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# PHYSIOLOGICAL RESPONSES OF GRACILARIOPSIS LONGISSIMA TO COPPER EXPOSURE

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University of Plymouth

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**PHYSIOLOGICAL RESPONSES OF**

***GRACILARIOPSIS LONGISSIMA***

**TO COPPER EXPOSURE**

by

**JAMES EDWARD NEWMAN**

A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

**DOCTOR OF PHILOSOPHY**

Department of Biological Sciences

Faculty of Science

**March 1998**

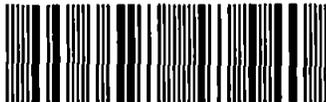
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**The author on an exploratory  
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## ABSTRACT

### Physiological responses of *Gracilariopsis longissima* to copper exposure

James Edward Newman

Seaweeds fulfil many of the criteria regarded as being important for a good biomonitor. They are known to accumulate trace metals and the concentration of these metals measured in the thalli of native populations has been related to the level of contamination in the surrounding water. However, this passive biomonitoring approach has been of limited use and the relationship between the concentration of metal in the seaweed and that in the surrounding environment is not always apparent.

The aim of this study was to investigate the response of the rhodophyte *Gracilariopsis longissima* to copper exposure, in the laboratory and field, in order to increase the understanding of the toxic effects of the metal and ultimately to assess the potential of this species as a biomonitor of trace metal pollution.

A series of comparative physiological experiments were performed to assess the relative effects of copper on the growth and physiology of the species. Growth was reduced at significantly lower levels of copper ( $12\mu\text{g L}^{-1}$ ) than any other physiological parameter measured. Photosynthesis, as measured by oxygen evolution and fluorescence measurements, was only reduced at high copper treatments ( $250\text{--}400\mu\text{g L}^{-1}$ ). This coincided with the actual shrinkage of the algal material, increased ion leakage and loss of pigmentation. One possible explanation for the uncoupling of growth from photosynthesis was investigated and rejected.

Copper accumulated in the thallus of exposed material as a linear function of the copper treatment. Algal material was able to recover, in terms of growth, following exposure to elevated levels of copper. This recovery coincided with a significant release of copper from the thalli and a resulting increase of copper in the recovery media. This discovery suggests that the accumulated metal is not irreversibly bound to the thalli, and has important implications in terms of using the species as a biomonitor of copper pollution.

Populations of *G. longissima* collected from sites known to differ in trace metal contamination were exposed to copper in a series of laboratory based experiments to measure the effect of the metal on their growth. Exposure to elevated levels of copper significantly reduced growth. However, no major difference existed between the response of the populations compared. Possible explanations for this are discussed. Considerable inter and intra-individual variability was found to exist within populations of *G. longissima* and causes and implications of this mostly overlooked source of variation are highlighted.

A field method of active biomonitoring using reciprocally transplanted individuals was developed as an alternative to passive biomonitoring and was found to offer a number of distinct advantages. In particular, the ability to observe responses to copper in areas where no natural population occurred was explored at a highly contaminated site.

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## AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Relevant scientific seminars and conferences were regularly attended, at which work was often presented; external institutions were visited for consultation purposes and to update ZENECA Limited on research progress.

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**J. E. Newman** and M. T. Brown. Inter-individual variation and its use in ecotoxicology: The advantages of studying seaweeds. *Oral paper* presented at: SETAC-Europe (UK Branch) 6<sup>th</sup> Annual meeting – Unifying Themes in Environmental Chemistry and Toxicology, 5-6 September 1995, Plymouth, UK.

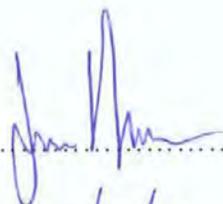
**J. E. Newman** and M. T. Brown. The responses of different populations of *Gracilariopsis longissima* to copper exposure. *Poster* presented at: 1<sup>st</sup> European Phycological Congress, Cologne, Germany, 11-18 August, 1996.

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**J. E. Newman** and M. T. Brown. Growth and photosynthetic responses of *Gracilariopsis longissima* to copper exposure. *Poster* presented at: 6<sup>th</sup> International Phycological Congress, Leiden, The Netherlands, 9-16 August 1997.

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Date

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## **Chapter 1**

# **GENERAL INTRODUCTION**

Contamination of the marine environment due to human activities occurs primarily via two main routes, land based run off and atmospheric input (GESAMP, 1990). The nature of these two sources varies considerably. Input from land occurs mainly via point sources whereas atmospheric inputs are usually more diffuse and dilute (GESAMP, 1990). Because of concentrated human activity along coastal areas it is readily apparent that, 'The coastal strip, encompassing the shallow water and intertidal area (including estuaries) along with adjacent land, is clearly the most vulnerable as well as the most abused marine zone' - GESAMP (1990).

### **1.1 Contamination and pollution**

Contamination has been defined as 'an increase in background concentration of a chemical or radionuclide' (GESAMP, 1995) whereas definitions of pollution usually include some reference to a deleterious effect upon organisms within the contaminated environment (Moriarty 1983, GESAMP 1995). This highlights an important distinction between the two terms - contamination may, or may not, lead to pollution (Perryman, 1996). Anthropogenic releases of many trace metals including Hg, Pb, Zn, Cd, and Cu have been estimated to have increased natural levels in the marine environment by between one and three orders of magnitude (Ramade, 1987). This does not exclusively result in pollution. Only by investigating the effect of elevated levels of trace metals on the biota is it possible to relate a level of contamination to a biological effect.

Contamination can be measured by chemical analysis of the water, but pollution can only be measured by assessing the effect of the contaminant on the biota. Research usually focuses around estuarine and coastal regions, reflecting the importance of this land ocean interface to humans. The usefulness of direct measurement of trace metals as an

indicator of pollution in the marine environment is further compounded by two additional factors.

- ◆ The marine environment (especially estuaries) can be extremely dynamic, with considerable oscillations in salinity, pH and dissolved organic matter as a result of geo-physio-chemical and biological mechanisms. These factors can combine to result in distinct short-term fluctuations of trace metal levels and speciation in the water column (Seeliger & Cordazzo, 1982).
- ◆ Levels of trace metals in seawater samples are technically difficult to quantify accurately, because of errors due to interference of major ions (such as Na) present in seawater (Churella & Copeland, 1978). Sample contamination is also a serious problem, since although the levels of a contaminant may be relatively high, absolute levels are often still very low.

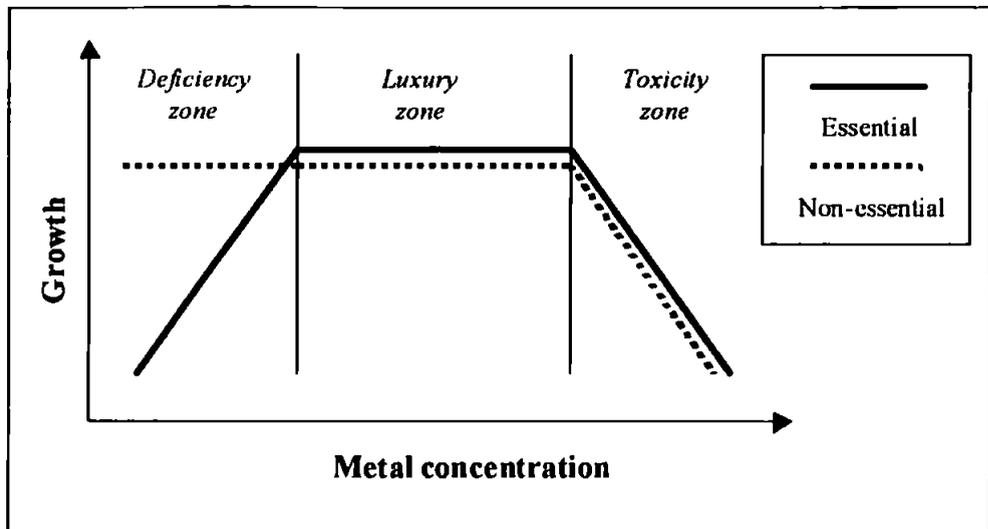
Direct measurement of trace metal contamination in the estuarine environment using analytical chemical techniques is time consuming, costly and does not necessarily provides an indication of the biological impact of the contaminant.

## **1.2 Trace metals in the marine environment**

Trace metals are normal constituents of the marine environment. The term 'trace metals' is used here in favour of the term 'heavy metals', both of which are commonly used as a description of all Class B and intermediate or transitional metal ions (Rainbow, 1993). As the name suggests, these metals are normally present in the environment at very low concentrations (traces). Trace metals are considered here to include both essential and non essential metals, although the term is sometimes used to describe only essential metals.

Many trace metals including Fe, Cu, Zn, Co, Mn, Cr, Mo, V, Se, Ni, and Sn are known to be essential micronutrients for living organisms (Bryan, 1976). The understanding of the nutritional role trace metals play in normal cellular function has changed markedly over time. Bryan (1976) illustrated this fact by pointing out the change in statements from the same authority: - In 1956, Underwood stated that 'a diet well-balanced and adequate in other respects is likely on present evidence to provide the normal individual with an abundance of all the trace elements with little chance of deleterious excess'. This contrasts with the later statement that 'diets do not necessarily provide the normal individual with all the trace elements if proper levels of adequacy are employed, nor can we be so confident that there is little chance of deleterious excess' (Underwood, 1970).

The relationship between metal concentration and growth is now generally considered to be a dose response curve, of the form shown in Figure 1.1. For essential trace metals (Fig. 1.1) three main regions can be identified: the deficiency zone, where growth is limited by lack of the metal, the luxury zone, where levels are optimal for growth and the toxicity zone, where increasing concentrations cause reduced growth. Non-essential trace metals are considered to cause toxicity only at high levels (Fig. 1.1). Normal levels of essential trace metals in coastal seawater are generally considered not to be limiting (Bryan, 1976).



**Fig. 1.1.** Typical dose response curve for essential (solid line) and nonessential (dashed line) metals. Non-essential metals show no symptoms of deficiency at low concentrations.

Figure 1.1 highlights a significant change in thinking, which is reflected by considerable research into the effects of elevated levels of trace metals in the marine environment over the last two decades (for reviews see, Bryan 1976, Bryan & Langston, 1992 and Tessier & Turner 1995).

### 1.3 The use of biomonitors

Marine organisms are known to accumulate most trace metals to levels at least several orders of magnitude higher than those in the surrounding seawater. Because of this fact, biomonitors (also known as *biological indicators*, *bioindicators*, *bio-accumulative indicators* and *sentinel organisms* (Phillips, 1990)) have been used to monitor trace metal pollution for more than twenty years (for reviews see Depledge *et al.* 1994, Langston & Spence 1995 and Phillips 1990). The primary rationale for using these organisms as biomonitors of trace metal pollution is that they provide a time integrated indication of the level of pollution, rather than contamination, in a particular environment. A well

established list of criteria by which organisms are selected has been described (modified from Langston & Spence, 1995):

- ◆ Organisms should be sedentary – so as to be representative of the local environment.
- ◆ They should be geographically widespread, abundant, and easily collected and identified, providing adequate material for analysis.
- ◆ They must be reasonably tolerant of a range of metal concentrations and environmental conditions and be amenable to laboratory experimentation or transplantation.
- ◆ Populations should be relatively stable, allowing year round sampling.
- ◆ A correlation should exist between metal contamination in some compartment of the environment and concentrations in the tissues of the organism.
- ◆ Concentration factors should be similar at all sites.

Concern over the effect of elevated levels of trace metals in estuarine environments, has resulted in this list being expanded to include the criteria that the organism be tolerant of changes in salinity and turbidity (Phillips, 1990).

Benthic organisms spanning many phyla have been employed in trace metal biomonitoring studies. Molluscs (especially bivalves) and crustaceans feature prominently, due to concern over the risk posed to humans from consuming contaminated seafood. However, other species, including polychaetes, coelenterates, echinoderms and macrophytes (algae and seagrasses) have also been employed (Brown & Depledge, 1998).

#### 1.4 Algae as biomonitors

Seaweeds are generally considered to satisfy most of the requirements of a good biomonitor, with fucoids considered to be a particularly suitable group of species (Phillips, 1979). In temperate regions fucoid species (e.g. *Fucus vesiculosus* and *Ascophyllum nodosum*) are often the most abundant species in the intertidal zone and this has led to them being used in many biomonitoring studies (Bryan & Hummerstone 1973, Fuge & James 1974, Haug *et al.* 1974, Melhuus *et al.* 1978, Mykelstad *et al.* 1978, Bryan 1983, Ronnberg *et al.* 1990, Gibb *et al.* 1996 and Jayasekera & Rossbach 1996). Other brown and green macroalgal species (e.g. *Enteromorpha* sp.) have also commonly been used (Seeliger & Cordazzo 1982, Say *et al.* 1990, Karez *et al.* 1994 and Correa *et al.* 1996). There are relatively few examples of the use of red seaweeds (Phillips 1990, Fletcher 1991 and Malea *et al.* 1994).

There are many studies, including those cited above which consider algae, in general, to be useful monitors of trace metal contamination. This is based on the fact that: non-regulated accumulation of trace metals is thought to occur (Bryan 1976 and Phillips 1994) and the levels of metals in the algae are considered to reflect the level of environmental contamination; a linear relationship is often found between metal content of the alga and the seawater concentrations (in laboratory studies). However, the results from field studies can often show considerable error in this relationship (Phillips 1994 and Lobban & Harrison 1994) and the extent to which other factors influence the metal content in a particular species is often unknown (Langston & Spence, 1995).

Considerable differences have been reported between the accumulation of metals in different species. For example, comparative studies of several species sampled from sites differing in metal contamination indicate that not all species accumulate metals to the same

extent. Karez *et al.* (1994) sampled the metal content of nine species in two bays, one of which was contaminated with trace metal laden industrial wastes and found significant differences in the accumulation of metals in different species. Malea *et al.* (1994) sampled seven species of red algae and found '... entirely contrary behaviour', with significant interspecific differences in the accumulation of different metals.

The metal concentration factor (a ratio of thallus content to seawater content) found in many species has been reported to be three or four orders of magnitude higher than in the surrounding water (Bryan 1976, Bryan & Hummerstone 1973 and Edwards 1972) and may reach as high as six (Phillips, 1979). A constant concentration factor at sites varying in metal contamination is considered an essential prerequisite of a qualitative biomonitor (see list of criteria above) but differences are often found between sites (e.g. Bryan & Hummerstone 1983, Morris & Bale 1975 and Langston & Spence 1995). It could be argued that this makes it inappropriate to use the concentration of a particular metal in a seaweed species to indicate the level of contamination at one site relative to another.

Considerable effort has been placed into investigating factors that affect trace metal accumulation in macrophytes. By using both laboratory based and field experiments, numerous biotic and abiotic factors have been found to influence the accumulation of metals by seaweeds. These various factors and their implications for the suitability of seaweeds as biomonitors of trace metal contamination are discussed in more detail below.

## 1.5 Factors influencing bioaccumulation of metals by seaweeds

### 1.5.1 Speciation and bioavailability of trace metals

The concentrations of metals in water or adjacent sediments are used as indicators of the level of exposure to seaweeds, but it should be remembered that both measurements have their limitations. Quantifying the relationship between the concentration of a metal in seawater and that in the seaweed is complicated by the fact that it is not readily apparent what proportion of the total metal concentration in seawater is available to an organism. i.e. the term 'bioavailable' can be defined as the fraction of the total metal concentration that might be taken up by the seaweed (Brown & Depledge, 1998). For many trace metals this is often considered to be associated with the free activity of the free ion in solution (Romeo & Gnassia-Barelli, 1993). For example, the bioavailability of different species of copper ions has been investigated for phytoplankton and bacteria and it has been shown that the rate of uptake, and degree of toxicity, usually depend upon the free  $\text{Cu}^{2+}$  concentration (Gledhill *et al.* 1997). This dependency of copper toxicity on the  $\text{Cu}^{2+}$  is assumed to be the same for macroalgae.

If the bioavailable form of copper is free  $\text{Cu}^{2+}$  species then any factor that alters its equilibrium in seawater would be expected to affect copper accumulation. Factors known to influence the speciation of copper in the marine environment include temperature, salinity, pH and the concentration of inorganic and organic ligands. These factors can fluctuate considerably and often unpredictably in an estuarine environment. This can make direct measurement of the dose of a metal to which an organism is exposed very difficult to quantify (Luoma & Carter, 1991). Single measurements of seawater metal levels would represent only 'snapshots' of the concentrations at a particular site, which may vary considerably even over small time scales (hours, days). Sediments are often used as

indicators of the level of contamination at a particular site since they are considered to be less misleading in terms of short term fluctuations in metal concentrations.

The concentration of metals in the sediment can be less affected by short-term fluctuations than instantaneous water measurements, but may not necessarily reflect the fraction of copper available to the seaweed. The level of metal in sediments can however, be used to indicate the history of metal contamination in a particular area.

Seaweeds are considered good biomonitors because of their ability to integrate fluctuations in contamination levels in the environment over time. This assumed time integration is based on very little mechanistic data and changes in metal levels in seawater are not always reflected by corresponding changes in an organism's tissue concentrations (Brown & Depledge, 1998).

### *1.5.2 Regulation and accumulation*

It has been suggested that organisms be designated as having either accumulator or regulator strategies (Rainbow 1992 and Langston & Spence 1995). An accumulator is defined as an organism that does not appear to regulate uptake in any way. This classification has, however, been questioned for a number of reasons (see Depledge *et al.* 1994 and Brown & Depledge, 1998). The main criticisms are listed below.

- ◆ The classification disguises the fact that intermediate possibilities can exist.
- ◆ It fails to highlight the fact that an organism may differ in its regulation of different trace metals.
- ◆ The classification is misleading from a mechanistic point of view since the term 'strategy' implies some form of choice by the organism.

- ◆ It may obscure biologically significant events by overlooking mechanisms of regulation within the organism.

Regulation is better defined as the mechanisms involved in the handling and processing of metals, i.e. uptake, elimination and sequestration. Accumulation of a metal by an organism will occur when the rate of uptake exceeds the rate of elimination

### 1.5.3 Uptake and accumulation

It is a well established fact that the concentration of many metals in seaweeds is higher (usually by orders of magnitude) than in the surrounding seawater (Philips, 1994). Uptake and net accumulation of trace metals by macroalgae are usually attributed to passive diffusion across cell and organelle membranes, adsorption to the cell surface, reversible and irreversible binding and storage in vacuoles and inter- and intracellular fluids (Rice & Lapointe, 1981).

In the case of the essential metal copper, uptake is considered to occur via facilitated transport (Gledhill *et al.*, 1997), operating through some form of transport ligand ( $L_T$ ) and resulting in the metal being bound to some form of internal binding agent ( $L_I$ ) (Williams 1981, Fig 1.2). The internal binding of the metal maintains a lower internal free  $Cu^{2+}$  concentration compared to the water, thus allowing further uptake to occur. This is in agreement with the assumption that the bioavailable form of the metal is the  $Cu^{2+}$  ion, since it would be in the available form for binding to the transport ligand  $L_T$ .

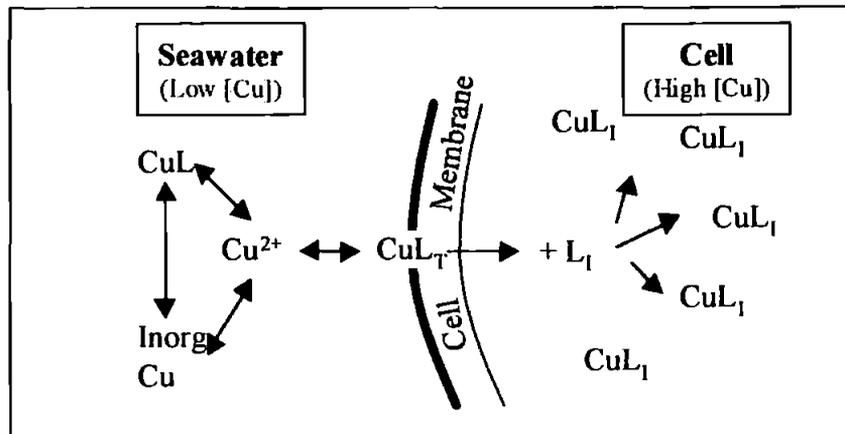


Fig. 1.2. Uptake of copper into an algal cell.  $\text{Cu}^{2+}$  = free copper,  $\text{CuL}$  = organically complexed copper,  $\text{Inorg Cu}$  = inorganically complexed copper,  $\text{L}_1$  = internal ligand,  $\text{L}_T$  = transport ligand. After Gledhill *et al.* 1997.

#### 1.5.4 Elimination

The net accumulation of a trace metal by an organism under a particular set of conditions does not necessarily mean that it lacks the ability to regulate the metal. It simply implies that the rate of uptake is higher than the rate of elimination or loss of the trace metal under current conditions. Claims that macroalgae lack the ability to regulate metals (Lovett Doust *et al.* 1994 and Langston & Spence 1995) are considered misleading, since it implies that macroalgae can only accumulate metals passively. The net accumulation of a metal in a seaweed's thallus, and thus its regulation, will be affected by any elimination mechanism that may be operating.

Information regarding elimination mechanisms has been obtained by studying the release of metals from tissue following exposure to metals. Seeliger & Cordazzo (1982) measured the concentration of copper in an *Enteromorpha* sp. during culture in control media following a pre-treatment with copper. They found a (non-significant) tendency for copper levels to decrease. This decrease may have been due to a dilution effect resulting from increased growth during the recovery phase (see allometry section below). Hall (1981) found a significant release of copper from the marine fouling alga *Ectocarpus*

*siliculosus*. Seip (1979) modelled the uptake and release of metals in *Ascophyllum nodosum* and estimated that release rates of zinc were small. This was in agreement with the previous work of Young (1975), who reported small losses of zinc following exposure and subsequent removal to 'neutral media'. Field transplantation experiments have also indicated that loss may occur in parts of *Ascophyllum nodosum* when moved from a polluted site to a cleaner site. Release rates can therefore vary significantly depending upon the species and the metal in question. More studies are required to establish the exact nature of trace metal regulation in individual species of macroalgae.

A better understanding of the mechanisms of metal uptake, exclusion and toxicity can only improve the usefulness of seaweeds as quantitative biomonitors of trace metal pollution. From the little information that does exist on the mechanisms of trace metal regulation it appears that different species regulate metals in different ways. The way in which a particular species regulates its uptake and loss of a metal will influence its degree of time integration. If populations of seaweed species are to be used as quantitative biomonitors of trace metal pollution, then more must be understood about the mechanisms by which species regulate trace metal concentrations.

#### 1.5.5 *Tolerance to trace metals*

The exposure to elevated levels of metals may lead to some form of physiological adaptation, which can result in increased tolerance. There is some confusion of the use of the term 'tolerance', which is commonly used but often not defined. For example, it can be taken to indicate differences in response between races which are assumed to be genetically determined (Foster, 1986) or simply a measured difference in response between two populations which may be either phenotypic or genotypic in origin (Correa *et al.*,

1996). Tolerance is used here as the more general term, which does not indicate a determined genetic basis for any difference unless this is stated.

A change in tolerance must function through a change in the mechanisms involved in metal regulation in the organism. This could have important implications for the use of the species as a biomonitor of trace metal contamination.

Physiological tolerance can be achieved by one or more of the following mechanisms (adapted from Fernandes & Henriques, 1991):

- ◆ Excretion of complexing compounds that reduce metal availability.
- ◆ Metal exclusion through selective uptake of elements.
- ◆ Metal immobilisation in the cell wall.
- ◆ Metal accumulation in vacuoles and other intracellular bodies.
- ◆ Increased production of intracellular metal-binding compounds.
- ◆ Development of metal tolerant enzymes.

This list was compiled for higher plants and is not exhaustive, although it still gives some indication of the mechanisms that can be expected to be found in algae. The results from various studies indicate that mechanisms can vary significantly from species to species. For example, some studies report no difference, or even increased metal levels in tolerant individuals exposed to metals (Reed & Moffat 1983, Bryan & Hummerstone 1973). In contrast, Foster (1986) reported that a copper tolerant strain of the green alga *Chlorella vulgaris* excluded copper more than a non-tolerant strain. Similarly, Hall *et al.* (1979) found evidence of an exclusion mechanism in a copper tolerant strain of *Ectocarpus siliculosus*.

Adaptive mechanisms known to reduce tissue accumulation of metals in macroalgae include the release of extracellular organic chelating compounds (Langston & Spence 1995). Seeliger & Edwards (1979) found that the release of dissolved organic material resulted in lower metal concentration in the algal tissue. In contrast, exclusion of copper by tolerant populations *Ectocarpus siliculosus* was found to be due to reduced permeability and not the complexation of copper with extracellular products (Hall, 1981). Lage *et al.* (1996) found evidence of two tolerance mechanisms in the dinoflagellate *Prorocentrum micans* exposed to sub lethal levels of copper, copper efflux and sequestration in polymeric substances.

Prolonged exposure to elevated levels of metals can lead to genetic adaptation in some cases. The majority of examples of contamination induced genetic adaptation to trace metals come from studies of higher plants (Macnair, 1993). Many populations of marine organisms have been found to exhibit increased tolerance to elevated levels of metals. In their review of genetic adaptation to heavy metals in aquatic organisms, Klerks & Weis (1987) concluded that 'most, but not all, populations in polluted areas do have an increased tolerance'. However, it was not possible to establish whether this tolerance had a genetic basis in every instance although it did in some cases.

In algae, most examples of differences in tolerance are from studies conducted on microalgal species. Some examples do however exist for macroalgae. By assessing the level of tolerance in the progeny of tolerant individuals, it is possible to establish whether the tolerance has any genetic basis. Genetically determined tolerance to copper has been found in *Ectocarpus siliculosus* (Russell & Morris, 1970). Reed & Moffat (1983) also found that copper tolerance was genetically determined in populations of the ship-fouling *Enteromorpha compressa*.

More recently, Correa *et al.* (1996) compared populations from two sites differing in copper contamination. They found increased tolerance to copper in the population from the more polluted site but the F<sub>1</sub> progeny of this tolerant population were as equally affected as the progeny of the population from the less polluted site. This suggests that increased tolerance was not genetically based; acclimation may play as important a role as heritable adaptation in providing increased tolerance.

When considering using a macroalgal species as a biomonitor of trace metal pollution, the possibility that different populations have adapted to metal contamination (genotypic or phenotypic) should not be overlooked. Differences in tolerance between populations could clearly have significant implications for the use of a species as a biomonitor. Since the fundamental purpose of trace metal biomonitoring is to establish a link between the level of a metal in an organism and the level of contamination in the environment, it will be necessary to measure metal levels from organisms from contaminated (or at least suspected to be contaminated) sites. There will always be some chance that individuals from the polluted sites will have adapted in some way to the elevated levels of metal in that environment. Judging from the available literature it is clearly inappropriate to assume that tolerant individuals will accumulate metals in the same way as non-tolerant ones. The usefulness of seaweeds as quantitative biomonitors of trace metal pollution requires a better understanding of the effect of the metals on the particular species involved and some assessment as to whether or not tolerance has been acquired.

As was stated above, establishing the existence of differences in tolerance between populations has other uses in addition to indicating the potential of a species as a biomonitor. Studying populations that differ in tolerance can be a very powerful tool to help understand mechanisms of trace metal accumulation and toxicity. By comparing the physiological responses of populations differing in tolerance, it may be possible to learn

more about functional significance of tolerance. For example, it has been proposed that increased tolerance may incur some form of cost on the individual (Macnair, 1993). If increased tolerance is due to reduced permeability of the cell to the metal then it is possible that this could result in reduced absorption of other essential elements. This may lead to deficiencies in some of these metals. Similarly, if increased tolerance is the result of some form of energy requiring process then this could incur a cost by diverting energy from other essential cellular process thus reducing overall fitness. These possibilities can be investigated by comparing populations differing in tolerance. Therefore, investigations of the response of different populations from sites known to vary in metal contamination is viewed as a beneficial line of investigation.

#### *1.5.6 Inter-individual responses to contaminant exposure*

Another important consideration when investigating the effects of a contaminant on a population is the response of the individual. Typically, comparative studies investigate the effect of a contaminant at the population or species level, or higher (Bennett, 1987). This approach overlooks the importance of the individual. It is erroneous to assume that all individuals from within a population will respond in a uniform way to contamination (Forbes *et al.*, 1995). By describing the response of the average organism from a population, the individual is neglected (Bennett, 1987).

Inter-individual variability is often overlooked but is an important source of variation. Selectional pressures caused by environmental stresses such as pollutants, are more likely to exert themselves upon individuals at the limits of the ranges of variability than upon those in the middle. This means that the toxic effect of a contaminant can be expected to alter the distribution of different 'types' of individuals within a population.

Concentrating upon the mean response of a population may overlook subtle effects of this kind.

Very few studies exist that have investigated inter-individual variability in relation to pollutants. Forbes *et al.* (1995) investigated the intra-population variability in response to trace metal exposure of gastropod populations. They found that sublethal exposure to cadmium reduced the mean growth rate, but increased the variability in growth rate within populations. Non genetic variation was found to be the most important component of the total phenotypic variance in growth rates.

One problem associated with such studies is the fact that in order to investigate inter-individual differences, some form of replication of an individual is necessary. The inter-individual variability in the response of the common shore crab *Carcinus maenas* has been investigated by classifying individuals into 'physiotypes' and measuring the effect of copper on the physiology of different types (Depledge & Bjerregaard, 1989). Organisms which can be vegetatively propagated are particularly amenable for this type of study, since ramets from a single genet can be used as replicates. Macroalgae therefore appear to be potentially useful organisms for this type of investigation. Despite this, no examples of this type of ecotoxicological study could be found that utilised seaweeds.

#### *1.5.7 Interactions between metals*

One important aspect that must be considered when considering the use of an organism for any form of biomonitoring, is that more than one contaminant may be present at the site(s) of interest and that these contaminants may interact. Several laboratory based investigations have found evidence that metal-metal interactions can occur. For example Bryan as early as 1969 found that the presence of high levels of cadmium, copper or

manganese reduced the net accumulation of zinc in *Laminaria digitata*. The accumulation of cadmium by *Fucus vesiculosus* was also found to be affected by copper, manganese and zinc (Bryan *et al.*, 1985).

Wang *et al.* (1995) studied the reciprocal effect of copper, cadmium, and zinc on the marine microalga *Phaeodactylum trinitum* and found evidence of instances of both synergistic and antagonistic interactions, depending upon the ratios of the different metals in the media. Zolotukhina *et al.* (1991) studied the interactions between six metals (Cu, Mn, Zn, Cd, Ni and Pb), seven species of macroalgae (including red, green and browns) and two light levels (light or dark) in a multifactorial experiment. A complex range of responses were found with more than 140 significant interactions, 44 of which were specific to a particular species. Interestingly the presence of light was found to change the interactions of some metals. They concluded that when analysing algal material for copper, zinc and manganese concentrations and using these as indications of seawater concentrations, corrections due to interactions were optional, since errors were likely to be small. However when considering nickel and cadmium, corrections were essential since estimates of contamination could be out by as much as 400%.

Interactions have also been inferred from field based studies. For example, Morris & Bale (1975) found reduced uptake of manganese in *Ascophyllum nodosum* and *Fucus vesiculosus* in the presence of copper, zinc and cadmium.

From the examples discussed above, it is clear that interactions can vary from species to species and metal to metal, and may be dependant upon other environmental factors such as light or nutrient levels. Most of the interactions found are considered to be caused by competition between metals for binding sites (or uptake sites) on the cell surface (Wang *et al.* 1995 and Brown & Depledge, 1998).

There is equally the possibility that metals can interact with other non-metal contaminants. Although little evidence exists for this possibility, Florence & Stauber (1986) did find that lipid soluble organic ligands could increase the toxicity of copper, suggesting that interactions with organic contaminants in the field could possibly occur.

#### *1.5.8 Seasonal variation*

Seasonal variations in the metal content of many species have been found to occur (Riget *et al.* 1995, Ronnberg *et al.* 1990, Fuge & James 1974, Bryan & Hummerstone 1973 and Phillips 1994). This is usually attributed to the dilution effects caused by seasonal growth patterns. Dilution occurs during periods of fast growth and concentration increases during periods of slow growth. In some cases, seasonal variation may reflect differences in metabolic processes (Eide *et al.*, 1980) or even temporal changes in metal concentrations dissolved in the water (Knauer & Martin 1973 and Langston 1984).

#### *1.5.9 Allometry – dilution of accumulated metal by growth*

The concentration of a metal in the algal thallus is usually expressed on a dry weight basis. Any factor that influences growth rate could potentially alter the concentration of metal in the tissue. Growth is a non-specific physiological parameter that is known to be altered by many biotic and abiotic factors, including exposure to elevated levels of trace metals. This has significant implications for the potential use of a particular species as a biomonitor of trace metal contamination. Differences in environmental factors such as light levels, temperature or nutrient levels may alter concentration factors, independently of trace metal levels. Rice & Lapointe (1981) measured the effects of nutrient and light levels and found that the latter exerted the greatest overall influences on

metal uptake and growth. Uptake of cadmium and rubidium decreased as the specific growth rate decreased, whereas, uptake of manganese increased with increasing growth rates. When this uptake data was expressed as biomass-specific uptake rate (i.e.  $\mu\text{g}$  uptake of the metal per gram biomass per day) it was found that the specific uptake rate of rubidium and cadmium was constant (Rice, 1984). Thus, cadmium and rubidium uptake could simply be explained by the differences in exposure times of tissue of different ages. In contrast specific uptake rates of manganese increased with increasing growth rate, indicating that increased incorporation of the metal must occur at higher growth rates.

There is no reason to expect that the specific requirements and tolerances of different species should be the same for any substance in their environment (Rice & Lapointe, 1981).

Another consideration is the age of the tissue, since younger sections are often more actively growing and have been present in the environment for less time. This can also lead to differences in metal levels within a single individual (Eide *et al.*, 1980). For example, zinc, lead and copper concentrations have been found to be higher in older regions of the brown algae *Ascophyllum nodosum* in comparison with younger tips (Myklestad *et al.*, 1978).

Equally, if growth can recover following exposure to elevated levels of a particular metal, then dilution effects may occur during the recovery phase. This has particular relevance because the metal levels in an estuarine environment can fluctuate considerably over short time periods.

Since it appears that the concentration of a metal in a given plant can be a function of both growth rate and metal bioavailability, (both of which may vary with time) then all

environmental parameters which influence growth may also influence the results of algal biomonitoring surveys.

#### *1.5.10 Summary of factors affecting bioaccumulation of metals in seaweeds*

The rational basis for biomonitoring using seaweeds must be questioned. Too many factors other than the bioavailability of the metal can clearly alter metal concentrations in macroalgae to be able to consider them quantitative indicators of contamination without a greater understanding of the underlying mechanisms. In summarising the factors affecting accumulation in macroalgae it can be seen that:

- ◆ A positive relationship has been found between the concentration of some metals in seawater and macroalgae in laboratory based studies. However, accumulation can vary significantly depending upon the species and the metal(s) in question and does not always reflect the contamination in the environment.
- ◆ Exposure to elevated levels of metals in macroalgae may result in increased tolerance and this tolerance may have a genetic basis. The accumulation of metals can be affected by increased tolerance.
- ◆ Metal concentrations in the seaweed can be related to growth rates, which are known to be affected by numerous abiotic and biotic factors, including metal bioavailability.
- ◆ Interactions may occur between metals, and possibly other contaminants resulting in alteration of metal accumulation.
- ◆ Little information exists concerning the physiological effects of trace metals on macroalgae. A greater understanding of these processes will help lead to a better understanding of the mechanisms of trace metal toxicity and tolerance.

It must therefore be concluded that simply measuring the levels of trace metals in a particular species may give some basic quasi-quantitative measure of contamination. However, knowledge of the accumulation strategy and the mechanisms involved enables a better understanding of the significance of a particular metal concentration. This not only provides a better understanding of the ecotoxicological effects of the metal but also could help improve the usefulness of the species as a biomonitor.

### **1.6 Active versus passive biomonitoring**

The majority of biomonitoring studies to date have involved sampling and analysis of individuals from their natural (if possibly contaminated) environment. This method has been termed 'passive biomonitoring' (Chaphekar, 1991). While passive biomonitoring does have the advantage of being relatively simple and quick, the factors discussed above illustrate that many confounding factors could potentially reduce its usefulness. An alternative that is considered much more beneficial and informative is 'active biomonitoring', the introduction of individuals for a defined period of time into a polluted environment (Lovett Doust *et al.* 1994 and de Kock & Kramer 1994).

Active biomonitoring offers several important advantages over passive biomonitoring:

- ◆ The period of time that the individuals are exposed for is known and can be manipulated depending upon the objectives of the particular study.
- ◆ Comparisons can be made between native and introduced individuals. This may indicate possible differences in tolerance that have developed.

- ◆ Similarly, reciprocal transplants can be made between sites thought to differ in contamination, thus providing further useful information on the rates of uptake, or release, of metals.
- ◆ Monitoring stations may be selected, independent of the natural (non) occurrence of the species being used.
- ◆ Resolution of statistical tests can be optimised by using similar groups of organisms.

Species used in transplants include; molluscs (see de Kock & Kramer 1994 and Perryman 1996), Gastropods (Bryan *et al.* 1983), Mosses (Evans & Hutchinson, 1995), and macroalgae (Mykkestad *et al.* 1978, Ho 1984 and Wilkinson *et al.* 1992).

Ho (1984) transplanted two species of macroalgae (*Fucus vesiculosus* and *Ascophyllum nodosum*) from a relatively clean site to a site known to be highly contaminated with metals and monitored the levels of zinc and copper for 60 days. The levels of both metals were found to increase very quickly. However, the rate of increase was different for each species with *F. vesiculosus* apparently accumulating the metals faster. A similar result was found by Langston (1984). It has been suggested that these differences in time integration in the two species may be due to differences in growth (Phillips, 1994).

Refinements of the technique, such as the use of genetically identical clones, can be made. For example, Stebbing *et al.* (1983) measured the responses of genetically identical clones of the hydroid *Campanularia flexuosa* to study the metal concentrations in the river Tamar. Using genetically identical clones helped eliminate inter-individual variability as a source of variation in the data analysis. Although genetically identical clones can often be cultured in many species, this advantage does not appear to have been fully explored.

Many species of macroalgae offer a similar but simpler method of reducing unwanted variation and increasing the statistical power of a transplant experiment – by splitting individuals and transplanting sections (ramets) of individuals to various sites. This can also reduce the number of samples required.

Active biomonitoring is seen as a more informative method of estimating contamination, with considerable advantages over passive biomonitoring. Macroalgae are seen as suitable organisms for active biomonitoring studies.

### **1.7 A need to link field and laboratory based studies**

Passive biomonitoring studies reveal little or no information about the mechanisms of toxicity of a particular pollutant. Active biomonitoring techniques may provide information about the mechanisms of metal uptake and regulation and possibly highlight differences in tolerance that may have developed between populations. Laboratory based investigations are however considered an essential requirement for furthering the understanding of the mechanisms of trace metal toxicity and tolerance. This has prompted a plea in recent years for laboratory based experiments to compliment field studies (Rainbow *et al.* 1990, Depledge 1990a and Forbes & Depledge 1992). This it is argued will place the experimenter in a better position to interpret the significance of the field data.

One further advantage of investigating the physiological and mechanistic effects of a toxicant is that it may indicate a potential biomarker of exposure. A 'biomarker' has been defined as a molecular or cellular early warning distress signal of contamination (Moore, 1993) which can provide a measure of either exposure and possibly of effect (Peakall, 1994). The usefulness of a biomarker will largely depend upon its specificity. A better

understanding of the various effects of particular metals is considered an essential step in developing a metal specific biomarker of exposure.

### **1.8 The use of lower levels of contaminants in ecotoxicological studies**

Numerous toxicological studies have clearly shown that elevated levels of many trace metals have deleterious effects upon many marine organisms. These laboratory based toxicological experiments were designed primarily to provide guidelines for regulatory bodies concerned with the effect of chemicals on humans. More recently, emphasis has shifted to the ecological impact of pollutants in the natural marine environment. Ecotoxicology is gaining more importance, as the need to extrapolate from the laboratory to the field becomes more apparent. Many toxicological studies have used excessively high levels of pollutants (Munda & Hudnik 1986 and 1988, Plotz 1991 and Zolotukhina *et al.* 1991). It can be argued that these levels bear little or no relevance to those experienced in the field and provide little or no valuable information on the ecological impact a particular contaminant. Nevertheless, short term, toxicity studies can provide useful information on the physiological effect of a pollutant. By assessing the effect of lower, more realistic, levels of contamination in the laboratory, one can try to gain a better indication of the likely response of the organism in the field.

### **1.9 Physiological effects of copper toxicity on algae**

Copper is an essential trace metal required for normal cellular function. It has however, been shown to be a particularly toxic metal to plants and algae when present at elevated levels (Rai *et al.*, 1981). Because of this, the toxic effect of this metal on many species of plants and algae has received considerable attention. The most commonly reported effect of copper on algae is the reduction of growth (Fernandes & Henriques,

1991). Moreover, the presence of a toxicant, such as copper, may also induce a range of physiological responses in an organism (Fernandes & Henriques, 1991). Copper toxicity has also been shown to reduce photosynthesis (Stauber & Florence 1987, Cid *et al.* 1995 and Vavilin *et al.* 1995), as well as decreasing chlorophyll *a* content and the rate of cellular division (Rijstenbil *et al.*, 1994a).

The most detailed studies investigating the physiological effects of copper in algae have involved the use of microalgae species (see reviews by Rai *et al.* 1981 and Fernandes & Henriques 1991). As well as reducing growth, copper has been reported to interfere with cell permeability and the uptake of other metals (Sunda & Huntsman, 1983). Following entry into the cell, copper has been shown to react with –SH enzyme groups and thiols, thus inhibiting enzyme function (Stauber & Florence, 1986). In the complexed form Cu(I) can react with H<sub>2</sub>O<sub>2</sub> forming damaging oxygen free radicals (Florence *et al.*, 1985).

Physiological responses to elevated cellular copper concentrations may include the production of enzymes for metabolising and detoxifying the metal (Lovett Doust *et al.* 1994, Lage *et al.* 1994 and 1996). Lage *et al.* (1996) provided evidence for this by looking at changes in polypeptides profiles in the marine dinoflagellate *Prorocentrum micans* following exposure to elevated levels of copper exposure. They found significant changes including the induction of a new 25kda protein in the copper treated cells.

Further information about the physiological effects of copper has been gained from assessing differences in response to exposure between tolerant and non-tolerant strains. Tolerance mechanisms found to occur in microalgae include; accumulation of copper in polyphosphate bodies, storage in membrane bound vesicles, chelation by phytochelatin and exudation by organic compounds (Lage *et al.*, 1996). Hall *et al.* (1979) found that copper-tolerant strains of the brown seaweed *Ectocarpus siliculosus* excluded excess

copper better than non-tolerant ones, with evidence that this exclusion mechanism was membrane based. Reed & Moffat (1983) found evidence of an internal detoxification mechanism in a copper tolerant strain of *Enteromorpha compressa*.

Despite numerous field studies investigating the ability of seaweeds to accumulate trace metals, little information exists on the physiological effects of copper. For example, Berail *et al.* (1991) found that high copper concentrations ( $500 \mu\text{g L}^{-1}$ ) resulted in copper being found in all protein fractions of *Cystoseira barbata*, which resulted in the loss of enzyme function. Copper has also been reported to reduce photosynthesis in the macroalgae, *Enteromorpha compressa* (Reed & Moffat, 1983) and *Fucus vesiculosus* (Plotz, 1991).

#### **1.10 Localisation of metals within the cells**

The localisation of the metal within the thallus has been investigated to help gain insight into the effects of copper. The exact location may depend upon the species and the metal in question. For example, many authors have noted a high affinity of trace metals for algal polysaccharides such as alginate, agar and carrageenan (Phillips, 1994), which are common components in the cell walls of many species of brown and red macroalgae including gracilarioids. Johnson (1987) concluded that the majority of bound chromium was on the plant surface, while others have found the metals localised within the cells. For example, McLean & Williamson (1977) found that cadmium was localised within the nuclei of the red macrophyte *Porphyra umbilicalis*, while in fucoids, the majority of metals were found to be principally located intra-cellularly, associated with polyphenols (Ragan *et al.*, 1979).

X-ray microanalysis techniques (XMA) may be used to indicate sites of intra or extra cellular metal localisation, which can reveal important information about the mechanisms of metal accumulation and detoxification. XMA investigations have been used to reveal the chemical binding and compartmentalisation that marine invertebrates use to detoxify metals (Nott, 1991) as well as the localisation of metals within higher plants (Lichtenberger & Neumann, 1997)

In reviewing the information available for marine invertebrates, Nott (1991) found that there may be considerable inter-species variation in the processes involved. This also appears to be the case in algae. Qureshi & Stokes (1985) used XMA to investigate the mechanism of metal tolerance in the green alga *Scenedesmus* sp. They found that one species showed predominant intranuclear inclusions of copper while another showed cytoplasmic deposits when exposed to copper in the laboratory. No involvement of the cell wall in metal detoxification could be found. Holmes *et al.* (1991) found that high concentrations of zinc were associated with epiphytic bacteria on the thallus surface of *Gracilaria sordida* following exposure in laboratory studies. They concluded that the presence or absence of such bacteria could have significant implications for the concentration of the metal by the alga.

### **1.11 Aims**

This thesis investigates the effects of copper on the growth and physiology of several populations of the rhodophyte *Gracilariopsis longissima* from sites varying in trace metal contamination. Comparisons are also made of the accumulation and regulation of copper by different populations of this species, both in the field, using active biomonitoring techniques, and in the laboratory.

Emphasis was placed on assessing the physiological effects of environmentally relevant levels of copper exposure in different populations of *G. longissima* using a range of test end points including, growth, photosynthesis, respiration and membrane permeability. Investigations are also conducted into the accumulation and loss of copper during and after an exposure event. These laboratory based investigations are complimented with a series field studies in which the active biomonitoring approach is used. The information gained will further the understanding of the physiological effects of copper toxicity and accumulation in *G. longissima* and this will enable more informed appraisal of the appropriateness and usefulness of this species as a biomonitor of trace metal pollution.

#### **1.12 Reason for choosing the species**

In addition to the advantages of using seaweeds in general, *G. longissima* was selected for this study for the following reasons:

- ◆ Relatively little information exists on the use of red macroalgae as biomonitors of metal contamination.
- ◆ The genus is found in the estuarine environment in temperate waters worldwide.
- ◆ Information already exists on growth and physiology of the genus because of the commercial interest for agar production.
- ◆ The ramets from individuals can be vegetatively propagated which meant that genetically identical clones could be used in active biomonitoring studies.

### **1.13 Reasons for focusing on copper**

Copper was selected as the metal to investigate for three main reasons:

- ◆ Elevated levels of copper are generally considered particularly toxic to plants and algae (Bryan 1976, Rai *et al.* 1981 and Fernandes & Henriques 1991).
- ◆ Copper is one of the main contaminants of the study site - the Fal Estuary, Cornwall, which is the most trace metal contaminated estuary in the UK (Bryan & Gibbs, 1983).
- ◆ There is a large database available on the physiological effects of copper on microalgae to refer to. Information on macroalgae is far less.

## **Chapter 2**

# **SITE AND SPECIES DESCRIPTION, GENERAL MATERIALS AND METHODS**

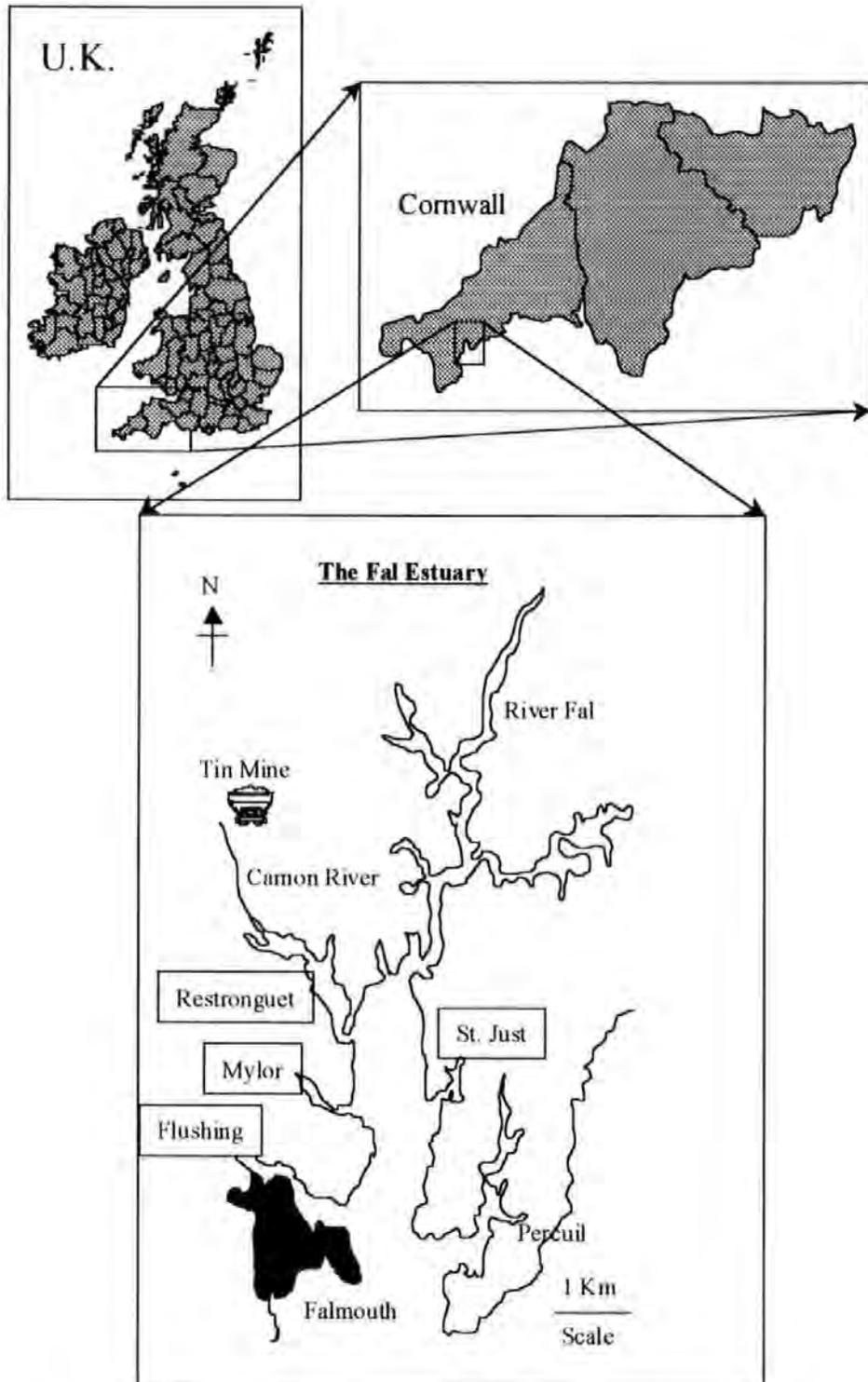
## 2.1 Introduction

### 2.1.1 General site description – The Fal Estuary

The area surrounding the Fal Estuary in Cornwall, south-west England, has a history of trace metal mining spanning over two hundred years and as a result is considered the most contaminated estuary within the UK (Bryan & Gibbs, 1983). Although there is currently no commercial mining activity within the area, a legacy of trace metal contaminated sediment remains. Mining activity was concentrated on the west side of the estuary with the main source of metal contamination entering Restronguet Creek. Water containing elevated levels of some metals still enters the estuary from the recently closed (in 1991) Wheal Jane tin mine; metal rich floodwater from the mineshafts leaks into the tailing dam which drains into the Carnon River, and enters the estuary via Restronguet Creek (see Fig 2.1).

The estuary is contaminated by high concentrations of metals including copper, zinc and arsenic (Bryan & Gibbs 1983 and Somerfield *et al.* 1994). Copper is considered to have the largest impact upon the flora and fauna within the estuary although zinc is also considered to occur at toxic levels (Austen & Somerfield, 1997). The freshwater entering the estuary from Restronguet Creek has been reported to contain  $550\mu\text{g L}^{-1}$  soluble copper and  $660\mu\text{g L}^{-1}$  total copper (Bryan & Hummerstone, 1973) and is acidic, pH 4 (Perryman, 1996). The concentration of metals in water samples is largely dependent upon the salinity and pH. As the freshwater from Restronguet Creek mixes with the seawater, the pH and salinity rapidly rises. This results in the precipitation and deposition of the majority of the metals from the water to the sediment within Restronguet Creek. Despite this precipitation, the level of copper in the water at the estuarine end of Restronguet Creek is still high and has been reported as  $11\mu\text{g L}^{-1}$  of total soluble copper (Bryan & Hummerstone, 1973). High

levels of copper have also been recorded in water samples from Restronguet Creek more recently which indicate that similar conditions still exist within the creek (Bryan & Gibbs 1983 and Hubert 1995).



**Fig. 2.1.** Map showing the location of the Fal Estuary and the creeks within it and the Wheal Jane tin mine from which trace metals still enter the estuary today.

The estuary has been the focus of numerous studies on the effects of elevated levels of metals on the flora and fauna living within it (e.g. Bryan & Gibbs 1983, Bryan & Langston 1992, Somerfield *et al.* 1994 and Perryman 1996). From the analysis of sediment, water and marine organisms it has been established that there is a considerable trace metal gradient within the estuary (Bryan & Hummerstone 1973, Bryan & Gibbs 1983 and Perryman 1996). A copper gradient exists in surface sediments taken from within the estuary (Bryan & Gibbs, 1983). The most contaminated site within the estuary is Restronguet Creek, where levels of copper in the sediment can exceed  $2000\mu\text{g g}^{-1}$ . Mylor Creek is the second most contaminated site ( $1117\mu\text{g g}^{-1}$ ), followed by St Just ( $356\mu\text{g g}^{-1}$ ) and Flushing ( $322\mu\text{g g}^{-1}$ ) with Percuil ( $<150\mu\text{g g}^{-1}$ ) being the least contaminated site (Fig 2.1). Non-contaminated estuaries, by comparison, contain sediment levels of copper of about  $10\mu\text{g g}^{-1}$  (Bryan & Langston, 1992). A similar copper gradient has also been found in the shellfish, *Mytilus edulis* (Perryman, 1996), the polychaete *Nephtys hombergi* (Williams, 1994) and the brown macroalga *Fucus vesiculosus* (Bryan & Hummerstone, 1973). The meiofaunal communities within the estuary also appear to reflect this metal gradient (Somerfield *et al.*, 1994).

No other major forms of contamination are thought to occur in the estuary (Somerfield *et al.*, 1994) and other environmental variables such as salinity and pH are relatively uniform between creeks (Perryman, 1996).

Because of the gradient of trace metal contamination in the Fal Estuary, supported by the information available from previous studies of the area it was considered the most appropriate study site. Population differences in metal tolerance, if they exist, were thought to be most likely found between different populations from within the gradient in the Fal Estuary, or between populations from the Fal Estuary and other clean estuaries in the south west of England.

## 2.2 The study organism – *Gracilariopsis longissima*

### 2.2.1 Taxonomy of the species

The genus *Gracilaria* is comprised of over 100 species and is widely distributed in temperate waters worldwide. Globally, the genus is of considerable economic importance as a source of the phycocolloid, agar (Jensen, 1979). Agar extraction from the genus accounts for more than half of the world's agar production, an industry worth in excess of US\$200 million per annum (Critchley, 1993). In addition, many species have been used directly as a vegetable for human consumption and incorporated in animal feed. The majority of raw material is harvested from natural standing crops, although over-harvesting and increased demand has led to the development of mass culturing techniques (Critchley, 1993). Most recently, interest has focused on their potential to provide specific chemicals for the pharmaceutical industry.

Despite industrial interest, the taxonomy of the family Gracilariaceae (Gracilariales, Rhodophyta) can, at best, be described as problematic, with considerable debate as to the correct identification and nomenclature of many species (Bird & Rice, 1990). Much of the confusion can be attributed to extreme morphological polymorphism within the genus. Since industry is often interested in specific characteristics of a phycocolloid, which can vary from species to species, it is not surprising that there has been considerable effort to address the taxonomic anomalies. Many attempts have been made to reassess the taxonomy of the gracilarioids (Bird, 1995). Traditional approaches such as comparing morphological and reproductive differences have been supplemented with more modern methods such as biochemical, genetic, and molecular biological comparisons. The current taxonomy in the British Isles is still under review.

During the initial phase of the current study, the species *Gracilaria verrucosa* was proposed as the study organism. However, an extensive taxonomic review of this species by Steentoft *et al.* (1995), resulted in the species being split into two. Highlighting the considerable confusion that exists within the genus and detailing an extensive survey of morphological characters, they rejected the name *G. verrucosa* in favour of two novel ones, encompassing two genera, *Gracilaria gracilis* and *Gracilariopsis longissima*. In the light of the confusion, the relevance of much previously reported experimental data on *Gracilaria verrucosa* must be questioned, as it is often impossible to distinguish from the literature which of the two species researchers have used (Steentoft *et al.*, 1995).

### 2.2.2 *Development of methods and optimisation of culture conditions*

Difficulties encountered during initial attempts to culture *G. longissima* indicated that many of the reported successful culture methods for *Gracilaria verrucosa* were inappropriate for *G. longissima*. The aim of this section is therefore to describe the development of the methods for culturing and measuring growth in the species for experimental purposes.

### 2.2.3 *Measurement of growth*

The measurement of growth impairment is a common end point in toxicological studies (Lovett Doust *et al.*, 1994). It is also considered to be one of the most convenient methods available (Fletcher, 1991), although it represents the integrated response of numerous abiotic and biotic factors (Lovett Doust *et al.*, 1994). Methods employed to measure growth in macroalgae vary considerably depending upon the species, the method of culture and the purpose of the study. Typically, they involve measuring an increase in length or surface area of excised sections or discs of thallus, measuring fresh weight

(following blotting to remove excess water), dry weight or even changes in volume (Fletcher, 1994).

For seaweeds with apical growth, excised apical tips are frequently used. Much use has been made of using growing tips of algae with an apical mode of growth (Fletcher, 1994). Calibrated ocular micrometers (Russell, 1963) and even laser diffraction techniques (Stromgren, 1975) have been used to measure growing apical tips, although these techniques can be impractical and time consuming. In this study, it was desirable to be able to measure the growth of small sections of tissue (such as apical tips and fragments of individuals) accurately and quickly, without contaminating samples or damaging material. For this purpose, the use of an image analyser to measure changes in various growth-related parameters was tested.

A method for acquiring and processing images of portions of algal material using a Quantimet Image analysis system was developed and compared with the more traditional method of measuring fresh weight.

As well as developing the most suitable method for measuring growth, it was also necessary to standardise, as much as possible, the material being used for experimentation. Space restrictions meant it was impractical to use whole plants in experiments, therefore the type and amount of tissue used needed to be determined. Large sections (~1g) of thallus have been grown (in large glass flasks with continuous aeration) using other gracilariod species (Dawes, 1994). This method was attempted with limited success and a new miniaturised method was adopted using polyethylene petri dishes (Rueness & Tananger, 1984). Petri dishes were considered to have several specific advantages: they require less algal material, media, and cabinet space, aeration is not necessary (providing

the tissue to volume ratio is kept low) due to the large surface area of the dishes, they are sterile and relatively cheap.

The morphology of *G. longissima* plants is relatively simple (Fig 2.2). Each individual may contain hundreds of apical tips. This permits either excised apical tips or sections of thallus to be used for culturing. The growth characteristics of both tissue types were investigated to assess which would be the most appropriate for subsequent experimental work.



**Fig. 2.2** Typical morphology of *Gracilariopsis longissima*

#### 2.2.4 *Optimising the growth conditions within the controlled environment cabinet*

In addition to developing the most appropriate method for measuring growth of material, experiments were performed to assess the optimal conditions for growth within controlled environment cabinets (CEC), which were to be used throughout for copper exposure experiments.

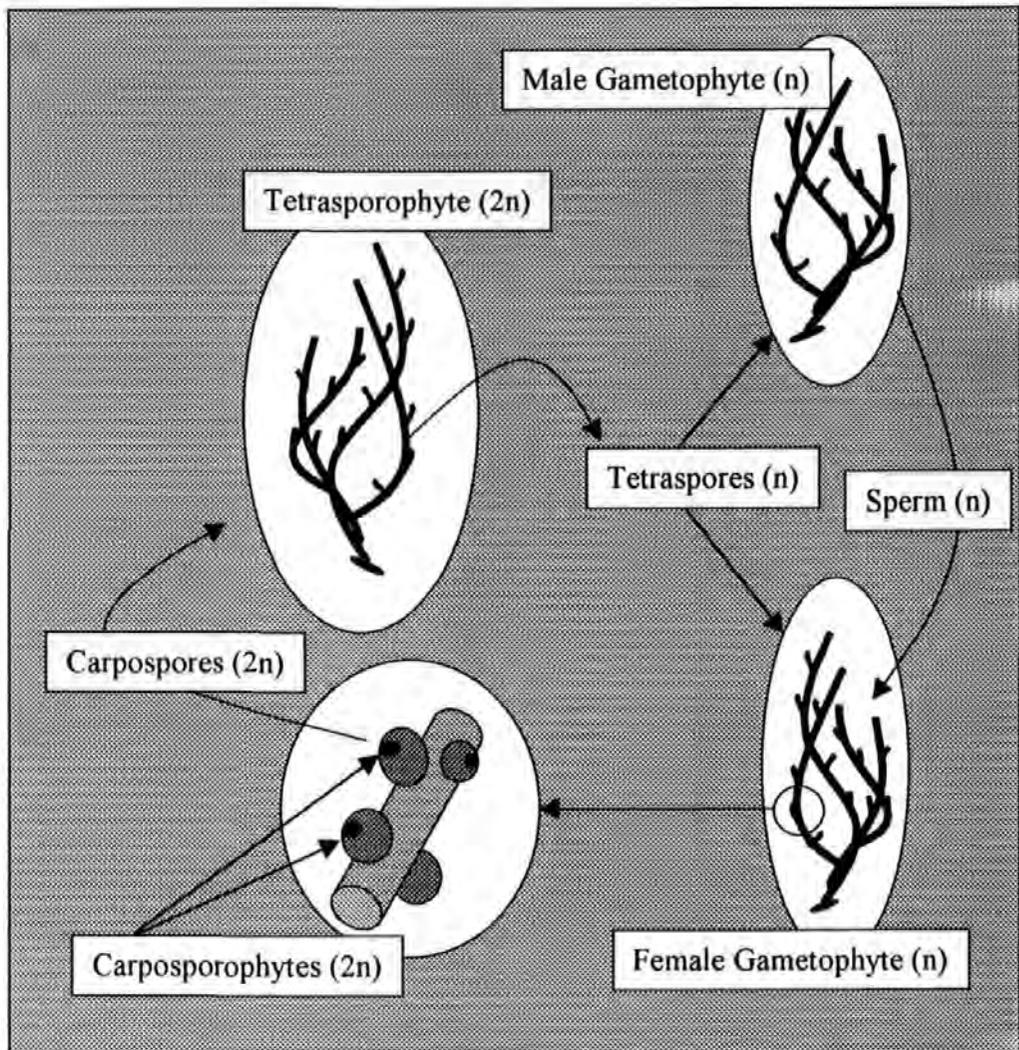
Growth can be considered to be the integrated response of numerous physiological and biochemical processes (Lovett Doust *et al.*, 1993). As such, many variables are known to affect the growth and metabolism of seaweeds (see Lobban & Harrison, 1994). It was therefore desirable to replicate normal growth conditions as much as possible, while at the same time minimising the influence of other environmental variables. The controlled environment chambers (Sanyo MLR-350HT) have the facilities to program several environmental factors: temperature, irradiance levels, humidity and day-length. The effect, of varying temperature and light on the growth of *G. longissima* were investigated. Both factors have been previously reported to significantly affect growth and metabolism in macroalgal species including *Gracilaria sp.* (Lapointe *et al.* 1984, Lignell *et al.* 1987 and Friedlander *et al.* 1993).

#### 2.2.5 *Determination of life cycle phases*

*Gracilariopsis longissima* has a relatively complex triphasic life cycle, with three isomorphic phases and two ploidy levels (see Fig. 2.3). Depending upon the reproductive status of individuals, it is possible to determine their sex and ploidy level. When reproductive, female gametophytes (n) are distinguishable by eye because of the presence of carposporophytes (Fig. 2.3). Tetrasporophytes (2n) can be identified under a binocular-dissecting microscope by the presence of tetrads of spores covering the thallus, forming

thousands of dark spots. Male gametophyte tissue is considerably harder to identify, requiring histological preparation, staining and sectioning of samples followed by the use of a compound microscope (Steentoft *et al.*, 1995). When tissue is not in a reproductive state there is no reported method of sexing it (pers. comm. C. Destombe).

Opinion has been divided as to whether or not ecological differences should be expected between isomorphic haplo-diploid phases. The maintenance of a haplo-diploid life cycle is hard to explain (Valero *et al.*, 1992) and it has been suggested that differences might be expected between the growth of different phases (Dyck & de Wreede, 1995). In practice, some differences between isomorphic phases have been found in some species of red algae (Destombe *et al.* 1993 and Dyck & de Wreede 1995), while in others studies no differences have been found between phases (Zhang & van der Meer 1987 and Hannach & Santelices 1985). Growth responses of gametophytic and tetrasporophytic tissue of *G. longissima* to a range of irradiances and temperatures were investigated.



**Fig. 2.3.** Typical life cycle of a gracilarioid species such as *Gracilariopsis longissima*. This incorporates three isomorphic stages, the male and female gametophytes, both haploid (n) and the diploid (2n) tetrasporophyte stage. Sperm from the male gametophyte fuse with the female gametophyte to form diploid carposporophytes (or cystocarps). The carposporophyte produces thousands of diploid carpospores through mitotic amplification from a single fusion event. These carpospores form into tetrasporophytes which when mature release tetrads of haploid tetraspores which develop into the male and female gametophytes completing the life cycle.

## 2.3 General methods

### 2.3.1 Field Sampling

Because of the habitat of *G. longissima* (always submersed and usually found on or below the mean low water mark), it was only possible to search for and sample populations on spring lows tides, approximately once monthly. All individuals collected were taken from submerged populations sampled at the low water line, within one hour of a spring low tide. Individuals were placed in clean plastic bags containing seawater and returned to the laboratory within 4 hours of sampling. On return, samples were cleaned of all visible epiphytes, rinsed in filtered seawater, and placed in stock cultures prior to experimentation.

### 2.3.2 Species identification

According to Steentoft *et al.* (1995) *Gracilariopsis longissima* is usually to be found in estuaries attached to small stones or shells where as *Gracilaria gracilis* is typically found attached to bedrock along coastlines. However, the two species can sometimes be found at the same site. To avoid any possible confusion over the identification of samples, specimens of each population were checked to confirm that they were *G. longissima* by using visual and textural tests (pers. comm. M. Steentoft). In addition, DNA from specimens of both species was compared using a molecular RAPD test. Comparison of the DNA banding patterns in the two species indicated that the two species are genetically distinct (pers. comm. C. Destombe).

### 2.3.3 *Stock culture conditions*

Stock cultures were held in 15L clear plastic tanks containing 8-10L of aerated filtered natural seawater. Biomass density was maintained at approximately  $8\text{g L}^{-1}$ . Media was supplemented with nitrate and phosphate ( $100\mu\text{M NaNO}_3$ ,  $10\mu\text{M Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) and changed weekly. The covered tanks were kept in a constant temperature room at approximately  $15^\circ\text{C}$ , under ambient light levels ( $30\text{-}40\mu\text{mol m}^{-2}\text{ s}^{-1}$  PAR) as provided by a mix of warm white and cool white fluorescent tubes).

### 2.3.4 *Standard experimental culture conditions*

Unless stated otherwise all experiments were carried out in Sanyo CECs (Sanyo MLR-350/HT) under the following environmental conditions:  $15^\circ\text{C}$ , 90% relative humidity, 12:12 light:dark as supplied by cool white fluorescent tubes at a irradiance level of  $\sim 30\mu\text{mol m}^{-2}\text{ s}^{-1}$  PAR as measured by a Quantimet PAR meter.

### 2.3.5 *Culture media for experiments*

During experimentation unless stated otherwise all algal material was cultured in natural seawater that was filtered ( $0.45\mu\text{m}$ ), microwave sterilised as per the method of Keller *et al.* (1988) and supplemented with nitrate and phosphate ( $100\mu\text{M NaNO}_3$ ,  $10\mu\text{M Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ).

### 2.3.6 *Pooling of samples*

A primary objective in this study was to investigate possible population differences in response to copper exposure. It was important to reduce as much as possible any

variation due to differences between individuals. This was especially important since sexing individuals was not always possible and the growth of different sexes may vary considerably. For this reason, apical tips (or sections) were excised and pooled from a number of individuals (typically about 10) from each population. Ten apical tips were then randomly selected from the pool of tips and placed in each petri dish. The mean response of the ten tips was then used as the data point for each replicate. To reduce the possibility of wound effects influencing the experiment the apical tips were normally excised and pooled in a beaker containing prepared media for at least 12 hours prior to the start of each experiment.

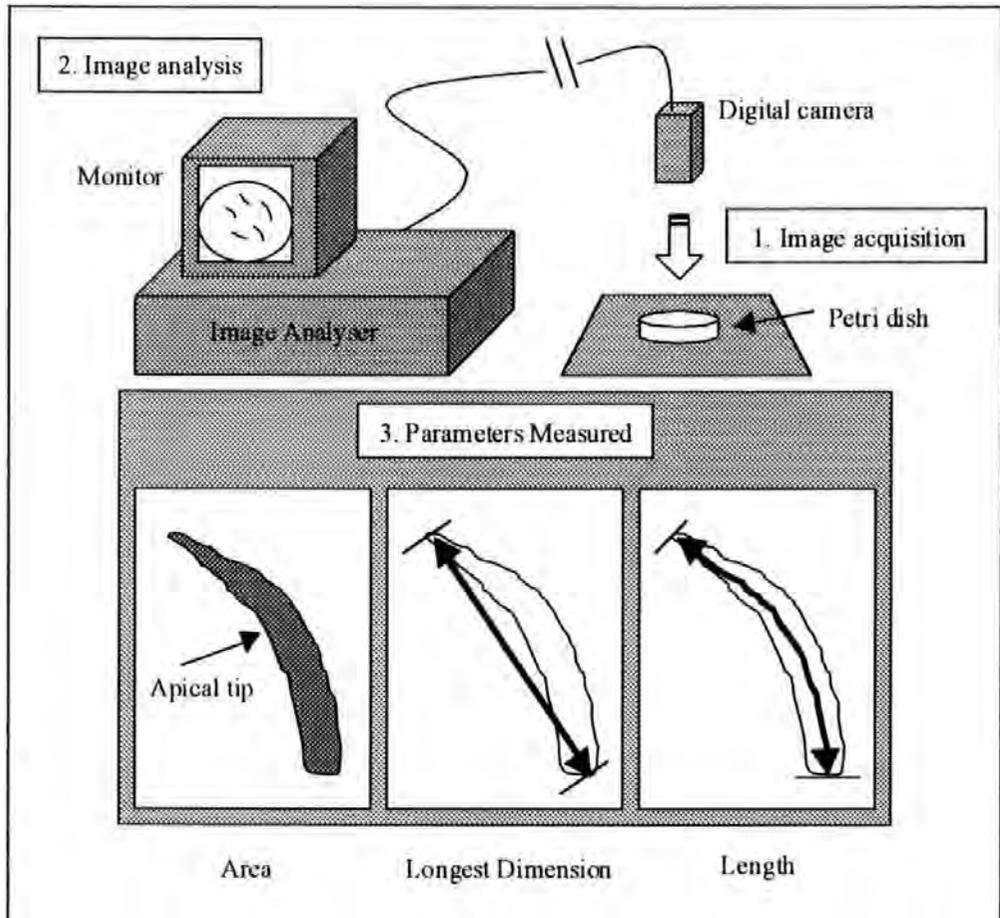
### 2.3.7 *Randomisation of replicates*

For each experiment, replicate dishes were laid out in fully randomised designs within the CEC to eliminate any possible effects from unknown environmental gradients within the cabinets.

### 2.3.8 *Image analysis*

A high contrast digital image of individual petri dishes containing apical tips was acquired using a light table and a 12.5 - 77mm zoom lens attached to a CCD video camera. This image was imported to a Quantimet image analyser where images were then calibrated, filtered and processed using a purpose written programme (see Appendix A for details). Using this method, parameters could be obtained simultaneously and compared between treatments (Fig. 2.4). Three main parameters were measured from each object, *total area*, *longest dimension* and *length* (Fig. 2.4). The *total area* is the area (in mm<sup>2</sup>) each object covered when lying horizontally in the dish while the *longest dimension* is the straight line distance (in mm) between the furthest two points on the object. The *length*

parameter was the total length (in mm) of an imaginary line drawn along the centre of the objects image. Any branching of the object would increase this parameter. This meant that the *length* of a curved apical tip could be significantly longer than its *longest dimension* (see Fig. 2.4)



**Fig.2.4.** Technique used to capture and process images of apical tips using the Quantimet image analyser. 1) Image capture using a light table and digital camera. 2) Image processing and filtering using the image analyser and associated software. 3) Parameters recorded for each algal section. Note that with a curved apical tip, the '*length*' will be longer than the '*longest dimension*'.

### 2.3.9 Relative growth rate calculations

Relative growth rates (RGR) of the tips in each dish were calculated using the formula (Hunt, 1982):

$$\text{RGR (\% d}^{-1}\text{)} = \frac{\text{Ln (L}_f\text{)} - \text{Ln (L}_i\text{)}}{d} * 100$$

Where  $L_f$  = the final length and  $L_i$  = the initial length of the sample in mm. The same formula was used to calculate growth using biomass data, substituting fresh weight for length.

### *2.3.10 Method for tissue digestion and measurement of copper content*

#### *2.3.10.1 Preparation for sample digestions*

Sample preparation: Frozen (-5°C) material was freeze-dried (Edwards, Super Modulyo). Following freeze drying, samples were weighed and digested in nitric acid using a microwave sample preparation system MDS-2000. This technique allows the use of two different digestion vessels depending upon the amount of material available, large digestion vessels (120ml) and small digestion vessels (7ml).

Where possible, sample containers used were plastic (polyethylene or Teflon). All containers were washed in detergent, rinsed in MilliQ 'ultrapure' water (Millipore), placed in an acid bath (2M HCl) for at least 24hrs and subsequently rinsed again in MilliQ water. All samples were handled with acid washed plastic tweezers and gloves were worn throughout. All reagents used were high grade, at least Analar and typically Aristar quality. Procedural blanks to check contamination levels were run with each digest and blanks were subtracted from samples.

#### *2.3.10.2 Large digestion vessels*

Approximately 0.2g of freeze dried material was added to 5ml of nitric acid (Aristar) and 0.5ml hydrogen peroxide (Aristar) in acid washed 120ml lined large digestion

vessels (LDVs) and pre-digested overnight. Samples were then run twice through the microwave in batches of 12 using the following program, allowing cooling between runs.

Step	Power (%)	Time (seconds)
1	20	200
2	40	200
3	60	130
4	80	100
5	100	15

Microwave digestion program for large vessels

### 2.3.10.3 *Small digestion vessels*

Approximately 70mg of tissue (FDW) was added to 2.5ml of nitric acid (Aristar) and pre-digested overnight. Samples were then microwaved twice (40 samples per run) using the following program, allowing cooling between runs.

Step	Power (%)	Time (seconds)
1	20	30
2	40	30
3	60	15
4	80	15
5	100	15

Microwave digestion program for small vessels

Following digestion, samples were rinsed out of the digestion vessels and made up to appropriate volumes with MilliQ water in acid washed volumetric flasks, then analysed for copper using atomic absorption spectrophotometry (AAS) in either flame or graphite furnace mode. Calibration and procedural blanks were run with each data set and the mean of the procedural blanks was subtracted from each sample. Copper standards were made using a BDH 'spectrosol' certified copper standard solution, acidified with nitric acid and made up with MilliQ water so that the standard contained the same concentration of acid as

the samples. This process, called 'matrix matching' minimises the possibility of introducing errors during calibration.

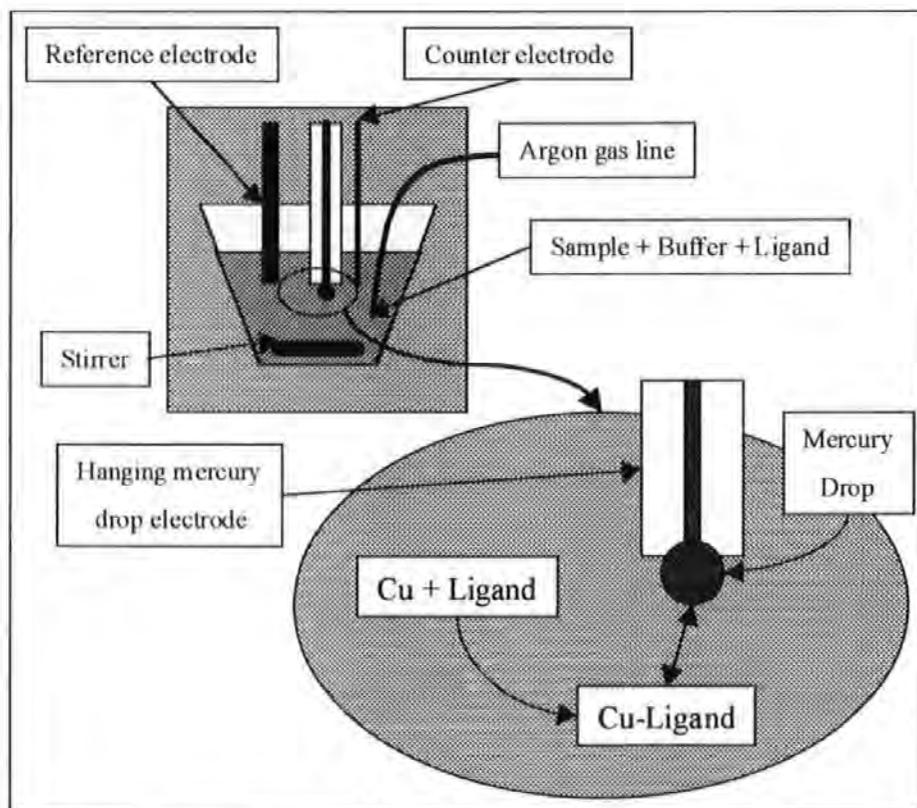
Contamination of samples during trace metal analysis can represent a serious problem. During sample preparation and analysis, extreme care was taken and 'clean' techniques were used throughout. The sample preparation and digestion methods used were tested comparing percentage extraction of copper with the reported values for the reference material *Ulva lactuca* (CRM # 279, Griepink & Muntau 1987). The technique was found to perform adequately with 104% ±12 (95% CI) recovery obtained.

### 2.3.11 Seawater analysis

The ambient concentrations of copper in oceanic seawater are very low. The mean Cu concentration in fully saline water (34 ppt) is reported to be 0.25 µg L<sup>-1</sup> (4nM) (Chester, 1990). In freshwater from the contaminated Restronguet Creek the copper concentration may be as high as 660 µg L<sup>-1</sup> dropping to around 11 µg L<sup>-1</sup> at the mouth of the creek (Bryan & Hummerstone, 1973). At intermediate creeks within the estuary, this concentration will be considerably lower. Since absolute levels are low, contamination of samples during collection and sampling can represent a serious problem.

The analysis of seawater samples for trace metals is technically difficult. Few analytical techniques available for measuring metal levels are sensitive enough to detect metal levels in seawater. Many methods, such as atomic absorption spectrophotometry, are subject to interferences caused by other ions such as sodium present at high concentrations in seawater (Churella & Copeland, 1978). The two main methods used for the analysis of trace metals in seawater samples are stripping voltammetry and solvent extraction.

Voltammetry is generally accepted to be the better method for measuring metal levels and their speciation in seawater samples (Romeo & Gnassi-Barelli, 1993).



**Fig. 2.5.** The principles of voltammetric analysis. See text for details.

The basic principle of cathodic stripping voltammetry (CSV) is shown in Fig. 2.5. During analysis a measured volume of the sample (typically 10ml) is placed into the clean glass cell and sealed to the voltammeter. The cell is then purged with an inert gas to remove oxygen (usually with nitrogen or argon gas). A metal complexing ligand is added to the solution that chelates the metal. The metal-ligand complex is then allowed to adsorb onto the mercury drop on the end of the electrode (cathode) for a defined period at a set potential. A potential scan is then made in a negative direction as the voltage is decreased at a constant rate. As the specific potential of each species of metal present is reached the metal will leave the electrode and a change in current can be measured relative to a reference electrode within the cell. Ligands of varying complexing strength and concentration can be added to the sample to enable the quantification of the different

fractions of the dissolved metal. Freshly collected samples are used to determine the amount of electrochemically labile copper using a relatively weak ligand which is used to estimate the amount of free  $\text{Cu}^{2+}$  and weakly bound copper in solution. The total amount of dissolved copper is usually determined by breaking down all of the natural organic ligands within the sample using UV irradiation.

By measuring the peak height for a particular metal and then making a standard addition of the same metal, the original amount of metal in the sample can be estimated from the resulting change in peak height, using the following calculations.

The change in current per nM of metal added in the standard addition is calculated as:

$$s (\mu\text{A nM}^{-1}) = (i_x - i_o) / c_a$$

where  $i_o$  ( $\mu\text{A}$ ) is the average initial peak height and  $i_x$  ( $\mu\text{A}$ ) is the average height of the peak following the standard addition of  $c_a$  nM of metal.

The reciprocal of  $s$  gives the amount of metal per change in current ( $\text{nM } \mu\text{A}^{-1}$ ). Multiplying this by  $i_o$  gives the amount of metal present in the initial sample in nM.

Seawater samples were collected from sites in acid washed HDPE 'Nalgene' bottles. Samples were filtered through  $0.45\mu\text{m}$  nitro-cellulose filters and refrigerated. Labile copper measurements were made within 24hrs of collection. Following the measurement of labile copper levels, samples were acidified ( $1\mu\text{L}$  of 50% Aristar HCl added per ml) and frozen, until analysed for total copper concentration. To measure total copper levels in samples, 30ml aliquots were placed in acid washed silica glass tubes and irradiated for 2.5hrs under a 1000W UV lamp to remove all natural organic ligands from the media. Total copper levels were then measured in the irradiated samples using CSV.

Water samples were analysed for labile copper using cathodic stripping voltammetry (CSV) using a EG&G Princeton Applied Research 384B polarographic analyser connected to a EG&G PARC 303 hanging mercury drop electrode (HMDE). Some samples were analysed using anodic stripping voltammetry ASV due to the limited availability of instruments. Samples were still collected and prepared in the same way as for CSV, and the majority of the principles of measurement remain the same. However, when using ASV metals were deposited on an anode and scans were made in the opposite direction (positive) to CSV scans. Both methods produce comparable results for copper analysis.

### 2.3.12 Statistical analysis

Data sets were analysed by appropriate statistical tests using the statistical software package 'STATSGRAPHICS Plus for Windows' (v2.1). Actual tests performed and any transformations made are stated with each analysis. All two-way Duncans multiple range tests were made using the statistical software package 'Teddybear' (Wilson, 1991). Tests for normality and variance checks were made prior to all parametric analyses. All errors are displayed graphically as 95% confidence intervals ( $\pm t_{(n-1)} \times \text{S.E.}$ ) since this was considered most appropriate for allowing comparisons between treatments.

## 2.4 Aims

The purpose of this series of preliminary experiments was to:

- ◆ Establish the best region of tissue to use for measuring growth (i.e. apical tips or thallus sections).
- ◆ Establish the best method of measuring growth of *G. longissima* material in a miniaturised culturing system.

- ◆ Optimise the environmental conditions for culturing *G. longissima*, within the range normally experienced in the field.
- ◆ Assess differences in growth between different isomorphic phases.

## **2.5 Experiment 1. Comparison of fresh weight and length as measures of growth**

### *2.5.1 Materials and methods*

*Gracilariopsis longissima* material was collected from the Fal Estuary, Cornwall. On return to the laboratory, individuals were cultured for one week in the laboratory. Subsequently, more than 120, 10mm tips were excised and pooled in a beaker. For each replicate ten tips were randomly selected, blotted dry in tissue paper then weighted (fresh weight). After weighing, the tips were placed into petri dishes containing 25ml of experimental culture media (described in the General Methods above). Each dish was then processed using image analysis.

The experimental design was comprised of four replicates of three treatments (control, 25 and 50 $\mu\text{g L}^{-1}$  copper) - 12 petri dishes in total. Dishes were placed in a CEC for two weeks under standard experimental culture conditions. After one week, all tips were remeasured using image analysis and blotted dry for fresh weight determinations. Material was then replaced into each dish and returned to the plant growth chamber for a further week. Image analysis and fresh weight determinations were repeated at the end of the second week.

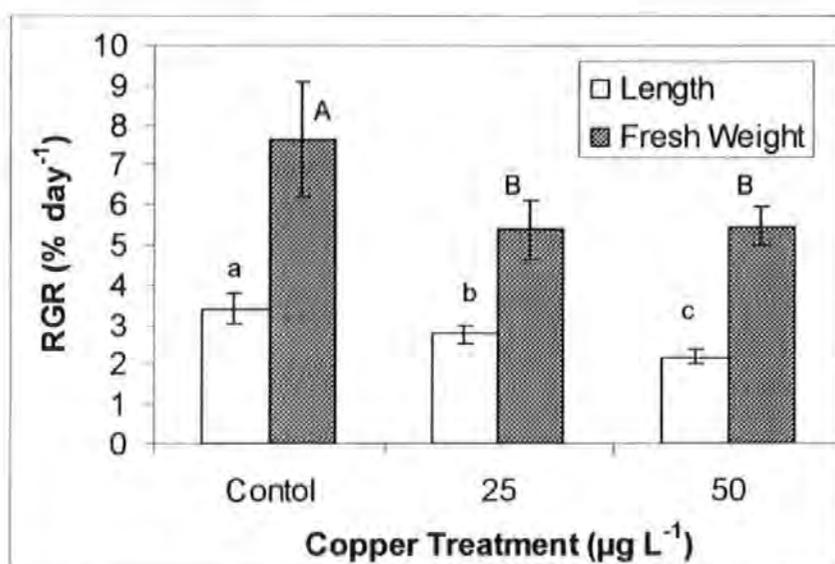
### *2.5.2 Results*

Length and fresh weight data sets were analysed using one way ANOVA (Table

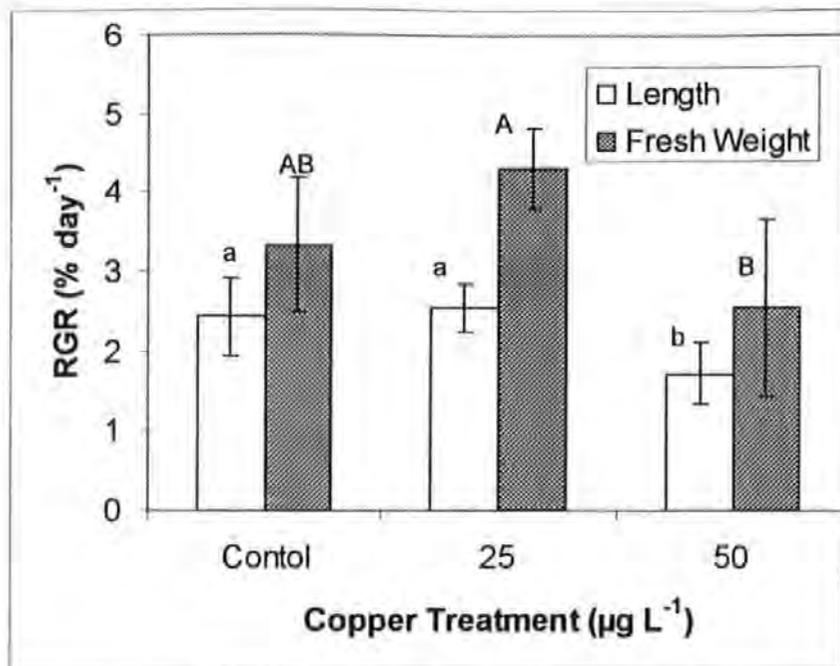
2.1). The addition of copper caused a significant reduction in RGR during the first week when measured as length ( $P=0.001$ ), and as fresh weight ( $P=0.0175$ ) (Fig.2.6). A Duncan's multiple range test found that all treatments, as measured by length, were significantly different from each other (Fig 2.6). Analysis of the fresh weight data found the control to be statistically different from the two copper treatments, but there was no statistical difference between the 25 and 50  $\mu\text{g L}^{-1}$  copper treatments (Fig. 2.6).

Length	Source	SS	DF	MS	F-ratio	p-value
(Week 1)	Between groups	3.018	2	1.509	20.12	0.001
	Within groups	0.675	9	0.075		
	Total (Corrected)	3.693	11			
Fresh weight	Between groups	13.041	2	6.520	6.560	0.018
(Week 1)	Within groups	8.946	9	0.994		
	Total (Corrected)	21.986	11			
Length	Between groups	1.571	2	0.786	4.610	0.042
(Week 2)	Within groups	1.535	9	0.171		
	Total (Corrected)	3.106	11			
Fresh weight	Between groups	6.231	2	3.116	4.050	0.056
(Week 2)	Within groups	6.917	9	0.769		
	Total (Corrected)	13.148	11			

**Table 2.1** One way ANOVA summaries for the two methods of measuring growth rate over the two weeks of culture.



**Fig. 2.6.** Mean ( $n=4$ ) RGR of *G. longissima* apical tips exposed to three treatments of copper for one week, calculated from the changes in length measured by image analysis and by fresh weight. Letters designate statistically significant groupings using a Duncan's multiple range test (lower case for length and upper case for fresh weight). Error bars show 95% confidence intervals.



**Fig. 2.7.** Mean ( $n=4$ ) RGR for the second week of apical tips exposed to three treatments of copper for two weeks, calculated from the changes in length measured by image analysis and by fresh weight. Letters designate statistically significant groupings using a Duncan's multiple range test (lower case for length and upper case for fresh weight). Error bars show 95% confidence intervals.

In the second week, growth was significantly affected as measured by length ( $P=0.042$ ). The Duncan's multiple range test found that there was no significant difference between control and  $25\mu\text{g L}^{-1}$  copper treatment but both were significantly different from the  $50\mu\text{g L}^{-1}$  copper treatment. The fresh weights were not significantly different at the 95% level ( $p=0.056$ ) although the trends appear the same for both fresh weight and length measurements (Fig. 2.7). The RGR of controls in the second week was lower than in the first week,  $\sim 70\%$  of the first week as measured by length and  $\sim 44\%$  as measured by fresh weight.

The fresh weight and length measurements for each dish were compared using RGRs from the two weeks. A significant ( $p<0.001$ ,  $R^2=63.49\%$ ) positive relationship was found between the two variables. The  $R^2$  explains 63.49% of the variation in the data indicating a moderately strong relationship between the two variables.

### 2.5.3 Discussion

Copper was found to significantly reduce the growth of apical tips, as estimated by length and fresh weight. The length of tips correlated reasonably well with fresh weight measurements. Considering the small amount of biomass, error was more likely in the measurement of fresh weight by blotting, than in measuring length using image analysis. The measurement of growth by fresh weight was considered to have some subjectivity since the weight will be affected by the length of time the apical tips have been removed from the media and the amount of blotting used. The handling of the apical tips was also considered likely to damage to the tips and increase the likelihood of cross contamination of the samples. By comparison, image analysis was found to be faster and less likely to contaminate or damage samples by handling. Because of these considerations, the change in length (as measured by apical tip elongation using image analysis) was adopted as the standard method for measuring growth in preference to measuring fresh weight.

Material in the control and  $25\mu\text{g L}^{-1}$  copper treatments grew similarly during the second week of the experiment but the RGR of the controls was much lower than during the first week. This was probably because the media was not changed during the experiment and so other factors such as nutrient limitation may have been limiting growth. It was therefore decided that in future experiments, responses would be measured after one week of copper exposure. In longer experiments, the culture media would be changed at least once a week to avoid nutrient limitation.

## **2.6 Experiment 2. Comparison of the growth of apical tips and mature thallus sections**

### *2.6.1 Materials and methods*

Material collected at the same time as that for Experiment 1 was also used for the following experiment. Approximately 40, 10mm apical tips were excised and pooled and 40, 10mm intercalary thallus sections of the same material were excised 4-5cm from the apical tips and pooled. Four replicate dishes each containing 10 tips or 10 sections in 25ml of culture media were placed in a CEC for one week under standard experimental conditions.

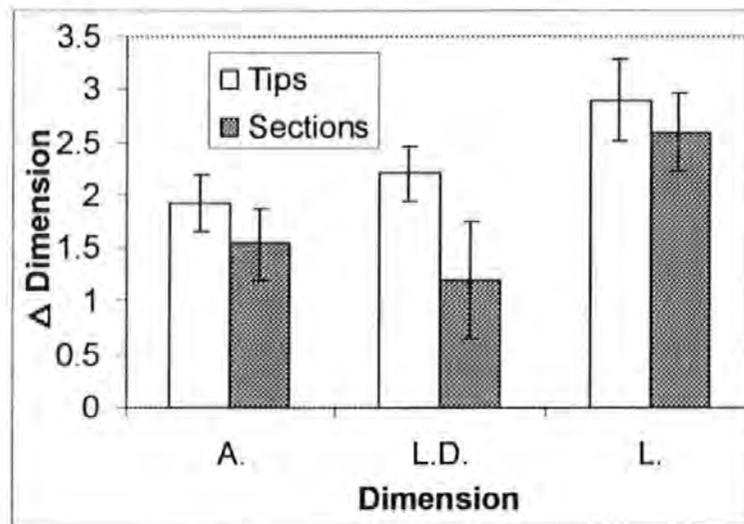
Tips and sections were measured at the beginning and end of the week using image analysis. The three parameters – '*total area*', '*longest dimension*', and '*length*' were calculated using image analysis.

### *2.6.2 Results*

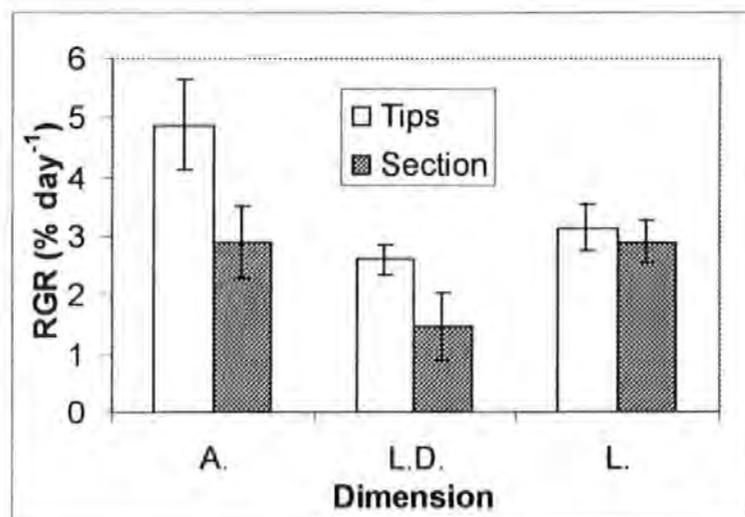
Figure 2.8 shows the increases in the three measured dimensions of the apical tips and the intercalary sections. Comparison of the means using student *t*-tests (Table 2.2) found that the only parameter to show a significant difference between the apical tips and the intercalary sections was the '*longest dimension*'. The same dimensions calculated as RGR are also displayed (Fig. 2.9). Comparisons using *t*-tests (Table 2.2) found significant differences between apical tips and intercalary sections, as calculated using the '*total area*' and the '*longest dimension*' parameters.

		D.F.	t-value	P-value
$\Delta$ Dimension (Fig. 2.8)	Area	6	-1.763	0.128
	Longest Dim.	6	-3.248	0.018
	Length	6	-1.109	0.310
RGR (Fig. 2.9)	Area	6	-4.013	0.007
	Longest Dim.	6	-3.530	0.012
	Length	6	-0.865	0.420

**Table 2.2** Results of *t*-tests comparing mean changes in dimension and RGRs of apical tips and sections of *G. longissima* using the alternate hypothesis that the two means are not equal.



**Fig. 2.8.** Mean ( $n=4$ ) change in 5 dimensions, as measured by image analysis, of apical tips and sections of *G. longissima* cultured for one week. Error bars show 95% confidence intervals. A. = Area ( $\text{mm}^2$ ), L.D. = Longest Dimension (mm) and L. = Length (mm).



**Fig. 2.9.** Mean ( $n=4$ ) RGR for 5 dimensions measured by image analysis from apical tips and sections of *G. longissima* cultured for one week. Error bars show 95% confidence intervals. A. = Area ( $\text{mm}^2$ ), L.D. = Longest Dimension (mm) and L. = Length (mm).

### 2.6.3 Discussion

These comparisons between the growth of apical tips and intercalary sections highlight some differences in the growth characteristics of the apical tips and sections. While the change in 'total area' (Fig. 2.8) between the tips and sections was not significantly different after one week of culture, the RGR calculated using change in 'total area', was significant (Fig. 2.9). The higher RGR for apical tips was due to the fact that 10mm sections had larger initial areas than the 10mm apical tips. The significant difference between the change in 'longest dimension' for the two tissue types would seem to indicate greater linear growth of apical tips.

Some thallus sections were observed to have small lateral branches. Elongation of these branches could explain the fact that no significant difference was obtained for apical tips and sections using the 'length' parameter. Lateral branching would not have contributed to the 'longest dimension' but would have increased the 'length'.

The results of the image analysis indicate that growth of apical tips is primarily linear elongation from the apex. However, since it was observed that some tips were curved, the 'length' of apical tips was considered the most appropriate measure of growth. Growth from any secondary apical buds along an apical tip would also be incorporated in this parameter. Jones (1959a) considered changes in length to be a good estimate of growth in *Gracilaria verrucosa* but considered lateral branching a potential problem. The method employed here overcomes the potential problems associated with lateral branching and curving of apical tips during growth.

## 2.7 Experiment 3. Optimal temperature and light conditions for growth

### 2.7.1 Materials and methods

*Gracilariopsis longissima* individuals were collected from the Helford Estuary (SW Cornwall) on a spring low tide (September 1996). On return to the laboratory the material was cleaned of visible epiphytes rinsed in filtered seawater and cultured under standard stock culture conditions for two weeks prior to undertaking the following two experiments which were run one week apart.

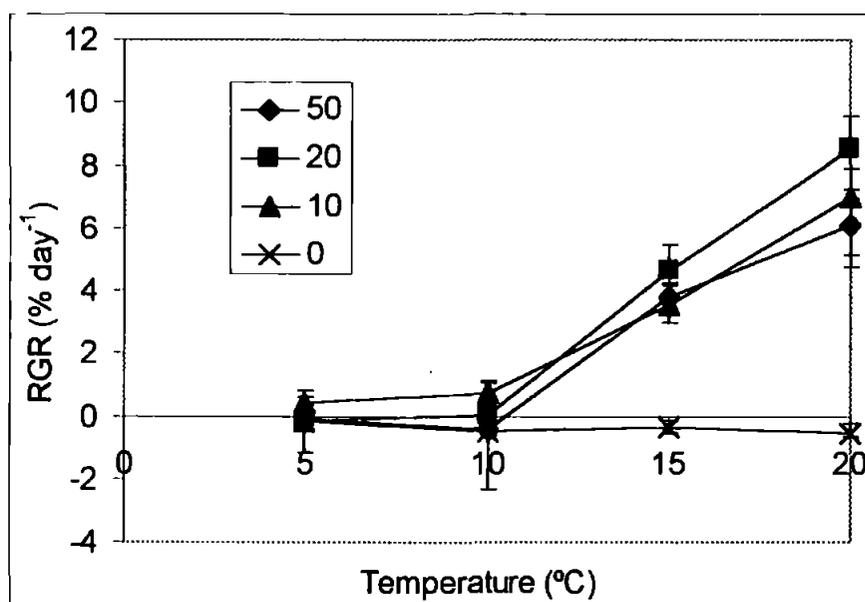
#### 2.7.1.1 Optimal growth conditions for female gametophytes

Female gametophytes were identified (by the presence of cystocarps) and sorted from the stock culture. Approximately 100, 10mm apical tips were excised and pooled. Individual apical tips were randomly selected and placed in separate compartments of three-well 90mm petri dishes. Each well contained 8ml of culture media enriched with twice the normal amount of nitrate and phosphate (200 $\mu$ M NaNO<sub>3</sub>, 20 $\mu$ M Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O). Dishes were placed in one of four CECs set at 90% RH and 16:8 light:dark for one week. Each cabinet was set to one of the following four temperatures; 5, 10, 15 and 20°C. Four irradiance levels: 0, 10, 20 and 50 $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> PAR (as measured by a Quantimet PAR meter) were set for individual dishes using layers of neutral density filter or black polythene sheets (for the dark treatment). Each treatment was replicated six times. The lengths of individual tips were measured at the beginning and the end of the week by image analysis.

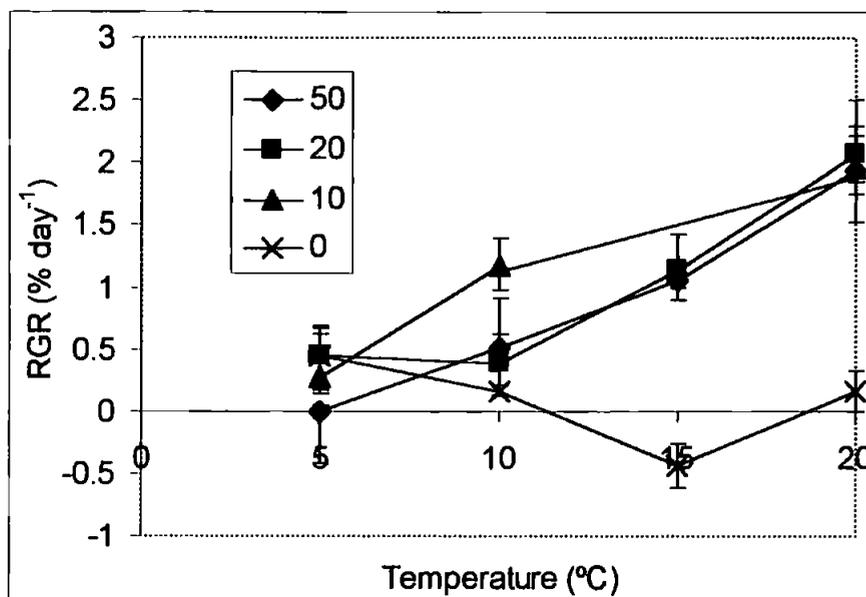
The following week, individual tetrasporophytes were identified from stock culture using a binocular dissecting microscope. An identical experiment to that described above was run using tetrasporophyte material.

### 2.7.2 Results

The growth rates of tetrasporophyte and female gametophyte material were significantly affected by temperature and light (see Figs. 2.10 & 2.11). The most noticeable effect was due to temperature. There was virtually no growth observed at 5°C and the highest growth occurred at 20°C for both tissue types.



**Fig. 2.10** Median ( $n=6$ ) RGR of female gametophyte apical tips cultured for one week under different light levels and temperatures. Legend shows the four irradiances used in  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. Error bars show interquartile ranges.



**Fig. 2.11** Median (n=6) RGR of tetrasporophyte apical tips cultured for one week under different light levels and temperatures. Legend shows the four irradiances used in  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. Error bars show interquartile ranges.

This experiment was designed so it could be statistically analysed using a two way ANOVA in order to assess interactions between light and temperature. However, the data set was found to not be normally distributed (even when transformed) and variances were significantly different between treatments. Therefore, it was only possible to perform less powerful non-parametric tests with the data. Displaying the median and interquartile range was considered the most appropriate method of displaying this non-parametric data.

Individual Kruskal-Wallis tests for each temperature range (See Table 2.3) confirmed that at 5°C no effect could be seen due to irradiance levels for either tetrasporophyte (P=0.193) or female gametophyte material (P=0.619), the RGR in both cases being approximately zero at all irradiance levels. At higher temperatures (10, 15 and 20°C) there were significant irradiance treatment effects for both tetrasporophyte and female gametophyte material (Table 2.3). Figure 2.10 and 2.11 show clearly that these treatment effects were due to the zero irradiance treatment in all cases. The zero irradiance treatment resulted in approximately zero growth regardless of the temperature for both life cycle phases. There was no significant difference between the higher 10, 20 and

50 $\mu\text{mol m}^{-2} \text{s}^{-1}$  irradiances at higher temperatures in either of the two life cycle phases.

Nevertheless, the maximum RGR obtained in both life cycle phases was at 20°C and 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Under these conditions the tetrasporophyte material had a median RGR of ~2.1% day<sup>-1</sup> (with an interquartile range of 0.7) while the female gametophyte material was considerably higher with a median RGR of 8.6% day<sup>-1</sup> (with an interquartile range of 2.6).

Tissue	Temperature	Kruskal – Wallis Test		
		N=	Test Statistic	P-value
Tetrasporophyte	5°C	24	4.722	0.193
	10°C	24	10.126	0.018
	15°C	18	6.035	0.049
	20°C	24	13.193	0.004
Female Gametophyte	5°C	24	1.780	0.619
	10°C	24	7.687	0.053
	15°C	24	13.841	0.003
	20°C	24	11.549	0.009

**Table 2.3** Summary of Kruskal-Wallis tests, testing for differences between light levels at each different temperature.

### 2.7.3 Discussion

It is well established that temperature can significantly influence seaweed growth rates (Lobban & Harrison, 1994); the growth rates of many species increase with temperature. This is attributed to higher temperatures resulting in faster metabolic rates. The genus *Gracilaria* is thought to originate from the tropics (McLachlan & Bird, 1984) with many gracilarioid species still found there. Optimal temperatures for many gracilarioid species are typically in the 20-25°C range (McLachlan & Bird 1984 and Edelstein *et al.* 1976). Friedlander *et al.* (1993) have reported *Gracilaria verrucosa* to have an optimal growth temperature range in the laboratory of between 25-30°C. Field culture studies have found maximal growth for this species to be between 12-20°C (Li *et al.*, 1984). Lapointe *et al.* (1984) found that in *Gracilaria tikvahiae* optimal growth occurred at 25°C and would not grow below 15°C.

The results obtained in this experiment are generally in agreement with these findings and clearly demonstrate that higher temperatures result in higher growth rates for both female gametophytic and tetrasporophytic tissue types. The highest growth rates were obtained at 20°C, although the optimum temperature may be higher than this, since there was no indication of growth rates reaching a plateau (see Figs. 2.10 & 2.11).

The temperature range used in this experiment was chosen to reflect a larger range than the species is likely to experience at the field sites. Individuals collected for this study originated from temperate surface waters with an annual water temperature range of 9-16°C (Bowden, 1983). For this reason 15°C was chosen as the most suitable temperature for subsequent growth experiments. At this temperature reasonable growth rates were obtained, while still remaining within the temperature range experienced by the species in the field. Furthermore, the constant temperature culture room used for maintaining stock cultures was pre-set to 15°C. Carrying out experiments at this temperature reduced the chances of large temperature fluctuations inducing stress responses in the algae which could mask treatment effects.

The effect of light on growth for both tissue types was only apparent at temperatures above 5°C. For both types of material it can be clearly seen that the absence of light significantly reduced growth at higher temperatures. No significant difference could be found between the 10, 20 and 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  treatments. This suggests that the low levels of irradiance (in the range 10-50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) are adequate for maximal growth without any reduction in growth due to excess light causing photoinhibition. Lignell *et al.* (1987) reported very high growth rates (47%  $\text{day}^{-1}$ ) of *Gracilaria secundata* cultured in a specially developed growth chamber. This particularly high growth rate was exceptional however, compared with only 3%  $\text{day}^{-1}$  found in tank cultivation. Photon irradiance was found to be limiting growth at levels of 1450  $\mu\text{mol m}^{-2} \text{s}^{-1}$  which was considerably higher

than the levels used here but they do point out that the species grows inter-tidally and such species are known to have very high light optima for growth.

Due to the design of the experiments, a direct comparison between the growth rates of the two life cycle phases is not strictly possible. The two experiments were run one week apart and any differences observed could be attributed to the longer acclimation period of material used in the second experiment rather than any differences due to life stages *per se*. Nevertheless differences in growth rates are apparent and it is likely that these can be attributed to differential responses of female gametophyte and tetrasporophyte material. Such differences in growth between different ploidy levels have been reported previously in *Gracilaria verrucosa* (Destombe *et al.*, 1993), but were not found in *Gracilaria tikvahiae* (Zhang & van der Meer, 1987). The possibility of differential responses between life cycle phases is investigated further in Chapter 8.

## 2.8 General discussion

The results obtained from the series of experiments described in this chapter provide the justification for the methodologies adopted in the experimental methods used in the remainder of this study. Preliminary investigations found cultured material grew better in supplemented, filtered natural seawater (personal observation) than artificial media (such as Instant Ocean). This was considered advantageous since the maintenance of stock cultures required large volumes of media and filtered seawater was readily available in the laboratory. While more defined artificial media have been used to successfully culture macroalgae (Stein, 1975), many species are often found to grow better in natural seawater, due largely to the extremely complex nature of natural seawater, which is difficult to accurately replicate artificially in the laboratory. Moreover, one of the objectives of this research was to relate laboratory and field based responses of *G.*

*longissima* to copper exposure, and it was therefore considered most appropriate to culture in supplemented natural seawater.

The results from the comparison of apical tips and sections did indicate that mature sections also continue to elongate and expand, suggesting some intercalary growth in the species. This is consistent with the findings of Santelices & Varela (1995) who found that sections of *Gracilaria chilensis* with and without apical tips grew at a similar rate, indicating that intercalary growth can be important. Apical tips were considered the tissue of choice for further growth experiments since growth appears to occur primarily by linear elongation from an apical meristem. Using apical tips was also considered advantageous since it provided a method of standardising the age of tissue used in subsequent experiments, and was possible to obtain large numbers of them.

Image analysis enabled the accurate and reproducible measurement of large numbers of samples over a short period of time without having to remove tissue from the media. Reduced handling and exposure to air were considered to limit the likelihood of damaging the algal material through drying which is a real possibility when measuring growth by fresh weight.

The experiments investigating the optimal light and temperature for culturing, found that while differences may exist between life history phases, low light ( $\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and high temperatures ( $20^\circ\text{C}$ ) are the most appropriate combination for optimal growth in both phases. However, since this temperature is outside the range experienced by the species at the field sites, and because a key aspect of the work reported here is to relate laboratory and field based studies, it was considered more appropriate to use  $15^\circ\text{C}$  which is at the upper end of the temperature range experienced *in situ*.

## **Chapter 3**

# **THE RESPONSE OF DIFFERENT POPULATIONS TO COPPER EXPOSURE**

### 3.1 Introduction

In the general introduction (Chapter 1) a number of factors were highlighted that could potentially influence the accumulation of trace metals in marine macroalgae. One factor, adaptation of an organism to elevated levels of trace metal exposure, was shown to be an important consideration as it could potentially limit the effective use of macroalgae as biomonitors. It was also highlighted that comparisons between the response of tolerant and non-tolerant populations could provide important information about the mechanisms of toxicity and tolerance that may be involved. Such mechanisms are known to vary considerably between different species.

This chapter details the comparisons made between a number of populations to ascertain whether any differences in tolerance could be found between populations of *Gracilariopsis longissima* originating from sites differing in metal contamination. As well as the implications such differences may have for the species use as a biomonitor, differential responses can be used to provide valuable insight into the mechanisms of copper toxicity and accumulation in *G. longissima*.

#### 3.1.1 Population differences

Numerous cases of increased tolerance to trace metals have been reported in various species of algae (e.g. see review by Klerks & Weis, 1987). It has however been pointed out that the degree of tolerance is relative (Correa *et al.*, 1996). Table 3.1 highlights this fact by comparing the levels of copper exposure, which have been used to establish differences in tolerance in different populations of a number of macroalgal species.

Species	Origin of tolerant population	Range of copper levels used	Effect on non-tolerant population	Effect on tolerant population	Reference
<i>Enteromorpha compressa</i>	Copper treated hull	0-610 $\mu\text{g L}^{-1}$ (0-9.6 $\mu\text{mol L}^{-1}$ )	Growth affected at 114 $\mu\text{g L}^{-1}$ (1.8 $\mu\text{mol L}^{-1}$ )	Unaffected at 610 $\mu\text{g L}^{-1}$ (9.6 $\mu\text{mol L}^{-1}$ )	Reed & Moffat, 1983
<i>Enteromorpha compressa</i>	Minc contaminated site	0-6354 $\mu\text{g L}^{-1}$ (0-100 $\mu\text{mol L}^{-1}$ )	6354 $\mu\text{g L}^{-1}$ (100 $\mu\text{mol L}^{-1}$ ) resulted in 50% reduction of growth	No reduction in growth at 6354 $\mu\text{g L}^{-1}$ (100 $\mu\text{mol L}^{-1}$ )	Correa <i>et al.</i> , 1996
<i>Ectocarpus siliculosus</i>	Copper treated hull	0-10000 $\mu\text{g L}^{-1}$ (0-157 $\mu\text{mol L}^{-1}$ )	10 $\mu\text{g L}^{-1}$ (0.16 $\mu\text{mol L}^{-1}$ ) resulted in 50% reduction of growth	Growth ceased at 500 $\mu\text{g L}^{-1}$ (7.87 $\mu\text{mol L}^{-1}$ )	Russell & Morris, 1970
<i>Ectocarpus siliculosus</i>	Copper treated hull	0-500 $\mu\text{g L}^{-1}$ (0-8.88 $\mu\text{mol L}^{-1}$ )	Did not grow in 250 $\mu\text{g L}^{-1}$ (4.44 $\mu\text{mol L}^{-1}$ )	250 $\mu\text{g L}^{-1}$ (4.44 $\mu\text{mol L}^{-1}$ ) resulted in 50% reduction of growth	Hall <i>et al.</i> , 1979
<i>Fucus vesiculosus</i>	Restronguet Creek	0-250 $\mu\text{g L}^{-1}$ (0-4.44 $\mu\text{mol L}^{-1}$ )	25 $\mu\text{g L}^{-1}$ (0.44 $\mu\text{mol L}^{-1}$ ) resulted in 60% reduction of growth	250 $\mu\text{g L}^{-1}$ (4.44 $\mu\text{mol L}^{-1}$ ) resulted in 60% reduction of growth	Bryan & Gibbs, 1983

**Table 3.1** Summary of examples of copper tolerance found in species of macroalgae.

It can be seen that populations classified as non-tolerant by one author may not have been considered so by another. For example, the non-tolerant *Enteromorpha compressa* population described by Correa *et al.* (1996) is likely to have been classified as tolerant by Reed & Moffat (1983). The relative scale of tolerance is apparently quite different, although it should be highlighted that the experimental procedures used in each comparison do differ. A few studies have also been published which report little or no differences between populations (Fisher & Froud 1980, Edwards 1972 and Anderson *et al.* 1990). For example, Edwards (1972) compared two populations of *Callithamnion hookeri* from polluted and unpolluted sites and found no significant differences in tolerance to copper.

Regardless of the mechanisms involved, increased tolerance could have serious implications for the usefulness of a particular species as a biomonitor of contamination. For example, increased tolerance can lead to differences in accumulation, exclusion or increased uptake of the metal. In light of this, it was considered important to characterise the response of populations of *G. longissima* from a range of sites differing in

contamination. The Fal Estuary was considered an ideal study site since it represents a large gradient of trace metal contamination that has been present for some time.

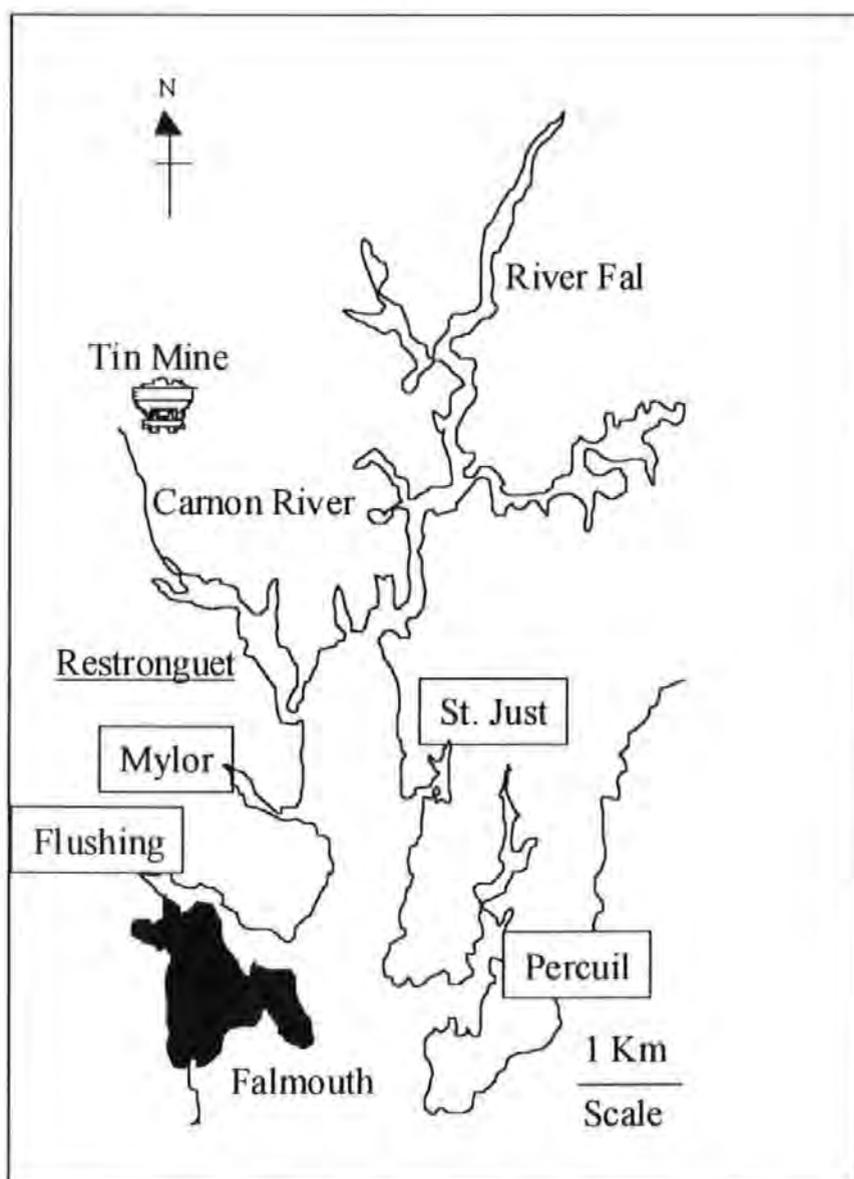
### 3.1.2 Site selection

In the four population comparison experiments described in this chapter, samples from five different populations are compared.

The invertebrate fauna of Restronguet Creek is limited but richer than might be expected by toxicity data (Bryan & Langston, 1992). This is thought to be due to populations being adapted to the elevated levels of metals in the creek. Bryan (1976) reported an increased tolerance of the polychaete *Nereis diversicolour* population from Restronguet Creek compared with samples from the cleaner Avon Estuary. Somerfield *et al.* (1994) found evidence that some nematode species may have developed different tolerance mechanisms for survival in the more contaminated areas within the estuary. Bryan & Gibbs (1983) found evidence of increased tolerance in a population of *Fucus vesiculosus* from Restronguet Creek in comparison to a population from a clean site.

During a number of exploratory searches of the Fal Estuary, three populations of *G. longissima* were located. These were in Mylor (Grid Ref. SW 820 354), St Just (Grid Ref. SW 847 358) and Flushing (Penryn Creek, Grid Ref. SW 804 343, Fig. 3.1). No populations of *G. longissima* could be located within Restronguet Creek, although one small individual was found on one occasion. The analysis of surface sediments for metal content (summarised in Bryan & Gibbs, 1983) ranks these three sites in the following decreasing order of copper contamination Mylor ( $1117\mu\text{g g}^{-1}$ ) > St Just ( $356\mu\text{g g}^{-1}$ ) > Flushing ( $322\mu\text{g g}^{-1}$ ). Somerfield *et al.* (1994) reported similar levels of  $1272\mu\text{g g}^{-1}$  for Mylor and  $332\mu\text{g g}^{-1}$  for St Just. The Mylor site was therefore considered the most

contaminated site to have a population of *G. longissima* and the Flushing and St Just sites were considered to be of a similar, but considerably lower level of contamination. It has been noted that even in relatively less contaminated sites from within the Fal estuary, contamination may still be relatively high in comparison to completely uncontaminated sites (Bryan & Gibbs, 1983) where levels in the sediment may be as low as  $10\mu\text{g g}^{-1}$ . Two other sites were also included in some of the comparisons. These were a population from the neighbouring Helford Estuary (Grid Ref. SW 758 268) and one from Chesil Fleet, Portsmouth, Dorset (Grid Ref. SY 625 800).



**Fig. 3.1.** Map of the Fal Estuary illustrating three (St Just, Mylor and Flushing) of the five sites used for population comparisons in this chapter.

In the studies outlined above it should be noted that the comparisons are between biota and sediment metal levels and not water levels. This is probably due to the fact that it can be technically difficult to obtain meaningful measurements of metal levels in seawater and also because in some species, such as *Nereis diversicolour*, the available fraction is considered to be more related to the particulate and sediment bound metals.

### **3.2 Aims**

This chapter outlines the investigations of the effects of copper on the growth of different populations of *G. longissima* in the laboratory. Four separate population comparisons were made. The purpose of these experiments was to:

- ◆ Determine the effect of copper on the growth of *G. longissima* and to establish the extent to which growth is reduced.
- ◆ Investigate the possibility that differences in response may exist between populations from sites differing in levels of trace metal contamination.
- ◆ Investigate the relationship between copper treatment and accumulation.

The results of four population comparison experiments are presented following a description of the methods employed. The results are described and discussed after individual experiments in order to highlight the rationale for each subsequent experiment. A general discussion is provided at the end.

### **3.3 Experiment 1. Comparison of growth and copper accumulation between Mylor and St Just populations following copper exposure in conical flasks**

Unless stated otherwise the field sampling, stock culturing, experimental culturing, tissue digestion and copper measurements, for all experiments, are the same as those described in Chapter 2.

#### *3.3.1 Materials and methods*

Material from two populations within the Fal estuary, Mylor and St Just (Fig. 3.1) was sampled on a spring low tide (June 1995). Sub-samples were frozen ready for digestion and analysis for copper content. The remaining material was held overnight in tanks of aerated, filtered seawater. Samples were used the following day.

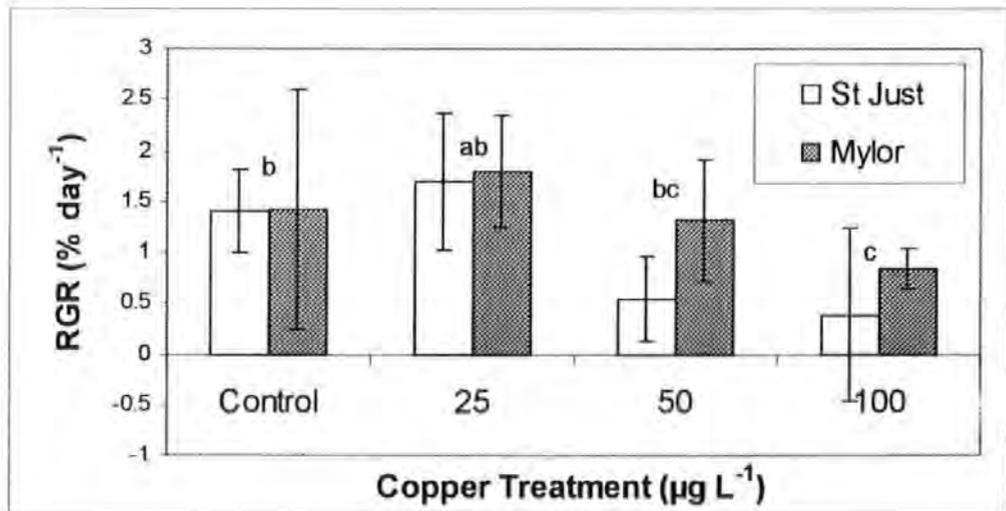
Four treatments (control, 25, 50 and 100 $\mu\text{g L}^{-1}$  copper) and five replicates from each population were prepared making 40 samples in total. Each replicate was comprised of approximately 0.5g of algal material (~8cm section of thallus), blotted dry with tissue paper and weighed accurately (FW) using a Sartorius balance. These sections of algal material were placed in 250ml conical flasks (acid washed and rinsed with MilliQ ultrapure water) containing 200ml of aerated filtered seawater and cultured in a controlled environment cabinet (CEC) under standard experimental culture conditions. After one week of culture, samples were re-weighed, frozen, freeze-dried and re-weighed (Freeze Dry Weight, FDW), acid digested (in large digestion vessels) and analysed for copper content using Atomic Absorption Spectrophotometry AAS (flame mode).

### 3.3.2 Results and discussion

The growth of *G longissima* sections cultured in flasks for one week was significantly reduced by the addition of copper ( $p=0.011$ , Fig. 3.2 & Table 3.2). No significant difference could be found between the growth response of the Mylor and St Just populations ( $P=0.677$ , Fig. 3.2 & Table 3.2).

Source	SS	DF	MS	F-ratio	P-value
Populations	1.151	1	1.151	1.980	0.170
Treatment	7.563	3	2.521	4.330	0.011
Interaction	0.894	3	0.298	0.510	0.677
Residual	18.643	32	0.583		
Total (Corrected)	28.251	39			

**Table 3.2** Two way ANOVA for the RGR of two populations (Mylor and St Just) of *G. longissima* exposed to a range of copper treatments for one week.



**Fig. 3.2.** Mean ( $n=5$ ) RGR (as measured by fresh weight) of sections of *G. longissima* from two populations (Mylor and St Just) exposed to a range of copper treatments for one week. Error bars show 95% confidence intervals. Letters designate statistically significant groupings using a Duncan's multiple range test (pooled population data).

A Duncan's multiple range test (using the pooled data from both populations) indicated that the only significant difference in RGR occurred between the highest copper treatment ( $100\mu\text{g L}^{-1}$ ) and the control and  $25\mu\text{g L}^{-1}$  treatments (Fig. 3.2). The RGR at  $100\mu\text{g L}^{-1}$  was approximately half that of the control ( $\sim 1.5\% \text{ day}^{-1}$ ). There was however, a

considerable amount of variation within treatments, as can be seen by the large error bars in Fig. 3.2. This was considered to be due to one or more of three possible factors.

- ◆ Sections of thallus in flasks broke up during the course of the week's culture. The fragmentation did not appear to be a function of copper treatment since it was seen to occur in some replicates from all treatments and was observed to occur in other samples when cultured this way (personal observation). It is not known what caused the fragmentation of the material when cultured in flasks, one possibility could be the mechanical movement of the material due to the method of aeration.
- ◆ The sections originated from single plants and inter-individual variability between plants could have been a major source of error.
- ◆ Errors that occurred during fresh weight measurement, due to the difficulty of accurately blotting dry the sections without damaging the tissue.

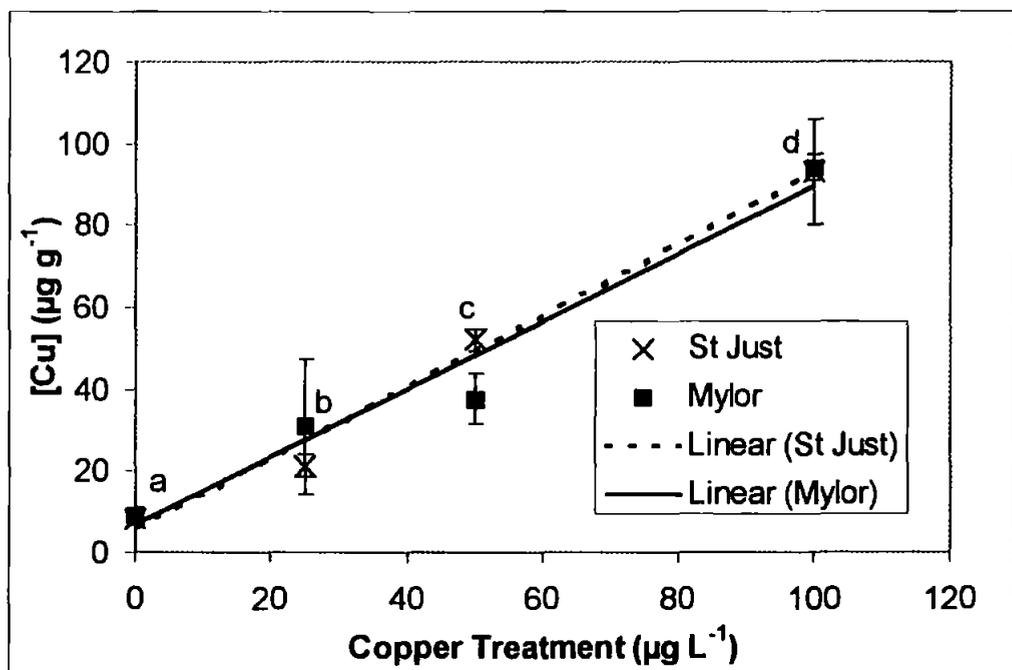
Analysis of tissue samples from the field sites revealed a significant difference in copper content between the two populations (students t-test,  $n=6$ ,  $p<0.001$ ). Algal material sampled from Mylor Creek contained  $22.6 \pm 6.9 \mu\text{g g}^{-1}$  copper (mean  $\pm 95\%$  C.I) whereas samples from St Just contained only  $6.5 \pm 1.2 \mu\text{g g}^{-1}$ , approximately 3.5 times less. This result reflects the copper gradient previously reported to exist in the sediment within the estuary. Somerfield *et al.* (1994) reported 3.8 times less copper in the sediment from St Just than that from Mylor.

Analysis of the sections following one week of exposure revealed that copper content increased with increasing copper treatment ( $P<0.001$ , Table 3.3 & Fig. 3.3).

No significant difference was found between the accumulation rates of the two populations ( $P=0.823$ ). The copper content of sections exposed to  $100\mu\text{g L}^{-1}$  copper for one week resulted in an increase in the copper concentration from a control level of  $\sim 10\mu\text{g g}^{-1}$  to over  $90\mu\text{g g}^{-1}$ .

Source	SS	DF	MS	F-ratio	P-value
Populations	1.715	1	1.715	0.050	0.823
Treatment	16023.300	3	5341.090	165.710	<0.001
Interaction	304.456	3	101.485	3.150	0.086
Residual	257.851	8	32.231		
Total (Corrected)	16587.300	15			

**Table 3.3** Two way ANOVA for the copper concentration of two populations (Mylor and St Just) of *G. longissima* exposed to a range of copper treatments for one week.



**Fig. 3.3.** Mean ( $n=2$ ) copper content of sections of *G. longissima* from two populations following copper treatment for one week. Error bars show 95% confidence intervals. Lines represent linear regressions for each population data set. Letters designate statistically significant groupings using a Duncan's multiple range test (pooled population data).

Regression analysis of the pooled data set from the two populations found a linear relationship best explained the relationship between copper treatment and the copper concentration within the seaweed sections. The concentration of copper accumulated in the

sections of thallus can be expressed as a function of the treatment by the following equation:

$$[\text{Cu}_{\text{tissue}}] (\mu\text{g g}^{-1}) = 0.853 \times [\text{Cu}_{\text{treatment}}] (\mu\text{g L}^{-1}) + 5.98 \quad (n= 16, R^2 = 0.959, P < 0.001)$$

This equation indicates a constant concentration factor (in equivalent units) from seawater to thallus section of approximately 853\* times over the range of copper treatments used in this experiment (\*Note that weight in  $\text{ng g}^{-1}$  is equivalent to volume in  $\mu\text{g L}^{-1}$ ).

### **3.4 Experiment 2. Comparison of the growth of four populations exposed to a range of copper concentrations**

In the previous experiment, a 50% reduction of the RGR of *G. longissima* sections was achieved with the addition of  $100\mu\text{g L}^{-1}$  of copper. This method of comparing populations was unsatisfactory because there was a large amount of variability within the data. This was thought to be partly due to the glass flask culturing technique. In this experiment, the effect of copper on apical tips was measured using the petri dish culturing technique and the image analysis method of measuring growth developed in Chapter 2. Because  $100\mu\text{g L}^{-1}$  of copper only reduced growth in the sections by 50%, a wider range of copper treatments was used ( $50\text{-}500\mu\text{g L}^{-1}$ ). The responses of four populations were compared, using three different populations collected from sites within the Fal estuary (Mylor, St Just and Flushing) and one from Chesil Fleet.

### 3.4.1 *Materials and methods*

*Gracilariopsis longissima* individuals from populations at Mylor, St Just and Flushing were collected on the same spring low tide (September 1995) and returned to the laboratory where they were held in filtered seawater overnight. Individuals from Chesil Fleet were collected ten days earlier and cultured in the laboratory under standard stock culture conditions. Approximately 150 apical tips (~10mm long) from each population were excised and pooled in separate beakers containing filtered seawater. Four treatments were used – control, 50, 100 and 500 $\mu\text{g L}^{-1}$  copper, with three replicates per treatment. For each replicate ten tips (nine for the Chesil population due to limited number of tips) were randomly selected from the pool of tips. Dishes were placed in a CEC under standard experimental culture conditions for one week.

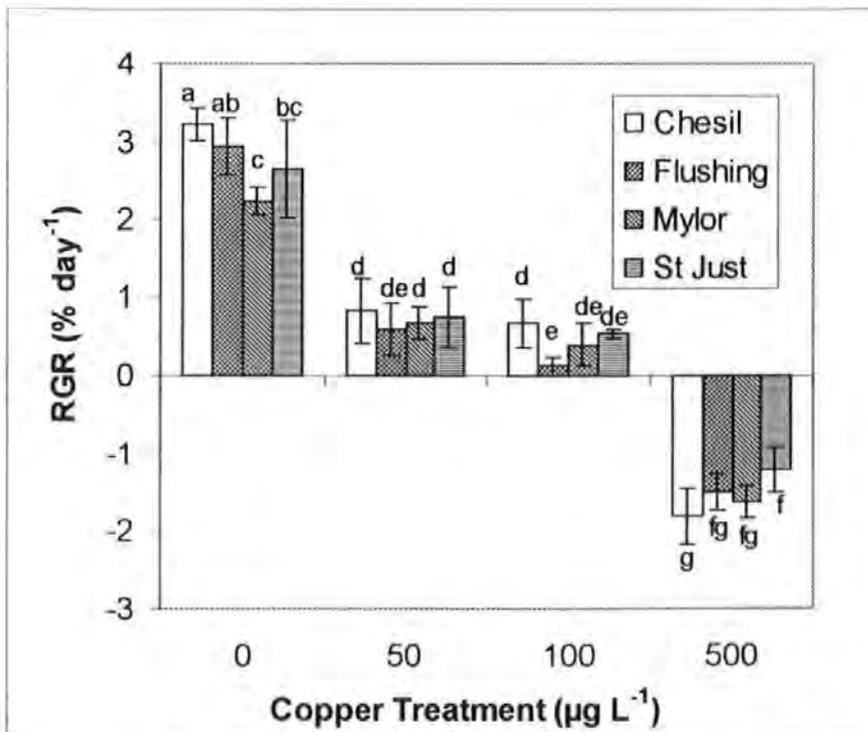
### 3.4.2 *Results and discussion*

The RGR data was analysed using a two way ANOVA (Table 3.4). Copper was found to significantly affect growth ( $P < 0.001$ ). The addition of copper significantly reduced the RGR of apical tips from all four populations (Table 3.4 & Fig. 3.4). The 50 $\mu\text{g L}^{-1}$  copper treatment resulted in an approximately 80% reduction in growth after one week, the 100 $\mu\text{g L}^{-1}$  copper treatment on average resulted in marginally more reduction and the 500 $\mu\text{g L}^{-1}$  copper treatment resulted in a negative RGR (i.e. shrinkage of tissue) of approximately  $-1.5\% \text{ day}^{-1}$ . Some shrinkage was observed when growth was limited by low temperature and no light ( $-0.45\% \text{ day}^{-1}$ , see Figs. 2.10 & 2.11, Chapter 2), but it was considerably less than seen here.

Source	SS	DF	MS	F-ratio	P-value
Populations	0.684	3	0.228	3.004	0.045
Treatment	111.647	3	37.216	490.076	<0.001
Interaction	2.007	9	0.223	2.937	0.012
Residual	2.430	32	0.076		
Total (Corrected)	116.769	47	2.484		

**Table 3.4** Two way ANOVA for the RGR of four populations (Mylor, St Just, Flushing and Chesil) of *G. longissima* exposed to a range of copper treatments for one week.

No fragments of tissue were visible in the dishes following shrinkage. These levels of shrinkage were higher than those reported in the previous chapter when growth was reduced by the removal of light or reducing temperature. Negative growth rates have been observed at high levels of trace metal exposure in other species. Bryan & Gibbs (1983) found the growth of *Fucus vesiculosus* was -30% of the control when cultured in  $250\mu\text{g L}^{-1}$  copper. One explanation of the observed shrinkage could be the loss of membrane integrity causing ion leakage and the loss of cell turgor. This possibility is investigated in more detail in Chapter 5.



**Fig. 3.4** Mean ( $n=3$ ) RGR of four populations of *G. longissima* exposed to four copper treatments for one week. Error bars show 95% confidence intervals. Letters designate statistically significant groupings using a Duncan's multiple range test.

A significant difference was found between the growth response of the four populations to copper exposure ( $P=0.045$ ) and the interaction between the two factors was also found to be significant ( $P=0.012$ , Table 3.4). A two way Duncans multiple range test was used to indicate significantly distinct groups within the treatments and populations (Fig. 3.4). The Mylor controls grew significantly less than both the Chesil and Flushing controls and Flushing material at  $100\mu\text{g L}^{-1}$  copper grew significantly less than the Chesil material at  $100\mu\text{g L}^{-1}$  copper. Similarly, the growth observed in the St Just material at  $500\mu\text{g L}^{-1}$  copper was significantly less affected than that of Chesil material at the same copper treatment (Fig. 3.4).

These results highlight some differences between the responses of the four populations when exposed to copper. The significant interaction between the two factors appears to be due to individual populations responding differently in some treatments. These differences appear to vary from treatment to treatment and population to population and no real evidence for any major difference in response between any population could be found. The groupings from the Duncans multiple range test confirm that overall all four populations responded similarly with the primary effect clearly being copper treatment.

### **3.5 Experiment 3. Comparison of growth between three populations exposed to a range of copper concentrations**

The previous experiment gave no clear indication of increased tolerance to copper by any population following exposure to copper in the laboratory. It did however indicate that the growth of apical tips in petri dishes was greatly reduced by  $50\mu\text{g L}^{-1}$  of copper. Hence, the population comparison was repeated (using the three Fal estuary populations only) using a lower range of copper treatments ( $25\text{-}100\mu\text{g L}^{-1}$ ) to determine whether a similar response in RGR would be observed.

### 3.5.1 Materials and methods

Material from three populations from within the Fal estuary (Mylor, St Just and Flushing) were collected on the same day (November 1995) and returned to the laboratory where they were cultured for four days prior to experimentation under standard stock culture conditions. Approximately 150 apical tips (~10mm long) were taken from individuals from each population, excised and pooled in beakers containing filtered seawater. Four treatments were used – control, 25, 50 and 100 $\mu\text{g L}^{-1}$  copper - with three replicates per treatment. For each replicate, ten tips were randomly selected from the pool of tips and placed in a petri dish. Dishes were placed in a CEC under standard experimental culture conditions for one week.

### 3.5.2 Results and discussion

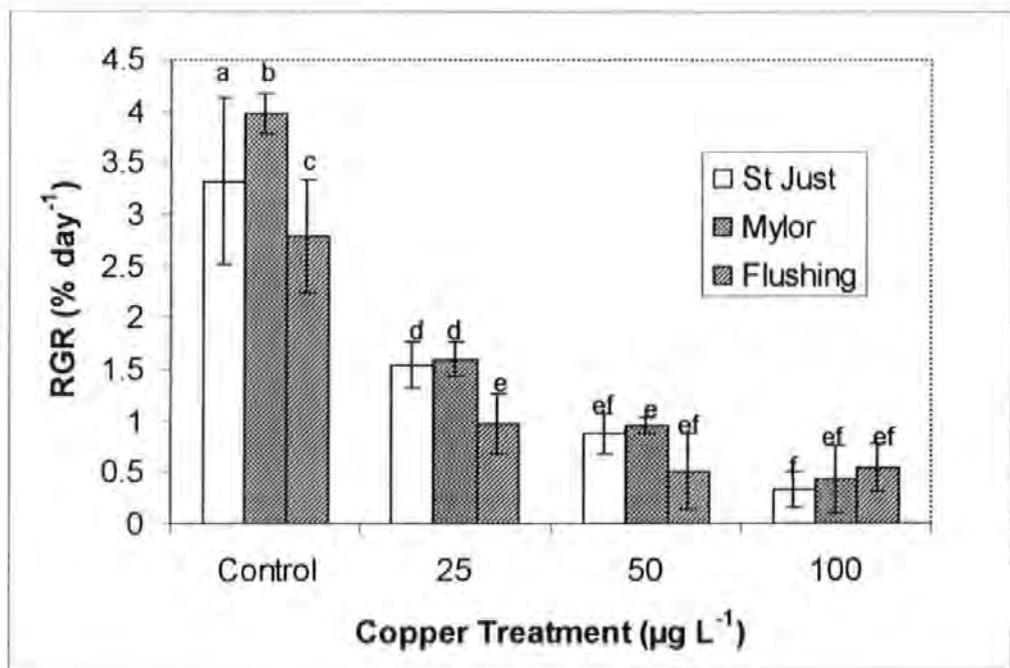
The results from this experiment were consistent with the findings of the previous experiment. The reduction of RGR by the addition of copper was analysed using a two way ANOVA (Table 3.5). Copper treatment was found to be highly significant ( $p=0.001$ ). The RGR of *G. longissima* apical tips were significantly reduced by the addition of copper (Fig. 3.5). A significant population effect ( $p=0.001$ ) and interaction between the two factors ( $p=0.045$ ) were also found.

Source	SS	DF	MS	F-ratio	P-value
Populations	1.760	2	0.880	8.864	0.001
Treatment	46.266	3	15.422	155.327	<0.001
Interaction	1.542	6	0.257	2.588	0.045
Residual	2.383	24	0.099		
Total (Corrected)	51.952	35	1.484		

Table 3.5 Two way ANOVA for the RGR of three populations (Mylor, St Just and Flushing) of *G. longissima* exposed to a range of copper treatments for one week.

A two way Duncans multiple range test (Fig. 3.5) highlighted that the major factor influencing growth is copper treatment. The addition of  $25\mu\text{g L}^{-1}$  of copper reduced growth by approximately 50%, the  $50\mu\text{g L}^{-1}$  copper treatment by approximately 70% and the  $100\mu\text{g L}^{-1}$  copper treatment by marginally more (Fig. 3.5). Differences in response between populations in some treatments were significant. In this instance, all populations grew differently in the control treatments and the Flushing population grew significantly less than the Mylor and St Just populations in the  $25\mu\text{g L}^{-1}$  copper treatment (Fig. 3.5).

It can be concluded that overall, no major difference exists between the response of the three populations to copper exposure. All three were similarly reduced by the same levels of copper exposure. This indicates that the interpretation made in the previous experiment, that there is no real difference in tolerance between populations tested was correct.



**Fig. 3.5.** Mean ( $n=3$ ) RGR of three populations of *G. longissima* exposed to copper for one week. Error bars show 95% confidence intervals. Letters designate statistically significant groupings using a Duncans multiple range test.

### **3.6 Experiment 4. Comparison of growth and accumulation of copper following low levels of copper exposure in two populations**

Another population, located within the Helford Estuary was found. A comparison of this Helford population was made with the population from the most contaminated site in the Fal Estuary (Mylor). The levels of copper used so far, had failed to reveal any differences in tolerance between populations and had shown a marked decrease in growth. For this reason, even lower levels of copper were used in this experiment to determine whether any differences could be revealed. A range of copper levels from 12-50 $\mu\text{g L}^{-1}$  was used. The lowest treatment (12 $\mu\text{g L}^{-1}$ ) was comparable to the levels reported to be in the seawater in Restronguet Creek (the most contaminated creek in the Fal Estuary).

#### *3.6.1 Materials and methods*

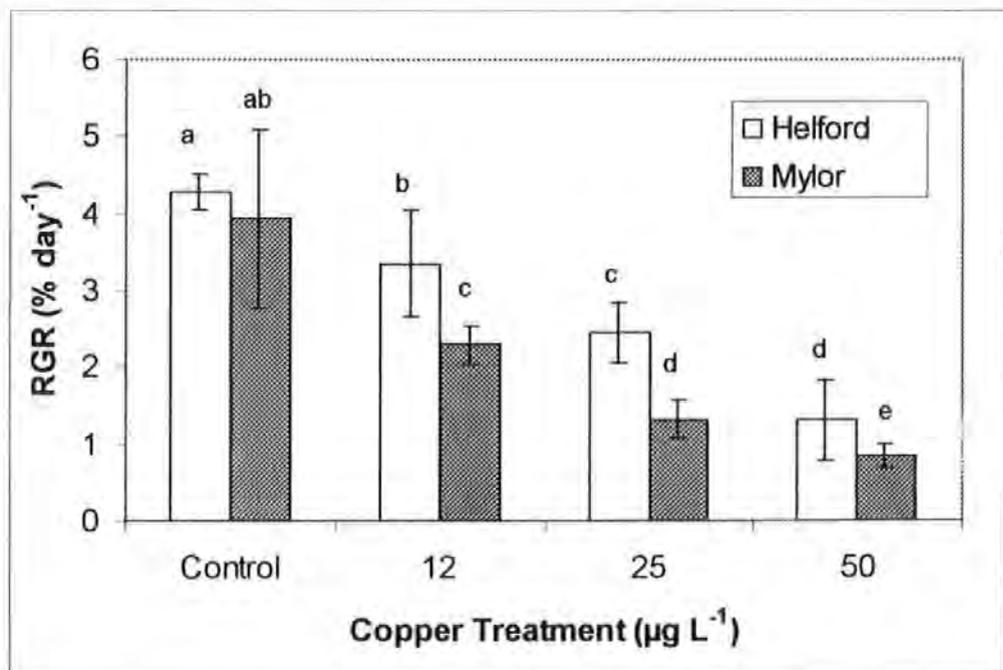
Material from the Mylor and Helford populations was collected on the same day (April 1996) and returned to the laboratory where they were cultured for five days under standard stock culture conditions. Water samples were collected from each site at low tide and used to measure the labile and total copper content using the methods described in Chapter 2.

In this experiment approximately 150 apical tips (~10mm long) from each population were excised and pooled in beakers containing filtered seawater. Four treatments were used – control, 12, 25 and 50 $\mu\text{g L}^{-1}$  copper - with four replicates per treatment. For each replicate, ten tips were randomly selected from the pool of tips and placed in a petri dish. Dishes were placed in a CEC under standard culture conditions for one week. After image analysis, three of the four replicates were frozen, freeze-dried,

weighed using a micro-balance (Cahn) and acid digested using small digestion vessels and analysed for copper content using AAS (graphite furnace).

### 3.6.2 Results and discussion

Inspection of the raw data indicates some small differences between the response of each population to some of the copper treatments and increasing copper treatments clearly reduced growth (Fig. 3.6). However, the data failed tests for normality, namely error variance and kurtosis tests. A natural log transformation had to be used to normalise this data set. A two-way ANOVA of the  $\text{Log}_{\text{natural}}$  transformed data (Table 3.6) found that the addition of copper significantly reduced the RGR of apical tips ( $p < 0.001$ ). There was also a significant difference between populations ( $p < 0.001$ ) and the interaction between the two factors ( $p = 0.011$ ). The lowest copper treatment,  $12 \mu\text{g L}^{-1}$ , significantly reduced the RGR in comparison with the control by about 25% in both populations. The higher copper treatments resulted in further reduction. The significantly different groupings found using a



**Fig. 3.6** Mean ( $n=3$ ) RGR of two populations of *G. longissima* exposed to copper for one week. Error bars show 95% confidence intervals.

two-way Duncan's multiple range test are shown in Fig. 3.6. The interaction can be explained by the fact that the population controls had similar growth rates, while each successive copper treatment reduced the growth of the Mylor material more than the Helford material. This analysis should be treated with some caution since there is clearly a large amount of variation in the Mylor control samples in comparison with the other samples (Fig. 3.6). Overall, it is clear that the Mylor material did not grow as well as the Helford material but these differences are relatively small and do not appear to indicate any major difference in response to copper exposure.

Source	SS	DF	MS	F-ratio	P-value
<b>Populations</b>	1.185	1	1.185	56.629	<0.001
<b>Treatment</b>	8.364	3	2.788	133.240	<0.001
<b>Interaction</b>	0.289	3	0.096	4.609	0.011
<b>Residual</b>	0.502	24	0.021		
<b>Total (Corrected)</b>	10.341	31	0.334		

**Table 3.6** Two way ANOVA for the RGR (Ln transformed) of two populations (Mylor and Helford) of *G. longissima* exposed to a range of copper treatments for one week.

The analysis of the apical tips for copper content after one week of culture, found significant population ( $P=0.004$ ) and treatment effects ( $P<0.001$ ) but no interaction ( $P=0.427$ , Table 3.7).

The significant population effect appears to be due to Mylor apical tips containing more copper than the Helford apical tips in all treatments.

Source	SS	DF	MS	F-ratio	P-value
<b>Populations</b>	3484.45	1	3484.45	11.280	0.004
<b>Treatment</b>	29111.2	3	9703.74	31.420	<0.001
<b>Interaction</b>	908.117	3	302.706	0.980	0.426
<b>Residual</b>	4941.19	16	308.824		
<b>Total (Corrected)</b>	38445.0	23			

**Table 3.7** Two way ANOVA for the copper concentration of apical tips from two populations (Mylor and Helford) of *G. longissima* exposed to a range of copper treatments for one week.

Since there was no evidence of an interaction between the copper treatment and populations, it can be concluded that the accumulation (concentration factor) of copper was the same for both populations over a period of seven days.

The best fitting model, describing the copper accumulation in the apical tips of both populations was found to be a positive linear regression (Fig. 3.7). The equation for each population was:

Helford population:

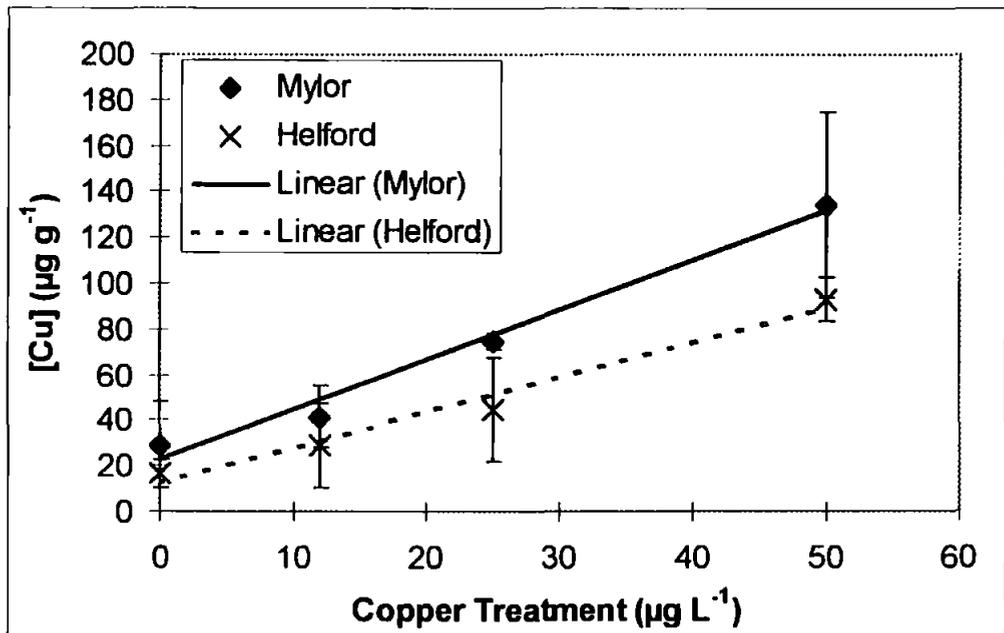
$$[\text{Cu}_{\text{tissue}}] (\mu\text{g g}^{-1}) = 1.54 \times [\text{Cu}_{\text{treatment}}] (\mu\text{g L}^{-1}) + 12.17 \quad (n=12, R^2=84.72, p<0.001)$$

Mylor population:

$$[\text{Cu}_{\text{tissue}}] (\mu\text{g g}^{-1}) = 1.76 \times [\text{Cu}_{\text{treatment}}] (\mu\text{g L}^{-1}) + 26.47 \quad (n=11, R^2=90.52, P<0.001)$$

The constants (slope and intercept) for the two equations are summarised in Table

3.8.



**Fig. 3.7.** Mean ( $n=3$ ) copper content of *G. longissima* apical tips from two populations following copper treatment for one week. Error bars show 95% confidence intervals. Lines represent linear regressions for each population data set.

Population	Slope (x1000 = concentration factor in ppb )	Intercept ( $\mu\text{g g}^{-1}$ )
	Estimate ( $\pm$ 95% CI)	Estimate ( $\pm$ 95% CI)
Helford	1.54 ( $\pm$ 0.46)	12.17 ( $\pm$ 12.92)
Mylor	1.76 ( $\pm$ 0.42)	26.47 ( $\pm$ 10.78)

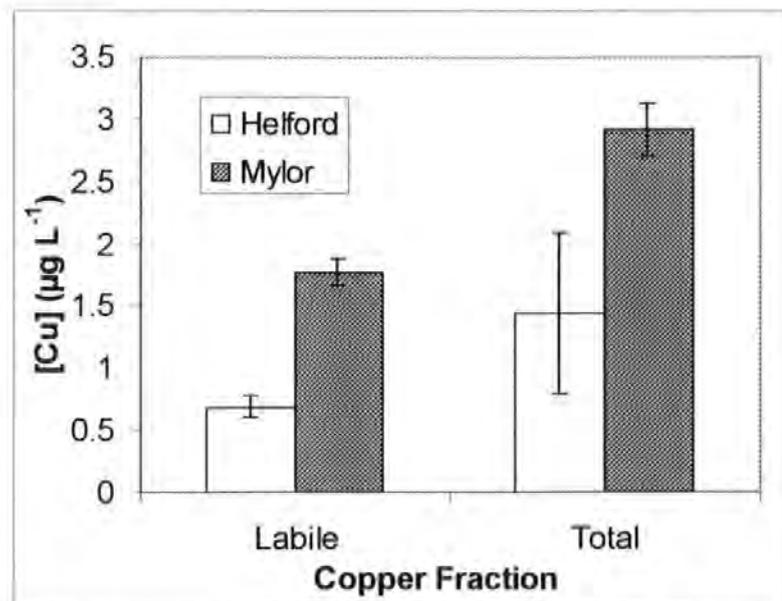
**Table 3.8** Estimated slope and intercept from the modelled linear relationship between copper treatment and accumulation in the apical tips from two populations (Mylor and Helford) of *G. longissima*.

By comparing the 95% confidence intervals for the two regression lines, it can be seen that the slopes for each equation were not significantly different, confirming that the accumulation was essentially the same for the two populations (Table 3.8). This can clearly be seen by the fact that the regression lines for the two populations are close to parallel (Fig. 3.7) and is in agreement with the two-way ANOVA (Table 3.7) which found no interaction between population and copper treatment. The intercepts of the two equations do not appear to be significantly different although the two-way ANOVA (Table 3.7) found a significant population effect. This effect is most likely due to Mylor material having higher initial copper concentrations than the Helford apical tips. Although accumulation for the two populations was similar, Mylor apical tips contained consistently more copper than the Helford samples at all treatments (Fig. 3.7).

The concentration factors for the two populations were approximately 1600. This is higher than the value estimated for the thallus sections in Experiment 1 (~850), in which the thallus sections were cultured under different conditions. Despite this, in both cases a positive linear relationship was found between copper treatment and copper concentration in the algal material. It therefore appears that tissue saturation did not occur and that higher tissue concentrations are possible.

Reed & Moffat (1983) found a similar result when comparing two populations of *Enteromorpha compressa*. They found that the accumulation was the same in both populations, despite the fact that one population contained significantly more copper than the other, at all points during the experiment due to higher initial copper concentrations on one population. However, they did find significant differences in tolerance between the populations as measured by growth.

Water samples collected at the same time as algal material and analysed for copper levels indicate that there is a difference in both total and labile copper concentrations between the two sites, with the Helford site being less contaminated (Fig. 3.8). The only available data on the level of contamination in the Helford Estuary (Bryan *et al.* 1980) appears to agree with this finding, suggesting that the Helford Estuary has a similar level of sediment contamination ( $328\mu\text{g L}^{-1}$ ) to that reported for St Just ( $356\mu\text{g L}^{-1}$ , Bryan & Gibbs 1983).



**Fig. 3.8.** Labile and total copper content from filtered water samples from Mylor and Helford. Error bars show 95% confidence intervals.

As has been pointed out earlier, water samples provide single 'snapshots' of the two sites and a detailed chemical analysis of the two sites could reveal significantly different results. A study of this nature was beyond the scope of this work. However, differences in metal concentrations for (the control) algal samples collected from the two populations do agree with the water sample results. This suggests that there is a positive relationship of some form between the concentration of copper in the water and tissue at the field sites.

In this experiment, the two populations were exposed to even lower levels of copper than had been used previously. The hypothesis being tested was that if a difference in tolerance did exist between two populations then it may only be observed at lower, more environmentally realistic levels (i.e. those more likely to be experienced at contaminated estuaries). The lowest copper treatment used ( $12\mu\text{g L}^{-1}$ ) is comparable to the reported levels of total copper found in Restronguet Creek (Bryan & Hummerstone, 1973). Growth in both populations was found to be significantly reduced by this treatment after only seven days. This suggests that prolonged exposure to this level of contamination may be detrimental to the survival of these populations.

### **3.7 Dose response curves**

The RGR data in the four experiments above were presented in the most appropriate way to allow differences between populations to be assessed. However, this method of displaying the data does not best illustrate the type of dose response relationship between the level of copper treatment and the reduction in growth. This relationship was investigated by determining the best fitting regression equation for each population in Experiments 2,3 and 4. The constants (intercept and slope) are summarised in Table 3.9.

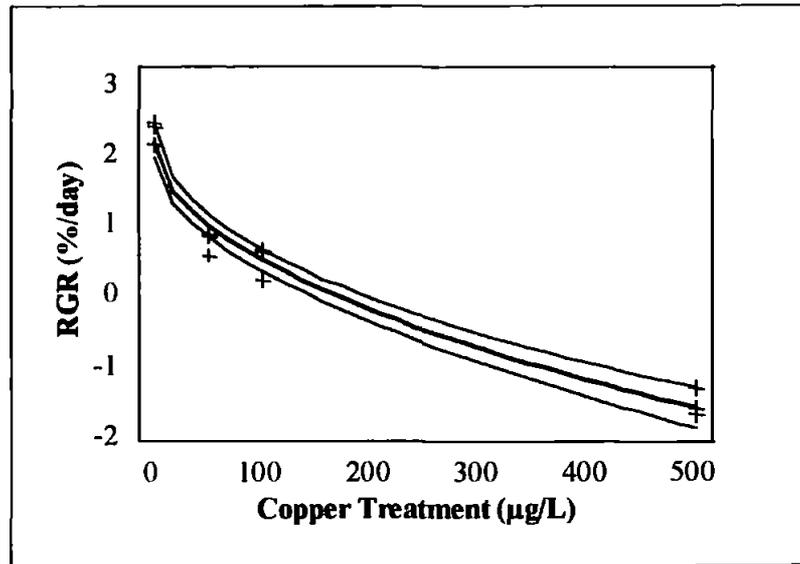
Note that the data from Experiment 1 was excluded because it used sections of algal material rather than apical tips and a different culturing technique.

Experiment (copper level $\mu\text{g L}^{-1}$ )	Population	Square root X model i.e. $Y = a + b\sqrt{X}$		Fit of model		
		Intercept 'a' ( $\pm$ 95% CI)	Slope 'b' ( $\pm$ 95% CI)	N	P-value	R <sup>2</sup>
#2 (50-500)	Chesil	2.86 ( $\pm$ 0.44)	-0.22 ( $\pm$ 0.03)	12	<0.001	95.06
	Flushing	2.40 ( $\pm$ 0.59)	-0.19 ( $\pm$ 0.04)	12	<0.001	90.97
	Mylor	2.09 ( $\pm$ 0.52)	-0.17 ( $\pm$ 0.02)	12	<0.001	97.60
	St Just	2.08 ( $\pm$ 0.99)	-0.15 ( $\pm$ 0.03)	11	<0.001	94.60
#3 (25-100)	Flushing	2.51 ( $\pm$ 1.22)	-0.24 ( $\pm$ 0.06)	12	<0.001	79.39
	Mylor	3.75 ( $\pm$ 0.89)	-0.36 ( $\pm$ 0.06)	12	<0.001	94.62
	St Just	3.21 ( $\pm$ 0.98)	-0.31 ( $\pm$ 0.08)	12	<0.001	91.03
#4 (12-50)	Helford	4.40 ( $\pm$ 0.64)	-0.41 ( $\pm$ 0.06)	15	<0.001	93.80
	Mylor	3.88 ( $\pm$ 0.62)	-0.46 ( $\pm$ 0.06)	14	<0.001	95.61

**Table 3.9** Summary of the regression models fitted to the RGR data from three sets of population comparisons.

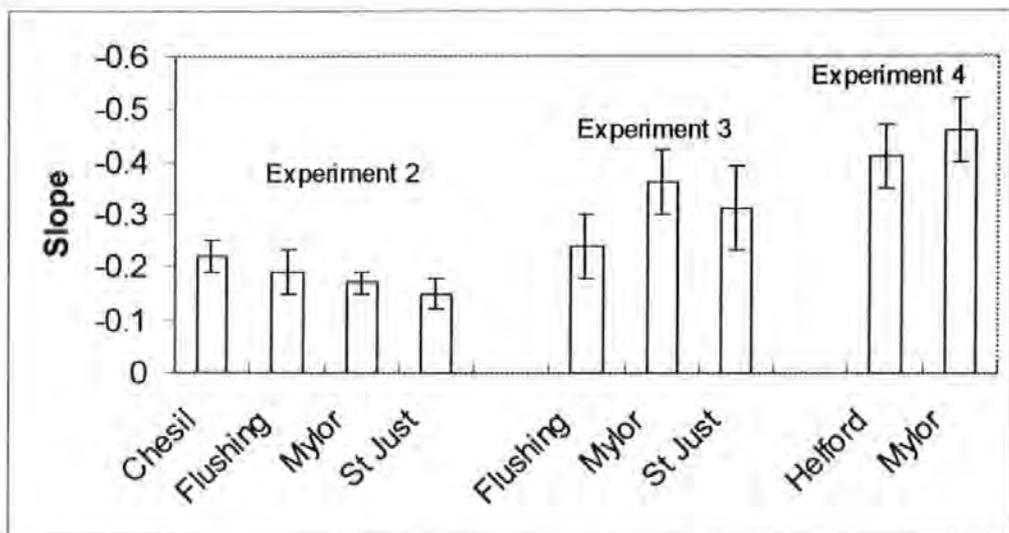
Separate regressions made using the nine population data sets from the three experiments found that a model of the form  $Y = \sqrt{X}$  best explained the data in eight of the nine cases. In the final set (Experiment 4, Helford population) this model was the second best fitting model (by less than 0.5%) and still explained more than 93% of the variability in the data. Figure 3.9 illustrates the form of the relationship between the level of exposure and the reduction of growth using this model, fitted to the data from the Mylor population in Experiment 2. It can be seen from this model that the rate of RGR reduction decreases, with increasing copper concentration.

Figures 3.10 & 3.11 show the slopes and intercepts estimated from the three

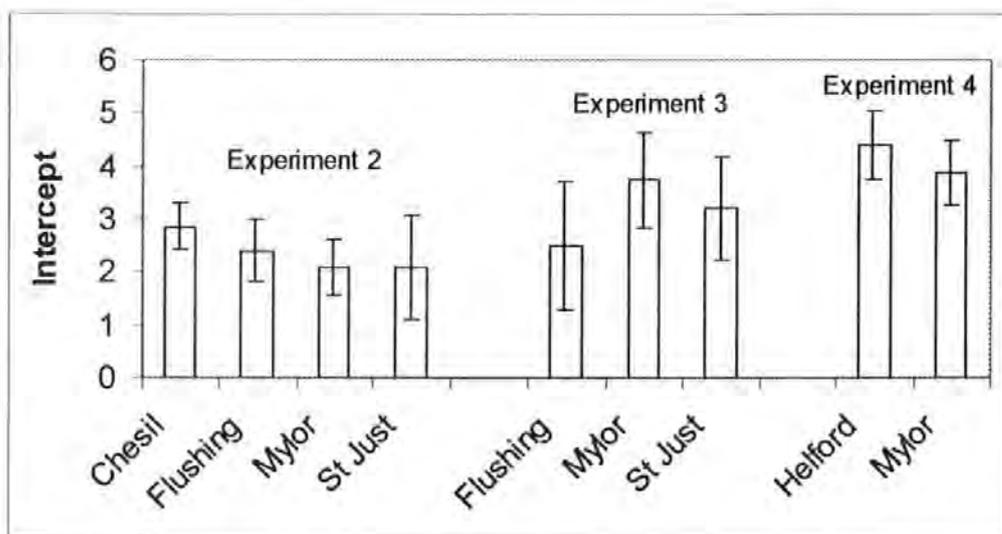


**Fig. 3.9** Example of the dose response curve fitted to *G. longissima* apical tips exposed to a range of copper treatments for one week. Values shown here originate from the Mylor data set from population comparison Experiment 2. The equation describing this relationship is shown in Table 3.9.

experiments. Allowing for the 95% confidence intervals, the values for these slopes and intercepts overlap for each population comparison. This suggests that the modelled dose response of the different populations in each experiment is not significantly different. This finding is in agreement with the interpretations made previously, that no major difference in response could be found between the different populations tested. The variation in the values found between experiments 2, 3 and 4 could be due to seasonal variation in the growth of the populations, since the three experiments were run during different times of the year (September, November and April respectively). Considering the different ranges of copper treatments used in the three experiments, the results are remarkably similar.



**Fig. 3.10** Slopes calculated from the regression model of the general form  $y = a + b\sqrt{x}$  fitted to each population in three separate population comparison experiments. The regressions are summarised in Table 3.9. Error bars show 95% confidence intervals.



**Fig. 3.11** Intercepts calculated from the regression model of the general form  $y = a + b\sqrt{x}$  fitted to each population in three separate population comparison experiments. The regressions are summarised in Table 3.9. Error bars show 95% confidence intervals.

It is clear from the equation used to describe the dose response curve that low levels of copper exposure can reduce growth significantly. Concentrations that result in 50% reduction in growth ( $EC_{50}$ ) can be estimated for each data set. These values are shown in Table 3.10 along with the concentration estimated to cause zero growth ( $EC_{100}$ ).

Experiment	Population	Copper EC <sub>50</sub> (µg L <sup>-1</sup> )	Copper EC <sub>100</sub> (µg L <sup>-1</sup> )
#2	Chesil	42	169
	Flushing	40	160
	Mylor	38	151
	St Just	48	192
#3	Flushing	27	109
	Mylor	27	108
	St Just	27	107
#4	Helford	29	115
	Mylor	18	71

**Table 3.10** One-week EC<sub>50</sub> and EC<sub>100</sub> values calculated from the dose response equations described in Table 3.9.

As would be expected the EC<sub>50</sub> and EC<sub>100</sub> values are similar for each population within each experiment. It appears that a concentration in the region of 30-40µg L<sup>-1</sup> results in a 50% reduction in growth, whereas a concentration in the region of 110-170µg L<sup>-1</sup> results in no growth. The values are also relatively similar between the three experiments.

Regardless of the small differences between experiments, these models concur with the ANOVA of the data sets. That is, increasing levels of copper exposure reduces the RGR of *G. longissima* in a non-linear fashion and this reduction in growth is essentially the same for each population tested.

### 3.8 General discussion

The exposure of apical tips and sections of *G. longissima* to copper in the laboratory significantly reduced growth rates. This reduction could be observed at concentrations as low as 12µg L<sup>-1</sup> (Fig. 3.6) where approximately 25% reduction in growth was found and at levels as high as 500µg L<sup>-1</sup> of copper (Fig. 3.4), where significant shrinkage of the apical tips could be observed.

A small amount of variation was found in the response of different populations to individual copper treatments. However, the greatest effect was due to the level of copper exposure. When the dose response of all the populations were compared, it became apparent that the inhibition of growth could be best described by a regression equation of the form  $Y = a + b \sqrt{x}$  (Fig. 3.9). This model predicts that low levels of exposure can have the greatest effect on growth, with increasingly higher levels having relatively less effect. This model does not take into account the fact that at very low copper concentrations, growth would be expected to be impaired due to copper deficiency. However, no evidence of copper deficiency was found in these experiments.

The fitted models also indicated that there was no major difference in response between the different populations in each experiment. These dose response curves do however, highlight the fact that differences in slopes and intercepts could be seen between the same populations sampled at different times for separate experiments. This indicates that although the same type of relationship existed during all of the experiments, the actual level of response may vary significantly with time. These differences are most likely to be due to seasonal variation in growth patterns which have been found in many species (Lobban & Harrison 1994) including *Gracilaria verrucosa* (Jones 1959b). Anderson *et al.* (1990) found significant temporal differences in the response of *Macrocystis pyrifera* to copper. The differences in the ranges of copper levels may have also caused some of the variation between the three experiments.

It is therefore concluded that no major difference in tolerance between populations used in this study, as measured by growth, could be found. Populations from sites differing in contamination within the Fal Estuary responded in a similar way, as did two populations from outside the estuary. This finding is consistent with Edwards (1972) who found no

significant difference in copper tolerance between two populations of the intertidal red algae *Callithamnion hookeri* from a polluted and unpolluted site.

Comparing these results with the reported effects of copper on other macroalgal species (Table 3.1) highlights some similarities. For example  $12\mu\text{g L}^{-1}$  of copper was reported to result in a 50% reduction in growth of a non-tolerant population of *Ectocarpus siliculosus* (Russell & Morris, 1970). In this study  $12\mu\text{g L}^{-1}$  of copper resulted in approximately 25% growth reduction (Experiment 4). Similarly Hall *et al.* (1979) found that  $250\mu\text{g L}^{-1}$  stopped a non-tolerant population of *E. siliculosus* from growing, which is similar to the level which caused zero growth in *G. longissima* (Experiment 2). Bryan & Gibbs (1983) found that a non-tolerant population of *Fucus vesiculosus* had 60% reduced growth at  $25\mu\text{g L}^{-1}$ , which is similar to the level of reduction seen in Experiments 3 & 4. Their finding that the tolerant population of *F. vesiculosus* from Restronguet Creek grew at 40% of the control in  $250\mu\text{g L}^{-1}$  indicates that it is more tolerant than any of the populations of *G. longissima* used in this study. This may explain why no population of *G. longissima* could be found in Restronguet Creek. The effects of copper on *G. longissima* reported here are comparable with those reported by Edwards (1972) for *Callithamnion hookeri* who found that  $10\mu\text{g L}^{-1}$  of copper significantly reduced growth while  $500\mu\text{g L}^{-1}$  was 'completely toxic'.

By contrast both the tolerant and non-tolerant populations of *Enteromorpha compressa* used by Reed & Moffat (1983) and Correa *et al.* (1996) appear to be considerably more tolerant than the populations of *G. longissima* used in this study. The so-called non-tolerant populations, tolerated considerably higher levels of copper. It should be stressed however, that experimental procedures do vary from study to study and direct comparisons of results from these different studies are not strictly possible.

No population of *G. longissima* could be found in the most heavily contaminated site (Restronguet). This creek has considerably higher levels of copper in the sediment ( $\sim 2500\mu\text{g g}^{-1}$ ) than in the neighbouring Mylor Creek ( $\sim 1100\mu\text{g g}^{-1}$ ) and a significantly reduced flora and fauna (Sommerfield *et al.* 1994 and Bryan & Gibbs 1983). Despite this level of contamination, some macroalgae such as the *Fucus sp.* and *Enteromorpha sp.* can be found in this creek and in both species some evidence of increased tolerance has been found (Bryan & Gibbs 1983 and Lewis pers. comm.). Sommerfield *et al.* (1994) found that the nematode and copepod communities in Restronguet Creek were very different from those in all the other creeks. Perryman (1996) also found in her multivariate analysis of macrofauna community structures within the estuary, that Restronguet Creek communities were significantly different from communities in other creeks in the estuary. The increase in metal concentrations from Mylor to Restronguet appears to represent some form of threshold that many species have not been able to cross (Sommerfield *et al.*, 1994). The dose response curves from these experiments indicate that very low levels of copper (below  $12\mu\text{g L}^{-1}$ ) can have an effect on the growth of *G. longissima*. This species therefore seems to be relatively sensitive to copper exposure. Concentrations of copper in Restronguet Creek water have been recorded as high as  $10\mu\text{g L}^{-1}$ . It seems likely that *G. longissima* (and many other species) have not (yet) developed the tolerance required to exist in this environment.

### 3.9 Conclusions

In summary, these experiments indicate that:

- ◆ Copper reduces growth in *G. longissima* in a non-linear fashion. Significant reduction in growth can be seen at low, environmentally realistic, levels of contamination ( $12\mu\text{g L}^{-1}$ ).

- ◆ At exceptionally high levels ( $500\mu\text{g L}^{-1}$ ), copper results in the shrinkage of algal material.
- ◆ Apical tips accumulate copper in a linear fashion over a large range of copper concentrations ( $12\text{-}100\mu\text{g L}^{-1}$ )
- ◆ Despite small differences in response to certain treatments, no major differences could be found in the responses of populations, from within and outside the Fal Estuary to copper exposure.
- ◆ Accumulation of copper also did not differ between populations tested.

## **Chapter 4**

# **COMPARATIVE STUDIES ON THE EFFECT OF COPPER ON THE GROWTH, PHOTOSYNTHESIS AND RESPIRATION OF *GRACILARIOPSIS LONGISSIMA***

## 4.1 Introduction

The previous chapter provides, no evidence of a major difference between the growth response of different populations exposed to copper. Copper did however significantly reduce growth and accumulate within the tissue. This indicated that the metal is having a significant effect upon the metabolic processes of the alga.

As stated previously, copper is an essential trace metal required for normal cellular function that is also considered one of the most toxic metals (Gledhill *et al.*, 1997). Many reports exist of copper toxicity to plants and algae at elevated levels (Rai *et al.* 1981 and Fernandes & Henriques 1991). The toxic effect of copper has received considerably more attention in species of plants and microalgae than macroalgae. The most commonly reported effect of copper on algae is the reduction of growth (Rai *et al.* 1981 and Fernandes & Henriques 1991). Nevertheless, the presence of a toxicant (such as copper) may induce a range of physiological responses in an organism. For example, copper is often reported to inhibit photosynthesis (Fernandes & Henriques, 1991). Copper plays an essential role in photosynthesis, where it is known to be a constituent of the primary electron donor (plastoquinine) in Photosystem I (Bishop 1964 and Baron *et al.* 1995). It may also play an important role in other photosynthetic pathways and could be a constituent of Photosystem II, although this has been questioned by Baron *et al.* (1995).

Irrespective of the normal function of copper, elevated levels have been shown to reduce photosynthesis in microalgae (Stauber & Florence 1987, Cid *et al.* 1995, Vavilin *et al.* 1995, Abalde *et al.* 1995 and Nalewajko & Olaveson 1995), as well as decreasing chlorophyll *a* content and cellular division (Rijstenbil *et al.*, 1994 a & b). Copper has also been reported to reduce photosynthesis in the macroalgae *Enteromorpha compressa* (Reed & Moffat, 1983) and *Fucus vesiculosus* (Plotz, 1991).

The net rate of photosynthesis is a fundamental aspect of plant growth and physiology since it provides the energy required for all cellular processes. In microalgae, photosynthesis has been reported to be more sensitive to copper toxicity than other metabolic processes but this is usually measured in isolated chloroplasts (Sheoran & Singh, 1993). However, Nalewajko & Olaveson (1995) point out that this apparent sensitivity has not been demonstrated in whole algae.

Few comparative studies have been carried out where the effects of copper on growth and photosynthesis have both been measured simultaneously during the same experiment. In microalgae, the few examples of comparative studies that exist, all found growth was more sensitive than photosynthesis (Fisher *et al.* 1981, Lumsden & Florence 1983, Stauber & Florence 1987, Cid *et al.* 1995, Abalde *et al.* 1995, Nalewajko & Olaveson 1995). Of the relatively few studies investigating the effect of copper on photosynthesis in macroalgae, none have used a comparative approach, so differences between growth and photosynthetic responses cannot be directly assessed.

When Reed & Moffat (1983) compared a range of physiological responses including net photosynthesis in two populations of the ship-fouling *Enteromorpha compressa* exposed to copper, significant differences were found between the two populations. Copper had no effect upon the more resistant population over the range used (0-610  $\mu\text{g L}^{-1}$ ). Nevertheless, in the less resistant population, net photosynthesis was shown to be affected to a greater extent than cell viability and changes in the intracellular concentration of the organic solute DMSP and potassium. Net photosynthesis could be seen to be reduced by concentrations of copper of 203  $\mu\text{g L}^{-1}$  and above after only 24 hours at which time no effect upon the other parameters could be seen. Thallus growth was also found to be reduced by copper exposure levels of 114  $\mu\text{g L}^{-1}$  and above after nine days. However, this effect was observed in a separate experiment that used different methods

and therefore does not enable a direct comparison between growth and photosynthesis to be made. The authors did however consider that the reduction in net photosynthesis might partly explain the reduction in growth.

Plotz (1991) also found that exposure to copper reduced oxygen release in *Fucus vesiculosus*. However, very high levels of copper were used ( $10 \text{ mg L}^{-1}$ ) and no growth measurements were recorded, making a comparison between the sensitivity of the two factors impossible.

By taking a comparative approach, it is possible to estimate the relative sensitivity of photosynthesis and growth to copper exposure. This can provide basic information on mechanisms of copper toxicity, by indicating to what extent the metal impacts upon the photosynthetic apparatus.

#### 4.2 Aims

In an attempt to further understand the mechanisms of copper toxicity in *G. longissima*, the effect of copper on a range of physiological test endpoints was assessed. To enable direct comparisons, a series of studies measuring, growth, photosynthesis and respiration were carried out.

Although the results from the previous chapter indicated no obvious differences in copper tolerance between populations (as measured by differences in growth or copper accumulation), there is still a possibility that differences do exist. This was investigated further in two experiments comparing growth, photosynthetic and respiratory responses of two populations. Each experiment is described and discussed separately with the rationale

for the next experiment given. The results of all the experiments are summarised in the general discussion.

### **4.3 Materials and Methodology**

#### *4.3.1 Method for oxygen evolution measurements*

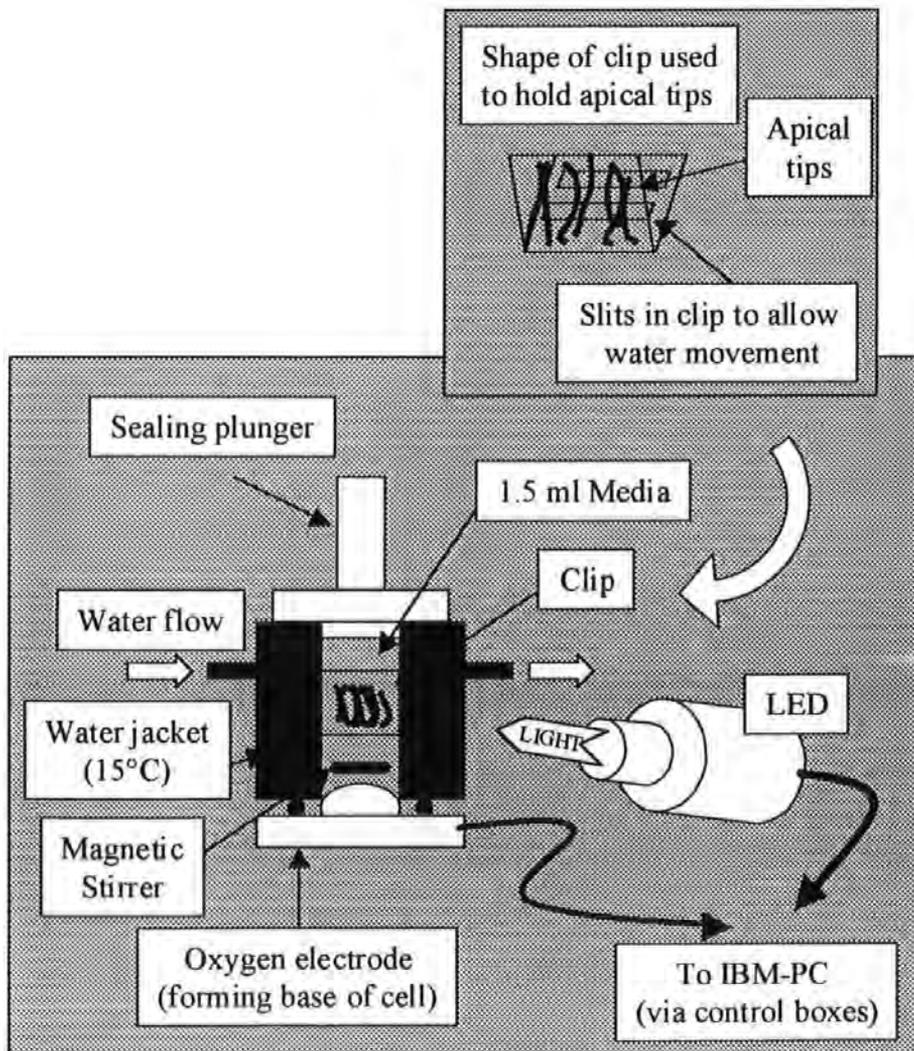
Photosynthesis and respiration rates were measured by changes in oxygen evolution using a modified Clark type oxygen electrode (see Fig. 4.1). The apparatus comprised of a variable volume (0.2-2.5ml) sealed chamber (Hansatech DW2) containing a magnetic stirring bar, controlled by a motorised base plate. The electrode, which seals to the chamber sides forming the base of the chamber, was connected to an IBM PC via an external control box and an internal PC card (Hansatech IF 2). Light to the chamber was provided via two banks of 'Ultra bright' red light emitting diode (LED) arrays. Light levels were controlled from the IBM-PC via the light control box (Hansatech CBD-1) and were calibrated from within the chamber using the Hansatech Quantitherm Light meter and thermometer (QRT 1). The water temperature was regulated using a circulating water jacket surrounding the chamber connected to a thermostatically controlled water bath and calibrated from within the chamber using the Quantitherm meter.

The apparatus was controlled from the IBM-PC using the software package 'Leafdisc'. This enabled the programming of a number of light steps. The duration of each light step was set at 120 seconds.

Prior to each experiment, the electrode was cleaned and calibrated. The electrode was polished using an aluminium oxide paste. A potassium chloride (50% saturated) electrolyte solution was used. The system was calibrated using the nitrogen purge method

(Hansatech Instruments Ltd., 1995). Oxygen concentrations were calculated using Green & Carritt's (1969) oxygen content for seawater tables.

Samples were placed in the chamber containing 1.5ml of aerated culture media and left in the dark to stabilise prior to recording (typically ~5 minutes.). Oxygen evolution rates were then calculated from a 100-second rate window taken from the last section of each 120-second light step.



**Fig. 4.1** Apparatus used to measure the rate of oxygen evolution of *G. longissima* apical tips. See text for details.

The chamber for the oxygen electrode was designed primarily for use with extracted chloroplasts and microalgal samples. Therefore, it was necessary to develop a

method of maintaining the *G. longissima* apical tips in position within the chamber, preventing them from coming into contact with the stirrer and the electrode membrane on the base of the chamber.

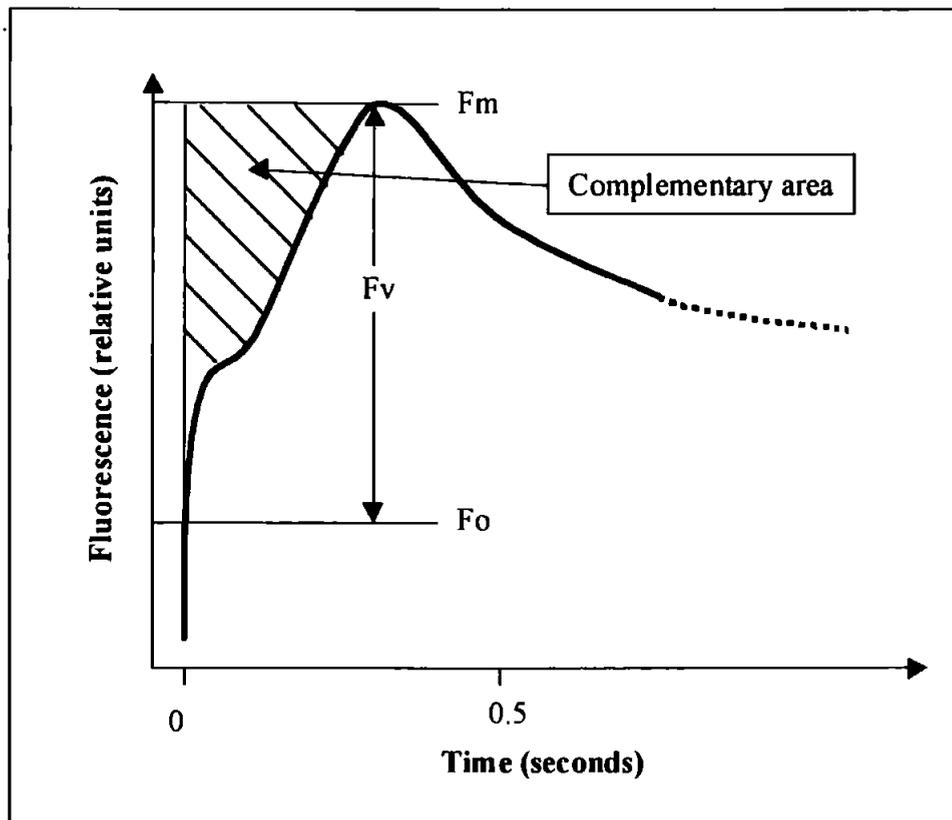
The technique used for these experiments was to hold the tips in a purpose built acetate 'clip' that could be lowered into the chamber and orientated so that the tips were receiving the maximum light from the two light sources. The light sources were positioned on each side of the chamber, thus causing the minimum amount of self shadowing. The clip was perforated with slits so that adequate circulation within the chamber could be maintained (see Fig. 4.1).

#### 4.3.2 *Chlorophyll fluorescence measurements*

When the photosynthetic apparatus (either in a whole plant or in an extracted chloroplast suspension) is illuminated the absorbed energy flows through one of three pathways. It is either utilised in photochemistry or re-emitted as heat or fluorescence. The measurement of chlorophyll fluorescence induction kinetics has become a relatively common method of assessing photosynthetic efficiency in higher plants (Bolhar-Nordenkampf *et al.*, 1989). Studies investigating the relationship between the amount of light absorbed and the changes in chlorophyll fluorescence have led to a series of fluorescence parameters being defined that are considered to relate to the photosynthetic efficiency of the plant. Dark adapting a leaf in total darkness for several minutes and then illuminating it produces a characteristic fluorescence induction curve, often termed the Kautsky curve or effect after its discoverer (Kautsky & Hirsch 1931, Fig. 4.2). The changes in fluorescence during the initial illumination have been shown to be related to the photochemical processes of PSII. Points along this simplified curve are defined as follows: The initial fluorescence ( $F_0$ ) which is considered to represent the emission of open (fully

oxidised) reaction centres in PSII prior to illumination.  $F_m$ , the maximal fluorescence observed when all of the reaction centres are closed (fully reduced) due to saturating light.  $F_v$ , variable fluorescence, is simply the difference between  $F_o$  and  $F_m$  ( $F_m - F_o$ ) and  $F_v/F_m$  is simply the calculated ratio  $(F_m - F_o)/F_m$ .  $F_v/F_m$  is considered an estimate of photosynthetic efficiency, specifically the function of PSII. The area above the Kautsky curve between  $F_o$  and  $F_m$  (complementary area) is considered to be proportional to the pool size of the electron acceptors on the reducing side of PSII (Bolhar-Nordenkamp *et al.*, 1989).

A Hansatech Plant Efficiency Analyser (PEA) was used to measure the chlorophyll fluorescence of *G. longissima*, as another measure of photosynthetic function following copper exposure. This apparatus is a fast (0.01ms) time resolving non-modulated fluorometer, which can measure, among other parameters,  $F_o$  and  $F_m$ . The apparatus



**Fig. 4.2.** Kautsky curve showing characteristic fast phase kinetics of light induced chlorophyll fluorescence of dark adapted leaves. The fast phase typically occurs during the first second of illumination.  $F_o$  = initial fluorescence,  $F_m$  = maximal fluorescence and  $F_v$  = variable fluorescence.

consists of a variable actinic light source ( $0\text{-}3500\mu\text{mol m}^{-2} \text{s}^{-1}$ ), which comprises of an array of Red LEDs (650nm maximum) and fluorescence detector which is fitted with a high pass filter, only allowing light of a wavelength of 700 or higher to pass. This design ensures no actinic light from the LEDs is detected by the fluorescence sensor.

Because of the morphology of *G. longissima*, it was not thought to be possible to obtain a standardised surface area. This meant that only the area independent parameter  $F_v/F_m$  could be calculated. A preliminary evaluation of the technique looking at the effect of desiccation on *G. longissima* did however indicate that it was possible to show a considerable reduction in  $F_v/F_m$  due to water stress.

Prior to measurements, tips were dark adapted for at least 20 minutes using light tight clips and a light setting of 60% maximum was used (Both of these parameters were tested independently using algal material prior to experimentation). Because of the relatively small size of the single apical tips, the signal from the sensor required pre-amplification (Hansatech pre-amplifier).

#### **4.4 Experiment 1. The relationship between photosynthesis and irradiance in *G. longissima*.**

The purpose of this preliminary experiment was to obtain estimates of the photosynthetic parameters, which can be calculated by plotting the rate of oxygen evolution against irradiance (a so-called Photosynthesis versus Irradiance (PI) curve - see Fig. 4.3). Parameters calculated were:  $P_{\text{max}}$ , the maximal rate of photosynthesis;  $\alpha$ , the initial slope over the light limiting part of the curve;  $I_k$ , the saturating irradiance level for photosynthesis;  $I_c$ , the light compensation point and  $R$ , the rate of dark respiration.

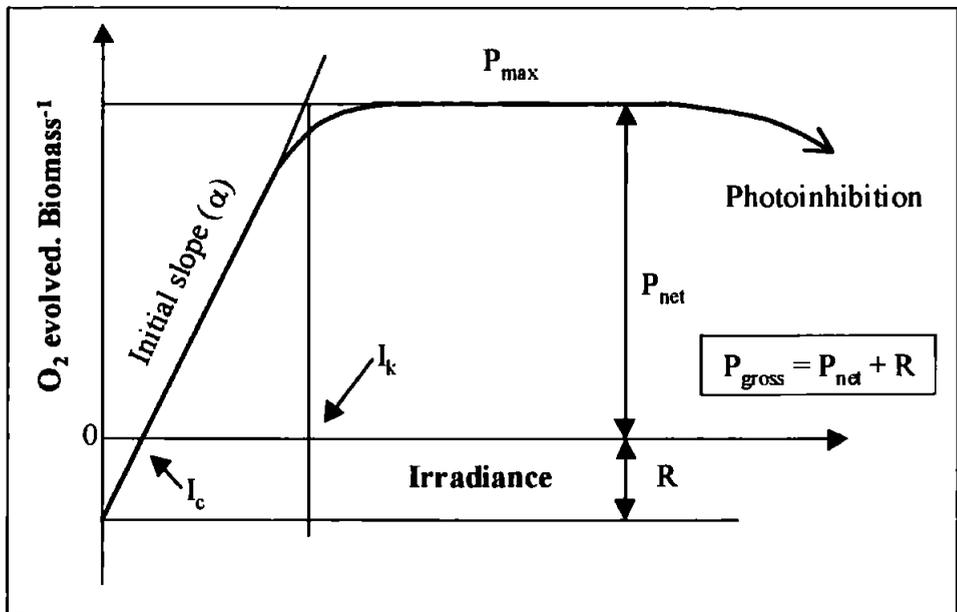


Fig. 4.3 A typical photosynthesis versus irradiance (PI) curve. The parameters shown are described in the text above.

#### 4.4.1 Materials and Methods

Material collected from St Just and held in the laboratory under stock culture conditions for five days was transferred to petri dishes and cultured under standard experimental conditions in a controlled environment chamber (CEC) for one week. There were three replicates, each containing ten 10mm apical tips and two treatments (control and 25 $\mu\text{g L}^{-1}$  copper). The 25 $\mu\text{g L}^{-1}$  copper treatment was chosen since this had been found to significantly reduce the relative growth rate (RGR) of *G. longissima* by approximately 50%. Growth rates were measured using image analysis. Oxygen evolution rates (expressed as  $\mu\text{mol O}_2 \text{ g}^{-1} (\text{fw}) \text{ min}^{-1}$ ) were measured using the ten apical tips at each of 12 light steps which increased from 0 to 642 $\mu\text{mol m}^{-2} \text{ s}^{-1}$  PAR. Following the measurement of oxygen evolution, apical tips were blotted dry and the fresh weight of samples measured.

#### 4.4.2 Results and discussion

Growth data from the seven day experiment revealed a significant reduction in RGR due to the copper ( $n=6$ ,  $P=0.002$ , student t-test). Controls grew  $4.52\% \text{ day}^{-1}$  while the  $25\mu\text{g L}^{-1}$  treatments grew  $3.02\% \text{ day}^{-1}$ , a 33% reduction in growth.

No significant difference could be found between the control and copper treatments for any of the parameters calculated from the PI curves (Fig. 4.4, Table 4.1).

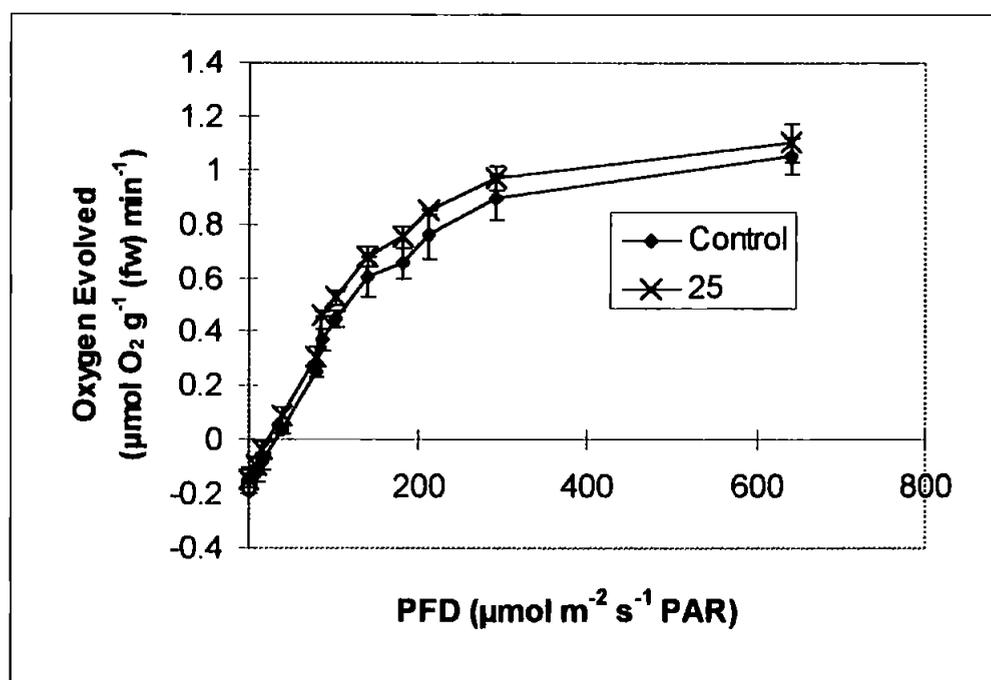


Fig. 4.4 Mean ( $n=3$ ) oxygen evolution of *G. longissima* apical tips over a range of irradiances following exposure to control or  $25\mu\text{g L}^{-1}$  copper treatment for one week. Error bars show 95% confidence intervals.

Treatment	$I_c$	$I_k$	$P_{max}$	$\alpha$	R
	PFD ( $\mu\text{mol m}^{-2} \text{s}^{-1} \text{ PAR}$ )		$(\mu\text{mol O}_2 \text{ g}^{-1}(\text{fw}) \text{ min}^{-1})$		
Control	31.6	206	0.970	0.005	0.145
Copper ( $50\mu\text{g L}^{-1}$ )	23.1	201	0.971	0.005	0.150

Table 4.1 Photosynthetic parameters from *G. longissima* calculated from the PI curves measured after exposure to two treatments for one week as shown in Fig. 4.3.

No evidence of photoinhibition at high irradiances could be found. As a result of these estimates,  $400\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR was chosen as a suitable level of irradiance for estimating  $P_{\text{max}}$  in the subsequent experiments.

Exposure of apical tips for one week to  $25\mu\text{g L}^{-1}$  of copper inhibited growth, a finding similar to that found previously (Chapter 3). However, exposure to this level of copper had no significant effect on oxygen evolution. These results indicate that growth was more susceptible to this level of copper exposure than was oxygen evolution.

The purpose of this preliminary experiment was to establish the optimal irradiance to use for net photosynthesis measurements over a wider range of copper treatments. While it would have been preferable to measure the various parameters from the PI curves, the time required to do this made it impractical. Hence subsequent comparisons were made using  $P_{\text{max}}$  which was measured at  $400\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR.

#### **4.5 Experiment 2. The effect of copper on $P_{\text{max}}$ and respiration.**

The previous experiment's findings were expanded upon by exposing *G. longissima* material to a greater range of copper treatments in order to investigate the comparative level at which oxygen evolution was affected.

##### *4.5.1 Materials and methods*

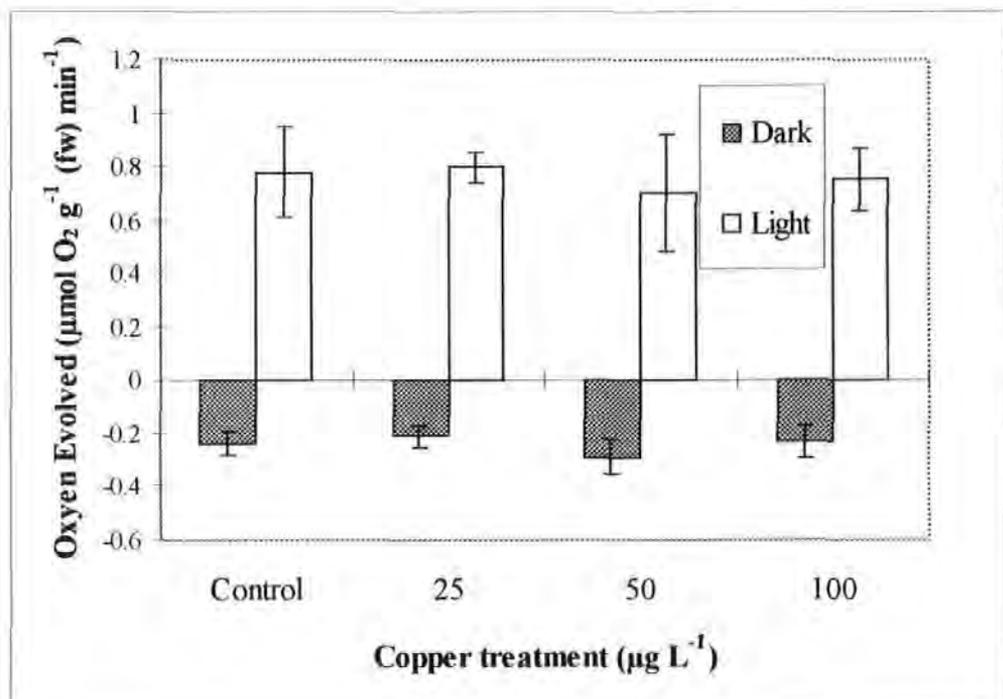
*Gracilariopsis longissima* material collected from Mylor creek and cultured for seven weeks under standard stock culture conditions was used in the following experimental design. Four replicates of four treatments (Control, 25, 50,  $100\mu\text{g L}^{-1}$  copper) were cultured under standard experimental culture conditions for one week. Each replicate

contained ten 10mm apical tips placed into petri dishes. Growth rates were calculated using image analysis.

Following one week's culture, oxygen evolution rates were measured at 0 (Respiration) and  $400\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR ( $P_{\text{max}}$ ). Samples were blotted dry and their fresh weights measured. Sub-samples from each replicate were randomly selected and used to measure fluorescence ( $F_v/F_m$ ) using the Hansatech fluorometer (PEA).

#### 4.5.2 Results and discussion

Exposure to the range of copper treatments from  $25\text{-}100\mu\text{g L}^{-1}$  failed to result in any significant change in the photosynthetic parameter  $P_{\text{max}}$  ( $P=0.834$ , Fig. 4.5, Table 4.2), a similar oxygen evolution rate ( $\sim 0.8\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$ ) to that estimated from the PI curve in Experiment 1. There was also no significant difference in the respiration rate ( $P=0.282$ , Table 4.3) which was  $\sim 0.2\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$  for all treatments (Fig. 4.5).



**Fig. 4.5** Mean ( $n=4$ ) oxygen evolution in the dark (respiration) and in the light ( $P_{\text{max}}$ ) of *G. longissima* apical tips following exposure to a range of copper treatments for one week. Error bars show 95% confidence intervals.

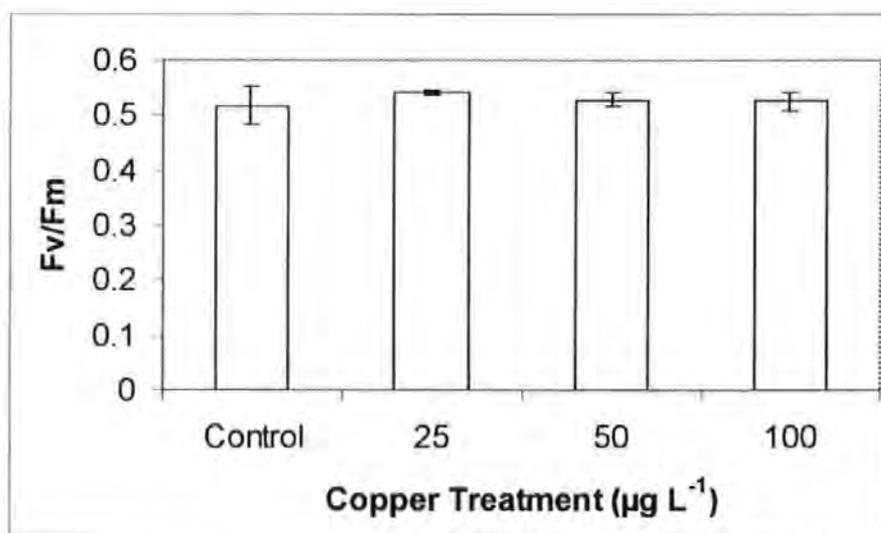
Source	SS	DF	MS	F-ratio	P-value
Between Treatments	0.021	3	0.007	0.290	0.834
Within Treatments	0.291	12	0.024		
Total (Corrected)	0.312	15			

**Table 4.2** One-way ANOVA for the net photosynthesis ( $P_{max}$ ) of *G. longissima* apical tips following exposure to a range of copper treatments for one week.

Source	SS	DF	MS	F-ratio	P-value
Between Treatments	0.013	3	0.004	1.43	0.282
Within Treatments	0.037	12	0.003		
Total (Corrected)	0.051	15			

**Table 4.3** One-way ANOVA for the dark respiration of *G. longissima* apical tips following exposure to a range of copper treatments for one week.

Similarly no difference could be found in the chlorophyll fluorescence parameter  $F_v/F_m$  (Kruskal-Wallis test,  $n=16$ , test statistic = 3.203, P-value = 0.361), with the apical tips being found to have a mean  $F_v/F_m$  value of 0.528 (Fig. 4.6).



**Fig. 4.6** Mean ( $n=4$ )  $F_v/F_m$  of *G. longissima* apical tips after exposure to a range of copper treatments for one week. Error bars show 95% confidence intervals.

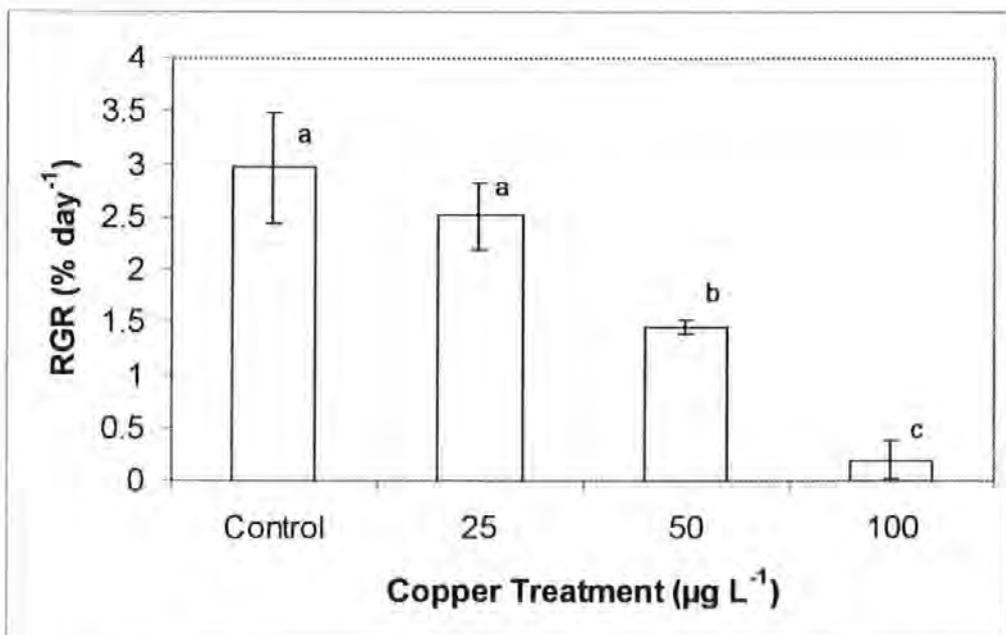
By contrast, growth rates were significantly reduced by increasing copper treatments ( $P<0.001$ , Table 4.4). A Duncans multiple range test found significant differences between three of the four treatments. In this instance, no significant difference could be found between the control and  $25\mu\text{g L}^{-1}$  copper treatment (Fig. 4.7). The RGR of

the  $100\mu\text{g L}^{-1}$  copper treatment ( $0.198\% \text{ day}^{-1}$ ) was approximately 7% of the control ( $2.969\% \text{ day}^{-1}$ ).

Source	SS	DF	MS	F-ratio	P-value
Between Treatments	2.903	3	0.968	75.63	<0.001
Within Treatments	0.141	11	0.013		
Total (Corrected)	3.044	14			

**Table 4.4** One-way ANOVA for the RGR of *G. longissima* apical tips exposed to a range of copper treatments for week. Data was square root transformed.

The aim of this experiment was to make a comparison between the growth, photosynthesis and respiration rates measured over a range of copper exposures. The results show that despite growth being greatly reduced by increasing copper exposure, photosynthesis ( $P_{\text{max}}$ ) and dark respiration were not affected. Similarly no significant effect on fluorescence ( $F_v/F_m$ ) could be seen (Fig. 4.6). These results indicate that growth is affected at considerably lower levels of copper exposure than photosynthesis.



**Fig. 4.7** Mean ( $n=4$ ) RGR of *G. longissima* apical tips exposed to a range of copper treatments for one week. Error bars show 95% confidence intervals. Letters designate statistically significant groupings using a Duncans multiple range test.

The chlorophyll fluorescence results are consistent with the oxygen evolution results, indicating that PSII is functioning normally in the copper exposed material over the range of copper treatments used. The results are consistent with the view that copper is causing an uncoupling of photosynthesis from growth. While  $F_v/F_m$  is considered an estimate of photosynthetic efficiency (Krause & Weiss, 1991), there is some debate over the measurement and definition of fluorescence parameters and their interpretation (Strasser & Strasser 1995). The Hansatech PEA was developed primarily for use with higher plants and little information exists on its functional applicability to macroalgae. In light of this, the interpretation of these chlorophyll fluorescence measurements is made with caution although in this case they do appear to correlate with the oxygen evolution measurements.

#### **4.6 Experiment 3. Comparison of the photosynthetic response of two populations following 20 days exposure to copper.**

The previous experiment demonstrated that growth was affected at significantly lower levels of copper exposure than photosynthesis or respiration and that neither photosynthesis or respiration are affected by levels of copper as high as  $100\mu\text{g L}^{-1}$ . One possibility considered was that the same levels of exposure for a longer time period might be sufficient to induce a photosynthetic response. It was thought possible that extended exposure could reveal differences between populations, in either growth or copper accumulation that had not previously been observed. To test these hypotheses, apical tips from two populations were exposed to copper for 20 days and the effects of this extended exposure period on growth, photosynthesis and copper accumulation were measured.

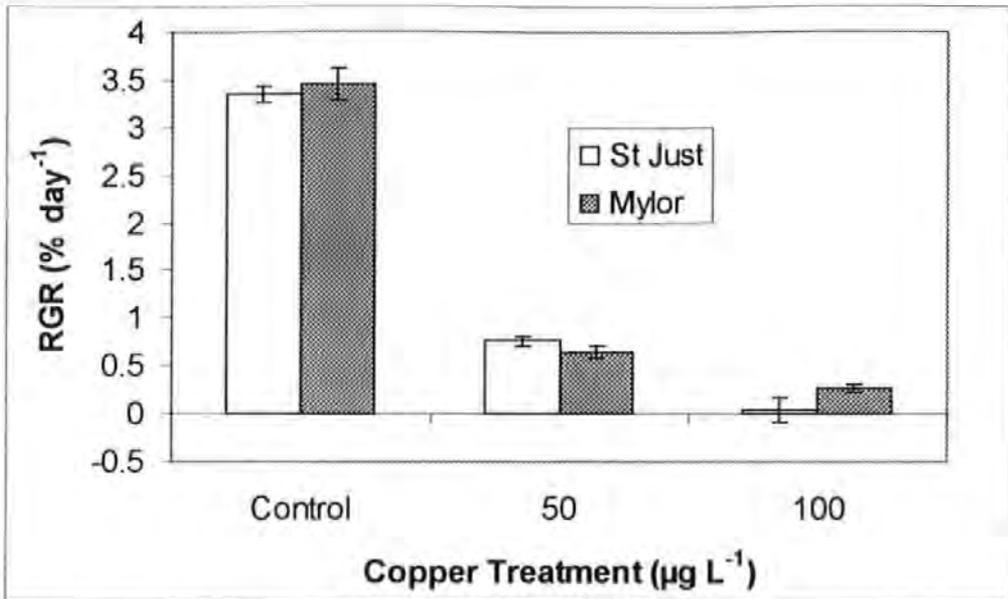
#### 4.6.1 *Materials and Methods*

Apical tips from samples of two populations from within the Fal Estuary (Mylor and St Just) which had been in stock culture for 10 weeks were used in the following experimental design. Five replicates of three treatments (control, 50 and 100 $\mu\text{g L}^{-1}$ ) were placed in a CEC for 20 days. Each replicate consisted of ten, 10mm apical tips placed in a petri dish. Media solutions were changed on every third day. Growth rates were measured using image analysis on every third day (days 0,3,6,9,12,15, and 18), prior to each media change. On Day 20, oxygen evolution rates were calculated at 0 and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR.

Following experimentation, samples were blotted dry and their fresh weights measured. Samples were subsequently frozen, freeze dried, digested and analysed for copper content using the small digestion vessel method, as described in Chapter 3.

#### 4.6.2 *Results and discussion*

Culturing the apical tips in elevated levels of copper for 20 days caused a significant reduction in growth ( $P < 0.001$ , Fig. 4.8, Table 4.5). After 18 days, the 50 $\mu\text{g L}^{-1}$  copper treatment resulted in a reduction of RGR of approximately 80%. The growth reduction in the 100 $\mu\text{g L}^{-1}$  treatment was more than 90%. This reduction was similar to the level found previously after only seven days. No significant interaction ( $P = 0.195$ ) or differences between the response of the two populations ( $P = 0.391$ ) could be found (Table 4.5).



**Fig. 4.8** Mean (n=5) RGR of apical tips from two populations of *G. longissima* exposed to a range of copper treatments for 18 days. Error bars show 95% confidence intervals.

Source	SS	DF	MS	F-ratio	P-value
Treatment	60.268	2	30.134	647.820	<0.001
Population	0.035	1	0.035	0.760	0.391
Interaction	0.163	2	0.082	1.75	0.194
Residual	1.116	24	0.047		
Total (Corrected)	61.583	29			

**Table 4.5** Two-way ANOVA for the RGR of two populations of *G. longissima* exposed to a range of copper treatments for 18 days.

When the increase in length of the apical tips is plotted over the 18 days it can be seen that the effect of each treatment was relatively constant (Fig. 4.9). No change in response can be seen over the 18 days.

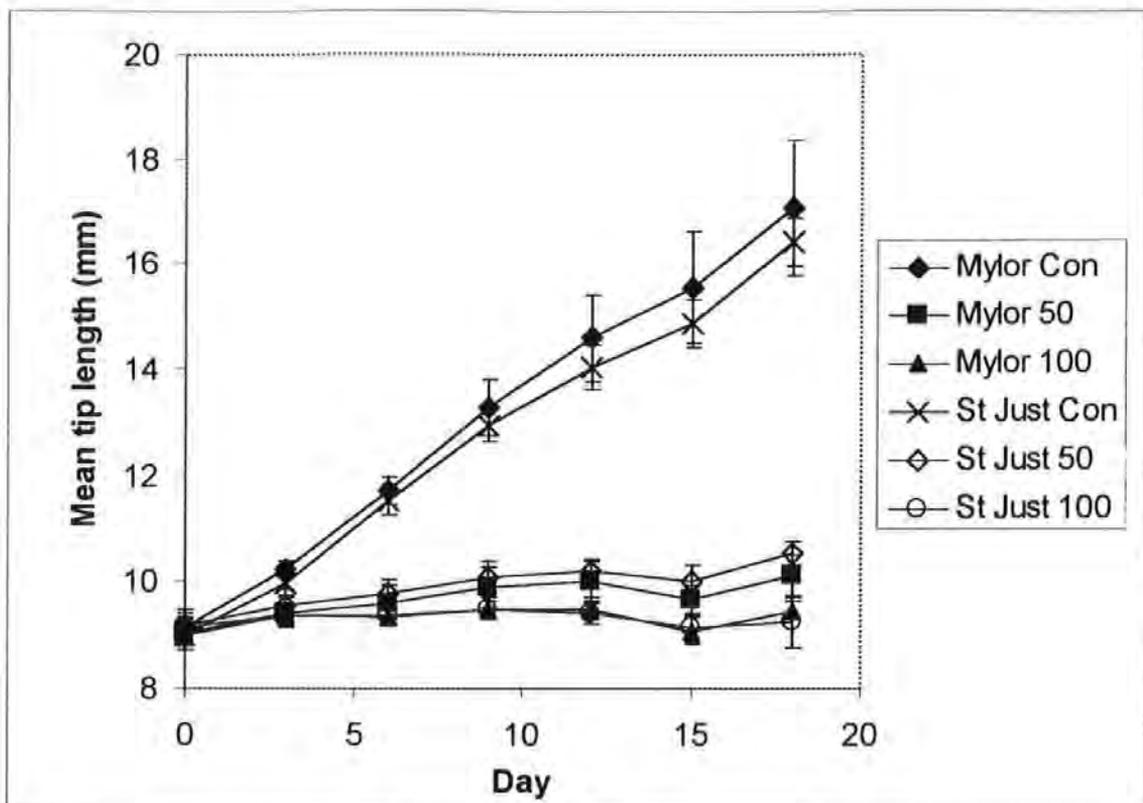
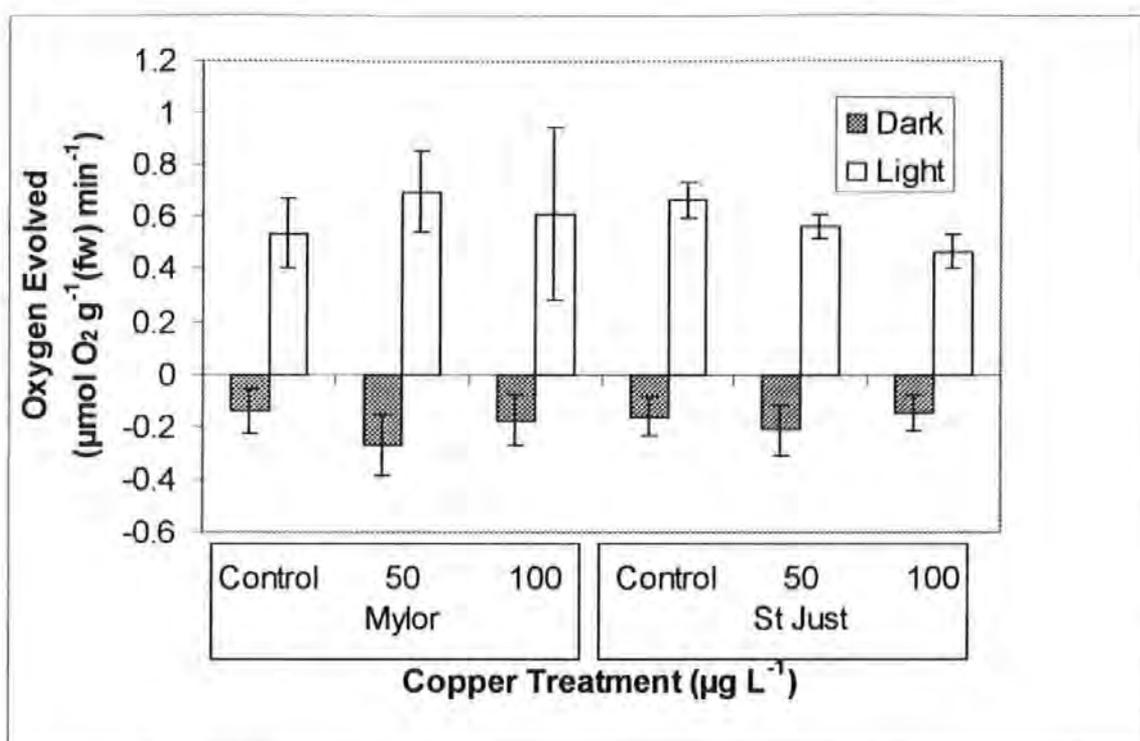


Fig. 4.9 Mean (n=5) length of apical tips from two populations of *G. longissima* exposed to a range of copper treatments for 18 days. Con = Control, 50 & 100 = copper treatments in  $\mu\text{g L}^{-1}$ . Error bars show 95% confidence intervals.

Despite this large reduction in growth, oxygen evolution rates at  $P_{\text{max}}$  ( $P=0.571$ ) and respiration ( $P=0.134$ ) were found to be unaffected by copper (Fig. 4.10, Table 4.6 & 4.7). Both  $P_{\text{max}}$  ( $\sim 0.6 \mu\text{mol O}_2 \text{ g}^{-1} (\text{fw}) \text{ min}^{-1}$ ) and respiration ( $\sim 0.2 \mu\text{mol O}_2 \text{ g}^{-1} (\text{fw}) \text{ min}^{-1}$ ) were similar to the levels found in the previous experiment.

Source	SS	DF	MS	F-ratio	P-value
Treatment	0.025	2	0.013	0.590	0.571
Population	0.011	1	0.01	0.490	0.497
Interaction	0.072	2	0.036	1.68	0.227
Residual	0.258	12	0.021		
Total (Corrected)	0.365	17			

Table 4.6 Two-way ANOVA for net photosynthesis ( $P_{\text{max}}$ ) of two populations of *G. longissima* following exposure to a range of copper treatments for 18 days.

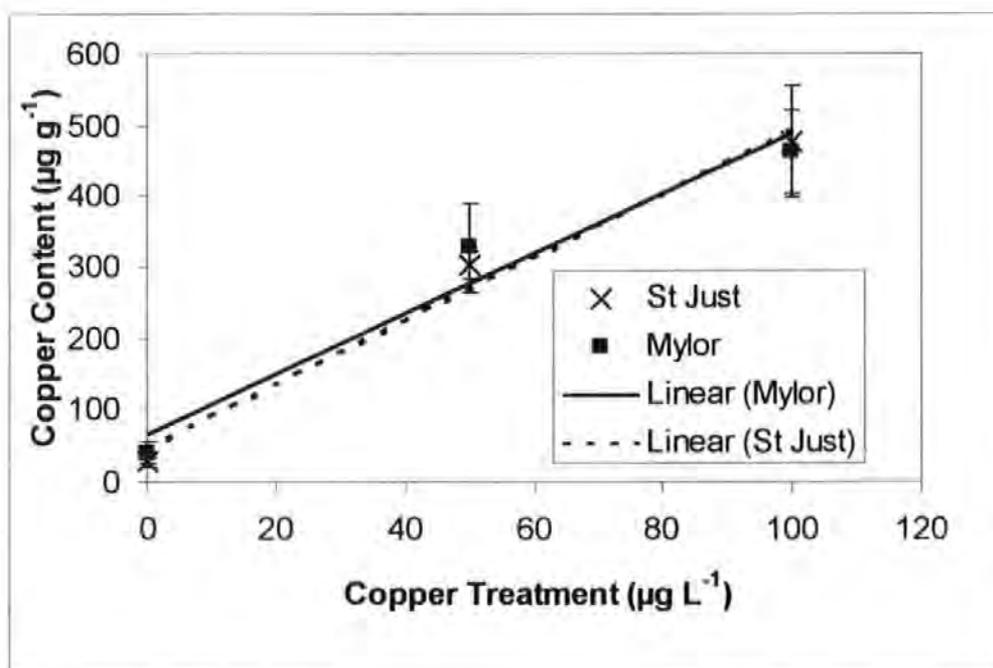


**Fig. 4.10** Mean (n=3) oxygen evolution of apical tips from two populations of *G. longissima* following exposure to a range of copper treatments for 20 days.  $P_{max}$  (Light) and dark respiration (Dark) rates were calculated. Error bars show 95% confidence intervals.

Source	SS	DF	MS	F-ratio	P-value
Treatment	0.030	2	0.015	2.390	0.134
Population	0.002	1	0.002	0.360	0.562
Interaction	0.005	2	0.002	0.400	0.681
Residual	0.075	12	0.006		
Total (Corrected)	0.113	17			

**Table 4.7** Two-way ANOVA for the dark respiration of two populations of *G. longissima* following exposure to a range of copper treatments for 20 days.

Analysis of tissue samples for copper content following oxygen measurements found that the concentration of copper in the apical tips increased significantly with increasing copper exposure ( $P < 0.001$ , Fig 4.11, Table 4.8). After 20 days the concentrations increased from a control level of  $\sim 40 \mu\text{g g}^{-1}$  to over  $400 \mu\text{g g}^{-1}$  in the  $100 \mu\text{g L}^{-1}$  treatment. No significant difference could be found however between the copper accumulation in the two populations.



**Fig. 4.11** Mean (n=4) copper concentration in apical tips from two populations of *G. longissima* following exposure to a range of copper treatments for 20 days. Error bars show 95% confidence intervals.

Source	SS	DF	MS	F-ratio	P-value
Treatment	31.895	2	15.948	264.830	<0.001
Population	0.136	1	0.136	2.260	0.150
Interaction	0.196	2	0.098	1.630	0.224
Residual	1.084	18	0.060		
Total (Corrected)	36.781	23			

**Table 4.8** Two way ANOVA for the copper concentration in apical tips of two populations of *G. longissima* following exposure to a range of copper treatments for 20 days. Data was Ln transformed.

Pooled data from the two populations revealed a significant positive linear relationship between the concentration in the media and the concentration in the tissue.

$$[Cu_{\text{tissue}}] (\mu\text{g g}^{-1}) = 4.394 \times [Cu_{\text{treatment}}] (\mu\text{g L}^{-1}) + 51.84 \quad (n=24, R^2=92.039, p=0.001)$$

This equation indicates a constant concentration factor of approximately 4400. This concentration factor is considerably higher than the 1600 estimated in the accumulation experiment in the previous chapter (Chapter 3, Experiment 4) after one week of exposure.

This represents a relatively linear increase in concentration factors over time ( $1600/7(\text{days}) \times 20(\text{days}) = 4571$ ). The concentration factor appears to be a function of the duration of the exposure.

Prolonged exposure to elevated levels of copper that are clearly reducing growth and resulting in significant accumulation of the metal could not be seen to be altering photosynthesis or respiration. This is consistent with the findings of the previous experiments.

#### **4.7 Experiment 4. Photosynthetic response following high levels of copper exposure.**

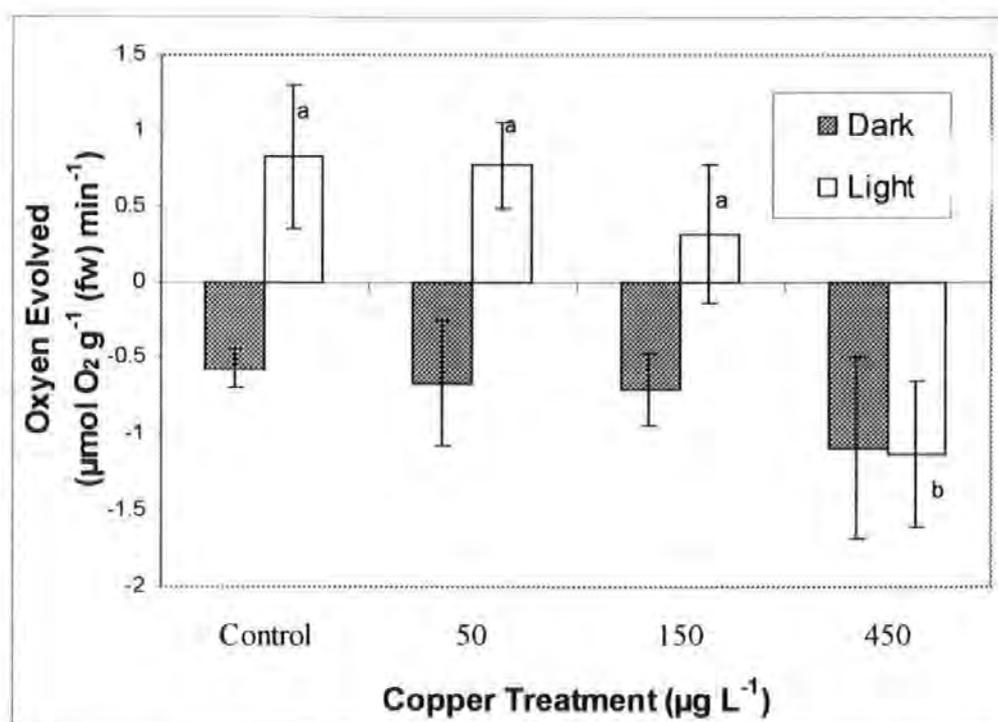
Since no inhibition of photosynthesis due to copper exposure had been recorded, a further experiment was run using a greater range of copper treatments. This included a much higher level ( $450\mu\text{g L}^{-1}$ ) similar to that which had been observed to result in the shrinkage of apical tips in previous growth inhibition experiments (Chapter 3).

##### *4.7.1 Materials and Methods*

Using the same culturing conditions and media as described for Experiment 3, apical tips from the Helford estuary (previously stock cultured for four weeks) were exposed to a wider range of copper levels (Control, 50, 150 &  $450\mu\text{g L}^{-1}$ ) for one week. Growth rates were measured using image analysis and oxygen evolution rates were calculated in the dark and under  $400\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. Following oxygen measurements samples were blotted dry and their fresh weights measured.

#### 4.7.2 Results and discussion

$P_{\max}$  was found to be significantly reduced ( $P=0.001$ ) following the exposure of apical tips to high levels of copper ( $450\mu\text{g L}^{-1}$ ) for one week (Fig. 4.12, Table 4.9). The  $P_{\max}$  of  $0.82\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$  in the control is comparable with previously recorded levels. By contrast, the  $P_{\max}$  of the  $450\mu\text{g L}^{-1}$  copper treatment was in fact negative ( $-1.08\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$ ) indicating that this level of copper had completely disrupted net photosynthesis. Respiration was not significantly affected by any copper treatment ( $P=0.335$ , Fig. 4.12, Table 4.10). There was some decrease in the amount of oxygen evolved in the  $150\mu\text{g L}^{-1}$  copper treatment, although this was not significant, possibly due to large amount of variability in the response.



**Fig. 4.12** Mean ( $n=3$ ) oxygen evolution in the dark (respiration) and in the light ( $P_{\max}$ ) of *G. longissima* apical tips following exposure to a range of copper treatments for one week. Letters designate statistically significant groupings using a Duncan's multiple range test (for photosynthesis). No significant treatment effect was found for respiration.

Source	SS	DF	MS	F-ratio	P-value
Between Treatments	7.159	3	2.506	17.360	0.001
Within Treatments	1.155	8	0.144		
Total (Corrected)	8.674	11			

**Table 4.9** One-way ANOVA for net photosynthesis ( $P_{max}$ ) of *G. longissima* apical tips following exposure to a range of copper treatments for one week.

Source	SS	DF	MS	F-ratio	P-value
Between Treatments	0.450	3	0.153	1.320	0.335
Within Treatments	0.931	8	0.116		
Total (Corrected)	1.391	11			

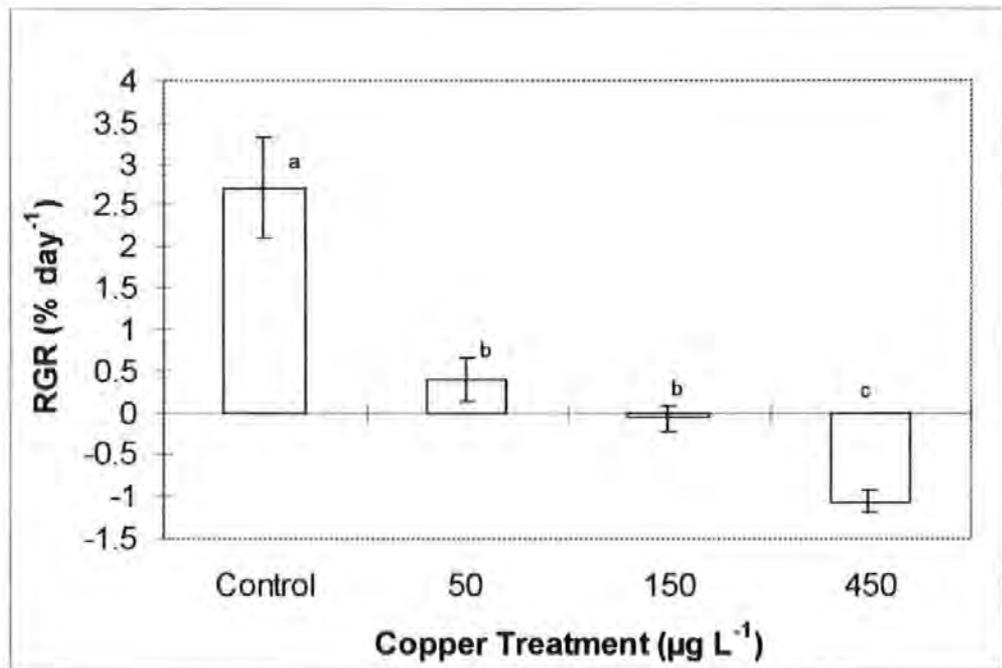
**Table 4.10** One-way ANOVA for the dark respiration of *G. longissima* apical tips following exposure to a range of copper treatments for one week.

RGRs were significantly reduced by copper treatment as low as  $50\mu\text{g L}^{-1}$  ( $P < 0.001$ , Fig. 4.13, Table 4.11) The level of exposure, which reduced photosynthesis, corresponded with actual shrinkage of apical tips (negative growth rate of  $-1.06\% \text{ day}^{-1}$ ) a similar amount to that seen previously at high levels of copper exposure.

Source	SS	DF	MS	F-ratio	P-value
Between Treatments	22.948	3	7.649	79.730	<0.001
Within Treatments	0.767	8	0.096		
Total (Corrected)	23.716	11			

**Table 4.11** One-way ANOVA for the RGR of *G. longissima* apical tips exposed to a range of copper treatments for one week.

These results indicate that copper only inhibits photosynthesis at exceptionally high copper treatments where considerable damage to apical tips can be seen in the form of shrinkage and pigment bleaching. This would suggest that the toxic effect of copper is acting directly upon oxygen evolution. Growth and photosynthesis (as measured by oxygen evolution) are clearly uncoupled by copper in *G. longissima*.



**Fig. 4.13** Mean ( $n=3$ ) RGR of apical tips from *G. longissima* exposed to a range of copper treatments for one week. Letters designate statistically significant groupings using a Duncans multiple range test. Error bars show 95% confidence intervals.

#### 4.8 Experiment 5. Measurement of PS using an alternate light source.

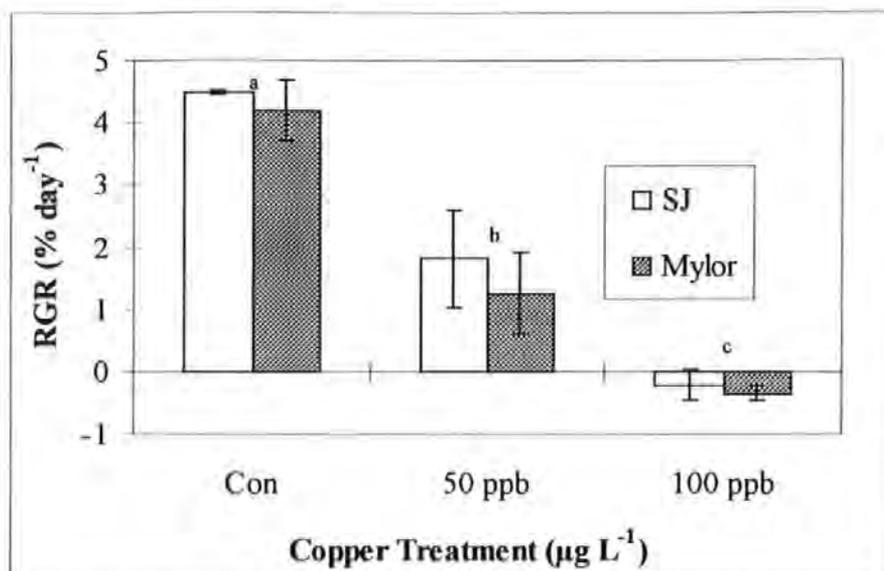
In the first four experiments material was exposed to copper under standardised experimental culture conditions (described in Chapter 2) but photosynthesis was then measured at  $400\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR using ultra bright red LEDs. It was hypothesised that an effect of copper exposure on photosynthesis might only be apparent at the same irradiance and spectral quality of lights under which the apical tips were cultured. The measurement of photosynthesis using red LEDs may have been giving misleading information compared with the actual photosynthetic response occurring during culture. To test this possibility, oxygen evolution was measured at a similar level and quality of light to which the material was cultured, using a small fluorescent tube instead of the LED array.

#### 4.8.1 *Materials and methods*

Material from the Mylor and St Just populations was collected and cultured under stock culture conditions for two weeks prior to experimentation. For each population, three replicates of three treatments were used; control, 50 and 100  $\mu\text{g L}^{-1}$  copper. Samples were cultured for one week under standard experimental culture conditions, with two alterations; the day length was changed from 12 to 16 hours (i.e. 16:8 light dark) and the irradiance increased to 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. Growth rates were calculated using image analysis. The standard red ultra-bright LED light source used previously for the photosynthesis measurements was replaced with two fluorescent tubes which provided the same level of irradiance as in the growth cabinets. Oxygen evolution rates were then measured in the dark and under fluorescent light.

#### 4.8.2 *Results and discussion*

Growth was significantly reduced by copper treatment ( $P < 0.001$ , Fig. 4.14. Table 4.12). The addition of 50  $\mu\text{g L}^{-1}$  copper resulted in more than 60% reduction in growth compared with the controls (which had high growth rates of over 4%  $\text{day}^{-1}$ ). By comparison, the 100  $\mu\text{g L}^{-1}$  copper treatment resulted in a RGR slightly less than zero indicating that some shrinkage was occurring. This RGR in the 100  $\mu\text{g L}^{-1}$  copper treatment is comparable with the reduction found in the other experiments in this chapter at 100  $\mu\text{g L}^{-1}$ .

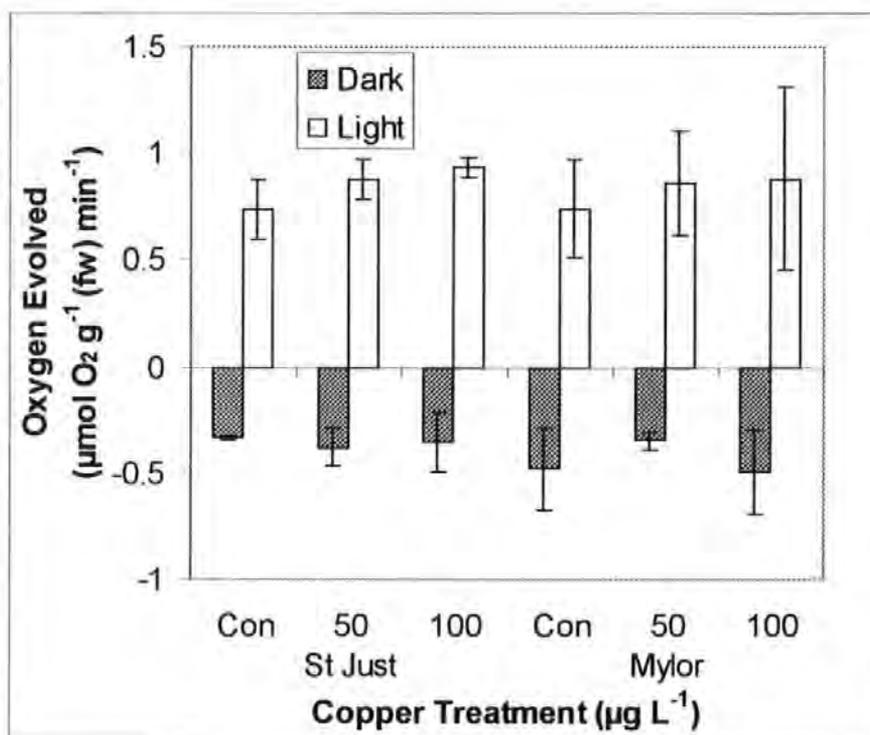


**Fig. 4.14** Mean (n=3) RGR of apical tips from two populations of *G. longissima* exposed to a range of copper treatments for one week. Letters designate statistically significant groupings using a Duncans multiple range test (pooled data). Error bars show 95% confidence intervals.

Source	SS	DF	MS	F-ratio	P-value
Treatment	65.162	2	32.581	185.330	<0.001
Population	0.473	1	0.473	2.690	0.127
Interaction	0.139	2	0.069	0.390	0.683
Residual	2.110	12	0.176		
Total (Corrected)	67.883	17			

**Table 4.12** Two-way ANOVA for the RGR of two populations of *G. longissima* exposed to a range of copper treatments for one week

Measuring oxygen evolution using fluorescent tubes of the same irradiance and spectral quality as in the growth cabinets failed to indicate any difference in photosynthesis due to copper treatments (Fig. 4.15). No significant difference could be seen in either net photosynthesis (P=0.364) or respiration (P=0.652) over the range of copper treatments used (Tables 4.13 & 4.14). No population effect for either parameter could be seen (Tables 4.13 and 4.14). The fact that the net photosynthetic rate ( $\sim 0.8 \mu\text{mol O}_2 \text{ g}^{-1} \text{ (fw) min}^{-1}$ ) was comparable to that measured using the ultra-bright Red LEDs indicates that the level of lighting used in the CEC was saturating for growth.



**Fig. 4.15** Mean ( $n=3$ ) oxygen evolution of apical tips from two populations of *G. longissima* following exposure to a range of copper treatments for one week.  $P_{\max}$  (Light) and dark respiration (Dark) rates were calculated. Error bars show 95% confidence intervals.

Source	SS	DF	MS	F-ratio	P-value
Treatment	0.094	2	0.047	1.100	0.364
Population	0.023	1	0.023	0.050	0.821
Interaction	0.023	2	0.011	0.030	0.974
Residual	0.511	12	0.043		
Total (Corrected)	0.609	17			

**Table 4.13** Two-way ANOVA for the net photosynthesis ( $P_{\max}$ ) of two populations of *G. longissima* following exposure to a range of copper treatments for one week.

Source	SS	DF	MS	F-ratio	P-value
Treatment	0.012	2	0.006	0.440	0.652
Population	0.031	1	0.031	2.220	0.163
Interaction	0.030	2	0.015	1.080	0.372
Residual	0.167	12	0.014		
Total (Corrected)	0.240	17			

**Table 4.14** Two-way ANOVA for the dark respiration of two populations of *G. longissima* following exposure to a range of copper treatments for one week.

The results concur with the findings of the previous photosynthesis and growth response experiments i.e. no measurable change in photosynthesis or respiration was found at copper levels as high as  $100\mu\text{g L}^{-1}$ , despite significant reduction in growth.

#### 4.9 Discussion

The method developed to measure changes in oxygen production in the apical tips was successful. The shape of the PI curve obtained from exposing apical tips to a sequence of increasing irradiance levels is similar to the 'classic' PI response shown in Fig 4.3. The estimated  $P_{\text{max}}$  of about  $0.8\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$  is higher than a previous estimate of  $P_{\text{max}}$  (measured at a PFD of  $250\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in the closely related species *Gracilaria verrucosa*, of  $0.38\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$  (Levy *et al.*, 1990). Their reported respiration rate,  $0.12\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$ , was comparable with the value found in this study ( $0.15\mu\text{mol O}_2 \text{g}^{-1} (\text{fw}) \text{min}^{-1}$ ).

In all five experiments described in this chapter, growth was found to be affected by lower copper concentrations than photosynthesis. In the only experiment where photosynthesis was found to be affected (Experiment 4), levels of copper exposure were very high ( $450\mu\text{g L}^{-1}$ ) which also resulted in considerable shrinkage of the apical tips. This finding is consistent with the level reported to inhibit photosynthesis ( $203\mu\text{g L}^{-1}$ ) in a non-resistant strain of *Enteromorpha compressa* (Reed & Moffat, 1983). The finding that fluorescence ( $F_v/F_m$ ) was not affected by copper treatments as high as  $100\mu\text{g L}^{-1}$  (Experiment 1) was consistent with oxygen evolution results.

$F_v/F_m$  is the only area independent induction fluorescence parameter that can be measured. Although this parameter is considered a rapid means of assessing damage to PSII, if the area of thallus being used to measure fluorescence is standardised then a

number of other parameters can be compared which may be used to indicate the relative effect of copper on particular aspects of the photosynthetic pathway (Bolhar-Nordenkampf, *et al.*, 1989). This possibility is explored in the next chapter.

No significant difference could be found between the response of the two populations to RGR, copper accumulation or oxygen evolution (Experiments 3 and 5). This is consistent with the previous experiments (Chapter 3) which found no major differences in growth or copper accumulation between the Mylor and St Just populations, despite the Mylor population originating from a more contaminated site than the St Just population. The long period of culturing prior to the start of Experiment 3 (ten weeks) may have also resulted in the two populations responding similarly. During this period, the two populations were cultured under the same conditions and it is possible that this led to the acclimated samples starting with similar copper concentrations.

The prolonged exposure to copper in Experiment 3 resulted in more copper accumulation in the apical tips than in previous experiments together with further growth reduction, but still no significant change in photosynthesis or respiration was recorded. The higher concentration factor after 20 days of exposure to copper implies that longer periods of exposure could result in further accumulation in the apical tips than observed here.

Bryan (1969) found that concentration factors of zinc in *Laminaria digitata* increased almost linearly over 32 days and showed no sign of saturation. Amado Filho *et al.* (1996) also found no evidence of zinc saturation after 21 days exposure in the brown alga *Padina gymnospora*. This was consistent with Karez *et al.* (1994) who found that the accumulation of zinc was still increasing over a shorter period in the same species. At some stage however, some form of saturation or equilibrium might be expected between the seawater and algal metal concentrations. Hall (1981) found that copper accumulation in

*Ectocarpus siliculosus* ceased after periods of incubation varying between 2 and 14 days, depending upon the copper concentration in the medium. It appears that in *G. longissima*, saturation does not occur for as least 20 days at either 50 or 100 $\mu\text{g L}^{-1}$  copper.

The hypothesis that oxygen evolution is only affected when measured using the same irradiance under which algae are cultured, was rejected. Photosynthesis rates measured under illumination from the fluorescent tube were comparable to those measured using the LED array.

#### 4.9.1 *Uncoupling of Growth from Photosynthesis*

The results of these experiments clearly show that levels of copper, which reduce growth rates in *G. longissima*, do not affect photosynthesis or respiration – as measured by oxygen evolution or Fv/Fm. Excessively high levels of copper exposure are required before any reduction in photosynthesis could be seen. This uncoupling of growth from photosynthesis has not been reported before in macroalgae and suggests that the initial site of action of copper toxicity in *G. longissima* does not occur in any part of the pathways involved in oxygen evolution.

Copper has been reported to uncouple growth from photosynthesis in microalgae. For example, Stauber & Florence (1987) found that copper concentrations that were inhibitory to cell division in the marine diatom *Nitzschia closterium*, had no effect on photosynthesis or respiration. These results were consistent with Lumsden & Florence (1983) who had previously reported that although growth rates in *N. closterium* were halved by 20 $\mu\text{g L}^{-1}$  copper, photosynthesis was not affected until copper concentrations were above 100 $\mu\text{g L}^{-1}$ . Other studies of microalgae have also found similar uncoupling (Fisher *et al.* 1981, Cid *et al.* 1995, Abalde *et al.* 1995 and Nalewajko & Olaveson 1995).

In some of these examples growth rates were represented as a function of cell division and the apparent uncoupling was explained by increased cell volumes (Fisher *et al.* 1981, Lumsden & Florence 1983 and Stauber & Florence 1987). For example, Fisher *et al.* (1981) showed that copper exposed cells of *Asterionella japonica* photosynthesised at a normal rate and cells increased in volume but could not divide. It has been suggested that copper may inhibit cell division independently of the production of new cell material (carbon fixation), Stauber & Florence (1987). This is thought to occur by copper interacting with -SH binding sites and interrupting enzyme active sites (Stauber & Florence 1987 and Romeo & Gnassia-Barelli 1993).

Inhibited cell division alone can not explain the findings reported here for *G. longissima*, because the reduction in growth as measured by tip elongation was also found to be reflected in the fresh weight of samples. If copper was halting cell division but cells were continuing to fix carbon normally, then the fresh weight of samples would be expected to reflect this. One possible explanation for the uncoupling of growth from photosynthesis could be increased carbon exudation of dissolved organic carbon from copper exposed material. This possibility and other possible mechanisms of copper toxicity are explored in the next chapter.

#### **4.10 Conclusions**

The results of this chapter can be summarised as follows:

- ◆ Growth is considerably more sensitive to copper exposure than photosynthesis as measured by oxygen evolution and Fv/Fm (Experiments 1-5). This indicates some form of uncoupling of growth from photosynthesis and respiration.

- ◆ This uncoupling is still apparent after 20 days of exposure to copper, when considerable accumulation of copper in apical tips is seen (Experiment 3) and also when photosynthesis is measured under the same irradiance used for culturing (Experiment 5).
  
- ◆ Relatively high levels of copper exposure are required before any reduction in oxygen evolution can be seen (Experiment 4). These levels correspond with actual shrinkage and bleaching of the apical tips.
  
- ◆ No significant difference in response could be found between the RGR or oxygen evolution of samples from the two populations, Mylor and St Just (Experiments 3 & 5).

## **Chapter 5**

# **THE PHYSIOLOGICAL EFFECTS OF COPPER IN *GRACILARIOPSIS LONGISSIMA*: PIGMENTATION, FLUORESCENCE, ION LEAKAGE AND DISSOLVED ORGANIC CARBON EXUDATION**

## 5.1 Introduction

In the preceding chapters, copper was found to clearly inhibit growth at levels as low as  $12\mu\text{g L}^{-1}$ , while at high levels ( $>450\mu\text{g L}^{-1}$ ) shrinkage of algal material was found. A comparative assessment of the effects of copper on growth, photosynthesis and respiration found that growth was affected at considerably lower levels of exposure; photosynthesis (as measured by oxygen evolution) was only affected at levels that resulted in actual shrinkage of algal material. This uncoupling of growth from photosynthesis at environmentally relevant copper concentrations implies that some other physiological process(es) are being affected by copper prior to any noticeable effect on oxygen evolution. This chapter details investigations on the effects of copper on other processes which might shed some light on this uncoupling. The effects of copper on dissolved organic carbon exudation, membrane damage and thallus pigmentation are investigated. The effect of copper on chlorophyll fluorescence is also investigated in more detail. Since no major differences in resistance could be found between the different populations in the previous experiments (Chapters 3 & 4), no population comparisons are included in these investigations.

Many primary producers including seaweeds are known to release photosynthetically produced organic compounds into the surrounding seawater (Romeo & Gnassia-Barelli, 1993). This exudate can represent 1-5% of the carbon fixed by photosynthesis and in some extreme cases can be as high as 40% (Schramm, 1993). Pregnall (1983) found *Enteromorpha prolifera* could release up to 16% of the carbon it fixed as dissolved organic carbon (DOC). The range of compounds found to be released include polysaccharides, polyphenols, polypeptides and amino acids (Langston & Bryan, 1984)

The potential role of these exuded organic substances in complexing metals, and subsequently influencing their toxicity, has been investigated (see Romeo and Gnassia-Barelli, 1993 for review). Some organic compounds have been shown to have high metal complexing capacity (van den Berg *et al.* 1979 and Gledhill *et al.* 1997). Whether this release can be considered a protective mechanism against heavy metal toxicity *in situ*, is not clear as release of organic matter by healthy cells is a normal event (Romeo & Gnassia-Barelli, 1993). Florence *et al.* (1983) demonstrated that the diatom *Nitzschia closterium* only produces exudate when exposed to copper. Rijstenbil *et al.* (1994b) also found evidence that the diatom *Thalassiosira pseudonana* released organic ligands in response to exposure to trace metals. Schramm (1993) investigated the toxic effects of copper on the growth of two rhodophytes *Delesseria sanguinea* and *Phycodrys rubens*, in the presence of organic substances extracted from a number of macrophyte species. Using “ecologically relevant copper concentrations ( $1\text{-}2\mu\text{g L}^{-1}$ )”, they found that organic substances could reduce the toxicity significantly. There were significant differences in the detoxification ability of extracts from different species. Noticeably, extracts from red algae had the least effect. Exuded organic material may not necessarily provide protection from the toxic effects of metals. For example, Hall *et al.* (1979) found that DOC from a copper-tolerant strain of *Ectocarpus siliculosus* did not protect non-tolerant strains from copper toxicity.

Irrespective of the exact role that DOC exudation may play in detoxifying copper, it was considered possible that the uncoupling of photosynthesis and growth reported in the previous chapter, might be explained by increased DOC exudation from copper exposed tissue resulting in less energy being invested in growth. To test this hypothesis, the amount of DOC exuded into the media was measured following copper exposure.

### 5.1.1 Ion leakage

Ion leakage is considered to be an indicator of damage to the plasmalemma (Axelsson & Axelsson 1987 and Wu *et al.* 1995) and has been used as a method of assessing the extent of cellular damage from various pollutants (Koch *et al.*, 1995). Copper has been shown to induce potassium leakage in a number of instances. Lage *et al.* (1996) found that sub-lethal levels of copper induced potassium leakage in the marine dinoflagellate *Prorocentrum micans*. Wu *et al.* (1995) found sub-lethal levels of copper increased potassium leakage and enhanced proline levels in the cyanobacterium *Anacytis nidulans*. This leakage was reduced by the addition of proline to the media. Overnell (1975) found that potassium release in two species of marine algae, *Dunaliella tertiolecta* and *Phaeodactylum tricorutum* occurred at a marginally lower level than the inhibition of photosynthesis as measured by oxygen evolution.

The possibility that copper affects permeability of the plasmalemma was explored by measuring ion leakage from the alga using a conductivity meter. This technique has been recommended as “a rapid and reliable method to quantify environmental effects...” (Axelsson & Axelsson, 1987).

### 5.1.2 Pigmentation

Copper has been reported to alter the pigmentation of algae. Cid *et al.* (1995) found that increasing copper treatments at first increased and then decreased the chlorophyll *a* concentration in the marine diatom *Phaeodactylum tricorutum*. Abalde *et al.* (1995) found that copper reduced chlorophyll *a* and carotenoid concentrations in the marine microalga *Dunaliella tertiolecta* but not chlorophyll *b* concentration. Carbon assimilation was found to be affected at a slightly lower level of copper exposure but the measurements were made

at different times. Gupta (1986) reported that the synthesis/degradation of chlorophyll *a* was more affected by copper than other pigments in the cyanobacterium *Anacystis nidulans*. The effect of copper on pigmentation (chlorophyll *a*,  $\beta$ -carotene and phycobiliproteins) was measured in *G. longissima*, to allow comparison with the other observed physiological effects.

### 5.1.3 Chlorophyll fluorescence

In Chapter 4, the effect of copper on the fluorescence parameter  $F_v/F_m$  was measured. Other parameters such as  $F_o$ ,  $F_v$ ,  $F_m$  the complementary area can also be measured and calculated using the Hansatech PEA fluorescence meter. These parameters can be used to assess other aspects of photosynthetic function, but are all area dependant. In an attempt to assess the effect of copper upon these other parameters, a method of standardising the area of *G. longissima* material exposed to the meter was developed. The results of these further measurements are discussed.

## 5.2 Experiment 1a. The effect of copper on ion leakage and chlorophyll fluorescence

This experiment aimed to establish the relative effect of increasing copper treatments on the growth, membrane permeability and chlorophyll fluorescence of *G. longissima*. In addition to aiding the understanding of the effect of copper on these parameters, this experiment also enabled the potential of measuring ion leakage or fluorescence as an indicators of copper toxicity to be assessed.

### 5.2.1 Materials and methods

*Gracilariopsis longissima* material collected from St Just and cultured for 7 weeks under stock culture conditions was used in the following experiment. Under standard experimental culturing conditions (as described in Chapter 2) apical tips were cultured for one week using an experimental design of; four replicates of five treatments (control, 25, 50, 250 and 500  $\mu\text{g l}^{-1}$  copper). Growth rates were measured by image analysis. Following one week of culturing, one tip per dish was randomly selected and used to measure chlorophyll fluorescence while the remaining nine tips were used for measuring ion leakage.

Ion leakage was calculated using the method of Axelsson & Axelsson (1987). Samples of *G. longissima* (9 apical tips) were briefly rinsed in distilled water for a few seconds (to rinse off any salts) and placed in test tubes containing 5ml of distilled water for 4 minutes. All samples were then transferred to boiling tubes containing another 5ml of distilled water. The boiling tubes were sealed with marble lids and placed in a boiling water bath. After 5 minutes the apical tips were removed, discarded and the conductivity of the two water samples was measured. An index of ion leakage was then calculated using the following formula:

$$\text{Ion leakage (\%)} = C_{\text{distilled water}} / (C_{\text{distilled water}} + C_{\text{boiled water}}) \times 100$$

Where C is the conductivity of the sample in  $\mu\text{S}$ .

A portable fluorometer (Hansatech PEA) was used to measure fast kinetic fluorescent parameters. The amount of thallus exposed to the PEA sensor was standardised by using single apical tips (randomly selected from dishes) which were measured for thickness (using a micrometer) and orientated across the middle of the sensor. This enabled

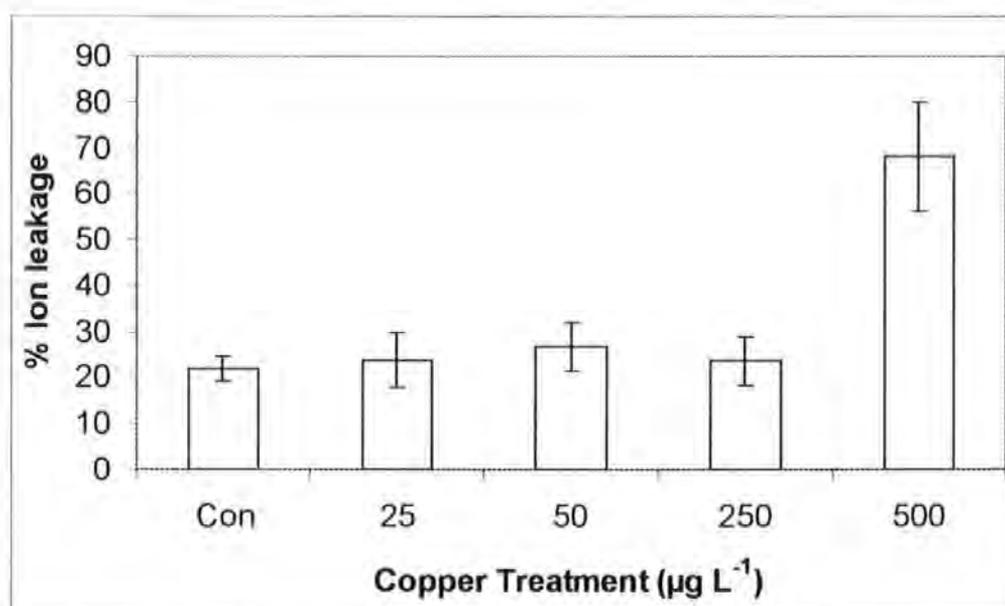
the comparison of four area dependent parameters  $F_o$ ,  $F_m$ ,  $F_v$  and complementary area, not previously measured, as well as the area independent parameter  $F_v/F_m$ .

### 5.2.2 Results and discussion

The results of all the one way ANOVAs performed on each of the parameters measured are summarised in Table 5.1. Copper was found to have a significant effect on the amount of ion leakage from the apical tips. However, a Duncan's multiple range test revealed that the addition of copper only caused significantly more ion leakage (68%) at the highest treatment level ( $500\mu\text{g l}^{-1}$ ). Ion leakage at lower copper treatments was indistinguishable from the control (22%) (Fig 5.1).

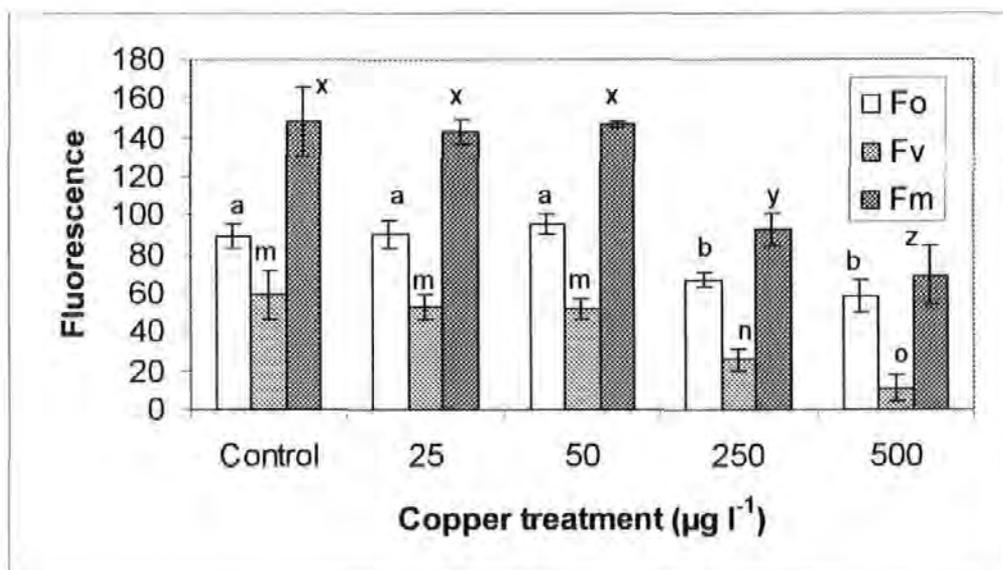
Parameter	f-ratio	P-value
Ion leakage	32.18	<0.001
Thickness of apical tips	0.25	0.904
$F_o$	24.16	<0.001
$F_v$	37.62	<0.001
$F_m$	26.88	<0.001
$F_v/F_m$	16.23	<0.001
Complementary Area	3.71	0.029
RGR	162.81	<0.001

**Table 5.1.** Summary of the one-way ANOVA for each of the parameters measured in the *G. longissima* apical tips following exposure to a range of copper treatments for one week. The degrees of freedom for each analysis were 4, 15.

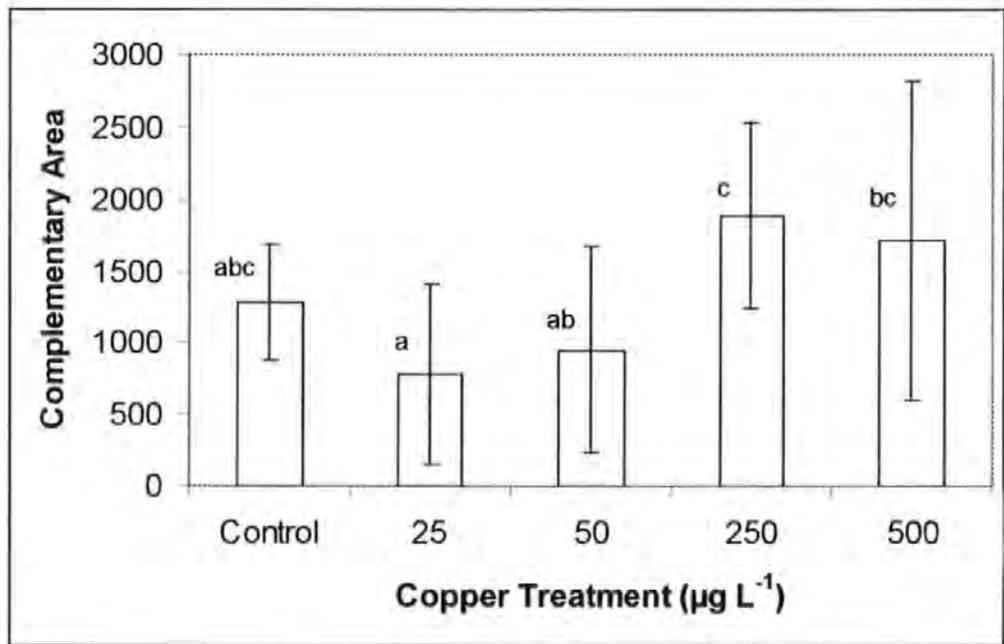


**Fig. 5.1** Mean ( $n=4$ ) percentage ion leakage from *G. longissima* apical tips exposed to a range of copper treatments for one week. Error bars show 95% confidence intervals

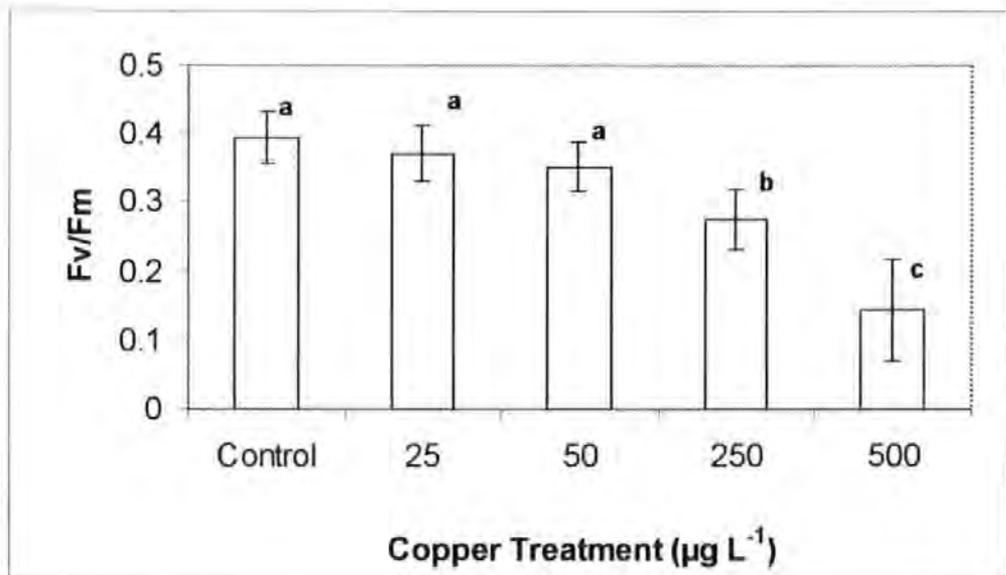
No significant difference could be found between thickness of apical tips used for fluorescence measurements (Table 5.1) and it was therefore assumed that there was no significant difference between area of each tip used. This validated the comparison of the four area dependent fluorescence parameters,  $F_o$ ,  $F_v$ ,  $F_m$  and complementary area. Figure 5.2 shows the mean  $F_o$ ,  $F_m$  and  $F_v$  values for each copper treatment. A separate Duncan's multiple range test was made for each parameter. The parameters  $F_o$ ,  $F_m$ ,  $F_v$  and  $F_v/F_m$  (Fig. 5.3), were significantly reduced by the two highest copper treatments (250 & 500  $\mu\text{g L}^{-1}$ ) - the biggest reduction in the 500  $\mu\text{g L}^{-1}$  treatment, while the lower copper treatments (including and below 50  $\mu\text{g L}^{-1}$ ) were not significantly different from the control values. Although copper treatment had a significant effect on the complementary area parameter (Table 5.1), there was a large amount of variation in the data and no copper treatment was significantly different from the control (Fig 5.3). The  $F_v/F_m$  had a mean of 0.275 (31% reduction) and 0.144 (64% reduction) in the 250 and 500  $\mu\text{g L}^{-1}$  copper treatments respectively, in comparison with a control mean of 0.396 (Fig. 5.4).



**Fig. 5.2** The effect of copper on the three fluorescence parameters  $F_o$ ,  $F_m$  and  $F_v$  in *G. longissima* apical tips. Letters designate statistically significant groupings for each parameter (ab, mno and xyz respectively) using a Duncan's multiple range test. Error bars show 95% confidence interval.



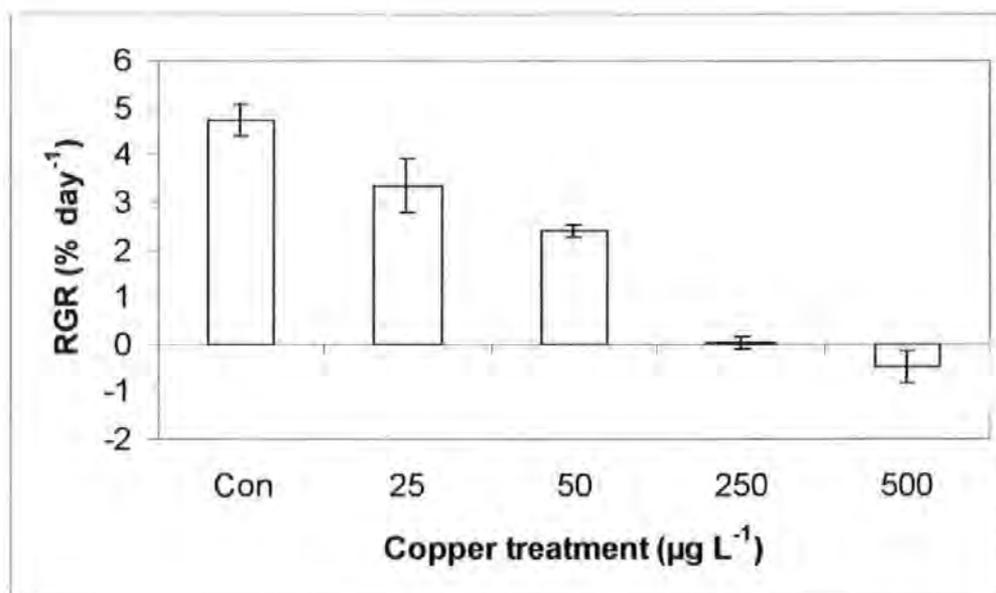
**Fig. 5.3** The effect of copper on the fluorescence parameter, complementary area, in *G. longissima* apical tips. Letters designate statistically significant groupings using a Duncans multiple range test. Error bars show 95% confidence intervals.



**Figure 5.4** Mean ( $n=4$ ) fluorescence ( $F_v/F_m$ ) of *G. longissima* apical tips exposed to a range of copper treatments for one week. Letters designate statistically significant groups using a Duncans multiple range test. Error bars show 95% confidence interval.

The RGR of the apical tips was significantly reduced by increasing copper treatments. The mean RGR of the apical tips in the control treatment ( $4.73\% \text{ day}^{-1}$ ) was significantly higher than the RGR in all the other treatments. Each increasingly higher

copper treatment significantly reduced growth. The highest copper treatment ( $500\mu\text{g L}^{-1}$ ) resulted in a negative mean RGR of  $-0.47\% \text{ day}^{-1}$  (Fig. 5.5).



**Figure 5.5** Mean ( $n=4$ ) RGR of *G. longissima* apical tips exposed to a range of copper treatments for one week. Error bars show 95% confidence intervals.

### 5.3 Experiment 1b. The effect of copper on tissue pigmentation

The aim of this experiment was to investigate the effect of elevated levels of copper on the pigmentation of *G. longissima*, to allow comparison with the other observed effects.

#### 5.3.1 Materials and methods

Material collected from the St Just population and held under stock culture conditions for 9 weeks was used for this experiment. The measurement of pigments required a relatively large mass of algal material. Consequently sections of *G. longissima* were cultured for one week using the following experimental design: Three treatments: control, 50 and  $500\mu\text{g L}^{-1}$  copper, were used each containing three approximately 30 mm sections of *G. longissima*. There were three replicates of each treatment.

Samples were placed into acid washed 200ml plastic beakers, each containing 100ml of standard experimental culture media. Beakers were placed in a controlled environment cabinet (CEC) and grown under standard experimental culture conditions for one week. Individual beakers were aerated via a pump and fine tubing. After one week, samples were used to measure pigment concentrations using the following methods.

The three sections from each replicate were cut in half. Each half was blotted dry and weighed (fresh weight). One half from each sample was then used for a dimethyl-sulfoxide (DMSO)/acetone pigment extraction for the determination of chlorophyll *a* and  $\beta$ -carotene (after Seely *et al.*, 1972) and the other half used for a phosphate buffer extraction of phycobiliproteins (after Beer and Eshel 1985).

For chlorophyll *a* and  $\beta$ -carotene determinations, samples were cut into 1-2mm segments and placed into 0.4ml DMSO in the dark for 30 minutes after which the supernatant was pipetted off and replaced with a fresh 0.3ml of DMSO for a further 15 minutes. This second process was subsequently repeated so that the final total volume of DMSO used equalled 1ml.

Following removal of the last 0.3ml of DMSO, 0.6ml of 90% acetone was added to the remaining algal material for 30 minutes and then replaced by another 0.6ml for a further 30 minutes. Samples were refrigerated in the dark until chlorophyll *a* and  $\beta$ -carotene concentrations (expressed per unit fresh weight) were determined using the following equations:

$$\text{DMSO Extract:} \quad [\text{chl } a] = A_{665}/72.8$$

$$\text{Acetone Extract:} \quad [\text{chl } a] = A_{661}/83.3]$$

$$[\beta\text{-car}] = (A_{480} - 0.033 A_{661})/193$$

(where A = absorbance at each wavelength)

For phycobiliprotein determination, samples were placed in 5 ml of 0.1M (pH 6.8) phosphate buffer, ground using a mortar and pestle (with a little acid washed sand), and rinsed with another 5ml of buffer. The sample was then spun at 1000g in a centrifuge for 10 minutes and phycobiliprotein concentrations (per unit fresh weight) in the supernatant were calculated using the following formulae:

$$\text{Phycoerythrin:} \quad [\text{R-PE}] = [(A_{546} - A_{592}) - (A_{455} - A_{592}) 0.20] 0.12$$

$$\text{Phycocyanin :} \quad [\text{R-PC}] = [(A_{618} - A_{645}) - (A_{592} - A_{645}) 0.51] 0.15$$

(where A = absorbance at each wavelength)

All spectrophotometric readings were made using 1ml quartz cuvettes in a Unicam UV/vis spectrometer and Unicam Vision software (v3.20). Fixed values for pigmentation calculations were measured using one second iterations and three cycles (replications) per value.

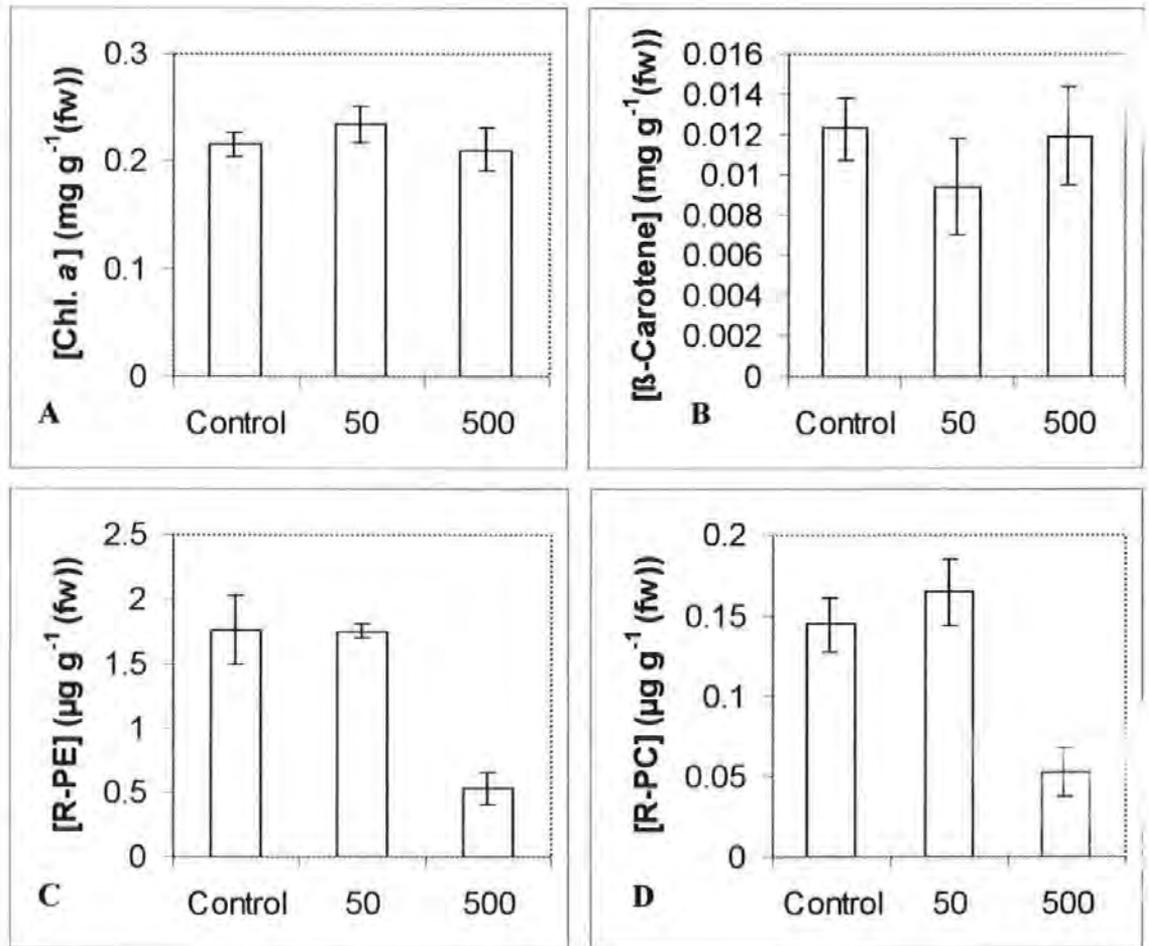
### 5.3.2 Results and discussion

Copper was found to significantly affect the concentrations of some pigments (Table 5.2). The mean chlorophyll *a* and  $\beta$ -carotene concentrations in the control samples were 0.216 and 0.012mg g<sup>-1</sup> respectively. These concentrations were not significantly affected by the highest copper treatment (500 $\mu$ g L<sup>-1</sup>, Fig 5.6A & 5.6B). In comparison both phycobiliprotein levels (R-PE and R-PC) were found to be significantly lower in the high (500 $\mu$ g l<sup>-1</sup>) copper treatment (Table 5.2, Fig. 5.6C & 5.6D).

The mean control R-PE concentration of 1.766 $\mu$ g g<sup>-1</sup> in the controls was reduced by 70% to 0.527 $\mu$ g g<sup>-1</sup> in the 500 $\mu$ g L<sup>-1</sup> copper treatment. Similarly, the mean R-PC concentration of 0.144 $\mu$ g g<sup>-1</sup> in the controls was reduced by 63% to 0.053 $\mu$ g g<sup>-1</sup>.

Factor	f-ratio	P-value
Chlorophyll- <i>a</i>	2.37	0.175
$\beta$ -carotene	2.01	0.215
R-PE	66.91	<0.001
R-PC	43.72	<0.001

**Table 5.2.** Summary of the one-way ANOVA for each pigment concentration measured in *G. longissima* sections following exposure to a range of copper treatments for one week. The degrees of freedom for each analysis were 2,6.



**Figs. 5.6 A-D** Mean (n=3) pigment concentration of *G. longissima* sections, following treatment with a range of copper concentrations (control, 50 and 500  $\mu$ g L<sup>-1</sup>) for one week. **A** – chlorophyll *a*, **B** –  $\beta$ -carotene, **C** – phycoerythrin, **D** – phycocyanin. Error bars show 95% confidence intervals.

## 5.4 Experiment 2. The measurement of DOC exudation

In order to investigate the potential role of DOC in explaining the uncoupling of growth from photosynthesis, the following experiment was designed whereby the amount of DOC exuded from copper treated algal material was measured.

### 5.4.1 *Materials and methods*

Using material collected from the St Just population and stock cultured under standard conditions for one week, the amount of DOC released from algal material was estimated using the following experimental procedure. Three replicates each containing 20, ~18mm apical tips were placed into petri dishes containing 20ml of DOC free media. Three treatments were used; control, 25 and 50 $\mu\text{g L}^{-1}$  copper. Dishes were cultured under standard experimental culture conditions (described in Chapter 2) in a CEC for 6 days. The DOC free media was prepared by irradiating the standard culture media (microwaved sterilised filtered seawater) with a UV lamp (400w medium pressure mercury vapour, Photochemical Reactors Ltd.) to remove dissolved organic carbon.

After six days culture, the amount of inorganic (IC) and total carbon (TC) in the media was measured using high temperature catalytic oxidisation and non-dispersive infrared detection (Shimadzu Carbon Analyser, TOC 2000). The amount of DOC in each sample was calculated by subtracting the amount of inorganic carbon from the total carbon.

The length of the apical tips was measured using image analysis on Day 0 and Day 6 and used to calculate RGR. Following image analysis on day 6 the apical tips were removed from the media, blotted dry, weighed (fresh weight - FW), dried for 48hrs at 40°C, and re-weighed (dry weight - DW).

#### 5.4.2 Results and discussion

All of the measured parameters (RGR, FW, DW, and DOC) were significantly reduced by copper treatment (Table 5.3). In terms of RGR, both the 25 and 50  $\mu\text{g L}^{-1}$  copper treatments significantly reduced RGR in comparison with the control (Fig. 5.6.A). The RGR in the 25  $\mu\text{g L}^{-1}$  copper treatment was 2.23%  $\text{day}^{-1}$ , a reduction of 42% from the control RGR of 3.84%  $\text{day}^{-1}$ , the higher 50  $\mu\text{g L}^{-1}$  copper treatment reduced RGR by 54% (1.76%  $\text{day}^{-1}$ ).

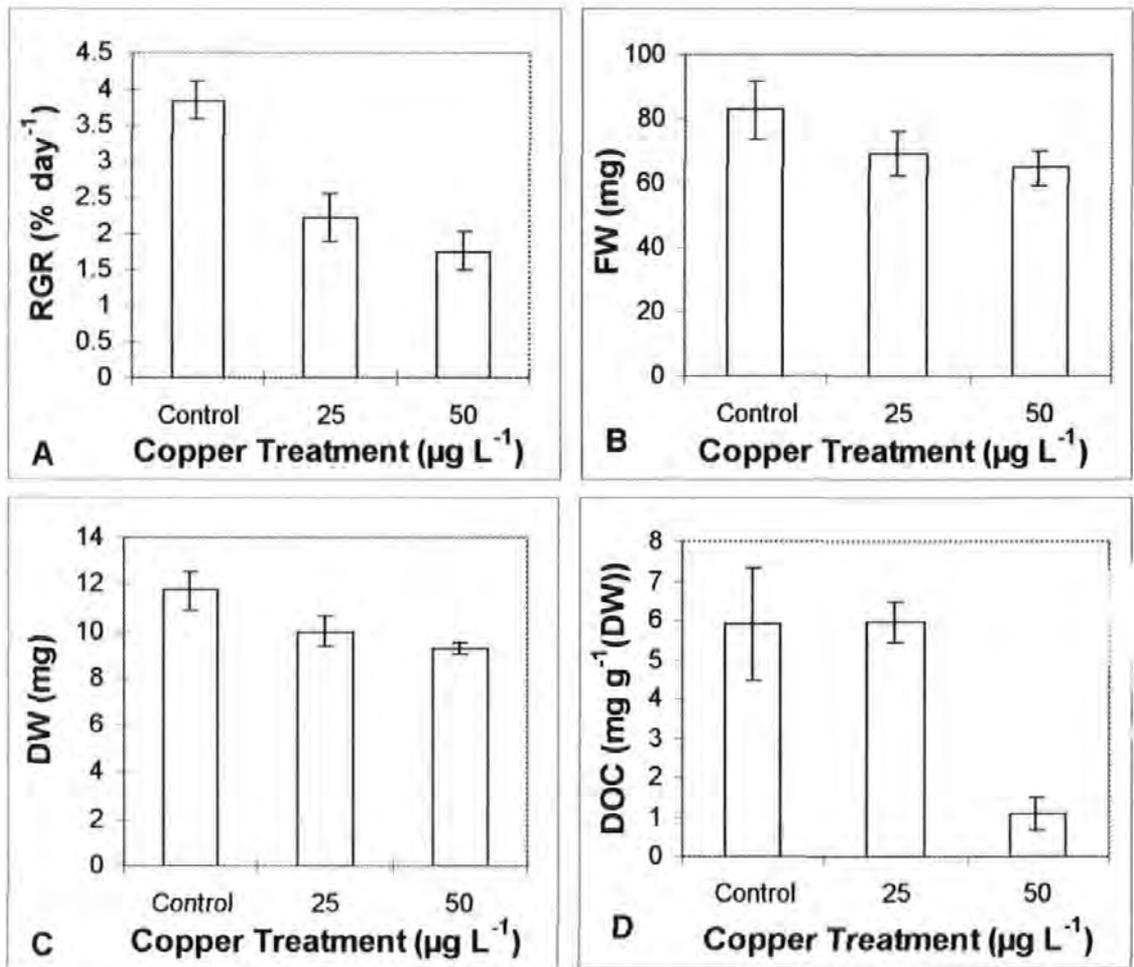
A similar pattern of reduction, was also found in the FW and DW measurements (Figs. 5.7.B & 5.7.C). Fresh weights were reduced from 83mg in the control to 65mg in the 50  $\mu\text{g L}^{-1}$  copper treatment, a 22% reduction. Dry weights were reduced from 12mg to 9mg, a 25% reduction.

Factor	f-ratio	P-value
DOC	9.21	0.015
RGR	56.09	<0.001
FW	8.12	0.020
DW	17.06	0.003
FW/DW	0.10	0.909

**Table 5.3** Summary of one-way ANOVA for each parameter measured in the DOC experiment. The degrees of freedom for each analysis were 2,6.

Significant positive correlations were found between the RGR, FW and DW (Table 5.3). No difference could be found between the FW:DW ratio of the different treatments (Table 5.3). DOC exudation in the highest copper treatment (1.09mg  $\text{g}^{-1}$  DW) was found to be significantly reduced by 81%, in comparison with the control (5.89mg  $\text{g}^{-1}$  DW). DOC exudation was positively correlated with DW (Table 5.4, Fig. 5.6.D).

No significant relationships could be found between the FW or RGR and DOC exudation (Table 5.4).



**Figs. 5.7 A-D** Mean (n=3); **A** – RGR (as measured by change in length), **B** – FW, **C** – DW and **D** – DOC exudation from *G. longissima* apical tips exposed to a range of copper treatments for six days. Error bars show 95% confidence intervals.

Comparison	Correlation coefficient	R <sup>2</sup>	P-value
RGR-FW	0.77	60%	0.046
RGR-DW	0.88	77%	0.002
RGR-DOC	0.51	26%	0.162
FW-DOC	0.56	31%	0.117
DW-DOC	0.67	46%	0.046

**Table 5.4** Summary of comparisons between RGR, FW, DW and DOC exudation of *G. longissima* apical tips following exposure to a range of copper treatments for 6 days.

## 5.5 Discussion

Ion leakage was found to be increased only by very high levels of copper exposure ( $500\mu\text{g l}^{-1}$ ) suggesting that lower levels are not damaging the functional integrity of the plasma membrane. Increased ion leakage was not observed until copper treatments were elevated to a similar level to those that were found to inhibit oxygen evolution. This finding is similar to that reported previously by Overnell (1975) in the marine microalgae, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. Increased potassium leakage was found to occur at a marginally lower copper concentration than that required to inhibit photosynthesis. Increased ion leakage did not occur at levels of copper exposure below  $500\mu\text{g L}^{-1}$ . Therefore it appears that measuring ion leakage would not be a good indicator of copper exposure, due to the relative insensitivity of this parameter to the metal. This is contrary to the suggestion of Axelsson & Axelsson (1987) who considered ion leakage may be used to detect harmful substances such as trace metals.

The increased ion leakage at the high copper treatment coincided with a reduction in the concentration of phycobiliproteins in the thallus and the shrinkage of the apical tips. The fact that there was no corresponding reduction in chlorophyll *a* or  $\beta$ -carotene concentrations is contradictory to Gupta (1986) and Abalde *et al.* (1995) who both reported decreased levels of chlorophyll *a* following copper exposure. Gupta (1986) also found no reduction in carotenoid concentration, as was found in this study, but no reduction in phycobiliproteins, unlike in this study. Abalde *et al.* (1995) showed that photosynthesis was effected in *Dunaliella tertiolecta* by copper at about the same time as changes in pigmentation could be seen, similar to the findings reported here. It may be that in *G. longissima*, copper has a greater binding affinity for phycobiliproteins than for chlorophyll and  $\beta$ -carotene, thus causing more damage to these pigments.

The fluorescence parameter  $F_v/F_m$ , which is considered to be a measure of the functional efficiency of PSII (Bolhar-Nordenkampf *et al.*, 1989, Davison & Pearson 1996), was not affected at low levels of copper exposure ( $<250\mu\text{g L}^{-1}$ ), indicating that PSII is functioning normally at these levels. This finding is consistent with the results of Experiment 2 in Chapter 3, which found no significant effect on  $F_v/F_m$  over a range of copper treatments up to  $100\mu\text{g L}^{-1}$ . At the higher copper treatments ( $250\text{-}500\mu\text{g L}^{-1}$ ),  $F_o$ ,  $F_m$  and  $F_v$  were all found to be significantly reduced.

The parameter  $F_o$  is thought to relate to the amount of fluorescence emitted by the PSII reaction centres when they are fully oxidised (open) and when photochemical quenching of fluorescence is maximal. Increases in this parameter would be expected if the reaction centres are damaged or if the transfer of excitation energy from the antenna to the reaction centres is impeded (Bolhar-Nordenkampf *et al.*, 1989). The fact that  $F_o$  decreased in *G. longissima* apical tips exposed to high copper treatments ( $>250\mu\text{g L}^{-1}$ ) may be explained by the observed loss of phycobiliproteins. Typically red macroalgae have lower 'unstressed'  $F_v/F_m$  values than higher plants and it has been suggested that this could be due to fluorescence contributions from phycobiliproteins (Franklin and Forster, 1997). The reduction of  $F_v/F_m$  at higher copper treatments was however, due to reduction in the variable fluorescence ( $F_v$ ), which indicates an increase in photoinhibition (Bolhar-Nordenkampf *et al.*, 1989). This would suggest that the remaining pigments in the thallus also have a reduced efficiency at high levels of exposure above  $250\mu\text{g L}^{-1}$ .

The area over the curve (complementary area) is proportional to the PSII electron acceptor pool and can be greatly reduced by blocking the electron transfer from the reaction centres to the quinone pool (Samson & Popovic 1988). The use of this parameter has been proposed over  $F_v/F_m$  as a sensitive indicator of phytotoxicity (Samson & Popovic 1988). Although the ANOVA found that copper treatment had a significant effect

on the complementary area, the amount of variability in the data was considerable and the high copper treatments actually increased the size of this area. This would suggest that this parameter would not be a good indicator of copper toxicity in *G. longissima*.

These findings are consistent with the oxygen electrode data indicating that photosynthesis is only disrupted by very high levels of copper exposure, which also damages other essential cellular functions such as membrane integrity. Ciscato *et al.* (1997) studied the effects of copper on the photosynthetic apparatus of Durum wheat, *Triticum durum*. They found that copper affected photosynthesis in a mainly indirect way and that photochemical efficiency, as indicated by Fv/Fm, was mostly preserved. Further information could possibly be obtained from investigating other fluorescence parameters, for example, quenching analysis using a modulated fluorometer (Bolhar-Nordenkamp *et al.*, 1989).

The results from the DOC experiment clearly show that the exposure of apical tips to copper does not result in a higher production of DOC. In fact the opposite appears to be occurring, as the addition of  $50\mu\text{g l}^{-1}$  of copper significantly reduced the amount of DOC exuded per gram DW of algal tissue. If increased DOC exudation was sufficient to explain the observed uncoupling of growth and photosynthesis, then a considerable amount of DOC would have been expected to be exuded into the media.

Using the length, fresh weight and dry weight data from this experiment, it is possible to make an estimate of the amount of DOC exudate expected to be in the media if DOC exudation explained the uncoupling. The mean length of the 20 tips in the control treatments was 18.2 mm on Day 0 and 22.8mm on Day 6, giving a mean of 4.7mm new growth over the 6 days. Since the DW of the tips on Day 6 was 11mg, this represents an increase in dry weight of 2.4mg. An estimate of the amount of carbon present can be made

by assuming approximately 50% of the DW is carbon (pers. comm. R. Gieder). The increase in carbon can therefore be estimated to be 1.2mg. Repeating these calculations for the  $50\mu\text{g L}^{-1}$  copper, results in 0.46mg of carbon present in the new growth. This represents a difference of 0.74mg. If all this carbon was exuded into the media, then this would be expected to result in an increase in the concentration of DOC of  $36\text{mg L}^{-1}$ . There was no evidence of any increase in DOC exudation and the predicted increase in DOC exudation is well above the detection limit of the carbon analyser ( $\sim 10\mu\text{g L}^{-1}$ ). It can therefore be concluded that the uncoupling of photosynthesis from growth cannot be explained by increased exudation of DOC.

These findings are contrary to Florence *et al.* (1983) and Rijstenbil *et al.* (1994b) who observed that several species of diatom released organic ligands in response to exposure to trace metals. The results do however, indicate that DOC exudation is not a copper protection mechanism in *G. longissima* and thus supports the finding of Hall *et al.* (1979), that DOC from a copper-tolerant strain of *Ectocarpus siliculosus* did not protect non-tolerant strains from copper toxicity.

There was also no significant difference between the FW:DW ratio of the different copper treatments, the dry weight being approximately 14% of the fresh weight at all times. In several of the examples of uncoupling in microalgae, it was found that copper uncoupled photosynthesis from growth by inhibiting cell division. Fisher *et al.* (1981) found that DOC exudation was depressed by copper treatment in the diatom *Asterionella japonica*. This resulted in enlarged cells since cell division was also inhibited but photosynthesis and carbon fixation was not. Similarly, Ciscato *et al.* (1997) found starch accumulated in the copper treated plants which was assumed to be a consequence of the diminished requirement of photosynthetic products. The fact that the addition of copper did

not alter the FW:DW ratio and that DW decreased with increasing copper treatment, means that cell enlargement cannot be used to explain the uncoupling found in *G. longissima*.

The oxygen evolution rates in the previous chapter were expressed as a function of the FW of the tissue. Photosynthetic rates are often expressed per unit dry weight or per unit chlorophyll (Dudgeon *et al.* 1995). Since low levels of copper did not result in any change in the pigmentation of apical tips or in the FW:DW ratio, then expressing photosynthesis rates as per unit of chlorophyll *a* or per unit dry weight would not make any relative difference to the results. i.e. oxygen production is unaffected by low levels of copper exposure whereas growth clearly is.

A summary of all the observed effects of copper on *G. longissima* found in the last three chapters shows clearly that the most sensitive responses are reduction of growth and accumulation of copper which were both evident after one week's culture in  $12\mu\text{g l}^{-1}$  copper. The next observable effect was reduction in DOC exudation which could be seen at  $50\mu\text{g l}^{-1}$  followed by a reduction in fluorescence and photosynthesis at around  $250\mu\text{g l}^{-1}$ . High levels of copper where oxygen evolution was reduced (above  $250\mu\text{g l}^{-1}$ ) coincided with increased ion leakage, and a reduction in the concentration of phycobiliproteins. This suggests that disruption of photosynthesis at this level of exposure was occurring when most cellular functions have been impaired. This indicates that reduction in photosynthesis is a secondary effect of copper toxicity and that the effect of copper on *G. longissima* is not initially on the chloroplasts, mitochondria, or membranes. These findings are similar to those of Stauber & Florence (1987) in several species of marine diatom.

In the previous chapter, evidence for the uncoupling of photosynthesis and growth was found. This indicated that carbon fixed by photosynthesis was not being utilised for growth. One hypothesis for this uncoupling was increased exudation of fixed

carbon as DOC following copper exposure. This chapter found no evidence for this mechanism and no physiological cause for the uncoupling could be established.

One explanation could be that low levels of copper exposure result in the diversion of the energy captured by the light reactions away from carbon assimilation in the dark reactions. In the light reactions (PSII and PSI), H<sub>2</sub>O is oxidised, releasing O<sub>2</sub> and resulting ultimately in the reduction of adenosine diphosphate (ADP) to the higher energy adenosine triphosphate (ATP). Low levels of copper exposure could feasibly result in more of this energy being used to maintain cellular integrity and essential metabolic pathways in the alga and less being available for growth. Cid *et al.* (1995) provide support for this theory. In their study on the effects of copper on the marine microalga *Phaeodactylum tricornutum* they measured among other factors ATP concentration, growth and photosynthetic rates. At low levels of copper exposure photosynthesis was uncoupled from growth, however, intracellular ATP content was found to be reduced at even lower levels of copper than growth. Their explanation for this finding, was that the higher ATP consumption was caused by the cell 'avoiding' the effects of copper. A recommended line of further research would be to measure the levels of intracellular ATP over a range of copper levels.

Copper is known to bind to -SH groups which are of functional importance in many metabolic pathways (Fisher *et al.* 1981) and this binding may reduce their function. Exposure to elevated levels of copper can also affect the synthesis of proteins. Examples of protective mechanisms could include new protein synthesis (such as metallothioneins), production of secondary metabolites such as phytochelatins, or increased production of stress reducing enzymes such as glutathione peroxidase and superoxide dismutase (SOD), which can reduce oxidative stress. There is evidence that trace metals can induce such activity in algae; for example, Berail *et al.* (1991) isolated metal induced proteins in *Cystoseira barbarta* and Rijstenbil *et al.* (1994a) found that copper stress induced the

production of SOD in *Ditylum brightwellii*. Lage *et al.* (1994) found that exposure to sublethal levels of copper in the marine dinoflagellate *Prorocentrum micans*, not only significantly reduced growth, but also altered the polypeptide profile in the culture. In comparison with the control, both increases and decreases of different sized polypeptides was found when the cells were exposed to copper. Investigations into the effect of excess copper on the production and function of other cellular metabolites is considered to be a beneficial line of future investigation.

## **Chapter 6**

### **RESPONSES FOLLOWING THE REMOVAL OF COPPER**

## 6.1 Introduction

It has been stated that one of the primary reasons organisms are chosen as biomonitors of contamination in the aquatic environment is that they are “considered to live in equilibrium with the surrounding water and may, thus, serve as an integrating sampling device”, (de Kock & Kramer, 1994). It is, nevertheless, imperative to take account of the fact that rates of uptake and excretion of trace metals differ between organisms and metals, resulting in different time integrations (Lovett Doust *et al.*, 1994). This can lead to considerably different trace metal concentrations in different species, despite being exposed to the same regime of contamination.

In the introductory chapter, the need to understand how a species can regulate metals was highlighted. Regulation is defined here as all mechanisms involved in the handling and processing of metals, including uptake, elimination and sequestration. The bioaccumulation of a trace metal within an organism must be the sum of any uptake and loss mechanisms that a particular species employs. In Chapter 3 it was clearly demonstrated that *G. longissima* can accumulate copper to considerable levels during short term, seven day, exposure experiments. While these experiments served the purpose of illustrating that uptake and accumulation of the metal occurs, they did not provide information as to whether or not loss of copper is possible from *G. longissima*. To more fully understand the regulation and time integration of copper in *G. longissima* it is not only desirable to know if accumulation can occur, but it is necessary also to determine the extent of elimination (or loss) of the metal from the tissue.

Laboratory experiments often find net accumulation of trace metals by an organism, which in case of seaweeds is assumed to indicate unregulated uptake is occurring (see Philips, 1994). This assumption may be incorrect. These results simply indicate that the

indicate that the rate of accumulation is higher than any rate of release or loss of the trace metal by the organism, in the controlled environmental conditions under which the particular experiment was carried out. Claims that macroalgae lack the ability to regulate metals (Lovett Doust *et al.* 1994, Philips 1994 and Langston & Spence 1995) are considered misleading, because they imply that macroalgae can only accumulate metals passively. This is further compounded by claims that accumulated metals are irreversibly bound within the seaweed and cannot be released. While this may be correct for certain metals in some species (Bryan 1969, Skipness *et al.* 1975, Cullinane *et al.* 1987 and Karez *et al.* 1994) it may not always be the case.

Evidence that seaweeds can eliminate accumulated metals does exist. For example, Seeliger & Cordazzo (1982) measured the concentration of copper in *Enteromorpha* sp. during culture in recovery (control) media following a pre-treatment with copper and found a (non-significant) tendency for copper levels to decrease. It was suggested that this decrease may have been due to a dilution effect as a result of new growth during the recovery phase. Hall (1981) also found a significant release of copper from the marine fouling alga *Ectocarpus siliculosus* but this loss could not be explained simply by growth.

Field transplantation experiments have also provided information that loss of accumulated zinc may occur in *Ascophyllum nodosum* when moved from a contaminated site to a cleaner site (Myklestad *et al.*, 1978). This release could not be explained by growth (dilution) effects alone. Similarly, when Ho (1984) transplanted two fucoids from the heavily contaminated Restronguet Creek to a cleaner site, a significant loss of copper and zinc was found to occur. These examples all provide evidence that trace metal regulation can occur in seaweeds and that not all metal(s) taken up by macroalgae are irreversibly bound in the thallus.

Rainbow *et al.* (1990) stated that, "Knowledge of the accumulation strategy of a particular trace metal is a prerequisite for understanding the significance of an observed metal concentration in a particular marine invertebrate, especially from the aspect of biomonitoring." Although the use of the term 'strategy' in this statement is misleading and should be avoided since it implies the organism has a choice (Brown & Depledge, 1998), the important point that needs emphasising is that the mechanisms of uptake and regulation of trace metals must be better understood before an organism can be used as a quantitative biomonitor of trace metal contamination.

This statement equally applies to macroalgae. If a metal is simply accumulated in an unregulated way by the seaweed, then the amount of metal within it should be a function of the uptake rate (or any factor which affects it), the growth rate (due to dilution effects) and the duration and level of contamination that the species has been exposed to. By contrast, if the seaweed can regulate the internal levels of a metal, then the concentration can be considered a function of the concentration of the contaminant in the environment and any factor which affects the rates of uptake and loss of the metal(s).

Another important consideration is to what extent growth can recover once the level of contamination is reduced. Exposure to elevated levels of copper clearly reduces growth in many species of seaweeds, including *G. longissima*, but the length of time this toxic effect lasts, following removal of the contaminant is not known. This aspect of trace metal pollution is considered of particular interest for three reasons:

- ◆ It can indicate the ability of a species to recover following a short term toxic pollution event, thus giving some indication of any long lasting effects of short term trace metal contamination.

- ◆ It can provide information about any growth related dilution effects, which can lead to an apparent reduction in trace metal concentration within the thallus without any actual loss of metal from it.
- ◆ Investigating the ability of a species to recover, following exposure, may provide some insight into the mechanisms of trace metal regulation.

The recovery of growth and loss of copper from the seaweeds can be investigated using a similar experimental approach, by exposing the seaweed to a toxic level of copper and then measuring the growth response during the subsequent recovery phase. It is also possible that differences in recovery may exist between populations. This possibility was explored by comparing the recovery response of two populations (Mylor and Helford) whose growth and copper accumulation response to elevated levels of copper have been compared previously (Chapter 3).

## 6.2 Aims

The main aims of this chapter were:

- ◆ To establish if the growth of *G. longissima* can recover following exposure to toxic levels of copper, and if so, to what extent this varies between populations.
- ◆ To ascertain whether or not recovery of growth results in a corresponding reduction in the concentration of copper within the tissue.
- ◆ To determine whether any detected decrease in the copper concentration in the alga is due to dilution (growth effects) and/or actual loss of copper from the thallus and to assess the relative importance of these two potential causes for any decrease.

In order to investigate these questions, three recovery experiments were carried out whereby the recovery of apical tips following removal from a high level of copper exposure was monitored.

### 6.3 Experiment 1. Growth recovery

In this first recovery experiment apical tips were exposed to copper for one week and then placed into control media for a further week.

#### 6.3.1 Materials and methods

*Gracilariopsis longissima* material was collected from Helford (April 1996) and cultured in the laboratory under standard stock culture conditions (as described in Chapter 2) for six weeks. Apical tips were excised and pooled in a beaker containing standard culture media. Ten tips were then randomly selected and placed in petri dishes containing 25ml of standard culture media. The apical tips were then exposed to a range of treatments (control, 25 and 50  $\mu\text{g L}^{-1}$  copper) using standard experimental culturing conditions in a controlled environment cabinet (CEC) for one week. After one week, all material was placed into fresh media and dishes using the experimental design described in Table 6.1. The apical tips were cultured for a further week. There were four replicates of each treatment. The lengths of the apical tips were measured initially and at the end of each week using the image analysis protocol described previously.

Treatment name	Copper treatment	
	Week 1	Week 2
Con-Con	Control	Control
25-Con	25 $\mu\text{g L}^{-1}$	Control
25-25	25 $\mu\text{g L}^{-1}$	25 $\mu\text{g L}^{-1}$
50-Con	50 $\mu\text{g L}^{-1}$	Control
50-50	50 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$

**Table 6.1** Experimental design for Recovery Experiment 1. Values refer to copper concentrations ( $\mu\text{g L}^{-1}$ ). Four replicates of each treatment were used. See text for details.

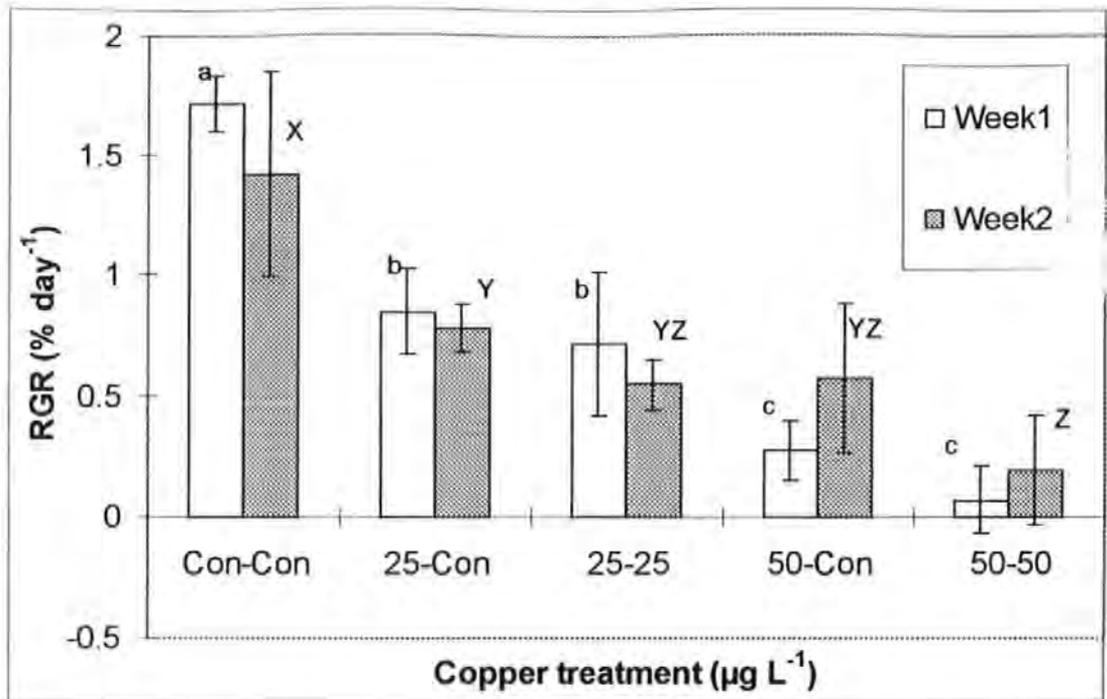
### 6.3.2 Results

Since the RGR of the apical tips in Week 2 is not independent of the RGR of the same apical tips in Week 1, the data was analysed separately using a one way ANOVA for each week. The results of the analyses are summarised in Table 6.2 and results of the growth measurements are summarised in Fig. 6.1. Duncan's multiple range tests were performed on each week's RGR data (Fig. 6.1). These groupings only strictly allow comparison within the treatments from each week.

	Source	SS	DF	MS	F-ratio	P-value
Week 1	Between Treatments	6.560	4	1.640	46.420	<0.001
	Within treatments	0.530	15	0.035		
	Total (Corrected)	7.090	19			
Week 2	Between Treatments	3.311	4	0.827	11.300	<0.001
	Within treatments	1.099	15	0.073		
	Total (Corrected)	4.410	19			

**Table 6.2** Summary of the three one-way ANOVAs performed on the RGR data from each week.

The one way ANOVA performed for each of the two weeks growth data found significant differences between the copper treatments (Week 1,  $P < 0.001$  and Week 2,  $P < 0.001$ ). The copper treatments significantly lowered the RGR in comparison with the controls. After one week, the RGR of the controls had a mean value of  $1.7\% \text{ day}^{-1}$ . This contrasts with a RGR of  $0.8\% \text{ day}^{-1}$  (50% reduction) in the  $25\mu\text{g L}^{-1}$  copper treatment and a RGR of  $0.3\% \text{ day}^{-1}$  (85% reduction) in the  $50\mu\text{g L}^{-1}$  treatment (Fig. 6.1).



**Fig. 6.1** The mean ( $n=4$ ) RGR of *G. longissima* apical tips measured over two consecutive weeks. The experimental design is described in Table 6.1. Con = control, 25 =  $25\mu\text{g L}^{-1}$  and 50 =  $50\mu\text{g L}^{-1}$  copper. Letters designate statistically significant groupings using a Duncans multiple range test for each weeks data (a-c = Week 1, X-Z = Week 2). Error bars show 95% confidence intervals.

At the end of the second week, the RGR of the control samples was similar to that of the first week. There was no significant difference between samples replaced in the same copper concentration media and those placed into control (recovery) media (Fig 6.1). It does appear, however, that tips transferred to the recovery media in the second week did have higher growth rates than those maintained in copper. At  $25\mu\text{g L}^{-1}$ , there was an indication that recovery had occurred. Growth in the 25-25 treatment ( $0.54\% \text{ day}^{-1}$ ) was lower (not significantly) than the 25-C treatment ( $0.78\% \text{ day}^{-1}$ ). At  $50\mu\text{g L}^{-1}$ , there does appear to be some recovery of the 50-C treatment ( $0.57\% \text{ day}^{-1}$ ) in the second week, compared to the 50-50 treatment ( $0.19\% \text{ day}^{-1}$ ), although this was also not significant. The reason these differences were not significant is probably due to the large amount of variation within treatments.

## 6.4 Experiment 2. Growth recovery

In Experiment 1, considerable variability in growth within treatments and the relatively low growth rate of the control material ( $1.7\% \text{ day}^{-1}$ ) made it difficult to categorically state that recovery was occurring. While the results of experiments in previous chapters also found some variability in the growth rates of control samples, values were typically around  $4\% \text{ day}^{-1}$  or higher (Chapter 3, 4 & 5). In light of this, a second recovery experiment was designed. In this second experiment, the length of the recovery period was extended by a week and the response of two populations was compared. This longer recovery period would establish to what extent recovery was possible and whether any differences in response were apparent between populations.

### 6.4.1 Materials and methods

*Gracilariopsis longissima* material from the Helford and Mylor populations was collected (July 1996) and returned to the laboratory where it was cultured for one week under standard stock culture conditions. Two hundred apical tips were excised and pooled from each population sample and used in a three week experiment with the following experimental design (see Table 6.3).

Population	Treatment name	Copper treatment		
		Week 1	Week 2	Week 3
Mylor	C-C-C (M)	Control	Control	Control
	25-C-C (M)	$25\mu\text{g L}^{-1}$	Control	Control
	25-25-C (M)	$25\mu\text{g L}^{-1}$	$25\mu\text{g L}^{-1}$	Control
	50-C-C (M)	$50\mu\text{g L}^{-1}$	Control	Control
	50-50-C (M)	$50\mu\text{g L}^{-1}$	$50\mu\text{g L}^{-1}$	Control
Helford	C-C-C (H)	Control	Control	Control
	25-C-C (H)	$25\mu\text{g L}^{-1}$	Control	Control
	25-25-C (H)	$25\mu\text{g L}^{-1}$	$25\mu\text{g L}^{-1}$	Control
	50-C-C (H)	$50\mu\text{g L}^{-1}$	Control	Control
	50-50-C (H)	$50\mu\text{g L}^{-1}$	$50\mu\text{g L}^{-1}$	Control

**Table 6.3** Experimental design for Recovery Experiment 2. Four replicates of each treatment were used. See text for details.

Apical tips were placed into one of three initial treatments, control, 25 and 50 $\mu\text{g L}^{-1}$  of copper and cultured for one week. All media and dishes were changed at the start of Week 2 at which time the 25-C-C and 50-C-C copper treatments were placed in control media. At the end of the second week, all copper treatments and dishes were replaced with control media.

The experiment was carried out under standard experimental culture conditions in a CEC with four replicates per treatment. Each replicate contained 25ml of standard culture media and 10 apical tips selected randomly from the pooled samples. All media and dishes were changed at the end of each week. The lengths of the apical tips were measured initially and after each week using image analysis.

#### *6.4.2 Results*

As in Experiment 1, it was not possible to incorporate the data from each week into one multivariate analysis, since each week's RGR is not strictly independent of the other two weeks RGR. A two way ANOVA was performed on the data sets collected at the end of each week, the two factors being population and treatment (copper) as described in Table 6.4.

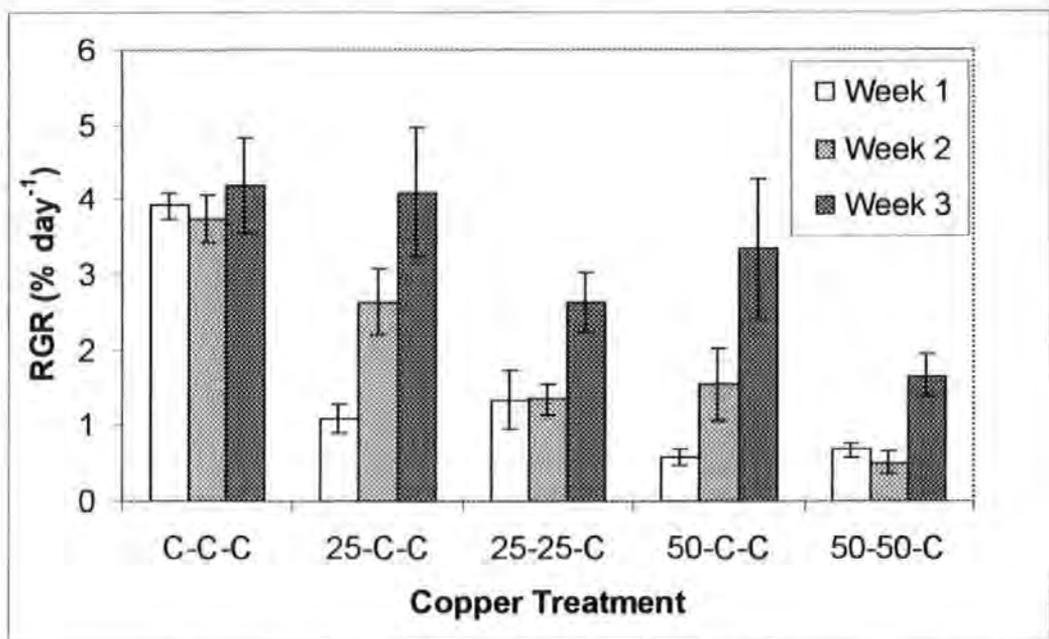
	Source	SS	DF	MS	F-ratio	P-value
<b>Week 1</b>	<b>Population</b>	0.819	1	0.819	26.773	<0.001
	<b>Treatment</b>	13.662	4	3.416	111.593	<0.001
	<b>Population * Treatment</b>	0.943	4	0.236	7.699	<0.001
	<b>Residual</b>	0.888	29	0.031		
	<b>Total (Corrected)</b>	16.312	38	0.429		
<b>Week 2</b>	<b>Population</b>	0.008	1	0.008	0.053	0.820
	<b>Treatment</b>	39.609	4	9.902	68.221	<0.001
	<b>Population * Treatment</b>	3.541	4	0.885	6.099	0.001
	<b>Residual</b>	4.354	30	0.145		
	<b>Total (Corrected)</b>	47.512	39	1.218		
<b>Week 3</b>	<b>Population</b>	0.395	1	0.395	1.035	0.317
	<b>Treatment</b>	15.914	4	3.978	10.428	<0.001
	<b>Population * Treatment</b>	7.020	4	1.755	4.600	0.005
	<b>Residual</b>	11.446	30	0.382		
	<b>Total (Corrected)</b>	34.775	39	0.892		

**Table 6.4** Summary of the two-way ANOVA performed on each week's RGR data. The RGR data from Week 1 was Ln transformed.

#### 6.4.2.1 *Recovery of growth*

Since the primary objective was to determine if any recovery of growth occurred, the data from all three weeks is presented on a separate figure for each population. This best illustrates the recovery response of each population.

Figure 6.2 summarises the recovery response of the Helford population samples over the three weeks. The growth of the control samples during the course of the three week experiment remained at a constant rate of  $\sim 4\% \text{ day}^{-1}$  (more than twice the RGR of the control in Experiment 1). Exposure to  $25\mu\text{g L}^{-1}$  copper for one week reduced growth, by  $\sim 75\%$  ( $1.08\% \text{ day}^{-1}$ ). Prolonging the exposure of the apical tips to  $25\mu\text{g L}^{-1}$  copper for two weeks resulted in a similar low RGR in the second week ( $1.34\% \text{ day}^{-1}$ ).

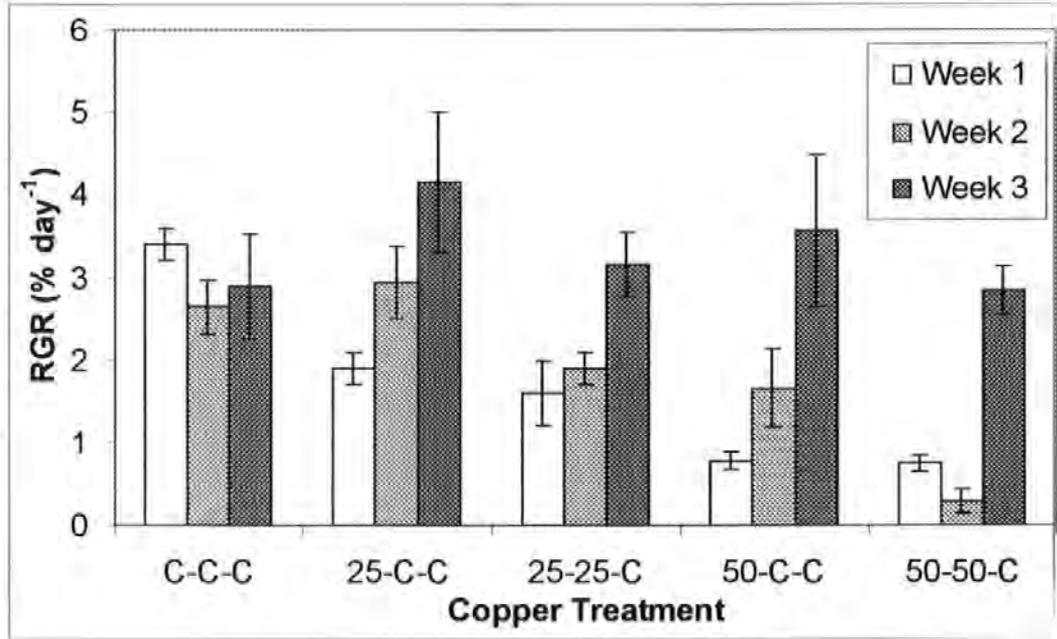


**Fig. 6.2.** Mean ( $n=4$ ) RGR of *G. longissima* apical tips from the Helford population exposed to a range of copper treatments over three weeks. The experimental design is described in Table 6.3. C = control, 25 =  $25\mu\text{g L}^{-1}$  and 50 =  $50\mu\text{g L}^{-1}$  copper. Error bars show 95% confidence intervals.

Significant recovery of growth occurred in the 25-C-C during the subsequent two weeks culture in control media (see Fig 6.2). By the end of the third week, the RGR of the apical tips in this treatment ( $4.10\% \text{ day}^{-1}$ ) was similar to the control. Significant recovery was also found to occur during the third week of culture in the 25-25-C treatment (Fig 6.2). The RGR in Week 3 of the 25-25-C treatment was similar to that of the RGR in Week 2 of the 25-C-C treatment (Fig. 6.2). The rate of recovery after two weeks copper exposure is similar to that after one week of exposure.

A similar pattern of growth reduction and recovery was found to occur in the  $50\mu\text{g L}^{-1}$  copper treatments (50-C-C & 50-50-C), although the actual reduction in growth in the higher copper treatment was significantly greater,  $0.59\% \text{ day}^{-1}$  (85%). The growth rates in weeks 1 and 2 of the 50-50-C treatment were similar ( $\sim 0.51\% \text{ day}^{-1}$ , Fig 6.2).

A similar pattern of recovery was found in the Mylor population (compare Figs. 6.2 & 6.3). The most notable difference was that the growth rate of the Mylor control samples over the three weeks was more variable and on average lower (2.97% day<sup>-1</sup>) than the Helford controls (3.95% day<sup>-1</sup>). The mean RGR at 25µg L<sup>-1</sup> after one week was 1.75% day<sup>-1</sup>. A similar RGR was found after two weeks exposure to 25µg L<sup>-1</sup>.



**Fig. 6.3** Mean (n=4) RGR of *G. longissima* apical tips from the Mylor population exposed to a range of copper treatments over three weeks. The experimental design is described in Table 6.3. C = control, 25 = 25µg L<sup>-1</sup> and 50 = 50µg L<sup>-1</sup> copper. Error bars show 95% confidence intervals.

The recovery following one or two weeks exposure to 25µg L<sup>-1</sup> was similar to recovery of the Helford samples. After exposure for one week, the RGR in Week 2 was 2.93% day<sup>-1</sup> and 4.15% day<sup>-1</sup> in Week 3. After two weeks exposure the RGR in Week 3 was 3.16% day<sup>-1</sup>, a similar rate to that in Week 2 of the 25-C-C treatment.

The reduction in RGR in 50µg L<sup>-1</sup> copper after one week was 0.77% day<sup>-1</sup> and this was reduced further after two weeks exposure (0.29% day<sup>-1</sup>). When transferred to control media after one week of exposure RGR recovered to 1.66% day<sup>-1</sup> in Week 2 and 3.58%

day<sup>-1</sup> in Week 3. After two weeks exposure RGR recovered to 2.84% day<sup>-1</sup> in Week 3, a similar rate to that of 25-25-C in Week 3 (Fig. 6.3).

The significant population effect found in Week 1, and the significant interactions found in all three weeks (Table 6.4) are further illustrated by the Duncan's multiple range tests performed on each weeks growth data (Table 6.5).

Week	Population	Treatment				
		C-C-C	25-C-C	25-25-C	50-C-C	50-50-C
Week 1	Helford	A	D	CD	F	EF
	Mylor	A	B	BC	D	E
Week 2	Helford	A	B	C	C	D
	Mylor	B	B	C	C	D
Week 3	Helford	A	A	B	B	C
	Mylor	B	A	B	AB	B

**Table 6.5** Summary of the two-way Duncans multiple range tests comparing populations and treatments, performed on the RGR data. Each week's data was analysed separately. Letters designate statistically significant groupings within each week.

Certain specific differences were found between the two populations. In the control treatment (C-C-C) the RGR of Mylor material was significantly less than Helford material in Weeks 2 and 3. In Week 1, Growth of the Helford material was significantly more affected by 25 and 50µg L<sup>-1</sup> than the Mylor material. In Week 3 the RGR of Mylor material after two weeks exposure at 50µg L<sup>-1</sup> was significantly greater than that of the Helford material. When considered overall these findings indicate that there were some small but significant differences between the response of the two populations in some treatments. These differences are however not consistent with the similarly small differences found between populations in Chapter 3 and are not considered to indicate any major difference in response between the two populations recovery.

## 6.5 Experiment 3. Copper accumulation and loss

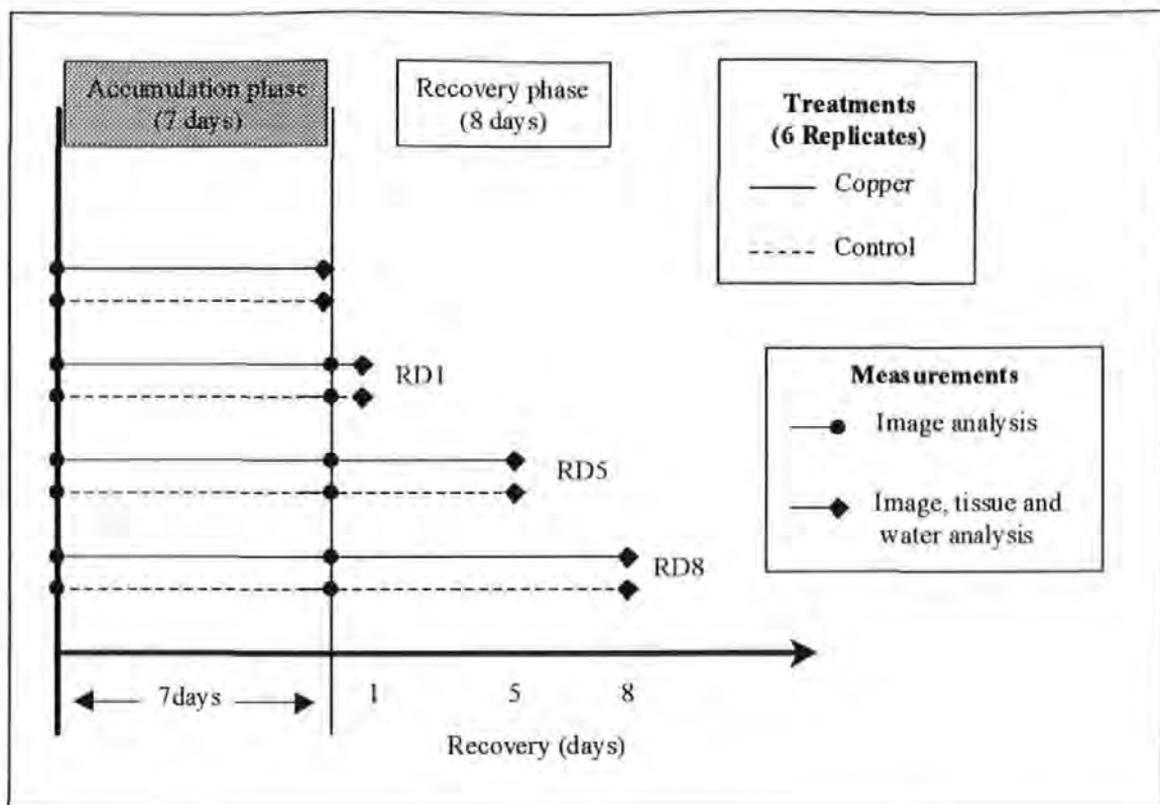
Having clearly established that growth can recover after exposure to copper, this next experiment was designed to establish whether or not there was a corresponding reduction in copper concentration measured in the algal material and to determine if this was due to dilution (i.e. growth effects) or to an actual loss of copper from the tissue.

To investigate these possibilities, changes in copper concentrations in apical tips during the recovery phase were measured, following exposure to a high level of copper. The concentrations of copper in the media both during the accumulation (exposure) and recovery phases were also measured in order produce a basic copper budget, between the media and the tissue.

### 6.5.1 *Materials and methods*

Five hundred and forty 10mm apical tips were excised and pooled from *G. longissima* samples which had been collected from St Just (January 1997) and cultured for 4 months under standard stock culture conditions.

These tips were used in the following 'two phase' experimental design, comprising of an accumulation phase followed by a subsequent recovery phase. A summary of the experimental design is presented in Fig. 6.4.



**Fig. 6.4** Experimental design for Recovery Experiment 3. Apical tips were cultured for one week in an accumulation phase and then transferred to recovery media for up to 8 days. Samples were removed from the experiment on Accumulation day 7 and recovery days (RD) 1, 5 and 8. Two treatments (control and  $145.6\mu\text{g L}^{-1}$  copper) and six replicates of each treatment were used. See text for details.

#### 6.5.1.1 Accumulation phase

During the accumulation phase, 24 replicates for each of two treatments, control and  $145.6\mu\text{g L}^{-1}$  copper (measured in media on day 0 using CSV – see below) were cultured in a CEC under standard culture conditions for one week. A high copper concentration was used in this experiment (in comparison to the previous two) to ensure that a relatively large amount of copper accumulated in the apical tips during the exposure period. For each replicate, ten apical tips were randomly selected and placed in a petri dish containing 25ml of standard culture media and copper. The lengths of the apical tips in each replicate were measured using image analysis at the start and end of the week.

At the end of Week 1, six replicates from each treatment were removed from the experiment and used for copper analysis. The algal material from the six replicates of each treatment were blotted dry and pooled (1+4, 2+5 & 3+6) to provide sufficient material for analysis. Samples were frozen, freeze dried and then digested using the small digestion vessel method. Copper analysis of digested algal material was carried out using AAS-GF (see Chapter 2 for details). Three replicates each containing 20 apical tips from the initial pool were also collected on Day 0 and analysed for copper in the same way.

Ten ml of media from three of the six replicate petri dishes were also used to determine dissolved copper concentration. The concentration of copper ( $Cu_{labile}$ ) in each sample was determined using the CSV method described in detail in Chapter 2. Samples were also taken from the media prepared for each treatment on Day 0 to provide an indication of the actual copper concentration in the media at the beginning of the experiment. Three petri dishes of each treatment containing no apical tips were also prepared on Day 0, kept under the same culture conditions as the algal material for a week and analysed for copper content at the end of the week. These blanks were used in order to account for any changes in copper concentration in the media during the accumulation phase, which were not due to accumulation by the apical tips.

#### 6.5.1.2 *Recovery phase*

Following the week-long accumulation phase, all remaining 18 replicates were removed and placed into new petri dishes containing fresh control media. All apical tips were rinsed in fresh control media, before transfer to the new media to remove any copper not bound to the tips. The recovery phase lasted eight days. Six replicates from each of the two treatments were removed on each of Days 1, 5 and 8 of the recovery period. These were used to determine the copper concentration in the apical tips and the media, in the

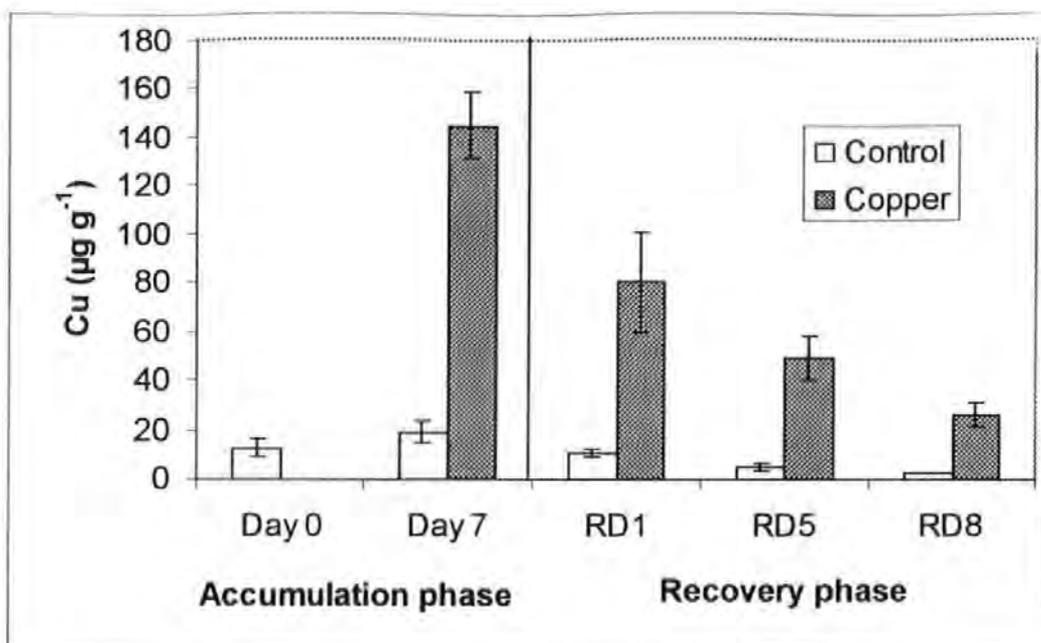
same way as described above for the accumulation phase. The lengths of the apical tips in each of the six replicates were also measured using image analysis prior to processing the tips for copper analysis.

### 6.5.2 Results

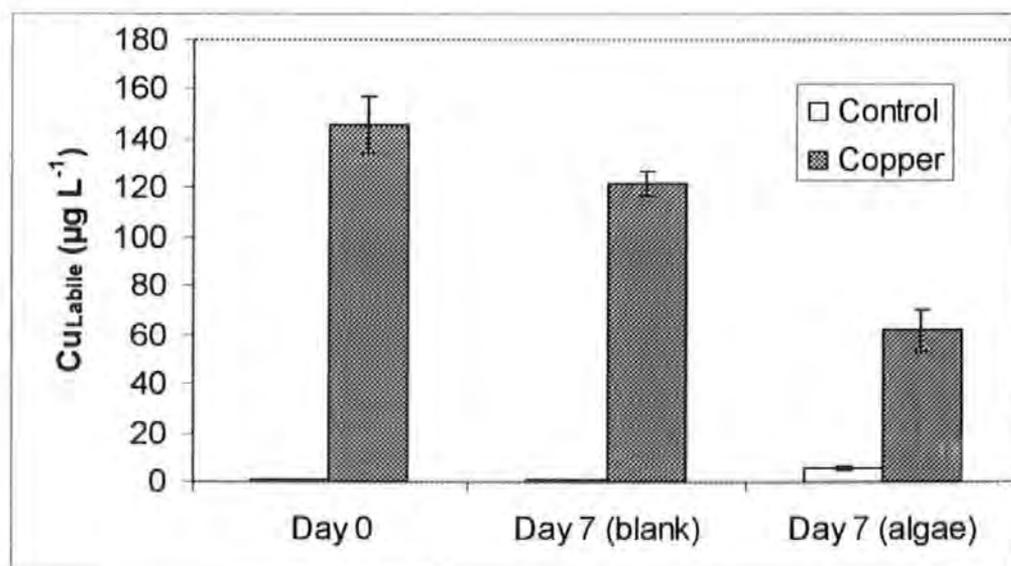
The pattern of growth reduction and recovery was similar to that found in the previous experiment. The addition of  $145.6\mu\text{g L}^{-1}$  copper resulted in a significant reduction of growth of the apical tips compared to the controls during the seven days treatment (t-test,  $n=48$ ,  $t=18.118$ ,  $P<0.001$ ). The copper treatment RGR,  $3.1\% \text{ day}^{-1}$ , was 40% less than the control RGR of  $5.1\% \text{ day}^{-1}$ . After eight days recovery, there was no significant difference in the RGR of the control and copper treatments. The average RGR during the recovery phase was  $4.20\% \text{ day}^{-1}$ .

The initial mean copper concentration of the apical tips was found to be  $12.4\mu\text{g g}^{-1}$ . This increased significantly (t-test,  $n=5$ ,  $t=19.963$ ,  $P<0.001$ ) to  $145.0\mu\text{g g}^{-1}$  over the week of exposure to copper (Fig 6.5). The concentration of copper in the control samples on Day 7 remained comparatively low ( $19.1\mu\text{g g}^{-1}$ ) and was not significantly different from that of copper concentration in the control samples on Day 0 (Fig. 6.5).

In the media, the copper treatment contained  $145.6\mu\text{g L}^{-1}$  of copper, on Day 0 whereas the control media contained less than  $1\mu\text{g L}^{-1}$ . The blank copper sample (containing no algal sample) on Day 7 of the accumulation phase contained  $121.9\mu\text{g L}^{-1}$  of copper. Although lower, this was not significantly different from the initial concentration (Fig 6.6). By comparison the copper concentration in the media, in which the tips were cultured for one week, was significantly reduced to  $61.8\mu\text{g L}^{-1}$ , 50% less copper than in the Day 7 copper blank treatment (t-test,  $n=6$ ,  $t=5.698$ ,  $P=0.005$ , Fig 6.6).

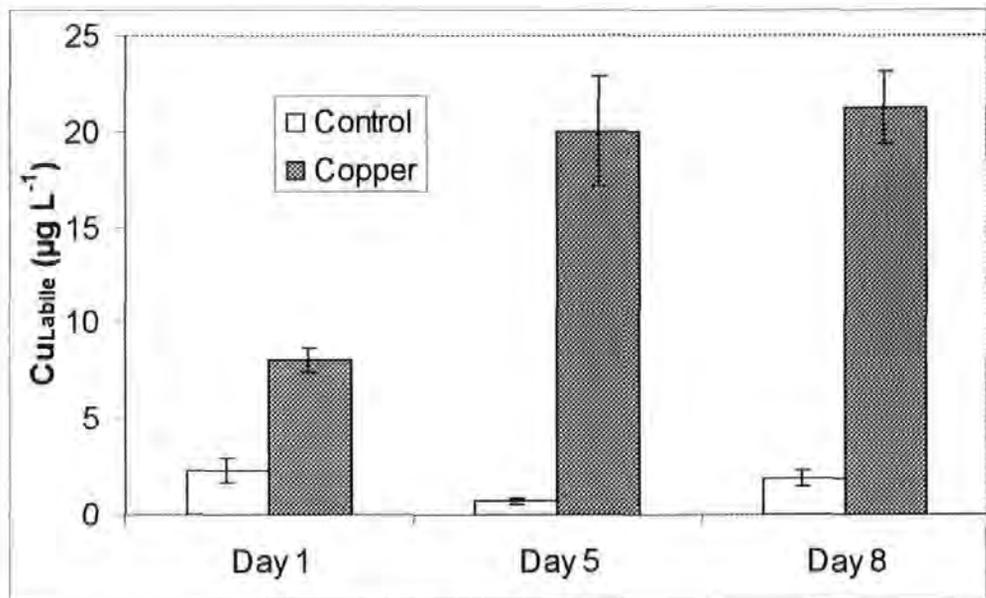


**Fig. 6.5** Mean (n=3) copper concentration of *G. longissima* apical tips during accumulation and recovery phases. Error bars show 95% confidence intervals.



**Fig. 6.6** Mean copper concentration (Cu<sub>labile</sub>) in the media during the accumulation phase. The Day 7 blank contained no apical tips. Error bars show 95% confidence intervals.

During the recovery phase the concentration of copper in the apical tips which had been exposed to copper was reduced. From an initial mean concentration of  $145.0\mu\text{g g}^{-1}$  at the end of the accumulation phase, the concentration of copper decreased to  $80\mu\text{g g}^{-1}$  after one day of recovery. By Day 5, the copper concentration in the apical tips was found to be  $49.1\mu\text{g g}^{-1}$  and by Day 8 it was  $26.1\mu\text{g g}^{-1}$ . This represented a significant reduction (t-test,  $n=5$ ,  $t=16.914$ ,  $p<0.001$ ) of 82% from the concentration at the start of the recovery phase (Fig. 6.7). The growth during this recovery phase averaged  $4.20\% \text{ day}^{-1}$ .



**Fig. 6.7** Mean copper concentration ( $\text{Cu}_{\text{labile}}$ ) in the media used during the recovery phase. Error bars show 95% confidence intervals.

The relative importance of dilution of copper concentration due to growth effects over the recovery phase was also estimated. The mean length of the apical tips in the copper treatment on Recovery Day 0 was 12.7mm, and by the end of the recovery phase this had increased to 17.8mm. Assuming that no loss of copper from the apical tips occurred over this period and that tip length relates to biomass (as was found in Chapter 5), this would represent a 30% reduction in copper concentration during the recovery phase due to growth. Therefore, dilution alone cannot explain the 82% reduction in copper concentration during this phase.

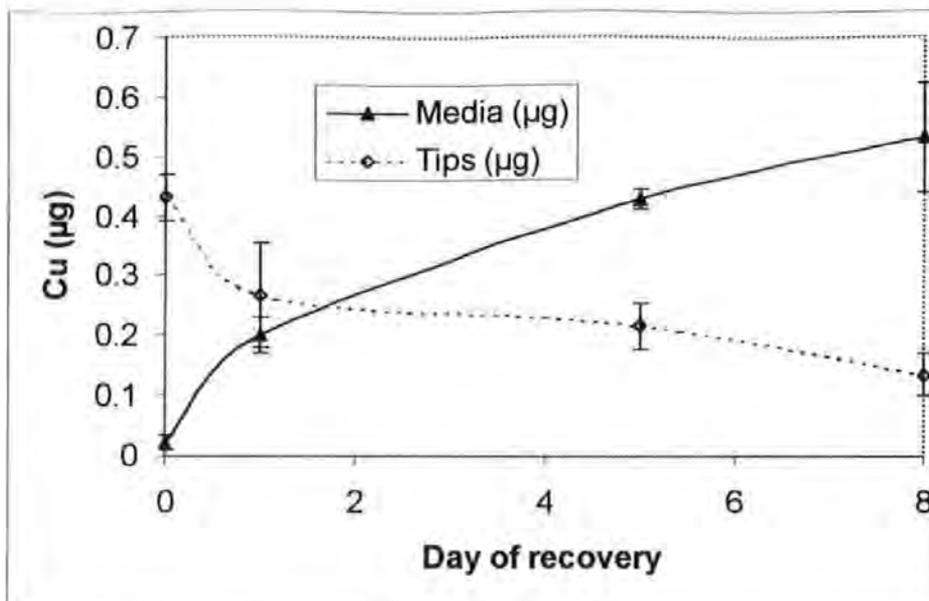
After one week of exposure to copper, the apical tips were removed from the treatment and placed in the control recovery, media. The copper concentration in the recovery media increased significantly from a control concentration of  $0.9\mu\text{g L}^{-1}$  on Day 0, to  $21.3\mu\text{g L}^{-1}$  on Day 8 (t-test,  $n=6$ ,  $t=10.896$ ,  $P<0.008$ , Fig. 6.7). This second, independent, approach also provides good evidence to indicate that there was significant release of copper into the media from the apical tips during the eight day recovery phase.

To establish whether or not copper was being lost from the apical tips, and not just diluted by growth during recovery, the total amount of copper in the apical tips in each petri dish ( $\mu\text{g}$ ) was calculated (Fig. 6.8). Since this value is independent of growth, it would not be expected to vary if the change in copper concentration was due to growth alone. The total amount of copper in the apical tips decreased considerably over the eight days, decreasing from an initial (start of recovery)  $0.431\mu\text{g}$  to  $0.148\mu\text{g}$ , a loss of 66%. This provides good evidence that the total amount of copper present in the algal tissue is significantly reduced over the recovery period and copper loss is occurring.

A comparison of different regression models found that the equation best fitting this loss of total copper from the apical tips was a model of the form;  $y = a + b \sqrt{x}$ .

$$\begin{aligned} \text{Equation:} \quad \text{Total Cu in tips } (\mu\text{g}) &= 0.399 - 0.092 \sqrt{(\text{Recovery}_{\text{days}})} \\ & (n=12, R^2=79.95\%, P<0.001) \end{aligned}$$

Where  $\text{Recovery}_{\text{days}}$  = the number of days in the recovery media.



**Fig. 6.8** Mean ( $n=3$ ) amount of copper ( $\mu\text{g}$ ) in found in the media and *G. longissima* apical tips during the recovery phase. Error bars show 95% confidence intervals.

#### 6.5.2.1 Copper accumulation in the media during the recovery phase

The loss of copper from the tissue during the recovery phase corresponded with the significant increase of copper in the media (Fig 6.8). A comparison of regression models indicated that the best model to fit the rate of accumulation of copper into the recovery media was also an equation of the form  $y = a + b \sqrt{x}$ .

$$\text{Equation: Total Cu in media } (\mu\text{g}) = 0.021 + 0.181 \sqrt{(\text{Recovery}_{\text{day}})}$$

$$(n=12, R^2=96.85\%, P<0.001)$$

Both the concentration of copper in the media and in the apical tips were measured during accumulation and recovery. It is therefore possible to relate the two. Table 6.6 summarises the gains and losses of copper (in  $\mu\text{g}$ ) from both the media and the algal material during the accumulation and recovery phase. The change in copper content in the media during the accumulation phase was estimated using the difference between the Day 7 blank and the Day 7 algal media samples (Fig. 6.6). The estimates of the amount of

copper lost from the apical tips during the recovery phase were made relative to the amount gained by the copper treated material on accumulation Day 7.

Treatment	Media	Apical tips
	<i>Copper Lost (µg)</i>	<i>Copper Gained (µg)</i>
Accumulation (Day 7)	1.501±0.492	0.431±0.106
	<i>Copper Gained (µg)</i>	<i>Copper Lost (µg)</i>
Recovery Day 1	0.201±0.049	0.164±0.193
Recovery Day 5	0.429±0.037	0.213±0.088
Recovery Day 8	0.531±0.148	0.297±0.094

**Table 6.6** Summary of the copper budget made between the culture media and algal tissue. The mean amount of copper (µg) ± 95% confidence intervals are shown.

Over the eight days of recovery there was reasonable agreement between the amount lost from the apical tips and the amount gained to the media (Table 6.6). While it is unrealistic to expect an exact correlation between the two measurements, the results suggest that the copper lost from the apical tips can be accounted for in the media.

## 6.6 Discussion

The results of three experiments in this chapter show that growth can recover following a significant reduction, due to exposure to toxic levels of copper. Growth rates following one week of exposure to 25µg L<sup>-1</sup> copper recovered to those of the controls, after two weeks culture in recovery media. In comparison, apical tips exposed to the 50µg L<sup>-1</sup> treatment for one week only recovered to approximately 80% of the controls after two weeks culture in the recovery media. This indicates that higher levels of copper not only result in increased reduction in growth but may also increase the time required to recover. Recovery was still possible following two weeks pre-treatment with copper and despite sustained low growth rates during exposure.

It is clear from these results that valuable information may be obtained from investigating the growth response of seaweeds during a recovery phase, following trace metal exposure.

No major difference could be seen between the recovery pattern of the two populations, Helford and Mylor (Experiment 2). The significant interaction found between the two populations and copper treatments, appears to be due to the lower growth of the Mylor controls over the duration of the three week experiment. Over this time, the growth of the Mylor controls was considerably variable, but on average lower, than the Helford controls which were more constant. The Mylor 25-C-C treatment recovered to a growth rate higher than the Mylor controls and similar to the Helford controls. This suggests that the population effect observed in Week 1 and the interactions found over the three weeks, are not indicative of any major differences in recovery between the populations. These results are consistent with the conclusions made in Chapter 3, that no major difference exists between the response of any of the different populations compared.

The results obtained in Experiment 3 provide good evidence that the apical tips that accumulated copper during exposure to high levels can be rapidly lost when moved into cleaner media. This was reflected by both the media and the apical tips. The loss of copper after eight days recovery totalled 66% of that accumulated over seven days. Dilution effects due to growth were estimated. According to these calculations, the elongation of the apical tips during the recovery phase may contribute towards the reduction in the concentration of copper in the apical tips, but would not explain any more than 30% of the observed reduction in apical tips. The rate of copper loss from the apical tips was non-linear, initially fast and slowing with time. The rate of increase of copper in the media appears to also follow a similar pattern, indicating that the majority of the copper released by the apical tips can be accounted for by its presence in the recovery media. The relatively

large discrepancies in the copper budget (Table 6.6) are not surprising considering the amounts of copper being quantified. These errors are thought to be mainly due to the fact that digested algal samples were pooled from several dishes and possibly differences between the techniques used to measure the copper in the media and apical tips.

The limited number of studies which have considered the release of trace metals from macroalgae have varied in their findings depending upon the algal species, metal and method of investigation. Two early studies by Bryan (1969, 1971) on the absorption of the zinc radioisotope  $^{65}\text{Zn}$  by the brown seaweed *Laminaria digitata*, found that the metal gradually accumulated in the alga over time and that it was irreversibly bound within living algal cells. The accumulated  $^{65}\text{Zn}$  was rapidly lost by killing the algal material. From this they concluded that there was no evidence of regulation and that the metal was gradually accumulated within the cells by the alga throughout its life and that extracellular polysaccharides play little or no role in this binding. Skipness *et al.* (1975) used a similar method to investigate the uptake of strontium ( $^{85}\text{Sr}$ ) and zinc ( $^{65}\text{Zn}$ ) in the brown seaweed *Ascophyllum nodosum*. They concluded that while strontium was reversibly bound, probably via ion exchange with extra cellular polysaccharides, zinc was mostly irreversibly bound intra-cellularly with only a small fraction of the uptake due to the ion exchange with the extracellular polysaccharides. In a more recent study Karez *et al.* (1994) studied the release of zinc in *Padina gymnospora* following exposure to  $^{65}\text{Zn}$  and found no significant decrease in the metal content of the alga over more than ten days of recovery in non-radioactive medium. They also concluded that zinc is strongly bound to cellular sites within the algae. However, these results are contrary to the findings of Mykelstad *et al.* (1978 & 1979) and Eide *et al.* (1980) who found evidence for the release of zinc in *Ascophyllum nodosum* using field transplant experiments. This release could not be explained by dilution effects due to growth alone and it was suggested that zinc could be excreted, bound to algal polyphenols (Eide *et al.*, 1980).

By comparison, Hall (1981) found a significant release of copper from the marine fouling alga *Ectocarpus siliculosus*. Although no growth data during this recovery phase was provided, it was claimed that this copper loss could not be explained by dilution effects. She found that the percentage of copper measured in the cells rapidly reduced during incubation in copper free seawater following pre-incubation in high copper treatments. Although the experimental methods differ from those used in this study, losses of more than 80% after nine days incubation were found which is comparable with those reported here. The slight (non significant) loss of copper reported in *Enteromorpha* sp. by Seeliger & Cordazzo (1982) was thought more likely to have been due to a dilution effect, as a result of new growth during the recovery phase, although they also do not provide any growth data to support this claim.

These contrasting findings could be due to a number of factors such as the metal and species being investigated, the concentrations involved or even differences in the methods employed (for example field or laboratory).

The relationship between copper content of the apical tips and time during the recovery phase in Experiment 3 indicates an initially fast release rate over the first day followed by a slowing of the release rate. This may be explained by an initially fast loss of copper weakly bound to the cell wall and extracellular polysaccharide layers, followed by a slower release from inside the cell. A comparison of the release rate of copper from living and dead algal material such as that used by Bryan (1969) and Skipness *et al.* (1975) may help to indicate the localisation of copper in the alga.

While it is not possible to establish the exact mechanism(s) involved in the release of copper from *G. longissima*, some inferences can be drawn from the results using the data obtained from Experiment 3. The copper measurements from the media were all

measures of the electrochemically labile copper ( $\text{Cu}_{\text{labile}}$ ) using a relatively strong complexing ligand (8-hydroxyquinoline). The mass balance results indicate that most of the copper released by the apical tips could be measured in the media using this technique. This suggests that the copper being released back into the media was either free ( $\text{Cu}^{2+}$ ), inorganically bound ( $\text{CuI}$ ) or bound to organic ligands ( $\text{CuL}$ ) which were weaker at complexing copper than 8-hydroxyquinoline. A measure of the  $\text{Cu}_{\text{total}}$  in the media may have accounted for the missing copper. Further experiments incorporating voltametric analysis could be used to further distinguish the chemical speciation of the released copper. This novel method of assessing the loss of copper from algal material by measuring the concentration released into the media, is considered to be a method with much potential.

As was stated above, determining the exact localisation of the metals accumulated by *G. longissima* may help indicate the mechanisms of metal exchange that are occurring. Previous investigations into the localisation of trace metals in algae using x-ray microanalysis have found varying results, depending upon the species and metal in question. For example, Qureshi & Stokes (1985) found that copper was mostly bound in cytoplasmic deposits and intra-nuclear inclusions in two species of the green algae *Scenedesmus sp.*, whereas Brown *et al.* (1988), found evidence for copper being bound externally in *Amphora coffeaeformis*. This was thought to be due to binding to the outer cell surface or mucilage, although metal hydroxides have been shown to coat cells and bind ionic copper (Stauber & Florence, 1985). Importantly, Zolotukhina & Gavrilenko (1992) found that when they exposed *Gracilaria verrucosa* to a high concentration of copper ( $300\mu\text{g L}^{-1}$ ), polysaccharides were involved in binding the excess copper.

Zinc has also been shown to be mainly accumulated in the cell walls and extracellular matrix of *Gracilaria sordida* (Holmes *et al.*, 1991) and *Padina gymnospora*

(Amado Filho *et al.*, 1996). In the example of *Gracilaria sordida*, a considerable amount of the zinc was also shown to be associated with bacteria surrounding the alga.

#### 6.6.1 Implications for biomonitoring

The results presented here suggest that the sampling of native populations of *G. longissima* for use as a biomonitor of copper contamination may be of limited value. Apical tips were found to release a large percentage of accumulated copper over a relatively short period (66% in eight days). This indicates that the analysis of native material collected from sites suspected to be contaminated could be misleading, unless the level of contamination is known to be constant. This is an assumption that is unlikely to be true all of the time and would need to be verified. Lovett Doust *et al.* (1994) suggested an alternative approach that may be more informative and has greater potential, i.e. the use of seaweeds in an active biomonitoring role. This approach is described and explored in detail using *G. longissima* in the next chapter.

### 6.7 Conclusions

The results clearly show that recovery of growth following exposure to high levels of copper is possible. Complete recovery of RGR could be seen following exposure to copper levels as high as  $145\mu\text{g L}^{-1}$  for one week.

The copper concentration in the apical tips was found to be significantly reduced once removed from media containing a high concentration of copper. This reduction in copper concentration was not explained solely by dilution due to growth. A significant loss of copper from the algal material (66% over eight days) was found to occur during the recovery phase and this was reflected by an increase in copper within the external media.

Both the rate of copper loss from the apical tips and the rate of increase of copper in the media were found to be non-linear and reduce with time. In relative terms, the copper lost from the tissue could be accounted for by copper recovered in the external media. The loss of copper from *G. longissima* apical tips demonstrates that both accumulation and elimination of copper can occur in this species, indicating that copper is not irreversibly bound by *G. longissima* and suggesting some form of regulation may be occurring, which is worth investigating.

## **Chapter 7**

# **ACTIVE BIOMONITORING: ASSESSING THE FEASIBILITY OF USING *GRACILARIOPSIS LONGISSIMA***

## 7.1 Introduction

In the introductory chapter, the advantages of active biomonitoring (ABM) over the more commonly used passive biomonitoring approach were described. These advantages can be summarised as follows:

- ◆ The period for which the individuals are exposed for is known and can be adjusted depending upon the objectives of the particular study.
- ◆ Comparisons can be made between native and introduced individuals. This may indicate possible differences in resistance that may have developed.
- ◆ Similarly, reciprocal transplants can be made between sites thought to differ in contamination, thus providing further useful information on the rates of uptake (or release) of metals.
- ◆ Monitoring stations may be selected, independent of the natural (non) occurrence of the species being used.
- ◆ Resolution of statistical tests can be optimised by using similar groups of organisms.

This chapter details investigations carried out using the ABM approach to assess copper contamination using *G. longissima*.

Numerous studies, in both the terrestrial and aquatic environment, have employed different species of flora and fauna for an ABM approach to monitor contaminants (Lovett Doust *et al.* 1994 and de Kock & Kramer 1994).

Many examples exist of bivalve molluscs being used for ABM (for review see de Kock & Kramer, 1994) with the approach often being found to be beneficial. For example,

Gibb *et al.* (1996) transplanted the mussel *Mytilus edulis* (for seven weeks) from a control site to sites known to differ in mine derived trace metal contamination. They found that the concentrations of zinc and lead increased in transplanted mussels placed in the contaminated sites and these concentrations correlated with those in mussels native to the sites. Metal concentrations in transplanted mussels were nevertheless still lower than in native mussels after the seven week period, suggesting that a longer period may be required to equilibrate with the higher metal concentrations at the contaminated sites. These findings were in agreement with Simpson (1979) who found that it took three months for transplanted mussels to equilibrate to higher zinc concentrations and even longer to equilibrate for lead.

The exact methodology employed, including the duration and method of transplantation varies considerably between different studies depending upon the species used and the particular metal(s) under investigation. For example, Evans & Hutchinson (1995) transplanted two species of lichen (*Cladonia rangiferina* and *Hypogymnia physodes*) and one species of moss (*Pleurozium schreberi*), for 20 months, from a control site to a range of sites thought to differ in mercury contamination. Significant differences in mercury concentration of *C. rangiferina* could be seen at some sites after 12 months while *P. schreberi* differences were not apparent until 20 months. By contrast, no differences in the mercury concentration of *H. physodes* could be seen over the same period.

Despite the apparent advantages of ABM techniques over passive biomonitoring, surprisingly few pollution studies using seaweeds to monitor trace metal contamination have employed this approach. Langston (1984) transplanted a number of organisms including several gastropods, molluscs and two species of brown seaweeds (*Ascophyllum nodosum* and *Fucus vesiculosus*) from a relatively clean site, in the Tamar Estuary, to Restronguet Creek and monitored arsenic concentrations in the organisms, in comparison

with the natives. Ho (1984) also transplanted two of the same species, *Fucus vesiculosus* and *Ascophyllum nodosum*, from the Tamar Estuary to Restronguet Creek and monitored the change in concentration of copper and zinc in the transplanted material over 60 days. The levels of metals in the transplanted species from these studies can be compared with the work of Bryan & Gibbs (1983) who surveyed the concentration of many trace metals in the sediment, flora and fauna of the Fal Estuary.

The use of transplanted seaweeds to monitor polluted estuaries has been recommended by Wilkinson *et al.* (1992) who measured the growth and survival of three intertidal seaweed species transplanted to polluted sites to gauge the effects of different effluent discharges.

Most ABM studies involve the transplantation of individuals from a clean (control) site to other site(s) thought to be contaminated. Mykkestad *et al.* (1978) did the opposite, transplanting the brown seaweed *Ascophyllum nodosum* from a locality with high trace metal contamination to a locality of low contamination for five months and measured the change in metal concentrations in different age classes of algal material over time. The concentration of zinc in young apical tips of transplanted individuals was approximately the same as native plants after two months. Concentrations in older parts also decreased over the same period. Even accounting for growth (dilution effects) this reduction in older plants suggested a release of at least 20-30% of the accumulated zinc following transfer. This finding is contrary to the view held by Skipnes *et al.* (1975) that in *A. nodosum* zinc is irreversibly bound.

It is sometimes possible to transplant samples both to and from different sites i.e. reciprocal transplanting. Reciprocally transplanting samples between populations, has the advantage of allowing an evaluation of each populations response at the different sites.

Although such transplant studies have commonly been used in macroalgae for other purposes such as to evaluate interpopulation phenotypic variation (see review by Mathieson *et al.*, 1981), only one example could be found that used macroalgae with this approach to investigate trace metal contamination. Eide *et al.* (1980) following on from the results of their one way transplants (Myklestad *et al.*, 1978) reciprocally transplanted *Ascophyllum nodosum* between two sites which differed in trace metal contamination.

A reciprocal transplant approach has been employed to study the Fal Estuary using animals. Langston (1984) reciprocally transplanted individual *Scrobicularia plana* between a control site and the contaminated Restronguet Creek for one year and monitored changes in arsenic concentrations. The author found that the concentration of arsenic in the transplanted individuals (to both sites) was the same as that in the native individuals after four months.

Given that ABM has considerable advantages over passive biomonitoring, it was decided to evaluate the use of *G. longissima* in this role. Since two populations of *G. longissima* (Mylor and St Just) had already been investigated from within the Fal Estuary, it was decided to transplant samples of *G. longissima* between the two sites, as well as transplanting samples to the most contaminated creek (Restronguet). These transplants were designed to enable comparisons between the response of the two populations, as well as differences in copper contamination between the different creeks.

In all of the transplantation studies described above (which transplanted from a control site into contaminated sites), some attempt was made to standardise the individuals transplanted between sites in order to reduce inter-individual variability. For example, de Kock & Kramer (1994) describe the use of mussels from an 'appropriately composed selected length class'. To minimise this variation, physiologically identical individuals

should ideally be used. These could be obtained from culturing genetically identical clones in a uniform environment. Although difficult in many animals and plants this option is possible in various species of seaweed where the life cycle can be manipulated to produce many clones from one individual. Unfortunately, attempts to obtain genetically identical clones of *G. longissima*, using carpospores from the carposporophyte phase, were unsuccessful. Reliable spore release and settlement could not be achieved. Thus, it was not possible to use laboratory reared clones in an ABM role.

In light of this, a different approach was developed using ramets of individuals as replicates. *Gracilariopsis longissima*, like many species of seaweed, can be propagated vegetatively for culturing purposes. This feature was utilised to increase the statistical resolution of the transplants used for ABM. By splitting individuals at the holdfast into ramets, it was possible to effectively transplant the same individual (genet) into different sites. Comparisons between the ramets from the same individuals could then be made statistically using matched pairs analyses. This novel transplanting approach was tested using *G. longissima* individuals from two different populations. Using ramets from individuals also has the added advantage that it is possible to describe and discuss the range of responses each individual exhibits in the different sites.

As has been highlighted previously (Chapter 1), it is possible for changes in trace metal concentration to occur due to growth effects (dilution). In order to investigate this possibility, the RGR of transplanted material can be measured. If ramets are used then this allows comparison between the metal accumulation and RGR of each individual to be made.

## 7.2 Aims

The main aim of this work was to establish the feasibility of using *G. longissima* in an active biomonitoring role. In order to do this, the reciprocal transplants were made between the Mylor and St Just populations using matched pairs of samples from individuals split into ramets. Ramets were also transplanted into Restronguet Creek, to establish how much information could be gained from transplanting into a contaminated site where no native population of *G. longissima* could be found. The growth rate of each ramet was measured so comparisons could be made between RGR and metal concentration of each individual.

## 7.3 Experiment 1

The purpose of this first experiment was to assess the practicalities of using ramets of *G. longissima* individuals as active biomonitors of copper contamination. Due to the limitations in sampling (only possible on spring low tides) material was transplanted for one month. Reciprocal transplants were made between the populations from Mylor and St Just, and some ramets were placed in Restronguet Creek.

The three sites are described in detail in chapter 2, but can be summarised as follows. Levels of copper contamination, as measured by sediment analysis (Bryan & Gibbs, 1983), show that the least contaminated site is St Just ( $356\mu\text{g g}^{-1}$ ) followed by Mylor ( $1117\mu\text{g g}^{-1}$ ), with Restronguet ( $>2000\mu\text{g g}^{-1}$ ) being considerably more contaminated. These measurements were thought to give the best indication of the relative amounts of copper which might be present in the water at each site.

### 7.3.1 Materials and methods

Large individuals (still attached to small stones or shells) from the Mylor and St Just populations were collected from below the waterline on a spring low tide (January 1996) and placed in clean plastic bags. Samples were then taken to the laboratory where they were prepared for transplanting.

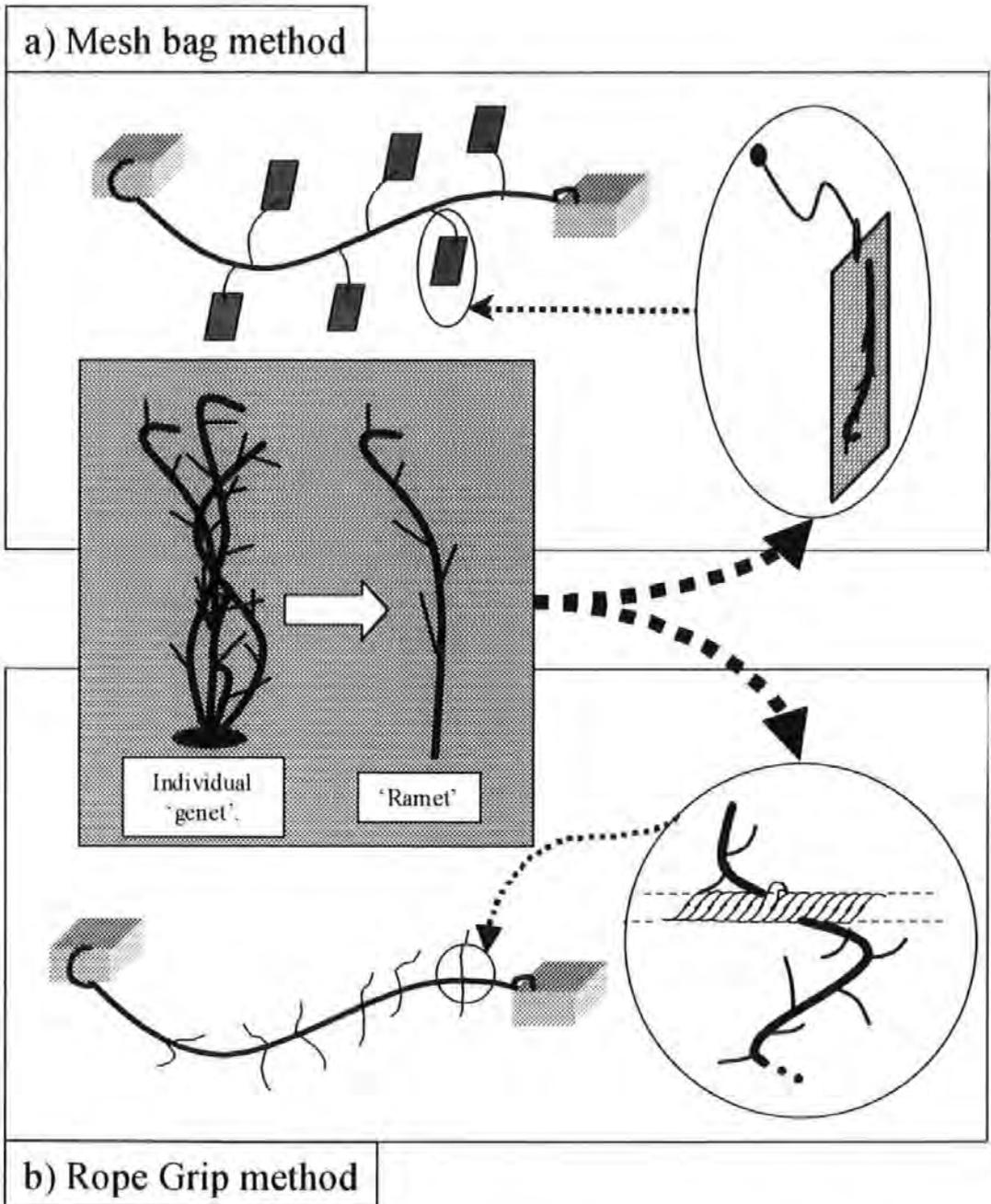
A method for transplanting *Gracilaria chilensis* described by Pilai (1992) was employed to reciprocally transplant ramets of *Gracilariopsis longissima* individuals from the Mylor and St Just sites.

All visible epiphytes were removed from each individual. Six individuals from each population were rinsed in filtered seawater and split (at the holdfast) into two ramets of approximately equal size which were then accurately weighed (using a Sartorius balance) after removing excess water using a salad drier (ten spins per ramet).

A ramet from each individual was placed inside a plastic mesh bag (~15x4cm) and attached to one of two nylon rope lines (4mm thick, 2m long, see Fig. 7.1a). Each line was arranged so that a ramet from each of the six individuals, from the two populations was attached to it in a random order (12 ramets in total per line). This resulted in two treatments for each population.

Origin of individual	Site in which ramet was placed for one month	Treatment name	Number of samples
Mylor	Mylor	Mylor Control (MC)	6
Mylor	St Just	Mylor Transplant (MSJT)	6
St Just	St Just	St Just Control (SJC)	6
St Just	Mylor	St Just Transplant (SJMT)	6

**Table 7.1** Experimental design used in the reciprocal transplant Experiment 1.

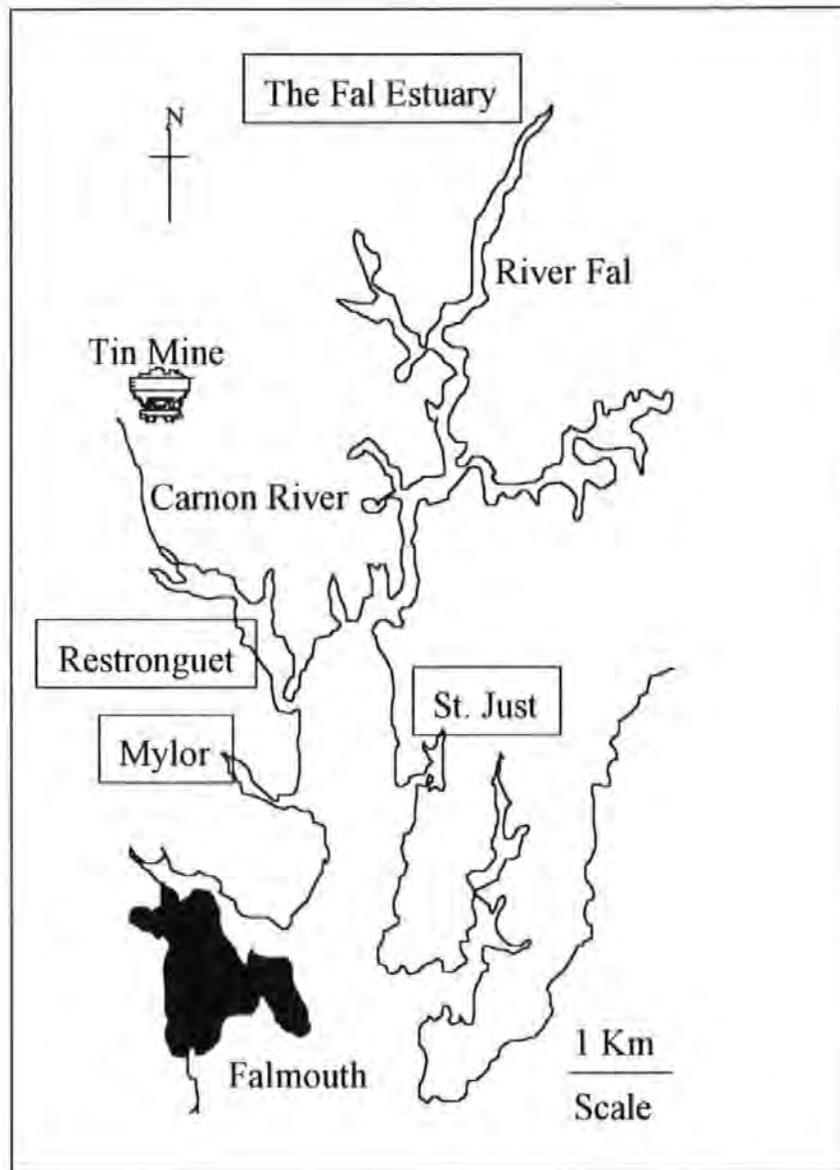


**Fig. 7.1** Methods used to transplant ramets from *G. longissima* individuals. Individuals (genets) were split apart at the holdfast and ramets from each individual attached to nylon rope using either the mesh bag method (a) or the rope grip method (b). Nylon lines were then weighted down on each end with a brick and transplanted for one month.

In addition to the above samples, two further lines were prepared.

- ◆ Ramets from another five individuals from each population were also rinsed in filtered seawater, spun dry, weighed and placed in nylon mesh bags which were attached in a random order to another nylon rope line (4mm thick, 2m long). This line was placed in Restronguet Creek. The Mylor and St Just samples transplanted into Restronguet were labelled MRT and SJRT respectively.
  
- ◆ In order to test the rope grip method of transplanting Gracilarioids previously described by Critchley (1993), five individuals from the Mylor population were rinsed, and weighed in the same way as described above. Instead of being placed in a mesh bag attached to a rope line, they were inserted between the strands of the nylon rope itself (Fig 7.1b).

All rope lines containing ramets of algae were returned to the Fal Estuary the following day. For the reciprocal transplants, one line was placed in each of Mylor and St Just creeks. Each line was placed in the same area as the native population (submersed) at low tide. Bricks were tied to each end of the lines to anchor them in place. The rope grip test line was also placed in the St Just site. The Restronguet Creek samples were placed at the mouth of the creek just below the waterline at low tide (see Fig. 7.2).



**Fig. 7.2.** Map of the field sites used in this chapter. Individuals sampled from the Mylor and St Just populations were used for reciprocal transplants and were also transplanted into a site in Restronguet Creek.

All lines were left at the sites for one month. When they were collected from the water and returned to the laboratory all samples were rinsed in filtered seawater, spun dry, and re-weighed. Samples from the reciprocal transplants and the Restronguet Creek line were then frozen and freeze dried in preparation for acid digestion using the microwave digestion technique for small digestion vessels. Digested algal samples were then assayed for copper content using AAS-GF. Fresh weight values were used to calculate RGRs (described in Chapter 2).

### 7.3.2 Results

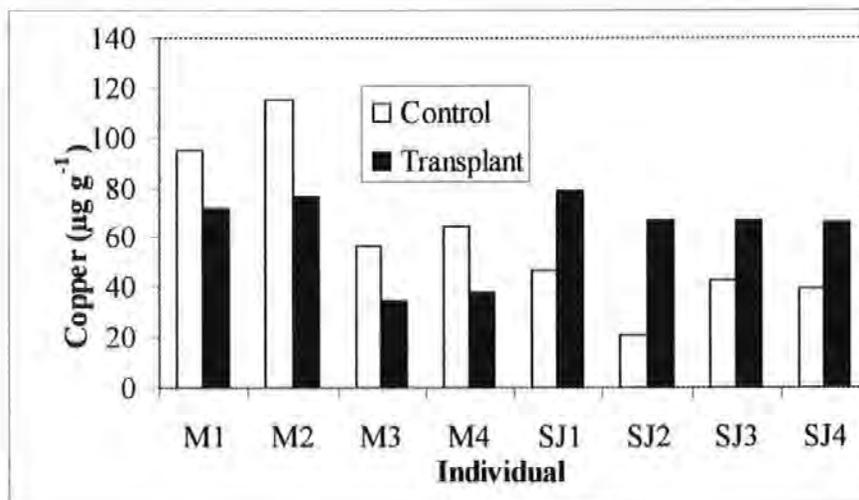
Upon retrieval of the lines, it was found that some mesh bags had become detached resulting in the loss of some samples. However, matched pairs of samples from four of the six individuals from each population were still attached. In the case of the line transplanted to Restronguet Creek only eight of the original ten mesh bags remained – four samples from each population. These samples were digested (small digestion vessels) and copper concentrations measured, following the procedures outlined in Chapter 2.

Copper concentrations of Mylor individuals transplanted to the St Just site for one month, were significantly lower than those of the control samples. (Comparison MC-MSJT, Table 7.2). This reduction can be seen by comparing the control and transplant copper concentrations for Mylor individuals in Figure 7.3. The mean copper concentration in the Mylor controls after one month was  $82.8 \mu\text{g g}^{-1}$  whereas it was only  $55.0 \mu\text{g g}^{-1}$  for the transplants (Fig 7.4). This represents an average loss of  $27.8 \mu\text{g g}^{-1}$  of copper (34% of controls) from each individual over the month.

Conversely, transplantation of St Just individuals into Mylor for one month, resulted in a significant increase in the copper concentrations of all individuals (Comparison SJC-SJMT, Table 7.2, Fig. 7.3). This can be seen clearly by the fact that the St Just transplants contained on average  $69.6 \mu\text{g g}^{-1}$  copper when transplanted to Mylor, whereas the controls contained on average only  $37.7 \mu\text{g g}^{-1}$  of copper (Fig. 7.4). This represents a gain in copper over the month of an average  $31.9 \mu\text{g g}^{-1}$  (84.6% of controls) an amount similar to that lost by the reciprocal transplant from Mylor.

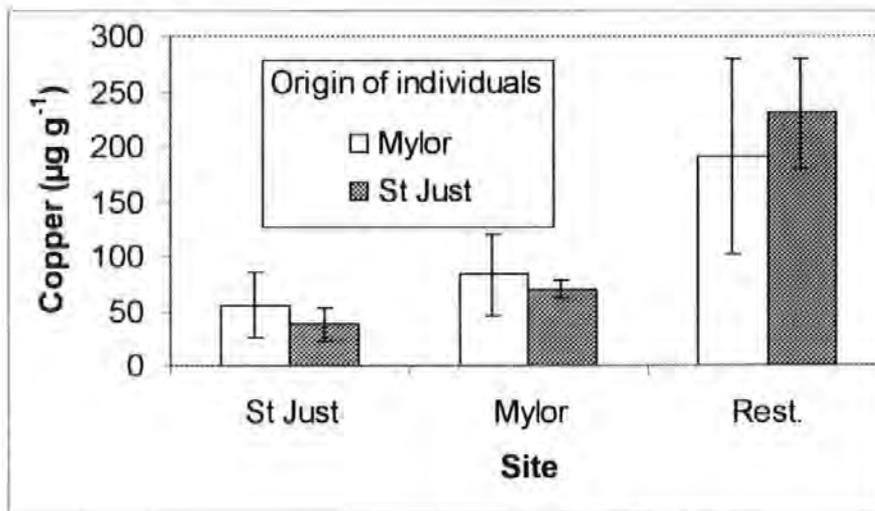
Comparison	Difference	P-value
MC-MSJT (Matched Pairs)	MC>MSJT	0.005
SJC-SJMT (Matched Pairs)	SJC<SJMT	0.008
SJC-MC	SJC<MC	0.022
SJC-MSJT	Not significant	
SJMT-MC	Not significant	
SJMT-MSJT	Not significant	
SJRT-MRT	Not significant	
MC-MRT	MC<MRT	0.012
SJMT-SRT	SJMT<SRT	<0.001
MSJT-MRT	MSJT<MRT	0.004
SJC-SJRT	SJC<SJRT	<0.001

**Table 7.2** Summary of comparisons between copper concentrations of the different transplant treatments in reciprocal transplant Experiment 1. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.



**Fig. 7.3** Copper concentration in ramets of *G. longissima* from four Mylor (M) and four St Just (SJ) individuals, reciprocally transplanted for one month. Control ramets from each individual were replaced back into the site they originated from, while transplanted ramets were placed in the opposite site.

The Mylor controls were found to contain significantly more copper than the St Just controls (Comparison SJC-MC, Table 7.2, Fig. 7.4). The copper concentrations of samples from both populations growing in the same site for one month were not significantly different (Comparisons SJC-MSJT and MC-SJMT, Table 7.2, Fig. 7.4).



**Fig. 7.4** Mean (n=4 for St Just and Mylor sites and n=5 for Restronguet site) copper concentration of *G. longissima* individuals from St Just and Mylor populations transplanted into three sites; Mylor, St Just and Restronguet. Error bars show 95% confidence intervals.

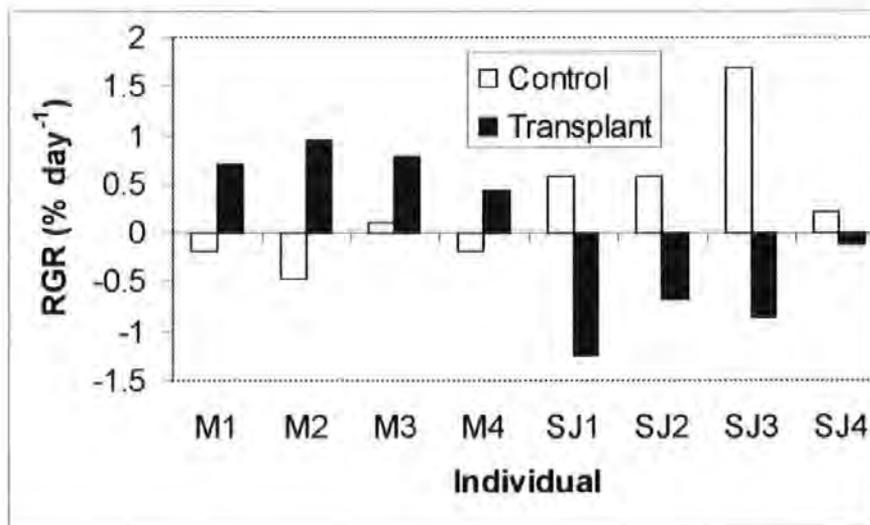
The samples from the two populations that were transplanted to Restronguet Creek (MRT & SJRT) accumulated copper to a similar level, approximately  $200\mu\text{g g}^{-1}$ . This concentration was significantly higher than that accumulated by material growing at Mylor (Fig. 7.4). The difference is verified by the fact that the SJMT-SJRT and MC-MRT comparisons were both significant, while the SJRT-MRT comparison was not (see Table 7.2).

The RGRs of the transplanted ramets were highly variable with considerable differences between individuals. The analysis of the RGR data from the reciprocally transplanted individuals indicates that the Mylor ramets transplanted to St Just grew significantly more than their paired controls in Mylor (Comparison MC-MSJT, Table 7.3, Fig. 7.5). The mean RGR of the Mylor controls was in fact negative ( $-0.19\% \text{ day}^{-1}$ ) while the mean of the Mylor transplants was positive ( $0.71\% \text{ day}^{-1}$ ). Conversely, the St Just ramets transplanted to Mylor all grew significantly worse ( $-0.73\% \text{ day}^{-1}$ ) than their paired controls in St Just ( $0.77\% \text{ day}^{-1}$ ). The St Just controls had a significantly higher growth rate

than the Mylor controls and the Mylor transplants grew significantly more than the St Just transplants.

Comparison	Difference	P-value
MC-MSJT (Matched Pairs)	MC<MSJT	0.016
SJC-SJMT (Matched Pairs)	SJC>SJMT	0.050
SJC-MC	SJC>MC	0.031
SJC-MSJT	Not significant	
SJMT-MC	Not significant	
SJMT-MSJT	SJMT<MSJT	0.001
SJRT-MRT	Not significant	
MC-MRT	Not significant	
SJMT-SJRT	Not significant	
MSJT-MRT	MSJT>MRT	0.002
SJC-SJRT	Not significant	

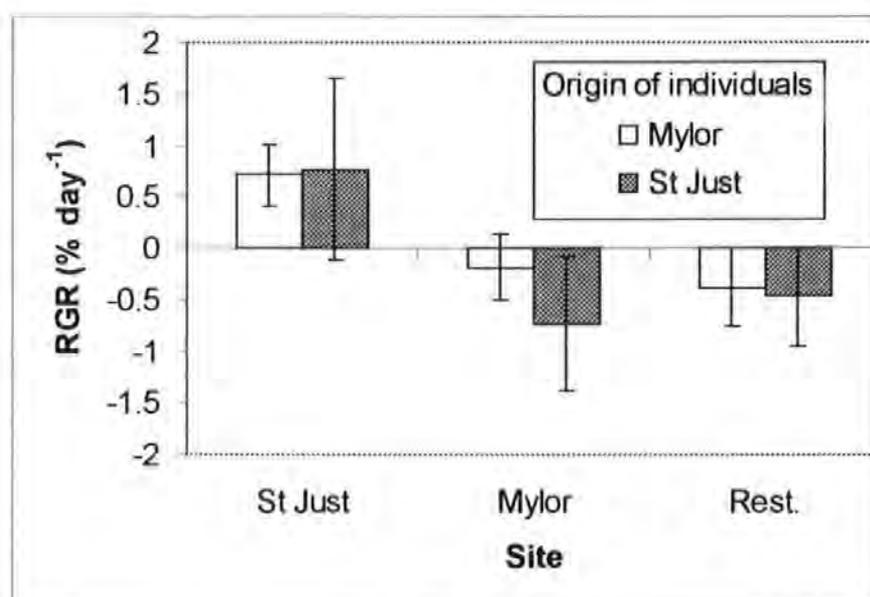
**Table 7.3** Summary of the comparisons made between RGRs of the different treatments in reciprocal transplant Experiment 1. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.



**Fig. 7.5** Relative growth rates of *G. longissima* ramets from four Mylor and four St Just individuals, reciprocally transplanted for one month. Control ramets from each individual were replaced back into the site they originated from, while transplanted ramets were placed in the other site.

There was no significant difference between the growth rate of Mylor and St Just individuals transplanted to Restronguet (Table 7.3, Fig. 7.6). The mean growth rate of the samples from both the Mylor and St Just populations transplanted to Restronguet for one month was  $\sim 0.43\% \text{ day}^{-1}$ , which was similar to the RGR of the individuals placed in Mylor (Fig. 7.6). The RGR of the Mylor individuals transplanted to Restronguet was significantly

lower than the RGR of the growth of the Mylor individuals transplanted to St Just (Comparison MSJT-MRT, Table 7.3,  $P=0.002$ , Fig. 7.6).



**Fig. 7.6** Mean ( $n=4$  for St Just and Mylor sites and  $n=5$  for Restronguet site) RGR of *G. longissima* individuals from St Just and Mylor populations transplanted into three sites: Mylor, St Just and Restronguet. Error bars show 95% confidence intervals.

After one month all the samples used to test the rope grip method were still present. The mean RGR of the five Mylor individuals transplanted to St Just for one month using the rope grip method was  $0.96\% \text{ day}^{-1}$ . This was higher, but not significantly different from the mean RGR of the Mylor individuals transplanted to St Just using the mesh bag method ( $0.71\% \text{ day}^{-1}$ ).

Although there was no replication of the analysis of copper concentrations in individual ramets used in this study (the freeze-dried samples were too small to divide further), it does appear that some consistent differences exist between individuals. For example, Mylor individuals 1 and 2 appear to contain more copper than Mylor individuals 3 and 4 (see Fig. 7.3). The differences are apparent – both in control and transplanted material. The Mylor individual with the highest copper concentration of  $115\mu\text{g g}^{-1}$  at the Mylor site (Individual M2, Fig. 7.3) also contained the highest copper concentration when

transplanted to St Just,  $76\mu\text{g g}^{-1}$ . The individual with the lowest copper concentration at the Mylor site ( $56\mu\text{g g}^{-1}$ ) also contained the lowest copper concentration when transplanted to St Just,  $34\mu\text{g g}^{-1}$  (Individual M3, Fig. 7.3). Similarly, the individual that contained the highest copper concentration at the St Just site ( $47\mu\text{g g}^{-1}$ ) also contained the highest copper concentration after transplanting to Mylor,  $79\mu\text{g g}^{-1}$  (Individual SJ1, Fig. 7.3). The individual that contained the least copper in St Just  $21\mu\text{g g}^{-1}$  (Individual SJ2, Fig. 7.3) was not the one with the least copper after transplanting to Mylor although it was similarly low.

Attempts were made to relate the concentration of copper and the RGR of each ramet in all the individuals. No obvious relationship could be found between the copper concentration and the RGR of each individual (Fig. 7.3 & 7.5). For example, individuals SJ3 and SJ4 were found to have very different growth rates but similar copper concentrations when comparing control and transplanted ramets.

#### **7.4 Experiment 2**

The results of the previous experiment indicated that the reciprocal transplantation of ramets of individuals was a more informative alternative to the commonly used passive biomonitoring approach. With the use of relatively few samples, it was possible to compare not only differences between sites but also the response of the two populations. The use of matched pairs of ramets from the same individuals, also allowed some comparisons to be made between the different individuals as well as increasing the statistical power of the experiment.

In light of the results from the first experiment, a further series of reciprocal transplants were carried out. The rope grip method of transplanting was adopted in favour of the mesh bags. Similar growth rates were achieved using the rope grip and mesh bag

method of attaching ramets of material to the nylon lines. The rope grip method of transplanting was, however, considered better for several reasons: it was quicker to assemble the lines, less intrusive on the natural movement of the plants, less likely to cause shading of the ramets by the mesh. In addition to this, no ramets were lost when testing this technique, which would suggest the rope grip method to be more secure.

Initial samples of algal material were also collected from each individual so that any change in copper concentration in controls over the month could also be monitored. To allow some comparison between copper concentrations in the water and the algal tissue, seawater samples were collected from each site at the start and finish of the month's transplant.

The Fal Estuary is known to be contaminated with other metals as well as copper. The availability of an Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES) meant it was possible to measure more than one metal, at the same time. Zinc concentrations were also measured in the digested algal samples as they have been previously reported to be present in the Fal at high concentrations (Langston, 1984).

In order to further investigate the effect of transplanting algal material into sites known to differ in metal contamination, the chlorophyll fluorescence parameter  $F_v/F_m$  was also measured. This non-obtrusive parameter can be measured on samples directly in the field using the portable hand held Hansatech PEA and any response compared with the RGR and metal concentration data.

#### 7.4.1 *Materials and methods*

Individuals from the Mylor and St Just populations were collected on a spring low tide (February 1997) and returned to the laboratory.

Five individuals from each population were cleaned of visible epiphytes, rinsed in filtered seawater and split (at the holdfast) into three ramets of approximately equal size. The three ramets were then accurately weighed (using a Sartorius balance) after being spun dry in a salad spinner (ten spins per ramet). One ramet from each plant was kept as an initial sample for copper and zinc analysis.

The remaining two ramets from each individual were then used in a reciprocal transplant as outlined in Experiment 1. One ramet from each individual was attached to a nylon rope line (4mm thick, 2m long) using the 'rope grip' method (Fig 7.1). Each ramet from the five individuals, from each population was attached in a random order, resulting in a total of ten ramets per line.

A further five individuals from each population were also cleaned and prepared as above and four ramets obtained. As before, one ramet from each plant was frozen and kept for copper and zinc determination and two ramets were used in the same reciprocal transplant design as above. The fourth ramet from each individual from the two populations was attached to a third nylon line, which was placed in Restronguet Creek.

Ten individuals in total were reciprocally transplanted between Mylor and St Just. Five of these individuals also had a ramet transplanted to Restronguet. Table 7.4 summarises this procedure and indicates the naming of the treatments created by this experimental design.

Origin of Individual	Site in which ramet was placed for one month	Treatment Name	Number of samples
Mylor		Mylor Initial (MI)	10
Mylor	Mylor	Mylor Control (MC)	10
Mylor	St Just	Mylor-St Just Transplant (MSJT)	10
Mylor	Restronguet	Mylor-Restronguet Transplant (MRT)	5
St Just		St Just Initial (SJI)	10
St Just	St Just	St Just Control (SJC)	10
St Just	Mylor	St Just-Mylor Transplant (SJMT)	10
St Just	Restronguet	St Just-Restronguet Transplant (SJRT)	5

**Table 7.4** Experimental design used in the reciprocal transplant Experiment 2.

The lines were placed in the field sites (Mylor, St Just and Restronguet) on the following day at low tide and left for one month after which time they were retrieved. Immediately following retrieval but whilst still in the field, the chlorophyll fluorescence parameter  $F_v/F_m$  was measured for each ramet of five individuals from both populations using the Hansatech portable Plant Efficiency Analyser (PEA). All material was then returned to the laboratory where they were cleaned, dried and weighed. The ramets were then frozen, freeze-dried and acid digested using small digestion vessels. Digested samples were analysed for copper and zinc using ICP-OES. A multi-elemental standard containing  $500\mu\text{g L}^{-1}$  copper and  $1000\mu\text{g L}^{-1}$  zinc was used.

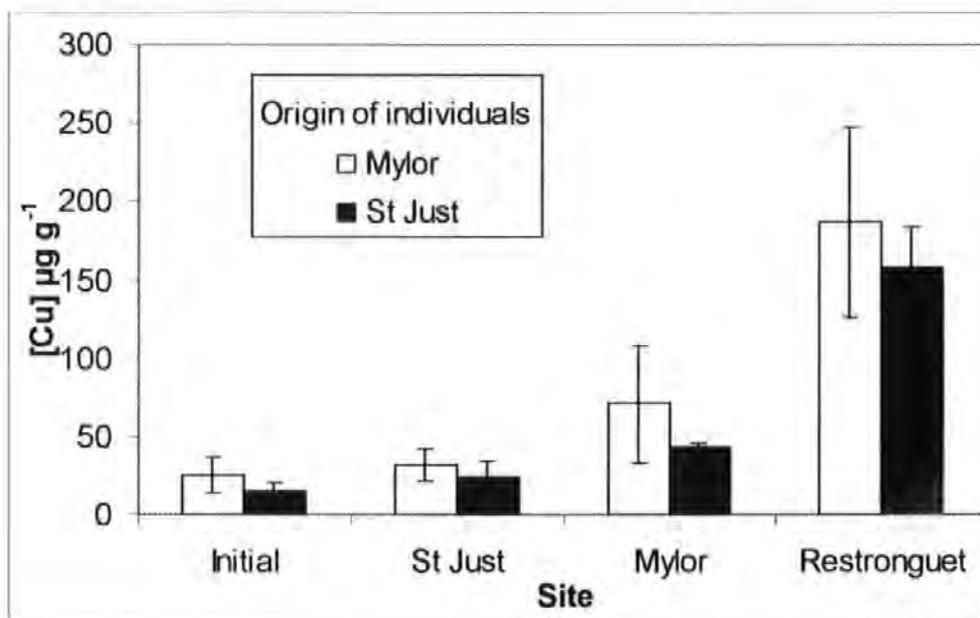
A water sample was collected from each of the three sites (Mylor, St Just and Restronguet) at the start and the end of the month in which algal samples were transplanted. These samples were filtered and analysed for copper content (Total Copper) using CSV as described in Chapter 2. The salinity of each water sample was measured (using a refractometer) at the same time that they were analysed for copper concentration. No measurements of zinc concentrations in seawater were made.

#### 7.4.2 Results

When the lines were retrieved from the three sites, algal material from each ramet was attached to the lines. When this material was digested, there was a significant increase in the copper concentration within individuals that remained in the same site over one month. This is evident from the comparison between the initial copper concentrations (MI and SJI) and their corresponding controls (MC and MSJC) after one month i.e. MI-MC & SJI-SJC (Table 7.5, Figs. 7.7). The copper concentration of the ramets from Mylor increased from an initial mean (MI) of  $25.5\mu\text{g g}^{-1}$  to  $70.9\mu\text{g g}^{-1}$  (MC) an increase of  $45.4\mu\text{g g}^{-1}$  (64%). The St Just samples increased from an initial mean (SJI) of  $15.2\mu\text{g g}^{-1}$  to  $23.9\mu\text{g g}^{-1}$  (SJC) over the month, an increase of  $8.7\mu\text{g g}^{-1}$  (57%). There was no significant difference between the initial copper concentration of ramets from the two sites SJI ( $15.2\mu\text{g g}^{-1}$ ) and MI ( $25.5\mu\text{g g}^{-1}$ ). However after one month the control samples from St Just (SJC) contained significantly less copper ( $23.9\mu\text{g g}^{-1}$ ) than the Mylor control ( $70.9\mu\text{g g}^{-1}$ ) (Table 7.5, Fig. 7.7).

Comparison	Difference	P-value
MI-MC (matched pairs)	MI<MC	0.019
MI-MSJT (matched pairs)	Not significant	
MI-MRT (matched pairs)	MI<MRT	0.002
MC-MSJT (matched pairs)	MC>MSJT	0.025
MC-MRT (matched pairs)	Not significant	
MSJT-MRT (matched pairs)	MSJT<MRT	0.001
SJI-SJC (matched pairs)	SJI<SJC	0.001
SJI-SJMT (matched pairs)	SJI<SJMT	<0.001
SJI-SJRT (matched pairs)	SJI<SJRT	<0.001
SJC-SJMT (matched pairs)	SJC<SJMT	0.002
SJC-SJRT (matched pairs)	SJC<SJRT	<0.001
SJMT-SJRT (matched pairs)	SJMT<SJRT	0.001
SJI-MI	Not significant	
SJC-MC	SJC<MC	0.020
SJRT-MRT	Not significant	
SJMT-MC	Not significant	
SJC-MSJT	Not significant	

**Table 7.5** Summary of comparisons between copper concentrations of the different transplant treatments in reciprocal transplant Experiment 2. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.



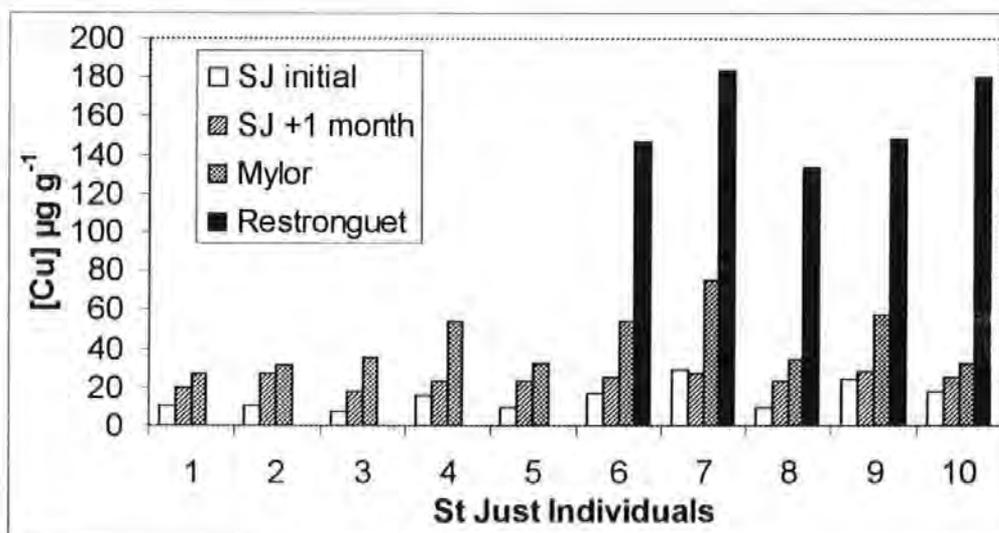
**Fig. 7.7** Mean (n=10 for St Just and Mylor sites, n=5 for Restronguet site) copper concentration of *G. longissima* ramets transplanted from St Just and Mylor to three sites (St Just, Mylor and Restronguet) for one month. Error bars show 95% confidence intervals.

The mean copper concentration of the Mylor samples transplanted into St Just for one month (MSJT) was,  $31.7 \mu\text{g g}^{-1}$ , significantly lower than the Mylor control (MC) but not significantly different from the initial Mylor (MI) concentration (Table 7.5, Fig. 7.7). The St Just samples transplanted to Mylor (SJMT) accumulated significantly more copper ( $43.4 \mu\text{g g}^{-1}$ ) than the St Just initial (SJI) or control (SJC) samples, indicating an increase in copper concentration of  $19.5 \mu\text{g g}^{-1}$  (82%), relative to SJC.

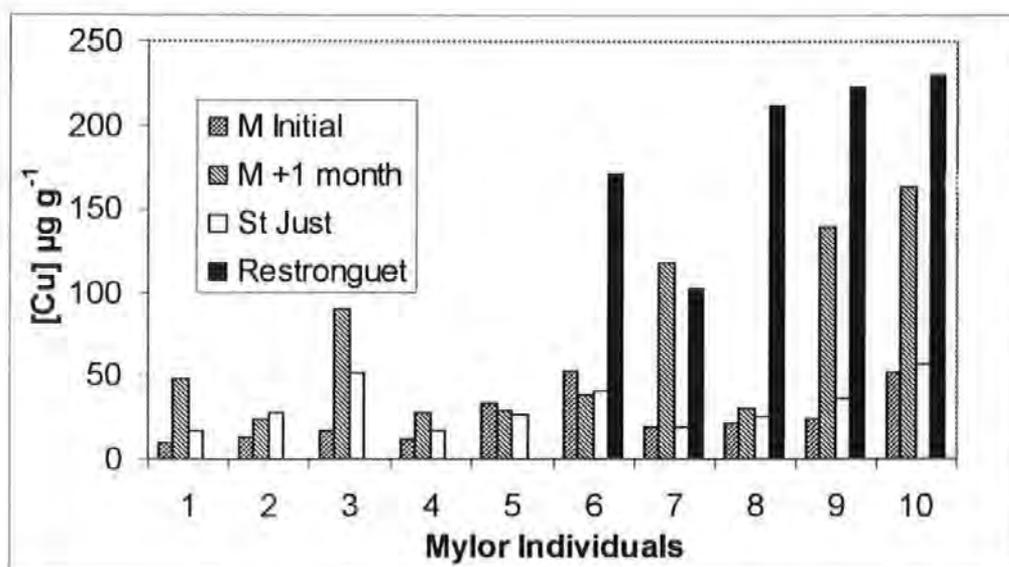
The ramets of Mylor and St Just individuals transplanted to Restronguet Creek had similar mean copper concentrations ( $187.0 \mu\text{g g}^{-1}$  (MRT) &  $158.7 \mu\text{g g}^{-1}$  (SJRT)); these were significantly higher than all other means except the Mylor control (MC) samples (Comparison MC-MRT, Table 7.5).

There were no significant differences between the copper concentrations of St. Just and Mylor individuals placed in the same site for one month, i.e. between SJT and MC, SJC and MSJT or between SJRT and MRT (Table 7.5, Fig. 7.7).

These transplant effects are evident when the copper concentrations in the ramets from each individual are inspected (Figs. 7.8 & 7.9).

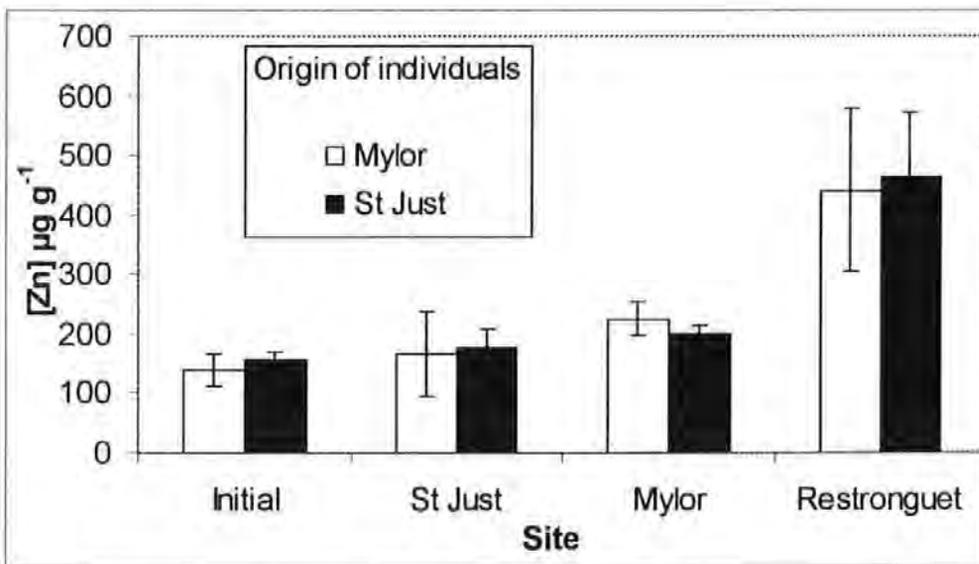


**Fig 7.8** Copper concentration of ramets from ten *G. longissima* individuals from the St Just population, transplanted to three sites (St Just, Mylor and Restronguet) for one month. Five individuals (6-10) had ramets transplanted to Restronguet. Ramets from each individual were also analysed at the start of the month (SJ Initial).



**Fig 7.9** Copper concentration of ramets from ten *G. longissima* individuals from the Mylor population, transplanted to three sites (St Just, Mylor and Restronguet) for one month. Five individuals (6-10) had ramets transplanted to Restronguet. Ramets from each individual were also analysed at the start of the month (M Initial).

The same comparisons as above were made for zinc content (Table 7.6). As for the copper data, the zinc concentrations in the control samples were found to increase significantly over the course of the month. The zinc concentration of the ramets from Mylor increased from an initial mean (MI) of  $138\mu\text{g g}^{-1}$  to  $225\mu\text{g g}^{-1}$  (MC) an increase of  $87\mu\text{g g}^{-1}$  (63%). The St Just samples increased from an initial mean (SJI) of  $155\mu\text{g g}^{-1}$  to  $176\mu\text{g g}^{-1}$  (SJC) over the month, an increase of  $21\mu\text{g g}^{-1}$  (14%) (Fig. 7.10). There was no significant difference between the initial zinc concentration of ramets from the two sites SJI ( $155\mu\text{g g}^{-1}$ ) and MI ( $138\mu\text{g g}^{-1}$ ). (Table 7.6, Fig. 7.10).



**Fig. 7.10** Mean ( $n=10$  for St Just and Mylor sites,  $n=5$  for Restranguet site) zinc concentration of *G. longissima* ramets transplanted from St Just and Mylor to three sites (St Just, Mylor and Restranguet) for one month. Error bars show 95% confidence intervals.

The mean zinc concentration of the Mylor samples transplanted into St Just for one month (MSJT) was,  $166\mu\text{g g}^{-1}$ , significantly lower than the Mylor control (MC), but significantly higher than the initial Mylor zinc concentration (MI). The St Just samples transplanted to Mylor (SJMT) accumulated significantly more zinc,  $198\mu\text{g g}^{-1}$ , than the initial (SJI) or control (SJC) zinc concentrations, indicating an increase in zinc of  $22\mu\text{g g}^{-1}$  (13%), Fig. 7.10.

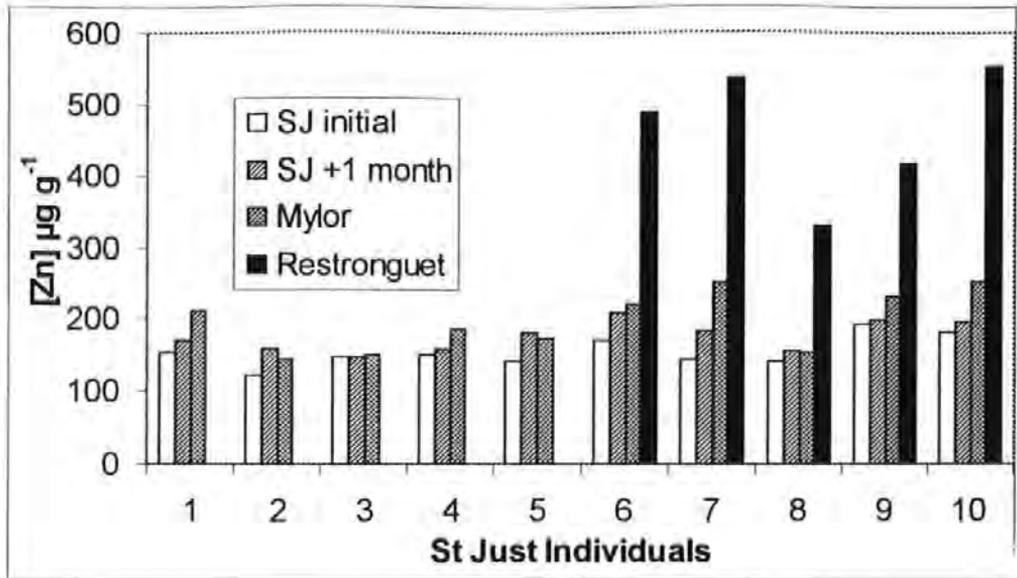
Comparison	Difference	P-value
MI-MC (matched pairs)	MI<MC	0.017
MI-MSJT (matched pairs)	MI<MSJT	0.041
MI-MRT (matched pairs)	MI<MRT	0.001
MC-MSJT (matched pairs)	MC>MSJT	0.030
MC-MRT (matched pairs)	Not significant	
MSJT-MRT (matched pairs)	MSJT<MRT	0.008
SJI-SJC (matched pairs)	SJI<SJC	0.001
SJI-SJMT (matched pairs)	SJI<SJMT	0.001
SJI-SJRT (matched pairs)	SJI<SJRT	0.002
SJC-SJMT (matched pairs)	SJC<SJMT	0.036
SJC-SJRT (matched pairs)	SJC<SJRT	0.002
SJMT-SJRT (matched pairs)	SJMT<SJRT	0.001
SJI-MI	Not significant	
SJC-MC	Not significant	
SJRT-MRT	Not significant	
SJMT-MC	Not significant	
SJC-MSJT	Not significant	

**Table 7.6** Summary of comparisons between zinc concentrations of the different transplant treatments in reciprocal transplant Experiment 2. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.

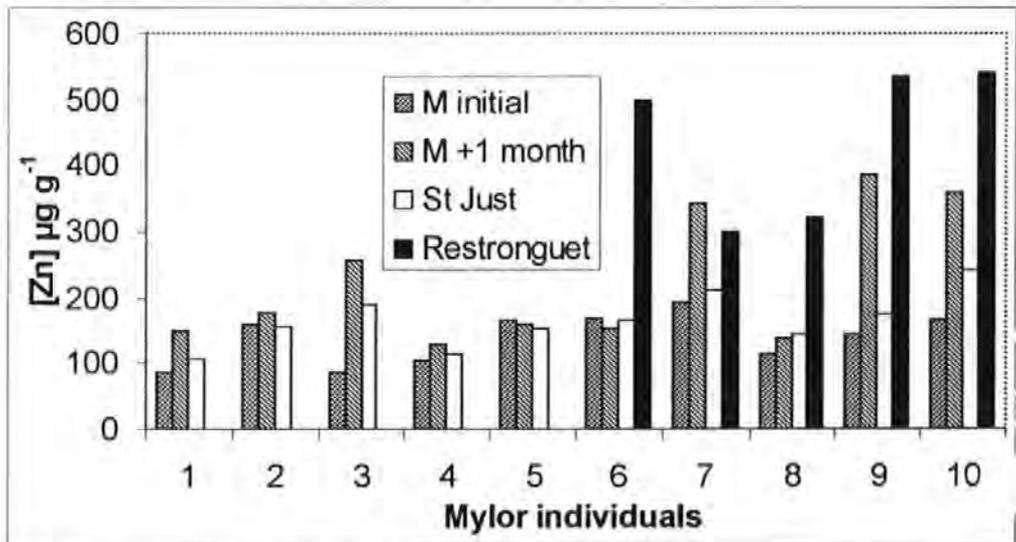
The ramets of Mylor and St Just individuals transplanted into Restronguet Creek accumulated zinc to a similar concentration ( $440\mu\text{g g}^{-1}$  (MRT) &  $464\mu\text{g g}^{-1}$  (SJRT), Fig. 7.10). This level of accumulation was significantly higher than the concentrations found in any of the individuals from the other samples except the Mylor control samples (comparison MC-MRT, Table 7.6, Fig. 7.10).

No significant differences could be found between the zinc concentration of ramets from the St Just and Mylor populations placed in the same site for one month, i.e. between SJT and MC, SJC and MSJT or between SJRT and MRT (Fig. 7.10, Table 7.6).

These zinc transplant effects are apparent in the zinc concentration in ramets of each individual (Figs. 7.11 & 7.12).

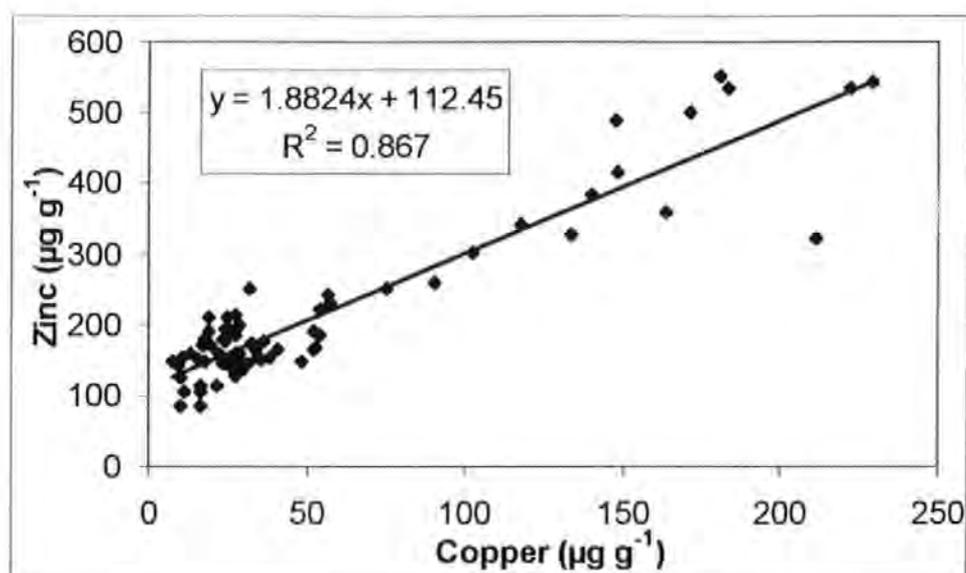


**Fig 7.11** Zinc concentration of ramets from ten *G. longissima* individuals from the St Just population transplanted to three sites (St Just, Mylor and Restronguet) for one month. Only five individuals (6-10) had ramets transplanted to Restronguet. Ramets from each individual were also analysed at the start of the month (SJ initial).



**Fig 7.12** Zinc concentration of ramets from ten *G. longissima* individuals from the Mylor population transplanted to three sites (St Just, Mylor and Restronguet) for one month. Only five individuals (6-10) had ramets transplanted to Restronguet. Ramets from each individual were also analysed at the start of the month (M initial).

A significant positive correlation of 0.93 ( $n=70$ ,  $R^2=86.7$ ,  $P<0.001$ ) was obtained between the zinc and copper concentrations of the ramets from all individuals from both populations (Fig. 7.13). Indicating that ramets which contained high concentrations of copper also had high zinc concentrations.

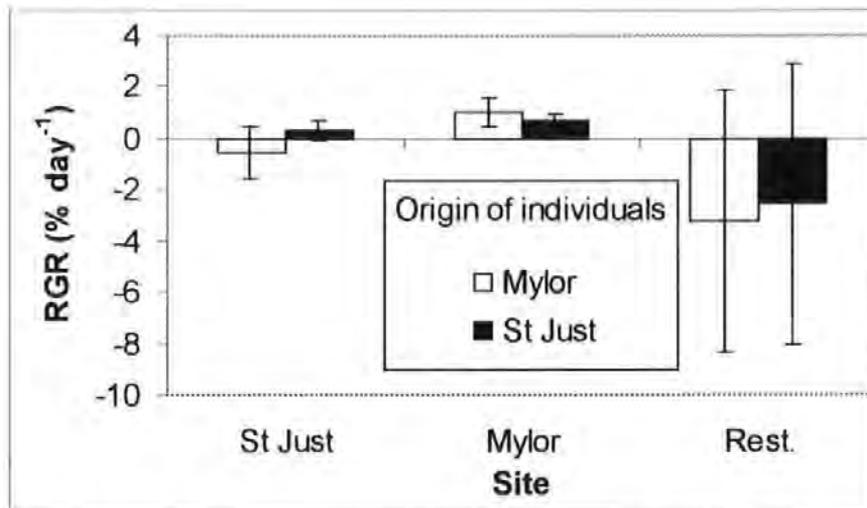


**Fig. 7.13** Relationship between the copper and zinc concentrations in all of the ramets analysed following reciprocal transplant Experiment 2.

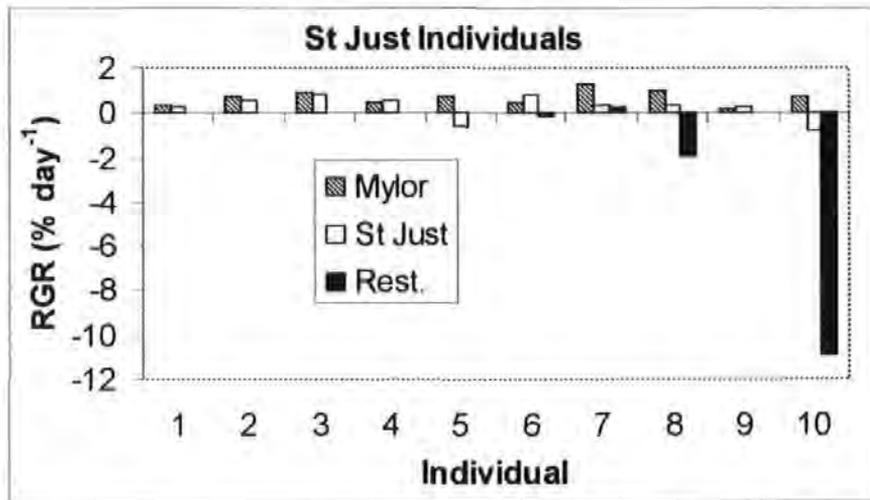
The growth rates of the samples measured over the one month transplant period were highly variable. The only pair-wise comparison that was found to be significant was the matched pair comparison between the growth rate of Mylor controls and their ramets transplanted to St Just. The RGRs of Mylor ramets in St Just were significantly lower than in Mylor ( $P=0.003$ , Table 7.7). The RGRs of the samples transplanted to Restronguet were the most variable, with some individuals having negative RGRs as high as  $-10\% \text{ day}^{-1}$  (Figs. 7.14, 7.15 & 7.16).

Comparison	Difference	P-value
MC-MSJT (Matched pairs)	MC>MSJT	0.003
MSJT-MRT (Matched pairs)	Not significant	
MC-MRT (Matched pairs)	Not significant	
SJC-SJMT (Matched pairs)	Not significant	
SJMT-SJRT (Matched pairs)	Not significant	
SJC-SJRT (Matched pairs)	Not significant	
MC-SJC	Not significant	
SJMT-MC	Not significant	
SJC-MSJT	Not significant	
SJMT-MSJT	Not significant	
MRT-SJRT	Not significant	
MRT-SJMT	Not significant	
SJRT-MC	Not significant	
MSJT-SJRT	Not significant	
SJC-MRT	Not significant	

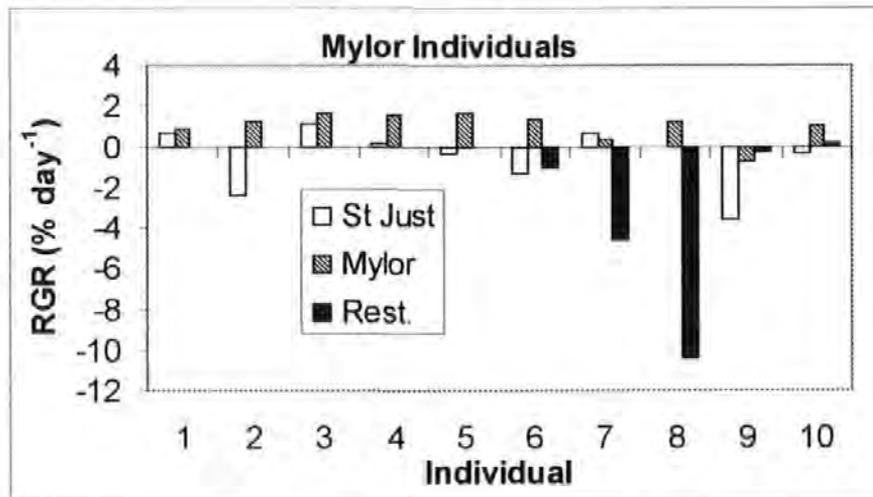
**Table 7.7** Summary of comparisons between RGR of the different transplant treatments in reciprocal transplant Experiment 2. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.



**Fig. 7.14** Mean (n=10 for St Just and Mylor sites, n=5 for Restronguet site) RGR of *G. longissima* ramets transplanted from St Just and Mylor to three sites (St Just, Mylor and Restronguet) for one month. Error bars show 95% confidence intervals.



**Fig 7.15** RGR of ramets from ten *G. longissima* individuals from the St Just population transplanted to three sites (St Just, Mylor and Restronguet) for one month. Only five individuals (6-10) had ramets transplanted to Restronguet.



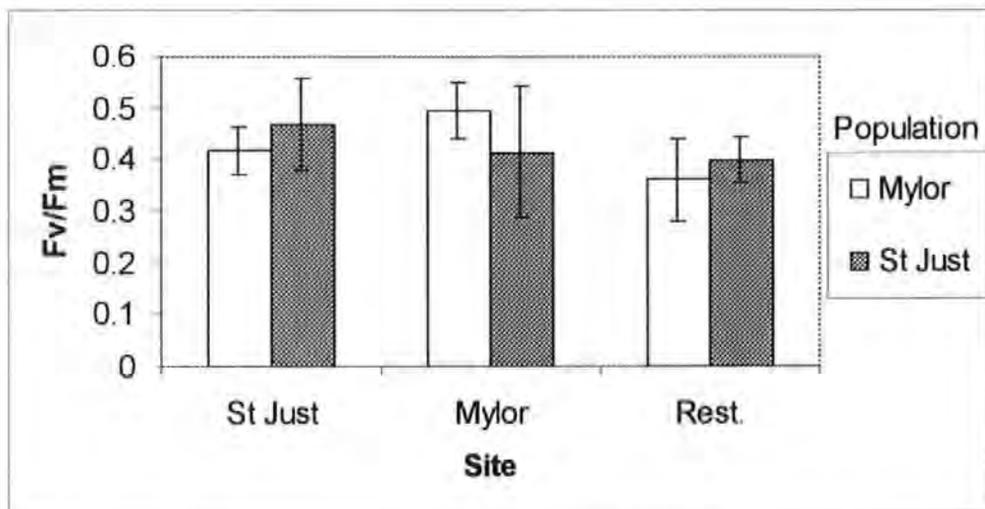
**Fig 7.16** RGR of ramets from ten *G. longissima* individuals from the Mylor population transplanted to three sites (St Just, Mylor and Restronguet) for one month. Only five individuals (6-10) had ramets transplanted to Restronguet.

No pattern could be found between the growth response of ramets from each individual at the different sites. Individuals which had relatively high growth rates at one site did not appear to have consistently high or low growth rates at another. Similarly, no relationship could be found between the RGR and copper (or zinc) concentrations of the ramets. Ramets which contained high concentrations of copper were not found to be consistently fast or slow growing.

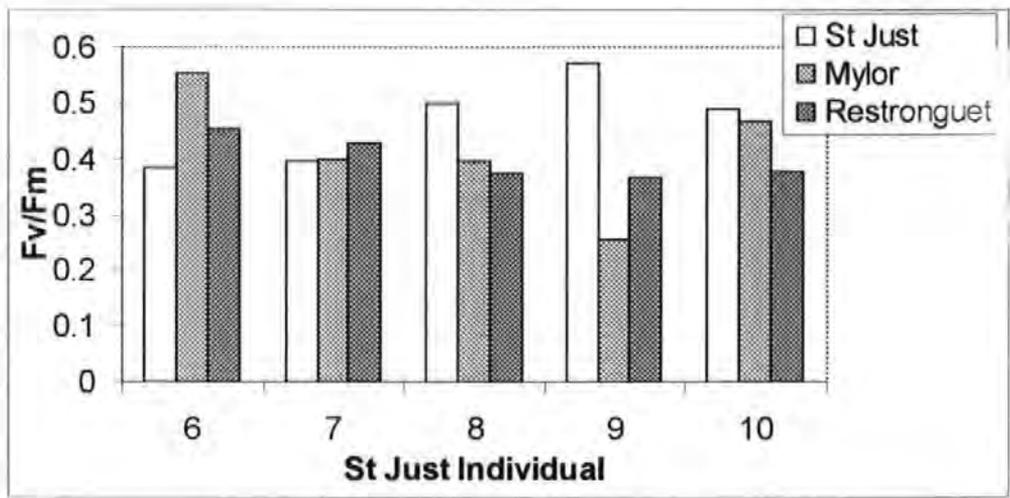
The comparisons of Fv/Fm made between the ramets of the five individuals transplanted from Mylor and St Just are summarised in Table 7.8 and Figs 7.17, 7.18 & 7.19. No significant differences could be found between any of the comparisons made between populations or between the ramets from the five St Just individuals. The mean Fv/Fm of the St Just individuals transplanted to St Just, Mylor and Restronguet was 0.468, 0.413 and 0.399 respectively.

Comparison	Difference	P-value
MC-MSJT (matched pairs)	MC>MSJT	0.005
MC-MRT (matched pairs)	MC>MR	0.043
MSJT-MRT (matched pairs)	MSJT>MR	0.001
SJC-SJMT (matched pairs)	Not significant	
SJC-SJRT (matched pairs)	Not significant	
SJMT-SJRT (matched pairs)	Not significant	
SJC-MC	Not significant	
SJRT-MRT	Not significant	
SJMT-MC	Not significant	
SJC-MSJT	Not significant	

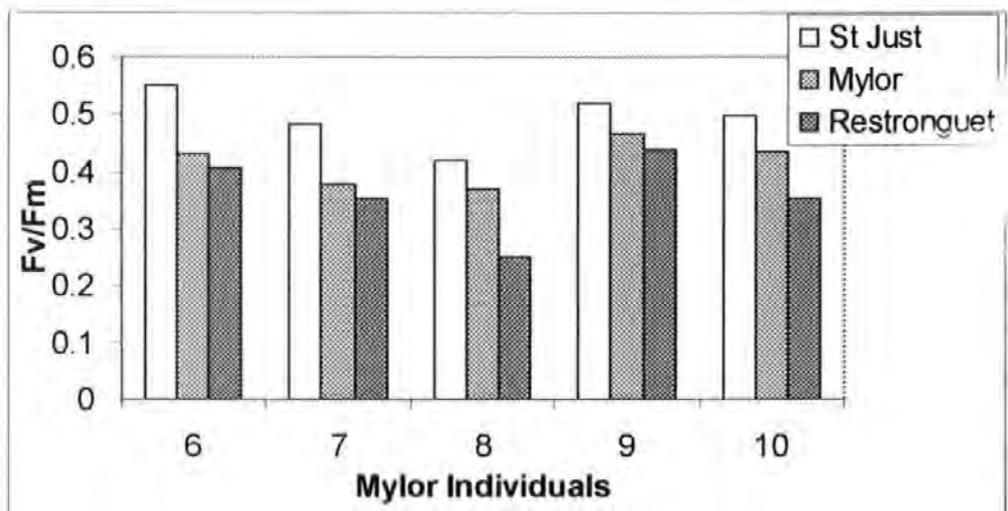
**Table 7.8** Summary of the comparisons made between Fv/Fm values taken from transplanted individuals in reciprocal transplant Experiment 2. Treatment means were compared using either a two sample comparison or a matched pairs comparison t-test. The direction of all significant differences (two tailed, 95% confidence interval) and their P-values are shown.



**Fig 7.17** Mean (n=5) Fv/Fm of *G. longissima* individuals from the St Just and Mylor populations following transplanting to three sites (St Just, Mylor and Restronguet) for one month. Error bars show 95% confidence intervals.



**Fig 7.18** Fv/Fm of ramets from five *G. longissima* individuals from the St Just population following transplanting into three sites (St Just, Mylor and Restronguet) for one month.



**Fig 7.19** Fv/Fm of ramets from five *G. longissima* individuals from the Mylor population following transplanting into three sites (St Just, Mylor and Restronguet) for one month.

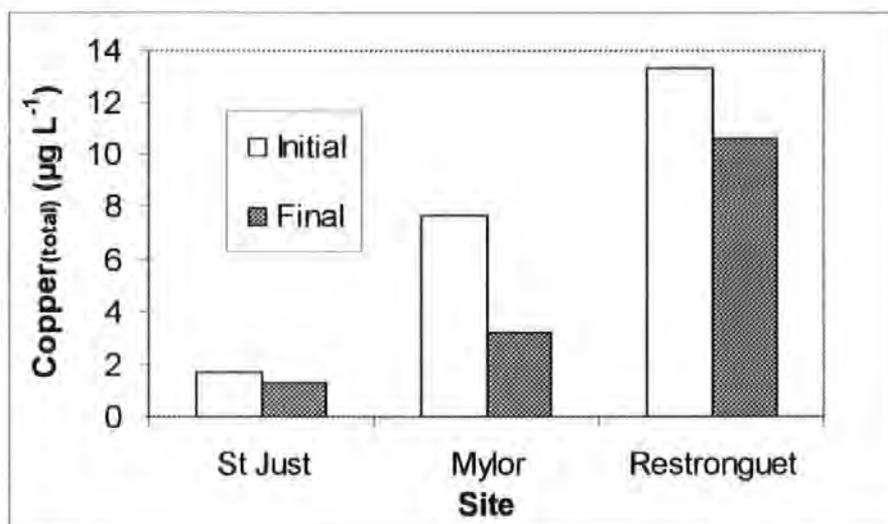
The pair-wise comparisons made between the different Mylor individual ramets transplanted to different sites did reveal some significant differences. The mean Fv/Fm of the Mylor control samples (MC = 0.494) was significantly higher than the Mylor samples transplanted to St Just (MSJT = 0.416). The Fv/Fm of the samples transplanted from Mylor to Restronguet (MRT = 0.360) was significantly lower than the control or St Just treatment (Table 7.8, Fig. 7.17).

The Fv/Fm values varied considerably between individuals, ranging from 0.467 (M8) to 0.372 (M9) amongst the Mylor control ramets (Fig. 7.19). Despite this, the Fv/Fm values of the ramets from the five Mylor individuals transplanted to the other sites show a consistent trend i.e. an increase when transplanted to St Just and a decrease when transplanted to Restronguet. By comparison, the ramets from the St Just individuals did not display a consistent pattern with large differences, both between and within individuals, with no clear treatment (transplant) or individual effect (Fig. 7.18).

The total copper concentration in the filtered water samples from the three sites at the start (Initial) and finish (Final) of the transplant is summarised in Table 7.9. The salinity of each sample is also shown. The concentration varies between collection times, in each case the initial sample being higher than the final sample. However, the gradient in copper levels does rank the sites in the same order as the analysis of algal material. The concentration of total copper in water from St Just was lower than Mylor which was in turn considerably lower than Restronguet (Table 7.9. Fig 7.20). The salinity can also be seen to vary between the sites. In the case of Restronguet the salinity of the sample taken from the end of the month was especially low (5ppt).

Origin of water sample	Cu <sub>total</sub> (µg L <sup>-1</sup> )	Salinity (ppt)
St Just Initial	1.65	28
St Just Final	1.30	30
Mylor Initial	7.64	29
Mylor Final	3.20	21
Restronguet Initial	13.27	14
Restronguet Final	10.60	5

**Table 7.9** Total copper and salinity values from the three sites. Samples were collected at the start (initial) and at the end of the month long transplant experiment.



**Fig. 7.20** Copper concentration ( $Cu_{total}$ ) in water samples taken from each site at the beginning (Initial) and end (Final) of the months transplant.

A number of estimates of the concentration factor between the algal samples and the water can be calculated. Table 7.10 lists the different values calculated for this factor, using the different estimates of copper concentration in the water and algal material.

Water sample	Seaweed sample	Concentration Factor
SJ Initial	SJI	9166
M Initial	MI	3307
SJ Final	SJC	18254
M Final	MC	21934
SJ Final	MSJT	24186
M Final	SJMT	13437
R Final	SJRT	14840
R Final	MRT	17512
	<b>Mean</b>	<i>15330</i>

**Table 7.10** Summary of the concentration factors calculated using the copper concentrations in the transplanted algal material and seawater samples.

The lowest concentration factor is 3307, calculated using the initial water copper concentration in Mylor and the initial Mylor tissue sample (Table 7.10). By contrast the greatest concentration factor was that estimated using the Mylor Final water sample and the Mylor Control algal samples (24186). The mean concentration factor from all estimates is 15330 (Table 7.10).

## 7.5 Discussion

The results of the transplantation experiments clearly demonstrate that the copper concentrations within the algal material alter significantly over the period of a month. The changes in accumulation reflect the gradient of contamination previously described within the Fal Estuary (Bryan & Gibbs, 1983). Material transplanted from St Just to Mylor accumulated copper to the level of the native Mylor population. Mylor individuals moved to St Just, had copper concentrations similar to those of the native St Just population and ramets of both populations transplanted to Restronguet Creek accumulated significantly higher levels of copper, up to  $200\mu\text{g L}^{-1}$ . These findings suggest that the two populations regulate copper in a similar way and after one month the transplanted material (in Mylor and St Just) is in equilibrium with the surrounding water. The same also appears true for zinc. There was a significant positive correlation between the two metals, indicating that both zinc and copper are under a similar form of regulation.

From comparisons between the copper concentrations in individuals prior to and after transplantation, it becomes apparent that the levels within individuals can vary considerably even within the same site. This variation in copper and zinc concentration over time may reflect seasonal variation in the bioavailability of the metals which has been reported previously. For example, Langston (1984), found that the concentration of arsenic in native and transplanted *Fucus vesiculosus* in Restronguet Creek fluctuated considerably over the course of a year. The concentration of arsenic in the algae reflected the concentration of dissolved arsenic in the water during this time, indicating that the concentration of arsenic in *F. vesiculosus* is related to the dissolved form in the water. Seasonal variation in metal concentrations have also been reported in other macroalgae (Fuge & James 1974 and Riget *et al.* 1995), where it has been considered to relate to seasonal changes in growth (dilution) and metabolic rates in the seaweed. Dilution does not

appear to be an adequate explanation for the changes in copper concentration seen here after transplantation, since growth rates did not correlate with accumulation rates of copper.

A large amount of variation was also found between the dissolved copper concentration in the water measured at the beginning and end of the experiment and highlights the fact that metal concentrations can vary significantly. These fluctuations may reflect seasonal changes but could equally be due to changes in other environmental factors fluctuating over different time scales. The measurement of the copper concentration in Restronguet Creek did however indicate a similar level of copper contamination in the water at the mouth of the creek ( $\sim 11 \mu\text{g L}^{-1}$  total copper), to that reported previously by Bryan & Hummerstone (1973) of  $11 \mu\text{g L}^{-1}$  total copper. This suggests that a high level of contamination still exists within the creek.

The two transplant experiments were run approximately one year apart. Despite this, the copper concentrations accumulated in the samples placed in Restronguet Creek were similar,  $\sim 200 \mu\text{g L}^{-1}$  in the first transplant and  $\sim 180 \mu\text{g L}^{-1}$  in the second. This is lower than the higher values of  $\sim 500 \mu\text{g L}^{-1}$  reported to have been found in native populations of *Ascophyllum nodosum* and *Fucus vesiculosus* within the creek (Ho, 1984). The zinc concentrations in the samples transplanted to Restronguet Creek were  $\sim 500 \mu\text{g L}^{-1}$  in the *G. longissima* samples. This is also lower than that found in the native populations of *A. nodosum* and *F. vesiculosus* of  $\sim 1100 \mu\text{g L}^{-1}$  (Ho, 1984). Although some differences may be expected between species, it is possible that the concentration of copper and zinc in the *G. longissima* samples transplanted to Restronguet, had not reached equilibrium. Given more time, metal levels may have reached similar concentrations to those found in the other species.

This suggestion is supported by the results from previous transplant experiments. For example, Langston (1984) found that the concentration of arsenic in *F. vesiculosus* transplanted to Restronguet Creek increased over two months to be similar to the concentrations of native plants. By comparison, the arsenic concentration in *A. nodosum* did not increase significantly over 223 days. Similarly, when Ho (1984) transplanted the same species to Restronguet Creek, it was found that the concentration of both copper and zinc increased in *F. vesiculosus* to approximate concentrations of native Restronguet plants after two months. The concentrations in *A. nodosum* did not increase as much over the same period. These findings suggest that given more time the concentration of copper and zinc in the *G. longissima* material placed into Restronguet may increase.

In both experiments, growth rates were measured. The measurement of RGR was considered important for two reasons:

- ◆ The laboratory based experiments clearly demonstrated that growth was sensitive to changes in copper exposure and by monitoring growth in the field it may be possible to see if a similar response occurs.
- ◆ By recording the changes in growth it would be possible to assess the relative importance of growth effects diluting metal concentrations in transplanted material.

Attempts were made to follow the changes in growth of the transplanted ramets but a considerable amount of variation was encountered. This variation could have been due to the method used to transplant the material. Two different transplanting techniques were tried, the mesh bag and the rope grip method. Although these two methods have been used previously to transplant gracilarioid species (Pilai 1992 and Critchley 1993), algal material can be lost from the transplanted sample if fragmentation of the algal material occurs.

Gracilarioid species are known to be able to use fragmentation as a form of vegetative reproduction (Santelices & Varela, 1995). Since growth rates were calculated using the change in fresh weight of the material over the month, some of the large decreases in growth could have been caused by fragmentation of tissue.

Despite these problems, some significant differences between the sites were evident. For example, in the first transplant, the highest growth rates were recorded in the ramets placed in St Just. Significantly lower growth rates of ramets occurred in Mylor (Fig. 7.6). The RGRs of the individuals from Mylor and St Just transplanted to Restronguet for one week were not significantly different from each other or the RGRs at Mylor. However, differing results were obtained in the second transplant. For both populations, RGRs were higher at Mylor than St Just. Although not significantly different from the RGR at other sites, the RGR of samples placed in Restronguet were lower and considerably more variable (Fig. 7.16).

Wilkinson *et al.* (1992) measured the growth of *Fucus vesiculosus*, *Ulva lactuca* and *Enteromorpha intestinalis* transplanted to polluted sites where these species were absent. They concluded that growth could be used to gauge survivability in unknown situations. The results from this study indicate that the growth of *G. longissima* would not be a good indicator of pollution in the Fal Estuary, due to the large amount of variation between the RGRs of individuals. It is considered important that future experiments should attempt to address this problem in measuring the growth rate of transplanted material, through refinement of transplantation and growth measurement techniques.

The Fv/Fm measurements were highly variable and did not seem to consistently reflect the metal gradient at the two sites. This is perhaps not surprising considering the

fact that the results from Chapter 5 indicate that high levels of copper ( $250\mu\text{g L}^{-1}$ ) are required to affect fluorescence.

The lower copper concentration in the ramets from the Mylor individuals transplanted to St Just in the first experiment suggested a loss of copper over the course of the month. However, the initial copper determinations of material at the start of the second experiment highlighted the fact that considerable fluctuations may occur within a site over time. Since there is also little confidence in the growth data, it not possible to eliminate dilution effects as a potential cause of changing metal concentrations. This makes it impossible to establish unequivocally whether the loss of copper from algal material occurred when ramets were transplanted from a more contaminated site to one less contaminated. However, given that the laboratory based experiment clearly established that accumulated copper can be released from apical tips (Chapter 6), it seems likely that some loss of copper would occur after transplantation to a cleaner site.

The loss of metals from seaweeds has been reported previously in field transplant experiments. Myklestad *et al.* (1978) transplanted *Ascophyllum nodosum* from a locality with high trace metal contamination to a locality of low contamination for five months, the concentration of zinc in young apical tips of transplanted individuals was approximately the same as native plants after two months. Concentrations in older parts also reduced over the same period. Even accounting for growth (dilution effects) this reduction in older parts of the plant suggested a release of at least 20-30% of the accumulated zinc following transfer. A consistent result was found when this experiment was repeated at a later date (Eide *et al.*, 1980).

There was difficulty in obtaining a meaningful measure of the levels of copper in the water column. This is because numerous other factors, such as salinity and the amount

of particulate matter present, make it very difficult to gain an accurate indication of the mean levels of metals in the water over time, using a few instantaneous measurements (Seeliger & Cordazzo, 1982). A detailed chemical analysis of the water was unfortunately outside the time limitations of this study, but would be recommended as part of future work. This could establish the range of fluctuations normally experienced in the sites and allow better relationships to be made between the concentrations of copper (and other trace metals) in the water and algae. The variation in water measurements obtained led to considerable doubt as to the reliability of the estimated concentration factors and illustrates the difficulty in quantitatively relating water and algal metal concentrations in the field. Despite the variation between estimates, the mean concentration factor from all of the sites (~15,000) is considerably higher than that found in the accumulation experiments in the laboratory (Chapter 3). This could be due to the fact that copper levels of the apical tips in the laboratory may not have been in equilibrium with the water concentration.

The novel use of individuals as matched pairs for transplanting purposes not only increased the statistical resolution of some of the comparisons made between transplants but also enables some discussion to be made about possible differences between individuals. In both of the reciprocal transplant experiments, large differences in the metal concentrations and growth rates of individuals from within the same site were found. This suggests that some individuals may grow and/or accumulate metals differently than others. Since no replication was made of each individual within each site, it is not possible to test this observation. There does however appear to also be considerable intra-individual variation in the growth and metal accumulation, since individuals from one population which contained high concentrations at one site, did not always contain relatively more at the other sites. Inter-individual variation has been found to be an important source of variability in the related gracilarioid, *Gracilaria chilensis* (Santelices & Varela, 1995), where significant variation in growth was found between ramets from the same individual

grown under similar culture conditions. These inter and intra-individual differences are investigated in more detail in Chapter 8.

## 7.6 Conclusions

The considerable variation between the RGR of each ramet made it difficult to relate changes in growth and metal accumulation. Nevertheless, using an active biomonitoring approach in conjunction with matched pairs allowed considerably more information to be obtained than using a passive biomonitoring approach.

The advantages of active biomonitoring were found to be:

- ◆ Fluctuations in metal concentration can be monitored.
- ◆ Matched pairs improved the statistical resolution.
- ◆ Samples can be placed into a site where they are not normally found.
- ◆ Reciprocal transplants allowed comparisons to be made between the response of different populations.

Refinement of the technique is still possible. The following lines of investigation may help improve the usefulness of any data obtained from this method of biomonitoring.

- ◆ Improving the accuracy of growth measurement.
- ◆ Lengthening the duration of the transplants, to allow investigation into the length of time required for equilibrium to be reached between the metal levels in the algae and water.
- ◆ More detailed analysis of environmental parameters at each site could be measured and compared.

- ◆ The use of ABM in conjunction with any available biomarker that can be developed for the species. Providing such a biomarker is specific for a particular contaminant then some scale of exposure or effect could be made.

In conclusion, ABM appears to be an informative method of monitoring copper contamination in the Fal Estuary. The copper concentrations in the transplanted individuals reflected the gradient known to exist between the three sites and the measured levels of copper in the water. Its use as an alternative to passive biomonitoring approaches is recommended, due to the advantages discussed above. In addition, the use of reciprocal transplants of ramets (matched pairs) is seen as an under-utilised technique that has some potential in seaweed biomonitoring studies. The use of ramets was considered to be an interesting advantage of using a seaweed species such as *G. longissima*, which can be vegetatively propagated.

**Chapter 8**

**INTER AND INTRA-INDIVIDUAL VARIATION  
IN *GRACILARIOPSIS LONGISSIMA***

## 8.1 Introduction

In all experiments described thus far in this thesis, any intra-individual variability was minimised as much as possible. This was achieved by pooling apical tips taken from a number of individuals prior to each experiment and by taking the mean response (usually of 10 apical tips grown in a petri dish) as a single replicate. While this served the purpose of minimising intra-population effects, it does not enable any discussion about the amount of variation that may exist between or even within individuals. Such variation is a valuable source of information, which is overlooked by simply comparing the mean response of a population.

Bennett (1987) has highlighted the importance of considering the individual in organismal physiology. He pointed out that comparative studies usually investigate the effect of a particular environmental variable at the species, population or higher level and that this approach overlooks the importance of the individual. Individuals from within a population would not be expected to respond in a uniform way to a particular environmental variable. It is likely that each individual may differ in its response to a contaminant. Selection must act upon the individual, with detectable changes at a population level occurring as a consequence of these changes at the individual level. This was highlighted by Depledge, (1990b) who stated that '...ecological disturbance (whether natural or man made) arises due to the differential effects of a disturbance on individuals constituting each population'.

In toxicological studies inter-individual variation is usually looked upon as a source of variation that needs to be minimised (Aldrich 1990 and Forbes *et al.* 1995). Yet, selectional pressures caused by environmental stresses such as pollutants, are more likely to exert themselves upon individuals at the extremes of the range of variability than upon

the mean, median or mode (Bennett, 1987). Hence, the toxic effect of a contaminant can be expected to alter the proportion of different phenotypes within a population. Concentrating upon the mean response of a population may overlook this effect. Despite the evolutionary significance, very few studies exist that have investigated inter-individual variability in relation to pollutants. This is perhaps not surprising, 'Given the general lack of interest in inter-individual variability...' (Bennett, 1987).

Investigating the effects of pollutants at the intra-population level could be one way of separating subtle toxic effects from normal phenotypic variation within an environment (Aldrich, 1990). By assessing not only the mean but the variance within a population it may be possible to determine the potential of the population to evolve tolerance to a particular environmental stress (Forbes & Depledge, 1992). It has been found that environmental stress may result in increased variance within a population (Forbes *et al.*, 1995). Such changes in the variance of a population may offer a sensitive approach for assessing the impact of pollutants (Forbes & Depledge, 1992).

In order to investigate inter-individual differences some form of replication of an individual is necessary. This limits the approaches that can be used for this type of study. In animals, the replication of an individual is not always possible. However, non-invasive comparative approaches can be used involving repeated measures on the same individual. For example, individual variability in the common shore crab *Cancer maenas* has been investigated using a non-invasive method of monitoring heart rates (Aagaard & Depledge, 1993).

An alternative approach is to employ clonal or self replicating organisms. In one of the few published studies on inter-individual variation with respect to trace metals, Forbes *et al.* (1995) investigated the intra-population variability in response to cadmium in two

species of gastropod, one reproducing sexually and the other asexually. Sublethal exposure to cadmium reduced the mean growth rate, but increased the variability in growth rate within populations. The intra-clonal variability in the asexual species *Potamopyrgus antipodarum* was comparable to that of the intra-population variability in the sexually reproducing *Hydrobia*. They therefore concluded that non-genetic variation was the most important component of the total phenotypic variance in growth rates within these gastropod populations. These results indicate that a consideration of the variation within a population would permit a better understanding of the effects of a contaminant upon a particular species.

Many species of plants are particularly suited to this type of approach since they can often be vegetatively reproduced and/or selfed to create genetically identical copies of an individual, which can be then used as replicates (Aldrich 1990 and Macnair 1993). Many species of macroalgae would therefore appear to be potentially useful organisms for this type of investigation since they can be replicated in this way. Despite this potential, no examples could be found that utilised seaweed in this way for ecotoxicological investigations.

## **8.2 Aims**

In order to investigate the significance of both inter and intra-individual variability in *G. longissima*, a series of three experiments was designed. In Experiment 1, the aim was to investigate if any difference in growth and responses to copper exposure exists between individuals of different life cycle phases from within a population (Helford). The aim of the second experiment was to assess differences in growth and responses to copper exposure of individuals from the two populations from within the Fal Estuary (St Just and Mylor). These two populations originated from sites that differ in metal contamination.

The third and final experiment was designed to determine whether ramets from a single individual respond differently to copper exposure.

### **8.3 Experiment 1. Inter-individual variation between life cycle phases**

In Chapter 2 some evidence was provided to suggest that female gametophytes may have higher RGRs than tetrasporophytes. In this experiment the growth responses of 14 individuals: 7 tetrasporophyte and 7 female gametophytes, were compared.

#### *8.3.1 Materials and methods*

Individuals were collected from the Helford population (October 1996) and cultured under stock culture conditions for 5 days. Seven female gametophytes identified by the presence of cystocarps, and seven tetrasporophytes identified by the presence of tetrasporangia (using a dissecting binocular microscope) were used.

Eight apical tips (~10mm in length) were excised from each of the 14 individuals and placed into separate beakers containing filtered seawater. Two treatments were used, a control and  $25\mu\text{g L}^{-1}$  of copper. Single apical tips were replicates for each individual (four per treatment), randomly selected from the eight excised. This resulted in a nested experimental design as summarised below:

- ◆ 2 life cycle phases (female gametophyte and tetrasporophyte)
- ◆ 7 individuals (nested within the phases)
- ◆ 2 treatments (control and  $25\mu\text{g L}^{-1}$  copper)
- ◆ 4 replicates (one apical tip per replicate)
- ◆ 112 samples in total

Because of the large number of samples in this experiment, ten 12 well (8ml volume) repli-boxes were used instead of the petri dishes, which had been used previously. Each well contained 5ml of standard experimental media with or without added copper. Since each well contained only one apical tip, this resulted in half the normal tissue to volume ratio of previous experiments (i.e. 1 tip per 5ml, compared with 10 tips per 25 ml). This reduced the likelihood of nutrient limitation during the experiment. All the apical tips were assigned to separate wells using a fully randomised design. The repli-boxes were placed into a controlled environment cabinet (CEC) under the standard experimental conditions (described in Chapter 2) except that the day length was increased to 16:8 Light:Dark.

The lengths of all apical tips were measured using image analysis at the start and again at the end of the week long experiment. RGRs were calculated as previously described (see Chapter 2 for details of protocol). A General Linear Model (GLM) was used to analyse the nested experimental design. This type of analysis allows for crossed and nested effects, such as individuals within life phases, as well as the use of categorical or continuous factors. The four factors in the model were: Life phase, Individual (nested within phase), Treatment (control or  $25\mu\text{g L}^{-1}$  copper), and Replicate.

### 8.3.2 Results

The GLM was significant ( $P < 0.001$ ) and had an  $R^2$  value of 57% (Table 8.1). The GLM analysis of the data is summarised in Table 8.2.

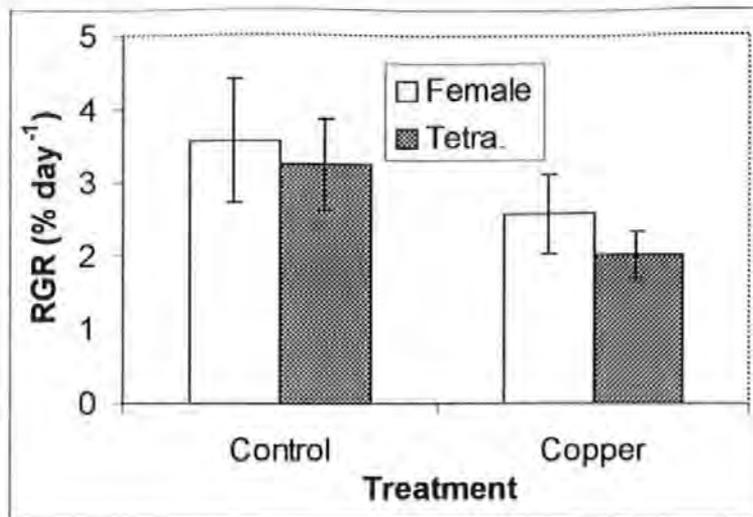
Source	SS	Df	MS	F-ratio	P-value
<b>Model</b>	189.132	30	6.304	3.59	<0.001
<b>Residual</b>	142.165	81	1.755		
<b>Total</b>	331.297	111			

**Table 8.1** Test for goodness of fit for the GLM describing the variation between the response of different individuals from two life phases to copper exposure.

Source	SS	Df	MS	F-ratio	P-value
<b>Individual {phase}</b>	119.856	12	9.988	9.68 (a)	0.044
<b>Life phase</b>	5.422	1	5.422	0.54 (b)	0.475
<b>Treatment</b>	35.588	1	35.588	20.28 (c)	<0.001
<b>Treatment * Individual {phase}</b>	24.760	12	2.063	1.18 (c)	0.475
<b>Treatment * phase</b>	0.411	1	0.411	0.23 (c)	0.630
<b>Replicate</b>	3.096	3	1.032		
<b>Residual</b>	142.165	81	1.755		
<b>Total (corrected)</b>	331	111			

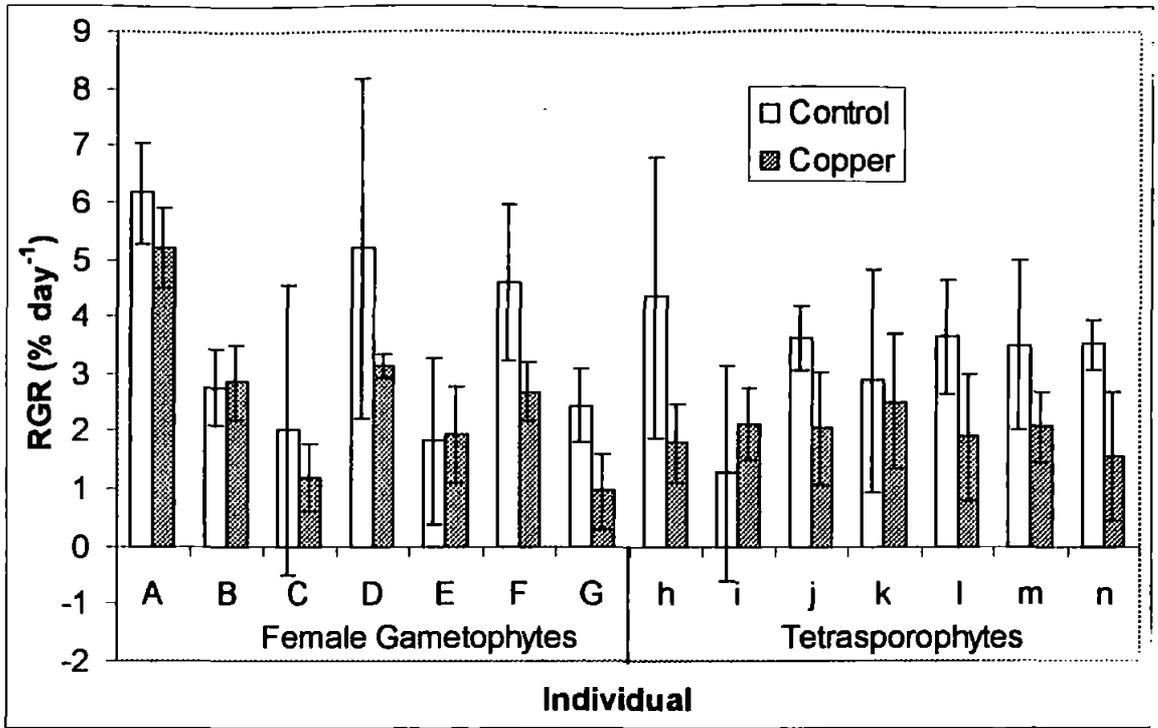
**Table 8.2** Summary of the GLM describing the variation between individuals from different life phases exposed to copper. Apical tips from seven individuals taken from two phases (female gametophyte and tetrasporophyte) were exposed to two copper treatments for one week. The F-ratios were based on the following mean squares: a = replicate, b = individual {phase} and c = residual. Brackets '{ }' indicate nesting i.e. individuals are nested within phases.

Copper treatment was found to be significant ( $P < 0.001$ ). The life phase and the interaction between the life phase and treatment were not significant (Table 8.2). This can be seen in Fig. 8.1, which shows that the mean response of both phases to copper were similar. The addition of  $25 \mu\text{g L}^{-1}$  copper reduced the RGR by approximately 30% in both phases, from a control level of  $3.4\% \text{ day}^{-1}$ .



**Fig. 8.1** Mean ( $n=28$ ) RGR of apical tips from seven female gametophyte and seven tetrasporophyte individuals, exposed to two treatments (control and  $25\mu\text{g L}^{-1}$  copper) for one week. Error bars show 95% confidence intervals.

When the responses of the individuals from within the life phases were analysed, it was found that there was a significant difference ( $P=0.044$ ) in growth response between individuals (Table 8.2). A summary of the responses of all 14 individuals to copper is shown in Fig. 8.2. No significant interaction was found between the copper treatment and the individuals (Table 8.2), although not all individuals appear to have responded in the same way to copper. For example, Individuals B and E were not affected by the  $25\mu\text{g L}^{-1}$  copper treatment, unlike Individuals G and j (Fig. 8.2). The absence of any significant interaction is most likely due to the large amount of variability in growth response within certain individuals. For example, Individual D (control) and Individual h (control) had large 95% confidence intervals (Fig. 8.2).



**Fig. 8.2** Mean ( $n=4$ ) RGR of each individual female gametophyte (A-G) and tetrasporophyte (h-n) exposed to two treatments (control or  $25\mu\text{g L}^{-1}$  copper) for one week. Error bars show 95% confidence intervals.

An attempt was made to compare the response of the individuals within each life phase separately. A two-way ANOVA of the tetrasporophytes found no significant differences between individuals. However, a two-way ANOVA was not possible for the female gametophyte data, as it was not normally distributed (Bartlett's test,  $P=0.002$ ) and could not be successfully transformed.

Individual female gametophytes do appear to be more variable in their growth than the tetrasporophytes. In the control media the mean RGR of female gametophytes varied from  $6.2\% \text{ day}^{-1}$  (Individual A) to  $1.8\% \text{ day}^{-1}$  (Individual E), a range of  $4.4\% \text{ day}^{-1}$ . Tetrasporophytes varied from  $4.3\% \text{ day}^{-1}$  to (Individual h)  $1.3\% \text{ day}^{-1}$  (Individual i) a range of  $3.0\% \text{ day}^{-1}$ . Female gametophytes had a larger range of responses to the  $25\mu\text{g L}^{-1}$  copper treatment, from  $5.2\% \text{ day}^{-1}$  (Individual A) to  $1.0\% \text{ day}^{-1}$  (Individual G) a range of  $4.2\% \text{ day}^{-1}$ , compared with the tetrasporophytes  $2.5\% \text{ day}^{-1}$  (Individual k) to  $1.6\% \text{ day}^{-1}$  (Individual n), a range of only  $0.9\% \text{ day}^{-1}$  (Fig. 8.2).

## 8.4 Experiment 2. Inter-individual differences between populations

In all population comparisons described thus far, no major difference in response to a given level of copper could be found. It is possible however, that by concentrating on the mean response of a population, subtle differences between populations could have been overlooked. In Chapter 7, evidence was presented, from the active biomonitoring study, for differences between individuals. There was considerable variation in growth and copper accumulation of each individual but because there was no replication of each individual, this observation could not be tested statistically. The possibility that the extent of variation between individuals varies between populations was explored in this experiment by comparing the inter-individual variability within two populations.

### 8.4.1 *Materials and methods*

Individuals from the St Just and Mylor populations were used in a similar experimental design to that described above. Five individuals from the two populations, Mylor and St Just, were used.

Ten apical tips (~10mm in length) were excised from each of 10 individuals and placed into separate beakers containing filtered seawater. The life phase of the individuals used could not be ascertained because reproductive tissue was not present. Two treatments were used, control and 50 $\mu\text{g L}^{-1}$  copper. Single apical tips were used as replicates for each individual, five per treatment, each randomly selected from the ten excised. This resulted in a nested experimental design summarised as:

- ◆ 2 populations (Mylor and St Just)
- ◆ 5 individuals (nested within populations)

- ◆ 2 treatments (control and 50µg L<sup>-1</sup> copper)
- ◆ 5 replicates (one apical tip per replicate)
- ◆ 100 samples in total

Nine, 12 well (8ml volume) repli-boxes were used to culture the apical tips. Each well contained 5ml of standard experimental media with or without added copper. All apical tips were randomly assigned to separate wells. The repli-boxes were placed into a CEC under standard experimental conditions as described above.

The lengths of the apical tips were measured at the start and end of the week long experiment using image analysis. A GLM was used to analyse the nested experimental design. The four factors in the model were: Population, Individual (nested within population), Treatment (control or 50µg L<sup>-1</sup> copper), and Replicate.

#### 8.4.2 Results

The GLM was significant ( $P < 0.001$ ) and explained 83% of the variability in the data (Table 8.3). A summary of the GLM analysis is shown in Table 8.4.

Source	SS	Df	MS	F-ratio	P-value
Model	272.734	23	11.858	16.12	<0.001
Residual	55.895	76	0.735		
Total	328.629	99			

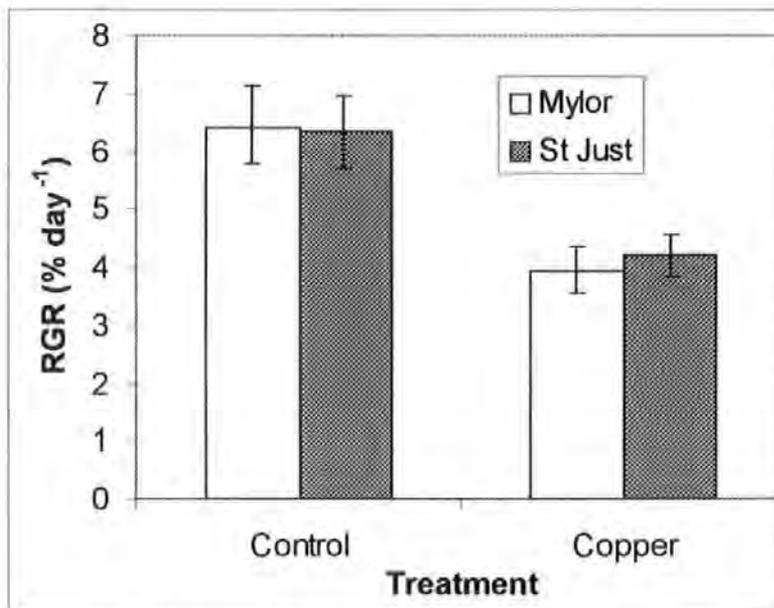
**Table 8.3** Test for goodness of fit for the GLM describing the variation between the response of different individuals from two populations to copper exposure.

Source	SS	Df	MS	F-ratio	P-value
Individual {Population}	108.185	8	13.523	42.07 (a)	0.001
Population	0.060	1	0.060	<0.01 (b)	0.9483
Treatment	134.377	1	134.4	182.71(c)	<0.001
Treatment * Individual {Population}	28.425	8	3.553	4.83 (c)	<0.001
Treatment * Population	0.894	1	0.894	1.22 (c)	0.274
Replicate	1.286	4	0.321		
Residual	55.895	76	0.735		
Total (corrected)	328.629	99			

**Table 8.4** Summary of the GLM describing the variation between individuals from different life phases exposed to copper. Apical tips from five individuals taken from two populations (Mylor and St Just) were exposed to two copper treatments for one week. The F-ratios were based on the following mean squares: a = replicate, b = individual (Population) and c = residual. Brackets '{ }' indicate nesting i.e. individuals are nested within populations.

A significant difference was found between copper treatments ( $P < 0.001$ ) although no population effect or interaction between the two factors were evident (Table 8.4).

Figure 8.3 shows the similarity of the response of both populations to the treatments. The RGR was found to be reduced by approximately 35% by the addition of  $50 \mu\text{g L}^{-1}$  of copper from a control value of over  $6\% \text{ day}^{-1}$ .

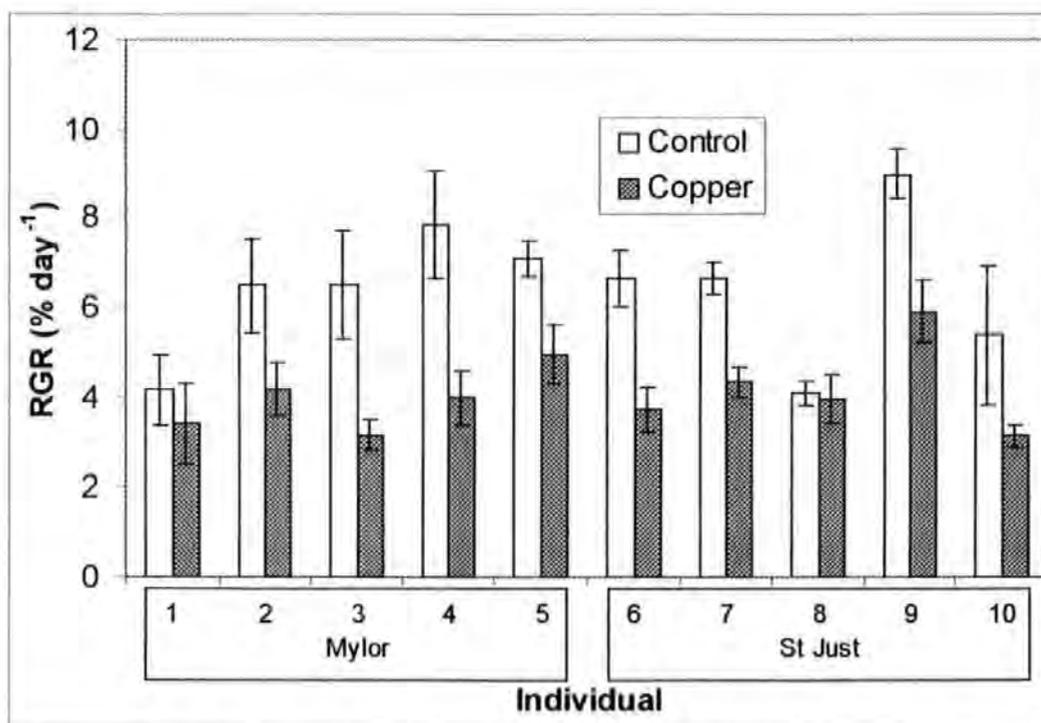


**Fig. 8.3** Mean ( $n=25$ ) RGR of apical tips from five Mylor and five St Just individuals exposed to two treatments (control and  $50 \mu\text{g L}^{-1}$  copper) for one week. Error bars show 95% confidence intervals.

A significant difference was found between individuals within populations ( $P=0.001$ ) and there was a significant interaction between copper treatment and individual ( $P<0.001$ ). This indicates that not all individuals within a population had similar control growth rates or responded in a similar way to the  $50\mu\text{g L}^{-1}$  copper treatment. To assess the individuals response further, a series of pairwise comparisons (students t-tests) were made between the control and  $50\mu\text{g L}^{-1}$  copper treatment for each individual (Table 8.5). In four of the five individuals from each population, exposure to  $50\mu\text{g L}^{-1}$  copper resulted in a significant reduction in growth. However, growth of Individual 1 from Mylor and Individual 8 from St Just were not significantly affected by copper. This interaction can be clearly seen when the response of each individual to the copper treatments is plotted (Fig. 8.4).

Individual	Population	Difference	P-value
1	Mylor	Not Significant	
2	Mylor	Control $>50\mu\text{g L}^{-1}$	0.005
3	Mylor	Control $>50\mu\text{g L}^{-1}$	0.004
4	Mylor	Control $>50\mu\text{g L}^{-1}$	0.001
5	Mylor	Control $>50\mu\text{g L}^{-1}$	0.001
6	St Just	Control $>50\mu\text{g L}^{-1}$	$<0.001$
7	St Just	Control $>50\mu\text{g L}^{-1}$	$<0.001$
8	St Just	Not Significant	
9	St Just	Control $>50\mu\text{g L}^{-1}$	$<0.001$
10	St Just	Control $>50\mu\text{g L}^{-1}$	0.045

**Table 8.5** Comparisons made between copper treatments for each individual. The control and  $50\mu\text{g L}^{-1}$  copper treatments for each individual were compared using students t-test with five replicates per treatment.



**Fig. 8.4** Mean (n=4) RGR of each individual from Mylor (1-5) and St Just (6-10) exposed two treatments (control or 50 $\mu\text{g L}^{-1}$  copper) for one week. Error bars show 95% confidence intervals.

The mean RGR of the controls for the individuals from each population had similar ranges, 4.1% day<sup>-1</sup> (M1) to 7.8% day<sup>-1</sup> (M4), a range of 3.7% day<sup>-1</sup> in the Mylor individuals and 4.1% day<sup>-1</sup> (SJ8) to 8.9% day<sup>-1</sup> (SJ9), a range of 4.8% day<sup>-1</sup> in St Just individuals. The variation in reduction due to copper in the two populations was also similar. In the Mylor individuals the percentage reduction due to copper ranged from 17% (M1) to 51% (M3), a range of 34%, while in the St Just individuals it ranged from 3% (SJ8) to 44% (SJ6), a range of 41%. It also appeared that individuals that had lower control growth rates, showed relatively less reduction in growth when exposed to copper. For example Individuals M1 & SJ8 had relatively low control RGRs and were affected by copper exposure the least, while individuals with high control RGRs, such as M4 and SJ9, showed greater reduction in RGR due to copper exposure.

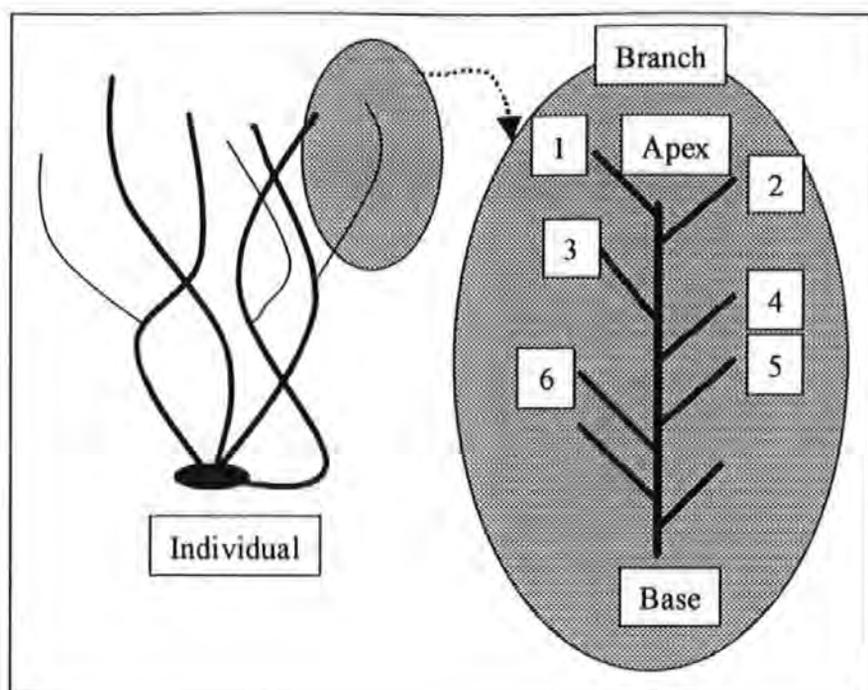
## 8.5 Experiment 3. Intra-individual variation

In order to investigate the relative importance of intra-individual variability, the growth of apical tips from different branches of the same individual were compared.

### 8.5.1 *Materials and methods*

Four individuals, which had been used in Experiment 2 (Individuals 4 & 5 (Mylor) and 7 & 9 (St Just)) and cultured under stock culture conditions for two months were used to investigate intra-individual variability. It would have been preferable to compare the individuals that showed the greatest differences in response from the previous experiment but this was not possible since there was insufficient material of some individuals. For each of the four individuals, five branches (only four from Individual 1) were selected and six apical tips (~10 mm in length) were removed in order from the apical region of each. Three apical tips from each branch were then used as replicates for two treatments, control and 50 $\mu\text{g L}^{-1}$  copper. The relative position on the branch from which each apical tip was taken was recorded (Fig. 8.5). This resulted in the following experimental design:

- ◆ 4 individuals
- ◆ 5 branches (nested within individuals)
- ◆ 2 treatments (control and 50 $\mu\text{g L}^{-1}$  copper)
- ◆ 3 replicates (one apical tip per replicate, with the relative position along the branch recorded)
- ◆ 114 samples in total



**Fig. 8.5** Diagram of *G. longissima* individual illustrating the method used to obtain apical tips for Experiment 3. From each of five branches six apical tips (~10mm long) were excised. For each branch, tips 1,3,5 were used as replicates for the control treatment and tips 2,4 & 6 were used as replicates for the copper treatment.

Ten, 12 well (8ml volume) repli-boxes were used to culture the apical tips. Each well contained 5ml of standard experimental media with or without copper. All of the apical tips were randomly assigned to separate wells. The repli-boxes were placed in a CEC under the same experimental conditions used in Experiment 1 and 2.

The lengths of all apical tips were measured at the start and end of the week long experiment, using image analysis. A GLM was used to analyse the nested experimental design. The four factors in the model were: Individual, Branch (nested within individual), Treatment (control or  $50\mu\text{g L}^{-1}$  copper), and Replicate.

### 8.5.2 Results

The data was analysed using a GLM that significantly ( $P < 0.001$ ) described 66% of the variability in the data (Table 8.6). The factors included in the GLM are summarised in Table 8.7.

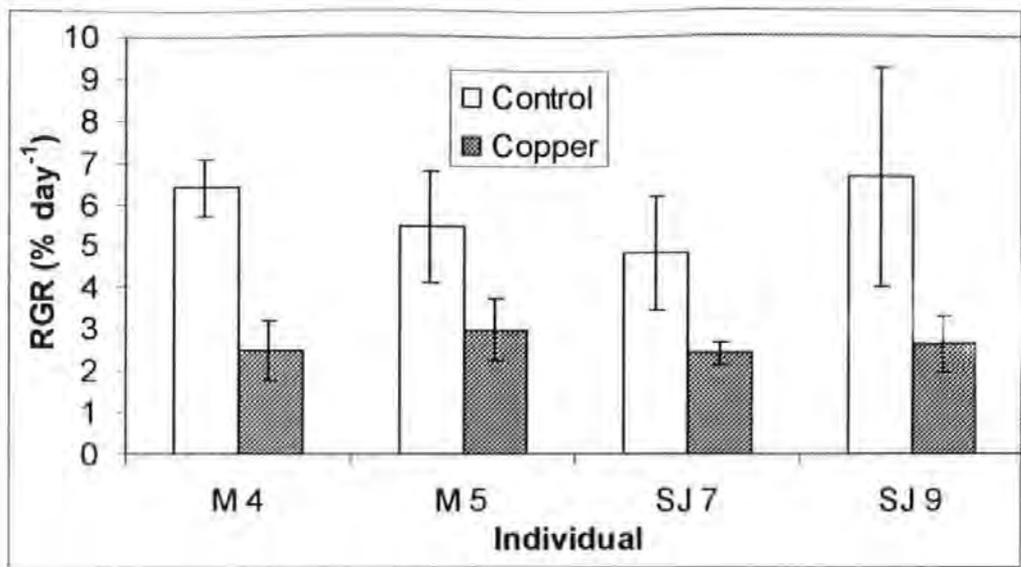
Source	SS	Df	MS	F-ratio	P-value
<b>Model</b>	459.782	39	11.789	3.71	<0.001
<b>Residual</b>	234.934	74	3.175		
<b>Total</b>	694.716	113			

**Table 8.6** Test for goodness of fit for the GLM describing the variation within and between different individuals exposed to copper.

Source	SS	Df	MS	F-ratio	P-value
<b>Branch {Individual}</b>	98.610	15	6.574	96.41 (a)	0.010
<b>Individual</b>	25.143	3	8.381	1.27 (b)	0.319
<b>Treatment</b>	262.856	1	262.856	84.62 (c)	<0.001
<b>Treatment * Branch {Individual}</b>	52.041	15	3.469	1.09 (c)	0.378
<b>Treatment * Individual</b>	21.135	3	7.045	2.22 (c)	0.093
<b>Replicate</b>	0.136	2	0.068	0.02 (c)	0.979
<b>Residual</b>	234.934	74	3.175		
<b>Total (corrected)</b>	694.716	113			

**Table 8.7** Summary of the GLM describing the variation within and between four individuals. Apical tips from five branches within four individuals were exposed to two copper treatments for one week. The F-ratios were based on the following mean squares; a = Replicate, b = Branch {Individual} and c = Residual. Brackets '{ }' indicate nesting i.e. branches are nested within individuals.

The copper treatment was significant ( $P < 0.001$ , Table 8.7). No significant difference could be found between the mean response of the four individuals to copper exposure nor was the individual x treatment interaction significant, indicating that all individuals responded to copper in the same way. The mean response of each individual is shown in Fig. 8.6. The controls grew at a mean RGR of approximately  $6\% \text{ day}^{-1}$ , while the  $50 \mu\text{g L}^{-1}$  copper treatment reduced growth by an average of 54%. These results were comparable to those obtained for the same individuals in Experiment 2 (Fig. 8.4).



**Fig. 8.6** Mean ( $n=15$ ) RGR of four *G. longissima* individuals exposed to two treatments (control or  $50\mu\text{g L}^{-1}$  copper) for one week. The labels refer to the population (M = Mylor, SJ = St Just) and number of the same individual used in the previous experiment. Error bars show 95% confidence intervals.

Growth responses of branches within each individual were significantly different ( $P=0.010$ , Table 8.7). When the RGRs of the branches from each individual are compared (Fig. 8.7) significant differences between some branches can be seen. For example, within Individual M5, the highest mean RGR of the controls was Branch C ( $7.9\% \text{ day}^{-1}$ ) whereas the lowest mean RGR of the controls was branch A ( $4.0\% \text{ day}^{-1}$ ). Similarly, within individual SJ7 the highest mean RGR of the controls was in branch D ( $6.9\% \text{ day}^{-1}$ ) which was more than twice as high as that of branch B ( $2.9\% \text{ day}^{-1}$ ). Within individual SJ9 the highest mean RGR of the controls was branch D ( $8.9\% \text{ day}^{-1}$ ), which was more than twice that in branch B ( $3.5\% \text{ day}^{-1}$ ). Contrary to these results, in individual M4, all branches grew at a similar rate in the control media ( $\sim 6\% \text{ day}^{-1}$ ).

No significant interaction was found between the response of each branch to copper treatment, indicating that all of the branches respond in the same way to copper (Table 8.7). In addition to the variation between branches, in many cases there was often considerable variation in growth between the replicates for each branch. Such variations are particularly evident in the Individual SJ7, Branches A, C & E.

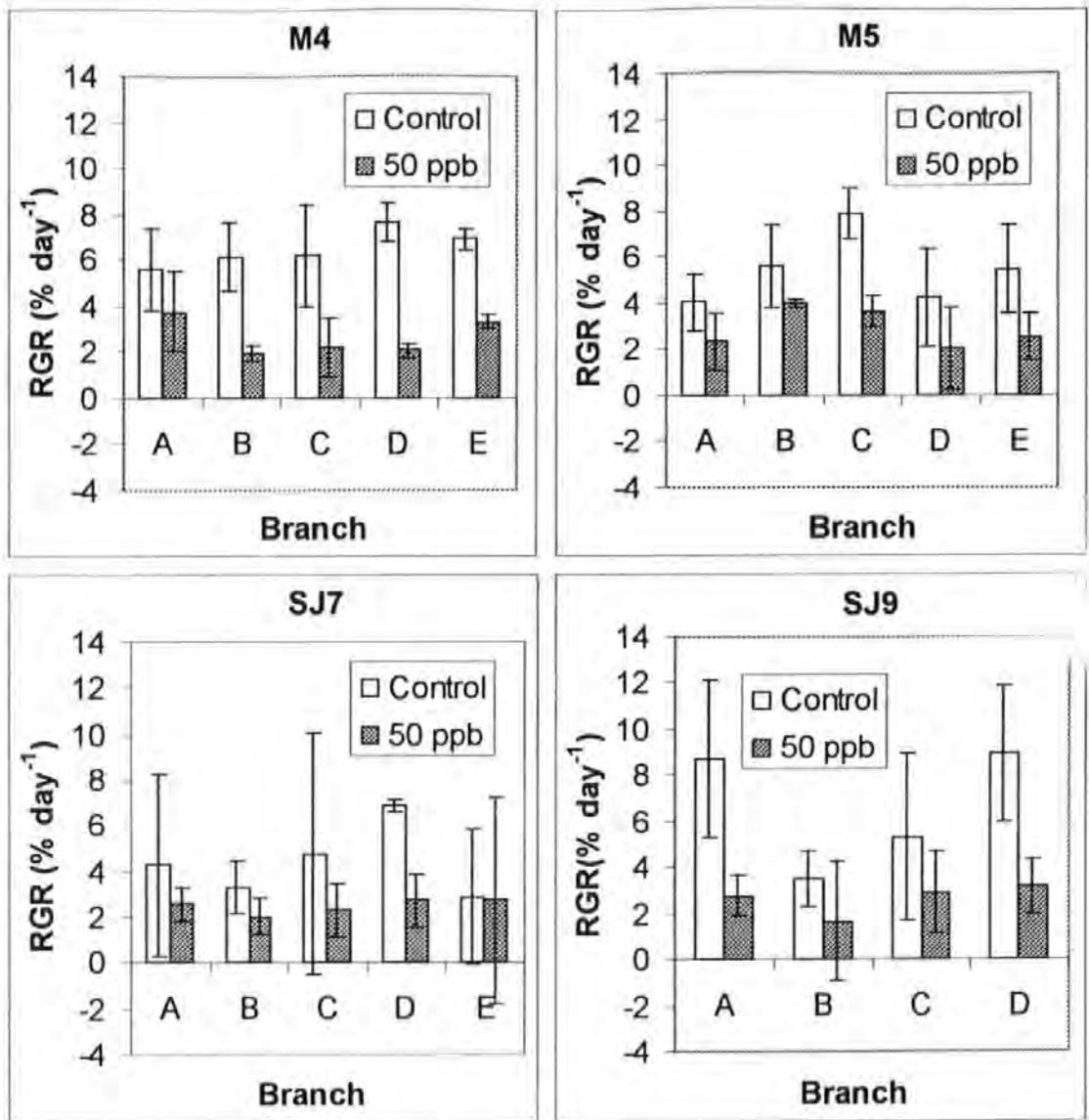


Fig. 8.7 A-D Mean (n=3) of each branch (A-E) from four *G. longissima* individuals exposed to two treatments (control and 50 $\mu\text{g L}^{-1}$  copper) for one week. Error bars show 95% confidence intervals.

Since the GLM adequately explained only 66% of the variability in the data, the intra-individual variability between branches, within each individual, was tested separately using a two-way ANOVA. Using Duncans multiple range tests, significant differences were found between the responses of branches from within each individual (Table 8.8). Within Individual M4 the control and copper treatments were not significantly different in Branch A whereas in Branches B-E the copper treatment was significantly lower. Within Individual M5 the control and copper treatments were only significantly different in

Branches C and E. Within Individual SJ9 the control and copper treatments were not significantly different in branches B & C, whereas in Branches A & D the copper treatment was significantly lower. In SJ7 the two treatments were not significantly different in any of the five branches, however this data set was not normally distributed due to significant differences in variation between branch treatment combinations (Bartlett's test,  $P=0.017$ ).

Individual	Treatment	Branch				
		A	B	C	D	E
M 4	Control	xy	x	x	x	x
	50 $\mu\text{g L}^{-1}$	yz	z	z	z	z
M 5	Control	yz	y	x	yz	y
	50 $\mu\text{g L}^{-1}$	z	yz	yz	z	z
SJ 7*	Control	xy	xy	xy	x	xy
	50 $\mu\text{g L}^{-1}$	xy	y	xy	xy	xy
SJ 9	Control	x	yz	y	x	-
	50 $\mu\text{g L}^{-1}$	yz	z	yz	yz	-

**Table 8.8** To assess treatment branch interactions, comparisons were made between treatments and branches within each individual using a two-way Duncans multiple range test. Statistically distinct groups (95% level) within each individual are represented by a different letter. \*There was a significant difference between error variances in the ten treatment branch combinations in this individual.

The absence of a significant difference between the RGR of the three replicates for each treatment (Table 8.7) indicates that the relative position of each apical tip along a branch does not affect its RGR. Apical tips taken from lateral branches near the apex apparently do not grow any differently than apical tips taken from lateral branches closer to the basal region of the branch.

## 8.6 Discussion

The results obtained from the three experiments in this chapter have provided evidence for significant intra and inter-individual variability in the growth of *C. i.*

*longissima*. This variation in growth was greater than the variation between life cycle phases (Experiment 1) and the variation between populations (Experiment 2).

No evidence of any population effects could be found. This is illustrated by the fact that in Experiment 2, the mean response and the amount of variation in growth between individuals was similar in the St Just and the Mylor population. This was consistent with the findings in previous chapters, where no major differences in response between populations was found. The experiments in this chapter did however, provide evidence for three important sources of variation in *G. longissima*. These are:

- ◆ Variation between the response of different individuals exposed to elevated levels of copper.
- ◆ Intra-individual variation in growth and even possibly in response to exposure to elevated levels of copper.
- ◆ Some suggestion of differences in the amount of variation between individuals in different life phases.

Evidence for inter-individual differences in tolerance was provided by Experiments 1 and 2. In Experiment 2, there was a significant interaction between individuals and the  $50\mu\text{g L}^{-1}$  copper treatment. Specifically, Individual 1 (Mylor) and Individual 8 (St Just) were both found to be unaffected by copper exposure, in terms of RGR, whereas all the other individuals showed various levels of reduced RGR in the copper treatment. There was also some indication that some of the individuals in Experiment 1 may have also responded differently to copper. However, no significant interaction was found between individuals and copper treatment in either life phase, possibly because of the large amount of variation within certain individuals or the lower level of copper exposure ( $25\mu\text{g L}^{-1}$ ) in this experiment compared with Experiment 2. These findings indicate that some

individuals may be more tolerant to copper exposure than others. Similarly, Reed & Moffat (1983) referred to the fact that different *Enteromorpha compressa* individuals responded differently when exposed to a range of copper treatments. They stated that some individuals possessed the ability to regenerate while others did not, but no data was provided to support this claim.

The fact that differences were found between individuals in Experiments 1 and 2 is not surprising. Since the individuals originated from natural populations, some differences would be expected between them. Macnair (1993) stated that 'differences in clones collected from the field may not be heritable, but simply reflect their individual histories and environmental experiences'. Similar or possibly even greater differences might be expected between a number of individuals randomly selected from a natural population (Depledge, 1990b), such as was the case in these experiments.

Evidence for a difference between life phases was provided in Experiment 1, where it was found that although both phases had the same mean response, female gametophytes appeared to be more variable in their growth than tetrasporophytes. As was highlighted in Chapter 2, opinion has been divided as to whether ecological differences should be expected to be found between isomorphic haplo-diploid phases (Valero *et al.*, 1992). Some differences between isomorphic phases have been found in species of red algae. For example, Destombe *et al.* (1993) found that juvenile haploid (male and female gametophytes) and diploid (tetrasporophyte) isomorphic phases of *Gracilaria verrucosa* responded differently to varying environmental conditions. Haploid individuals grew better under non-optimal culture conditions while diploid juveniles were found to have better resistance to lead. Santelices & Varela (1995) observed not only that female *Gracilaria chilensis* gametophytes grew significantly more than tetrasporophytes, but also that vegetative female gametophyte thalli grew significantly more than fertile thalli.

Conversely, Zhang & van der Meer (1987) found that adult phases of *Gracilaria tikvahiae* did not differ in growth rate. Differences between individual female gametophytes may be due to the reproductive status of the individuals (fertile or vegetative). The faster growing individuals could be vegetative, similar to the findings of Santelices & Varela (1995). Steentoft *et al.* (1995) reported that *G. longissima* can exist in both short and long growth stages and that female thalli in the long growth stage have at least three forms. These different forms could also possibly explain some of the variation in growth observed between female individuals. Further experimentation involving a greater number of individuals of each life phase should clarify whether or not differences in growth between phases do exist.

The final source of variability described in this chapter was intra-individual variability. A large amount of intra-individual variability was indicated by the relatively large error bars for the growth responses of some individuals in Experiments 1 and 2. This was investigated further by assessing the amount of variability between branches from within individuals. It was found that branches from within the same individual grew at different rates and responded differently to copper (Experiment 3), which indicates that intra-individual is an important source of variation in *G. longissima*.

Seaweed populations are often reported to exhibit considerable phenotypic plasticity. Gracilarioid species have been characterised as being amongst the most physiologically and morphologically variable of all seaweeds (Santelices & Varela, 1993). Typically, this phenotypic plasticity within populations is explained in terms of inter-population or inter-individual differences (Santelices & Varela, 1993).

When Santelices & Varela (1993) investigated causes for this phenotypic plasticity in *Gracilaria chilensis*, the authors found that *Gracilaria chilensis* spores from the same

cystocarp (which originate from one gametic fusion and were hence considered genetically identical), grew at significantly different rates even when cultured under similar growth conditions. Ramets from a single genet did the same. In another study of clonal organisms (Bryophytes), considerable variation has also been reported between individual ramets from a single genet (Shaw 1990). This may represent an important source of intra-population variation, which in some species may be larger than inter-population variability (Santelices & Varela, 1993) and thus possibly mask the latter.

Santelices *et al.* (1996) list a number of factors which may be expected to contribute to such intra-clonal variation including:

- ◆ physiological and developmental differences (possibly caused by microenvironmental differences between replicates)
- ◆ biotic factors (such as bacterial infections or pathogens infecting replicates)
- ◆ genetic mechanisms (such as mitotic recombinations or unstable mutations) which appear to be common among gracilarioid species (Van der Meer & Todd 1977 and Van der Meer & Zhang 1988)

In addition, a more recent study by Santelices *et al.* (1996) found that a single 'individual' could in fact originate from numerous different spores and suggested that this could explain some of the intra-individual differences found previously by Santelices & Varela (1993). Mechanisms such as spore coalescence that appear to cause intra-clonal variation in *Gracilaria chilensis* may also operate in *G. longissima*. This might explain why some branches from the same individual did not grow similarly or respond in the same way to copper exposure.

Recently the importance of phenotypic plasticity as a component of evolutionary change has been reconsidered. Contrary to the assumption that phenotypic plasticity

buffers the effects of natural selection and constrains evolutionary change, it is increasingly being considered an important evolutionary mechanism (Thompson, 1991). Phenotypic plasticity has been shown to be a heritable trait (see Thompson, 1991) but the exact mechanisms by which it is selected and the environmental conditions which favour it are not clear. It has been suggested that phenotypic plasticity may represent a mechanism by which fitness is maintained in a variable environment (Bradshaw, 1965). In a comprehensive study into the amount of phenotypic plasticity in the widespread annual *Polygonum persicaria*, Sultan & Bazzaz (1993) found that the species demonstrated a large range of adaptive phenotypic plasticity in response to major environmental factors such as light, nutrient conditions, and soil moisture. This variation in response was presumed to promote functional stability (homeostasis) despite large scale environmental variation.

Estuarine environments are, by their nature, extremely variable. If such an environment favours the selection of phenotypic plasticity then this may explain the large amount of morphological variation reported to exist in gracilarioid species. A large amount of phenotypic plasticity may also explain why no major differences could be found in copper resistance between populations of *G. longissima*, but significant differences could be seen between and even within individuals.

Since considerable intra-individual variability was found between ramets from each genet, it appears likely that the intra-individual differences described here for *G. longissima* may also be found intra-clonally, i.e. between spores from the same cystocarp, as was found to occur in *Gracilaria chilensis* (Santelices & Varela, 1995). This could invalidate the usefulness of rearing clones from carpospores in the laboratory and using them in active biomonitoring studies (as was proposed in Chapter 7) since it may not reduce variation. Studying the amount of variability that exists between spores from a single cystocarp would establish how informative this approach could be, as well as help to

indicate the importance of a largely overlooked source of variation. This was intended to be investigated in this study, but all attempts to culture spores from *G. longissima* cystocarps were unsuccessful.

Genetically uniform populations (clones) are used widely in laboratory based toxicological testing programmes. It is assumed that genetic uniformity minimises phenotypic variability. However, this rationale for using such populations has recently come under scrutiny. For example, Forbes *et al.* (1995) questioned the use of genetically uniform populations in ecotoxicology for two reasons. Firstly, the relative ranking of chemical toxicities does not appear to be genotype dependent. Secondly, phenotypic plasticity is not always tightly coupled to genetic variability. Evidence for this claim was provided by their study (Forbes *et al.*, 1995) which found that intra-clonal variation in the growth of *Potamopyrgus antipodum* was comparable to inter-population variability *Hydrobia*. This indicated that phenotypic plasticity may be considerable, even within an apparently genetically uniform clone. If there is significant intra-clonal variation in *G. longissima*, then the validity of using ramets from a single genet as replication units for minimising variation in ecotoxicological tests, would have to be questioned.

In most of the experiments described in this thesis, each replicate in an experiment comprised of ten apical tips selected randomly from a pool of tips, collected from a number of individuals within a population. This approach was used to reduce, as much as possible, any variation that was thought may occur between individuals from within a population. The results of the experiments reported in this chapter clearly illustrate that inter and intra-individual variation can be a significant source of variation. When attempting comparative physiological studies in future, the pooling approach described above is recommended, since it maximises the chance of observing other sources of variation such as treatment or population effects.

No significant difference was found between the RGR of apical tips from different positions along each branch. This indicates that apical tips from near the apex of a branch do not grow at different rates than apical tips originating from a more basal portion of the branch. This is consistent with the findings of Santelices & Varela (1993) who showed that apical portions of *Gracilaria chilensis* grew faster in some individuals but not in others and that overall there was no consistent difference in growth between basal and apical portions. Santelices & Varela (1995) also found no significant differences in the growth of ramets originating from proximal or distal parts of the same axis.

## 8.7 Conclusions

Inter and intra-individual variability in *G. longissima* represents a significant source of variation. The experiments described here, investigated a relatively small number of individuals. It is important for future studies to investigate these sources of variation in greater detail, among more individuals. Further investigations to evaluate the importance of this source of variation are considered worthwhile and could include;

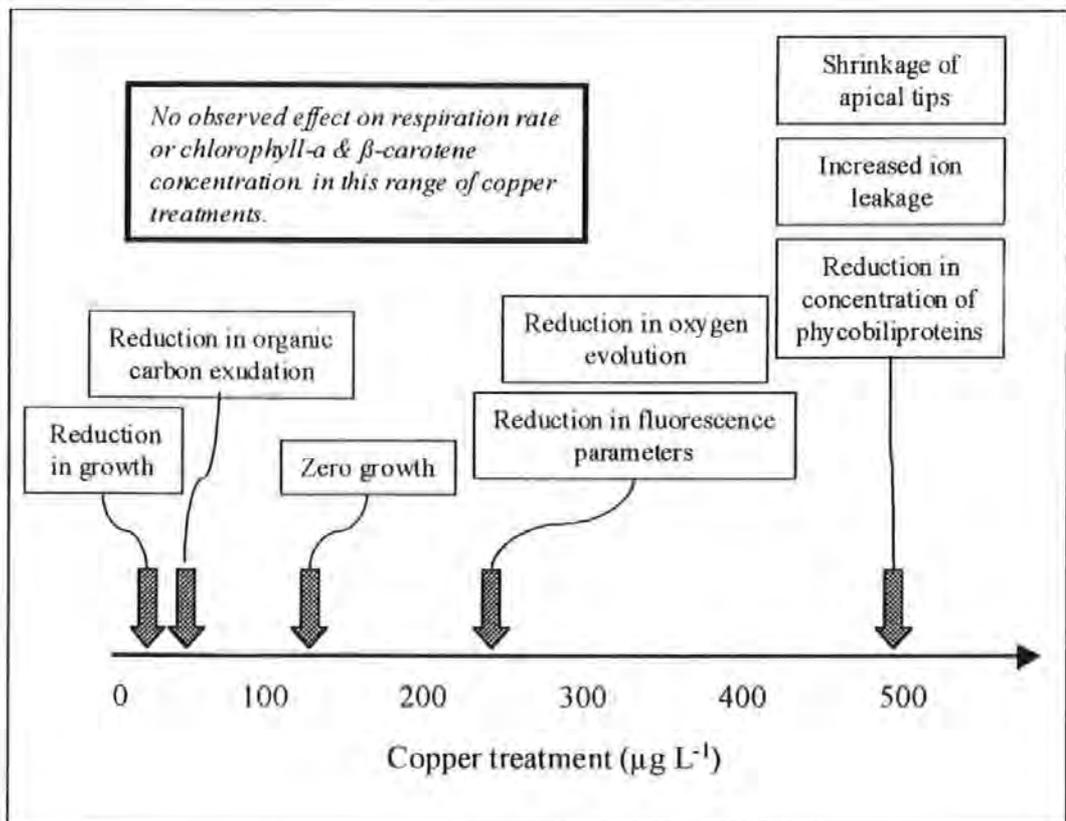
- ◆ Investigation into possible differences in response between life history phases in reproductive and vegetative states.
- ◆ Comparisons of the response of spores from individual cystocarps.
- ◆ Further investigation into the source of intra-individual variability.
- ◆ The relationship between growth rate and tolerance of individuals.

## **Chapter 9**

### **GENERAL DISCUSSION**

The objectives of this thesis were threefold: to investigate the effects of copper on the growth and physiology of populations of *G. longissima*, to relate these laboratory based findings to data obtained from field studies and to use all this information to assess the appropriateness of using the species as a biomonitor of (copper) contamination.

Throughout the course of this study a number of experiments were performed investigating various aspects of the effect of copper on the growth and physiology of *G. longissima*. These findings are summarised in Fig 9.1. When the relative effect of copper on growth and photosynthesis was tested in a series of comparative experiments, growth was affected at significantly lower concentrations than other physiological test end points.



**Fig. 9.1** Summary of the comparative effects of copper on the growth and physiology of *G. longissima* following one week of exposure.

Exposure to copper reduced growth considerably with copper concentrations as low as  $12\mu\text{g L}^{-1}$  causing a significant reduction in growth after one week of exposure. No evidence of growth inhibition due to copper deficiency was found. Symptoms of deficiency were unlikely since the levels of copper in uncontaminated seawater would be expected to be adequate for normal growth (Bryan, 1976). High levels of copper ( $>150\mu\text{g L}^{-1}$ ) caused shrinkage of the apical tips; such shrinkage has been reported previously in *Fucus vesiculosus* following exposure to high levels of copper (Bryan & Gibbs, 1983).

Growth was found to recover following exposure to copper and the rate of recovery was dependent upon the level of exposure. Apical tips exposed to the same level of copper for one or two weeks recovered at a similar rate, whereas increasing the copper concentration from  $25\mu\text{g L}^{-1}$  to  $50\mu\text{g L}^{-1}$  resulted in a longer recovery period being required. It took two weeks for growth rates to recover to those of the controls after one week of exposure to  $25\mu\text{g L}^{-1}$  copper. The fact that the rate of recovery appeared to be related to the level of contamination and not the duration of exposure suggests that some form of regulation was occurring. However, the algal material was only exposed to copper for two weeks and a longer period of exposure is required to further investigate the relationship between exposure level, duration and growth reduction.

Photosynthesis, as measured by oxygen evolution and chlorophyll fluorescence, was eventually affected at high levels of copper exposure, which were also shown to be causing shrinkage of the algal material. Uncoupling of growth from photosynthesis has been reported previously in a number of microalgal species (Cid *et al.* 1995, Abalde *et al.* 1995 and Nalewajko & Olaveson 1995), and has explained by the inhibition of cellular division and the swelling of cells (Fisher *et al.* 1981, Lumsden & Florence 1983 and Stauber & Florence 1987). Measurement of growth parameters indicated that this explanation could not account for the uncoupling found in *G. longissima*. This indicates

that the primary site of copper toxicity in this species is not in any part of the photosynthetic pathway that affects oxygen evolution or fluorescence.

One hypothesis which was proposed to explain the uncoupling was the release of organic exudate from the apical tips. In some species of algae the release of dissolved organic carbon (DOC) can represent a significant proportion of the photosynthetically fixed carbon (Schramm, 1993) and has been shown to be induced by exposure to copper (Romeo & Gnassia-Barelli, 1993). The release of DOC by algae may also function as a mechanism for detoxifying copper. Some organic exudates are known to chelate copper and therefore potentially limit the toxic effect of the metal. Regardless of its detoxifying ability, the release of DOC from *G. longissima* apical tips could explain why growth is reduced, with no apparent effect on photosynthesis or respiration. However, exposure to elevated levels of copper was found to significantly reduce the amount of DOC exuded by *G. longissima*, suggesting that this hypothesis does not explain the uncoupling of growth and photosynthesis.

In order to further the understanding of this uncoupling of growth from photosynthesis, a further series of comparative experiments was carried out. The effect of copper on other physiological test end points was measured including ion leakage, fluorescence and tissue pigmentation. All of these parameters were affected at significantly higher copper concentrations than growth. The fluorescence parameters  $F_0$ ,  $F_m$  and  $F_v$  were all found to be reduced at copper concentrations above  $250\mu\text{g L}^{-1}$  and ion leakage was only significantly increased at the highest copper treatment ( $500\mu\text{g L}^{-1}$ ). Of the four photosynthetic pigments measured, phycoerythrin and phycocyanin were reduced at  $500\mu\text{g L}^{-1}$ , but not at  $50\mu\text{g L}^{-1}$ , while chlorophyll-*a* and  $\beta$ -carotene were not significantly effected by either concentration (Fig 9.1).

It is apparent that all of the physiological parameters measured were affected at much higher levels of copper than growth, corresponding approximately with the actual shrinkage of the apical tips. It is most likely that at this level of copper exposure, membrane integrity has been damaged resulting in the loss of cell function. This can be seen by the increased leakage of ions from the cells, the impairment of photosynthetic function and the shrinkage of the tissue, probably due to loss of turgor and damage to pigments in the cells. No physiological parameter was measured that was affected at a similar copper level to that which first reduced growth. It is suggested that the measurement of ATP within the cell may be as sensitive to copper as growth, as has been reported previously in the marine microalga *Phaeodactylum tricorutum* by Cid *et al.* (1995). Investigating other aspects of the photosynthetic pathway, for example fluorescence quenching analysis, may also provide more information as to the cause of the uncoupling. One likely explanation for this uncoupling of growth from photosynthesis is that energy harvested in the photosynthetic light reactions is being diverted from normal energetic requirements of growth and development (i.e. carbon fixation) and is being used instead to counter the effects of copper toxicity (Cid *et al.*, 1995). This toxicity could be due to a number of factors including the reduction in enzyme function due to the copper binding to -SH groups or by the metal inducing oxidative stress. Another suggested mechanism by which growth is impaired at low levels of copper exposure, could be that copper interferes with the passage of other essential ions into the cells by inhibiting their uptake and regulation.

The results of the copper accumulation and recovery experiments indicate that copper is accumulating in apical tips but can be rapidly released back into the media and that this release is associated with the recovery of growth. This important finding contradicts the common assumption that metals are irreversibly bound in seaweeds (Philips, 1994). The method of measuring the metal concentration in both the algal

material and in the media allowed a budget to be calculated. This technique is worth developing further, possibly by more detailed analysis of the speciation of the copper released by the algae. The rate of release of copper from the apical tips was initially fast and then slowed over an eight day period. It therefore appears likely this release involves copper that is weakly bound to extracellular ligands such as polysaccharides, as well as intra-cellular binding sites. Further work is required to establish the kinetics of uptake and release of copper, since this has important implications for the use of *G. longissima* as a biomonitor. Establishing the relative importance of the cell wall polysaccharides in copper accumulation is considered as a beneficial line of future research since this would indicate the relative importance of intra and extracellular bindings sites for copper.

Throughout this study, a considerable amount of effort was placed on comparing the responses of different populations of *G. longissima* to elevated levels of copper exposure. The populations used, originated from sites within the Fal Estuary (Mylor, St Just and Flushing) which are known to differ in trace metal contamination. Two other sites from outside the Fal (Helford and Chesil) were also included.

In a series of different experiments, populations of *G. longissima* were exposed to a range of copper treatments and growth responses and copper accumulation measured. In several of these experiments, small, but significant, copper treatment x population interactions were found to occur at one or more copper treatment. However, these differences were not considered to be indicative of any major differences in response between populations since the effects of copper were essentially the same for each population. Moreover, the growth response of a population often altered in different experiments. Such differences in response between populations are considered most likely to be due to seasonal variation in the growth of *G. longissima* and even perhaps the length of time material was maintained in culture prior to experimentation.

Several different experiments also indicated that populations accumulate copper and recover from exposure to the metal similarly. In addition, no significant difference was found in the effect of copper on oxygen evolution (both photosynthesis and respiration) between different populations. All the evidence from these laboratory based experiments indicate that no major differences in response to copper existed between any of the populations compared in this study. This conclusion was further supported by the reciprocal transplant field experiments, where there was no evidence of any difference in the response of the two populations from sites differing in contamination.

While there was no evidence of consistent differences in the degree of copper tolerance between any of the populations tested, an interesting observation was the absence of *G. longissima* in the most contaminated site in the study area (Restronguet Creek). It was concluded that the levels of metal contamination within this creek may exceed the threshold concentration that *G. longissima* can tolerate. Support for this view comes from laboratory experiments, which indicated that levels of copper similar to those encountered within Restronguet Creek, could significantly reduce the growth of apical tips after only one week of exposure. In addition, field experiments indicated that although the growth of transplanted ramets varied considerably, the mean RGR was lowest for ramets transplanted to Restronguet Creek.

This lack of any major differences in response between populations eliminates the risk of population effects potentially influencing copper accumulation, when comparing copper levels in native algal material with the level of contamination. However, as was highlighted in the introduction, numerous other factors such as the growth rate, or interaction with other contaminants, could possibly affect copper accumulation in these populations.

The fact that it was not possible to differentiate populations on the basis of copper tolerance, did unfortunately eliminate the possibility of using contrasting differences in tolerance to further investigate copper tolerance mechanisms. Future comparisons could include the use of *G. longissima* populations located in other sites that are known to be more or less contaminated than those used here, to establish whether or not more or less copper tolerant populations do exist. An alternative approach could be to investigate one of the few species of macroalgae that can be found to survive naturally within Restronguet Creek. A previous study has found more copper tolerant species in this more contaminated site (Bryan & Gibbs, 1983), although mechanisms are likely to differ between species.

Active biomonitoring, suggested as an alternative to normal passive biomonitoring techniques, was found to be a successful form of assessing copper (and zinc) contamination in the Fal Estuary. This method, not normally performed using seaweeds, provided more information than would have been obtained by simply measuring the concentration of native plants. It also had the added advantage of being able to monitor a site such as Restronguet Creek, which is heavily contaminated and where no native populations of *G. longissima* could be found. Despite temporal fluctuations in the copper levels in *G. longissima* and the water within each site, overall the data reflects the gradient that has been previously shown to exist within the estuary. Zinc concentrations were tightly correlated with copper concentrations and also reflected the gradient.

Growth rates of transplanted material were highly variable and could not be used to predict the levels of contamination. This is perhaps not surprising considering the fact that growth is known to be altered by many biotic and abiotic factors, such as irradiance levels, water flow, nutrient availability and interactions with other species, which may also have differed between the sites. The relatively poor growth of the transplanted material may have been due to *G. longissima* ramets fragmenting during the course of the month. In the

future, using a species that has a more robust morphology or reducing the amount of fragmentation of *G. longissima* by improving the method of transplanting the species may remove such problems. In spite of the difficulties encountered, this technique could be developed further and successfully employed as a practical way of monitoring trace metal contamination.

The novel approach of using ramets from individuals as matched pairs was seen as a powerful tool for assessing responses of individuals and increased statistical resolution of the comparisons made between sites. The results also indicated that there was variation in the concentration of copper and zinc in ramets from individuals exposed to the same level of exposure. This part of the study has provided good evidence for the view that differences between individuals are an important source of variation that needs to be taken into account.

The significance of this source of variation was explored further by assessing the degree of inter and intra-individual variation which exists within different populations of *G. longissima*. By comparing the responses of individuals, it was found that some grew at different rates and responded differently to copper under standardised laboratory conditions. This intra-population variation, which is typically ignored in ecophysiological studies was comparatively much greater than the inter-population variation observed. The amount of variation between individuals of each population was similar, with no significant difference between the two populations studied (St Just and Mylor). A comparison between female gametophyte and tetrasporophyte life cycle phases found some evidence that differences in the degree of variation between individuals in each phase may exist, with the female tetrasporophytes being more variable.

Some differences would be expected between the growth and response of individuals when randomly selected from within native populations, due to factors such as genotypic or developmental differences between individuals. What was perhaps more surprising was the amount of variation found within some individuals, when grown under the same standard laboratory conditions. Branches from some individuals grew and responded quite differently to copper exposure than other branches from the same individual, with some displaying a greater degree of tolerance to copper. Such intra-individual variation has been found previously in *Gracilaria chilensis* by Santelices & Varela (1993), where a contributing factor to this variability was a number of spores coalescing to form one 'individual' (Santelices *et al.*, 1996). Causes for this phenotypic plasticity were not established in *G. longissima* but it would seem likely that those found to contribute in closely related gracilarioid species, such as spore coalescence and genetic mutations, may also be important here.

### **9.1 Use of *G. longissima* as a biomonitor of trace metals**

In the introduction, the rationale for using seaweeds as biomonitors of trace metal contamination was stated. In addition, a number of factors that could potentially interfere with the use of seaweeds as informative biomonitors, were highlighted. Active biomonitoring was suggested as a considerably more informative method than the more conventional passive approach. A series of field transplants proved this to be the case.

The need for a better understanding of the mechanisms of trace metal regulation and toxicity in seaweeds was stated. It was felt that by better understanding these pathways through using laboratory based experiments, one may be in a stronger position to use these organisms as more informative biomonitors of trace metal pollution. Significant advances have been made in this respect and a greater appreciation of some of the physiological

effects of excess copper on *G. longissima* has been provided. It is however felt that considerably more information needs to be obtained regarding the effects of copper on this species before the mechanisms of copper toxicity are fully understood. This may eventually lead to the development of some form of biomarker of copper exposure or effect.

In terms of recommending the use of *G. longissima* as a biomonitor, a number of important aspects remain to be investigated. These include the investigation of some other factors considered to affect trace metal accumulation in seaweeds (as described in Chapter 1) such as any interactions that occur between different metals and the effect of growth rates on the accumulation and regulation of the metal.

Using *G. longissima* was found to be advantageous in terms of the ability to culture apical tips in petri dishes for laboratory based experiments. This culturing technique coupled with image analysis was very successful and should be used in future laboratory work. One negative aspect of using *G. longissima* was the inability to successfully culture sporelings which prevented studies into the effects of copper on the reproductive stages. It is felt that, given time, this could be overcome thus allowing a new range of possible experiments.

In conclusion, although significant advances have been made, further research is required before *G. longissima* can be proposed as an informative biomonitor of trace metal pollution.

## APPENDIX A

```
10 PANEL 0,0,80,79,1,14,3"WELCOME":COLTEXT 44:COLTEXT 33:COLTEXT 1
20 POSTEXT 12,14
30 PRINT "PLACE 3.5" DISK IN BOTTOM DRIVE THEN PRESS SPACE"
40 G$=INKEY$:IF G$="" THEN 40
50 TEST G$=" " THEN 60 ELSE 40
60 CLS
70 PANEL 0,0,80,79,1,14,3"FILE":COLTEXT 44:COLTEXT 33:COLTEXT 1
80 POSTEXT 14,5
90 INPUT "ENTER YOUR DATA FILENAME (8 CHARS MAX.):",N$
100 CLS
110 PANEL 0,0,80,79,1,14,1"PROCESSING":COLTEXT 44:COLTEXT 33:COLTEXT 1
120 POSTEXT 12,14:PRINT "PLEASE WAIT"
130 OPEN#1'B:'+N$+'.PRN'
140 camera 1
150 scanner 80.12 60.46
160 setlamps 0.00 0
170 mframe 44 78 347 430
180 iframe 0 0 512 512
190 qmenu 'image_setup'
200 qmenu 'calibrate'
210 RIASETTINGS 'CAL_VALUE' K
220 acquire 0
230 greyfill 0 1 2 1
240 greydetect 1 146 165 4 1 0
250 qmenu 'detect'
260 edgefeat 1 2
270 binmode 2 3
280 binerode 3 4 256 1
290 build 3 4
295 qmenu 'bin_edit'
300 setftrpar "1,8,15,2,3"
310 ftrgrey 0 : measfeat 4 1 100 300000 : clraccept
320 acceptfeat 15 4000 200000
```

```

330 acceptxfer 4 5
340 rfeatnum n(1)
345 DIM S(N(1),3)
350 for f=0 to n(1)-1
360 rfeatres f 1 a(1):S(F+1,1)=A(1)
370 rfeatres f 8 l(1):S(F+1,2)=L(1)
380 rfeatres f 15 r(1):S(F+1,3)=R(1)
400 NEXT F
410 binwskel 5 6
420 setftrpar "1,13,2,3"
430 ftrgrey 0 : measfeat 6 1 4 300000 : clraccept
440 acceptxfer 6 7
450 rfeatnum n(1)
460 for f=0 to n(1)-1
470 rfeatres f 13 p(1)
480 rfeatres f 1 as(1)
490 PRINT#1:K,N(1),K*K*S(F+1,1), K*S(F+1,2), S(F+1,3)/1000,K*K*AS(1),K*P(1)
500 NEXT F
510 CLOSE#1
520 END

```

## REFERENCES

- Aagaard, A. & Depledge, M.H. 1993. Inter-individual variability in the responses of *Carcinus maenas* to copper exposure. In: Aldrich, J.C. [Ed.] *Quantified Phenotypic Responses in Morphology and Physiology*. JAPAGA, Ashford Co. Wicklow, pp. 275-80.
- Abalde, J., Cid, A., Reiriz, S., Torres, E. & Herrero, C. 1995. Response of the marine microalga *Dunaliella tertiolecta* (Chlorophyceae) to copper toxicity in short time experiments. *Bull. Environ. Contam. Toxicol.* 54:317-24.
- Aldrich, J.C. 1990. How can toxic effects be separated from normal phenotypic variations with in the estuary? In: Chambers, P.L. & Chambers, C.M. [Eds.] *Estuarine Ecotoxicology*. JAPAGA, Ashford, Wicklow, pp. 29-41.
- Amado Filho, G.M., Karez, C.S., Pfeiffer, W.C., Yoneshigue-Valentin, Y. & Farina, M. 1996. Accumulation, effects on growth, and localization of zinc in *Padina gymnospora* (Dictyotales, Phaeophyceae). *Hydrobiologica*. 326/327:451-6.
- Anderson, B.S., Hunt, J.W., Turpen, S.L., Coulon, A.R. & Martin, M. 1990. Copper toxicity to microscopic stages of giant kelp *Macrocystis pyrifera*: interpopulation comparisons and temporal variability. *Mar. Ecol. Prog. Ser.* 68:147-56.
- Austen, M.C. & Somerfield, P.J. 1997. A community level sediment bioassay applied to an estuarine heavy metal gradient. *Mar. Env. Res.* 43(4):315-28.
- Axelsson, B. & Axelsson, L. 1987. A rapid and reliable method to quantify environmental effects on *Laminaria* based on measurements of ion leakage. *Bot. Mar.* 30:55-61.
- Baron, M., Arellano, J.B. & Lopez Gorge, J. 1995. Copper and photosystem II: a controversial relationship. *Physiol. Plant.* 94:174-80.

- Beer, S. & Eshel, A. 1985. Determining phycoerythrin and phycocyanin concentrations in aqueous crude extracts of red algae. *Aust. J. Mar. Freshw. Res.* 36:785-792.
- Bennett, A.F. 1987. Interindividual variability: an underutilized resource. In: Feder, M.E. [Ed.] *New Directions in Ecological Physiology*, pp. 147-69.
- Berail, G., Prudent, P., Massiani, C. & Pellegrini, M. 1991. Isolation of heavy metal-binding proteins from a brown seaweed *Cystoseira barbata* f. *repens* cultivated in copper or cadmium enriched seawater. In: Merian, E. & Hacrdi, W. [Eds.] *Chemical Speciation and Bioavailability, Metal Compounds in Environment and Life 4. Special Supplement*. Northwood, pp55-62.
- Bird, C.J. 1995. A review of taxonomic concepts and developments in the Gracilariaceae (Rhodophyta). *J. App. Phycol.* 7:255-67.
- Bird, C.J. & Rice, E.L. 1990. Recent approaches to the taxonomy of the Gracilariaceae (Gracilariales, Rhodophyta) and the *Gracilaria verrucosa* problem. *Hydrobiologica*. 204/205:111-8.
- Bishop, N.I. 1964. Site of action of copper in photosynthesis. *Nature*. 204:401-2.
- Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Adv. Gen.* 13:115-55.
- Bolhar-Nordenkamp, H.R., Long, S.P., Baker, N.R., Oquist, G., Schreiber, U. & Lechner, E.G. 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Funct. Ecol.* 3:497-514.
- Bowden, 1983. *Physical oceanography of coastal waters*. Ellis Horwood Publishers, 302pp.
- Brown, L.N., Robinson, M.G. & Hall, B.D. 1988. Mechanisms for copper tolerance in *Amphora coffeaeformis*-internal and external binding. *Mar. Biol.* 97:581-586.
- Brown, M.T. & Depledge, M.H. 1998. Determinants of trace metal concentrations in marine organisms. In: Bebianno, M.J. & Langston, W.J. [Eds.] *Metabolism of Trace Metals in Aquatic Organisms*. Chapman & Hall, London. pp185-217.

- Bryan, G.W. 1969. The absorption of zinc and other metals by the brown seaweed *Laminaria digitata*. *J. Mar. Biol. Ass. U.K.* 49:225-43.
- Bryan, G.W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proc. Roy. Soc. Lond. Ser. B.* 177:389-410.
- Bryan, G.W. 1976. Heavy metal contamination in the sea. In: Johnson, R. [Ed.] *Marine Pollution*. Academic Press, London, pp. 185-302.
- Bryan, G.W. 1983. Brown seaweed, *Fucus vesiculosus*, and the gastropod, *Littorina littoralis*, as indicators of trace-metal availability in estuaries. *Sci. Tot. Environ.* 28:91-104.
- Bryan, G.W. & Gibbs, P.E. 1983. Heavy Metals in the Fal Estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms. *Mar. Biol. Assoc. U.K. Occass. Publ.* 2:1-109.
- Bryan, G.W. & Hummerstone, L.G. 1973. Brown Seaweed as an indicator of heavy metals in estuaries in south-west England. *J. Mar. Biol. Ass. U.K.* 53:705-20
- Bryan, G.W., Langston, W.J. & Hummerstone, L.G. 1980. The use of biological indicators of heavy metal contamination in estuaries, with special reference to an assessment of the biological availability of metals in estuarine sediments from South West Britain. *Mar. Biol. Assoc. U.K. Occass. Publ.* 1:1-73
- Bryan, G.W., Langston, W.J., Hummerstone, L.G., Burt, G.R. & Ho, Y.B. 1983. An assessment of the Gastropod *Littorina littorea*, an indicator of heavy metal contamination in U.K. estuaries. *J. Mar. Biol. Ass. U.K.* 63:327-45.
- Bryan, G.W., Langston, W.J., Hummerstone, L.G. & Burt, G.R. 1985. A guide to the assessment of heavy-metal contamination in estuaries using biological indicators. *Mar. Biol. Assoc. U.K. Occass. Publ.* 4:1-92.
- Bryan, G.W. & Langston, W.J. 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environ. Pollut.* 76:89-131.

- Carroll, M.A. 1991. Distribution of morphological variation within populations of *Gracilaria*. *J. Phycol.* 21 (suppl.):13
- Chaphekar, S.B. 1991. An overview on bioindicators. *J. Environ. Biol.* 12:163-8.
- Chester, R. 1990. *Marine Geochemistry*. Chapman & Hall, London, 347 pp.
- Churella, D.J. & Copeland, T.R. 1978. Interference of salt matrices in the determination of copper by atomic absorption spectrometry with electrothermal atomization. *Anal. Chem.* 50(2):309-14.
- Cid, A., Herrero, C., Torres, E. & Abalde, J. 1995. Copper toxicity on the marine microalga *Phaeodactylum tricoratum*: effects on photosynthesis and related parameters. *Aquat. Toxicol.* 31:165-74.
- Ciscato, M., Valcke, R., van Loven, K., Clijsters, H. & Navari-Izzo, F. 1997. Effects of in vivo copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. *Physiol. Plant.* 100:901-8.
- Correa, J.A., Gonzalez, P., Sanchez, P., Munoz, J., Orellana, M.C. 1996. Copper-algae interactions: inheritance or adaptation? *Env. Mon. Assess.* 40:41-54.
- Critchley, A.T. 1993. *Gracilaria* (Rhodophyta: Gracilariales): An economically important agarophyte. In: Ohno, M. & Critchley, A.T. [Eds.] *Seaweed Cultivation and Marine Ranching*. Japan International Co-operation Agency, Yokosuka, Japan, pp. 89-112.
- Cullinane, J.P., Doyle, T.M. & Whelan, P.M. 1987. Uses of seaweeds as biomonitors of zinc levels in Cork harbour, Ireland. *Hydrobiologia* 151/152:285-90.
- Davison, I.R. & Pearson, G.A. 1996. Stress tolerance in intertidal seaweeds. *J. Phycol.* 32(2):197-211.
- Dawes, C.J. 1994. Physiological differentiation of the red seaweed *Gracilaria tikvahiae* from a mangrove estuary, exposed coast, and culture. *Bull. Mar. Sci.* 54(2):361-6.

- de Kock, W.C. & Kramer, K.J.M. 1994. Active biomonitoring (ABM) by translocation of bivalve molluscs. *In: Kramer, K.J.M. [Ed.] Biomonitoring of Coastal Waters and Estuaries*. CRC Press, Florida, pp. 51-78.
- Depledge, M.H. 1990a. Ecotoxicological relevance and significance of ecophysiological pollution effects. *Proc. Intern. Symp. Ecotox., Munich*.
- Depledge, M.H. 1990b. New approaches in ecotoxicology: can inter-individual physiological variability be used as a tool to investigate pollution effects? *Ambio*. 19(5):251-2.
- Depledge, M.H. & Bjerregaard, P. 1989. Explaining individual variation in trace metal concentrations in selected marine invertebrates: the importance of interactions between physiological state and environmental factors. *In: Aldrich, J.C. [Eds.] Phenotypic Responses and Individuality in Aquatic Ectotherms*. JAPAGA, Ashford Co. Wicklow, Ireland, pp. 121-6.
- Depledge, M.H., Weeks, J.M. & Bjerregaard, P. 1994. Heavy Metals. *In: Calow, P. [Ed.] Handbook of Ecotoxicology Vol II*, Blackwell Science, U.K, pp.543-69.
- Destombe, C., Godin, J., Nocher, M., Richard, S. & Valero, M. 1993. Differences in response between haploid and diploid isomorphic phases of *Gracilaria verrucosa* (Rhodophyta: Gigartinales) exposed to artificial environmental conditions. *Hydrobiologia*. 260/261:131-7.
- Dudgeon, S.R., Kubler, J.E., Vadas, R.L. & Davison, I.R. 1995. Physiological responses to environmental variation in intertidal red algae: does thallus morphology matter? *Mar. Ecol. Prog. Ser.* 117:193-206.
- Dyck, L.J. & de Wreede, R.E. 1995. Patterns of seasonal demographic change in the alternate isomorphic stages in *Mazzaella splendens* (Gigartinales, Rhodophyta). *Phycologia* 34(5):390-5.
- Edelstein, T., Bird, C.J. & McLauchlan, J. 1976. Studies on *Gracilaria*. 2. Growth under greenhouse conditions. *Can. J. Bot.* 54:2275-90.
- Edwards, P. 1972. Cultured red alga to measure pollution. *Mar. Pollut. Bull.* 3(12):184-7.

- Eide, I., Mykkestad, S. & Melsom, S. 1980. Long-term uptake and release of heavy metals by *Ascophyllum nodosum* (L.) Le Jol. (Phaeophyceae) *in situ*. *Environ. Pollut.* 23:19-28.
- Evans, C.A. & Hutchinson, T.C. 1995. Mercury accumulation in transplanted moss and lichens at high elevation sites in Quebec. *Water, Air and Soil Pollut.* 90:475-88.
- Fernandes, J.C. & Henriques, F.S. 1991. Biochemical, physiological and structural effects of excess copper in plants. *Bot. Rev.* 57:246-73.
- Fisher, N.S. & Froud, D. 1980. Heavy metals and marine diatoms: influence of dissolved organic compounds on toxicity and selection for metal tolerance among four species. *Mar. Biol.* 59:85-93.
- Fisher, N.S., Jones, G.J. & Nelson, D.M. 1981. Effects of copper and zinc on growth, morphology and metabolism of *Asterionella japonica* (Cleve). *J. Exp. Mar. Biol. Ecol.* 51:37-56.
- Fletcher, R.L. 1991. Marine macroalga as bioassay test organisms. *In: Abel, P.D. & Axiak, V. [eds.] Ecotoxicology and the Marine Environment*. Ellis Horwood Series in Aquaculture and Fisheries Support, pp. 111-31.
- Florence, T.M., Lumsden, B.G. & Fardy, J.J. 1983. Evaluation of some physio-chemical techniques for the determination of the fraction of dissolved copper toxic to the marine diatom *Nitzschia closterium*. *Anal. Chim. Acta.* 151:281-95.
- Florence, T.M., Stauber, J.L. & Mann, K.J. 1985. The reaction of copper-2,9-dimethyl-1,10-phenanthroline with hydrogen peroxide. *J. Inorg. Biochem.* 24:243-54.
- Florence, T.M. & Stauber, J.L. 1986. Toxicity of copper complexes to the marine diatom *Nitzschia closterium*. *Aquat. Toxicol.* 8:11-26.
- Forbes, V.E. & Depledge, M.H. 1992. Predicting population response to pollutants: the significance of sex. *Funct. Ecol.* 6:376-81.

- Forbes, V.E., Moller, V. & Depledge, M.H. 1995. Intrapopulation variability in sublethal response to heavy metal stress in sexual and asexual gastropod populations. *Funct. Ecol.* 9:477-84.
- Foster, P.L. 1986. Copper exclusion as a mechanism of heavy metal tolerance in a green alga. *Nature*, 269:322-3.
- Franklin, L.A. & Forster, R.M. 1997. Review: The changing irradiance environment: consequences for marine macrophyte physiology, productivity and ecology. *Eur. J. Phycol.* 32:207-232.
- Friedlander, M., Dawes, C. & Levy, I. 1993. Exposure of *Gracilaria* to various environmental conditions. I. The effect on growth. *Bot. Mar.* 36(4):283-8.
- Fuge, R. & James, K.H. 1974. Trace metal concentrations in *Fucus* from the Bristol Channel. *Mar. Pollut. Bull.* 5(1):9-12.
- GESAMP. 1990. *The State of the Marine Environment*. Blackwell Scientific Publications. Oxford, 111pp.
- GESAMP. 1995. *Biological Indicators and their Use in the Measurement of the Condition of the Marine Environment*. Rep. Stud., 65pp.
- Gibb, J.O.T., Allen, J.R. & Hawkins, S.J. 1996. The applications of biomonitors for the assessment of mine-derived pollution on the west coast of the Isle of Man. *Mar. Pollut. Bull.* 32(6):513-9
- Gledhill, M., Nimmo, M., Hill, S.J. & Brown, M.T. 1997. The toxicity of copper (II) species to marine algae, with particular reference to macroalgae. *J. Phycol.* 33:2-11.
- Green, E.J. & Carritt, D.E. 1969. New tables for oxygen saturation of seawater. *J. Mar. Res.* 25(2):140-7.
- Griepink, B. & Muntau, H. 1987. *The Certification of the Contents (Mass Fractions) of Arsenic, Cadmium, Copper, Lead, Selenium and Zinc in a Sea Lettuce (Ulva lactuca)*. CRM #279. Report: Eur 11185. Commission of the European Communities, Luxembourg, 54pp.

- Gupta, S.L. 1986. Copper intake and inhibition of growth, photosynthetic pigments and macromolecules in the cyanobacterium *Anacystis nidulans*. *Photosynthetica* 20:447-53.
- Gustavson, K. & Wangberg, S. 1995. Tolerance induction and succession in microalgae communities exposed to copper and atrazine. *Aquat. Toxicol.* 32:283-302.
- Hall, A. 1981. Copper accumulation in copper-tolerant and non-tolerant populations of the marine fouling alga, *Ectocarpus siliculosus* (Dillw.) Lyngbye. *Bot. Mar.* 24:223-8.
- Hall, A., Fielding, A.H. & Butler, M. 1979. Mechanisms of copper tolerance in the marine fouling alga *Ectocarpus siliculosus* – evidence for an exclusion mechanism. *Mar. Biol.* 54:195-199.
- Hannach, G. & Santelices, B. 1985. Ecological differences between the isomorphic reproductive phases of two species of Iridaea (Rhodophyta: Gigartinales). *Mar. Prog. Ser.* 22:291-303.
- Hansatech Instruments Ltd. 1995. *Operating Instructions for Plant Efficiency Analyser*. Hansatech Instruments Ltd, Norfolk.
- Haug, A., Melsom, S. & Omang, S. 1974. Estimation of heavy metal pollution in two Norwegian fjord areas by analysis of the brown alga *Ascophyllum nodosum*. *Environ. Pollut.* 7:179-92.
- Ho, Y.B. 1984. Zinc and copper concentrations in *Ascophyllum nodosum* and *Fucus vesiculosus* (Phaeophyta, Fucales) after transplantation to an estuary contaminated with mine wastes. *Conserv. Recycling* 7:329-337
- Holmes, M.A., Brown, M.T., Loutit, M.W. & Ryan, K. 1991. The involvement of epiphytic bacteria in zinc concentration by the red alga *Gracilaria sordida*. *Mar. Environ. Res.* 31:55-67.
- Hubert, E. 1995. *Nickel and Copper Speciation in the Fal Estuary, South West England*. Report of a practical period at the University of Plymouth for Environmental Studies (T32) at Wageningen Agricultural University. The Netherlands. 36 pp.
- Hunt, R. 1982. *Plant Growth Curves*. Edward Arnold Publishers Ltd, London, 248 pp.

- Jayasekera, R. & Rossbach, M. 1996. Use of seaweeds for monitoring trace elements in coastal waters, *Environ. Geochem. Health* 18:63-8.
- Jensen, A. 1979. Industrial utilisation of seaweeds in the past, present and future. *Proc. Int. Seaweed Symp.* 9:17-34.
- Johnson, I. 1987. *The Role of Bacteria in the Uptake of Metals in Otago Harbour*. PhD Thesis. University of Otago, New Zealand.
- Jones, W.E. 1959a. The growth and fruiting of *Gracilaria verrucosa* (Hudson) Papenfuss. *J. Mar. Biol. Ass. U.K.* 38:153-67
- Jones, W.E. 1959b. Experiments on some effects of certain environmental factors on *Gracilaria verrucosa* (Hudson) Papenfuss. *J. Mar. Biol. Ass. U.K.* 38:153-67.
- Karcz, C.S., Magalhaes, V.F., Pfeiffer, W.C. & Amado Filho, G.M. 1994. Trace metal accumulation by algae in Sepetiba Bay, Brazil. *Environ. Pollut.* 83:351-6.
- Kautsky, H. & Hirsch, A. 1931. Neue versuche zur kohlenstoffassimilation. *Naturwissenschaften* 19:964.
- Keller, M.D., Bellows, W.K. & Guillard, R.L. 1988. Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* 117(3):279-83.
- Klerks, P.L. & Weis, J.S. 1987. Genetic adaptation to heavy metals in aquatic organisms: a review. *Environ. Poll.* 45:173-205.
- Knauer, G.A. & Martin, J.H. 1973. Seasonal variations of cadmium, copper, manganese, lead, and zinc in water and phytoplankton in Monterey Bay, California. *Limnol. Oceanogr.* 18(4):597-604.
- Koch, U., Glatzle, D., Ringenbach, F., Dunz, T., Steger-Hartmen, T. & Wagner, E. 1995. Measurement of ion leakage from plant cells in response to aquatic pollutants. *Bull. Environ. Contam. Toxicol.* 54(4):606-13.

- Krause, G.H. & Weiss, E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 42:313-49.
- Lage, O.M., Parente, A.M., Soares, H.M.V.M., Vasconcelos, M.T.S.D. & Salema, R. 1994. Some effects of copper on the dinoflagellates *Amphidinium carterae* and *Prorocentrum micans* in batch culture. *Eur. J. Phycol.* 29:253-60.
- Lage, O.M., Parente, A.M., Vasconcelos, M.T.S.D., Gomes, C.A.A & Salema, R. 1996. Potential tolerance mechanisms of *Prorocentrum micans* (Dinophyceae) to sublethal levels of copper. *J. Phycol.* 32:416-23.
- Langston, W.J. 1984. Availability of arsenic to estuarine and marine organisms: a field and laboratory evaluation. *Mar. Biol.* 80:143-54.
- Langston, W.J. & Bryan, G.W. 1984. The relationship between metal speciation in the environment and bioaccumulation in aquatic organisms. In: Kramer, C.J.M. & Duinker, J.C. [Eds.] *Complexation of Trace Metals in Natural Waters*. Nijhoff/Junk Publications, The Hague, pp. 375-92.
- Langston, W.J. & Spence, S.K. 1995. Biological factors involved in metal concentrations observed in aquatic organisms. In: Tessier, A. & Turner, D.R. [Eds.] *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley & Sons Ltd, New York, pp. 407-78.
- Lapointe, B.E., Tenore, K.R. & Dawes, C.J. 1984. Interactions between light and temperature on the physiological ecology of *Gracilaria tikvahiae* (Gigartinales: Rhodophyta). *Mar. Biol.* 80:161-70.
- Levy, I., Beer, S. & Friedlander, M. 1990. Growth, photosynthesis and agar in wild-type strains of *Gracilaria verrucosa* and *G. conferta* (Gracilariales, Rhodophyta), as a strain selection experiment. *Hydrobiologia* 204/205:381-7.
- Lichtenberger, O. & Neumann, D. 1997. Analytical electron microscopy as a powerful tool in plant cell biology: examples using electron energy loss spectroscopy and x-ray microanalysis. *Eur. J. Cell Biol.* 73:378-86.

- Lignell, A., Ekman, P. & Pedersen, M. 1987. Cultivation technique for marine seaweeds allowing controlled and optimized conditions in the laboratory and on a pilotscale. *Bot. Mar.* 30:417-24.
- Lobban, C.S. & Harrison, P.J. 1994. *Seaweed Ecology and Physiology*. Cambridge University Press, New York. 366pp.
- Lovett Doust, J., Lovett Doust, L. & Schmidt, M. 1993. In praise of plants as biomonitors – send in the clones! *Functional Ecology*. 7:754-58.
- Lovett Doust, J., Schmidt, M. & Lovett Doust, L. 1994. Biological assessment of aquatic pollution: a review, with emphasis on plants as biomonitors. *Biol. Rev.* 69:147-86.
- Lumsden, B.R. & Florence, T.M. 1983. A new algal assay procedure for the determination of the toxicity of copper species in seawater. *Environ. Technol. Lett.* 4(6):271-6.
- Luoma, S.N. & Carter, J.L. 1991. Effects of trace metals on aquatic benthos. In: Newman, M.C. & McIntosh, A.W. [Eds.] *Metal Ecotoxicology: concepts and applications*. Lewis Publisher Inc., Michigan, pp. 261-300.
- Macnair, M.R. 1993. Tansley Review No. 49. The genetics of metal tolerance in vascular plants. *New Phytol.* 124:541-59.
- Malea, P., Haritonidis, S. & Stratis, I. 1994. Bioaccumulation of metals by Rhodophyta species at Antikyra Gulf (Greece) near an aluminium factory. *Bot. Mar.* 37:505-13.
- Mathieson, A.C., Norton, T.A. & Neushul, M. 1981. The taxonomic implications of genetic and environmentally induced variations in seaweed morphology. *Bot. Rev.* 47(3):313-47.
- McLean, M.W. & Williamson, F.B. 1977. Cadmium accumulation by the marine red alga *Porphyra umbilicalis*. *Physiol. Plant.* 41:268-72.
- McLachlan, J. & Bird, C.J. 1984. Geographical and experimental assessment of the distribution of *Gracilaria* species (Rhodophyta: Gigartinales) in relation to temperature. *Helgolander Meeresunters.* 38:319-34.

- Melhuus, A., Seip, K.L., Seip, H.M. & Mykkestad, S. 1978. A preliminary study of the use of benthic algae as biological indicators of heavy metal pollution in Sorfjorden, Norway. *Environ. Pollut.* 15:101-7.
- Moore, M.N. 1993. Biomarkers of contaminant exposure and effect: a way forward in marine environmental toxicology. *Sci. Tot. Environ. Supp.* 1993, pp. 1335-43.
- Moriarty, F. 1983. *Ecotoxicology: The Study of Pollutants in Ecosystems*. Academic Press, London, 233pp.
- Morris, A.W. & Bale, A.J. 1975. The accumulation of cadmium, copper, manganese and zinc by *Fucus vesiculosus* in the Bristol Channel. *Estuar. Cstl. Mar. Sci.* 3:153-63.
- Munda, I.M. & Hudnik, V. 1986. Growth response of *Fucus vesiculosus* to heavy metals, singly and in dual combinations, as related to accumulation. *Bot. Mar.* 29:401-12.
- Munda, I.M. & Hudnik, V. 1988. The effects of Zn, Mn, and Co accumulation on growth and chemical composition of *Fucus vesiculosus* L. under different temperature and salinity conditions. *Mar. Ecol.* 9(3):213-25.
- Mykkestad, S., Eide, I. & Melsom, S. 1978. Exchange of heavy metals in *Ascophyllum nodosum* (L.) Le Jol. *in situ* by means of transplanting experiments. *Environ. Pollut.* 16:277-84.
- Mykkestad, S., Eide, I. & Melsom, S. 1979. Heavy metal exchange by *Ascophyllum nodosum* (Phaeophyceae) plants *in situ*. 9<sup>th</sup> *International Seaweed Symposium*, pp. 143-51.
- Nalewajko, C. & Olaveson, M.M. 1995. Differential responses of growth, photosynthesis, respiration, and phosphate intake to copper in copper-tolerant and copper-intolerant strains of *Scenedesmus acutus* (Chlorophyceae). *Can. J. Bot.* 73:1295-303.
- Nott, J.A. 1991. Cytology of pollutant metals in marine invertebrates: a review of microanalytical applications. *Scann. Microsc.* 5(1):191-205.
- Overnell, J. 1975. The effect of heavy metals on photosynthesis and loss of cell potassium in two species of marine algae, *Dunaliella tertiolecta* and *Phaeodactylum tricorutum*. *Mar. Biol.* 29:99-103.

- Peakall, D.B. 1994. The role of biomarkers in environmental assessment (1). Introduction. *Ecotoxicol.* 3:157-60.
- Perryman, S.A.M. 1996. *The Effect of Heavy Metal Contamination on Estuarine Benthic Fauna at Varying Levels of Biological Organisation*. PhD Thesis. University of Plymouth, U.K.
- Phillips, D.J.H. 1979. Trace metals in the common mussel *Mytilus edulis* (L.) and in the alga *Fucus vesiculosus* (L.) from the region of the Sound (Oresund). *Environ. Pollut.* 18:31-43.
- Phillips, D.J.H. 1990. The use of macroalgae as monitors of metal levels in estuaries and coastal waters. In: Furness, R.W. & Rainbow, P.S. [Eds.] *Heavy Metals in the Marine Environment*. CRC Press, Florida, pp. 81-99.
- Phillips, D.J.H. 1994. Macrophytes as biomonitors of trace metals. In: Kramer, K.J.M. [Ed.] *Biomonitoring of Coastal Waters and Estuaries*. CRC Press, Florida, pp. 85-102.
- Pilai, A. 1992. *The Ecology and Physiology of Gracilaria chilensis*. PhD Thesis. University of Otago, New Zealand.
- Plotz, J. 1991. Effects of salinity and toxic heavy metals on oxygen release by *Fucus vesiculosus* L. *Acta Ichthyologica et Piscatoria* 21(Suppl):283-90.
- Pregnall, A.M. 1983. Release of dissolved organic carbon from the estuarine intertidal macroalga *Enteromorpha prolifera*. *Mar. Biol.* 73:37-42.
- Qureshi, J.A. & Stokes, P.M. 1985. Mechanism of heavy metal tolerance in a green alga (*Scenedesmus* sp.) ...an ultrastructural view. In: Leukas, T.D. [Ed.] *Heavy Metals in the Environment, Vol II*, pp. 156-8.
- Ragan, M.A., Ragan, C.M. & Jensen, A. 1980. Natural chelators in sea water: detoxification of  $Zn^{2+}$  by brown algal polyphenols. *J. Exp. Mar. Biol. Ecol.* 44:261-7.

- Ragan, M.A., Smidsrod, O. & Larsen, B. 1979. Chelations of divalent metal ions by brown algal polyphenols. *Mar. Chem.* 7:265-71.
- Rai, L.C., Gaur, J.P. & Kumar, H.D. 1981. Phycology and heavy metal pollution. *Biol. Rev.* 56:99-151
- Rainbow, P.S. 1992. I. The significance of trace metal concentrations in marine invertebrates. In: Dallinger, R. & Rainbow, P.S. [Eds.] *Ecotoxicology of Metals in Invertebrates*. Lewis Publishers, Florida, pp. 3-23.
- Rainbow, P.S. 1993. The significance of trace metal concentrations in marine invertebrates. In: Newman, M.C. & McIntosh, A.W. [Eds.] *Metal Ecotoxicology; concepts and applications*. Lewis Publishers Inc., Michigan, pp. 261-300.
- Rainbow, P.S., Phillips, D.J.H. & Depledge, M.H. 1990. The significance of trace metal concentrations in marine invertebrates: a need for laboratory investigation of accumulation strategies. *Mar. Pollut. Bull.* 21(7):321-24.
- Ramade, F. 1987. *Ecotoxicology*. John Wiley & Sons, New York.
- Reed, R.H. & Moffat, L. 1983. Copper toxicity and copper tolerance in *Enteromorpha compressa* (L.) Grey. *J. Exp. Mar. Biol. Ecol.* 69:85-103.
- Rice, D.L. 1984. A simple mass transport model for metal uptake by marine macroalgae growing at different rates. *J. Mar. Exp. Biol. Ecol.* 82:175-82.
- Rice, D.L. & Lapointe, B.E. 1981. Experimental outdoor studies with *Ulva fasciata* Delile. II. Trace Metal Chemistry. *J. Exp. Mar. Biol. Ecol.* 54:1-11.
- Riget, F., Johansen, P. & Asmund, G. 1995. Natural seasonal variation of cadmium, copper, lead and zinc in brown seaweed (*Fucus vesiculosus*). *Mar. Poll. Bull.* 30(6):409-13.
- Rijstenbil, J.W., Derksen, J.W.M., Gerringa, L.J.A., Poorvliet, T.C.W., Sandee, A., van den Berg, M., van Drie, J. & Wijnholds, J.A. 1994a. Oxidative stress induced by copper: defence and damage in the marine planktonic diatom *Ditylum brightwelli*, grown in continuous cultures with high and low zinc levels. *Mar. Biol.* 119:583-90.

- Rijstenbil, J.W., Sandee, A., van Drie, J. & Wijnholds, J.A. 1994b. Interaction of toxic trace metals and mechanisms of detoxification in the marine diatoms *Ditylum brightwellii* and *Thalassiosira pseudonana*. *FEMS Microb. Rev.* 14:387-96.
- Romeo, M. & Gnassia-Barelli, M. 1993. Organic ligands and their role in complexation and transfer of trace metals (micronutrients) in marine algae. *In: Rijstenbil, J.W. & Haritonidis, S. [Eds] Macroalgae, eutrophication and trace metal cycling in estuaries and lagoons: Proceedings of the Coast-48 Symposium of Sub Group III, Thessaloniki, pp. 121-35.*
- Ronnberg, O., Adjers, K., Ruokolaihti, C. & Bondestam, M. 1990. *Fucus vesiculosus* as an indicator of heavy metal availability in a fish farm recipient in the Northern Baltic Sea. *Mar. Pollut. Bull.* 21(8):388-92.
- Runess, J. & Tananger, T. 1984. Growth and culture of four red alga from Norway with potential for mariculture. *Hydrobiologia* 116/117:303-7.
- Russell, G. 1963. A study in populations of *Pilaiella littoralis*. *J. Mar. Biol. Ass. U.K.* 43:469-83.
- Russell, G. & Morris, O. 1970. Copper tolerance in the marine fouling alga *Ectocarpus siliculosus*. *Nature* 228:288-9.
- Samson, G. & Popovic, R. 1988. Use of algal fluorescence for determination of phytotoxicity of heavy metals and pesticides as environmental pollutants. *Ecotox. Environ. Safety* 16:272-8.
- Santelices, B., Correa, J.A., Meneses, I., Aedo, D. & Varela, D. 1996. Sporling coalescence and intraclonal variation in *Gracilaria chilensis* (Gracilariales, Rhodophyta). *J. Phycol.* 32:313-22.
- Santelices, B. & Varela, D. 1993. Intra-clonal variation in the red seaweed *Gracilaria chilensis*. *Mar. Biol.* 116:543-52.
- Santelices, B. & Varela, D. 1995. Regenerative capacity of *Gracilaria* fragments: effects of size, reproductive state and position along the axis. *J. App. Phycol.* 7:501-6.

- Say, P.J., Burrows, I.G. & Whitton, B.A. 1990. Enteromorpha as a monitor of heavy metals in estuaries. *Hydrobiologica* 195:119-26.
- Schramm, W. 1993. Investigations on the influence of organic substances produced by seaweeds on the toxicity of copper. In: Rijstenbil, J.W. & Haritonidis, S. [Eds.] *Macroalgae, eutrophication and trace metal cycling in estuaries and lagoons: Proceedings of the Coast-48 Symposium of Sub Group III, Thessaloniki*, pp. 106-20.
- Seeliger, U. & Cordazzo, C. 1982. Field and experimental evaluation of *Enteromorpha* sp. as a qualitative monitoring organism for copper and mercury in estuaries. *Environ. Pollut.* 29:197-206.
- Seeliger, U. & Edwards, P. 1979. Fate of biologically accumulated copper in growing and decomposing thalli of two benthic red marine algae. *J. Mar. Biol. Ass. U.K.* 59:227-38.
- Seely, G., Duncan, M.J. & Vidaver, W.E. 1972. Preparative and analytical extraction of pigments from brown algae with dimethyl sulfoxide. *Mar. Biol.* 12:184-188.
- Seip, K.L. 1979. A mathematical model for the uptake of heavy metals in benthic algae. *Ecol. Modelling* 6:183-97.
- Shaw, A.J. 1990. Intraclonal variation in morphology, growth rate, and copper tolerance in the moss, *Funaria hygrometrica*. *Evolution* 44(2):441-7.
- Sheoran, I.S. & Singh, R. 1993. Effect of heavy metals on photosynthesis of higher plants. In: Abrol, Y.P., Moherty, P. & Govindjee [Eds.] *Photosynthesis: photoreaction to plant productivity*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 452-68.
- Simpson, R.D. 1979. Uptake and loss of zinc and lead by mussels (*Mytilus edulis*) and relationships with body weight and reproductive cycle. *Mar. Pollut. Bull.* 10:74-8.
- Skipnes, O., Roald, T. & Haug, A. 1975. Uptake of zinc and strontium by brown algae. *Physiol. Plant.* 34:314-20.

- Somerfield, P.J., Gee, J.M. & Warwick, R.M. 1994. Soft sediment meiofaunal community structure in relation to a long-term heavy metal gradient in the Fal estuary system. *Mar. Ecol. Prog. Ser.* 105:79-88.
- Stauber, J. L. & Florence, T.M. 1985. The influence of iron on copper toxicity to the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquat. Toxicol.* 6:297-305
- Stauber, J. L. & Florence, T.M. 1986. Reversibility of copper-thiol binding in *Nitzschia closterium* and *Chlorella pyrenoidosa*. *Aquat. Toxicol.* 8:223-29.
- Stauber, J.L. & Florence, T.M. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. *Mar. Biol.* 94:511-9.
- Stebbing, A.R.D., Cleary, J.J., Brinsley, M., Goodchild, C. & Santiago-Fandino, V. 1983. Responses of a hydroid to surface water samples from the River Tamar and Plymouth Sound in relation to metal concentrations. *J. Mar. Biol. Ass. U.K.* 63:695-711
- Steenftoft, M., Irvine, L.M. & Farnham, W.F. 1995. Two terete species of *Gracilaria* and *Gracilariopsis* (Gracilariales, Rhodophyta) in Britain. *Phycologica* 34(2):113-27.
- Stein, J.R. 1975. *Handbook of Phycological Methods: culture methods and growth measurements*. Cambridge University Press, London, 448pp.
- Strasser, B.J. & Strasser, R.J. 1995. Measuring fast fluorescence transients to address environmental questions: the JIP-test. In: Mathis, P. [Ed.] *Photosynthesis: from Light to Biosphere Vol 5*. Kluwer Academic Publishers, The Netherlands.
- Stromgren, T. 1975. Linear measurements of growth of shells using laser diffraction. *Limnol. Oceanogr.* 20:845-8.
- Sultan, S.E. & Bazzaz, F.A. 1993. Phenotypic plasticity in *Polygonum persicaria*. I. Diversity and uniformity in genotypic norms of reaction to light. *Evolution* 47(4):1009-31.

- Sunda, W.G. & Huntsman, S.A. 1983. Effect of competitive interactions between manganese and copper on cellular manganese and growth in estuarine and oceanic species of the diatom *Thalassiosira*. *Limnol. Oceanogr.* 28:924-34.
- Tessier, A. & Turner, D.R. 1995. *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley & Sons Ltd, New York, 679pp.
- Thompson J.D. 1991. Phenotypic plasticity as a component of evolutionary change. *Tree* 6(8):246-9.
- Underwood, E.J. 1956. *Trace Elements in Human and Animal Nutrition*. Academic Press, London.
- Underwood, E.J. 1970. In: Mills, C.F. [Ed.] *Trace Element Metabolism in Animals*. Proc. WAAP/IBP Int'l Symp. Aberdeen, Scotland. E. & S. Livingstone, Edinburgh.
- Valero, M., Richard, S., Perrot, V. & Destombe, C. 1992. Evolution of alternation of haploid and diploid phases in life cycles. *Tree* 7(1):25-9.
- van den Berg, C.M.G., Wong, P.T.S. & Chau, Y.K. 1979. Measurement of complexing materials excreted from algae and their ability to ameliorate copper toxicity. *J. Fish. Res. Board Can.* 36:901-5.
- van der Meer, J.P. & Todd, E.R. 1977. Genetics of *Gracilaria* sp. (Rhodophyceae, Gigartinales). IV. Mitotic recombination and its relationship to mixed phases in the life history. *Can. J. Bot.* 55:2810-7.
- van der Meer, J.P. & Zhang, X.C. 1988. Similar unstable mutations in three species of *Gracilaria* (Rhodophyta). *J. Phycol.* 24(2):198-202.
- Vavilin, D.V., Polynov, V.A., Matorin, D.N. & Venediktov, P.S. 1995. Sublethal concentrations of copper stimulate photosystem II photoinhibition in *Chlorella pyrenoidosa*. *Biochem. Physiol. Mol. Biol. Plants* 146(5/6):609-14.
- Wang, J., Zhang, M., Xu, J. & Wang, Y. 1995. Reciprocal effect of Cu, Cd, Zn on a kind of marine alga. *Wat. Res.* 29:209-14.

- Wilkinson, M., Telfer, T., Cruz, R., Conroy-Dalton, S., Cunningham, E. & Grundy, S. 1992. The utility of field transplants of seaweeds in the study of polluted estuaries. In: Dyer, K.R. & Orth, R.J. [Eds.] *Coastal Fluxes in Estuaries: Implications from Science to Management*. Olsen & Olsen Academic Publishers, Denmark, pp. 257-60.
- Williams, P. 1994. *The Bioaccumulation and Bioavailability of Heavy Metals (Mn, Zn, Cu, Pb, Fe) within the Fal Estuary System*. MSc Thesis, University of Plymouth, U.K., 90pp.
- Williams, R.J.P. 1981. Physio-chemical aspects of inorganic element transfer through membranes. *Phil. Trans. R. Soc. London B* 294:57-74.
- Wilson, J.B. 1991. *Teddybear; A Statistical System*. Manual Version 3.0. University of Otago, New Zealand, 83pp.
- Wu, J.T., Chang, S.C. & Chen, K.S. 1995. Enhancement of intracellular proline level in cells of *Anacyclops nidulans* (Cyanobacteria) exposed to deleterious concentrations of copper. *J. Phycol.* 31:376-9.
- Young, M.L. 1975. The transfer of  $^{65}\text{Zn}$  and  $^{59}\text{Fe}$  along a *Fucus serratus* (L.)  $\longrightarrow$  *Littorina obtusata* (L.) food chain. *J. Mar. Biol. Ass. U.K.* 55:583-610.
- Zhang, X.C. & van der Meer, J.P. 1987. A study of heterosis in diploid gametophytes of the marine red algae *Gracilaria tikvahiae*. *Bot. Mar.* 30:309-14.
- Zolotukhina, Ye. Yu., Gavrilenko, Ye. Ye. & Burdin, K.S. 1991. Interactions among metal ions during uptake by marine macroalgae. *J. Hydrobiol.* 26(6):56-62.
- Zolotukhina, Ye. Yu. & Gavrilenko, Ye. Ye. 1992. Distribution of copper and cadmium in aquatic macrophytes. *J. Hydrobiol.* 28(5):7-16.

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