STUDIES OF *ALEXANDRIUM CATENELLA* (DINOPHYCEAE) GAMETES

by

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ABSTRACT

The life cycle of *Alexandrium catenella* (a Paralytic Shellfish Poison-producing dinoflagellate) facilitates bloom initiation, bloom decline, and species dispersal. Little is known of their gametes because they are indistinguishable from vegetative cells by morphology or ploidy. In this thesis study, (+) and (-) mating *A. catenella* cultures (NEPCC #743 and #744) were isolated from Toquart Bay, Barkley Sound, BC. These were maintained in enriched Barkley Sound seawater: artificial seawater medium induced aberrant cell forms and natural water from a local site produced slower growth and reduced the percentage of culture in chains.

The sexual cycle was induced by transferring cultures into N/10 medium. Cultures were mixed in stationary phase and cysts appeared in 10-12 d, suggesting that gametes were present after ~6 d. Encystment rates were highest in N-deplete medium (15.0%); but encystment was possible in N-replete medium, in many containers, at all times of the year, and was not affected by salinity (26 vs. 31). Blue light and germanium dioxide appeared to reduce encystment.

Single cells were isolated into single wells and videotaped daily. The following was learned by comparing non dividing single cells (ND) which are potentially gametes, and dividing cells (D) which are therefore vegetative cells: 1) 17 of the 92 isolated single cells were ND; 2) ND cells survived for 2-26 d; 3) the size range of ND cells (27-51 x 24-48 μm) overlaps that of D cells (25-55 x 24-49 μm); 4) ND and D cells before their division were longer than wide, and D cells after division were wider than long; 5) before dying, 4 ND cells gained in volume and 7 ND cells lost volume, whereas all D cells gained in volume prior to dividing; 6) on average, #744 cells were longer and wider than #743 cells, but the ranges overlapped. These findings explain why pairing has been infrequently observed between unequal sized gametes, but confirms that *A. catenella* pairing should be classified as isogamous.

Single cells were isolated into single wells. After 5-7 d, ND and D cells were fixed and then labeled with the lectin FITC-Con A. No surface differences in labeling were observed between ND and D cells or between mating type. Labeling was typically pale and diffuse with infrequent bright spots and no flagellar labeling. Differences may be discovered if studies were repeated at lower lectin concentrations, cells were labeled live, or larger sample sizes were used.

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CHAPTER 1 GENERAL INTRODUCTION

Alexandrium catenella (Whedon and Kofoid) Balech [synonyms: *Protogonyaulax catenella* (Whedon and Kofoid) Taylor 1979; *Gonyaulax catenella* Whedon and Kofoid 1936] is a thecate marine photosynthetic dinoflagellate. Single cells are typically 28-34 x 30-36 μm (Cembella 1986). When growing rapidly, long chains are formed, up to 128 cells *in situ* (R. Haigh, pers. comm.), and up to 32 cells in culture (pers. obs.). When growing slowly, both cultures and natural assemblages are dominated by single cells.

A. catenella was the first dinoflagellate to be linked to Paralytic Shellfish Poisoning (PSP); in 1927 near San Francisco, six people were killed and 102 people became ill (Sommer and Meyer 1937). Circannual blooms which impact economy and human health, are endemic to the British Columbia coast. Evidence suggests that these have occurred for thousands of years but recently, have "increased in frequency, severity, and duration" (Taylor and Horner 1994). Worldwide, the genus *Alexandrium* is widely distributed (Taylor 1984). The spread of this genus is possibly facilitated by anthropogenic transport of resting cysts in ballast water of ocean vessels (Hallegraeff *et al.* 1988), or by dredging, dumping, or shellfish transplantation (Anderson and Wall 1978).

Harmful dinoflagellate blooms can be thought of as occurring in four stages: initiation, development, aggregation, and dissipation (Steidinger 1973, Evans and Taylor 1980, Taylor and Pollingher 1984). Dissipation can be caused by many factors which may act singly or in concert, and those specific to *Alexandrium* have been identified by Rensel (1993 and refs. therein). These include the restriction of growth by temperature minima, mechanical stress, and copper toxicity. Bloom decline can also occur more suddenly via predation, parasite or viral infection, dilution by physical processes, or a shift to sexual reproduction.

The life cycle of *A. catenella* (see Figure 1.1) begins when a hypnozygote (2n) excysts to form a motile planomeiocyte (2n). This then divides to produce two vegetative cells (each n) which grow and divide independently. When the sexual cycle is induced, vegetative cells become

gametes (still n). Two gametes fuse to produce a planozygote which then develops into a hypnozygote (= resting cyst). While vegetative cells can be singles or in chains, gametes are always single cells. Most commonly, this cycle is completed once a year, but excystment can be induced by both temperature increases and decreases and therefore spring and fall blooms are possible (Anderson and Morel 1979, Anderson 1980).

Sexuality of dinoflagellates was first reported in 1879 when Joseph described pairing and fusing of *Peridinium stygium* (Joseph 1879 cited in Beam and Himes 1980). Today, little is known of dinoflagellate gametogenesis. In an *A. tamarense* study, Fritz *et al.* (1989) stated that "sexuality begins with the appearance of fusing gametes" and the only mention of gametogenesis is that it "involves the formation of motile gametes". The fact that gametogenesis is not described in this recent paper which claims to "present the entire sexual life history of one dinoflagellate" reveals the degree of uncertainty surrounding this process. The essential problem is that dinoflagellate gametes cannot be distinguished from vegetative cells by either the DNA quantity or morphology. Additionally, the slow progress in this field has been compounded:

> "Whereas the study of other microorganisms readily yielded a body of reproducible, consistent phenomenology including sexual life histories for many algae and protozoa, the record of dinoflagellate sexuality was from the first so sporadic, contradictory, uncertain, and peculiar that serious consideration even by algologists and protozoologists has been widely withheld." (Beam and Himes 1980).

In general, protistan sexual cycle themes are highly variable (Margulis *et al.* 1990). When groups closely related to dinoflagellates are examined closely (Figure 1.2), it becomes apparent that there are many unknowns. Also, there is no apparent evolutionary trend for the sexual cycle characteristics of i) timing of meiosis, ii) isogamy, anisogamy, and oogamy, and iii) morphological differentiation of gametes. Based on morphology and molecular techniques, ciliates and apicomplexa are the two groups most related to dinoflagellates. Based on Figure 1.2, chrysophytes share all traits, but closer scrutiny reveals differences. For example, *Dinobryon cylindricum* mating occurs between a loricate cell, who emits a chemical cue, and a non-loricate mate (Bold and Wynne 1985) and neither the production of extracellular chemicals nor differences

in mating type morphology have been identified for dinoflagellates. In addition to the variability between groups, there is variability between dinoflagellate sexual cycles (Pfiester and Anderson 1984). For these reasons, literature on other dinoflagellate genera and non-dinoflagellate groups may not be a helpful surrogate for *Alexandrium* research.

The literature is complex due to past and ongoing name changes. To reduce confusion and retain taxonomic sense, all original published names have been updated to reflect current understanding. Original names and their translations appear in Appendix 1. Changes include the well accepted incorporation of past *Gonyaulax* and *Protogonyaulax* genera into *Alexandrium*, as well as species changes still under debate. It is important to note that although *A. catenella* and *A. tamarense* appear here as separate species, the minor differences between them suggests that they are morphotypes along a *tamarense/catenella* species complex (Cembella 1986).

The impetus for this thesis grew from three components. First, *Alexandrium* is locally and globally important as a PSP producer. Second, the sexual cycle is an integral part of dinoflagellate ecology because it is tied to bloom initiation, bloom decline, protection for the gene pool during adverse conditions, and species dispersal. Third, there is little known of dinoflagellate gametes because they are indistinguishable from vegetative cells.

To learn more about *A catenella* gametes: new *A. catenella* clonal cultures capable of mating were first isolated (Chapter 2); secondly, a strategy to induce the sexual cycle was developed to approximate life stage durations and measure encystment rates (Chapter 3); thirdly, a method was devised to identify potential gametes whose volume change over time, size, shape, and longevity could then be measured (Chapter 4); fourthly, a protocol for labeling fixed cells with FITC-Con A was developed and used to test whether surface receptors would differ between mating types or life cycle stages (Chapter 5).

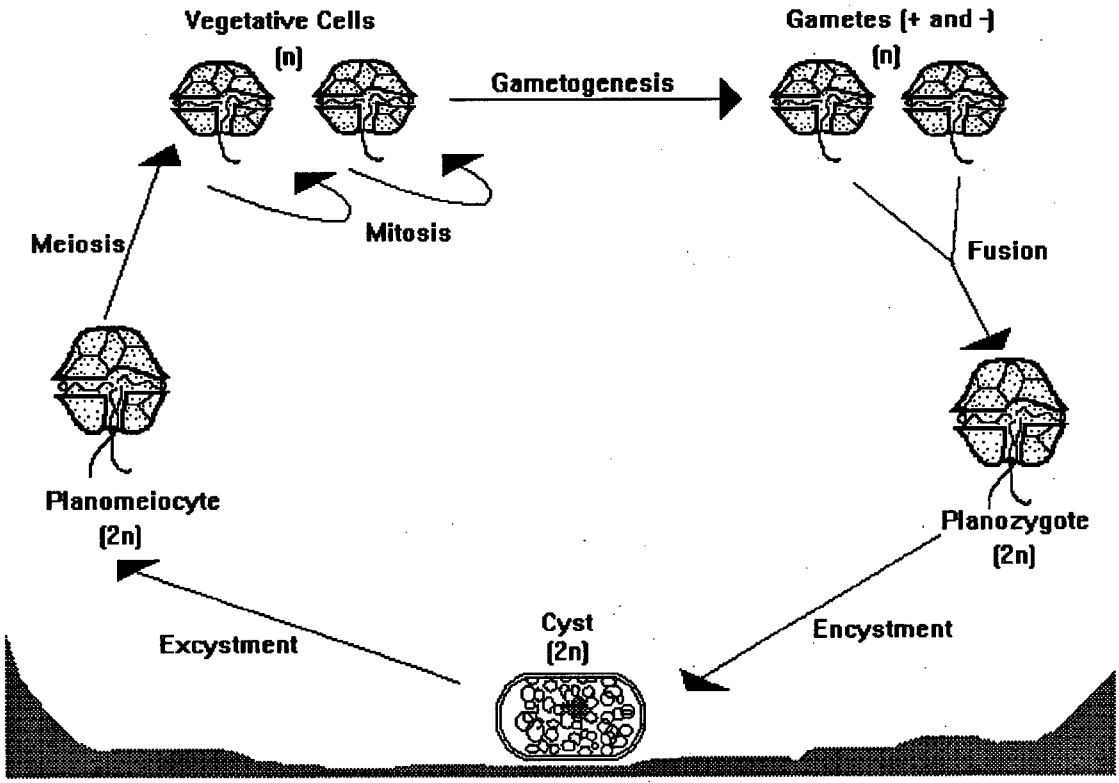


Figure 1.1 *Alexandrium catenella* Life Cycle.
A resting cyst (2n) hatches to form a motile planomeiocyte (2n) which divides to produce two vegetative cells (each n) which are likely (+) and (-) mating types. These grow and divide independently. When the sexual cycle is induced, vegetative cells become gametes (still n). Two gametes fuse either through heterothallic pairing (+ with -) or homothallic pairing (one mating type). Fusion produces a triflagellated or quadriflagellated planozygote which then develops into a resting cyst (hypnozygote). While vegetative cells can be singles or in chains, gametes are always single cells.

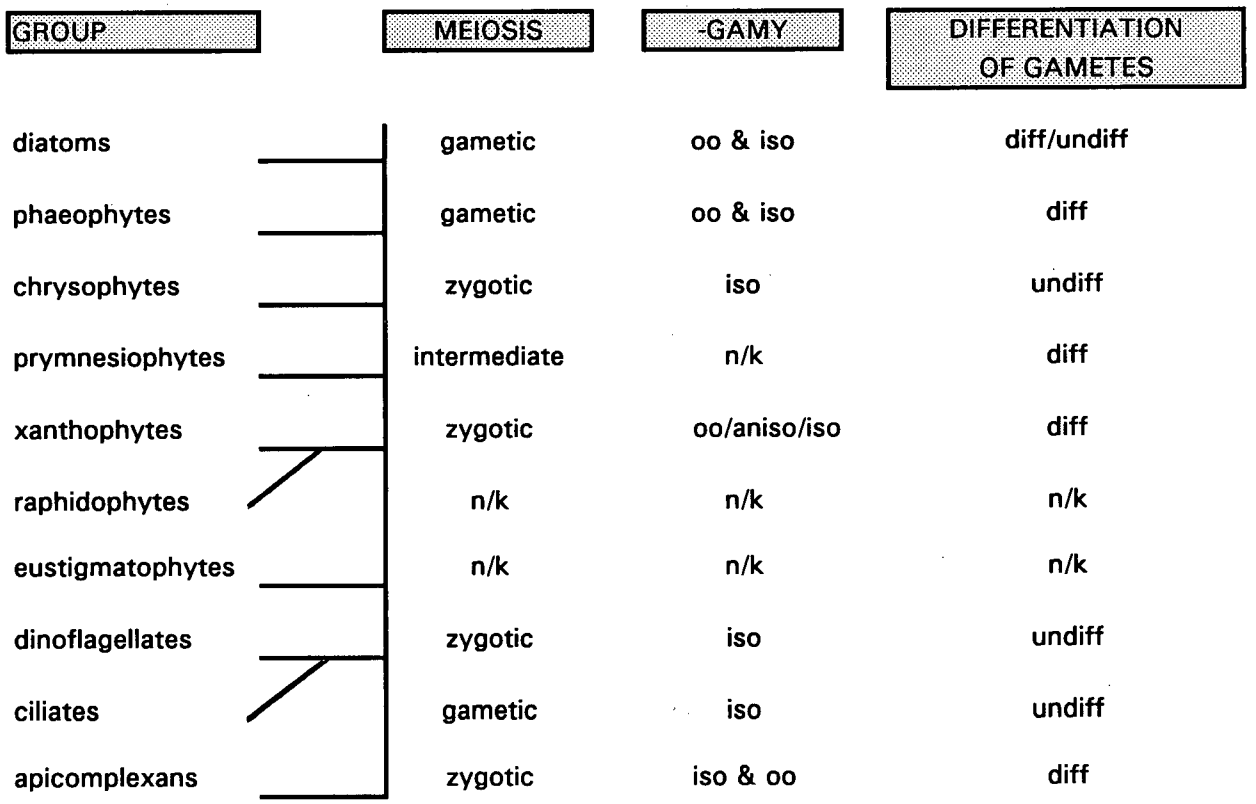


Figure 1.2 Sexual Cycle Characteristics For Groups Related to Dinoflagellates.
This assemblage is drawn from the tubular cristae branch of the "Taylor Tree" (Taylor 1978). No clear evolutionary trend is apparent in any of the three characteristics. Data compiled from Bold and Wynn (1985), Hibberd (1980), and *The Handbook of Protoctista* (1990) [ed. Margulis *et al.*] including entries by M.N. Clayton, J.C. Green *et al.*, P. Heywood, D.J. Hibberd, J. Kristiansen, D.H. Lynn & E.B. Small, and E. Vivier & I. Desportes.

CHAPTER 2 ISOLATION AND MAINTENANCE OF MATING CULTURES

1.0 Introduction

The first requirement in this series of thesis studies was to obtain two new clonal *Alexandrium catenella* isolates that could fuse, and maintain them in culture. The use of new *A. catenella* isolates was deemed necessary to minimize age-induced population shifts. Changes that have been attributed to culture effects include: fewer chains and a more isodiametrical shape for *A. catenella* (Cembella 1986), morphology changes in *Amphidinium carterae* that underwent prolonged exposure to 10.5 mM NaF (Klut *et al.* 1984), a doubling of the DNA content of *Ptychodiscus brevis* in a 23-year old culture (Loper *et al.* 1980), increased incidence of anisogamous pairing for *Gyrodinium uncatenum* (Coats *et al.* 1984), and an increase in homothallic over heterothallic pairing for *A. tamarense* (Destombe and Cembella 1990). Although two Northeast Pacific Culture Collection (NEPCC, UBC, Vancouver, BC) *A. catenella* isolates were available for study, they had been in culture for 10 and 14 years, and may have metamorphosed. Given the shifts reported, two of which involve the sexual cycle directly, new isolates were sought to ensure applicability of laboratory results to the field.

(+) and (-) cultures were needed. Since gametes are indistinguishable from vegetative cells, it was necessary to see a subsequent life cycle stage to confirm their presence. This required isolates that were capable of mating, thus (+) and (-). Additionally, these cultures needed to be separate. Since *Alexandrium* pairing is heterothallic, clonal cultures would produce gametes that would be unable to fuse and therefore, these gametes would be available for harvest.

A. catenella mating type is determined after the first post-hypnozygote division (Yoshimatsu 1984, 1985). Excystment yields one cell which divides to form a two-cell pair within 24 hours, which then doubles daily until it splits in half to form separate chains. The number of cells in the chain when this split occurs is variable (pers. obs.). Mating type tests indicate that the first division produces one (+) and one (-) cell, and with subsequent division, chains are formed with one mating type in the anterior half and the other mating type in the posterior half. Thus, *in situ* and in the laboratory, chains formed after the first split are unisexual.

Although the mating type is determined soon after excystment, the expression of this character may not appear until gametogenesis. What form this expression takes is not known, but it does not involve a change in DNA quantity or morphology.

It is generally accepted that *Alexandrium* species exhibit heterothallic pairing between (+) and (-) gametes. When eight clones of *A. catenella* were crossed, pairing only occurred between (+) and (-) and not within one mating type (Yoshimatsu 1981). Also, heterothallism has been observed in the closely related species, *A. tamarense* (Yoshimatsu 1985). Interestingly, it has been suggested that the evolution of mating types ensures that mating occurs within one species, but allows for genetic recombination (Goodenough 1985).

However, a detailed study of 12 *A. tamarense* isolates suggests that pairing may not simply be (+) and (-) (Destombe and Cembella 1990). One of the isolates was not auto-compatible but was able to mate with all other (+) and (-) isolates. Seven isolates were auto-compatible (homothallic) but some of these showed a tendency to mate with (+) or (-) isolates. Although problems in the research include low replicability and the observation of hypnozygotes in crosses where no fusing gametes were observed, this research indicates that the concept of mating type needs further study.

This chapter describes the isolation of clonal cultures and their identification as (+) and (-). Also, the effect of seawater source on *Alexandrium* cultures was tested to determine the optimal medium for maintenance and experimentation.

2.0 Materials and Methods

2.1 Incubator Conditions

All experiments and stock maintenance took place in one incubator. The light cycle was 14:10 light:dark. Temperature was 16±1°C. Light was 166 μmol quanta·m^{-2}·s^{-1} as measured with a model QSL-100 4π light meter, Biospherical Instruments Inc., San Diego, CA.

2.2 Isolation of Cultures

An Ekman grab was used to collect soft sediment from Toquart Bay (49°0'97"N, 125°19'74"W) and Macoah Passage (48°59'7"N, 125°21'9"W) in Barkley Sound, BC on July 07, 1993. These locations were chosen because Toquart Bay had been established as a "hot spot" for dinoflagellates (Haigh and Taylor unpubl.). During transit, samples were kept in the dark on ice until being put in a dark 5°C incubator.

The sediment was sieved to collect the 20-100 μm fraction using netting siliconed to polycarbonate cylinders. The sediment (~1.5 mL) was added to a Petri dish containing ~0.1 g of germanium dioxide (to deter diatom growth) and medium. The halves were sealed with silicone grease (Dow Corning Corp., Midland, Michigan) to prevent evaporation and the Petri dish put in the incubator. The sediment slurry was scanned daily under a dissecting scope at 10-80X. Single cells or short chains of *A. catenella* were collected with fine drawn micro-pipettes, transferred through at least 2 media washes, and then allowed to grow in 1 well of a 4-well Nunclon® multidish. Since the life span of temporary cysts is only a few weeks (Anderson and Wall 1978), the isolates here must be from zygotic cysts, since sediment was collected in July 1993, and isolations began nine months later in April. Isolates may be clonal (+ or -) or mixed (+ and –) depending on when they were captured post-excystment.

Six cultures of *A. catenella* were produced using this method. From these cultures, one chain of four cells was checked daily. After 6 d, the chain split to form 2 chains of 16. These were separated and grown into 2 clonal cultures. Thus a total of seven isolates came from the sediment samples.

One *A. catenella* culture was isolated from a plankton sample. Surface water was collected from Toquart Bay, Barkley Sound, in May 1993 in a 20 L Nalgene® carboy that had been rinsed and then filled to capacity. This was placed in a 16°C incubator upon returning to the laboratory. Contents of the carboy were siphoned through a 100 μm mesh and then reverse filtered to remove the fraction smaller than 10 μm. This concentrated the 10-100 μm fraction 10-fold, which could then efficiently be scanned under a dissecting microscope (10-80X).

Dinoflagellate chains or single cells were collected with a finely drawn micro-pipette, washed at least twice, and allowed to grow in 1 well of a 4-well multidish.

Together, the seven sediment cultures, one plankton culture, and one culture from the NEPCC totaled nine isolates.

2.3 Cultureware

All glassware was soaked, scrubbed with a brush (no soap), and rinsed 3 times in hot tap water. After being in 10% HCl overnight, they were soaked in distilled water, air dried, and autoclaved prior to use.

New polystyrene incubation wells were used without treatment to reduce the chance of contamination and also because even gentle washing produced scratches that would obscure observations.

2.4 Stock Maintenance

Stock cultures were kept in 250 mL Erlenmeyer Pyrex® flasks covered with 50 mL Pyrex® beakers. Transfers were completed every 10-14 d by pouring a small amount (~5mL) of culture into a fresh flask containing 50 mL medium. Transfers were done in a laminar flow hood and the flask mouths were flamed before their lids were replaced.

2.5 Artificial Medium vs. Natural Seawater Medium

Medium was made according to Harrison *et al.* (1980), with minor modifications (see Appendix 2). Typically, flagellates are cultured without added silicate, but since energy dispersive X-ray analysis detected silicon in *A. catenella* cysts (Meksumpun *et al.* 1994), it was added to all media used in these experiments. Two volumes of media were prepared according to Appendix 2, one with artificial seawater (AW) (modified ESAW, see Appendix 3, Harrison *et al.* 1980, courtesy of Anne Fisher) and one with seawater collected from the surface of Toquart Bay in Barkley Sound (BS). Both seawaters had comparable salinities (~30 and 26 respectively) and initial nutrient differences would have been overwhelmed by the nutrients added (for example, ~550 μM nitrate).

One mL of *A. catenella* stock was inoculated into 50 mL Pyrex® tubes containing 40 mL of the test medium. The tubes were arranged in random order (determined by generating random numbers) along the long edges of a culture rack and placed in the incubator.

For one month at the same time every 1-4 days, chl *a* was measured using a Turner Designs Model 10-AU Fluorometer (Sunnyvale, CA). Using this method to measure biomass was justified by the finding that chlorophyll and cell number were parallel for the dinoflagellates *A. tamarense* (Yentsch and Mague 1980) and *Peridinium gatunense* (Lindström 1991), and that brief insertion of *A. tamarense* cultures into a fluorometer did not affect their growth (Brand *et al.* 1981). Since a particle counter (Coulter Electronics) was unable to distinguish between large single cells, empty theca, and short chains of two, it could not be used.

To measure fluorescence, caps were tightened, tubes were gently inverted 3 times, and the first stable reading was recorded. After the caps were loosened, the tubes were placed into the rack one position to the right of their original positions. Equal lighting for all tubes was ensured by placing tubes midlength and equidistant between two pairs of lights and using only the long edges of the racks. Randomization of initial position, and moving tubes after measurement one position to the right were extra precautions.

The natural log of the fluorescence measures were plotted against time in days. Maximal growth was calculated in Lotus 123 via regression through the maximum slope, according to the formula:

$$F_f = F_o \cdot e^{\mu t}$$

where F is the fluorescence (f=final, o=initial) and μ is the intrinsic rate of increase (d^{-1}). Exponential growth is defined as time over which the plot produced a straight line with a positive slope, and stationary phase as the time where the curve flattened to a slope of 0. The highest fluorescence reading over the experiment duration was used as a measurement of maximum biomass.

2.6 Native vs. Non-native Sources of Seawater

HESNW (see Appendix 2) was prepared using either seawater collected from a 15 m intake in Burrard Inlet, courtesy of West Vancouver Laboratory, Department of Fisheries and Oceans (WV), or from seawater collected from the surface of Toquart Bay, Barkley Sound (BS). Again, both had comparable salinities (~26) and any nutrients present in the natural waters were minimal in comparison to the nutrients added.

Three *Alexandrium* isolates were tested: one *A. catenella* culture that had been isolated in this thesis study (now NEPCC #743) along with an *A. catenella* and an *A. tamarense* from the NEPCC (see Table 2.1). All stocks were acclimated in BS medium for at least 3 weeks prior to the experiment.

Table 2.1 Isolate Information of *Alexandrium* species used in Barkley Sound (BS) vs. West Vancouver (WV) Seawater Study.

Species	Isolation Location	Native Water	Culture Medium
#743 *A. catenella*	Toquart Bay	BS	BS since 1993
#574 *A. catenella*	Bamfield	BS	WV since 1984
#403 *A. tamarense*	Jericho Beach	WV	WV since 1981

A 1-3 mL inocula (depending on stock culture density) of #743, #574, or #403 were added to 50 mL Pyrex® tubes containing 40 mL of either BS or WV media. Incubation, chl *a* measurements, and calculations of μ_{max} were as described in Section 2.5 above.

When cultures were in exponential growth and had sufficient biomass, 1-3 mL was transferred to another tube containing the same type of medium (BS or WV), and a 10 mL sample from the host tube was fixed with acid Lugol's. The host tube biomass was followed for 1-2 days further to confirm that transfers had been done in exponential phase.

Using an inverted microscope and settling chambers, the number of singles, chains of two, etc. were counted in transects until at least 500 cells had been counted. Percent of cells in chains was calculated by dividing the total number of cells in chains by the total number of cells.

Maximum growth and percent of cells in chains were only compared for the last two transfers of four as it was found that dinoflagellate cultures take 5-20 generations to acclimate (Brand *et al.* 1981).

2.7 Identification of Mating Cultures

In the stock cultures, a culture named "Joe" began to produce cysts indicating that either (+) and (-) mating types were present, or that homothallic pairing was occurring. "Joe" was subcultured by isolating 10 chains of 4. 10 was chosen because in 100 trials of random numbers, a minimum of eight clonal isolates would guarantee at least one (+) and (-) culture. To be safe, 10 were isolated and were called J1-J10. The cultures were grown in nitrate-reduced medium and once in early stationary phase, cultures were combined in a 10x10 mating grid as follows. First, one drop (~20 μL) of medium was added to each of 55 flat-bottomed wells of a 96-well polystyrene Cell Wells™ culture cluster (Corning, New York). Then, either 3 drops of one clone and 3 drops of another clone or six drops of one clone were added to the wells to cover all combinations and clonal incubations. Interwell spaces were filled with distilled water, the cluster edges sealed with transparent tape, and the cluster put into the incubator. In another mating grid, stock cultures of all nine isolates (see Section 2.2) were similarly combined. Periodic observations using an inverted microscope were made and after one month, the number of hypnozygotes per well were tallied.

3.0 Results

3.1 Natural vs. Artificial Seawater Media

A. catenella had the same maximum growth rate whether in natural seawater or artificial medium (see Figure 2.1). The lower biomass maxima seen in artificial water was due to the

formation of pellets of cells in the tube bottoms that could not be mixed into solution. When a sample of the pellet was viewed, it contained mostly ragged irregular cells and vegetative cysts.

3.2 Native vs. Non-Native Natural Seawater Media

Isolate #743 grew faster and had a larger portion in chains when grown in native Barkley Sound seawater than in non-native West Vancouver seawater (see Figure 2.2a). Water source did not affect either growth or chain formation for #403 or #574 (see Figure 2.2b/c).

3.3 Mating Cultures

The intercrosses yielded many peculiar results. Mixing of the two isolates that were generated by separating a chain that had split soon after excystment, never produced hypnozygotes. In the 10x10 mating grid of "Joe" subcultures, cysts were found in crosses of J2/J4 and J3/J4. Because J3/J4 produced more cysts quicker, they were chosen as the cultures for work to follow. However, all subsequent mixing of J3/J4 failed to produce cysts. In the 9x9 stock culture mating grid, cysts were observed in 3 wells. One cyst was in a well containing the culture named "Joe" and another culture (note that all crosses containing "Joe" should have produced cysts since "Joe" was capable of producing cysts alone). A cross of another two isolates produced one cyst. One cross produced five cysts, and this pair has continued to produce cysts (and therefore gametes) under a variety of conditions. These two isolates have been entered into the NEPCC as #743 and #744 and are the subjects for Chapter 3, 4, and 5. Clone #743 was isolated from a plankton sample and #744 from a sediment sample.

4.0 Discussion

4.1 The Reduction of Mechanical Stress in Culture

Although the effect of mechanical stress on gametogenesis has not been studied, its effect on dinoflagellate growth is well documented. Growth rates of *A. tamarense* were inversely proportional to rotary shaker rpm with cell disintegration occurring at 125 rpm (White 1976). *Amphidinium carterae* cultures could not survive aeration or mixing (Galleron 1976). *Gonyaulax*

polyedra could not be grown in a reciprocal shaker or a chemostat, and was more sensitive to small-scale turbulence than either diatoms, blue-greens, or greens (Thomas and Gibson 1990a, 1990b). In light of these findings, several precautions were taken to minimize physical stress.

Stocks were maintained in 250 mL Erlenmeyer flasks and when growth was measured, the largest tubes the fluorometer would accommodate were used to reduce the surface:media ratio. Many dinoflagellates will not thrive in small spaces (E. Simons, NEPCC, pers. comm.). To homogenize cultures for fluorescence measures, tubes were gently inverted three times, rather than vortexing, because swirling may kill dinoflagellates (Karentz 1983). Batch cultures, rather than chemostats were used to avoid stirring.

In contrast, two personal observations exemplify *A. catenella*'s hardiness. First, after cultures had been centrifuged for 10 minutes at 180xg, chains of cells were intact and still motile. Second, a culture that had been stored in complete darkness for four months had motile cells, although weak and chlorotic. Gentle methods are necessary to maximize culture growth, but dinoflagellates are not as fragile as commonly believed.

4.2 Field Conditions Compared to Laboratory Conditions.

Although laboratory and field conditions will never be complete analogs, some precautions were taken to minimize differences. These included keeping isolates carbon replete, not axenic, in batch cultures, and at a temperature comparable to those typical in the field.

Whether cultures were carbon-limited was a concern. Culture pH was measured with a Radiometer PHM62 Standard pH Meter (Bach-Simpson Ltd., London, ON). For stock cultures, the maximum pH measured was 8.5. For tube cultures where nitrate was the limiting nutrient (details in Chapter 3), pH went as high as 8.9 in stationary phase. Several attempts to measure total carbon dioxide in the tubes according to Parsons *et al.* (1984), failed because the addition of acid aliquots did not drop the pH below the requisite 4.00. Possibly this was due to the buffering capacity of phosphate or borate in the medium, but this could also be construed as evidence that cultures were not carbon-limited. For comparison, when nutrient-replete tube cultures were grown into stationary phase (a condition presumed to induce carbon limitation), the pH soared to

9.12. Thus, it was presumed that stock cultures and nitrate-limited tube cultures were not carbon-limited.

Frequent transfers in sterile conditions were done to minimize contaminants but axenic cultures were not sought. Axenic dinoflagellate cultures experience longer lag phases (Cole *et al.* 1975, Achiha and Iwasaki 1990). Also, it was found that *A. tamarense* cyst yield was maximized by minimizing bacteria through frequent transfers, but not by making cultures axenic (Anderson and Lindquist 1985). Since the effect of micro-organisms on the sexual cycle is unknown, *in situ* conditions were approximated by minimizing but not eliminating bacteria, fungi, viruses etc.

Where nutrient limitation was required, batch cultures were used to approximate the nutrient drawdown that might typify the end of a bloom. Although it is recognized that diel migration, dark uptake of nitrate by dinoflagellates (Dortch and Maske 1982), and complex nutrient field dynamics may preclude extended periods of nutrient limitation *in situ*, it is possible that these conditions occur over short time scales.

Culture temperature was comparable to those in Toquart Bay (Haigh and Taylor unpubl.). Figure 2.3 shows that the incubator temperature (16°C) is between *in situ* maximum and minimum temperatures for all but possibly winter months.

The maximum abundance measured in Toquart Bay was $7x10^3$ cells·L^{-1}, which is lower than the $5x10^6$ cells·L^{-1} used in the mating experiments. However, Toquart Bay samples were integrated over a 3 m depth, and therefore any patchiness may have been made homogeneous. Whether any of these patches was $5x10^3$ cells·mL^{-1} is unknown.

4.3 Choice of Medium

The advantage of using artificial medium for experiments is control and replicability, and it has been used successfully to grow *A. catenella*. For example, ASP_7 but not ASP_{12} was used with success (Norris and Chew 1975). However, when *A. catenella* was grown in artificial medium in this thesis study, vegetative cysts and aberrant cells were formed. Others have found that *A. catenella* formed "warty" cells (Haigh unpubl.) and had diminished growth and biomass

(Cembella 1986, Richoux 1994) when grown in artificial medium. These findings imply that our knowledge of dinoflagellate growth requirements is incomplete.

When natural seawater is used as a medium base, it is often collected from a convenient location. For example, the NEPCC maintains isolates from around the world in water collected from Burrard Inlet. Although the logistics of maintaining all isolates in their native water is overwhelming, the findings of this thesis study show that the common use of local water may require further study.

Water source affected growth rate and chain formation for #743 but not #574, although both of these isolates are *A. catenella* from Barkley Sound. This difference could be ascribed to the fact that #574 has been in WV medium for 10 years longer than #743, but could also be clonal variability. Others have reported that medium affected *Gymnodinium catenatum* (Blackburn *et al.* 1989), and *A. tamarense* (Prakash 1967, Yentsch and Mague 1980), but more work may be needed to elucidate the effects of water source.

Chain formation has been found to increase throughout exponential growth and decrease thereafter (Tomas 1974). Also, salinity, temperature, and growth were found to affect the number of cells in chains of *Gymnodinium catenatum* (Blackburn *et al.* 1989). Thus, it should be noted that chain length and growth may not be independent measurements and are used in this chapter as indicators of culture welfare.

Although marine algae are fairly tolerant of wide ranges of media (McLachlan 1973), seawater contains over 50 known elements and a large and variable complement of organics (McLachlan 1973). These experiments have shown that artificial seawater cannot be used for all species, and that source of natural seawater can affect growth and chain length.

4.4 Confirmation of Sexuality

Given that lone gametes cannot be distinguished from vegetative cells, the presence of sexuality needed to be confirmed by the presence of a subsequent stage: dancing pairs, fusing pairs, planozygotes, or cysts. However, problems exist in the identification of each of these stages for *Alexandrium*.

A. catenella gametes "dance" when first attaching (Sawayama *et al.* 1990, 1993a, 1993b, 1993c). Although this stage has been personally witnessed, its short duration makes it likely to be missed in even diligent observations.

Fusing gametes are often found on the bottom of the culture container (Fritz *et al.* 1989, Destombe and Cembella 1990) swimming in tight circles (Anderson and Lindquist 1985, Fritz *et al.* 1989). They can be distinguished from dividing gametes by their oblique cingula (Walker and Steidinger 1979, Anderson and Lindquist 1985, Fritz *et al.* 1989, Destombe and Cembella 1990). However, parallel cingula have been observed in fusing pairs of *A. catenella*, joined epitheca to epitheca (Yoshimatsu 1981). Also, many pairs with oblique cingula that were isolated in this thesis study formed chains of two rather than planozygotes.

Although there should be temporal separation of fusing pairs and dividing pairs since division is restricted to one daily period (Chisholm 1981), dividing cells are common up to four hours into the light cycle during exponential growth, and on into the afternoon in stationary cultures (pers. obs.). Additionally, fusion is short-lived for *A. tamarense* (Anderson and Lindquist 1985) lasting only hours (Fritz *et al.* 1989). Therefore, the short duration of this stage coupled with identification problems make it a poor choice to confirm sexuality.

Planozygotes are larger and darker than vegetative cells (Yoshimatsu 1981, Anderson *et al.* 1983, Fritz *et al.* 1989) and have been described as having two longitudinal flagella (Walker and Steidinger 1979, Anderson 1980) or as being quadriflagellates (Doucette *et al.* 1989, Fritz *et al.* 1989, Destombe and Cembella 1990). However, stationary-phase vegetative cells can become up to double the volume of exponential-phase cells (Watras *et al.* 1982). Thus, the differences between enlarged vegetative cells and planozygotes can be indistinct.

Quantification of cellular DNA using DAPI staining and a computerized photomultiplier system with digital readout of relative fluorescence (courtesy of Dr. J. Berger, Zoology, UBC) was successful in distinguishing haploid cells (vegetative cells and possibly gametes) from diploid cells (planozygotes and hypnozygotes). However, even if fixation is done in the afternoon on exponential-phase cultures, 2% of the *A. catenella* population could be G2 or M cells (Tomas 1974) which would hamper the identification of planozygotes. Further, in stationary cultures,

nuclear division and cellular division may be poorly coupled. Although nuclear division commits *G. polyedra* to cellular division (Cetta and Anderson 1990), this has not been proven for *A. catenella*. In sum, planozygotes cannot be reliably identified with either light or quantitative epifluorescence microscopy.

Although *Alexandrium* species produce both temporary (asexual) and resting (zygotic) cysts, they can be readily distinguished (Anderson *et al.* 1984). The resting cysts of *A. catenella*, and *A. tamarense* are large (45-55 x 25-30 μm) non-motile ovoids containing granular cytoplasm, and possibly starch, lipid, and a red-pigmented body (Dale 1977). Their 2-5 μm multi-layered wall is usually coated with mucilage (Anderson and Wall 1978). The presence of hypnozygotes is thus a very reliable indicator of the sexual cycle, and because they have a 2-6 month mandatory dormancy (Anderson 1980), there is no chance that they will germinate in the study duration. A two week time lag was introduced into the experimental design by waiting for cysts but since the stage is distinct and long-lived, it is the most reliable way to confirm sexuality.

4.5 Homothallic or Heterothallic Pairing

With few exceptions, cysts were never formed in any clonal incubation of the nine stock cultures used in these thesis studies. The first exception is that the culture "Joe" produced cysts. This is likely because it was not a clonal culture. Isolations from excysting dinoflagellates should have both mating types if captured chains have not yet split (Yoshimatsu 1984, 1985). It appears that the culture "Joe" had been started by a chain containing both mating types since stock cultures could encyst, but none of the 10 subcultured clones from stock "Joe" ever encysted. The other exception is that two cysts were found in clonal cultures; an extremely rare event in 1.5 years of culturing nine isolates. Although it was found that more than two mating types may exist for *A. tamarense* (Destombe and Cembella 1990), evidence from this thesis study supports the findings of others (Yoshimatsu 1981, 1985): *A. catenella* is heterothallic.

4.6 Changes in Mating Characteristics With Time

Although work with *A. tamarense* (Destombe and Cembella 1990) showed that cultures would become increasingly homothallic with time, no evidence of this was found in these

experiments. Clonal incubations in many media, growth stages, and containers failed to produce any cysts in 16 months (Jan. 94-May 1995).

Recently excysted *A. tamarense* cells divide ~6 times before entering the sexual cycle (Anderson *et al.* 1983). It was proposed that the depletion of a stored product may initiate sexuality. In this thesis study, cultures #743 and #744 were isolated from sediment in July 1993. Inocula from stock cultures were mixed in February 1994, but no cysts were produced. However, when they were crossed in July 1994, again from stock inocula, cysts were formed. Although the date cannot be accurately determined, the pairs became mating-reactive sometime between 7 and 12 months from excystment.

In one study of *A. tamarense*, it was discovered that crosses (frequently homothallic) that successfully produced cysts were often unable to excyst to form viable planomeiocytes (Destombe and Cembella 1990). To test whether the cysts from #743 and #744 were completely viable, they were stored in the dark at 5°C for four months, and then returned to the 16°C incubator. Motile cells appeared five days later. After 30 days, there were still ungerminated cysts remaining, but less than 100% excystment also occurs for *in situ* populations (Anderson *et al.* 1983).

5.0 Chapter Summary

- Natural seawater is better than artificial seawater for culturing *A. catenella*.
- Growth rates and proportion of cell in chains were higher for #743 when cultured in medium made from native natural seawater.
- #743 and #744 were chosen as mating pairs because initial fusion rates were high and all subsequent pairings have produced cysts.

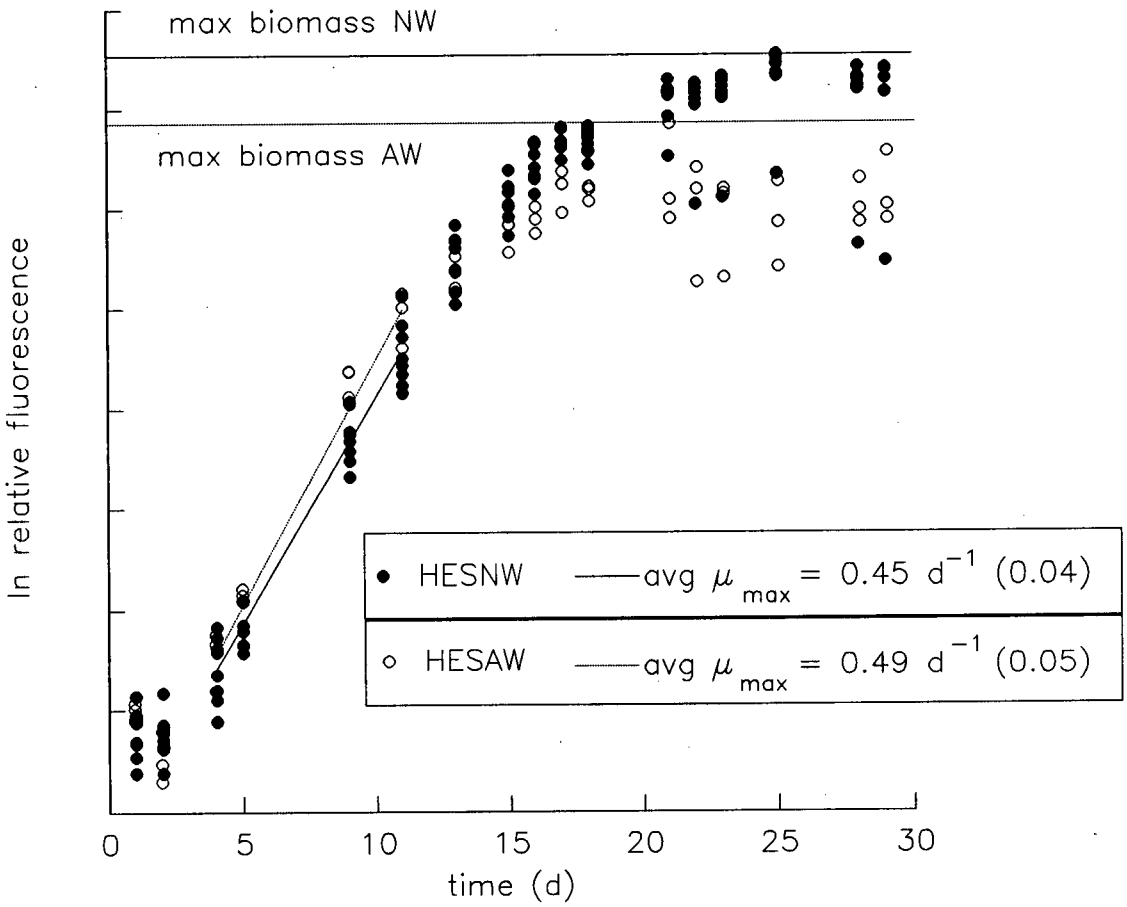


Figure 2.1 *Alexandrium catenella* in Artificial (AW) and Natural (NW) Media.
Growth was not affected by media source (t-test; $p=0.4$), but maximum biomass was higher in AW than NW (t-test; $p<0.05$). Individual regressions on each tube were calculated, and the averaged μ_{max} is plotted as a line.

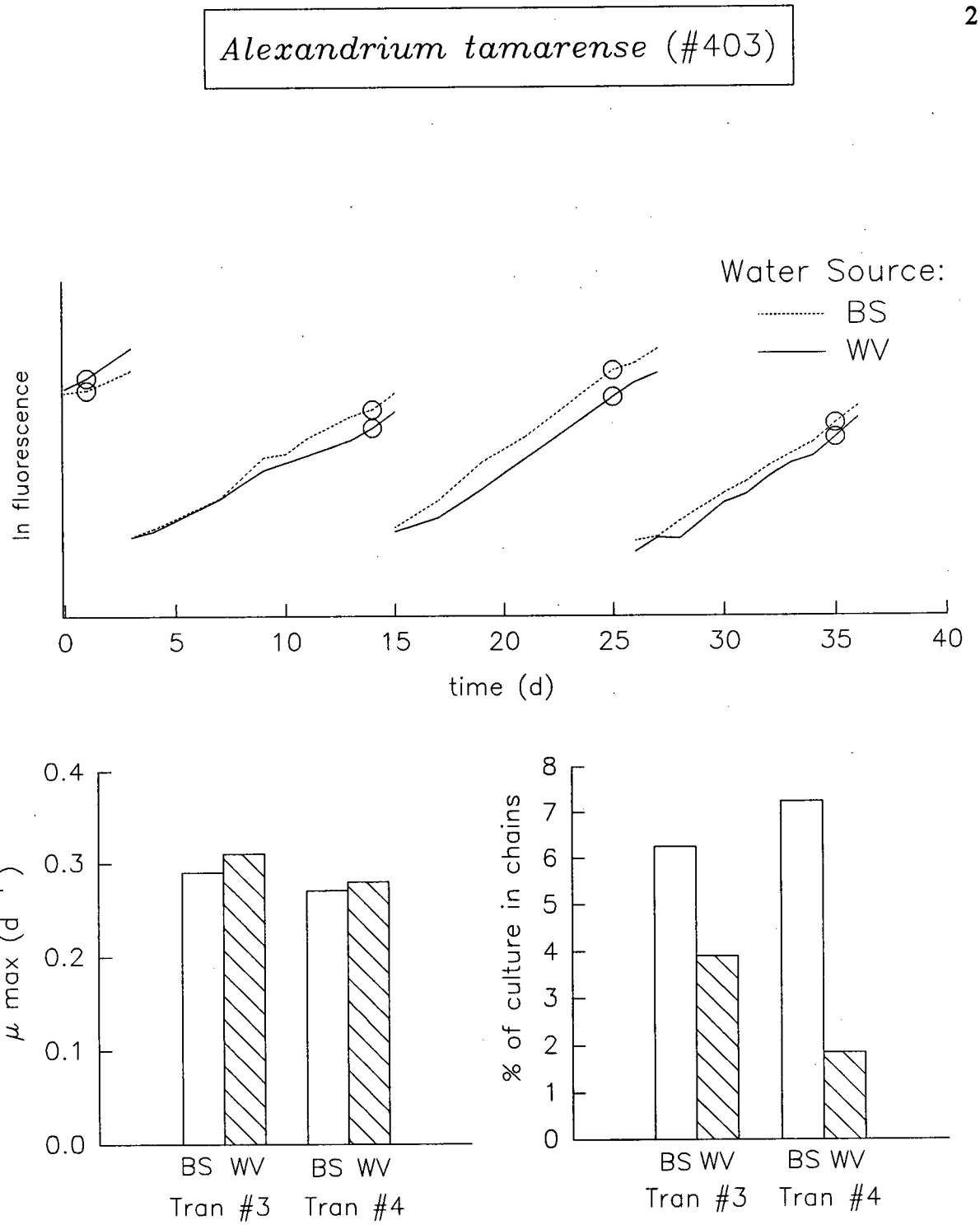


Figure 2.2a *Alexandrium tamarense* (#403) in Barkley Sound (BS) and West Vancouver (WV) Media. Culture was isolated from Jericho Beach, Vancouver, BC and therefore its native water would be WV. It has been in WV water since 1981. Top panel shows succession of four tube transfers and circles are sampling/transfer points. Data for last two tubes are shown in bottom panel. Neither growth nor proportion of culture in chains was affected by choice of natural water (t-test; p=0.6 and p=0.1).

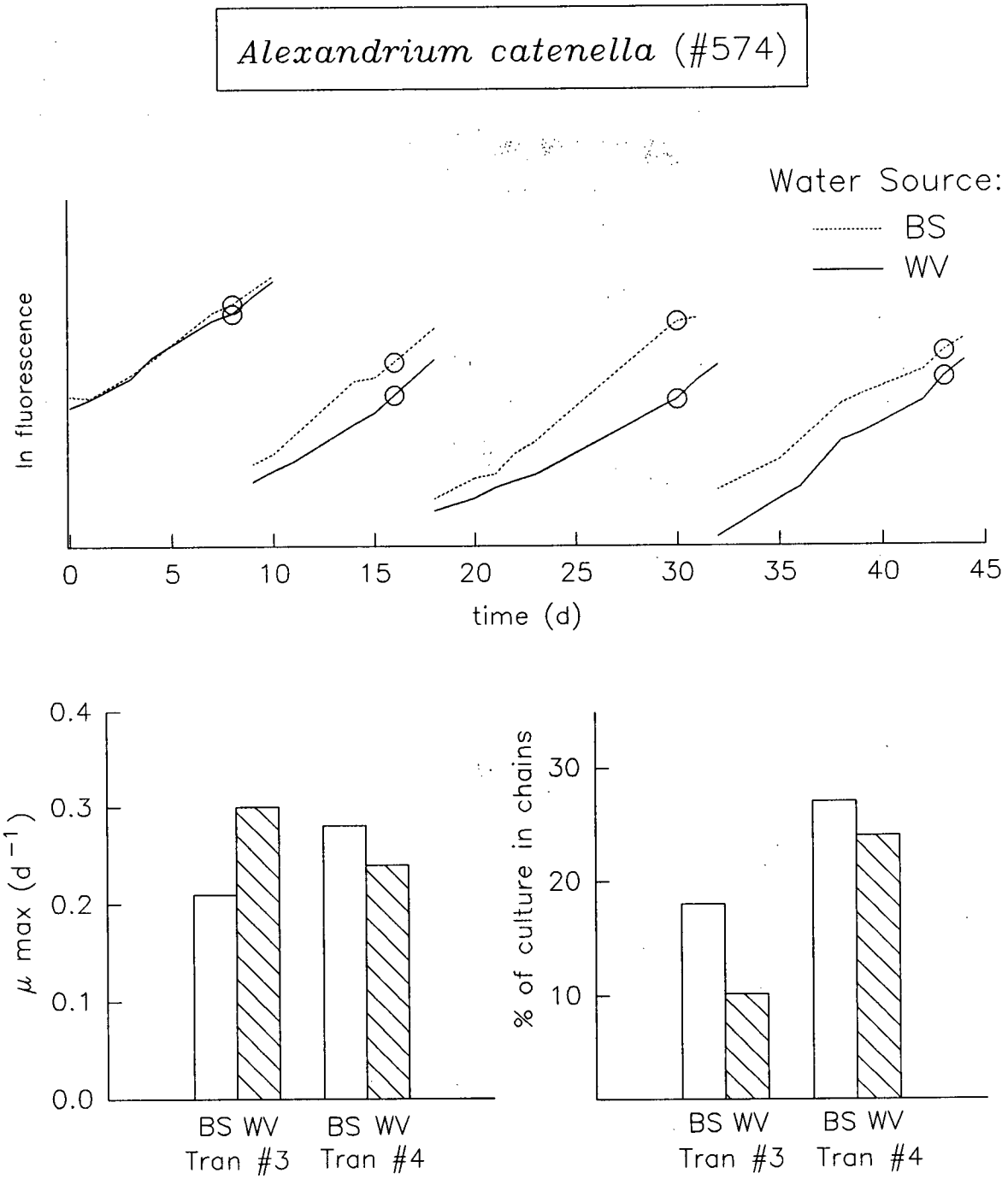


Figure 2.2b *Alexandrium catenella* (#574) in Barkley Sound (BS) and West Vancouver (WV) Media. Culture was isolated from Bamfield, BC and therefore its native water would be BS. It has been in WV water since 1984. Top panel shows succession of four tube transfers and circles are sampling/transfer points. Data for last two tubes are shown in bottom panel. Neither growth nor proportion of culture in chains was affected by choice of natural water (t-test; p=0.6 and p=0.6).

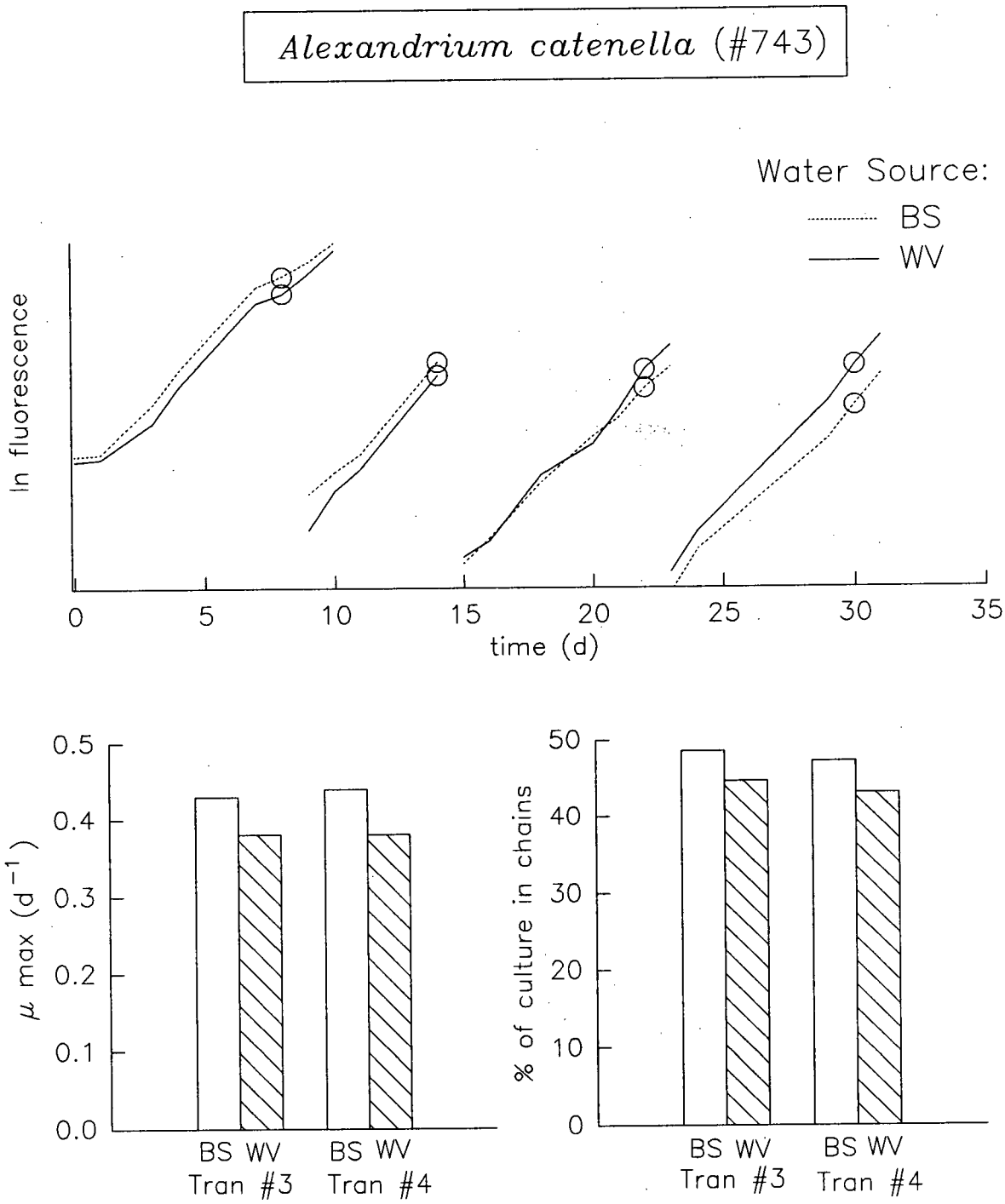


Figure 2.2c *Alexandrium catenella* (#743) in Barkley Sound (BS) and West Vancouver (WV) Media. Culture was isolated from Toquart Bay, BC and therefore its native water would be BS. It has been in BS water since 1993. Top panel shows succession of four tube transfers and circles are sampling/transfer points. Data for last two tubes are shown in bottom panel. Growth and proportion of culture in chains was affected by choice of natural water (t-test; $p=0.01$ and $p=0.06$).

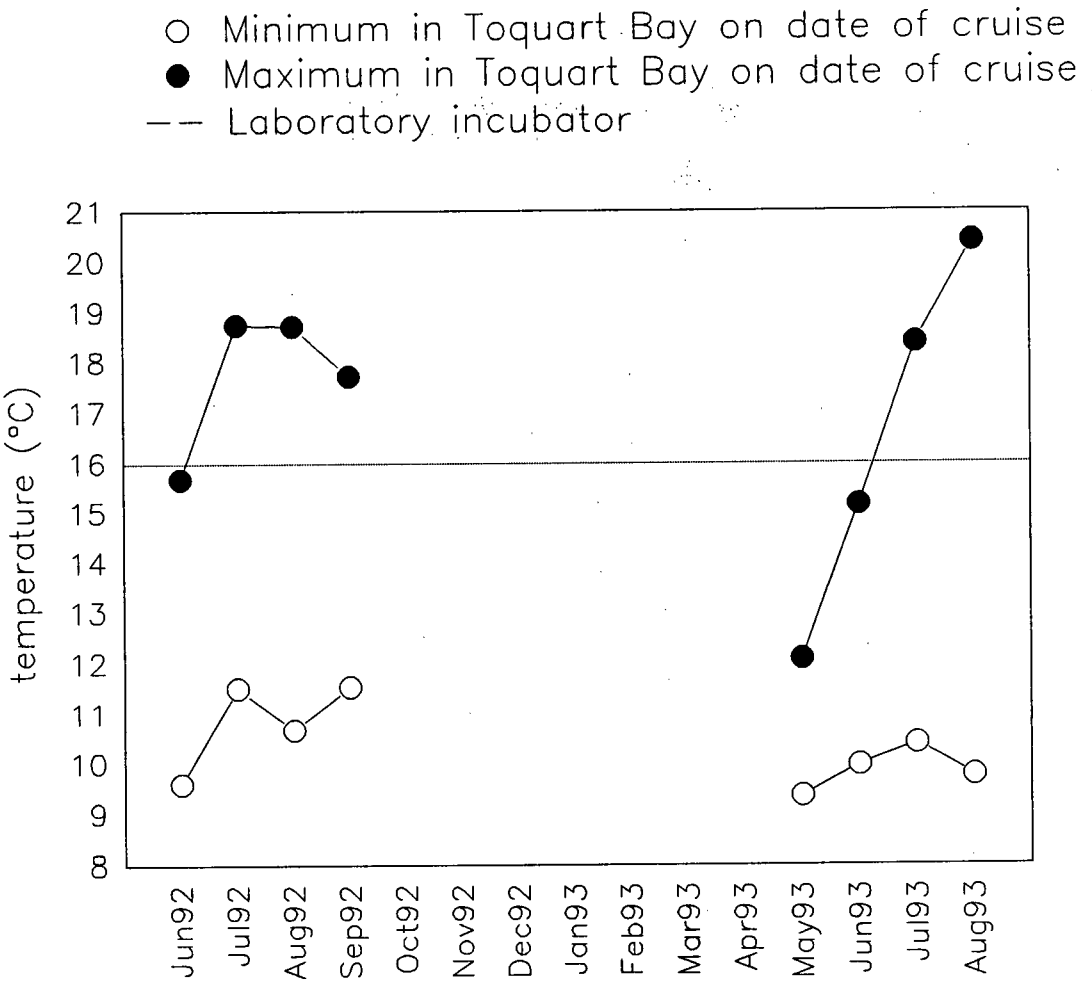


Figure 2.3 Temperatures in Culture and in Toquart Bay.
The temperature in Toquart Bay was profiled with an S4 or thermometer readings of integrated samples. For each of eight trips, the minimum and maximum temperature over the surface 30 m are plotted here. Incubator temperature was at a constant 16°C for all experiments.

CHAPTER 3 SEXUAL INDUCTION AND ENCYSTMENT RATES

1.0 Introduction

1.1 Methods of Sexual Cycle Induction

For heterotrophs such as ciliates, "vegetatively growing cells are displaced from asexual reproduction by the environmental message "food is no longer available"." (Ricci 1981). This heterotrophic concept that starvation causes cells to become mating-reactive can also be applied to nutrient limitation for autotrophs.

A variety of methods have successfully induced dinoflagellate sexual reproduction. These include nitrogen limitation (Turpin *et al.* 1978, Walker and Steidinger 1979, Anderson *et al.* 1984, Fritz *et al.* 1989, Sawayama 1990), phosphate limitation (Anderson *et al.* 1984, Anderson and Lindquist 1985), iron limitation (Doucette *et al.* 1989), dilution of all nutrients (Destombe and Cembella 1990), and no intentional limitation (Yoshimatsu 1981, Sawayama *et al.* 1993b, 1993c). Over time, nitrogen limitation has become the most common method since the goal of many studies has been to document the presence and process of the sexual cycle. Therefore, the method with historical success, nitrate limitation, was used and the testing of other nutrients, precluded (Pfiester and Anderson 1984).

Although many studies used nitrogen limitation to induce the sexual cycle, this was carried out in different ways. For example, *Gyrodinium uncatenum* was kept in exponential growth through several transfers in nitrate-free medium containing half the usual ammonium, and planozygotes appeared in stationary phase (Cetta and Anderson 1990). In contrast, others have successfully induced the sexual cycle by putting cultures in medium with zero nitrate (Pfiester 1975, Turpin *et al.* 1978), or reduced nitrate (Anderson *et al.* 1984, Fritz *et al.* 1989), which caused mating to begin within days.

1.2 Biology of Nitrogen Limitation as a Sexual Cycle Inducement

Before discussing nitrogen and the sexual cycle, the unique nitrogen biochemistry of dinoflagellates and particularly *Alexandrium,* makes an interesting preface. Arginine, which has

the lowest C:N ratio of the amino acids (6 C and 4 N) is very concentrated in dinoflagellates, even when N-starved (Flynn *et al.* 1993). In contrast, arginine is a minor component of other algae. This is noteworthy since arginine is a PSP toxin precursor (Shimizu *et al.* 1990). Furthermore, fundamental N-biochemistry is different for *Alexandrium* in comparison to six other dinoflagellates in that *A. tamarense* and *A. minutum* always maintain high levels of glutamine (Flynn *et al.* 1993).

Internal and external nitrogen were measured as a nitrogen-limited *A. tamarense* culture became sexual (Pfiester and Anderson 1984). On the day of inoculation, external N was 70 μM, internal N was 371 pg·cell^{-1}, and the C:N ratio was 4.1. By the time zygotes appeared 12 days later, external N was 0.2 μm, internal N was 188 pg·cell^{-1}, and the C:N ratio had more than tripled to 13.8. Internal pools were found to diminish one day after external depletion. Thus, in response to external N depletion, a rapid drop in cellular N happens concurrently with the development of sexual stages. However, there is little known of the metabolic path that begins with nitrogen depletion and results in encystment.

For *A. tamarense,* the optimal temperature for encystment is 20°C, whereas the optimal temperature for vegetative growth is 16°C (Anderson *et al.* 1984). From this, it was proposed that some metabolic process must be unique to gametogenesis, fusion, or encystment that requires higher temperatures than those processes involved in the biochemical processes of asexual growth.

1.3 Typical Encystment Rates

Table 3.1 was generated from a literature search for laboratory and field rates of sexuality. Generalizations are difficult because studies deal with different stages, species, and methodologies, but several conclusions are plausible. First, because the rate of dancing and fusing pairs is low at any one time, it is likely that these stages are short-lived. Second, there may be different rates for different species and/or genera. Finally, field populations may have higher proportions in the sexual cycle than laboratory populations.

Table 3.1 Percent of Dinoflagellate Population Entering Each Sexual Stage.
Literature values have been adjusted to genome equivalents whereby one gamete or vegetative cell = 1 genome and one dancing pair, fusing pair, planozygote, or cyst = 2 genomes. For example, if 4 cells produce 1 cyst, this would be an encystment rate of 50%, once corrected to genome equivalents.

Species	Dancing or Fusing Pairs	Planozygotes	Cysts	Time Frame	Reference
A. catenella	0.2-1.1%[1]			in 1 min	Sawayama *et al.* 1993b
A. catenella	0.2-1.3%[1]			in 1 min	Sawayama *et al.* 1993c
Coolia monotis	almost 100%			over time	Faust 1992
A. tamarense	<2%[2]			any 1 time	Turpin *et al.* 1978
A. tamarense	few	29%[3] then	21%	maxima	Anderson & Lindquist 1985
A. tamarense		6%[4] and	15%[4]	maxima	Doucette *et al.* 1989
A. tamarense *F		50%[4]		2 yr. peak	Anderson *et al.* 1983
A. monilata		67%[4]		maximum	Walker & Steidinger 1979
G. uncatenum *F		80-85%		maximum	Coats *et al.* 1984
G. uncatenum		55-85% then	9-13%	sums	Anderson *et al.* 1985
A. tamarense			20-40%	sum	Anderson *et al.* 1984

*F = field studies
Conversions to genome equivalents:
[1] Number of pairs observed x2 in total of 6,000-10,000 cells.
[2] "The incidence of pairing was very low (<1%)...".
[3] 1130 planozygotes/mL (=2260 genomes/mL) in population of 7700 genomes/mL.
[4] Percent given as x becomes (x+x)/(100+x).

Some of the variability in encystment rates seen in Table 3.1 may be explained by differences in culture conditions. For example, in studies of *A. catenella*, ES-Okaichi medium with germanium dioxide in continuous illumination produced more successful crosses than sterilized sea water with continuous or 14:10 lighting (Yoshimatsu 1981). Also, the number of cysts produced by *A. tamarense* using "normal" culturing techniques was less than half those produced under a "clean" regimen to "avoid precipitation, adsorption, desorption, or chemical contamination" (Anderson *et al.* 1984). Because culture conditions can affect encystment, the differences between reported rates is understandable, albeit poorly understood.

In one study of *A. tamarense,* encystment was a relatively consistent 20-40% over a wide range of cell concentrations (Anderson *et al.* 1984). This finding indicates that gametes are

efficient at finding and fusing with a mate regardless of the collision rate, and that the observed variations cannot be explained by culture concentrations.

1.4 Chapter Objectives

This thesis study develops a strategy to induce the sexual cycle using nitrate limitation and isolates #743 and #744 (see Chapter 2). The development of this protocol was necessary to approximate when gametes would be present (information used in Chapters 4 and 5). It also enabled the measurement of encystment rates with respect to the variables of salinity, well size, nutrient replete and nitrate deplete conditions, blue light, and the presence of germanium dioxide.

2.0 Materials and Methods

2.1 Induction of the Sexual Cycle

To induce the sexual cycle, 1 mL of 7-day old stock #743 and #744 cultures were added to separate 50 mL Pyrex® tubes. Each contained 30 mL of "induction medium" which incorporates HESNW (Harrison *et al.* 1980), F/2 (Guillard and Ryther 1962), NWSP-7 (Cembella 1986), and the "clean" protocol of Anderson *et al.* (1984). The main modification was to use HESNW/10 nitrate. Also, nutrient stocks and pre-filtered seawater were autoclaved separately in Teflon™ bottles, and then plunged into an ice bath to prevent precipitation (Anderson *et al.* 1984). A full description of the medium appears in Appendix 4.

The chosen nitrate enrichment was derived through trials to achieve good initial exponential growth that would yield a relatively high biomass of nitrate-limited culture. The initial nitrate in the tubes would be the sum of the enrichment (~55 μM), the nitrate in the seawater, and the residual nutrients from the stock inoculum.

Pyrex® tubes were pretreated according to the "clean" method of Anderson *et al.* (1984). Tubes were scrubbed in hot tap water, soaked in 10% HCl overnight, air dried, and then coated with 1% Surfasil (Pierce Chemical Co.) in acetone. After air-drying again, they were rinsed in distilled water, half-filled with distilled water, and autoclaved. Prior to use, the distilled water was discarded. Acid washing and Surfasil coating may be particularly important for dinoflagellate

cultures given their sensitivity to metals. For example, total inhibition of *A. catenella* occurred at copper concentrations that only partially inhibited the growth of other species studied (Anderson and Morel 1978).

Tube incubation and growth assessment were carried out as described in Chapter 2. Cultures grew exponentially for 7-10 d and then became stationary. That this phase was induced by nitrate limitation was confirmed in two ways. First, nitrate was very near zero in these stationary cultures, as measured with an AutoAnalyzer® (see Figure 3.1). Secondly, when nitrate stocks were added to stationary cultures, they bounced back into exponential growth (see Figure 3.2).

2.2 Encystment Rates

Once cultures were in stationary phase, a 1 mL sample of each isolate was fixed in acid Lugol's and counted in settling chambers using an inverted microscope to get cells·mL^{-1}. To measure encystment, equal measured amounts of each culture were mixed in either a 4-well or a 6-well cluster. In the same cluster and separate wells, #743 and #744 were added to provide parallel cultures of the unmixed mating types. This tested for homothallic pairing, and also provided stock to isolate gametes from (see Chapter 4). The interwell spaces were filled with distilled water, the edges sealed with transparent tape, and the clusters put into the incubator.

After 30 d, an inverted microscope was used to count cysts over the entire well bottom. Transects were not adequate due to the tendency of cysts to aggregate in clusters, notably near well edges. In one trial, number of cysts did not change substantially from four to seven weeks (93 and then 103 cysts). Therefore, 30 d incubations were used for all cases. Also, others have identified that cyst counts do not increase after 30 d (Anderson *et al.* 1984).

Encystment rates were calculated from cell concentrations and cyst counts. Since cultures were in stationary phase, the cell counts from Lugol's samples would not change appreciably up to encystment. Counts were converted to cells·well^{-1}. The lower concentration of #743 or #744 was used for calculations because pairing occurs 1:1. These values were used to calculate encystment rate (%) as follows:

$$\frac{(100)(\#\text{ of cysts per well})}{\text{total \# of \#743 or \#744 cells per well}}$$

Note that this calculation is based on genome equivalent, and therefore indicates the percentage of the gene pool that becomes encysted, rather than the percentage of the population that becomes encysted. For now, the calculation assumes that the great majority of pairing is homothallic. This will be substantiated later.

Encystment rates were calculated for crosses of nitrate-limited #743 and #744 cultures in large (35 mm) and small (16 mm) wells with typical (26) and high salinity (31). Also, nutrient replete crosses were done in small wells under white light, blue light (clusters underneath a #69 "brilliant blue" filter, Roscolux®), and with dissolved germanium dioxide.

3.0 Results

3.1 Time Course of Encystment

Early trials done this way produced cysts in the mixed wells beginning after 10-12 d; therefore, gametes must be present some days before. Since *A. tamarense* planozygotes mature for several days to one week (Anderson *et al.* 1983, Anderson and Lindquist 1985, Destombe and Cembella 1990), it was determined that gametes must have been present by day 6.

3.2 Success in Inducing Sexual Cycle

Cysts were formed under a variety of conditions including typical (26) and high (31) salinity, nitrate deplete and nutrient replete medium made with and without "clean" techniques, under blue light and white light, at all times of the year, in the following containers: 250 mL Erlenmeyer flasks, 50 mL Pyrex® tubes, a settling chamber, and polystyrene culture wells (diameters of 35 mm, 16 mm, and 6 mm). In fact, the trials where cysts were not produced could be attributed to incompatibility of the isolates or gross culture problems such as incubator failure or media contamination.

3.3 Encystment Rates

Table 3.2 shows encystment rates for several conditions tested. The highest encystment rate (12.3%) was seen when cultures were N-limited. Effect of well size was tested in two separate trials; one had higher encystment in the small well (t-test; $p<0.001$), and the other trial showed the reverse (t-test; $p<0.05$). The data are presented separately, but the only logical conclusion is that well size does not affect encystment. Salinity had no effect. Blue light produced lower encystment rates than white light (t-test; $p<0.05$), but this could also have been due to the reduction in the quantity of light, since this variable was not controlled. There were no cysts formed in any of the four wells with germanium dioxide.

Table 3.2 Encystment rates of *Alexandrium catenella.*
Percents are in genome equivalents calculated as number of cysts in well divided by number of cells of one mating type in well. Significant differences are shown and additionally, nitrate deplete encystment was found to be greater than nutrient replete encystment rates (t-test; $p<0.001$).

Nitrate	Well Size	Salinity	Light	Replicates	Encystment	SD	Results
-	large	26	full	2	12.3%	5.5	
-	small	26	full	2	11.3%	0.9	
-	small	31	full	2	11.0%	0.7	small>large
-	large	31	full	2	6.1%	1.3	t-test ($p<0.01$)
-	large	26	full	2	6.1%	1.2	
-	large	26	full	2	4.9%	0.4	large>small
-	small	26	full	4	3.7%	0.5	t-test ($p<0.05$)
-	small	26	full	2	3.0%	0.7	
+	small	26	full	4	0.4%	0.2	full>blue
+	small	26	blue	4	0.1%	0.1	t-test ($p<0.05$)

4.0 Discussion

4.1 Circannual Variability

Although circannual variability in dinoflagellate growth (Yentsch and Mague 1980, Costas and Varela 1989, Costas *et al.* 1990) and excystment (Anderson and Keafer 1987) are well documented, it is not clear whether this cycle also occurs for encystment.

Scrippsiella trochoidea over a two year period had a circannual rhythm in growth and cyst production under constant laboratory conditions for three of five cultures (Costas and Varela 1989). Although this finding is interesting for dinoflagellate ecology, the authors do not define "cyst". The protocol involves 15 d transfers of clonal cultures and the counting of cysts "just before transfers". The cysts reported in this experiment could be hypnozygotes, but only if the species is homothallic and produces cysts during what is presumably exponential phase growth.

In a similar study, Yentsch and Mague (1980) found that over five years, *A. tamarense* was most likely to form cysts in the winter. While these cysts are called "temporary cysts" in the results section, the abstract refers to them as "resembled resting cysts". Additionally, peak growth was two doublings per day and rates this high have not been corroborated for *Alexandrium*. These discrepancies preclude conclusions about hypnozygote formation rhythm.

Cysts were produced at all times of the year tested. The only months not tested were January, June, and October. Although encystment rates may have varied marginally over the year, the differences were not dramatic enough to be obvious, and since counts were not done on all crosses, fine scale differences could not be resolved. However, the finding that there were no times when cysts were not produced has implications for field encystment; without annual variability, an external cue such as nutrient depletion may be an important factor for *in situ* encystment.

4.2 Container Effects

Given that polystyrene is a charged material, there was initially some concern that this might interfere with fusion if gamete attraction was ionic. For example, mating-reactive

Paramecium stick to polystyrene (Hiwatashi 1988). However, cysts were successfully formed in 4, 6, and 96-well clusters. Again, if there was an effect, it was only in magnitude, and it was small enough to not be readily apparent.

Physical stimulation in the form of stirring was found to impair fusion for *A. tamarense* (Anderson and Lindquist 1985). However, in these thesis studies, cysts formed in very small wells (6 mm diameter and 140 µL volume), and there was no encystment rate difference between 35 and 16 mm diameter wells. Given that many dinoflagellates do not grow well in small spaces (E. Simons, NEPCC, pers. comm.), it was surprising that container size did not affect encystment. From this, it was concluded that mechanical stress in the form of small container:volume ratios does not arrest pairing.

4.3 Ecology of Limited Encystment Rates

The highest encystment rate in any one well (15%) occurred under nitrate limitation. This is much lower than the 40% encystment rate reported for *A. tamarense* (Anderson *et al.* 1984). Both of these studies used nitrate limitation and "clean" techniques, and therefore it was unlikely that medium caused the difference. However, the studies used different species. Although both species are very similar, possibly even morphotypes in the *tamarense/catenella* complex (Cembella 1986), they are different isolates, from different coasts, possessing different morphology. The second and more probable reason for the difference in encystment rates is temperature. Anderson *et al.* (1984) found that encystment peaked at 20°C and was much lower at the temperature used in these thesis studies, 16°C. A 16°C incubator was used here for convenience and because maximizing rates was not the focus of these thesis studies. However, by repeating these experiments at 20°C, the encystment rates might also increase.

The rates observed in this thesis study are much lower than those observed in the field: 50% for *A. tamarense* (Anderson *et al.* 1983). Observed encystment in laboratory studies is commonly lower than rates seen *in situ*. The reason for this difference is unknown, but the discrepancy indicates that current culturing protocols are not representative of field conditions.

The finding that the highest proportion of *A. catenella* or *A. tamarense* in the sexual cycle is 50% (Anderson *et al.* 1983) raises an interesting question. How is it that only some of the population encysts when the entire population is exposed to equivalent external environmental conditions? The mechanism behind this selection process is unknown, but the adaptive purpose it serves is a topic for speculation.

Since gametogenesis is irreversible and mandatory dormancy exists for cysts, it is probably adaptive to have only a portion of the gene pool committed to this shunt. If conditions improve, the motile population can continue to reproduce asexually. Ecologically, it makes sense to relegate a proportion of the gene pool to benthic storage whenever nutrient exhaustion is imminent. Thus, this strategy would provide safe storage to a qualitatively changed gene pool, while also allowing the remaining vegetative population to quantitatively increase.

There is evidence that a similar safety net system occurs during excystment. Anderson *et al.* (1983) observed that a "large residual cyst population carried over from year to year without germination, even during bloom years". If only a proportion of cysts germinate under equal conditions, this would provide a carryover population that could seed future blooms even after non-bloom years.

4.4 Ecology of Differing Encystment Observed

Blue light and high salinity were used as experimental variables to test a hypothesis; when surface *in situ* nitrogen is low, cells might sink/swim downward and be exposed to proportionately more blue light and higher salinity, and that one or both of these factors would provide an external cue to encyst. Blue light has been identified as a sexual cycle inducement for *Ptychodiscus breve* (Walker 1982), and also for *Acetabularia* (D.F. Mandoll, UW, pers. comm.). In this thesis study, encystment was not different for the two salinities (26 and 31), and blue light depressed rather than enhanced encystment. However, the blue light experiments were done in nutrient replete medium and total light was not controlled. The encystment rate of this trial could thus be due to the reduced quantity of light, 15 μmol quanta·$m^{-2}s^{-1}$ compared to the others at 61

μmol quanta·$m^{-2}s^{-1}$. The effect of blue light should be retested under nitrate deplete conditions where total light is controlled.

Since Meksumpun *et al.* (1994) found that the principle component of *A. catenella* cysts is silicon, it was tested whether cysts would form when germanium dioxide was in the medium. No cysts were formed in these wells. However, the presence of precipitate and general aberrant appearance of the cells suggests that the depression of encystment may have been due to a generalized effect on cellular processes rather than an effect specific to the sexual cycle.

4.5 The Ecology of Using Nitrate Limitation To Induce The Sexual Cycle

One protocol for limiting the nitrate concentration was used as a standard for these experiments without tests of other concentrations or other nutrient depletions for two reasons. First, this protocol was successful. Secondly, enrichment experiments and N:P ratios indicate that coastal marine waters are nitrogen-limited (Valiela 1991). In Toquart Bay, surface nitrate concentrations were undetectable even when phosphate was present (Haigh and Taylor unpubl.). Thus, nitrogen limitation was chosen because it could trigger the sexual cycle in pre-trials, and because it had *in situ* relevance.

Originally it was postulated that gametogenesis is stimulated by short-term nutrient limitation. As gametes fuse and begin to sink, they would gain access to nutrients needed to complete the encystment process. However, in these experiments, cysts appeared in cultures that had been nitrate starved for 12 d. This suggests that the nitrate requirements for encystment are low and/or that internal stores are sufficient.

Laboratory studies use nutrient limitation to induce sexuality, but field studies have failed to correlate nutrient depletion with the onset of the sexual cycle. For example, in a two year study of *A. tamarense* growing in salt ponds, it was concluded that "encystment was not linked to any obvious environmental cue" (Anderson *et al.* 1983). However, upon close examination of the data presented, ambient nitrogen dips 2-4 weeks prior to the appearance of planozygotes. Since *A. tamarense* planozygotes mature for several days to one week (Anderson *et al.* 1983, Anderson and Lindquist 1985, Destombe and Cembella 1990), it is possible that field encystment was

triggered by environmental nitrogen. However, the findings in these salt ponds may not be applicable to coastal embayments.

Although laboratory studies have successfully used nutrient limitation to induce sexuality, it is important to question whether *in situ* nutrient limitation occurs for more than short periods given sources and sinks present in field nutrient dynamics. Batch cultures are exposed to rapid reduction of nutrients but this may never occur in the field owing to much more complex nutrient dynamics (Anderson *et al.* 1984). Isolates #743 and #744 came from Toquart Bay. Whether their host population could experience nitrate limitation will be considered next by looking at data from this location.

Nutrient drawdown could occur in a dense concentration of dinoflagellates. Gametic pairing may be facilitated two-fold by these aggregations in that nutrient limitation would induce sexuality, and the chance of collision with a mate would be enhanced. In general, high concentrations of dinoflagellates are common due to group processes such as migration and phototaxis, as well as hydrographics and wind factors (Pfiester and Anderson 1984). In Toquart Bay, *A. catenella* biomass peaked at 7.5×10^3 cells$\cdot L^{-1}$ (Haigh and Taylor unpubl.). Additionally, 2×10^3 cells$\cdot L^{-1}$ of *A. catenella* were recorded in Toquart Bay in July 1992 where nitrate concentrations down to 30 m were less than 1 μM (Haigh and Taylor unpubl.). From this evidence, it is possible that *in situ* dinoflagellates are exposed to reduced nitrate.

It could be argued that with dinoflagellate diel migration and dark uptake of nitrate (Dortch and Maske 1982), populations would never be constrained to an area with dwindling nutrients. In general, migration has been reported for *A. tamarense* (Anderson and Stolzenbach 1985, Santos and Carreto 1992) and *A. catenella* (Takeuchi 1988), where upward movement started before sunrise and downward movement began before sunset. Maximum migration speeds range from 1-2.5 m$\cdot h^{-1}$ (Eppley *et al.* 1968, Kamykowski *et al.* 1988, Takeuchi 1988), and migration is not impeded by a 6°C thermocline (Santos and Carreto 1992), strong pycnoclines (Rasmussen and Richardson 1989) nor water movements such as upwelling (Blasco 1978). In Toquart Bay, peak *A. catenella* biomass was at or near the nitricline at times ranging from 0730 to 1635 (Haigh and Taylor unpubl.). Since nitrogen limitation has been found to affect diel

migration (Heaney and Eppley 1981, Rasmussen and Richardson 1989, Santos and Carreto 1992), a possible explanation for the absence of obvious diel migration may be that the resident population was responding to near zero surface nitrate levels.

In sum, although general indications are that field dinoflagellate populations should not be nitrogen-limited due to complex nutrient dynamics and migration, evidence indicates that this may have occurred for *A. catenella* in Toquart Bay. Therefore, the use of this as a sexuality induction mechanism in the laboratory had ecological saliency.

Parenthetically, it should be noted that although the highest encystment rates found in this thesis study were under N-limited conditions, cysts were found in nutrient replete medium in this thesis study and elsewhere (Anderson *et al.* 1984, Blackburn *et al.* 1989). The best synthesis may be that encystment is always possible even in conditions ideal for asexual growth, but is greatly augmented by nutrient limitation.

5.0 Chapter Summary

- Cysts form 10-12 days after nitrate-limited #743 and #744 cultures are mixed, and therefore gametes should be present about six days earlier.
- Encystment is possible under a wide variety of conditions (salinity, container, nutrients, light, and time of year).
- The highest encystment rate observed was 15.0%.

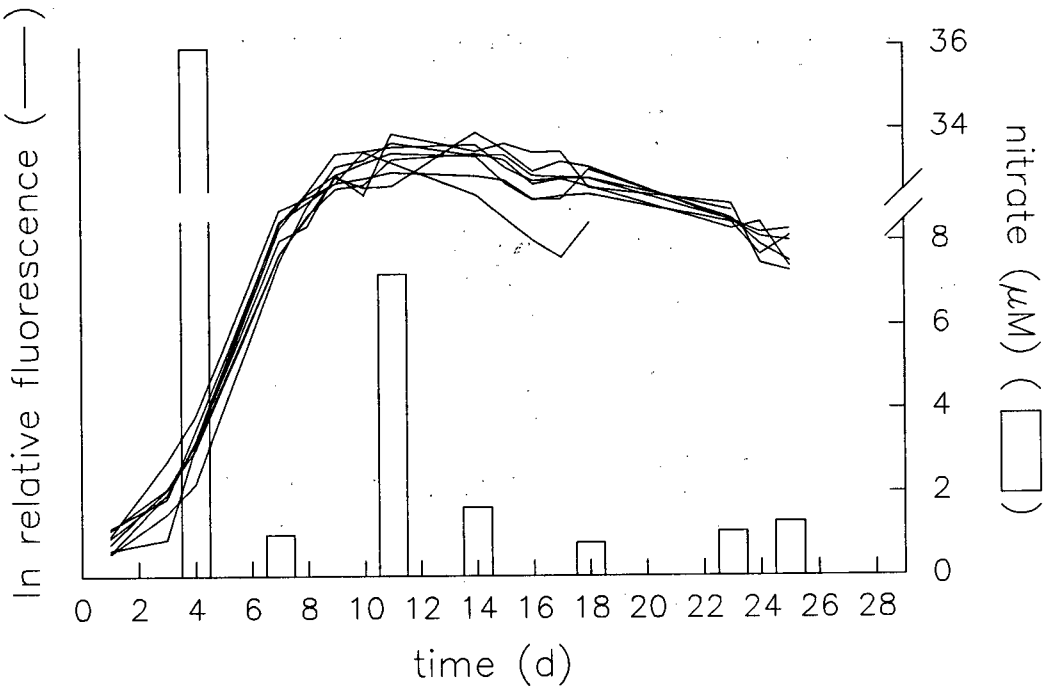


Figure 3.1 Nitrate In N-limited *Alexandrium catenella* Cultures.
Lines track *in vivo* fluorescence of eight tube cultures. Each nitrate measurement (bar graph) is from a different tube. The cultures become stationary concurrently with the reduction of available nitrate. The apparent rebound of nitrate on day 12 over day 8 may be due to the release of nutrients from cells, or is an artifact of using buddy tubes for samples.

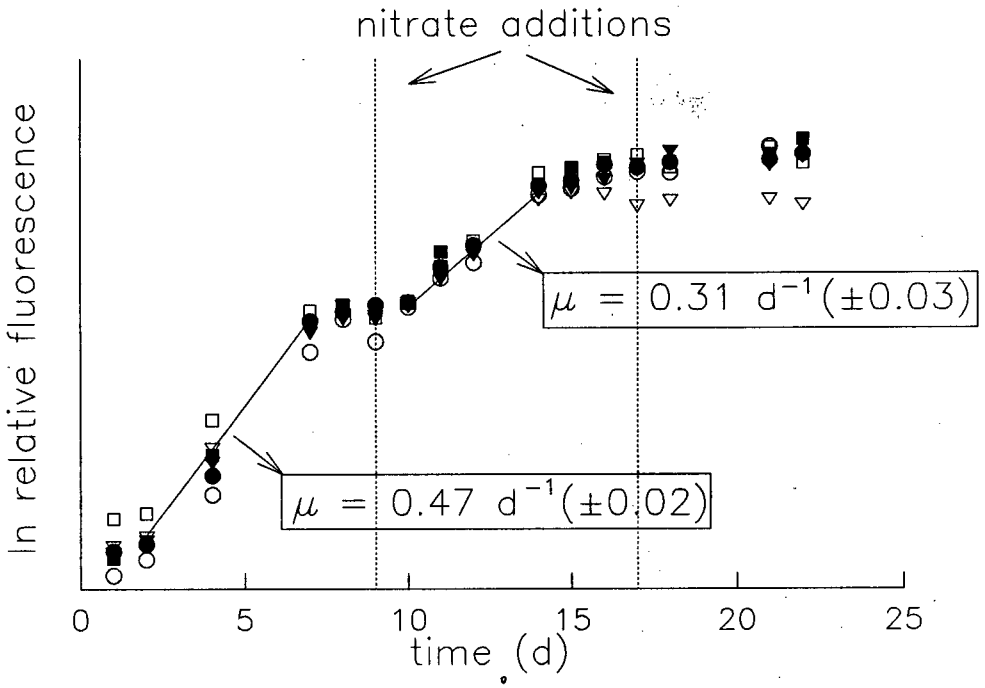


Figure 3.2 Growth response of N-limited *Alexandrium catenella* to Nitrate Addback. Points track *in vivo* fluorescence of culture in HESNW with N/10 nitrate. Solid lines are average of separate linear regressions through data, and growth rates are shown in the boxes. Stock nitrate solution is added on days 9 and 17 marked by dotted lines. Based on culture response, it is assumed that cultures were N-limited during the first stationary phase, and not limited by nitrate in the second stationary phase.

CHAPTER 4 SIZE, SHAPE, GROWTH, AND SURVIVAL OF NON-DIVIDING SINGLE CELLS COMPARED TO VEGETATIVE CELLS

1.0 Introduction

Although gametes and vegetative cells of most protists are distinguished by ploidy or morphology, most dinoflagellates, including *Alexandrium* species, are among the exceptions. Gametes and vegetative cells are both haploid and morphologically similar, but early studies found size differences between these life stages, and between mating types. These ideas continue to permeate the literature.

Early dinoflagellate studies described gametes as small, pale cells (von Stosch 1973; Pfiester 1975, 1976, 1977). However, in all of these studies, nitrogen limitation was used for sexual induction which itself may produce small cells unable to divide, and pale cells where nitrogen-rich pigments have been recycled for cell metabolism. Although these small, chlorotic cells may have been gametes, nutrient depletion, rather than gametogenesis may have generated the morphological change. As examples, nitrogen limitation induced pigment loss for two species of diatom (La Roche *et al.* 1993), the prymnesiophyte *Pavlova lutheri* (Madariaga and Joint 1992), and the dinoflagellate *Heterocapsa* sp. (Berdalet *et al.* 1994).

In an *A. tamarense* study where daily photographs of an encysting population were examined (Anderson and Lindquist 1985), the onset of gametogenesis was inferred to occur when the average population cell size decreased based on corroborating evidence: immediately before this time, there was a drop in internal phosphorus (the limiting nutrient) and a concomitant division rate surge; and mean cell size increased thereafter as fusion occurred. Since cell volume decreases in response to nitrate limitation have been reported for *Prorocentrum minimum* (Sciandra and Amara 1994) and *Heterocapsa* sp. (Berdalet *et al.* 1994), the mean cell size decrease may have been induced by the phosphorus limitation, rather than gametogenesis.

Individual gametes within fusing pairs of *A. tamarense* have been measured. Comparisons of these and vegetative cells have led to the conclusion that gametes are morphologically similar to vegetative cells (Doucette *et al.* 1989) and therefore on an individual basis, indistinguishable on

the basis of size (Anderson *et al.* 1983, Anderson and Lindquist 1985). To illustrate, sample measures for *A. tamarense* appear in Table 4.1.

Table 4.1 Lengths of Gametes and Vegetative Cells of *Alexandrium tamarense*. Gamete Sizes Measured From Fusing Pairs.

Gamete Length (µm)	Vegetative Cell Length (µm)	Reference
--	35	Anderson & Wall 1978
25-30 and 50	--	Turpin *et al.* 1978
--	25-30	Anderson 1980
27-36 (mean = 31)	33.5-36.5	Anderson & Lindquist 1985
--	25-48	Fukuyo 1985
--	25-35	Doucette *et al.* 1989
30-35	"somewhat" larger	Fritz *et al.* 1989

Recently Fritz *et al.* (1989) described *A. tamarense* gametes as being "somewhat smaller" than vegetative cells. However, the overlapping size ranges seen in Table 4.1 support Anderson and Lindquist's (1985) earlier assertion that the long-held belief in small gametes was just an impression.

Both anisogamy (Turpin *et al.* 1978) and isogamy (Walker and Steidinger 1979, Anderson 1980, Fritz *et al.* 1989) have been reported for *Alexandrium* species. A viable explanation for the observation of isogamy and anisogamy being described for one species comes from a comprehensive study of *Gyrodinium uncatenum* (Coats *et al.* 1984), where isogamy was the most common form but anisogamy occurred in aging laboratory cultures and only rarely in field populations.

In one study where measures were taken for *A. tamarense*, the mean size difference between two fusing gametes was 1.9 µm (Anderson and Lindquist 1985). Typical size variation overshadows this small difference, but if this difference was consistent between mating types, it would be evidence of anisogamy. Therein lies the problem. Anisogamy is where one of the uniting pair is consistently smaller (Bold and Wynne 1985), and not merely when gamete sizes are

unequal. To assert that a fusing pair is anisogamous, the mating type of each gamete must be known.

Thus, fusing pair evidence describes gametes as comparable in size and pigmentation to vegetative cells, and each other. However, no studies have been done on lone gametes because of the difficulty in identification.

One stage where lone gametes are identifiable is when they are in a pre-fusion "mating dance". Unfortunately, isolation of these pairs is complicated. The percentage of dancing pairs at any one time in a mating culture is extremely low, as seen in a study of *A. catenella* where dancing pairs made up only 0.2-1.3% of the population at any one time (Sawayama *et al.* 1993b, 1993c). Personal observations have confirmed that dancing pair percentages are low, but a second factor makes the capture of this rare event, very unlikely. Careful attempts to micro-pipette one dancing pair from a dense culture have always captured other nearby cells. Since physical stimulation disrupts the fusion, this process yields several individual cells, only two of which are the gametes. Thus, the event rarity and isolation factors make this method impractical to identify lone gametes.

Another way to identify lone dinoflagellate gametes may be by their inability to divide, but this has not yet been proven. The conclusion that non-dividing gametes were present in a field population of *A. tamarense* was made when the weekly cell counts disagreed with mitotic index data (Anderson *et al.* 1983). However, all or some of the non-dividing cells might have been aberrant vegetative cells rather than gametes.

Typically, gametogenesis is a commitment to the sexual cycle, whereby fusion is the only pathway back into the asexual cycle. In contrast, groups such as dinoflagellates that have zygotic meiosis, theoretically may not have hurdles between their sexual and asexual cycles, since vegetative cells and gametes have the same ploidy. However, parthenogenesis (the ability of gametes to grow into asexual individuals without sexual union) among the algae has only been reported for one macrophyte, *Ulva lactuca* (Bold and Wynne 1985). Although there is no direct proof that dinoflagellate gametes are unable to divide, the lack of precedent in the algae for parthenogenesis, makes it a plausible assumption.

In this thesis study, a new technique was used to identify non-dividing single cells. Measures of size, shape, growth, and survival of these 'potential gametes' enabled comparisons with vegetative cells and between mating types.

2.0 Materials and Methods

2.1 Induction of Sexual Cycle

Nitrate-limited #743 and #744 cultures in stationary phase were mixed in equal amounts in 2 wells, and added individually to 2 other wells of a 4- or 6-well culture cluster (see Chapter 3 for details of sexual cycle induction). The interwell spaces were filled with distilled water, the edges were sealed with transparent tape, and the cluster was placed in the incubator. In previous trials, cysts appeared 10-12 d later (see Chapter 3). Because planozygote maturation takes several days to one week (Anderson *et al.* 1983, Anderson and Lindquist 1985, Destombe and Cembella 1990), gametes should be present in this thesis study by day 6, and may begin to die thereafter.

2.2 Isolation of Potential Gametes

To prepare the potential gametes for isolation, 1 mL of each clone was transferred into ~20 mL fresh nitrate replete medium in separate glass Petri dishes. An acclimation period of several hours made the cells more tolerant of the next step.

Culture density and depth were first adjusted by adding/removing medium to facilitate quick and efficient isolation work. The Petri dish of culture was viewed under a dissecting microscope using 10-80X power. A finely drawn Pasteur pipette fitted with mouth apparatus was used to first take in a plug (~1μL) of cold medium and then gently pick up one single motile cell at a time and place it into 3 drops (~60 μL) of fresh nitrate replete medium in one well of a flat-bottomed 96-well culture cluster (see Figure 4.1). Since all gametes are single cells, this isolation step concentrated the number of gametes, slightly. All interwell spaces had previously been filled with distilled water. The first 48 wells were for clone #743 and the second 48 wells, for clone #744. The cluster was then sealed with transparent tape and placed into the incubator.

To prevent overheating of the cells during isolation work, the culture clusters and medium for pipette plugs were kept on a metal tray with a cold pack and the Petri dish of culture was returned to the incubator frequently.

2.3 Videotaping Cells in Wells

Isolated cells were videotaped with a Sony Trinicon (DXC 1850) camera and camera control unit, a JVC high resolution video cassette recorder, a Zeiss Axiovert 10 inverted light microscope, and a Sony Trinitron monitor (model KX-1901). Taping was done 8 h into the light cycle every day, beginning the day of isolation until each cell died or up to and including the first division. Each cell was first located under low power (100X) and low light, then videotaped at 200X and higher light until the cell had been recorded with the cingulum in view, in focus, and in the center of the screen. The cell cluster was returned to the incubator periodically to reduce overheating.

2.4 Cell Measurements

Cells were measured from the videotaped images. A piece of acetate inscribed with a grid was affixed to the monitor and aligned on each cell with the cingulum as one axis. The apical to antapical distance and the outer reaches of the girdle lists were recorded directly into Lotus 123 as length and width of each cell. When making these measures, care was taken to keep the viewer's head in one central position to avoid distortion caused by parallax.

Cell volume was calculated from measured length and width using the formula for an ellipse:

$$\text{volume } (\mu m^3) = (4\pi/3)(\text{length}/2)(\text{width}/2)(\text{depth}/2)$$

The width measurement was also used for depth. In fixed samples, width and depth were equal or minimally different. *A. catenella* is not a perfect ellipse but the introduced error is acceptable since volumes were not used as absolute measures.

2.5 Method Evaluation

Two tests before this trial and one test after the completion of all trials were done to examine aspects of this single cell isolation protocol.

First, in order to determine whether cell size was related to dividability, single cells upon isolation were crudely assessed as relatively large or relatively small. At the end of the incubation, large cells were more likely to divide (t-test; $p<0.001$, $n=26$). Therefore, only small cells were isolated in this and all subsequent runs to maximize the percentage of non-dividing single cells.

Second, to test whether cells swimming near the bottom of the Petri dishes were more or less likely to divide was tested. Although it is easier to pipette one cell at a time if the cells are near the bottom, there was concern that cells near the bottom may be weaker or somehow different from cells swimming near the surface. Ability to divide was not related to vertical position in the medium (t-test; $p<0.3$; $n=30$), so cells were isolated from near the bottom of the Petri dish where possible.

Third, whether well position within the cluster affected dividability was tested after all isolation runs were completed. There was concern that the manufacturing of wells, uneven lighting, or the practice of filling the wells in order might cause high or low dividability sections in the clusters. There was no effect of row or column on the position of the 57 non-dividing cells (chi-square; $p<0.05$).

3.0 Results

3.1 Optimal Days to Fixation for Subsequent Trials

Of dividing cells, #743 cells had a longer lag phase than #744 cells (t-test; $p<0.001$) (see Figure 4.2). This lag time difference occurred even though the two clones had comparable growth rate and biomass before isolation. Some non-dividing cells survived only 2 d. Remarkably, 1 non-dividing cell survived for 26 d.

The optimal date for fixation of subsequent runs was determined to be 7 d for #743 and 5 d for #744. These times were selected to maximize the number of non-dividing singles and

minimize the number of vegetative cells that had not yet divided. For later trials, after 5 or 7 d, each cluster would have wells that were empty (had been missed on isolation), wells with dead cells, wells with cells that had not yet divided, and wells with cells that had divided 1-4 times.

3.2 Length, Width and Shape Measures for Isolates

The cell measurements were pooled into three categories: all daily measures of cells that never divided (ND), all daily measures of cells up to the day before division (BD), and cells after the first division (AD). The box and whisker plot seen in Figure 4.3 reveals a considerable overlap in data among these three groups, between the two clones, and between length and width. However, there are differences between the means. Clone #744 cells are longer and wider than #743 cells (ANOVA; $p<0.001$). The other relationships are more complex (see Figure 4.4).

Length divided by width provides an indication of cell shape. An isodiametric cell would thus have an index of 1. Figure 4.5 shows that cells after division are wider than long, and that both non-dividing and cells before division are longer than wide. There is no shape index difference between the two clones.

3.3 Growth of Non-Dividing and Dividing Cells

Regressions of ln transformed volume measurements provided growth measures of cells up to their division or death (see Figure 4.6). All cells up to the time of their first division gained in volume or, in the case of one cell, maintained the same volume. In contrast, the mean volume change up to non-dividing cell death was negative. However, the raw data shows that both growing and shrinking cells were present in the non-dividing group.

4.0 Discussion

4.1 Growth of Pre-division Vegetative Cells and Non-dividing Single Cells

Although growth and division often co-occur, they are independent processes. For example, over the daily cycle, cell division and cell growth are not coupled for *A. tamarense* (Watras *et al.* 1982, Karentz 1983). In this thesis study, cell counts and chl *a* fluorescence curves

would overlap during exponential growth and then become disparate beginning in early stationary phase. Although this is at least in part due to changes occurring in cell biochemistry because of nitrogen limitation, this illustrates how cellular growth and cellular division can act independently. Some interesting results came from measuring cellular growth before cells divided, or died. The way that *A. catenella* is able to increase or decrease its volume given the seeming rigidity of the dinoflagellate amphiesmal plate arrangement, is not known.

It was found that cells which would eventually divide had a net gain in volume up to the day of division. While not surprising, it is interesting that the highest rate of volume change (0.45 d^{-1}) is comparable to μ_{max} rates based on chl *a* fluorescence measures (0.4-0.6 d^{-1}).

In a *Prorocentrum minimum* study, a pulse of nitrate to a nitrate-limited culture would cause an increase in average cell volume (Sciandra and Amara 1994). This finding is in agreement with the finding in this thesis study for dividing cells. All cells that would divide gained in volume. In contrast, some non-dividing cells increased in volume and some lost volume in the presence of replete nutrients.

In response to dwindling nitrate, average cell volume of *P. minimum* decreased (Sciandra and Amara 1994). This finding is doubly important to this thesis study. First of all, it supports the finding of this thesis study that cells could decrease in cell volume. Secondly, it indicates that the observations of volume increase/decrease observed in this thesis study in response to nitrate renewal could have been preceded by shrinking since the cells were previously nitrate-limited. The mechanisms involved are beyond the scope of this thesis study and how these volume changes relate to gametogenesis remains the topic of further study.

Based on the findings of this thesis study, moribund cultures could contain both very large and very small cells, neither of which are necessarily involved in the sexual cycle. Slowed growth has also been associated with an increase in large cells in *A. tamarense* (Prakash 1967, Anderson and Lindquist 1985, Watras *et al.* 1982). Although the Prakash (1967) study may have been observing planozygotes, the other two studies were of non-sexual cultures. These size changes in response to slowed growth may complicate observations of sexual cultures.

4.2 Identification of Non-dividing Single Cells as Gametes

It seems maladaptive for gametes to form when fusion is impossible, so the reliance in this thesis study on clonal cultures producing gametes must first be justified.. Sawayama (1993a, 1993b) mixed clonal (+) and (-) *A. catenella* cultures and observed them dancing within 10 minutes. Thus, gametes can form in clonal cultures, and the protocol of this thesis study is valid.

An important caveat is that the mixed clones did not produce cysts in this run, likely because of an incubator failure. Gametogenesis can occur without subsequent encystment, but without encystment in this trial, the conclusion that non-dividing single cells in this thesis study were gametes, becomes more tenuous.

The non-dividing single cells in this thesis study may have been gametes, aberrant vegetative cells, or both. Considering that some cells increased in volume and some lost volume until their death, this may indicate that two populations of non-dividing cells were present, at least one of which may have been gametes.

In this isolation, 27% of #743 and 11% of #744 were non-dividers. Encystment rates for this trial were not obtainable, but the rate of non-dividing single cell formation in this trial and encystment rates for other trials, up to 15% (see Chapter 3), are comparable. However, factors that complicate such a comparison include the unequal time scales, the fact that cysts only reflect successful fusion and not total gametes, and the fact that the isolation population as single motile cells was only a subset of the population that encystment calculations were based on. Although complicating factors exist, the percentages of non-dividing cells in this run and encystment rates of other runs are comparable which may indicate that the non-dividing single cells here were gametes.

It was noticed that in nitrate addback experiments, growth rates before the culture became nitrate-limited were higher than the second exponential rise after nitrate was added. One explanation for the reduced growth in the second exponential period, may be that non-dividing cells were present. Back calculations suggest that growth rates for the two exponential periods would be equal if 50% of the cells at the first stationary phase were non-dividing cells. This

provides theoretical evidence that nitrogen limitation induces non-dividing cells but whether these are gametes is only a possibility.

4.3 Differences Between Non-dividing Single Cells and Vegetative Cells

One difference between *Alexandrium* gametes and vegetative cells may be shape. Since *A. catenella* gametes are always single cells, it was postulated that their shape may differ from the shape of cells in chains. Previously, it was noted that *A. catenella* cells in chains are anterio-posteriorly compressed while single cells are isodiametrical (Hallegraeff *et al.* 1991). Since all gametes are single cells and all cells in chains are vegetative cells, this shape difference may be salient. For *Gyrodinium uncatenum,* lengths and widths overlapped for vegetative cells and gametes, but gametes were narrower in width (Coats *et al.* 1984). Also, *A. catenella* in culture was found to reduce chain length and produce more tamarensoid-shaped cells (Cembella 1986). Indeed, this thesis study found that post-division cells had a shape index <1 (cells wider than long), whereas both pre-division and non-dividing cells were longer than wide. Thus, if gametes were formed in an exponential culture where vegetative cells are dividing rapidly, the two groups may have recognizably different shapes.

In this thesis study, nitrate limitation was used to induce the sexual cycle. However, nitrate limitation also brought the cultures into stationary phase. Many of the measures could distinguish differences between post-division cells and potential gametes. Therefore, these size differences may distinguish gametes and vegetative cells in exponential growth.

Although stationary cultures were used in this thesis study, mating may occur during exponential growth. Mating has been observed in late exponential phase *A. catenella* cultures (S. Yoshimatsu, pers. comm.), and *A. hiranoi* (Kita *et al.* 1993). Also, *Peridinium trochoideum* encystment is "favoured rather than inhibited by optimal conditions for vegetative growth" (Wall *et al.* 1970). Finally, crosses of aging stock #743 and #744 cultures produced cysts, although they began in nutrient replete medium. Thus, the sexual cycle may occur in exponential growth and therefore the size and shape differences between gametes and vegetative cells would be apparent.

In one trial, many single cells divided to form two separate cells rather than a chain of two cells. This was intriguing since others had shown that gametes were formed after 1-2 divisions (Coats *et al.* 1984). To test whether these newly formed singles were gametes, they were separated to see if they were able to divide. All of them formed chains of two by the next day. Thus, division products of two cells or a chain of two cells seem to be natural variations of vegetative division, and not related to gametogenesis.

4.4 Differences Between Gamete Mating Types

Non-dividing single cells from #744 were longer and wider (41.4x37.7 μm vs. 37.2x34.8 μm) on average than #743 (ANOVA; $p<0.001$). However, the considerable overlap in length and width measures would make it impossible to identify any cell in a mixed assemblage as a particular mating type. Although this size difference may at first be seen as support for anisogamy, it is important to consider that the ranges of #744 lengths and widths fall within the ranges of #743. Therefore these differences in means will not likely translate to consistent size differences in mating pairs and therefore, anisogamy. However, the data suggest that unequal sized pairs are possible.

Clonal size variability was found in *A. catenella* and *A. tamarense* (Cembella 1986). Therefore, the size differences in this thesis study may be clonal differences and not mating type differences. Further studies of size differences between mating clones are necessary.

Since gametes are increasing or decreasing in volume until their death, unequal sized pairs could occur from the union of these very large and very small cells. The usual case *in situ* is that gametes from both mating types are formed in the same time frame, find each other, and fuse. However, size changes with age may lead to unequal sized pairs of old and new gametes. Again, the pairing is isogamous, but infrequently, pairing of unequal sized gametes would occur.

No consistent differences in pigmentation distinguished mating type or life stage in this or other trials. Although zygotes maintain chloroplasts from only one gamete (F.J.R. Taylor, pers. comm.), this evidence suggests that plastid degradation must occur post-fusion.

5.0 Chapter Summary

- Fixation times for subsequent trials should be 5 d for #744 and 7 d for #743.
- On average #744 is longer and wider than #743. For shape index, the two do not differ.
- Some length, width, and shape index differences exist among BD, AD, and ND which may be more apparent when gametogenesis occurs in exponential growth.
- Cells that are going to divide increase in volume, whereas both increases and decreases in volume occurred for non-dividing single cells (potential gametes).
- Pigmentation differences could not be assigned to mating type.
- Infrequently, unequal sized pairs may occur because there is a large size range for non-dividing singles, and gamete age affects size. However, one mating type would not consistently be larger in these pairs. Thus, pairing is isogamous.

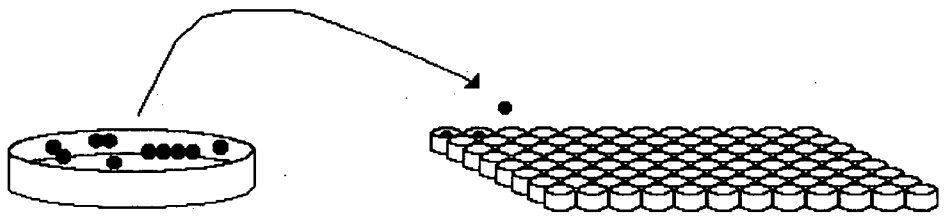


Figure 4.1 Single Cell Isolations.
After an acclimation period in N-replete medium, single motile cells are micro-pipetted into single wells of a 96-well cluster.

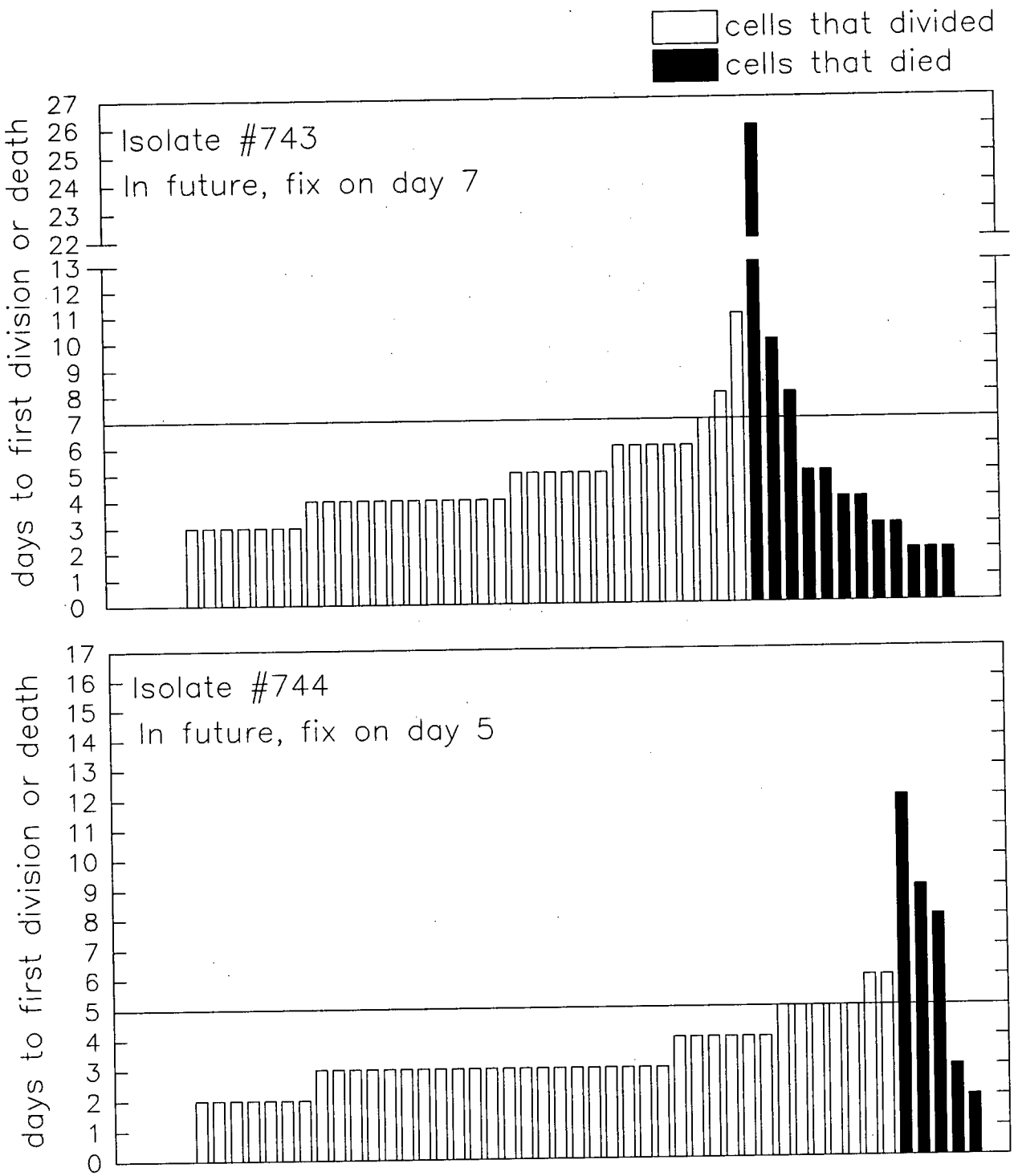


Figure 4.2 Days to Division or Death for Isolated Single Cells from #743 and #744. Each cell isolated appears as a white bar if it divided and a black bar if it died without dividing. They are ordered to illustrate how the optimal day for fixation (shown as a horizontal line) was chosen to maximize the number of non-dividing single cells and minimize the cells not yet divided.

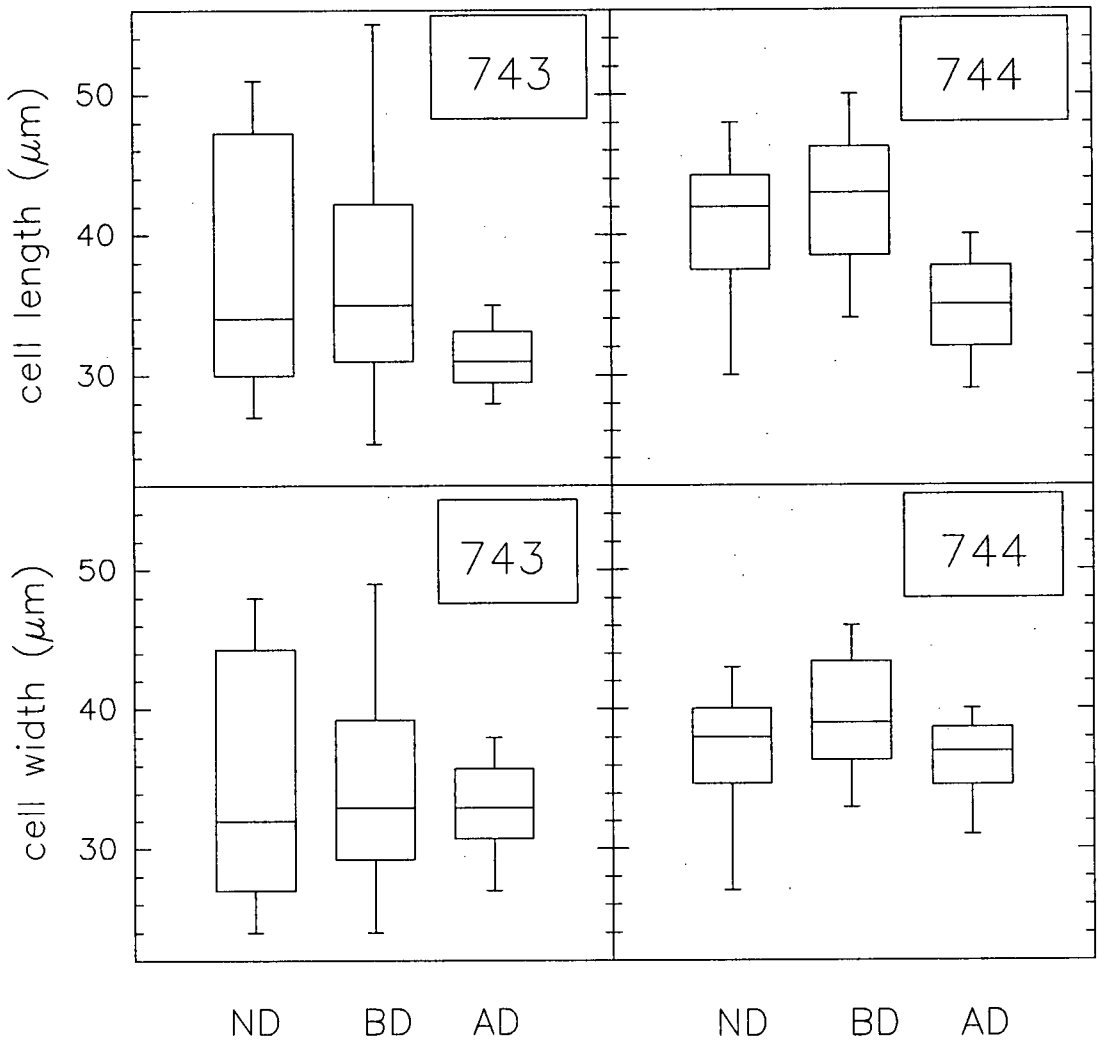


Figure 4.3 Cell Sizes for Dividing and Non-dividing Cells.
ND = all daily measures of non-dividing cells up to their death (#743 = 29; #744 = 45).
BD = all daily measures of dividing cells up to their division (#743 = 74; #744 =90).
AD = measure of 1 cell after first division (posterior cell if in chain) (#743 = 23; #744 = 26).
In this box and whisker plot, central line is median, box edges are first and third quartiles, and bars show data range. #744 cells are longer and wider than #743 cells (ANOVA; $p<0.001$).

	743	744
length	ND = BD > AD >	ND = BD > AD >
width	ND = BD > AD =	ND < BD = AD =

Figure 4.4 Relationships Between Life Stage Length and Width.
Greater than (>) and less than (<) symbols indicate a significant difference (t-tests; $p<0.05$) and equal to (=) symbol indicates that there was no difference between the means.

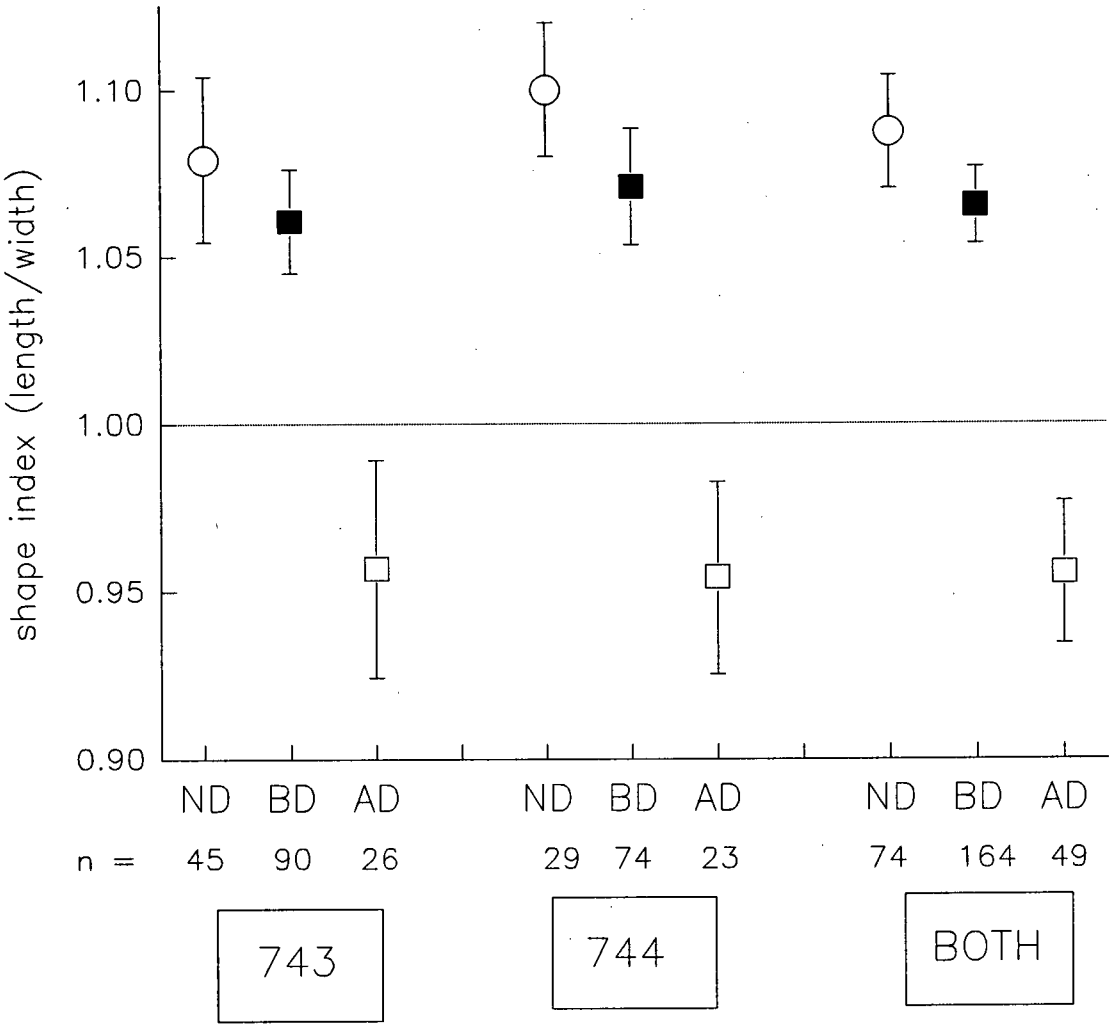


Figure 4.5 Shape Index of Cells. Index = Length/Width.
There is no shape difference between #743 and #744. When #743 and #744 data are pooled ("BOTH"), ND>BD>AD (t-tests; $p<0.05$). Points indicate data mean and error bars outline 95% confidence limits around the mean.
ND = all daily measures of non-dividing cells up to their death.
BD = all daily measures of dividing cells up to their division.
AD = measure of 1 cell after first division (posterior cell if in chain).

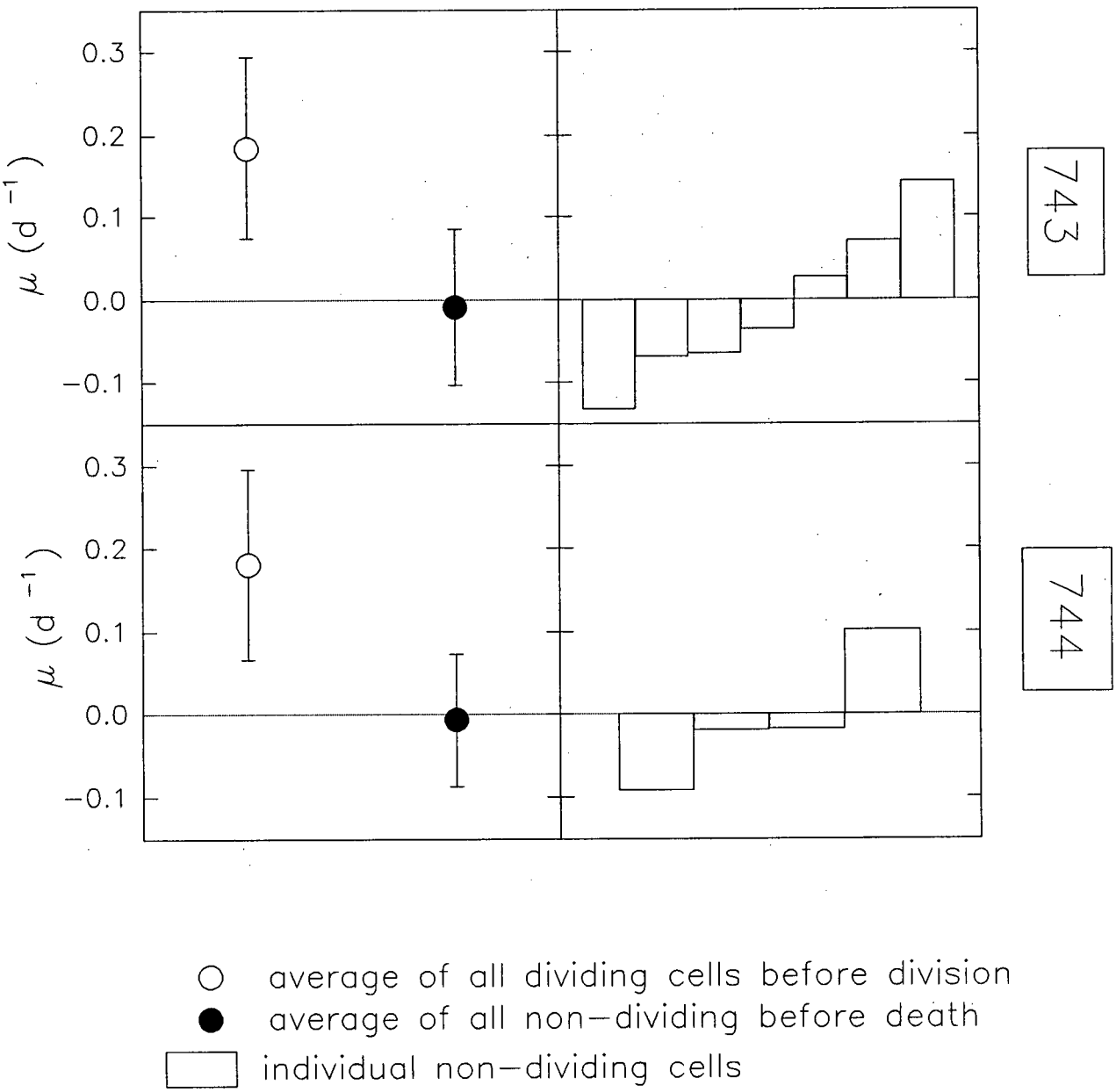


Figure 4.6 Growth of Non-dividing and Dividing Cells.
Regressions through ln transformed volume measurements provide presented measures of growth. The left panel shows that the average growth of the dividing cells is positive and the average growth of non-dividing cells is negative. Points are the mean of all observations and error bars are ± 1 SD. The right panel shows the volume change for the individual non-dividing cells, and shows that some gained and some lost volume before they died.

CHAPTER 5 SURFACE EXAMINATION OF *Alexandrium catenella* CELLS

1.0 Introduction

1.1 Cell-Cell Interaction

In general, carbohydrates covering the cell surface can form the first interaction between cells, and thereby define the identity of the cell (reviewed in Berger *et al.* 1982). For gametes, evidence from a number of studies shows that recognition is mediated by surface-localized complementary macromolecules (Bolwell *et al.* 1980), which are often surface glycoproteins (Kemp *et al.* 1973). Interestingly, the preliminary step in protist fusion involves a change to these complementary cell surface macromolecules that subsequently act in specific recognition and adhesion processes (Goodenough 1985).

1.2 The Lectin Concanavalin A

Lectins are large molecular weight proteins or glycoproteins that have multiple binding sites for carbohydrates. They have been isolated from plants, animals, microorganisms, viruses, and recently from the red alga *Porphyridium cruentum* (Mockler *et al.* 1992). All lectins bind exclusively to the non-cytoplasmic side of membranes, i.e. the outer surface of the plasma membrane and the inner surface of organelle membranes (Sharon and Lis 1989). The first lectin was discovered in 1888 and 100 years later, publications on these proteins approached 2000 per year (Sharon and Lis 1989).

Concanavalin A (Con A), originally isolated from *Canavalia ensiformis* (Jack Bean), is the most widely known and extensively studied lectin. The three dimensional structure of this protein is dependent on both pH (Kalb and Lustig 1968) and temperature (Huet *et al.* 1974), but likely exists as a tetramer (MW=112 000) or as higher aggregates, as used in these thesis studies. Each subunit has a binding site for calcium, manganese, and sugars with terminal mannosyl or glucosyl residues according to the following binding affinity: Manα1,2Manα1,2Man > Manα1,2Man > α-Man > α-Glc > α-GlcNAc (Goldstein and Poretz 1986).

1.3 Lectins as Research Tools

Surface glycoproteins of cells ranging from bacteria to humans have been identified, mapped, or quantified using lectins. For example, in one large undertaking, binding patterns of seven lectins were found to be unique and specific to group, species, structure, or developmental stage in 101 isolates from the groups cyanobacteria, cryptophytes, raphidophytes, euglenoids, chrysophytes, xanthophytes, diatoms, and chlorophytes (von Sengbusch and Müller 1983). Lectins have been used to distinguish species or strains of fungi (Robin *et al.* 1986), marine amoebae (Rogerson *et al.* 1992), the dinoflagellate *Prorocentrum* (González-Gil *et al.* 1992), and members of the cyanobacteria, Dinophyceae, and Conjugatophyceae (Costas *et al.* 1993). Also, lectin binding was dissimilar for strains of differing pathogenicity in *Trichomonas vaginalis* (Warton and Honigberg 1980) and the human parasite *Entamoeba histolytica* (Trissl *et al.* 1977).

Lectins cause agglutination (many cells sticking together) in *Euglena gracilis* (Sharabi and Gilboa-Garber 1980), the slime mold *Dictyostelium discoideum* (Weeks 1973, Gillette and Filosa 1973), *Leishmania donovani* (Dwyer 1974), forms of *Trypanosoma lewisi* (Dwyer 1976), *Chlamydomonas* species (Wiese and Shoemaker 1970, Wiese 1974, Musgrave *et al.* 1979, Sharabi and Gilboa-Garber 1980), the amoeba *Naegleria gruberi* (Josephson *et al.* 1977), and enveloped viruses (Klenk *et al.* 1974). Lectins also agglutinate a variety of metazoan cells transformed by carcinogens and viruses but not normal cells (Ben-Bassat *et al.* 1971, Klenk *et al.* 1974, Nicolson 1975). Additionally, binding of the lectin Con A induces abnormal morphology in the fertilized egg of the mollusc *Nassarius reticulatus* (Speksnijder *et al.* 1991), increases neurite outgrowth (Lin and Levitan 1991), enhances glucose transport (Cuatrecasas and Tell 1973), causes mitogenic stimulation of lymphocytes (Hesketh *et al.* 1983, Tregear *et al.* 1991) and causes an increase in cytoplasmic Ca^{2+} (Hesketh *et al.* 1983, Tregear *et al.* 1991, Ramaschi *et al.* 1993).

The above lists, far from being complete, give a good indication of the breadth of lectin applications and the multitude of cells which this tool has been used on. Most important to this

research however, is that lectins have been used successfully to distinguish developmental stages in many eukaryotic cellular events including division, maturation, and differentiation.

1.4 Developmental Stages - Ciliates

Ciliate research is relevant to this thesis study because, as in dinoflagellates, gametes are undifferentiated from vegetative cells by ploidy or gross morphology. Also, molecular techniques and morphology indicate that this group is closely related to dinoflagellates. Ciliate conjugation occurs when starved cells from complementary mating types are mixed (Hiwatashi 1988).

Mating is mediated by ciliary contact for *Oxytricha bifaria* (Ricci 1981) and *Paramecium caudatum* (Kitamura and Hiwatashi 1976). Although it was initially concluded that *Paramecium* species fusion involved glycoproteins, experimental results were later reinterpreted to indicate that glycoproteins are not essential to mating for this species (Hiwatashi 1988). In contrast, evidence that glycoproteins were involved in fusion for other ciliates was first provided by the finding that Con A (25 $\mu g \cdot mL^{-1}$) completely inhibited conjugation for *Tetrahymena pyriformis* (Ofer *et al.* 1976).

For at least three ciliates, lectin binding differences have been identified between conjugating and lone cells. Frisch and Loyter (1977) studied *T. pyriformis* preconjugates and found that Con A first binds to the oral region and then is taken into vacuoles. When Con A was added to fusing pairs, binding would appear as a ring surrounding the conjugating area. Similarly, Con A binding to *T. thermophila* was hardly detected until complementary mating types were mixed (Watanabe *et al.* 1981). In early conjugation, binding would begin at the anterior cell surface and then progressively change until a ring of intense binding would appear between conjugants. Also, Con A binding sites appear during the "courtship stage" of *Euplotes vannus*, undergo characteristic changes during the conjugation process, and disappear after pronuclei exchange (Lueken *et al.* 1981).

1.5 Developmental Stages - *Chlamydomonas*

Chlamydomonas gametes also have the same ploidy and morphology as their vegetative cells. Nutrient stressed mating types begin fusion instantaneously upon mixing, beginning with

flagellar tip adhesion. Large clumps of cells gradually reassociate to form pairs which then develop into quadriflagellated zygotes (Snell 1976a, 1976b; van den Ende 1981).

Glycoproteins are involved in *Chlamydomonas* cell contact (Hiwatashi 1988) as evidenced by the early finding that gametes but not vegetative cells agglutinate in the presence of Con A (Wiese and Shoemaker 1970, Millikin and Wiese 1984). Quantitative differences may not be important since vegetative cell flagella of *C. eugametos* had more ^{125}I-Con A labeling than gamete flagella (Musgrave *et al.* 1979). However, qualitative differences were discovered: all cells have Con A receptors on their flagella, but for gametes, these receptors are localized to the flagellar tips (Millikin and Wiese 1984). Interestingly, no binding differences were noted between mating types (Musgrave *et al.* 1979).

1.6 Developmental Stages - The Slime Mold *Dictyostelium discoideum*

In response to starvation, slime mold non-social amoebae stream together into aggregates which then differentiate into fruiting bodies. The early discovery that exponentially-growing cells were more Con A-agglutinable than stationary phases of *Dictyostelium discoideum* (Weeks and Weeks 1975) indicated glycoprotein involvement. It is now accepted that species-specific cell-cell adhesion involves both an endogenous lectin and glycoproteins (Springer and Barondes 1988).

Two interesting discoveries have come from the *D. discoideum* literature. First, when cells were labeled live, the binding of ^{125}I-Con A was diffuse for vegetative cells and patchy for aggregation phase cells (Weeks 1975). In contrast, if cells were fixed before they were labeled, both cell stages had uniform labeling (Molday *et al.* 1976). Based on these two studies, receptors are mobile only on live aggregation phase cells, and therefore, a change in membrane fluidity is likely involved in slime mold differentiation.

The second discovery was that glycoprotein changes occur between vegetative and aggregation phases of *D. discoideum*, then an almost complete glycoprotein changeover occurs between aggregation and pre-culmination phases (Hoffman and McMahon 1977). More than 35 Con A-binding molecules in the plasma membrane of *D. discoideum* have been identified, 12 which diminish and 12 which become increasingly important as receptors during differentiation

from vegetative to preculmination cells (Hoffman and McMahon 1977). Although earlier it was discovered that more ^{125}I-Con A bound to *D. discoideum* aggregation phase cells than to growing cells (Weeks 1975), the process is clearly quite complex.

1.7 Developmental Stages - The Kinetoplastid *Trypanosoma cruzi*

T. cruzi is a parasitic protist that causes Chagas' disease common in Central and South America (Snary and Hudson 1979). Its three forms include epimastigotes which multiply in insect guts, intracellular amastigotes which multiply in mammalian cells, and trypomastigotes which are non-dividing cells that circulate in the bloodstream. Con A agglutinates epimastigotes and not trypomastigotes (Alves and Colli 1974). While Con A-binding carbohydrates are present for all three life stages of *T. cruzi* (Snary and Hudson 1979), differences in binding exist (Araujo *et al.* 1980).

1.8 Developmental Stages - Other Groups

Although less is known for other groups, lectin research has identified glycoprotein changes in response to differentiation for 28 conjugatophyceans (von Sengbusch *et al.* 1982), the desmid *Cosmocladium saxonicum* (Surek and von Sengbusch 1981), *Euglena gracilis* (von Sengbusch and Müller 1983), and the rust fungus *Euromyces fabae* (Kapooria and Mendgen 1985). The gamut of research suggests that glycoprotein changes in response to differentiation is universal.

1.9 Developmental Stages - Metazoans

Lectin research in metazoans may seem far removed from dinoflagellate research, but the use of Con A across this evolutionary expanse underscores a striking commonality: mannose/glucose receptors change during maturation, particularly relating to the sexual cycle.

Maturational changes in lectin binding have been observed for the fertilized egg of *Ascidia malaca* (O'Dell *et al.* 1973), 10-13 day old mouse embryos (Herken *et al.* 1991), second stage juveniles of the nematode *Meloidogyne javanica* (Ibrahim 1991), and human erythrocytes

(Gutowski 1991a, 1991b). Also, cell cycle changes occur in Con A binding for transformed cells (Collard *et al.* 1975) and mouse embryo fibroblasts (Noonan *et al.* 1973).

Con A binding qualitatively and quantitatively changes during differentiation in the rat ventral prostate (Hauke *et al.* 1989) and also through maturation of epididymal sperm for rats (Olson and Danzo 1981) and goats (Sarkar *et al.* 1991). These sperm changes include the addition of glycoproteins to the membrane and a change in membrane fluidity (Hall *et al.* 1991). Also, Con A-binding of mouse vagina epithelium undergoes cyclic changes (Vrčić *et al.* 1991).

1.10 Receptors and *Alexandrium catenella* Fusion

Acridine orange and FITC-Con A both bind to surfaces of *Amphidinium carterae* and *Prorocentrum micans* (Klut *et al.* 1988). This indicates that at least these two dinoflagellates have acid polysaccharides on their surfaces including glucose/mannose residues.

In a study with *A. catenella* +/- mixtures, hypnozygote formation was reduced by 98% with 128 units·mL^{-1}, and by 79% with 64 units·mL^{-1} of "MI" (Sawayama *et al.* 1990). MI (=MINT in Sawayama *et al.* 1993a) is an extract from *Bacillus brevis,* a gram negative bacterium isolated from a high altitude lake. Extracts from the other 149 bacteria isolated at the same time did not affect *A. catenella* pairing. The finding that MI inhibits protein synthesis in *Chlamydomonas reinhardtii* gametes (Sawayama *et al.* 1993a) suggests that protein synthesis may be critical to *A. catenella* gamete formation.

In another study, 74 bacterial strains were isolated from an atoll. Extracts from 24 of these suppressed hypnozygote formation of *A. catenella* by more than 80% (Sawayama *et al.* 1993c). The greatest suppression was caused by an extract (MIMB) from *Altermonas* sp. (strain MBI). Pre-incubation with the extract was not necessary to block hypnozygote formation; the effect was immediate. Analysis revealed that the extract was a large molecular weight protein. These tests suggested that the extract may be lectin-like.

In a logical follow-up, the lectin Con A was added to +/- cultures of *A. catenella* (Sawayama *et al.* 1993b). The authors found that 0.005% Con A greatly suppressed sexual attachment to 0.33% of the control, and 0.01% Con A prevented all sexual attachment. This

effect was due to specific receptor interaction since additions of 10 mM glucose or mannose reversed the effect. The authors concluded that glucose and/or mannose sugar chains are involved in gamete attachment.

Interestingly, studies indicate that there are biochemical difference between *A. catenella* mating types. Tunicamycin (a potent protein glycosylation inhibitor of asparagine-linked sugar chains) completely inhibited attachment when added at 1 $\mu g \cdot mL^{-1}$ to (+) cultures, 12 hours before mixing with (-) cultures (Sawayama *et al.* 1993b). The same treatment of (-) did not inhibit attachment. Also, the bacterial extract MI (=MINT) reduced growth of (+) by 35% and (-) by 7% (Sawayama *et al.* 1990).

1.11 The Metabolic Pathway From Nitrogen Limitation to Surface Sugars

There is an interesting connection between the finding that nitrogen limitation (see Chapter 3) induces the sexual cycle and the evidence from the above survey, that the sexual cycle involves surface glycoprotein changes. There is evidence that proteins are affected by nitrogen limitation. For example, nitrogen (as well as phosphorus and iron) limitation was found to induce the synthesis of unique proteins in two species of diatom (La Roche *et al.* 1993). Also, nitrogen starvation reduced protein and RNA pools of the dinoflagellate *Heterocapsa* sp. (Berdalet *et al.* 1994). However, this effect may be mediated by the reduction of growth, i.e. nitrogen limitation reduces growth which in turn reduces the production of RNA and protein.

One suggestion is that the shift to sexuality may occur because exponentially growing cells transferred to an N-free medium continue to assimilate carbon. Possibly, a metabolic shift occurs when this carbon is diverted to non-nitrogenous compounds (Fogg 1959 cited in Pfiester 1975). In a study of the prymnesiophyte, *Pavlova lutheri*, it was found that N limitation increased cellular carbohydrate concentrations significantly (Madariaga and Joint 1992). In studies of *Heterocapsa niei*, it was found that carbohydrate synthesis and use are balanced during exponential growth, but in stationary phase, where division and synthesis may be uncoupled, it is possible to generate a carbohydrate surplus (Loeblich 1977). However, cellular starch was seen

to wax and wane on a diurnal cycle (Loeblich 1977), and therefore it is more likely that any carbohydrate surplus produced because of a synthesis/division uncoupling would likely be stored as starch rather than as surface carbohydrates for dinoflagellates.

Although some evidence exists to link N limitation and changes to glycoproteins, this is likely an over-simplification of the probably complex biochemical pathway to the production of specific receptors. For example, the glycocalyx of *Amphidinium carterae* was reported to increase with culture age (Klut *et al.* 1985), but this gross change may be unrelated to possibly fine-scale glycoprotein changes. Additionally, since both N and P reduction induced sexuality in *A. tamarense,* a common mechanism in the N and P metabolic pathway (Anderson *et al.* 1984), rather than one specific to N alone may be most important. Finally, the literature more frequently reports a qualitative change in glycoproteins and membrane fluidity rather than a simple increase in glycoproteins in response to N limitation.

1.12 Chapter Objectives

For many protistan groups, the discovery that lectins prevented fusion, or that lectin-induced agglutination was dependent on life stage was used to springboard into further glycoprotein research, which then uncovered the concurrent involvement of glycoproteins in differentiation. Since Con A has been found to prevent *A. catenella* fusion (Sawayama *et al.* 1993b), this thesis study used Con A tagged with the fluorochrome fluorescein isothiocyanate (FITC), to look for qualitative or quantitative differences in the surface carbohydrates of gametes and vegetative cells.

2.0 Materials and Methods

2.1 Isolation of Potential Gametes

The sexual cycle was induced as in Chapter 3 by inoculating 1 mL of a 7-day old stock culture into 30 mL of induction medium (see Appendix 4). Once the cultures were in stationary phase, measured volumes of #743 and #744 were mixed, and double aliquots of each mating type incubated separately. After ~6 d, single motile cells were micro-pipetted into 3 drops (~60 µL) of

nutrient-replete medium in single wells of a 96-well cluster. The interwell spaces were filled with distilled water, the edges sealed with transparent tape, and the clusters put into the incubator.

From the information obtained in Chapter 4, clusters containing #744 isolates were fixed after 5 d, and clusters with #743 were fixed after 7 d. These times reflect the different lag phases and growth rates of the clones, and were chosen to maximize the number of non-dividing single cells and minimize the number of cells that would divide, but had not yet divided. On their respective days, all wells were examined under a dissecting microscope using 10-80X or an inverted microscope at 200X to count and record the number of cells in each well. This was done after 1100 because cell division was observed to occur up to this time (light cycle began at 0800). Each well was identified as containing either a single cell (ND), or vegetative cells (D) as chains, multiple single cells, or combinations.

2.2 Fixation

The fixation method was chosen based on literature information and pretests to find a fix that would 1) preserve the membrane in its most natural state without changing its permeability or extracting membrane components, 2) preserve the cell in its natural state without shrinkage, swelling, and with a minimum of flagellar shedding, 3) not fluoresce, 4) not cause the cell to fluoresce and optimally cause the cell's autofluorescence to fade, 5) not impede the visualization of the surface, and 6) not block the label. Fixes that were tried in various combinations, strengths, buffers, pH's, and osmolarities were formaldehyde, glutaraldehyde, osmium tetroxide, acid Lugol's iodine (with and without sodium sulfite), heat shock, pH shock, and paraformaldehyde with and without methanol extraction. Of these, the best system was a weak paraformaldehyde-glutaraldehyde fix followed by washes in blocker medium.

Fix was prepared frequently in small batches: 2.00 g of paraformaldehyde powder (Fisher) and 1.07 g sodium cacodylate (J.B. EM Services Inc.) were added to 48 mL medium. To dissolve, the solution was either gently heated for ~10 min or allowed to stand at room temperature for ~1 d. After 0.25 mL of 10% glutaraldehyde was added, the solution was filtered

through a 0.22 μm GS Millipore® filter and then stored in the dark at 5°C. The unadjusted pH was 8.2.

Every ND cell per cluster and several D chains for comparison were fixed by adding 3 drops (~60 μL) of cold fix to their wells. The high density fix was observed to sink through the culture and mix and therefore, stirring was not required. This 1:1 fix:medium mix made the final concentration 2% paraformaldehyde, 0.025% glutaraldehyde, 0.05M sodium cacodylate buffer in medium. The fix concentrations used here are lower than those typically used. The success of the combination may be due to the presence of high osmolarity medium. After fixation, wells were scanned using an inverted microscope to ensure that only one cell was in each well identified as ND. Clusters were incubated for 30 min in the dark at room temperature.

The cells were then flushed with blocker medium (1 g of glycine in 200 mL of medium) which had been filtered just prior to use with a 0.22 μm GS Millipore® filter. Since aldehydes cross-link proteins (Steedman 1976a), this step cleared any unbound fix. In this thesis study, the next step was the application of a protein label, and thus it was critical to remove the free aldehydes. Washing was accomplished by first locating the cell(s) within the well using an inverted microscope (100X power). Most of the liquid was micro-pipetted out and then the wells were topped up with blocker medium. This was repeated 3 times and cells were left in a minimal volume of blocker medium.

2.3 FITC-Concanavalin A Labeling

FITC-Con A (Sigma) that had been stored frozen in the dark, was dissolved in medium just prior to use. Solutions were made in a test tube by adding ~100 μg of lectin plus 2 drops (~40 μL) of medium per well to be labeled. Although this method seems lax, it fulfilled three important criteria. First, the final concentration would be well above the typical 50-100 $\mu g \cdot mL^{-1}$ commonly used and therefore variations in the well volume and number of cells per well would be inconsequential. Second, solutions could be made fresh every trial which would eliminate the need for freezing which possibly was the cause of previous poor labeling. Finally, there was no waste of the costly lectin.

Once the solution was mixed thoroughly, it was drawn into a pipette and 2 drops (~40 μL) were added to each well. Incubation was at room temperature in the dark for 60 min. This time had been determined experimentally (see Figure 5.1). Also, the relationship of fluorescence to concentration of FITC-Con A had been tested (see Figure 5.2), and found to be linear.

Each well was then filled with medium to make the concentration of Con A as low as possible to slow the labeling reaction. Next, each ND cell or D chain was located using an inverted microscope with very low light and room lights out, as the fading of FITC in response to high levels of microscope light had been shown to be rapid and marked (see Figure 5.3). Sequentially, the volume in each well was reduced using a finely drawn micro-pipette, and then again sequentially, the wells topped up with fresh medium. Each cell(s) was washed this way twice.

Each well was exposed to the same concentrations of Con A over the same time period within 30 s, i.e. the time to complete one round of additions. The total time required to wash all cells was often up to 30 min, but during this time the concentrations in each well would be roughly equal, only the volumes would differ. In addition, all cells would have been exposed to the same light and therefore any fading incurred would be equal for all cells. Since differences between ND and D cells were sought, it was imperative that they all receive the same concentration of lectin for the same amount of time.

In general, all washes were done with great care. Fluid was removed slowly by micro-pipette, and fluid was added by gently micro-pipetting fluid down the well wall opposite the cell(s). With these precautions, perturbation was minimized and the cell's flagella remained attached.

2.4 Mounting Onto Slides

The mounting method had to fulfill several requirements. First, slides should have only one cell or one chain to minimize their exposure to blue light (see Figure 5.3 for fading potential). Secondly, because one ND cell was to be mounted per slide, it was imperative that the cell would not be lost, squashed, or move too close to the edge of the slide to be scanned. With each cell so

valuable, the method had to be fail-safe. Third, only a minimum of mountant could be used in order to keep the cells close to or touching the cover slip for optical clarity; the scanner can only penetrate 10-20 μm of opaque matter (M. Weis, EM lab UBC, pers. comm.). Fourth, the cells needed to be stable as the scanner focused through the cell. If there is any shift, the image is blurred. Finally, the mountant could not fluoresce itself, affect the label, or alter the cell membrane.

The mountant, glycerine jelly (Kaiser 1880 cited in Steedman 1976b) was made in a 40 mL medicine dropper-equipped bottle on a hot plate (~60°C). First, 10 mL of distilled water and 1.5 g of gelatin were heated until dissolved. Then, 10 mL of glycerol (=glycerin) was added and the mixture heated and stirred until homogenous and small air bubbles had escaped.

To prepare the slides, #1 cover slips were affixed to each end with 0.5% low melting point agarose, and warm mountant was pipetted to cover the middle. Air bubbles were pipetted back off, and the slide was placed briefly on the hot plate to smooth the surface. Prepared slides were used while they were still warm as it was discovered that this caused less distortion of the cells.

Cell(s) were micro-pipetted up in a small volume of fluid (~5 μL) and the droplet placed in the center of the mountant. The slide was then put on the hot plate long enough (2-10 s) to collapse the bubble and mix the edges into the mountant. Under a dissecting scope, the cell(s) were located and tracked while a #1 cover slip was gently applied. FISHER*finest*™ cover glass was used because they lacked fluorescent specks observed in other brands. A felt marker was used to encircle the cell(s) to make it easy to find them under the scanner. After the mountant had cooled to room temperature, the edges were sealed with nail polish. Slides were stored flat at room temperature in the dark, and were viewed the same day they were made.

2.5 Confocal Laser Scanning

Several methods to image/measure surface labeling were attempted and abandoned. No differences were observed in cell surface label between ND and D cells using epifluorescence microscopy. This was in part due to the poor image resolution and secondly because of plastid autofluorescence. A quantitative epifluorescence system was used to measure the total photons

per unit area on ND versus D cells. This method failed because variability was high even in measurements of one cell. Also, fluorescence of plastids and accumulation bodies (= lysosomes; Zhou and Fritz 1994) interfered with accurate measures of surface fluorescence.

Finally, confocal laser scanning was tried and seemed to solve all previous problems. One cell could be imaged into optical slices that could be manipulated so that plastids and other fluorescent inclusions could be removed via the computer program Adobe Photoshop™. In addition, the high resolution of the system enabled the clear visualization of surface detail and flagella.

Imaging was done with a MRC-600 confocal system (Bio-Rad, Microscience Division, Watford, Herts.) attached to a Nikon Optiphot-2 microscope equipped with a 60X oil immersion objective, an argon ion laser, a 490 nm excitation filter, and a short pass 510 nm emission filter. With each run, a D chain was used to adjust the black level and gain so that there was at least one bright spot. Cells were located and positioned using light from a tungsten bulb and the cell's position was preliminarily assigned as dorsal, ventral, apical, or antapical. The pre-scan set up was done quickly, turning on the laser for short pulses only as absolutely necessary to center the cell on the screen, and to set z coordinates. A reduced box size of 49x49 µm helped reduce duration of scan. Kalman filtering was used to integrate the signal from 2 scans to help reduce noise. Scans started at mid-depth in the cell and stopped just above the cell's surface closest to the objective. With the focus step at 0.5 µm, about 30 images per cell were generated.

2.6 Epifluorescence Photography

After scanning, photographs were taken at 1000x with a Zeiss microscope fitted with an HBO light source, a band pass 450-490 nm excitation filter, a FT510 beam splitter, and a long pass 520 nm emission filter. A 35 mm camera recorded images on Kodak Ektachrome 100+ Daylight Professional Color Reversal film.

3.0 Results

Over a number of trials, more than 20 ND cells and a like amount of D cells for comparison, were viewed under the confocal laser scanner. In general, surface labeling was pale and diffuse for both ND and D cells (see Figure 5.4). Sometimes evenly spaced points of more intense labeling were observed (see Figure 5.5), probably associated with trichocyst pores. Where flagella were clearly visible, no labeling was detected for either ND or D cells (see Figure 5.6).

Often a small mass of highly labeled material appeared on cells (see Figure 5.7 and 5.8). It could not be satisfactorily determined whether these were tangled flagella, hot spots on cell surfaces, or extracellular particles that had become attached to cells. This last explanation is the most likely since labeled particles of similar shape and size were seen in the medium. These hot spots of labeling could not be related to ND, D, or cells of either mating type.

4.0 Discussion

4.1 How Membrane Glycoproteins May Change

Con A binding sites have been localized on the cisternal side of rough endoplasmic reticulum (Hirano *et al.* 1972). This indicates that Con A binding sites are probably added to cell surfaces along the well-described pathway whereby glycoproteins are transferred from the rough endoplasmic reticulum to the Golgi complex and then when vesicles fuse with the plasma membrane, they become incorporated into the cell surface (reviewed in Berger *et al.* 1982). In a perpendicular track from the RER to the outer *A. catenella* membrane are: two membranes, thecal plates, and a pellicle. Therefore, the potential sites of new receptor addition are limited. However, at least three possible sites are interesting to consider. Trichocysts are membrane-bound ejectosomes lying perpendicular to the cell surface which may join the outer membrane through trichocyst pores (Dodge and Greuet 1984). A second site is the pusules which are membrane-bound "bagpipe-like" structures that meet the surface near the basal bodies (Dodge

and Greuet 1984). The third, and most probable possibility is that new receptors are added during cell division.

In one study it could not be determined whether *A. tamarense* vegetative cells became gametes through division, or if vegetative cells without internal reserves or external supplies become gametes without a division (Anderson and Lindquist 1985). It makes energetic sense for vegetative cells to become gametes without a division since the process usually occurs in response to nutrient limitation (in the laboratory at least). However, most evidence indicates that division is required to transform vegetative cells into gametes (for example *A. monilata* (Walker and Steidinger 1979), *A. tamarense* (Destombe and Cembella 1990), and *Gyrodinium uncatenum* (Coats *et al.* 1984)). If gametes are formed by division, this may provide an opportunity for membrane component changes.

In addition to the formation of new receptors, quantitative changes in lectin binding per cell may arise in at least three other ways: a reduction in cell size (Ben-Bassat *et al.* 1971); the uncovering of existing receptors by a change in a neighbouring moiety; or by a configuration change in an existing receptor. Given that tunicamycin blocked mating for *A. catenella* (see Section 1.10 above, Sawayama *et al.* 1993b), it seems likely that receptor addition is part of gametogenesis and from the above evidence, this addition likely occurs during division.

4.2 Potential Reasons No Difference in Labeling Was Observed

Although lectins had identified differentiation changes for many organisms in other studies, no differences were observed in the binding of FITC-Con A to ND versus D cells. Rather than belabour this fact, the possible reasons for this failure will be quickly reviewed to provide a framework for anyone who continues the search. These experiments were not undertaken for this thesis due to time constraints.

First, it should be noted that the category ND cells was actually a mixture of cells that would never divide before dying and vegetative cells that had yet to divide (see Chapter 4). This may have clouded any real differences. Secondly, non-dividing cells were considered potential

gametes. Non-dividing cells may be aberrant cells. Also, although it is improbable (see Chapter 4), it is possible that dinoflagellate gametes can divide.

Given that no cells have been found that are completely resistant to Con A binding, it has been proposed that the presence of the oligosaccharide that binds Con A is essential for viability (Sharon and Lis 1989). Thus, both ND and D cells should be labeled, but qualitative or quantitative differences were expected. Although membranes are fluid, concentration and composition heterogeneity of some plasma membrane receptors can occur (Frisch and Loyter 1977). However, observations in this thesis study were that with few exceptions, binding was typically pale and diffuse.

Flagellar labeling was expected because of the involvement of these organelles in fusion of ciliates and *Chlamydomonas*. Also, in a study of *Scrippsiella* sp., fusion was proposed to occur as "the transverse flagellum of one gamete migrates out of the girdle and grasps the longitudinal flagellum of the other gamete" (Xiaoping *et al.* 1989). However, when the flagella were lying beside the cells (still attached), no labeling of either ND or D cells was observed.

Many studies suggest that the inhibitory action of Con A on conjugation may be due to binding of soluble glycoproteins (for example, Ofer *et al.* 1976). In a study of *Tetrahymena pyriformis*, 90-95% of Con A bound to glycoproteins in the medium. However, the use of antibodies showed that soluble glycoproteins were not involved in fusion (Frisch *et al.* 1977). The finding that *A. tamarense* has constant encystment rates across all cell concentrations was interpreted as evidence that extracellular chemicals were not important (Anderson *et al.* 1984). In addition, although symbiotic dinoflagellates exude glycoconjugates (Markell and Trench 1993), there is no evidence of this for free-living species. Therefore, differences in the lectin-induced binding inhibition of *A. catenella* (Sawayama *et al.* 1993b) and the failure in this thesis study cannot be explained by soluble glycoproteins.

Typically Con A is used at concentrations of 50-100 $\mu g \cdot mL^{-1}$ but some have found that lower concentrations produce better results. Differences in Con A binding between normal and transformed, interphase and mitotic, and trypsinized versus untrypsinized hamster and mouse cells were possible only at low concentrations of lectin, and not when concentrations over 25 $\mu g \cdot mL^{-1}$

were used (Shoham and Sachs 1972). Similarly, Con A mitogenic stimulation of mouse spleen cells was diminished at concentrations above 10 $\mu g \cdot mL^{-1}$ (Gunther *et al.* 1973). In this thesis study, perhaps labeling differences would have been apparent under lower lectin concentrations.

This thesis study was restricted to very small samples and unless large differences existed, it would be difficult to differentiate between non-dividing and vegetative cells since biological variability is expected. For example, in studies of hamster and mouse cells, all populations exhibited a range of fluorescence in response to equal FITC-Con A labeling (Shoham and Sachs 1972). Similarly, in Chapter 4, significant differences between mean sizes were seen, although ranges greatly overlapped. The number of non-dividing cells per trial minus the number that were actually vegetative cells not yet divided minus the few that were lost in handling, often left extremely small samples (n = 1-8). With larger samples, statistical differences between the two populations may have become apparent that were not observed in the comparison of individuals.

If bulk quantitative measures of labeling difference were possible, statistical differences might be realized. Indeed, an early trial labeled an exponential and a stationary culture and found more binding per cell in the stationary culture. However, this method is not feasible because in dinoflagellate cultures, gametes make up a small percentage of populations. Also, sexual cultures are typically stationary phase cultures which are littered with spent thecae, flagella, and vegetative cysts.

One difference between ND and D cells may be membrane fluidity. For example, the reason that Con A agglutinates transformed cells and not normal cells is due to increased receptor mobility (Inbar *et al.* 1973, Rosenblith *et al.* 1973, Poste and Reeve 1974, Poste 1975). This proposal is inviting because membrane fluidity is temperature dependent (reviewed in Kleinsmith and Kish 1988) and encystment rates of *A. tamarense* are higher at 20°C than at 16°C (Anderson *et al.* 1984). In this thesis study, fixed cells were labeled to see the *in vivo* Con A receptor pattern. Live labeling of ND and D cells may reveal a fluidity difference because Con A is a multivalent molecule and therefore, receptors can migrate into patches or even caps.

Further, this fluidity change may only occur for one mating type. In studies on virally infected mouse lymphoma cells (Rutishauser and Sachs 1974), it was found that cells could bind

together if only one cell had mobile receptors capable of short-range movements. The proposed model is that if receptors on one or both cells are mobile, they can move to form multiple bridges which then become irreversible. Thus, it is possible that gametes and not vegetative cells may have mobile receptors, and only one mating type need be involved in this change. Labeling of live cells, with careful comparisons of mating types, may be illuminating.

The point of initial contact is not known for *A. catenella*. Although, *A. catenella* gametes "dance" when first sexually attaching (Sawayama *et al.* 1990, 1993a, 1993b, 1993c), the point of contact has not been identified. This is possibly because it is variable and possibly because it is a short-lived stage. If the initial point of contact were known, increased focus in this area may be informative.

In ciliate studies, gamete and vegetative cell Con A binding differences were apparent only when conjugation had begun (Lueken *et al.* 1981, Watanabe *et al.* 1981). If a more productive method of isolating *A. catenella* mating pairs could be devised, Con A binding may be recognizably different circa pairing.

The impetus for this thesis study came from the finding that Con A inhibited fusion in *A. catenella* (Sawayama *et al.* 1993b) and evidence from other groups where lectins could distinguish surface glycoprotein differences for maturational stages. The discussion above gives possible explanations for the failing of this thesis study to find differences between ND and D cells. All will need further study before the null hypothesis (no differences in Con A receptors) can be confidently accepted.

4.3 Immunochemical Labeling

Currently, there is a growing field of immunochemical labeling. For example, genus and some species distinctions were possible among six ultraplankton groups using polyclonal antisera (Shapiro *et al.* 1989). Immunochemical labeling with polyclonal sera or monoclonal antibodies have been used to make genus, species, and/or clone distinctions for the marine algae *Prorocentrum* (de Chavarri *et al.* 1992, Costas *et al.* 1993), *Chattonella* (Nagasaki 1993), *Alexandrium* (Taylor and Lewis 1993), and *Gyrodinium/Gymnodinium* (Vrieling *et al.* 1994).

Based on monoclonal antibody research, it was found that *A. tamarense, A. catenella,* and *A. fundyense* could be considered varieties of a single species (Adachi *et al.* 1993b), which had also been supported by isozyme and toxin evidence (Cembella 1986). Further, monoclonal antibodies raised for *A. catenella, A. tamarense,* and *A. affine* reacted with vegetative cells in all growth phases, but most strongly with cells in exponential growth (Adachi *et al.* 1993a). Since these antibodies were not reactive to planozygotes or hypnozygotes, it may be fruitful to test them against gametes to pinpoint the stage where the cell surface differentiation occurs.

5.0 Chapter Summary

- Labeling for all cells was (with few exceptions) pale and diffuse.
- No flagellar labeling was detected
- No differences between D and ND cells, or between mating types were seen.
- Differences may exist but were masked by the use of too high a Con A concentration, very small sample sizes, fixed rather than live cells, and/or isolated cells rather than fusing pairs.

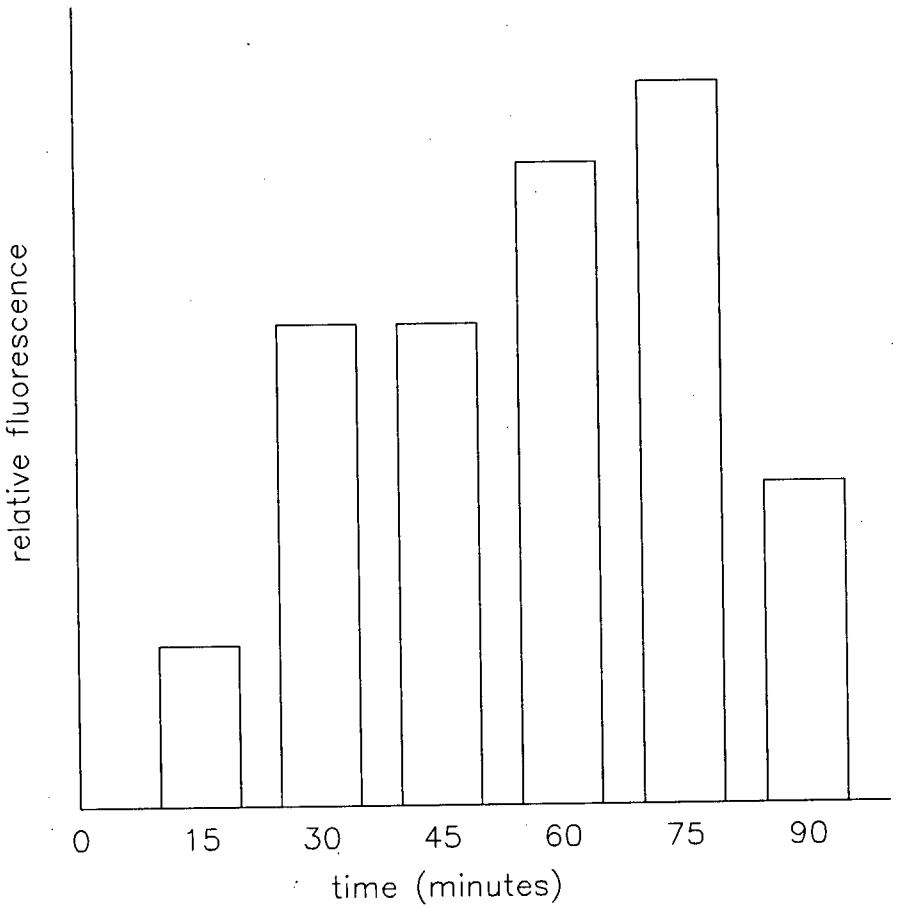


Figure 5.1 Optimal Incubation Time to Maximize FITC-Con A Labeling.
Six vials each containing 10 mL of *Alexandrium catenella* culture and 10 mL of 100 $\mu g \cdot mL^{-1}$ FITC-Con A were incubated in the dark at room temperature. Every 15 min for 90 min, the contents of 1 vial was collected on a 3 μm filter, and this filter was placed into a scintillation vial with 10 mL of 90% acetone. After 24 h, the fluid was decanted into a test tube and the fluorescence measured with a fluorometer (G.K. Turner & Associates model #111) equipped with FITC sensitive excitation and emission filters (Turner S60 and S22 respectively). 60 min incubations were used for subsequent labeling to stay clear of the rapid drop-off in binding seen here between 75 and 90 min.

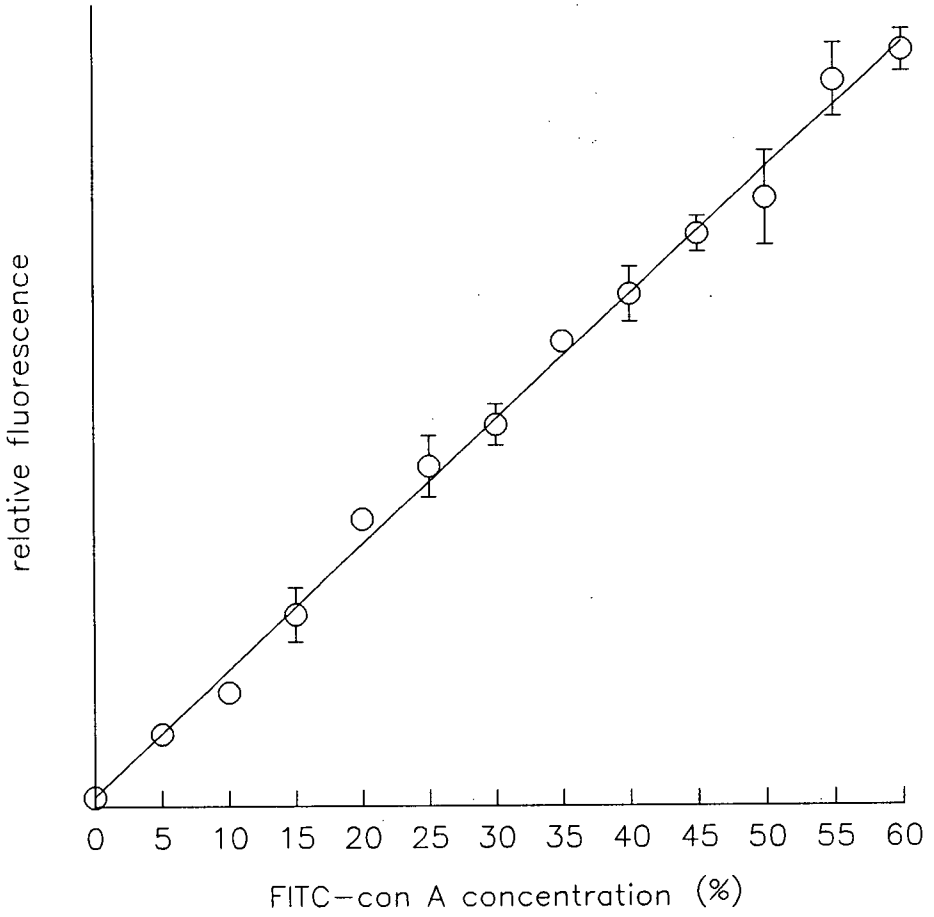


Figure 5.2 Linearity of Fluorescence Versus FITC-Con A Concentration.
100 $\mu g \cdot mL^{-1}$ FITC-Con A was first prepared in medium, and then diluted in test tubes with distilled water to obtain concentrations from 0-60%, each in triplicate. After inverting 3 times to homogenize, the fluorescence of each tube was measured in a fluorometer (G.K. Turner & Associates model #111) equipped with FITC sensitive excitation and emission filters (Turner S60 and S22 respectively). Error bars are smaller than the symbol where not seen.

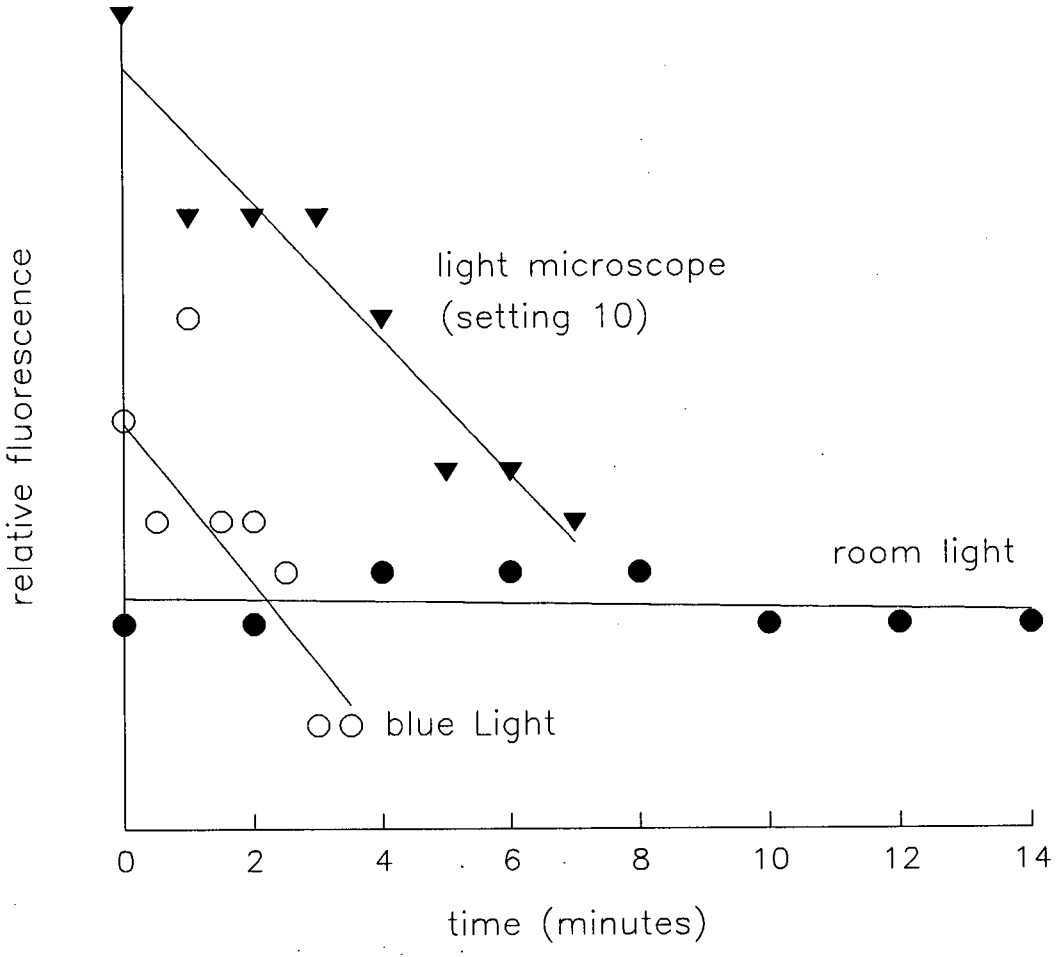


Figure 5.3 Fading of FITC-Con A With Exposure to Room, Blue, and Light Microscope Light.

Figure 5.4 Non-dividing Single Cell Showing Pale, Diffuse Surface Labeling with FITC-Con A. This photograph shows the labeling typical of most ND and D surfaces of both mating types. Cell body and FITC-Con A labeling of surface are yellow due to fading and colour distortion caused by the film.

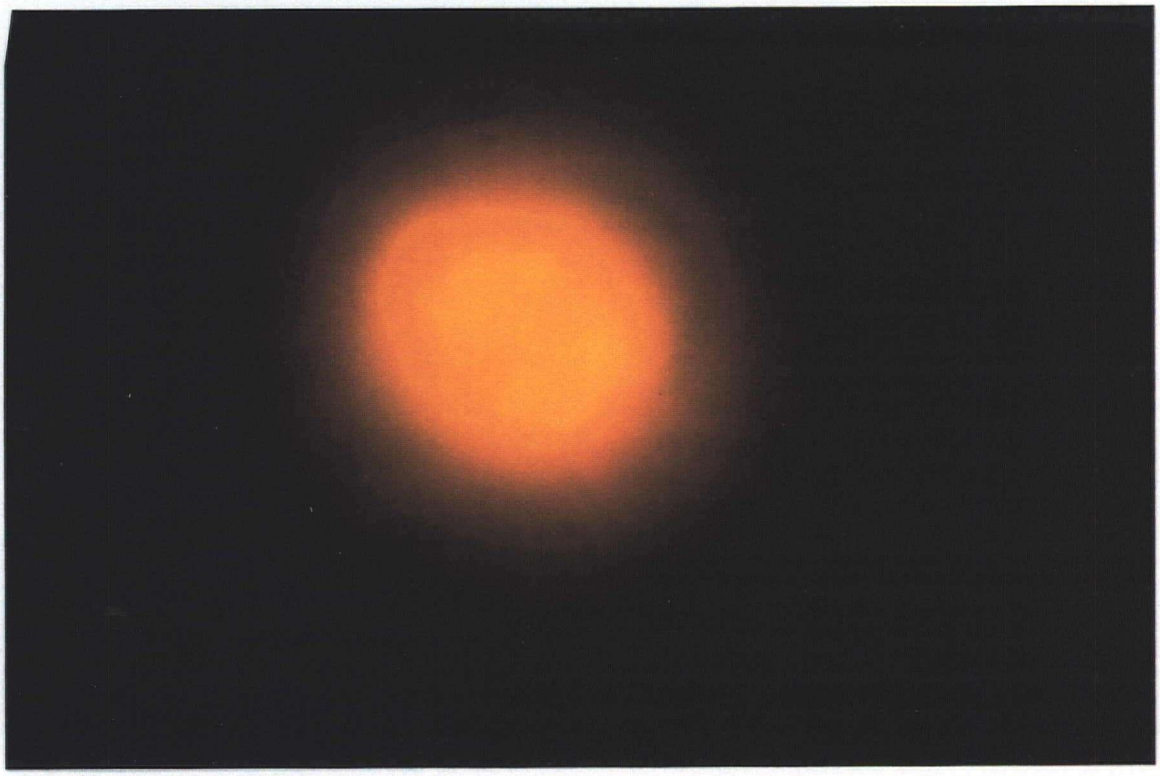
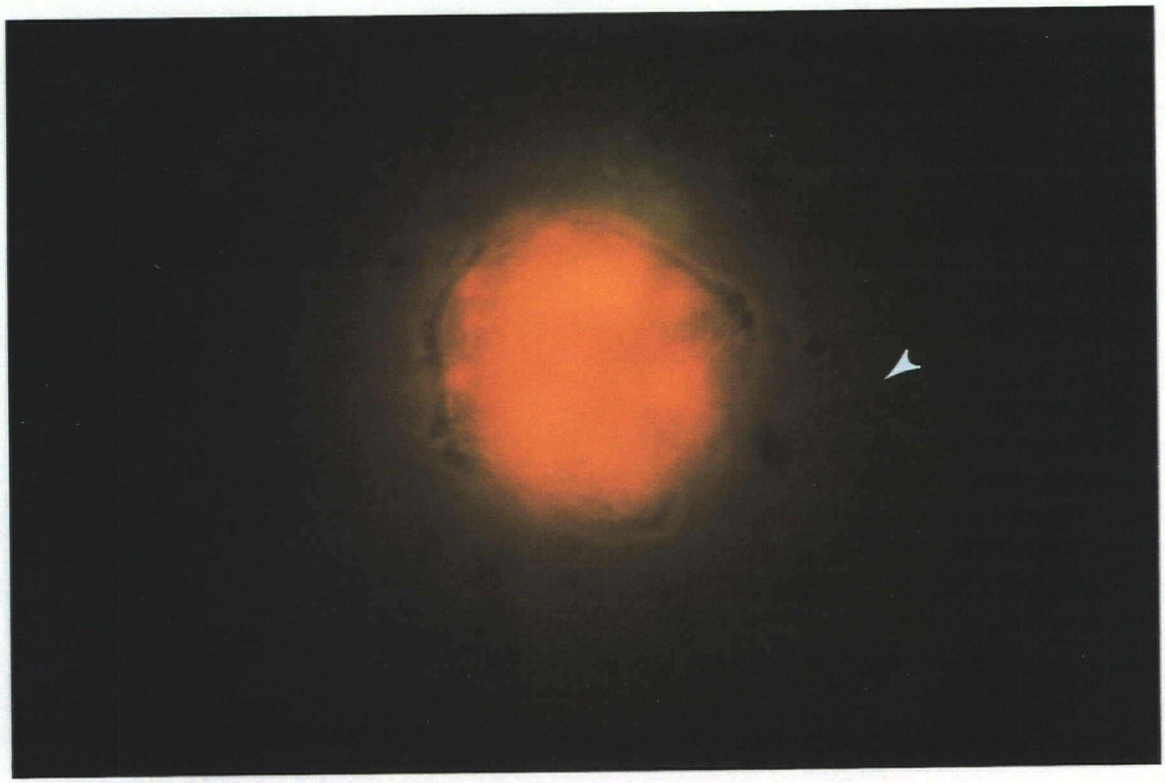


Figure 5.5 Non-dividing Single Cell Labeled with FITC-Con A Showing Bright Discreet Points. Sometimes bright points of labeling were seen on surface. Here in profile, it appears these points may be trichocysts. Cell body is yellow due to fading and colour distortion caused by the film.

Figure 5.6 Non-dividing Single Cell With Girdle Flagellum Attached.
Photograph is double exposed with both blue and white light to show a green mass of labeling present on the cell surface and the flagellum without any label.



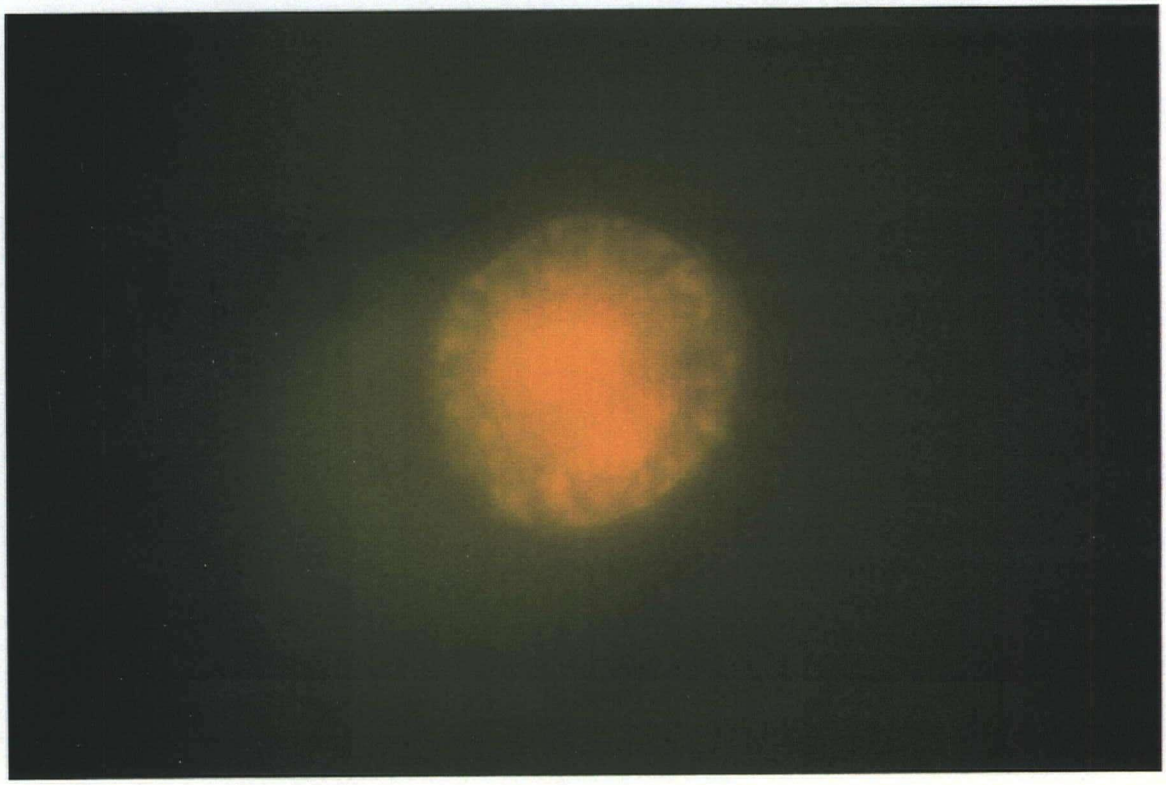
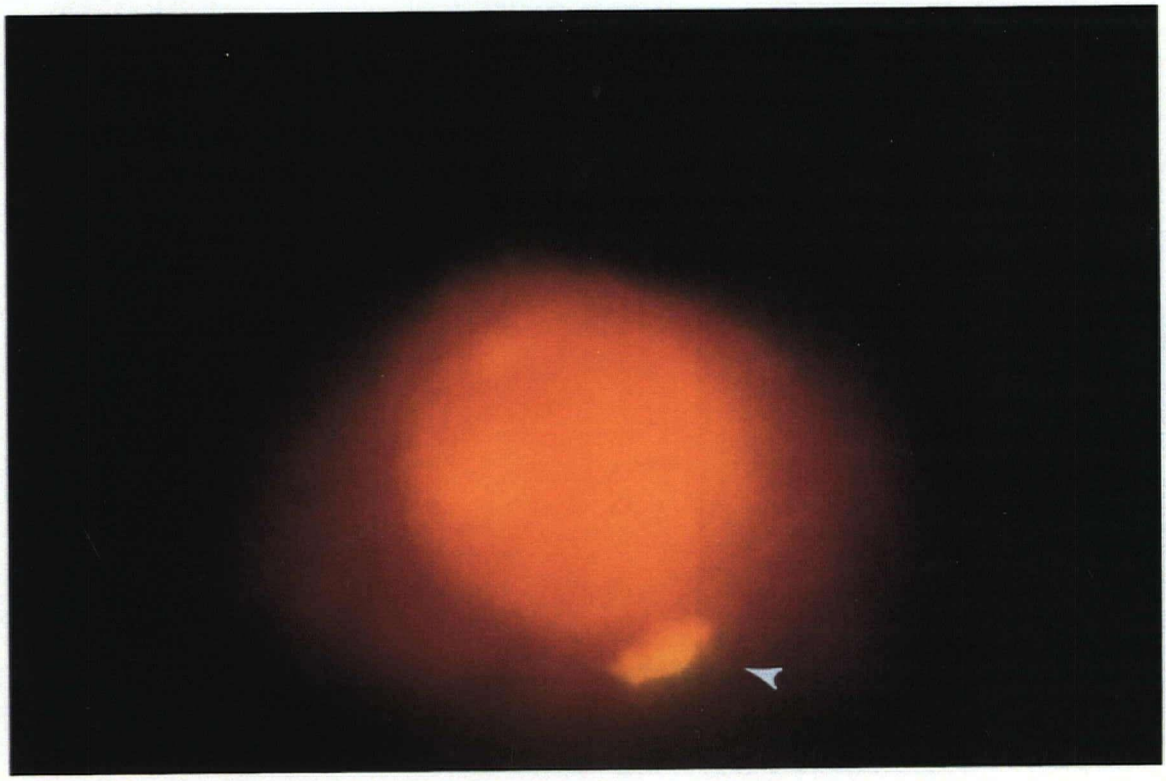
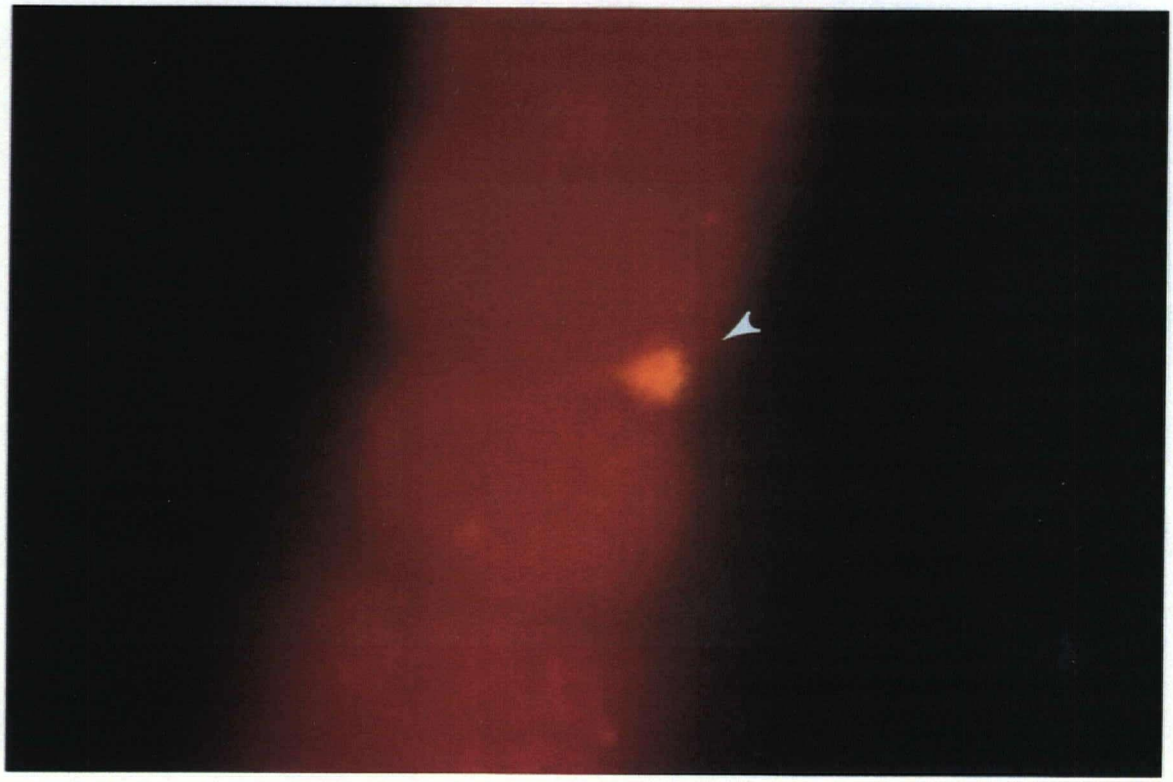


Figure 5.7 Non-dividing Single Cell With Mass of Highly Labeled Material.

Figure 5.8 Chain of Vegetative Cells With Mass of Highly Labeled Material.
The photograph also shows the typical brilliant red chl *a* autofluorescence that often obscured the relatively weak fluorescence of FITC.





CHAPTER 6 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

1.0 Conclusions

1.1 General Information

- For at least isolate #743, growth and proportion of culture in chains is higher when in natural seawater based medium from the native Barkley Sound location compared to water collected from Burrard Inlet.
- Artificial seawater based medium produced aberrant cells.
- It is possible to obtain new dinoflagellate cultures by incubating sediment from regions where planktonic populations have been identified.
- Single cells (including D before division and ND before death) are longer than wide, and cells after division are wider than long.
- Not surprisingly, maximum volume changes of D cells before division are comparable to growth rates of exponential cultures.
- Moribund cultures can contain very large and very small vegetative cells. It is possible that in other studies that used nutrient limitation, these were erroneously interpreted as planozygotes and gametes, respectively.

1.2 Mating and Encystment

- Nitrate limitation was used to induce the sexual cycle because it worked and it had ecological saliency, both in general, and when applied to the host population in Barkley Sound.
- Mating is heterothallic.
- Once cultures were nitrate-limited, crosses of #743 and #744 produced cysts in 10-12 d The presence of cysts is the best way to determine if cultures have become sexual.
- On average, #744 cells are longer and wider than #743 cells, but the ranges overlap.
- Some ND cells (potentially gametes) grow and some shrink before dying or fusing.

- Because of these variabilities in ND cell size, unequal sized fusing pairs are possible. Since one mating type would not be consistently larger, pairing is not anisogamous. Although unequal sized pairs occur, by definition, pairing is isogamous.
- Maximum encystment was 15% for nitrate-limited crosses at 16°C and 166 μmol quanta·m^{-2}·s^{-1}. Encystment was not affected or weakly affected by salinity, time of year, or container. Encystment occurred under nutrient-replete conditions, but at a much lower rate.
- Low encystment is speculated to serve the adaptive purpose of storing a qualitatively changed gene pool, and allowing remaining planktonic vegetative cells to make quantitative increases.

1.3 Gamete Characteristics

- Gametes may begin to appear ~6 d after cultures become nitrate-limited based on the appearance of cysts after 10-12 d.
- Identifying ND single cells as potential gametes allowed for the study of lone gametes that had previously not been possible.
- On average, ND cells had a similar shape index and the same size as D cells that had not yet divided. This suggests that in stationary cultures where vegetative growth is slowed, gametes cannot be identified by morphology alone.
- Lectins have revealed cell surface carbohydrate changes during differentiation for many groups (notably ciliates). No differences were observed between ND cells and D cells. Before it can be accepted that no differences really exist, further studies are needed using lower Con A concentrations, labeling of live ND and D cells, labeling circa pairing, and/or immunochemical labeling.

2.0 Suggestions for Future Research

- It would be interesting to videotape gametes and vegetative cells to analyze whether they have different swimming speeds or patterns. Gametes may adopt a different travel pattern that would optimize mate contact.
- Identify culture methods to maximize laboratory encystment rates.

- Develop a method to remove pigment fluorescence which would aid epifluorescence and confocal experiments.
- Labeling live cells may reveal a difference in membrane fluidity between gametes and vegetative cells.
- The use of other lectins and this protocol may reveal different binding patterns.
- The use of other species and this protocol may reveal different binding patterns.
- The application of this protocol to heterotrophic dinoflagellates would minimize autofluorescence problems encountered here.
- A method is needed to concentrate non-dividing singles so that bulk methods to withstand statistical tests could be performed.
- Using ferritin or gold conjugated Con A, the path of glycosylation from the Golgi apparatus to the cell surface could be traced. In *Alexandrium*, this is a puzzle since the cell bodies are bound by three membranes and thecal plates.
- If one or both mating types have Con A receptors, it is possible that one or both cells also have Con A-like surface lectins. Lectin isolation methods could be tried.

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APPENDIX 1

Taxonomic Translations (Hansen 1989, Moestrup and Hansen 1988, F.J.R. Taylor pers. comm., Wells *et al.* 1991). *A.* = *Alexandrium, G.* = *Gonyaulax, P.* = *Protogonyaulax*

Reference	Published Name	Translation
Anderson 1980	*G. tamarensis*	*A. tamarense*
Anderson & Lindquist 1985	*G. tamarensis*	*A. tamarense*
Anderson & Morel 1978	*G. tamarensis*	*A. tamarense*
Anderson & Stolzenbach 1985	*G. tamarensis*	*A. tamarense*
Anderson & Wall 1978	*G. excavata*	*A. tamarense*
	G. tamarensis	*A. tamarense*
Anderson *et al.* 1983	*G. tamarensis*	*A. tamarense*
Anderson *et al.* 1984	*G. tamarensis*	*A. tamarense*
Anderson *et al.* 1985	*Gyrodinium* in title *et al.* and *Gymnodinium* in discussion	*Gyrodinium*
Brand *et al.* 1981	*G. tamarensis*	*A. tamarense*
Cembella 1986	*P. catenella*	*A. catenella*
	P. tamarensis	*A. tamarense*
Dale 1977	*G. excavata*	*A. tamarense*
Destombe & Cembella 1990	*A. excavatum*	*A. tamarense*
Fritz *et al.* 1989	*G. tamarensis*	*A. tamarense*
Fukuyo 1985	*P. catenella*	*A. catenella*
	P. tamarensis	*A. tamarense*
Haigh unpubl.	*P. tamarensis*	*A. tamarense*
Karentz 1983	*G. tamarensis*	*A. tamarense*
Norris & Chew 1975	*G. catenella*	*A. catenella*
Pfiester & Anderson 1984	*G. tamarensis*	*A. tamarense*
Prakash 1967	*G. tamarensis*	*A. tamarense*
Santos & Carreto 1992	*A. excavatum*	*A. tamarense*
Sommer & Meyer 1937	*G. catenella*	*A. catenella*
Takeuchi 1988	*P. catenella*	*A. catenella*
Taylor 1984	*Protogonyaulax*	*Alexandrium*
Tomas 1974	*G. catenella*	*A. catenella*
Turpin *et al.* 1978	*G. tamarensis*	*A. tamarense*
Walker & Steidinger 1979	*G. monilata*	*A. monilata*
Watras *et al.* 1982	*G. tamarensis*	*A. tamarense*
White 1976	*G. excavata*	*A. tamarense*
Yentsch & Mague 1980	*G. excavata*	*A. tamarense*
Yoshimatsu 1981	*P. catenella*	*A. catenella*
Yoshimatsu 1984	*P. catenella*	*A. catenella*
Yoshimatsu 1985	*P. tamarensis*	*A. tamarense*

APPENDIX 2

HESNW - As Modified from ESAW (Harrison *et al.* 1980) for Use by the Northeast Pacific Culture Collection

	Enrichment Stocks	Stock Conc. (g/L)	Final Conc. (µmoles/L)
1)	$NaNO_3$	46.67	549.09
2)	Na_2glycerophosphate	6.67	21.79
3)	$Na_2SiO_3 \cdot 9H_2O$	30.00	105.60
4)	$Na_2EDTA \cdot 2H_2O$	3.64	9.81
	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	2.34	5.97
	$FeCl_3 \cdot 6H_2O$	0.16	5.92×10^{-1}
5)	$MnSO_4 \cdot 4H_2O$	0.54	2.42
	$ZnSO_4 \cdot 7H_2O$	0.073	2.54×10^{-1}
	$CoSO_4 \cdot 7H_2O$	0.016	5.69×10^{-2}
	$Na_2MoO_4 \cdot 2H_2O$	0.126	5.2×10^{-1}
	$Na_2EDTA \cdot 2H_2O$	1.89	5.05
6)	H_3BO_3	3.80	61.46
7)	Na_2SeO_3	0.00173	1.0×10^{-2}

For stocks #4 and #5, add $Na_2EDTA \cdot 2H_2O$ before trace metals.
For stock #4, heat solution to dissolve the iron and adjust pH to 6.

Vitamin Stock	Stock Conc. (g/L)	Final Conc. (µmoles/L)
Thiamine	0.1	2.97×10^{-1}
Vitamin B_{12}	0.002	1.47×10^{-3}
Biotin	0.001	4.09×10^{-3}

Vitamin stock should be stored frozen. Enrichment stocks can be refrigerated.

To Make 1 L of Media:
Filter natural seawater through 0.45 mm membrane filter with a glass fiber prefilter.
Pour 1 L. filtered seawater into flask. Add the following while mixing with a stir-bar:

a) 1 mL of stocks #1, 2, 4-7)
b) 1.45 mL 1N HCl
c) 1 mL of stock #3
d) 2 mL vitamin stock
e) 0.12 g sodium bicarbonate

Autoclave 30 min.
Let stand for two d to allow pH to equilibrate (should be 8.2).
Filter aseptically through a glass fiber filter to remove precipitate.

APPENDIX 3

Modifications to ESAW (Harrison *et al.* 1980).

Equimolar Changes:
- sodium glycerophosphate ⇒ sodium phosphate
- ferrous ammonium sulphate ⇒ ferric chloride

Additions (1 nM):
- selenite
- nickel
- molybdate

APPENDIX 4

Sexual Induction Media.
A fusion of media enrichments (Cembella 1986, Guillard and Ryther 1962, Harrison *et al.* 1980) and prepared according to the "clean" method of Anderson *et al.* (1984).

Soak Teflon™ bottles in 1% HCl and then rinse in distilled water.

With caps loose, autoclave for 20 min:
- 500 mL of pre-filtered (GF/A) natural seawater
- ~50 mL of each of the seven HESNW nutrient stocks in individual Teflon™ bottles
- 0.06 g sodium bicarbonate in a scintillation vial
- ~10 mL 1N HCl in a 50 mL Erlenmeyer flask

As soon as depressurized, plunge seawater and stocks into an ice bath.
After 24 h, add the following to the 500 mL of seawater:
- 0.05 mL of stock 1
- 0.5 mL of stocks 2,3,5,6, and 7
- 1.0 mL of stock 4
- sodium bicarbonate (see above)
- 1 mL of vitamins pre-filtered through a 0.22 μm GS filter
- 1N HCl to pH 8.2

Media and stock solutions should be stored at 16°C in the dark.