An investigation into the population genetics and ecology of *Gracilariopsis longissima* (Gracilariales, Rhodophyta) around the South West Peninsula of Britain

by

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The author at St Just in Roseland with her favourite seaweed

"They certainly *taste* like seaweed," agreed Rincewind, thinking they certainly taste like seaweed *would* taste if anyone was masochistic enough to eat seaweed!

Terry Pratchett, The Colour of Magic

ABSTRACT

An investigation into the population genetics and ecology of *Gracilariopsis longissima* (Gracilariales, Rhodophyta) around the South West Peninsula of Britain

TEAN JANE MITCHELL

For the conservation and management of natural resources, a detailed knowledge of the ecology and genetics of individual species is essential. Red seaweeds are an important component of marine ecosystems but few species have been the focus of research.

Gracilariopsis longissima is a poorly studied member of a family of agarophytes of commercial importance, although it is thought to occur commonly around the coast of Britain. This report provides new information about the ecology and population genetics of the species with the identification of 11 populations around the coasts of Devon and Cornwall, seven of which were in the Fal Estuary and Helford River complex. Site characteristics are described, in particular with regard to substrata which were found to be significantly different between sites. New ecological data about algal assemblages in which *Gs. longissima* occurs is reported for spring (four sites) and summer (five sites), with significant differences seen for all except two sites.

Anecdotal reports that *Gs. longissima* grows mainly on smaller substrata were investigated but were unsupported by the evidence, although a non-random distribution with respect to substrate size was found, which needs further investigation.

Microsatellites were newly developed for the species but were thought to be monomorphic and were not pursued. Cross-genera amplification with *Gracilaria gracilis* microsatellites did not provide sufficiently reliable data. A number of molecular methods were optimised and used to identify populations and investigate the genetics of three populations. These results are the first report of population genetics in the species. Intra-population genetic variation was seen to be high when estimated using RAPD primers and was accompanied by significant differentiation between the populations investigated.

Biofilms occur on almost all submerged aquatic surfaces, including living organisms. *Gs. longissima* is no exception: epiphytes and biofilms were investigated and found to be highly diverse and well-attached, with some thalli in some populations completely obscured by overgrowth. Cleaning methods were tested, with mechanical removal proving to be the most successful. Biofilms were also found to affect RAPD profiles, confirming that cleaning of wild collected specimens was essential for reliable RAPD data to be obtained.

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Chapter 1:
General Introduction

Seaweeds are the macroscopic algae found in marine habitats occurring throughout the world's oceans and seas (Dring 1982). Particularly in coastal zones, the seaweeds are a major component of the marine ecosystem, providing food, shelter and attachment substrate for a multiplicity of other marine organisms (Littler and Littler 1985).

There are many good reasons why the macroalgae should attract formidable research effort:

* their importance as primary producers in the sea
* their role as CO_2 sinks (Hanelt *et al* 2003)
* the many high-value commercial species worth more than US$3 billion annually in foodstuffs alone (Jensen 1993)
* their potential as a source of "nutraceuticals" (Douglas *et al* 2003)
* their potential as indicators of ecosystem health (McCormick and Cairns 1997)
* the extraordinary diversity of organisms found within the polyphyletic group "macroalgae".

Despite this the macroalgae have tended to receive less research attention than they deserve when considering their ubiquity and diversity (Andersen 1992), especially when compared with research effort in other groups of organisms (Littler and Littler 1985, Norton *et al* 1996). Van Oppen *et al* (1996) pleaded for the importance of algae in the marine ecosystem to be recognised and for more work

on biodiversity and, in particular, on genetic variation below species level. To increase knowledge about this under-represented polyphyletic group and to ensure its inclusion in international conservation policies, Norton *et al* (1996) put a strong case for urgent research into algal diversity, geographical distribution and phenotypic plasticity.

The majority of seaweeds are red algae with species numbers estimated between 2,500 and 6000 (Woelkerling 1990). The phylum Rhodophyta includes the red seaweeds. It is among these that we find some of the most important economic species. In particular, two genera of the Family Gracilariaceae, *Gracilaria* and *Gracilariopsis*, are important commercial sources of agar for the food and chemical industries around the world (Armisen 1995). This family of agarophytes (agar-producing seaweeds) accounts for over 50 per cent of current worldwide agar production and, therefore, at least half the annual US$132 million which this trade represents (McHugh 2003).

Gracilaria verrucosa was originally regarded as a commonly-occurring, spaghetti-like red alga with a world-wide distribution, cultivated in tropical seas as a commercial crop.

However, in 1995, as a result of studies of European populations of gracilarioid algae, *Gracilaria verrucosa* was reclassified by Steentoft *et al* as two species from different genera, *Gracilaria gracilis* and *Gracilariopsis longissima* (Steentoft *et al* 1995). A reassessment of existing knowledge of the species was needed and new

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NB: To avoid confusion in this report, abbreviations, where used, were *Gs.* sp. when referring to *Gracilariopsis*, as used by Iyer *et al* (2005a, b) and *G.* sp. for *Gracilaria* sp.

research, to establish greater understanding of how the two species differed, whether physically, ecologically or genetically. However, gross morphological similarities between the two species made field identification difficult and presented barriers to research which must first be overcome.

Gs. longissima is known to occur in some sites around Britain (Steentoft *et al* 1995, Steentoft and Farnham 1997, Newman 1998), and has been the subject of some ecotoxicological research (Newman 1998, Brown and Newman 2003). The species exhibits many of the characteristics which make red algae particularly interesting to researchers: a complex tri-phasic life cycle, systematic controversy, few existing records, morphological variability, inhabiting harsh environments, unknown population dynamics and probable importance in marine ecosystems.

A recent search of electronic databases (http://wok.mimas.ac.uk/, http://biblioline.nisc.com/, http://www.scopus.com/scopus/home.url) found, for the last five years, nearly 600 research papers on English oak, *Quercus robur*, an iconic species. In that same period, the favoured genetics research model, the fruit fly *Drosophila*, warranted over 25,000 papers. But, for the whole of the multi-million dollar commercial rhodophyte genus *Gracilaria*, in the same five years, a search found less than 300 publications. For *Gracilariopsis*, another commercially-harvested genus, this drops to just 50 papers. The study species in this report, *Gs. longissima* (S.G.Gmelin) Steentoft, L. Irvine & Farnham, warranted mention in only 13 research papers in the last 15 years. While these figures are not comprehensive they give an indication of the relative neglect of the macroalgae as a group and of *Gs. longissima* in particular.

Taxonomic difficulties over many years (Garbary and Gabrielson 1990, Maggs and Gabrielson 2003) may have contributed to this paucity of publications: of the 13 *Gs. longissima* papers, 10 are concerned with taxonomy and distribution (e.g., Steentoft *et al* 1995, Steentoft and Farnham 1997, Oliveira *et al* 2000, Iyer *et al* 2005a,b).

1.1. Background to the study

The globally important agarophyte, *Gracilaria verrucosa*, had been regularly recorded for British coasts since at least the 1880s (Dixon and Irvine 1977, ERCCIS Database, Norton 1985, Tregelles 1952). However, Bird and Rice (1990) recognised that the taxon *G. verrucosa* in northern Europe was, in fact, likely to encompass at least one species of *Gracilariopsis*. As described above, Steentoft *et al* (1995) were able to confirm that the taxon *G. verrucosa* was not valid for British terete gracilarioid species. It was proposed that two species *G. gracilis* and *Gs. longissima* occurred around the Southern British coasts in unispecific or mixed stands of individuals. Despite their apparent ubiquity (Hardy and Guiry 2003, Steentoft and Farnham 1997), the British populations remain largely unstudied.

However, one set of interesting observations about *Gs. longissima* have been made since that reassessment. To investigate the usefulness of *Gs. longissima* as a biomonitor of copper contamination in the Fal Estuary, Cornwall, UK, Newman (1998) measured the effects of copper on the growth and physiology of *Gs. longissima*, using individuals collected from established wild populations from sites with differing levels of copper concentration around South West Britain (Table 1.1).

Table 1.1: Copper concentrations in sediments of sites in South West Britain with established populations of *Gs. longissima*

Site	Location	Copper (µg g^{-1} dry weight sediment)
Mylor Creek, Fal Estuary	SW 820 354	1117
St Just Creek, Fal Estuary	SW 847 358	356
Flushing River, Fal Estuary	SW 804 343	322
Helford Estuary, Cornwall	SW 758 268	328
Chesil Fleet, Dorset	SY 625 800	32

The samples encompassed one population exposed to low concentrations of copper (Chesil Fleet) , three exposed to higher concentrations (Flushing, Helford, St Just) and one exposed to very high levels of copper contamination (Mylor). Newman also investigated the responses of individuals transplanted between sites of low and high copper concentrations to relate laboratory data to field studies. He measured responses to a variety of elevated copper concentrations (12.5- 500µg Cu l^{-1}) in a number of ways by assessing growth, photosynthesis and respiration.

Newman found copper affected the growth and physiology of all individuals from all sites. He also found that photosynthesis, measured as O_2 evolution and chlorophyll fluorescence, was decoupled from growth in individuals subjected to copper concentrations above 12.5µg Cu l^{-1} in the growth medium. Newman postulated that photosynthetic light reactions were being used not solely for growth or development but were being diverted to counter the effects of copper toxicity (Newman 1998, Brown and Newman 2003). His findings are summarised in Figure 1.1.

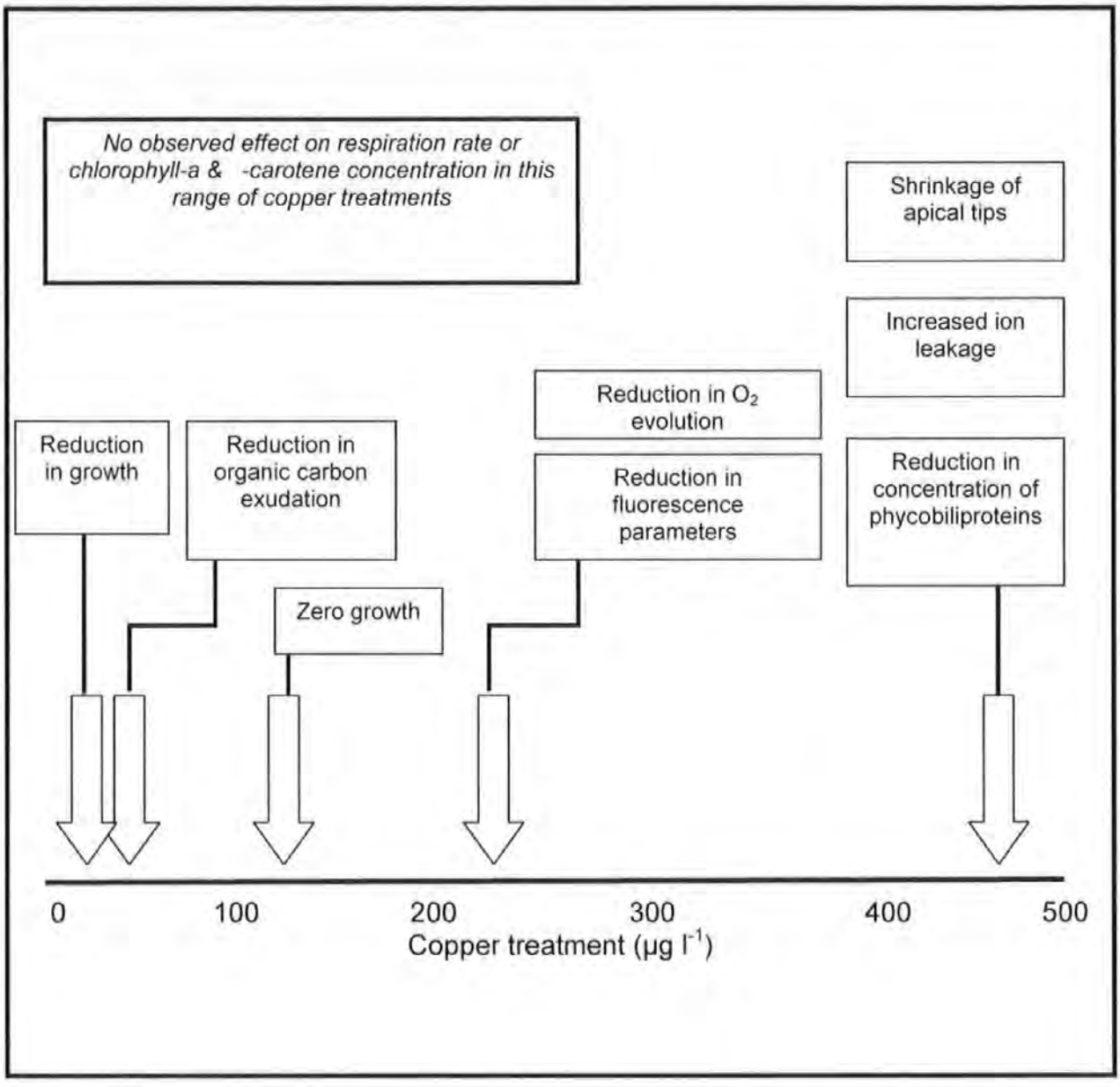


Figure 1.1: Summary of comparative effects of copper on growth and physiology of *Gs. longissima* following one week of exposure (Figure 9.1 from Newman 1998)

Newman (1998) carried out a set of comparative experiments to investigate differences between geographically separated populations. The experimental populations, as described above, originated from sites with differing levels of existing trace metal contamination. All the evidence from the laboratory experiments indicated no real differences between the populations studied. This conclusion was further supported by his reciprocal transplant field experiments, where there was no evidence of any difference in the response of populations from

sites differing in contamination. Newman therefore concluded that populations of *Gs. longissima* could not be differentiated on the basis of copper tolerance. His results, in fact, showed greater overall variation in responses between individuals from the same population than between individuals from different populations.

Newman also found a high degree of variation within particular individuals even though they were grown under the same standard laboratory conditions. He found that branches from some individuals grew and responded quite differently to copper exposure from other branches of the same individual, with some displaying a greater degree of tolerance to copper. He suggested that some of this inter and intra-individual variation could be due to genotypic and developmental differences.

Other authors have observed phenotypic variation within clonal populations of seaweeds (Santelices and Varela 1993, Santelices *et al* 1995, Meneses 1996, Meneses *et al* 1999, Meneses and Santelices 1999). In *Gracilaria chilensis*, Santelices and Varela (1993) found significant variation in terms of elongation in germlings derived from spores from the same cystocarp. The rates of branching between ramets derived from single individuals were also found to vary significantly. Further work on a group of vegetative clones of this species (Meneses and Santelices 1999) demonstrated dynamic genetic changes in individuals strongly affected by the external environment. They concluded that ramets resulting from thallus fragmentation of *G. chilensis* (a common method for commercial propagation) could not be assumed to be genetically stable and that mitotic recombination could be occurring continually, perhaps as a result of continuous reaction between environment and genotype.

Van der Meer and Todd (1977) and van der Meer (1981), investigating mixed phase individuals and polyploids in *Gracilaria* sp., reported that mitotic recombination was a regular occurrence. Meneses *et al* (1999) measured genetic variability in clones of *Gracilaria chilensis* with RAPD markers and observed changes with growth and biomass. They attributed the changes in genetic variability to mitotic recombination and other, unspecified, types of DNA-turnover mechanisms. Whether these processes occur in *Gs. longissima* is not known. As in *Gracilaria* species, *Gs. longissima* reproduces sexually, asexually and vegetatively (through fragmentation of thalli) but rates of recruitment from these processes are not known. The potential consequences of adopting a particular reproductive strategy (e.g. sexual versus vegetative) may influence how populations respond and adapt to pollution. In *Gracilaria* species the phase balance and therefore type of reproduction may depend on habitat (Kain and Destombe 1995).

If mitotic recombination is taking place in *Gs. longissima* during growth this may provide some explanation for Newman's findings. Alternatively, inherently greater levels of variation within populations, might allow some individuals to settle and survive in areas with differing metal exposure profiles from their site of origin. Populations may be characterised by high levels of gene flow which depend on life cycle traits such as dispersal (Destombe *et al* 1990) or reproductive strategies (Richerd *et al* 1993). Such a situation could account for the results obtained by Newman (1998) with most of his study populations being a mix of genetically tolerant and non-tolerant individuals.

Newman's finding that intra-population variation in copper tolerance was as great as that found between populations suggests that variation in physiological responses to contaminants cannot necessarily be predicted from exposure history. He was unable to identify tolerant or non-tolerant populations. These results are anomalous, as natural selection in response to contaminant exposure might be expected to produce distinct populations of tolerant and non-tolerant individuals as reported in some terrestrial plants (Mengoni *et al* 2000). With regard to the intra-population and intra-individual variation, Thompson (1991) proposed that phenotypic plasticity in seaweeds could be a heritable trait which could respond to natural selection.

Newman's work raises many questions about diversity, adaptation and gene flow in *Gs. longissima* populations. Newman (1998) originally chose *Gracilaria verrucosa* for his study because of its apparent ubiquity around the coast in South West Britain. The reassessment of the species led to him choosing to focus on *Gs. longissima* as there were established populations recorded for sites in areas of interest. However, the usefulness of any species for biomonitoring or other studies relies heavily on ease of identification.

1.2. Molecular markers in systematics, species identification and population genetics of algae

The development of molecular tools to investigate DNA for biological research has revolutionised studies of taxonomy, population genetics, biodiversity and evolution in a wide range of organisms from humans to bacteria, from fungi to flying fish. New techniques allow researchers to examine DNA directly or through allozymes,

to answer questions which previously could only be addressed using visible polymorphisms at macro and micro level.

Eukaryotic algal cells contain three potential sources of genomic DNA: the nuclei, the plastids (chloroplast) and the mitochondria (Coleman and Goff 1991). All three present opportunities for molecular level investigations. It is normally impossible to examine an entire genome for reasons of cost and time. Therefore, molecular markers which examine a region of DNA which can act as a "sample" of DNA, are commonly used.

Mutations within the region under examination can be used to investigate relationships at different levels within and between organisms by visualising base pair mutations as differential migration of DNA fragments through electrophoretic gels. This differential migration can be caused by fragment size or shape differences.

It was the advent of the polymerase chain reaction (PCR) method which amplifies many millions of copies of the DNA region under investigation, which first enabled visualisation of DNA through staining and electrophoresis. This apparently simple technique presents many challenges, as any factor or component of the protocol can lead to failure. Before applying these tools to biological questions, it is essential to assess the suitability of any particular marker. Also, molecular techniques usually need to be optimized for each species and marker combination.

Wattier and Maggs (2001) reviewed the potential of molecular tools for phycological research, in particular the potential for the development of algal markers to investigate questions of intraspecific variation and population genetics. Both PCR and sequencing of species-specific and other markers, have allowed access to detailed information about genomes to help answer questions about the ecological and population genetics of seaweeds.

Phycological research has embraced the new technologies and many researchers use these methods to study aspects of algal biology. Molecular tools are now used to research algal taxonomy (e.g., Byrne *et al* 2002), phylogenetics (e.g., Gurgel *et al* 2003, Iyer *et al* 2005b), alien introductions (e.g., Rueness 2005) and population genetics (e.g., Engel *et al* 2004). Molecular information can potentially support and confirm morphological and physical evidence of phylogenies, and provide insights into species and intra- and inter-population relationships. A wide variety of molecular methods and markers have been developed for different purposes including species identification, taxonomy and population genetics.

Over the last decade, phycologists have developed skills in molecular techniques leading to an increase in the development of species-specific markers, mainly microsatellites, for ecologically important macroalgae, including *Laminaria digitata* (Billot *et al* 1998), *Fucus serratus* (Coyer *et al* 2002, Engel *et al* 2003), *F. evanescens* (Coyer *et al* 2002), *F. vesiculosus* (Engel *et al* 2003), *Ascophyllum nodosum* (Olsen *et al* 2002, Engel *et al* 2003), *Cladophoropsis membranacea* (Van der Strate *et al* 2000), *Enteromorpha intestinalis* (Alstrom-Rapaport and Leskinen 2002) and *Postelsia palmaeformis* (Whitmer 2002).

The development of markers for loci with high degrees of polymorphism (Wattier *et al* 1997) has allowed fascinating new discoveries about breeding systems in algae (Engel *et al* 1999, 2002, 2004). Some of these markers have been used subsequently to investigate questions of phylogeography (Van der Strate *et al* 2002a), population structures and diversity (Billard *et al* 2005, Van der Strate *et al* 2002b, 2003), post-glacial recolonisation of Europe by *F. serratus* (Coyer *et al* 2003, Coyer *et al* 2006), and asexual reproduction in *F. vesiculosus* in the Baltic Sea (Tatarenkov *et al* 2005). In particular, such methods have helped researchers to investigate the highly complex taxonomy of the algae.

A wide variety of molecular methods and markers have been developed for different purposes including species identification, taxonomy and population genetics.

1.2.1. DNA sequencing

An entire genome can vary in length from a few thousand base pairs (some viruses) to billions (humans, fish and some amoebae). The time and resources needed to sequence an entire genome make it impractical for most cases. However, it is possible to isolate regions of DNA of varying length which can be sequenced to provide sufficient data to examine questions of relationships between species or populations.

Eukaryotes share many genes, reflecting the common biochemical functions essential to life. Humans share genes with not only closely-related species such as chimpanzees but even with the nematode worm *Caenorhabditis elegans* (Ashrafi *et al* 2003). This means that once the DNA sequence for a gene has been

found and sequenced in one organism, it is possible, with limited adjustment of priming sites, to use the PCR process to amplify the region sufficiently to be able to detect the same region in other organisms. Universal primers make it is possible to amplify and thus sequence at least some regions of DNA for most taxa with no prior knowledge of their DNA. Such "universal" primers for eukaryotic genomic regions have contributed greatly to the ability to investigate new species (Sogin 1990).

Sequence information already known in other organisms is available on databases such as the National Centre for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html).

A number of nuclear, plastid or mitochondrial genes have been used by phycological researchers. Zuccarello *et al* (1999a) developed primers for a mitochondrial marker from existing sequence data for three rhodophyte species published in GenBank. Primers were designed for the amplification of the intergenic spacer region between the genes for cytochrome oxidase subunit 2 (*cox2*) and subunit 3 (*cox3*) and the region was sequenced. The variability of the sequences was examined to assess potential use for intraspecific and population level studies in a number of distantly related red algae. Gene positions were found to be conserved in a number of rhodophyte orders including the Gracilariales, Bonnemaisoniales and Ceramiales. It was proposed that the marker could be used successfully for population studies in red algae. The sequences of the *cox2-3* spacer were subsequently used to help elucidate relationships among species of *Bostrychia* in North America (Zuccarello and West 2003).

Sequences of the large subunit of the gene that codes for Rubisco (ribulose bisphosphate carboxylase/oxygenase), the enzyme responsible for CO_2 fixation in plants, have been used to investigate the origins of plastids (Douglas *et al* 1990), and in population studies in *Gracilaria verrucosa* (Destombe and Douglas 1991) and *Polysiphonia harveyi* (McIvor *et al* 2001). This region of DNA (*rbc*L) was used by Maggs *et al* (1992) to help resolve the taxonomy of *Gymnogongrus* in the North Atlantic. Byrne *et al* (2002) used both the Rubisco spacer and *cox2-3* spacer to investigate species of *Gracilaria* from Australia, successfully identifying a new species of the genus.

The internal transcribed spacer (ITS) is a region of eukaryotic ribosomal DNA which includes a first spacer (ITS1), between the 18S gene and the 5.8S gene, plus a second intergenic region (ITS2) between the 5.8S and 28S genes. Sequencing this region has allowed researchers to propose new ideas about systematics and to confirm identifications of isomorphic species of Gracilariales (Goff *et al* 1994).

Sequence data can be applied to many questions as it is "complete" information about a given region of the genome under investigation. However, knowledge of mutation rates for any given region will indicate the usefulness of sequence data depending on the taxonomic level. For example, nuclear ribosomal DNA genes may not provide sufficient resolution for questions of population genetics (Medlin 2003). Sequencing gives high quality information but is expensive if comparing large numbers of samples and some genomes may not be straightforward to align due to secondary structure (Lowe *et al* 2004).

In particular, these techniques have helped to investigate the highly complex taxonomy of the algae. With much debate about species divisions, cryptic and sibling species, molecular data can help elucidate theories concerning diversity and variation. Universal primers for eukaryotic genomic regions such as the cox 2-3 spacer (Zuccarello and West 2003), *rbc*L (McIvor *et al* 2001) and internal transcribed spacers (Goff *et al* 1994) have allowed researchers to propose new ideas about systematics and phylogeny.

The use of sequenced regions of DNA in taxonomy and species identification is discussed further below (1.3 Taxonomy, 1.4. Species identification).

1.2.2. Restriction fragment length polymorphisms (RFLPs)

The discovery of restriction enzymes revolutionised molecular biology. These enzymes are able to cut doubled-stranded DNA at particular oligonucleotide sequences (restriction sites). Several hundred such enzymes cutting DNA at different restriction sites have been isolated for use (Avise 1994).

By cutting DNA (digesting it) with a number of difference restriction enzymes it is possible to break it up into fragments of differing molecular weight. The fragments can then be separated by electrophoresis where the fragments migrate differentially through a gel according to size and visualised following staining with a fluorescent dye (Sambrook *et al* 1989). With no knowledge of a genome, it is possible to use these enzymes to create a pattern of bands of different size depending on the number of restriction sites in the target DNA. Different species should produce different patterns if their DNA differs sufficiently to contain varying

numbers of restriction sites. These differences are called restriction fragment length polymorphisms (RFLPs).

RFLP markers are reliable and repeatable but do require large amounts of DNA. Restriction enzymes are expensive to purchase and it may be necessary to screen many before finding those with sufficient restriction sites to gain adequate data (Lowe *et al* 2004). They have been used with success in algae. For example, Rice and Bird (1990) proposed that restriction endonuclease profiles could prove useful in delineating different taxa and used plastid DNA restriction enzyme profiles to investigate relationships between populations of *G. verrucosa*. Goff and Coleman (1998) used restriction endonuclease patterns of plastid DNA from various species of seaweeds to distinguish species within a genus. This method is expensive for large numbers of samples normally required for population genetics studies. The need for large amounts of template DNA compared with some other methods, also makes it problematic where only small amounts are available.

1.2.3. Amplified fragment length polymorphism (AFLP)

A DNA fingerprinting method was proposed by Vos *et al* (1995) which allowed researchers to obtain information about unknown genomes. The stringent reaction conditions used were thought to confer reliability and repeatability. This is a modified RFLP method where the genomic DNA is first digested with one common site restriction enzyme (RE) and one rare site RE. The ends of the fragments then have adapters of specific sequence added to them to allow particular primers to be used for selective amplification using the PCR technique. The resulting fragments are either radiolabelled or stained with fluorescent dye for visualisation on denaturing polyacrylamide gels. Polymorphisms are then detected as differences

in size of the fragments separated by differential migration through the gel (Matthes *et al* 1998).

The use of amplified fragment length polymorphisms (AFLP) for population genetics research has met with mixed success (De Bruin *et al* 2004, Donaldson *et al* 2000). Although it does allow the investigation of unknown genomes, it is a relatively complicated method and requires large amounts of DNA, as well as the screening of potentially large numbers of expensive restriction enzymes.

1.2.4. Randomly Amplified Polymorphic DNA

In 1990, two groups of researchers, (Williams *et al* 1990, Welsh and McClelland 1990) described a new molecular polymorphism assay using single primers of arbitrary nucleotide sequence. Both sets of authors showed that arbitrary primers could be used successfully to create molecular fingerprints for unknown genomes.

In particular, Williams and his colleagues synthesised primers of 50-80% G+C content, of 9-10 nucleotides in length which contained no sequences which were complementary and could therefore form dimers preferentially during the PCR process. Where areas of the genome contained the complementary sequences on opposite strands at sites which were no more than 3000bp apart, it was possible for both areas to anneal to primers and amplification of the intervening sequence could take place. The resulting banding patterns did not reveal where on the genome the loci occur but were proposed as particularly useful for fingerprinting and population genetics.

Since then, RAPD markers have lost popularity to locus-specific markers for many researchers asking genetic questions, as RAPDs do present a number of disadvantages. As multi-locus, dominant markers, it is not possible to distinguish homozygotes from heterozygotes (Conner and Hartl 2004). RAPDs are therefore unsuitable for use with specimens of unknown ploidy, because haploids can be interpreted as diploid homozygotes which renders the haploid and diploid individuals as indistinguishable from each other.

Ellsworth *et al* (1993) warned of unwanted artefacts where methodologies were not carefully standardised. Davin-Regli *et al* (1995) showed that variations in DNA template concentration affected the reproducibility of RAPD fingerprints. Mizukami *et al* (1998) found that extraction method and purity of DNA extractions affected the reproducibility of RAPD fingerprints. Power (1996) warned of the need to use the same thermal cycler, reagents and visualisation techniques to be sure of valid results. Power (1996) also suggested that the very low annealing temperatures advised (37-40°C) could allow annealing in regions which were not fully complementary to the primers, increasing difficulties of reproducibility.

However, for species where there is little or no information about the genome, RAPDs can still provide a useful tool (Lowe *et al* 2004, Bouza *et al* 2006). They need very little genomic DNA and relatively straightforward techniques (PCR, agarose gel electrophoresis) to generate large amounts of data (Wattier and Maggs 2001). Compared with the resources needed to develop species-specific markers such as microsatellites, RAPDs are quick and cheap. RAPDs can be used to investigate the entire genome of an organism, thereby providing valuable

preliminary data for pilot studies or to explore initial hypotheses (O'Hanlon *et al* 2000).

1.2.5. Single-Stranded Conformational Polymorphism (SSCP)

After denaturation, and under appropriate conditions, single-stranded DNA can undergo folding to create different conformations depending on the sequence present. Even a single nucleotide difference between DNA samples of identical length can lead to this conformational polymorphism. These differences in shape affect the rate at which the DNA fragments migrate through an electrophoretic gel. This characteristic is termed single-stranded conformational polymorphism (SSCP) and can be used to investigate population genetics (Sunnucks *et al* 2000). However, conformational states are subject to many experimental conditions and sequence differences may or may not be detected (Beebee and Rowe 2004). Also, the electrophoretic conditions under which SSCPs can be detected must be determined empirically for each set of mutations under analysis (Jordan *et al* 1998).

Some phycologists have developed the use of SSCP in the Rubisco spacer region for algae (Zuccarello *et al* 1999a,b), and applied it to the study of phylogeography in *Bostrychia* (Zuccarello and West 2003), and population structure and genetics in *Caloglossa leprieurii* (Zuccarello *et al* 2000, 2001). These researchers found that SSCP of the Rubisco spacer provided a useful tool for the rapid screening of plastid variation in seaweeds but suggested that the method needed further development before it could be used for population genetics studies, although they subsequently used this marker to identify haplotypes of *Caloglossa leprieurii* in an Australian mangrove (Zuccarello *et al* 2000). This method was in its infancy at the

start of the study reported here and was therefore not considered for use, although subsequent work as noted above has shown that it can be used successfully for a number of applications.

1.2.6. Microsatellites

Microsatellites are areas of the genome of unknown functional significance, assumed to occur throughout genomes in all eukaryotes, characterised by a variable number of tandem repeats (VNTR) (Goldstein and Schlotterer 1999). A repeat sequence of 2 to 6 nucleotides can occur any number of times in a microsatellite, and it is that variation in the number of repeats, a size polymorphism, which is usually used to reveal underlying relationships in populations. It is the inherent instability of microsatellite loci that make them particularly useful for evolutionary and genetic studies (Goldstein and Schlotterer 1999).

Variously referred to as VNTRs, simple sequence repeats (SSRs), simple sequence repeat polymorphisms (SSRPs) and short tandem repeats (STRs), microsatellites have become the marker of choice for many researchers as they can reveal a tremendous amount of variation (Conner and Hartl 2004) including the detection of heterozygotes because of their codominant nature (Wattier *et al* 1998). They are excellent markers for the detection of genetic diversity, population differentiation, gene flow and, when there are exceptionally high levels of variation, individual genotyping (Lowe *et al* 2004). Wattier *et al* (1997) found they were able to characterise 93% of individuals in a population with a unique genotype using just two microsatellite loci.

Although costly and time-consuming to develop, microsatellites can offer a number of benefits, such as the small quantities of template DNA needed to amplify loci, once primers have been designed. Because the primers are site-specific, well-optimised PCR protocols and highly reproducible amplification can be developed. Microsatellites are popular markers for population genetics studies as they can be highly polymorphic, allowing deep insights into diversity and inter-population relationships.

Microsatellites have been reported for few species of macroalgae: *Enteromorpha intestinalis* (Alstrom-Rapaport and Leskinen 2002), *Fucus vesiculosus, F. serratus* and *Ascophyllum nodosum* (Engel *et al* 2003), *Gracilaria chilensis* (Guillemin *et al* 2005), *Fucus serratus* and *F. evanescens* (Coyer *et al* 2002), *Chlamydomonas* (Kang and Fawley 1997), *Gracilaria gracilis* (Lou *et al* 1999, Wattier *et al* 1997), *Laminaria digitata* (Billot *et al* 1998), *Ascophyllum nodosum* (Olsen *et al* 2002), *Cladophoropsis membranacea* (van der Strate *et al* 2000), and *Postelsia palmaeformis* (Whitmer 2002).

A group of researchers in France, studying *G. gracilis* populations on the coast of Brittany over many years, have used the species-specific microsatellite markers developed by Wattier *et al* (1997) and Lou *et al* (1999) to investigate questions of breeding success, haploid-diploid success and population dynamics in the species (Engel *et al* 1997, 1999, 2001, 2002, 2004). These studies have revealed the intricacies of mating patterns, the potential for mate choice and male competition, and measured gene flow, demonstrating high fertilisation rates between nearby individuals, adding important new information about red algal genetics.

The expense involved in developing microsatellites has led to attempts to amplify markers developed for one species in closely-related species. Cross-species amplification of microsatellite markers has shown some success in birds (Dallimer 1999, Field and Scribner 1997, Petren 1998, Primmer *et al* 1996), fish (Liu *et al* 1999; Smith *et al* 1998), wasps (Ezenwa *et al* 1998), mammals (e.g., Coote and Bruford 1996, Balloux *et al* 1998, Zeiss *et al* 1998) and plants (e.g., Kijas *et al* 1995; Lubbersted *et al* 1998). Field and Scribner (1997), Petren (1998) and Smith *et al* (1998) suggested that cross-species amplification could only be achieved with closely related species. Primmer *et al* (1996) concluded that results among related species would provide an excellent method for answering phylogenetic questions.

There are several examples of successful cross-species amplification of microsatellites in algae of the same genus (Billard *et al* 2005, Engel *et al* 2003). Limited success had been reported for cross-genera microsatellite markers when microsatellites found in *G. gracilis* were successfully amplified in some samples of *Gs. longissima* in France (Wattier *et al* 1997).

1.2.7. Allozymes

Proteins extracted from organisms and applied to a starch or polyacrylamide gel will migrate differentially when an electrical current is applied because of differences in net charges according to the amino acid sequence, conformation and size of the protein molecule. The resulting migrated bands of protein can be visualised using histochemical stains (Wendel and Weedon 1989).

Enzymes are proteins that can be investigated in this way. Although the terms have not been used with complete consistency, enzymes that share a biochemical function but are encoded by different gene loci are generally referred to as isozymes, while variants encoded by different alleles at the same locus are termed allozymes (Sosa and Lindstrom 1999; Lowe *et al* 2004). Different alleles arise as a consequence of mutations to the relevant coding regions of the genome, and the resulting differences in allozyme character have been used to investigate genetic differences between individuals or populations of particular species (Cheney 1985), genetic structure in plant populations (Hamrick 1989) and in plant systematics (Crawford 1989).

In 1998, allozymes were the most widely used marker system being applied to ecological genetic studies (Butlin and Tregrenza 1998). While they are cheap and simple to develop, they represent a very small proportion of the genome and may have low levels of polymorphism (Lowe *et al* 2004). The number of loci that can potentially be scored is limited by the availability of suitable staining techniques. Lowe *et al* (2004) also state that allozymes do not represent a random sample of a genome as they reflect only coding regions. This constraint may introduce bias to genetic inferences from allozyme data (Aagaard *et al* 1998). Cheney (1985) also noted that allozymes underestimate genetic differentiation among populations and that only soluble proteins can be detected, undermining their use as random markers.

Wendel and Weeden (1989) and Sosa and Lindstrom (1999) suggest that isozymes can be used to study large numbers of genes and alleles from different individuals and populations at one time. Cheney (1985) described the use of

electrophoresis of proteins for the study of population genetics in algae, highlighting the need for well-developed expertise in the technique and that developing assays for new species requires a lot of time and resources.

An important factor in successful protein electrophoresis is the development of suitable extraction buffers as each taxon poses specific problems with regard to endogenous compounds which can interfere with protein extraction (Wendel and Weeden 1989). For instance, the presence of polysaccharides, found in many macroalgae, can create problems for this technique (Cheney 1985, Sosa and Lindstrom 1999, Wendel and Weeden 1989). The ability of polysaccharides to bind to proteins may influence the number of bands and, therefore, number of loci visualised.

Sosa and Lindstrom (1999) reported at least 35 isozyme loci for macroalgal species. However, they also reported that macroalgal species maintain relatively low levels of detectable genetic variation using this method, compared with other plants. Aagaard *et al* (1998) compared allozyme and RAPD data for genetic variation in populations of Douglas fir (*Pseudotsuga menziesii*) and found them to be similar but the authors echoed concerns about lack of randomness for protein electrophoresis and constraints of selection which apply far less to RAPDs which sample largely from non-coding regions. Butlin and Tregenza (1998) stated that allozyme heterozygosity within species was not necessarily a good guide to other types of genetic variation. Newbury and Ford-Lloyd (1993) advocate the use of RAPDs, which avoid environmental influences on gene expression, rather than protein electrophoresis which they say is constrained by the number of resolvable protein species that can be visualised as clear bands and by the number of plant

enzymes that can be made to produce a suitable colour reaction for detection on a gel. However, allozymes can be a useful tool and should be considered when investigating population genetics in algae.

1.3. Molecular markers for population genetics studies in *Gracilariopsis longissima*

For *Gs. longissima* there were only 20 sequences registered with GenBank (Table 1.2), before the work reported in this study (Chapter 4). The sequences listed in Table 1.2 have been used mainly to investigate questions of taxonomy and species delineation. In particular, the sequences have been use to help resolve the complex taxonomy among members of the Gracilariales (Gurgel and Fredericq 2004, Iyer *et al* 2005b, Rueness 2005). These sequences, however, do not appear to provide sufficient variation for the investigation of relationships within species or between populations unless those populations are at large spatial scales.

1.3.1. Microsatellites

Microsatellites have become very popular in the study of population genetics because of their high levels of polymorphism, reliability, simplicity of use (once developed) and ability to amplify with very small amount of DNA. However, they are expensive and time-consuming to develop. While there is some evidence of cross-amplification between closely related species, most microsatellite markers must be developed independently for each species.

This study reports the development of microsatellite markers for *Gs. longissima* and the amplification success of microsatellite markers developed for a closely related species, *G. gracilis* (Lou *et al* 1999, Wattier *et al* 1997).

Table 1.2: DNA sequences isolated from *Gracilariopsis longissima* and published in GenBank (www.ncbi.nlm.nih.gov)

Sequence type	Length (bp)	Reference	Accession number
Chloroplast *rbc*L	714	Unpublished	AY049424
Chloroplast *rbc*L	1430	Unpublished	AY049404
Chloroplast *rbc*L	1440	Gurgel *et al* 2003	AY049410
Chloroplast *rbc*L	1427	Gurgel *et al* 2003	AY049420
Chloroplast *rbc*L	1410	Gurgel *et al* 2003	AY130244
Chloroplast *rbc*L	1410	Gurgel *et al* 2003	AF527881
Mitochondrial *cox2-3*	375	Rueness 2005	AY725149
Mitochondrial *cox2-3*	377	Rueness 2005	AY725148
18S ribosomal RNA	1807	Zuccarello *et al* 2004	AY617148
18S ribosomal RNA	1756	Iyer *et al* 2005	AY204161
18S ribosomal RNA	1756	Iyer *et al* 2005	AY204160
18S ribosomal RNA	1756	Iyer *et al* 2005	AY204158
18S ribosomal RNA	1756	Iyer *et al* 2005	AY204159
Chloroplast *rbc*L, *rbc*S & spacer	374	Iyer *et al* 2005	AY241174
Chloroplast *rbc*L, *rbc*S & spacer	374	Iyer *et al* 2005	AY241170
Chloroplast *rbc*L, *rbc*S & spacer	374	Iyer *et al* 2005	AY241146
Chloroplast *rbc*L, *rbc*S, & spacer	374	Iyer *et al* 2005	AY241142
Chloroplast *rbc*L, *rbc*S, &spacer	374	Iyer *et al* 2005	AY241141
Chloroplast *rbc*L, *rbc*S, & spacer	374	Iyer *et al* 2005	AY241139
Chloroplast *rbc*L, *rbc*S & spacer	374	Iyer *et al* 2005	AY241135

1.3.2. Randomly amplified polymorphic DNA

The inexpensive and simple to use randomly amplified polymorphic DNA markers developed by Williams *et al* (1990) and Welsh and McClelland (1990) have been popular with phycologists interested in revealing population genetics and variation in algae, in dinoflagellates such as *Prorocentrum micans* (Shankle *et al* 2004), diatoms such as *Skeletonema marinoi* (Godhe *et al* 2006), chlorophytes such as *Chlamydomonas eugametos* (Haring *et al* 1996), rhodophytes such as *Gracilaria lemaneiformis* (Wang *et al* 2007) and *Gelidium canariense* (Bouza *et al* 2006), and to large brown macrophytes such as *Postelsia palmaeformis* (Coyer *et al* 1997).

Gs. longissima is commonly found in a vegetative state and may be haploid or diploid depending on life stage. However, Bouza *et al* (2006) point out that, in organisms which show a haploid-diploid life cycle, complications caused by the dominant nature of RAPD markers can be avoided by selecting haploid individuals.

1.3.2.1. Biofilm effects on RAPD-PCR profiles

The use of non-specific primers makes randomly amplified polymorphic DNA (RAPD) analysis particularly vulnerable to contamination, as the process cannot discriminate between target and non-target DNA. PCR-based techniques are designed to amplify even tiny amounts of DNA. Bands produced by non-target DNA amplification cannot be distinguished from those produced by the target DNA, thereby confounding results or producing false positives (Newbury and Ford-Lloyd 1993). Forensic entomologists warn that DNA from the gut contents of fly larvae could lead to contamination of RAPD profiles (Benecke 1998).

When using RAPD primers for fingerprinting, Chiang *et al* (1993) found that fungal contamination of DNA extractions from a group of grass species (*Miscanthus*) produced bands subsequently identified as originating from the fungus. They warned that lack of attention to this potential problem could lead to an overestimation of genetic diversity or unsound conclusions. They suggest screening for the fungus by using a universal ITS spacer primer with known fungal profile to establish presence or absence of the fungus in specimens.

PCR with RAPD primers is thought to be a competitive process (Chan *et al* 1994, Williams *et al* 1993, Zwartes and Hnida 2000) which might suggest that very low concentrations of biofilm DNA would make its amplification much less likely. Chiang and colleagues (1993), however, do not specify the relative DNA concentrations of *Miscanthus* leaf to fungus which brought about the results they report.

However, the competitive nature of the markers can lead to preferential priming of a locus even when at a low concentration where other criteria, such as genome complexity, lead to differential affinity of primers for particular loci (Williams *et al* 1993). Thus any non-target DNA found in extractions used for RAPD markers can lead to false positive results or incorrect data.

1.4. Taxonomy

While the reassessment of the species by Steentoft *et al* (1995) has been widely accepted, there is much ongoing debate about the systematics of the Rhodophyta (Maggs and Gabrielsen 2003), the Gracilariaceae (Gurgel and Fredericq 2004,

Liao and Hommersand 2003) and species of *Gracilaria/Gracilariopsis* (Gurgel *et al* 2003a,b).

While it is likely that *Gs. longissima* shares many life history traits with *G. gracilis* (Kain and Destombe 1995), there is little research specifically on *Gs. longissima*, perhaps due to the difficulty of attributing research on *G. verrucosa* to either species. Furthermore, the taxonomy of the family to which the species belongs, the Gracilariaceae, is constantly under review. The current state of the taxonomy of *Gs. longissima* is discussed below.

1.4.1. Taxonomy of the Phylum Rhodophyta

Red algal systematics provides fertile ground for ongoing debate (Liao and Hommersand 2003, Maggs and Gabrielsen 2003). Even at Kingdom level there is an unresolved debate about whether the red algae should be placed in the plants or the protists (Cavalier-Smith 1998, Woelkerling 1990). A recent paper authored by a committee of the International Society of Protistologists has proposed that red algae be placed in one of six new proposed "super-groups" of eukaryotes called the Archaeplastida which includes the Plantae, green and red algae, and Glaucophyta (Adl *et al* 2005). The authors reject the current Linnean taxonomic hierarchy of formal rank definitions (Class, Order, Family etc) in favour of a system closer to the recently proposed PhyloCode system of taxonomy (http://www.ohiou.edu/phylocode/). However, this new system is not in common usage and, therefore, is not used in this report.

Two recent proposals for the Phylum Rhodophyta (Yoon *et al* 2006, Saunders and Hommersand 2004) place the red algae in the Kingdom Plantae but then place

them in different subkingdoms: Biliphyta and Rhodoplantae, respectively. Both proposals agree a common Phylum: Rhodophyta. Subsequently, the Class Florideophyceae, which includes the family Gracilariaceae, is proposed to be in a sub-phylum of either Rhodophytina (Yoon *et al* 2006) or Eurhodophytina (Saunders and Hommersand 2004). Both groups of researchers agree the Class Florideophyceae.

1.4.2. Taxonomy of the Order Gracilariales

Morphological polymorphisms within species and high levels of similarity between diverse species of gracilarioids makes them very difficult to identify and classify, hampering research and fuelling the ongoing debate about taxonomy and the need for unambiguous methods for identification and classification of this complex group of algae.

The florideophycean family Gracilariaceae is no exception to the taxonomic debate (Bird 1995, Woelkerling 1990). For many decades, the family was included in the Order Gigartinales (Dixon and Irvine 1977). However, Fredericq and Hommersand (1989a) proposed that the family Gracilariaceae should be placed in an order of its own, the Gracilariales, to reflect a unique combination of characters in what was then classified as the species *Gracilaria verrucosa* which was the type species of the genus *Gracilaria*. In particular, they included (1) the absence of auxiliary cells in the process of gonimoblast formation and (2) a lack of connecting filaments or cells. Later Woelkerling (1990) suggested that there was no overall consensus on which orders or families should be included in the Rhodophyta. He indicated problems associated with the used of short-lived events in reproductive development which are difficult to observe as characteristics for the classification

of orders and families in the Rhodophyta. Garbary and Gabrielson (1990) also supported the creation of this new order.

1.4.3. Taxonomy of the Genus *Gracilariopsis*

Further to their work on the taxonomy of the Gracilariaceae, Fredericq and Hommersand (1989b) re-examined the taxonomic status of the genus *Gracilariopsis*, which had been lost as a separate entity two decades earlier. Comparing various reproductive structures in *G. verrucosa* (Fredericq and Hommersand 1989a) with those found in *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto et Foldvik (at the time classified as *Gracilaria lemaneiformis*), they were able to show that there were distinct differences which were sufficient to justify the separation of the two genera. For example, they were able to differentiate the two genera on the basis of spermatangial development and a number of post-fertilization events : (1) differences in fusion of flanking cells into the carpogonium;(2) the presence of nutritive tissue in the inner pericarp; (3) secondary fusions between carposporophytic and gametophytic cells; (4) carposporangia organised in comparatively straight chains; and (5) early linking of inner, sterile gonimoblast cells by secondary pit connections. They therefore proposed the resurrection of *Gracilariopsis* as a genus distinct from *Gracilaria* with at least four discernible species: *Gracilariopsis lemaneiformis, Gs. chorda, Gs. costaricensis* and *Gs. tenuifrons*.

Fredericq and Hommersand (1989b) also found that many specimens from the UK, Ireland and France identified as *G. verrucosa* were, in fact, identical in habit, spermatangial configuration and post-fertilization development to the representative specimens of *Gracilariopsis* from Pacific North and South America.

They suggested that the similarities of external morphologies and common co-occurrence in Atlantic European taxa had led to the confusion of *Gracilariopsis* sp. with *G. verrucosa*.

The suggestion that the taxon *G. verrucosa* was likely to contain more than one species has been made on several occasions (Yamamoto 1984, Fredericq and Hommersand 1989b, Bird and Rice 1990, Rice and Bird 1990). The use of *G. verrucosa*, therefore, as the type species for its genus came under attack (Bird and Rice 1990, Rice and Bird 1990).

Rice and Bird (1990) applied new molecular tools to the question and compared restriction enzyme profiles for plastid DNA from eleven populations of *G. verrucosa* from around the world. They found that two "strains" from Southern England and one from Northern France had markedly different profiles and were subsequently proven to be *Gracilariopsis*, although the species had yet to be identified fully. Bird *et al* (1990) compared sequences of 18S ribosomal DNA and found a wide divergence between *Gracilaria* and *Gracilariopsis* species. Subsequent studies on the variability of the 18S area of ribosomal DNA by Bird *et al* (1992) also clearly separated *Gracilaria* from *Gracilariopsis*, further supporting resurrection of the genus.

Ongoing difficulties with species determination in *Gracilaria* and *Gracilariopsis* by morphological features led to a number of attempts to use developing molecular tools for the purposes of species confirmation. In 1991, Destombe and Douglas noted that the work of Rice and Bird (1990) on restriction enzyme profiles had revealed differences in patterns between apparently conspecific populations of *G.*

verrucosa. They proposed that DNA sequencing would provide a better tool for the analysis of phylogenetic relationships. To that end they compared sequences of DNA which code for Rubisco (ribulose bisphosphate carboxylase/oxygenase), the enzyme responsible for CO_2 fixation in plants. They investigated inter and intra population variability and found some distinction between distant populations. The sequence did appear to be consistent within species while variable enough to distinguish different members of the Gracilariaceae with similar phenotypes. However, they did not find enough variability to separate populations of the same species. This region of DNA was subsequently used by Maggs *et al* (1992) to help resolve the taxonomy of *Gymnogongrus* in the North Atlantic.

Using a similar approach, Goff *et al* (1994) investigated two DNA regions for the molecular delineation of species and species relationships. These were the plastid Rubisco spacer region used by Destombe and Douglas (1991) and Maggs *et al* (1992), and an area referred to commonly as the internal transcribed spacer (ITS) region. The ITS is a region of eukaryotic ribosomal DNA which includes a first spacer (ITS1), between the 18S gene and the 5.8S gene, plus a second intergenic region (ITS2) between the 5.8S and 28S genes. Sequencing of the region also allowed species determinations to be made. Goff *et al* (1994) found that there were high levels of non-alignment (non-matching) in the ITS region of the genomes of *Gracilaria* compared with *Gracilariopsis*. They also discovered that there was a consistent difference in size in the ITS region between *Gracilaria* (850-1050bp) and *Gracilariopsis* (1100-1450bp). They noted the potential for this size difference to help resolve genus for morphologically similar species

In reviewing the taxonomy of the Gracilariaceae, Bird (1995) pointed out that discrimination of species within the group was particularly difficult because of the levels of variation within species of morphological features which were shared with other species.

In their examination of the systematics of *Gracilariopsis* based on *rbc*L (Rubisco Large subunit gene) DNA sequences, Gurgel *et al* (2003a) confirmed that *Gs. longissima* was present in Western Europe and the Mediterranean. In further work, Gurgel *et al* (2003b) suggested that the high degree of morphological similarity among species of *Gracilariopsis* may have prevented the recognition that there are more *Gracilariopsis* species than previously suspected.

It would seem that on the basis of the ongoing debate over systematics in the Gracilariaceae, further revisions to the group are likely in the future (Liao and Hommersand 2003). It is clear from the work of Gurgel and Fredericq (2004) that there is more work needed to sort out the phylogeny of this complicated group. This report does not attempt to resolve taxonomy but it is important to recognise that the debate about red algal phylogeny is far from over.

Therefore, leaving aside some more complex debate yet to be resolved, the current taxonomy of *Gracilariopsis longissima* is assumed to be:

Phlyum	Rhodophyta
Class	Florideophyceae
Order	Gracilariales
Family	Gracilariaceae
Genus	*Gracilariopsis*
Species	*longissima.*

1.5. Identification of *Gracilariopsis longissima*

Gracilaria gracilis and *Gracilariopsis longissima* are both perennial, variously branched, deep burgundy red to pale yellow-brown rhodophytes with small discoid holdfasts and a thallus of fine, cylindrical (terete), tapering axes.

Methods for the unambiguous identification of species are needed before any organism can be used in research. As has been discussed already, the taxonomy of the Gracilariales has posed problems for phycologists for many years. Because of the economic importance of many members of this family, researchers have attempted to develop stable taxonomies (Bird 1995, Iyer *et al* 2005b). However, high degrees of morphological similarity between species (Bird and Rice 1990, Goff *et al* 1994), added to the intraspecific morphological variation seen in the group of gracilarioid algae sometimes referred to as the *Gracilaria verrucosa* complex (Bellorin *et al* 2004), make classification and identification extremely difficult (Iyer *et al* 2004, Rueness 2005). Accurate identification of species is also essential for advances in other areas of research into this large and valuable group (Fredericq and Hommersand 1990).

Fredericq and Hommersand (1989a) pointed out that the name *G. verrucosa* had been used indiscriminately for terete, irregularly branched specimens of *Gracilaria* while, in fact, the Atlantic European taxa *Gracilariopsis* and *G. verrucosa* tended to overlap in their distribution. These authors also noted, as a result of their discovery of the superficial nature and configuration of the spermatangia in *Gracilariopsis* (Fredericq and Hommersand 1989b), that a number of specimens from the UK, Ireland and France had been wrongly attributed to *Gracilaria* (as *G. verrucosa*) when they were, in fact, a species of *Gracilariopsis*. Bird *et al* (1982) had similarly

noted that the British *G. verrucosa* populations were under characterized and needed further examination. Bird and Rice (1990) also reported the likely misidentification of *Gracilariopsis* sp. as *G. verrucosa* in the British Isles.

Rull Lluch (2002) noted the difficulties with morphological identification of *Gs. longissima* from other gracilarioids in Namibia and proposed that, because of this, there was a need to reassess its distribution worldwide. However, Gurgel *et al* (2003b) were not convinced that the *Gs. longissima* reported for Namibia was the same species as that found by Steentoft *et al* (1995) in Europe.

Despite the efforts of these many taxonomists, there are still parts of the world where researchers continue to refer to the gracilarioid alga with which they work as *G. verrucosa* (Aleksandrova *et al* 2003, Iknur and Cirik 2004). New potential complications have also arisen with the recognition of yet another morphologically similar gracilarioid, *Gracilaria vermiculophylla* in Europe (Bellorin *et al* 2004, Rueness 2005). There also appears to be an ongoing debate about how to separate *Gs. longissima* and *Gs. lemaneiformis* (Liao and Hommersand 2003, Gurgel *et al* 2003a, b).

However, based on the evidence of the majority of the various taxonomic publications referred to in section 1.2 of this report, the populations of gracilarioid algae found around the South West of Britain are most likely to consist of only two species, *G. gracilis* and *Gs. longissima*.

The study species in this report, *Gs. longissima*, can co-occur in nature with its isomorphic family member, *Gracilaria gracilis* making it particularly important to

find a suitable method for quick and simple separation of the two species. Two approaches were possible for identification: traditional morphological examination or the use of more recently developed molecular tools to investigate regions of DNA.

1.5.1. Morphological methods of identification of *Gracilariopsis longissima*

Gs. longissima is, as described above, perennial, variously branched, deep burgundy red to pale yellow-brown rhodophyte, reportedly up to 40 cm (Fredericq and Hommersand 1989b) or 1 m (Steentoft *et al* 1995) in length with a small discoid holdfast (often <1 mm diameter) and a thallus of fine, cylindrical (terete), tapering axes which may vary from 0.5 mm to 3 mm in diameter (Fredericq and Hommersand 1989b, Steentoft *et al* 1995). The species is morphologically highly variable from short and bushy to long and thin, with thalli of mature plants made up of axes which may vary widely in number, branching patterns and length (Fredericq and Hommersand 1989a, b, Steentoft *et al* 1995). A representative specimen is shown in Figure 1.2 below.

Bird and McLachlan (1984) had noted that identification of *Gracilaria verrucosa* by gross morphology was problematic, particularly as they had found spermatangia to be variable within the taxon as it then stood. Hoyle (1984) had noted that spermatangia, although very helpful in distinguishing different species of *Gracilaria*, were uncommon, occurring in approximately 10 per cent of specimens of *Gracilaria* collected in California, thereby rendering this feature of little practical use for taxonomic purposes.

Fredericq and Hommersand (1990) provided a key to the Gracilariaceae based on female reproductive structures but this method is of limited use in field collections where mixed stands of species with varying numbers of reproductive and non-reproductive individuals are present.

When Steentoft *et al* (1995) proposed reclassification of the commonly-occurring, spaghetti-like red alga *Gracilaria verrucosa* to two new species from different genera, *Gs. longissima* and *G. gracilis*, they suggested a number of gross anatomical features for distinguishing the two species in the field (Table 1.3). However, the qualitative and comparative nature of the listed characteristics makes allocation of individuals to species highly subjective.

Table 1.3: Field characteristics in *G. gracilis* and *Gs. longissima* proposed by Steentoft *et al* (1995) to allow determination of species

Structure	***Description***	
Species	***G. gracilis***	***Gs. longissima***
Holdfast	<10mm "thick" "crustose" compound (5-6 fused spores)	2-3mm thin" "discoid" simple (or 2-4 spores not fused)*
Thallus	leathery very resilient	leathery not resilient†
Pattern of "long growth" axes	Maintains "short growth" stage with "long growth"	Flagelliform branches at regular, longer, intervals
Cystocarp	Pronounced basal constriction	Slight or no basal constriction
(early stage)	Mamillate Pore asymmetrical	Domed Pore symmetrical
(late stage)	Spread laterally around axis	Spread along axis

*characteristic only revealed by histological investigation †Resilience is described as the ability to resist being reshaped by coiling or folding (Steentoft *et al* 1995)

Differences in Lugol's iodine staining of the gonimoblast cells between the two species (brown in *G. gracilis*, yellow in *Gs. longissima*) proposed by Steentoft *et al* (1995) also have limited use as this method cannot be applied to non-reproductive individuals, tetrasporophytes or males.

Liao and Hommersand (2003) included *Gracilariopsis* (as the "lemaneiformis group") in their morphological study of the Gracilariaceae again using mainly cytological reproductive features which are difficult to assess in large numbers of individuals which may not be reproductive at the time of collection. Spermatangial characteristics between *Gracilaria* and *Gracilariopsis* may be diagnostic for genus but present a number of problems for practical application. Yamamoto (1984) described five types, each type named for the species that exemplifies their structure. In *Gracilaria*, "*verrucosa*" type spermatangia are seen as simple pits with spermatia lining the walls, open to the surface of the thallus when mature). However, the "*corda*" type expected in *Gs. longissima* are very difficult to see, even with microscopy, making this method an unlikely candidate for field identification. Fredericq and Hommersand (1989b) show transverse sections of thallus cortical cells with minute spermatangium cells of <5 µm diameter. There is also the possibility of confusion with "*symmetrica*" type spermatangia (Bird 1995). Additionally, Steentoft *et al* 1995 note that male *Gracilaria gracilis* plants are rare and that male *Gs. longissima* in Britain occur only "in winter" but they only list 3 male specimens in their collection of representative specimens of *Gs. longissima* compared with seven males among their representative specimens of *G. gracilis*.

In reporting *Gs. longissima* from South Africa for the first time, Iyer *et al* (2004, 2005b) note the difficulty of confusing *G. gracilis* with *Gs. longissima* on the basis

of morphological characters and concluded that the morphology and anatomy of the two species were too similar for them to be distinguished on that basis. Certainly, the anatomical and morphological features used by Steentoft *et al* (1995) were not observed clearly in specimens collected for this study and were of limited use given the non-reproductive condition of many specimens.
As discussed above, *G. gracilis* and *Gs. longissima* are extremely difficult to separate morphologically, except by microscopic reproductive features. This presents major difficulties for researchers, especially as the microscopic features used to separate genera (Liao and Hommersand 2003, Steentoft *et al* 1995) are only present in fertile material. It is common to find populations of gracilarioids which are entirely vegetative (personal observation), sterile or seldom fertile (Rueness 2005).

1.5.2. Molecular methods for identification of *Gracilariopsis longissima*

Ongoing difficulties with species determination in *Gracilaria* and *Gracilariopsis* by morphological features led to a number of attempts to use emerging molecular tools for the purposes of species confirmation.

Rice and Bird (1990) were convinced that *G. verrucosa* comprised more than one species. They compared restriction enzyme profiles for plastid DNA from eleven populations of *G. verrucosa* from around the world. They found that two "strains" from Southern England and one from Northern France had markedly different profiles and were subsequently proven to be *Gracilariopsis*, although the species had yet to be identified fully. Bird *et al* (1990) compared sequences of 18S ribosomal DNA and found a wide divergence between *Gracilaria* and *Gracilariopsis* species. Subsequent studies on the variability of the 18S area of

ribosomal DNA by Bird *et al* (1992) also clearly separated *Gracilaria* from *Gracilariopsis*, further supporting resurrection of the genus.

Sequencing or amplifying particular regions of DNA has provided an invaluable tool for definitive separation of the genus *Gracilariopsis* from *Gracilaria* and confirmation of *Gs. longissima* as a distinct species found in Britain (Bird *et al* 1992, 1994, Goff *et al* 1994) although there may be further work needed to determine whether Atlantic and Mediterranean specimens are distinct (Gurgel *et al* 2003a, Gurgel and Fredericq 2004).

As discussed above (Section 1.4.3.), Goff *et al* (1994) found a consistent difference in size in the ITS region of the genome between *Gracilaria* (850-1050bp) and *Gracilariopsis* (1100-1450bp). In Britain, where *G. gracilis* and *Gs. longissima* often occur together, this difference presents an ideal tool for distinguishing between the isomorphic species,

Species confirmation for all samples used in the research reported in this thesis was by amplification of the ITS region of the ribosomal DNA (Goff *et al* 1994) and examination of a selection of sample material by an expert taxonomist (M. Steentoft, personal communication).

1.6. Ecology of *Gracilariopsis longissima*

The ecology of many red seaweeds is poorly known. However, some of the Gracilariales have received particular attention due to their economic importance as a source of agar and other important food and chemical products (Kain 1995,

Mollet *et al* 1998). Sub-tidal marine macroalgae are difficult to access and study in their natural habitat.

The recognition that *G. verrucosa* in Britain comprised two species created the need to reassess populations of gracilarioids formerly thought to be *G. verrucosa*. Pre-1995 records for *Gracilaria verrucosa* proposed a general distribution around the South West Peninsula of Britain (ERCCIS Database, Tregelles 1952, Norton 1985). Since the recognition that "*Gracilaria verrucosa*" included two distinct species from different genera, *G. gracilis* and *Gs. longissima* (Steentoft *et al* 1995), very little pre-1995 research can be allocated to one or other species, although some European populations have been subsequently confirmed under the new taxonomy, such as populations of *G. gracilis* found at Cap Gris Nez, France formerly classified as *G. verrucosa* (Destombe *et al* 1992, Engel *et al* 2001). Subsequently, Newman (1998) indentified a number of sites where populations of *Gs. longissima* or *G. gracilis* could be found.

While a number of populations of *Gs. longissima* have been recorded around South West Britain (Newman 1998, Steentoft *et al* 1995, Steentoft and Farnham 1997), the list is unlikely to be comprehensive. However, it is likely that the species occurs commonly around the coast (Hardy and Guiry 2003), which means it could be an important component of algal assemblages in Britain. Newman (1998) recorded the species growing in harsh environments of unstable substrates within muddy estuaries. Populations grow unispecifically or co-occur with the isomorphic species *G. gracilis* (Steentoft *et al* 1995).

While some theories about the ecology and habitat of *Gs. longissima* had been proposed by Steentoft *et al* (1995) and Steentoft and Farnham (1997), in particular in relation to temperature tolerances, there is little other information about the ecology which could help inform the genetics and biogeography of the species in Britain.

1.6.1. Distribution

The Gracilariaceae, as a group, are usually described as predominantly warm temperate to tropical species (Iyer *et al* 2004) or are found in "warmer" waters (Steentoft *et al* 1995). As described by Steentoft *et al* (1995), the first records of *Gs. longissima* in the UK are from samples found in a variety of south coast of England sites, the Scilly Isles, Wales, Scotland and Ireland, and the Channel Isles. These authors also report samples from France and Spain. The 2003 Check-List and Atlas of the Seaweeds of Britain and Ireland (Hardy and Guiry 2003) shows only one site in Southern Ireland for *Gs. longissima* but observes that the species is probably widely distributed.

The world-wide distribution reported for *Gracilaria verrucosa* (www.algaebase.org) cannot be attributed reliably to either species except in a few cases of well-studied populations of mainly *G. gracilis* (Engel *et al* 2001). Rueness (2005) has recently confirmed *Gs. longissima* in Portugal.

In 1997, Steentoft and Farnham (1997) reported the northernmost distribution for *Gs. longissima* as Western Scotland while Lein *et al* (1999) updated their list from Western Norway to include *Gs. longissima*, pointing out however that the area

experiences "relatively warm" winter temperatures which may explain how this warm water species is present.

Steentoft and Farnham (1997) describe *Gs. longissima* and *G. gracilis* as colder water species compared with *G. bursa-pastoris* and *G. multipartita*. While *Gs. longissima* commonly co-occurs with *G. gracilis*, Steentoft and Farnham believe that it is more likely to attach to mobile substrates which may lead to passive transport. The ability of *G. gracilis* to survive and grow at 5°C may not be shared by *Gs. longissima*. During this study, *Gs. longissima* specimens survived and grew, slowly, in low light culture at 10°C (personal observation).

It was suspected that *Gs. longissima* would be found in the candidate Special Area of Conservation (cSAC) of the Fal and Helford estuaries in South West England, recently characterised as a European Marine Site (EMS) by Langston *et al* (2006). They note the need for benthic community data and for ecological surveys of abundance and diversity of species. For conservation and management of natural resources, a detailed knowledge of the distribution, ecology and genetics of individual species is essential (Wang *et al* 1997).

1.6.2. Habitat

Steentoft *et al* (1995) describe *Gs. longissima* as inhabiting relatively sheltered sites in the lower tidal and subtidal, down to 15m, attached mainly to small pebbles or shells. There is no other information published about the ecological niche occupied by *Gs. longissima*.

1.6.3. Algal assemblages

There has been a large amount of work concerning the theory of zonation of seaweeds on the rocky shores of the world (Dring 1982). Most studies on algal assemblages focus on community change or responses to such things as physical disturbance (Hily *et al* 1992), other physical factors, such as temperature and salinity, herbivory (Kaehler and Williams 1998), and competition with invading species (Arenas 2006). While there is an extensive list of species and proposed distributions of marine algae around Britain (Hardy and Guiry 2003), there is little information about the relative abundances of species occurring together in specific sites.

1.6.4. Attachment

Most species of macroalgae attach to substrates by means of holdfasts of varying design. The process of attachment is one of the most critical stages in the life history of many benthic algae (Dring 1982) and macroalgal attachment conditions are significant factors in macroalgal production patterns (Trancoso *et al* 2005, Thomsen *et al* 2007). The composition and texture of the substrata can therefore influence the type and diversity of seaweed communities.

Benthic organisms which attach to substrata are normally considered to be static but this is dependent on the stability of the substrate to which the organism attaches. The size and surface characteristics of the organism itself can affect friction forces which may increase the likelihood of the organism being passively transported through the water column.

Once attached, algal spores may be subject to a certain amount of abrasion and disturbance as they are very small objects which may be easily dislodged (Thomsen and Wernberg 2005) or buried. There is evidence that some macroalgae can survive long periods of light deprivation (Chapman and Fletcher 2002) as would occur with burial.

Gs. longissima is thought to attach preferentially to small stones and shells (Steentoft *et al* 1995, Steentoft and Farnham 1997). However, this view was based on anecdotal evidence. No published experimental evidence was available to support or undermine this hypothesis.

1.6.5. Epibiota

Biofilms form rapidly on most submerged aquatic surfaces (Armstrong *et al* 2001, Freeman and Lock 1995). Marine algal thalli are no exception (Anderson *et al* 1998, Holmes *et al* 1991, Murray *et al* 1986, Weinberger *et al* 2001). Competition for attachment space leads to macroalgae of many species using other species, or even their own, as a substrate for support (see Figure 3.12; Arrontes 1990, Bown *et al* 2003, Dawes *et al* 2000, Harder *et al* 2004, Kain and Norton 1990, Rindi and Guiry 2004, Steinberg and Nys 2002, Wahl and Mark 1999).

The presence of bacteria on marine surfaces may have potential "signalling" properties which actively encourage the settlement of other organisms, including macroalgae (Joint *et al* 2002, Steinberg and Nys 2002) on other macroalgae. Steinberg and Nys (2002) also propose that macroalgae themselves may be responsible for signalling which encourages biofilm establishment.

There is also a large body of research on the potential anti-fouling properties of macroalgal exudates (e.g., Amsler *et al* 2005, Bhadury and Wright 2004, Boyd *et al* 1999, Harder *et al* 2004, Kim *et al* 2004, Nys *et al* 1998, Steinberg *et al* 1997, Weinberger *et al* 2001). It has been suggested that the bacterial biofilm may act as an anti-macrophyte fouling layer which may be advantageous for the host in repelling other, more detrimental, epiphytism (Armstrong *et al* 2001).

1.7. Life history of *Gracilariopsis longissima*

Red algae have complex and flexible life histories (Maggs 1988). *Gs. longissima* (Figure 1.2) is no exception with a complex tri-phasic life cycle, usually described as "*Polysiphonia* type" (Kain and Destombe 1995). The three phases are a diploid (tetrasporophyte) phase, a haploid (dioecious gametophyte) phase and a second diploid, zygote-derived sporangium (the carposporophyte) phase. The gametophytes and tetrasporophyte are isomorphic, free-living plants. The

Figure 1.2: A vegetative specimen of *Gs. longissima* from St Just in Roseland, in the Fal Estuary. This specimen is deeply pigmented and has many branches arising from a single main axis. While this individual may be considered typical, the morphology of the species is highly variable and may be widely different from the specimen shown here. Species confirmation was by amplification of the internal transcribed spacer region of the genome.

carposporophyte remains attached to the female following fertilisation, held in the protective cystocarps which appear as small (approximately 1mm diameter) swellings irregularly spaced along the thallus surface (e.g., Figure 1.4). The carposporophyte is totally dependent on the female.

The three phases are linked through syngamy and meiosis as shown in Figure 1.3. Haploid male gametophytes release non-flagellated spermatia into the water column where they may encounter trichogynes protruding from the thallus surface of the haploid female gametophytes. *In situ* fertilisation gives rise to a zygote which develops into a diploid carposporophyte: a spore-producing structure, entirely dependent on the female gametophyte (Figure 1.4). The carposporophyte (Figure 1.5) produces thousands of clonal diploid spores by mitosis. These are

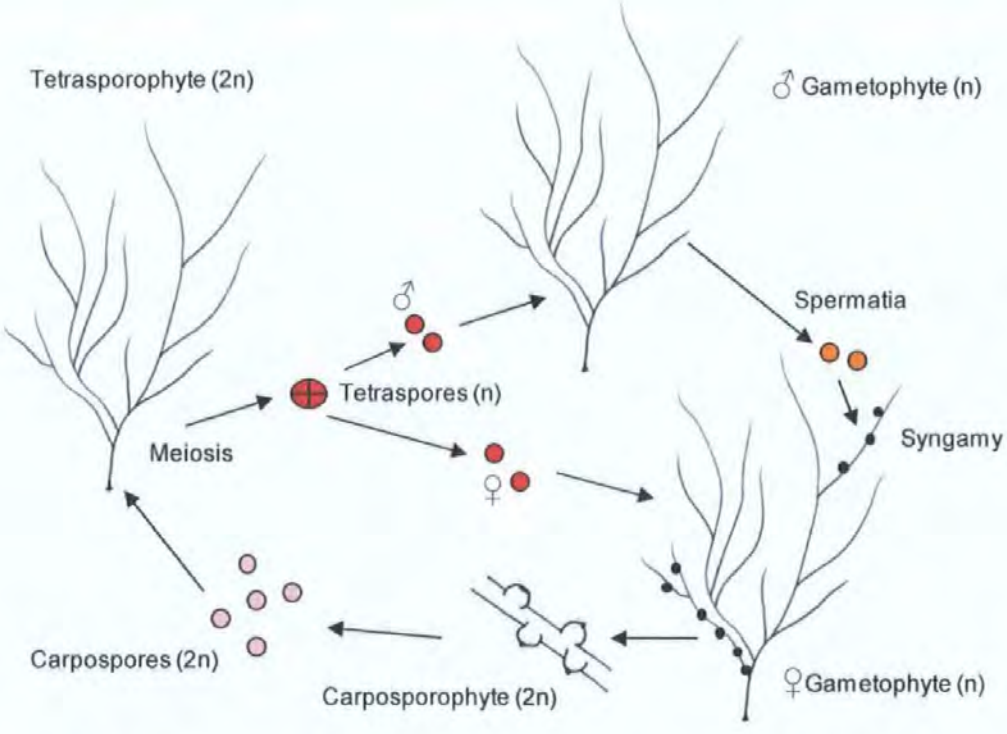


Figure 1.3: Life cycle of *Gs. longissima*. Tetraspores give rise to free-living isomorphic male and female gametophytes. Spermatia fertilise the female carpogonium which remains attached to the female to become a spore-producing carposporophyte which releases carpospores which give rise to the isomorphic, free-living tetrasporophyte.

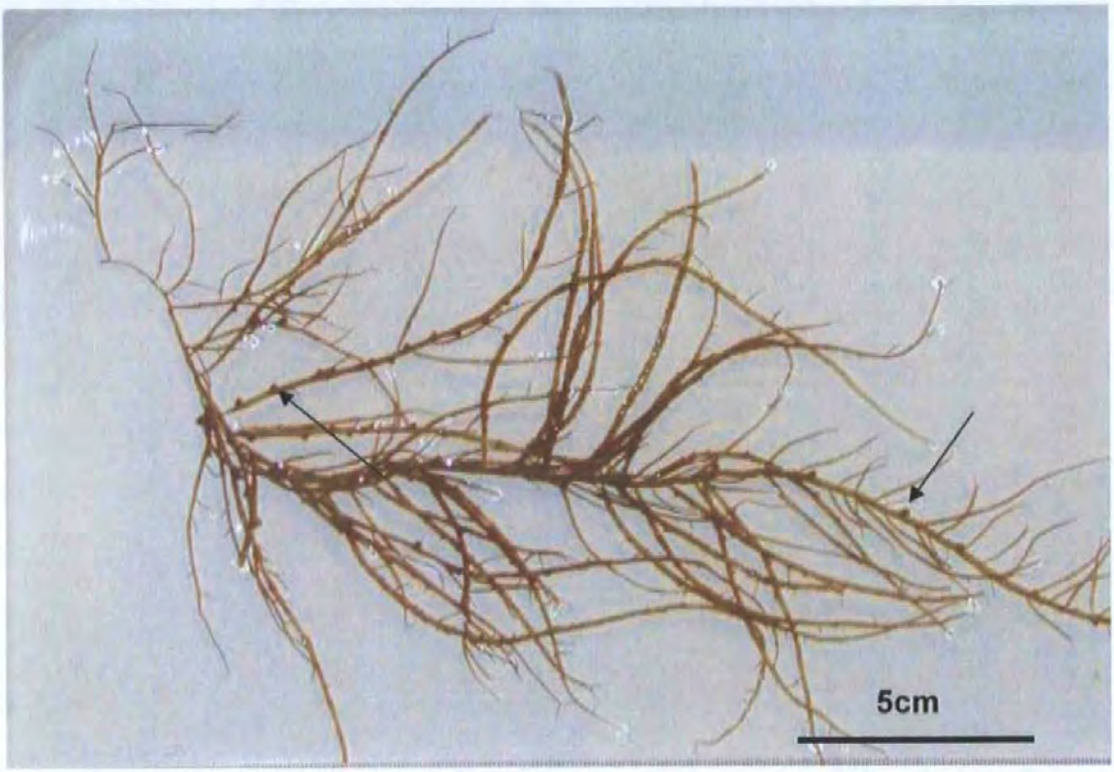


Figure 1.4: Female gametophyte of *Gs. longissima* from Braunton Burrows with clearly visible carposporophytes (examples arrowed) throughout the thallus. Species confirmation was by amplification of the ITS region.

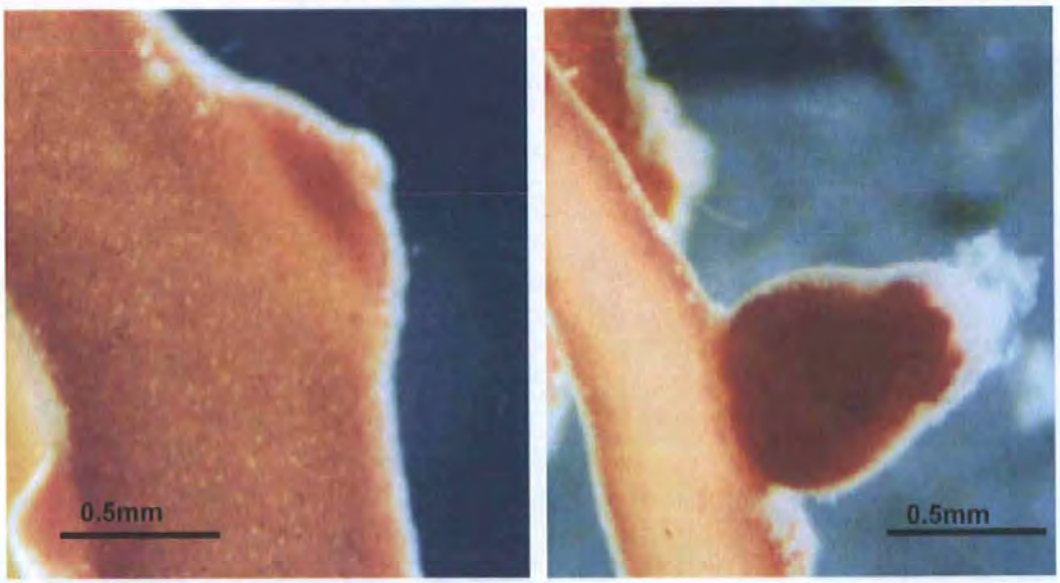


Figure 1.5: Diploid carposporophytes attached to haploid female *Gs. longissima* gametophyte from St Just in Roseland, Cornwall. LEFT– developing carposporophyte. The small white dots on the thallus surface may be empty tetrasporangia and the individual may be mixed-phase (see Section 1.7.4.). If so, this individual may be a chimera either resulting from mitotic recombination (a haploid plant developing diploid areas) or a diploid individual undergoing somatic meiosis creating areas of haploid tissue (C. Maggs, pers comm.). RIGHT– mature carposporophyte releasing spores in mucus. The rim of the cystocarp is everted as described by Steentoft *et al* (1995). Species confirmation for both these individuals was by amplification of the ITS region of the genome.

released into the water column in a mucous film. Successful settlement and germination give rise to diploid tetrasporophyte plants. The tetrasporophyte is able to reproduce by meiosis, producing numerous tetrasporangia (Figure 1.6), each of which contains four haploid spores which are released to establish new male and female gametophytes.

The isomorphy of mature plants and the non-reproductive state of varying proportions of different populations can make discrimination between life stages difficult and characterisation of populations extremely challenging.

1.7.1. Female gametophyte identification

When fertile, females can be discerned with the naked eye by the presence of subspherical, mamillate structures on the thallus, the cystocarps, usually <1 mm in diameter (Figures 1.4 & 1.5). The cystocarp is made up of the carposporophyte surrounded by supporting, protective female tissue.

1.7.2. Male gametophyte identification

The highly cryptic nature of the spermatangia in *Gs. longissima* makes identification of males extremely difficult. The spermatangia occur in a superficial layer and are composed of colourless cells, approximately 3 µm in diameter, each derived from a single spermatangial parent cell and do not form conceptacles (Fredericq and Hommersand 1989b, Steentoft *et al* 1995). They are therefore difficult to see without cytological examination (Nelson and Knight 1997). This form of spermatangia is found in a number of gracilarioid species and is described as "corda" type. The name relates to different types of spermatangia used to diagnose genera as described by Yamamoto (1984) and Bird (1995).

Bird *et al* (1982) noted the rarity of male plants in *Gracilaria verrucosa*. Steentoft *et al* (1995) noted that purely male thalli of *Gracilaria gracilis* were rare (only three individuals are listed in their 33 representative specimens). In *Gs. longissima* they do not comment on rarity but report that males of were seen "in winter", though there were also only three males listed in the collection of representative specimens used for differentiation between the two species.

1.7.3. Tetrasporophyte identification

Tetrasporangia, present as small, highly-pigmented red dots, can be seen under the dissecting microscope when the tetrasporophyte is fertile (Figure 1.6). Under high magnification, the cruciate arrangement of the four haploid spores is often visible. Each tetrasporangium contains two male and two female gametophyte spores. Once the spores have been released, it is possible to discern tetrasporophytes by the presence of empty tetrasporangia which appear as small white dots all over the surface of the thallus.

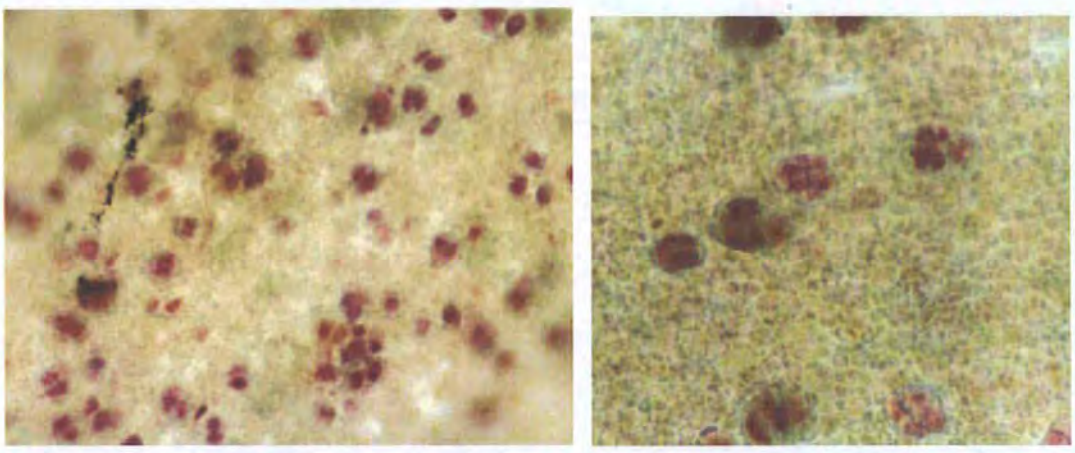


Figure 1.6: Tetrasporangia 20-30 µm in diameter near the surface of a mature tetrasporophyte of *Gs. longissima* from St Just in Roseland, Cornwall, photographed under high power (x 100). Species was confirmed by amplification of the internal transcribed spacer region of the genome.

1.7.4. Mixed-phase individuals and polyploids

Cases of life history variation within species are known in a number of members of the Rhodophyta (Maggs 1988). Failure of cytokinesis during the second meiotic division in tetrasporangia , mitotic recombination during normal mitosis and somatic meiosis are some of the phenomena which may be responsible for observations of mixed-phase, bisexual and unusual ploidy levels in species of red algae (Maggs 1988).

Mixed-phase individuals – where gametangia and tetrasporangia occur on the same individual - have been recognised in *Gracilaria* for some time (van der Meer and Todd 1977). The discovery of mitotic recombination in *Gracilaria* was reported by van der Meer and Todd (1977) who proposed this phenomenon as the underlying mechanism for the occurrence of mixed phase individuals, in particular male gametophytic reproductive structures on diploid tetrasporophytes. However, these authors found that all the gametophytic tissue derived in this way was diploid rather than haploid.

Recombination or crossover can occur in diploid somatic cells during mitosis leading to the production of homozygous allele combinations (van der Meer and Todd 1977). This potentially explains the occurrence of diploid gametophytic tissue in *Gracilaria* tetrasporophytes found by van der Meer and Todd (1977), van der Meer (1981), van der Meer and Patwary (1983) van der Meer *et al* (1984) and Zhang and van der Meer (1988). They found this phenomenon mainly as tetrasporangia occurring with male gametangia rather than female. Polypoid plants could therefore result from diploid spermatia fertilising haploid females.

Maggs (1998) reviewed life history variability in the Florideophycidae and reported a number of new observations. Possible mechanisms responsible for mixed phases and polyploidy included meiosis in tetrasporangia giving rise to haploid spores, somatic diploidization during spore germination or tetrasporangia which are mitotic but with meiosis possibly occurring during germination. Maggs (1988) also suggests that environmental conditions during sporeling development in *Atractophora hypnoides* might influence ploidy levels. Maggs highlights cytokinetic failure during meiosis as the most likely origin of polyploids.

"Bi-sexual" *Gracilaria* plants (van der Meer *et al* 1984) can take the form of female gametophytes bearing male spermatangial pits or vice versa. These were also thought to result from somatic mutation although spore coalescence, which is common in gracilarioids, may give rise to apparently bi-sexual plants where haploid male and female fronds are found in one plant. Incomplete cytokinesis during meiois in tetrasporophytes may also lead to spores containing more than one nucleus, giving rise to "sexual mosaics" (van der Meer 1977). Also, failure of cytokinesis in tetrasporangia after meiosis could lead to plants with male and female fronds, both arising from the same basal disc (van der Meer 1977).

Van der Meer (1981) showed that diploid male and female gametophytes could result from mitotic recombination which may explain the apparently diploid tissue on the female in Figure 1.5. Van der Meer and Todd (1977), van der Meer and Patwary (1983) and Zhang and van der Meer (1988) noted that diploid gametes formed by mitotic recombination were a potential source of polyploidy, a not uncommon occurrence in some groups of red algae (Cole 1990).

Somatic meiosis, where somatic cells undergo reduction division, is another possible source of mixed-phase individuals whereby a diploid individual can develop haploid tissue. Somatic meiosis was described by Huskins (1948) in the onion *Allium cepa* and proposed as a much more common occurrence in plants than had previously been thought. This process has been also noted in red algae (Thirb and Benson-Evans 1982, Hawkes 1990, Lobban and Harrison 1997, van den Hoek *et al* 1995, Maggs 1988) and may be responsible for haploid tissue found in some apparently diploid individuals. In Figure 1.5, the female plant appears to have white spots which could be interpreted as empty tetrasporangial pits on the thallus. If this was the case, it would not be possible to discern purely from visible characteristics whether this individual is a diploid plant with female tissue (either n or 2n) or a haploid plant (female) with diploid tissue which has become reproductive.

The level of any of these potential phenomena in *Gs. longissima* is unknown. Steentoft *et al* (1995) list 33 individuals of *Gracilaria gracilis* in their collection of representative specimens of which only two were purely male while seven were mixed phase individuals. Four of these had male tissue. In their collection of *Gs. longissima*, they list 36 individuals of which only 3 were male and none were mixed phase.

Mixed phase individuals with areas of variable ploidy or variable genetic make-up present additional challenges to researchers attempting to elucidate the population genetics of a species.

1.8. Population genetics

Research on economically important species of the Gracilariales has concentrated mainly on taxonomy and on the biochemistry and physiology of cultivated species, in particular the genus *Gracilaria* (e.g., Gurgel and Fredericq 2004, Rueness 2005). Perhaps due to the taxonomic uncertainty associated with the Gracilariales, the population genetics of most species has received little attention, although, as mentioned earlier, there are some exceptions. In particular, *Gracilaria gracilis* populations in northern France have attracted a large research effort (eg., Engel *et al* 2003).

Populations are important because evolutionary processes occur primarily within populations (Conner and Hartl 2004). Population genetics research can help identify biodiversity and throw light on gene flow between populations. With suitable genetic markers it is possible to investigate breeding systems (Engel *et al* 2004).

In many organisms it is possible to detect genetic differences through obvious morphological or other phenotypic variation, such as colour (e.g., Mendel's peas), physiology or size. However, in the highly morphologically variable seaweeds, such features are not usually diagnostic for genetic relatedness although van der Meer successfully used colour mutants in crossing experiments to determine inheritance of certain traits in *Gracilaria tikvahiae* (van der Meer 1979, 1984, 1986, 1988).

The physiological responses to copper contamination seen in *Gs. longissima* by Newman (1998) suggested that this species would be an interesting candidate for

population genetics research to see whether variation within and between populations might have any genetic basis. As discussed above, a number of markers for molecular genetics studies were investigated.

1.8.1. Molecular markers for population genetics studies in *Gracilariopsis longissima*

The vast majority of macroalgal species continue to have unknown genomes and, where resources are limited, non-specific markers provide the only possibility for genetic studies. Three molecular methods were developed for and applied to population studies in *Gs. longissima*.

* Species-specific microsatellites
* Cross-amplification of microsatellites found in a related species
* RAPDs

Microsatellite development was felt to be the best option but these markers are relatively rare in plants (Zane *et al* 2002) and algae (Wang *et al* 1994) compared with animals. Microsatellites are also laborious and expensive to develop. RAPDs on the other hand offer a quick and simple technique with good opportunities to generate large amounts of data. Existing microsatellites for *Gracilaria gracilis* also provided a ready-made and inexpensive opportunity to generate data of interest if the markers could be amplified in *Gs. longissima* as proposed by Wattier *et al* (1997). All three approaches were developed for use in population studies in *Gs. longissima* with varying degrees of success, as reported in later chapters of this report.

Optimisation of molecular techniques for individual species and circumstances is crucial to the generation of accurate and useable data. DNA extraction methods, PCR conditions and scoring of gels are all important parameters to be considered when developing protocols for molecular techniques. Therefore optimisation of methods was carried out for all aspects of the molecular techniques used in this study.

1.9. Research aims

Gs. longissima is worthy of research attention for a number of reasons.

(1) Newman's work highlighted unusual phenotypic variability in the species which warrants further investigation.

(2) The species is probably ubiquitous around southern Britain but has a largely unknown distribution.

(3) It is known to occur in a nationally important marine Special Area of Conservation (SAC): the Fal Estuary and Helford River.

(4) It occurs in interesting and harsh habitats such as muddy estuaries.

(5) It may be an important component of estuarine or coastal ecosystems.

(6) It exhibits a complex tri-phasic, haplo-diploid life history.

(7) It is one of a group of economically important species.

Despite this there is little information about the distribution, ecology and population dynamics. It is therefore timely to consider further research to reveal details of this recently recognised agarophyte of British waters.

Garbary and Gabrielson (1990) stated that taxonomic studies need to be combined with phylogenetic, biogeographic and ecological studies, to be able to

interpret evolutionary hypotheses. While taxonomy continues to be a focus of research (e.g.,Gurgel *et al* 2003a, b), the ecology of red seaweed species remains a neglected area (Kain and Norton 1990).

Research aims were:

(1) To identify populations of *Gs. longissima* in South West Britain

(2) To investigate the habitat and community ecology of the populations of *Gs. longissima* identified.

(3) To test the hypothesis that *Gs. longissima* attached preferentially to small stones and shells even where large substrates are present in greater proportions

(4) To identify and optimise molecular methods for population genetics studies of *Gs. longissima*

(5) To begin to use molecular genetics methods to investigate the genetic variability of *Gs. longissima* and genetic variation between populations.

The molecular investigations required a number of protocols and markers to be optimised.

1. Extraction of DNA from red algae for use in PCR

2. PCR amplification of the internal transcribed spacer region of the algal genome for species identification

3. Randomly amplified polymorphic DNA markers for population genetics research in *Gs. longissima*

4. Removal of epibiota to exclude non-target DNA from samples

4. Microsatellites in *Gs. longissima*

5. Cross-species amplification of *G. gracilis* microsatellite loci in *Gs. longissima* and other related Gracilariales

Chapter 2:

Ecology of *Gracilariopsis longissima* around the South West Peninsula of Britain

2.1. Introduction

Due to the taxonomic confusion surrounding this species, the distribution and ecology of *Gracilariopsis longissima* as a distinct entity is mainly unexplored in the UK. Little research has been reported on the community and population ecology of *Gracilariopsis longissima* in Britain since the new taxonomy in 1995. Steentoft and Farnham (1997) and Steentoft *et al* (1995) report that *Gs. longissima* can be found "occasionally in summer" in the intertidal but mostly submerged on mobile substrates, attached to small stones and shells down to 15m. Steentoft and Farnham (1997) note that its distribution is likely to be determined by temperature with a northern limit at around 58°N latitude.

There are few confirmed reports of the occurrence of *Gracilariopsis longissima* in Britain, although five sites in the South West were identified by Newman (1998) and samples have been identified from Portland Harbour and Chesil Fleet in Dorset, Sidmouth, Torbay, and the River Yealm in Devon, and Kingsand in Cornwall (Steentoft *et al* 1995). The extent of these populations is not known, although the species is thought to be widespread (Hardy and Guiry 2003). Certainly, early records describe *Gracilaria verrucosa* – which would have comprised both *Gracilariopsis longissima* and *Gracilaria gracilis* – from sites all around the peninsula (ERCCIS Database). Other pre-1995 records for *Gracilaria verrucosa* proposed a general distribution around the South West Peninsula of Britain (Tregelles 1952, Norton 1985).

The difficulty of positive identification in the field (Chapter 1) and the knowledge that *Gracilariopsis longissima* and *Gracilaria gracilis* commonly co-occur (Steentoft *et al* 1995) makes confirmation of wild populations problematic. Samples must be collected throughout a site and taken back to the laboratory for identification to be confirmed by the use of molecular methods as described in Chapter 1. This may explain the lack of ecological information about an apparently ubiquitous species which may play an important role in the intertidal and subtidal coastal ecosystem of southern Britain.

To make a definitive morphological or anatomical identification of *Gracilariopsis longissima*, individuals must be mature and in their reproductive state. However, as discussed in Chapter 1, males are highly cryptic due to the microscopic nature of the spermatangia. Also, females and tetrasporophytes are not reproductive at all times and may be encountered in their vegetative states when collections are being made from the field. Identification by molecular methods therefore provides useful tools as they do not rely on individuals being reproductive.

To determine which species had been found at sites where gracilarioids occurred, it was necessary to carry out molecular identification as described below by PCR amplification of the internal transcribed spacer region of the genome to determine the genus of each specimen using the size differences for this region between the two genera (Goff *et al* 1994).

This study aims to establish some basic information about the ecology of *Gracilariopsis longissima* with regard to its distribution and habitat in South West Britain. The study aims were:-

Part I: Identification and distribution

(1) To identify populations of *Gracilariopsis longissima* using molecular identification methods

(2) To establish the distribution of *Gracilariopsis longissima* around the South West Peninsula of Britain.

Part II: Ecology

(3) To investigate the nature of the physical habitat occupied by the species

(4) To determine the algal assemblages in which populations of *Gracilariopsis longissima* occur.

2.2. Part I: Identification and distribution of populations of *Gracilariopsis longissima* in South West Britain

2.2.1. Materials and Methods

2.2.1.1. Preliminary site surveys

Twenty sites around the South West Peninsula of Britain, covering South and North Devon and Cornwall (Table 2.1, Figure 2.1), were chosen for survey on the basis of one of three criteria.

(1) Reported to have existing populations of gracilarioid algae present in recent years (Newman 1998) – 5 sites.

(2) Previous reports of the presence of *G. verrucosa* (ERCCIS Database) – 15 sites

(3) Habitat characteristics similar to those of known existing sites where the species occurs – e.g., low energy estuaries with sediment rather than rocky substrates

Table 2.1: Twenty coastal and estuary sites around the South West Peninsula of Britain surveyed for the presence of populations of gracilarioid rhodophytes

	Site name	Map Ref	Description
1	Braunton Burrows, Bideford	SS 460 320	Open sea, estuary mouth, rock/shale substrate
2	Instow Sands, Bideford	SS 470 311	Open, tidal river confluence, estuary
3	Bantham, Bigbury Bay	SX 662 437	Sheltered, sandy estuary
4	Newton Ferrers, River Yealm	SX 551 478	Sheltered, silty tidal river
5	Millandreath Beach, Looe	SX 268 539	Open sea, rocky with sand
6	Looe River	SX 255 530	Sheltered tidal river mouth shale/mussel beds
7	Hannafore Point, Looe	SX 257 522	Open rocky shore
8	Polridmouth Beach, Fowey	SX 104 504	Sheltered, rocky/sandy bottom
9	Spit Point, Par	SX 076 524	Exposed rocky shore
10	Pentewan Beach, Mevagissey Bay	SX 468 021	Sandy beach, rocky outcrops
11	Towan Beach, Roseland Peninsula	SW 870 328	Open rocky shore, rock pools
12	Cellars Beach, Place, Fal Estuary	SW 854 323	Sheltered, muddy tidal inlet, land drain outfall
13	Froe, Porth Creek, Percuil River, Fal Estuary	SW 867 333	Sheltered, very silted, tidal stream with outfall from historic dammed fishpond
14	St Just Creek , Fal Estuary	SW 847 358	Sheltered, mud/shale tidal inlet with stream
15	Turnaware Point, River Fal	SW 835 383	Stony bank, tidal river
16	Restronguet Creek, Fal Estuary	SW 815 375	Sand/mud tidal river mouth
17	Mylor Creek, Fal Estuary	SW 820 354	Creek mouth, boat quay, muddy
18	Flushing, Penryn River, Fal Estuary	SW 807 338	Sheltered, mud/shale tidal river mouth
19	Helford Passage, The Bar, Helford River	SW 758 267	Sheltered, mud/sand/shale tidal river
20	Rock/Padstow, Camel Estuary	SW 923 763	Sandy, sheltered, tidal river

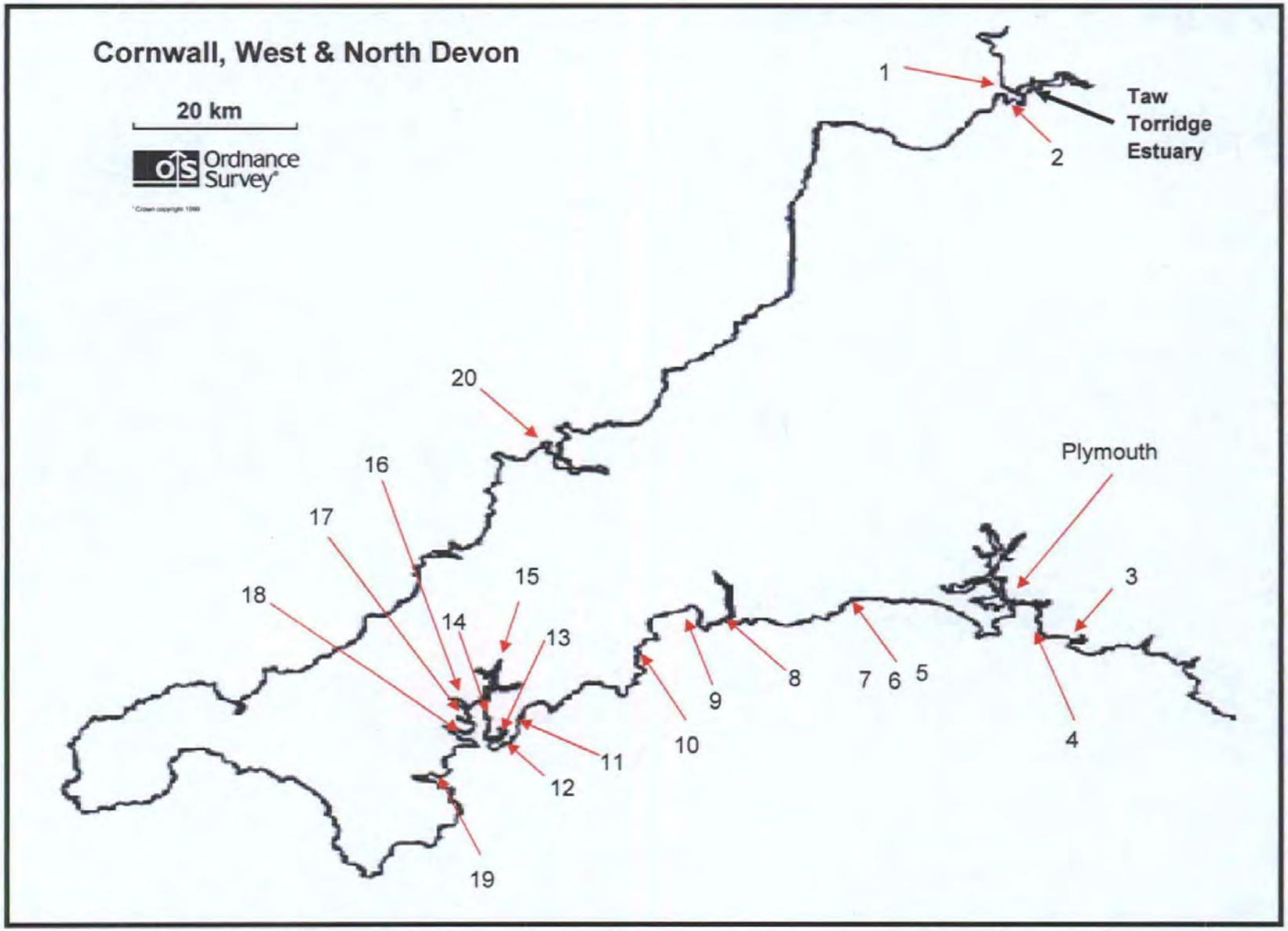


Figure 2.1: Map of Cornwall, West and North Devon to show approximate positions of sites surveyed for the presence of gracilarioid algae (arrowed) around the south west peninsula of Britain from just east of the City of Plymouth round the coast to the Taw Torridge estuary in north Devon. Numbers relate to numbering of sites in Table 2.1.

The sites were spread around the coast from Looe in the south east, to Bideford in the north east, including estuarine and open sites, rocky shores, sandy bays, silty inlets, and sand and shale beaches. Though mainly sub-tidal (Steentoft *et al* 1995), populations of *Gracilariopsis longissima* and *Gracilaria gracilis* can be accessed at their upper limits on the shore in shallow water left at low tide.

Each site was surveyed for the presence of gracilarioid species, which could be either *Gracilariopsis longissima* or *Gracilaria gracilis,* during extreme low water spring (ELWS) tides. Presence or absence was recorded and the extent of intertidal populations of gracilarioids throughout each site was noted within limits determined by safe access and physical barriers such as deep water or mud.

Specimens were also collected randomly throughout accessible areas for species identification to determine which sites hosted populations of *Gracilariopsis longissima*. The number of specimens collected for this investigation varied from less than 10 to over 30 individuals. The number collected was determined to some degree by abundance on site, i.e., more were collected from sites where gracilarioids were common or widespread. Specimens were transported back to the laboratory in seawater. Once cleaned of obvious epiphytes, specimens were air dried, labelled and stored in silica gel before identification by molecular methods, as described below (Section 2.2.1.2).

2.2.1.2. Molecular identification of populations of *Gracilariopsis longissima*

The identification of specimens of gracilarioids collected was carried out by amplification of the internal transcribed spacer (ITS) region of the genome as discussed in Chapter 1. The method used in this study is given here.

2.2.1.2.1.DNA extraction

Extraction buffer was freshly made up as described in Goff and Moon (1993): 5% (w/v) Chelex 100 Resin (Bio-Rad, Hemel Hempstead, UK), in a sterile solution of 90 mM Tris HCl (pH 8) and 50 µM ethylenediaminetetraacetate (EDTA). The Chelex beads were kept in suspension by stirring while pipetting. The extraction protocol of Wattier *et al* (1997) was adapted.

Three small tips of axes from each gracilarioid specimen were placed in labelled, sterile 1.5 ml Eppendorf tubes and kept on ice. Chelex suspension (350 µl) was added. Sterile micropestles were used to bruise but not crush the tips to reduce polysaccharide release (C. Destombe, personal communication). Samples were then boiled in a water bath for 10 min. After cooling to room temperature, samples were centrifuged for 2 min at 13,000 rpm in a bench centrifuge at 4°C. Being careful not to disturb debris at the bottom of the tube, 250 µl of supernatant was pipetted into a fresh tube, using a 1000-µl tip with the end cut to create a large opening to reduce shearing of DNA.

2.2.1.2.2. PCR of ITS region

The ITS region was amplified using primers designed by Goff *et al* (1994). These were TW81 (5'GGG ATC CTT TCC GTA GGT GAA CCT GC 3') which anneals to the 3' end of the small-subunit of the spacer, and AB28 (5'GGG ATC CAT ATG CTT AAG TTC AGC GGG T 3') which anneals to the 5' end of the large-subunit of the spacer (Goff *et al* 1994). A 3 µl aliquot of DNA extraction was added to 22 µl of reaction mix (Table 2.2) in a 0.5 ml thin-walled PCR reaction tube and topped with a drop of sterile mineral oil to prevent evaporation.

Table 2.2: Reaction mixture for PCR amplification of the ITS region in samples of gracilarioids from sites around the south west of Britain

Reagent	*Concentration*
Proprietary buffer *	x 1
dNTP mixture (MBI, Fermentas, York, UK)	200µM
Primer (MWG Biotech, Milton Keynes, UK)	400nM
$MgCl_2$ *	1.5mM
Taq polymerase*	0.625 units
Template DNA	3µl

*Roche Diagnostics Ltd, East Sussex, UK

Amplification through PCR was carried out in a Perkin Elmer 9700 Thermal Cycler. Following a 3 min initial denaturation step at 97°C, a three-step cycle of 1 min at 95°C (denaturing step), 30 s at 60°C (primer annealing temperature) and 2 min at 72°C (primer extension step) was repeated 37 times, followed by a final elongation step of 10 min at 72°C. Two positive controls (one for each species) and one negative control, to ensure integrity of results, were included in each set of reactions.

For visualisation of the resulting PCR product, 2 µl were added to 5 µl of 6x loading buffer (MBI Fermentas, York, UK) and loaded onto a 1% agarose gel stained with ethidium bromide, and then visualised with ultra-violet light. A 100bp ladder was included on every gel for sizing of fragments obtained. Specimens were then allocated to species according to the fragment size seen for each sample. Those amplifying a fragment of 1107bp were allocated to *Gracilaria gracilis*, those with fragments of 906bp were allocated to *Gs. longissima*. In total, 228 specimens of gracilarioid algae were collected and tested.

2.2.1.3. Study site determination

Sites which had (1) *Gs. longissima* (2) *G. gracilis*, and (3) both species present were identified.

2.2.2. Results

2.2.2.1. Preliminary site surveys

Of the twenty sites visited (Table 2.1, Figure 2.1), 13 were found to support populations of gracilarioid rhodophytes (Figures 2.2 to 2.8). The sites ranged from very open sites such as Braunton Burrows in North Devon (Figure 2.2), to very sheltered sites such as Froe on a small creek in the Fal Estuary on the south coast of Cornwall (Figure 2.7). A reported population of what was previously identified as *G. verrucosa* at Towan Beach (C. Destombe, personal communication) was not located during this survey. No gracilarioids were found at seven of the sites surveyed (Tables 2.3 and 2.4).

Areas surveyed at each site hosting gracilarioid populations (Figures 2.2 to 2.8) were determined by physical features. Upper limits were set by such factors as a fish pond dam at Froe (Figure 2.7), a land retaining wall at Place (Figure 2.6) and increasing mud depth at St Just (Figure 2.8). Full area surveys were not possible at Instow Sands and Braunton Burrows where sites were very large. The former was up to 1 km^2 in size and the latter may extend to over 1 km^2 (Figure 2.2). Survey areas at these sites were determined by walking to where was judged, subjectively, to be the nearest low water mark from the site access point, in an area previously identified as supporting gracilarioid algae, and then working back up the shore from that point searching for and recording the presence of gracilarioids.

2.2.2.2. Distribution of gracilarioids within sites

Where populations of gracilarioids were found they were distributed throughout sites where standing or flowing water remained at low water, as far as could be investigated by foot to the upper or lower reaches of each site.

This means that survey areas varied widely in size from the smallest at Froe (approximately 200 m x 50 m) (Figure 2.7) to the largest at Camel (1100 m x 200 m) (Figure 2.3), Braunton and Instow (Figure 2.2). The extent of survey areas was also determined by emersion times, with the least time available at Flushing and Helford (approximately 0.5 -2 hours) and the longest available time at the Camel, Instow and Braunton (up to 4 hours). All sites were subject to very fast reflooding.

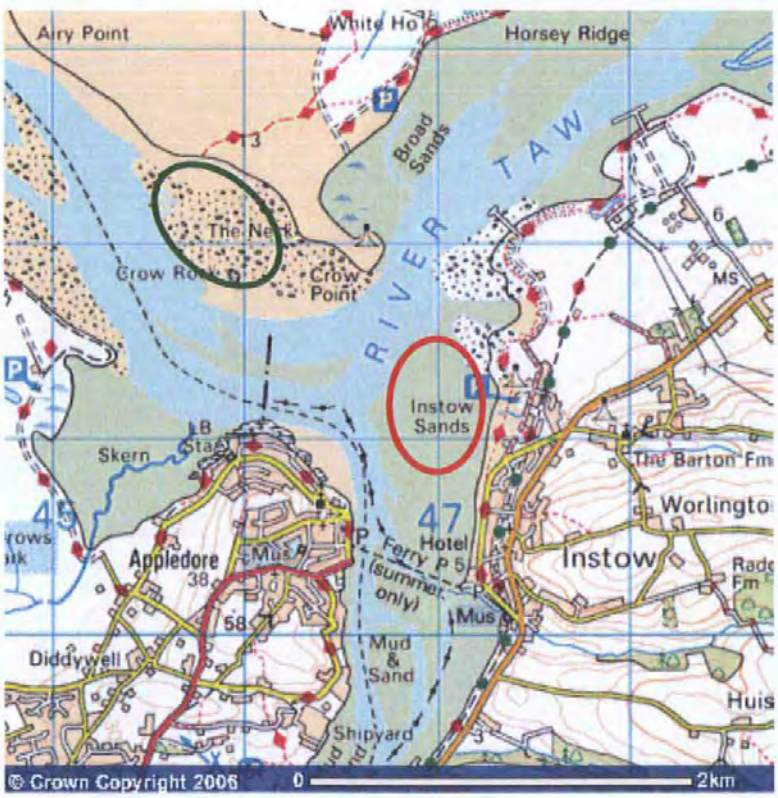


Figure 2.2: Map of approximate areas surveyed for the presence of gracilarioids during initial site surveys at Instow Sands (red oval) and Braunton Burrows (green oval)

Figure 2.3: Map of approximate initial survey area for the presence of gracilarioid algae in the Camel Estuary close to Padstow and Rock (blue oval)

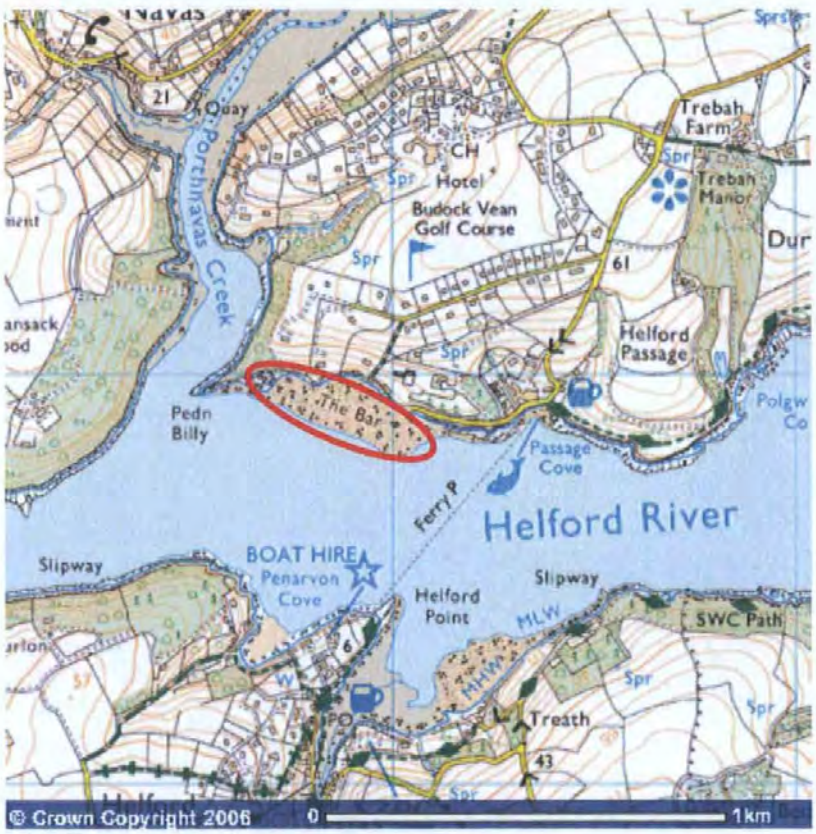


Figure 2.4: Map of approximate general area of initial survey for the presence of gracilarioid algae in Helford Passage on the Helford River (red oval)

Figure 2.5: Map of approximate initial survey area when investigating the presence of gracilarioid algae at Flushing on the Penryn River (red oval), opposite the city of Falmouth

Figure 2.6: Map of approximate general area of initial survey for the presence of gracilarioid algae at Cellars Beach, Place near St Anthony in the Fal Estuary

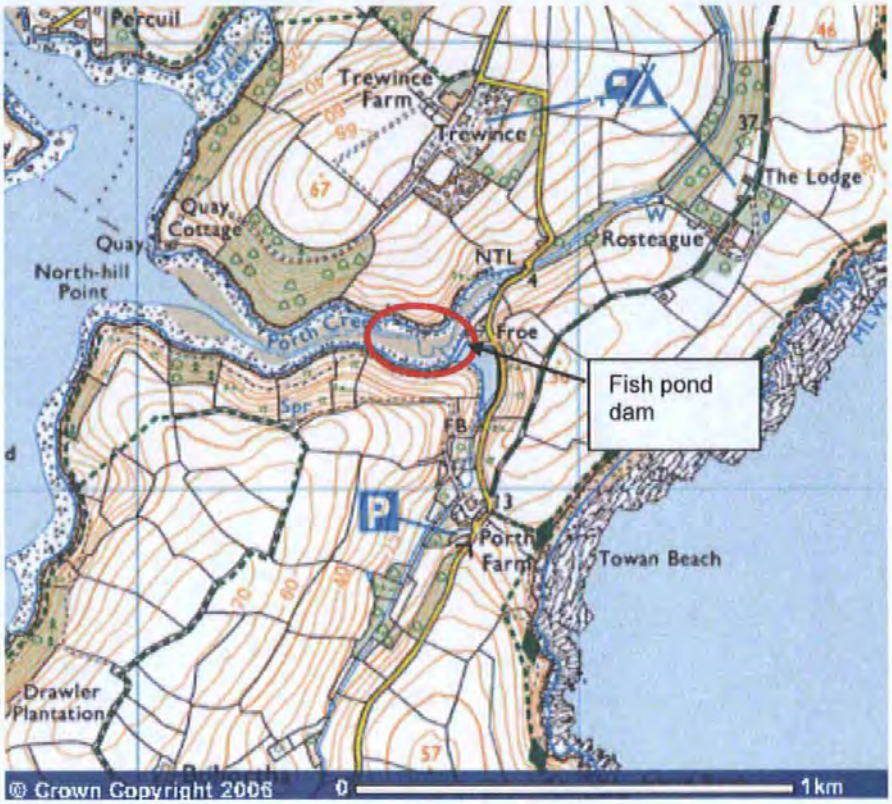


Figure 2.7: Map of area accessed at Froe, on the Percuil River, in the Fal Estuary system, during initial survey for the presence of gracilarioid algae (red oval)

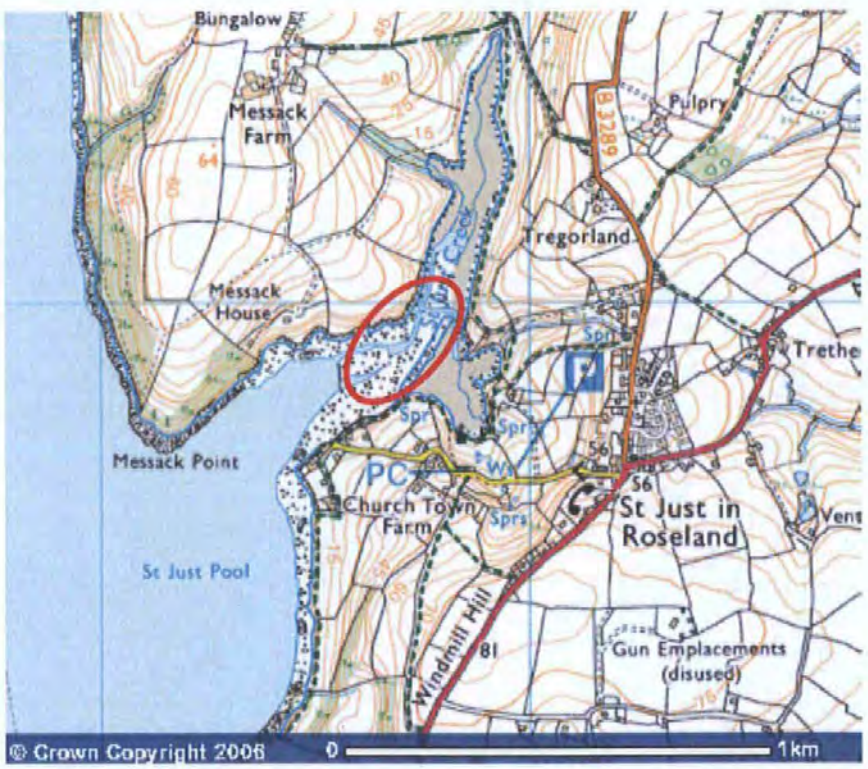


Figure 2.8: Map of area of St Just Creek in the Fal Estuary surveyed initially for the presence of gracilarioid algae (red oval)

All maps: Image produced from the Ordnance Survey Get-a-map service. Image reproduced with kind permission of Ordnance Survey and Ordnance Survey of Northern Ireland.

The population of gracilarioids found at Newton Ferrers was not attached and therefore its extent could not be assessed.

At Flushing, Helford and the Camel, the foreshores were mainly exposed during low water, with only the central stream or river providing immersed areas, which meant that gracilarioids were accessed almost entirely at the water's edge during extreme low water springs when they became accessible, apart from limited shallow puddles which were probably only exposed on the ELWS and during less extreme tides.

2.2.2.3. Molecular identification of populations of *Gracilariopsis longissima*

Gracilarioid algae collected were successfully allocated to species by amplification of the ITS region of the genome. The difference in size of the region reported by Goff *et al* (1994) was clearly visible thus enabling allocation to species according to genus confirmation. Figure 2.9 shows a representative agarose gel with amplified product from eight samples. Four have amplified a product of 1107bp (Lanes 2-5) which identifies those samples as *Gracilaria gracilis*. Three samples have amplified fragments of 906bp (Lanes 6, 8 & 9) which identifies them as *Gs. longissima*. The sample in lane 7 failed to amplify despite repeated attempts.

As can be seen in Figure 2.9, the strength of the signal varied considerably from individual to individual but the size difference between the two genera was clear and unambiguous. Where there was a failure of amplification, a dilution of template by x10 or x100 times usually brought about successful amplification.

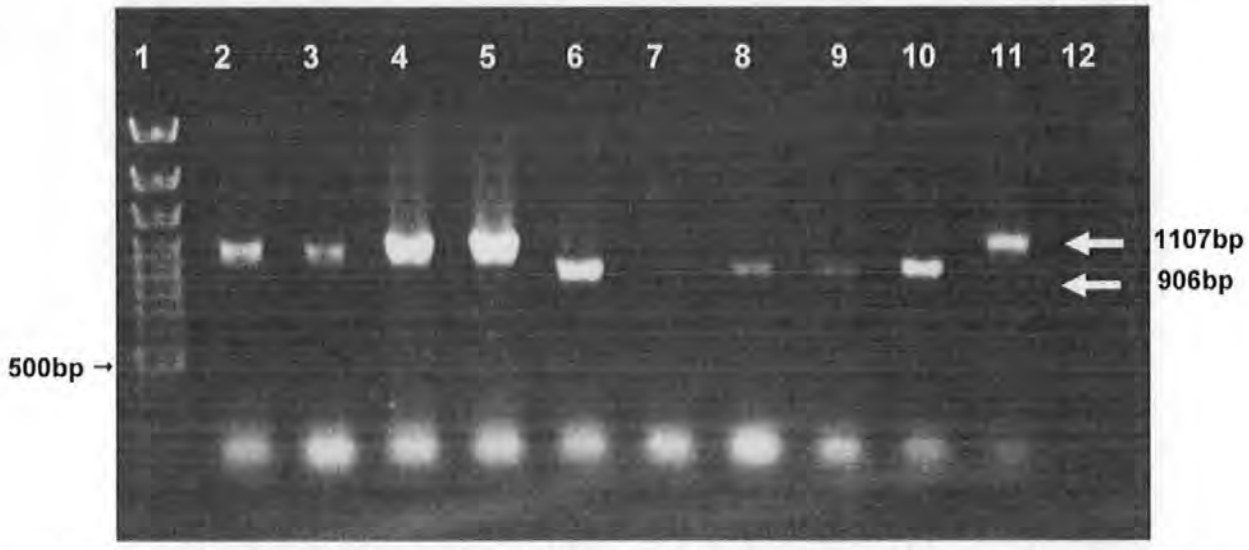


Figure 2.9: Representative gel of amplification of ITS region for 8 specimens of gracilarioids used in this study. Lanes 10 and 11 are positive controls for *Gs. longissima* and *G. gracilis* respectively. Lanes 2-5 are from Millandreath (L1,2,3,4) and are seen to be *G. gracilis*. Lanes 6, 8 & 9 are all from Mylor (M1) and Helford (H3, H7) and are seen to be *Gs. longissima*. Lane 12 - negative control. Lane 1 - 100bp ladder. Specimen H2 (lane 7) failed to amplify.

Table 2.3: Numbers of randomly collected samples of gracilarioid from 13 sites around the South West Peninsula of Britain and allocated to species by amplification of the ITS region of the genome

	Site name	Numbers of specimens	*Gracilariopsis longissima*	*Gracilaria gracilis*
1	Braunton Burrows, Bideford	22	22	0
2	Instow Sands, Bideford	23	8	15
3	Bantham, Bigbury Bay	10	0	10
4	Newton Ferrers, River Yealm	8	8	0
5	Millandreath Beach, Looe	7	0	7
6	Cellars Beach, Place, Fal Estuary	31	18	13
7	Froe, Porth Creek, Percuil River, Fal Estuary	26	15	11
8	St Just Creek , Fal Estuary	30	30	0
9	Restronguet Creek, Fal Estuary	6	6	0
10	Mylor Creek, Fal Estuary	8	8	0
11	Flushing, Penryn River, Fal Estuary	19	19	0
12	Helford Passage, The Bar, Helford River	28	28	0
13	Rock/Padstow, Camel Estuary	10	10	0

2.2.2.4. Study site determination

Of the 13 sites found to support gracilarioid populations, eight had unispecific populations of *Gracilariopsis longissima* (Braunton Burrows, St Just, Newton Ferrers, Restronguet, Mylor, Flushing, Helford and the Camel), two had unispecific populations of *Gracilaria gracilis* (Bantham and Millandreath) and three (Froe, Place and Instow) supported mixed populations of both species (Tables 2.3 & 2.4 and Figure 2.10).

From the sampling carried out at that stage, it was not possible to determine whether the two species were distributed differentially within sites or whether they were randomly mixed throughout sites.

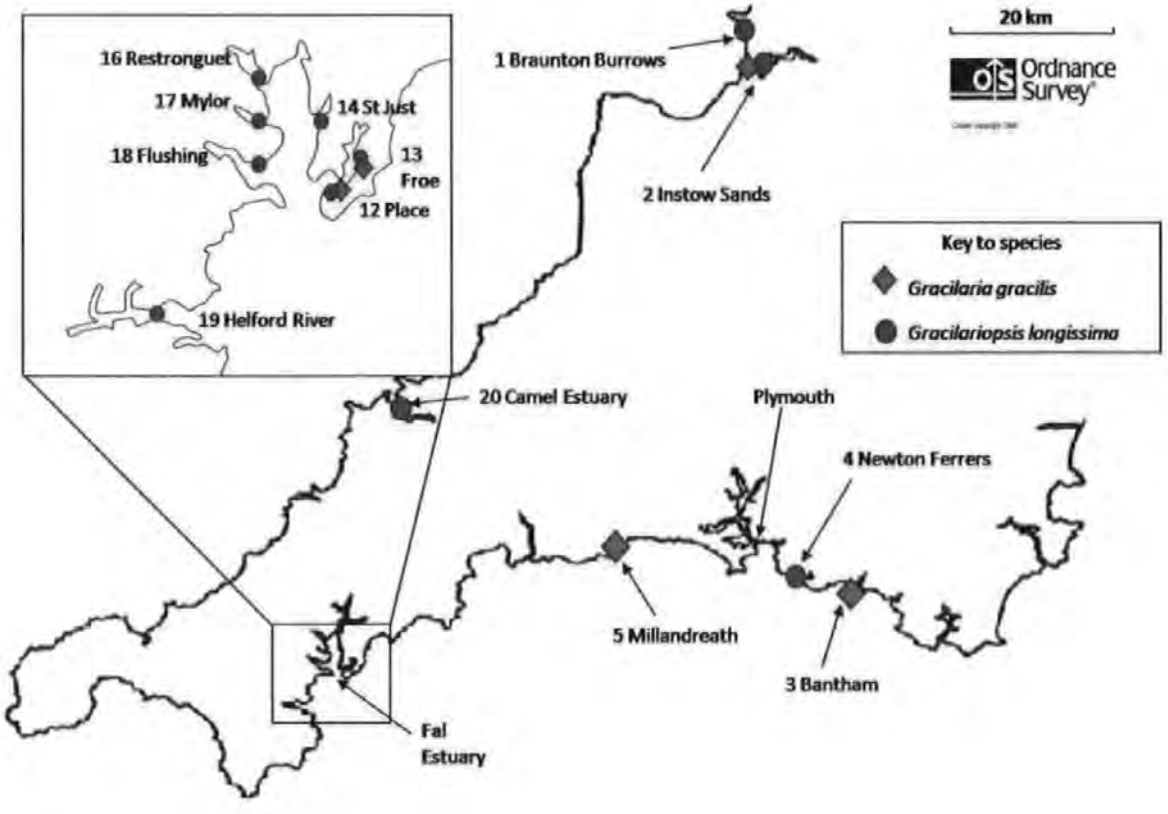


Figure 2.10: Coastline map of Cornwall and West and North Devon showing locations of sites found to have populations of *Gs. longissima* (green circles), *G. gracilis* (red diamonds), or both during this study. The inset box shows populations in the Fal Estuary. Sites are numbered as in Tables 2.1 and 2.4. The city of Plymouth is indicated for orientation.

Table 2.4: Results of surveys of 20 sites around the South West Peninsula of Britain listing those where gracilarioid populations which were confirmed as either *Gs. longissima* or *G. gracilis* with sites chosen for further ecological investigations highlighted in bold type.

	Site name	Species of gracilarioid found
1	**Braunton Burrows, Bideford**	***Gracilariopsis longissima***
2	**Instow Sands, Bideford**	***Gs. longissima*** ***Gracilaria gracilis***
3	Bantham, Bigbury Bay	*G. gracilis*
4	Newton Ferrers, River Yealm	*Gs. longissima*
5	Millandreath Beach, Looe	*G. gracilis*
6	**Looe River**	**Neither**
7	Hannafore Point, Looe	Neither
8	Polridmouth Beach, Fowey	Neither
9	Spit Point, Par	Neither
10	Pentewan Beach, Mevagissey Bay	Neither
11	Towan Beach, Roseland Peninsula	Neither
12	**Cellars Beach, Place, Fal Estuary**	***Gs. longissima*** ***G. gracilis***
13	**Froe, Porth Creek, Percuil River, Fal Estuary**	***Gs. longissima*** ***G. gracilis***
14	**St Just Creek , Fal Estuary**	***Gs. longissima***
15	Turnaware Point, River Fal	Neither
16	Restronguet Creek, Fal Estuary	*Gs. longissima*
17	Mylor Creek, Fal Estuary	*Gs. longissima*
18	**Flushing, Penryn River, Fal Estuary**	***Gs. longissima***
19	**Helford Passage, The Bar, Helford River**	***Gs. longissima***
20	**Rock/Padstow, Camel Estuary**	***Gs. longissima***

2.2.3. Discussion and Conclusions

2.2.3.1. Preliminary site surveys

This research study was not comprehensive and, as proposed by Hardy and Guiry (2003), there is likely to be wide distribution of *Gs. longissima* around the British coast. Despite superficial site similarities between all those surveyed, not all were found to have gracilarioid populations.

It was established that gracilarioids grow commonly around the South West Peninsula of Britain, though *Gs. longissima* was not found at most of the more exposed, high energy sites surveyed such as Hannafore Point or Par. It is possible that physical damage from wave action precludes colonisation of such shores by this organism. *Gracilaria* species are known to have good regenerative abilities (Santelices and Varela 1995) but it is not known whether *Gs. longissima* shares this ability.

2.2.3.2. Distribution of gracilarioids within sites

Gracilarioids were found throughout the intertidal sites where they occurred as submerged populations which suggests they can colonise and occupy estuarine, open coast and other habitats successfully where water remains at low tide and survive the fluctuating conditions experienced by organisms living in the intertidal zone.

2.2.3.3. Molecular identification of populations of *Gracilariopsis longissima*

The simplicity and speed of the ITS amplification method for species determination made it very useful to delineate the populations. However, occasional specimens consistently failed to amplify (e.g. H2, Figure 2.9), despite carrying out several

extractions. Individuals which behaved in this way could not, therefore, be confirmed as one species or another. Even where they originated from a site which appears to host a unispecific *Gs. longissima* population, such individuals had to be rejected from any further study.

Reasons why some individual specimens failed to amplify repeatedly, including some extracted with more sophisticated methods, need further investigation. It is unclear whether this is a problem of some individuals with high concentrations of inhibitory products which are not neutralised by dilution or sufficient removal during extraction, or whether it suggests a major difference in priming sites which render these individuals impossible to analyse with existing markers.

ITS amplification is a simple and straightforward method of genus confirmation which was used over and over again successfully. Problems of the presence of inhibitory products for successful PCR were not a major concern. Dilution was usually a successful strategy for successful amplification.

The ITS protocol allowed the researcher to verify species with confidence and to assess the contribution of each species to the populations where they co-occurred, assuming there is no contaminating DNA present. For researchers with limited field botany experience or cytological skills in plant identification, the development of molecular markers to resolve taxonomic questions with a lack of ambiguity is an extremely useful tool.

2.2.3.4. Study site determination

Populations of both *Gracilariopsis longissima* and *Gracilaria gracilis* can be found unispecifically and co-occurring. From the molecular identification, it was possible to identify sites that hosted populations of *Gracilariopsis longissima*, and these were the sites that were used for further investigations. Out of the 11 sites found to support populations of *Gracilariopsis longissima* (Table 2.4) only eight were chosen for further ecological studies (Figure 2.10). This was for a number of reasons. Newton Ferrers was excluded because all specimens found at this site were unattached. Therefore, it was impossible to determine their origin.

Despite the presence of *Gs. longissima* at Restronguet and Mylor, these sites were also not included in the study. Restronguet Creek has very high levels of pollution (Bryan and Gibbs 1983, Newman 1998) and only four specimens of *Gs. longissima* were found there. These specimens had damaged thalli and were weakly pigmented with areas of green colouration, which may indicate poor health. At Mylor Creek the construction of a new marina prevented access for surveying.

Gs. longissima occurs unispecifically at Braunton while it grows in a mixed population only a kilometre away in the pools of Instow Sands. This suggests a difference in conditions that somehow precludes *Gracilaria gracilis* from surviving at Braunton. None of the data reported here is able to explain this finding.

Where the two species occurred together, it would be interesting to find out how they were distributed within each site and in relation to each other. Future work could encompass this research.

2.3. Part II: Ecological investigation

2.3.1. Materials and Methods

As discussed above, eight sites hosting populations of *Gracilariopsis longissima* were chosen for further investigations of the ecology of the species, in particular with regard to the nature of its habitat and the algal assemblages in which it occurred.

A ninth site, Looe (Figure 2.11), where the species did not occur was included in the research site list for comparison. Superficially meeting the criteria described by Steentoft *et al* (1995) as suitable for *Gs. longissima*, Looe is a sheltered river estuary with shallow shores accessible at low tide.

Generalised descriptions of each site were recorded, including photographs of sites and sampling quadrats.

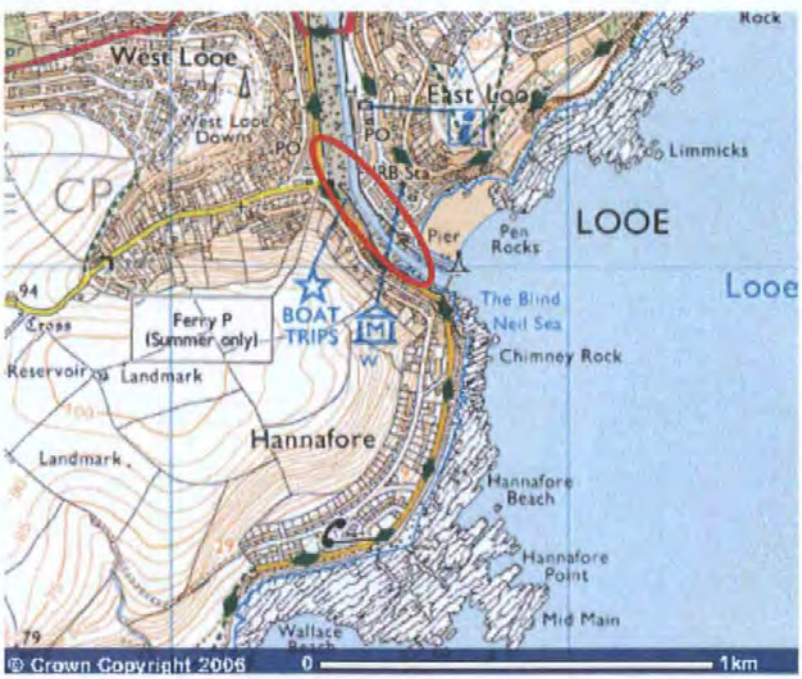


Figure 2.11: Map of area of Looe on the south coast of Cornwall initially surveyed for the presence of gracilarioid algae (red oval)

2.3.1.1. Sampling design

Sites were visited on extreme low water springs (ELWS) to allow access to lowest parts of the shore and longest possible access period on site. Due to tide constraints, four sites were surveyed in March and April and five in July and August (Table 2.5).

To some degree sampling design was determined by access and extent of remaining water cover at low water as both species occur only submerged. At each site 10 x 1 m^2 quadrats were sampled. This was a number which could be surveyed successfully in the between-tide time available. As *Gs. longissima* is not normally found emersed, sampling was restricted to either (1) stream flow or (2) standing water left in natural pools in substrates. An additional quadrat was sampled at the Camel site (quadrat 11) in error but the data has been included in this report as the statistical package used was able to deal with data sets of unequal numbers (see below).

Table 2.5: Survey dates for the collection of algal assemblage data at eight sites where populations of *Gracilariopsis longissima* had been recorded and one site (Looe) where abiotic conditions were superficially similar for no gracilarioid populations were found

Site	Site code letter	Date of data collection
Braunton Burrows	A	4 April 2003
Froe	F	3 April 2003
Place	P	21 March 2003
St Just	J	20 March 2003
The Camel	C	17 July 2003
Instow	S	1 August 2003
Flushing	G	30 July 2003
Helford	H	31 July 2003
Looe	L	29 August 2003

The sampling method was designed in a hierarchy using the same number of sampling points (quadrats) spread in a similar way through each site where access permitted. A first quadrat was assigned randomly along the low water mark (LWM) at the "bottom" of each site as determined subjectively to be the lowest seaward point accessible. Two further quadrats were then placed 5 m from that original position at the same shore level. A second set of three quadrats were surveyed at a point between 60 and 100 m up the shore. A third set of three was positioned at the "top" of the site. The top of a site for the purposes of this study was either determined by physical features, such as the fish pond dam at Froe or the retaining wall at Place, or by the need to confine the study area to a manageable intertidal size within a much larger site - such as at Instow or Braunton. This sampling design is outlined in Figure 2.12.

The approximate positions of the sampling quadrats for each site are indicated in the large scale maps shown in Figures 2.13 to 2.21. Quadrats were numbered as shown in Figure 2.12. The areas marked in these maps relate closely to those shown as approximate survey areas in the maps given in Figures 2.2 to 2.8. The pattern of quadrats at three sites, the Camel, Helford, and Flushing, was constrained by the fact that the populations of gracilarioids were found only along the water margins which were, effectively, the stream/river edges. This is illustrated in the maps for these sites (Figures 2.17, 2.19 and 2.20, respectively).

Braunton Burrows and Instow Sands were very large sites where it was not possible to mark exactly the positions of quadrats without the benefit of global positioning system (GPS) equipment which was not available. Neither site had easily recognisable landmarks of suitable size or structure to stand out in the

landscape to be able to locate positions accurately. However, quadrats were positioned as closely as possible to the pattern described in Figure 2.12.

At Looe (Figure 2.21), sampling was limited to the west bank by deep water at its lower reaches but the area of shallow stream flow in the mid region of the site was accessible at low water.

Photographic records of general site characteristics were taken where possible.

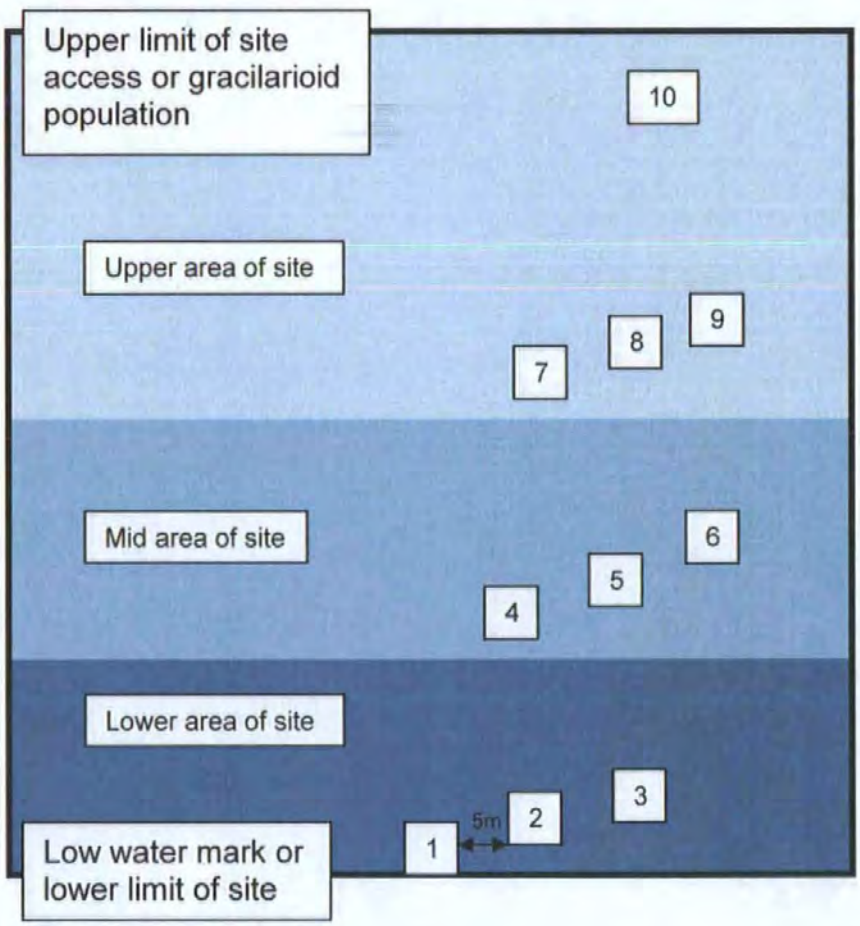
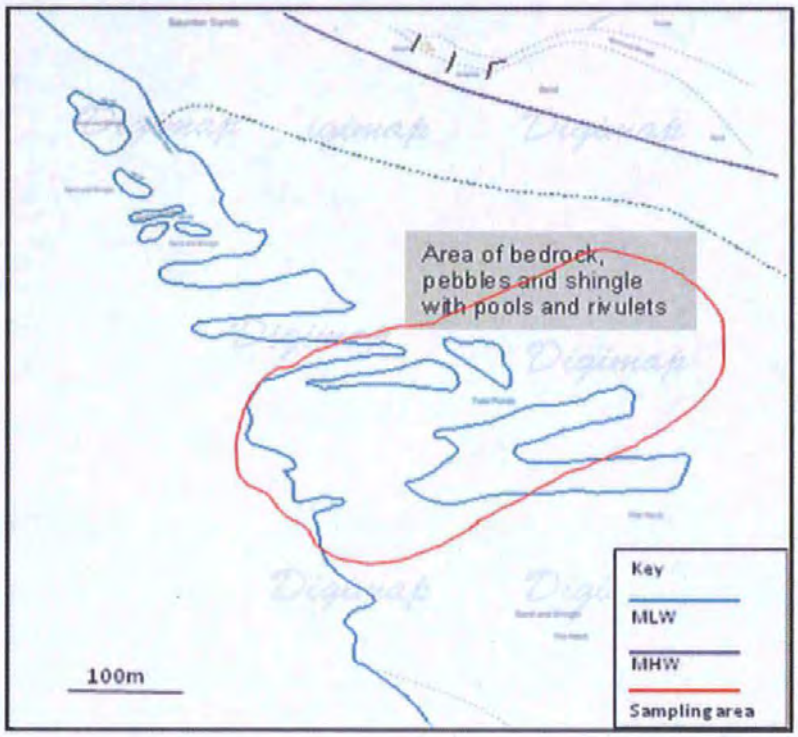


Figure 2.12: Diagrammatic representation of sampling strategy for algal assemblage data collection at nine sites around the coast of South West Britain. Quadrats are represented as numbered boxes in groups of three set approximately 5 m apart. Three quadrats were positioned at or close to the low water mark, 3 in the middle of the site and 3 in the upper region of each site. A final quadrat was placed close to the upper access limit of the site or upper limit of gracilarioid populations.



Figs 2.13: Map of algal assemblage sampling area at Braunton Burrows (red outline) surveyed on April 4, 2003. Standing water and rivulets were found all over the rocky area (between the green dotted line and mean low water). With such a large site individual quadrat positions could not be accurately located without GPS instrumentation.

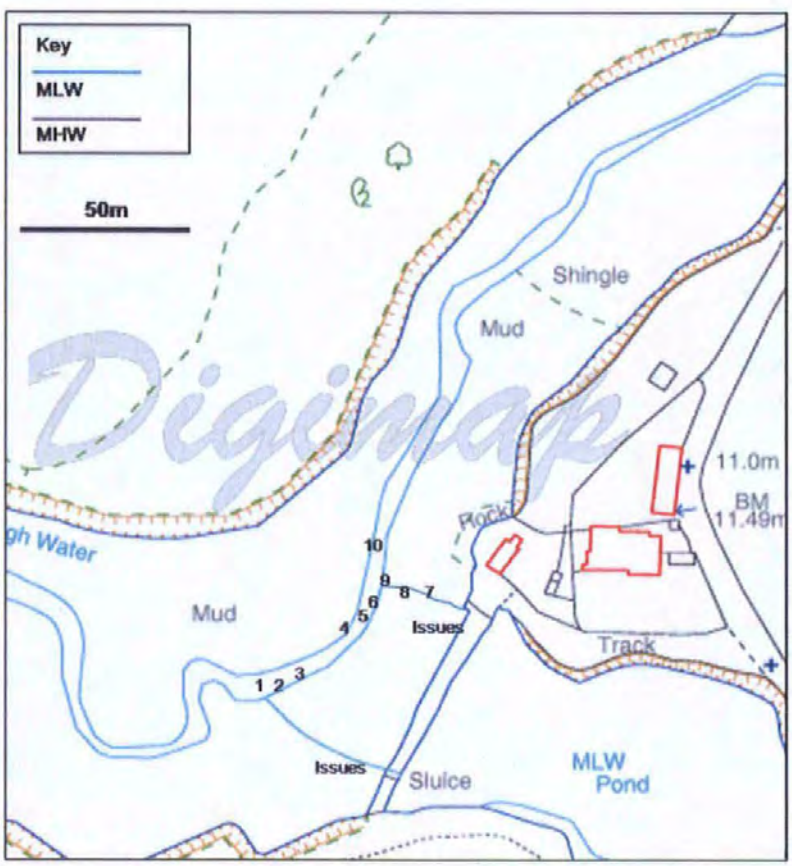


Figure 2.14: Map of Froe creek showing numbered positions of quadrats sampled for algal assemblage data on April 3, 2003. Quadrat numbers are shown in relation to position as described in Figure 1.12.

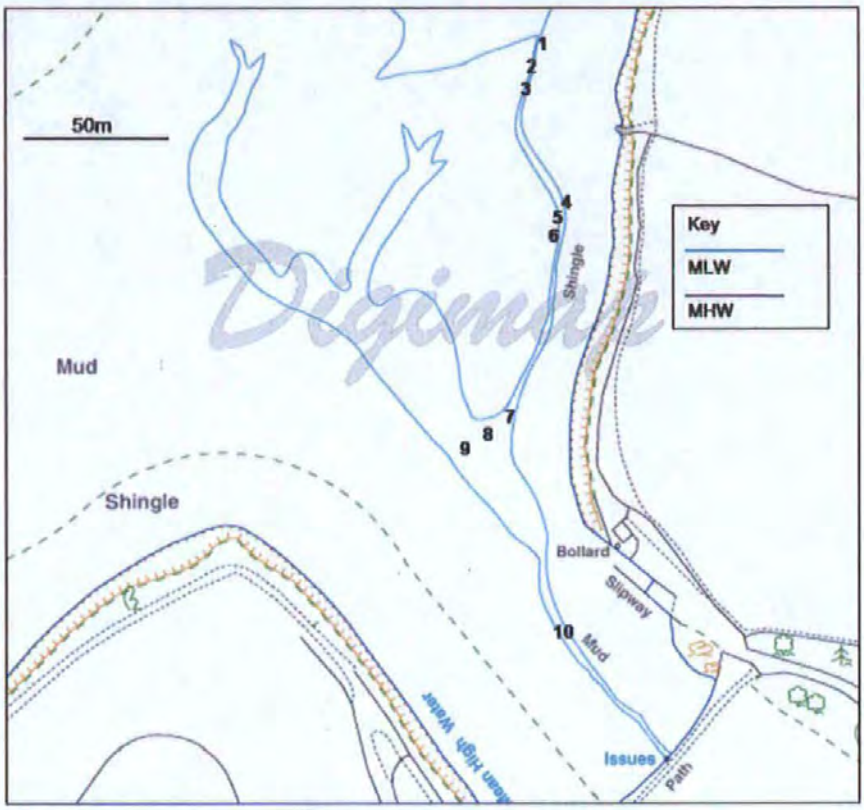


Figure 2.15: Map of Place showing positions of numbered quadrats surveyed for algal assemblages on March 21, 2003. Quadrat numbers are shown in relation to position as described in Figure 1.12.

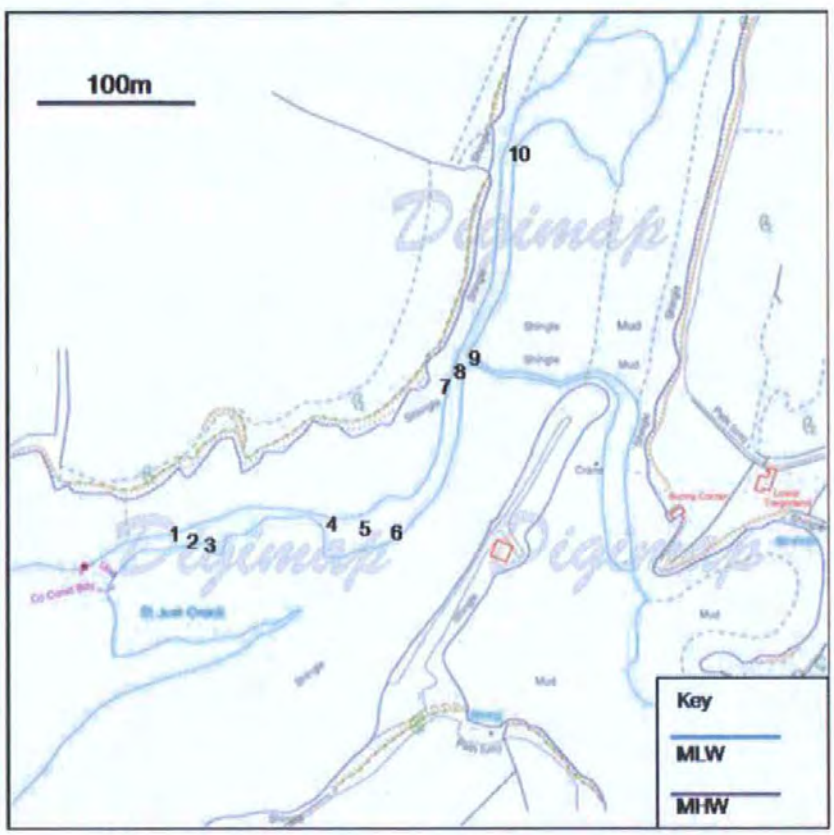


Figure 2.16: Map of St Just Creek showing numbered positions of algal assemblage data quadrats sampled on 20 March 2003. Quadrat numbers are shown in relation to position as described in Figure 1.12.

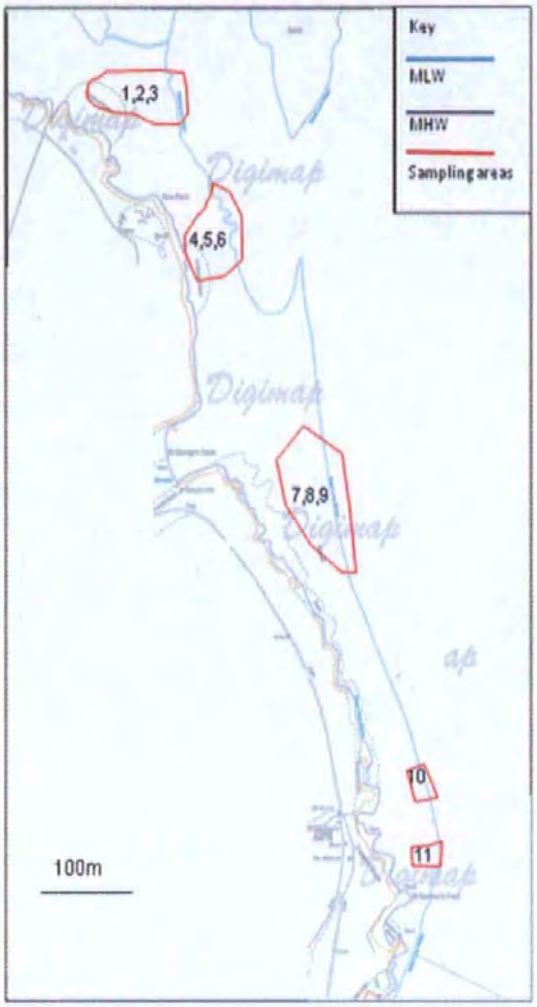


Figure 2.17 (left): Map of the Camel Estuary indicating the areas of numbered quadrats sampled for algal assemblages on July 17, 2003 (red outlines). It was possible to show approximate positions of quadrats due to easy to locate rocky outcrops. Quadrat numbering is as shown in Figure 1.12.

Figure 2.18 (below) : Map of Instow Sands showing approximate area surveyed for algal assemblage data on 1 August 2003 (red outline). It was not possible to mark exact positions of each sampling quadrat without GPS instrumentation.

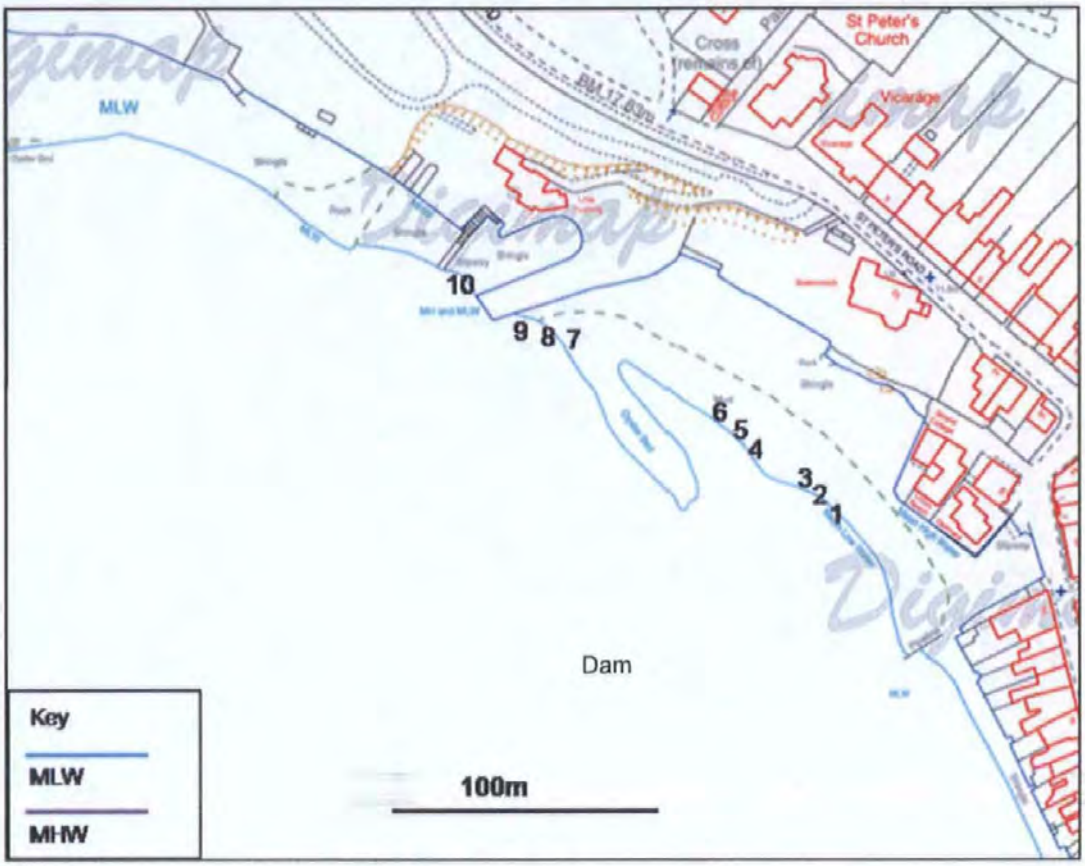
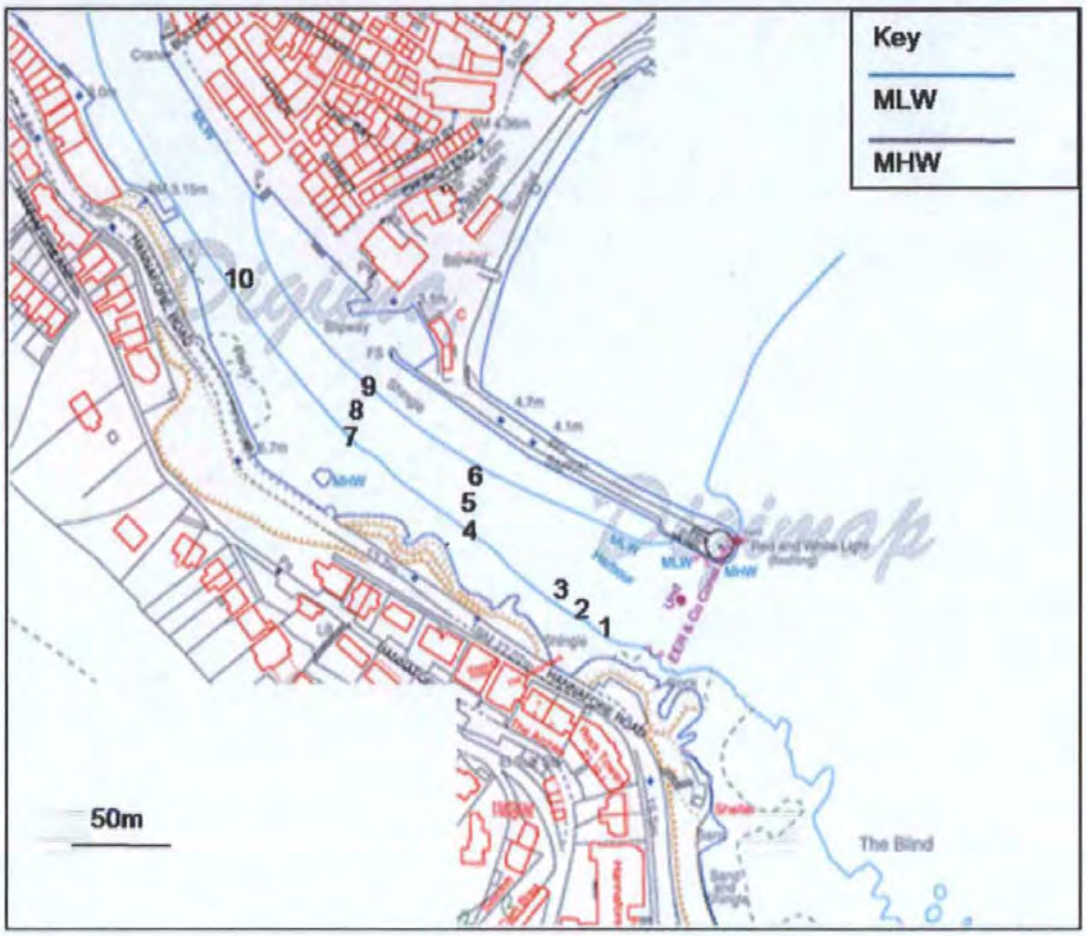


Figure 2.19: Map of Flushing showing positions of numbered quadrats surveyed for algal assemblages on 30 July 2003. The foreshore was a narrow strip of land with very little water left at low water. Quadrats were therefore sited where the stream flow continued at low water. Quadrat numbers followed those given in Figure 1.12.

Figure 2.20: Map of Helford Passage showing positions of numbered quadrats sampled for algal assemblage data on 31 July 2003. The site was made up of the area between low and high water where pools remained at low water, and the main stream flow at and below low water. Quadrat numbers followed those given in Figure 1.12.



Figs 2.21: Map of Looe river showing positions of numbered quadrats sampled for algal assemblage data on 29 August 2003. The area below low water mark is a swift flowing stream until it meets the open sea Quadrat numbers followed those given in Figure 1.12.

2.3.1.2. Physical habitat characteristics

Sediment samples (approx 250-500 ml) were collected from each quadrat where possible. Size partition was determined by drying samples before dividing into size classes by sieving. The weight in each size class was measured and recorded as a percentage of the whole sample. Particles below 2 mm in size were further analysed by digestion with hydrogen peroxide to remove all organic matter, followed by analysis with a laser particle size analyser (Malvern Long-bed Mastersizer X). For comparison between samples, size data was expressed on the phi (Φ) scale where phi = - $\log_2$ of particle size in mm (Gray 1981).

A YSI Model 63 System Meter was used to measure temperature, salinity and pH for each quadrat. Some recordings were not possible where the depth of water was insufficient for immersion of the instrument's probe.

2.3.1.3. Algal assemblage data collection

Macrophyte species which could be easily identified on site were recorded. Where filamentous red algae were not identified as *Ceramium* sp. or *Polysiphonia* sp., they were recorded as unknown red filamentous species and grouped together. Full identification of *Ectocarpus*-like species in the field was not attempted with such species recorded as "ectocarpoid".

The abundance of each species was recorded as estimated percentage cover in individual quadrats. The percentage of bare substrate was also recorded. Where multi-layering of algae occurred, values of over 100% cover for an individual quadrat were recorded. Macroscopic animals were recorded when seen.

2.3.1.4. Statistical analysis of data

Statistical analysis was carried out using the PRIMER V computer program which was developed for statistical analysis and interpretation of changes in marine communities (Clarke and Warwick 1994, 2001).

2.3.1.4.1. Physical habitat characteristics

Sediment characteristics were compared by site to detect whether there were any significant differences in conditions experienced by the algal populations. Sediments were categorised by particle size percentages for each quadrat sampled. Site data were then compared by analysing within and between site data

using a one-way analysis of similarities using the ANOSIM routine provided in PRIMER V (Clarke and Warwick 2001).

Other abiotic factors – temperature, salinity and pH – were snapshot measurements taken on the day of survey and could not be examined statistically.

2.3.1.4.2. Algal assemblages

As there are potentially large effects of season on the composition of algal communities (Dring 1982), sites were considered in seasonal groups for direct between-site analyses. Data for sites surveyed in March and April (Braunton, Froe, Place and St Just) were grouped as "Spring" data. Data for sites surveyed in July and August (Camel, Instow, Flushing, Helford and Looe) were grouped as "Summer" data.

A number of routines in PRIMER V were used to compare floristic data within and between sites, to investigate a number of potential differences, as follows:

(1) Within-site variation

(2) Between sites grouped by season

(3) Between seasons

(4) Between sites where *Gracilariopsis longissima* was found alone and those where it occurred with *Gracilaria gracilis*.

Data was first prepared according to the constraints of the PRIMER V program. Clarke and Warwick recommend leaving out data for very rare species – those representing less than 4% of abundance/cover - as they warn that their inclusion can affect the robustness of the results as the program can give too much weight

to rare species when included (Clarke and Warwick 2001). The data was therefore adjusted accordingly with all species which were recorded only once as a single individual for the whole data set, omitted from calculations. However, some species which were rare at one site, i.e., less than 4% cover, were sometimes common at others and were therefore included in the floristic data set. For example, tubular *Ulva* species represented over 30% of the algal cover at the Camel but was present as only just over 3% at Helford. The species was therefore included in the data. By additionally carrying out square root transformations of percentage cover data throughout the data sets, it was possible to reduce the effect of the most common species and to allow inclusion of most of the rarer species (Clarke and Warwick 2001).

A few quadrats (one each at Froe, Helford and Instow) were recorded as completely devoid of algae, i.e., 100% bare substrate. The way that the similarity matrices are calculated in PRIMER V means that records of 100% for any "species", e.g., bare substrate, had to be omitted to avoid skew. These quadrats therefore were omitted from the data set. However, as with the additional quadrat at Camel, the PRIMER program treats individual quadrats as independent data, allowing inclusion of uneven numbers of data sets from different sites. The program compares data from individual quadrats independently in relation to all other quadrats in the data set.

Within-site quadrat data for algal assemblages was investigated for similarities using the cluster analysis routine in PRIMER V (Clarke and Warwick 2001).

Differences in algal assemblages were analysed by creating similarity matrices using Bray-Curtis similarity coefficients and square-root transformed percentage cover data as described above. Relationships within these similarity matrices were then displayed as multi-dimensional scaling (MDS) and cluster plots (using group average clustering). These plots provide a two-dimensional display of how individual quadrats relate to one another in terms of their algal assemblages and can be used to investigate comparisons within and between sites with regard to any of the factors under consideration such as season or site. This is done by labelling data prior to calculation of similarity matrices.

Matrices were further used to analyse the statistical significance of differences between algal assemblages with a one-way analysis of similarities (ANOSIM) calculation, using the same factors as above, at a significance level of $p < 0.05$.

2.3.1.4.3. Correlations between algal assemblages and habitat characteristics

Correlations between floristic assemblages and abiotic factors such as temperature, salinity and pH, could not be tested with the restricted data collected for these variables.

To investigate potential correlations between floristic data and sediment characteristics, the Bray-Curtis (dis)similarity matrix for algal data was subjected to a rank correlation comparison with a Euclidean (dis)similarity matrix for sediment in combination for each quadrat. The BIOENV routine in PRIMER V then compares the similarity matrices using a Spearman rank correlation which calculates a value for the best measure of how much the environmental variable, in this case sediment, correlates with the floristic differences.

2.3.2. Results

2.3.2.1. Study site descriptions

Table 2.2 includes brief descriptions of all sites. The three study sites on the north coast differed considerably from the southern study sites in being more open sites, with larger areas exposed at low water.

North Coast

Braunton Burrows is a flat, rocky shore mainly composed of bedrock abutting a sandy shore at high water level (Figure 2.22). Pools and rivulets remain at low water, often with a bed of shale and gravel. Figure 2.23 shows a typical quadrat at Braunton with clear water and well spaced individual algae with large areas of bare substrate.

Figure 2.22: Photograph of Braunton Burrows looking north west towards the sea. Pools of water with rivulets running through are typical of this rocky site overlain with shale and areas of gravel.

Figure 2.23: Photograph of typical quadrat (1m^2 white plastic frame) at Braunton Burrows, with stones and shells overlying sandy substrate with scattered algae. A specimen of *Gs. longissima* is indicated (arrow).

Instow Sands is a large, flat site separated from Braunton Burrows to the north west by the wide tidal confluence of the Taw and Torridge rivers (Figures 2.2 and 2.13). The substrate was almost entirely sand, with some small stones and shells. Pools of water were left in sand depressions at low water. Figure 2.25 shows the clear water of a pool with gracilarioids easily distinguished.

Figure 2.24: Photograph of Instow Sands looking north west towards Braunton Burrows across the wide confluence of the Taw and Torridge rivers. Large specimens of gracilarioids can be seen clearly (arrowed) standing pools of water left at low water

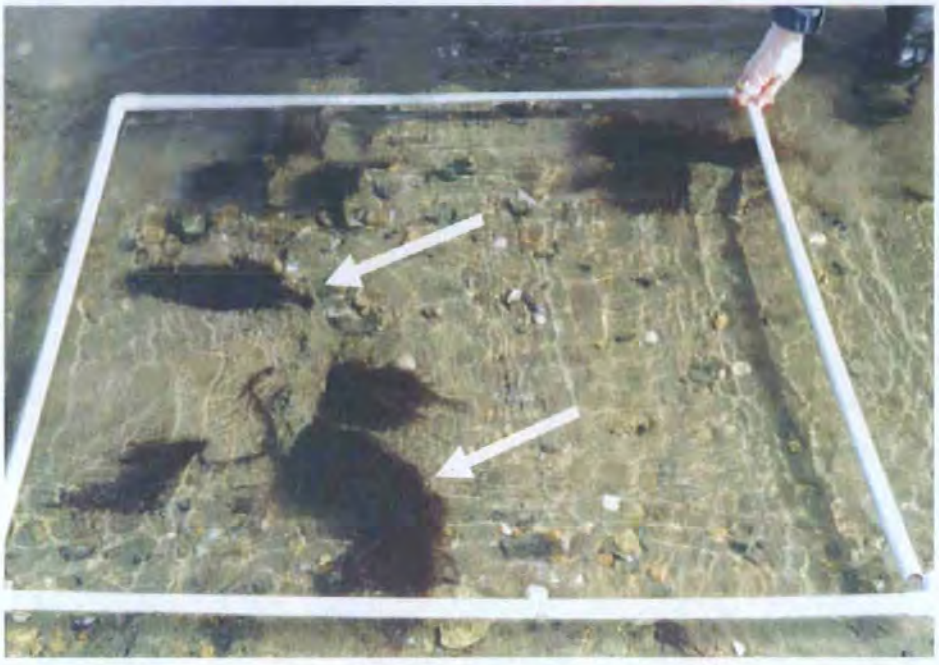


Figure 2.25: Photograph of typical quadrat (1m^2 white plastic frame) at Instow Sands illustrating the sandy substrate with stones and shells. Large, healthy gracilarioids are clearly seen in the shallow standing pools of water (white arrows).

The *Camel Estuary* was characterised by almost entirely sandy substrate with a few rocks and rock pools where specimens of algae were found. Deep, wide (>300 m) river flow remained at low water with a narrow eastern bank for more than 1km from the access point at Padstow (Figure 2.3).

South Coast

Sites on the south coast were mainly characterised as low energy tidal inlets or river valleys with varying degrees of freshwater input. Many were in steep valleys, with muddy substrates, mixed with small stones and shells.

Helford River retained a central deep river flow at low water (Figure 2.4). A muddy, stony bank with areas of fine silty sand, Helford Bar was exposed at low water with algae visible either just submerged in the river at the edges or in very shallow areas of standing water (<3 cm). *Gs. longissima* was found attached to stones buried in the soft, sandy sediments (Figure 2.26).

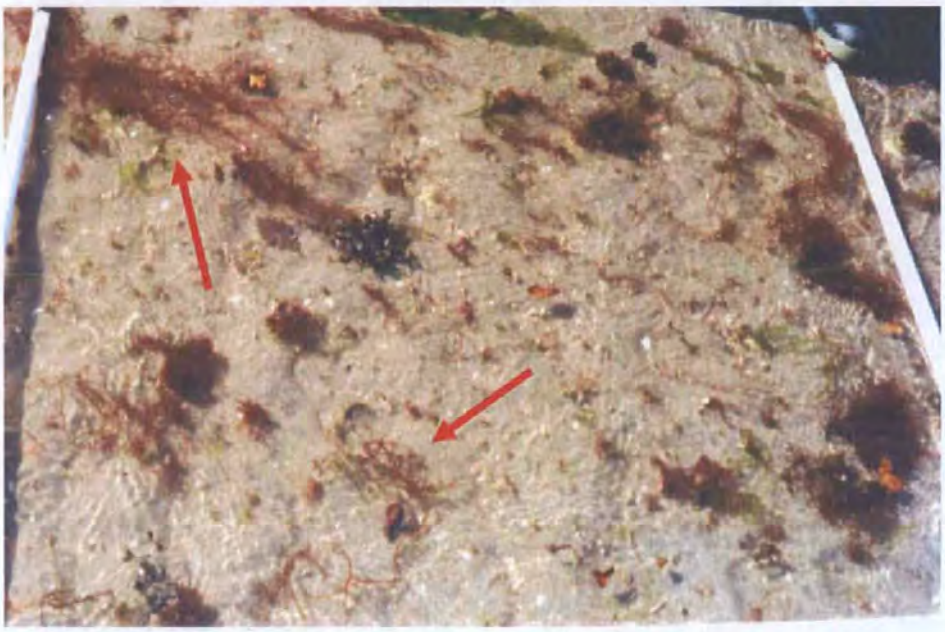


Figure 2.26: Photograph of typical quadrat at Helford ($1m^2$ white plastic frame) showing gently flowing water area retained even at ELWS with specimens of *Gs. longissima* (arrows indicate two specimens) attached to stones buried in the soft, sandy sediments. Specimens tended to be very bright red at this site

Flushing had a firm stony substrate overlain with silt on a narrow foreshore (Figure 2.5). The main river water was opaque with disturbed silt and mud. Once away from the immediate exposed shore, the river was too deep and opaque to be waded to observe submerged algae, as was possible at Place, Froe and St Just (see below).

Surveying was therefore restricted to the water's edge and standing water. *Gs. longissima* was found here almost emersed (Figure 2.27). As these observations were made at ELWS, it is likely that most algae were exposed only occasionally. A single individual of a locally rare species, *Gracilaria multipartita*, was found at this site.

Place changed from a firm stony substrate at the south east end of the site where a small freshwater stream entered the site through a tunnel in a land-retaining wall (Figure 2.6), to deep silt and fine gravel at the low water mark. Specimens of

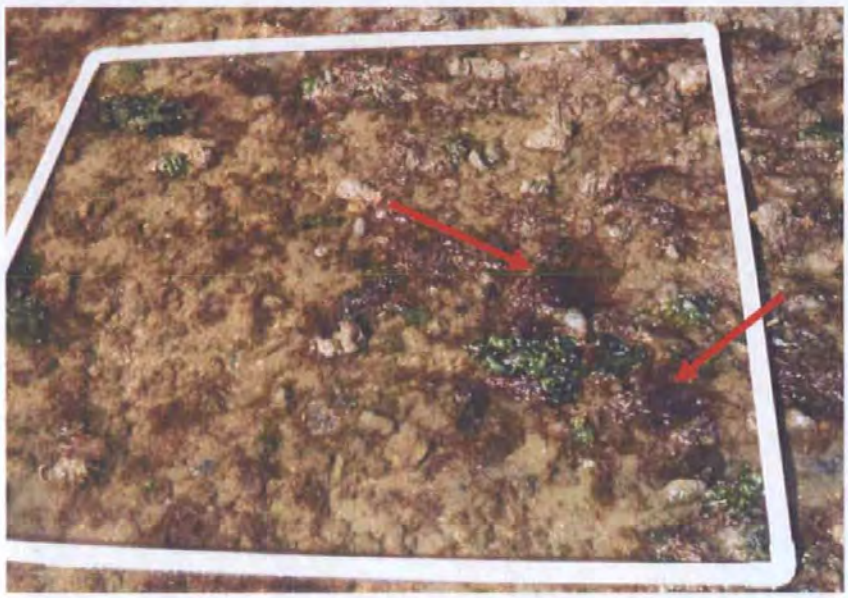


Figure 2.27: Photograph of typical quadrat (1 m^2 white plastic frame) at Flushing, where specimens of *Gs. longissima* were often only just covered with water during ELWS. Individuals seen here are partially out of the water altogether (arrows) although this was an unusual site.

gracilarioids were found in the stream several metres inside the tunnel which extended into the retaining wall at the south western limit of the site (Figure 2.6). Specimens were also found throughout the fast flowing central stream from halfway down the site (Figure 2.28) and in the slow flowing water or pools among multi-layered algal assemblages (Figure 2.29).

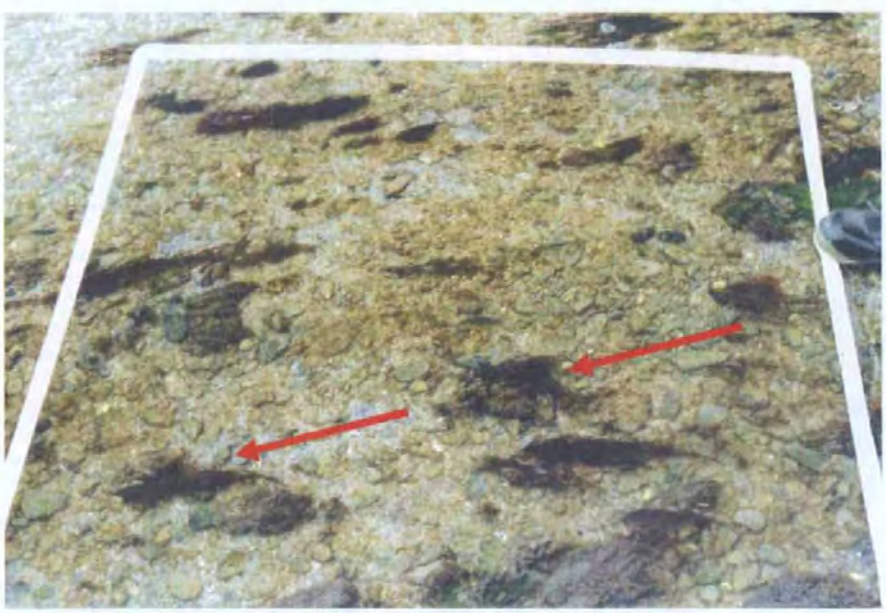


Figure 2.28: Photograph of typical quadrat (1 m^2 white plastic frame) in an area of fast flowing water in the central stream at Place. The dominant algae in these clear areas are the gracilarioids (red arrows)

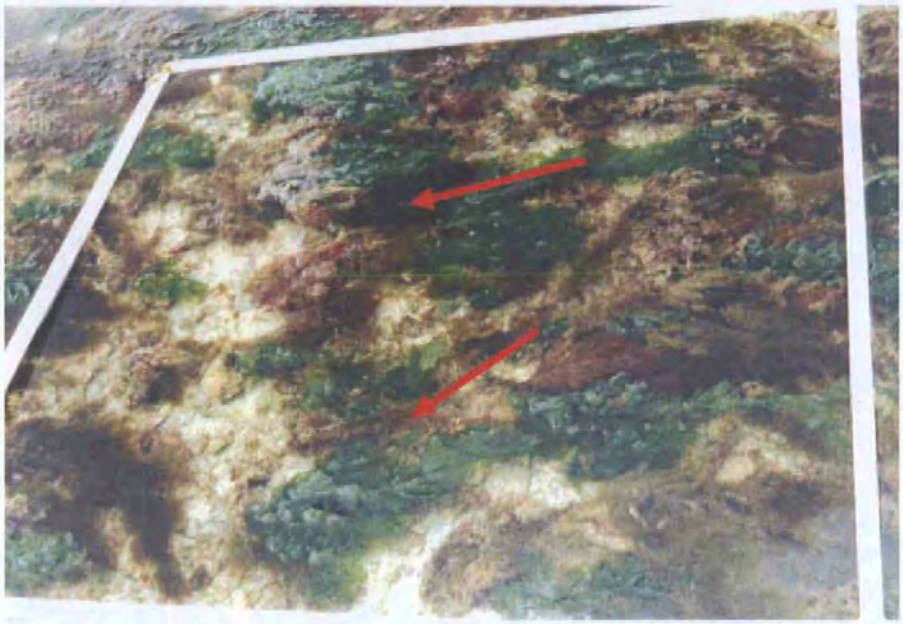


Figure 2.29: Photograph of the type of algal community found in areas of slow or still water at Place (edges of central stream, pools) (1 m^2 white plastic frame). Gracilarioids can just be detected where strands of underlying plants are poking through to the surface (red arrows)

Froe was a particularly muddy site with deep silt preventing access to any area other than where flowing water washed silt away, leaving a soft fine gravel substrate, or below the fish pond sluice gates (Figure 2.30) where rocks and

Figure 2.30: Photograph showing a general view of the sampling area at Froe, with the north eastern outfall from the freshwater fishpond seen in the background

stones overlie the gravel (Figures 2.31). Gracilarioids were commonly found attached to small stones and buried up to 3-4 cm in the silt substrate (Figure 2.32). They commonly had a heavy burden of epiphytes and silt which can be seen in Figure 2.32.

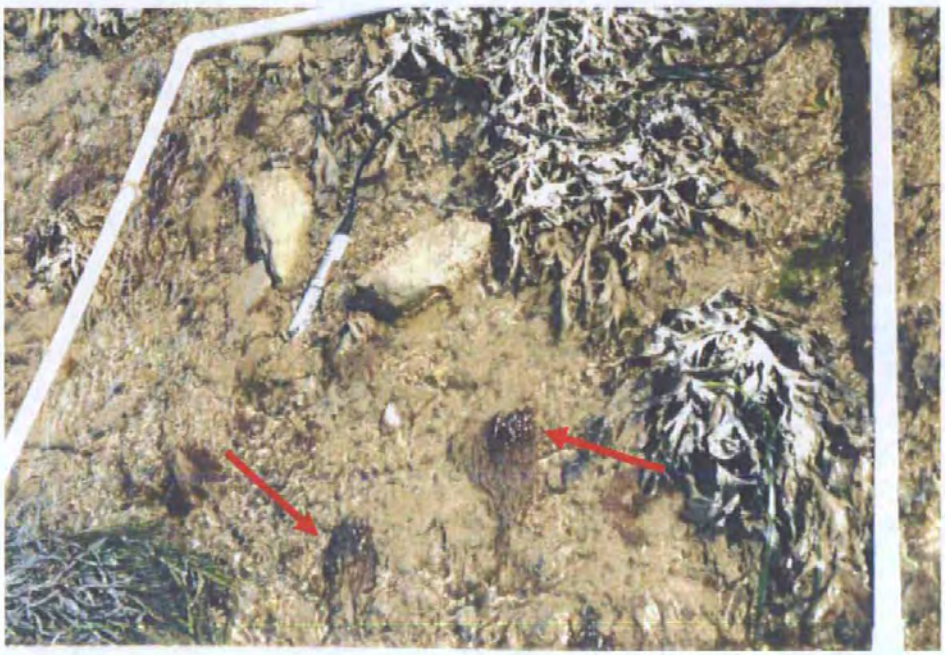


Figure 2.31: Photograph of representative quadrat (1 m^2 white plastic frame) near the sluice gates at Froe where a more diverse algal community than those in the midstream flow could be seen. Specimens are heavily overgrown with epiphytes

Figure 2.32: Photograph of representative quadrat (1 m^2 white plastic frame) in the midstream flow at Froe where few algae grew. Two specimens of gracilarioids can be seen overgrown with epiphytes (arrows)

St Just Creek is a sheltered river valley with a 1930s man-made bar separating a large silted pool from the main creek (Figures 2.8, 2.16, 2.33). The site was also characterised by muddy substrates but of less depth than Froe. Conditions varied across the site from silted in slow flowing areas (Figure 2.34) to relatively clear with 100% algal cover (Figure 2.35) or nearly bare stony areas (Figure 2.36).

Figure 2.33: Photograph of general view of St Just Creek at mid-low water from the causeway to the boat yard (see Figure 2.8). Central stream reduces to <30cm deep and about 3 m wide at low water

Figure 2.34: Photograph of a St Just quadrat (1 m^2 white plastic frame) showing *Gs. longissima* specimens covered in ectocarpoids and with trapped silt (arrows) seen in areas of slow flow and sedimentation

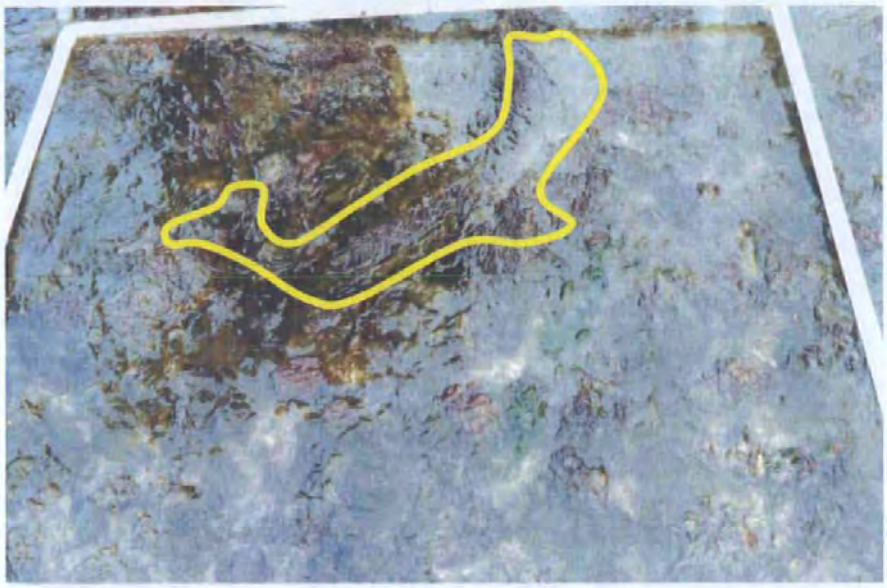


Figure 2.35: Photograph of quadrat (1 m^2 white plastic frame) demonstrating that algal cover in some quadrats at St Just was multi-layered with *Gs. longissima* growing to >1 m in length (individual outlined in yellow)

Figure 2.36: Photograph of a quadrat (1 m^2 white plastic frame) at St Just showing the stony substrate seen in some areas of the site

At St Just the substrate became firmer towards the sea while at Place the opposite applied. *Gs. longissima* occurred throughout the site at St Just with some very large specimens, as can be seen in Figure 2.35.

Looe is a river constrained by man-made banks as it reaches its mouth, with a fast flowing central stream at low water, approximately 15-20m wide (Figure 2.9). Looe

was unique in having extensive mussel beds and the lowest salinity of all the sites (mean 11‰). Looe also had mainly solid bedrock rather than mobile substrates.

In general, Froe, St Just and Place all reduced to shallow streams (<30 cm deep) between 1 and 5m wide at low water allowing access to flowing water areas. The Camel, Helford, Flushing and Looe sites all had sizeable rivers of varying depths and widths which prevented recording of algae in the flowing water except on the edges of banks.

2.3.2.2. Distribution of gracilarioids within sites

In all sites, except Looe, gracilarioids were found distributed throughout sites where standing or flowing water remained during low water. At the largest sites, at Braunton Burrows (Figure 2.13) and Instow Sands (Figure 2.18) it was not possible to survey the entire exposed areas during this study. The extent of distributions within these sites, therefore, could not be determined. However, individuals were found at the highest shore level where water remained and at the lowest accessible areas that were surveyed. It is therefore possible that the populations at these two sites do extend throughout the areas where flowing and standing water remain at low water.

Spaces between individuals or groups of individuals varied from site to site. There were areas of sites where no individuals were found.

2.3.2.3. Physical habitat characteristics

The sites supporting populations of *Gs. longissima* were mainly as described as typical for the species by Steentoft *et al* (1995), such as Helford, Flushing, Place,

Froe and St Just which were all sheltered sites with mobile substrates. However, other sites where *Gs. longissima* was found, such as Instow and Braunton Burrows were very different as described above.

2.3.2.3.1. Sediment

The beaches and shores where *Gs. longissima* was found varied in substrate characteristics. Table 2.6 describes superficially observable sediment characteristics for each site. Sites varied from fine silt at Froe, through mixtures of silt and stones (Flushing and Place), to almost pure sand (Camel) and bedrock overlain with large stones and gravel (Braunton).

Instow and the Camel were sandy sites hosting very scattered populations of algae. Braunton appeared to be the least typical site with large rocks and areas of bedrock. However, *Gracilariopsis* was found in pools where the mixed substrate included small stones and shells. Statistical analysis of sediments is given below (Section 2.3.2.5.1.)

Table 2.6: Sediment descriptions for nine sites surveyed to determine algal assemblages

Site	Sediment character
Braunton	Bedrock with shale and gravel
Instow	Sand with stones, gravel and shells
Camel	Coarse sand
Helford	Fine sand and silt
Flushing	Silt with stones and shells
Place	Silt with stones and shells
Froe	Fine silt (with fine gravel in flowing water)
St Just	Stony with silt and gravel
Looe	Bedrock with stones and mussels

2.3.2.3.2. Salinity, temperature and pH

Table 2.7 lists the means, maxima and minima for salinity, temperature and pH recorded from all quadrats at each site but collected on a single day per site. Looe had the lowest mean salinity at 10.7‰ and a minimum of 7.1‰, while Flushing had the highest mean at 35.1‰ and a maximum of 46.2‰ which suggests very high evaporation on a sunny day. Temperatures recorded varied from a minimum at Helford of 9.6°C to a maximum of 24°C at Camel. pH varied from a minimum of 7.3 at St Just to a maximum of 12.7 at Froe.

While these data give a snapshot picture of possible conditions experienced by algae living in these sites, the highly variable nature of these parameters over the year makes it impossible to compare values between sites. However, one measurement does seem to stand out. The salinity at Looe, measured at low water, as were all the sites, was less than half that found at all the other sites. The river flow at Looe comes mainly from a central, deep stream while stream flow at other sites was observed to be much lower and fed partly by run-off of seawater from silt and sand banks alongside the streams. This may explain the difference seen at Looe.

Froe also experienced very low salinities in parts of the site. This may be explained by the constant infall from a dam sluice which is almost never inundated from the sea (personal communication from landowner) situated at one of the highest shore positions of the sample area.

The range of conditions experienced across sites can vary quite considerably, even on a single day, but it is not possible to extrapolate this data to understand

Table 2.7: Maximum, minimum and mean measurements for salinity, temperature and pH

Site	Salinity ‰			Temperature °C			pH		
	Min	*Max*	***Mean***	*Min*	*Max*	***Mean***	*Min*	*Max*	***Mean***
Braunton	19.3	34.4	**28.4**	18.7	19.4	**19.1**	8.5	9.7	**9.1**
Instow	27.8	35.4	**31.8**	18.2	19.5	**18.9**	8.3	8.7	**8.4**
Camel	25.3	35.5	**29.5**	19.0	24.0	**20.0**	8.1	8.9	**8.4**
Helford	27.6	35.6	**33.8**	9.6	21.3	**18.6**	8.5	9.1	**8.8**
Flushing	32.9	46.2	**35.1**	19.5	21.7	**20.6**	7.9	8.9	**8.6**
Place	16.0	28.4	**20.9**	12.9	14.8	**13.6**	8.1	9.6	**9.0**
Froe	19.0	31.9	**27.2**	10.9	12.7	**11.9**	8.7	12.7	**10.2**
St Just	13.5	30.1	**21.6**	13.8	16.1	**15.1**	7.3	9.3	**8.3**
Looe	7.1	16.5	**10.7**	16.7	18.6	**17.5**	8.6	9.0	**8.8**

the full range or general conditions experienced on these sites. Environmental parameters at each site may be significantly different but it is not possible to calculate the probability from the data set collected here.

2.3.2.4. Algal assemblage data

In both data collection periods (spring and summer), sites where *Gs. longissima* was found varied in the amount and variety of other algal species found. Cover in individual quadrats varied from 0-100%, or more where multi-layered assemblages were recorded. Mean algal cover varied from 23% (Camel) to 78% (Place), with significant areas of uncolonised substrate in all sites.

The macrophyte assemblages varied from single-layered, where individual plants were separated across the substrate as at Braunton (Figure 2.23), to multi-layered communities where species such as *Gs. longissima* were found in the understorey. In Figure 2.29 it is just possible to see strands of thalli of underlying gracilarioids at Place. Three sites (Instow, Place and Froe) were found to have both species of gracilarioid present in varying numbers (Table 2.3). However,

without further sampling designed for the purpose, it was not possible to extrapolate this information to determine the relative contribution of each species in terms of relative abundance in the sites where they co-occur.

2.3.2.4.1. Algal assemblage data recorded during spring surveys

Tables 2.8 lists the macrophyte species identified at each site surveyed in spring – Braunton, Place, Froe and St Just – as mean percentage cover including the percentage mean area of bare substrate averaged across all quadrats, with the relative abundance as a percentage contribution to total algal assemblage. Froe and Place had mixed populations of *G. gracilis* and *Gs. longissima* but it was not possible to determine what contribution each species makes to the total gracilarioid cover. The two species are therefore grouped together for those sites.

Place supported the least number of species (9) with Braunton, Froe and St Just supporting 13, 13 and 14 species respectively. Place also had the most algal cover with only 20% bare substrate. St Just also had extensive algal cover with only 30% bare substrate. Froe and Braunton both had much greater areas of bare substrate at 72% and 65% respectively.

Two species were found uniquely at Braunton: *Blidingia minima* and *Fucus spiralis*. *Fucus vesiculosus* and *Chondrus crispus* were the most abundant species there, accounting for >40% of all algal cover. *Gs. longissima* accounted for 7% of the total.

Ectocarpoids dominated at Place (39%), Froe (46%) and St Just (39%). Many appeared to be epiphytic on other species, in particular Gracilariales. *Bifurcaria*

bifurcata and *Sargassum muticum* were found only at St Just. *Pterothamnion plumula* and *Colpomenia peregrina* were only found at Froe. *Ulva* was a major species at Place (18%).

At Place and Froe, sites with co-occurrence of both species, *Gs. longissima* and *G. gracilis* together made up the second most abundant taxon, with 29% at Place and 18.5% at Froe.

Table 2.8: Algal species found at sites surveyed in spring. Mean percentage contribution of each species to total space occupancy (including bare substrate) averaged across all quadrats is given (in bold) with percentage relative abundances of each species (as percentage of total algal cover) shown in brackets.

Species	Braunton	Place	Froe	St Just
*Pterothamnion plumula**	–	–	**0.1** (0.4)	–
Ascophyllum nodosum	–	–	**1.7** (6.0)	**0.6** (0.8)
*Bifurcaria bifurcata**	–	–	–	**0.1** (0.1)
*Blidingia minima**	**0.3** (0.9)	–	–	–
Ceramium sp	**2.6** (7.4)	**3.8** (4.7)	**0.2** (0.7)	**5.5** (7.6)
Chondrus crispus	**7.2** (20.6)	**0.4** (0.5)	–	**4.6** (6.4)
*Colpomenia peregrina**	–	–	**0.1** (0.4)	–
Corallina officinalis	**3** (8.6)	–	–	–
Dumontia contorta	**3** (8.6)	**1.3** (1.6)	**0.5** (1.8)	**6.2** (8.6)
Ectocarpoids	**0.5** (1.4)	**30.9** (38.6)	**12.8** (45.6)	**28.2** (39.1)
Tubular *Ulva spp*	–	**3.9** (4.9)	**0.2** (0.7)	–
Fucus serratus	**3.5** (10.0)	**1** (1.2)	**2.5** (8.9)	**10.7** (14.8)
*F. spiralis**	**1** (2.9)	–	–	–
F. vesiculosus	**7.0** (20.0)	–	**0.9** (3.2)	**2.7** (3.7)
Gracilariales	**2.5** (7.1)	**23.2** (29)	**5.2** (18.5)	**4.7** (6.5)
Osmundea pinnatifida	**0.7** (2.0)	–	–	–
Palmaria palmata	–	–	–	**1.3** (1.8)
Polysiphonia sp	**2.5** (7.1)	–	**0.8** (2.8)	**2** (2.8)
*Sargassum muticum**	–	–	–	**0.3** (0.4)
Filamentous red	–	**1.4** (1.7)	**3** (10.7)	**4.4** (6.1)
Ulva lactuca	**1.2** (3.4)	**14.2** (17.7)	**0.2** (0.7)	**0.8** (1.1)
Bare substrate	**65**	**20**	**72**	**30**
Total species	**13**	**9**	**13**	**14**

*single plant found at one site

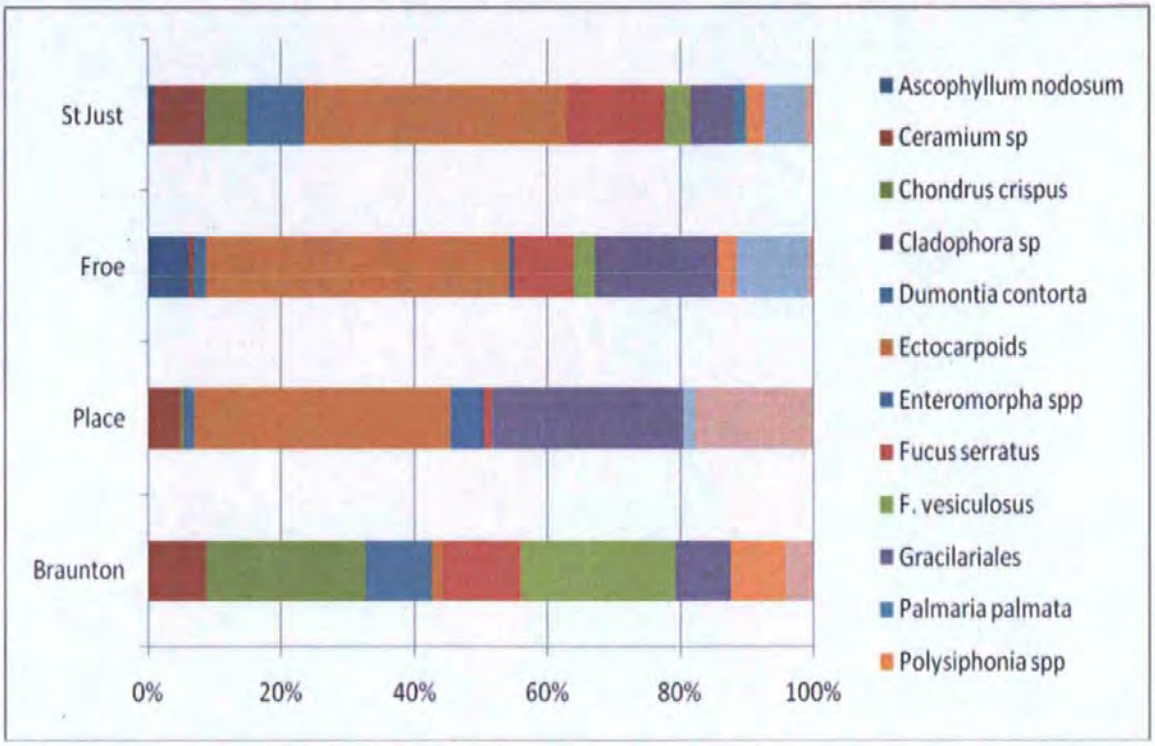


Figure 2.37: Relative abundances (%) of main species of algae found in spring at each site averaged across all quadrats. Ectocarpoids dominate several sites (St Just, Froe and Place) but mainly as an epiphyte on gracilarioids. The key lists species in the order they appear in bars from left to right. *Enteromorpha* in this key relates to tubular *Ulva spp.*

2.3.2.4.2. Algal assemblage data recorded during summer surveys

Tables 2.9 lists the macrophyte species identified at sites surveyed in summer – Looe, Instow, Camel, Helford and Flushing – as mean percentage cover averaged across all quadrats including the percentage mean area of bare substrate, with the relative abundance as a percentage contribution to total algal assemblage.

Helford and Flushing hosted greater numbers of species (12 and 14 respectively), while the two sandy sites in the summer survey, Instow and the Camel, hosted only eight species each. Looe also hosted eight species.

At Instow and the Camel, tubular *Ulva* spp. were dominant (>40% and 77%, respectively). At Instow, Gracilariales were next in importance at nearly 24%. At

Helford, *Ceramium* (23%), *Ulva* (26%) and *Gs. longissima* (21%) were the major species.

Gs. longissima was the dominant species at Flushing but ectocarpoids, mainly as epiphytes on *Gs. longissima*, accounted for 35% of the macrophyte assemblage. *Chorda filum, Lomentaria articulata* and *Polyoides rotundus* were all found uniquely at Flushing.

Table 2.9: Algal species found at sites surveyed in summer. Mean percentage contribution of each species to total space occupancy (including bare substrate) averaged across all quadrats is given (in bold) with relative abundances of each species (as percentage of total algal cover) shown in brackets.

Species	Looe	Instow	Camel	Helford	Flushing
Ascophyllum nodosum	–	**0.6** (1.8)	–	**0.2** (0.4)	–
Ceramium sp	**0.1** (0.2)	**1.3** (4.4)	**0.2** (0.5)	**10.3** (22.6)	**5.5** (8.6)
Chondrus crispus	**8.3** (15.3)	–	–	**0.5** (1.1)	**0.5** (0.7)
Cladophora sp	**0.4** (0.7)	**1.2** (4.0)	**2.1** (5.3)	–	–
*Chorda filum**	–	–	–	–	**0.5** (0.7)
Dumontia contorta	**22** (40.6)	–	–	–	–
Ectocarpoids	–	**0.8** (2.6)	–	**6** (13.2)	**22.5** (35.3)
Tubular *Ulva sp*	**11.3** (20.8)	**12.8** (42.2)	**30.5** (76.9)	**3.3** (7.3)	**0.5** (0.7)
Fucus serratus	**10.5** (19.4)	–	**3.5** (8.7)	**1** (2.2)	**0.5** (0.7)
F. vesiculosus	–	**2.8** (9.2)	**0.7** (1.8)	**1.5** (3.3)	–
*Gracilaria multipartita**	–	–	–	**0.1** (0.1)	–
Gracilariales	–	**7.2** (23.8)	**2.1** (5.3)	**9.5** (20.9)	**24** (37.6)
*Lomentaria articulata**	–	–	–	–	**0.6** (0.9)
Palmaria palmata	–	–	–	**0.4** (0.9)	**0.1** (0.2)
*Polyides rotundus**	–	–	–	–	**1.5** (2.4)
Polysiphonia sp	**0.9** (1.7)	**3.7** (12.1)	–	**1** (2.2)	**4** (6.3)
Porphyra umbilicalis	–	–	**0.5** (1.1)	–	**0.1** (0.2)
Filamentous red	–	–	–	–	**0.5** (0.8)
Ulva lactuca	**0.7** (1.3)	–	**0.2** (0.5)	**11.8** (25.9)	**3.5** (5.5)
Bare substrate	**46**	**70**	**60**	**55**	**38**
Total species	**8**	**8**	**8**	**12**	**14**

*single plant found at one site

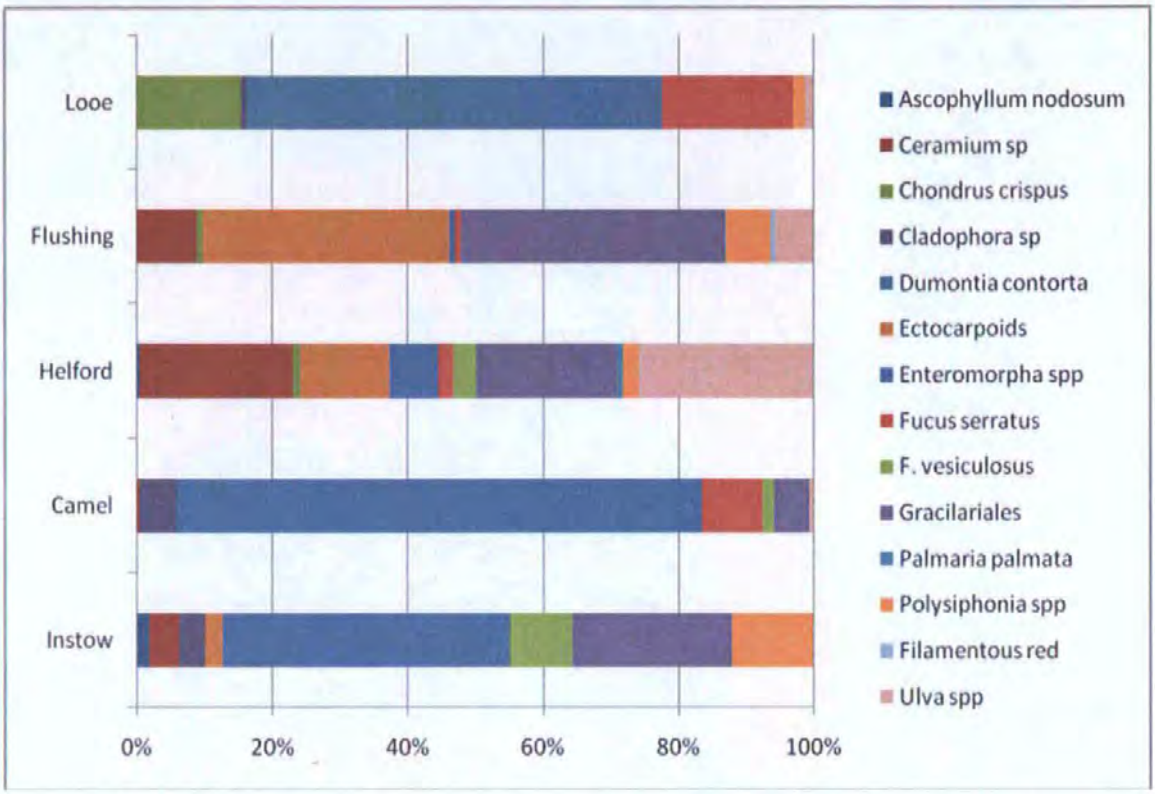


Figure 2.38: Relative abundances (%) of main species of algae found in summer at each site averaged across all quadrats. Ectocarpoids occurred mainly as an epiphyte on gracilarioids. The key lists species in the order they appear in bars from left to right. *Enteromorpha* in this key relates to tubular *Ulva spp.*

Ectocarpoids and Gracilariales dominated at Flushing (22.5% and 24% respectively) while *Ulva* was a major species at Helford (26%). *Dumontia contorta* is an annual species commonly found in spring and occurred in all sites surveyed in spring. However, in summer, *Dumontia contorta* was recorded only at Looe, where it accounted for 41% of macrophyte cover with *F. serratus* (19%), tubular *Ulva* (21%) and *C. crispus* (15%) all major species.

2.3.2.5. Statistical analysis of data

2.3.2.5.1. Statistical analysis of sediment data

An analysis of sediment character using relative mean abundances of size fractions across sites using the ANOSIM program, reveals statistically significant differences between all sites when compared with one another ($p<0.01$) , except

for Flushing and St Just which are not shown to be statistically significantly different from one another ($p=0.3$) at a significance level of $p<0.05$. This suggests some greater similarity between the sediment characteristics of the two sites.

The MDS and cluster plots of similarities between sediment characteristics from each site are shown in Figure 2.39 and Figure 2.40 respectively. These provide a diagrammatic representation of comparisons between sediment sizes for each site analysed with relation to one another and between sites.

Quadrats in St Just seem to be closely clustered, as do those for Flushing and the Camel. This suggests a degree of homogeneity for sediment characteristics within these sites. It can be seen that quadrat data for St Just (yellow diamonds) cluster quite closely among themselves and with a number of quadrats from Flushing which is a reflection of the ANOSIM statistic.

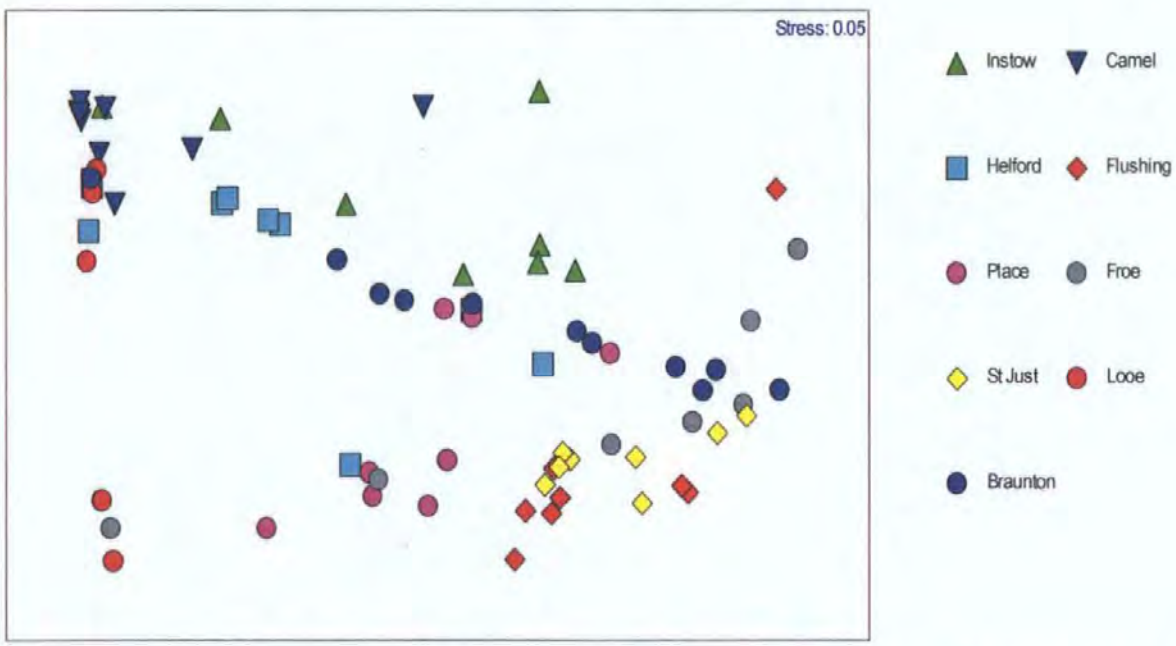


Figure 2.39: MDS plot of sediment comparisons between all sites investigated. Each symbol represents a single quadrat. The spread of the quadrats apparently randomly throughout the plot suggest a high degree of heterogeneity within sites. The apparent pattern created by differences between quadrats within sites does not correlate with quadrat position.

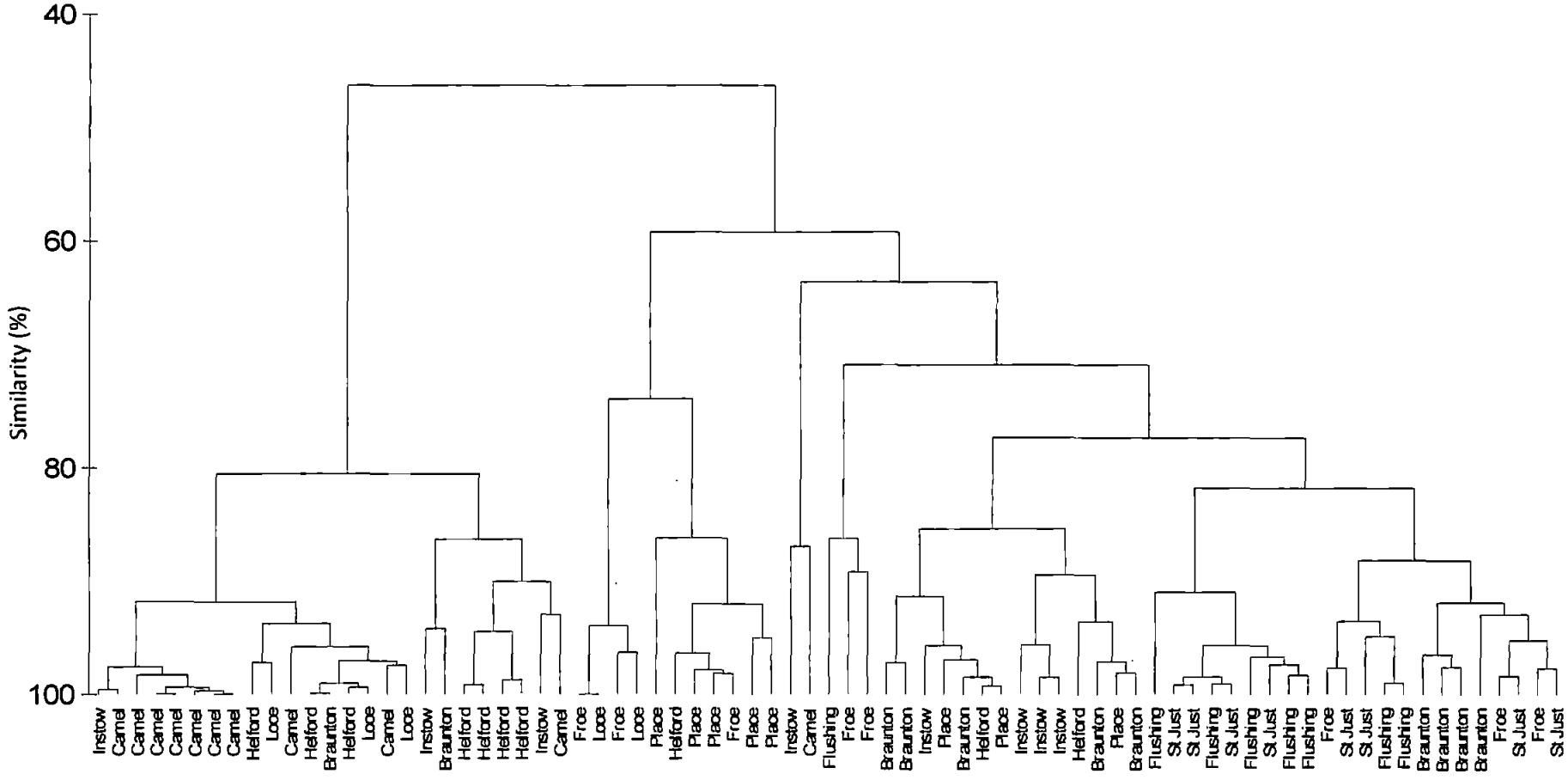


Figure 2.40: Cluster plot of relationships between quadrats from all sites with regard to sediment data. Site quadrats can be seen to be relatively randomly distributed rather than segregated into site, although there are some groupings of quadrats from individual sites such as the seven quadrats from the Camel which are grouped together with >90% similarity between the quadrats.

Quadrats for Braunton, Looe and Instow appear to vary quite significantly within sites suggesting more heterogeneous sediment characteristics across these sites. The presence of widespread mussel beds at Looe makes it very different from any of the other sites.

The cluster plot of sediment data also indicates that, while some sites have several quadrats with very similar properties (e.g., seven quadrats from the Camel are around 95% similar), other sites have individual quadrats that may be very different (e.g., one quadrat from Braunton appears very distant from a group of other Braunton quadrats, sharing only around 45% similarities).

Differences between numbers of quadrats for individual sites used for statistical analysis are due to the omission of those recorded as 100% bare substrate to accommodate constraints of the statistics program used (Clarke and Warwick 2001).

The associations between individual quadrats from different sites show no obvious pattern. This is supported by the analysis of similarity statistics discussed above.

2.3.2.5.2. Statistical analysis of algal assemblage data

A simple analysis of similarities between individual sites showed that algal assemblages were significantly different when comparing across all sites ($p<0.01$ for all comparisons). However, a number of other statistical analyses which indicated other factors affecting the interpretation of data are shown below.

2.3.2.5.2.1. Statistical analysis of spring assemblages

Within-site variation

Degrees of local homo- or heterogeneity of algal assemblages vary from site to site, as illustrated by the percentage distances between quadrats within each site as shown in the cluster plots (Figures 2.41 to 2.44).

Braunton

Similarity between quadrats was high with a 60% similarity between the algal community found at the highest (8, 9, 10) and lowest (1,2,3) positioned quadrats (Figure 2.41).

Place

The cluster plot of algal assemblage data (Figure 2.42) shows no clear relationships between quadrats with regard to position. However, quadrats 4 & 6, which are 5m apart are very similar (75%), while quadrat 5 is more like quadrat 7 despite the distance between them. Quadrats 2, 8,9 and 10 are around 75% similar too.

Froe

Quadrat 8 is seen isolated from all other quadrats sharing only 20% similarity with a group including its closest spatial neighbour, quadrat 9 which is closer in character to quadrat 4 which is more distant in the site. This site also has potentially complex reflooding patterns but quadrats at this site were positioned solely in one streamflow.

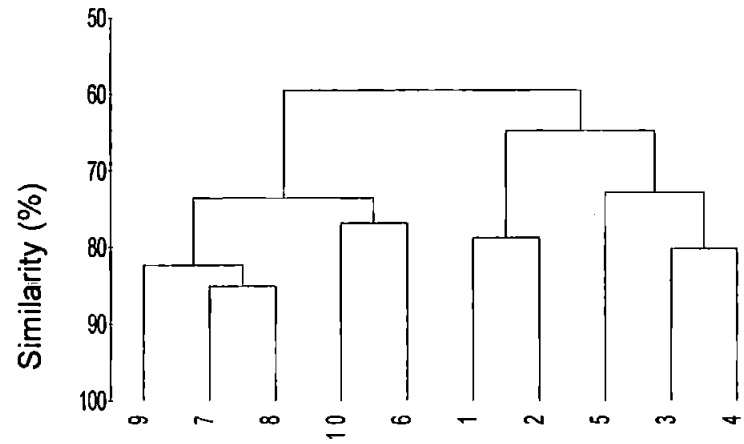


Figure 2.41: Cluster plot for algal assemblage data for Braunton Burrows showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

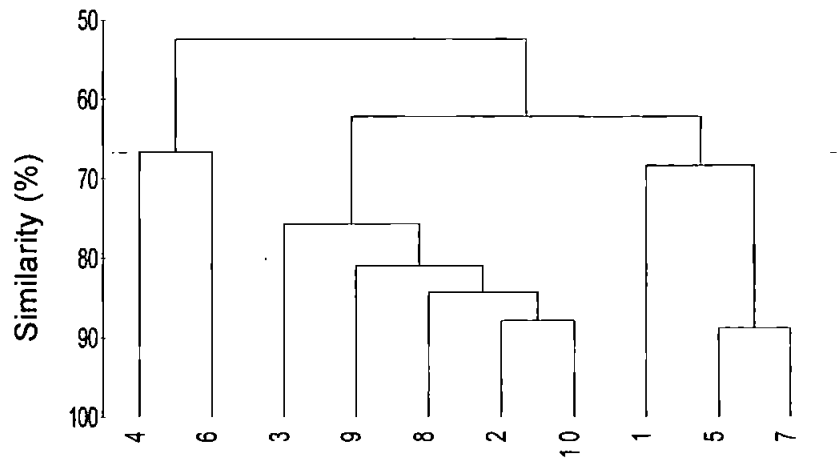


Figure 2.42: Cluster plot for algal assemblage data for Place showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

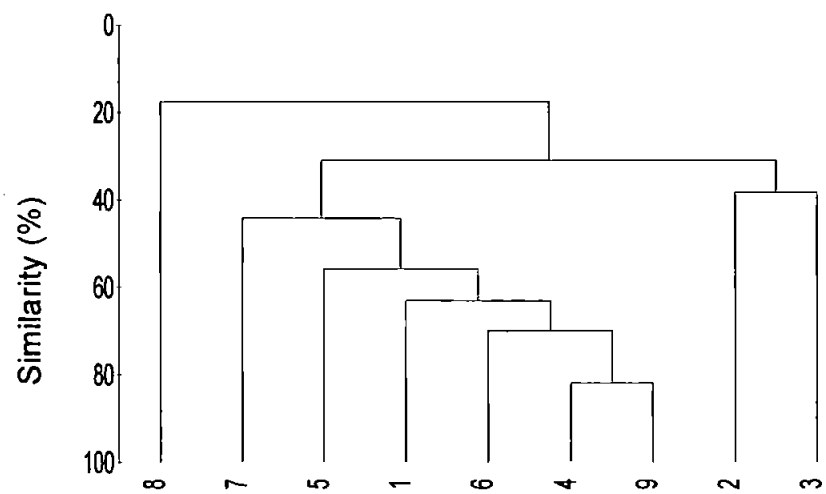


Figure 2.43: Cluster plot for algal assemblage data for Froe showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

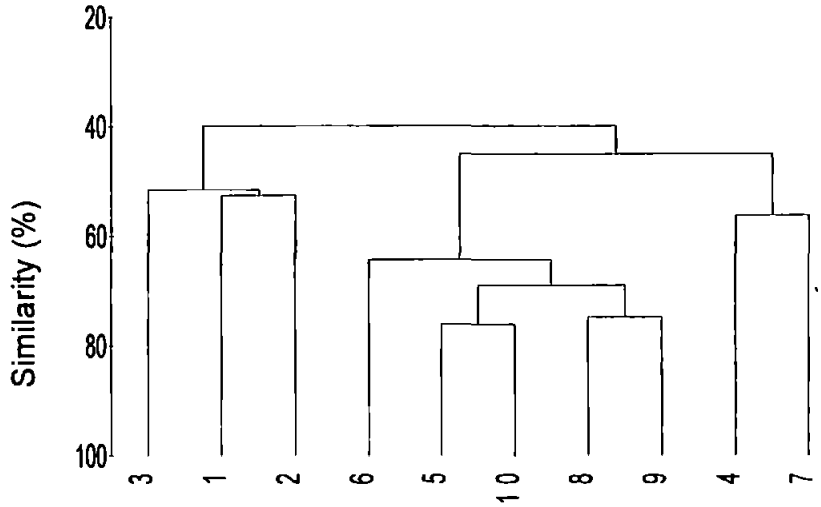


Figure 2.44: Cluster plot for algal assemblage data for St Just showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

St Just

Quadrats from the three different levels of the site fall into loose groups with at least 50% similarity with one another (Figure 2.44). The three quadrats from the top of the site have at least 70% similarity with one another. While quadrat 4 seems to be an outlier, across the site there is a minimum 40% similarity between communities from different parts of the site.

Between-site variation

Analysis of differences between the sites surveyed in spring using the ANOSIM programme making pairwise comparisons in Primer V shows that the algal assemblages found at the different sites were significantly different from one another ($p<0.05$ for all cases Table 2.10).

The cluster plot of similarities between sites (Figure 2.45) illustrates that quadrats from the same site are, mainly, more similar to one another than to quadrats from other sites. For example, 8 quadrats from Froe are closely clustered with a

Table 2.10: Significance values (p) for ANOSIM analysis for pairwise comparisons between algal assemblages recorded at sites surveyed in spring

Site	Braunton	Place	Froe
Braunton			
Place	0.001		
Froe	0.001	0.002	
St Just	0.001	0.001	0.002

similarity of at least 70%. However, some individual quadrats can be very different from other quadrats from the same site. For example, one quadrat from Froe associates closely with a single quadrat from St Just but only shares around 45% similarities with the cluster of eight other Froe quadrats.

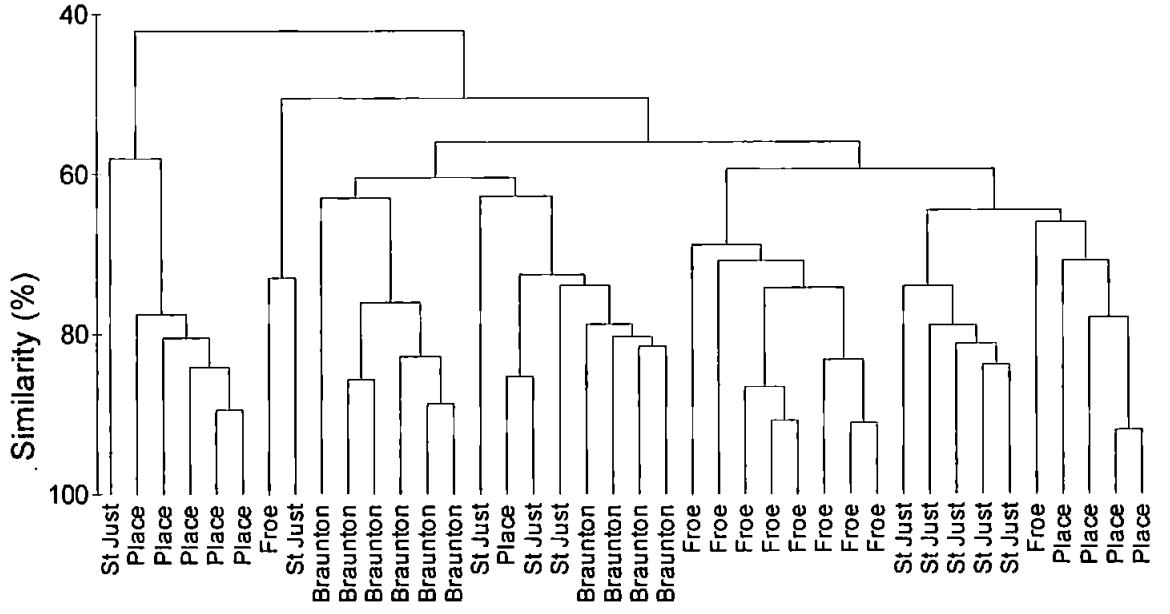


Figure 2.45: Cluster plot of similarities of algal assemblages between sites surveyed in spring (Braunton, Froe, Place and St Just). Sites cluster strongly together and are significantly different from one another ($p<0.05$)

The MDS plot (Figure 2.46) illustrates the data in a more graphic way. Each coloured shape represents an individual quadrat. The outlying Froe quadrat (blue squares) can be seen positioned far away from the main grouping of the remaining quadrats for the site.

The diagram also demonstrates the general similarity of quadrats within sites and segregation of quadrats to their sites with no overlapping of quadrats from different sites suggesting dissimilarity between sites. However, in the centre of the MDS plot quadrats from St Just and Place appear to be mixed together, suggesting that algal assemblages within these two sites may be more alike. Despite this apparent similarity, the ANOSIM analysis shows St Just and Place to be significantly different ($p=0.001$).

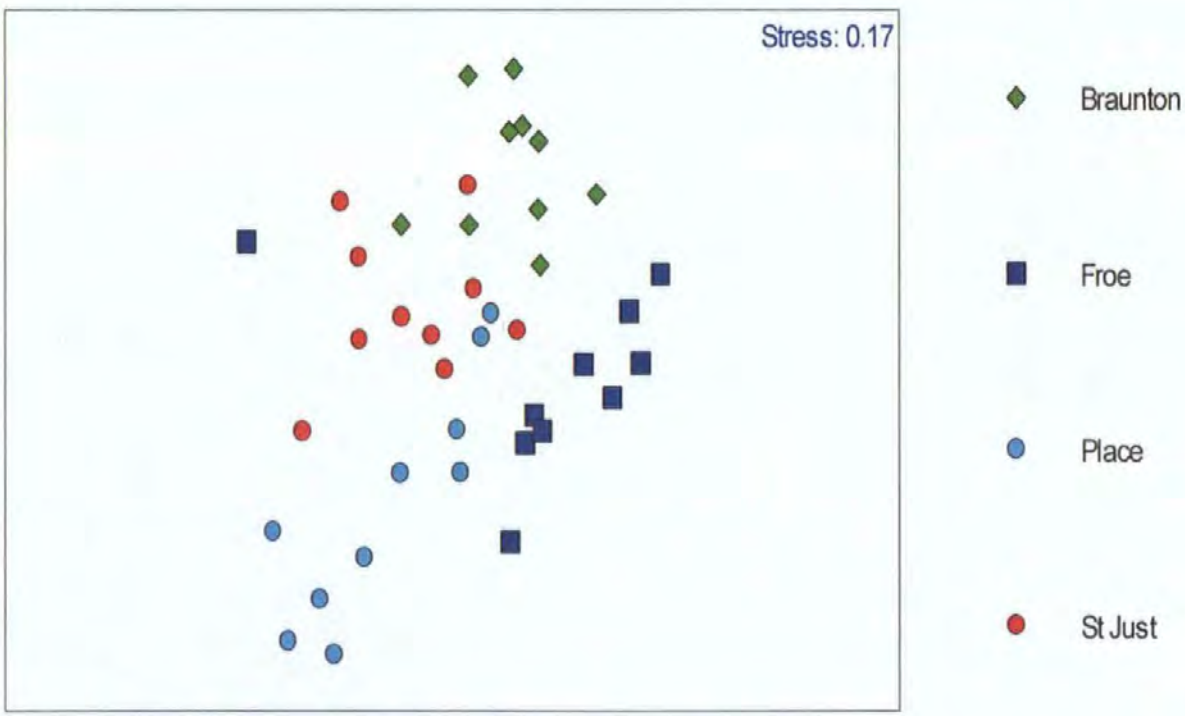


Figure 2.46: MDS plot of similarities of algal assemblages between sites surveyed in spring (Braunton, Froe, Place and St Just). Sites cluster strongly together and are significantly different from one another ($p<0.05$)

2.3.2.5.2.2. Statistical analysis of summer assemblages

Within-site variation

Instow

Figure 2.47 shows that some quadrats at Instow (2,7 & 8) showed remarkable degrees of similarity (90%) but the two closest quadrats on the low shore (1 & 2) were seen to be much less similar (40%), with quadrat 2 clustering with three higher shore quadrats (6,7& 8). Quadrats 5 and 6 (within 5m of one another) also clustered more closely with quadrats from different heights on the shore than with each other (Figure 2.47).

Camel

The cluster plot (Figure 2.48) shows similarity between quadrats ranging from around 60% between two of the lowest shore quadrats, 1 and 2, approaching 95% for quadrats 3 and 4, and quadrats 9 and 10.

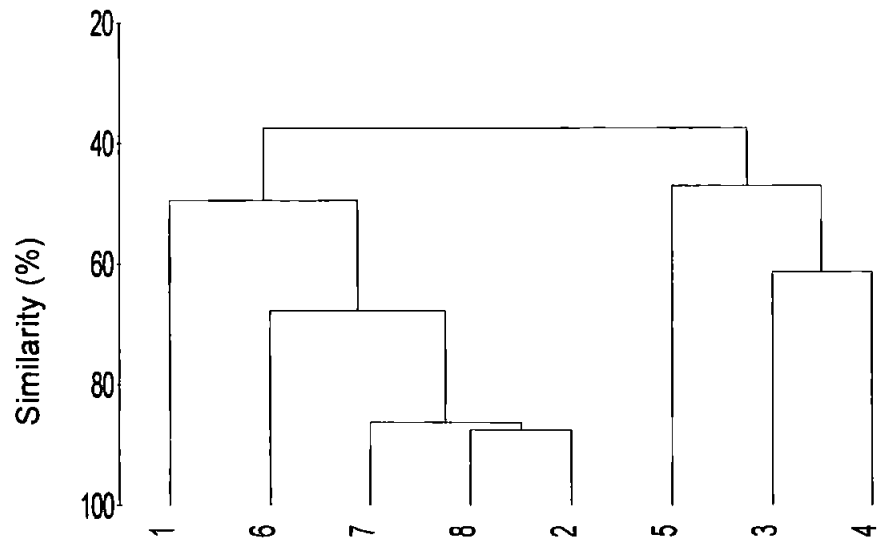


Figure 2.47: Cluster plot for algal assemblage data for Instow Sands showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

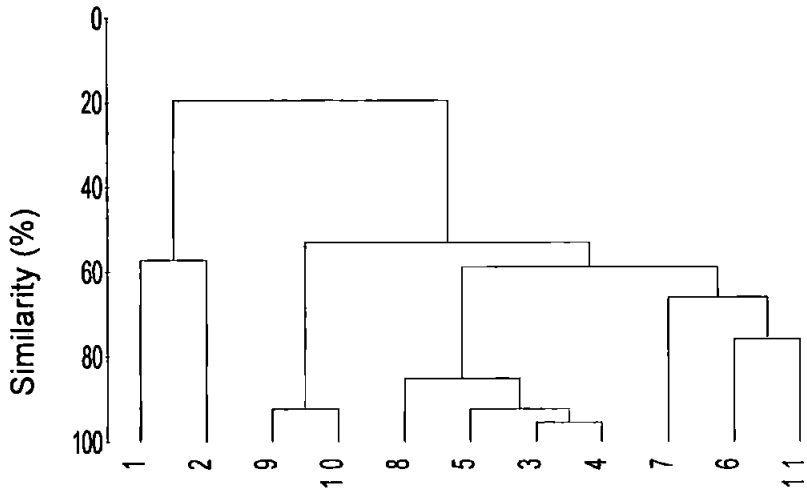


Figure 2.48: Cluster plot for algal assemblage data for Camel showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design). The additional quadrat surveyed (11) is included.

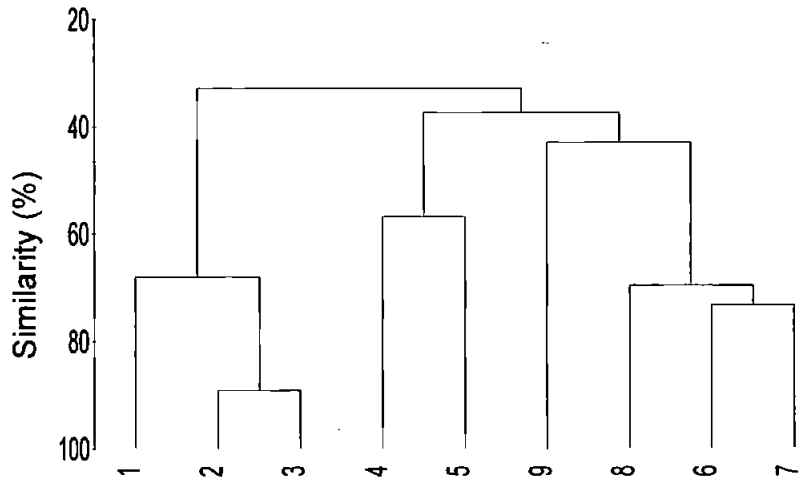


Figure 2.49: Cluster plot for algal assemblage data for Helford showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

Helford

From the cluster plot (Figure 2.49) it can be seen that levels of heterogeneity within the site were high with only two quadrats (2 & 3) showing a high degree of similarity (90%). Quadrats 6, 7 and 8 were clearly grouped together and quite distant from other groups (e.g., 4 & 5). Quadrats 4 and 5 were only 5m apart at same shore level but were only 55% similar.

Flushing

As can be seen from the cluster plot (Figure 2.30), the algal community at Flushing is up to 95% similar between quadrats in similar positions (quadrats 7 & 8) while quadrats at the top of the site, e.g., quadrat 10 is around 40% similar to that at the lowest (quadrat 1).

Looe

The cluster plot for Looe (Figure 2.51) shows that quadrat 7 was the only one at Looe which did not have any mussels present, which probably explains its position as an outlier with a 45% similarity with the remaining quadrats for the site.

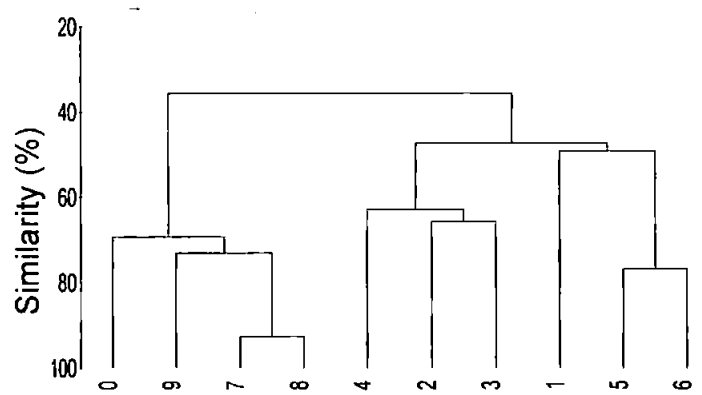


Figure 2.50: Cluster plot for algal assemblage data for Flushing showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

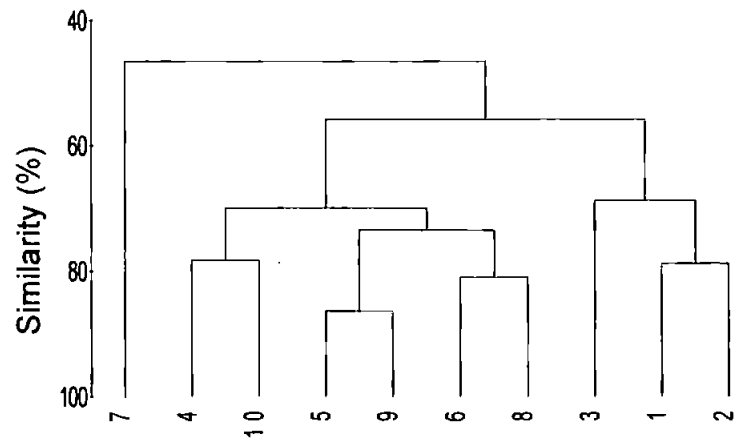


Figure 2.51: Cluster plot for algal assemblage data for Looe showing percentage similarity between algal assemblages found in each quadrat (numbered as shown in sampling design)

Between-site variation

Analysis of differences between the sites surveyed in summer using the ANOSIM routine making pairwise comparisons in Primer V shows that the algal assemblages found at the different sites were significantly different from one another ($p<0.05$) except for the comparison between Instow and The Camel sites (Table 2.11). The algal assemblages at these two sandy sites do not differ significantly if applying a significance level of 95%. However, the probability value (p) rises to only 0.09, still giving a 90% probability that the sites are significantly different with only a 10% probability of the sites being genuinely similar.

Cluster and MDS plots (Figures 2.52 and 2.53) illustrate that the quadrat data for algal assemblages within sites are, on the whole, more similar to each other than to quadrats from other sites, although there is some apparent clustering for Instow and the Camel, which might be expected in view of the ANOSIM results. However, while the cluster plots have quadrats from the two sites mixed together, there is no overlapping, which might be expected where there are similarities between sites. In fact, there appears to be more clustering (Figure 2.52) and mixing (Figure 2.53) between Flushing and Helford, while the ANOSIM statistics support the hypothesis that these two sites are significantly different.

Table 2.11: Significance values (p) calculated by the ANOSIM routine for pairwise comparisons between algal assemblages recorded at sites surveyed in summer

Site	**Instow**	**Camel**	**Helford**	**Flushing**
Instow				
Camel	0.09			
Helford	0.004	0.001		
Flushing	0.001	0.001	0.05	
Looe	0.001	0.001	0.001	0.001

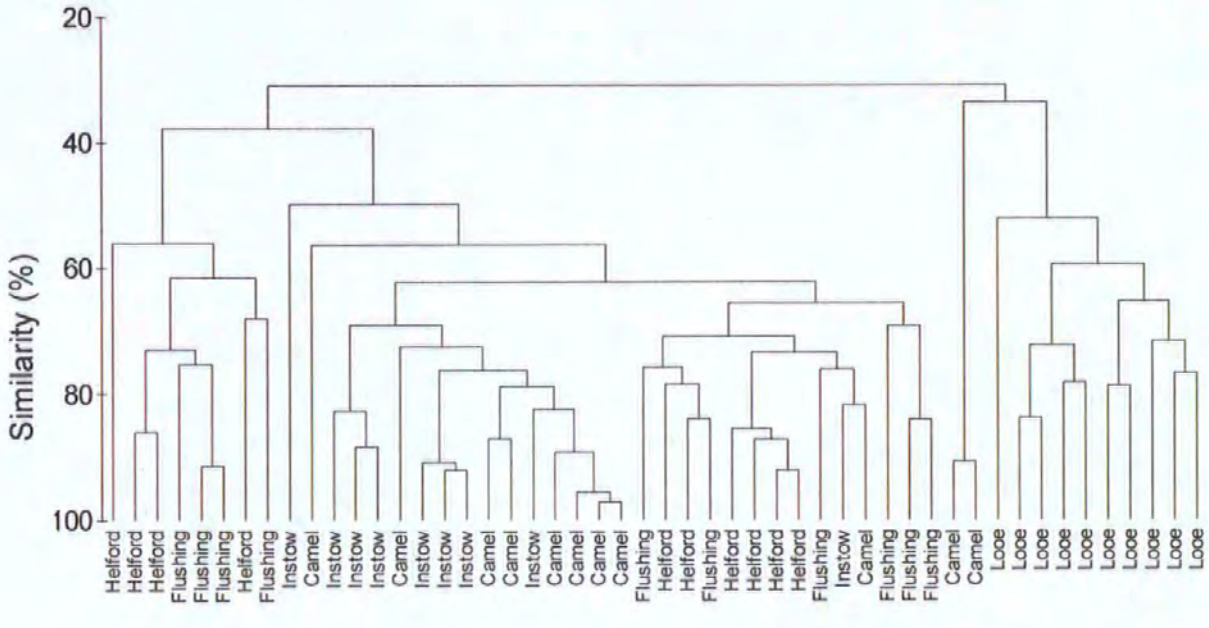


Figure 2.52: Cluster plot of similarities between algal assemblages between sites surveyed in Summer (Instow, Camel, Helford, Flushing and Looe). Some mixing of quadrats can be seen, in particular between Camel and Instow. Looe segregates strongly from the other sites.

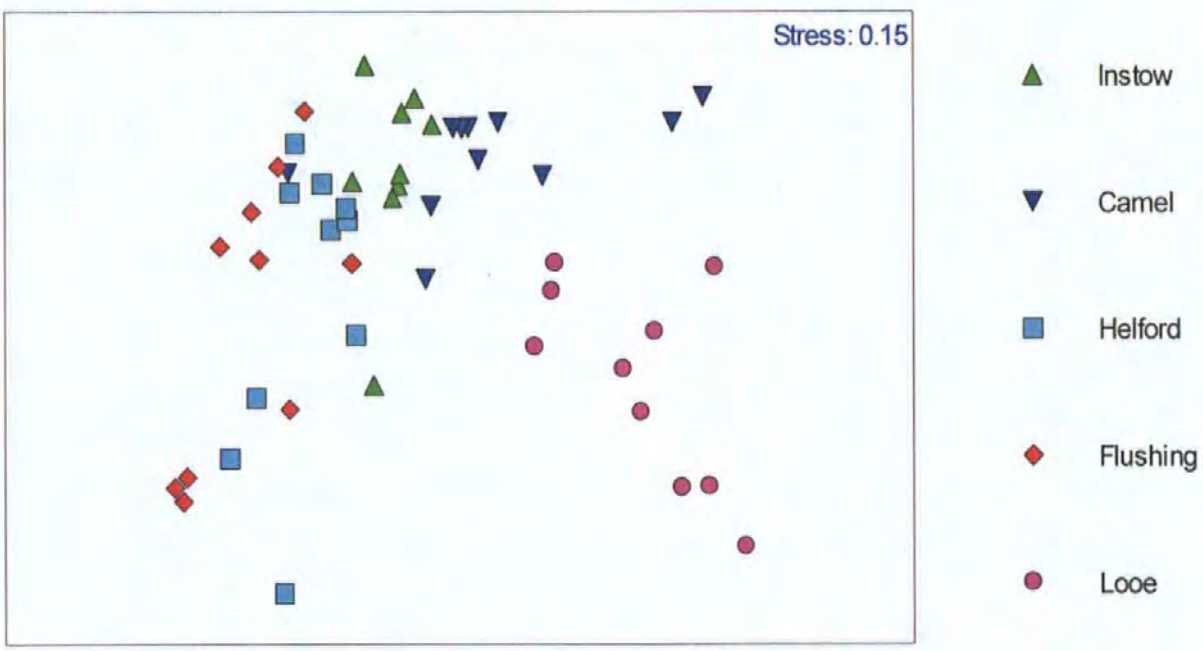


Figure 2.53: MDS plot of similarities of algal assemblages between sites surveyed in Summer (Instow, Camel, Helford, Flushing and Looe). Each symbol represents a single quadrat.

2.3.2.5.2.3. Statistical comparison between seasonally grouped sites

Analysis of similarities (ANOSIM) using season as the factor for calculation (Primer V) gives a p value of 0.001. This supports the hypothesis that spring and summer algal assemblages are significantly different from one another (at a confidence limit of $p<0.05$).

The MDS and cluster plots of data from all the sites (Figures 2.54 and 2.55) demonstrate a degree of segregation between spring and summer algal assemblages. As was seen in Tables 2.8 and 2.9 and Figures 2.37 and 2.38, the presence or absence of some species could have a large effect on the data. For example, *Dumontia contorta*, which is usually only seen in its erect form, and therefore recorded, in spring and early summer, was recorded as more prevalent at sites surveyed in spring (Table 2.7) and will contribute to the seasonal differences seen.

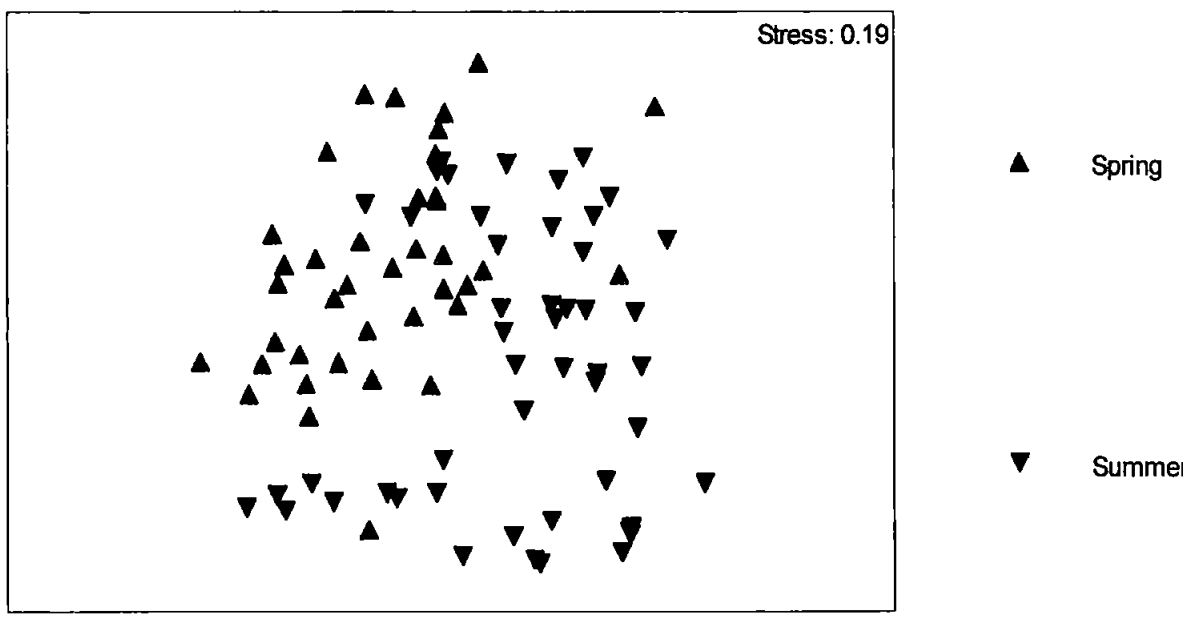


Figure 2.54: MDS plot of similarities of algal assemblages for sites surveyed in spring (green triangles) compared with those surveyed in summer (blue triangles). Each symbol represents a quadrat.

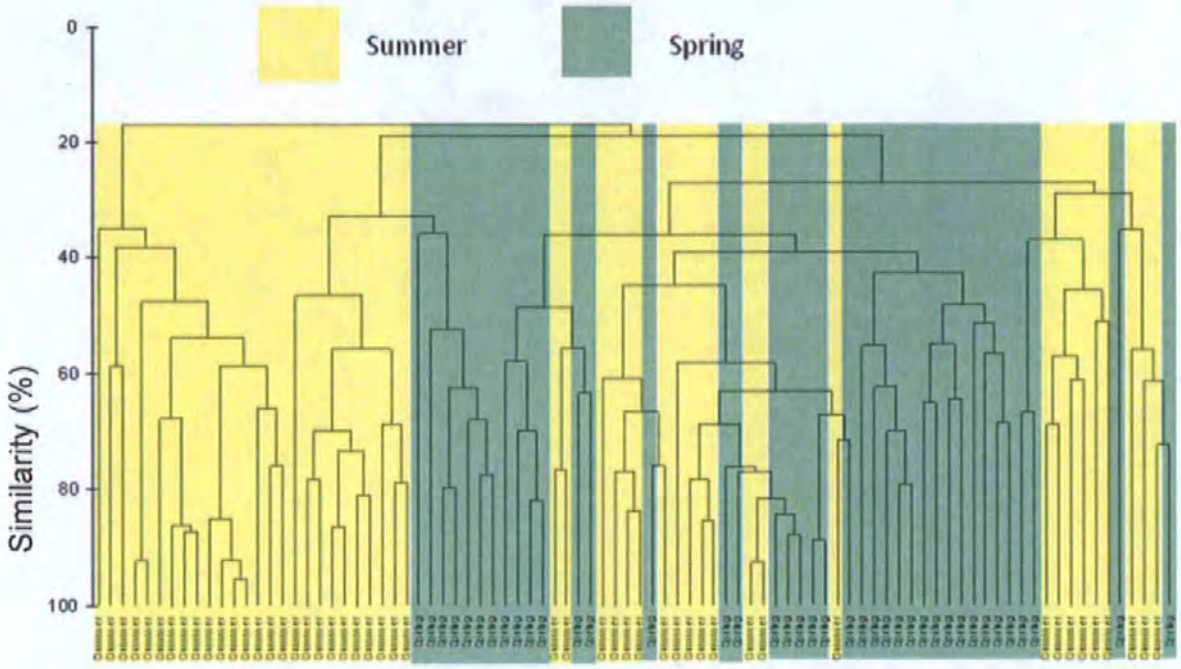


Figure 2.55: Cluster plot of similarities of algal assemblages between sites surveyed in spring (green) and those surveyed in summer (yellow). Site quadrats are each labelled with the site name.

Although there appears to be a certain amount of clustering of the two seasonal groups, with significant differences between all sites, it might be unwise to assume that the spring and summer differences were purely due to season rather than site.

Apparent seasonal differences, suggested by the ANOSIM analyses, are possibly compromised by being potentially confounded with other between-site differences in character, such as exposure or nutrient levels, since each site was only sampled in one season.

2.3.2.5.2.4. Statistical comparison of sites hosting unispecific Gs. longissima *populations with sites hosting both gracilarioid species*

ANOSIM pairwise comparisons of sites where *Gs. longissima* occurred unispecifically with sites where it grows alongside populations of *G. gracilis*,

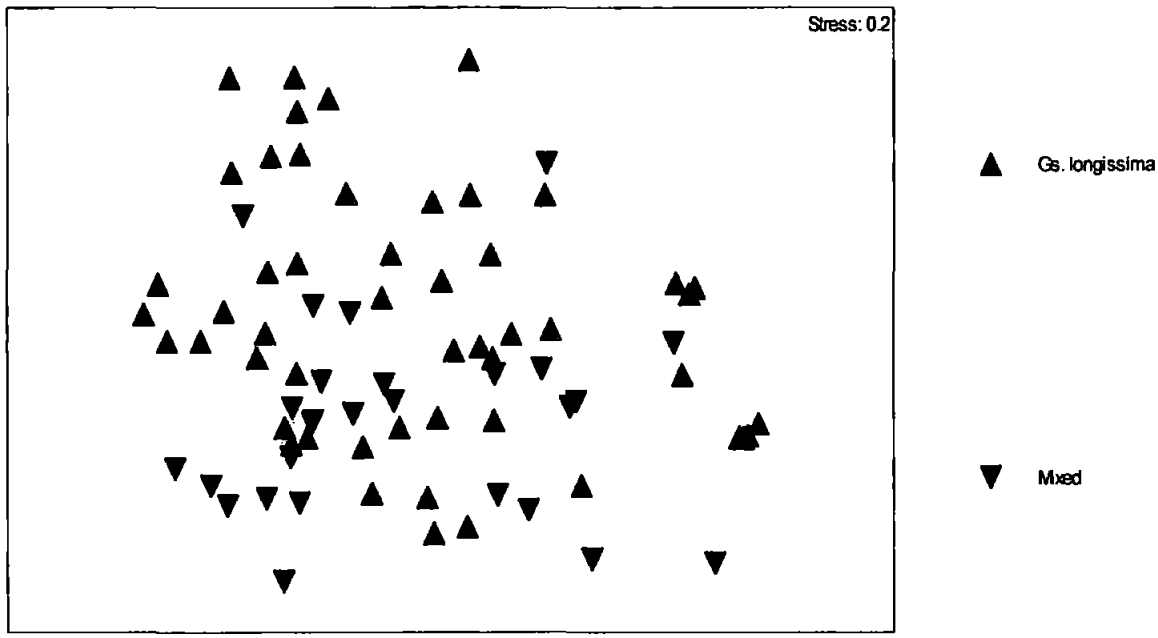


Figure 2.56: MDS plot of all floristic data from all sites shown as symbols according to whether *Gs. longissima* was present unispecifically (green triangles) and in mixed populations with *G. gracilis* (blue triangles). Each symbol represents a quadrat. The sites with *G. gracilis* do not appear to segregate significantly from those hosting *Gs. longissima* unispecifically.

indicate that there is no significant difference between these categories of site (p=0.4). Figure 2.56 is an MDS plot which illustrates the unsegregated nature of sites with regard to whether or not they host one or both species of gracilarioid.

2.3.2.5.2.5. Statistical analysis of correlation between sediment characteristics and algal assemblages

Despite the significant differences in sediment characteristics between all sites apart from Flushing with St Just, as seen above, no correlations could be found between sediment differences and floristic ones (maximum Spearman rank correlation = 0.164), as calculated by the BIOENV program in PRIMER.

Therefore, sediment characteristics did not appear to be the cause of floristic differences between sites.

2.3.2.6. Animal biota found at all sites

Although animal biota were not specifically surveyed, it was noted that Looe had up to 95% of substrate occupied by *Mytilus edulis*. Mussels were not present at any other site, except Instow where <5 were recorded in two quadrats.

At Helford, populations of *Lanice conchilega* were present at high densities in most quadrats (10-150 individuals per m^2). These Terebellidae and at least two species of sabellid tube worm were found in varying numbers (1-10 per m^2) at other sites (Froe, St Just and Place). An orange sponge (*Suberites* sp.) was found in low numbers (<1 per m^2) at two sites (St Just and Place).

2.3.3. Discussion

With almost no information about wild populations of *Gs. longissima* formerly available, the data presented here raises many questions for further research.

2.3.3.1. Distribution of gracilarioids within sites

Gs. longissima is considered to be a mainly sub-tidal organism (Steentoft *et al* 1995, Steentoft and Farnham 1997) and it certainly appears to survive only if immersed (personal observations). In both unispecific and mixed populations, gracilarioids were found throughout sites where either standing or flowing water remained. They did not appear to tolerate desiccation as individuals were never found emersed except on very rare occasions of extreme low water when exposure would possibly be for just minutes rather than hours. How long either species can withstand desiccation is not known.

The abundance of *Gs. longissima* at the highest points of most sites (furthest from the open sea or low tide mark) in both shallow pools and running streams,

suggests that it successfully tolerates a wide range of temperatures, salinities and light levels. From the measurements made in this study, *Gs. longissima* individuals situated in stream flow, while subjected to low salinity (e.g., 13.5‰ at St Just), experienced less temperature fluctuation – the water temperature was 16.1°C at 2pm on a hot summer's day (ambient air temperature 23°C). Experiments to establish environmental ranges for this species would provide useful information.

A question arises why both species can be found at Froe and Place but only *Gs. longissima* at St Just. With no correlations between any of the abiotic factors and algal assemblages, and no significant differences in abiotic factors between sites identified, it is not possible to identify the reasons why some sites have both species and others have only one.

Neither species colonised all sites which appeared to be suitable for gracilarioids, i.e., those where at least one species was found. This may be due to specific conditions which were not apparent, such as local micro habitat differences, failure of dispersal or survival, competition or predation. The ability of both *Gs. longissima* and *G. gracilis* to tolerate sites with highly mobile substrates and silting which commonly covered algae, may confer some competitive advantage over other species which are less able to tolerate low light and burial.

Although not within the scope of this study, it would be interesting to determine the distributions of the two gracilarioid species within each site where they co-occur to investigate whether they are randomly mixed or differentially distributed within sites.

2.3.3.2. Physical habitat characteristics

The main features of the climate of the South West Peninsula are an early spring, with a prolonged summer, later autumn, and short winter. Climatic conditions for all sites are similar (www.metoffice.com). However, local variation in estuarine and open sea sites can be extreme. As with most intertidal zones, parameters such as temperature, light and salinity, can fluctuate widely from day to day and season to season.

Local differences measured one day, as reported here, may give a very biased picture of differences between sites. The fact that none of the parameters measured was correlated with algal assemblage data might change with a full range of environmental data available.

All floristic data in this report was collected from immersed populations and the conditions experienced by those in flowing water versus standing pools may be vastly different. Plants in flowing water are likely to be buffered from extremes of temperature and from raised salinities, although they may experience lowered values. Those found in standing water may be subject to fast and large temperature changes.

As Dring (1982) points out, in a particular locality, EL(H)WS tides will occur at roughly the same time of day throughout the year. If that time is early in the morning or evening, emersion conditions are likely to be less dramatic than if that time falls in the middle of the day when the sun is at its strongest. In the study area, ELWS tides are normally experienced in the middle of the day. This will have a significant effect on the light, temperature and salinity ranges experienced by the

intertidal algae. Exposure times for *Gracilariopsis longissima* plants will also vary with datum point on the shore. Plants low down might only experience 30 min of extreme temperature, salinity, pH or light, while those high on the shore might have to withstand extreme conditions for around 4 hours (personal observation).

Measurement of dissolved organic carbon and nutrients might provide useful information for discovering the determining factors for differences between sites.

2.3.3.3. Sediments

Substrate characteristics, though not shown to be significant with relation to algal assemblages, do vary between sites. Many sites had mobile sediments with very fine particles which easily covered algal surfaces, as could be seen from some quadrat photographs.

The presence of widespread, unbroken beds of *Mytilus edulis* at Looe was not repeated anywhere else in the study except the River Taw, upstream from the survey sites at Instow and Braunton. Mussels are major competitors of intertidal seaweeds (Kain and Norton 1990) and Buschmann *et al* (1995) reported that mussels outcompeted commercial beds of *Gracilaria* (species not reported) in Chile. However, no data reported here is able to correlate the presence of mussels at Looe with the absence of *Gs. longissima.*

2.3.3.4. Algal assemblages

The seasonal nature of algal assemblages is well documented (Dring 1982, Lobban and Harrison 1994) and this researcher had observed apparent differences in assemblages when visiting sites on other occasions. For example,

at St Just in early spring, it was observed that *Dumontia contorta* was present with fewer *Gs. longissima* than found at other times of year. Subsequently, at the same site, in autumn, a subjective assessment was made that *Gs. longissima* was dominant in all strong water flow areas.

The data reported here was divided into seasons to remove effects of season as far as possible. However, it should also be borne in mind that the region of the study enjoys a mild climate with a smaller range of temperature variation between seasons than other parts of Britain which may reduce the effects of season on species presence and absence within algal assemblages.

Notwithstanding possible local climate effects, further surveys covering all seasons over at least a two-year period would provide a much more informative set of data for site comparisons of the algal assemblages and potentially provide more opportunities for linking data to determining factors.

The sites which appeared to have high levels of within-site heterogeneity, such as Froe and Flushing, suggest that there are patterns of algal distribution which were not identified by the sampling regime used here. Notwithstanding the seasonal effect, the significant difference seen between almost all sites, suggests high degrees of heterogeneity in the fluctuating conditions of estuarine sites or those subject to highly variable environmental conditions. A wider-ranging biotic investigation may reveal details of the relationships affecting community structures.

Competition for space is commonly held to be the main factor influencing sessile biotic assemblages. However, in many of the sites investigated, large areas of bare substrate were seen. Potentially, the ability of *Gs. longissima* to use small stones and shells which subsequently appear to anchor in soft sediments, may allow it to colonise very mobile sediments which other species find hard to do. Whether competition or some other factor creates the conditions that allow it to settle and colonise only small pieces of substrate is unclear. *Gs. longissima* was observed in some sites (Froe, Instow, Helford, Place) to be a major coloniser and provided the main substrate for epiphytic ectocarpoids. Markers of plastic pipes with luminous orange tapes attached, placed in sites to locate permanent quadrats (St Just and Place only), were colonised by many species of algae but never by gracilarioids (personal observation).

Field ID skills are a pre-requisite for accurate recording of species abundance on site. Without these skills, the quality of the data may be compromised. Identification and quantification of rarer species that contribute to the overall community structure might have generated useful additional data.

Extensive areas of bare substrate were unhelpful components of quadrat data. Mussels are known to affect the ability of algae to establish (Durr and Wahl 2004), which may explain the significant differences between Looe and all other sites. However, separate investigations to identify and correlate any specific interactions at Looe would be required. Removal of areas of *Mytilus edulis* and examination of subsequent colonisation would answer the question of whether this is the causative factor in the absence of specific algae.

2.3.3.5. Statistical analyses of data

Lobban and Harrison (1994) warn of the difficulties of obtaining meaningful data describing seaweed communities. In particular, they highlight the problem of gathering data randomly.

It should be considered that the number of sample points scored in the present study may have not been sufficient to reveal underlying patterns. The choice of recording only easily identified dominant macrophytes may have lost important information about algal communities or animal communities associated with them which might account for differences seen between sites. Also, a more comprehensive and clearer picture of the algal communities on the sites may have been better produced by using a more conventional approach to sampling design, such as several line transects from low to high water marks through areas of continued immersion at low water. Accurate data of available time between tides could be helpful. GPS instrumentation for surveying larger sites would be invaluable.

Relative abundance measurements of conspicuous organisms, e.g., large macrophytes, are likely to be overestimates and to underestimate the contribution of inconspicuous species. Also, the data is based on subjective estimates which might change with different observers. However, it is a useful technique for initial studies of patterns of distribution, as reported here.

2.4. Conclusions

New information about the distribution and ecology of *Gs. longissima* as a British species has been recorded which can definitely be attributed to the species.

However, the data reported here represents only a snapshot. The lack of correlation between algal assemblage data and sediment characteristics, despite both sets of data showing significant differences for a variety of sites, needs further investigation. Structures of marine algal communities are determined by environmental conditions, competition, herbivory, physical forces and substrate characteristics, along with levels of recruitment and survival. A deeper understanding of these factors, as they affect the sites and species studied in this report, would be useful.

Gs. longissima can be found in algal assemblages dominated by ectocarpoids (Froe, St Just) or *Ulva* (Instow, Camel) or can dominate assemblages itself (Helford). However, the specific conditions which regulate those assemblages need to be examined further. Figures for ploidy frequencies, differences between the structures of populations in terms of life-history stage, seasonal effects and identification of males would be of particular interest. Interesting questions are also raised about the causative factors for *Gs. longissima* and *G. gracilis* to occur together or unispecifically.

Algal assemblages can be used to assess the health of ecosystems (Wells *et al* 2007), so new data for sites around Britain all add to the potential for their use as research tools. By establishing a benchmark set of data, future surveys can be compared with these data to investigate changes to the ecosystems around the Fal Estuary and other sites, which may be of interest to local environmental management programmes.

Chapter 3:
A quantitative evaluation of substrate size preference in populations of *Gracilariopsis longissima* from the South West Peninsula of Britain

3.1. Introduction

Attaching to suitable surfaces or remaining mobile are potential alternative strategies of marine organisms. Different species of red algae adopt both strategies (Kain and Norton 1990). In some cases, the same species may apparently adopt either strategy. For example, both attached and apparently floating populations of *Gracilariopsis longissima* had been identified in this study (Chapter 2) and Iyer *et al* (2004) found *Gs. longissima* in South Africa anchored in sediment with no apparent holdfasts.

In describing *Gs. longissima* and its habitat, Steentoft *et al* (1995), proposed that the species is likely to be found attached to small pebbles and shells. In 1997, Steentoft and Farnham noted that *Gs. longissima* may also occasionally be found attached to bedrock and boulders in summer in the intertidal but stated that the species is more likely to be found submerged, attached to mobile sediments. They also suggested that *Gs. longissima* is more likely to attach to mobile substrata than *Gracilaria gracilis* with which it often co-occurs. They proposed that the variable ability of both species to settle and survive on mobile sediments accounts for the wider distribution of these two species compared with other species of *Gracilaria* (for example, *G. bursa-pastoris* and *G. multi-partita*).

As has been seen in this study, *Gs. longissima* successfully colonises sites with significantly different sediment characteristics, with a wide variety of particle sizes

available for attachment (Chapter 2, Section 2.3.2.1.3.). The aim of this study was to test whether *Gs. longissima* preferentially attached to specific particle sizes when presented with a variety of sizes.

3.2. Materials and Methods

3.2.1. Sites

Three sampling sites around the South West Peninsula of Britain – Helford, St Just and Braunton – were chosen on the basis of two criteria.

(1) Each site hosted populations of *Gs. longissima* where it occurred as the only gracilarioid present.

(2) The sediment characteristics of each site with regard to size had been shown to be significantly different from one another (Chapter 2, Section 2.2.2.3.1.). Qualitatively, the sediment at Helford had been found to be mainly fine sand with silt while St Just was characterised by stones with silt and gravel. Braunton was a site with large areas of exposed bedrock and areas of shale, pebbles and gravel (Chapter 2, Table 2.6).

3.2.2. Sampling

Each site was visited during extreme low water spring tides (ELWS) to allow maximum time on the shore. At each site 30 1 m^2 quadrats were sampled. The positioning of sampling quadrats was determined by two factors: (1) *Gs. longissima* only occurs immersed and (2) areas of water remaining during low tide occurred non-randomly within sites.

Taking into account these factors, the first quadrat (quadrat 1) at each site was randomly thrown along the low water mark at the most seaward position

accessible. The remaining 29 quadrats were then positioned haphazardly by walking randomly away from the original quadrat landward in each site at least 5 metres from the previous quadrat, staying within water-covered areas, covering as much of the site as possible, until a total of 30 quadrats had been sampled, ending as close as possible to the upper limit (landward) of the *Gs. longissima* population on the site.

In each quadrat an estimation of the percentage surface area occupied by each of eight sediment size categories (2 mm, 2.8 mm, 4 mm, 5.6 mm, 8 mm, 16 mm, 32 mm and 64 mm) was made subjectively by eye using a see-through sizing gauge (Figure 3.1) as a guide. These size classes were chosen as they represent the normal size range used for the assessment of sediment characteristics and are feasible measurements to make in the field. Larger sizes were, for the purposes of this study, classed as bedrock.

Figure 3.1: Photograph of a study plot at Braunton, showing 1 m^2 buoyant plastic quadrat used to collect sediment size data with the sizing gauge, used to estimate percentage surface area occupied by the different size categories of substrata, visible in the bottom left hand corner.

In each quadrat, at least 10 specimens of *Gs. longissima* were collected, each with its attachment stone or shell, taking care to avoid separation of the plant from its substrate. Specimens were transported back to the laboratory where they were rinsed to remove unattached detritus. Where multiple plants were attached to a single substrate, these plants were counted. Plants were then removed and the stones left to dry before being measured by size class as described in Table 3.1.

3.2.3. Analysis of data

The data was analysed using a Chi-square goodness-of-fit test (X^2) to determine whether *Gs. longissima* occurred randomly on all sizes of sediment available (H_0, the null hypothesis) or whether, in fact, it occupies substrates in a non-random way.

For each site, the data was prepared for input to the Chi-square routine in the Minitab 15 statistical program by the following steps:

(1) calculation of the mean percentage surface area for each sediment size class for the site (data from the 30 quadrats);

(2) calculation of the number of plants found attached to each size class (Observed frequencies) and overall;

(3) calculation of the (Expected) frequencies of plants attached to each size class, according to the formula below:

(Estimated % surface area for size class X total no. of plants for all size classes)/100

This apportions the total number of plants in all quadrats to each particle size class in proportion to the estimated percentage surface area for each size class.

The X^2 routine in Minitab was then used to calculate the contribution of each category (size class) and the overall value of X^2.

3.3. Results

3.3.1 Sites

Gs. longissima specimens were often found with their attachment point buried up to 3 or 4 cm deep (personal observation). At Braunton Burrows, with underlying solid rock and pools with deep gravel/pebble substrata, it was difficult to dig down to find the origins of attachment.

The general characteristics of the three sites used in this experiment are described in Chapter 2 (Section 2.3.2.1.). Maps of each site, illustrating the approximate extent of remaining water cover during low tide are also given in Chapter 2 in Figure 2.13 (Braunton), Figure 2.16 (St Just) and Figure 2.20 (Helford).

3.3.2. Sample data

Sediment particles at or below 2 mm were well represented at all sites (Braunton >25%, St Just 10% and Helford <18%) but attachment to this category was virtually absent with only three plants out of a total of 1486 attached to particles in this size category.

To allow concentration of the analysis on more commonly colonised size categories, the 2 mm size class was omitted from the calculations. The mean estimated percentage surface area occupied by sediment size categories for each site, omitting 2 mm data, calculated from the data from all 30 quadrats at each site are given in Table 3.1.

Table 3.1: Mean estimated percentage surface area occupied by each sediment size category at each site, calculated from 30 samples

Size category (mm) (maximum diameter)	Mean estimated surface area occupied (%)		
	Helford	St Just	Braunton
2.8	23.28	9.81	12.33
4.0	14.58	9.53	9.42
5.6	15.18	9.91	8.97
8.0	12.96	23.14	9.19
16.0	15.38	29.08	21.52
32.0	12.55	16.11	21.75
64.0	6.07	2.41	16.82
Total	100	100	100.00

During the course of data collection, it was noted that there were a number of *Gs. longissima* individuals found buried in sediments at both Helford and St Just but without being attached. These individuals were not included in the study. Many specimens found attached were also buried to some degree with their attachment substrate and had to be dug out.

Gs. longissima was not found epiphytic on other algal species at any of the sites investigated. However, at Helford, it was found occasionally attached to the cases of tube worms. This observation was not investigated further at this point.

3.3.3. Analysis of data

Data for observed and expected results for plants attached to different categories of sediment size are illustrated in Figure 3.2 (Helford), Figure 3.3 (St Just) and Figure 3.4 (Braunton). Chi-square goodness-of-fit results are given in Table 3.2 with the contribution of each category to the overall values displayed in bar charts in Figure 3.5 (Helford),

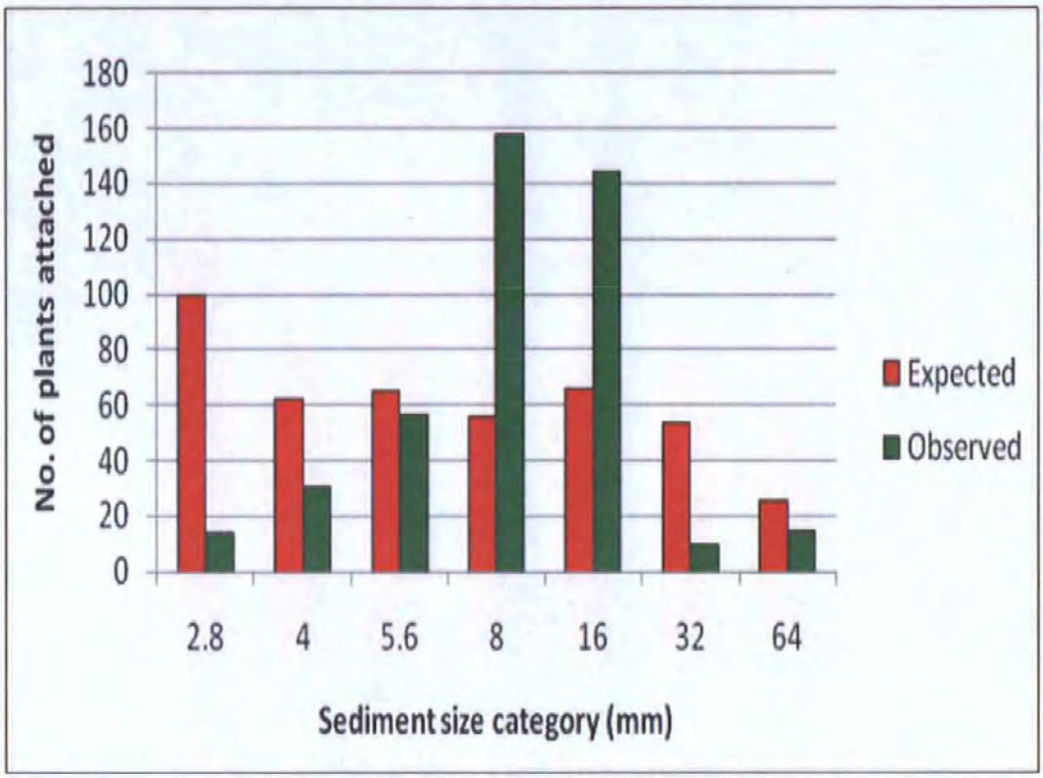


Figure 3.2: Bar chart illustrating the observed and expected numbers of plants attached to seven size categories of sediment particle at Helford

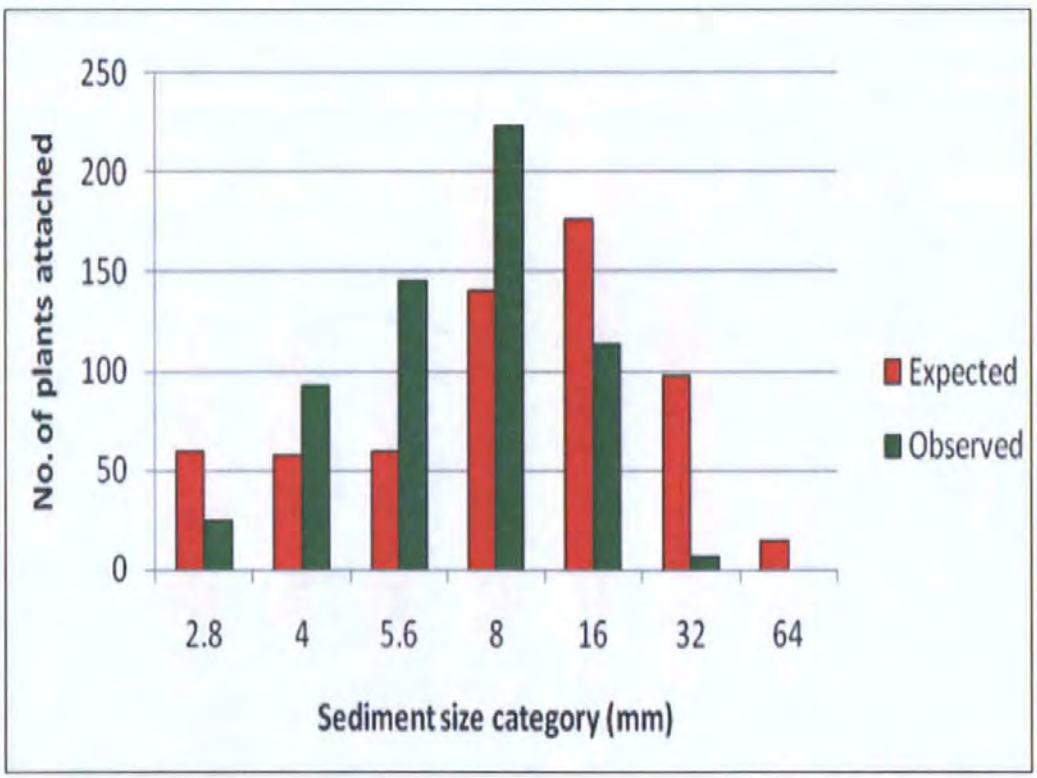


Figure 3.3: Bar chart illustrating the values for the observed and expected numbers of plants attached to seven size categories of sediment particle at St Just

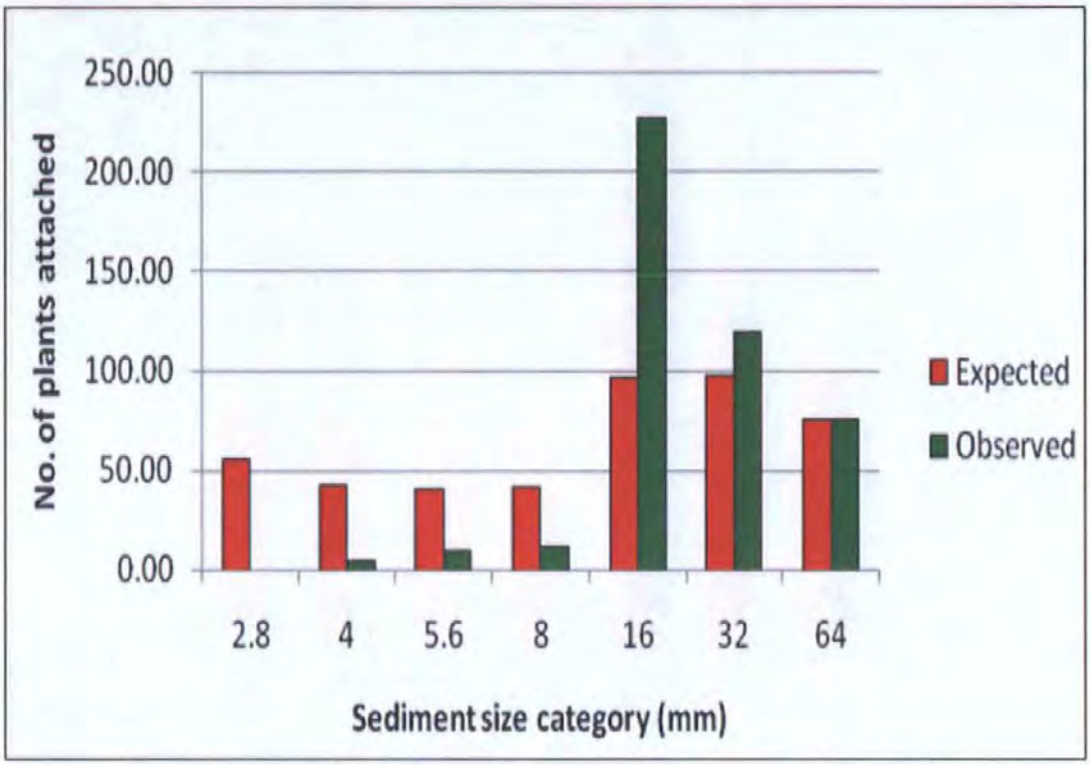


Figure 3.4: Bar chart illustrating the values for the observed and expected numbers of plants attached to seven size categories of sediment particle at Braunton

Figure 3.6 (St Just) and Figure 3.7 (Braunton). For each site, the probability that the plants are distributed at random with respect to particle size (i.e., in proportion to the percentage cover of each size category) is <<0.001. The critical value of X^2 for p=0.001 for 6 degrees of freedom is 22.46 (Rohlf and Sokal 1995).

From the X^2 figures, therefore, it can be seen that, for all of the three sites investigated, there were significant differences between the observed and expected numbers of plants attached to any category of substrate size.

In Figures 3.2 and 3.5, it can be seen that, at Helford, there were many more plants observed in the 8 mm and 16 mm size categories than expected. At St Just there were many more plants observed in the 5.6 mm and 8 mm size categories than predicted by the expected results but far less than expected in the 16 mm

Table 3.2: X^2 statistics for the expected versus observed numbers of *Gs. longissima* plants attached to seven size categories of sediment found at three sites showing the contribution of each category to the total X^2 value

Size category (mm) (maximum diameter)	*Helford*	*St Just*	*Braunton*
2.8	73.852	20.046	55.490
4.0	15.899	21.356	32.970
5.6	1.015	119.640	22.838
8.0	188.593	48.440	20.851
16.0	92.295	22.133	174.855
32.0	35.688	84.293	5.004
64.0	4.687	14.640	0.001
X^2 value	412.028	330.548	312.008
p value	<<0.001	<<0.001	<<0.001

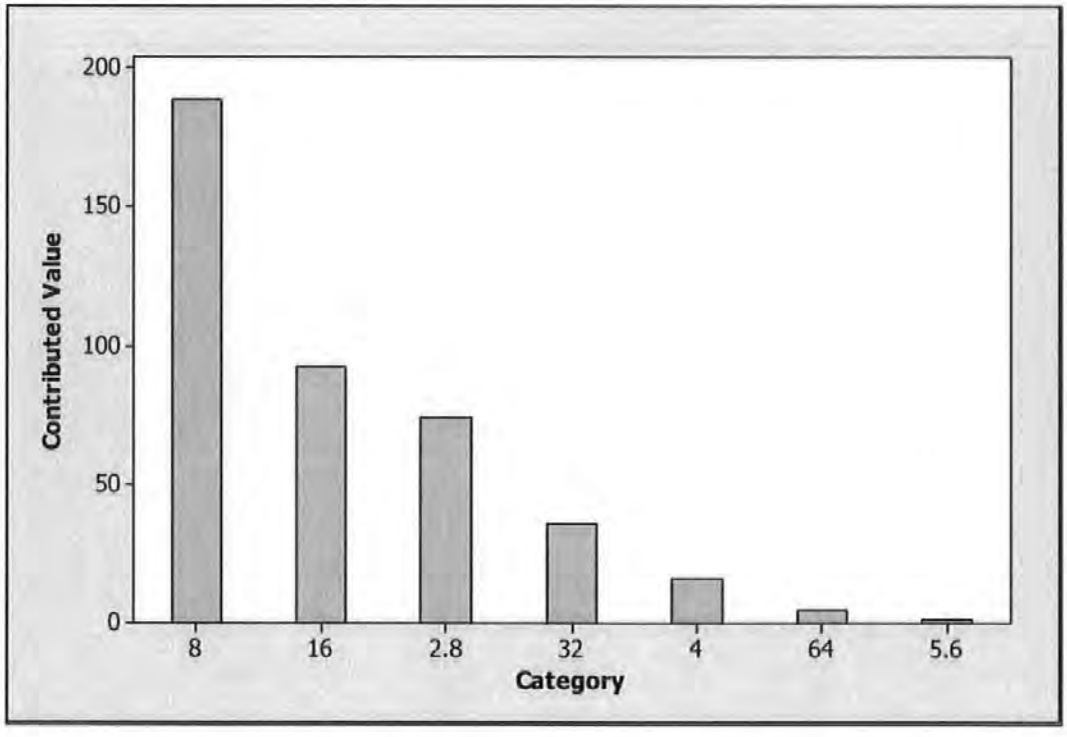


Figure 3.5: Bar chart illustrating the values for the contribution of each sediment size category to the overall value for X^2 for Helford.

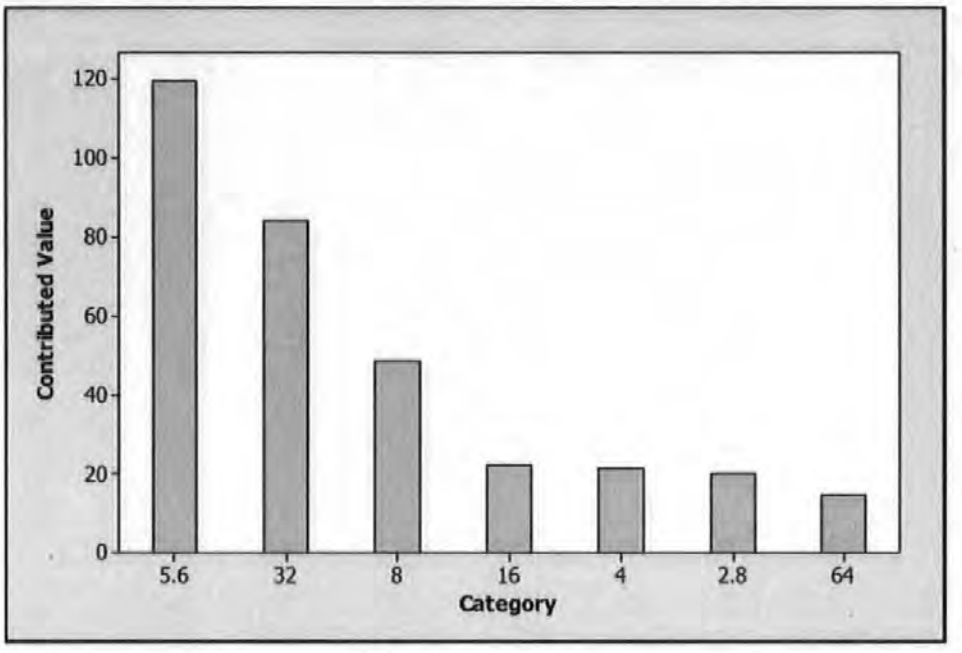


Figure 3.6: Bar chart illustrating the values for the contribution of each sediment size category to the overall value for X^2 for St Just

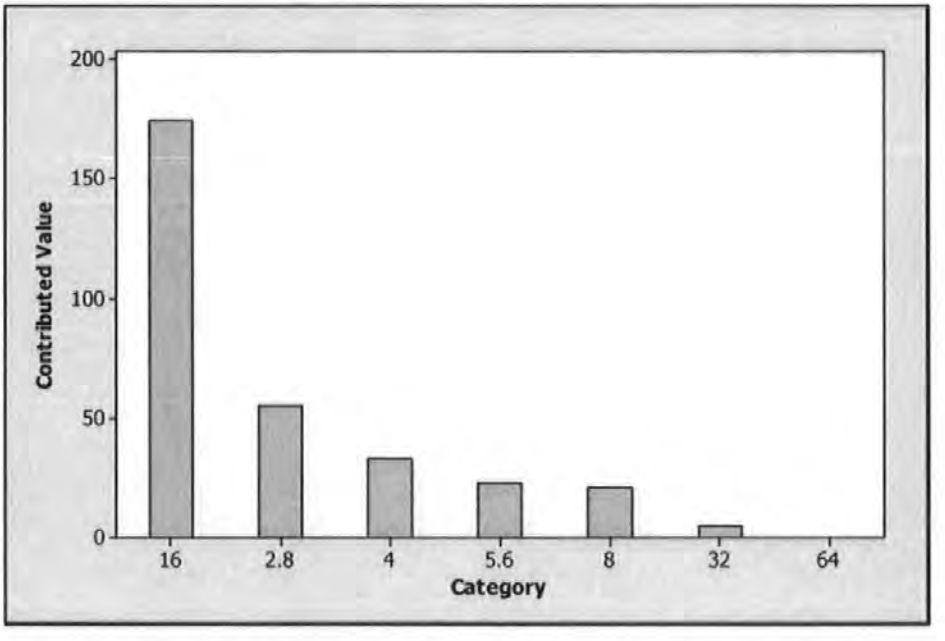


Figure 3.7: Bar chart illustrating the values for the contribution of each sediment size category to the overall value for X^2 for Braunton

size category (Figures 3.3 and 3.6). For Braunton, there appears to be a shift towards the larger size substrata (Figures 3.4 and 3.7) with extreme over-representation observed in the 16 mm size category. At St Just, there were far less plants attached to 32 mm sized stones than the expected value which also contributed considerably to the X^2 value.

3.4. Discussion

The results presented here clearly demonstrate that *Gs. longissima* does not grow randomly on all sizes of sediment available in the three sites studied. There appears to be an over-representation in size categories 8 mm and 16 mm at Helford, in 5.6 mm and 8 mm at St Just, and in16 mm and 32 mm at Braunton. The shift of preference to larger sizes at Braunton, where there were generally more of those sizes available, is unexplained.

Spores for *Gs. longissima* are non-motile and commonly released with a mucous sheath, creating possibilities for groups of spores moving together which would remove the independence of individual plants with regard to settlement, creating bias in results. Also, small indentations in the surfaces of sediment particles may cause micro effects in slowing water movements to allow sinking. On larger stones, such as at Braunton, there may be greater numbers of indentations and cracks providing opportunities for improved settlement conditions. However, the hydrodynamics of each site studied here are likely to be very different, with Braunton experiencing far greater energy forces than either St Just or Helford.

The estimation of percentage surface areas of sediment size categories in the field became more difficult as the size category decreased. In particular, it was hard to distinguish between 2 mm and 2.8 mm by eye. However, as the differences between observed and expected values were concentrated in the mid-range, and by leaving out the 2 mm size category, there is some confidence that values have not been unduly affected. Photometric data could prove useful if images were taken at a sufficiently high resolution for later analysis. However, photographs

taken during this study were subject to a number of problems, notably disturbance of the water's surface by wind and algal cover obscuring underlying substrata.

Determination of sediment character by surface examination may be valid for the purposes of estimating available settlement space but *Gs. longissima* specimens were commonly found buried with their substrate attachment covered by other sediment particles. Therefore, the validity of using percentage surface area of sediment size categories may be undermined. Further details of depth of burial, frequency of burial and mobility of sediments would be needed to determine the contribution of sub-surface sediment particles to the availability of surfaces for attachment of *Gs. longissima* spores. It is notable that red algae are able to tolerate lower light conditions which may allow burial for short periods (Kain and Norton 1990). As noted in Chapter 2, *Gs. longissima* successfully colonises sites with very different substrata. Observations in the field (and comments by Steentoft *et al* 1995) suggested that *Gs. longissima* remained potentially mobile even when attached by a holdfast to a substrate.

The presence of *Gs. longissima* plants on particular particles implies not only successful establishment but later survival. *Gs. longissima* spores have a mucilage coat which aids adhesion to surfaces. Once attached, many red algal spores are able to withstand high degrees of shear forces (Thomsen 2004) which may help to explain how the species survives on highly mobile substrata. Being attached to such small items, suggest Steentoft *et al* (1995), allows the plants to drift into areas where no suitable substrate exists allowing them to live in such places where the lack of suitable substrate would otherwise legislate against establishment. The ability of *Gs. longissima* to successfully colonise mobile

substrata is of particular interest as such conditions are thought to be particularly hard for macroalgal survival (Chapman and Fletcher 2002).

The sites visited in this study are subject to highly variable abiotic conditions which must influence settlement, attachment and survival but it was not possible in this study to identify the causative factors involved in the non-random distribution of *Gs. longissima* on substrata at these three sites.

Speculation by Steentoft *et al* (1995) regarding possible drift and recolonisation through attachment to small sediment particles is not addressed by the results seen here. Data concerning the mobility of specific sizes of sediment and drag forces operating on differing sizes of plant would be needed to draw any specific conclusions. However, the fact that many plants were found attached to the smallest sizes of sediment (2.8 m and 4 mm) suggests there is potential for the mechanisms of drift proposed by Steentoft *et al* (1995).

The mechanism by which *Gs. longissima* grows in the non-random way revealed by this study, therefore, remains unknown but would be worthy of further investigation.

3.5. Conclusions

This experiment has demonstrated that *Gs. longissima* does not grow randomly on all sizes of substrata available at three sites in South West Britain. In particular, algal presence was higher than expected on three particle size categories, 5.6 mm, 8 mm and 16 mm. So, there was no evidence of preferential growth on the

smallest feasible particles. Nevertheless, *Gs. longissima* can grow attached to particles of 2.8 mm, 4 mm and even, very rarely, 2 mm.

From the data presented here, the possibility cannot be excluded that initial settlement is random with respect to sediment size. The eventual over-representation of plants on particles in the 5.6 mm, 8 mm and/or 16 mm size classes could result from differential growth and survival, with medium-size particles offering a partial refuge from both physical disturbance, which could be expected to be greater for smaller substrate sizes, and grazing pressure, which could be expected to be greater on larger particles and bedrock.

Chapter 4:

Investigation and development of molecular methods for population genetics studies in *Gracilariopsis longissima*

4.1. Introduction

Molecular tools provide an excellent opportunity to investigate the population genetics of *Gracilariopsis longissima*. However, molecular techniques need to be optimised for each species and marker combination.

In this study, a number of molecular techniques was investigated and optimised for use in studies into the diversity and population genetics of *Gs. longissima*. The molecular methods investigated were:

1. Protocols for the extraction of DNA from red algae for use in PCR
2. PCR amplification of the internal transcribed spacer region of the algal genome for species identification
3. The development of randomly amplified polymorphic DNA markers for population genetics research in *Gs. longissima*
4. The development and screening of microsatellites in *Gs. longissima*
5. Cross-species amplification of *G. gracilis* microsatellite loci in *Gs. longissma* and other related Gracilariales.

4.2. DNA extraction and quantification

4.2.1. Introduction

DNA extraction from plants has always been complicated by the co-extraction of other complex compounds such as polysaccharides and phenols. Apart from

problems of increased viscosity, a variety of plant metabolites can interfere with DNA isolation procedures (Sharma *et al* 2002). Persistent polysaccharide and polyphenolic compounds can affect downstream applications such as DNA restriction, amplification and cloning. Authors have proposed a variety of methods for removal of polysaccharides from plant DNA extractions, including salt precipitation (Dellaporta *et al* 1983, Fang *et al* 1992), centrifugation (Coyer *et al* 1991, Hu and Zhou 2001) and post-isolation purification (Doulis *et al* 2000, Do and Adams 1991, Saunders 1993).

The problem of isolating high-quality DNA from large numbers of individuals may have delayed the development of population genetics research in algae (Chai-Ling *et al* 1996, Wattier and Maggs 2001). Rhodophytes present particular problems because many commercially-important species, such as *Gs. longissima* and *G. gracilis*, contain large amounts of soluble phycocolloids (Saunders 1993, Wattier *et al* 2000). These high molecular weight acidic polysaccharides are known to have an inhibitory effect on the enzyme (*Taq* polymerase) needed to catalyse the polymerase chain reaction (PCR) (Jin *et al* 1997, Demeke and Adams 1992). The very compounds which make these species of worldwide economic importance, therefore, also cause practical problems for researchers attempting to use molecular methods for a closer look at population genetics and phylogeny.

The removal of inhibitory compounds during DNA extraction is essential for successful PCR. The difficulty of extracting good quality, pure, large size genomic DNA from red seaweed has been addressed by many authors (Hu *et al* 2004, Sharma *et al* 2002, Wattier *et al* 2000). Extraction methods vary from the very simple, quick and cheap (Wattier *et al* 1997), to the long, complicated and

expensive (Doulis *et al* 2000). There are also large numbers of commercially available extraction kits (e.g.,Biorad, Qiagen) with, recently, a number designed specifically for plant material (e.g., Qiagen Plant DNEasy, Sigma Extract-N-Amp Plant PCR Kit, Whatman FTA cards), many of which claim to provide efficient polysaccharide removal or neutralization (Sigma Extract-N-Amp).

With sample amounts for this study being limited, it was important to identify the most efficient, least wasteful method which produced high enough quality DNA for the various molecular methods used in this study, including:

(i) Species confirmation by amplification of the nuclear ribosomal DNA internal transcribed spacer region of the genome

(ii) Investigation of population genetics and biofilms using randomly amplified polymorphic DNA (RAPDs) primers

(iii) Creation of DNA libraries to probe for microsatellites

(iv) Testing cross-genera amplification of microsatellite markers

For each of these procedures, it was necessary to consider which of the following criteria also applied:

(i) Nuclear, organellar or whole genomic DNA

(ii) Purity required by downstream procedures

(iii) Quantity of starting material available

(iv) Quantity of DNA required for procedures

(v) Stability of preparation

(vi) Cost and difficulty of extraction method

(vii) Use of hazardous reagents

There are a number of commercially available kits which claim to provide rapid and simple plant DNA extraction suitable for PCR of which four were made available at no cost for testing with the study species (Table 4.1). Successful published protocols developed by phycologists also offer potentially suitable methods, especially those which have already been used successfully with rhodophyte species. Three such methods were tested (Table 4.1). High quality, high molecular weight DNA is required for microsatellite isolation. Following problems of degradation of DNA, nuclear extraction was also proposed as a suitable method (Dr P. Samitou-Laprade, University of Lille, France, personal communication).

Table 4.1: DNA extraction methods investigated for suitability for use with a variety of molecular investigations in *Gracilariopsis longissima* and the rationale for testing.

	Method	*Characteristics of method*	*Rationale for testing*
1	Chelex 100 chelating resin	Very time and cost efficient but low DNA concentration and possible problems of contamination	To determine suitability for use in PCR reactions
2	Whatman FTA	Very simple, one-step method but unknown suitability for PCR	To determine suitability compared with (1)
3	Qiagen DNeasy Plant Kit	Very simple kit for plant material but unknown yields and quality.	As alternative to (1) if good quality DNA obtained
4	DNAmite Plant Kit	Simple procedure designed for plant material	As alternative to (1) if results provide good quality DNA
5	Sigma Extract-n-Amp Plant PCR Kit	Simple kit for plant material	As for (3) and (4)
6	Wattier *et al* (2000) rhodophyte protocol	Designed specifically for rhodophytes but longer process than Chelex or commercial kits	For comparison with other methods for quality and PCR suitability
7	CTAB	Established protocol used by algal researchers (modified from Doyle and Doyle 1987). Longer than 6 and uses some toxic chemicals	To test quality and quantity of product
8	Nuclear extraction (Lille University)	Delivers high quality, high molecular weight DNA but time consuming and requires expensive machinery	To overcome problems with quality and quantity needed for genomic DNA library creation

These eight methods were investigated and tested with a view to (a) simplicity and speed for processing high numbers of samples (b) highest yield (c) successful PCR with a variety of primers (d) suitability for DNA library creation (e) lowest toxicity hazard (f) cost. DNA quantitation was used to identify the best extraction process, to assess the quality of DNA and to standardise concentrations across extractions for some PCR protocols (Davin-Regli *et al* 1995).

The aim of the present study was to identify the most appropriate DNA extraction method from the eight tested, for molecular studies in *Gs. longissima*.

4.2.2. Materials and Methods

4.2.2.1. Sample material

Dried specimens which had been cleaned by vigorous brushing under a microscope were used. All specimens were rinsed in distilled water at least twice before air drying and storing in silica gel. Live cleaned sample was used for the Sigma Extract-N-Amp protocol.

Material was very limited with many individuals providing as little as 10-20 mg in total. Figure 4.1 shows a 10 mg piece of *Gs. longissima*.

4.2.2.2. Extraction protocols

Four proprietary kits (provided as free samples), two established methods (CTAB and Chelex), one rhodophyte-specific protocol (Wattier *et al* 2000) and one adapted nuclear extraction method (Lille University) were tested (Table 4.1). The extraction protocols tested were as follows:-

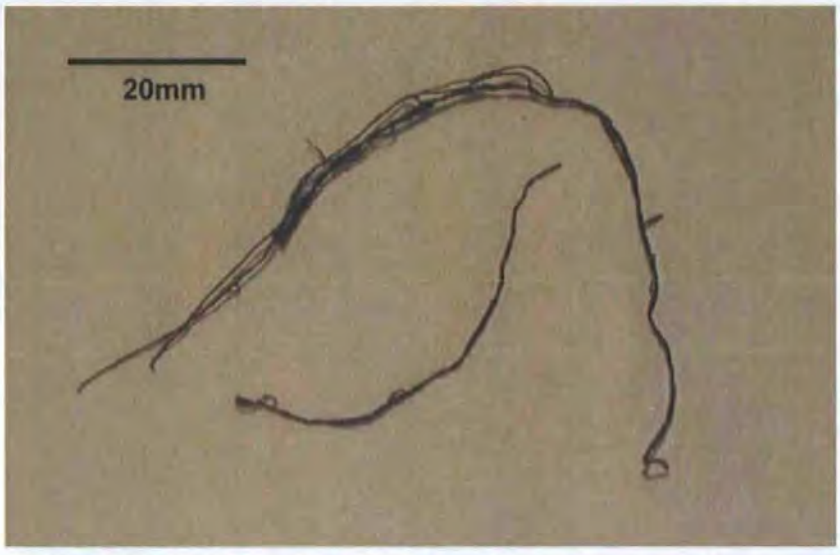


Figure 4.1: A piece of dried *Gs. longissima* sample of approximately 10 mg weight

1. Chelex 100 Chelating Resin

Extractions were carried out as described in Chapter 2 for species confirmation. Samples were stored at -20°C or kept at 4°C rather than frozen if they were to be used repeatedly within a short space of time.

2. Whatman FTA cards

FTA Classic Cards (Whatman International Ltd, Maidstone, UK) are designed to immobilise nucleic acids in a chemically-impregnated physical matrix on a card. Two methods are recommended by the manufacturer. (1) Fresh specimens were crushed and pressed directly onto cards (Figure 4.2). (2) Dried sample material (10 mg) was ground with phosphate buffered saline at an estimated ratio of 1:5 volumes as recommended by the manufacturer. Homogenate (25 µl) was applied to the proprietary cards. Cards were air dried and sealed in plastic snap top bags with silica gel and stored at room temperature.

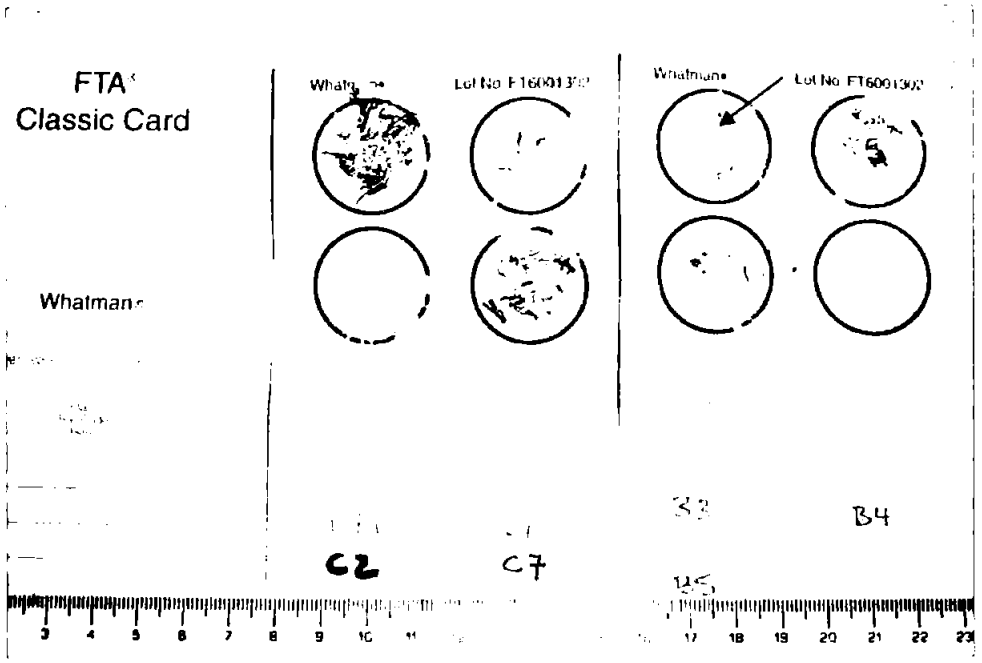


Figure 4.2: Fresh material squashed onto FTA card (left) and 25 µl of homogenate pipetted onto a card (right). Discs (2 mm diameter) were punched out (arrow) for use in PCR reactions

3. Qiagen DNeasy Plant Kit

Dry sample (20 mg) was ground to a powder in liquid nitrogen and processed with a Qiagen DNeasy Plant Kit (Qiagen, West Sussex UK) following the proprietary protocol.

4. DNAmite Plant Kit

Approximately 5mg of dried specimen were ground to powder in liquid nitrogen and processed following the proprietary protocol for the DNAmite Plant DNA Extraction Kit (Microzone Ltd, Haywards Heath, West Sussex, UK). Pellets were resuspended in 30 µl x1 TE.

5. Sigma Extract-N-Amp Plant PCR Kit

Because of the nature of the material (terete thalli of approximately 1-2 mm diameter), it was not possible to use the recommended paper punch to select specimen material for extraction. An equivalent was estimated from the surface

area of fresh material to equate with a 5 mm diameter disc. The protocol was then followed and the plant material left in the resulting solution as instructed. Samples were stored at 4°C.

6. Rhodophyte protocol

Wattier *et al* (2000) modified the method of Dellaporta *et al* (1983), seeking to minimise polysaccharide co-isolation. Dried sample (5 mg) was ground to a powder in liquid nitrogen and mixed vigorously with 1ml extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 2% SDS by weight, 250 µg proteinase K).

Proteins were digested by incubation for 30 min at 37°C. Cell debris and putatively high molecular weight polysaccharides are removed by centrifugation (15 min at 13,000 rpm). RNA was removed by incubation of the supernatant with ribonuclease A (30 min at 37°C). A 30 min period on ice with occasional agitation helped to precipitate more high molecular weight polysaccharides and proteins, followed by centrifugation (15 min at 13,0000 rpm at 4°C).

DNA was precipitated from the supernatant with 1:1 volume of ice-cold isopropanol, mixed gently and incubated at -20°C overnight. Pelleted DNA (centrifugation at 13,000 rpm for 30 min at 4°C) was washed in 1ml ice cold 70% ethanol three times including a 10 min, 3000 rpm centrifugation after each wash. The air-dried pellet was resuspended in 250 µl TE x 1 buffer and stored at -20°C.

7. Cetyl Trimethyl Ammonium Bromide (CTAB)

Approximately 5 mg of dried specimen was ground to powder in liquid nitrogen and added to 500 µl of CTAB buffer (2% w/v CTAB; 100 mM Tris-Cl, pH 8.0; 20

mM EDTA, pH 8.0; 1.4 M NaCl) plus 10 µl proteinase K (20 mg/ml) and incubated at 57°C for a minimum of 2 h.

The sample was incubated with 3 µl ribonuclease (10 mg/ml) for 30 min at 37°C. Samples were cooled to room temperature, 500 µl of chloroform:isoamylalcohol (24:1) was added and mixed vigorously. After spinning at 13,000 rpm in a bench centrifuge for 5 min, the floating aqueous phase was transferred to a new tube and the chloroform step repeated. The resulting aqueous phase was transferred to a clean tube and DNA precipitated with 1/10 volumes of 3M sodium acetate and 2 volumes of ice-cold 100% ethanol.

The pellet remaining after centrifugation (20 min at 13,000 rpm) was washed in 70% ice-cold ethanol and air dried. After resuspension in 100 µl 1x TE, samples were stored at -20°C.

8. Nuclear extraction

Adapted from a protocol used by Lille University (Dr P. Samitou-Laprade, personal communication), this method was used to obtain nuclear DNA. At least 35 g of dried material (pooled specimens) were ground to a powder in liquid nitrogen and mixed with 100 ml of extraction buffer (20 mM Tris HCl, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM KCl, PVP 2% w/v, Glycerol 40% v/v, 0.25% Triton X-100 v/v, 0.1 - mercaptoethanol v/v).

Major debris was removed by filtering through Miracloth (Calbiochem, Nottingham, UK). Centrifugation at 10,000 rpm for 15 min at 4°C in a Sorvall SS34 left the mitochondria in suspension while pelleting the nuclei. The crude nuclear pellet

was resuspended in a further 100ml of extraction buffer, followed by centrifugation at 6,500 rpm for 15 min. This cycle of resuspension and repelleting was repeated twice more to concentrate nuclei. The pellet was resuspended in 10ml of lysing solution (50 mM TrisHCl, 50 mM NaCl, 100 mM EDTA, 0.5% w/v SDS) and incubated with 5 mg proteinase K at 40°C for 2 hours with gentle agitation.

DNA extraction was performed with an equal volume of phenol/chloroform/ isoamylalcohol (25:24:1) mixed gently for 15 min. After centrifugation (10 min at 12,000 rpm), the top aqueous layer was removed carefully with a wide ended tip, leaving behind the denatured proteins in a solid middle phase and other unwanted organic molecules in a lower phase. The phenol/chloroform/isoamyalcohol extraction step was repeated.

Chloroform was used to remove phenols by adding 1 volume, mixing and spinning (twice) before removal of the final aqueous phase. DNA was precipitated with 1/10 volumes of sodium acetate (3.3 M, pH 5.3) and 1.5 volumes of ice-cold ethanol. Pellets were washed in 70% v/v ice-cold ethanol and dried in air, before being resuspended in 1ml TE (x1 pH 8) and stored at -20°C.

4.2.2.3. DNA quantification

DNA was quantified via two methods. Template DNA was visualised by electrophoresis on agarose gels. Extraction product (2 µl) was mixed with 2 µl x 6 loading buffer (MBI Fermentas,York, UK) and loaded onto an ethidium bromide (1%) stained 40 ml agarose gels (0.8%) in x1 TBE running buffer. Gels were run at 60V for 1-2 h. Standards of known concentration were run on each gel in various concentrations to assess concentration of extractions. Gels were visualised with

an ultraviolet transilluminator with a UVItec Gel Documentation System (UVItech, Cambridge, UK) which allowed both visual and computer-aided assessment of concentrations.

Optical density measurements were also made (Beckman Spectrophotometer) at 260nm and 280nm for a number of samples (Sambrook *et al* 1989). 100 µl of extraction sample was added to 900 µl of double-distilled water. The spectrophotometer was calibrated against blanks of appropriate extraction solutions. Concentrations and purity (from the ratio between the two absorbance measurements) was calculated and compared with results from ethidium bromide stained gel electrophoresis.

Quantification of DNA available from FTA cards and Sigma Extract-N-Amp templates was not possible due to extractions being bound to solid media.

4.2.2.4. Molecular tests

DNA extractions were investigated for suitability for molecular protocols used in this study. Template DNA was tested for a variety of molecular methods. For ITS and microsatellite amplification, the PCR conditions were as described for the ITS region amplification in Chapter 2 (Section 2.2.1, Table 2.2). For testing the suitability of extractions for RAPD marker amplification the PCR conditions were modified from Atienzar (2000b) (See 4.4, Table 4.3).

The Sigma Extract-N-Amp kit is designed to be used in conjunction with Sigma proprietary PCR Ready-Mix. As this is very expensive compared with standard

reagents, a test of Extract-N-Amp extractions with ReadyMix and with the standard PCR mix used with Chelex or Wattier extractions was carried out.

DNA templates from samples of known genotype for markers under investigation were used as positive controls and for comparison of results. PCR conditions used in these tests are described elsewhere in this report (Chapter 4.3, 4.4, 4.5)

4.2.3. Results

4.2.3.1. DNA quantification

Discrepancies between agarose gel measurements and absorbance measurements made DNA quantification problematic. For example, a DNA sample from St Just (J1) which was judged to be very pure (A_{260}/A_{280} ratio 1.87) at an approximate concentration of 3.5 ng μl^{-1} by absorption measurements could not be visualised at all when 20 µl was loaded onto an ethidium bromide stained gel. However, as little as 5 ng can be detected on ethidium bromide stained gels (e.g., Figure 4.3). This anomaly suggested that there was a problem with the spectrophotometric method. This may be due to interference from contaminants such as RNA, as described by Sauer *et al* (1998). Therefore quantification given in Table 4.2 was assessed using comparative intensity measurements of ethidium bromide fluorescence on agarose gels.

4.2.3.2. Yield and quality of DNA

An overview of the success of the various extraction methods is given in Table 4.2. DNA amounts of 5 ng provided reproducible visualisation. However, Chelex extractions proved impossible to visualise on agarose gels even when loading up to 20 µl.

The quantity and quality of DNA bound by the Whatman FTA cards could not be assessed. The Qiagen kit produced good quality DNA but the yield was low (50 ng/mg dry weight). DNAmite extractions produced good quality DNA but low yields (80 ng/mg dry weight) compared with the Wattier or CTAB methods. Samples also showed some degree of smear when visualised on an agarose gel (Figure 4.3).

The rhodophyte-specific extraction process (Wattier *et al* 2000) gave the highest yields (200-400 ng/mg dry weight) and was of consistently good quality (Figure 4.4). Other gels of Wattier extractions run against Lambda phage DNA standards showed the DNA obtained was approximately 45 kb in size.

CTAB extractions produced good yields of DNA – up to 200 ng/mg dry weight – but smearing was seen on some gels (Figure 4.5), along with a large amount of low molecular weight product which may have been RNA, despite digesting extractions with ribonuclease A as part of the protocol.

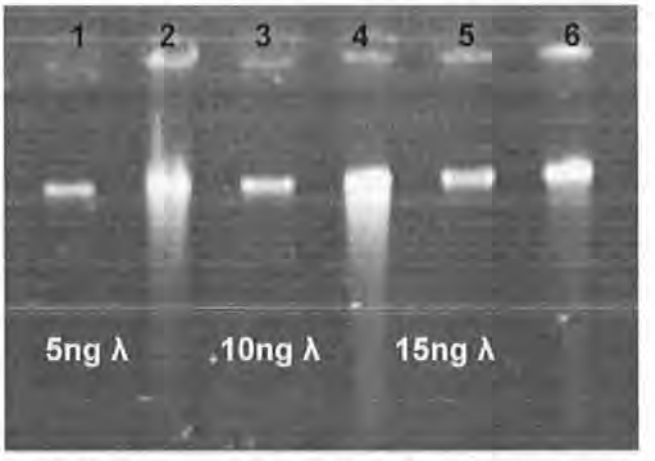


Figure 4.3: Microzone's DNAmite extraction method yielded an average of around 80 ng/mg dry weight of *Gs. longissima* sample with some smearing of product. Samples were from Braunton: A32 (lane 2), A40 (lane 4) and A14 (lane 6) . Lanes 1,3 & 5 are lambda standards as marked of approximately 45 kb in size.

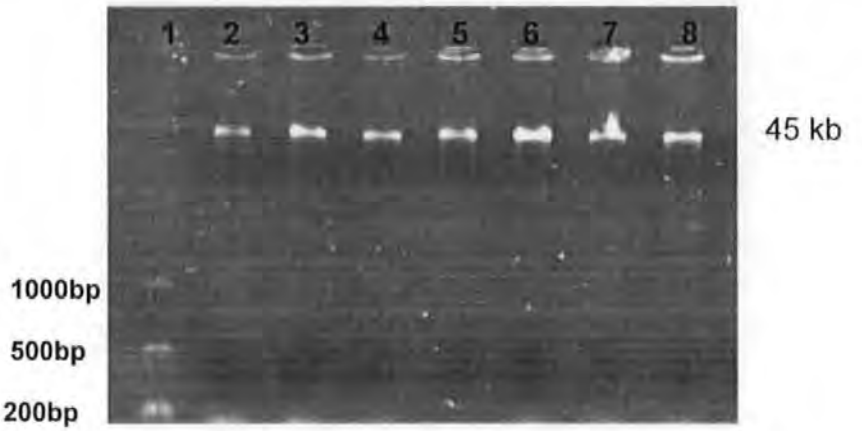


Figure 4.4: Seven samples (2 µl) of Wattier-extracted *Gs. longissima* DNA from Braunton: A30 A36, A40 (lanes 2-4), from Helford: H12, H26, H30 (lanes 5-7) and Place: P72 (lane 8) visualised with ethidium bromide-stained 0.8% agarose gel. Lane 1 is a quantitation standard (ABgene, Epsom, UK) with lambda quantities shown. Extractions were of a consistent yield of 200-400 ng/mg dry wt of sample. DNA extraction fragments were approximately 45 kb in size.

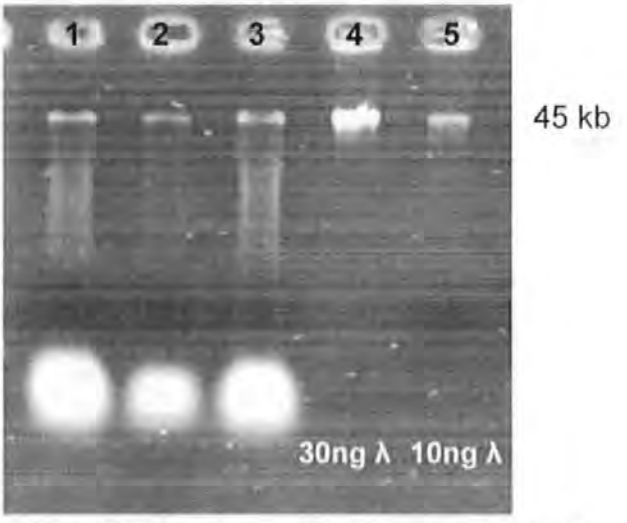


Figure 4.5: Three samples (5 µl) of CTAB-extracted *Gs. longissima* DNA from samples A57 (Braunton), H45 (Helford) and P83 (Place) (Lanes 1-3) were loaded onto a 0.8% agarose gel stained with ethidium bromide showing large amounts of unidentified low molecular weight compound. Lanes 4 & 5 are lambda standards of 45 kb size.

The long and complicated nuclear extraction method produced DNA which was successfully fully digested with Sau3AI and HaeIII but suffered quite severe degradation for reasons that were not identified (personal communication, Dr. D. Dawson, Sheffield Molecular Genetics Facility).

As can be seen from Table 4.2, the Wattier method delivered the best yields with high quality DNA. With the failure of the complex nuclear extraction to provide

Table 4.2: DNA yield, quality and outcomes of molecular tests from eight DNA extraction methods tested for use in molecular techniques for the investigation of the taxonomy and genetics of *Gracilariopsis longissima*

	Method	Starting material	Average total DNA (µg)	Mean yield (ng/mg dry wt)	Quality assessment	PCR marker amplification			DNA library creation
						ITS	RAPD	Microsatellites	
1	Chelex 100 resin	3 x 5mm dry tips	Not detectable	-	-	✓	✓	✓	-
2	FTA cards	25 µl homogenate	Unquantifiable	-	-	**X**	-	-	-
3	Qiagen DNeasy Plant Kit	20 mg dry wt	1	50	Good	✓	-	-	-
4	DNAmite Plant Kit	5 mg dry wt	0.4	80	Good but with high degree of smearing	✓	✓	-	-
5	Sigma Extract-N-Amp	<3 mg dry wt	Unmeasured	-	-	-	**X**	-	-
6	Wattier *et al* 2000	5 mg dry wt	1.5	200-400	Good	✓	✓	✓	✓
7	CTAB	5 mg dry wt	0.75	150	Good but with some smearing	✓	✓	-	-
8	Nuclear extraction	40 g dry wt	100	2.5	High degree of degradation	-	-	-	**X**

Key: ✓ - successful, **X** – limited or no success, - not tested

DNA of suitable quality (D.Dawson, personal communication), Wattier-extracted DNA was used successfully for the preparation of enriched microsatellite libraries.

4.2.3.3. Amplification success

The purpose of DNA extractions was to investigate molecular questions. The most important aspect of any extraction was its success as a template for amplification of chosen molecular markers for other investigations reported here. Details of molecular protocols are given elsewhere in this report. Results are summarised in Table 4.2.

Internal transcribed spacer (ITS) All extractions tested, apart from Whatman FTA cards, were successfully amplified using ITS primers. Failure of amplification of FTA disc templates was confirmed by the inclusion of positive controls in all amplifications. Chelex extractions sometimes failed to amplify ITS markers at the first attempt but were usually successfullly amplified with x10 or x100 dilutions of original extractions.

Randomly amplified polymorphic DNA (RAPD) markers RAPD primers (OPB5-9, OPR8) successfully amplified with Chelex extractions, with very similar amplicon profiles to those obtained with the cleaner, quantifiable Wattier extractions (Figure 4.6). However, there were subtle differences, with less well amplified products in the higher weight bands. Some bands are also more clearly defined in the Wattier extractions than the Chelex, e.g., 1150bp with primer OPR8 (arrowed in Figure 4.6) and vice versa. There was some difference in the banding patterns which is unexplained.

Wattier extractions consistently reproduced banding patterns. Chelex extractions also showed a high degree of repeatability. When optimising RAPD protocols (Chapter 4.4), Wattier extractions were seen to amplify more product than Chelex in six samples of *Gs. longissima* with primer OPB5-5 (Figure 4.13). CTAB and DNAmite extractions also successfully amplifed RAPD markers.

Results with Sigma extractions did not produce clear bands nor the full range expected for two RAPD primers (OPB7-6, OPR8) tested. The profile obtained from three samples with Wattier extractions amplified alongside Sigma extractions and standard PCR mix matched that obtained previously with this primer. Figure 4.7 demonstrates the results obtained for RAPD primer OPR8.

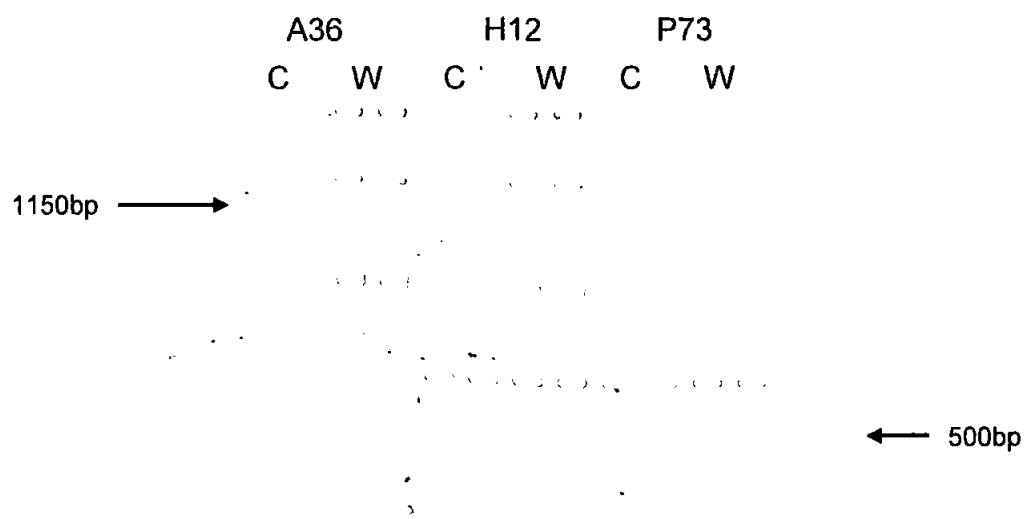


Figure 4.6: Ethidium bromide stained agarose gel (1.5%) Shown in negative exposure for clarity, showing amplification products of RAPD primer OPR8 using Chelex (C) and Wattier (W) DNA templates for three specimens of *Gs. longissima* (A36 Lanes 2-5, H12 – Lanes 6-9, P73 – Lanes 10-13) Lane 14: negative control. Lanes 1 & 15 100bp DNA ladder. Each amplification is duplicated. The arrow highlights a band at 1150bp which demonstrates the variation in amplification strength seen in some bands between the two different types of extraction.

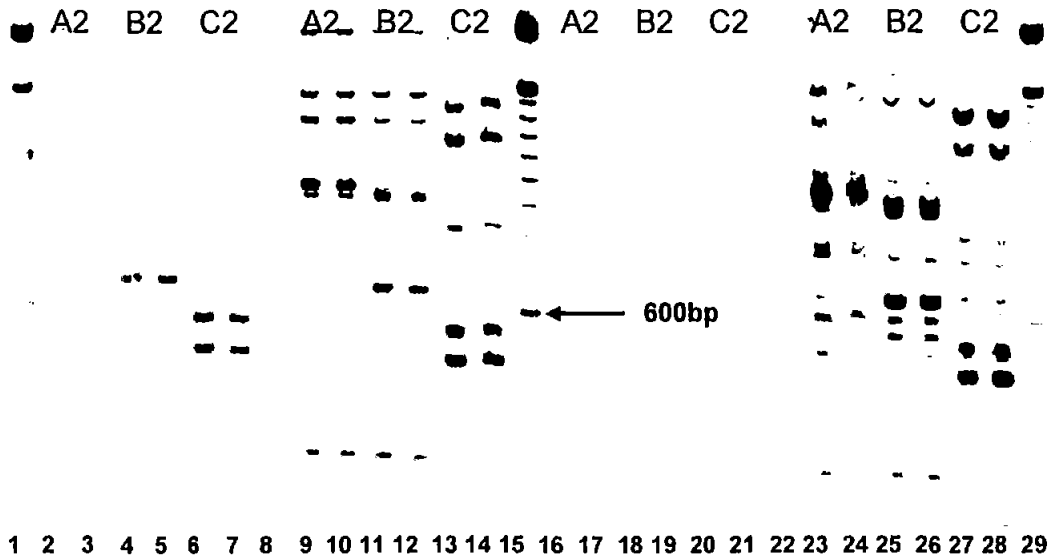


Figure 4.7: Amplification of RAPD primer OPR8 with template from two specimens of *Gs. longissima* (A2, B2) and one specimen of *G. gracilis* (C2). Gel is shown in negative exposure for clarity. Lanes 2-7: Sigma extractions amplified with Sigma Ready Mix. Lanes 9-14: Wattier extraction amplified with Sigma ReadyMix. Lanes 16-21: Sigma extraction amplified with standard mix. Lanes 23-28: Wattier extraction amplified with standard mix. Lanes 1, 15, 29 are 100bp ladder, 600bp is arrowed.

Microsatellites An investigation of cross-species amplification of *G. gracilis* microsatellite markers in *Gs. longissima* was successful in a number of samples using Chelex and Wattier extractions (see Chapter 4.5). *Gs. longissima* microsatellites were successfully amplified using Wattier extractions (see Chapter 4.6). Chelex extractions were not used for the latter PCRs.

Microsatellite DNA library creation DNA microsatellite library creation for *Gs. longissima* was not successful with the nuclear DNA extraction but finally succeeded with a second extraction using the Wattier method.

4.2.4. Discussion

In this study, several criteria were applied to decisions about which extraction protocols were best suited for which purpose. Ploidy determination is essential when using RAPD markers. High numbers of vegetative individuals whose ploidy could not be determined, combined with the resource implications of biofilm

removal, reduced the availability of suitable sample material. Cost was a further factor in the choice of extraction method.

Chelex resin extraction is the simplest and quickest method. It proved perfectly adequate for PCR with ITS primers allowing confirmation of species identification. The advantage of needing only very small amounts of material for extraction was particularly attractive. The many Chelex DNA extractions lasted very well without apparent degradation as they were amplified successfully up to three years after extraction (unreported data). This is contrary to reported problems with the crudeness of Chelex extractions which allow nucleases to remain active to degrade DNA very quickly (Wattier and Maggs 2001). Shivji *et al* (1992) also note the possible problem of high nuclease activity in algal DNA extractions leading to degradation of product.

The fact that dilutions of Chelex extractions produced amplification in otherwise recalcitrant samples suggests the presence of inhibitory compounds such as polysaccharides (C. Destombe, personal communication). The most effective way to eliminate polysaccharide inhibition is to dilute the DNA extracts, thereby diluting the polysaccharide inhibitors (Pandey *et al* 1996). For reasons of cost, ease of execution, lack of toxic chemicals and high rates of success, Chelex extractions were used for ITS amplification.

With concerns about the effects of concentration on amplification results for RAPD primers (Davin-Regli *et al* 1995, Power 1996) it is important to quantify and standardise DNA templates for RAPD amplification. The Chelex method using 3 thallus tips and 250 µl of DNA solution, is thought to produce around 2 ng/µl (Dr C.

Engel, personal communication). However, if this were the case, loading 20 µl on an ethidium bromide-stained agarose gel should produce highly visible bands – which it did not.

A recent Measurement for Biotechnology (MfB) Conference (http://www.mfbprog.org.uk/publications/publicationdocs/1215.PDF 2006) highlighted the difficulty of standardising DNA quantitation methods and the high degrees of variability found between different measurement methods. Also, the Chelex method did not remove potential *Taq* polymerase inhibitors, making these extractions a less robust choice for RAPD amplification which is purportedly susceptible to variations in DNA quality as well as concentration. For these reasons, Chelex extractions were rejected for use with RAPD investigations.

Most other methods required grinding in liquid nitrogen before processing. With 5-10 mg of dry material, grinding in an Eppendorf tube, even when cooled in liquid nitrogen first, losses due to boiling over were difficult to avoid, leading to highly variable final yields. However, the Wattier *et al* (2000) method was favoured as it used no expensive or noxious chemicals, was simple in execution and produced good, consistent yields which successfully amplified in all situations. Extractions were done repeatedly and were always reliable. These were therefore used for all RAPD amplifications. The CTAB method was rejected because it used noxious chemicals and produced slightly lower yields than the Wattier method.

As will be seen later (Section 4.5), success with inter-genera microsatellite amplification using Chelex extractions was mixed and it might be worth trying again with the higher quality, cleaner DNA extractions obtained with the Wattier method.

Microzone's DNAmite proprietary method produced good quality DNA which successfully amplified both ITS region and RAPD markers. However, the yield was very low compared with the Wattier or CTAB extraction methods (Table 4.1).

Nuclear extractions used the most sample material (>35 g) which meant pooling material from several samples and was a long process using toxic chemicals. The resulting DNA seemed to degrade very quickly and was found to be of no use for enriched DNA library creation for which it was intended.

4.2.5. Conclusions

Comparison of DNA extraction methods for a variety of purposes allows researchers to be certain that DNA templates are suitable for the purpose for which they are required. Before embarking on expensive and time-consuming molecular investigations, it is worthwhile to carry out some pilot amplifications and to check the optimal DNA templates for any particular protocol.

4.3. Molecular identification of study species

4.3.1. Introduction

To make a definitive morphological or anatomical identification of *Gracilariopsis longissima*, individuals must be mature and in their reproductive state. However, as discussed above (Chapter 1), males are highly cryptic due to the microscopic nature of the spermatangia. Also, females and tetrasporophytes are not reproductive at all times and may be encountered in their vegetative states when collections are being made from the field. Therefore, identification of study

samples was made by the proven molecular method of amplification of the ITS spacer region of the genome.

This method is described in full in Chapter 2 where it was used to identify populations of *Gs. longissima* and confirm where mixed populations with *G. gracilis* occurred. As described in Chapter 2, the method is simple and quick to use with reliable results giving unambiguous identification. The protocol allowed the researcher to verify species with confidence and was used throughout the study.

4.4. Development of method for randomly amplified polymorphic DNA (RAPD) markers in *Gs. longissima*

4.4.1. Introduction

RAPD markers, as discussed in Chapter 1, provide molecular markers which do not require any preknowledge of the genome being investigated. Existing molecular data for *Gs. longissima* was focussed on taxonomy and inter-specific relationships with, probably, less variation than needed for investigations at a population level. Therefore RAPD markers were proposed to investigate variation within and between populations of *Gs. longissima*.

As with most molecular markers, the PCR process is used to amplify regions of the genome so they can be visualised by gel electrophoresis. All factors in the PCR process are thought to influence RAPD profiles. Therefore, before screening primers for levels of variability, the PCR process was optimised for DNA extraction

type, concentration of template, *Taq* polymerase, $MgCl_2$, primers and dNTPs, plus annealing temperature.

4.4.2. Optimisation of RAPD PCR protocol

4.4.2.1. Materials and Methods

All products from the experiments below were visualised by gel electrophoresis, using 1.5% agarose gels stained with ethidium bromide. All tests, except those comparing DNA templates, were carried out using randomly chosen DNA extractions from a group of *Gs. longissima* specimens which had been successfully amplified on at least one previous occasion. All extractions were carried out using the Wattier method (Wattier *et al* 2000).

4.4.2.1.1. Primers

A number of commercial Operon decamer primers (Operon Technologies, California, USA) from the 'B' and 'R' groups were used, along with a group designed by Atienzar (2000) which were surplus to his requirements but still met the RAPD criteria as described above (Chapter 4.4.1).

4.4.2.1.2. Thermal cycling conditions

For optimisation of PCR parameters, RAPD primers OPB5-9 and OPB10 were used. Initial PCR conditions were derived from those published in 25 publications of RAPD priming in various organisms, including those described in Williams *et al* 1990. In detail, DNA template (20 ng) was added to a reaction mix (25 µl) containing: 1X PCR buffer (10X is 20 mM Tris-HCl pH 8.0, 0.1 M KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% v/v Nonidet P40, 0.5% v/v Tween 20, 50% v/v glycerol : Roche Diagnostics Ltd, Lewes, UK), 2 mM Mg Cl_2, 100 µM dNTPs, 1 µM

primer, and 0.5 U *Taq* polymerase (Roche Diagnostics Ltd, Lewes, UK) . Thermal cycling conditions were initial denaturation for 5 min at 94°C, followed by 45 cycles of a denaturing step (1 min at 94°C), an annealing step (1 min at 36°C), and an extension step (2 min at 72°C), followed by a final extension period of 10 min at 72°C.

Additional trials were conducted with raised concentrations of *Taq* polymerase (1, 1.5 and 2 U per reaction) and $MgCl_2$ (3 mM, 4 mM, 4.5 mM and 5 mM). The annealing temperature was raised to 50°C as described by Atienzar *et al* (1998, 2000b). The extension step in the thermal cycling programme was reduced to 1 min and the number of cycles reduced to 40.

The use of an oil overlay is not obligatory with heated-lid thermal cyclers or amplification plates where fixed foil lids are provided. However, many protocols recommend its use. PCR reaction mixtures may be mixed by pipetting or centrifugation prior to cycling. An experiment to investigate the combinations of oil or no oil with mixing with pipette or mixing by centrifugation for PCR plates and tubes was carried out with one Wattier-extracted DNA template (A3), RAPD primer OPB5-9, using PCR conditions as for RAPD primer screening (Table 4.3). Each reaction was duplicated.

4.4.2.1.3. Comparison of thermal cyclers

Three machines were available for use: an MWG AG Biotech Primus 96 Plus, a PE Applied Biosystems GeneAmp PCR system 9700 and a Perkin Elmer 480 DNA Thermal Cycler. Amplification plates which could take up to 96 samples (Camlab,

Cambridge UK) or single sample 0.2 ml Eppendorf tubes could be used with the first two. The PE 480 could only take 0.5 ml Eppendorf tubes.

Five *Gs. longissima* DNA templates (sample A61, A62, H46, H52, J70) were amplified with RAPD primer OPB5-9, using PCR conditions as for RAPD screening (Table 4.3) in all three machines, using one master mix of reagents. Each reaction was carried out in duplicate. Tubes were used in all machines to match conditions as closely as possible for comparison.

4.4.2.1.4. Reaction vessels

Comparisons of amplification using 0.2 ml thin-walled tubes versus 96-well amplification plates (Camlab, UK; ABgene, UK) in the MWG thermal cycler were carried out, repeating the samples and PCR conditions as for the thermal cycler test (Table 4.3).

4.4.2.1.5. DNA extraction methods and concentration

DNA isolation method and template concentration are thought to influence the number, reproducibility and intensity of bands in RAPD profiles (Davin-Regli *et al* 1995). Two samples (A57, H45) were extracted by CTAB and Wattier methods. One sample (P83) was extracted with the DNAmite kit and Wattier methods. All three were amplified in duplicate with RAPD primer OPB5-9 using the conditions used for RAPD primer screening (Table 4.3). Three samples (A36, H12, P73) were extracted by the Chelex and Wattier methods and amplified in duplicate with OPBR8 using conditions as for RAPD primer screening (Table 4.3).

Table 4.3: Optimised PCR conditions for the amplification of RAPD markers in samples of *Gs. longissima*

PCR parameter	*Optimised conditions*
Reaction mix	
Proprietary PCR buffer*	x1
$MgCl_2$	4.5 mM
dNTPs	0.3 mM
RAPD primer	2 µM
Taq* polymerase	1.5 U
DNA template	10 ng
Oil overlay	
Thermal cycling conditions	
Denature	1 min at 94°C
Cycle (40) - Denature	1 min at 94°C
- Anneal	1 min at 50°C
- Elongate	1 min at 72°C
Final elongation	10 min at 72°C

* Taq from Roche Diagnostics Ltd was used for all PCR reported here except for RAPD PCR investigating population genetics where GoTaq Flexi DNA Polymerase (Promega, Southampton, UK) was used.

The ability of DNA extracted by different methods to amplify successfully for RAPD profiling was tested. Three specimens of *Gs. longissima* were extracted in four ways - Chelex, CTAB, DNAmite and Wattier (see Chapter 4.2.). Amplifications were carried out as in Table 4.3 with all samples replicated.

An examination of 25 publications using RAPD loci was carried out to investigate success for other authors with different DNA template concentrations. Subsequently, DNA template from sample A3 from Braunton, was added to a 25 µl reaction mix in quantities of 5, 10,15, 20, 30 and 40 ng total, representing 0.2, 0.4, 0.6, 0.8, 1.2 and 1.6 ng DNA template per µl reaction mix. Amplification conditions with primer OPB10 were as in Table 4.3.

4.4.2.2. Results and Discussion

4.4.2.2.1. Thermal cycling conditions

Figure 4.8 illustrates the very poor results of amplification using PCR conditions as described by Williams *et al* (1990). The PCR conditions used by Atienzar (2000b) for RAPD profiling, adapted slightly for the work reported here (Table 4.3), produced much better results (Figures 4.11, 4.12, 4.13 and 4.14). The use of oil appeared to give an improved result. Mixing also appeared to give more product, with little difference detectable between pipetting or spinning (Figure 4.9). Therefore, subsequently, all PCR mixes were routinely overlain with oil and mixed by spinning.

With the many potential variables within any PCR process, optimisation could be an almost endless task. Once clearly scorable results were obtained, conditions were assumed to be optimised sufficiently for the purposes required. PCR conditions shown in Table 4.3 were used for all further RAPD PCRs in this study.

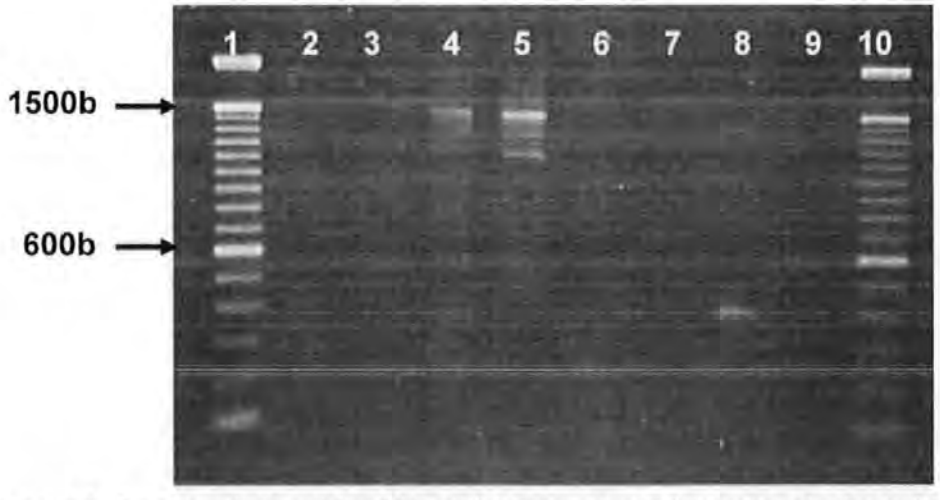


Figure 4.8: Results obtained for PCR of OPB10 using PCR protocol adapted from Williams *et al* (1990) on a 1.5% agarose gel. All template DNA was from three specimens of *Gs. longissima* from the same site (Braunton). All PCRs were carried out in duplicate. Lanes 2&3: A32, 4&5: A40, 6&7: AZ1). Lanes 8 and 9 are negative controls. Lanes 1 and 10 are 100bp ladders. Bold fragments (arrows) denote 600bp and 1500bp.

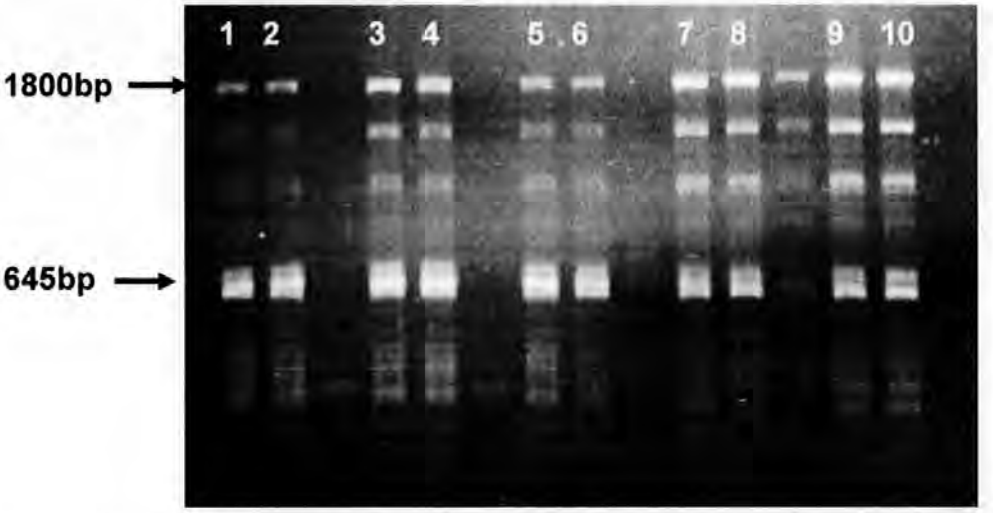


Figure 4.9: PCR results from tests using RAPD primer OPB5-9 with *Gs. longissima* sample A57 from Braunton to find out whether oil and mixing or spinning plates is a better combination. Each combination is replicated in pairs. From L to R: 1&2 no oil or mixing; 3&4 oil, no mixing; 5&6 no oil, mixing; 7&8 oil & pipette mixed; 9&10 oil & spun to mix. There appears to be some overspill of product into empty lanes.

4.4.2.2.2. Thermal cycler

No difference in results from thermal cyclers could be detected. For convenience, 96-well amplification plates were used for all PCRs which restricted use to the MWG or the Applied Biosystems machines. To avoid potential variation in results from using different machines, and for reasons of availability, all further RAPD PCRs were carried out in the Applied Biosystems thermal cycler.

4.4.2.2.3. Reaction vessels

A run of failed PCR reactions led to a realisation that the plates being used (Camlab, Cambridge, UK) were faulty in some way (Figure 4.10). This unexpected problem only became apparent after many repeated failures could not be corrected by changing reagents being used in a process with a history of success. A new set of plates from a new supplier (ABgene, Epsom, UK), tested against thin-walled tubes showed very little difference between the two (Figure 4.11). Plates were found to be more convenient in handling large numbers of samples for population genetics studies.

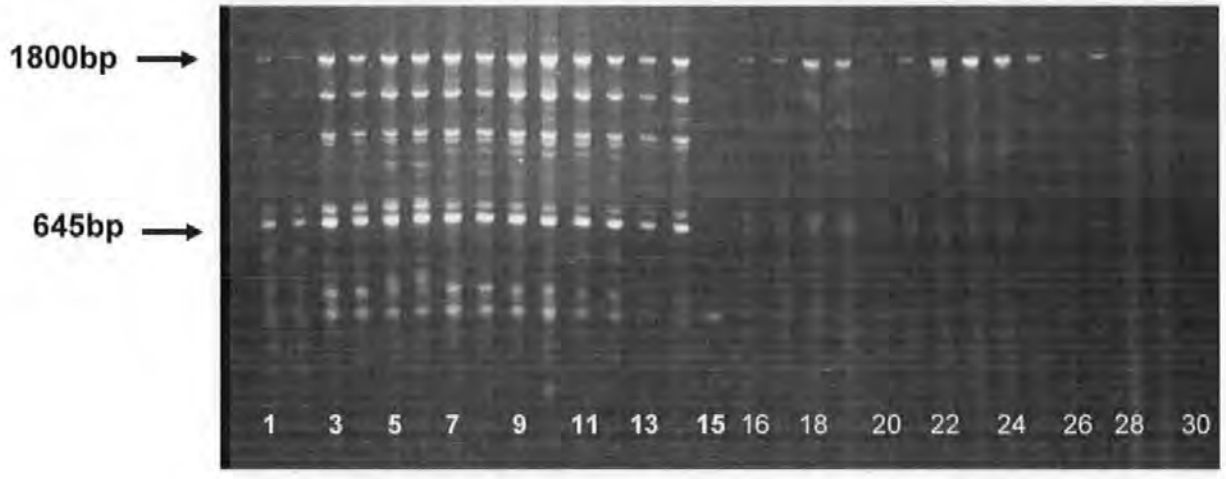


Figure 4.10: First test of thin-walled tubes (left hand side of gel) versus 96-well plates (right hand side of gel) revealed a problem with the amplification plates from Camlab. This PCR was with RAPD primer OPB5-9 and five samples of *Gs. longissima* from Braunton (A6 & A57), Helford (H45) and Place (P83). Each sample was amplified in duplicate and run next to each other on the gel. Two samples (A57 and H45) were amplified from both CTAB (CT) and Wattier (W) extractions) Samples from L to R: 1&2: A6 (W), 3&4: A57 (CT), 5&6 A57 (W), H45 (CT), H45 (W), P83 (W), A51 (DNAmite). Lanes 16 to 29 are in the same order. Lanes 15 and 30 are negative controls.

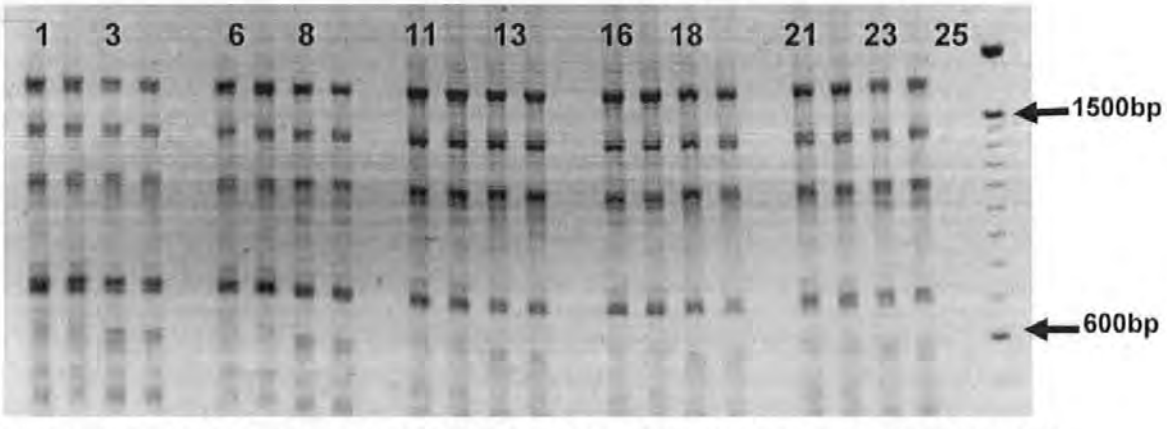


Figure 4.11: A second test of tubes (lanes 11 to 24) versus new plates (lanes 1 to 9) purchased from ABgene. Primer OPB5-9 amplfied with two samples of *Gs. longissima* used in duplicate for each test. Sample M3 from Mylor is in Lanes 1&2, 6&7, 11&12, 16&17, 21&22). Sample M4 is in lanes 3&4, 8&9, 13&14, 18&19, 23&24. Lanes 5, 10, 15, 19 and 25 are negative controls for each amplification group. Lane 26 is 100bp marker. A negative image is provided for clarity.

The experience reported here clearly demonstrates the need to investigate all possible factors when optimising PCR protocols or investigating failures in amplification.

4.4.2.2.4. DNA extraction methods and template concentration

Results with CTAB extractions compared with Wattier and DNAMite are shown in

Figure 4.12. Comparison of Wattier versus Chelex is shown in Figure 4.13. Differences between the CTAB and Wattier extractions are small. The DNAmite extraction gave weaker and less clear products. In Figure 4.13 the Wattier extractions amplify a greater number of bands, more clearly than Chelex

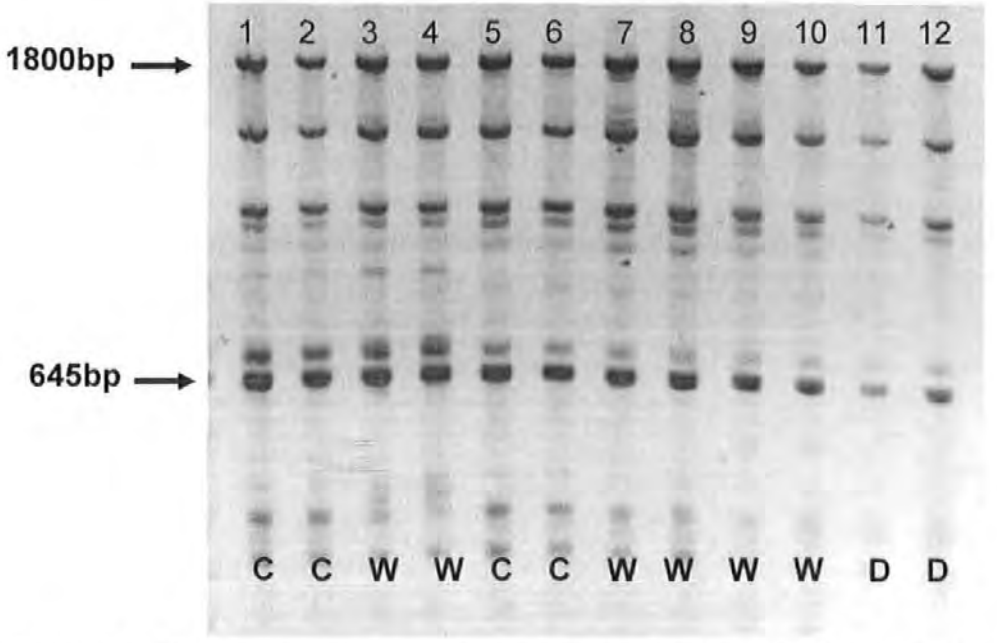


Figure 4.12: DNA extraction effects on PCR product (Primer OPB5-9) for three samples of *Gs. longissima* from Braunton (A57) in lanes 1-4, Helford (H45) in lanes 5-8 and Place (P83) in lanes 9-12. CTAB extractions (C) in lanes 1,2,5,6 produced very similar profiles to Wattier extractions (W) in lanes 3,4,7,8,9,10. The DNAmite extraction (D) in lanes 11 & 12 performed least well. A negative image is provided for clarity

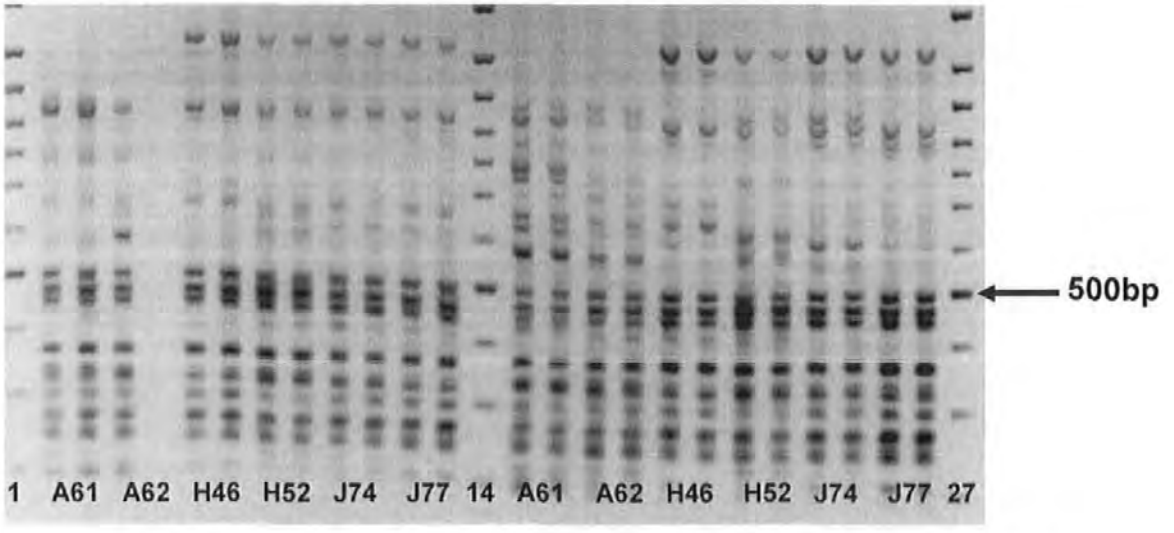


Figure 4.13: Differences between Chelex (lanes 2-13) and Wattier (lanes 15-26) DNA extraction methods for RAPD banding profiles for primer OPB5-5 in 6 samples of *Gs. longissima* from Braunton (A62, A62), Helford (H46, H52) and St Just (J74, J77) Duplicates of each amplification are run alongside one another. The A62 duplicate has failed to amplify demonstrating the importance of duplication. Lanes 1, 14 & 27 are 100bp markers. A negative image is provided for clarity.

extractions. For this reason and because the Wattier extraction method also allowed standardisation of DNA concentrations, used no noxious chemicals and gave consistent results, this method was used for all RAPD PCR procedures.

In the various papers consulted, DNA concentrations for RAPD PCRs varied from 80 pg/µl reaction mix (Faugeron *et al* 2001) to 4 ng/µl reaction mix (Haring *et al* 1996, Marston and Villalard-Bohnsack 2002). The test conducted here showed similar results for all concentrations of DNA template (Figure 4.14). For practical reasons of ease of standardisation, 20 ng per reaction mix, which had given a good, clear result, was used for all RAPD PCR procedures.

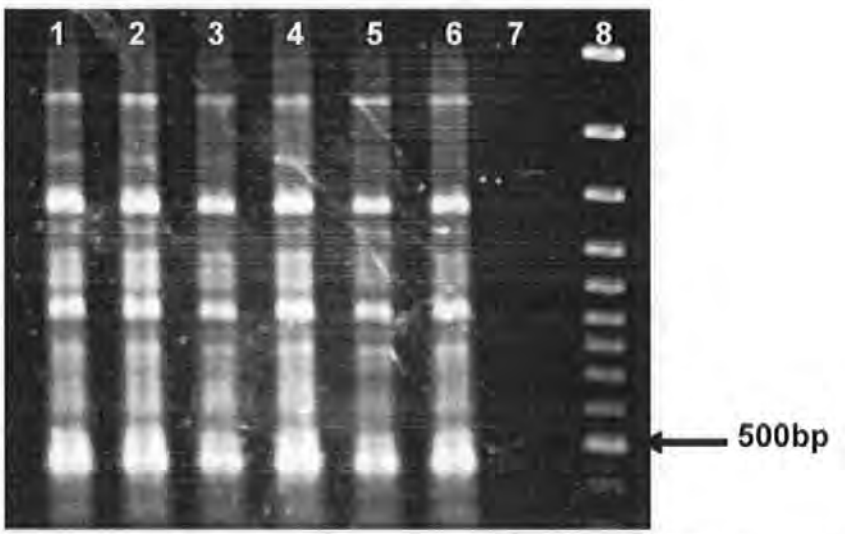


Figure 4.14: Effect of DNA template concentration on results for Primer OPB10 in a sample of *Gs. longissima* from St Just (J21) extracted by the Wattier method. Lanes 1 to 6 were amplification products from 5, 10, 15, 20, 30 and 40 ng template DNA respectively in a reaction volume of 25 µl. There is little difference between the results although concentrations below 30 ng gave marginally better results. Lane 7 - control. Lane 8 – 100bp marker. Lower bold band is 500 bp.

4.4.2.2.5. Reproducibility

Using the same primer many times for different tests demonstrated clear reproducibility in the case of the test primer (OPB5-9) despite the wide variation in test conditions. Substantially the same banding patterns were seen on each occasion (Figures 4.9., 4.10, 4.11, 4.12).

4.4.3. Screening of RAPD primers for variation

4.4.3.1. Materials and Methods

Arbitrary primer sets, each containing different 10-mers are available commercially (Operon Technologies, California, USA). Operon Technologies also publish lists of 10-mer primer sets with letter/number codes (www.operon.com). A total of 26 Operon primers were synthesised for this study by Sigma-Genosys (Sigma-Aldrich, Haverhill, UK) as shown in Table 4.4. A further 20 primers were provided by F. Atienzar who had created additional primer sets with single base substitutions (Atienzar 2000) while still meeting the criteria of RAPD primers (70% GC content, no sequences which could be complementary in reverse) . The Atienzar primers are highlighted in italics in Table 4.4. All primers were diluted to 10 µM and stored as 500 µl aliquots to avoid primer degradation through repeated freeze-thawing.

4.4.3.1.1. Samples

A special collection of haploid specimens females indentified by the presence of cystocarps (as seen in Figure 1.5) was made from three sites spanning the full geographic spread of sites in this study, known to support uni-specific populations of *Gracilariopsis longissima*. Six samples were chosen: two from Braunton Burrows (A61, A58), two from Helford (H46, H52) and two from St Just (J70, J75). Specimens were cleaned by brushing as described elsewhere in this report (Chapter 5) to remove any contaminating biofilm. All cystocarps were excised from samples to remove any diploid material.

DNA extractions (Wattier *et al* 2000) were quantified and amplified with ITS region primers as described in Chapter 4.3 to confirm species and test the ability of the extractions to amplify successfully.

4.4.3.1.2. PCR reaction conditions

PCR conditions for RAPDs were as described in Table 4.3. All amplifications were carried out in 96-well PCR plates (ABgene, Epsom, UK) in a PE Applied Biosystems GeneAmp PCR system 9700.

4.4.3.2. Results and Discussion

Results are given in Table 4.4. In summary, of 46 10-mer primers, 32 amplified successfully, producing a total of 387 scorable bands. Of those, 78 bands, from 13 primers, were observed to be polymorphic, in terms of presence or absence. Some primers produced large numbers of bands but often proved to have few which were polymorphic as can be seen in primer OPR4 (Figure 4.15) where many bands have been amplified but only three proved to be polymorphic (Table 4.4).

A wide variation in the strength of amplification was seen between samples, despite measures to standardise each amplification by using the same concentrations of DNA template in all reactions and carrying out all PCR processes at the same time with common reagents. However, scoring by use of computer analysis and visual assessment allowed for reproducible scoring.

Table 4.4: Amplification success and numbers of polymorphic loci amplified by 46 10-mer RAPD primers in *Gs. longissima*

Code	*Scorable bands*	*Number polymorphic*
B1	No amp	
B5	7	0
B5-1	13	4
B5-2	7	0
B5-3	18	8
B5-4	Poor amp	
B5-5	16	6
B5-6	18	5
B5-7	18	6
B5-8	15	5
B5-9	21	10
B5-10	Poor amp	
B6	19	12
B7	8	0
B7-1	13	6
B7-2	Poor amp	
B7-3	13	0
B7-4	11	0
B7- 5	10	0
B7- 6	17	7
B7- 7	9	0
B7- 8	12	0
B7- 9	No amp	
B7- 10	13	0
B8	5	0
B10	13	0
R1	11	0
R2	10	2
R3	Poor amp	
R4	14	3
R5	6	0
R6	Poor amp	
R7	No amp	
R8	15	4
R9	Poor amp	
R10	No amp	
R11	Poor amp	
R12	8	0
R13	13	0
R14	No amp	
R15	7	0
R16	4	0
R17	No amp	
R18	No amp	
R19	14	0
R20	9	0
Primers	**32**	**13**
Bands	**387**	**78**

Primers shown in italics are those designed by Atienzar (2000). Each is based on the Operon original (ie, B5 and B7) with a single base substitution

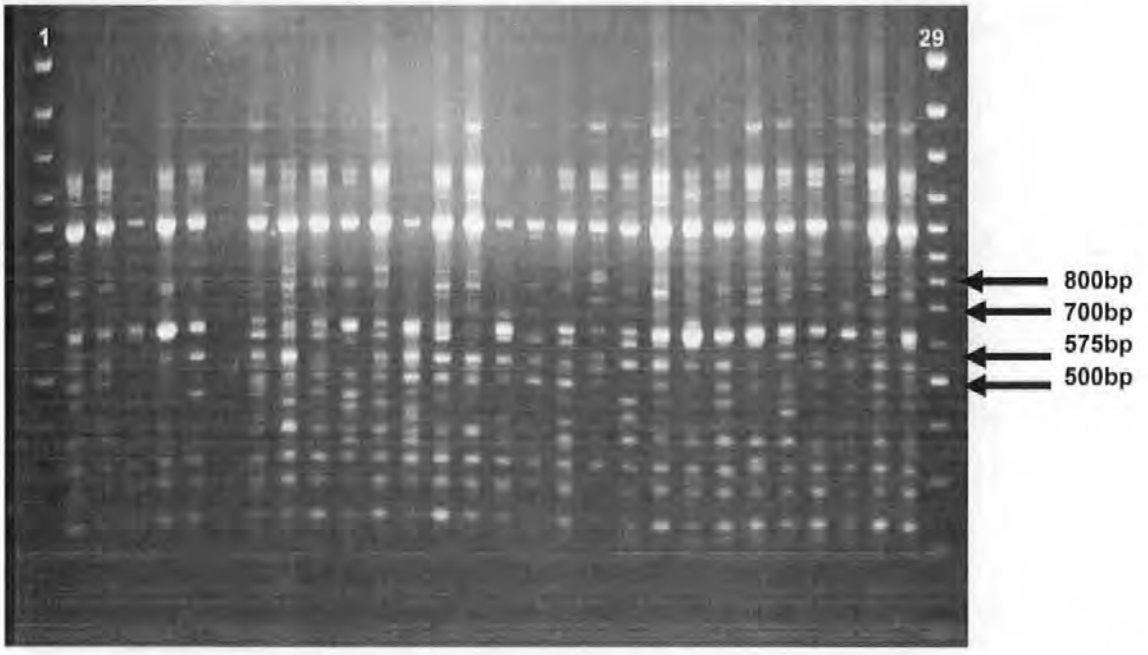


Figure 4.15: Agarose gel of amplification products for RAPD primer OPR4 for haploid female samples of *Gs. longissima.* Lanes 1 and 29 are 100bp markers. Samples in lanes 2 to 28 are J70, J73, H42, J76, A60, H49, A54, H54, J78, A50, H40, H53, J77, H44, A61, A58, H51, A55, H46, J74, J72, H53, A53, A56, J79, A63, J71. Three polymorphic bands were seen at 575bp, 700bp and 800bp (arrowed). The 500bp band in the marker lane is arrowed.

4.4.4. Conclusions

With careful attention to optimisation, PCR conditions and DNA template quality and quantity, RAPD banding profiles can be reproduced successfully and reveal information about completely unknown genomes. Even two years post extraction, this researcher was obtaining the same profiles, time after time for individual primers (as shown above).

However, there were also unexpected failures. Primer OPR8, which produced clear bands with some polymorphism (Table 4.4), failed to amplify on other occasions (data not presented). Some primers amplified well, others were more recalcitrant, suggesting that optimisation for each individual primer/template pair might produce worthwhile results, if resources are available. An option is to screen

many more primers using the existing protocol until an adequate set for the purpose required is obtained.

Unexpected problems experienced with a set of amplification plates were detected after extensive testing of all other components of the protocol. This experience provided a useful lesson in how every aspect of the PCR process can be liable to failure and very careful examination of every part should be separately investigated where failures are experienced.

4.5. Development and screening of microsatellites in *Gracilariopsis longissima*

4.5.1. Introduction

The availability of macroalgal DNA sequence information is limited. Therefore the development of species-specific markers is highly desirable. Finding microsatellites in plants is difficult (Goldstein and Schlotterer 1999). Their abundance in seaweeds appeared to be less than in higher plants when all records for published sequences were examined (Wang *et al* 1994). Wang *et al* also reported that all tandem repeats in algae contained G-C base pairs whereas only between 15 and 50% in higher plants did so.

Table 4.5 lists the results obtained by a variety of authors looking for microsatellites in five species of algae. It has been suggested that algae may have few microsatellites and many of these may be monomorphic (Billot *et al* 1998). Certainly the number of polymorphic loci found seems few but the number of alleles found in some species, for example *Enteromorpha intestinalis* (Alstrom-

Rapaport and Leskinen (2002) suggests potentially high levels of polymorphism in those microsatellites successfully isolated.

4.5.2. Materials and Methods

Although tetra-nucleotide repeats are easier to score than di- or tri- repeats, they represent less than 5% of microsatellites in *G. gracilis* (R. Wattier, personal communication) and may be similarly poorly represented in *Gs. longissima*.

4.5.2.1. Sample selection

For initial microsatellite isolation, DNA from five specimens of *Gs. longissima* was used. Two specimens from Braunton Burrows (A2, A6), two from Froe (F18, F19) and one from Helford (H7) were chosen to provide DNA from the geographically most distant populations available. For screening, an additional seven samples from St Just (J1, J2, J3, J4, J12, J21A, J23A) were used.

Table 4.5: Microsatellite abundances and polymorphisms found in genomic DNA libraries enriched for repeat motifs in 7 species of alga (NP- information not published)

Author	***Species***	*Colonies cloned*	*Positive clones*	*Number polymorphic*	*Number of alleles*
Alstrom-Rapaport and Leskinen (2002)	*Enteromorpha intestinalis*	230	15	5	67
Engel *et al* (2003)	*Fucus vesiculosus*	-	28	6	30
Guillemin *et al* (2005)	*G. chilensis*	1500	273	6	18
Van der Strate *et al* (2000)	*Cladophoropsis membranacea*	5088	174	8	46
Olsen *et al* (2002)	*Ascophyllum nodosum*	NP	70	6	93
Lou *et al* (1999)	*Gracilaria gracilis*	15,000	1000	9	41
Whitmer (2002)	*Postelsia palmaeformis*	-	-	6	21

4.5.2.2. DNA isolation

Genomic DNA (30µg) was isolated from each sample using the method of Wattier *et al* 2000 as described in Section 4.2.

4.5.2.3. Microsatellite sequence isolation

The NERC Sheffield Molecular Genetics Facility developed microsatellite primers for *Gs. longissima* using the method of Armour *et al* (1994) as modified by Gibbs *et al* 1997.

In summary, 5 µg genomic DNA was digested overnight at 37°C with 10 units of restriction enzyme *Mbo* I (ABgene, Surrey, UK) which cuts double stranded DNA at 5'/GATC 3'(3' CTAG/ 5') sequences. Since the *Mbo* I fragments created were approximately of the size required (200 to 600 bp) size selection was not performed. The digested DNA was then precipitated using 3M sodium acetate (pH 5.5) and absolute ethanol, washed with 70% ethanol and then resuspended in double distilled autoclaved water to a concentration of approximately 1 µg/µl.

Resulting fragments were ligated to *Sau*-LA-B linkers (*Sau*-L-A GCGGTACCCGGGAAGCTTGG, *Sau*-L-B GATCCCAAGCTTCCCGGGTACCGC) (Royle *et al* 1992) to provide priming sites to allow for amplification of the fragments obtained from digestion. Linkers also protect the ends of digested DNA fragments. Ligation was performed by incubating approximately 2.5 µg of DNA (which had been *Mbo*I digested and precipitated) with approximately 5 µg of the annealed linkers (ie, *Sau*-L-A annealed to *Sau*-L-B linker) and T4 DNA ligase enzyme (Gibco BRL, Invitrogen, Paisley, UK) at 15°C overnight.

Resulting fragments were enriched for the following di-, tri- and tetra-nucleotide sequences:-

Dinucleotides	$(GC)_n$, (AC)n, (GA), (GT)n,
Trinucleotides	(CAA)n, (GCC)n, (CTG)n, (CAG)n
Tetranucleotides	(CTAA)n, (GTAA)n, (TTTC)n, (GATA)n.

The di-, tri- and tetra-nucleotides are all double stranded, therefore in each case the complement is also being enriched for. The dinucleotides were purchased as double stranded from Pharmacia and the tri- and tetra-nucleotide sequences were ordered as two complementary short single strands and extended and made double-stranded as in Armour *et al* (1994).

Enrichment was achieved by a non-radioactive hybridization incubation between denatured digested DNA and single-strand probes of the selected sequences (as listed above) bound to 1 cm square pieces of nylon Hybond N filters (Amersham Pharmacia Biotech). The enrichment hybridization was performed overnight at 55°C.

Genomic DNA not bound to the filter was then washed from the mix before the DNA bound to the filters (ie, to the target sequences) was then stripped from the nylon filter with a wash solution of 50 mM potassium hydroxide and 0.01% SDS incubated at room temperature for 30 min. DNA in the wash solution was precipitated with absolute ethanol and 3 M sodium acetate (pH 5.5). This DNA was now enriched for the target sequences (ie, microsatellite repeat patterns).

The algal DNA was PCR amplified using the *Sau*L A linker sequence as priming sites to convert the single stranded DNA to double stranded. The linkers were then removed by digesting again with *Mbo* I ready for ligation into the vector. The vector (pUC18 New England Biolabs, Hitchin, Hertfordshire, UK) was prepared by digestion with *BamH* I to create suitable ends for ligation with the genomic DNA and dephosphorylation with shrimp alkaline phosphatase (SAP). Ligation of the microsatellite-enriched genomic DNA to the vector was carried out by incubation of 1 µg of DNA with vector DNA (<100 ng) and DNA ligase overnight at 15°C.

The genomic DNA ligated into the pUC18-BamHI-SAPed vector was then transformed into XL1 Blue (Stratagene, La Jolly, CA, USA) competent cells and plated out on agar plates containing ampicillin, tetracycline, X-gal and IPTG to grow colonies. After incubation overnight at 37°C, colonies were screened and those which were white in colour picked into Luria broth (LB) microtitre plates containing ampicillin and 20% glycerol for plating onto Hybond using a metal "hedgehog" colony transfer device and for storage at -80°C. The colonies which were white (as opposed to blue) should contain an insert (microsatellite) of interest.

Two thousand colonies were screened with radioactively labelled (alpha dCTP labelled) repeat motifs (as used earlier for the non-radioactive enrichment hybridisation) which identified 21 colonies that produced a positive signal on the autoradiograph. DNA was isolated from these 21 colonies using a miniprep kit and then sequenced in both directions using M13 universal sequencing primers (M13F: 5-'GTAAAACGACGGCCAGT-3', M13R: 5'-CAGGAAACAGCTATGAC-3'

(Pharmacia, Sandwich, Kent, UK) on an ABI 377 Sequencer (PE Applied Biosystems, Warrington, UK).

From these, 14 sequences contained microsatellite repeat regions and were unique. These sequences were submitted to the EMBL database (Accession numbers: AJ512800 to AJ512813). Primer sets (Table 4.6) were designed in Sheffield using Primer (version3) (Rozen and Skaletsky 1996). These were used to determine whether they successfully amplified microsatellite loci in *Gs. longissima* specimens from the study area and to detect any allelic variability.

4.5.2.4. Microsatellite screening for polymorphisms

Screening for polymorphisms was carried out at Sheffield NERC Molecular Genetics Facility and Plymouth University. To screen for polymorphisms, each of the 14 microsatellite primer pairs were tested with DNA template from 12 specimens of *Gs. longissima* from four geographically separated sites (St Just, Braunton, Froe and Helford) as described above. PCR amplifications were carried out. Template (10 ng) was added to a reaction mix containing buffer (ABgene, Epsom, UK), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM each primer (forward and reverse), and 0.5 units of Taq polymerase (ABgene, Epsom, UK). Each reaction was carried out in duplicate. Negative controls were included with each PCR run.

Amplification was carried out in an MWG AG Biotech Primus 96 Plus. Conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 30 sec at 60°C (annealing), 30 sec at 72°C (extension). Products were visualised initially on a 1% agarose gel stained with ethidium bromide.

Where potential polymorphism was seen as size variation in fragments between samples, higher resolution of bands was attempted with silver-stained, 6% polyacrylamide gels (19:1 acrylamide:bisacrylamide, AccuGel, National Diagnostics, Yorkshire, UK). Gels were run in x1 TBE buffer at 150 v for 5 h. Gels were fixed/stopped by covering with 10% glacial acetic acid for 30 min. After rinsing with distilled water, gels were stained for 30 min with 200 ml silver nitrate solution (10% w/v) with 300 µl formaldehyde which is used to reduce the silver nitrate to metallic silver ions in the solution. A further rinse with distilled water was followed by the addition of a developer solution of anhydrous sodium carbonate (30g l^{-1}) with 300 µl of formaldehyde and 40 µl of sodium thiosulphate (0.2g l^{-1}). Silver DNA bands developed over 5-10 min. A further 200 ml of fix/stop solution was added to the developing solution and left for at least 3 min. Gels were cleaned of all staining chemicals with distilled water and left to dry at room temperature before scoring and photographing.

4.5.3. Results

Many primers failed to amplify clear product despite several attempts. Several loci amplified poorly, producing smeary product which was difficult to resolve (see Table 4.6). Some samples failed to amplify or amplified very poorly compared with other samples. For example, sample J4 failed to amplify at all with primer SWD3D12 and sample J12 amplified less strongly than other samples (Figure 4.16). Results are summarised in Table 4.6.

Figures 4.16 and 4.17 are representative agarose gels showing the apparently monomorphic nature of the microsatellites isolated in this study. Silver-stained

Table 4.6: Microsatellite sequences isolated in *Gs. longissima* with primer sequences for amplification, amplification results and number of alleles for each locus (N - intervening non-repeat regions)

Locus	*Core sequence*	*Primer sequences (5'-3')*	*Expected product size (bp)*	*No. of alleles*
SWD3D02	$(CA)_7(TA)(CA)_2(TA)_2N_{59}(CA)_{13}$	F: CAG CAC TTC TCC AAA CGC CC R: TCA GAC TCG ACA AGT GCC GC	161	No product
SWD3D12	$(GT)_2GCAT(GT)_4CT(GT)_6$	F: CGT CTT TTG CAG ATT CTG TAC GC R: GTA CGC CAC CAG GGC CTA AC	166	1
SWD5H10	$(CT)_2GC(GT)_3GC(GT)_5CA(CT)_6$	F: ATC AAG CGG GCA TAA GCG G R: CTC CGT GCT TCC CTA CCT GC	152	1
SWD9E01	$(CAA)_6$	F: GAT ACA AAC ATC GGC ACC TCT G R: CAA TGG CTG CTT CTA GTT TGG TAG	175	Unresolved product
SWD9F06	$(GT)_{13}$	F: TCC TAC GAC GTT TTT GAA GGA GG R: TGC CCA TCA AGC AAA GGA CA	343	Unresolved product
SWD9G06	$(GCT)_5$	F: CCC TCC GCC GAC AAG CTC R: GGG AGC AAC GTT GGA CAC GA	168	Unresolved product
SWD10B09	$(CA)_{13}$	F:TAG CCA CCG AAC TTC CAC GC R:GTG GAC GGT TAC TGC GGT CC	213	1
SWD11A01	$(CT)_2(GT)_2T(GT)_9CT(GT)_2CTGTGG(GT)_3$	F:GAT CGC AAT AGC AGT GGA GGG R:CTC CGT ATC ATC GCG CTC AC	141	1
SWD12D04	$(TC)_6N_{25}(TC)_6$	F: CGG GTT TCT TTC TCC CTT CTC C R CGC CAT CAG CAG CGT AAC TG:	253	1
SWD17D01	$(GA)_{12}$	F: CAG GCG ACC AAA CGT CAT ACC R: TCC AAG ATG CTT AAG ACG GCT G	135	Unresolved product
SWD20F03	$GTT(GTG)_9GTT$	F: CGG CGA TAT CGG ATA GGT GC R: ATC CAA TCG CTG AAC CGC TC	326	1
SWP21E06	$(CAG)_9$	F: CTC AGC CAC CGG TGT AGT GC R: GCT GGC ACT GCA GCT AAT GAG	159	1
SWP21G07	$(CAG)_8$	F: ATC CGG TTG CAG AGC AAA GG R: GCT ACA ACT GCC CGA GTC GC	214	1
SWP48C02	$(CA)_{19}$	F: ATC TTC GCA AGC ACC AGC G R: CCG AAG ACT TTG CTT TGC CG	172	No product

polyacrylamide gels with higher resolution of band sizes, confirmed this result (Figures 4.18 and 4.19) where clear amplification could be detected. However, the quality of the silver stained polyacrylamide gels made scoring difficult. Stutter bands as seen on Figures 4.18 and 4.19 are characteristic of microsatellites (Schlotterer and Tautz 1992).

Figure 4.16: Amplification product from 12 *Gs. longissima* samples for microsatellite locus Hae III marker. Lanes 2 to 13 are, from L to R, samples A2, A6, F18, F19, H7, J1, J2, J3, J4, J12, J21A, J23A. Bands for all samples appear to be the same size (152bp). Lane 14 is the negative control.

Figure 4.17: Amplification product from 12 *Gs. longissima* samples for microsatellite locus SWD3D12 visualised on an ethidium bromide stained agarose gel (1.5%). Lane 1 is 123bp marker. Lanes 2 to 13 are, from L to R, samples A2, A6, F18, F19, H7, J1, J2, J3, J4, J12, J21A, J23A. Bands for all samples appear to be the same size (160bp). Sample J4 has not amplified. Numbers written on the gel are secondary lab codes for individual samples as listed.

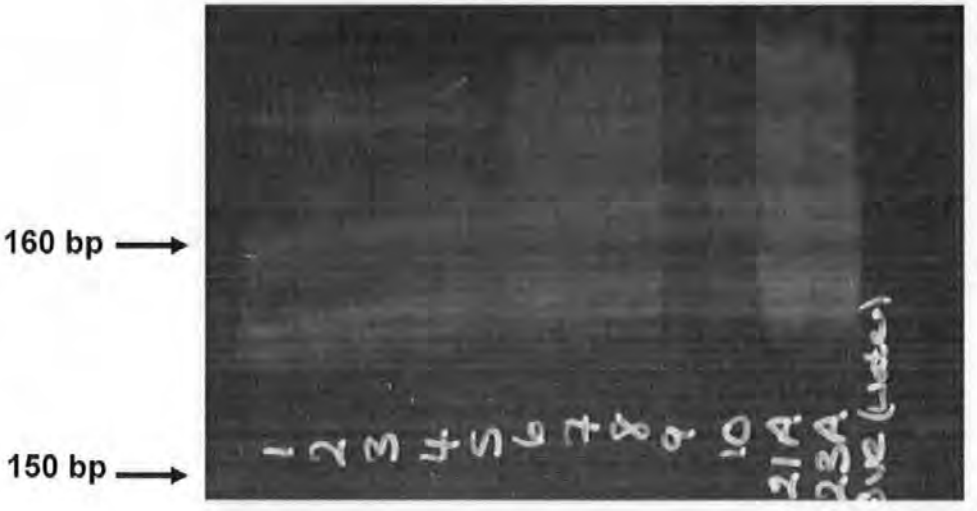


Figure 4.18: Amplification results obtained with locus SWD3D12 in 12 samples of *Gs. longissima* run on a polyacrylamide, silver-stained gel. The locus is monomorphic with one allele at 160bp, with stutter bands characteristic of microsatellite loci. Lane 1 is a 100bp ladder. The 150bp marker is arrowed although it is not very clear to see. Lanes 2 to 13 are, from L to R, samples A2, A6, F18, F19, H7, J1, J2, J3, J4, J12, J21A, J23A. Lane 14 is the negative control. Numbers written on the gel refer to secondary lab codes for samples.

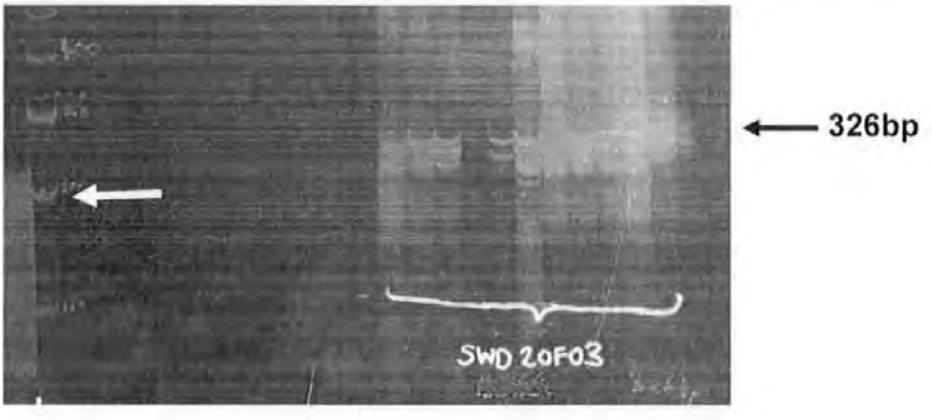


Figure 4.19: Amplification results obtained with locus SWD20F03 in 12 samples of *Gs. longissima* run on a polyacrylamide, silver-stained gel. The locus is monomorphic with one allele of 326bp, with stutter bands characteristic of microsatellite loci. Lane 1 is a 100bp ladder which is difficult to see. 300bp marker (white arrow) in the 50bp ladder on the far left. Lanes 1 to 12 are, from L to R, samples A2, A6, F18, F19, H7, J1, J2, J3, J4, J12, J21A, J23A.

Of the 14 primers tested, 10 produced resolveable product. Seven were monomorphic and three appeared to be polymorphic but this was unconfirmed as size differences were not well resolved on silver-stained acrylamide gels. Further attempts to see polymorphisms proved unsuccessful at this time. Varying conditions for PCR did not produce any noticeably different results. Sequence data have been published on the EMBL Nucleotide Sequence Database under accession numbers AJ512800- AJ512813.

4.5.4. Discussion and Conclusions

The apparently monomorphic nature of the microsatellites isolated in this study requires further investigation. Although samples from most distant populations were included, the numbers of samples was low and it may be that polymorphisms could have been revealed if many more individuals had been included. The use of agarose gels, which are unable to provide the same level of resolution of band sizes as, say, polyacrylamide gels, for some initial screening may also have led to some variation remaining undetected.

However, the lack of polymorphism seen may be real in these particular microsatellites. The maximum of 3 potentially polymorphic loci was less than those found by other researchers in other algae (for example, Olsen *et al* 2002, Wang *et al* 1994, Guillemin *et al* 2005, Alstrom-Rappaport and Leskinen 2002, Engel *et al* 2003). This low level of polymorphism may be partly due to the length of the repeat sequences in the microsatellites reported here compared with those found in other species (e.g., Wattier *et al* 1997). Only four of the microsatellites isolated had more than ten consecutive repeat motifs (Table 4.6). Less than ten repeats of a microsatellite are thought to be associated with low numbers of alleles (e.g., Akkaya *et al* 1992). However, the inclusion of greater numbers of samples in tests could have possibly revealed so far undetected polymorphisms. With further resources, this course of action would be worthwhile.

Since the work reported here was carried out many more macroalgal species have been screened and microsatellites markers found, as phycologists have developed skills in molecular techniques, as discussed in the introduction to this thesis.

4.6. Evaluation of cross-species amplification of *Gracilaria gracilis* microsatellites in *Gracilariopsis longissima* and other red algae

4.6.1. Introduction

As described earlier (Chapter 1.2.6), microsatellites have been developed for a number of macroalgae in recent years including *G. gracilis* (Luo *et al* 1999, Wattier *et al* 1997). Some researchers have attempted cross-species (Billard *et al* 2005) and cross-genera (Engel *et al* 2003, Martinez *et al* 2005) amplification with mixed results. Wattier *et al* (1997) had some success in amplifying *G. gracilis* microsatellites in other *Gracilaria* species (*G.pacifica*, *G. tikvahiae*) and in *Gs. longissima*.

Many researchers have found a strong relationship between evolutionary distance and microsatellite conservation, others (e.g., Dallimer 1999) did not. Balloux *et al* (1998) found, in shrews, that phylogenetic distance correlated with success of cross-species amplification. Therefore, expecting microsatellites found in one genus (*Gracilariopsis*) to amplify in another (*Gracilaria*) may seem optimistic. Given the results reported by Wattier *et al* (1997) and the amount of work and expense involved in developing new markers, an experiment to test *G. gracilis* markers in other species, including *Gs. longissima*, was considered worthwhile, particularly if they could be used to investigate the genetics of *Gs. longissima* populations found in estuaries in south west England.

To investigate the suitability of these markers for studies of the population genetics of *Gracilariopsis longissima*, screening of six microsatellite markers was carried out and the results are reported here. The availability of samples of other species

related to *Gracilaria gracilis* allowed a wider investigation of the potential cross-species amplification in these markers.

4.6.2. Materials and Methods

Thirteen species of Gracilariales were used (Table 4.7) although, for most, only one or two individual specimens were available. All samples, apart from *Gs. longissima* collected as part of this study, were provided by C. Destombe. For *G. chilensis*, three specimens from different sites were used.

For *Gs. longissima*, 32 individuals from four British populations (St Just, Froe, Appledore, Newton Ferrers) and one from France (Lonnodec, Britanny) were included in the study, of which up to 22 were used for any one set of PCR reactions. The ploidy of individual specimens was unknown.

Table 4.7: List of species of Gracilariales used to test the potential for cross-species amplification of microsatellite markers isolated from *Gracilaria gracilis*

Species	No of specimens
G. gracilis	4
G. chilensis	3
G. multipartita	2
G. bursa-pastoris	2
G. cervicornis	1
G. tikvahiae	2
G. pacifica	1
G. crassissima	1
G. cornea	1
Gs. longissima	32
Gs. lemaneiformis	1
Curdiea flabellata	1
Melanthalia obtusata	1
M. abscissa	1

The identity of all *Gs. longissima* samples was confirmed by the ITS amplification method of Goff *et al* (1994) as described in Chapter 2. Two samples of *G. gracilis* of known genotype with regard to the microsatellites were used as positive controls in all experiments. DNA was extracted using the Chelex method of Goff and Moon (1993).

Microsatellite loci primers were chosen from those already isolated for *Gracilaria gracilis* (Luo *et al* 1999, Wattier *et al* 1997). Repeat motifs and primer sequences of those used in this study are listed in Table 4.8.

Amplification by PCR was carried out using the method used by Wattier *et al* (1997) for *G. gracilis*. Most stringent conditions were defined as those standard for *G. gracilis*: these were 2 mM $MgCl_2$, annealing temperature of 55 to 60°C, and 30 reaction cycles. Stringency was reduced to increase the likelihood of amplification. Therefore, $MgCl_2$ concentration was increased to 2.5 or 3 mM, annealing temperature was lowered by 5°C, and the number of cycles increased by 5. In addition, 10 mg/ml bovine serum albumin (BSA) was added to all reaction mixtures to prevent PCR inhibition with potential impure DNA samples and PCR cycles were begun with a 5-cycle "touchdown" programme. This programme starts at the standard annealing temperature and declines by one degree per cycle for the first five cycles to reach the lower annealing temperature before commencing the main programme at the lower temperature. This helps to reduce the number of fragments produced which may be artefacts rather than good quality, scorable bands (M. Valero, personal communication). At least two PCR amplifications were carried out for each locus with a full set of samples as listed in Table 4.7.

Table 4.8: Microsatellite sequences isolated in *G. gracilis* and primer sequences for their amplification with expected band sizes and number of alleles already found in *G. gracilis* (Lou *et al* 1999), used in this study to investigate the potential for cross-amplification in other species of red algae from the Order Gracilariales (N-intervening non-repeat regions)

Locus	Core sequence	Primer sequences (5'-3')	Expected product size (bp)	No. of alleles found in *G. gracilis*
Gg121	$(CT)_{17}$	F:TTG ATT CTT CGC TGG TGT CC R: ATT TGC TGC CCA TTT TCT TG	201-230	7
Gg122	$(GGC)_{11}(GA)_4N_{12}(GA)_6AA(GA)_2N_{25}(GCA)_4N_{84}(GT)_{12}$	F: CGC GCT GAC GTA AGC TT R TCG TCC GTG AGC TGC ATG G	236-250	7
Gg155	$(CT)_{11}$	F: GAA GAT GCA ACC AGT CGT GA R: ACC TCA ACT CGG GTA CAT GC	201-205	3
Gg173	$(TC)_{12}TG(TC)_{13}$	F: AGA ATC GCA GGA AGC CGT AT R: CTC CAC TTA CTA CGA GCG CC	164-174	4
Gg182	$(GA)_7N_{19}(CA)_{12}$	F: AAG TTG TGT TTG TGT GCG T R: AAT CGG AGT ACC GAC GAG	127-141	6
Gg202	$(CT)_{11}$	F: TGA CAT TCG TTC ATG CAG R: TGC TGG CCT TCT TAT TGA	168-178	4

Products were analysed by loading on a Li-Cor automated DNA sequencer using fluorescence-labelled primers ID 700 or 800 (MWG Biotech) on a denaturing polyacrylamide gel. Initial experiments suggested that there were six potentially useful loci.

4.6.3. Results

As with other cross-species research described above, some loci in this study amplified in a wide range of species, while others amplified in only a few. Three species failed to amplify any of the microsatellites investigated: *Curdiea flabellata*, *Melanthalia obtusata* and *M. abscissa*. *G. cornea* showed doubtful amplification at one locus (202). Figure 4.20 demonstrates the variable success of amplification in locus Gg121, where specimens of *Gracilaria gracilis* have all amplified but only one of the other species (*G. chilensis*) amplified, although very weakly. Locus Gg155 was more successful with six different species amplifying successfully (*G. gracilis, Gs. longissima, G. chilensis, G. multipartita* and *Gs. lemaneiformis*) (Figure 2.22). Table 4.9 summarises the results for all loci.

Gel quality was highly variable. Also, very variable amplification strength was seen between species within loci which made exposure of gels for scoring very difficult. This can be seen clearly in Figure 4.22 where specimens of *Gracilaria gracilis* have amplified very strongly (lanes 4&5) while specimens of *Gracilaria bursa-pastoris* have amplified weakly in comparison (lanes 1 &2). Some loci amplified successfully in *Gs. longissima* but not in all individuals. For example, out of 22, only 9 individuals produced bands for locus 155 (Figure 4.21 and 4.24). Also, certain individual specimens of *Gracilariopsis longissima* amplified differing numbers of microsatellites, for example, one individual (F21) amplified only locus

Table 4.9: Numbers and sizes of alleles amplified in fifteen species of gracilariales using six microsatellite markers isolated in *Gracilaria gracilis*

Locus	Gg121	Gg122	Gg155	Gg173	Gg182	Gg202	*Total loci*
Expected allele sizes (bp)	**203-245**	**236-250**	**201-205**	**164-174**	**125-141**	**166-178**	
Species							
G. gracilis	203	250	201-207	166-168	124-144	166-170	**6**
G. chilensis	203-235	238	201-209	162&170	128&131	?	**5**
G. multipartita	-	-	205	164&175	125&141	168&170	**4**
G. bursa-pastoris	?	?	?	168&175	115&144	?	**2**
G. cervicormis	?	238	-	?	127	?	**2**
G. tikvahiae	?	-	?	162-164	142	-	**2**
G. pacifica	-	-	203	-	-	-	**1**
G. crassissima	-	?	203-205	-	-	-	**1**
G. cornea	?	?	-	-	-	?	**0**
Gs. longissima	-	?	201-207	164-173	110-214	?	**3**
Gs. lemaneiformis	-	?	207	164	?	?	**2**
Curdiea flabellata	-	-	-	-	-	-	**0**
Melanthalia obtusata	-	-	-	-	-	-	**0**
M. abscissa	-	-	-	-	-	-	**0**
Total alleles amplified	**2**	**2**	**5**	**5**	**5**	**5**	
Total species amplified	**2**	**3**	**7**	**7**	**6**	**3**	

Key: - no amplification ? doubtful amplification

155, F22 only locus 173, while in F24 both 155 and 173 amplified. Some species produced unexpected band sizes such as *G. bursa-pastoris* which amplified an allele of 143bp for locus Gg202 when the expected range for *G. gracilis* was 166-176. Also, while *Gs. lemaneiformis* successfully amplified an allele at 207bp for

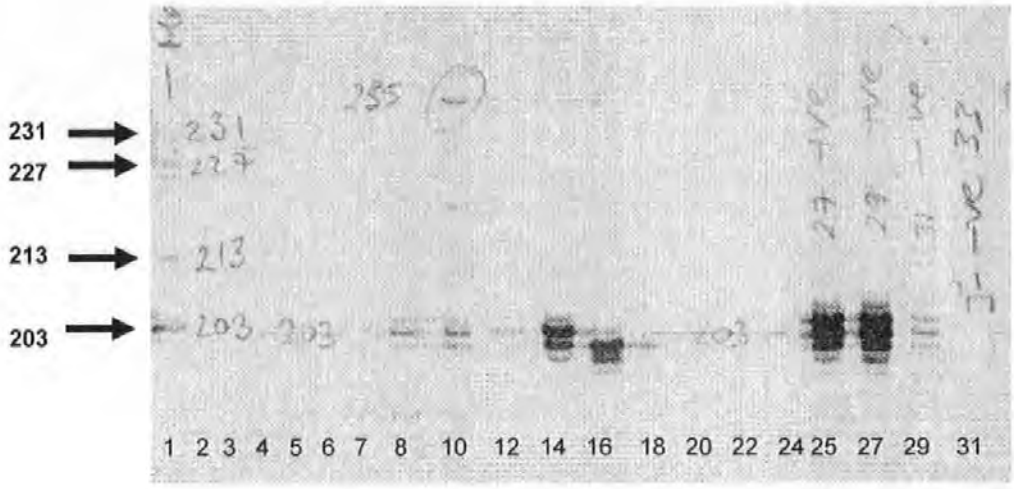


Figure 4.20: Representative polyacrylamide gel of amplification of microsatellite locus Gg121 in a number of species of Gracilariales. Amplification is successful in *G. gracilis* controls (lanes 25 and 27) and less strongly in 3 other specimens of *G. gracilis* in lanes 12, 14 and 16. Lane 1 is a marker with bands as arrowed. The faint product seen in lanes 8 & 10 is from two specimens of *Gracilaria chilensis*. No other specimens have successfully amplified. The product seen in one of the two negative control lanes (29 and 31) may be overspill from lane 27 as the other negative control is clean.

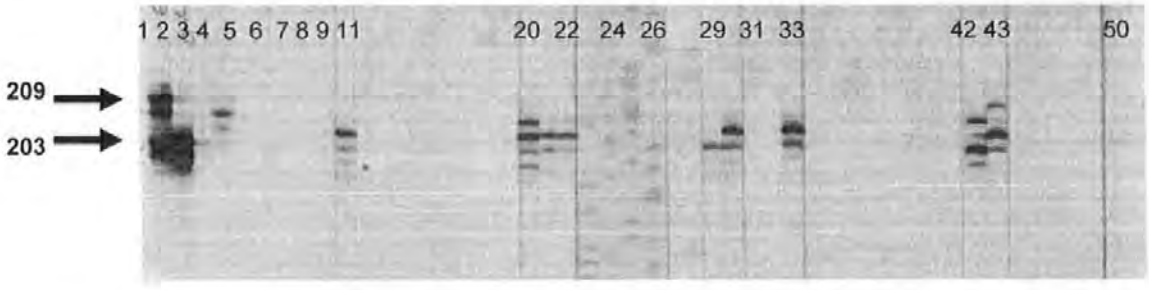


Figure 4.21: Representative polyacrylamide gel of amplification success of microsatellite locus Gg155 in a number of species of Gracilariales. Specimens of 6 species have amplified successfully. Lanes 2 &3 are *G. gracilis*. Lanes 11,20, 21, 22, 42 &43 are *Gs. longissima*. Lane 29 is *G. chilensis*. Lane 30 is *G. multipartita*. Lane 33 is *Gs. lemaneiformis* (207bp). Lanes 23 to 26 are pUC (R) which allows individual base pair counting to size bands accurately. Other lanes represent all other samples tested where no product was amplified for this locus. For example, lanes 34 to 41 are all specimens of *Gs. longissima*.

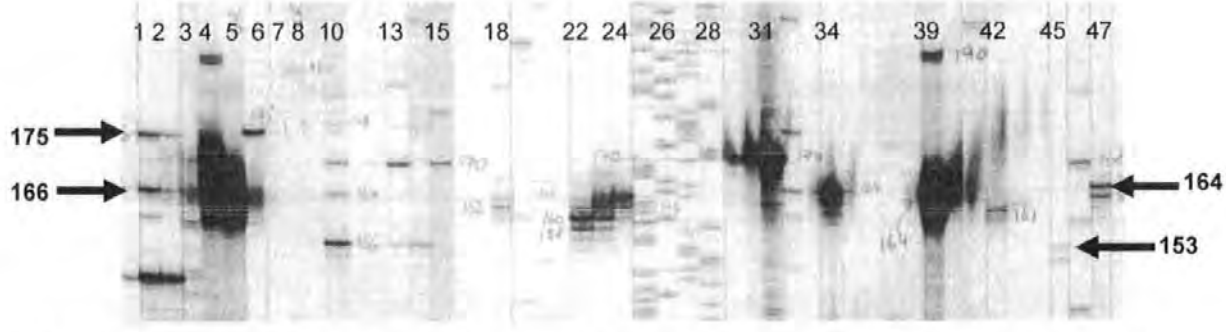


Figure 4.22: Representative polyacrylamide gel of amplification success of microsatellite locus Gg173 in a number of species of Gracilariales. This gel demonstrates the wide variation in product strength between species with *G. gracilis* (lanes 4,5&39) amplifying very strongly compared with *G. bursa-pastoris* (lanes 1 &2). 11 specimens of *Gs. longissima* have successfully amplified (lanes 10,13,14,1518,22, 23,24,42,45,46,47, although some sizes were outside the expected range of 164-174bp (e.g., lane 45, 153bp). Charateristic stutter bands can be seen in lanes 22,23,24&47.Lanes 25-28 are pUC(R).

locus Gg155 (Figure 4.21) from one PCR, on another run with this locus, it produced two spurious bands outside the expected size range at 178bp and 219bp (Figure 4.24).

Gs. longissima seemed to show some population-specific variation in amplification. For example, all individuals (5) from one population (Lonnodec, France) amplified at locus 155 (Figure 4.21 lanes 20-22), while only some individuals from Newton Ferrers, St Just and Froe amplified for that locus (Figure 4.21, lanes 11, 42, 43). Individuals from Appledore did not amplify at all for locus 155. For Locus 173, again, not all individuals amplified but one individual from Appledore did. For Locus 202 only Froe and Appledore individuals amplified. Overall frequencies of success for the three amplified loci were: Gg155 - 58%; Gg173 - 30%; Gg202 - 14%.

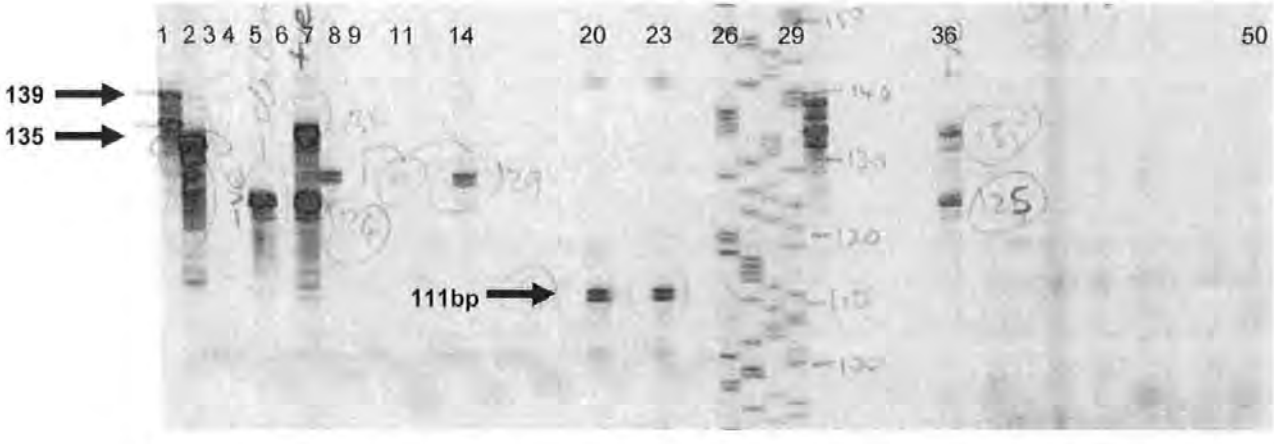


Figure 4.23: Representative polyacrylamide gel of amplification of microsatellite locus Gg182 in a number of species of Gracilariales. Lanes 1&30 are markers of 135 and 139bp. Species which amplified successfully are shown in lanes 2, 5, 7 & 36 (*G. gracilis*), lanes 8,11 &14 (*G. chilensis*) and lanes 20 &23 (*G. bursa-pastoris*). Lanes 26-29 are pUC (R) which allows individual base pair counting to size bands accurately. Other lanes represent all other samples tested where no product was amplified for this locus. For example, lanes 31-35, 37-50 are all samples of *Gs. longissima*. The specimen in lane 36 has amplified two alleles at 125 and 135 bp.

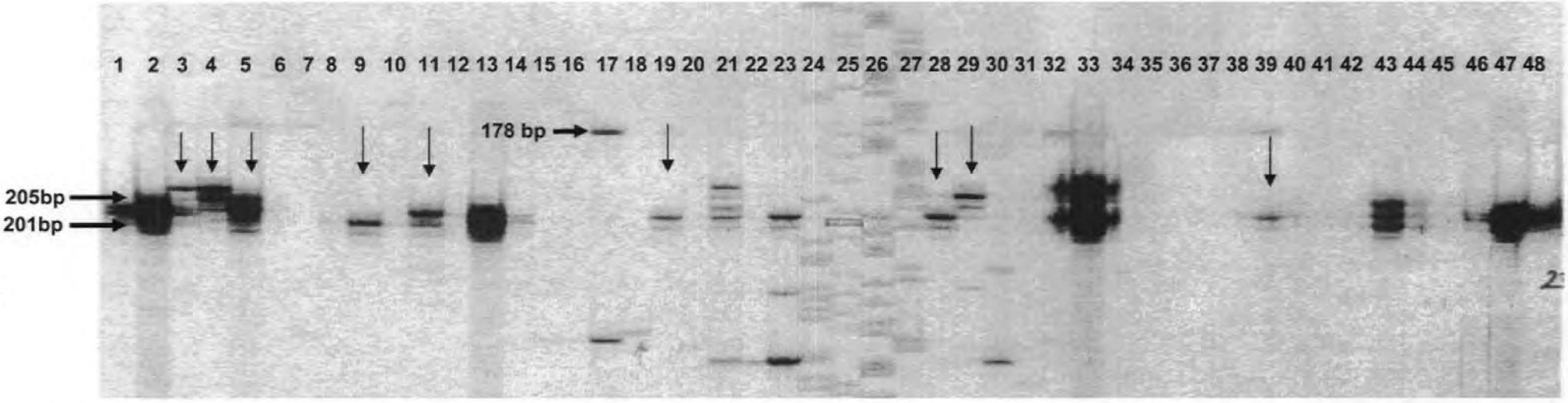


Figure 4.24: Representative polyacrylamide gel of amplification of microsatellite locus Gg155 in a number of species of Gracilariales. Those which have successfully amplified are *G. gracilis* (+ve controls and other specimens, lanes 2,13, 33, 47), *Gs. longissima* (lanes 3,4,5,9,11,28,29,39 – arrowed), *G. pacifica* (lane 19), *G. chilensis* (lanes 21,23) and *G. crassissima* (lane 43). A specimen of *Gs. lemaneiformis* in lane 17 has amplified some bands of unexpected size which may not be the locus under investigation (178bp & 219bp). Lanes 1 & 12 are negative controls. The product in lane 1 is probably overspill from lane 2 which entered the wrong lane when the gel was loaded. Lanes 24 to 27 are pUC (R) which allows individual base pair counting to size bands accurately. Other lanes represent all other samples tested where no product was amplified for this locus. For example, lanes 6,7 and 8 are all specimens of *Gs. longissima*.

4.6.4. Discussion

For cross-species amplification of microsatellites to work, flanking regions of the microsatellite need to be conserved in the species being tested. The variable results obtained with markers designed for *Gracilaria* but used in *Gracilariopsis* is not particularly surprising, given that they are from different genera rather than just different species. Sequencing of amplified products might provide a clue as to why this variability is seen, and indicate whether further investigation is worthwhile. The patchy amplification could also be the result of null alleles associated with differences between geographically distant populations.

Ongoing debate about red algal phylogenies makes it hard to be entirely confident about relationships among the Gracilariales. However, there does seem to be common consensus about some of the species tested here. Iyer *et al* (2005b), Gurgel and Fredericq (2004) and Byrne *et al* (2002) all propose that *G. pacifica* is more closely related to *G. gracilis* than *G. tikvahiae*, followed by *G. chilensis*, with *G. cornea* and *G. crassissima* the most distantly related species. Bird *et al* (1992), Goff *et al* (1994), Rice and Bird (1990) demonstrated that *Gracilariopsis* species were clearly phylogenetically distant from *Gracilaria*.

In this study, more *G. gracilis* microsatellites were amplified in *G. chilensis* than any other species (5), followed closely by *G. multipartita* (4). Amplification in *Gs. longissima* was the next most successful (3). These results do not support the theory that relatedness determines the success of cross-species amplification suggested by other researchers. It is possible, however, that the systematics for this group of taxonomically challenging species is still not fully resolved and therefore it would be misguided to expect a close relationship between

amplification of these microsatellites and current estimates of relatedness. However, as only one specimen was available for most species, conclusions from this study are provisional and it was not possible to estimate polymorphisms or investigate the possibility of null alleles. Null alleles (ie, where the sequence of interest may be present but fails to amplify) may be due to a number of reasons. In particular, point mutations or deletions in one or both primer annealing sites can lead to failure to amplify (Dakin and Avise 2004). Variation in size of alleles may also lead to differential amplification where smaller alleles are more efficiently amplified than larger ones (Wattier *et al* 1998). PCR failure due to inconsistent template DNA quality or quantity is also thought to be a potential source of apparent null alleles (Dakin and Avise 2004).

It is interesting to note that the five *Gs. longissima* samples from France amplified more successfully than those from Britain. This may suggest that the French population had a better match in flanking primer sites for *G. gracilis* microsatellites. However, this would need further investigation by sequencing flanking regions to be able to discover the real cause. Further research linked to geographical factors, e.g., isolation by distance, might reveal more information about the conservation of these microsatellites in *Gs. longissima*.

4.6.5. Conclusions

Cross-species amplification of microsatellies in the Gracilariales is possible with loci apparently conserved throughout the group. By optimising PCR conditions, it may be possible to amplify a larger number of loci than was seen here. Sequencing of bands obtained with unexpected band sizes might reveal non-target loci of interest, ie those which may reveal genetic information.

However, results obtained here are not clear and further optimisation or sequencing of PCR product for *Gracilariopsis* is needed to understand better the reasons for the data obtained. Insertions or deletions in priming regions, for example, would explain the highly unpredictable results experienced in attempting to amplify *G. gracilis* microsatellites in *Gs. longissima*. *G. gracilis* is polymorphic for all loci, which can then be used for population studies in that species. In this study, some degree of polymorphism was seen in *G. chilensis* and *Gs. longissima*. Whether this would be revealed in other species, were more samples available, is unknown. More data is needed for detailed analysis.

Chapter 5:
Biofouling and epiphytism in gracilarioids: Effects on RAPD profiling

5.1. Introduction

Macroalgae commonly play host to epiphytes and, in common with other submerged marine surfaces, accumulate biofilms of varying diversity and amount (Anderson *et al* 1998, Holmes *et al* 1991, Murray *et al* 1986, Weinberger *et al* 2001). While gross epiphytism may be visible to the naked eye and successful removal clearly seen, microbial biofilms may be missed due to their microscopic nature.

If macroalgae from which DNA is to be extracted are not cleaned of all epibiota, DNA from the epibiota will be unavoidably co-extracted, contaminating the algal DNA sample. This is of importance when using non-specific molecular markers such as RAPDs. Results are compromised as banding patterns cannot be guaranteed to represent solely the DNA of the target organism. Competition for priming is thought to reduce the likelihood of amplification of non-target DNA where it is present in much lower concentrations than the target DNA (Zwartes and Hnida 2000). However, without definitive quantification of non-target to target DNA ratios or empirical evidence that non-target DNA does not affect DNA profiles obtained, the validity of results is questionable.

Phycologists working on phytoplankton species can isolate and culture their study organism in unispecific conditions (De Bruin *et al* 2004, Shankle *et al* 2004) while some authors working on macroalgal species are able to grow plants from spores under clean laboratory conditions (van Oppen *et al* 1996, Santelices *et al* 1996).

However, those working on macrophytes face the fact that their wild-collected specimens will almost invariably be host to a varying amount of epiphytism (Rindi and Guiry 2004, Steinberg and Nys 2002). Phycological researchers have cleaned macrophytes for RAPD investigation with varying degrees of effort, such as washing and blotting (Coyer *et al* 1997, Faugeron *et al* 2004, Gonzalez *et al* 1996), brushing and rinsing (Martinez *et al* 1999), freshwater rinsing with detergent (Alberto *et al* 1997), sonication (Kim *et al* 2004), sonication and brushing (Faugeron *et al* 2001), treating tips with bleach and antibiotics before growing on for DNA extraction (Patwary *et al* 1993) or, apparently, not at all (Atienzar *et al* 2000). Hu and Zhou (2001) cleaned "thoroughly in seawater" and then resorted to unicell isolation and low speed centrifugation. Patwary *et al* (1993) report the use of microscopy to check the efficacy of cleaning. Using RAPD markers in land plants, Muller-Scharer and Fisher (2001) only rinsed leaves before extraction.

In this study, RAPDs were chosen to investigate the population genetics of *Gs. longissima*. All samples for this research were collected in the wild and were thus likely to be contaminated with epibiota. Cleaning is extremely time-consuming and, from the existing literature, there was no universally accepted cleaning method or any quantitative data on the efficiency of a particular protocol. In the present study, a method for cleaning large numbers of samples was required as population genetics research required tens of samples from which uncontaminated DNA could be extracted. Integrity of the thallus wall was not of pre-eminent importance as for other researchers (e.g., Gledhill *et al* 1998), as the removal of contaminating non-target DNA was the prime purpose. However, breakage of samples was to be avoided to reduce loss of sample material.

Therefore, before using RAPD markers to investigate the population genetics of *Gs. longissima*, it was important to (1) confirm the presence or absence of epibiota and biofilms, (2) develop successful removal methods and (3) establish whether presence or absence affects RAPD profiles. Therefore, an initial assessment of biofilm and epiphyte cover of samples of gracilarioid rhodophytes found at sites where *Gs. longissima* populations were being studied was made. Three experiments were then carried out:

(1) An initial evaluation of seven potential methods for the removal of biofilm from thallus surfaces of gracilarioid algae.
(2) A quantitative evaluation of epibiont removal efficiency from gracilarioid algae, using four cleaning methods tested in (1).
(2) An investigation into the effect of epibiont removal on RAPD profiles of gracilarioid algae using successful protocols and markers from Chapter 4.

5.2. Qualitative assessment of epibiota on gracilarioids from the South West Peninsula of Britain

The presence or absence of epibiotic contamination on gracilarioids from sites around the South West Peninsula of Britain, where populations of *Gracilariopsis longissima* had been confirmed (Chapter 2), was first assessed.

5.2.1. Materials and Methods

Samples of gracilarioids (*G. gracilis* and *Gs. longissima*) collected randomly from eight sites around the South West Peninsula of Britain (Table 5.1) were transported to the laboratory in seawater in plastic snap-seal

Table 5.1: Numbers of gracilarioid samples collected randomly at eight sites around the south west peninsula of Britain examined for epibiota under a dissecting microscope. Species confirmation was carried out as part of ecological studies (Chapter 2).

Site	*Gs. longissima*	*G. gracilis*	Total
Braunton	33	0	33
Flushing	19	0	19
Helford	28	0	28
Camel	10	0	10
St Just	52	0	52
Froe	15	11	26
Place	18	13	31
Instow	8	15	23
Total	183	39	222

bags. Samples were stored in filtered seawater at 10°C in a 12 h in 24 h very low light (2.5-5 µmoles m^{-1} s^{-1}) regime.

Before confirmation of species by amplification of the internal transcribed spacer region of the genome (as described in Chapter 2), each individual was examined under a light microscope for the presence of epibiota. Epibiota present were recorded and photographs were taken for later identification for as many organisms as possible. Visible epiphytes were then removed by gentle brushing with a toothbrush and identified where possible.

Three thalli from St Just which appeared to be "clean", that is, free of gross contamination, were prepared for scanning electron microscopy and viewed at high magnification.

Sample Preparation for Scanning Electron Microscopy

Pieces 1 cm long were cut randomly from the central area of three axes from each of the three St Just thalli (species was confirmed as *Gracilariopsis longissima* by amplification of the ITS region of the genome). The 1 cm pieces of thallus were fixed in a chemical fixative and preservative, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), for 1 h followed by rinsing in 0.1 M sodium cacodylate three times, for 15 min each time. They were then gradually dehydrated in sequentially increased concentrations of ethanol as shown in Table 5.2 for a minimum of 15 min per treatment.

The pieces were subjected to final dehydration in a Tousimis Samdri 780 Critical Point Dryer which creates the conditions at which CO_2 can change from a liquid to a gas without any surface tension forces causing damage to the specimen. This

Table 5.2: Sample preparation protocol for imaging samples of gracilarioid algae at high magnification using a scanning electron microscope

Step	Procedure	Treatment	Time (min)
1	Fixing	Soak in 3% glutaraldehyde	60
2	Wash 1	Soak in buffer 0.1M sodium cacodylate	15
3	Wash 2	Repeat 2	15
4	Wash 3	Repeat 3	15
5	Dehydration	Soak in 30% ethanol	15
6	Dehydration	Soak in 50% ethanol	15
7	Dehydration	Soak in 70% ethanol	15
8	Dehydration	Soak in 90% ethanol	15
9	Dehydration	Soak in 100% ethanol	60
10	Dehydration	Soak in 100% ethanol	o'night

process first removes the ethanol by replacing it with liquid CO_2 and then drives off the CO_2 as gas, leaving the samples completely dehydrated and stable.

Each piece of thallus was then attached to 10mm cylindrical metal stubs for placement in the SEM. Once fixed to the stubs, the pieces were sputtered with gold in an Emitech K550 Gold Sputter Coater.

Scanning

Samples were examined in a Jeol JSM 5600LV Scanning Electron Microscope. Thallus surfaces were scanned to see whether there was any epibiota present which had not been visible under the light microscope. A number of micrographs were taken to provide some representative images of biofilms found. Full identification of biofilm components was not attempted.

5.2.2. Results

A wide variety of epibiota which could be seen with the naked eye or under a light microscope was found attached to the gracilarioid thalli, including:

(1) Macroalgal germlings or small epiphytes

(2) Microscopic animals

(3) Diatoms

(4) Parasites

Varying levels of epibiotic infestation were found from site to site. Samples collected from muddy, silty sites, with low energy wave action and limited perturbation (e.g., Froe, Place, St Just), had surfaces covered with epiphytic growth almost obscuring the underlying algae (Figures 5.1 and 5.2).

Macrophyte representatives from the three main eukaryotic divisions of the algae were found attached to gracilarioids (Table 5.3). Most were found only as small germlings of up to 1cm long. Many plants were so small or undeveloped that they were difficult to indentify. At Froe, this growth was mainly made up of a filamentous tangle of ectocarpoids mixed with diatoms and other algal epiphytes (Figure 5.3). Many plants had an obvious covering of detritus and epiphytes on

Table 5.3: Macrophyte genera found attached to gracilarioid algae collected at eight sites around the south west peninsula of Britain

Division	*Genus*
Chlorophyta	*Cladophora* *Ulva*
Phaeophyceae	*Desmarestia* Ectocarpoids
Rhodophyta	*Antithamnion* *Ceramium* *Gelidium* Gracilarioids *Palmaria* *Phyllophora* *Polysiphonia*

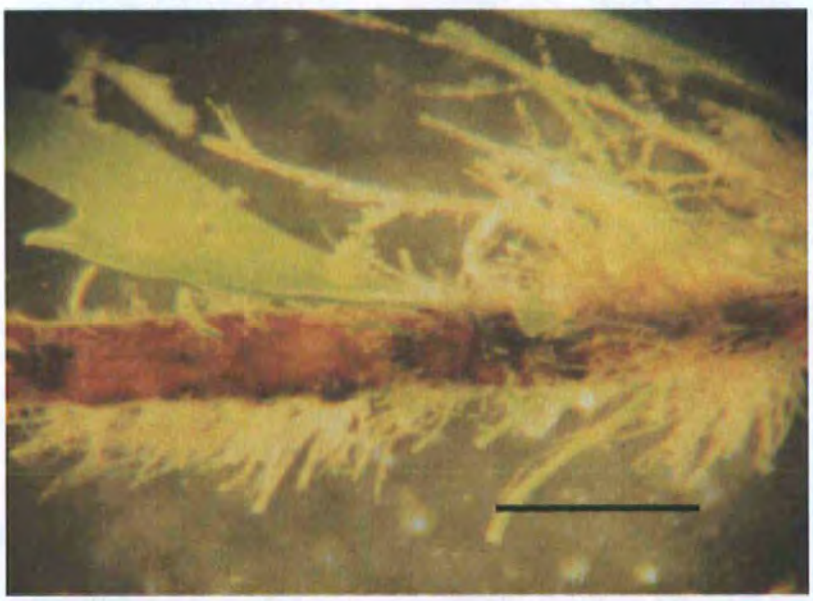


Figure 5.1: Photography taken under a light microscope, showing gracilarioid specimen from Froe almost entirely obscured by overgrowth of epibionts including a small green alga, possibly *Ulva sp.* Scale bar is 2 mm

Figure 5.2: Photograph taken through the light microscope showing a close-up of heavily infested gracilarioid thallus from Place showing filamentous rhodophyte epibiont. Species was confirmed as *Gs. longissima* by amplification of the ITS region of the genome. Scale bar is 1 mm.

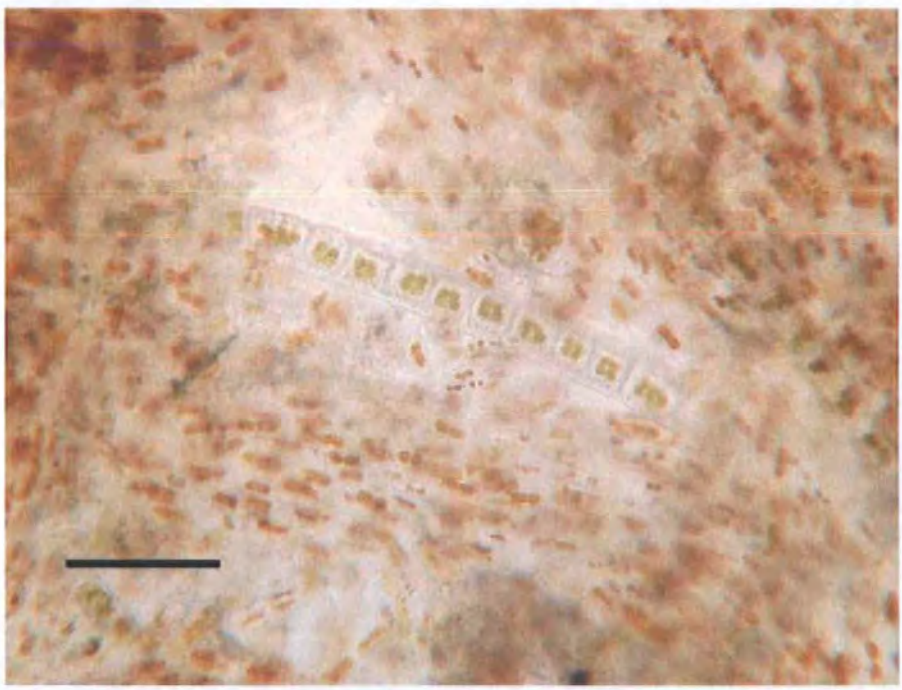


Figure 5.3: Photograph taken through a light microscope showing a thick mass of ectocarpoid filaments mixed with chain diatoms; together these were the most common epiphytes found on gracilarioids from all sites. Scale bar is 100 µm

the very lowest part of the thallus, suggesting that these parts of the plant had survived from the previous season, while less overgrown, apparently clean, areas higher up the plant may have grown that season.

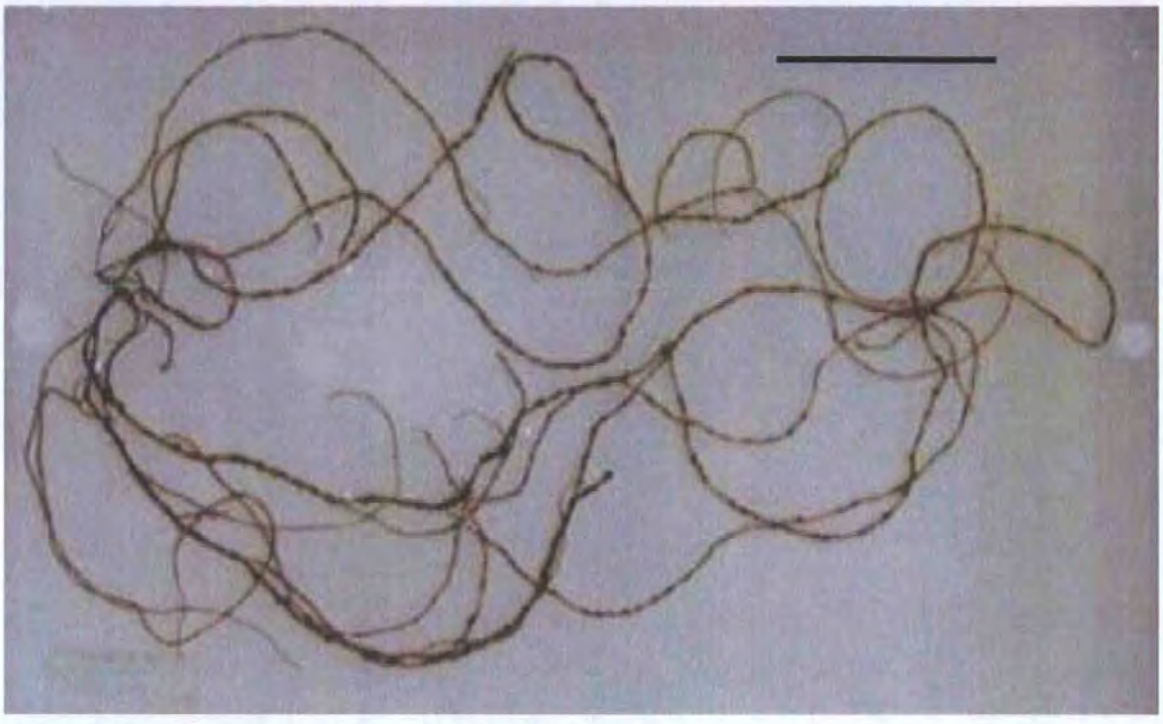


Figure 5.4: An apparently "clean" female individual of *Gracilariopsis longissima* from Braunton Burrows with no obvious epibiota visible. Scale bar represents 5 cm

Samples from sandy or more energetic sites such as the sandy Camel estuary, or Instow where the substrate is compacted sand, or Braunton Burrows which is more exposed than the silty sites, with sandy pools and widespread shingle, appeared clean to the naked eye (Figure 5.4) although occasional single epiphytes of genera such as *Ulva* or *Palmaria* were seen. Although not quantified here, a wide variety of species and numbers were found on samples collected from silty sites where sandy sites were less contaminated. Photographs of organisms allowed some identification of some groups of organism found.

A huge range of diatoms was found, including solitary pennate species (Figures 5.5A&B), colonial chain forming species (Figures 5.6. 5.7, 5.8), tube-dwelling species such as *Navicula* (Figure 5.9A) and other species which could clearly be seen gliding through their tubes (Figure 5.9B).

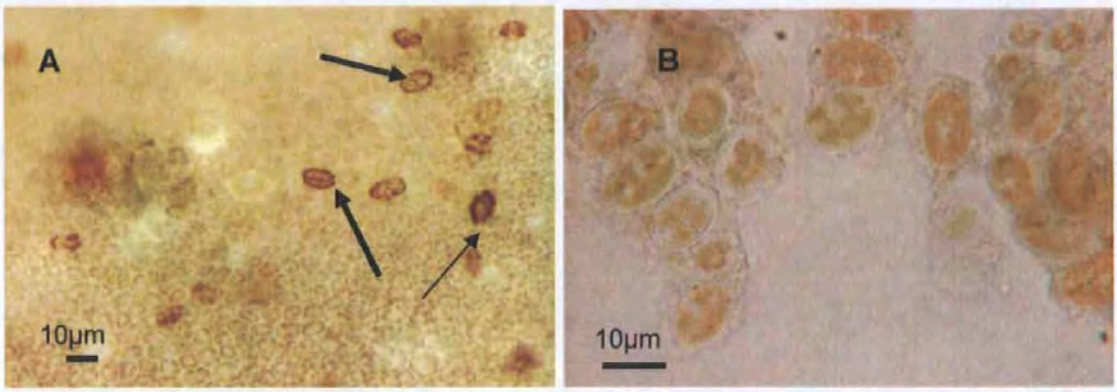


Figure 5.5A & B: Representative photographs of pennate diatoms found on the surface of *Gs. longissima* samples from St Just. A shows a scattering of the diatoms on the surface of a specimen of *Gs. longissima* from St Just. B shows the diatoms at higher magnification. This species were present in large numbers even after superficial cleaning. The species pictured was particularly common and was one of the largest species within the biofilm community.

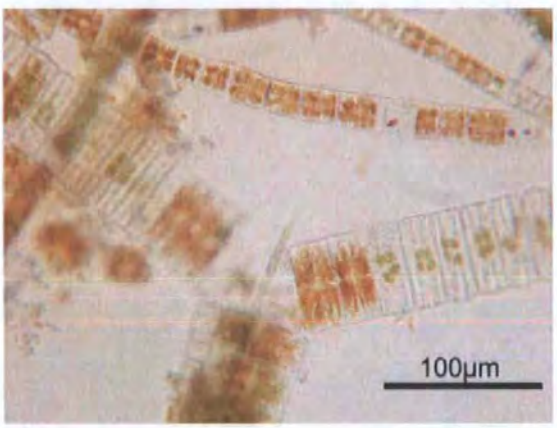


Figure 5.6: Photograph showing chains of diatoms from different species found on a sample of *Gracilariopsis longissima* from Helford.

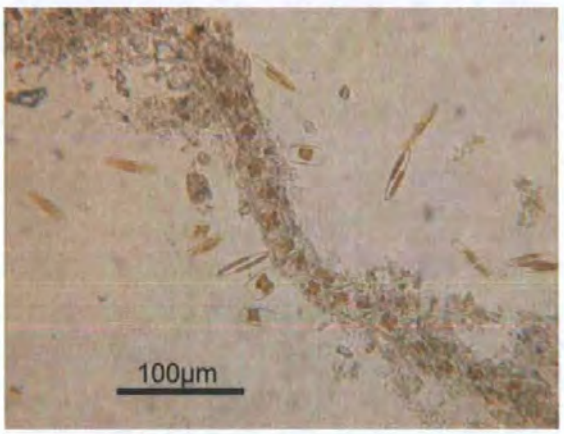


Figure 5.7: Photograph showing chain diatom species and a number of free-living species found on a specimen of gracilarioid from Place.

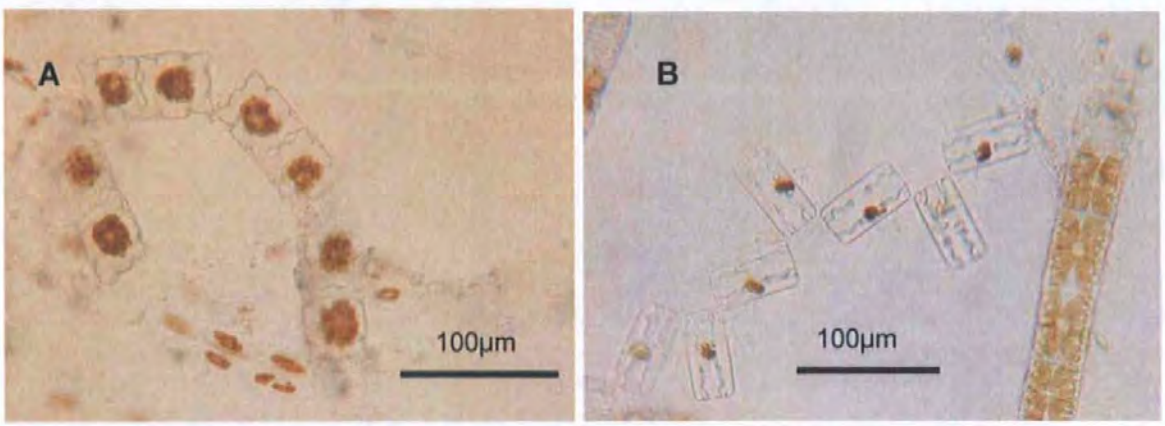


Figure 5.8: Two photographs of species of chain diatom found on specimens of gracilarioids from Froe. The species on the right (A) is *Biddulphia sp.* Running across the centre of (B) is *Tabellaria sp.* with a second, unidentified chain species on the right

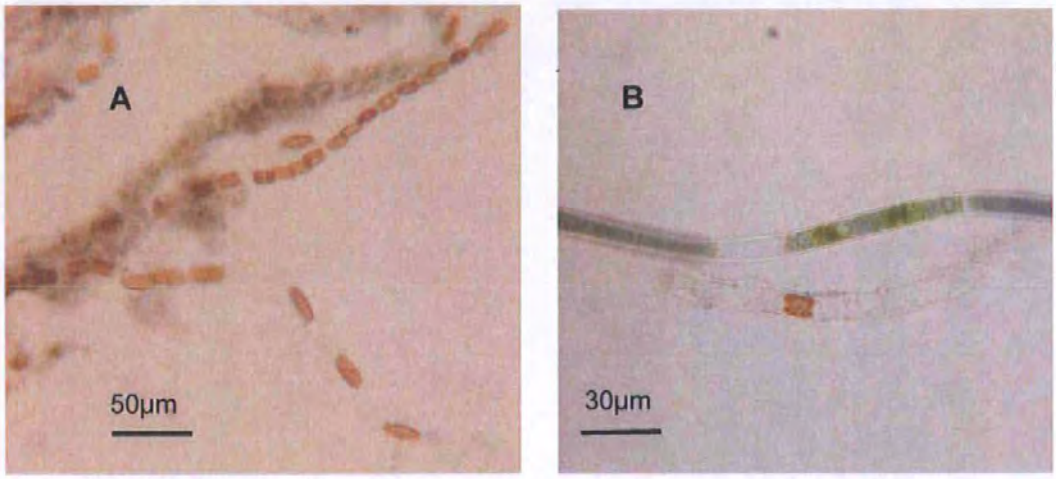


Figure 5.9: A & B Photographs of tube-dwelling diatoms of various species, including *Navicula* (A), were found on samples from St Just and could be seen moving along inside their protective tubes

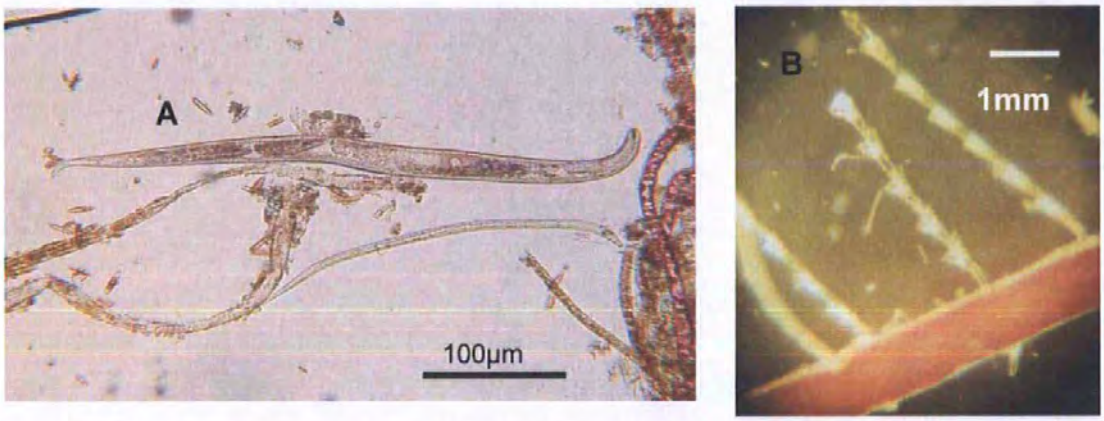


Figure 5.10: A- Nematode worms were seen in great numbers on the surfaces of thalli from several sites, in particular from Place, especially where algae had damaged thalli.
B - Hydroids which were almost invisible to the naked eye could be seen under high power magnification were found on specimens from Flushing

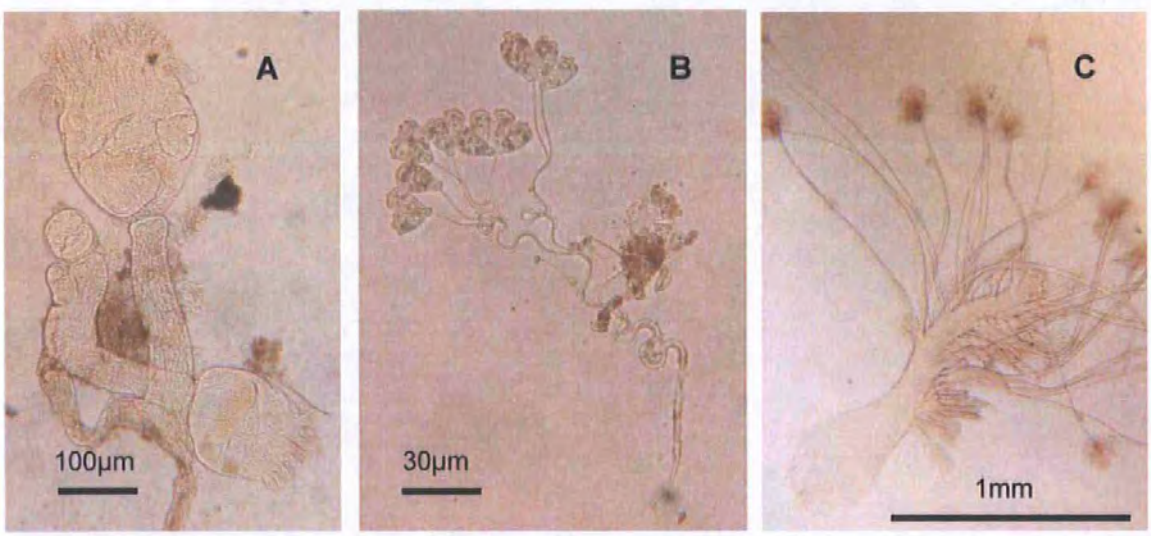


Figure 5.11: A wide variety of life, including many microscopic organisms such as (A) entoprocts (B) vorticellids ciliophorans and (C) unidentified organisms, was found in the biofilm community on gracilarioids from sites around South West Britain.

Many microscopic animals were seen on the surfaces of thalli, either free living or attached, including nematode worms (Figure 5.10A), nemerteans, molluscs, and at least two species of hydroids, of which one is illustrated in Figure 5.10B. Other cnidarian species, bryozoans, entoprocts (Figure 5.11A), vorticellids (Figure 5.11B) ascidians and copepods were also represented. Other organisms remain unclassified at this stage (Figure 5.11C).

Under scanning electron microscopy, it was revealed that apparently "clean" individuals of *Gs. longissima* could still be host to diverse biofilms (Figure 5.12). The representative electron micrographs taken showed a range of organisms on the thallus surface embedded in a polysaccharide matrix as a well-adhered biofilm,

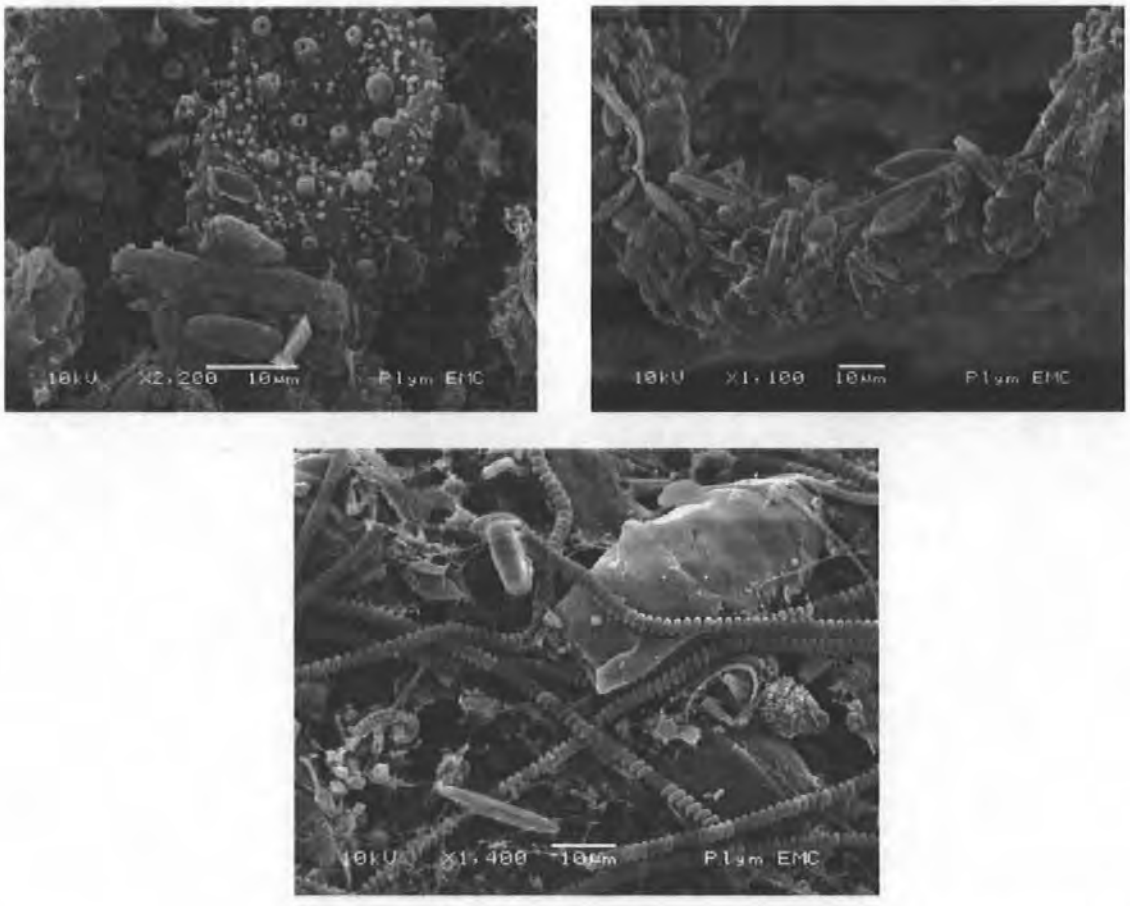


Figure 5.12: Representative electron micrographs of areas of gracilarioid thalli from sites around South West Britain, demonstrating the wide diversity of taxa which were found in the biofilm on these algae

including diatoms, bacteria, fungi, cyanobacteria, spores, microscopic animals and egg cases. Identification of these organisms was not attempted at this stage.

Many specimens were found to be parasitised by a penetrating, red, filamentous alga (Figure 5.13). From photometric information, positive identification was not possible but it is likely to be a species of *Aglaothamnion* (Professor C. Maggs, personal communication), possibly *A. pseudobyssoides*, a red alga known to parasitise other red algae. One specimen from St Just was found to be infected with a white, pustular organism (Figure 5.14) which looks very like the parasitic red alga *Gracilariophila oryzoides*, which looks colourless as it lacks pigment..

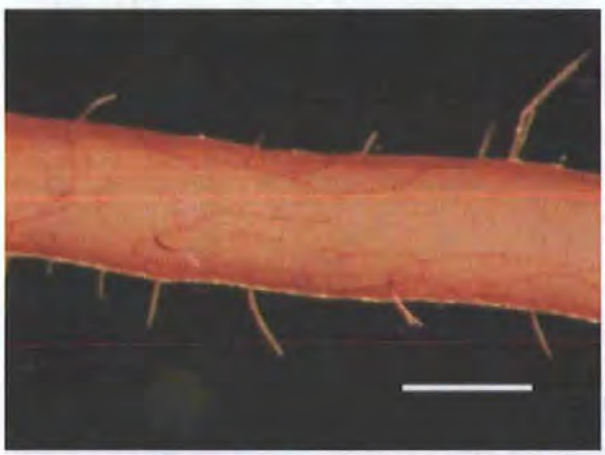


Figure 5.13: Photograph of a red filamentous endophyte (*Aglaothamnion* sp.) found on specimens of *Gs. longissima* from Helford. Scale bar represents 1mm

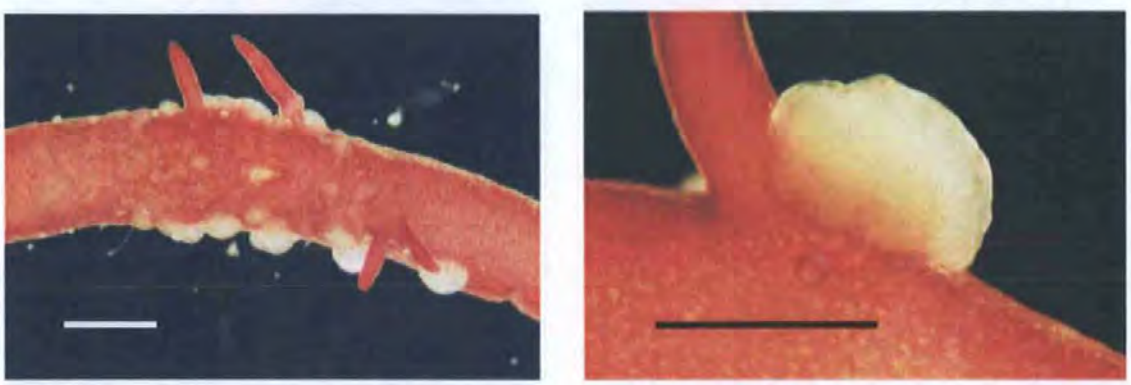


Figure 5.14: Two representative photographs of a specimen of *Gs. longissima* from St Just found to be infected with a colourless, pustular organism that is likely to be a species of parasitic red alga but is currently unidentified. The blotchy appearance of the specimen on the right may be due to damage to the thallus caused by the parasitic infection or some other factor. Scale bars represent 1mm.

However, that species is thought to be highly host-specific infecting only *Gs. lemaneiformis* (Goff and Zuccarello 1994). The blotchiness seen on the thallus may be due to the infection. While it may appear similar to the artefacts seen in *Gracilaria gracilis* with empty spermatangia, this is unlikely here as the specimen was confirmed as *Gracilariopsis* by amplification of the ITS spacer as discussed elsewhere (Chapter 1 and Chapter 2) and the spermatangia of *Gracilariopsis* spp are very much less obvious.

Holmsella pachyderma is another unpigmented parasitic red alga which forms rounded, pustular growths known to infect species of *Gracilaria* (Goff 1982, Evans *et al* 1973) and *Gracilariopsis* including *Gs. longissima* (Zuccarello *et al* 2004). Without further investigation of live material it is not possible to confirm species. It is possible that this species is one so far unreported in Europe (G. Zuccarello, personal communication).

5.2.3. Discussion

5.2.3.1. Epibiota

This initial examination demonstrated that gracilarioids growing in a number of sites around the south west of Britain provided a substrate for extensive epibiota vsble to the naked eye as well as less obvious but well-developed biofilms which included an impressive array of phyla. The extracellular polysaccharides which are exuded by both bacterial biofilms and the thalli themselves may provide added attractions for living and non-living detritus allowing things to "stick" to thalli strongly. Whether or not any signalling operates in the process of epiphytic colonisation in either of the gracilarioid species seen here would be an interesting area for further investigation.

There is a large body of research on the possible anti-fouling properties of macroalgal exudates (e.g., Amsler *et al* 2005, Bhadury and Wright 2004, Boyd *et al* 1999, Harder *et al* 2004, Kim *et al* 2004, Nys *et al* 1998, Steinberg *et al* 1997, Weinberger *et al* 2001) and the potential for bacterial biofilm to act as an anti-macrophyte fouling layer which may be advantageous for the host in repelling other, more detrimental, epiphytism (Armstrong *et al* 2001). From the presence of biofilms including macrophyte germlings seen on specimens from all sites, there was no obvious anti-fouling demonstrated in *Gs. longissima* or *Gracilaria gracilis*, particularly as it appears that only specimens subject to abrasion, such as those in sandy sites, appeared to be less contaminated.

The effects of these epibiota on the growth and function of either of the gracilarioid species found at the sites visited is unknown, although research has shown that the presence of the chlorophyte *Ulva* as an epiphyte on *Gracilaria* can have an adverse effect on growth in commercial cultivation (Friedlander *et al* 2001). It is interesting to note that individuals from Froe and Place, where epiphytism was particularly well developed, did seem able to grow and remain healthy despite heavy burdens of epiphytes. Further research is needed to elucidate the effects of these heavy epiphytic burdens.

The relatively low levels of epiphytism seen on specimens from the Camel, Braunton and Instow Sands, compared with those from Place and St Just or Helford River may be due to abrasion by the sand or shingle. Sites with higher epibiotic burdens were those where muddy substrates were likely to harbour much higher nutrient levels than the sandy and rocky sites.

Identifying epibiota requires well developed botanical and zoological skills, and plenty of time to devote to examination of samples. Photographic records may prove to be a valuable tool in capturing information for later examination where live samples could not be preserved.

5.2.3.2. Parasites

Confirmation of species was not possible solely by photographic methods. As the red endophyte found was common on individuals from Helford, further samples can be obtained for examination of live specimens in more detail.

Although the white, pustular parasite looks similar to *G. oryzoides*, its specificity on only *Gs. lemaneiformis* makes it an unlikely candidate. However, *Gs. lemaneiformis* has been confused with *Gs. longissima* at some sites (Iyer *et al* 2004). Other candidate parasitic species are equally difficult to confirm. As with the filamentous endophyte recorded here, live samples need to be examined in more detail to allow full identification. This is particularly worthwhile given the exciting possibility that this is a new report for Britain (G. Zuccarello, personal communication).

5.3. Experiment 1: Qualitative assessment of seven potential methods for cleaning algal thalli of epiphytes and biofilms

5.3.1. Introduction

Cleaning methods were first assessed qualitatively, by looking briefly at a wide range of possibilities before undertaking more rigorous quantitative research to indentify the most efficient and practical method to be used for the remainder of

the study. Gledhill *et al* (1998) had evaluated a number of methods, concluding that scraping surfaces with PTFE spatulas while soaking samples in 10% ethanol was most effective. However, these authors were cleaning a species with wide, flat thalli (*Fucus*) which may render it less successful for cleaning the fine, terete thalli of *Gs. longissima*.

5.3.2. Materials and Methods

The methods chosen for this stage of the experiment were sonication, agitation in a stomacher, soaking in disinfectant, abrasion with sand, and brushing/scraping either with or without ethanol or disinfectant. The protocols for each are listed below.

5.3.2.1. Samples

A large healthy vegetative thallus from St Just was used. Approximately 30 sections of axes, each 3 to 5 cm long, were cut randomly from the central regions of the thallus (Figure 5.15) and examined under a binocular dissecting microscope (Olympus SZ-PT) to confirm that there was at least some fouling of the thallus before treatment. Up to three pieces were then subjected to one of the cleaning methods as described below. Three pieces of uncleaned material were retained as controls.

Following cleaning, all samples were then re-examined under the light microscope to determine whether biofouling could still be seen. At this stage, evaluation of the effectiveness of any particular method was by subjective observation.

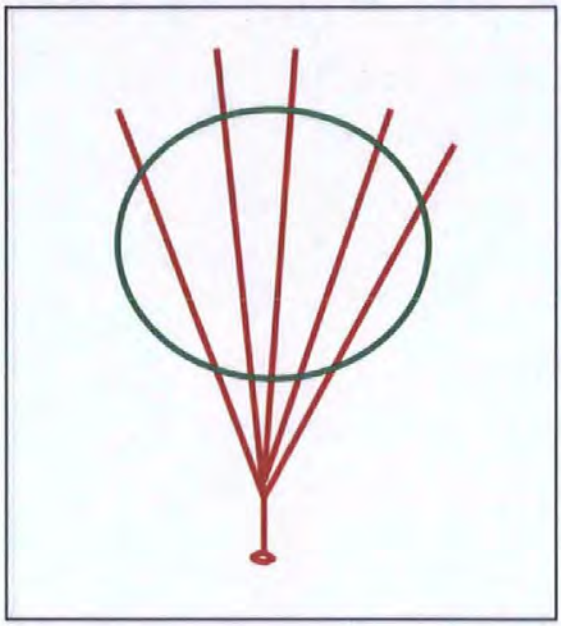


Figure 5.15: Diagrammatic representation of region of sampling for investigation of cleaning methods for the removal of epibiota from gracilarioid algae. Short lengths (3 to 5 cm long) were cut from central areas of the axes (green circle) chosen randomly throughout the thallus (red).

5.3.2.2. Cleaning protocols

(1) Sonication

Sonication is used commonly to shake microorganisms off substrates including algae (Espeland *et al* 2001, Gledhill *et al* 1998, Kim *et al* 2004) and terrestrial plants (Morris *et al* 1998). Three 5 cm sections of contaminated thallus were placed in a 30 ml thin-walled bijou bottle filled with filtered seawater. An Elma D78224 Singen Sonicator was used to sonicate samples at a frequency of 35 kHz for 1 min. After examination the samples were sonicated for a further 2 min, re-examined, sonicated for a further 5 min, then re- examined for a final time.

(2) Agitation in a stomacher

Stomachers imitate the peristalsis of the stomach with paddles pummelling a bag containing samples in liquid media. Fry *et al* (1985) used stomaching to remove biofilm from watercress with some success but did not achieve complete removal. This method could potentially handle large scale processing as the stomacher

bags can hold approximately 0.5 l of media. Three 5 cm sections were placed in a stomacher bag with filtered seawater. The bag was agitated for 5 min and then the samples were examined before being pummelled for a further 5 min before final inspection.

(3) Soaking in a detergent disinfectant

Detergents and disinfectants lyse cells of bacteria, fungi and other microbes and destroy the thick mucus surface layer carrying the contaminating biofilm. Hycolin is a phenol-based compound kept in aqueous solution by a detergent and commonly used as a disinfectant in research laboratories (This product has now been discontinued but was available at the time). Despite the relative permeability of algal cell walls and membranes (Cole and Sheath 1990), destruction of the biofilm layer could potentially be achieved by soaking in detergent/disinfectant for a period short enough to avoid penetration. Three sections of thallus were soaked in 0.1% Hycolin for 15 mins.

(4) Shaking in sand

Algal thalli from sandy sites had been observed to have much reduced epiphyte burdens (Section 5.2). Agitation of samples with sand may therefore abrade biofilm from algal surfaces, removing biofilm. Trachoo (2004) vortexed bacterial cultures on stainless steel coupons with 5g of sand in 10ml of water and successfully removed up to 90% of the biofilm. Three sections of gracilarioid thallus were placed in a 25ml bijou bottle filled with 50% sand and 50% filtered seawater. The bijou was shaken on a hammer drill for 1 min, removed and examined under the light microscope, shaken for a further 3 mins and re-examined.

(5) Brushing (scraping) in filtered seawater

Scraping with a PTFE spatula had been shown to be successful in removing bioiflm from *Fucus* (Gledhill *et al* 1998). A spatula was not practical for dealing with the small surface area of *Gs. longissima* so small artists' paintbrushes were used. Under the dissecting microscope, paintbrush bristles could be seen to have rounded ends. These failed to remove well-adhered material (personal observation). Ends were "sharpened" by cutting at an angle with a scalpel. The new shape turns the brush into a scraper (Fig 5.16).

Three sections of thallus were scraped with this brush while submerged in filtered seawater in a Petri dish. Recalcitrant material observed under the microscope was scraped until it was seen to have been dislodged. The whole surface of the thallus was scraped regardless of whether material could be seen or not. This method was very time consuming as the terete nature of *Gs. longissima* meant that only small areas of surface were scraped for each stroke of the brush. It was necessary to turn the sample round by minute degrees while scraping the surface.

(6) Brushing as (5) with 10% ethanol in filtered seawater

Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective

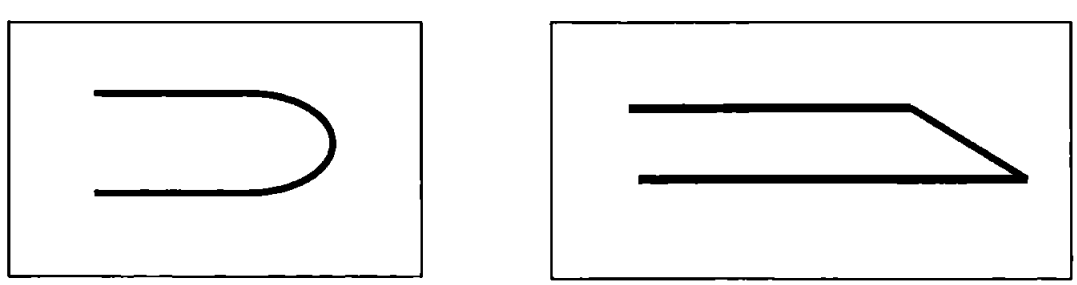


Figure 5.16: Bristles on toothbrushes and artist's brushes normally have rounded ends (left) but these can be "sharpened" by cutting at an angle with a scalpel (right)

against bacterial spores (Sykes 1965). Sissons *et al* (1996) found that 15% was sufficient to kill most bacteria but 40% was more effective. Gledhill *et al* 1998 reported enhanced biofilm removal in ethanol over mechanical removal alone. Hellio *et al* (2000) removed surface microflora from macroalgae by washing in 30% ethanol for 10 min but this researcher felt that this would cause unwanted dehydration damage to samples at this stage of the process. Using the same brush as for (5), three sections of thallus were scraped while submerged in seawater mixed with ethanol (10%).

(7) Brushing as (5) after soaking in detergent/disinfectant

Three sections of thallus were brushed in filtered seawater after soaking in Hycolin as in (3).

5.3.3. Results and Discussion

Under the microscope, it was seen that thallus surfaces of controls were subject to at least some biofouling.

Outcomes of the various cleaning treatments are reported in Table 5.4. In brief, sonication, stomaching and soaking in disinfectant had no visible effect on biofouling. The vigorous (non-adjustable) action of the stomacher tended to break up samples while having no noticeable effect on the biofouling. Samples soaked in Hycolin were observed to lose pigment and became flaccid.

Shaking in sand did appear to remove some material but only a proportion. The available machinery could only accommodate samples sizes up to 4 cm in length

Table 5.4: Qualitative observations of cleaning success of methods tested

Cleaning method	Comparison with control
1. Sonication	No noticeable difference
2. Agitation in stomacher	Damage to samples and small effect
3. Soaking in disinfectant	No noticeable effect. Potential damage to sample
4. Shaking in sand	Some gross epibiont removed
5. Brushing/scraping in seawater	Noticeable removal of gross epibionts
6. Brushing/scraping in seawater +ethanol	Similar result to 5
7. Disinfectant soak, then brushing in seawater	Similar result to 5

and tended to overheat as well as being extremely noisy. Confirmation of cleaning success would still require microscopic examination.

Only methods involving abrasion or scraping were observed to have had any substantial effect on the biofilm. All three methods using brushing/scraping with the sharpened paintbrush had visibly removed material and the thallus looked clean under the optical light microscope.

5.3.4. Conclusions

Overall, mechanical removal by brushing/scraping or abrasion was observed to be most effective. Therefore it was decided to conduct the second, quantitative, experiment with only four methods: (1) brushing/scraping (2) brushing/scraping with ethanol (3) brushing/scraping with Hycolin (4) sand abrasion.

5.4. Experiment 2: Quantitative evaluation of four methods for epibiont removal from thallus surfaces of gracilarioid algae

5.4.1. Materials and Methods

Scanning electron microscopy (SEM) was used to make a quantitative assessment of the four methods listed above in removing biofilm and other microscopic contaminants from *Gs. longissima* thallus surfaces. SEM can provide high resolution images at high magnification, allowing fine detail of biofilms to be resolved.

5.4.1.1. Samples

Three live individuals were assigned codes A, B and C.

A: *Gracilariopsis longissima* from Instow Sands in the Taw Torridge Estuary, Bideford (OS map reference SS470314)

B: *Gs. longissima* from Flushing in the mouth to the Fal Estuary (SW777251)

C: *Gracilaria gracilis* from Place on the Fal River (SW852323)

From each thallus, pieces of axes were taken randomly from the central region of axes to provide at least 10 cm length in total. This material was then divided into five pieces for each thallus. One piece from each was kept as a control (uncleaned) (Coded A1, B1, C1). The other four pieces were randomly allocated for treatment by one of the cleaning methods as described in the first experiment (coded A2,3,4,5, B2,3,4,5, C2,3,4,5) (Table 5.5).

After treatment, samples were kept in filtered seawater until prepared for SEM examination.

Table 5.5: Coding of samples of three gracilarioid thalli subjected to various cleaning methods for epibiota removal. Three replicates for each sample were coded X, Yand Z, ie, A1X, A1Y, A1Z.

Sample Code	Treatment
A1, B1, C1	Control - uncleaned
A2, B2, C2	Brushing
A3, B3, C3	Brushing in 10% ethanol
A4, B4, C4	Brushing in disinfectant (Hycolin)
A5, B5, C5	Sand abrasion

5.4.1.2. Scanning Electron Microscopy

Treated samples (4) and controls (1) for each thallus were prepared for scanning electron microscopy as described in Section 5.2.1.

Samples were examined in a Jeol JSM 5600LV Scanning Electron Microscope. Because scan areas were very small (77x77 µm at x 2000), dispersed replicate scans were used to ensure that data representative of each sample were generated (Figure 5.16). First, a full-screen area at x250 (e.g. Sample A, replicate X, area b in Figure 5.16) was chosen haphazardly; and images were captured to record the amount and type of epibiosis. Within this area, three non-overlapping x2000 images were taken haphazardly (Xb1, Xb2, Xb3) using a 10x10 grid overlay of approximately 77x77µm to count how many of the hundred squares contained biofilm. This whole sequence was repeated for two other areas (e.g., Xa and Xc) on the same sample, to give a total of three main areas (e.g., Xa, Xb, Xc), with bioifilm cover data collection from three sub-areas (e.g., Xb1, Xb2, Xb3) in each main area, giving a total of nine grid counts per sample. This data collection hierarchy is illustrated in Figure 5.17. This procedure was repeated for all three replicate sections (e.g., A1X, A1Y, A1Z) per sample/treatment combination.

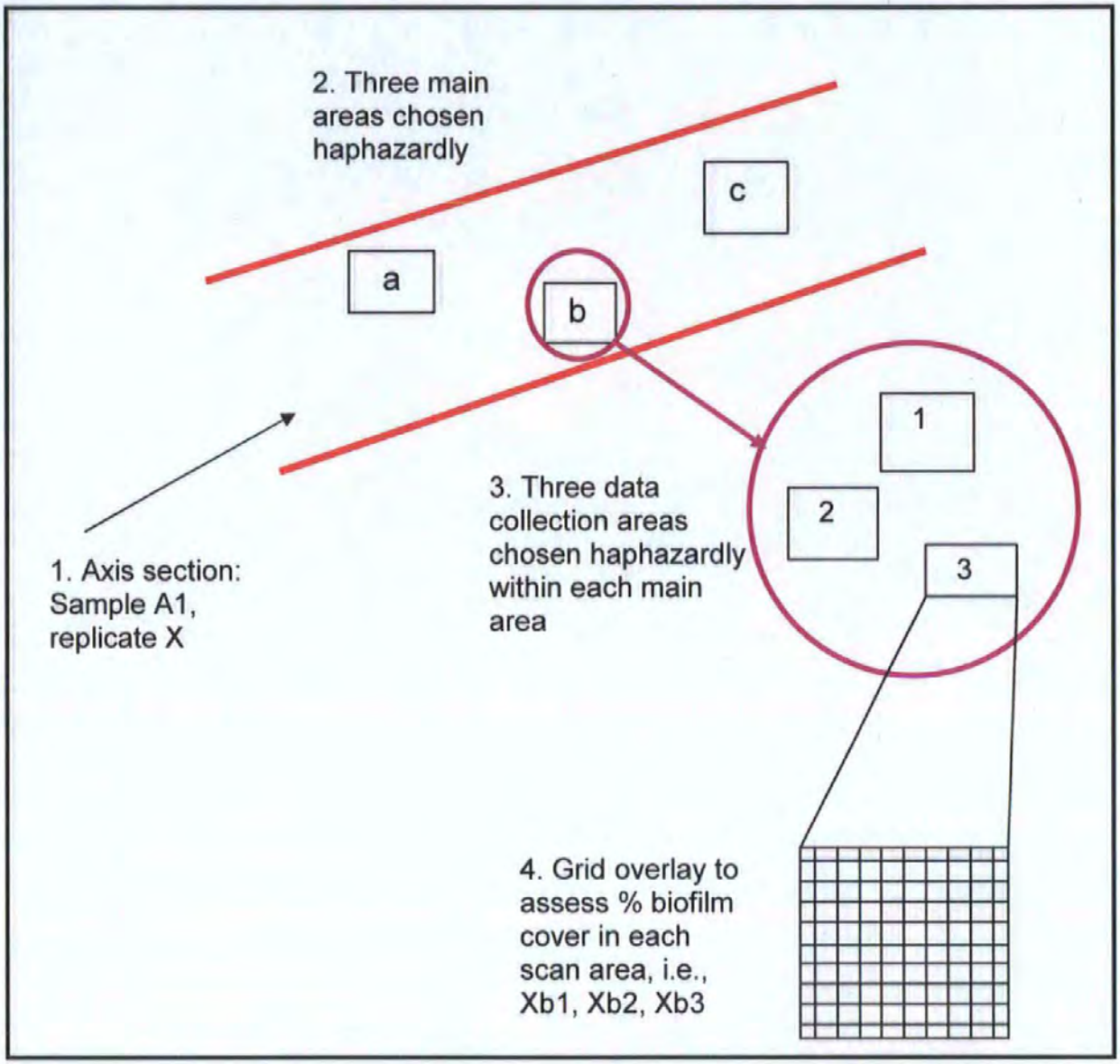


Figure 5.17: Diagram of representative sample (A1, replicate X) showing scanning procedure provide haphazardly-collected sets of data. Three areas of each replicate were scanned 3 times in small areas using a 10x10 grid to give nine data points of percentage biofilm cover for each replilcate, for each treatment/sample combination.

5.4.1.3. Data analysis

The means and standard deviations of the 27 grid coverage counts averaged across all replicates, for each treatment and thallus combination, were calculated for an overview of the results. The entire data set was then arcsine transformed to normalise the data before it was input to Minitab software as a general linear model for comparison of treatments. Sample differences were not important but

the statistical analysis used automatically calculates that variance too. Minitab was then used to calculate a two-way analysis of variance (ANOVA) for treatments and samples. To investigate which treatments, if any, differed significantly from one another, a Tukey test was used to analyse significant differences between pairs of treatments.

5.4.2. Results

Quantitative data for cleaning success is given in Table 5.6 and Figure 5.18 with mean percentage cover averaged across all replicates, recorded for controls and all four treatments.

Controls had between 94 and 100% biofilm cover. Samples cleaned with scraping alone had a mean of between 0.41 and 25% biofilm cover remaining with many replicate grids at zero. However, one axis section for Sample C (sample C1, replicate Z) showed between 23 and 100 per cent cover still present. If that one set of readings is omitted from the data, the mean level of remaining cover for that sample is 6%, and across all samples the mean remaining cover after treatment

Table 5.6: Mean biofilm cover (%) and standard deviations averaged across all replicates, recorded for algal thalli left uncleaned (controls) or treated with a four different cleaning regimes when examined by SEM

Treatment		*Sample*		
		A	**B**	**C**
1	Controls	98.8 ± 3.5	94.0 ± 16.5	100.0 ± 0.0
2	Brushing	8.0 ± 9.8	0.4 ± 0.7	24.9 ± 32.6
3	Brushing + ethanol	6.6 ± 6.3	4.7 ± 10.1	1.4 ± 1.9
4	Brushing + Hycolin	6.5 ± 5.8	1.0 ± 1.7	17.0 ± 28.6
5	Sand abrasion	2.1 ± 2.8	8.7 ± 12.5	71.3 ± 38.7

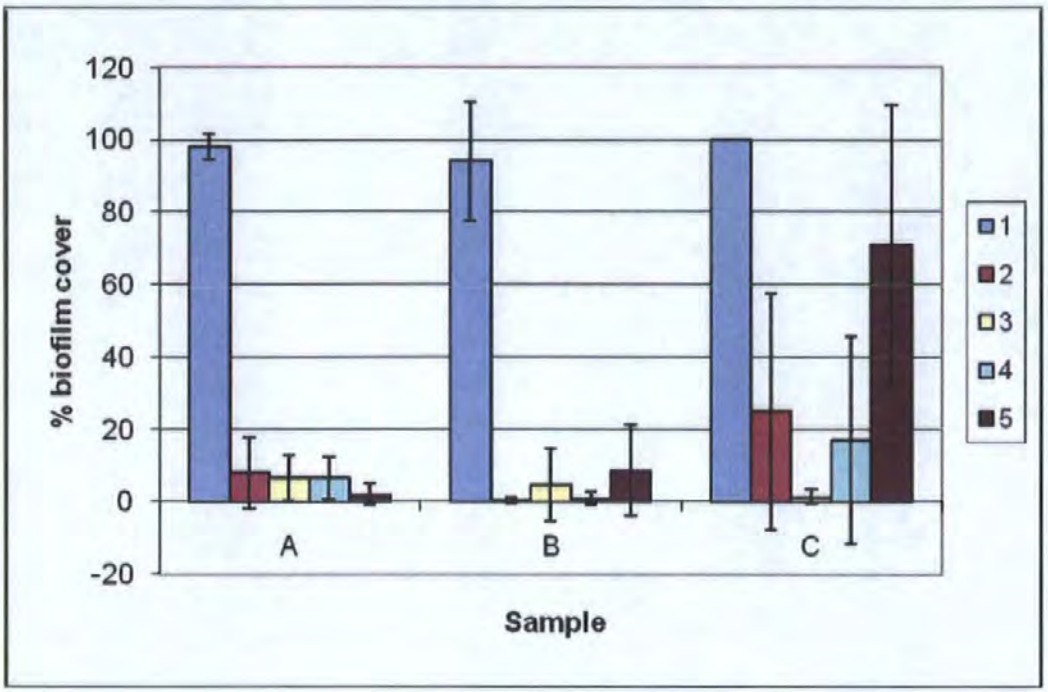


Figure 5.18: Bar chart of percentage biofilm cover found on controls and on cleaned samples averaged across all replicates, as shown in Table 5.5. Codes are 1 – controls, 2- brushing, 3 – brushing & ethanol, 4 – brushing & Hycolin, 5 – sand abrasion. Standard devistion bars are included.

by brushing is less than 5%. Sand abrasion appeared to have failed to remove significant amounts of biofilm in Sample C. However, this method had successfully removed nearly 95% of biofilm from the other two samples.

Possibly because of the anomalous results for sand abrasion in sample C, the statistics also show that the results for sample C were significantly different from the other two samples ($p<0.001$). The ANOVA output in Minitab identifies outliers as "unusual observations". A large number of "unusual observations" were identified for Sample C and abrasion, and for two control readings for sample B, at 40 and 45% cover versus 100% recorded for 23 of the remaining 25 readings.

Analysis of variance for arcsine-transformed data showed that there were significant differences between controls and treatments ($p<0.001$). The Tukey test pairwise comparisons demonstrated significant differences between controls

and all treatments ($p<0.001$) and significant differences between sand abrasion and all other treatments ($p<0.001$) . No significant difference was found when the other three treatments were compared. Therefore, the three brushing/scraping methods were equally efficient at biofilm removal.

A few samples were "wrinkled" in appearance but most were less so (Figure 5.19), suggesting that the latter were fixed more successfully before dehydration. However, the degree of wrinkling did not affect the ability to see whether surfaces were clean or covered in material as can be seen from these images. Although this could affect the size of area scanned in grids, the clear results overall suggest this has had little or no effect on the statistics.

Images show that while all sample controls were recorded as having between 90 and 100% cover with biofilm, there were qualitative differences in the way the biofilm was constructed. The biofilm varied in assemblage, extent and thickness from sample to sample.

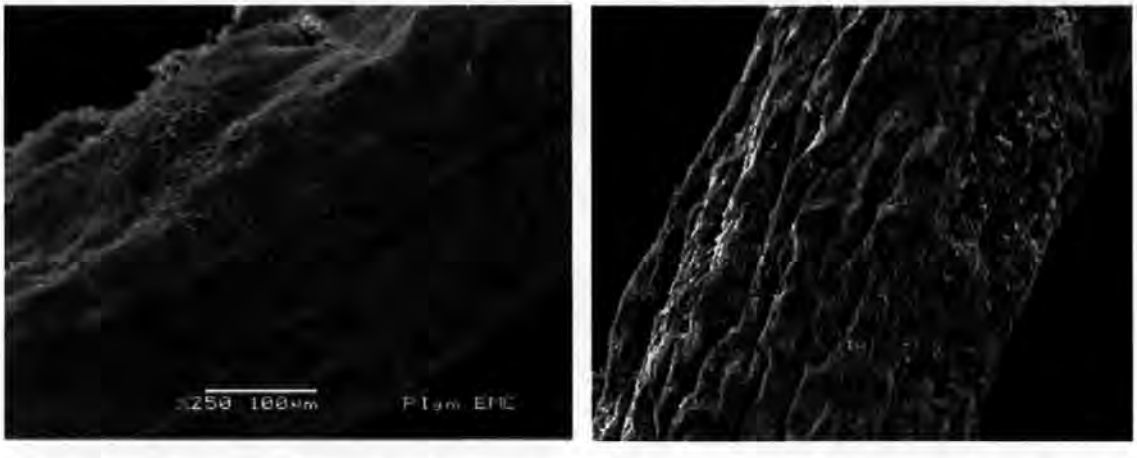


Figure 5.19: Scanning electron micrographs of Sample A1, replicate Z control thallus taken at at x250 magnification showing little evidence of dehydration (left). The right hand (Sample A replicate X) micrograph shows some wrinkling caused by SEM dehydration process. Scale bar applied to both images.

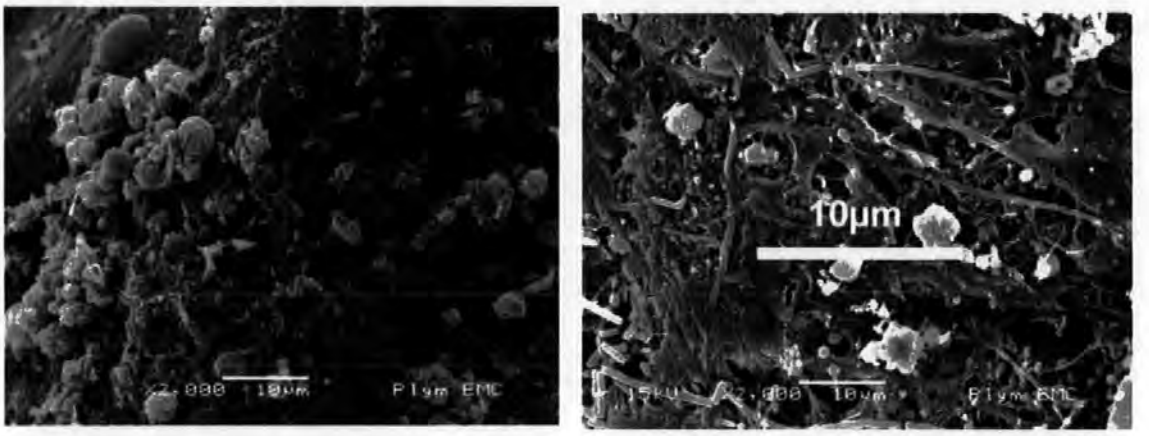


Figure 5.20: SEM images showing details of biofilm in controls with non-continuous cover and the thallus surface visible in places (left Sample A1, replicate Y) and with well-developed mucilage matrix (right Sample B1, replicate Z) Both images were taken at x2000 magnification

For example, the mucilage matrix was less well developed in Sample A (Figure 5.20, left hand image) than in Samples B and C (Figure 5.20 right hand image, Figure 5.21). Although not quantified in this study, the variety of organism present in the more developed biofilms in thalli B and C, compared with thallus A.

Most treated thalli had no visible biofilm remaining after cleaning (Figure 5.22), which demonstrated quite clearly that cleaning with brushes was highly effective.

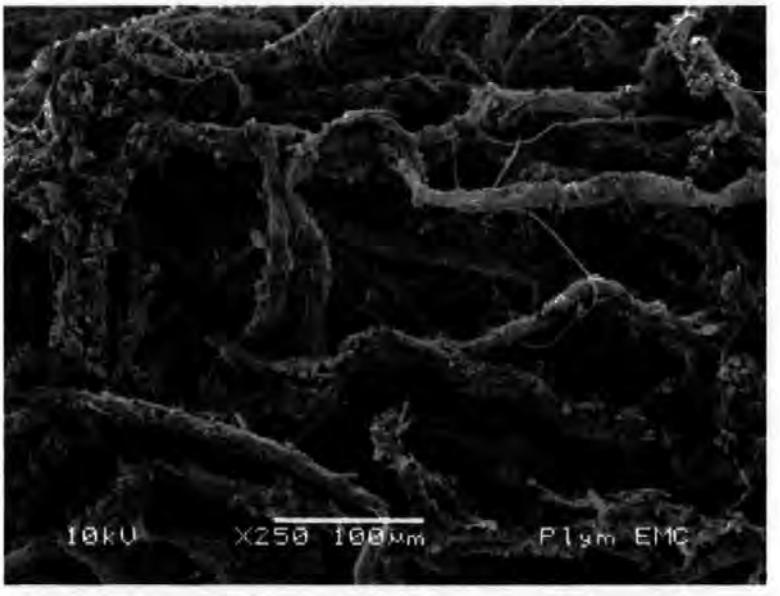


Figure 5.21: SEM image of Sample C1, replicate Y, control with mucilage covered with ectocarpoid filaments completely obscuring the *Gracilaria gracilis* thallus taken at x 250

Images of cleaned samples showed up to 100% removal of fouling biofilm with no visible material still attached.

Biofilm characteristics varied between samples. Before cleaning, the biofilm on thallus A was not always continuous over the whole surface (Figure 5.19) with individual organisms in a single layer with some areas of thallus surface visible among the mucilage (figure 5.20, left hand image). The biofilm found on uncleaned thallus B, on the other hand, was like a thick "coat" which was almost completely unbroken and up to 10 µm thick (Figure 5.20 left hand image, Figure

Figure 5.22: SEM image of an area of Sample A2, replicate X, after treatment by brushing/scraping taken at x2000 magnification showing clean surface of thallus

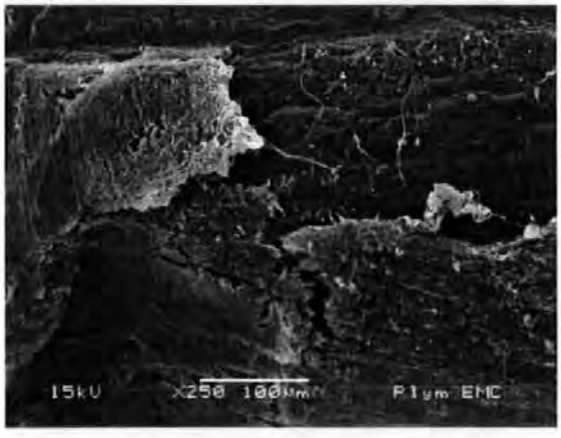


Figure 5.23: SEM image of broken edges of biofilm "coat" seen in Sample B1, replicate Y

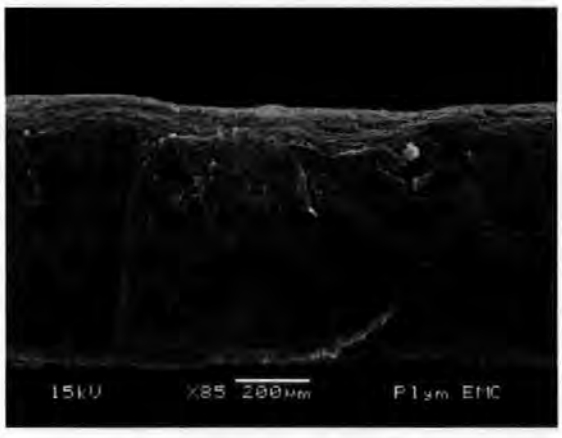


Figure 5.24: SEM of Sample B1, replicate Y control showing marks caused by forceps

Figure 5.25: SEM image to show scraping damage to the cleaned thallus surface in Sample C2, replicate Z

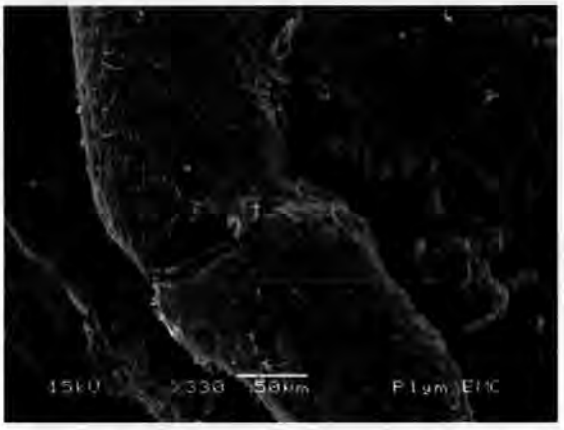


Figure 5.26: SEM image of thallus B biofilm cover showing little or no shrinkage in the mucus matrix after dehydration for SEM

5.26). Breaks seen (Figure 5.23, 5.24) may have been the result of physical damage caused during handling. There was also sporadic damage to the surface of the thallus (Figure 5.25) where scraping had scored deeply and removed part of the thallus cuticle and surface layers. Most samples were otherwise fully intact apart from removal of side branches which commonly snapped off during brushing/scraping.

5.4.3. Discussion

Scanning electron microscopy revealed the rich diversity of epibiota in the biofilms found on *Gracilariopsis longissima* and *Gracilaria gracilis* (see above, Section 5.2). It was also clear that even apparently "clean" samples – those where no obvious epiphytes were observed – could still be covered with mucilage embedded with numerous species of microbe or particulate matter of indeterminate origin.

The images show almost unbroken polysaccharide matrices of the biofilm found in thallus C and much of thallus B. The more interrupted cover seen in thallus A and some of thallus B may be due to dehydration of the mucilage which may be up to 97% water (Sutherland 2001). However, the fixing procedure used here is

designed to minimise effects of dehydration by fixing the sample before removal of water thereby enabling observation of biofilms in close to their original state. Law *et al* (2001) suggest that the very high water content of biofilm matrices does lead to major dehydration effects during preparation with the result that mature biofilms look continuous when they were not. This statement seems at odds with the likely result of dehydration which is that of pulling apart of the mesh-like structure of the adhesive polysaccharide mucilage. It can be seen from Figure 5.26 that this is not the case. Breakages in biofilm were more likely to be due to damage during handling. Forceps pressure grooves can be seen on some samples (Figure 5.24). This image also confirms the absence of any apparent biofilm shrinkage. Biofilm appears to be up to 10 μm thick (Figures 5.23 and 5.26) where the surface of the thallus is completely hidden from view.

The purpose of this experiment was to find the most efficient and practical method for the removal of all extraneous material on the alga. Scraping with a suitable brush was efficient at biofilm removal but very time-consuming.

Sand abrasion was mainly successful except for the anomalous results in sample C5 which are unexplained. The machinery available was not suitable for the throughput of samples required for the main part of the research reported in this thesis. The sample-holding bottle was too small to hold more than a few strands of sample. The vibration and noise caused by the shaking drill action was unacceptable to other laboratory users. The drill tended to heat up quite quickly and had then to cool down before reloading. Each sample had to be shaken several times to ensure removal of the biofilm. It would require much more work to perfect the protocol to be sure of 100% removal. It would possibly be worth

designing and building equipment capable of holding whole samples, shaking for long enough periods (yet to be determined) and not causing damage to samples. Shaking in sand could be a very useful way to clean samples but, as suitable equipment was not available to process the size and numbers of samples required, this method was abandoned, along with those which appeared ineffective on visual examination. However, it would very probably be possible to devise machinery and regimes that could deliver a high degree of cleaning given time and resources. Possibly the use of the stomacher with sand included might have produced a suitable combination but this method was not tested.

As there was no significant difference between any of the cleaning methods that employed brushing/scraping, it can be assumed that physical removal is the most important factor. While ethanol may have some disruptive potential for the mucilage matrix, the outcome for scraping with or without ethanol was not sufficiently different to make it worthwhile to subject the samples to potential damage or the researcher to fumes from ethanol. (Setting up the microscope for this procedure in a fume hood was not possible.)

Samples soaked in Hycolin appeared to be softened and exhibited some loss of pigment. This suggests damage to cell walls and membranes. While no samples appeared to die (ie, complete pigment loss and disintegration were not observed), once cell wall and membrane integrity has been damaged, death is likely to follow. The use of detergents and disinfectants therefore may be contraindicated for biofilm removal in organisms with membranes that are potentially highly permeable to such compounds.

Although the numbers of replicates for treatments was small, the very clear results with highly significant differences between clean and uncleaned samples, show that, while there is an apparent difference between the thallus C and the other two thalli, this was irrelevant in terms of the question posed by the experiment. There were a number of anomalous results such as the failure of sand abrasion in thallus C, and one or two individual readings which varied widely from the majority of readings for any particular treatment/sample combination. Explanations are hard to find. Thallus C was a different species from the other two. Perhaps some unseen quality of biofilm "stickiness" determined by species differences could have influenced the results.

The sampling design is also confounded by treatments being restricted to one axis from each thallus. A randomized block design, with one of each treatment carried out on random sections from a single axis, would overcome potential differences between biofilm cover on individual axes on the same thallus. However, there is no evidence that axes from individual thalli were significantly different from one another, although it would be worthwhile to carry out an experiment to test the theory that there is no difference between individual axes, as long as they are from the same region of the thallus, e.g., the central sections.

5.4.4. Conclusions

The only successful cleaning method to be sure of removing all biofouling from algal surfaces is mechanical brushing/scraping with a suitable implement. Unless a suitable abrasion machine can be devised, hand brushing under a microscope remains the only option if the presence of epibiota is considered a problem for

other research parameters. Whether or not the presence of biofilms presents problems must be decided by researchers based on the protocols they are using.

5.5. Experiment 3: Effects of biofilm removal on RAPD profiles of gracilarioid algae

5.5.1. Introduction

As discussed earlier, the non-specific nature of RAPD markers means that profiles obtained from DNA samples potentially contaminated with non-target DNA may be compromised. However, there is no existing evidence about whether biofilm DNA interferes with RAPD banding patterns in macroalgae.

In this study, it was not possible to quantify the contribution of alga versus biofilm to total DNA extracted, either before or after the process of extraction. With the unknown nature of the target DNA, it is also impossible to distinguish between bands produced by DNA from different sources. It was not possible to screen for the presence of biofilm with other primers as Chiang *et al* (1993) had done, as biofilms can be extremely phylogenetically diverse, with representatives from many different phyla. The assemblage could also be different for every site. Therefore, total removal of biofilm would be the only option, if biofilm presence was found to affect RAPD banding patterns in algae.

A simple test comparing RAPD profiles from cleaned samples with uncleaned controls from the same thalli should establish whether biofilm removal, which is resource intensive, is actually needed to obtain meaningful data for RAPD studies. If profiles are seen to be different, then wild-collected samples of species where

external surfaces remain intact for DNA extraction will need to be cleaned using a method proven to remove biofilms efficiently.

5.5.2. Materials and Methods

5.5.2.1. Samples

Three specimens, chosen on the basis of their apparent healthy state and available material, were as described in the cleaning experiment above. The three live thalli were assigned codes A, B and C.

A: *Gracilariopsis longissima* from Instow Sands in the Taw Torridge Estuary, Bideford (OS map reference SS470314)

B: *Gs. longissima* from Flushing in the mouth to the Fal Estuary (SW777251)

C: *Gracilaria gracilis* from Place on the Fal River (SW852323)

From each individual, pieces were taken randomly from the central region of axes. This material was then divided randomly into four samples for each individual. One length from each was kept as a control (uncleaned) (Coded A1, B1, C1). The other three samples were treated by one of cleaning methods in the same way as described in the first experiment, except for sand abrasion, using the same codes (Table 5.5). Sand abrasion was not included in this experiment as the method had not removed the biofilm reliably. After treatment, samples and controls were air dried and stored wrapped in acid-free tissue, in silica gel, in sealable plastic wallets.

5.5.2.2. DNA extraction

Approximately 5 mg of dry material was used to extract total genomic DNA from

each sample, using the method developed by Wattier *et al* (2000) as described in Chapter 4, with the final pellet resuspended in 150 µl 1x Tris-EDTA.

Concentrations of DNA were estimated by placing 10 µl of each extraction on a 40 ml 1.5% TBE agarose gel stained with ethidium bromide which was run for 50 min at 100 volts. Despite starting with approximately equal amounts of original material, concentrations of DNA extracted varied considerably from approximately 10ng/µl to 60ng/µl. Extractions were therefore standardised to a concentration of approximately 10ng/µl.

5.5.2.3. RAPD amplification

A number of RAPD primers had been screened to identify those suitable for investigations into the population genetics of *Gs. longissima* (Chapter 4). The development and optimisation methods are given in Chapter 4. In brief, 14 RAPD markers found to produce a range of bands were used to produce fingerprints for the controls and treatments in the three specimens.

Based on preliminary trials, approximately 30 ng of template DNA was found to be a suitable amount for successful amplification and was used in 25 µl PCR reaction mixes. PCR reaction mixes and amplification programmes were as described in Chapter 4. Reaction mixes without DNA template were run as negative controls to check for any non-attributable priming. All reactions were run in duplicate.

5.5.2.4. Visualisation of RAPD banding patterns

Amplification products from each primer were loaded onto a single gel to avoid any effects of possible gel to gel variability. A 10 µl aliquot for each product was run

on a 250 ml TBE 1.5% agarose gel, pre-stained with ethidium bromide (0.5 µg/ml) at 100 volts for 5 h in 1% TBE buffer. 100bp DNA ladders (MBI Fermentas, York, UK) were run in the two outside lanes of the gel to enable sizing of products. RAPD patterns were visualised on an ultra-violet transilluminator with a camera and digital image capture software as described in Chapter 4. Gel images were adjusted to provide the most even image across the whole gel.

5.5.2.5. Band scoring and analysis

To avoid bias, scoring was done blind (ie, with no knowledge of the layout of samples across gels). All gels were subjected to analysis by evaluating band size by eye and using computer-aided weight calculations (Uvitec Gel Documentation System). All bands present were assessed for size and then recorded as present or absent for each sample.

Band presence/absence data for each specimen, combining results from all markers, were constructed. From this presence/absence data, a triangular Bray Curtis similarity matrix comparing every pair of samples was constructed in PRIMER V software (Clarke and Warwick 1994, 2002). Factors within the data were labelled as "thallus" (A, B and C were respectively 1, 2, and 3) and "treatment" (control - 1, treatments - 2,3,4).

The similarity matrix was then used with the ANOSIM2 routine in PRIMER for a two-way crossed analysis with no replication. This program is able to analyse the similarities (by comparing means in the light of variance) between the treatments. The similarity matrix was also used to carry out hierarchical cluster analysis and to produce multi-dimensional scaling (MDS) plots of results.

5.5.3. Results

RAPD banding patterns for thallus C were very different from A or B even where profiles for A and B were almost identical (Figure 5.27). This was because C was *G. gracilis* rather than *Gs. longissima* which was confirmed by amplification of the internal transcribed spacer (ITS) area of the genome as described elsewhere (Chapter 1).

It was observed that banding patterns for controls (ie, uncleaned material) were often characterised by poor amplification, even where bands were shared throughout the rest of the treatments. Figure 5.28 illustrates this in the case of primer OPR4. It can be seen that all three controls have amplified many of the same bands as the cleaned samples but less strongly. The same phenomenon is demonstrated in Figure 5.27 for primer OPB5-4 for three bands at 780, 800 and 850bp in thalli A and B.

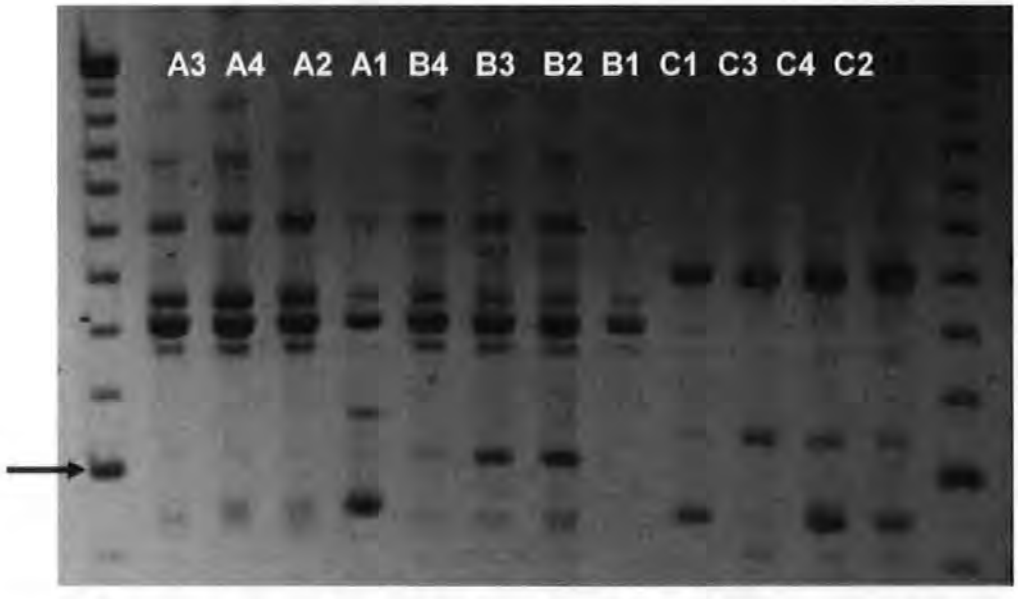


Figure 5.27: Banding pattern for RAPD primer OPB5-4 illustrating the very different profile produced by thallus C (*G. gracilis*) compared with thalli A and B (*Gs. longissima*). Lanes 1 and 14 are 100bp markers (arrow indicates 600bp)

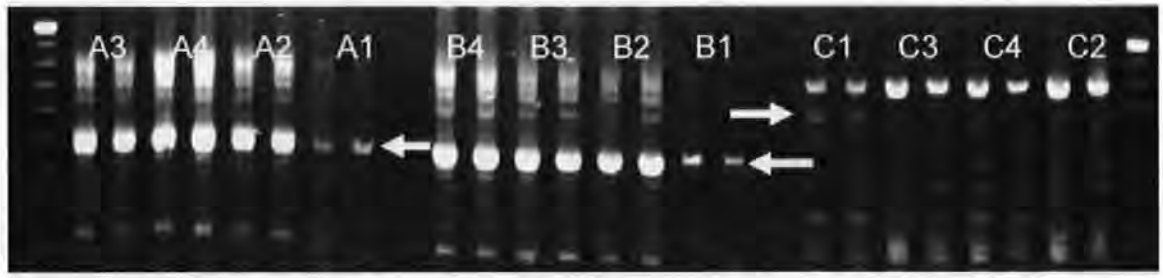


Figure 5.28: Banding pattern for RAPD primer OPR4. Where controls (A1, B1, C1) have produced the same bands as treatments many have amplified comparatively weakly. This can be clearly seen in the locus at 970bp in A and B (arrows), and for a band at 1280bp in thallus C (arrow)

Another common observation was that bands which amplified strongly in the controls might be faintly present in treatments, whereby the human eye could detect a presence but the computer program would fail to do so. There was a wide range of band strength which made it difficult to produce a single image for band analysis. Strong bands were sometimes overexposed if settings were adjusted to be able to visualise weaker bands. Weaker bands can fade to invisibility if adjustments are made to enable easier assessment of very strongly amplified bands.

For example, primer OPB5-5 (Figure 5.29) produces a band at about 750 bp which is almost invisible in Samples A and B for treatments but quite strongly amplified in the two controls. The computer analysis software did not detect those weaker bands. This sort of variation across gels made scoring difficult and a subjective view had to be taken. In general, if a band varied in strength from clearly visible, through just visible, to absent, it was not used. The view was taken that loci amplified this ambiguously could lead to a score of absent where that locus could, in fact, be present but undetected under the conditions of the image capture.

In total, of the 14 markers tested, 13 produced bands suitable for analysis. A total

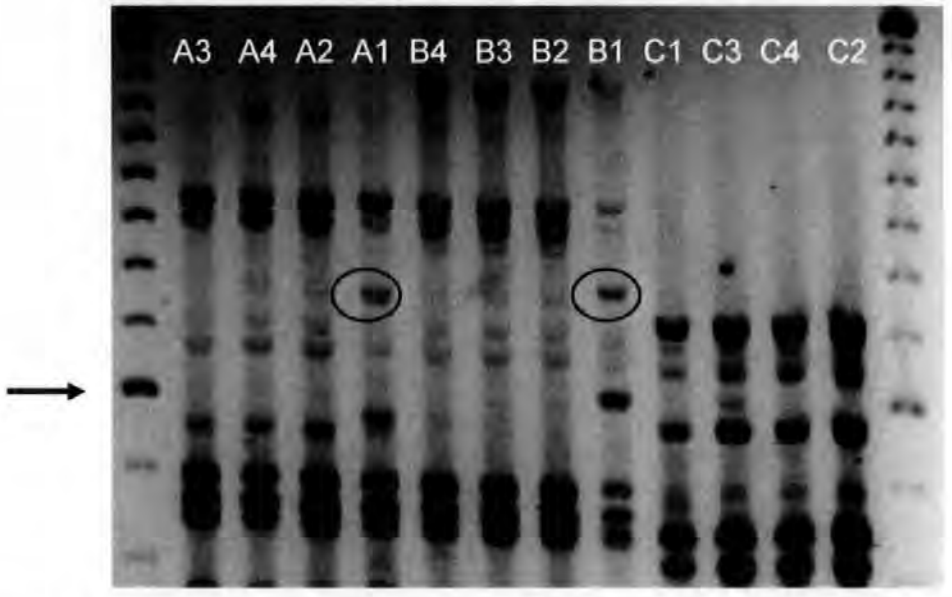


Figure 5.29: Primer OPB5-5 amplified a strong band at 750bp in A1 and B1 (circled). In the treatments, it was amplified weakly in A4 and A2, and possibly in B3 and B2, but appeared to be absent in A3 and B4. The far left and right hand lanes are 100bp marker with an arrow indicating the 600bp band in the far left hand lane.

of 253 distinct bands were scored for presence or absence. Table 5.7 shows the number of bands amplified for each sample and treatment. For thalli A and C, controls amplified a greater number of bands than treatments. In thallus B, the control amplified fewer than treatments. As described above, this may be a scoring

Table 5.7: Numbers of bands amplified by 13 RAPD markers in controls (A1, B1, C1) and treatments (A2-4, B2-4, C2-4) for gracilarioid thalli A, B & C

Thallus	A				B				C			
Treatment	1	2	3	4	1	2	3	4	1	2	3	4
Primer												
B5-2	7	7	7	7	7	7	7	7	7	5	5	6
B5-3	10	10	10	10	12	10	10	10	11	9	9	10
B5-4	6	6	6	6	6	8	8	8	2	3	2	3
B5-5	13	12	12	12	8	12	12	12	8	9	10	9
B5-6	12	12	12	11	12	13	13	13	15	13	13	13
B5-7	11	8	8	8	9	8	8	8	8	7	6	7
B5-8	7	7	7	7	10	8	8	9	8	11	12	10
B5-9	8	6	6	6	5	5	5	6	6	8	7	8
B6	13	13	13	13	10	12	12	12	9	9	9	9
B7-1	8	6	6	6	8	8	8	8	11	8	8	8
B8	9	8	8	8	9	9	9	9	6	5	5	5
R2	10	9	9	9	8	10	10	10	8	7	7	7
R4	6	7	7	7	3	7	7	7	3	2	2	2
Total	120	111	111	110	107	117	117	119	102	96	95	97

artefact due to the generally poor amplification of controls compared with treatments.

5.5.3.1. Data analysis

The ANOSIM2 analysis in PRIMER V treats the data as blocks (thalli A, B and C) and treatments (control and three cleaning methods) which allows us to concentrate on the internal relationship between treatments which is the question of interest. The Spearman rank correlation statistics for thalli is = 1 with $p<0.01$ which indicates a significant difference between the thalli. This is to be expected as thallus C is a different species from A and B and it produced an almost completely different set of banding patterns. However, using the same analysis comparing the controls and treatments, = 0.859 with $p<0.05$, which supports the hypothesis that there is a significant difference in the treatments and controls.

While this analysis does not allocate that difference, the additional analyses shown below provide supporting evidence that this difference is due to controls differing with treatments rather than treatments differing from one another.

The cluster analysis (Figure 5.30) shows a clear distance between the controls (A1, B1, C1) and the cleaning treatments (A2,3,4, B2,3,4, C2,3,4) in both species, while results for the cleaning treatments for each thallus cluster together strongly. Again, the difference between C and the other two samples is clear but this is does not affect the clarity of the separation of controls from treatments. All the treatments for sample A are 100% similar. B and C have a 1-2% difference between treatments. Controls are between 10 and 20% different from their

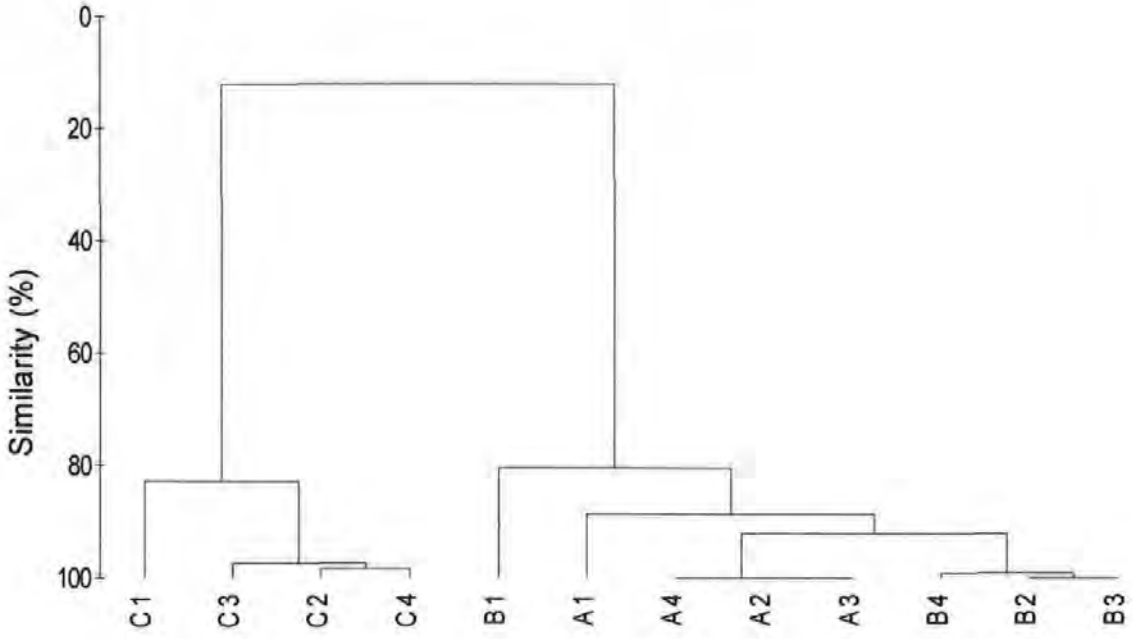


Figure 5.30: Hierarchical cluster analysis comparison of sample results. Similarity is shown as percentage. Results for thallus C are grouped clearly on the left with the cleaned samples (C2,3,4) grouped together. For thalli A and B a similar pattern is seen where the cleaned samples group closely together and the uncleaned controls are demonstrably different. For thallus A there is no difference between different cleaning regimes. For thalli B and C there is a very small (1-2%) difference between cleaning methods.

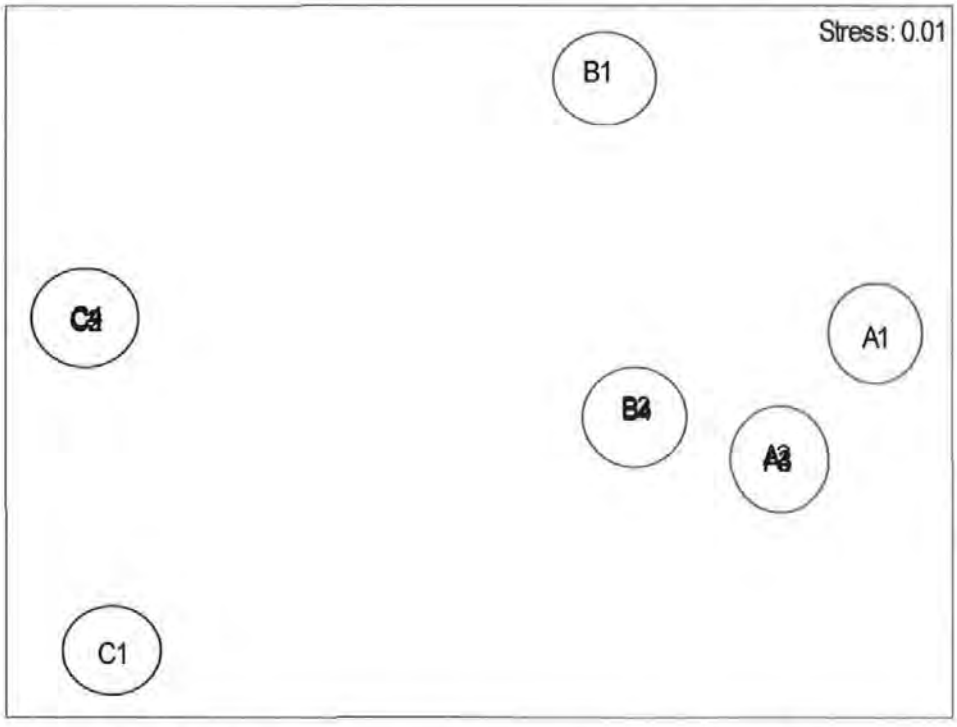


Figure 5.31: MDS plot of treatments and controls for thalli A (red), B (green) and C (blue). Results for controls (A1, B1, C1) can be seen to be well separated from their respective cleaning treatments (A2-4, B2-4, C2-4). Results for cleaning treatments for each thallus cluster so strongly together, that they are superimposed.

cleaned counterparts. A multi-dimensional scaling (MDS) plot also illustrates the clear differences between uncleaned and cleaned RAPD profiles for each thallus (Figure 5.31). Controls A1, B1, C1 are all isolated strongly from their respective groups of treatments. Cleaning treatments cluster so strongly together that it is impossible to distinguish the individual treatments.

5.5.4. Discussion

Some subjectivity in scoring was inevitable (as discussed in the Methods section). By using large numbers of primers, producing many polymorphic bands, it is possible to reduce any "noise" created by inter-specimen variation. With 253 scorable bands, a large amount of data was available for individual specimens. Although the ANOSIM2 test does not have a lot of power here, because of the relatively small number of specimens and treatments used, the clear and consistent treatment effect is sufficient to give a significant result.

Since the purpose of the experiment is to test the difference between cleaned and uncleaned samples, and the three methods using brushing/scraping were statistically the same, the method of removal is also not relevant to the question of biofilm effects. With regard to the effect of biofilm DNA on the DNA profile of individual algae, the fact that one sample was of a different species also did not prevent the data being included for the purposes of the analysis because of the strength of the signal.

However, if the results had been less clear, the inclusion of many more samples would have been needed. Fortunately, the ANOSIM2 test removes the initial differences in the underlying "community type" (banding) for each specimen and

concentrates on the internal relationship amongst the treatments for each "community" type (ie, specimen) (K.R. Clarke, personal communication).

As the outcome of the different cleaning methods is effectively the same with regard to biofilm removal, the clustering of the various cleaning methods for each specimen is hardly surprising. The varying but clear distance of the controls in each case is consistent with the hypothesis that the presence of biofilms can and does affect RAPD profiles.

Polysaccharides are thought to have an inhibitory effect on PCR amplification (Jin *et al* 1997). As biofilm is embedded in a mucus matrix, one might hypothesise that the polysaccharide bound up with the biofilm was contributing to the variation seen in the RAPD profiles. However, the DNA extraction method used in these experiments (Wattier *et al* 2000) was designed with the removal of polysaccharides specifically in mind, as rhodophytes commonly contain high levels of hydrocolloids used for commercial purposes. Therefore, it is likely that mucilage associated with the biofilm is also removed during the extraction process, thereby eliminating any influence on the PCR process.

While it is not possible to quantify the level of contamination required to affect RAPD profiles, this is a key criterion with regard to possible false positives during RAPD amplification (Zwartes and Hnida 2000, Williams *et al* 1993). The thickness and complexity of biofilms on the specimens as seen in Section 5.2 suggests that there could be any number of contributors to total DNA extracted and each component could vary widely in the proportion it contributes to the whole.

Identifying which components are important in terms of the RAPD profiling results would require a significant programme of research which would be difficult to justify.

5.5.5. Conclusions

The presence of biofilm has been shown to have a potentially significant effect on RAPD banding patterns. It seems advisable, therefore, to ensure that specimens are carefully cleaned to remove all traces of biofilms before DNA extraction. This should be taken into consideration when using RAPD markers or methods employing other non-specific PCR primers, for the investigation of wild-collected specimens of marine macroalgae and, potentially, any other organisms which are subject to biofilm formation and adhesion.

Chapter 6:

The use of randomly amplified polymorphic DNA (RAPDs) to investigate the population genetics of *Gracilariopsis longissima* in the South West of Britain

6.1. Introduction

Gracilariopsis longissima has only been recognised and recorded in the South West of Britain recently (Steentoft *et al* 1995). As described elsewhere (Chapter 1), *Gs. longissima* and *Gracilaria gracilis* were formerly classified together as *G. verrucosa*. This means that many previous publications citing *G. verrucosa* may not be attributed to either species with any certainty. While the re-assigned *G. gracilis* has been the subject of a number of genetic studies in Europe since 1995 (e.g., Engel *et al* 1997, 1999, 2001, 2002, 2004), research on *Gs. longissima* has been largely taxonomic.

Difficulties with isolating suitable quality DNA from macroalgae have probably hampered investigations into their population genetics until relatively recently (Wattier and Maggs 2001). As discussed in Chapter 4, new methods have now been developed. Suitable molecular markers for algae have also been slower to develop than for other taxa. Questions of taxonomic importance are still unanswered or uncertain in many algal groups (Bellorin *et al* 2004, Engel *et al* 2003), making this area of investigation high on the list of priorities for phycological researchers. There is, therefore, a paucity of information about much of the biology of *Gs. longissima*, including its population genetics.

Gs. longissima is thought to occur in sheltered sites in the lower intertidal and sub-tidally to about 15m around the British coast (Steentoft *et al* 1995). Due to its

relatively recent reclassification, there are few published records of its geographical distribution in Britain, with none confirmed in the recent edition of the Check-List and Atlas of the Seaweeds of Britain and Ireland (Hardy and Guiry 2003).

Surveys carried out as part of this study found a number of populations of *Gs. longissima*, *G. gracilis* and mixed assemblages as described in Chapters 1 and 2. As survey information was gathered from the intertidal zone, the degree of continuity of populations subtidally is unknown. At all the sites studied, populations of *Gs. longissima* were accessed in shallow water but populations could normally be seen to continue into deeper water. Levels of gene flow between sites and populations, intraspecific diversity and the limits of populations are all unknown for *Gs. longissima*.

Gene flow describes the exchange of genetic material between spatially separated populations. Whether that exchange is frequent or rare will affect the degree of genetic similarity between what might appear to be geographically separate populations (ie, separated by distance). Theoretically, gene flow estimates can be based on a continuous population with isolation-by-distance as the underlying source of variation, or as island populations isolated by barriers to gene flow where variation arises as a result of intrapopulation mutation and genetic drift (Lowe *et al* 2004).

For molecular genetics research, a lack of existing DNA markers for *Gracilariopsis longissima* means either developing new markers or using universal ones which can successfully measure variation. Randomly amplified polymorphic DNA

(RAPD) markers can be amplified in any DNA and can detect polymorphisms in DNA sequences which can be used as raw data to compare plant genotypes. They require no preknowledge of the genome under investigation and generate large amounts of data at relatively low cost (Lowe *et al* 2004). With RAPD markers alone, however, it is not possible to identify the gene, or position, although it is possible to clone and sequence the resulting bands.

RAPDs can provide an indirect estimate of gene flow using patterns of genetic structure in adult populations. Although not ideal for measuring changes in contemporary gene flow, they can give an indication of historical genetic connections between individuals within a population or between populations (Lowe *et al* 2004).

Despite a number of drawbacks, described in Chapter 4, many authors continue to use RAPDs for molecular ecology. While recognising the importance of avoiding artefacts which can result from inconsistencies in methodologies, Ali *et al* (2004) found RAPDs particularly useful in population genetics investigations in fish. Peakall *et al* (1995) compared RAPD results with allozyme data in buffalograss and found they were able to reveal comparable but greater genetic variation which the authors suggest is explained by the advantage that RAPDs reflect the whole genome, while allozymes are restricted to protein coding loci.

Kjolner *et al* (2004) compared RAPDs with amplified fragment length polymorphism (AFLP) in the herbaceous perennial *Saxifraga cernua* and found that both methods produced similar estimates of genetic diversity. Van Oppen *et al* (1996) found RAPDs useful for large-scale biogeographic intraspecific

investigations in two red algae (*Lophocladia trichoclados* and *Digenea simplex*). However, they warned that the resolving power of RAPD data can be limited at lower geographic scales although they suggest that increased numbers of specimens can enhance resolution. Hadrys *et al* (1992) warned of the problems of scoring diploids, and the sensitivity of the method to changes in reaction conditions such as DNA template concentration.

RAPDS have also continued in use for population genetics in algae, including rhodophytes such as *Gracilaria lemaneiformis* (Wang *et al* 2007), *Gelidium canariense* (Bouza *et al* 2006), *Gigartina skottsbergii* (Faugeron *et al* 2004), *Delisea pulchra* (Wright *et al* 2000), *Mazzaella laminarioides* (Faugeron *et al* 2001) and *Grateloupia doryphora* (Marston and Villalard-Bohnsack 2002). Alberto *et al* (1997) successfully used RAPDs to assess genetic variation in natural populations of *Gelidium sesquipedale*. Pakker *et al* (1996) found RAPDs well suited for biogeographic studies in *Digenea simplex*. Meneses (1996) detected variation in *Gracilaria chilensis* which had been undetected with RFLPs of organellar DNA.

In Chapter 4, it was shown that RAPD molecular markers could be used to detect variation in *Gs. longissima*. This study uses that variation, seen as presence/absence polymorphisms in RAPD fingerprints (Welsh and McClelland 1990, Williams *et al* 1990) to assess:

(1) genetic diversity within three populations of *Gracilariopsis longissima* from the South West Peninsula of Britain, including the prevalence of clonal propagation (from the frequency of identical fingerprints among thalli) and

(2) genetic differentiation among the same populations.

6.2. Materials and Methods

6.2.1. Samples

As discussed in Chapter 4, the dominant nature of RAPD markers makes them problematic for use with diploid specimens as homozygous individuals cannot be distinguished from heterozygous individuals, resulting in misleading allele frequency counts. To overcome this problem, special field collections of female gametophytes, the only haploid plants easily identified in the field by the presence of cystocarps (Figure 1.5), were made from three sites. Specimens arising from a single axis from the holdfast were used to avoid any potential chimeric individuals (section 1.7.4) arising from spore coalescence. Ten specimens were collected from each site. All samples were confirmed as *Gs. longissima* by amplification of the ITS region of the genome as described in Chapter 2, before use in this experiment.

The sampling design took into account a number of constraints in this study:
(1) To avoid collecting samples of *G. gracilis* which cannot be distinguished in the field, samples were collected solely from sites where unispecific populations of *Gs. longissima* had been found (as demonstrated in Chapter 2).
(2) Banding profiles obtained may not necessarily represent entire populations as access was restricted to individuals in intertidal zones and by physical barriers, such as deep water or unstable substrates.
(3) The distribution of fertile female individuals within sites could not be predicted and sampling was therefore haphazard, determined by visual identification of suitable plants. Spatial relationships between individuals from each site were not recorded.

(4) Concerns about RAPD profile susceptibility to small changes in either amplification or gel electrophoresis conditions, restricted numbers of sites and individuals to a total of 30 samples, as this was the maximum number which could be compared alongside each other on a single gel using the equipment available for this study. This number allowed ten individuals and three sites to be studied at one time which was the minimum acceptable number of samples for statistical analysis using PRIMER V (R. Clarke (PRIMER author) personal communication).

One site from each of the three main geographical areas of study – the North Devon coast, the Fal Estuary, the Helford River – was chosen on the basis of three criteria:

(1) It had been established that these sites hosted unispecific populations of *Gs. longissima* (Chapter 2).
(2) Sites had safe and easy access.
(3) Individuals of *Gs. longissima* were found commonly throughout the sites which made them easy to find.

The three sites were: Braunton on the north Devon coast (sample code A), Helford Passage on the Helford River (sample code H), and St Just in Roseland in the Fal Estuary (sample code J). A map of sites is provided in Chapter 2 (Figure 2.3). The northern site at Braunton was the most distant from the Helford River site, at approximately 300km if travelling by sea around the coast (as algal propagules would be transported), while the St Just site was approximately 15km east of the Helford site.

Samples were coded by site and number for identification as shown in Table 6.1. Specimens were cleaned thoroughly as described in Chapter 5 and all cystocarpic material was removed with a scalpel to avoid contamination with diploid material. Samples were air dried and stored in silica gel.

6.2.2. DNA extraction

DNA was extracted from approximately 5 mg of dried material for each specimen using the method of Wattier *et al* (2000) as described in Chapter 4.

6.2.3. RAPD PCR

RAPD PCR was carried out using the optimised PCR conditions as described in Chapter 4 (Table 4.3) using 12, 10-mer primers which had been screened for amplification success and polymorphisms. Table 6.2 lists the primers and their sequences. Each reaction was carried out in duplicate.

Table 6.1: Codes for individual female specimens of *Gs. longissima* collected from three sites around the South West Peninsula of Britain for population genetics studies

	Site	
Braunton Burrows	**Helford River**	**St Just in Roseland**
A50	H40	J70
A52	H42	J71
A54	H44	J72
A55	H46	J73
A56	H47	J74
A58	H49	J75
A60	H51	J76
A61	H52	J77
A62	H53	J78
A63	H54	J79
Total 10	**10**	**10**

Table 6.2: RAPD primers used to produce DNA profiles for 30 *Gs. longissima* individuals from three populations around the South West Peninsula of Britain

Code	Sequence 5'-3'	Scorable bands	Number polymorphic
B5-1	AGCGCCCTTC	12	3
B5-3	TGGGCCCTTC	18	11
B5-5	TGCGGCCTTC	16	5
B5-6	TGCGCGCTTC	17	5
B5-7	TGCGCCGTTC	18	7
B5-8	TGCGCCCATC	15	6
B5-9	TGCGCCCTAC	20	9
B6	TGCTCTGCCC	16	6
B7-1	CGTGACGCAG	13	6
B7- 6	GGTGAGGCAG	17	9
R2	CACAGCTGCC	10	2
R4	CCCGTAGCAC	14	5
Total		**186**	**74**

The sensitivity of RAPD PCR to small variations in amplification conditions is well documented. Therefore all reactions for a single primer were processed in exactly the same way, at the same time, using the same instrumentation, reagents and processes to avoid any anomalies in results. One duplicate of each primer/specimen result were then compared on the same gel.

6.2.4. Electrophoresis

Standardisation of electrophoresis was achieved by running the amplification product (10 µl) from one duplicate of each primer/specimen pair on an agarose gel (1.5%) with a negative control and 100 base pair DNA ladders (MBI Fermentas, York, UK) in the two outermost lanes, to allow identification of each product by size.

For accurate band scoring across a number of individuals, it is essential to run amplification products from individual primers on a single gel. However, available

gel size was limited to 30 lanes. Because there were duplicate reactions for each primer/specimen pair, it was necessary to run two gels simultaneously for each primer, with one set of the duplicate PCR products on each. With a 30-lane limit for each gel, 27 primer/specimen products were run on each gel with three size markers. This meant that, for each PCR amplification, three primer/specimen products were run on only one of the duplicate gels. Each amplification product could therefore be scored.

6.2.5. Band scoring

To avoid the possibility of bias in scoring bands, amplification products were allocated to gel lanes randomly. This was achieved by drawing specimen ID codes out of a "hat"; the first individual picked was run in lane 1, the next in lane 2 and so on until all had been picked. Duplicate gels were loaded with specimens in the same positions to allow comparison between gels. Specimens omitted from the first gel occupied the same lane as the specimen left out of the second, so that all other specimens stayed in the same position for the duplicate gel. This randomization procedure was repeated for each primer so particular specimens did not always appear at the same position on the gel.

Bands were scored using a combination of computer program detection (UviMap Gel Documentation System, Uvitech Inc., Cambridge) and visual assessment. The computer program was used to determine band size according to base pair standard markers. Visual assessment was used to confirm presence/absence, as variation in strengths across gels sometimes led to inconsistent detection by the computer program.

Bands were recorded as one of two "alleles", present or absent, represented digitally as 1 or 0 respectively. Bands which varied in strength from apparently absent, through just visible, to very strong were, along with monomorphic bands, excluded from the analysis.

6.2.6. Data analysis

Allele frequences for each putative locus (band) at each collecting site were calculated and used to construct a comparative bar chart for each site.

Two matrices representing haplotype were constructed for each specimen by the presence or absence of each band formatted to conform to data input requirements for two different statistical programs. Matrix 1, for the ARLEQUIN program, combined all bands as a single profile creating a binary string of 0s and 1s for each specimen, coded for site. Matrix 2 for PRIMER listed each band separately as either 0 (absent) or 1 (present) coded for specimen and site. These matrices were used to analyse the variation within and between sites.

(i) Analysis of molecular variance (AMOVA) was calculated using ARLEQUIN V. 3 (Excoffier *et al* 2005). AMOVA is analgous to standard analysis of variance (ANOVA) statistics which are used to partition variation to the different components of a set of data, in this case within and between sites or populations. ARLEQUIN also calculates Wright's Fixation Index Statistic (Fst), treating the data from each site as a subpopulation of a whole. The analysis was applied to all three sites together and between pairs of sites, ie, Braunton with Helford, Braunton with St Just, and Helford with St Just.

(ii) A similarity matrix of individuals at all sites was created in PRIMER V (Clarke and Warwick 2001) using Bray-Curtis similarity indices. This program is able to treat each band as a separate "species" and the frequencies of the two possible alleles (present/absent) as occurrence data. The matrix was used to calculate multi-dimensional scaling (MDS) ordination and cluster plots for individuals. Labelling data (as described above) by individual and by site (population) as a factor allows the program to create a cluster plots at the level of individual or site.

The Bray-Curtis similarity matrix was also used to carry out an analysis of similarities (ANOSIM) in PRIMER. Where R = 1 all specimens within a site have greater similarity to one another than with any specimens from other sites, ie, populations are likely to have low levels of genetic exchange. Where R = 0, this indicates that similarities between and within sites are the same on average, ie, populations are likely to experience high levels of gene flow between them.

6.3. Results

Of thirteen 10-mer primers previously found to produce polymorphic bands in *Gs. longissima* (cf Table 4.4), one (OPR8) failed to amplify when used in this experiment. The remaining 12 produced a total of 186 scorable bands of which 74 were polymorphic (Table 6.2) in the 30 specimens used in this experiment. Scorable amplification products ranged in size from 200bp to 2500bp.

Of the 74 polymorphic bands amplified, 58 were found in individuals from Braunton, 56 in individuals from Helford and 60 in individuals from St Just. Allele frequencies for each site are shown in Figure 6.1. Among all the specimens and primers, despite a large number of bands shared among individuals and sites, no

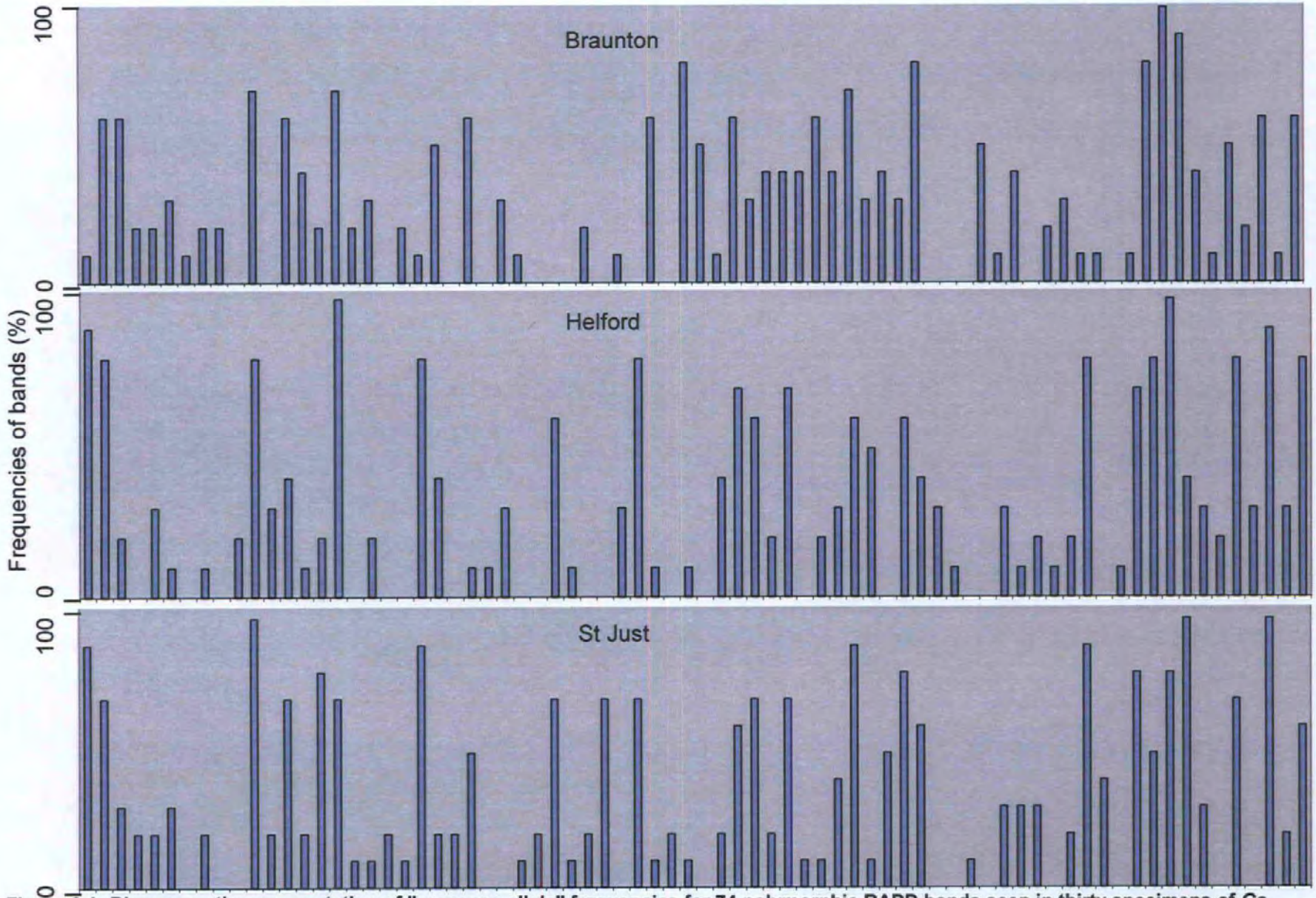


Figure 6.1: Diagrammatic representation of "presence allele" frequencies for 74 polymorphic RAPD bands seen in thirty specimens of *Gs. longissima* made up of ten specimens from each of three sites - Braunton, Helford and St Just - arranged to allow comparison. Of the 74 bands, 58 were found at Braunton, 56 in individuals from Helford and 60 in individuals from St Just.

identical haplotypes were recorded either within or between sites, with each individual from each site having a distinct combination of alleles.

Bands showed highly variable strength across specimens as illustrated in Figure 6.2 by the band at 1520bp (green arrow) which appears absent in some individuals but only where amplification of the entire fingerprint is weaker than in other individuals. The potential for mis-scoring such bands as present or absent erroneously made it safer to exclude them entirely. No ambiguities between duplicate gels were found.

Partitioning of variance over all three sites, as determined by the AMOVA program in Arlequin, is shown in Table 6.3. Variation within populations accounted for almost 84% of differences seen, while between population variation accounted for only just over 16%, at a significance level of <0.001. Populations were therefore significantly different from one another. The Fst index for all sites was 0.16

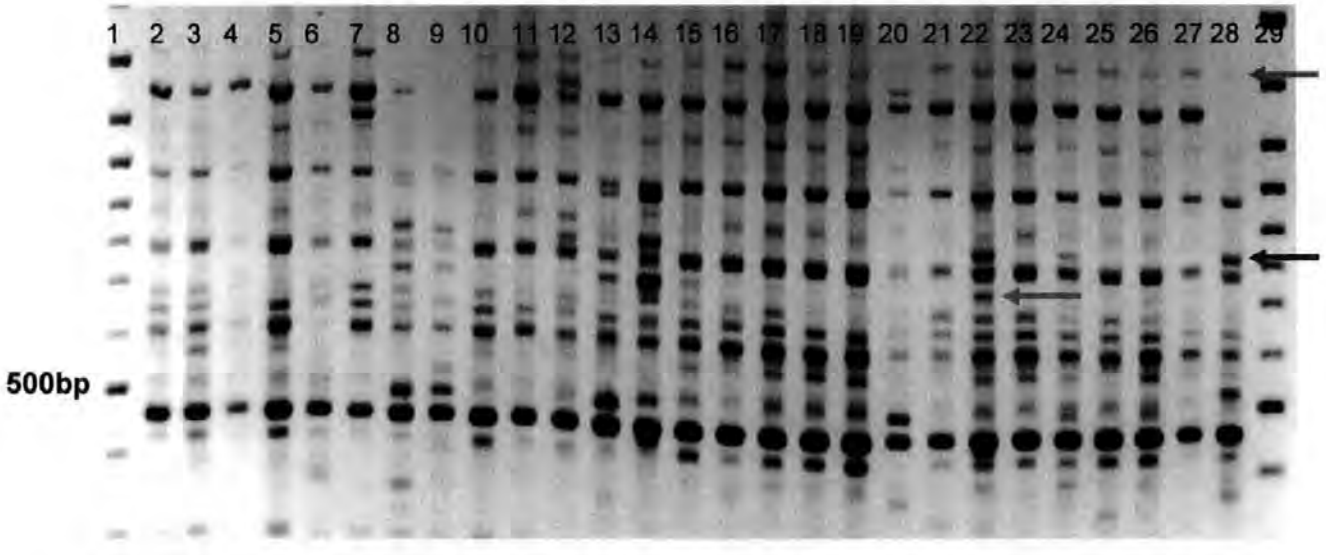


Figure 6.2: Reverse image of results obtained for RAPD primer OPB5-3 in samples A54, J74, A61, A56, J76, A60, A55, J77, H40, J72, A63, H49, A62, H47, J73, J79, H44, H52, J70, J75, H53, H51, H46, J78, H54, A58 (lanes 2-28). Product size varied from 450bp up to 1520bp. Two polymorphic bands are arrowed (blue – 820bp, red – 700bp). Band 1520 (green arrow) was unscorable as it appeared faintly in less well amplified product and disappeared in very poorly amplified ones. Lanes 1 & 29 are 100bp marker lanes with the bolder 500bp band labelled. Products for A52, H49 and J71 were run on the duplicate gel for this primer.

(p <0.001), also indicating that differentiation between all sites considered together was moderate to high (Wright 1978).

Analysis of variance between pairs of sites identifies a large proportion of variance within sites (Tables 6.4, 6.5, 6.6) but differences between populations are still significant. For comparisons between Braunton and the two southern sites, the difference is highly significant (p <0.001). The Fst for Braunton versus Helford of 0.19 (p< 0.001) suggests large differentiation between these sites. For Braunton

Table 6.3: Analysis of Molecular Variance (AMOVA) for 30 individuals of *Gs. longissima* from three sites in the South West of Britain, using 74 RAPD loci

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*Percentage of variation*	*Significance level (p)*
Between populations	2	63.000	2.06926	**16.07**	<0.001
Within populations	27	291.800	10.80741	**83.93**	
Total	29	354.800	121.87667		
Fixation index	Fst:	**0.16**	p <0.001		

Table 6.4: Analysis of Molecular Variance (AMOVA) for 20 individuals of *Gs. longissima* from Braunton and Helford using 74 RAPD loci

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*Percentage of variation*	*Significance level (p)*
Between populations	1	37.15	2.62	**19.35**	<0.001
Within populations	18	196.70	10.93	**80.65**	
Total	19	233.85	13.55		
Fixation index	Fst:	**0.19**	p < 0.001		

Table 6.5: Analysis of Molecular Variance (AMOVA) for 20 individuals of *Gs. longissima* from Braunton and St Just using 74 RAPD loci

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*Percentage of variation*	*Significance level (p)*
Between populations	1	39.40	2.83	**20.36**	<0.001
Within populations	18	199.40	11.08	**79.64**	
Total	19	238.80	13.91		
Fixation index	Fst:	**0.20**	p < 0.001		

Table 6.6: Analysis of Molecular Variance (AMOVA) for 20 individuals of *Gs. longissima* from Helford and St Just using 74 RAPD loci

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*Percentage of variation*	*Significance level (p)*
Between populations	1	17.95	0.75	**6.74**	<0.003
Within populations	18	187.50	10.42	**90.26**	
Total	19	205.45	11.17		
Fixation index	Fst:	**0.07**	p< 0.003		

compared with St Just, the Fst index is 0.20 (p< 0.001), suggesting similar levels of differentiation between Braunton and this second southern site.

Between Helford and St Just the significance level for variation between populations falls to p < 0.003, which is less than for other comparisons but still significant within 95 % confidence limits. The Fst index falls to 0.07 (p < 0.003) indicating, in Wright's interpretation, that there is much less differentiation between these two populations than any other pair.

The cluster (Figure 6.3) and MDS (Figure 6.4) plots, despite a few specimens which seem to fall outside the main groupings (H52, H53, J72, J76), suggest that geographic distance between sites is reflected in their population genetics with Braunton being separated clearly from the two southern sites, for which individual specimens are more closely related. Helford and St Just are also clustered more closely together suggesting a greater level of similarity of allele frequencies than between Braunton and the two southern sites.

The cluster plot calculated from allele frequencies for each site clearly separates the northern shore site (Braunton) from two southern sites (Helford and St Just) (Figure 6.5). In this calculation, Helford and St Just show around 75% similarity based on band frequencies while their joint similarity with Braunton is reduced to 62%.

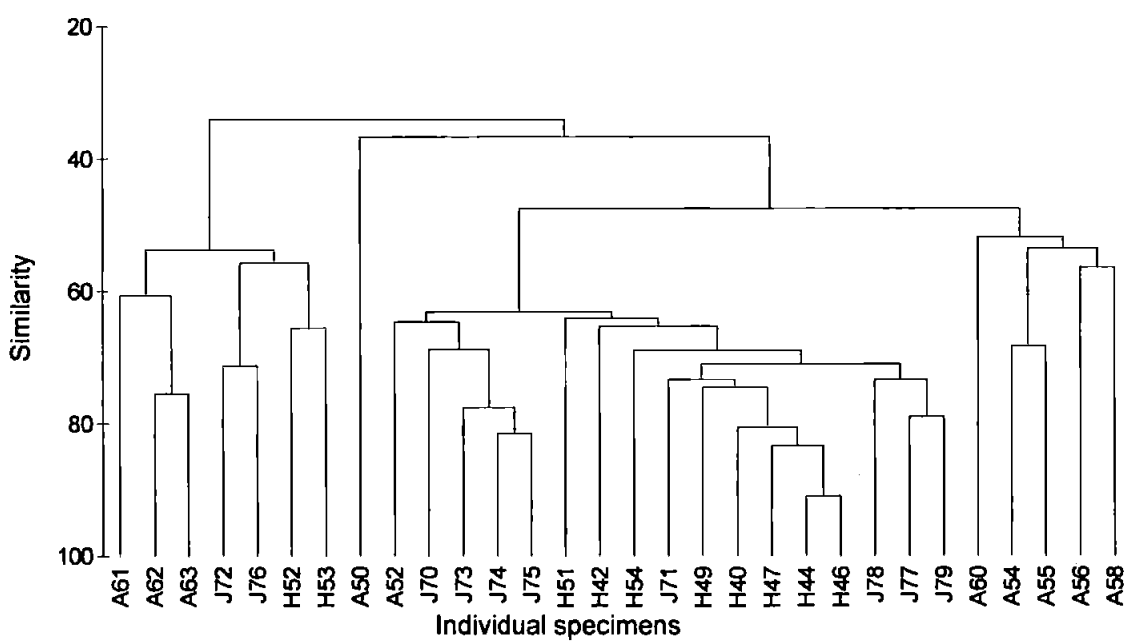


Figure 6.3: Cluster analysis of 30 specimens of *Gs. longissima* from three sites (A-Braunton, H-Helford, J-St Just) based on similarities between RAPD profiles from 74 bands

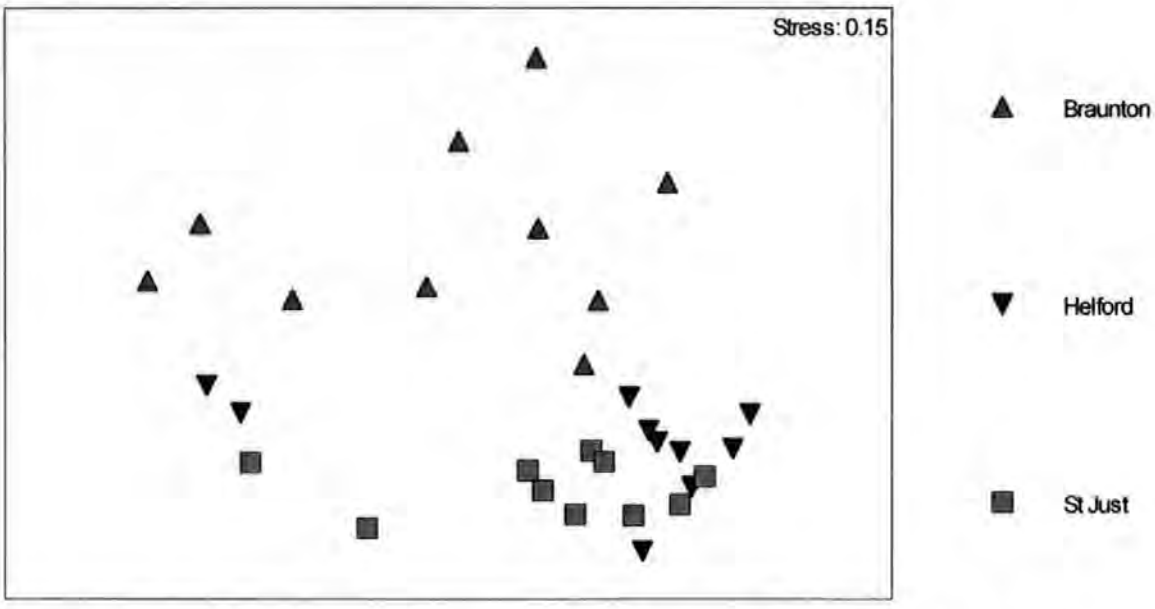


Figure 6.4: MDS plot from a similarity matrix of individuals of *Gs. longissima* from three sites based on banding patterns from 74 RAPD markers

The analysis of similarities calculated with the PRIMER program yields pairwise comparisons (Table 6.7), giving indications of the relationships between individual sites. In Table 6.7, when Braunton is compared with Helford or St Just, R = 0.371 ($p < 0.001$) and 0.456 ($p < 0.001$) respectively. This confirms the AMOVA results, with Braunton differing significantly from both southern sites. With $p = 0.044$ for the comparison between Helford and St Just (R = 0.126), these two populations are still just found to be significantly different, even though at a reduced level.

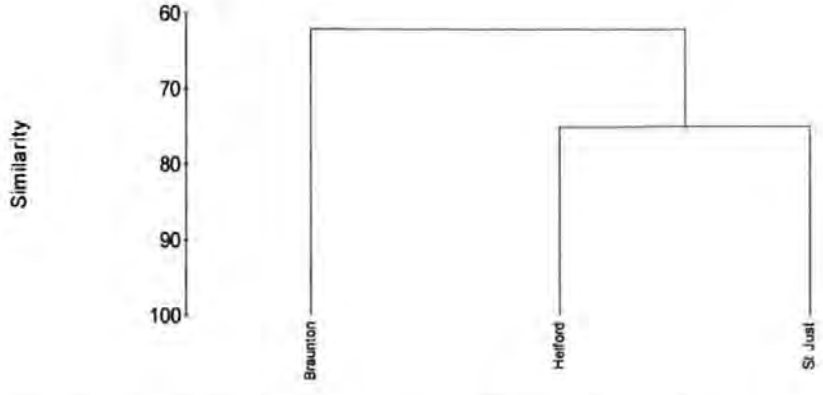


Figure 6.5: Cluster plot of sites for the 74 RAPD loci as calculated by the PRIMER program using Bray-Curtis similarity indices

Table 6.7: ANOSIM calculations with significance levels between sites calculated from the RAPD profiles of 10 individuals from each site

Group comparisons	*R*	*Significance level*
Braunton with Helford	0.371	$p < 0.001$
Braunton with St Just	0.456	$p < 0.001$
Helford with St Just	0.126	$p = 0.044$
Global R	0.294	

In summary, all pairs of sites are significantly different but Helford and St Just are only marginally so.

6.4. Discussion

Gracilariopsis longissima is thought to be mainly subtidal and to be ubiquitous around the South West Peninsula of Britain, which suggests the possibility of a panmictic population, assuming no major discontinuities nor major barriers which would create "islands" or metapopulations. However, the data reported here is sufficient to conclude that the sites investigated, with significant variation seen between sites, do not support the hypothesis of a panmictic population. Although there may be gene flow between populations it could be limited with separation by distance being a factor. However, without additional sites and individuals included in the study, it is difficult to say why the most distant site is most dissimilar.

There are many potential complicating factors, as gene flow is strongly affected by reproductive and dispersal systems in red algae. Differences in dispersal can

have a huge impact on gene flow and genetic diversity in populations. As details of this process in *Gs. longissima* are almost entirely unknown it is difficult to comment on the contribution of spore, gamete and ramet dispersal to gene flow between sites.

In *G. gracilis* (as *G. verrucosa*), Destombe *et al* (1990) established that sperm survival times could be as short as 6 hours. Studies of sperm dispersal in *G. gracilis* populations in France (Engel *et al* 1999) suggest that distances travelled are very limited, perhaps as little as 1 m. Haploid and diploid spores have been shown to have different dispersal abilities (Destombe *et al* 1992) but most appeared to have settled within 2m of the parent plant.

Species of *Gracilaria* are known to regenerate easily from thallus fragments (Santelices and Varela 1995) and, during this study, specimens of *Gs. longissima* were found apparently fixed but actually held in place by thalli being partially buried in silt and without holdfasts, nor attached substrates (personal observations). This indicates that *Gs. longissima* is also able to regenerate or grow from broken off vegetative material which would represent clones of the parent plants and lead to many clonal individuals being present. If fragmentation were contributing significantly to propagation in the populations studied here, high degrees of similarity might be expected in RAPD banding patterns for individual sites. However, with the very high levels of variation seen in this study, with distinctive haplotypes found for every individual in the study, there is no evidence for the presence of clonal individuals. The RAPD patterns indicate that fragmentation and regeneration are not prevalent in these populations. As with the

between population variation, much greater numbers of individuals would need to be studied for a firm hypothesis to be determined.

As has been seen (Chapter 2), *Gs. longissima* attaches commonly to very small stones and fragments of shell at sites with mobile substrates. They are easily dislodged by disruption to substrates but settle or snag again very quickly (personal observation). The potential for fragments to drift over long distances to resettle away from their origin as suggested by Steentoft *et al* (1995) seems small, but the potential contribution to gene flow of dispersal by passive transport of broken fragments is unknown.

Again in *G. gracilis*, Engel *et al* (1999, 2002) demonstrated that non-random mating (sexual selection by females) takes place. Such a process taking place in *Gs. longissima* would also be expected to influence genetic structures.

The high levels of intra-population variation found in this study of populations from sites experiencing great fluctuations in conditions, daily, monthly and seasonally, may accord with Maynard Smith's (1998) statement that "it seems only common sense that a population should be more diverse genetically if it lives in a variable environment". Putting aside the normal diurnal variations experienced by all estuarine organisms, there is still potential patchiness in the environment in which *Gs. longissima* occurs.

A spore released from a parent plant living in the highest reaches of an estuary may settle in the lower reaches or subtidally. If so, it will experience very different fluctuations in salinity, temperature, light and nutrient levels from the parent plant.

The same is true for spores of sub-tidal origin carried on the flood tide up to the highest reaches of the estuary. Whether all individuals are able to survive throughout the range of conditions is unknown and therefore no correlation between the high levels of variation seen in these populations and the potential for patchy habitats is possible.

Meneses and Santelices (1999) found that *Gracilaria chilensis* was capable of demonstrating significant RAPD profile changes when transferred from the wild to experimental laboratory culture, with greater degrees of variation seen in the wild populations. They concluded that *G. chilensis* demonstrated a continuous reaction between genotype and the environment, possibly through mitotic recombination. These findings could undermine the use of RAPDs in population genetics studies but, if enough samples are collected at the same time, presumably any changes over time would not confound the data but could confound comparisons between sites.

Santelices *et al* (1996) and Meneses (1996) suggest that sporeling coalescence in *Gracilaria chilensis* may be a source of high variation in individuals. Certainly, there is good evidence that *Gs. longissima* spores also coalesce (Section 1.7.4) as specimens were seen during the course of sample collection, although this does not necessarily mean that individuals exchange or fuse their nuclei or DNA. However, in such an individual with multiple axes, it would not be possible to distinguish which axes had arisen from which spore and DNA extractions would include all genomes present, leading to variation of non-specific origin.

The analyses of molecular variance (AMOVA) and analysis of similarities (ANOSIM) detected significant differences between populations. As there are no known physical barriers between populations, it is only limitations to spore, sperm and thallus fragment dispersal (as seen in *G. gracilis*) that affects the potential for the exchange of genetic material on a regular basis. Therefore, it may be that the differentiation between populations is a case of isolation by distance with attenuation of gene flow with increasing distance. However, as stated above, the use of only three sites and 30 samples precludes a conclusion of isolation by distance. Any direction of flow is undetectable in this data set and would need many more sites at different spatial scales would be needed to provide information of that nature. Greater numbers of sites and specimens would be needed before any such conclusion could be reached.

There are a number of problems arising from the constraints of this study, which must be considered when evaluating the results. First, there were low numbers of specimens from each site. Although other researchers have used similarly low numbers with other species (e.g.,Wolff *et al* 1997), most RAPD studies in algae have used larger numbers of specimens. For example, Meneses (1996) used 35 individuals from each site; Faugeron *et al* (2004) used between 22 and 100 per site. Increasing the number of individuals used in this study could have revealed patterns not seen with low numbers and increased the robustness of the statistical analyses.

Second, using only females collected in a relatively non-random way could have created a bias in allele frequencies, if there were sex-linked loci, which cannot be quantified. A similar species, *Gracilaria gracilis*, has been shown to have a

population structure of 25% female, 25% male and 50% tetrasporophyte (Engel *et al* 2001). If a similar population structure is true for *Gs. longissima*, the study group of individuals may not represent the true allelic frequencies of the population as a whole. However, other researchers have adopted a similar strategy when using RAPDs for questions of population genetics in algae (e.g., Faugeron *et al* 2001, Bouza *et al* 2006).

Third, the high numbers of invariant bands experienced with RAPD markers, highlighted by Wattier and Maggs (2001), and the high levels of variation between individuals from the same site create increased "noise", decreasing the strength of the signal from RAPD profiles. In this study, as in others (Huff *et al* 1993), all bands which were monomorphic for the entire data set were omitted from calculations. However, it is possible that some degree of genetic variability may be associated with apparently invariant bands. It was not in the scope of this study to pursue this question.

A further complicating factor is that when scoring RAPD profiles, an assumption of uniqueness for each band is made. However, two fragments of the same molecular weight are not, necessarily, the same in sequence. Two distinct fragments of the same size or within a couple of base pairs may be indistinguishable on an agarose gel. It is also possible that bands of different size actually represent allelic variants. Such occurrences are undetectable but could undermine data if they were common.

It is not practical to sequence each band, so using a large number of primers, combined with statistical tests of similarities/differences to elucidate relationships, is a reasonable alternative.

G. gracilis, for which there are established microsatellite markers, was found at a number of the sites surveyed during the course of this study. An investigation into the population genetics of this species around the South West Peninsula might reveal some interesting details of gene flow for this very similar species. As passive transport in water currents around the area is the only method of dispersal for sperm, spores or ramets for both species, notwithstanding any differences in sexual strategies as yet undiscovered, it might be expected to find similar potentials for gene flow in both species.

Many fascinating questions remain about the life history and reproductive strategies of *Gs. longissima*. Whether the species proves to be similar or very different in these respects from *G. gracilis*, further questions will arise about what separates the two species and enables them to co-exist.

While this small study has revealed some interesting information about just three populations of *Gs. longissima* in Britain, there is no question that the development of specific markers would provide the best tool for unravelling the complexities of the population genetics of *Gs. longissima*.

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