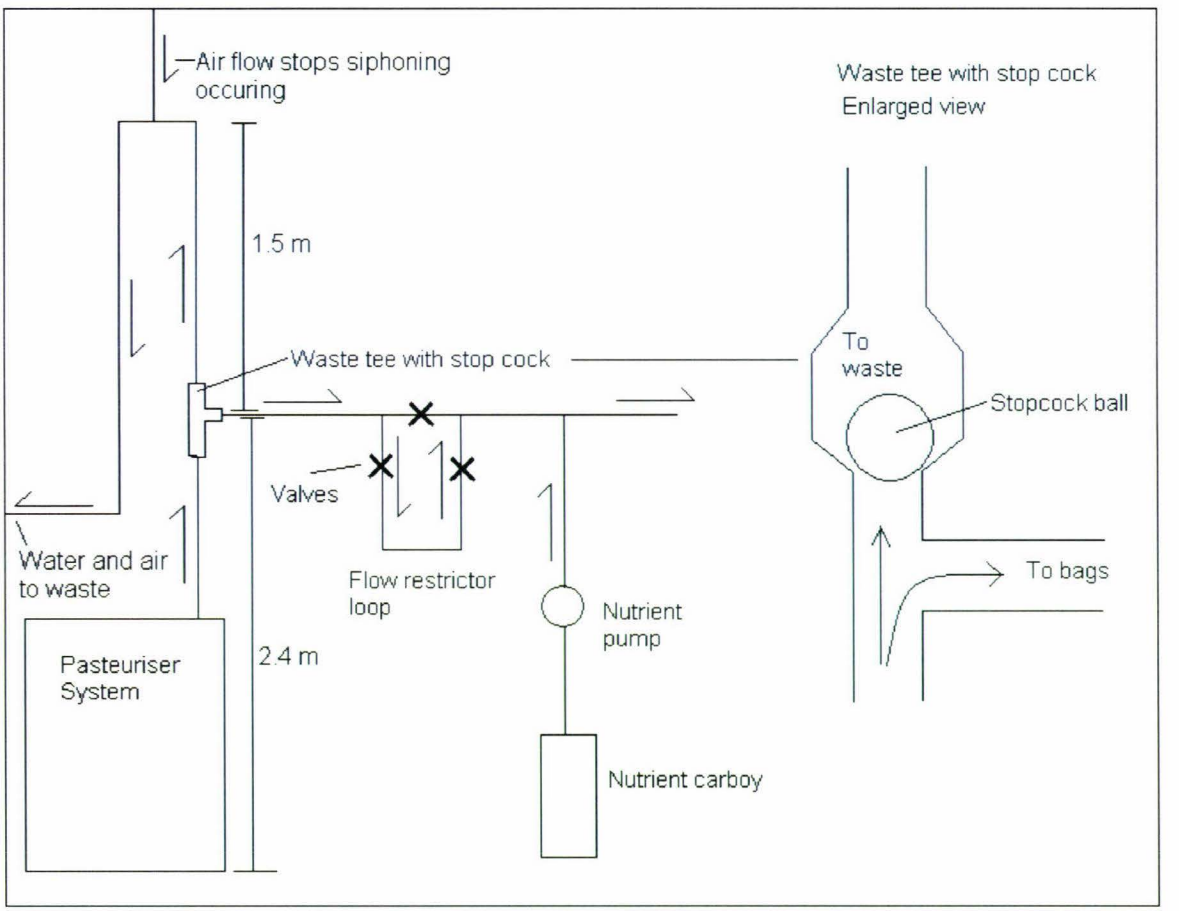


Figure 29 – Schematic drawing of the waste overflow and flow restriction systems.

The water flowing to the bags is initially the full 1.3 L/min to ensure fast and easy filling of all the silicon hosing that makes up the reticulation to all bags. Once bags are attached to the reticulation system and have begun filling, the valves at the flow restrictor loop are opened or closed so the water flows through the loop and the flow is reduced to the

desired 0.26 L/min. The flow restrictor loop consists of two straight (600 mm long) glass tubes of 6 mm diameter and 3 mm bore connected with silicon hosing.

The bags are set out with 8 bags per row. The main reticulation line is silicon hose with 10 mm i.d. and each branch to a bag consists of silicon hose with a 5 mm i.d.. The 'T' junctions are all glass pieces (Figure 30).

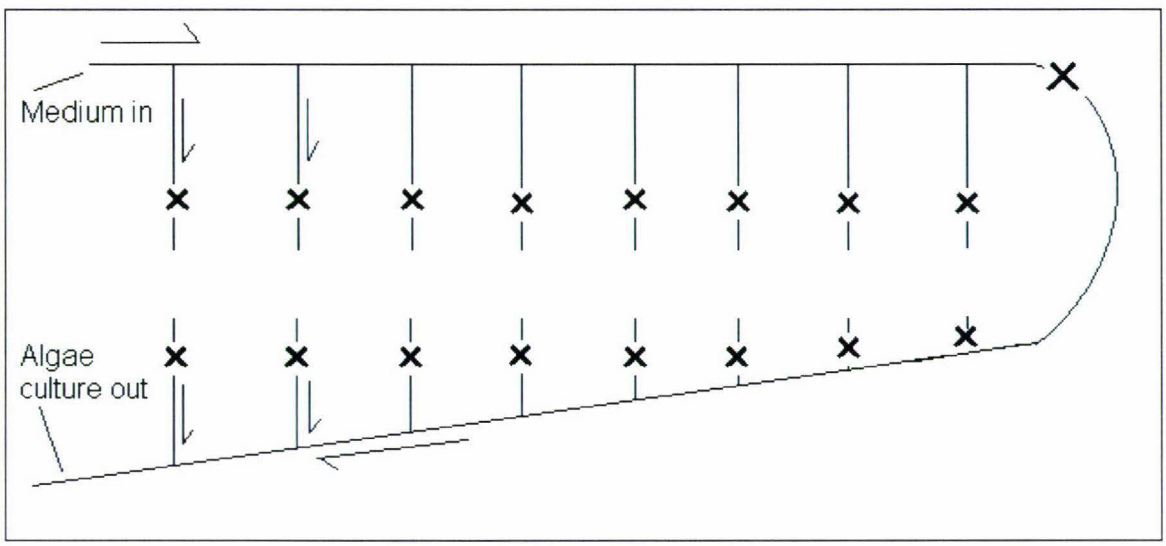


Figure 30 – Schematic drawing of the reticulation for a row of 8 bags in the continuous culture system.

Each bag has an inlet tip with a connector tube that has a 0.5 mm bore. This small bore tube assists in the even distribution of medium to the bags. The 'algae out' line is inserted into the bag approximately 75 mm below the inlet tip. The bag fills up with medium to the level of the outlet. Due to air sparging the medium dripping in is quickly mixed with the culture in the bag. The addition of medium gives cause for a similar volume of homogenous algal culture to drain from the bag through the outlet (Figure 31).

Sodium metasilicate was provided to the bags once per day by an injection of 20 mL/bag/day [120 g/L]. A 30 mL syringe was filled with 20 mL of the silicate solution. A hypodermic needle was fitted and the syringe was autoclaved. The silicate injection port was sprayed with 70% ethanol: 30% water solution to disinfect the port septa (Figure 31).

The bags are made of 230 mm layflat plastic and are sealed using a CD-300 bag sealer. The bottom of the bag is shaped to a point and air is injected into the bag at this point. The air is supplied through a 0.2 μm Midisart filter. The aeration rate is initially set at

0.9 L/min for *C. calcitrans* and additional CO_2 is added at a rate of 2% v:v. The flow rate of the air is controlled via a manifold. Each bag is connected to the manifold via a Festo brand needle valve. After the needle valve a series of tees and valves allow the air flow from one bag to be diverted through a rotameter so the air flow can be measured. The air then flows through the filter and through a silicon hose to the base of the bag (Figure 32).

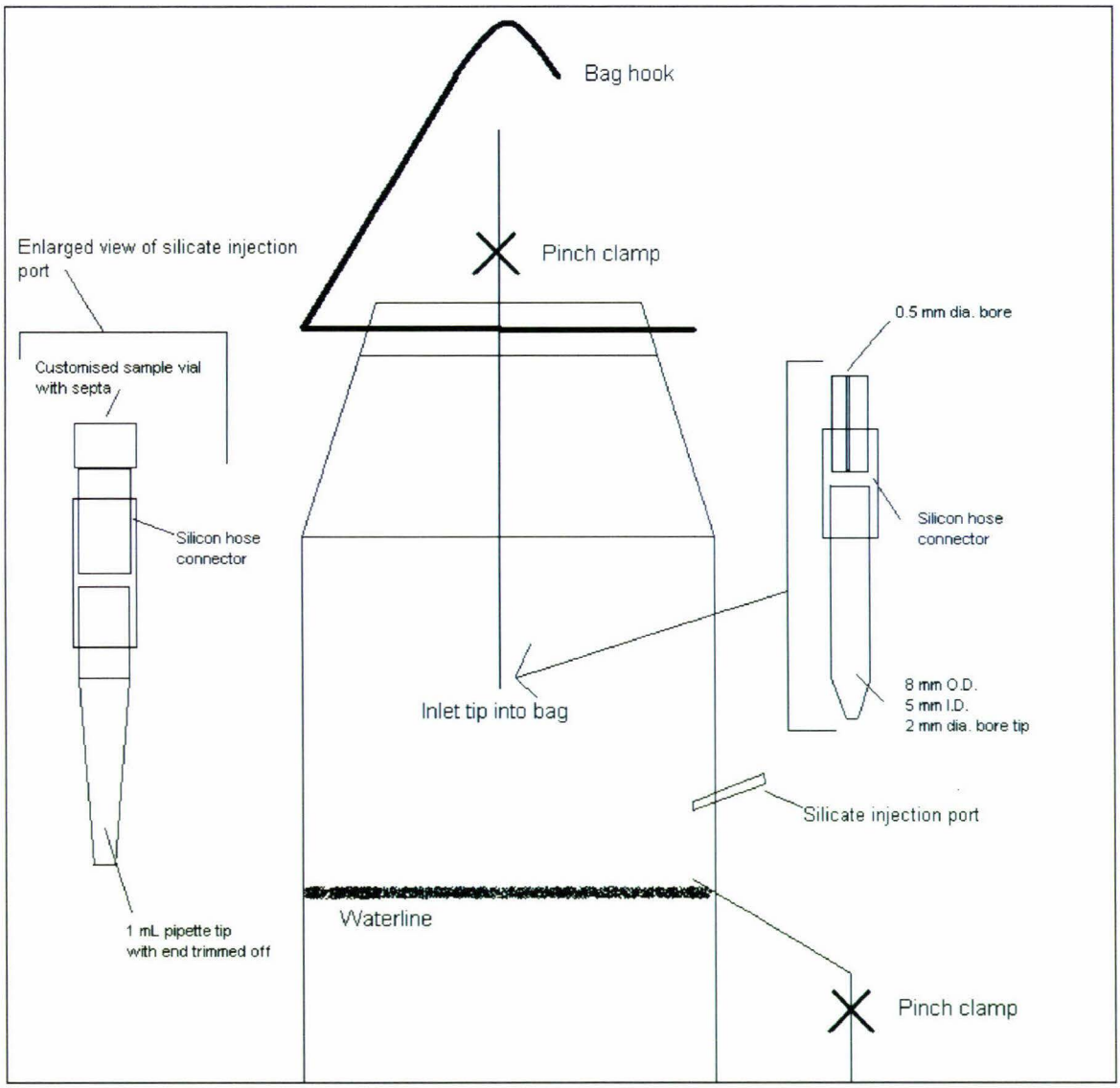


Figure 31 – Schematic drawing of the top of a plastic bag used for the continuous culture of algae. The total length of the bag is 2.2m.

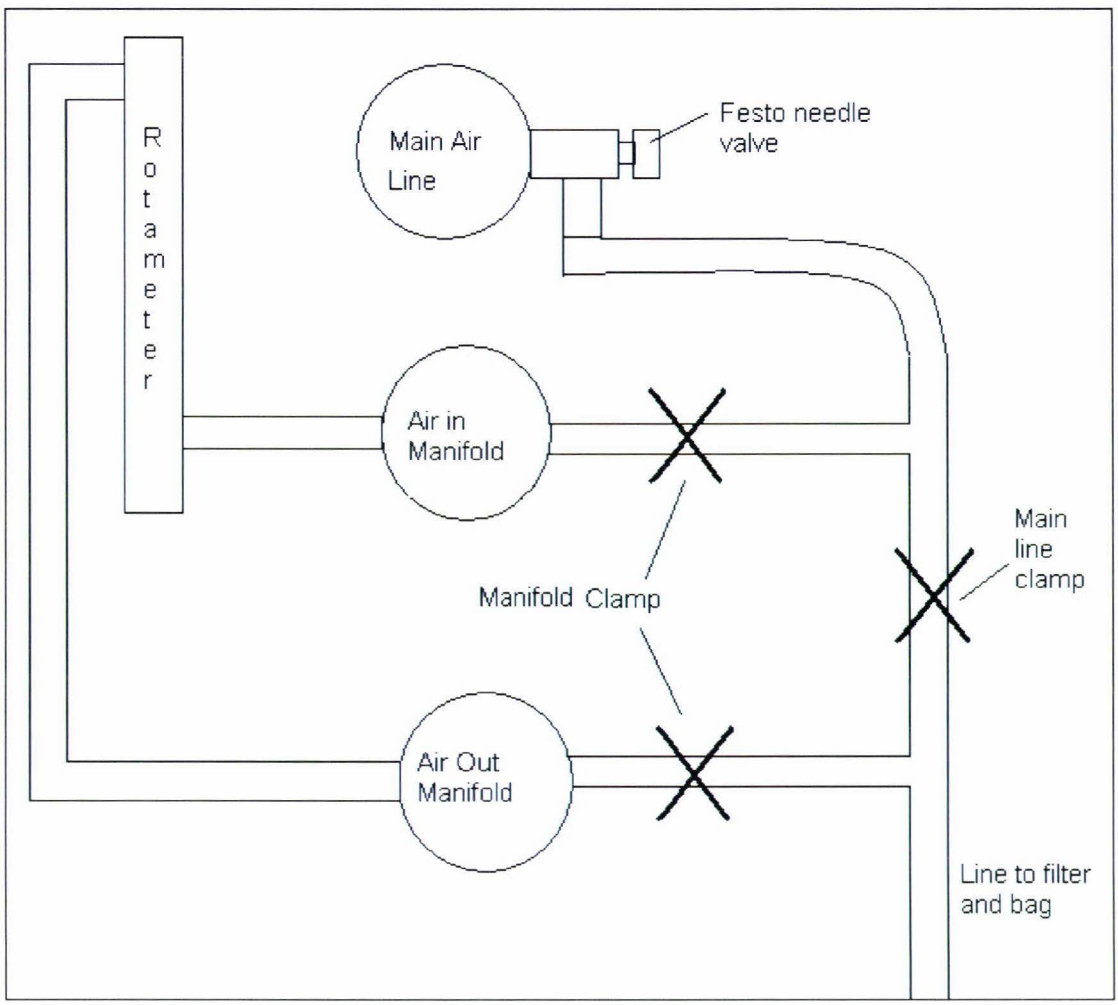


Figure 32 – Schematic drawing of the reticulation for supplying air to bags. The air can be diverted through the manifold and rotameter so air flow can be measured before it enters the bag.

Light is supplied to the bags from a bank of 12 cool white fluorescent tubes spaced vertically 240 mm apart. The centre of each bag is 260 mm away from the surface of the tube.

The cell concentration was monitored over 38 days. The pH of the bags was also monitored as the average value of algal culture from the eight bags in each row. The pH was monitored using the same equipment outlined in 3.1.

After 15 days of continuous culture the setting on the nutrient pump was altered to increase the rate of nutrient addition by 75% and the injections of sodium metasilicate was altered from 20 mL/bag/day [120 g/L] to 30 mL/bag/day. Cell concentration and pH were monitored as normal. On Day 23 the nutrient and silicate injection rates were returned to the original level for 3 days before the nutrient recipe supplied to the bags was altered. From Day 26 the media used was Glenhaven media, the pump rate remained the same. The sodium metasilicate rate was altered to 20 mL/bag/every 2nd day and the

concentration was changed to 30 g/L. These nutrient and silicate rates are the same as the *Chaetoceros muelleri* cultures in the Seasalter pasteuriser system at GACL receive. Cell concentration and pH were monitored as normal.

3.3.2.11 Quality of *C. calcitrans* grown continuously in bags with different rates of aeration

As mentioned in the review of literature, air sparging in bags has been a source of cell damage in the past. The flow rate of air into the bags in the Seasalter system at the GACL normally approximates 4 L/min. As this is already known to be damaging, an initial flow rate for air of 0.9 L/min was adopted for bags of *C. calcitrans* grown in the medium treated by the glass tube and silicon hose pasteuriser. The correct rate of additional CO_2 to control the pH was found through trial and measurement. As the new rate of aeration has done no damage to cells in culture, a comparison of this new rate and that of the rate previously used in the Seasalter system was performed to qualitatively assess any potential damage done to cells.

Bags were inoculated with 1.25 L of exponentially growing *C. calcitrans* in autoclaved medium. Four bags were set at 0.9 L/min air flow and four other bags were provided with air from the other system. This air initially entered the bag at 4 L/min and this rate reduced over time as the bag filled with culture medium and the head pressure of the bag increased. Any damage that occurred to the cells in culture was qualitatively assessed by the appearance of dead cells accumulating at the waterline when the bag was full.

3.3.2.12 The effect of water treated by the Seasalter pasteuriser system upon *C. calcitrans* grown continuously in the glass tube and silicon hose pasteuriser system

The purpose of this work has been to develop a system that will culture *C. calcitrans* continuously in bags. The water treatment system currently used at the GACL was supplied by Seasalter Shellfish Ltd. It was necessary to confirm that the new glass tube and silicon hose pasteuriser system developed in this work was the reason that *C. calcitrans* was growing continuously in a stable culture for more than four days, and not that the raw seawater being used was simply void of harmful bacteria or their spores.

To establish this point, four bags of *C. calcitrans* growing continuously in the new system were seeded with 1 L of water that had been treated by the Seasalter system. Initially, *C. calcitrans* was inoculated into new bags and then filled over the next 2 days as described in Section 3.3.2.10. Once the bags had reached the 'algae out' line, samples were taken to measure the density of bacteria and cell concentration. A 1 L aliquot of water treated in the Seasalter system was then added to each of four bags. Four other

bags (every second bag in the row) did not receive an aliquot and were the control for this trial. Cell densities were recorded each day in all bags sampled and bacterial density was again measured on Day 4 of the trial.

Chapter 4 Results and Discussion

4.1 Assessment of conditions for producing optimal cell concentration of *C. calcitrans*

4.1.1 Assessment of biomass production

4.1.1.1 The relationship between spectrophotometer absorbance readings and cell concentration

There was a good correlation between counted cell concentration and the absorbance readings of the spectrophotometer ($R^2 = 0.9177$; Figure 33). Because of the high level of correlation between these two values, most of the subsequent experiments were monitored by measuring the absorbance readings of algal culture samples and converting them to cell number equivalents. This conversion from absorbance value to cell concentration number entailed applying the following equation:
Cell number = Absorbance reading × 26,993.
The resulting sum was in cells/µL. There was no repeat of this experiment at a later stage to make certain that this correlation was still correct or that the conversion factor had changed.

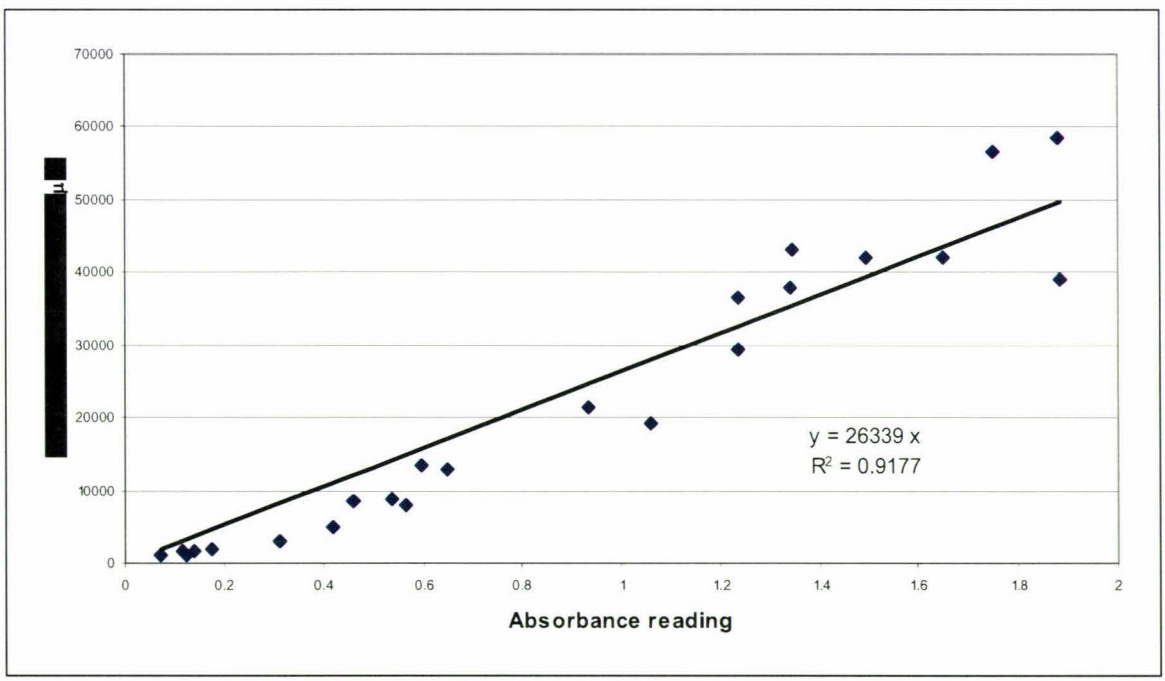


Figure 33 - Spectrophotometer absorbance readings versus counted cell concentration (cells/µL) in *C. calcitrans* cultures over the duration of the growth period of a trial.

4.1.1.2 The biomass produced by *C. calcitrans* in batch culture

The weight of biomass produced by *C. calcitrans* was measured. One litre of *C. calcitrans* culture, grown in 15‰ salinity seawater with Glenhaven medium, contained 1.1147 g/L of dry biomass after five days of culture (Table 7). This experiment was performed in the "pigeon–hole" system outlined in Section 3.1. At the completion of these experiments (Sections 4.1.3 - 4.1.6) and those involving Pluronic F-68 (Section 4.1.7) various parts of this system were dismantled and used for the continuous culture bag system in later experiments (Sections 4.3.2.10, 4.3.2.11 and 4.3.2.12). Following the dismantling of the pigeon–hole system it was realised that a comparison of the biomass produced in cultures grown with Glenhaven and CAW media had not been completed. As the pigeon-hole system had already been dismantled this trial was completed in the algae culture room of the GACL. The conditions for culture (light quality and intensity, temperature, aeration) are all very similar to the pigeon-hole system.

Although light intensity at the front of the culture flask was very similar (100 $\mu mol.m^{-2}.s^{-1}$) in both growing rooms, it is likely that the amount of reflected light from the walls of the compartments in the pigeon-hole system provided, overall, more indirect light to the culture flask. This difference must be taken into consideration in comparing the results.

The cell concentration (calculated using a haemocytometer - section 3.1.1.1) of the Glenhaven medium culture (at GACL) reached a similar level to the cultures grown in Glenhaven medium in the pigeon-holes, but the CAW medium produced a lower cell concentration (Table 7). Not only did it fall short of the cell concentration seen in the last of the optimum nutrient level experiments (84,832 cells/µL at 40 g/L (sodium dihydrogen orthophosphate) in the phosphate experiment (Section 4.1.5)), it was lower than the Glenhaven medium culture grown at GACL. The cell concentrations obtained in these experiments were not significantly different at the 5% level (5 replicates).

Sample source	Cell concentration (Cells/µL)	Cell biomass (g/L)	Mass per cell (pg/cell)
Glenhaven (Pigeon hole cultured)	38,586	1.1147	28.8
Glenhaven (GACL cultured)	39,250	0.7660	19.5
CAW (GACL cultured)	35,500	0.6380	17.9

Table 7 – The cell concentration (cells/µL), biomass (g/L) and mass per cell (pg/cell) of *C. calcitrans* grown in Glenhaven and CAW medium in two similar culture environments.

The reason for the poor result of the CAW medium culture is not clear. Light limitation is the most likely reason. The poorer level of indirect light, due to the absence of the surrounding white walls, as in the pigeon-holes, may have led to the observed low level of cells.

The values for dry weight per cell (pg/cell) presented in Table 7 also convert directly to a different presentation of the results as dry weight per one million cells (μg/10^6 cells). For example the first treatment "Glenhaven (Pigeon hole cultured)" has a value of 28.8 μg/10^6 cells. This is approximately twice as much as Laing (1979) reported for 2 L of *C. calcitrans* grown in a 3 L flask. When *C. calcitrans* was grown in 2.5 L volumes in 3L flasks the dry weight was 31.3 μg/10^6 cells. The level of illumination in Laing's work was 25,000 lux. This comparison is one of very few in the literature that can be used to evaluate the performance of these trials. The majority of experiments in the literature are evaluating physiological or biochemical responses to ecological or environmental conditions. In these instances the algae are cultured in media with very low concentrations of nutrients so as to obtain results about responses to minimum or limiting conditions. In other works that are evaluating aquaculturally interesting species, the other conditions are too disparate from those found in this work e.g. no CO_2 supplementation.

4.1.2 The effect of salinity upon cell concentration of *C. calcitrans*

Cultures at different salinities grew in similar patterns, in regard to lag and exponential phases. There were two time periods when there was a significant difference between some of the treatments in relation to cell concentration (Figure 34).

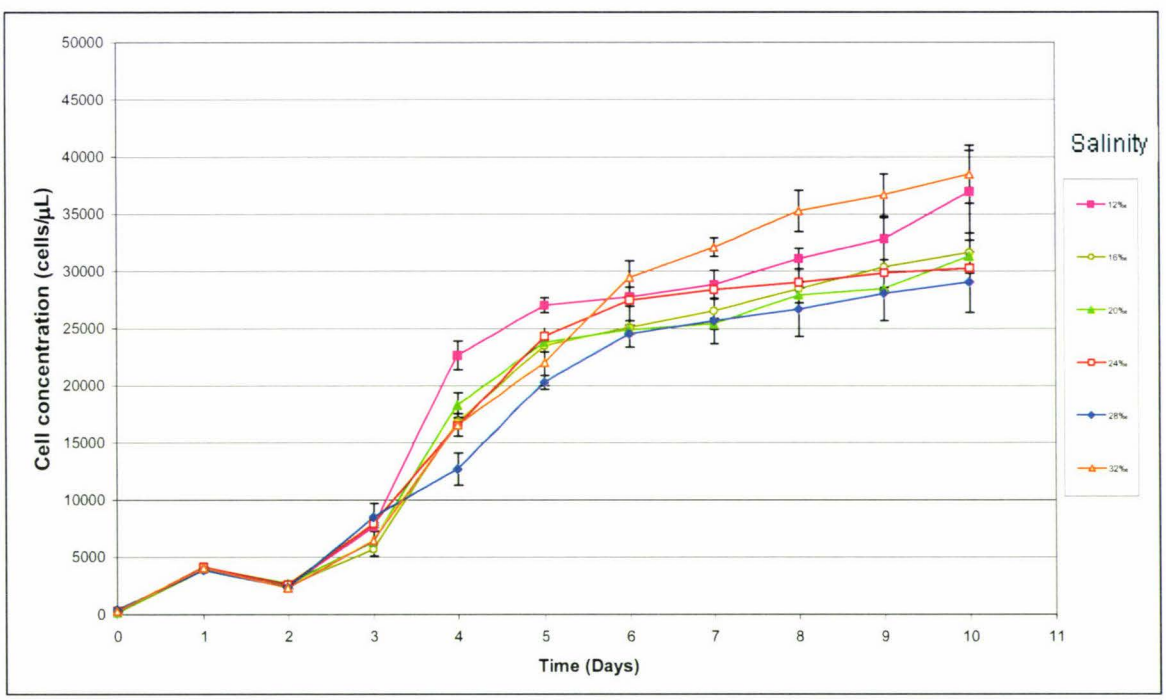


Figure 34 - The average cell concentration (cells/μL) of *C. calcitrans* grown in Glenhaven medium at different salinities (‰). Error bars represent the standard error (n=3).

At Day 4 and 5 of culture, the cell concentration of the 12‰ treatment had risen significantly more than the others. Also at Day 7 – 9 the 32‰ culture was significantly

higher in regard to cell concentration than the other salinity treatments. The variability among treatments was moderate and the data allow the conclusion that there was no significant difference of *C. calcitrans* growth performance at salinities between 12 and 32‰.

Despite there being only the two periods of difference in treatments, as shown by the results, there was a visible difference in the appearance of the cultures. The cultures grown at 12 and 16‰ were darker than the rest, but this difference was not due to cell number. The denser appearance of a culture may relate to chlorophyll *a* and other pigments. The absorbance wavelength for measuring cell concentration is 625 nm (Section 3.1), but this is not the wavelength of maximum absorbance of chlorophyll *a*. The peak absorbance wavelengths for chlorophyll *a* are 440 nm and 680 nm (Suzuki *et al.* 1998; Millie *et al.* 2002). Because of this difference, cell density cannot be assessed by a qualitative 'eyeball' assessment. The absorbance level of the treatments at the wavelengths of maximum chlorophyll *a* absorbance was not measured, but this could be investigated in any future trial where cell densities by spectrophotometer and by 'eye' do not agree.

Laing (1979) decided that 15‰ salinity was best for the culture of *C. calcitrans* and this experiment agrees with his results. All further work within this thesis was carried out with water at 15‰ salinity unless otherwise indicated.

4.1.3 The effect upon cell concentration of *C. calcitrans* at different concentrations of sodium nitrate in the Glenhaven medium

The concentration of sodium nitrate in the culture medium was varied to establish the optimal level for exponential growth. Within the timeframe of exponential growth (50-100 hours) the cultures with 130 and 160 mg/L of sodium nitrate were the best performing treatments (no significant difference; Figure 35). The 200mg/L treatment had a higher standard error and the average was lower than the other two aforementioned treatments until about 80 hours, when the average cell number was higher in the 200 mg/L treatment.

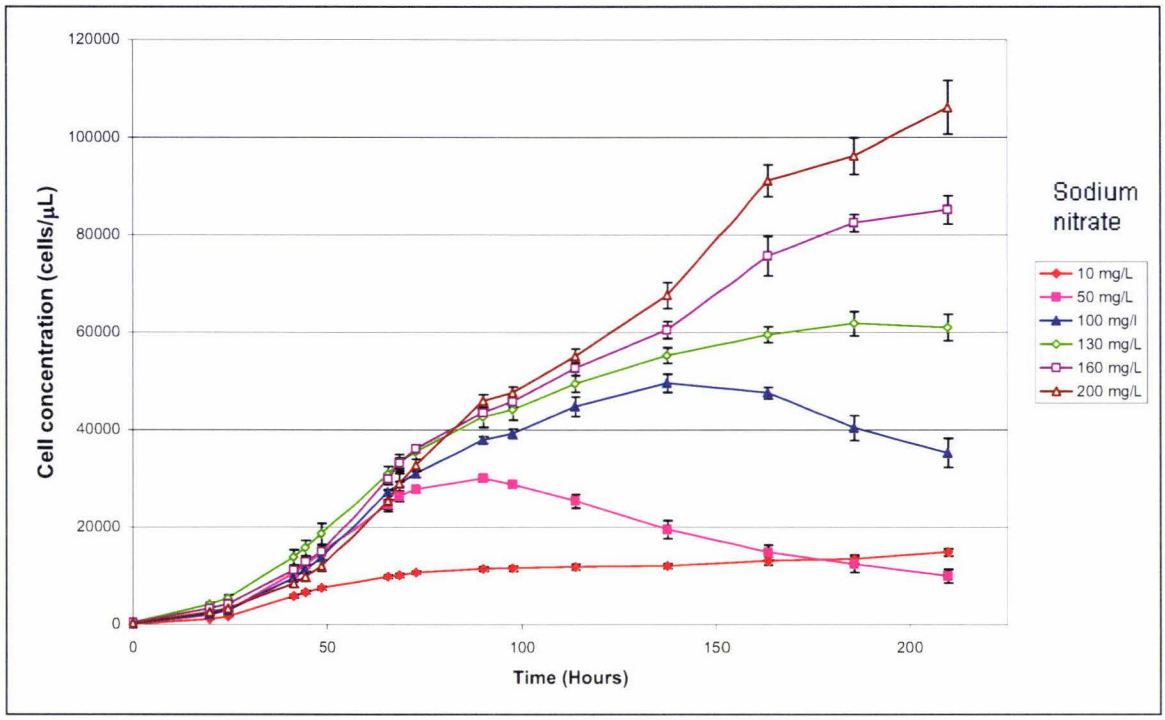


Figure 35 - The average cell concentration (cells/µL) of *C. calcitrans* grown in Glenhaven medium at different sodium nitrate concentrations (mg/L). Error bars represent the standard error (n=4).

As could be expected, the lower concentrations of sodium nitrate did not perform well in respect to cell concentration. What is interesting to note is the performance of the 100 mg/L treatment, as this is the concentration of sodium nitrate that has been used to culture *C. calcitrans* at the GACL. As mentioned at the start of Section 3.1 the nutrient recipe in use at GACL is not Conway medium and is referred to as Glenhaven medium.

The Conway medium has a sodium nitrate concentration of 200 mg/L (Table 11). The higher concentrations of sodium nitrate produced higher cell concentrations, and in the final stages of the experiment it was clear that cell concentration was directly related to the concentration of sodium nitrate in every instance except the 10 mg/L treatment. From the results of this trial and in close agreement with Laing (1979) it was concluded that optimum concentration of sodium nitrate for *C. calcitrans* culture is 160 - 200 mg/L. Although the final cell concentration was clearly highest in the 200 mg/L treatment, the main focus of this work relates to the growth of *C. calcitrans* in the exponential phase, where the culture will be maintained in a continuous culture system. For this reason, 160 mg/L sodium nitrate was selected for further experiments as it is a compromise between the best performing treatment in the exponential phase (130 mg/L) and the most productive treatment overall (200 mg/L). This is a 60% increase over the nitrate level recommended by Walne (1966) for many algae, but less than the 200 mg/L recommended by Laing (1979) for general cultures. However, Laing has also published a recipe specific to *C. calcitrans* and recommends 150 mg/L (1991).

4.1.4 The effect upon cell concentration of *C. calcitrans* by altering the Si:N molar ratio in the Glenhaven medium

The concentration of sodium nitrate in the culture medium was altered following experiments in Section 4.1.3. With the new concentration of sodium nitrate set at 160 mg/L, it is logical that alterations will also need to be made to the concentration of sodium metasilicate. The molar ratio of Si:N is an important aspect of diatom culture because cells grow poorly without silicate (Kudo, 2003), and too much can alter the culture environment (e.g. pH) deleteriously (Laing, 1985).

The ratios of Si:N in the growth medium were set empirically following the current ratios used at the GACL and those recommended by Laing (1979) as a guide. The Si:N ratios at GACL are 0.36 (3.0 L flask) and 0.24, (20 L carboy) and in Conway medium it is 0.16 (Laing, 1979). The difference in Si:N ratio between the 3.0 and 20 L vessels used at GACL was because the 20 L vessel did not produce the same cell density as smaller flasks due to light limitation caused by the lower surface area:volume ratio, and the translucent nature of the walls of the carboy itself. Therefore, maintaining the concentration of nutrient supplementation as a 3.0 L flask would be inefficient. The volume of each supplement in carboys was lowered by an approximated estimate rather than calculating the correct value and this is the reason for the discrepancy.

After 44 hours of culturing the cell concentrations of the 0.25 and 0.35 molar ratio cultures were significantly different from the 0.6 and 1.0 molar ratios. After 50 hours of culturing the 0.25 and 0.35 molar ratio treatments were significantly different from all other treatments in terms of cell concentration (Figure 36).

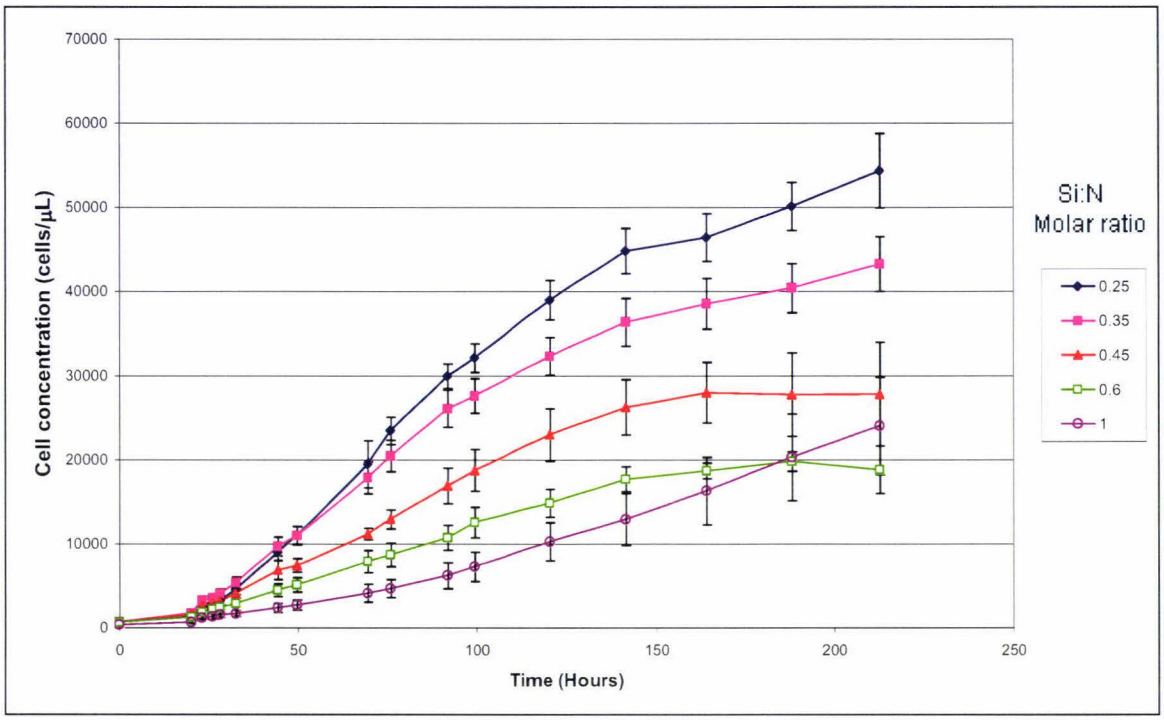


Figure 36 - Average cell concentration (cells/µL) of *C. calcitrans* grown in Glenhaven medium at different molar ratios of silicon to nitrogen (Si:N) ($NaNO_3$ = 160 mg/L). Error bars represent the standard error (n=5).

The specific growth rate (µ) was calculated from the results and this demonstrated which treatments were optimal (Table 8). The results show that in the Glenhaven medium the molar ratio of Si:N that was best for growth was 0.25, and for that reason this molar ratio was utilised for all media in subsequent experiments.

Treatment (Si:N molar ratio)	Specific Growth Rate (µ hr^{-1})	R^2 value
0.25	0.059	0.992
0.35	0.047	0.986
0.45	0.040	0.992
0.60	0.029	0.986
1.00	0.034	0.906

Table 8 - Specific growth rate for *C. calcitrans* grown in Glenhaven medium at different molar ratios of silicon to nitrogen (Si:N) ($NaNO_3$ = 160 mg/L).

The Conway medium (Laing, 1979) has a Si:N of 0.16 and the Glenhaven medium has a ratio of 0.36 (Table 9). The Conway medium Si:N ratio is lower than that tested in this trial and Laing (1979) does not elucidate upon how this ratio was selected. Laing (1979) reports a "typical growth curve" for *C. calcitrans* in autoclaved sea water medium. From this graph a maximum specific growth rate of 0.0164 hr^{-1} was calculated. In Laing's trial

(1985) of different rates of silicate (350, 950 and 1400 $\mu mol.L^{-1}$) he maintained the same concentration of sodium nitrate for each treatment.

This meant that not only did the concentration of available Si increase, but the Si:N altered between treatments. The Si:N ratio for the 1400 $\mu mol.L^{-1}$ silicate treatment was 0.59. This was similar to the second highest ratio in this trial (i.e. 0.60). Laing found that the *C. calcitrans* cells in this treatment (1400 $\mu mol.L^{-1}$ silicate) had the smallest cells by volume (~ 40 μm^3) and the largest cells were from the 350 $\mu mol.L^{-1}$ treatment (~75 μm^3 at the beginning and end of the trial). There was a direct correlation between silica limitation and increase in cell volume (Laing, 1985).

	Si:N	$\mu mol.L^{-1}$ Si	mg/L Si	$Na_2SiO_3.5H_2O$ mg/L
Laing	0.15	350.0	9.8	74.2
(1985)	0.4	950.0	26.7	201.5
	0.59	1400.0	39.3	296.9
Trial	0.25	471.0	13.2	99.9
(Section 4.14)	0.35	659.1	18.5	139.8
	0.45	848.7	23.8	180.0
	0.6	1131.5	31.8	240.0
	1.0	1884.0	52.9	399.6
Glenhaven medium	0.36	424.6	11.9	90.0
Conway (Laing, 1979)	0.16	377.0	10.6	80.0

Table 9 – Comparison of the Si:N ratios, $\mu mol.L^{-1}$, and the actual weight of silicon and sodium metasilicate from the work of Laing (1979, 1985), the Glenhaven medium and the recipes used in this trial (Section 4.1.4).

In this trial, cell volume was not a parameter that was measured but further investigation would be worthwhile as cell size is an issue with regard to this microalga as it is grown so as to be to be fed to small shellfish larvae.

4.1.5 The effect upon cell concentration of sodium dihydrogen orthophosphate concentration in the Glenhaven medium

The concentrations of sodium nitrate (160 mg/L) and sodium metasilicate (99.9 mg/L) were chosen based on the results of the experiments described in Sections 4.1.3 and 4.1.4. Even though the ratio of Si:N was almost the same as it was previously, the concentration of sodium metasilicate was also increased to maintain the same ratio, because the concentration of $NaNO_3$ had been increased.

One of the other important elements in a medium for cell growth was phosphorus (Walne, 1966; Facialtore and Bowler, 2002), and in Glenhaven medium this was supplied to the algae in the form of sodium dihydrogen orthophosphate. With the concentrations of nitrogen and silicate having been increased there is the potential for other elements becoming a limiting factor in biomass production. As phosphorus is also an important

element in cell growth generally, the optimum concentration of sodium dihydrogen orthophosphate was investigated under these new conditions.

The results of average cell concentration showed that the maximum concentration (40 mg/L) of sodium dihydrogen orthophosphate trialled in this experiment produced a higher cell concentration over the latter part of the trial. The appearance of the improved performance in this treatment began after 50 hours of culturing (Figure 37).

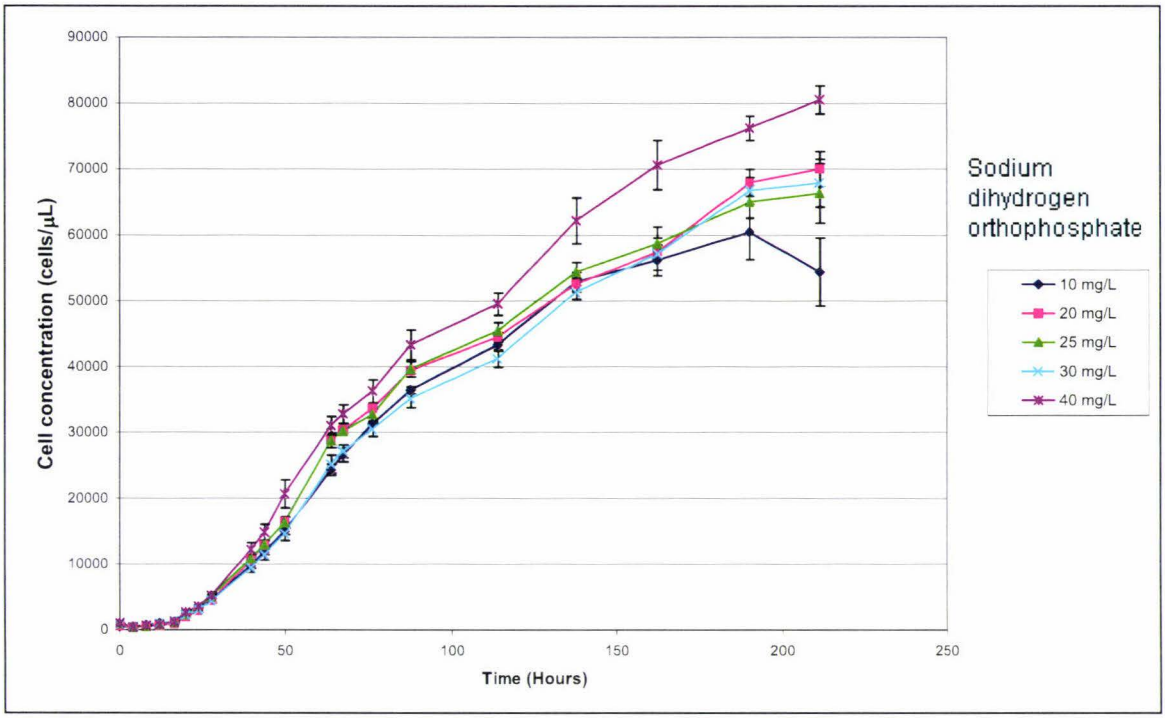


Figure 37 - The average cell concentration (cells/µL) of *C. calcitrans* at different concentrations of sodium dihydrogen orthophosphate (mg/L). Error bars represent the standard error (n=5).

In regard to the other phosphate concentrations the results indicate that there is little difference between cell concentrations. The maximum specific growth rate (µ) was highest for the 20 mg/L treatment and the 30 and 40 mg/L treatments were very similarly the lowest rates (Table 10). Despite this, the two values were still 42% higher than the highest maximum specific growth rate found in Section 4.1.4 with the 0.25 Si:N treatment ($\mu = 0.59\ hr^{-1}$).

NaH_2PO_4 (mg/L)	Specific Growth Rate ($\mu\ hr^{-1}$)	R^2 value
10	0.091	1
20	0.104	1
25	0.097	0.999
30	0.084	0.998
40	0.086	0.998

Table 10 - Exponential growth phase - average cell doublings per day of *C. calcitrans* at different concentrations of sodium dihydrogen orthophosphate (mg/L).

Despite the 40 mg/L treatment not having the highest μ in this experiment it was still higher than the μ in the last Section (4.1.4), and it did produce a higher cell concentration, most notably after 50 hours of culture. For these reasons the 40 mg/L treatment was selected as the optimal rate of NaH_2PO_4 to continue using from this point for culturing *C. calcitrans*.

4.1.6 The comparative cell concentrations of *C. calcitrans* in two different nutrient recipes

The Glenhaven medium was enhanced to increase the cell concentration of *C. calcitrans* cultures. Table 11 outlines the differences in the media recipes of Walne (1966), Conway (Laing, 1979), Glenhaven, and the enhanced recipe developed in this work and referred to as CAW.

	Walne (1966)	Conway (1979)	Glenhaven	CAW Recipe
Solution A	**mg/L**			
$FeCl_3.6H_2O$	1.3	2.6	1.3	1.3
$MnCl_2.4H_2O$	0.36	0.72	0.36	0.36
H_3BO_3	33.6	67.2	33.6	33.6
$NaH_2PO_4.2H_2O$	20	40	20	40
EDTA (Na Salt)	45	90	45	45
$NaNO_3$	100	200	100	160
Trace elements* (L)	0.001	0.0015	0.001	0.001
Solution B	**mg/L**			
$ZnCl_2$	0.00021	0.000042	0.021	0.021
$CoCl_2.6H_2O$	0.0002	0.00004	0.02	0.02
$(NH_4)6Mo_7O_{24}.4H_20$	0.00009	0.000018	0.009	0.009
$CuSO_4.5H_2O$	0.0002	0.00004	0.02	0.02
Vitamins	**mg/L**			
B12	0.005	0.0004	0.005	0.005
B1 (Thiamine)	0.1	0.008	0.1	0.1
Sodium metasilicate	**mg/L**			
$Na_2SiO_3.5H_2O$	No addition	80	60	99.9

Table 11 - The concentrations of various compounds in seawater medium seeded with the nutrient preparations of Walne (1966), Conway (1979), Glenhaven and CAW. * The recipe for trace elements is given as Solution B in Appendix 1.

Before algal cultures were inoculated into 0.8 L or 3.0 L flasks they were grown in 0.2 L volumes of medium in a 0.5 L conical flask. The growth of *C. calcitrans* was monitored on a daily basis with the Glenhaven and CAW media. All flasks (2 treatments, 5 replicates) were inoculated with *C. calcitrans* from a stationary phase flask (as is normal practice for stock cultures).

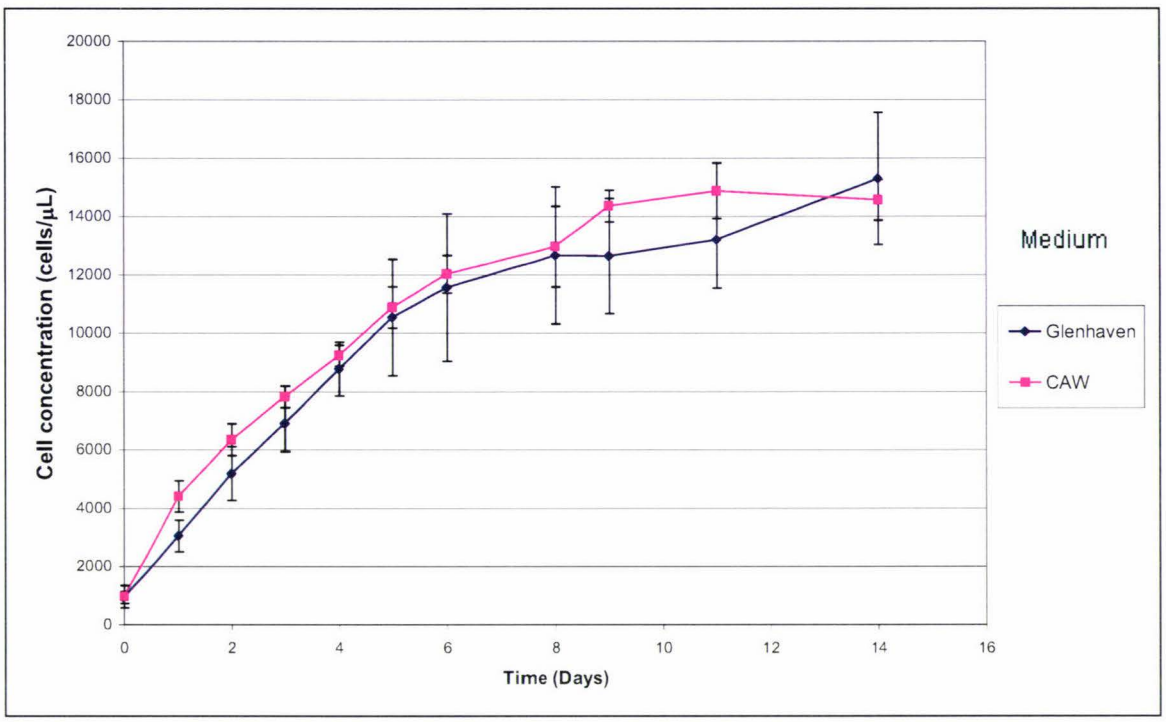


Figure 38 - The average cell concentration (cells/µL) of *C. calcitrans* grown in Glenhaven and CAW media in 200mL batches in 500mL conical flasks with no aeration. Error bars represent the standard error (n=5).

The inoculum volume was calculated to give an initial cell concentration of 750 cells/µL. The cell concentration of the flasks (Figure 38) indicated there was no difference between the two treatments across the course of this experiment. This result shows that both media are suitable for the maintenance of *C. calcitrans*. This is in keeping with the recommendations of Laing (1979) for starter cultures of *C. calcitrans*.

4.1.7 The effect of Pluronic F-68 on *C. calcitrans* cell concentration

The initial growth of the *C. calcitrans* cultures through the exponential phase was similar at a range of Pluronic F-68 concentrations. Clear differences only occurred after four days of culturing (Figure 39).

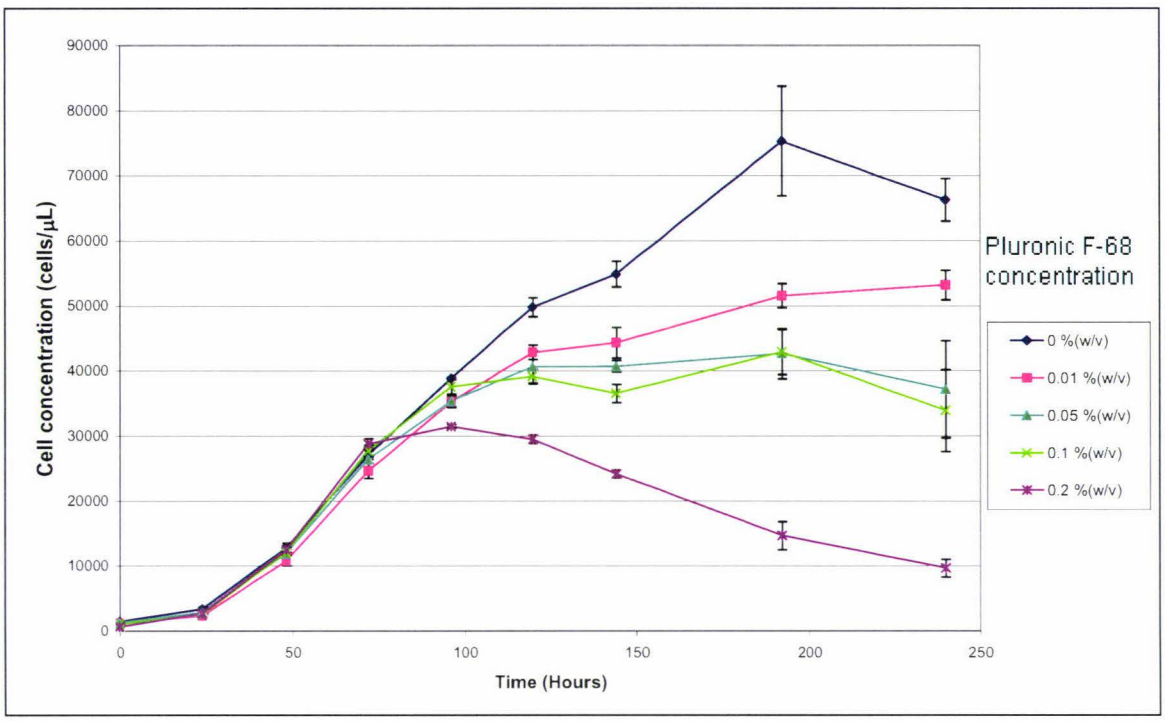


Figure 39 - The effect of various concentrations of the cell protectant Pluronic F-68 upon cell concentration of *C. calcitrans*. Error bars represent the standard error (n=5).

Observations of the cultures would suggest that the problem that arose was the occurrence of foaming in the flasks due to aeration. The excess foaming was, by qualitative assessment, directly related to the concentration of Pluronic F-68 in the medium. Despite the claim of several researchers (Murhammer and Goochee, 1990; Kioukia *et al.* 1996; Wu *et al.* 1997) that Pluronic F-68 reduces both, the contact of cells to gas bubbles, and cell entrainment into any foams formed by aeration, there was a visible biomass entrained in the foam above the medium. This biomass was assumed to be *C. calcitrans* cells. The poor state of all but the control culture after 72 hours made it evident that some form of antifoaming agent would be necessary for the quality of the culture to be maintained.

4.1.7.1 The effect of using Antifoam A in combination with Pluronic F-68 as additives to the culture of *C. calcitrans*

Due to the issue of cell entrainment in the foam of the Pluronic F-68 treated cultures (Section 4.1.7), the addition of Antifoam A to the medium was tested to investigate if this improved the cell concentrations of the cultures treated with Pluronic F-68.

The cell concentrations of the four treatments were similar for the first 136 hours (5½ days). This was approximately 64 hours longer than the previous experiment without Antifoam A. All cultures performed similarly over the lag, exponential and the beginning of the stationary phases (Figure 40).

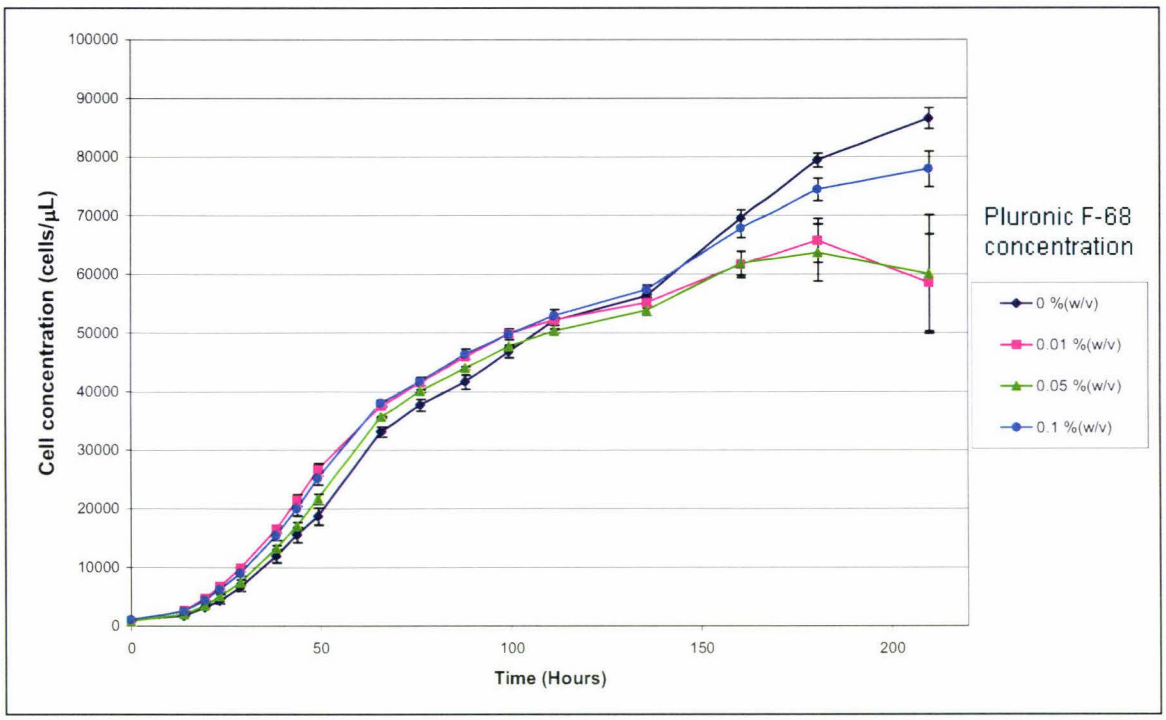


Figure 40 - The cell concentration (cells/µL) of *C. calcitrans* grown in CAW medium at different concentrations of the cell protective agent Pluronic F-68 (% w/v) and Antifoam A (100ppm). Error bars represent the standard error (n=5).

An interesting result from this experiment was the overall difference between all treatments and the control. From the first measurement at 14 hours, until the measurement at 50 hours, it was possible to visually identify the control replicates from the Pluronic F-68 + Antifoam A treatments, because the culture colour and apparent pigment density of the controls was so markedly different (lighter) than the Pluronic F-68 treatments. This difference, however, was not apparent between treatments in the comparison of spectral absorbance at 625 nm. The reason for the apparent visual difference between the control and treatments that contained additives is unknown, but

may be explained by relating the result to the absorbance peaks of chlorophyll *a* (Section 4.1.2 has further explanation).

The difference, visually, between these treatments may be worth investigating further. If there was a difference in cell pigment (e.g. chlorophyll *a*) production or stability, this may have an effect upon the lipids or metabolites in production during culture. If this were the case there are implications for algal culture, not only in aquaculture but also in the culture of microalgae for the production of eicosapentaenoic acid (EPA), lipids for bio-diesel and amino acids for cosmetics (Lebeau and Robert, 2003a).

4.2 Identification of agars best suited to culture the bacteria present in non-axenic cultures of the microalga *C. calcitrans*, as well as in treated and untreated sea water

Bacteria were isolated from pasteurised water (21 strains), from healthy *C. calcitrans* cultures (16 strains) and from crashed *C. calcitrans* grown in the Seasalter pasteurised water (32 strains; Table 12). These isolates were qualitatively assessed for their ability to grow on 9 types of agar media. The greatest numbers of isolates were culturable on Marine agar (MA) and Tryptone soy agar (TSSW) made with sea water. Only seven isolates would grow on Tryptone soy agar (TSA) or TSSW and not on MA. Eleven isolates grew on MA only and 19 isolates grew on MA and TSA or TSSW.

Source	Isolates on Agar Type									
	MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA	RAA	Total
Pasteurised water	8	-	-	8	-	6	-	-	-	22 (10)
C. calcitrans culture	-	-	8	9	1	1	-	-	-	19 (9)
Pasteurised water with crashed culture	19	-	-	3	-	1	-	-	-	23 (18)
Total	27	0	8	20	1	7	0	0	0	

Table 12 – Culturable bacterial isolates from different sources on a range of agar plate media. MA = Marine agar; MACc = Marine agar with lysed *C. calcitrans* cells; TSA = Tryptone soy agar; TSSW = Tryptone soy agar prepared in sea water (15‰ salinity); TSACc = Tryptone soy agar with lysed *C. calcitrans* cells; SBA = Sheep blood agar; TCBS = Thiosulphate citrate bile sucrose agar; VCA = Vibrio chromogenic agar; RAA = Ryan's aeromonas agar. Some bacteria have been tabulated as growing on more than one media type. Totals in parentheses are actual number of individual isolates.

Because the majority of isolates could be grown on MA this medium was selected as the agar to be used for the duration of this project when assessing bacterial densities in treated water or *C. calcitrans* cultures. When an algal culture had crashed but no bacteria had been detected a sample was reassessed by plating it upon TSA and TSSW.

The two agars prepared by the author (MaCc and TSACc) did not provide a good substrate for the bacteria present in the samples to grow upon. The MACc medium was prepared by mixing the Marine agar powder and with lysed *C. calcitrans* culture. The final preparation contained salt (NaCl) from the *C. calcitrans* culture and from the agar powder. This may have increased the salinity to greater than 36‰, which may have inhibited the growth of the bacteria. The issue of excessively high salinity is not relevant for TSACc however and the growth results for this preparation were also poor. There may have been some other indeterminate factor that resulted in the poor growth in both these preparations.

The isolates were described relative to density in initial culture and a range of biochemical (e.g. catalase test) and physical (e.g. shape) tests (Appendix 3). These characteristics are not however relevant to the thesis and are not presented here.

4.2.1 The effect of mono specific bacterial infections upon the growth of *C. calcitrans* in batch culture

All except one (Strain 49a) of the 22 bacterial isolates assessed in this trial caused detrimental effects to the *C. calcitrans* culture quality. The control grew normally and showed no signs of stress. Table 13 lists the observations made of the quality of the cultures, as well as the starting and finishing densities of the bacteria in the cultures.

Bacterial Strain/ Water Treatment	Start Density (cfu/mL)	Finish Density (cfu/mL)	Observations at end of culture period
31	62,500	12,900,000	Medium scum level and cell flocculation (drop out)
32	52,500	27,000,000	Medium scum, large cell flocculation
37	61,875	420,000	Mild scum, medium cell flocculation
36	62,500	26,200,000	Very large scum, medium cell flocculation
30	27,375	690,000,000	Medium/large cell flocculation
63a	7,000	2,200,000	Medium cell flocculation
64a	9,625	1,500,000	Some biofilm, large cell flocculation
53	15	500,000	Medium/large scum, medium cell flocculation
54	42	1,200,000	Large scum, medium cell flocculation
55	235	5,000,000	Very large cell flocculation
56	106	800,000	Medium scum and cell flocculation
57a	37,625	500,000	Large scum and cell flocculation
58a	21,750	2,400,000	Medium/large scum, some cell flocculation
59a	4,875	2,900,000	Some scum and cell flocculation
67a	1,462	46,000,000	Very slight cell flocculation
46a	27,250	75,000,000	Medium cell flocculation
72a	16,750	4,700,000	Large scum, medium/large cell flocculation
73a	17,375	240,000,000	Large scum and cell flocculation
49a	6,125	25,600,000	Minor cell flocculation, general appearance almost normal.
Pasteurised	6	100,000,000	Large scum and froth. Large cell flocculation
Untreated	62,500	5,800,000	Some scum, medium cell flocculation Remaining culture very light gold colour
Control	0	0	No adverse effects Normal appearance

Table 13 – Effect of water treatment and bacterial isolates from a crashed *C. calcitrans* culture on the quality of *C. calcitrans* cultures.

Figure 41 illustrates the differences in bacterial density of the bacterial isolates/water treatment methods at the start and finish of the culture period. The difference is shown by the natural log of the final density minus the start density. The control had no bacteria present and therefore no value is given in the graph. The pasteurised water treatment gave the 4th highest bacterial growth. There was no recognisable pattern between the *C. calcitrans* observations (Table 13) and the final concentration of bacteria.

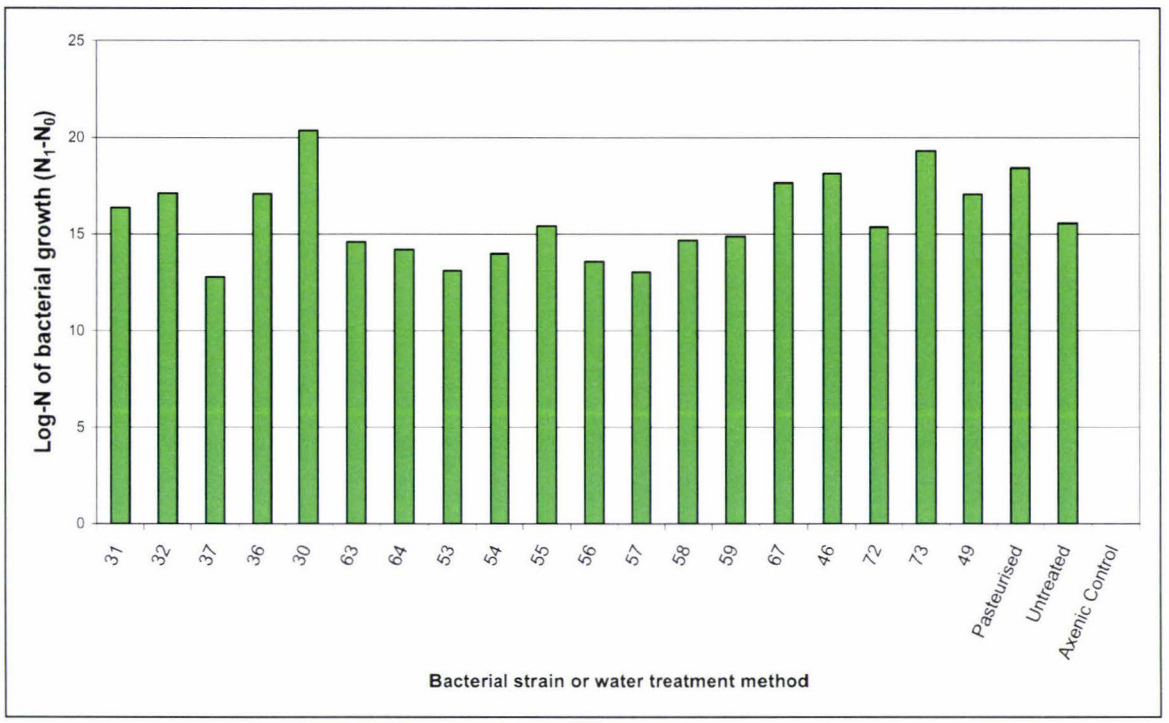


Figure 41 – Bacterial growth in *C. calcitrans* cultures inoculated with individual bacterial strains or in water that had been pasteurised (Seasalter system) or untreated.

The relationship between the presence of bacteria and the generally poor health of the *C. calcitrans* cultures confirmed the hypothesis that the bacteria from pasteurised seawater had the potential to be detrimental to *C. calcitrans* cultures. This result was similar to those of Baker and Herson (1978) and Hirayama & Hirayama (1993, 1996) who found that bacteria inoculated into cultures of microalgae (e.g. *Thallasiosira pseudonana* (3H), *Chaetoceros gracilis*) could be detrimental to the health of the culture. On the other hand, there are many accounts of isolated bacteria either promoting growth of algae cultures or improving culture stability in stationary phase of culture (Fukami *et al.* 1992; Fukami *et al.* 1997; Hirayama & Hirayama, 1997). The results of this study suggest that the uncontrolled presence of bacteria in *C. calcitrans* cultures is detrimental to the algal culture quality and should be avoided.

4.3 Water treatment methods for the growth of *C. calcitrans* in batch and continuous culture

4.3.1 The electrolysis of 15‰ seawater

4.3.1.1 Testing the Monarch Pool Systems' seawater electrolysis system for the level of sodium hypochlorite

The temperature of the water pumped through the electrolysis system in the 15‰ seawater had an effect upon the concentration of the sodium hypochlorite produced at the higher electricity input levels (Figure 42). The rate of hypochlorite production increased in an almost linear manner, notably at the lowest temperature trialled (14°C). The system used in this trial is not in common use in the aquaculture industry, and no literature was found with which to compare the results of electrolytic production of sodium hypochlorite relative to temperature.

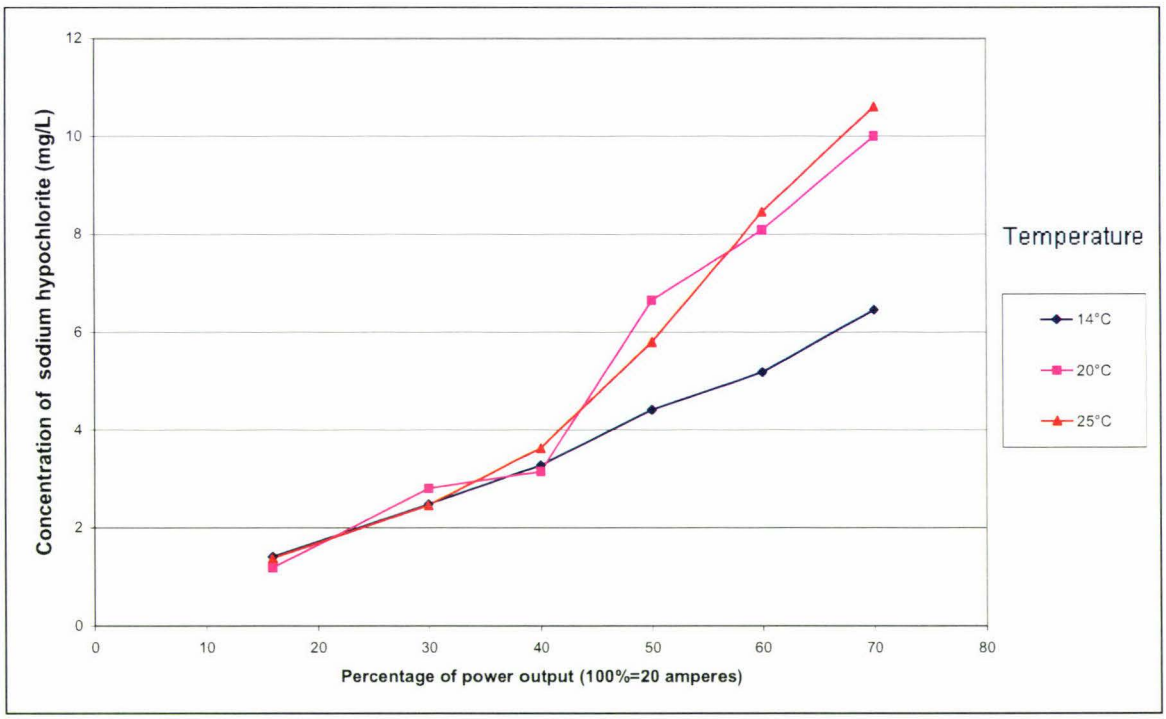


Figure 42 - The effect of temperature upon the concentration of sodium hypochlorite produced by the electrolysis system. (Salinity = 15‰. Flow rate = 30 L/min)

The concentration of electrolytically produced sodium hypochlorite required to disinfect or sterilise seawater was tested. Table 14 shows the concentration of hypochlorite produced at a range of power levels. Samples of water processed at each power level (Section 3.3.1.1) were alternately taken, immediately after dechlorination or 48 hours

after dechlorination. The number of colony forming units (cfu) cultured in each sample is presented. Initial bacterial concentration of the water to be treated was 17800 cfu/mL.

% Power level (50% = 10 Amperes)	[OCl^-] (mg/L)	[OCl^-] after treatment and aeration (mg/L)	Bacterial density (cfu/mL) 0 hours after treatment	Bacterial density (cfu/mL) 48 hours after treatment
0 (Control)	0.07	0	17800	
20	1.54	0	0	
25	1.81	0		0
30	2.54	0	0	
35	2.59	0		0
40	3.53	0	0	
45	3.61	0		0
50	3.82	0	0	

Table 14 - The concentration of electrolytically produced sodium hypochlorite at different power input levels and the density of culturable bacteria immediately and 48 hours after dechlorination.

All treatments except the control showed a total elimination of colony forming units at each power level and time interval tested. The results of Jorquera *et al.* (2003) are in agreement to those shown here. The work of these researchers generally related reductions in bacterial activity to the power level of the electrolysis system they were using, rather than the concentration of hypochlorite ions. They did note that the system produced free chlorine in concentrations from 0.39 – 6.5 mg/L at power levels of 0.3 – 2.0 Amperes (salinity = ~35‰). The power level of 1.3 A produced 2.13 mg/L of free chlorine and no culturable bacteria were detected in any sample treated with this or a greater power level (Jorquera *et al.* 2003).

The above results showing no culturable bacteria in the samples were unexpected. "Yes/No" culturable bacteria tests (Section 3.3.1.1) were completed with 20mL samples. Table 15 shows the results of samples of electrolytically treated water (50% power level) from the 20L carboy growth trial.

Carboy	Yes/No after 1 day of culturing	Yes/No after 7 days of culturing
E9	No	Yes
E10	No	No
E11	No	Yes
E12	No	No
Control	No	No
E13	Yes	Yes
E14	No	No
E15	No	No
E16	No	No
Control	No	Yes

Table 15 - Yes/No results of samples from 20 L carboys of electrolytically treated water (50% power level = 10 Amperes) in double strength marine broth after 1 and 7 days of culture. Controls are autoclaved seawater (15‰).

The results from the Yes/No tests showed that 3 of the 8 samples did grow bacteria after 7 days culturing. One of the two controls also grew bacteria after 7 days. This result is most likely due to accidental contamination during handling of the samples. The possibility of infection during handling applies to all "Yes" results, not solely the control.

The length of time the state of disinfection lasted for carboys filled with electrolysed water was tested. The results showed that 66% of carboys (6) were still disinfected five days after they were treated with sodium thiosulphate and aerated to remove the hypochlorite ions. The carboys that were treated with thiosulphate 5 days after they were prepared showed a 27% reduction in the mean concentration of total hypochlorite present. Despite this, these carboys still showed a very high level of disinfection. Table 16 shows the density of bacteria (cfu/mL) in carboys sampled on Day 0 and Day 5 after the dechlorination step.

	Carboy	[OCl⁻] (mg/L) Day 0	[OCl⁻] (mg/L) Day 5	Cfu/mL Day 0 after dechlorination	Cfu/mL Day 5 after dechlorination
Carboys treated with thiosulphate 24hrs after electrolysis	A	4.25		0	0
	B	4.00		0	0
	C	3.88		200	$62*10^5$
	D	3.71		0	0
	E	4.29		200	0
	F	4.85		0	$33*10^5$
Carboys treated with thiosulphate 6 days after electrolysis	G		2.76	0	
	H		2.58	0	
	I		3.61	0	
	J		3.17	0	
	K		2.62	0	
	L		3.67	0	

Table 16 - The concentration of hypochlorite in water treated with electrolysis (70% power level) after 24 hours (Day 0) and 6 days (Day 5) and the density of bacteria in the water (cfu/mL) after dechlorination. Samples were taken immediately after dechlorination (Day 0) and five days after dechlorination (Day 5).

4.3.1.2 Cell concentration of *C. calcitrans* cultured in electrolysed water

Following the time interval experiment in Section 4.3.1.1, five carboys from each treatment (24 hours and 6 days after electrolysis) were inoculated with *C. calcitrans*. All ten cultures grew rapidly and the cell concentration was not different from cultures of *C. calcitrans* inoculated into carboys of autoclaved water at the same time. Two of the algal cultures, in carboys F and L, from Section 4.3.1.1 did crash on the 5th day of culture. It is assumed that this was due to bacterial levels. Figure 43 shows that there was no difference between the cell concentrations in the two treatments.

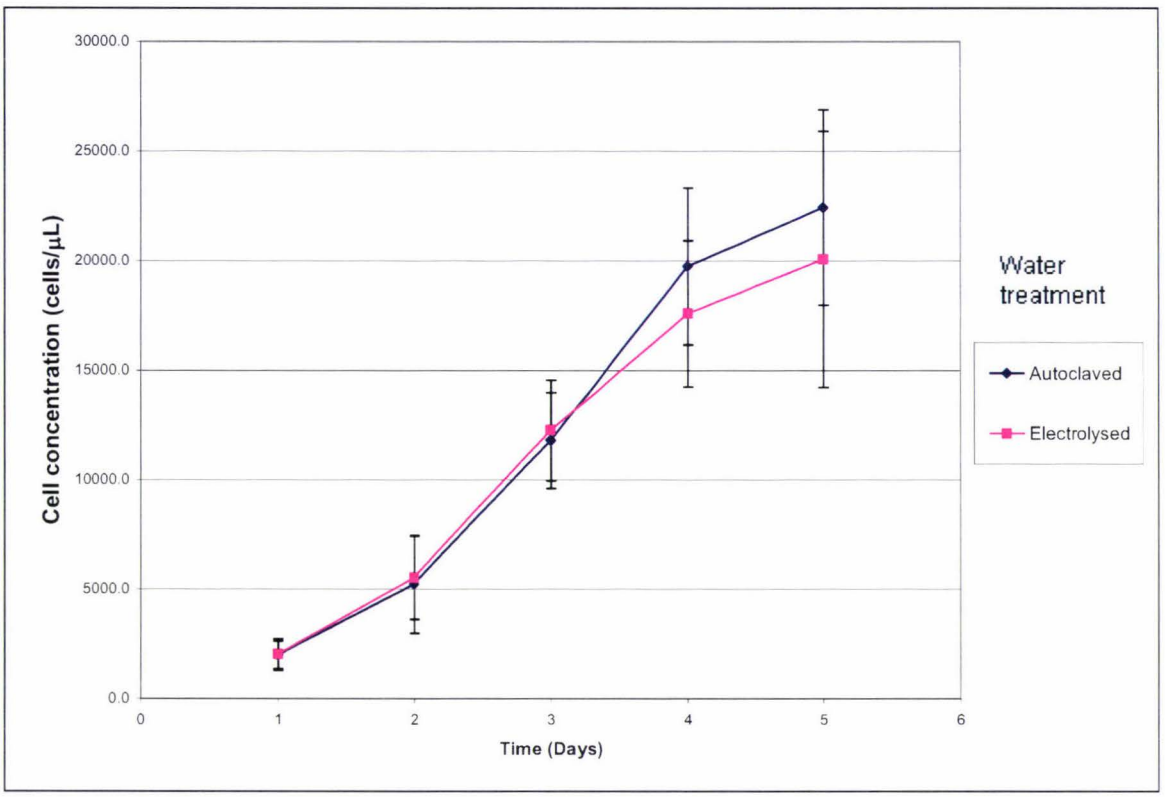


Figure 43 - The growth of *C. calcitrans* in 20 L carboys disinfected by autoclaving or electrolysis of the water. Error bars represent the standard error (n=5).

Despite the growth of the two treatments being very similar there were differences in the suitability of the algae to feed larvae of the New Zealand Greenshell mussel (*Perna canaliculus*). The staff at the GACL that were using the algae grown in this experiment considered that larvae that were fed *C. calcitrans* grown in electrolysed water were not growing as well as those being fed the algae grown in the water that was autoclaved. The general consensus of these workers was that the larvae appeared to be suffering from higher levels of bacteria in the tanks that were being fed cultures from electrolysed treatments.

4.3.1.3 The growth of bacteria in electrolysed water

The two control and one autoclaved treatments had similar bacterial densities at the start of this experiment. The other two treatments (electrolysed and electrolysed plus nutrients) had significantly lower initial densities. The inoculum from the *C. calcitrans* supernatant provided enough bacteria to raise the density of all treatments to at least 44,250 cfu/mL. After 24 hours, the bacterial density of the electrolysed treatments was at least 1.9 times higher than the next highest treatment (Control + Nutrients). The treatment with the lowest bacterial density (Autoclaved) was more than four times higher than the initial inoculum density.

After 96 hours the bacterial densities were similar for the Control, Control + Nutrients and Electrolysed treatments. The Electrolysed + Nutrients treatment had nearly four times the bacterial density of the three previously mentioned treatments and more than eighty times the density of the autoclaved treatment (Figure 44). No similar work was found in the literature with which to compare these results.

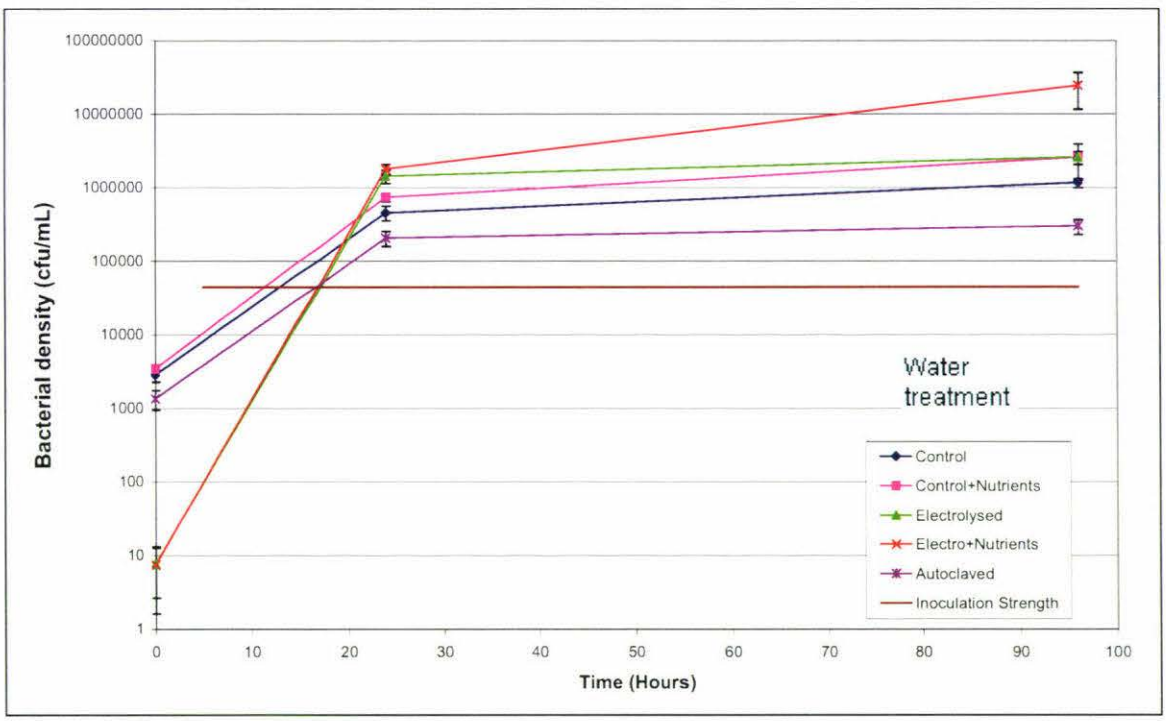


Figure 44 - The average bacterial density of water treatments before and after inoculation with a suspended bacterial culture. Error bars represent the standard error (n=5).

Although the results show that the electrolysis of seawater using the described method is not suitable for the culture of microalgae there is still scope for water treated this way to be of use in aquaculture hatcheries. There are many areas of hatchery husbandry where the reduction of bacteria is important and the use of electrolytically treated water containing hypochlorite would be suitable as a cheap, bulk cleaning solution. For

example, the cleaning of pipe work, pumps and tanks would be much easier using water treated electrolytically than having to handle concentrated solutions of bleach.

4.3.1.4 The effect of activated carbon filtration upon the level of organic carbon in seawater

Results from previous experiments (Section 4.3.1.3) showed increased bacterial densities in media prepared with electrolysis. Pre-treatment removal of organic carbon was trialled as a method of reducing the bacterial densities.

The activated carbon filter removed approximately 40% of the organic carbon in the sample. This was in line with the manufacturer's expectation of performance (Table 17).

Sample	Non-Purgeable Organic Carbon (NPOC) ($g.m^{-3}$)
Untreated	1.2
Activated carbon filtered	0.7

Table 17 - The concentration of organic carbon in 15‰ seawater before and after passing the water through an activated carbon filter at 8 L/min.

The second trial of this experiment provided results that supported the hypothesis that the electrolysis process released bound organic carbon to a state of dissolved organic carbon in the water. As occurred previously, when water was filtered through the activated carbon filter, approximately 40% of the organic carbon was removed by the process. The sample that provided results supporting the above hypothesis was Sample F. This water was filtered and then electrolysed and the final organic carbon level was higher than the original concentration – (samples D, E and F in Table 18).

Sample	Non-Purgeable Organic Carbon (NPOC) ($g.m^{-3}$)
Sample A - Control (Batch 1)	1.3
Sample B - Electrolysed then filtered	0.8
Sample C - Electrolysed, filtered then electrolysed	1.4
Sample D - Control (Batch 2)	1.2
Sample E – Filtered	0.7
Sample F – Filtered then Electrolysed	1.5

Table 18 - The concentration of organic carbon in 15‰ seawater at various stages during a filtration and electrolysis process. (See Figure 12 – Schematic drawing of the sample points in the water treatment process for the removal of organic carbon and water electrolysis experiment. for sample points.)

In the sample that was first electrolysed and then filtered, which would presumably release more organic carbon and then remove it during filtering, the final organic carbon concentration was still higher than the original, untreated sample – (samples A, B and C in Table 18).

Repeating the trial of Section 4.3.1.3, the growth of bacteria in the samples A, C and F gave similar results to the previous experiment. The samples that had been electrolysed promoted bacterial growth and the untreated water (Sample A) decreased in bacterial numbers over the growth period. The electrolysed treatment with the lower of the two bacterial densities (Sample F) was nearly 12 times greater than Sample A, the untreated water (Figure 45).

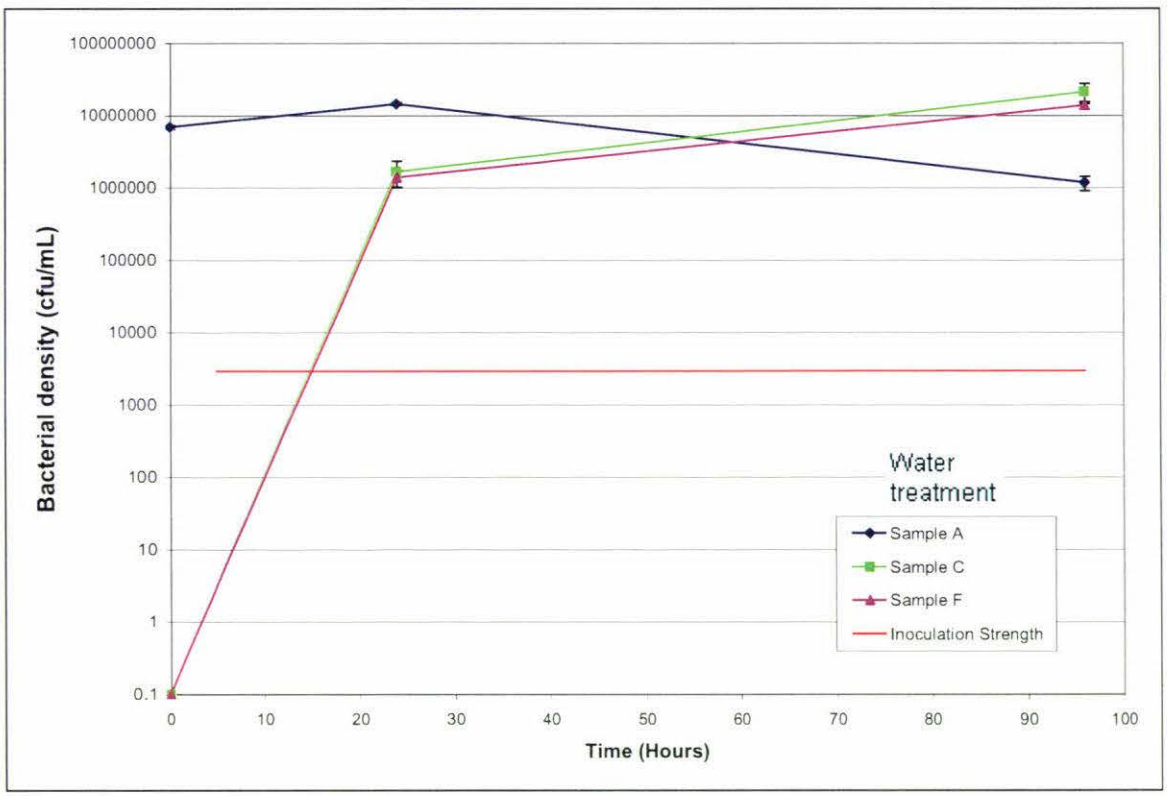


Figure 45 - The average bacterial density in electrolysed and carbon filtered water treatments before (Time = 0) and after inoculation with a suspended bacterial culture. Error bars represent the standard error (n=5).

4.3.2 Heat treating water for culturing *C. calcitrans*

4.3.2.1 The effect upon bacterial activity of heat treating seawater

The heat treated samples showed a large (99.99%) reduction in bacterial density after five minutes of heating at 85°C. This reduction was even greater at 10 minutes. The results presented in Figure 46 suggest that the bacterial density remains around 10-100 cfu/mL beyond this time. However, these numbers are from averaged results and in all except one case, four of the five replicates after the five minute sampling time gave a 0 cfu/mL result and 1 replicate showed some bacteria present. The treatment for 35 minutes had two of five replicates with some bacteria present. Overall, the heat treatments removed most if not all culturable bacteria, and it is possible that the replicates with bacteria present were from spurious infections.

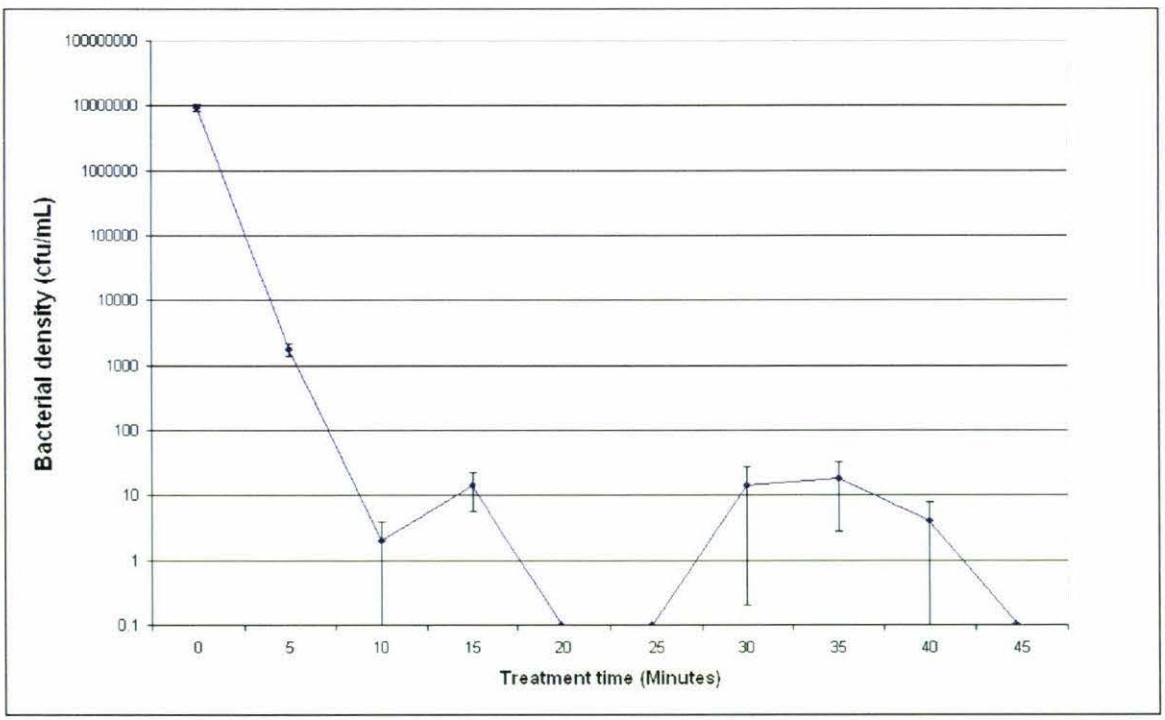


Figure 46 - The effect of heat treatment (85°C) over time upon bacterial density in 15‰ water. Error bars represent the standard error (n=5).

The results are in general agreement with those of Moats (1971) and Kilsby *et al.* (2000), where heat treatment over time reduces the viability of the bacteria present. Although the exact kinetics of this thermal death have not been investigated in any detail (Moats, 1971) the results are sufficient for this project i.e. indicating that there is a 99.9% thermal death in water treated at 85°C for 10 minutes or more.

4.3.2.2 Evaluation of flow dynamics in the Seasalter pasteuriser system used at the Glenhaven Aquaculture Centre Ltd

The initial concentration of Rhodamine WT in the source tank was 473 ppb. After the dye had been added the first Rhodamine WT exiting the 60 L kettle was detected after one minute (34.0 ppb, Figure 47). Within five minutes the reading was 164 ppb. This was ⅓ of the total concentration of Rhodamine WT in the source water. The dye concentration continued to increase, with time, until approximately 25 minutes, when it began to plateau at about 375 ppb.

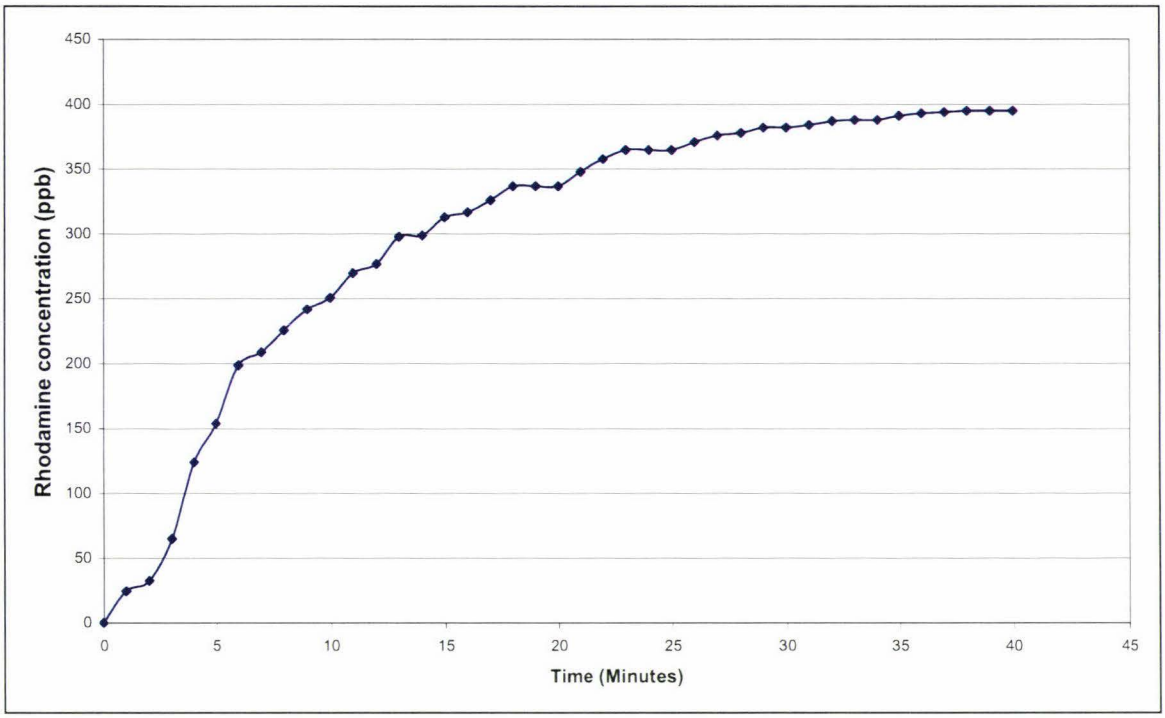


Figure 47 - The concentration of Rhodamine WT at the exit port of a 60L pasteuriser kettle with a 1.3 L/min flow rate. Initial concentration of Rhodamine was 473 ppb.

The concentration of Rhodamine WT at the exit port of the kettle was 34.9 ppb after one minute. This is 7.4% of the concentration of the Rhodamine WT in the source tank. If the assumption were made that the flow behaviour was not greatly different between ambient and 85°C, then 7.4% of the water exiting the kettle would have been heat treated for one minute or less. From the results of Section 4.3.2.1 (Figure 46) the density of bacteria that would still be viable after one minute at 85°C is 16% of the original density. In the untreated water (control) of Section 4.3.1.3 the bacterial density was 2400 cfu/mL. If 16% of this were to exit the kettle still viable, then the bacterial density of the water would be 295 cfu/μL in the water exiting the kettle at one minute. Water with a bacterial density such as this would not be expected to provide a good quality culture environment for microalgae (Gibson. L. pers. comm.). It was concluded that the Seasalter pasteuriser

as used at GACL does not provide sufficient reduction of bacterial activity for the reliable cultivation of *C. calcitrans*.

4.3.2.3 Rhodamine WT dye concentration in a ten-chamber flow through system

Water in one body e.g. 60 L kettle has been shown to have an uneven flow dynamic, and so some form of barrier or containment is necessary to make it impossible for water that has just entered the treatment system to exit the system in a short time (i.e. short-cutting the system).

In a ten-chamber system (9.6 L per chamber; Section 3.3.2.3) the concentration of Rhodamine WT flowing out of the tenth tank was monitored from the time the dye was added to the source tank (See Figure 13). The two trials were very similar in the time it took for the dye to pass through the tanks and out of the outlet on tank ten. The initial dye concentration in the source tank was higher in the second trial (670 ppb) than in the first trial (593 ppb) and this is reflected in the graphed result (Figure 48). The breakthrough time for the Rhodamine WT dye was 29 minutes in both trials.

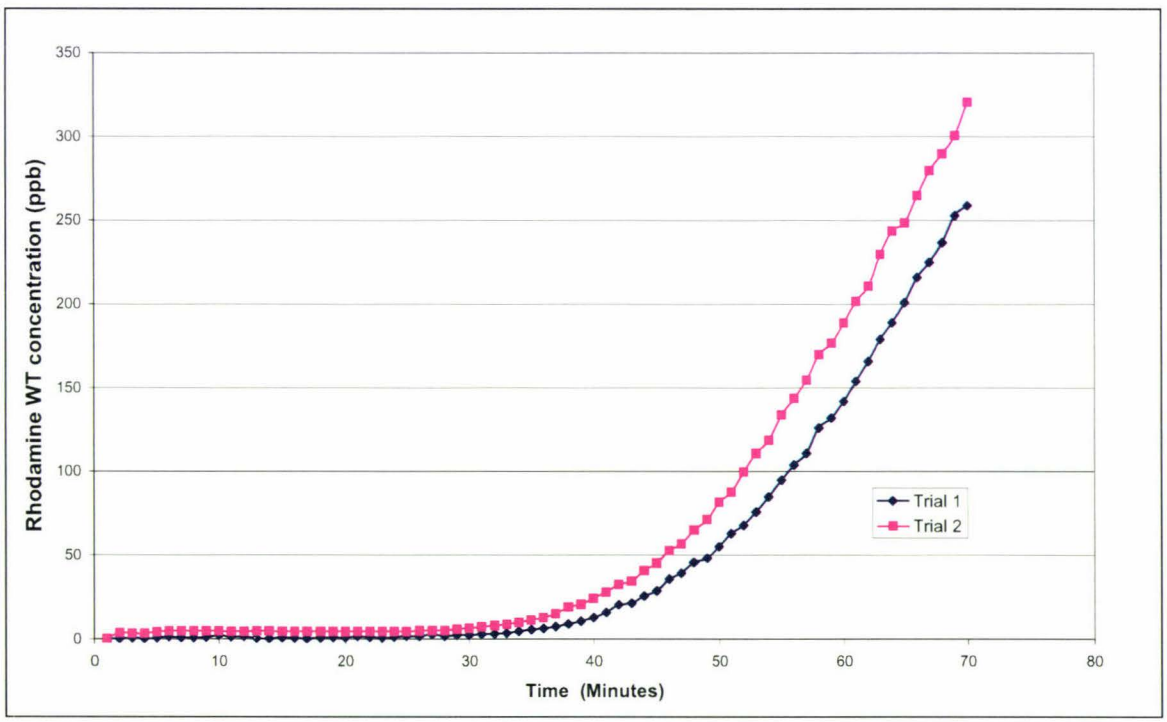


Figure 48 - The concentration of Rhodamine WT in the outflow at tank #10 of a ten tank flow through system (flow rate = 1.3 L/min).

In Trial 2, as well as recording the concentration of dye in the outflow, the dye concentration in each tank (1 – 10) was recorded every ten minutes. This measurement

clearly illustrated the passage of the dye through each tank and how this differed from tank to tank (Figure 49).

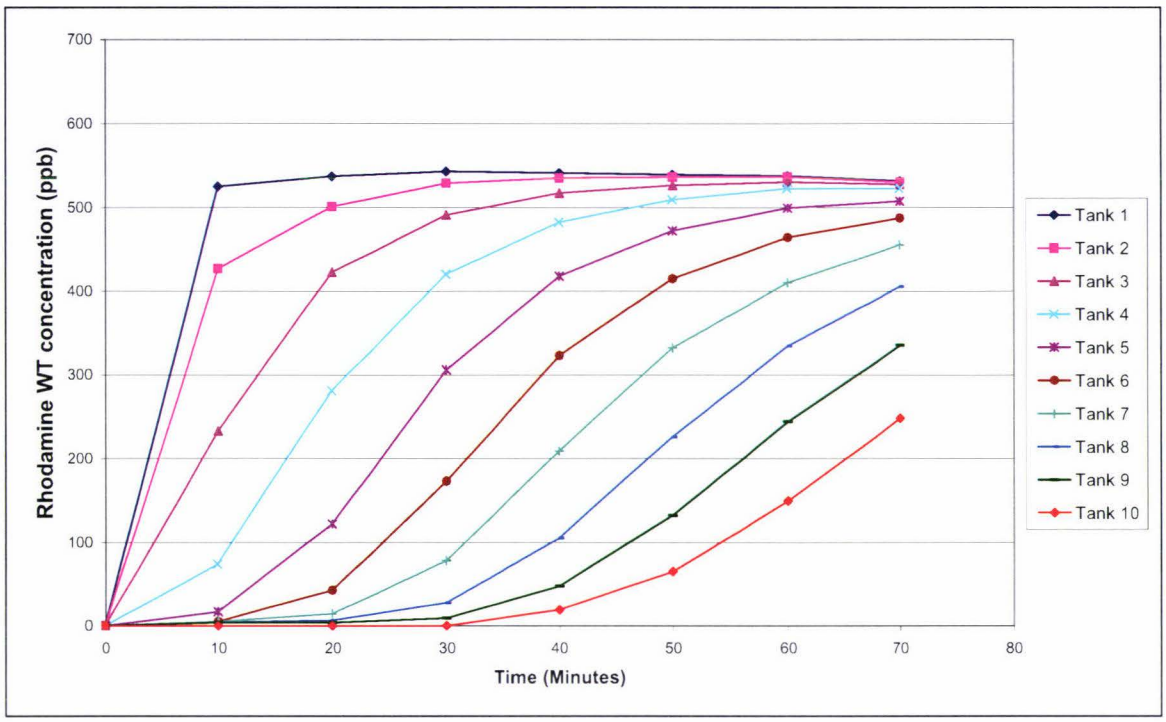


Figure 49 - The concentration of Rhodamine WT in each tank in a ten tank flow through system. Source tank Rhodamine WT = 670 ppb (Flow rate = 1.3 L/min).

A computer model of the system was written using the equations of Levenspiel (1999), and this agreed well with the results of the actual system (Figure 50). The most obvious value where the results and the model did not agree was at the exit port of tank 10. The model predicted that the dye would breakthrough the tank at a detectable level (i.e. 100 parts per trillion) after 23 minutes. In the actual system, the dye breakthrough time was not until 29 minutes. The difference was likely due to non-ideal mixing in the tanks of the actual system. The other difference between the two systems was the source tank of dye in the actual system received one aliquot of dye, and after that the dye concentration reduced in the source tank as it was being continuously refilled as water was pumped from it. In the computer model the programme stated the source dye concentration was constant at 670 ppb.

The experimental system did provide the result that it was designed to produce. However, as the set-up of this experiment was so simple it was built quickly, without enough forethought as to the necessary design of a working prototype. Following the positive result of this trial further thought was given to the design of a working prototype and a number of issues arose that were going to be problematic e.g. heating of individual chambers, mixing/agitation within chambers, cleaning. With so many difficulties being immediately apparent, the plans were put aside in favour of a simpler design.

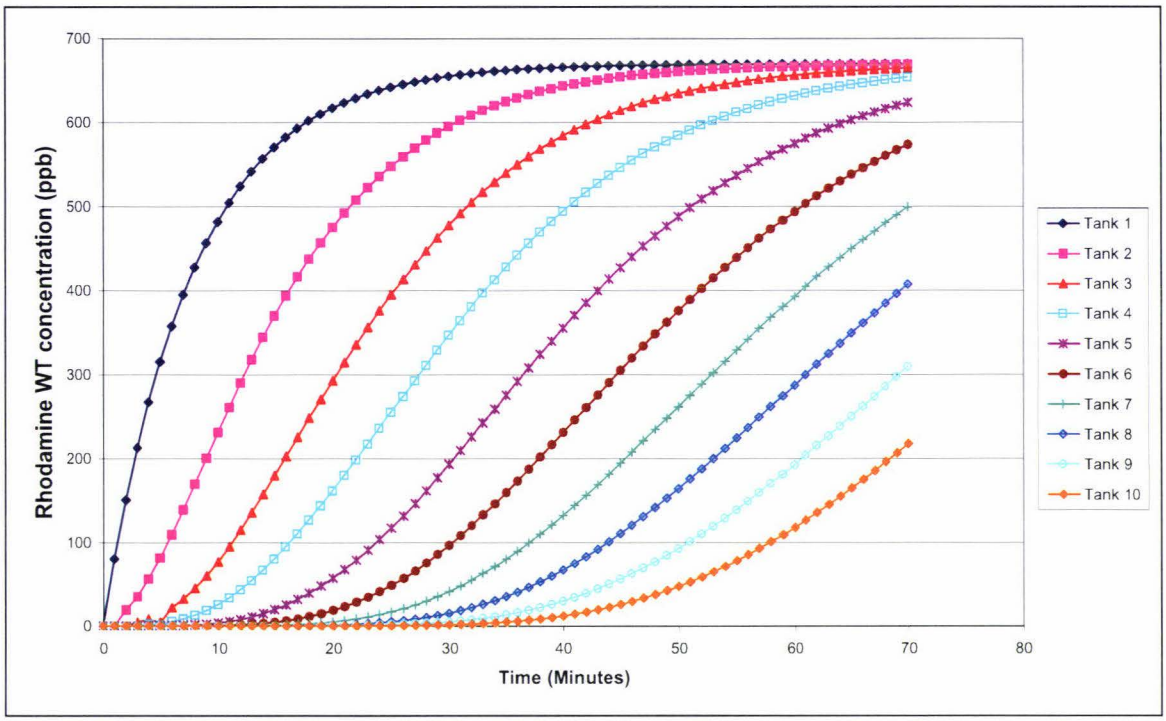


Figure 50 – A computer model of the 10-tank system suggested by Levenspiel (1999). Source tank Rhodamine WT = 670 ppb (flow rate = 1.3 L/min) .

4.3.2.4 Breakthrough time and concentration of Rhodamine WT dye in water flowing through a 200m long pipe

With a flow rate set at approximately 0.7 L/min the time before the dye concentration increased at the outlet of the coiled 200m long pipe was 27 minutes (Figure 51), about half that of the theoretical residence time of 50 minutes at that flow rate. The actual breakthrough time was not the same as the theoretical value because of the occurrence of laminar flow rather than plug flow which the theoretical value was based upon. The Reynolds number ($Re = \rho u_m D_h/\mu$) where ρ=fluid density, u_m = fluid mean axial velocity, D_h = hydraulic diameter of flow passages and μ = fluid dynamic viscosity) was calculated for this system to be 1947.5 (Shah and Sekulić, 2003). The other factor that must have some effect upon this result is that the flow rate was variable. For reasons that are not clear, the flow rate dropped below the set level several times and needed to be adjusted. Because of this repeated alteration to the flow rate, the result of 27 minutes can only be taken as an indication.

In the second trial of this experiment, where disc disrupters (Figure 14) were inserted into the pipeline every 50m, the time taken for the dye to breakthrough was 25 minutes. The disc disrupters were inserted with the intention of causing eddies and turbulence in the flow of the tube, thus creating a situation with more mixing and plug flow. This was not however the case. The uniformity of the flow rate over the time of the experiment was however an issue in the second trial as well and so there may have been very little effect

upon flow by the disrupters, and for this reason further investigations did not involve their use.

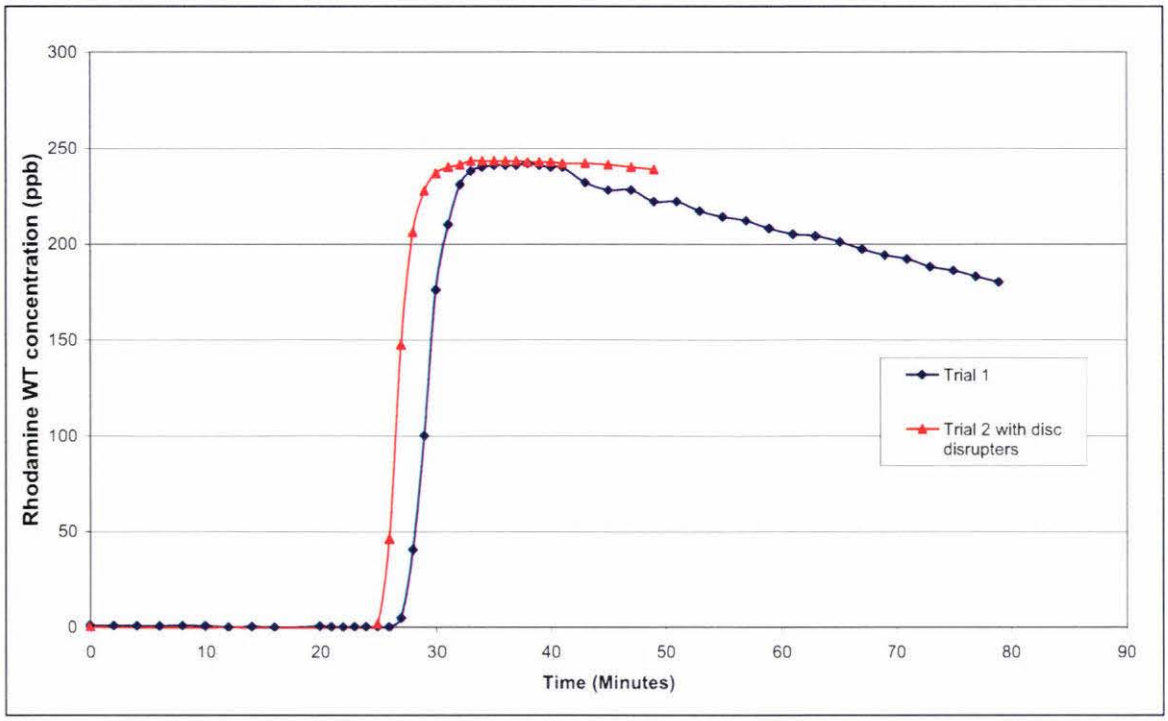


Figure 51 – The concentration of Rhodamine WT in a 200m long pipe with a flow rate of 0.7L/min. In the second trial flow disrupting discs were inserted into the line every 50m.

4.3.2.5 Breakthrough time and concentration of Rhodamine WT dye in a prototype of a glass tube and silicone hose system

The next step was to design a system that had a breakthrough time of approximately 30 minutes in a system that treated 1000 L of 15‰ seawater per day. This meant a flow rate of 0.694 L/min (rounded to 0.7 L/min). Due to the high capital cost of either silicon hosing (able to cope with the pressure and temperature required) or a plate heat exchanger, the decision was made to build the prototype system from glass tubes connected with segments of silicon hose (Section 3.3.2.5) because for the initial prototype this would be approximately half the price of the next cheapest option (silicon hosing.) The other considerations for not choosing silicon hosing were its poor heat transfer properties and the likelihood of the tube expanding upon heating and having kinks forming in the hose when laid out upon the support frame that would be required.

The longest breakthrough time obtained in this trial (Figure 52) was from the 'Hot - with clamps' (Figure 18). The time for this treatment was $9.^{10}/_{60}$ minutes. The time for all the dye to pass through the system (flushing time) in this treatment was the second fastest ($4.^{55}/_{60}$ minutes). The second longest retention time ($8.^{55}/_{60}$ minutes) was the 'Hot - no clamps on the tubes' treatment. This treatment had the fastest flushing time as well

(2.$^{45}/_{60}$ minutes). This result is reflected in the high peak concentration of Rhodamine WT as much of the dye passed through the system in a short space of time. The difference in breakthrough times between these two treatments was only 15 seconds, but the flushing times were 2.$^{10}/_{60}$ minutes apart.

The shortest breakthrough time occurred with the 'Cold - no clamps' treatment (7.$^{45}/_{60}$ minutes). The 'Cold - with clamps' treatment had a longer retention time (8.$^{30}/_{60}$ minutes) than the other cold treatment. Both these treatments had very long flushing times (8.$^{15}/_{60}$ and 8.$^{30}/_{60}$ minutes respectively).

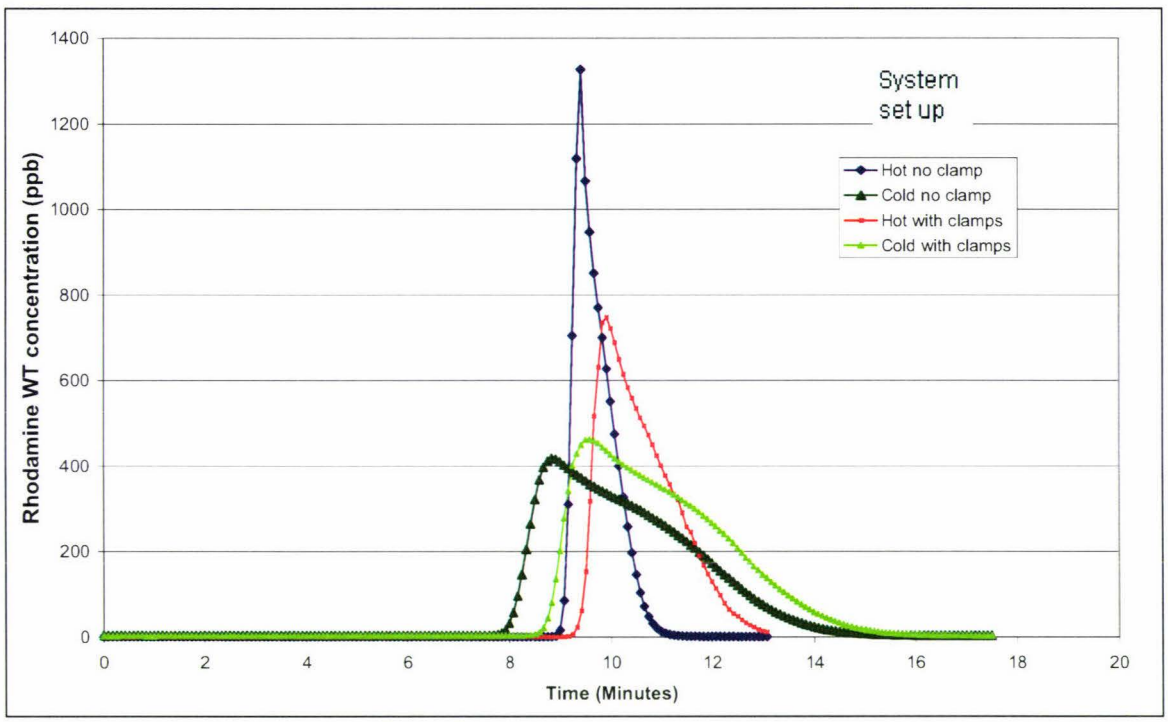


Figure 52 – The concentration of Rhodamine WT dye in a prototype glass tube and silicon hose system (Length 108.2 m) with hot or cold water and a flow rate of 0.7 L/min. Two treatments had clamps placed on the silicon hoses to alter flow dynamics.

A calculated estimate for the length of pipe needed to achieve a breakthrough time of 30 minutes was made from the results. The equations were formulated in the following way:

- The volume of the glass tube and silicon hose system is 6.88 L. ($\pi \times r^2 \times h$)
- At a constant flow rate of 0.7 L/minute the residence time would be 9.$^{49}/_{60}$ minutes. (6.88 L ÷ 0.7 L/minute)
- The observed breakthrough time from this trial was 8.$^{55}/_{60}$ minutes for the 'Hot - no clamps' treatment. This implies a working volume in the pipe system of 6.24 L. (8.$^{55}/_{60}$ minutes × 0.7 L/minute).

- If a pipe that was 108.2 m long had a volume of 6.24 L then its diameter would be 8.56 mm. This is considered to be the 'working' diameter:

$$d = \sqrt{\frac{\text{volume}}{h} \div \pi} \times 2$$

- For a system with a flow rate at 0.7 L/minute and a required breakthrough time of 30 minutes the length of the system would need to be 364.13 m long.
  0.7 L/minute × 30 minutes ÷ cross sectional area of working pipe.
  NB. r in this equation equals half the diameter of the 'working' diameter calculated above, not the actual diameter of the pipe.

$$h = \frac{\text{volume}}{\pi \times r^2}$$

The layout of the frame that held the glass tubes in this trial was utilised for the design of a larger frame to hold the glass tubes needed. With the frame layout as it was (Figure 15 and Table 6) there were 238 holes in the frame and this gave a total pipe length of 402 m of glass tubing and silicon hosing. This was 37.9 m longer than the above calculations specified but the prospect of increasing the breakthrough time seemed to be a desirable attribute.

4.3.2.6 Breakthrough time of Rhodamine WT dye in a full scale glass tube and silicon hose pasteuriser system

Once the glass tube and silicon hose system was fully assembled, the intention was to have it run for 24 hours before the dye was used to monitor breakthrough time. When the system was set to work at 0.7 L/min it maintained this flow rate for one to two hours. After this point the flow rate decreased with time and the working pressure increased. It seemed likely that without intervention the system would reach a point where the flow would stop. The valve on the pump was opened to increase the flowrate, and this initially caused the pressure to increase as well. After approximately five minutes the pressure decreased and the flowrate increased further. After re-setting the flowrate to 0.7 L/min the flow rate again reduced over time with a consequential increase in pressure. There was concern that there was chemical precipitation occurring in the tubes and the precipitate was collecting in the bottom loops of the silicon hose connectors. When the system was flushed again a 10 μm sieve was placed over the outlet and any solids were collected. The solid that was collected after the flushing period appeared to have a gelatinous and colloid-like quality to it. When left to sit in a beaker of water the solids separated from the bulk of the liquid. If left to sit for several hours the settled solids began to bind and required vigorous agitation to re-suspend them.

The solids were tested for the presence of calcium carbonate, ash content, protein and carbohydrate content, and were assessed for elemental content on an Inductively-Coupled Plasma spectrometer (ICP; this is a high temperature (7000-8000K) excitation source that

desolvates, vaporizes, excites, and then ionizes atoms. Molecular interference is markedly reduced with the excitation source but is not eliminated completely. ICP is used to excite atoms for atomic-emission spectroscopy and to ionize atoms for mass spectrometry). During the test for metal content, part of which is an acid digestion, the sample became a very thick rubber-like solution.

Quantitatively, the ICP result was indeterminate, but was consistent with silicon content in the sample. Given the results of the acid digestion and the ICP, the following explanation is given for the problems associated with the reducing flow/increasing pressure problem. The silicon hosing (~45 m) used in the glass tube and silicon hose system is coated with a silicon powder during the manufacturing process. Once the system was assembled, brought up to temperature and water was passed through it, the silicon powder on the inside of the hosing began to absorb water and formed a gelatinous like substance. Over time the particles detached from the surface of the hosing into the process water. Because of the slow flow rate the particles bound with others to form larger particles. These bound again with the hosing and thus restricted flow. As this occurred the problem was exacerbated and the pressure increased. When the flow rate was increased manually the particles were dislodged and passed through the system. When the flow was reduced again the process repeated itself. Further support for this explanation was found in the hot water cylinder (HWC). The freshwater in the heater contained large aggregates (20mm) floating in the water. A small quantity of the aggregates were collected in a fine sieve and allowed to dry. Once dry the aggregates collapsed upon contact and when rubbed on one's hand had the texture of silicon powder.

Part way through this process the mid line pump (Section 3.3.2.6) failed. The high temperatures most likely were too great for the diaphragms and one way valves. Due to the problems that were concurrently occurring with the gelatinous substance it was decided to remove the pump and use only one of the two divided glass tube and silicon hose systems. This meant that in all further assessments of water treatment times etc the flowrate would be reduced to half of the current rate i.e. 0.35 L/min. During the time it was not in use the other half of the system became blocked. It is assumed that the gelatinous silicon formed an immovable gel plug in one or more of the loops in that system.

After it was determined that the most likely explanation for the flow problem was the silicon powder on the hoses forming gelatinous particles and blocking the system, the system was flushed for 72 hours at a high (1.5 L/min) flowrate. The system was then operated at the desired flowrate (0.35 L/min).

The breakthrough times for the different flow rates through the glass tube and silicon hose system illustrated the actual behaviour of the system in comparison to the theoretical values.

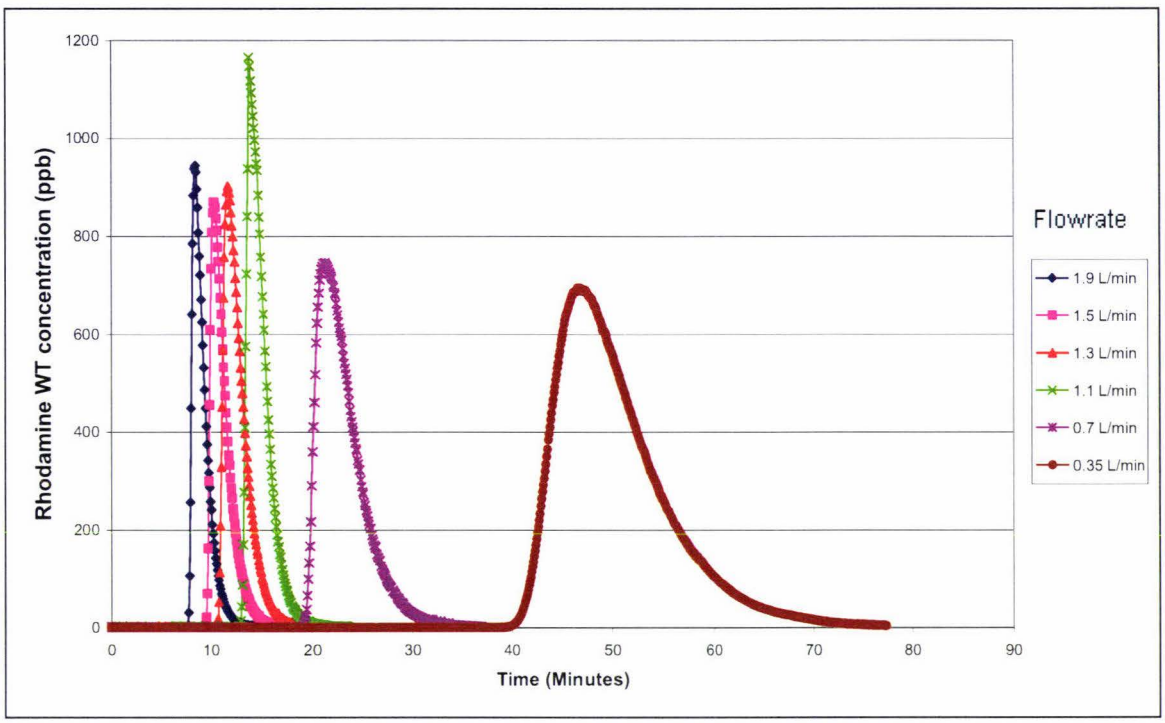


Figure 53 – The concentration of Rhodamine WT flowing from the exit point of the full scale glass tube and silicon hose pasteuriser system at 95°C.

The breakthrough times for the Rhodamine WT dye were directly related to the flow rates (Figure 53). The desired flow rate of 0.35 L/min had a breakthrough time of $39.^{30}/_{60}$ minutes. This was proportionately 69% of the theoretical breakthrough time of $57.^{6}/_{60}$ minutes. The proportional comparison gives an indication of the amount of laminar flow occurring in the system. With total plug flow the proportion would be 100% (Table 19).

Flowrate (L/min)	Actual breakthrough time (minutes)	Theoretical breakthrough time (minutes)	Proportion (%; Actual/Theoretical ×100)
1.9	$7.^{55}/_{60}$	9.0	86%
1.5	$9.^{30}/_{60}$	$11.^{25}/_{60}$	83%
1.3	$10.^{40}/_{60}$	$13.^{12}/_{60}$	80%
1.1	$12.^{50}/_{60}$	$15^{40}/_{60}$	82%
0.7	19.0	$24.^{30}/_{60}$	78%
0.35	$39.^{30}/_{60}$	$57.^{6}/_{60}$	69%

Table 19 – The actual and theoretical breakthrough times of Rhodamine WT dye in the full scale glass tube and silicon hose pasteuriser system at 95°C.

The increase in flow rate causes an increase in the Reynolds number (Re). There was an increase in the system pressure as the flow rate increased (Figure 54) as higher pressures are required to achieve increased flow in a given system.

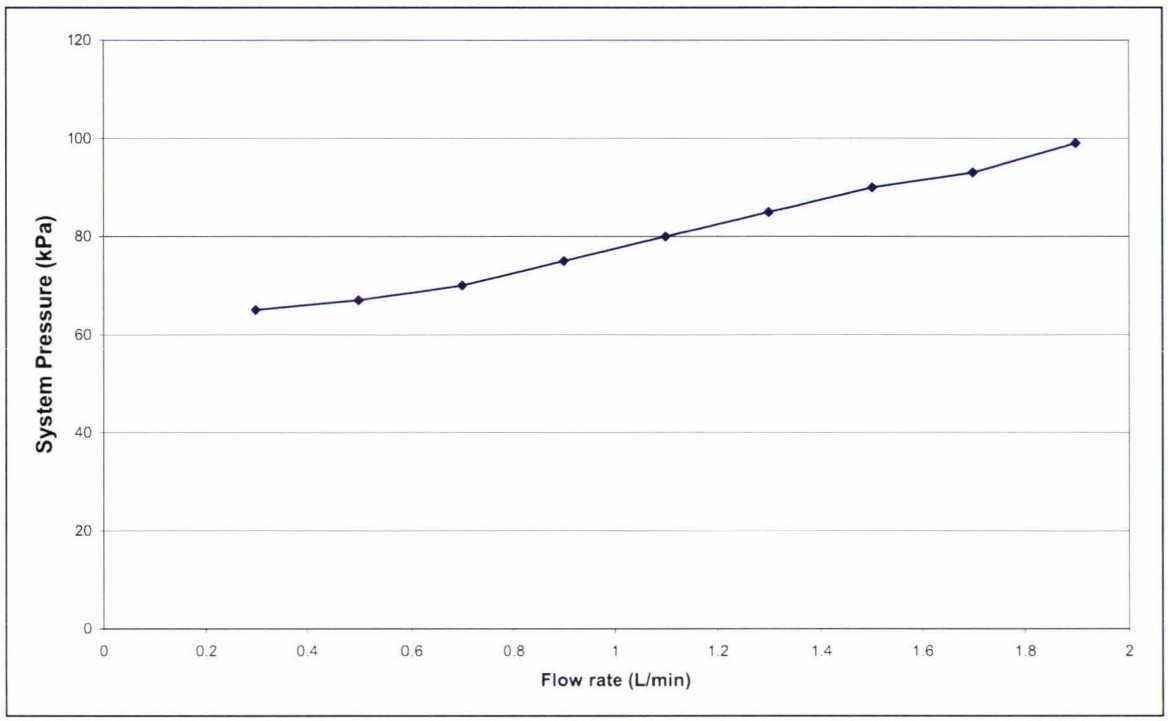


Figure 54 – The relationship between pressure and flow rate in the glass tube and silicon hose pasteuriser.

4.3.2.7 The effect of heat treatment of water in a full scale glass tube and silicon hose pasteuriser system upon bacterial density

The samples from the 0.4 – 1.3 L/min treatments did not contain any culturable bacteria (Figure 55). The other three treatments (1.5 – 1.9 L/min) contained 2000 – 3000 cfu/mL. This constituted two or three cfu upon an agar plate. The 26‰ raw seawater sample had a comparatively low bacterial density as well, especially when compared to the treated (Seasalter pasteurised) water which had a value 4 orders of magnitude higher than the untreated water. A possible cause of the unexpectedly high bacterial density in the water treated with the Seasalter system may be that when the sample was taken from this system it had been in operation for approximately one week since its last steam cleaning. If the bacteria and spores that survive the treatment process are able to settle in points of low flow within the reticulation system they may be able to grow on the dissolved organic matter present in the treated water. If this is the case then the sample taken could contain a large background density of bacteria.

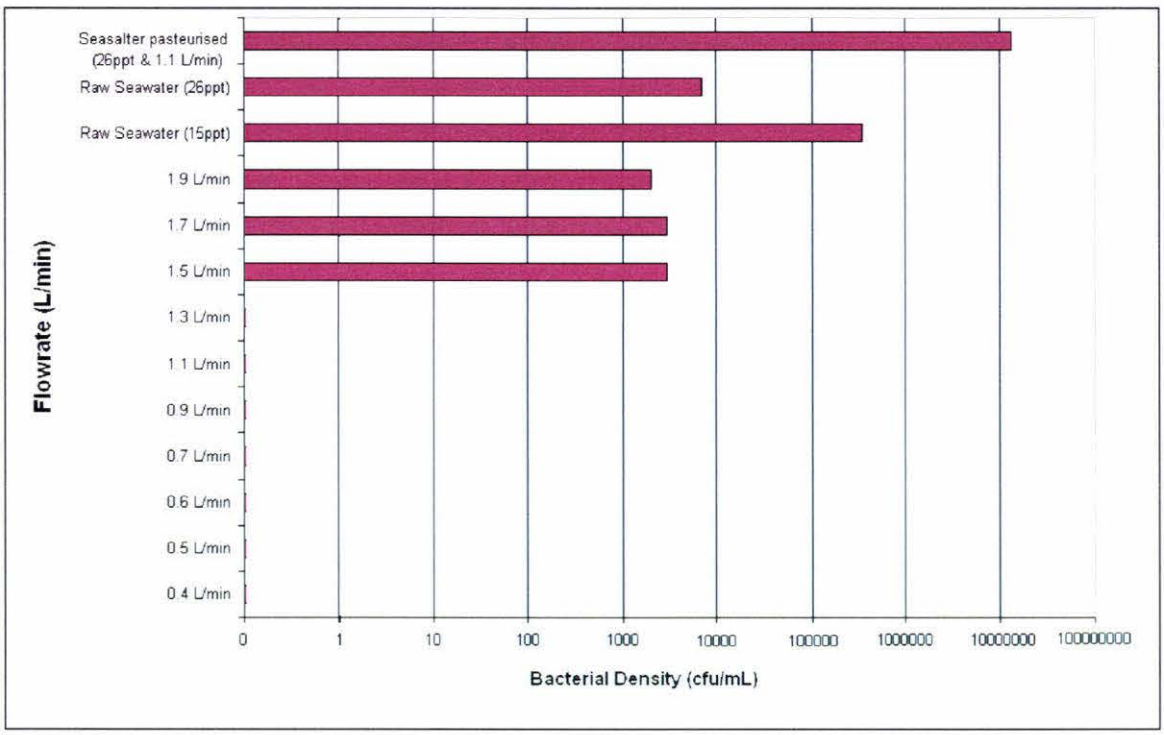


Figure 55 – The density of bacteria in treated and untreated seawater (15 and 26‰). Treated water was pumped through the full scale glass and tube pasteuriser at a range of flow rates.

4.3.2.8 Cell concentration of batch cultured *C. calcitrans* grown in water treated in the full scale glass tube and silicon hose pasteuriser system

The control cultures grew normally. The cultures grown it the treated water from the glass tube and silicon hose pasteuriser all showed a decrease in cell concentration over the first three days of culture. A possible explanation for this lack of growth was that the treatment process had removed some essential element from the water. This was considered a possibility as there were clearly crystals coming out of the system and collecting in the 1L vessel of the degassing system (Figure 27). The second possibility was that some constituent within the water was becoming toxic during the treatment process. A third possibility was that some material in the system was leaching into the water and had a toxic effect.

The only material that was novel to this system as compared to the Seasalter system already in use was the Selleys "Knead It" putty that was used to make the tubulations on the glass tubes. To test this possibility, a trial was set up to investigate this (Section 4.3.2.9). The second possibility, toxic effect of a water constituent, seemed the least likely as this problem would, most likely, have occurred also in water that was autoclaved or treated in the Seasalter system in the past. This left the possibility of the chemical state of some element being altered and made unavailable to the cells.

This hypothesis was tested by adding healthy culture from another source (pasteurised water and *C. muelleri* or autoclaved water and *C. calcitrans*) to the treated water culture that was not growing. On Day 2 after the cell readings were recorded, a further reduction in cell density was observed. Following this observation, 0.5 L of healthy culture was added to C1 and C2. The remaining 2.0 L of the control flask was allowed to grow on. The cell concentration of these two cultures began to increase within the following 24 hours and the growth was not dissimilar to a normal culture. The flasks (C3 and C4) that had not been supplemented did not show any improvement (Figure 56).

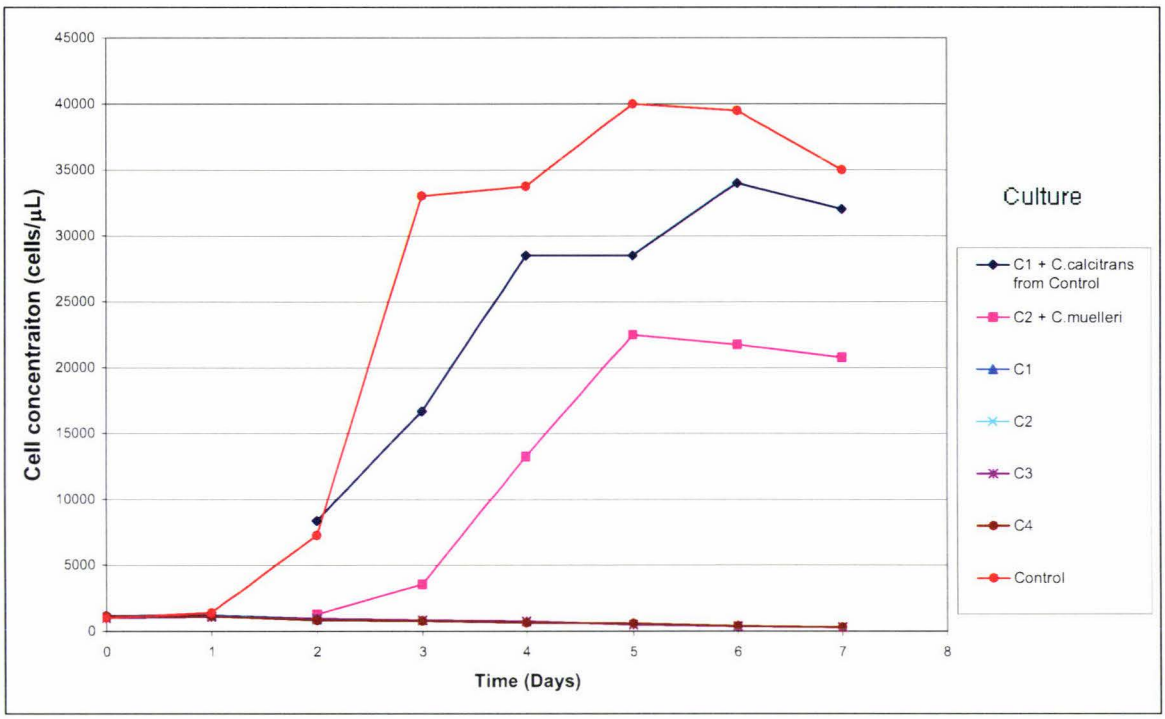


Figure 56 – The cell concentration of cultures of *C. calcitrans* and *C. muelleri* (Cm) cultured in water treated in the glass tube and silicon hose pasteuriser at 0.3 L/min at 95°C. Cultures C1 and C2 had a second inoculum from another source added to them on Day 2.

The result of this trial inferred that the algae would grow in the pasteurised water treated at 0.3 L/min if it was also seeded with a 20% aliquot of seawater from some other source.

To test this observation with more rigour, a second trial testing cell concentration in water of different flow rates was performed. In the first of two similar trials, the temperature of the pasteuriser system was also lowered to 75°C with the intention that the detrimental effect that was occurring may not be so pronounced and that some growth may be attained.

The results (Figure 57) clearly showed that the length of the breakthrough time within the system had a definite effect upon the growth of the *C. calcitrans* culture in the first 24 hours. However it was not expected that the cultures that did not grow well in the first 24

hours would eventually begin to grow and that the time to stationary phase was inversely related to the flow rate of the heat treatment.

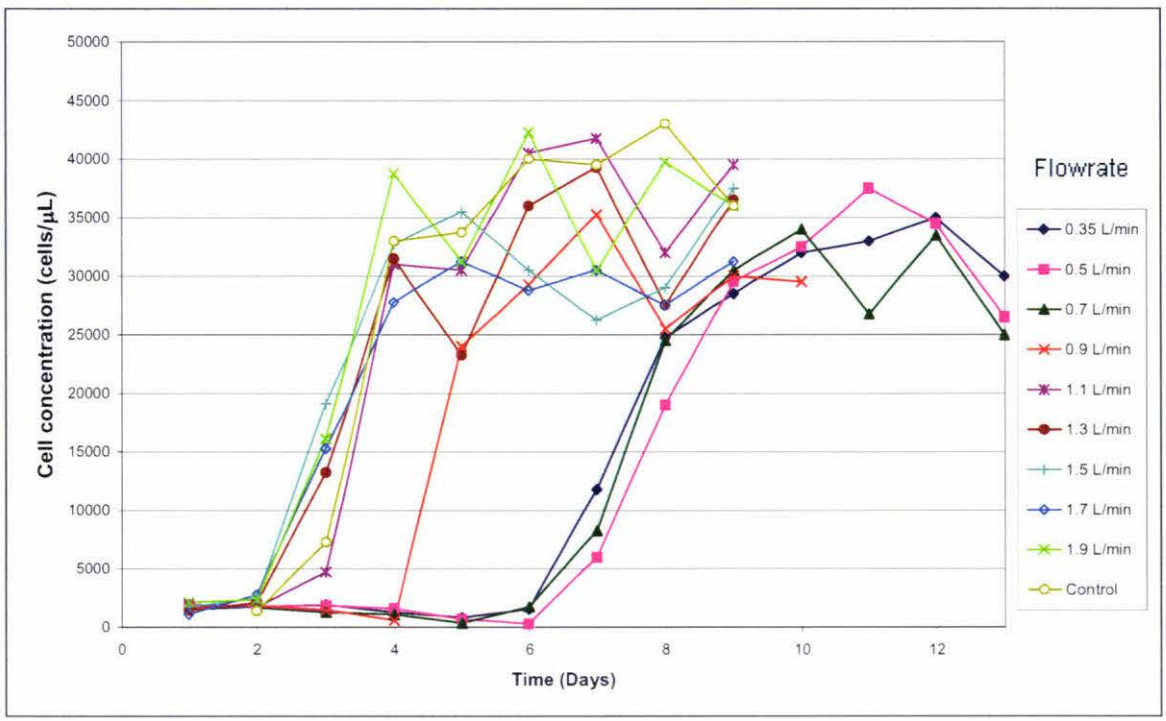


Figure 57 – The cell concentration of *C. calcitrans* grown in water treated at different flow rates (L/min) in the glass and tube pasteuriser (75°C)

For the repeat of this experiment, at 95°C, the flow rates started at 0.9 L/min as it seemed reasonable that if the culture did not grow immediately at 0.35 - 0.9 L/min and 75°C then it was unlikely that it would grow instantly at the same flow rate and a higher temperature. This reasoning proved to be correct (Figure 58) and the 0.9 and 1.1 L/min treatments did not grow as quickly as the other treatments in the first 24 hours after inoculation. Following the initial lag phase the slow to start cultures did grow almost identically to the other treatments.

The reason for this slow start is unclear and no literature has been found that describes this behaviour. The problem of extended lag phase in water treated at a lower flow rate is not considered to be related to pH as the pH of the water after treatment was ~8.2. The most plausible explanation would be that the water treatment is having an effect upon one or more elements in the water. The element(s) involved would need to be those that are not part of the CAW nutrient medium; as if they were one of these they would be replaced when the CAW medium was added. One of several hypotheses was that there was some element present in several chemical species in equilibrium. When the water was treated, this equilibrium was driven to one side or the other and there was a resultant dominance of one element species over the others. This species would not be bio-available and therefore no, or low, growth occurs. Over the ensuing days, the equilibrium

shifts back to its normal position and the element species that are bio-available occur again and the algae are able to grow.

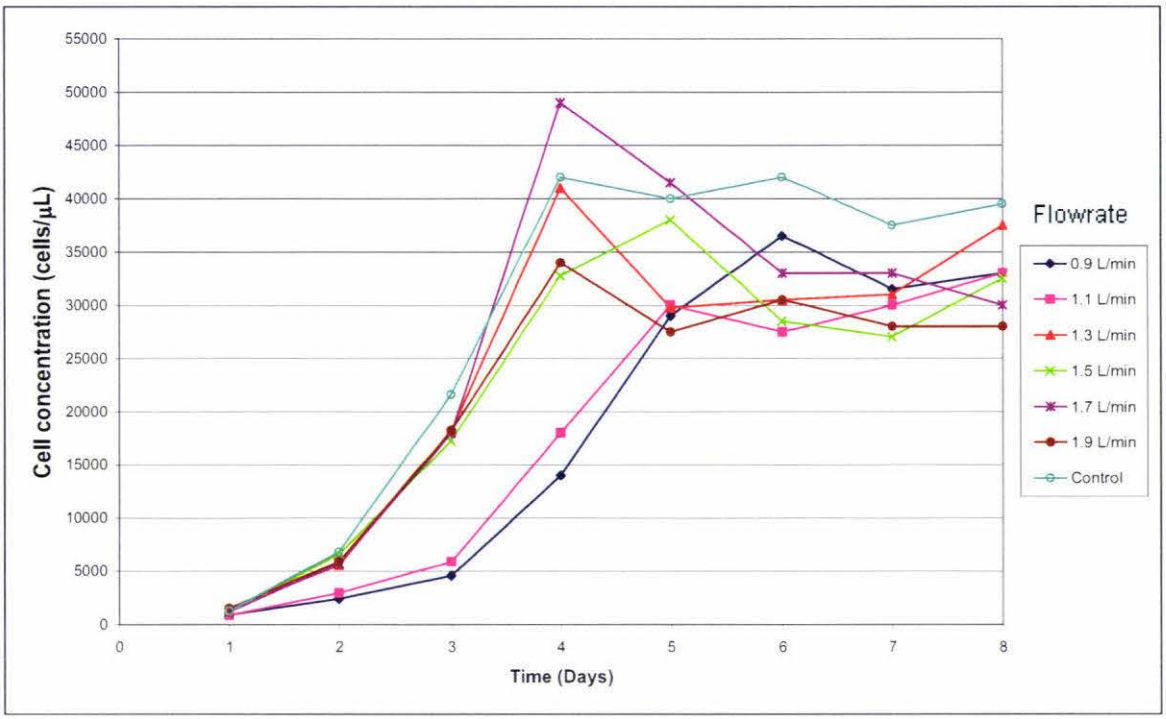


Figure 58 – The cell concentration of *C. calcitrans* grown in water treated at different flow rates (L/min) in the glass and tube pasteuriser (95°C).

This particular hypothesis was supported by the evidence of a 20% aliquot of water treated in the autoclave or Seasalter pasteuriser being sufficient to ensure growth in the first 24 hours. This slow/no growth effect does not occur in autoclaved water which is heated for longer and to higher temperatures (121°C). The difference between this and the pasteurised water is the pressure during treatment. Water heated in the pasteuriser was not under the same pressure and so a large proportion of the dissolved gases in the water are released from solution, hence the need for the degassing systems. This release of gases may affect the equilibrium of the element concerned.

The results of this experiment indicate that water at a flow rate of 1.1 L/min still causes some residual slow growth and so the system was run at 1.3 L/min for future experiments. The results in Section 4.3.2.7 show that there were no detectable bacteria at that flow rate (Figure 55) and therefore that 1.3 L/min is an acceptable flow rate to work with for future trials of continuous culture.

4.3.2.9 Cell concentration of *C. calcitrans* in autoclaved water in the presence of the proprietary product "Selleys Knead It" putty

The cell concentration of *C. calcitrans* cultures (Figure 59) that were grown in the presence of beads of "Knead It " putty were no different to those of the control (0 beads). This result enabled the issue of any toxic effect by the putty to be ruled out.

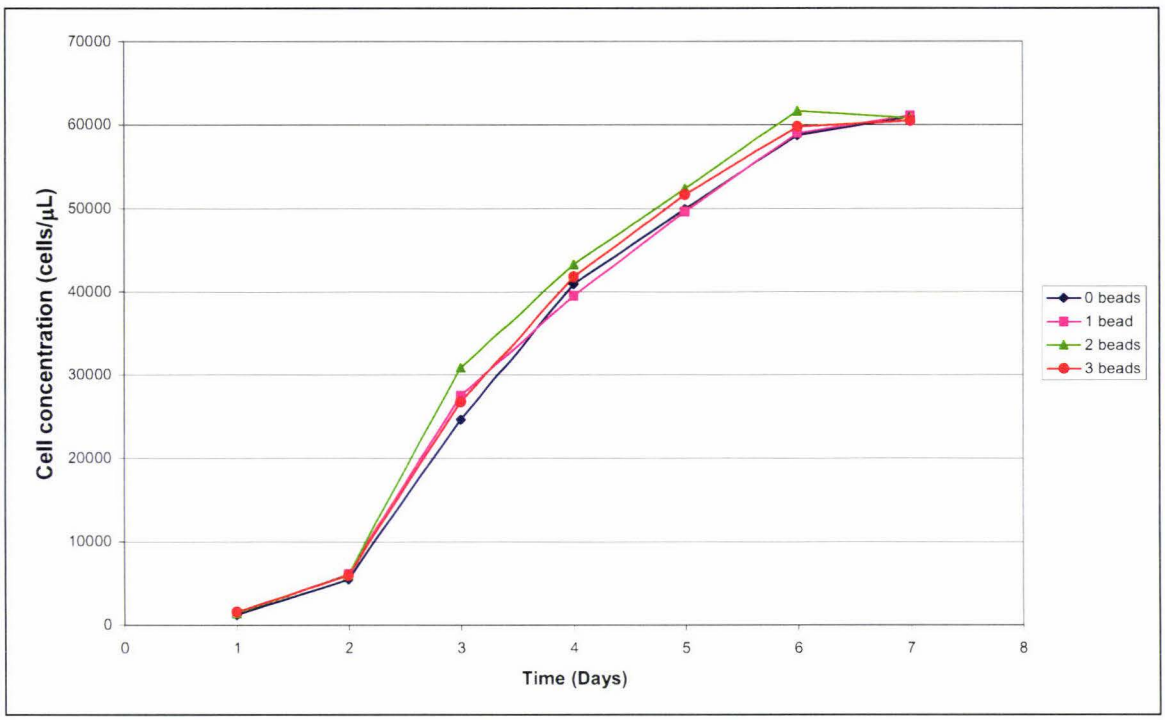


Figure 59 – The cell concentration of *C. calcitrans* cultures grown in the presence of beads of the proprietary product "Selleys Knead It" putty. Cultures contained between 0 and 3 beads of putty in the culture medium before autoclaving.

4.3.2.10 Continuous culture of *C. calcitrans* in bags with water treated in the full scale glass tube and silicon hose pasteuriser

The bags took 2 – 3 days to fill up to the point where the 'algae out line' was inserted into the bag. The day the culture began to flow out of the bags was the first day the pH and cell concentration were recorded. From this time (Day 1) the concentration of *C. calcitrans* in the culture bags continued to rise from approximately 9000 cells/µL to 17290 cells/µL for Row 17 and 20260 cells/µL for Row 18 after Day 5 up until Day 26

(Figure 60). The pH for the two rows averaged 7.85 and 7.89 respectively. On Day 15 the pulsing rate of the nutrient injection pump was altered to increase the amount of nutrients being added to the system by 75% for 8 days. There was no definite effect upon the cell concentrations, although the cell concentration in Row 18 did fluctuate more than Row 17. The pH levels were similar. The lack of increase in the cell concentrations in the rows would indicate that the bag cultures were at a point where further growth did not occur due to light limitation. Light is the energy source for photoautotrophic organisms and at the level of light supplied to these algae cultures (100 $\mu mol.m^{-2}.s^{-1}$) the rate of photosynthesis is directly proportional to light intensity (Pulz, 2001).

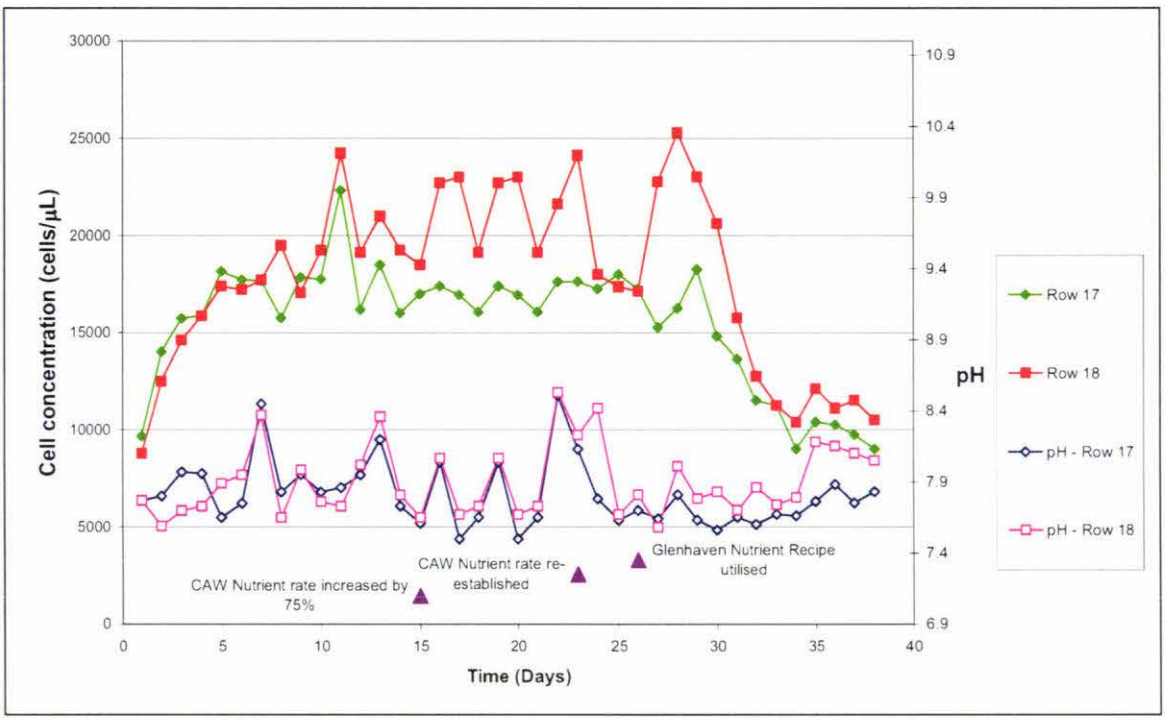


Figure 60 – The average cell concentration and pH level of two rows (8 bags/row) of *C. calcitrans* grown in continuous culture in water treated by the glass tube and silicon hose pasteuriser.

Although the cell concentration of Row 18 was not as steady over the growth period as Row 17 was, the overall impression was that for the 10 days after Day 13 the cell concentration of Row 17 stayed at an average of 17100 ± 1100 cells/µL. In Row 18 the ten day average was 20475 ± 3650 cells/µL. The specific growth rate (µ) in continuous culture conditions at steady state cell numbers is equal to the dilution rate (D = hr^{-1} (volume of bag (L)/bag flow rate (L/hr)); Rhee, 1980). Accepting that the cell averages per row are steady state values, then µ for the bags in continuous culture is 0.025 hr^{-1}. This compares with 0.086 hr^{-1} in the CAW medium in a 1 L flask.

In a semi-continuous system (Krichnavaruk *et al.*, 2005) μ was 0.0965 hr^{-1}. The cell concentration in this airlift photobioreactor (17 L) was 4086 cells/μL, less than a quarter of the density of the cell concentration of the bags in Row 17. The system was harvested (8.5 – 12.0 L) every 12 hours and refilled with new medium. The resultant productivity of this system was 9.45×10^9 cells/L day and produced 20 L of culture per day (Figure 61). Samonte *et al.* (1993) investigated the economic costs of *C. calcitrans* in batch culture. Their results indicated that they could produce a 200 L batch of algae with a productivity of 6.4×10^8 cells/L day. The daily productivity of one 38 L bag of *C. calcitrans* from the glass tube and silicon hose pasteuriser system is 1.12×10^{10} cells/L day. That is an increase of more than 15% upon the production of the 17 L airlift photobioreactor. The highest productivity was in the 20 L batch culture carboys produced at the GACL (1.49×10^{10} cells/L day).

Despite the better performance of carboys compared to bags there are several limitations related to carboys. The economic limitation of carboys is discussed later in this section, but it is suffice to say that despite the better performance, with respect to cell production, of a carboy compared to a bag, bag cultures are more than six times more efficient on a staff labour cost basis.

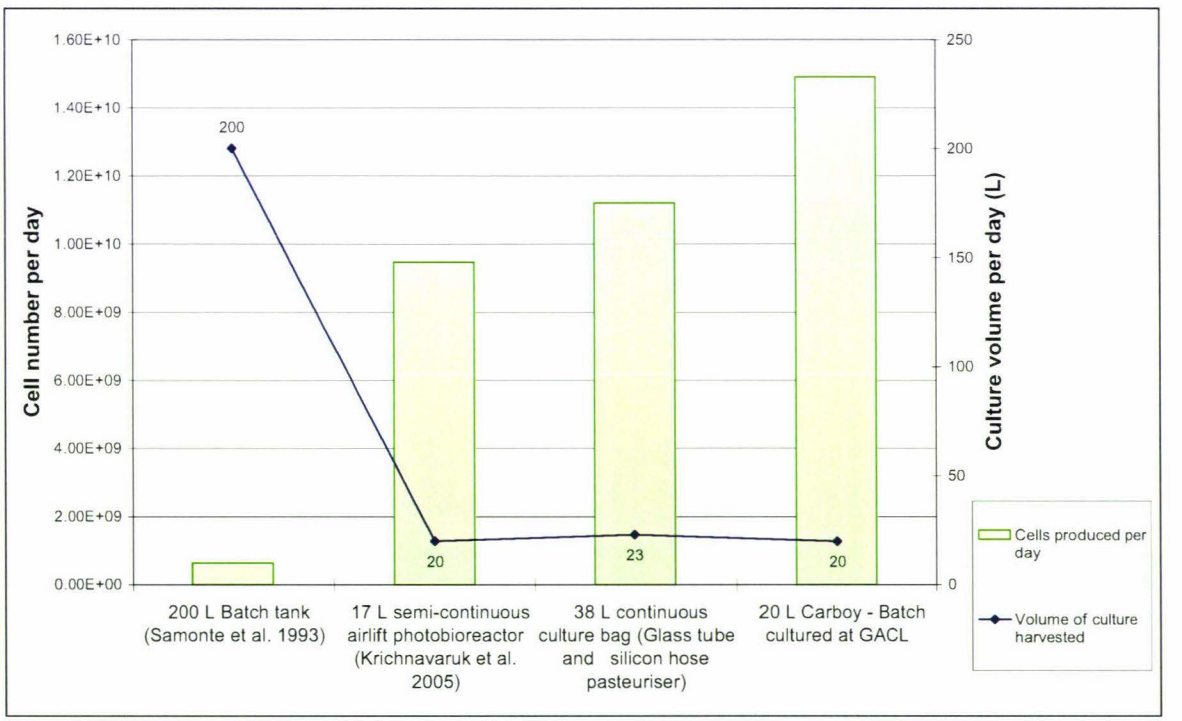


Figure 61 - The daily harvest volume and number of *C. calcitrans* cells harvested in 4 systems: 200 L batch culture, 17 L airlift photobioreactor, a 38 L 'bubble column' bag and a 20 L batch cultured carboy.

From an aquaculture perspective, where algal production is the most expensive part of a shellfish hatchery, the prospect of utilising approximately 50 airlift photobioreactors as used by Krichnavaruk *et al.* (2005) to produce 1000 L of *C. calcitrans* per day would appear to be very expensive. Not only are acrylic systems such as this expensive, they are difficult to clean between culture runs. There would also be considerable work involved in the harvesting and refilling of 50 such systems every 12 hours.

The alternative, developed in this work, would be to have 48 bags in 6 rows of 8. Each new bag is cheap and sterile upon filling. After setting up, such a system needs 11 minutes of staff labour per row per day. Further testing of the silicate concentration of the daily injections (Section 3.3.2.10) should be able to reduce the injections to every second day and consequently the staff labour cost to 11 minutes every second day.

In further work with the system at GACL it would be of interest to trial the addition of an extra light frame on the outside of a row of culture bags. Each outer light bank could be of such a height as to still allow access to the base of the bag for any maintenance to air injection ports as well as to the 'algae out lines'. Such a light bank would be approximately 1.4 m in height and could have a reflective surface on the side furthermost from the bags to ensure the maximum amount of light produced could be utilised. Trials would need to be performed to find if there was an improvement in algal biomass and if the level of improvement was economic given the extra energy inputs necessary (lights and air conditioning for heat removal).

The rate of nutrient injection was returned to the original level for the CAW medium 8 days after it had been increased and the cultures in both rows settled back to a stable level (~18,000 cells/ µL) within the next three days. At that time the nutrient media was changed from CAW to Glenhaven medium. The intention was to maintain the rate of nutrient injection; however a handling error caused the pump injection rate to be accidentally pushed up to 200% of the original rate. Because of that incident there was a fluctuation in the cell concentrations of both rows of *C. calcitrans* culture. Once this error was identified on the second day of the Glenhaven medium being used it was corrected and the cell concentrations in both rows dropped down in the following days to reach a level of about 11,000 cells/µL. The pH of Row 18 increased from an average of 7.8 in the days immediately after the nutrient recipe was changed to an average of 8.1 in the last four days that this experiment was monitored. The pH of Row 17 rose slightly over the period outlined above, changing from 7.6 up to 7.8.

In the first trial to culture *C. calcitrans* continuously it was observed that some cells altered morphology and size from the normally appearing cells (Figure 62), some to a great extent (Figure 63). The initial hypothesis was that a different alga had entered the system and infected the bags. This possibility was checked by taking samples of the algae in bags and re-culturing them in Glenhaven media in 12-well tissue culture dishes. The samples had individual cells of different shape "picked" from them and re-suspended

in fresh medium. These cells were cultured over ten days and then assessed for their appearance. Some of the cells were found to have divided and returned to the normal morphology for *C. calcitrans* cells whilst others still appeared distended (Figures 63, 64, 65 and 66). The appearance of setae on the differently shaped cells are an indication these cells are diatoms. Staff from the Phytoplankton Monitoring Group at The Cawthron Institute (Nelson) considered that the large cells were not consistent with any genus of algae that was common within the Nelson area, and were not consistent with any other alga they were familiar with (Gladstone, M. and Langi, V. pers. comm.).

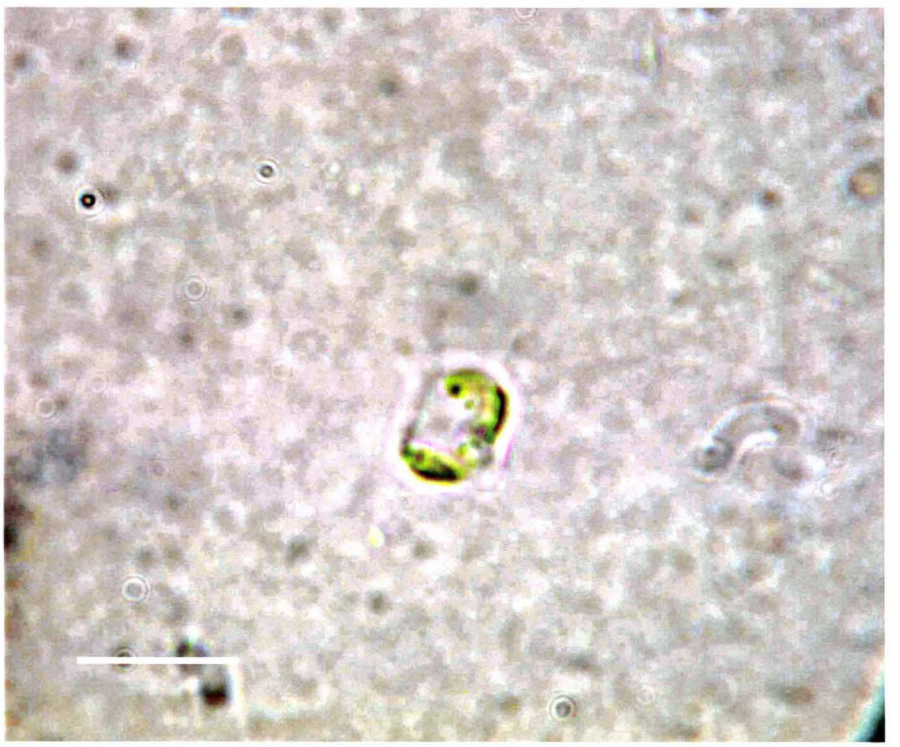


Figure 62 –A normal *C. calcitrans* cell from the continuous culture system. The setae can be seen on the top and bottom right corners. Setae on the left side were not in focus. (Bar = 10 µm)

Distended cells appeared when the supply of sodium metasilicate into the bags was below the level suited to the CAW medium. In the first trial of the glass tube and silicon hose pasteuriser system the amount of sodium metasilicate necessary for the CAW medium was calculated incorrectly by a factor of 2. Bags received a 20 mL injection of autoclaved sodium metasilicate [120 g/L] every second day. This error was detected after the first trial was completed. In the second trial the silicate injections were given every day and cells had a more normal appearance. The other instance where the metasilicate level was considered to be sub-optimal was during the second trial when the nutrient recipe was changed from CAW medium to Glenhaven medium on Day 26. The appearance of the distended cells occurred on Day 30, four days after the medium was changed. Sodium metasilicate injections (30 mL) were given every second day at a concentration of 30 g/L during this section of the trial. In CAW medium the bags

received injections every day. The injections were 20 mL and had a concentration of 120 mg/L. As previously noted (Section 4.1.4), Laing (1985) found a direct correlation between the limitation of silica and an increase in cell volume.

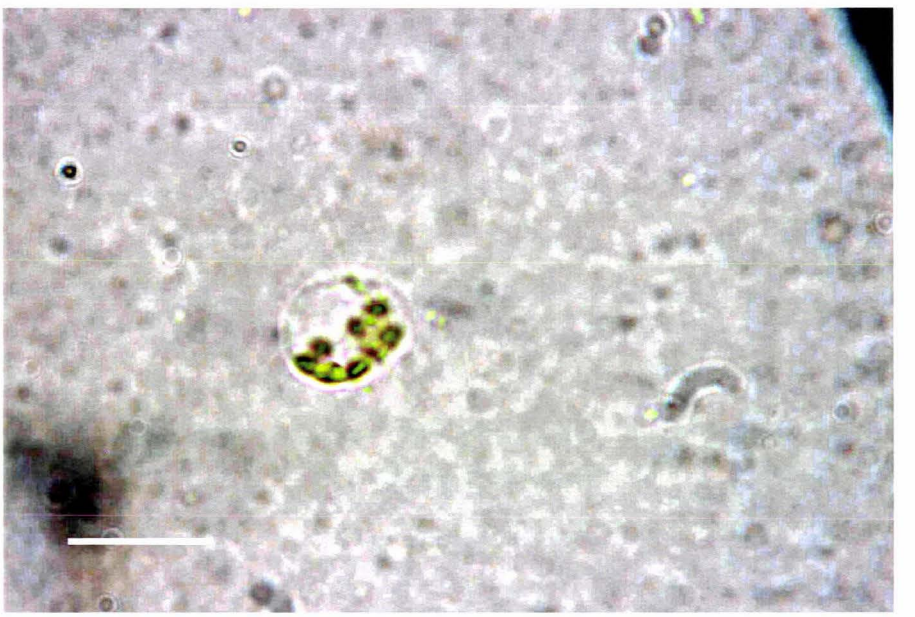


Figure 63 – A distended *C. calcitrans* cell. Two setae are visible. (Bar = 10 µm)

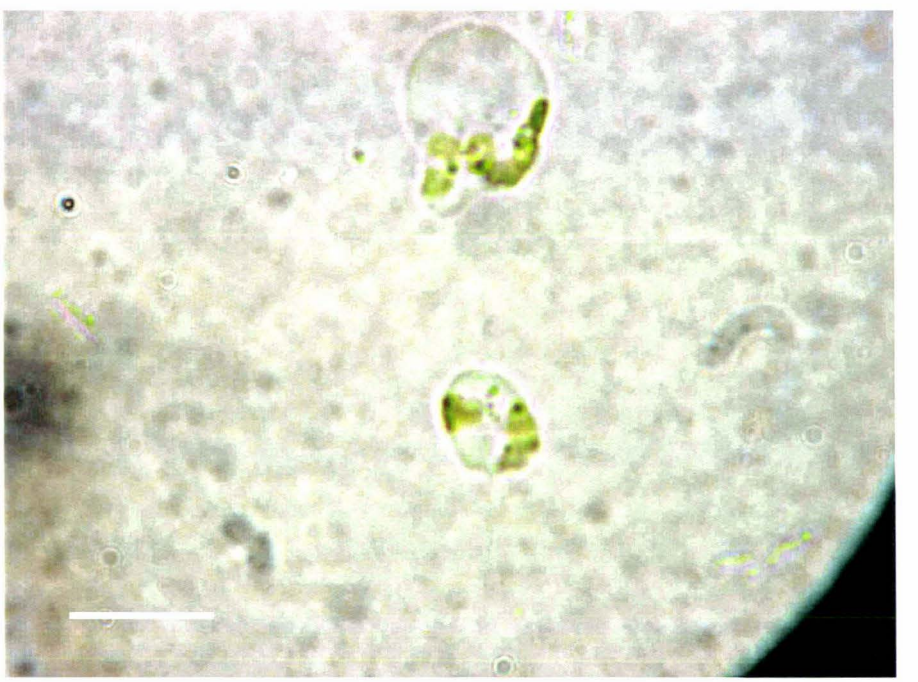


Figure 64 – Two cells from a bag of continuously cultured *C. calcitrans*. The cell at the top is distended. One seta is in focus in the cell at the bottom. (Bar = 10 µm)

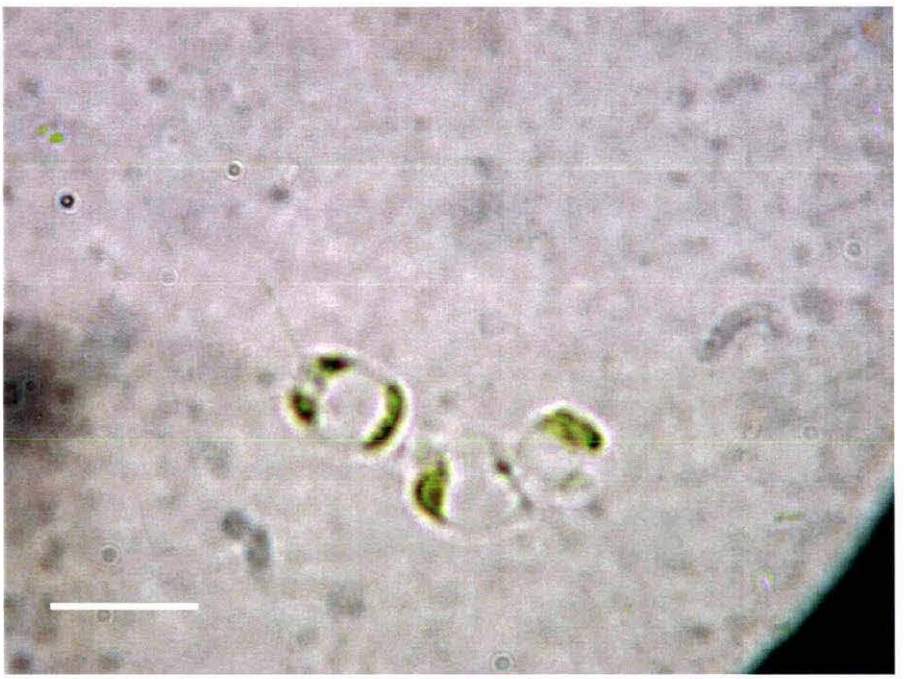


Figure 65 - Cells from a bag of continuously cultured *C. calcitrans*. The two cells to the left are distended more than a normally appearing cell (right). Setae are visible on the cell on the left. (Bar = 10 µm)

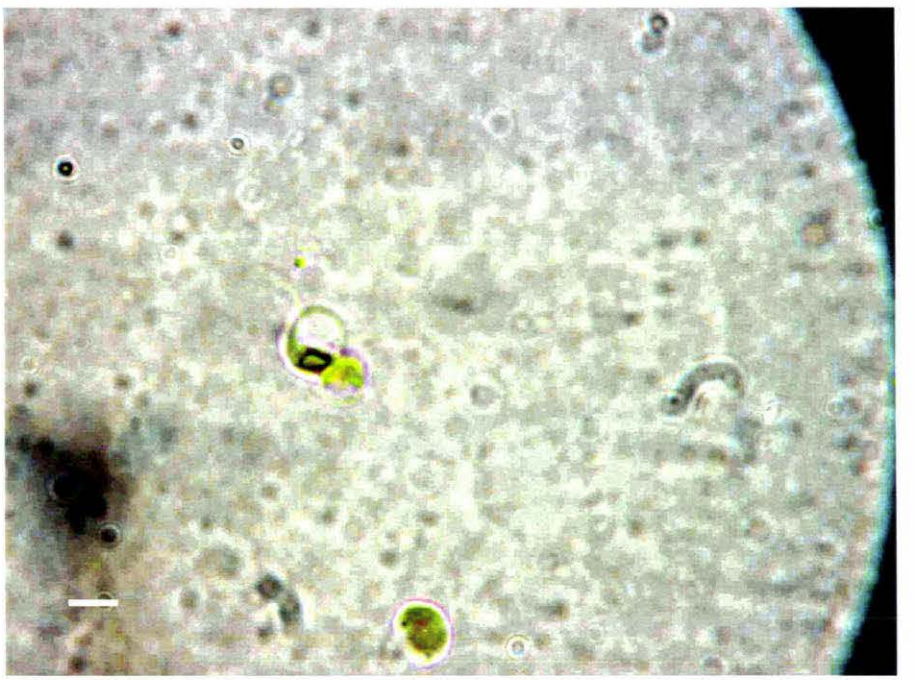


Figure 66 - Cells from a bag of continuously cultured *C. calcitrans*. The larger of the two cells in the centre of the image has visible setae. It has a distended morphology compared to the cell immediately beside it. (Bar = 10 µm)

A consideration of any commercial algae culture system is the economic cost of producing the algal biomass (Samonte *et al.* 1993). Laing (1985) determined that the cost of producing algal cultures in 80 L vessels (including labour and depreciation of capital items) was about 75% of the cost of producing it in 200 L vessels. A comparison of the time taken to produce *C. calcitrans* in 20 L carboys and in continuous culture bags identified the economic advantages as they relate to staff input of the continuous system (Table 20). From a breakdown of the time costs of preparing 4 × 20 L carboys/day for one month compared to the preparation of 1 row of 8 bags to be run for one month, on an hourly basis the continuous culture system produces more than six times as much *C. calcitrans* culture for each hour of staff labour.

This comparison does not take into account energy differences involved but it must be noted that, despite the cost of running the water heater in the continuous system most likely being higher than the running costs of the autoclave, all carboys used at GACL are treated at the Cawthron Institute which is 15 kilometres away from GACL. This is the reason for the high handling costs of carboy preparation. In the above comparison the running and capital costs of the van that was used have not been taken into account. In the instance where an autoclave was on-site at a hatchery, the capital cost of such equipment would be between $40k and $120k depending on whether the machine was second-hand or new. Over time this cost would reduce due to depreciation, but even if carboy preparation occurred on site the volume of algae per hour of input would only change to 87.3 L/hour. The continuous system would still be greater than 5 times more efficient in this circumstance.

In recent time, the shellfish aquaculture industry has begun to utilise flow-through systems for the rearing of larvae. This will require a continuous feeding system to ensure food (algae) levels are maintained at all times. To further enhance these systems will require feeding the larvae with fresh algae that have not been stored in 'feed-out' tanks for up to 24 hours. With a continuous culture algae system, the algae can be pumped from a harvest tank directly into the larval rearing tanks.

Process	Time allocation	Carboys		Continuous system		
		Daily Cost (minutes)	Monthly cost (minutes)	Daily Cost (minutes)	Weekly cost (minutes)	Monthly cost (minutes)
Wash (carboy + lid)	2 minutes/ea	8	248			2
Fill carboy (water, nutrients, assemble lid)	3 minutes/ea	12	372			3
Load carboys into van	2 minutes/ 4	2	62			
Unload at Cawthron - up to autoclave	4 minutes/ 4	4	124			
Load Autoclave	20 minutes/ 4	20	620			6
Load into van	4 minutes/ 4	4	124			
Unload at Hatchery	2 minutes/ 4	2	62			
Transfers (200mL – 3000mL – carboy)	15 minutes/ 4	15	465			10
Prepare nutrient carboy	7 minutes				7	31.5
Prepare Si injection	5 minutes/ 8			5		155
Perform Si injection	6 minutes/ 8			6		186
Steam clean	1 hr/fortnight				30	135
Preparation and inoculation of bags (8)	16 minutes/bag					128
Monthly cost (hrs)			34.6			10.9
Volume of algae produced/day (L)			80			160
Volume of algae produced/month (L)			2480			4960
L of algae per hour of input			71.6			453.3

Table 20 – Comparison of the cost in time for carboys (4/day) and one row of continuously cultured *C. calcitrans* on a monthly basis.

4.3.2.11 Quality of *C. calcitrans* grown continuously in bags with different rates of aeration

The assessment of cell aggregation at the waterline of bags of *C. calcitrans* grown continuously at two different rates of aeration identified differences in the quality of the cultures. The aggregations that occurred in the bags that were at the high rate of aeration (4 L/min) were not assessed for their composition, but were assumed to be *C. calcitrans* cells (Figures 67 and 68). The potential for these accumulated cells to be a substrate for bacteria growth would be high (Gibson, L. pers. comm.).

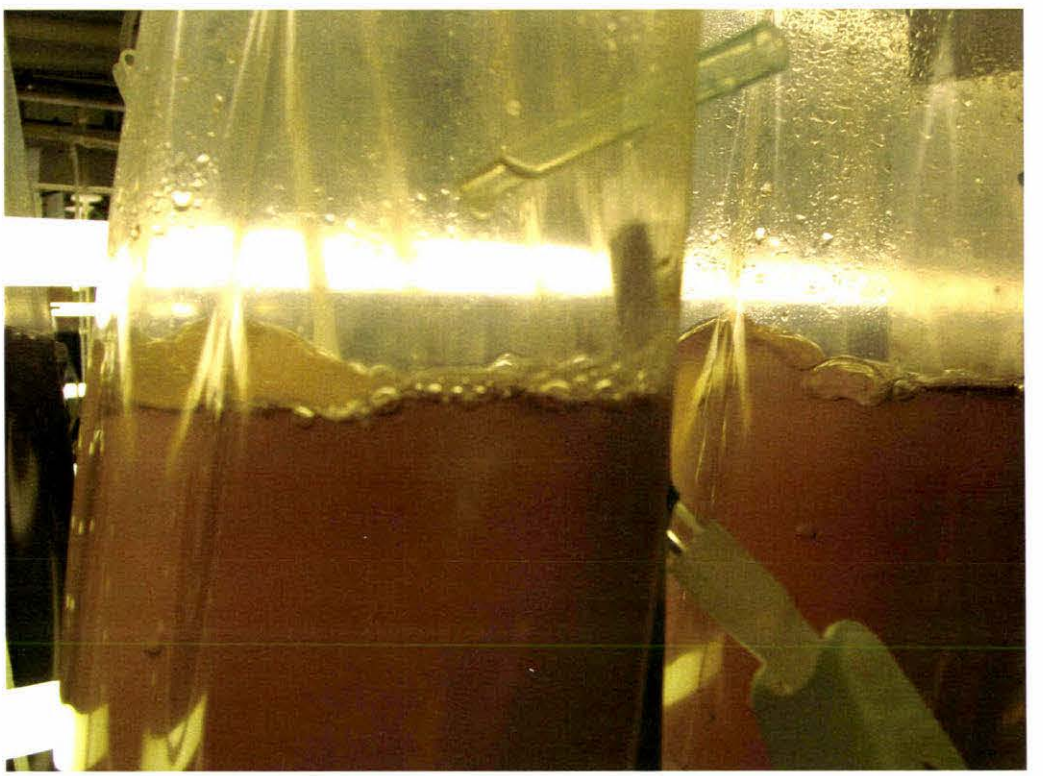


Figure 67 – The waterline of a bag of *C. calcitrans* growing continuously for two days with an aeration rate of 0.9 L/min.

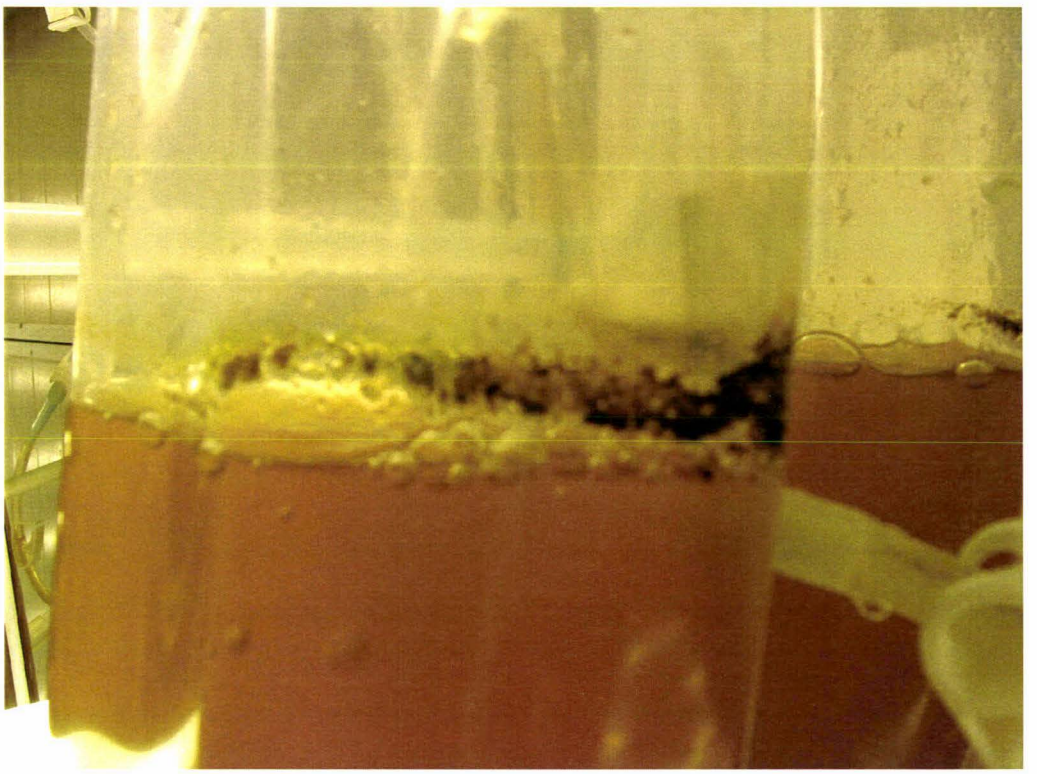


Figure 68 - The waterline of a bag of *C. calcitrans* growing continuously for two days with an aeration rate of 4.0 L/min.

The standard method of comparing air flow rates in culture vessels of different sizes is to compare the superficial gas velocity of each system. The superficial gas velocity was calculated using the equation:

$$U_t\ (m/sec) = \frac{\text{Flow rate } (m^3/sec)}{\text{Cross sectional area } (m^2)}$$

In regard to U_t in culture vessels for microalgae the units are commonly converted from m/sec to cm/sec. Table 21 compares the superficial gas velocities of the continuous culture bags in the Seasalter system at GACL (culturing *Chaetoceros muelleri* and *Isochrysis galbana* "Tahitian" strain), the continuous culture bags of *C. calcitrans* at GACL in the glass tube and silicone hose pasteuriser, *C. calcitrans* cultures in an airlift photobioreactor (Krichnavaruk *et al.* 2005), and a vertical tube reactor at two different flow rates (Miyamoto *et al.* 1988; culturing cyanobacteria, green algae and diatoms).

The aeration rate of the Seasalter system used at GACL has a U_t of 0.40 cm/sec. This has been shown to be acceptable for all species of microalgae (*Pavlova lutheri, Thalassiosira pseudonana, Tetraselmis suecica, Chaetoceros muelleri* and *Isochrysis galbana* "Tahitian" strain) cultured in this system except for *C. calcitrans*. When *C. calcitrans*

was cultured continuously aerated at U_t 0.09 cm/sec the waterline remained clean (Figure 67). When *C. calcitrans* was cultured with a U_t of 0.40 cm/sec an aggregation of biomass formed at the waterline of the bag that suggested a detrimental effect upon the cells (Figure 68).

Superficial Gas velocity (cm/sec)	**Seasalter continuous culture system bags at GACL (*C. muelleri, I. galbana, P. lutheri*)**	**Continuous culture bags of *C. calcitrans* at GACL**	***C. calcitrans* in an airlift photobioreactor (Krichnavaruk *et al.* 2005;**	**A vertical tube reactor at two settings (cultured cyanobacteria, green algae and diatoms; (Miyamoto *et al.* 1988)**	
U_t	0.40	0.09	3.0	0.08	0.42

Table 21 – A comparison of the superficial gas velocities (cm/s) in algae cultured in bags, an airlift photobioreactor and a vertical tube reactor.

The U_t for the *C. calcitrans* culture in bags grown in water treated by the glass tube and silicon hose pasteuriser was 0.09 cm/sec. Because it was comparatively low there was the need for the rate of additional CO_2 to be increased above the level used in the comparative Seasalter system (U_t = 0.40 cm/sec, CO_2 addition 1% v:v). The rate of CO_2 addition required to maintain the pH at between 7.6 and 8.00 in *C. calcitrans* was 2.14% v:v.

Miyamoto *et al.* (1988) cultured a range of cyanobacteria, green algae and diatoms in glass vertical tubes (internal diameter 50 mm) and found that a U_t of 0.08 to 0.42 cm/sec was sufficient for mixing of the culture and to ensure the pCO_2 was at a level that supported maximal productivity. The researchers did find that any U_t above the higher rate of 0.42 cm/sec caused serious foaming and a loss of culture.

In an airlift photobioreactor (17 L culture volume) a U_t of 3.0 cm/sec was observed to result in the highest cell concentration of *C. calcitrans* (9000 cell/µL; Krichnavaruk, 2005). Four superficial gas velocity rates (2, 3, 4 and 5 cm/sec) were compared and each incremental increase in U_t improved the cell concentration over time, with the exception of the 5 cm/sec U_t treatment. This treatment did not grow well and the final cell concentration was 2500 cells/µL. It is suggested that the high superficial gas velocity in the column damaged the cells in culture.

4.3.2.12 The effect of water treated by the Seasalter pasteuriser system upon *C. calcitrans* grown continuously in the glass tube and silicon hose pasteuriser system

The cell concentrations of the control bags rose in a similar pattern to those in Section 4.3.2.10. The cell concentration of the bags that were seeded with water from the Seasalter pasteuriser system rose on the second day and then reduced rapidly to 625 cells/μL by Day 4 (Figure 69).

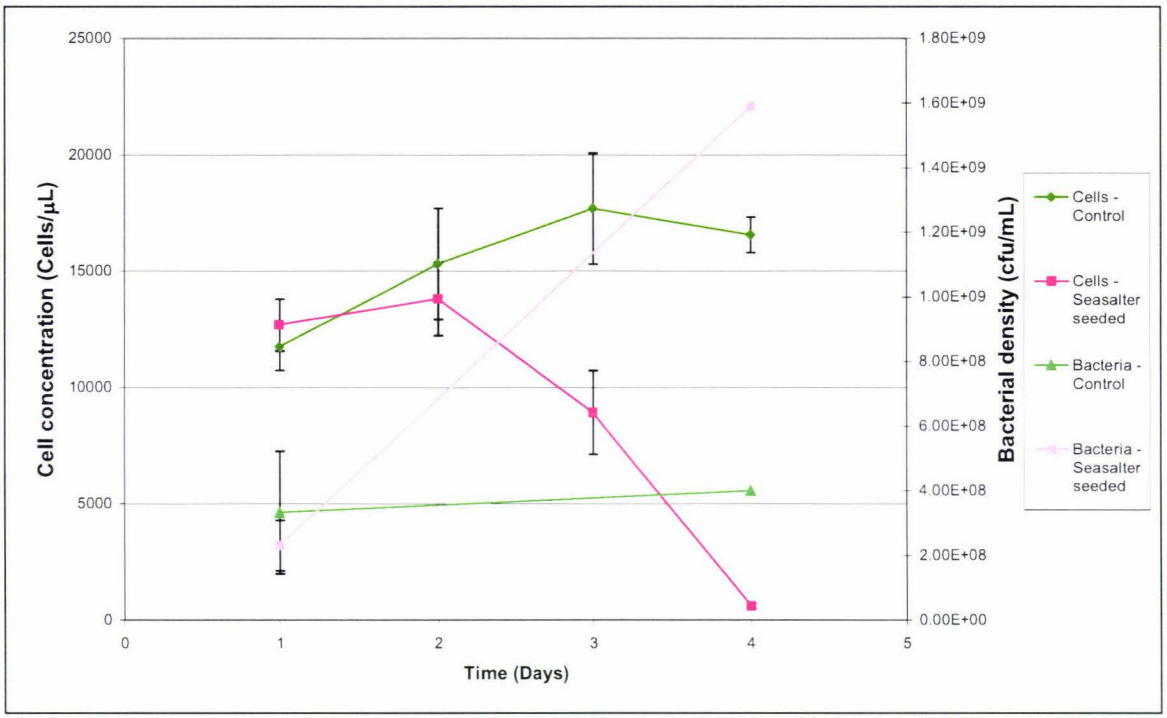


Figure 69 – The cell concentration of *C. calcitrans* and the bacterial density in bags cultured continuously in water treated by the glass tube and silicon hose system. Four bags were seeded with 1 L of water that was treated in the Seasalter pasteuriser system. Error bars represent the standard error (n=4).

The density of bacteria in the control bags remained at a similar level (4×10^8 cfu/mL), but in the Seasalter seeded bags the bacterial density increased by an order of magnitude (1.6×10^9 cfu/mL). The visual difference between the control and treated bags was marked (Figure 70).

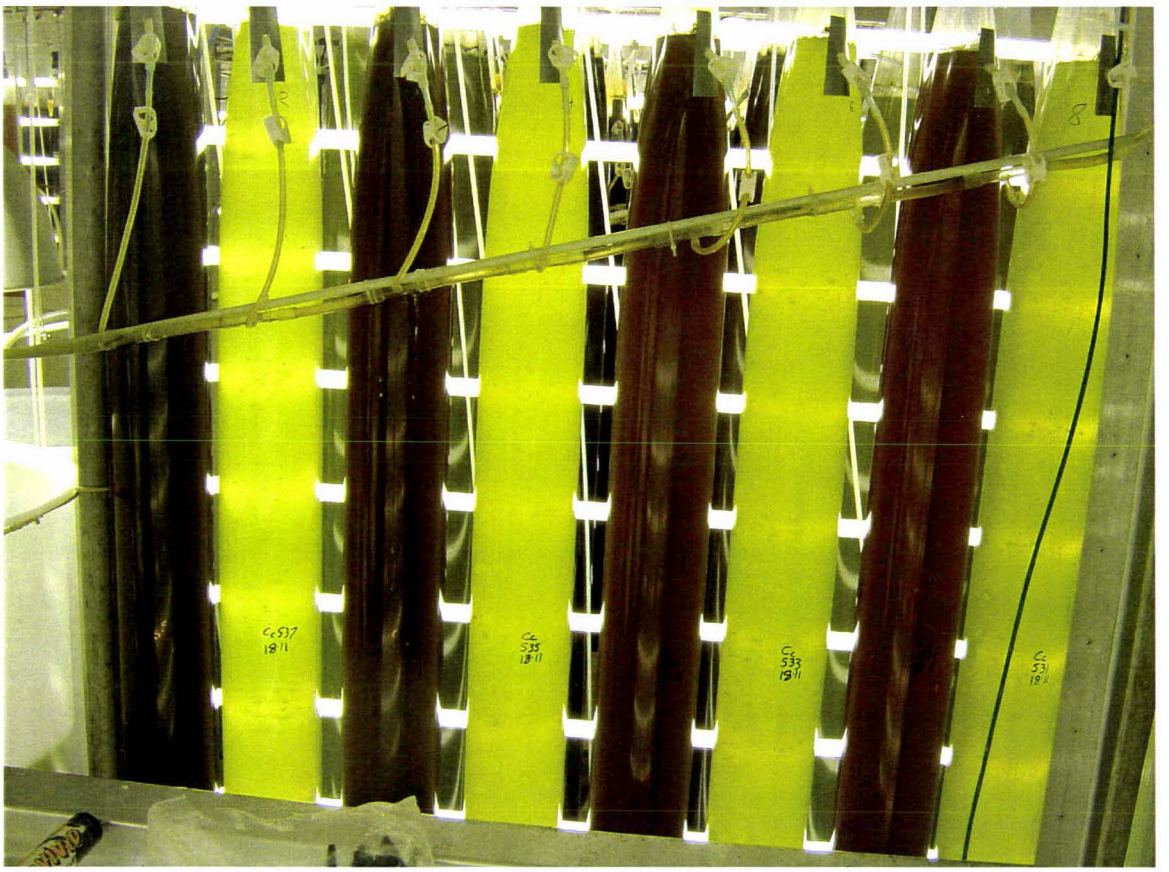


Figure 70 – Bags of continuously cultured *C. calcitrans*. Every second bag (from the left) was seeded with a 1 L aliquot of water treated in the Seasalter pasteuriser system four days previously.

Although no literature was available to compare the effects of bacteria in continuously cultured systems, many reports have been published of the detrimental effects of bacteria infecting algal cultures (Baker and Herson, 1978; Hirayama & Hirayama, 1993, 1996, 1997; Fukami *et al.* 1992, 1997).

Chapter 5 Conclusions

To the author's knowledge, this is the only report of successful continuous culture of *C. calcitrans* in a photobioreactor system that is simple and cheap enough for use in shellfish hatcheries at a realistically large scale.

5.1 Conditions for optimal growth

The improved medium (CAW) for *C. calcitrans* growth developed in this work is very similar to the Conway recipe of Laing (1979). The major difference between these two recipes is the greater amount of sodium metasilicate in the CAW medium. No pronounced salinity optimum was found, but results were in agreement with Laing (1979) and 15‰ was employed for most experiments. The use of Pluronic F-68 and Antifoam A did not have any deleterious effect on cell growth. Further work with these additives may be warranted where shear stress within a bioreactor is problematic.

C. calcitrans in bags must have correct nutrient medium supplied to it as nutrient depletion (e.g. sodium metasilicate) may cause cell morphology to alter. This aspect requires further confirmation.

Air sparging in culture vessels must be controlled to ensure shear stress, related to bubbles bursting, does not cause cell damage. Carbon dioxide addition must be regulated to give optimal pH at all air flow rates.

5.2 Bacteria and *C. calcitrans* culture

The presence of a range of bacterial strains was detrimental to the culture of *C. calcitrans*. Marine agar was acceptable for culturing many of those bacteria and is suitable for monitoring the level of bacterial infection in *C. calcitrans* cultures in a hatchery environment.

5.3 Water treatment methods for the batch and continuous culture of *C. calcitrans*

5.3.1 Water treated by electrolysis

The use of water that had been electrolytically treated to reduce bacterial activity was found to support *C. calcitrans* growth. However, the surviving bacteria or spores, and bacteria from the *C. calcitrans* inoculum were able to grow to higher levels in water treated in this way. The overall effect was detrimental upon the algal culture. Although water treated this way is not currently suitable for algal culture, there are many other aspects of hatchery work where this system could be useful.

5.3.2 Treating water with heat

The heating of water to 95°C for ten and a half minutes reduced bacterial activity to a level that is suitable for the batch and continuous culture of *C. calcitrans* in flasks, carboys and bags. Water treated in a 60 L heated kettle (95°C) did not provide sufficient reduction of bacterial activity due to non-uniform flow of the water in the kettle. The non-uniformity of flow meant that a portion of the water was treated at this temperature for one minute or less. Water that was heat treated whilst flowing through a 235m long pipe was more suitable for culturing *C. calcitrans*. The length of time the water was retained in the pipe system had an effect upon the growth of the algae culture and the activity of bacteria after the treatment process. Water treatment at 95°C for longer than 13 minutes caused a longer lag phase in the *C. calcitrans* culture. Water treated for less than nine and a half minutes contained bacteria or spores that were culturable on Marine Agar. Heat treated water (95°C) for ten and a half minutes was able to support the continuous culture of *C. calcitrans* for 38 days without bacterial infection causing the culture to crash. Continuous algae culture in long thin bags is a more efficient system in regard to cost of labour compared to batch systems used at GACL.

Appendix 1

Recipe for Glenhaven Medium

Solution A

$FeCl_3.6H_2O$	26.0 g
$MnCl_2.4H_2O$	7.2 g
H_3BO_3	672.0 g
Sodium EDTA (di-sodium salt)	900.0 g
$NaNO_3$	2000.0 g
Solution B	20.0 mL

Make up to 20 L with distilled water

Solution B (Trace metal solution)

$ZnCl_2$	21.0 g
$CoCl_2$	20.0 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	9.0 g
$CuSO_4.5H_2O$	20.0 g
HCl [37%]	100 mL

Make up to 1 L with distilled water.

Solution C

Vitamin B_{12}	50 mg
Vitamin B_1	1 g

Make up to 1 L with distilled water.

Solution D

$NaH_2PO_4.2H_2O$	20.0 g

Make up to 1 L with distilled water.

Solution E

$Na_2SiO_3.5H_2O$	30.0 g

Make up to 1 L with distilled water.

Culture medium preparation

To prepare 1 L of algae culture medium, add the following volumes of the 5 solutions and make up to 1 L with seawater of the desired salinity.

Solution A	1.0 mL
Solution C	0.1 mL
Solution D	1.0 mL
Solution E	3.0 mL

Appendix 2

Sodium thiosulphate calculator

The calculations required for determining how much sodium thiosulphate is needed for a given volume of seawater with a known concentration of sodium hypochlorite. By creating a spreadsheet with the formulas below in Microsoft Excel and then entering the known values (in bold) the calculator provides the volume of sodium thiosulphate stock needed.

	B	C	D
6	**Sodium Hypochlorite - Sodium Thiosulphate**	Hypochlorite Mol. Weight.	Thiosulphate Mol. Weight.
7	**Calculator**	g/mol	g/mol
8		74.442	160.1248
9	Enter the concentration of Total chlorine		
10	mg/L =	**5**	
11	Enter the volume of tank		
12	Total L of water	**1**	
13	Total mg of Cl	=+C10*C12	
14	Total mols of Cl	=+C13/1000/C8	
15	Stock solution of sodium thiosulphate (g/L)	**10**	Enter the concentration of thiosulphate
16	Total µL of thiosulphate stock	=+C17*1000	
17	Total mL of thiosulphate stock	=+C18/C15*1000	You need this many mLs of the stock solution
18	Total g thiosulphate	=+C19*D8	
19	Total mols of thiosulphate needed	=+C14*5/8	
20	**If solution is diluted**		
21	Level of Dilution	eg. 1:5 enter as =5/1	
22	Enter dilution ratio	**=5/1**	
23	mLs of thiosulphate stock	=+C22*C17	You need this many mLs of the stock solution

Appendix 3

Bacterial Strain Stock Sheets

Date 8.5

Strain 34 Source

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	Yes	Rod	31×10^4		

Name

Stock identity number

Date 10.5

Frozen	Date	Location	ID #
Yes	26.5	E10	DL 55 PC 26.5

34	44	44-2	55				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
6/10			3/10				

Date 8·5

Strain 30 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	-ve	+ve	No	Rod	3×10^4		

Name

Stock identity number

Frozen	Date	Location	ID #
Yes	13·5	A8	04 30 PC 13·5

Date

10·5

30							

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
3/10			6/10				

Date 8·5

Strain 31 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	-ve	+	Yes	Rod	7×10^4		

Name

Stock identity number

Frozen	Date	Location	ID #
Yes	13·5	A3	04 31 PC 13·5

Date

10·5

31							

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
4/10			OK 6/10				

Date 8/5

Strain 32 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	No	Rod	17×10^4		

Name

Stock identity number

Date 10/5

Frozen	Date	Location	ID #
Yes	13·5	A4	04 PC 32 13·5

32							

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
6/10			2/10				

Date 8·5

Strain 36 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	Yes	Rod	58×10^4		

Name

Stock identity number

Date 10·5

Frozen	Date	Location	ID #
Yes	13·5	A7	04 PC 36 13·5

36							

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
7/10			3/10				

Date 8/5

Strain 37 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	No	Cocci	53×10^4		

Name

Stock identity number				Frozen	Date	Location	ID #
Date				Yes	13·5	A5	04 37 PC 135
10·5							
37							

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
7/10			2/10				

Date 8.5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		Vibrio
-ve	+ve	+ve	mot	Rod	31×10^3		-ve

Name

Stock identity number				Frozen	Date	Location	ID #
Date				Yes	1·6	G7	04 46 PC 1·6
10·5	14·5						
34	46						

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
OK 5/10		0/10	Poor 3/10				

Date 8/5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	mot	Rod	66×10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10·5	14·5			Yes	6	H1	04 PC 44 2·6
35	49						

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
OK 5/10			Poor 2/10		Good 6/10		

Date 8/5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	Yes	Rod	66×10^5		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10/5				Yes	26·5	E8	04 PC 53 26·5
35	47	47·2	53				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
7/10							

Date 8/5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	Yes	Rod	31×10^4		

Name

Stock identity number

Date 10·5

				Frozen	Date	Location	ID #
				Yes	26·5	E9	04 54 PC 26·5
34	44	44-1	54				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
6/10			3/10				

Date 8·5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	Yes	Rod	31×10^4		

Name

Stock identity number

Date 10·5

				Frozen	Date	Location	ID #
				Yes	26·5	F1	04 56 PC 26·5
34	44	44-3	56				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
5/10			2/10				

Date 8/5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		Vibrio
-ve	+ve	+ve	mot	Rod	31×10^3	Comma	-ve

Name

Stock identity number

Date				Frozen	Date	Location	ID #
				Yes	1·6	G 9	04 73 PC 1·6
10/5	14/5	18/5	22-5	31·5	1·6		
34	45	45-2	45-2-2	45-2-2-1	73		

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 3/3			Poor 1/3		Poor 1/3		

Date 8/5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
+ve	+ve	+ve	mot	Rod	31×10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
				Yes	1·6	2F	04 57 PC 1·6
10/5	14/5	18/5	1·6				
34	45	45-1	57 To freeze				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
OK Good 3/3			Poor 1/3		Poor 1/3		

Date 8/5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
+ve	+ve	+ve	mot	Rod	31×10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
				Yes	1·6	F3	04 PC 58 1·6
10/5	14/5	18/5	22·5	1·6			
34	45	45-2	45-2-1	58			

Plate media tested — To freeze

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 5/10			Poor 2/10		Poor 2/10		

Date 8/5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
+ve	+ve	+ve	mot	Rod	66×10^3		

Name — some cocci

Stock identity number

Date				Frozen	Date	Location	ID #
				Yes	1·6	F4	04 PC 59 1·6
10·5	14·5	18·5	22·5	1·6			
35	47	47-1	47-1-2	59			

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 7/10							

Date 8-5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	mot	Rod	66×10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10-5	14-5	18-5	22-5	Yes	1-6	F8	04 PC 63 1-6
35	47	473	473-2	63 1-6			

Plate media tested — To freeze

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 6/10							

Date 8-5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	mot	Rod	66×10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10-5	14-5	18-5	22-5	Yes	1-6	F9	04 PC 64 1-6
35	47	47-3	47-3-1	64 1-6			

Plate media tested — To freeze

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 7/10							

Date 8/5

Strain 35 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
-ve	+ve	+ve	mot	Rod	66x10^3	comma	Vibrio -ve

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10·5	14·5	18·5	1·6	Yes	1·6	G2	04 PC 67 1·6
35	48	48-1	~~mot~~ 67				

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 5/10							

Date 8/5

Strain 34 Source PC

Tests Done

Gram	Catalase	Oxidase	Motility	Shape	Density at isolation		
+ve	+ve	+ve	mot	Rod	31x10^3		

Name

Stock identity number

Date				Frozen	Date	Location	ID #
10/5	14/5	18/5	22-5	Yes	1·6	G8	04 PC 72 1·6
34	45	45-2	45-2-2	31·5	1·6		
				45-2-2-2	72		

Plate media tested

MA	MACc	TSA	TSSW	TSACc	SBA	TCBS	VCA
Good 7/10			Poor 3/10		Poor 1/10		

Appendix 4

Operations Manual for the glass tube and silicon hose pasteuriser

Abbreviations used in this manual

HEx – Heat Exchanger
HWC – Hot Water Cylinder (Large blue heating cylinder)
FW – Freshwater
RHS – Right Hand Side

Initial start up

During this process it is essential that the working pressure of the system be monitored at all times. The water pressure gauge is between Valve 11 and the flow meter, which is on the wall to the left of Hex. The pressure on this system should not go above 150 kPa. If at any time the pressure rises above this point, quickly try to identify the cause. Should the pressure reach 180 kPa open Valve 8 and Valve 7 and shut off the pump or FW hose supplying the system with water. Open Valve 8 and Valve 7 immediately and then shut off the pump.

Shut all valves before beginning. Fill HWC with water. Turn on heater on computer control unit.

Connect Freshwater hose to inlet by Valve 12. Valve 10 should be set so flow is from the pump to Valve 11 (╣). Valve 10 is a T-valve, which means that it allows water to flow in one or two directions at the same time. The position of the T is stamped into the valve and indicates which way water will flow. Fully open Valve 11. Open the valve on the FW hose so water flows through the HEx and allow water to drain to waste through Valve 8. Valve 9 should be shut. After 1 minute of water flow open Valve 9 and close Valve 8. Open Valve 22 and Valve 7 as well. Once water is exiting Valve 7 run water through system for 5 minutes then close Valve 12 and leave until HWC has reached operating temperature of ~95°C. Leave other valves as they are at this point, do not open or close other valves.

HWC has reached temperature

Salinity batching system should be set at control computer to desired salinity. Open Valve 13 and allow water to flow through system (It may not). Flow meter will indicate when flow of water has stopped.

Turn Valve 10 (⊤) so it supplies water from the pump in both directions (left is to Valve 11 and right is a return to the seawater supply line). Lift the locking nut on Valve 17 and increase the air pressure on the gauge on Valve 17 to 0.22 Mpa. Open Valve 14 so the compressed air can flow to the pump. The pump should now operate (the pump operates with a piston and there are two part strokes to each pump cycle, similar to a heart beat; lub-dub, lub-dub). Close Valve 11 and then reopen three full turns. Turn Valve 10 clockwise so that the return supply line (RHS) begins to be shut off (⊣). To find the best position for the pump to operate regularly and not too fast is a matter of trial and error. With too much flow to Valve 11 and not enough to the main return line will make the pump work irregularly. Too much flow to the main return line and not enough to Valve 11 will mean the pump runs quickly without much flow showing on the flow meter. The desired flow rate for this system is 1.3 L/min. Valve 11 needs to be adjusted to get the flow rate. Once set, the pump should be running regularly at around 112 "beats" per minute. I.e. 224 actual strokes. Once the pump is running regularly close Valve 8 and open Valve 9. Water will now flow through the HEx and into the glass tube and silicon hose treatment system in the HWC. The pump is likely to need to be adjusted as the pressure changes when it begins to pump through the treatment system. Open Valve 24. Water should exit to waste through Valve 7. Open Valve 1 and then close Valve 7. Water will enter the other half of the HEx and then flow out Valve 1. Allow the system to run for ten minutes.

Before preparing the system for steam cleaning, ensure that all the reticulation in the algae room has been steam cleaned (see other algae system manual for this process) and there is no delay between shutting this system down for steam cleaning and the actual steam cleaning occurring. (It is important to ensure there is no delay. When the glass tube and silicon hose system in the HWC is filled with seawater and there is no flow in the pipe, crystals of calcium carbonate will form and precipitate into the pipes. These crystals increase the viscosity of the water and therefore the operating pressure. If left for too long the system could block. (See Blockage section for instructions on this.)

When the system is ready to be steamed, the pump must be shut down. Reduce the air pressure at Valve 16 and close Valve 9 and Valve 11. Go to Steam cleaning section for further instruction.

Steam Cleaning the Pasteuriser System's Heat Exchanger

The reticulation in the algae room has all been steamed as per the other manual. Open the dump valve on the cyclone detritus system (above the harvest bins at rows five and six in the algae room). Allow water to drain from the system and let steam run to waste

for 15 seconds. Close the cyclone system valve. Close Valve 22 and Valve 7. Open Valves 3, 2 and 1. When steam is exiting Valve 1 close Valve 3. Open Valve 6 and Valve 19. Allow steam to flow for 15 seconds. Close Valve 6 and Valve 19. Open Valve 4 and Valve 5. Allow steam to pass through the loop (Valves 4 and 5 are a bypass system to ensure the correct flow to the bags during normal operation). Close Valve 4 and Valve 5. Open Valve 3, let steam exit Valve 1 for 15 seconds. Close Valve 1. Open Valve 20 (positioned under the Pasteuriser system table). Once steam has flowed passed this point for 20 seconds, close Valve 20. Open Valve 7, close Valve 9 and open Valve 8. Ensure Valve 11 is closed completely. The steam passing through the Hex and out Valve 7 will heat the water in the other side of the Hex and more will pass through Valve 8. As the steam heats the HEx, the water in the other half of the HEx will heat and turn to steam. Take note of when steam is flowing out of the waste pipes from Valves 7 and 8. Leave to steam for 10 minutes then close Valve 7 and Valve 8. Close Valve 2 and Valve 3. Close the valves in the algae room as per the instructions in the manual for the other system.

Restarting the Pasteuriser after steam cleaning

During this process, on a regular basis check that the water pressure gauge does not exceed 150 kPa. Initially the flow rate to start the system can be 1.8 L/min. Wind the knob on Valve 16 to increase the compressed air pressure (0.22 MPa). Open Valve 11 and allow water pressure in the system to increase to ~80 kPa. Open Valve 8 and allow water to flush through the HEx. The water may initially be quite dirty. Valve 11 and Valve 10 may need adjustment to ensure regular pump operation. Once water is coming passed Valve 8 with few bubbles, close Valve 8 and open Valve 9. Water will now flow into the main system in the HWC. When water is observed to be flowing into the front degassing system flask open Valve 24 and allow flask to fill with water. Close Valve 24. Slowly open Valve 22. NB. The HEx and the hoses on that side of Valve 22 are under vacuum and will draw (suck) the water from the degassing flask. Control the opening of this valve or it will drain the flask. Once water has filled the HEx and the hoses, open Valve 1 and allow water to flush through. Water may initially be dirty. Close Valve 1 and open Valve 2 slowly. Allow water to flow into these hoses, which will also be under vacuum. When hoses are filled, open Valve 20. .

Some water may be in the narrow (8 mm) silicon hose up to Valve 23. Lift the locking knob of Valve 17 and raise the air pressure to 0.1 Mpa. Then slowly open Valve 15. Don't take too long to open Valve 23 after opening Valve 15 or the air pressure may build up and blow the silicon hose off the filter. On opening Valve 23, air will flow up through the silicon hose and into the 2nd T above Valve 2. If there is a lot of water in this silicon line, increase the flow of air (Valve 15) so that it blows this water out of the tube. Return the flow of air back to a low level. The rate of flow of air can be estimated by the effect it has on the wastewater at the top T. NB. This system is necessary to provide air to the wastewater to stop a siphoning effect occurring through the system. The reason for the high riser tube above the Valve 2 is so the water going to waste will provide head pressure to enable uniform flow to the algae room.

The hosing to the algae room will still be under vacuum. Open Valve 18 and Valve 6 and the nutrient carboy clamps to allow nutrient medium to be drawn (sucked) into the main line. Close Valve 6 when nutrient enters main line. Open Valve 5 and Valve 4 to allow water to be drawn into main line on algae room side.

Close Valve 5 and Valve 4. Open Valve 3 slowly. The water flowing out of the waste line can be seen at the top T above Valve 2. As Valve 3 is opened, be sure not to open it so fast that this water, at the top T, comes back down towards Valve 3 so air is drawn into the line going to the algae room. Once algae line is full, wastewater will return to flowing out the top T. In the algae room, slowly open the dump valve on the cyclone detritus system to remove any detritus or freshwater from steaming. Close valve on the cyclone system. Open the end clamp to run water to waste and remove bubbles. Don't fully open the clamp or water at the top T could again drop down and allow air to be sucked into the line towards the algae room. Once air is removed from the main line close this valve. Then open lines for bags as per other system manual. Once bags are running, open Valve 5 and Valve 4 and close Valve 3. This allows the correct flow rate for two rows of bags. Open Valve 6 and Valve 18 and turn on nutrient pump. Set to desired level.

Shutting down system for regular steam cleaning

Close Valve 3, Valve 6, Valve 18, Valve 5 and Valve 4. Unplug nutrient pump. Steam algae room system whilst leaving pasteuriser running. When algae room section of system is ready for steaming, close the following valves: 16 (reduce pressure to zero), 17 (reduce pressure to zero), 11, 22, 9 and 23.

Open Valve 3 and allow steam to pass out waste through Valve 20 (under table). Close Valve 20 after 20 seconds. Open Valve 1. Allow steam to run. Close Valve 3 and open Valve 6 and Valve 19. Once steamed for 20 seconds close Valve 6 and Valve 19. Open Valve 5 and Valve 4. Close once steamed. Open Valve 3 and close Valve 1. Open Valve 7 and Valve 8. Wait for steam to exit waste pipes and then allow to run for 10 minutes. Whilst the Hex is being steamed, complete the following part.

Remove air degassing float systems and disconnect the waste pipes. Replace the degassing floats with clean ones. Reconnect the waste pipes to the waste line.

Following the 10 minutes of steaming close Valve 7 and Valve 8. Close Valve 2 and Valve 3. Close algae room system as per the other system manual. Restart system as per section- **Restarting the Pasteuriser after steam cleaning.**

System is Blocked

If the glass tube and silicon hose system becomes blocked and flow does not occur up to a water pressure of 180 kPa then an attempt needs to be made to unblock it. Set the FW hose up to the inlet at Valve 12. Close Valve 13. Open Valve 7. Connect a Venturi pump and hose set-up to the waste pipe of Valve 7. This should be connected so the pump will suck the water from the system. Put a clamp between the glass T and the HEx on this line so the pump isn't sucking water from the HEx system. Remove the degassing float systems from the two 1 L flasks and replace the lids with standard flask lids. NB. This step is necessary as the suction from the Venturi pump suction could suck the degassing float into the flask and it may smash. Once the Venturi pump is sucking water from the system open Valve 12 and turn on the FW hose. Monitor pressure to ensure it does not go above 180 kPa. Set Valve 10 to only run to Valve 11 (⫤). Monitor water pressure very closely. If this process does not remove blockage, then glass tube and silicon hose system will need to be removed from the HWC and cleared manually.

In the instance where it is necessary to remove the glass tube and silicon hose system from the HWC, ensure the following steps are followed.

Turn off the heating elements in the HWC at the control computer. Turn off the FW supply to the HWC and drain the HWC and allow the system to cool. Disconnect the electrical connections to the elements of the HWC as the elements need to be removed and the HWC needs to be shifted. Once the system is cool disconnect the silicon hosing at the degassing flasks, not at the glass tube system. Extract the temperature probes and conductivity sensor from the HWC. Disconnect the freshwater supply to the HWC. Remove the heater elements. Remove the earthquake protection straps from around the HWC. The HWC should now have no connections or fittings attached to it.

On the roof of the Hatchery building remove the skylight above the air blowers. Place the support stand over the hole where the skylight was. Set up the chain pulley on the support stand so the chain hangs in the centre of the skylight. Move the HWC so it is directly below the skylight. Remove the Copper pipe 'T' that the stand is wired to at the top of the HWC. Get a torch with a strong light. Insert the first lifting cradle block into the centre of the copper stand. It is best to have two people do this. Insert the lowest block first and ensure it is sitting correctly in the bottom plate. The tension on this first rope must be maintained whilst the second (middle) block and then the top block are fitted. Once all three are fitted, connect the cradle rope up to the chain pulley hook and raise the hook to take up the tension on the cradle rope. Before beginning to lift the copper stand double check all cradle blocks are seated properly. Once ready to lift, four people are needed, one to operate the chain pulley and monitor the stand on one side. Someone on the other side on a ladder to monitor the stand on the other side of the HWC and two people to assist the moving of the HWC once the copper stand is lifted clear of the HWC (during this part the second person will assist with the moving). Once the HWC has been lifted and the HWC moved, lower the stand back down. Set the stand on a table or stand about 2 feet high as this makes attending to the silicon loops at the bottom considerably easier than if it is on the ground. Once the copper stand is resting on its feet

DO NOT remove all the tension from the chain pulley and the cradle by lowering the cradle further. The stand is not strong enough to support its own weight when outside of the HWC. Also, the cradle blocks could drop out of position if the tension was removed.

Attend to the silicon hose loops to identify and remove the blockage. If necessary hook the system up to a FW hose and push water through the system as tried previously. Ensure the cradle and chain pulley are upholding most if not all of the weight as with more weight from the added water, the legs of the stand could buckle. Once the blockage is removed, raise the stand up and place the HWC underneath and lower the stand back into the HWC. It should now be possible to work through this manual backwards and re-install and reconnect all parts that were detached or removed for the stand to be lifted. Ensure the cradle is removed from the system and the support T is reinstalled and the stand wired to it before moving the HWC with the stand in it.

Chapter 6 Bibliography

Arimoto, M., Sato, J., Maruyama, K., Mimura, G. and Furusawa, I., 1996. Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV) *Aquaculture*, 143, pp 15-22

Baker, K.H. and Herson, D.S., 1978. Interactions between the diatom *Thallasiosira pseudonana* and an associated pseudomonad in a mariculture system. *Applied and Environmental Microbiology*, 35, pp 791-796.

Bavarian, F., Fan, L.S. and Chalmers, J.J., 1991. Microscopic visualization of insect cell-bubble interactions. I: Rising bubbles, air-medium interface, and the foam layer. *Biotechnology Progress*, 7, pp 140-150.

Boulton-Stone, J.M. and Blake, J.R., 1993. Gas bubbles bursting at a free surface. *Journal of Fluid Mechanics*, 254, pp 437-466.

Chalmers, J.J. and Bavarian, F., 1991. Microscopic visualization of insect cell-bubble interactions. II: the bubble film and bubble rupture. *Biotechnology Progress*, 7, pp 151-158.

Chang, P.S., Chen, L.J. and Wang, Y.C., 1998. The effect of ultraviolet radiation, heat, pH, ozone, salinity and chemical disinfectants on the infectivity of white spot syndrome baculovirus. *Aquaculture,* 166, pp 1-17

Cherry, R.S. and Hulle, C.T., 1992. Cell death in the thin films of bursting bubbles. *Biotechnology Progress*, 8, pp 11-18.

Chisti, Y., 2000. Animal-cell damage in sparged bioreactors. *TibTech,* 18, pp 420-432.

Contreras, A., Garcia, F., Molina, E. and Merchuk, J.C., 1999. Influence of sparger on energy dissipation, shear rate, and mass transfer to sea water in a concentric-tube airlift bioreactor. *Enzyme and Microbial Technology*, 25, pp 820-830.

Corzo, A., Morillo, J.A. and Rodriguez, S., 2000. Production of transparent exo-polymer particles (TEP) in cultures of *Chaetoceros calcitrans* under nitrogen limitation. *Aquatic Microbial Ecology*, 23, pp 63-72.

Csordas, A. and Wang, J.K., 2004. An integrated photobioreactor and foam fractionation unit for the growth and harvest of *Chaetoceros* spp. in open systems. *Aquacultural Engineering*, 30, pp 15-30.

Falciatore, A. and Bowler, C., 2002. Revealing the molecular secrets of marine diatoms. *Annual Review of Plant Biology*, 53, pp 109-130.

Fernandez-Reiriz, M.J., Perez-Camacho, A., Ferreiro, M.J., Blanco, J., Planas, M., Campos, M.J. and Labarta, U., 1989. Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. *Aquaculture*, 83, pp 17-37.

Fredrickson, A.G., 1977. Behaviour of mixed cultures of micro-organisms. *Annual Review of Microbiology*, 31, pp 63-87.

Frerichs, G.N., 1990. Efficacy of chemical disinfectants against snakehead rhabdovirus. *Journal of Applied Ichthyology*, 6, pp 117-123.

Frerichs, G.N., Tweedie, A., Starkey, W.G. and Richards, R.H., 2000. Temperature, pH and electrolyte sensitivity, and heat, UV and disinfectant inactivation of sea bass (*Dicentrarchus labrax*) neuropathy nodavirus. *Aquaculture*, 185, pp 13-24.

Fukami, K., Nishijima, T., and Hata, Y., 1992. Availability of deep seawater and effects of bacteria isolated from deep seawater on the mass culture of food microalga *Chaetoceros ceratosporum*. *Nippon Suisan Gakkaishi*, 58, pp 931-936

Fukami, K., Nishijima, T. and Ishida, Y., 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia*, 358, pp 185-191.

Goksan, T., Durmaz, Y. and Gokpinar, S., 2003. Effects of light path lengths and initial culture density on the cultivation of *Chaetoceros muelleri* (Lemmermann, 1898). *Aquaculture*, 217, pp 431-436.

Goldblum, S., Bae, Y.K., Hink, W.F. and Chalmers, J., 1990. Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. *Biotechnology Progress*, 6, pp 383-390.

Guillard, R.R.L. and Ryther, J.H., 1962. Studies of marine planktonic diatoms I. *Cyclotella nana Hustedt*, and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology*, 8, pp 229-239.

Haas, C.N. and Engelbrecht, A., 1980. Physiological alterations of vegetative micro-organisms resulting from chlorination. *Journal of the Water Pollution Control Federation*, 52, pp 1976-1989.

Handa-Corrigan, A., Emery, A.N. and Spier, R.E., 1989. Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. *Enzyme and Microbial Technology*, 11, pp 230-235.

Harrison, P.J., Waters, R.E. and Taylor, F.J.R., 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. *Journal of Phycology*, 16, pp 28-35.

Harrison, P.J., Thompson, P.A. and Calderwood, G.S., 1990. Effects of nutrient and light limitation on the biochemical composition of phytoplankton. *Journal of Applied Phycology*, 2, pp 45-56.

Hirayama, S. and Hirayama, K., 1993. Relation between diatom growth and bacterial population in semi mass culture tanks of diatom. *Bulletin*. Faculty of Fisheries, Nagasaki University, 74/75, pp 37-41.

Hirayama, S. and Hirayama, K., 1996. Effects of bacterial coexistence on the growth of a marine diatom *Chaetoceros gracilis*. *Fisheries Science*, 62, pp 40-43.

Hirayama, S. and Hirayama, K., 1997. Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. *Hydrobiologia*, 358, pp 223-230.

Inouye, K., Ikeya, F., Yamazaki, T. and Hara, T., 1990. Virucidal activities of various germicides to infectious pancreatic necrosis virus (IPNV). *Fish Pathology*, 25, pp 81-86.

Jorquera, M.A., Valencia, G., Eguchi, M., Katayose, M. and Riquelme, C., 2003. Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment. *Aquaculture*, 207, pp 213-224.

Joshi, J.B., Elias, C.B. and Patole, M.S., 1996. Role of hydrodynamic shear in the cultivation of animal, plant and microbial cells. *The Chemical Engineering Journal*, 62, pp 121-141.

Kilsby, D.C., Davies, K.W., Mcclure, P.J., Adair, C. and Anderson, W.A., 2000. Bacterial thermal death kinetics based on probability distributions: the heat destruction of *Clostridium botulinum* and *Salmonella spp. Bedford. Journal of Food Protection*, 63, pp 1179-1203.

Kioukia, N., Nienow, A.W., Al-Rubeai, M. and Emery, A.N., 1996. Influence of agitation and sparging on the growth rate and infection of insect cells in bioreactors and a comparison with hybridoma culture. *Biotechnology Progress*, 12, pp 779-785.

Krichnavaruk, S., Loataweesup, W., Powtongsook, S. and Pavasant, P., 2005. Optimal growth conditions and the cultivation of *Chaetoceros calcitrans* in airlift bioreactor. *Chemical Engineering Journal*, 105, pp 91-98.

Kudo, I., 2003. Change in the uptake and cellular Si:N ratio in diatoms responding to the ambient Si:N ratio and growth phase. *Marine Biology*, 143, pp 39-46.

Kunas, K.T. and Papoutsakis, E.T., 1990. The protective effect of serum against hydrodynamic damage of hybridoma cells in agitated and surface-aerated bioreactors. *Journal of Biotechnology*, 15, pp 57-70.

Laing, I. 1979. Recommended procedure for the culture of *Chaetoceros calcitrans*. In *Culture of algae for larval fish and shellfish rearing*. Lowestoft : Ministry of Agriculture, Fisheries and Food. Directorate of Fisheries Research. Fisheries Research Technical Report no. 53

Laing, I., 1985. Factors affecting the large -scale production of four species of commercially important marine algae. *Aquaculture*, 44, pp 161-166.

Lebeau, T. and Robert, J.M., 2003. Diatom cultivation and biotechnologically relevant products. Part II: Current putative products. *Applied Microbiology and Biotechnology*, 60, pp 624-632.

Lebeau, T. and Robert, J.M., 2003. Diatom cultivation and biotechnologically relevant products. Part 1: Cultivation at various scales. *Applied Microbiology and Biotechnology*, 60, pp 612-623.

Levenspiel, O., 1999. *Chemical Reaction Engineering*, 3rd ed. Wiley, New York.

Liltved, H. and Cripps, S.J., 1999. Removal of particle-associated bacteria by pre-filtration and ultraviolet irradiation. *Aquaculture Research*, 30, pp 445-450.

Lourenco, S.O., Barbarino, E., Mancini-Filho, J., Schinke, K.P. and Aidar, E., 2002. Effects of different nitrogen sources on the growth and biochemical profile of 10 marine microalgae in batch culture: an evaluation for aquaculture. *Phycologia*, 41, pp 158-168.

MacLeod, R.A., 1965. The question of the existence of specific marine bacteria. *Bacteriological Reviews*, 29, pp 9-23.

Martin-Jezequel, V., Hildebrand, M. and Brzezinski, M.A., 2000. Silicon metabolism in diatoms: implications for growth. *Journal of Phycology*, 36, pp 821-840.

Meier, S.J., Hatton, T.A. and Wang, D.I.C., 1999. Cell death from bursting bubbles: role of cell attachment to rising bubbles in sparged reactors. *Biotechnology and Bioengineering*, 62, pp 468-478.

Merchuk, J.C., 1990. Why use airlift bioreactors? *TibTech*, 8, pp 66-71.

Merchuk, J.C., Ben-Zvi (Yona), S. and Niranjan, K., 1994. Why use bubble-column bioreactors? *TibTech*, 12, pp 501-511.

Michaels, J.D. and Papoutsakis, E.T., 1991. Polyvinyl alcohol and polyethylene glycol as protectants against fluid-mechanical injury of freely-suspended animal cells (CRL 8018). *Journal of Biotechnology*, 19, pp 241-258.

Millie, D.F., Schofield, O.M.E., Kirkpatrick, G.J., Johnsen, G. and Evens, T.J., 2002. Using absorbance and fluorescence spectra to discriminate microalgae. *European Journal of Phycology*, 37, pp 313-322.

Miyamoto, K., Wable, O. and Benemann, J.R., 1988. Vertical tubular reactor for microalgae cultivation. *Biotechnology Letters*, 10, pp 703-708.

Moats, W.A., 1971. Kinetics of thermal death of bacteria. *Journal of Bacteriology*, 105, pp 165-171.

Morris, C. (Ed.). (1992). *Academic Press Dictionary of Science and Technology*. San Diego: Academic Press.

Muller-Feuga , A., Moal, J. and Kaas , R., 2003. The microalgae of aquaculture. In Stottrup J.G. and McEvoy L.A. (eds.) *Live feeds in marine aquaculture.* Oxford, Blackwell Science. pp 207-252.

Murhammer, D.W. and Goochee, C.F., 1990. Sparged animal cell bioreactors: mechanism of cell damage and Pluronic F-68 protection. *Biotechnology Progress*, 6, pp 391-397.

Palomares, L.A., Gonzalez, M. and Ramirez, O.T., 2000. Evidence of Pluronic F-68 direct interaction with insect cells: impact on shear protection, recombinant protein, and baculovirus production. *Enzyme and Microbial Technology*, 26, pp 324-331.

Papoutsakis, E.T., 1991. Media additives for protecting freely suspended animal cells against agitation and aeration damage. *TibTech*, 9, pp 316-324.

Poulsen, B.R. and Iversen, J.J.L., 1998. Characterization of gas transfer and mixing in a bubble column equipped with a rubber membrane diffuser. *Biotechnology and Bioengineering*, 58, pp 633-641.

Pulz, O., 2001. Photobioreactors: production systems for phototrophic micro-organisms. *Applied Microbiology and Biotechnology*, 57(3): pp 287-293

Ramirez, O.T. and Mutharasan, R., 1990. The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. *Biotechnology and Bioengineering*, 36, pp 911-920.

Rasmussen, B., Davis, R., Thomas, J. and Reddy, P., 1998. Isolation, characterization and recombinant protein expression in Veggie-CHO: a serum-free CHO host cell line. *Cytotechnology*, 28, pp 31-42.

Rhee, G., 1980. Continuous culture in phytoplankton ecology. In Droop M.R. & Jannasch H.W. [Eds.] *Advances in Aquatic Microbiology.* London : Academic press. pp 151-203.

Sako, H., Ishida, N., Maeno, Y. and Sorimachi, M., 1988. Bacterial activities of five disinfectants on *Aeromonas salmonicida*, *Vibrio anguillarum* and *V. ordalii*. *Fish Pathology*, 23, pp 219-229.

Samonte, G.P.B., Espegadera, C.C. and Caturao, R.D., 1993. Economics of microalgae (*Chaetoceros calcitrans*) production using the multi-step method in the Philippines. *Aquaculture*, 112, pp 39-45.

Sanchez-Saavedra, M. and Voltolina, D., 1996. Effect of blue-green light on growth rate and chemical composition of three diatoms. *Journal of Applied Phycology*, 8, pp 131-137.

Shah, R.K. and Sekulic, D.P., 2003. *Fundamentals of heat exchanger design.* Hoboken, NJ: John Wiley.

Silva, H.J., Cortinas, T. and Ertola, R.J., 1987. Effect of hydrodynamic stress on *Dunaliella* growth. *Journal of Chemistry, Biochemistry and Biotechnology*, 40, pp 41-49.

Stabel, J.R., Steadham, E.M. and Bolin, C.A., 1997. Heat inactivation of *Mycobacterium paratuberculosis* in raw milk: are current pasteurization conditions effective? *Applied and Environmental Microbiology*, 63, pp 4975-4977.

Suzuki, K., Kishino, M., Sasaoka, K., Saitoh, S.I. and Saino, T., 1998. Chlorophyll-specific absorption coefficients and pigments of phytoplankton off Sanriku, north-western North Pacific. *Journal of Oceanography*, 54, pp 517-526.

Tramper, J., Williams, J.B., Joustra, D. and Vlak, J.M., 1986. Shear sensitivity of insect cells in suspension. *Enzyme and Microbial Technology*, 8, pp 33-36.

Tredici, M.R., 1999. Photobioreactors. In Flickinger M.C. and Drew S.W. (Eds.) *Encyclopaedia of Bioprocess Technology* v.1. New York: Wiley. pp 395-419.

Walne, P. R., 1966. Experiments in the large-scale culture of the larvae of *Ostrea edulis* L. London: HMSO. *Fishery Investigations* Series II, Volume XXV, Number 4.

Wang, N.S., Yang, J.D., Calabrese, R.V. and Chang, K.-C., 1994. Unified modelling framework of cell death due to bubbles in agitated and sparged bioreactors. *Journal of Biotechnology*, 33, pp 107-122.

Wedemeyer, G.A., Nelson, N.C. and Yasutake, W.T., 1979. Physiological and biochemical aspects of ozone toxicity to rainbow trout (*Salmo gairdneri*). *Journal of The Fisheries Research Board of Canada*, 36, pp 605-614.

White, G.C., 1999. *The handbook of chlorination and alternative disinfectants.* New York: J. Wiley.

Wu, J. and Goosen, M.F.A., 1995. Evaluation of the killing volume of gas bubbles in sparged animal cell culture bioreactors. *Enzyme and Microbial Technology*, 17, pp 241-247.

Wu, J., Ruan, Q. and Lam, H.Y.P., 1997. Effects of surface-active medium additives on insect cell surface hydrophobicity relating to cell protection against bubble damage. *Enzyme and Microbial Technology*, 21, pp 341-348.

Yanase, R. and Imai, T., 1968. The effect of light intensity and temperature on the growth of several marine algae useful for rearing molluscan larvae. *Tohoku Journal of Agricultural Research*, 19, pp 75-82.

Yura, T., Nagai, H. and Mori, H., 1993. Regulation of the heat-shock response in bacteria. *Annual Review of Microbiology*, 47, pp 321-350.

Zhang, Z., Al-Rubeai, M. and Thomas, C.R., 1992. Effect of Pluronic F-68 on the mechanical properties of mammalian cells. *Enzyme and Microbial Technology*, 14, pp 980-983.

Zhang, Z., Chisti, Y. and Moo-Young, M., 1995. Effects of the hydrodynamic environment and shear protectants on survival of erythrocytes in suspension. *Journal of Biotechnology*, 43, pp 33-40.