Toxin production in *Alexandrium tamarense*: Investigating clonal variation and extra-cellular bacterial effects

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Master Thesis

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Summary:

My thesis investigated the role of extra-cellular bacteria on the PSP toxin producing *A. tamarense*. Results showed that bacteria have no effect on the dinoflagellate growth rate. The toxin profile remained relatively constant throughout the experiment. Three samples exhibited low production of GTX1/4. This could be further investigated to determine if these cultures lose the ability to produce this STX derivative.

Bacteria have no effect on allelochemical produced by *A. tamarense*. This effect was seen in both lytic and non-lytic strains. The toxin profile remained constant for sub-clonal cultures, signifying that PSP toxin production is in actual fact a genetically determined characteristic. Variations in toxin content were observed, but they were not large enough to claim that PSP toxigenesis was non-chromosomally linked.

There is still
1) Introduction:

Marine ecosystems host algae that play a critical role as the source of primary production. They not only have a beneficial role, they can also be detrimental. One of the ways of doing this is by causing Harmful Algal Blooms (HABs). HABs are a widespread marine phenomena that consist of up to millions of cells per litre and cause numerous problems to human health, fisheries resources and marine ecosystems. They occur in coastal waters worldwide (Hallgraeff 1993) and exhibit varying effects on the area where they are found. Their effect ranges from stripping the water column of oxygen (due to the high biomass causing the death of microflora and macrofauna) to producing potent toxins that cause gastrointestinal and neurological illness in humans (Hallgraeff 1995).

Paralytic shellfish poisoning (PSP) is one such neurological illness. It is so called due to the symptoms exhibited by higher animals, particularly mammals and birds that ingest contaminated shellfish (i.e. contaminated through the accumulation of the causative algal species by suspension feeding into the tissues of shellfish). These toxins work by binding to receptors of muscle or nerve cells and disrupt the flow of sodium ions. This interruption of signal transmission is what causes paralysis. Due to its serious human health and economic impact it has been of great importance to study the causative microorganisms.

The causative toxins were found to synthesised by a group of microalgal species, namely: Gymnodium catenatum, Pyrodinium bahamense and the members of the genus Alexandrium (Cembella 1998)
These neurotoxins, including saxitoxin and 20 other chemically related derivatives differ in chemical structure (Gallacher & Smith 1999) and vary in toxicity.

The genus *Alexandrium* consists of 27 species, of which 9 are known to produce PSP toxins in varying amounts. Although it is arguably the best characterized harmful algal genus to date (Cembella 1998, Scholin 1998) little is known about the enzymes and genes involved in the synthesis of PSP toxins (Plumley 1997).

Some scientists have suggested eubacterial involvement in this process either directly (i.e. as autonomous PST producers) or indirectly (i.e. by affecting the growth rate, nutrient uptake, etc.). This concept is still somewhat controversial. The only non-dinoflagellate spp that are generally accepted to be able to produce PSP toxins autonomously are several species of cyanobacteria, namely; *Aphanizomenon flos-aquae* (Jakim and Gentil 1968; Mohood and Carmichael 1986), *Anabaena circinalis* (Humpage et al. 1994; Negri et al. 1997) and *Lyngbya wollei* (Carmichael et al. 1997; Onodera et al. 1997).

The notion of bacteria either producing or having an effect on PSP toxigenesis was first hypothesized by Silva (1962, 1982). It stemmed from the fact that she observed bacteria-like structures within *A. tamarense* cells. This was followed up by Nelinda (1985) who detected PSP toxins in a non toxic dinoflagellate culture after it was transferred into medium from toxic cultures. She attributed this to the extra cellular bacteria present in the medium. Further studies showed that there was not a significant amount of toxins transferred by extra-cellular bacteria. These findings did not refute Silva’s postulation due to the fact that her observations were based on intracellular bacteria. Kodama (1988; 1990) focused on intracellular
bacteria and was able to isolate a bacterium species called *Moraxella* from a toxic strain of *A. tamarense*. It showed the ability to autonomously produce PSP toxins. Several papers followed this by claiming proof of the ability of bacteria to produce PSP toxins (Kodama et al. 1988, 1990; Ogata et al. 1990; Doucette & Trick, 1995; Levasseur et al. 1996; Shimizu et al. 1996; Kopp et al. 1997; Gallacher et al. 1996, 1997). More recent papers refuting these claims followed by reporting the incorrect identification of bacterial metabolites as PSP toxins by HPLC analysis (Negri et al. 1997, Onodera et al. 1997, Sato et al. 1998, Pereira et al. 2000 and Baker et al. 2003). This has lead to the continual controversy surrounding autosomal or assisted bacterial PSP toxigenesis. Within this whole debate, a new approach was used to determine the effect of extracellular bacteria on dinoflagellate culture toxicity; namely the removal of the extracellular microflora were used. The methods employed to obtain axenic cultures were; sterile washing (Singh et al. 1982; Kim et al. 1993) antibiotics (Dantzer et al. 1997, Hold et al. 2001) and lysozyme/SDS treatment (Doucette and Powell 1998).

This has lead to an ongoing debate on the actual effects of bacteria on PST toxigenesis with papers professing and refuting this aspect.

Another unsolved aspect of PSP toxigenesis is the genetic control of toxin production. Few studies have been conducted to determine the genes involved in this. It is not clear whether chromosomal, mitochondrial, chloroplast DNA or nucleic acids from bacteria, plasmids or viruses are involved. One of the first studies to tackle this was conducted by Ogata et al. (1987). They looked at the toxicity of numerous sub-clones originating from one clonal culture and found variations. They attributed this to the fact that toxin
production is not hereditary, thus leads to the variation observed. Kodama (1990) observed the same differences and suggested that endonuclear bacteria where associated in toxin production.

Soon after, Sako (1995) and Ishida (1998) showed that toxigenesis of PSP toxins is chromosomally based, thus a hereditary character. This was done by conducting mating experiments on different strains of *A. tamarense* and *A. catenella* with differing PSP profiles and determining whether the PSP profile measured was a combination of both parents. The toxin profile was used as a phenotypic characteristic since the toxin profile is thought to be readily stable in *Alexandrium* species (Cembella., *et al* 1987; Ogata *et al*., 1987; Kim *et al* 1993). These studies showed that the progeny exhibited a combination of both parental toxins and most strains showed a Mendelian pattern of inheritance. This lead to the conclusion that the genes coding for PST producing enzymes where on nuclear DNA. Nonetheless, not all F1 progeny on these mating experiments exhibited the Mendelian pattern of inheritance. It was proposed that this was due to recombination between genes.

One more aspect of PSP production is the relevance of these toxins to the organism. Most scientists view these toxins as secondary metabolites (Plumley 1997). This term was coined by Vining in 1990 and defined as “compounds that do not fulfil a role in intermediary metabolism” Little is known as to the importance of toxin production with respect to the causative organism. It has been suggested that PSP toxins may play an intrinsic role in DNA metabolism (Mickelson & Yentsch 1979; Anderson & Cheng 1998) or aid in nitrogen storage (Anderson *et al* 1990) or act as pheromones (Wyatt and Jenkinson 1997) to promote mating or as anti-predation/allelochemical compounds (White & Maranda 1978; Haney *et al* 1995). Since the effectiveness of an allelochemical is
its ability to be released into the environment, PSP toxins do not fit into this definition (i.e. PSP toxins are kept in the cell and only released during senescence). Recent studies are indicating these allelochemicals also cause an effect on specific microorganisms. Allelochemical are defined as chemicals that cause inhibition or stimulation in another organism during their interaction (Molisch 1937). Arzul et al (1998) tested the allelochemical effects of three toxic *Alexandrium* species and found that the cell-free filtrates of these cultures had an allelochemical effect on *Chaetoceros gracile*, *Gymnodinium mikimotoi* and *Scrippsiella trochiodea*. They suggested that this effect was due to compounds other than PSP toxins. Several papers found that this was true in more recent case studies. (Ogata & Kodama 1986; Lush & Hallegraeff 1996; Tillmann & John 2002).

However nothing is known about the factors affecting production of these allelochemical by *A. tamarense*. One of the possibilities could be that extra-cellular bacteria are involved in this process. This question will be addressed in my thesis.
3. My thesis project deals with three aspects related to toxin production in *A. tamarense*, namely:

1) Determining the effects of extracellular bacteria on two *A. tamarense* stains by observing the growth rate, toxin profile and overall toxin content within axenic and xenic cultures.

2) Determining the possible genetic bias of toxigenesis by studying the phenotype (toxin profile) of sub-clones. Determining of this variation can be seen at a genetic level using AFLP.

3) Determining the allelochemical effects of the fore mentioned axenic, xenic and cultures using a bioassay with the Cryptophyte *Rhodomonas baltica* to determine the bacterial influence on the unknown allelochemical.
4) Materials and methods:

4.1 Sample site:

The *Alexandrium tamarense* strains used in these experiments were collected from two stations off the coast of Scotland denoted by S2 (56°N, between Dundee and Edinburgh) and S6 (57° 40’ N, north of Aberdeen). These cultures were isolated from natural plankton samples as single cells.

4.2 Strains and Culture conditions:

4.2.1 Axenic and xenic cultures

Two strains of *A. tamarense* were used for the bacterial experiments: S2-P4-D1 and S2-P4-F2.

K medium (Keller et al 1987) made with North Sea water adjusted to pH 8, with the addition of stock solutions (seen in Table 1). These cultures were then transferred to K medium containing an antibiotic cocktail (in Table 2). These cultures were maintained at 15°C, with artificial light at a photon flux density of 100μEm⁻²s⁻¹ and a light:dark cycle of 14:10. The xenic cultures were maintained in the same fashion with the omission of antibiotic medium. To reduce the amount of extracellular bacteria within the cultures, gauze filtering (using a 20 μm mesh) with sterile-filtered seawater was conducted during the second week. This entailed gently pouring the culture through sterile gauze, and rinsing three times with sterile-filtered seawater. These cultures were then transferred into K medium and maintained as previously described. After the second week, cultures were tested for bacterial
contamination (as described below) and used as inocula for growth bottles containing 5L of K medium (to ensure enough sampling volume). The inocula were used to allow for a final concentration of approximately 200cellsmL⁻¹. Each treatment was conducted in triplicate.

Table 1: List of reagents used in making K medium.

<table>
<thead>
<tr>
<th>Amount added/L</th>
<th>Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ml</td>
<td>NaNO₃ (75.0g/L H₂O)</td>
</tr>
<tr>
<td>1ml</td>
<td>NH₄Cl (2.68g/L H₂O)</td>
</tr>
<tr>
<td>1ml</td>
<td>NaH₂PO₄.H₂O (5g/L H₂O)</td>
</tr>
<tr>
<td>0.1ml</td>
<td>H₂SeO₃ (1.29mg/L)</td>
</tr>
<tr>
<td>1ml</td>
<td>Tris Base(pH7.2) (121.1g/L H₂O)</td>
</tr>
<tr>
<td>1ml</td>
<td>K Trace metal solution</td>
</tr>
<tr>
<td>1ml</td>
<td>F/2 Vitamin solution</td>
</tr>
</tbody>
</table>
Table 2: Antibiotic cocktail used in axenic medium.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock solution</th>
<th>Addition/L medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>15mg/ml</td>
<td>3.33ml</td>
</tr>
<tr>
<td>Gentamycine</td>
<td>1mg/ml</td>
<td>3.33ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>25mg/ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>34mg/ml</td>
<td>30ul</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1mg/ml</td>
<td>1ul</td>
</tr>
</tbody>
</table>

4.2.2 Sub-clone cultures

Single cells of *A. tamarense* from the strains: S2-P4-D1, S2-P4-F2, S2-P6-G12 and S6-P1-G7 were picked from existing cultures using a fine tipped plastic pipette attached to a rubber pipe. Cultures were diluted and placed in a Petri dish. Cells that were suspended within the medium or motile were picked. They were picked by applying a slight suction, which allowed the cells to be sucked into the pipette tip and transferred to a well of a 96 well plate containing 200 µl of diluted (1:10) K medium (Keller et al., 1987). The plates where then checked to confirm that only one cell per well was transferred. The plates for each strain were incubated and maintained at 15°C, 100 µE.m⁻².s⁻¹ photo flux density and a light:dark cycle of 14:10. Once the clones were formed they were transferred to a glass culture
vial containing 10 ml of undiluted K medium. Once the clones had reached substantial concentrations they were transferred into 200 ml fresh K medium with penicillin (0.1g/L) in 250ml culturing flasks and numbered 1 to 20 with duplicate samples from the culture where the single cells where picked (i.e. parent clone). They were maintained at the same growth conditions mentioned previously.

4.3 Sampling procedure:

4.3.1 Axenic and xenic cultures

Samples for cell counts and PSP measurements were taken every second day till the cultures reached the lag-phase of their growth curve (i.e. 20 days in total). Further samples were taken during three stages of the experiment for bacterial determination using Acridine orange. Samples for determining the allelochemical effect of the cultures were taken on days 8 and 10.

4.3.2 Sub-clone cultures

Cultures were harvested when cell number reached approximately 3000cells/ml. 1ml was used for cell counts, 40ml for PSP determination, 20ml for allelochemical tests and 100ml for DNA extraction for AFLP analysis.
4.4 Cell and bacterial counts:

4.4.1 Cell counts

The Utermohl-method was used for the axenic, xenic cultures and sub-clone samples to determine cell concentration. These results were used to determine the growth rate of each strain in the axenic and xenic cultures and to evaluate PST concentrations per cell for both experiments. Counting was done by mixing 1ml of sample with 20µl Lugol’s solution (to obtain a final Lugol’s solution of 2%). 100µl of this sample was then added to a counting chamber containing 200µl of K medium. The total number of cells was counted in the chamber with an inverted microscope (Zeiss Axiovert 40 C) and the numbers were converted to cells per ml. The growth rate was calculated by:

\[
\text{Growth rate} = \ln\left( \frac{N_t}{N_0} \right) \\
T_1 - t_0
\]

Where \(N\) refers to the cell concentration at time \(t\).

4.4.2 Bacterial counts:

Staining with Acridine orange (Hobbie et al, 1977) was used to determine the amount of bacteria in the Axenic and xenic cultures. This was done by first adding 20 µl Formaldehyde to 1ml of sample. These samples were stored in the fridge till further analysis. In order to begin staining, a cellulose nitrate filter (pore size 0.45 µm, Millipore) was mounted onto the filtering unit followed by a black Nucleopore filter (pore size 0.2 µm).

1ml of the fixed sample was added to the filtering unit with 100 µl of Acridine Orange (1mg/ml) and incubated for 3 minutes. Then the
sample was filtered off, the nucleopore filter was removed with pincers, mounted onto a slide with citifluor and covered with cover-slips. They were then stored in the fridge till analysis. The blank was prepared in the same manner with filtered Milli-Q water as the sample.

Bacterial counts of each sample were done as follows: 20 grids on each filter where counted under an epifluorescence microscope with 100X oil immersion objective.

4.5 PSP extraction and determination:

This method was applied to the axenic, xenic and sub-clone cultures. STX and its derivatives (Figure 1) were determined using known standards. The samples were prepared as follows:

Volumes ranging from 30ml to 900ml (i.e. to obtain approximately 3000 cells /ml) of the sample cultures were centrifuged at 13000 rpm, 4°C for 15 minutes to obtain a pellet from the culture. The pellet was then frozen and stored at -25 °C prior to extraction.

For PSP toxin extraction, 1ml of acetic acid (0.03N) was added to the cell pellet. This was then homogenized using lysing matrix D beads (Thermosavant) in a homogenizer (Fast Prep FP120, Thermosavant) for 40 seconds followed by centrifugation (Eppendorf 5415R) for 15 minutes at 13000 rpm. 500 µl of the supernatant was then passed through a 0.5ml spin filter (Millipore) and centrifuging for 30 sec at 3000rpm. The filtrate was collected in 2ml crimp glass vials (Agilent Technologies) and capped. A portion of the filtrate was hydrolyzed to determine the N-sulfocarbamoyl content (Table 3). This was done by adding 75µl
HCl (1M) to 300μl of the extracted sample followed by 90°C incubation for 15 min. 150μl of sodium acetate (1M) was then added after the sample cooled to room temperature. The N-sulfocarbamoyl content was determined indirectly by calculating the concentration difference between the hydrolyzed and non-hydrolyzed sample. All samples were kept at -25°C prior to analysis.

The HPLC method based on ion-pair chromatography with octanesulfonic acid, post-column derivatization followed by fluorescence detection (Hummert et al., 1997) was used to analyze the concentrations of PSP derivatives. The method used two aqueous eluents (Table 4A) with different gradients over time (Table 4B). The oxidizing reagent for the post column derivatization consisted of a 10mM periodic acid, 550mM NH₃ solution and 0.75M nitric acid. Fluorescent toxin derivatives were detected at excitation and emission wave lengths of 333nm and 395nm respectively.

Standards for saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins (GTX 1-4) and Decarbomoyl toxins (dcSTX, dcGTX 2 and dcGTX 3) purchased by the National Research Council Canada, Halifax, Nova Scotia, Canada, were used. These were used to calibrate the sample PSP toxin concentrations.
Figure 1: STX and its derivatives depicting the four major groups of PSP toxins. (depending on the group substituted on R4 position) namely, Carbamoyl, N-sulfocarbamoyl and Decarbamoyl toxins. Saxitoxin and NeoSTX are the most potent of these toxins, with N-sulfocarbamoyl being the least toxic and Decarbamoyl toxins having intermediate toxin content.
**Table 3:** Table of the N-sulfocarbamoyl compounds and the hydrolyzed derivatives they are hydrolyzed to indirectly determine their concentration.

<table>
<thead>
<tr>
<th>N-sulfocarbamyl compound formed</th>
<th>Hydrolysed compound formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>STX</td>
</tr>
<tr>
<td>B2</td>
<td>NEO</td>
</tr>
<tr>
<td>C1</td>
<td>GTX 2</td>
</tr>
<tr>
<td>C2</td>
<td>GTX 3</td>
</tr>
<tr>
<td>C3</td>
<td>GTX 1</td>
</tr>
<tr>
<td>C4</td>
<td>GTX 4</td>
</tr>
</tbody>
</table>
### Table 4A: Eluents used in liquid chromatography

<table>
<thead>
<tr>
<th>Eluent</th>
<th>Composition</th>
</tr>
</thead>
</table>
| A      | 6mM Octanesulfonic acid  
        | 6mM Tetrasulfonic acid  
        | 40mM Ammonium phosphate  
        | 0.75% Tetrahydrofurane |
| B      | 13mM Octanesulfonic acid  
        | 50mM Phosphoric acid  
        | adjusted to pH 6.9 with NH₃.  
        | 15% Acetonitrile  
        | 1.5% Tetrasulfonic acid |

### Table 4B: Eluent gradients used for chromatography. Injection: 5µl, flow 1.0 ml min⁻¹.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100% A</td>
</tr>
<tr>
<td>15</td>
<td>100% A</td>
</tr>
<tr>
<td>16</td>
<td>100% B</td>
</tr>
<tr>
<td>35</td>
<td>100% B</td>
</tr>
<tr>
<td>36</td>
<td>100% A</td>
</tr>
<tr>
<td>45</td>
<td>100% A</td>
</tr>
</tbody>
</table>

Concentrations of GTX1 and GTX4, GTX2 and GTX3, C1 and C2 and C3 and C4 were reported as the sum of the two individuals due to the potential of epimerization during the extraction process.
4.6 Allelochemical tests:

The allelochemical effects of *Alexandrium tamarense* cultures were quantified with a bioassay using the cryptophyte *Rhodomonas baltica*. After exposing *R. baltica* to *A. tamarense*, the target cells increased slightly in size due to swelling, formed blisters and finally lysed. Counting the number of intact *R. baltica* cells (with normal cell shape still visible) after a certain incubation period allowed for the quantification of the lytic effect.

The bioassay was performed as follows: the cell density of an exponentially growing culture of *R. baltica* (cultured at 15 °C, under artificial light at a photon flux density of 100 μE m⁻² s⁻¹ on a 16:8 h light-dark cycle) was estimated by microscope cell counts. A dilution to a final concentration of about 4 x 10⁵ ml⁻¹ cells was prepared. In triplicate, 3.9 ml of a predefined concentration of *A. tamarense* cells was pipetted into 6ml glass scintillation vials (Wheaton Scintillation Vials). Fresh K-medium was used as control. Vials were then spiked with 0.1 ml of the *R. baltica* stock culture (final concentration: 10 x 10³ ml⁻¹). After adding *R. baltica*, 0.5 ml of each control vial was fixed with 2% Lugol’s solution in order to determine the actual initial *R. baltica* concentration. Samples were then incubated at 15 °C at low light (about 5 μmol m⁻² s⁻¹) on a 16:8 h light-dark cycle. After 24 h, samples were fixed with 2 % Lugol’s solution and cell concentrations of both *A. tamarense* and *R. baltica* were determined. All counts of were performed with an inverted microscope (Zeiss Axiovert 40 C). The volume for cell counts was 0.5 ml. For *A. tamarense*, whole chambers were counted, except for the highly concentrated culture of S2-P4-F2, where representative sub-areas (> 400 cells per sample) were counted. A sub area of at least 800 cells in the control was counted for *R. baltica*. In order to quantify lytic effects, only
intact cells of the target species were scored. Final cell counts of *R. baltica* were converted to both concentration (cells ml$^{-1}$) and percent mortality (treatment compared to control).

In order to quantitatively compare lytic potency of antibiotic-treated and untreated cultures, dose-response curves covering a wide range of different *A. tamarense* concentrations were plotted. Before starting the experiment, current algal cell concentrations of the *A. tamarense* cultures (3 replicates antibiotic treated, 3 replicates untreated) were determined by algal counts and appropriate dilutions were prepared to gain a series of seven final cell concentrations ranging from 50 to 2000 cells ml$^{-1}$. Each dilution was set up in triplicate. In this experiment, six vials containing K-medium served as controls. The *Rhodomonas* bioassay was then performed as described above.

**4.7 Amplified Fragment Length Polymorphism:**

**4.7.1 DNA extraction**

DNA was extracted from 100ml of exponentially growing cultures. The cultures were filtered onto 3 µm pore size polycarbonate filters (Millipore) and washed with sterile-filtered seawater to remove extra-cellular bacteria. The filters containing the samples were placed in 2ml reaction vials (Eppendorf) and immediately frozen in liquid nitrogen and stored at -80°C until extraction. The DNA extraction was done following the instructions in the DNeasy Plant Mini kit (QIAGEN).

Before DNA extraction, the cells were disrupted using a tissuelyser(QIAGEN). A 0.5 mm stainless steel bead was added to the Eppendorf tube containing the frozen filters. The samples were put in liquid nitrogen for 30 sec and placed into the tissuelyser.
Cells were broken by vigorously shaking at an intensity of 30Hz for one minute. This was repeated three times, with refreezing after every cycle. The DNA preparation was conducted as per protocol with a minor adjustment. The DNA was eluted in a smaller volume than suggested to obtain higher concentrated DNA. The DNA was stored at -80 till further analysis.

4.7.2 DNA Restriction

The DNA was diluted accordingly to obtain 250ng/50μL. The DNA was digested using a Mastercycler (Eppondorph) with the following cycles: 16°C for 9 hours, 20°C for 6 hours and 65°C for 10 min (i.e. to inactivate the enzymes). The enzymes used for the digestion were 10 U EcoRI and 10U MseI (New England Biolabs). Samples where done in duplicate.

4.7.3 DNA Ligation

17μl of the ligated sample was added to a mixture of water (2.5μl), 10mM ATP(1.7μl), 10x T4 ligase buffer (0.8μl), 10 pmol μl⁻¹ EcoRI adapter (1μl), 50 pmol μl⁻¹ MseI adapter (1μl) and T4 DNA ligase (1μl). The ligation was carried out using a Mastercycler gradient at 16°C for 9h, 20°C for 6 hours and 65°C for 10 minutes. The ligated mixture was then diluted 1:5 with 0.1 x TE buffer and stored at −25°C.

4.7.4 Preamplification

10μl of the diluted ligated DNA was added to a reaction mixture of water (12.5μl), 10x Taq DNA polymerase buffer (3μl), 10 pmol/μl EcoRI+A primer (0.5μl), 10 pmol/μl MseI+C primer (0.5μl), 1 mM dNTPs (3μl) and Taq DNA ploymerase. The PCR reaction was carried out in a mastercycler gradient (Eppendorf,) with 22 cycles
of: 94°C for 1 minute (denaturation), 56°C for 30 seconds (annealing) and 70°C for 1 minute (extension). Followed by 70°C for 5 minutes. The product was diluted 1:10 with 0.1x TE buffer and stored at -25°C.

4.7.5 Amplification

5 µl of the preamplified mixture was added to a mixture of: water (9.9 µl), PCR-Buffer (2 µl), 1 mM dNTPs (2 µl), Primers MseI (0.6 µl), Primer EcoRI (0.1 µl) and Taq (0.4 µl)

Two primer combinations where used: MseI+CC, EcoRI+AA; EcoRI+AG and MseI+CT. The amplification was carried out in a Mastercycler gradient (Eppendorf) with 12 cycles of: 94°C for 1 min (denaturation), 65°C for 30 s (annealing) and 70°C for 1 min (extension) and 22 cycles of 94°C for 30 s, 65°C for 30 seconds and 70°C for 1 min. The product was stored at -25°C.

4.7.6 Capillary electrophoresis

1 µl of the amplified product was mixed with 15 µl of HiDi formamide (Applied Bios) and 0.5 µl Gene-Scan 500 marker (ABI). The mixture was denatured at 90°C for 3 minutes to obtain single stranded DNA. The samples were analyzed using capillary electrophoresis.

4.7.7 AFLP Analysis:

GeneMapper (Applied Biosystems) was used to analyse the data was analysed using BIONUMERICS software. This program calculated the distance matrix by the formula for the “simple match coefficient” (Kosman & Leonard 2005). The dendograms threshold of 400. The comparison was determined by looking at allele
similarities between sub clones. The allele size started at 75bp and was compared for smallest alleles.
5 Results:

5.1 Axenicity of cultures:

Bacterial contamination was determined using Acridine orange during 4 sampling periods throughout the whole experiment. These values are summarised in Table 6. The axenic cultures became contaminated with bacteria between days 15 and 20.

5.2 Growth of Axenic and Xenic cultures:

The growth of the xenic and axenic cultures was monitored by cell counts over the 20 day period.

Strain S2-P4-D1 (Figure2) showed a slight difference in the growth curves between the xenic and axenic cultures. The axenic cultures reached a higher cell concentration with their highest cell concentration at day 20 which corresponded to a cell concentration of $6 \times 10^4$ cells ml$^{-1}$. The xenic culture reached its highest concentration on day 10 which corresponded to a cell concentration of $4.2 \times 10^4$ cells ml$^{-1}$.

Strain S2-P4-F2 (Figure 3) showed a similar trend, with the axenic culture reaching higher cell concentration than the xenic culture. The highest concentration reached by the axenic culture was attained on Day 16. This corresponded to a cell concentration of $6.2 \times 10^3$ cells ml$^{-1}$. This then lead to a sharp decrease in cell concentration. The xenic cultures reached a maximum cell concentration of $5.4 \times 10^3$ cells ml$^{-1}$ on day 18 where it surpassed the cell concentration of the axenic cultures to reach a maximum of $5.8 \times 10^3$ cells ml$^{-1}$. 
Figure 2: Growth of the culture strain S2-P4-D1 over 20 days.

Figure 3: Growth of the culture strain S2-P4-F2 over the 20 day experiment in axenic and xenic cultures.

The growth rate varied slightly between the two S2-P4-D1 cultures (Figure 4). The axenic cultures had a growth rate of 0.42, while the
xenic cultures had one of 0.41. The axenic cultures had a 2 day lag phase and went into exponential growth (summarised in Table 6). The xenic cultures followed roughly the same pattern, with lower cell concentrations. The lag phase lasted till the fourth day, and then the cultures went into exponential growth. The stationary phase was similar for both cultures and lasted for the remainder of the experiment.

The growth rates of both axenic and xenic cultures of S2-P4-F2 (Figure 5) were virtually the same, namely 0.31 and 0.33 respectively. The defined growth phases are summarised in Table 6.

Both cultures reached the stationary phase at Day 14 and remained there with no significant cell concentration changes till the remainder of the experiment.

**Figure 4**: Growth curve of culture of strain S2-P4-D1 depicting the lag, exponential and stationary phases.
**Figure 5**: Growth curve of S2-P4-F2 showing the lag, exponential and stationary phases defined in table 6.

**Table 5**: Bacterial counts for axenic and xenic cultures using Acridine orange on four sampling days.

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<td>Day 14</td>
<td>Day 20</td>
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Table 6: The growth phases as defined. The growth rate was calculated using the defined exponential phases.

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<td>6-12</td>
<td>12-20</td>
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5.3 Production of PSP toxins by axenic and xenic cultures:

5.3.1 Toxin profiles

In order to determine the PSP toxin content, samples were taken every second day for a period of 20 days.

The axenic and xenic cultures of strain S2-P4-D1 (Figure 6A) produced the following PSP toxins: STX, NeoSTX, GTX1/4, dc STX, C1/2 and C3/4. STX was the most abundant, accounting for roughly 48 – 50% of the molar percent in the axenic cultures, while it accounted for slightly more in the xenic cultures (53-54 mol%). The second largest fraction was from NeoSTX which accounted for 31% in the xenic and 29% in axenic cultures. The C1/2 toxins accounted for 10 – 13 % in both cultures. GTX2/3, dc GTX2/3, dc STX and C3/4 were the least abundant and only accounted for 1, 0.5, 0.06 and 0.04 % respectively.

The axenic and xenic cultures of strain S2-P4-F2’s (Figures 7A & B) had a similar toxin profile to that of S2-P4-D1. STX accounted for roughly 33 -39 % in both treatments. NeoSTX followed with an abundance of 32-35% in both cultures. C1/2 was the third most
abundant toxin, accounting for 16-19% of the total molar concentration/percent. GTX1/4, GTX2/3, dcGTX2/3, dcSTX were the least abundant with average values of 9, 2, 1 and 0.05 respectively.

These profiles remained constant till the cultures had reached the stationary phase. The 20\textsuperscript{th} day of sampling showed a slight variation in both the axenic and xenic triplicates. The variation was in the form of a marked reduction in GTX1/4 production (Figure 6B). These levels where so low that they could not be quantified. S2-P4-F2 has saw variation in the xenic triplicate. It showed a high decrease in STX production (Figure 6). The molar percents and total toxicity differed over the course of the experiment. STX and NeoSTX showed the greatest variation where by the 20\textsuperscript{th} day the molar ratio of STX had decreased from 50 to 20% in both the xenic and axenic cultures of S2-P4-D1. The same was observed for S2-P4-F2 which saw the STX molar ratio decrease from 35 to 16%.
Figure 6 A and B: PST profile of S2-P4-D1 showing the molar percent composition of each PST at a) t = 0 (sampling time zero) and b) T=20 (end of the experiment)
**Figures 7 A and B:** PST profile of S2-P4-F2 in molar percent at a) $t = 0$ (sampling time zero) and b) $T=20$ (end of the experiment)
5.4 Toxin content verses cell growth in axenic and xenic cultures:

Total toxin content was plotted against the growth curve to determine the amount of toxin production at each growth phase. The axenic cultures of S2-P4-D1 (Figure 8a) showed a decrease in toxicity during the lag phase (from 90 fmol/cell to 60 fmol/cell). The PST concentration fluctuated during the exponential phase, reaching a high of 70 fmol/cell. The stationary phase showed a sharp decrease in toxin content (from 70 – 49 fmol/cell) followed by an increase to 60 fmol/cell, which was then followed by a permanent decrease for the remainder of the experiment to 25 fmol/cell. The variations in toxin content that were statistically significant were during the lag/exponential phase and the stationary phase. S2-P4-D1 xenic culture had a higher toxin content than the axenic culture. The lag phase saw a slight increase in cell toxin content (i.e. from 100-110 fmol/cell). The exponential phase generally saw an increase in toxicity. This corresponded to the highest toxin content reached by the culture of 210 fmol/cell at day 8. The stationary phase saw a continuous decrease in toxin content. There was a statistically significant difference between the three growth phases.

The axenic cultures of S2-P4-F2 (Figure 8B) showed an increase in toxicity from the lag to exponential phase that mimicked the growth curve. It increased steadily to 150 fmol/cell till the mid exponential phase where it dropped off slightly to 130 fmol/cell. It increased to 150 fmol/cell at the beginning of the stationary phase and steadily decreased there after till it reached a value of 50 fmol/cell. The toxin content of the xenic culture showed less variation. It increased evenly from the lag phase to the exponential phase to its maximum value of 400 fmol/cell. As the culture moved
into its stationary phase, the toxicity dropped off steadily till it reached the value of 70fmol/cell.

Figure 8 A & B: Changes in total PSP toxin concentration and cell concentration as a function of culture age of A) axenic and B) xenic S2-P4-D1.
Figure 9 A & B: Changes in total PST and cell concentration as a function of culture age of A) axenic and B) xenic S2-P4-F2.
Comparing the two strains in toxin production at the different growth phases yielded similar results (Figures 10 & 11). The xenic cultures showed a marked increase in toxicity during the lag and exponential phases. The stationary phase saw the total toxin production of the axenic cultures surpass that of the xenic cultures in both strains. These differences were only statistically significant in the exponential phase of the xenic cultures of both strains.

**Figure 10:** Total toxin content with respect to growth phase of strain S2-P4-D1 with a standard deviation of $6.4 \times 10^2$ (n=3).

**Figure 11:** Total toxin content as with respect to growth phase of strain S2-P4-F2 with a standard deviation of $1.2 \times 10^2$ (n=3).
**Total toxin content:**

The total toxicity for the axenic and xenic cultures of strain S2-P4-D1 (figure 12) where $6.4 \times 10^2$ and $1.3 \times 10^3$ fmol/cell respectively. The xenic cultures produced twice as much PSP toxin as the axenic cultures. The axenic and xenic cultures of S2-P4-F2 (Figure 11) where more toxic than S2-P4-D1 (Figure 10), with a total toxicity of $1.2 \times 10^3$ and $1.6 \times 10^3$ fmol/cell respectively. The xenic cultures where 1.3 times more toxic than the axenic cultures. This variation in toxin content was statistically significant.

![Graph showing total toxin content](image)

**Figure 12:** Total toxicity of both strains over the 20 day period.
Allelochemical tests:

Strain S2-P4-D1 was shown to have lytic potential. Cell concentrations over $4 \times 10^3$ saw no surviving *Rhodomonas* cells. This was seen in both cultures.

![Graph showing dose-response curve for Rhodomonas survival](image)

**Figure 13:** Dose-response curve depicting *Rhodomonas* survival with respect to changes in *A. tamarense* cell concentrations.

5.7 Sub-clones

5.7.1 Toxin profiles:

The toxin profile of 4 strains of *A. tamarense* was observed. All strains had different toxin profiles.

All 20 sub-clones of S2-P6-G12 (Figure 14) had similar toxin profiles to that of the parent clones (PC1 and PC2). This consisted of B1 (GTX 5), C1/2, GTX1/4, NeoSTX, STX, C3/4 (not present in all samples) and GTX2/3. The molar ratios were as follows: B1 (GTX 5): 32-54 %, C1/2: 20 – 30 %, GTX1/4: 3-9 %, NeoSTX: 8-20, STX: 4-9 %, C3/4: 1-8 % and GTX2/3: 1-7 %.
S2-P1-G7 (Figure) showed the same trend of the same toxin profile between PC’s and the 20 sub clones. The toxin profiles consisted of the following (with their average molar ratio in brackets): GTX 5 (17-40%), C1/2 (30-49), GTX1/4 (5-12), GTX 2/3 (4-10), NeoSTX (2-11) and STX (4-7). Low values of dcSTX and dcGTX2/3 were also observed and accounted for a molar percent ranging from 0.01 to 0.1.

Seven of the twenty S2-P4-D1 (Figure) sub clones showed differences in toxin profiles compared to PC1 and 2. The toxin profiles consisted of the following (with their average molar ratio in brackets): GTX 2/3 (33-40), NeoSTX (25-35), STX (20-28), GTX1/4 (2-6), with low levels of dcSTX and dc GTX 2/3.

**Figure 14:** Toxin profile showing the molar percent of all the toxins present in all sub clones of S2-P6-G12.
**Figure 15:** Toxin profile showing the molar percent of all the toxins present in all sub-cones of S1-P6-G7.

**Figure 16:** Toxin profile showing the molar percent of all the toxins present in all sub-cones of S2-P4-D1.
**Figure 17:** Toxin profile showing the molar percent of all the toxins present in all subcones of S2-P4-F2.

### 5.7.2 Variations in total toxicity:

The sub clones had varying toxicity (figure 18) with the highest and lowest values of 115 and 34 fmol/cell respectively. This accounts for a 3 fold difference in toxicity. 10 of the 22 sub clones had toxicity less than that of the average toxicity of all the sub clones (Figure 21).
Figure 18: Total toxicity of sub clones S2-P6-G12. Parent clones denoted as PC1 and PC2 with a standard deviation of 25.25 (n=20)

Figure 19: Variation in toxin content of S2-P6-G12 as a function of the difference between the average PSP toxin content of the parent clones and the 20 sub clones.
**Figure 20:** Total toxin content of 20 sub clones of S2-P1-G7. Parent clones denoted as PC1 and PC2, with a standard deviation of 10.48.

**Figure 21:** Variation in toxin content of S2-P1-G7 as a function of the difference between the average PSP toxin content of the parent clones and the 20 sub clones.
**Figure 22:** Total PST concentration of sub clones S2-P4-D1. Parent clones denoted as PC1 and PC2. With a STDEV of 38.16

![Bar graph showing total toxin content (fmol/cell) for subclones S2-P4-D1.](image)

**Figure 23:** Variation in toxicity of S2-P4-D1 as a function of the difference between the total toxicity of the individuals and average toxicity of the group.

### 5.6 AFLP:

Two primer combinations were used. The results of the different primer combinations are summarised in Table 6. The primer combination of E_AG_M_CT was used due to the higher values obtained when comparing the similarity of the sub-clones.

The dendogram of S2-P4-D1 (Figure) showed the most variation in sub clone grouping. It had a low similarity of 37%. Sub-clone 5 was the most obvious outlier. The remaining sub-clones had a similarity value of 82%. These where further divided into three
distinct groups with a similarity over 90%. The S2-P4-F2 sub-clones showed less variation and had a higher bootstrap value of 86% with one outlier sub-clone. The remaining sub-clones were divided into two distinct groups with a bootstrap value of 91%. S6-P1-G7 showed a higher level of similarity with a bootstrap value of 83%. They were further divided into 2 distinct groups with bootstrap values of 89 and 97% respectively. S2-P6-G12 had a bootstrap value of 89%. The sub-clone was divided into 2 different groups with bootstrap values of 97%. Due to time constraints this data could not be analysed further to determine whether the differences seen were statistically significant.
Table 6: Comparison of primer combinations used in AFLP.

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**Figure 24:** Dendogram depicting similarity between sub-clones of strain S2-P4-D1
Figure 25: Dendogram depicting similarity between sub-clones of strain S2-P4-F2

Figure 26: Dendogram depicting similarity between sub-clones of strain S6-P1-G7.

Figure 27: Dendogram depicting similarity between sub-clones of strain S2-P6-G12.
6. Discussion:

The extent of bacteria’s involvement in PSP toxin production is still continuing. It has been suggested that bacteria complement the dinoflagellate PSP producers by affecting the growth rate or the actual PSP toxin production. In order to assess this effect, experiments where conducted to determine which parameter is affected by bacteria.

Growth rate:

The growth rates of both strains of *A. tamarense* were very similar. The similarities in growth rate are consistent with Hold et al. (2001) findings on *A. tamarense*. Their experiments used *A. lustitancicum* and *A. tamarense* and compared the growth rate and toxin production between axenic and xenic cultures. Antibiotics were used to obtain axenic cultures. The axenic and xenic *A. tamarense* cultures showed equal growth rates. The authenticity of the axenic cultures was thoroughly investigated (solid and liquid media, epifluorescence microscopy with Sybr green 1 and PCR using universal eubacterial primers targeting 16S rRNA,).

Even though the xenic cultures of both strains took 4 days to reach their exponential growth phase; this did not seem to affect the overall growth rate. The difference in cell concentrations was probably due to the differences in initial inocula. When the axenic cultures were transferred into new medium, the xenic cultures where also transferred. This identical treatment reduced the chance of having cultures that were not in the same growth phase. The extended lag phase observed in the xenic cultures suggests that the bacterial micro flora in the medium might affect nutrient uptake initially due to the fact that the other factors that could affect
growth where constant (i.e. light intensity, temperature, salinity, pH). Though the bacteria affected the lag phase initially, they seem to have no effect on the growth rate of cultures after. This difference could also have been due to the lower cell concentrations used initially in the non-axenic cultures.

**Total toxin content:**

One of the first studies that compared axenic and xenic cultures with respect to growth and toxicity were reported by Dantzer & Levin (1997). They used *A. tamarense* cultures and found that there were no differences in toxicity between the axenic and xenic cultures. They then concluded that bacteria had no effect on toxicity. This is contradictory to my results because, overall the xenic cultures of both strains were more toxic than the axenic cultures. Xenic S2-P4-D1 was twice as toxic, while xenic S2-P4-F2 was 1.3 times as toxic. This is consistent with findings by Doucette & Powell (1998) and Hold (2001).

The uniformity of toxin content seen by Dantzer & Levin (1997) could have been due to the methods used to determine bacterial contamination. They grew their cultures in penicillin-rich medium and tested the axenicity of the cultures by using marine agar. This method was not very effective due to the fact that 95% of marine bacteria are unculturable (Schut et al 1993). So lack of bacterial growth could simply have been due to the unsuitability of the media used. Doucette and Powell (1998) used lysozyme/SDS to remove bacteria from their *A. lustitanicum* cultures. They found that the axenic cultures were less toxic than the xenic ones and that toxin production could be restored by the addition of bacteria believed to be PST producers. They concluded that the interaction between *A. lustitanicum* and bacteria accounted for 50% of the cellular toxins.
produced. This is consistent with the strain S2-P4-D1 which showed a two fold increase in the total toxin production. So this could point to the idea of bacteria enhancing toxin production. However the axenic nature of Dantzer & Levins (1997) cultures is questionable due to the methods used for bacterial contamination. They utilised epifluorescence microscopy and liquid medium to determine the extent of bacterial contamination. They did not report the frequency of their monitoring of bacterial growth. Another factor was that the observations did not take into account the effect lysozyme/SDS treatment might have had on A. *lustralitanicum*’s growth rate.

The highest toxin production occurred during the exponential phase for all the cultures. This is consistent with results by Boyer et al (1987) and Anderson et al (1990). SXT and NeoSTX in both xenic and axenic cultures increased during the exponential phase and showed a clear decrease during the stationary phase.

When comparing the total toxin production at the different growth phases, marked changes are observed. The differences in the exponential growth phase of two days in the axenic and xenic cultures could have lead to the difference in total toxin content. The xenic cultures spent longer in the exponential growth phase where toxin production is at its highest allowing them to produce more toxins. This observation was consistent with both strains. The axenic cultures in S2-P4-F2 showed a difference in the stationary phase where more toxins were produced than in the xenic cultures. This was probably due to the fact that they had a longer stationary phase. Even though toxin production decreases in this growth phase, it does so steadily (i.e. no sharp decreases). This enabled the axenic cultures to produce more toxins but this difference was not statistically significant.
Boyer et al (1987) conducted nutrient limitation experiments with nitrogen and phosphorus on A. tamarense. They found that the toxicity decreased in nitrogen limited cultures, while an increase was found in phosphorus limited cultures. The increase in PST productivity was due to the fact that the cells where unable to divide, causing cells to get bigger. PST production did not stop. So the PST concentration increases per cell, but the cell concentration remains constant leading to an observed PST increase. So it could be suggested that the bacteria might affect the nutrient uptake of the dinoflagellate, namely phosphorus since the xenic cultures where more toxic. This is unlikely since K medium is not nitrogen or phosphorus limited and the cell size between the axenic and xenic cultures did not vary extensively.

The differences in toxicity might not have been due to the bacteria, but due to the effect of the antibiotics used. Studies on the effects of streptomycin and chloramphenicol (which where both used in the antibiotic cocktail) showed harmful effects on some algal cells (Martins et al 2004). Streptomycin caused loss of pigmentation and changes in chloroplast morphology in Chlorella. In bacteria it inhibits protein synthesis in gram negative bacteria (Mckane and Kandel, 1996). While chloramphenicol caused inhibition of growth in Euglena as well as affecting chlorophyll formation. There is no documentation of the effects of antibiotics on dinoflagellates (Martins et al 2004). The possible effects on dinoflagellate could be different in the sense of affecting toxin production which would lead to a decrease in total toxicity. Or it could affect the cell wall by perforating it, causing leakage of some PSP toxins. Since the PST measured is from intact cells, the possible PSP toxins in the medium of the axenic cultures would not be detected. Or it could attack the organelles of endosymbiotic origin ("prokaryotic
components” These are believed to have been engulfed during the evolution of the eukaryotic cell.), namely the mitochondria and chloroplast. These vestigial organelles could be affected by the antibiotics that target the prokaryotic cells of bacteria causing an effect.

Anderson et al (1990) conducted experiments where they looked at the effect of N and P limitations as well as determining the rate of toxin production. They found that cells produced toxins at rates equal to those needed to replace losses to daughter cells during cell division (i.e. the “growth dilution” effect). I do not believe that this has an effect on the strains used due to the fact that if this loss was substantial, the greatest loss would be during the exponential phase since that is when the most daughter cells are produced, so PSP toxin content would decrease. But the highest amount of PSP toxin produced is in the exponential phase. They suggested that toxin production was complex and coupled to cell growth at certain conditions and unrelated in other conditions.

This could be due to the fact that the bacteria could play a role in remineralisation of nutrients. They could also produce metabolites that act as co-factors and stimulate PSP toxin production in that manner.

The PSP toxin profile remained constant through out the 20 day experiment although one of the triplicate samples of each strain showed the loss of a PSP toxin. This was seen in one triplicate of the samples: auxenic and xenic S2-P4-D1 and xenic S2-P4-F2. Since this difference was seen in both auxenic and xenic cultures, this effect cannot be attributed to bacterial influence.
Endonuclear bacteria could still have an effect on toxin production. This cannot be determined with the method used since we where focusing on extra-cellular bacteria.

My data suggests that the effect of bacteria in PSP production is significant. They probably influence PSP toxin production by producing compounds that stimulate PSP toxin production.

**Allelochemical tests on axenic and xenic cultures**

Numerous studies have been conducted investigating the allelochemical effect of *A. tamarense* on various phytoplankton species (Ogata & Kodoma 1986, Lush & Hallegraeff 1996, Tillmann & John 2002) and have shown that these lytic compounds are not related to PSP toxins. This lead to suggestions of other factors influencing the observed allelopathic effects. Cole (1982) suggested that bacteria may mediate this by affecting the metabolism of the causative species, or alternatively they could release allelochemical of their own. The results show that this is not true in the case of strain S2-P4-D1. These results are consistent with those reported by Tillmann & John (2002) and Suikkanen et al (2004). S2-P4-D1 showed no difference in lytic effect on *Rhodomonus* cells between the axenic and non-axenic cultures. This suggests that bacteria have no effect on the allelochemicals released. The medium is thought to have been axenic during the sampling time by. This means that the allelochemicals are produced by the dinoflagellate. The plot of dose curve showed that this effect is dependant on the cell concentrations. The non-lytic S2-P4-F2 strain showed no difference in lytic effect between axenic and non-axenic cultures. This gives further proof to the fact that bacteria do not produce these allelochemicals.
**Sub clones**

**Toxin profile:**
The toxin profile of four strains of *A. tamarense* where analysed to see if there is any variation in the toxins produced. Some authors have reported that these profiles remain constant (Boyer et al 1986) while others have reported variations (Boczar et al. 1988, Anderson et al 1990). Boczar et al (1986) suggested that the profile could be modified significantly with environmental changes. While Sako et al (1992) and Kim et al (1993) found that their toxin profiles did not change significantly under various growth conditions. They also conducted mating experiments and found that toxin profiles where inherited in a 1:1 Mendelian ratio. This allowed them to conclude that toxin composition is determined on a genetic basis. Ishida et al (1998) conducted mating experiments as well with both *A. tamarense* and *A. cantenella*. They took strains with different toxin profiles and mated them. They analyzed the toxin content to see if it would be a mixture of both parent strains and found that most of their strains exhibited a toxin profile of the combined parent strains. They suggested that

I determined the toxin profiles of single cells picked from four Parent strains. This would suggest that they where genetically identical due to their origin. Two strains namely, S2-P6-G12 and S6-P1-G7 showed some variations in toxin profile. On closer inspection it was found that these variations where due to the fact that some of the toxins where produced in such low amounts that they where unquantifiable (i.e. dcSTX). The introduction of C3/4 in 8 of the 40 sub clones which was not present in the parent clones could be explained as an error during hydrolysis. Strains S2-P4-D1 and S2-P4-F2 showed more differences in their toxin profiles. Where more that half of the sub clones had a differing toxin profile
to that of the parent clones. Seven of S2-P4-D1s sub clones
differed to that of the parent clone. These differences where due to
the introduction of both C3/4 and B2. B2 was not observed in the
original chromatogram. There was no B2 standard so its
concentration was measured like the other sulfocarbamoyl toxins by
determining the difference between the non hydrolysed and
hydrolysed peaks of NeoSTX. The harsh conditions of hydrolysis
(addition of HCl and the incubation at 90°C) such as an extended
amount of heat or the variations in pH most likely further converted
C3/4 into NeoSTX. This could have been done by cleaving the
sulfate group present on positions R2 and R3 respectively. As a
result the difference seen would be attributed to the presence of B2.

I believe that the variation seen in these strains can be accounted
for by methodological errors, so no variation in toxin profiles is
actually observed. This data supports the notion of toxin production
being a genetically determined stable characteristic.

The extent of this hydrolysing error can be determined by
conducting experiments with varying acid concentrations and time at
90°C to establish the optimal conditions for this method. The
samples could also be run once more to see of there is any
difference in chromatogram peaks. The minimal production of some
PSP toxins in the sub clones could also be investigated to see of this
will change over the time period. A recent paper (Martins et al
2004) reported the loss of toxin production in a once toxic strain of
A. lusitanicum. They attributed this to either genetic mutations or
effects from the prolonged treatment of the cultures by antibiotics.
It would be interesting to see of these cultures will follow the same
trend by not producing some PSP toxins or by loosing their PSP
toxin production completely.
Variations in toxin content:

The total toxin content was determined for the four strains. Total toxicity was determined and contrasted between the parent clones and the sub-clones.

Ogata et al (1987) conducted a similar experiment, using monoclonal cultures obtained from the same area during the same season. These clones and sub clones where cultured under the same conditions and their growth rate and PSP toxin producing capabilities where determined and contrasted. They found similarities in growth rates and toxin profiles. In addition, great differences in toxicity between the various sub clones where observed. This maximum difference in toxicity was 20 fold between some samples and 100 fold between clones. They attributed this difference to the fact that toxin production was not a hereditary characteristic. My cultures did not exhibit such a high degree of variation. The highest PSP toxin variation seen was 9 fold. This was only seen in 5 of the 22 sub clones observed. This is no where near what Ogata et al (1987) reported, but the source of this variation is still something to deliberate due to the fact that these presumed genetically similar strains where cultured under identical conditions, so it follows reason to expect modest variations between sub clones.

These variations in total cell toxin content could stem from the fact that dinoflagellate DNA is not equally shared/divided between daughter cells during asexual reproduction. Even though they probably have multiple copies of the same gene, some mechanism causes the production of PSP toxins to be non uniform.
This leads to questions as to what controls the PST production. Some produce PST in such low levels that they could not be quantified. What causes the reduction in the production of specific toxins? It would be interesting to analyse the toxin profile of the samples that showed the low production in some PSP toxins to see if these toxins are produced in greater quantities, or if they are lost entirely.

Due to time constraints, the AFLP data could not be sufficiently analysed to determine whether there where actual genetic differences within sub-clones. The preliminary data suggests that there is congruence within sub-clones of the same strain.
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References:


