The production of geosmin by *Anabaena circinalis* (Rabenhorst), and its measurement by sensory analysis.

Thesis submitted by

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Abstract

The Hay Weir Pool is a freshwater impoundment along the Murrumbidgee River near the township of Hay, New South Wales, Australia. Water quality was often degraded during warmer periods due to the formation of blooms of the cyanobacterium *Anabaena circinalis* which produces the odour-causing compound geosmin. Options for managing the drinking water supply were severely restricted because of the inability to rapidly measure geosmin concentration. Furthermore, the management of algal blooms was not possible because factors that affected the production of geosmin by *A. circinalis* were unknown.

The objectives of this study were two-fold: firstly, to develop a sensory method for the rapid assessment of geosmin in water that could be readily implemented by water authorities using untrained personnel; and secondly, to investigate the effect of light on the production of geosmin by *A. circinalis* (strain 852E) in laboratory culture experiments.

Five different sensory panels comprising untrained individuals were established. Testing of panels with geosmin solutions showed that panels consistently underestimated odour intensities across different panels and concentrations (attributed to 'nose fatigue' from repetitive smelling), but a correction factor was applied to compensate for this error. The overall variability associated with measuring solutions was 40% when panel sizes of at least 9 individuals were used, and increased markedly with decreasing panel size. A screening protocol was established to select individuals most sensitive to geosmin, however no statistical difference ($P<0.05$) was found between these and unscreened individuals that were also used. The success

of evaluating field and culture samples containing geomin varied when compared with the geosmin content measured using chemical analysis. This was thought to reflect the inexperience of panellists which may have been confused by the presence of other compounds competing with geosmin. Despite this, the ability of the panel to 'integrate' the total odour intensity and express it as geosmin equivalents on a consistent basis is important for identifying and quantifying off-flavours in water.

Laboratory cultures of *A. circinalis* 852E isolated from the Hay Weir Pool showed that the production of geosmin occurred continuously and that it was related with the synthesis of chlorophyll a. Cultures grown at different light intensities showed that the rate of geosmin production per unit chlorophyll a was not constant. At higher rates of culture growth (>1 ln units day^{-1}) the rate of geosmin production was 20 ng $\mu gChla^{-1}$ day^{-1}, and almost tripled at rates of less than 0.3 ln units day^{-1}. These results suggest either suppression of geosmin synthesis to divert cell energy into maintaining faster cell growth, or that geosmin synthesis may be directly linked to a different compound that is also affected by light intensity, most likely another photosynthetic pigment.

The rate of geosmin production obtained from this study was used to calculate predicted geosmin concentrations using data of *A. circinalis* density in the Hay Weir Pool obtained from the literature. This demonstrated that cell densities of almost 1,000 cells mL^{-1} could result in geosmin whose concentrations exceeding the odour threshold concentration. Furthermore, geosmin concentrations above 100 ng L^{-1} may be attained for periods of over 8 weeks due to levels of algal biomass consistently exceeding 6,000 cells mL^{-1}.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Some data in this thesis have been presented at the following forums:

Bowmer, K.H., Padovan, A., Oliver, R.L., Korth, W. and Ganf, G.G. (1992). Physiology of geosmin production by Anabaena circinalis isolated from the Murrumbidgee River, Australia. *Water Science and Technology*, **25**(2), 259-267.

Padovan, A., Korth, W., Oliver, R.L. and Bowmer, K. (1988). Production of an odoriferous compound, geosmin, by an *Anabaena* sp.. Australian Society for Limnology Conference, Magnetic Island, Queensland.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Armando Padovan

28 June 1995

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Chapter 1

1.1 Introduction

The presence of taste and odour-causing compounds in water supplies is of major concern to water authorities around the world, where consumer expectations of water quality are high for both aesthetic and economic reasons. Domestic users demand odour-free water as tainted waters are associated as being "dirty" or "unhealthy". In addition, various industries such as food processing and manufacturing, are dependent on a continuous and odourless water supply which is incorporated into edible products of high standards. These include canneries, breweries, and manufacturers of liquid refreshments. Contaminated waters are also of concern to the aquaculture industries, where water characteristics directly affect the eating quality of cultured fish, and therefore their market value (Persson, 1979; Ogata and Fujisawa, 1985; Martin and Suffet, 1992).

The origin of off-flavour compounds can be broadly divided as either man-made or natural. However, this distinction may not be clear-cut, as human interference may stimulate natural sources of odour. Amongst others, man-made sources include sewage effluent, industrial waste, and water treatment prior to reticulation (Lin, 1976 (Parts 1 and 2); Mallevialle and Suffet, 1987).

The release of treated sewage and industrial waste into coastal regions, although affecting the environment through eutrophication, does not pose problems to drinking water quality as discharge is directly into a marine environment. It is, however, important in inland waters as river and lake systems often share the dual role of absorbing waste and supplying water

for human consumption (Gutteridge *et al.*, 1976; Cullen and Rosich, 1980). Effluents may affect water quality directly through the introduction of odoriferous compounds including hydrogen sulfide, ammonia, mercaptans and organic acids (Lin, 1976, Parts 1 and 2), or indirectly by supplying nutrients that encourage the growth of aquatic organisms such as phytoplankton, bacteria and plants. These in turn are able to produce off-flavour compounds through decomposition and metabolic by-products.

The widespread practice of disinfecting water prior to reticulation to control bacterial activity often leads to taste and odour complaints (Muntisov, 1991). The oxidation of dissolved organic matter by chlorine-based compounds and ozone can result in the synthesis of nitriles, trihalomethanes and substituted benzenes which are easily detected by human senses (Kerslake, 1989). This is in addition to any traces of disinfectant remaining in the water, for example chlorine, which adds to the overall odour.

Natural processes leading to sensory problems can be of biological and geological origin. Depending on local geology, high salt and mineral concentrations may impart distinctive characteristics to drinking water, which are usually associated with taste rather than odour sensations. In some cases anaerobic bacteria are able to convert chemical species into problematic compounds with sulphur-reducing bacteria producing hydrogen sulphide being a well-known example (Lin, 1976 (Part 1)). Similar processes are of particular significance in Perth, Western Australia, where bacterial activity in ground-water results in the synthesis of problematic levels of dimethyl trisulphide (Wajon, 1988; Wajon *et al.*, 1986).

An additional biological source of tastes and odours is the decomposition of organic matter. Bacterial decomposition of cell constituents results in

the synthesis of several specific compounds with characteristic pungent or rotting odours. These include butanol, butanoic and hexanoic acids, and ester derivatives of these acids (Yasuhara and Fuwa, 1982). Furthermore, the oxidation of these cell constituents during water treatment may lead to the synthesis of additional odour-causing compounds. This is particularly a problem in eutrophic waters supporting high levels of plant biomass, especially phytoplankton. Under favourable conditions of light, temperature and nutrients, algal cells rapidly divide forming dense populations or "blooms". These populations are sustained until a limiting resource or unfavourable conditions cause the bloom to collapse, releasing high organic loads into the water as a result of cell lysis.

Tastes and odours can also result directly from algal populations prior to bloom formation, senescence and decomposition. Palmer (1962) was the first to correlate particular off-flavours to different algal populations, and was able to show that different algal groups were able to impart distinctive off-flavours. For example, grassy/musty odours were often associated with cyanobacteria (blue-green algae), and diatoms commonly resulted in fishy odours.

Advances in the chemical analysis of volatile organic compounds has enabled a number of other specific odour-causing compounds to be identified in natural waters (Juttner, 1983, 1984; Juttner *et al.*, 1986; Hayes and Burch, 1989). Furthermore, the analysis of algal mono-cultures derived from field populations has allowed the synthesis of certain compounds to be attributed to particular types of algae (Mohren and Juttner, 1983; Slater and Blok, 1983) and other aquatic microbes, in particular actinomycetes, a group of gram positive aquatic bacteria (Wood *et al.*, 1983; Kikuchi *et al.*, 1983; Gerber, 1979, 1983).

Early investigations into tastes and odours in water supplies were focused on bacterial rather than algal populations (Gerber, 1979). Actinomycetes were often associated with earthy/musty odours in both the United States and Europe. Attempts at identifying the odour causing compounds in odoriferous strains of *Streptomyces* using gas chromatography and sensory testing eventually led Gerber and Lechevalier (1965) to the discovery of geosmin. Gerber (1969) isolated a second compound producing earthy/musty odours, identified as 2-methylisoborneol (MIB).

Subsequent studies found that cyanobacteria were also able to produce geosmin (Safferman *et al.*, 1967) and MIB (Tabachek and Yurowski, 1976), as well as certain strains of fungi (Lechevalier, 1974; Kikuchi *et al.*, 1983). Since then it has been realised that earthy/musty tastes and odours resulting from the algal production of geosmin and MIB has been more problematic to water authorities than any other odour (Mallevialle and Suffet, 1987). In addition to their undesirable sensory properties, a strong focus on geosmin and MIB has developed because of the technical difficulty in their removal, and the potential for increasing occurrence.

Both geosmin and MIB have an easily distinguishable earthy/musty odour which gives water a dirty or unclean characteristic when swallowed. The odour threshold concentration (OTC: the concentration detected by 50% of the population) of geosmin and MIB are extremely low, in the order of 10 to 20 ng L^{-1} i.e. parts per trillion (ppt). In comparison, compounds produced in response to water disinfection and many industrial solvents and petrochemicals have OTCs measured in the range of micro to milligrams per litre (Mallevialle and Suffet, 1987).

Geosmin and MIB are monoterpene and sesquiterpene compounds respectively, which are synthesised by the isoprenoid metabolic pathway in cells (Bentley

and Meganathan, 1981). As they are both tertiary alcohols (Gerber, 1983), they are chemically very stable, and are not easily destroyed during conventional water treatment using chlorine, chlorine dioxide and ozone as oxidants (Lalezary *et al.*, 1986). Consequently, the removal of these compounds requires additional treatments including activated carbon and biological filters (McGuire and Gaston, 1988; Ando *et al.*, 1992; Egashira *et al.*, 1992), which require significant capital outlays and additional maintenance costs to water authorities.

Finally, there is the potential for increasing the geographical distribution and magnitude of problems resulting from geosmin and MIB. The increasing eutrophication of water resources has led to the proliferation of algal blooms. Coupled to this, the shift in algal dominance towards cyanobacteria (Steinberg and Hartmann, 1988; Reynolds, 1984), which are well documented producers of both geosmin and MIB, poses a greater threat to the quality of this resource. Although not all water bodies contain cyanobacteria capable of producing geosmin or MIB, the potential to support odour-producing strains still exists should they be introduced into these systems. This may be achieved via dispersal by birds, wind or recreational activities.

Genera of cyanobacteria which contain species known to produce geosmin or MIB in culture are summarised in Table 1 (a complete listing of species of cyanobacteria that produce geosmin and MIB is included in Appendix A). These taxonomic groupings were based on the classification of the Cyanophyta presented in Prescott (1954). This compilation not only identifies confirmed odour-producing genera, but may assist in identifying other problematic cyanobacteria. The majority of odour producing algae belong to the family Oscillatoriaceae, however, representatives are also found in the Nostocaceae, Stigonemataceae, and Chroococcaceae.

Order	Family	Genus		geosmin	MIB	toxins
Oscillatoriales	Oscillatoriaceae	Arthrospira				
		Borzia				
		Dasygloea				
		Hydrocoleum				
		Lyngbya		■	■	■
		Microcoleus				
		Oscillatoria		■	■	■
		Phormidium		■	■	
		Porphyrosiphon				
		Romeria				
		Schizothrix		■		
		Spirulina		■		
		Symploca		■		
		Trichodesmium				
Nostocales	Nostocaceae	Anabaena		■		■
		Anabaenopsis		■		
		Aphanizomenon		■		■
		Aulosira				
		Cylindrospermum				
		Nodularia				
		Nostoc				■
		Wollea				
	Stigonemataceae	Albrightia				
		Capsosira				
		Fischerella		■		
		Hapalosiphon	Stigonema			
		Nostochopsis	Thalpophila			
	Rivulareaceae	Amphithrix	Rivularia			
		Calothrix	Sacconema			
		Dichothrix	Scytonematopsis			
		Gloeotrichia				
	Scytonemataceae	Desmonema	Plectonema			
		Diplocolon	Scytonema			
		Fremyella	Tolypothrix			
		Microchaete				
	Hammatoideaceae	Hammatoidea	Raphidiopsis			
	Loriellaceae	Colteronema				
Chroococcales	Chroococcaceae	Microcystis			■	■
		Synechococcus				
		Aphanothece	Gloeochaete			
		Bacillosiphon	Gloeothece			
		Chondrocystis	Gomphosphaeria			
		Chroococcus	Holopedium			
		Coelosphaerium	Marssoniella			
		Dactylococcopsis	Merismopedium			
		Eucapsis	Rhabdoderma			
		Glaucocystis	Synechocystis			
		Gleoecapsa	Tetrepedia			
	Entophysalidaceae	Chlorogloea	Heterohormogonium			
		Entophysalis				
Chamaesiphonales	Chamaesiphonaceae	Chamaesiphon				
	Pleurocapsaceae	Hyella	Pleurocapsa			
		Myxosarcina	Xenococcus			
	Dermocarpaceae	Dermocarpa	Stichosiphon			

Table 1. Summary of algal genera known to produce geosmin, MIB and toxins (see Appendix A for details)

Although this representation of odour-producing genera suggests to some extent a phylogenic interaction, an element of chance is likely to affect the current status of this compilation. That is, species and genera that are not commonly problematic are less likely to be noticed and investigated, unlike the problematic species of *Anabaena* and *Oscillatoria* (*Planktothrix*) that received much attention. Furthermore, the presence of known producers of geosmin and MIB in water blooms may divert attention away from other species of cyanobacteria that form a minor component of the biomass.

Table 1 shows that some genera contain species that are only able to produce either geosmin (*Anabaenopsis*, *Symploca*, *Schizothrix*, *Fischerella*, *Aphanizomenon* and *Anabaena*) or MIB (*Synechococcus*). However, the genera *Lyngbya*, *Oscillatoria* and *Phormidium*, all belonging to the Oscillatoriaceae, contain species that produce both compounds.

The production of geosmin and MIB by members of the same genus in turn is a function of both the species and strain of the algal isolates. For example, isolates of *Oscillatoria agardhii* from Finland (Persson, 1979), Norway (Berglind *et al.*, 1983a, 1983b) and Canada (Tabachek and Yurkowski, 1976) are only known to produce geosmin. However *O. curviceps* (Izaguirre *et al.*, 1982; Izaguirre *et al.*, 1983) and *O. geminata* (Tsuchiya *et al.*, 1981; Matsumoto and Tsuchiya, 1988) to date are only known to produce MIB. On the other hand, different strains of the same species of *O. brevis* (Naes *et al.*, 1985; Berglind *et al.*, 1983b) and *O. tenuis* (Izaguirre *et al.*, 1982; Tabachek and Yurkowski, 1976) are able to synthesise geosmin or MIB, and particular cultured strains of *O. brevis* (Berglind *et al.*, 1983b), *O. tenuis* (Wu and Juttner, 1988a), *Oscillatoria sp.* (Matsumoto and Tsuchiya, 1988) and *Phormidium sp.* (Izaguirre, 1992) can produce geosmin

and MIB simultaneously. These strain-specific differences in geosmin and MIB synthesis is similar to known variations in toxin production by cyanobacteria (Collins, 1978; Gentile and Maloney, 1969). It is interesting to note that with the exception of the family Stigonemataceae, all other families that contain geosmin or MIB-producing genera also contain genera that produce various forms of toxins (Table 1).

Species of cyanobacteria that are able to synthesise geosmin and MIB are not restricted to fresh waters. *Lyngbya*, *Oscillatoria* and *Symploca* all contain salt water species that can synthesise one or both compounds (Appendix A). Furthermore, odour producing algae include benthic types such as *Oscillatoria*, *Phormidium* and *Schizothrix*. In some respects, the production of geosmin or MIB by benthic algae are more problematic as their submerged nature makes monitoring and treatment more difficult (McGuire *et al.*, 1984).

The occurrence of cyanobacterial blooms in Australia is well known and widespread in reservoirs, rivers and farm dams (May, 1974, 1981; Croome, 1980; Alpin, 1983; Falconer *et al.*, 1983; Hayes and Burch, 1989). The ability of some blooms to form toxins has been demonstrated. Although reports of tastes and odours associated with blooms are known, at the commencement of this study there were no confirmed reports on the production of geosmin or MIB in Australia.

Townships along the Murrumbidgee River in NSW often experience taste and odour problems. Algae were thought to be the origin of these earthy/musty flavours since they usually coincided with algal blooms, and tended to occur between late spring and autumn. This period is characterised by higher light intensities and warmer waters, both conducive to algal growth.

The cyanobacterium *Anabaena circinalis* is often the main bloom forming species along this river system, and overseas it is known to produce geosmin (Silvey *et al.*, 1970). Four strains were isolated in 1985 from the Hay Weir Pool (near Hay, NSW) by Dr R.L. Oliver of the CSIRO, and were successfully grown as monocultures. These cultures produced a strong earthy/musty odour, similar to that described in previous years.

Chemical analysis (Dr W. Korth, CSIRO Division of Water Resources Griffith Laboratory) confirmed the presence of geosmin, but not MIB. Although these cultures were not axenic, microbiological tests at the Murray Darling Freshwater Research Centre (Albury, NSW) showed cultures to be free of actinomycetes (Dr P. Boon, pers. comm.). Consequently, the production of geosmin could be solely attributed to *A. circinalis*. This was the first case of geosmin detection in Australia directly linked to a specific organism.

The regular occurrence of cyanobacterial blooms in Australia and the difficulties in treating geosmin in water supplies is therefore of great concern to water authorities. In addition, the increased potential for the occurrence and distribution of geosmin-producing blooms due to eutrophication and perhaps the spread of odoriferous strains necessitates a means of managing outbreaks of blooms and geosmin-contaminated waters.

Algal blooms and taste and odour problems have already affected populations relying on inland river systems for potable water. Contamination of the domestic water supply with geosmin or other odour-causing compounds has resulted in consumer complaints to local water authorities, and has raised concern over the health implications of ingesting tainted water.

The generation of off-flavours from algal blooms is particularly problematic because although they tend to occur on a seasonal basis, individual episodes of off-flavours are sporadic and unpredictable. In addition, although several townships may draw water from the same river source, the large distances between them, as well as barriers such as weirs, establishes geographical and physical divisions within the river so that a particular problem affecting one town may not affect those nearby. This requires many discrete stretches of water to be monitored for algal blooms and off-flavours.

Because of the unpredictable timing of odour problems associated with algal blooms, water managers are unable to anticipate and prepare for periods when water quality will be a problem. This inability to quickly react is due to irregular (if any) monitoring for geosmin or other taste and odour compounds in raw or treated waters, as such monitoring requires non-standard analytical methods. These methods are based on either chemical or sensory (smelling/tasting) techniques. Chemical analysis is restricted to larger urban research institutions because of the need for large capital requirements, and qualified operational personnel. In addition, the amount of time required for each analysis (2-3 hr per sample) confines such methods to non-routine applications.

Sensory assessment on the other hand, although more accessible to smaller water authorities, is either based on qualitative or comparative tests, or semi-quantitative tests that require initial and on-going training, and hence the availability of experienced instructors. This does not make current forms of sensory analysis an attractive alternative to most water managers.

Nevertheless, the advantages of sensory analysis over chemical methods are: low implementation costs; availability to a larger number of water managers; and ability to process samples rapidly. In addition, it is ultimately how individuals perceive any odours present that will determine the quality of the water, rather than a precise and detailed chemical analysis.

One aim of this study is to develop and test an alternative sensory protocol for measuring water odour. This method should be available to smaller or regional water managers at little cost and with minimal training, leading to the rapid assessment of water sensory quality on a routine and frequent basis. This method could also be used to construct records of water quality over time, and be applied as a research tool in investigating algal blooms and taste and odour problems over large areas.

In addition to the need for detecting and measuring the early occurrence of tastes and odours, the management of the water resource requires knowledge of the processes leading to geosmin production. Environmental factors that affect the synthesis of geosmin are an essential part in understanding geosmin formation by algal blooms. Interactions between various factors and algal cells that may stimulate or prevent geosmin synthesis not only presents opportunities in managing the water resource to minimise or prevent geosmin production, but may allow managers to anticipate problematic periods and plan accordingly. Furthermore, this may have important implications in the handling and storage of raw water which may encourage the release of geosmin.

The second aim of this study is to determine some of the factors that affect the synthesis of geosmin by *A. circinalis*, a common bloom-forming cyanobacterium found in Australian waters. Although the distribution of

A. circinalis is widespread, little is known of external factors that affect its ability to produce geosmin. That is, it is not known whether the synthesis of geosmin is a continuous process, or whether it is stimulated or suppressed under different environmental conditions or is dependent upon the stage of population growth. Furthermore, whether geosmin is cell bound or actively excreted is also undetermined. This aim will be achieved using a locally isolated strain of *A. circinalis* (852E)in controlled laboratory culture experiments to eliminate the many variables associated with field trials.

1.2 Objectives

In summary, the specific aims of this thesis are to:

1. Develop an alternative sensory protocol for estimating geosmin concentration in water, which can be used by water authorities to monitor water quality on a routine basis.

2. Investigate factors that affect the synthesis of geosmin by a strain of *A. circinalis* (852E) isolated from the Murrumbidgee River.

Chapter 2

Materials and Methods

2.1 Chlorophyll *a* Analysis

Depending on the density of algal cultures, 15-500 mL were concentrated onto 4.5 cm Whatman GF/C filters, homogenised in 90% acetone, and placed in the dark at 4°C for 12 hr. After extraction, samples were centrifuged at 1000 rpm for 20 minutes to attain optical purity. Chlorophyll *a* was measured as the optical density (O.D.) at 665 nm corrected for background absorption and scattering effects at 750 nm. Acetone extracts were measured in 1 cm quartz cuvettes using a Jasco UVIDEC-650 spectrophotometer. O.D. values were converted to chlorophyll *a* using the equation derived by Talling and Driver (1963).

2.2 Algal Dry Weights

A known volume of culture was concentrated onto a washed (200 mL MQ-water), dried (105°C for 24 hr) and pre-weighed (Mettler H54AR balance) 2.5 cm Whatman GF/C filter. Filtration funnels were rinsed with MQ-water to remove salts contained in W.C. Media (Guillard and Lorenzen, 1972) from the filter. Filters were dried at 105°C for 24 hr, cooled and then weighed.

2.3 Algal Biomass as Optical Density

A 3 mL culture sample was gently homogenised in a hand held glass/teflon homogeniser to break up large clumps into smaller fragments of uniform size. The O.D. of the suspension was measured at 438 nm (Stein, 1979) in a 1 cm cuvette using a Jasco UVIDEC-650 spectrophotometer. Dense suspensions were diluted two to four-fold and re-measured to ensure the sample was in the linear range of the O.D. versus concentration relationship.

2.4 Algal Biomass as Cell Density

30 mL of culture was sub-sampled and immediately preserved with Lugol's iodine. Five determinations (replicates) of cell density was made for each sub-sample by sedimenting separate 0.2 to 0.5 mL aliquots into 1 mL chambers and counting all cells individually over the entire bottom using an inverted microscope (x 400 magnification).

A target to count at least 1,000 cells per chamber was set. The first determination was used to calculate the aliquot volume required to ensure at least 1,000 cells were present in the remaining four replicates. Samples often needed to be diluted (10 to 200 fold) in culture media to avoid excessive counting. Cell counts were mostly in the range of 1,500 to 2,500 cells per chamber. Counts per chamber were adjusted for any sample dilution and aliquot volume and are presented as cells mL^{-1}.

2.5 Odour-free Water

Odour-free water (MQ-water) was directly obtained from a Millipore Milli-Q Reagent Water System, where distilled water was filtered through two ion exchange and two carbon cartridges.

2.6 Cleaning and Sterilisation of Glassware

Glassware was soaked overnight in detergent (Pyroneg), scrubbed and rinsed with warm tap water (5 times) and distilled water (3 times). They were acid-washed by either rinsing twice or soaking for 20-30 minutes with 15% HCl. Glassware was finally rinsed 5 times with distilled water, once with odour-free water, and dried in an oven (105°C, overnight).

Flasks and other items to be sterilised were stoppered with non-absorbent cotton wool or wrapped in aluminium foil, and were autoclaved at 121°C for 20-30 minutes.

2.7 Algal Culture Media

Algae were cultured in full-strength, modified W.C. Media (Guillard and Lorenzen, 1972). All compounds were dissolved in odour-free water. $FeCl_3$ was substituted with Fe-citrate and citric acid, and silica and buffers were omitted. All compounds except $(PO_4)^{3-}$ and $CaCl_2$ were combined, and the pH adjusted to between 7.2 and 7.5.

Flasks were stoppered with non-absorbent cotton wool, and the media autoclaved at 121°C for 40 minutes. On cooling, sterile solutions of

$(PO_4)^{3-}$ and $CaCl_2$ were added. Complete media was allowed to sit for 2 days before use to enable gases in solution to equilibrate with the air.

2.8 Culture Growth Environment

Cultures were grown in either a growth room or a growth cabinet, both with temperature maintained at 20°C. Light intensity (400-700 nm) could be varied, and was measured using a Li-Cor Quantum Meter (model LI-185). Cultures were kept at light intensities ranging from 70-100 $\mu M\ m^{-2}\ s^{-1}$ (PhAR). For details on the placement of the light sensor when measuring intensity, refer to the Methods section in the relevant chapter.

2.9 pH

100 mL or greater aliquots were measured using a Radiometer Copenhagen PHM 84 Research pH Meter calibrated in the range of 6.88 to 9.18 pH units. Samples were regularly swirled, and pH values recorded after the readout had stabilised.

2.10 Geosmin Analysis

Samples were taken immediately prior to analysis and stored in sealed glass flasks. Closed Loop Stripping Apparatus (CLSA) was used to concentrate geosmin and other volatile organic compounds from solution. A measured volume (200-500 mL) of sample was made up to 1 L using odour-free water in a short-form bottle, and a known volume (2-10 µL) of internal standard (1-chloro alkanes: 8, 10, 12, 14 carbons) was also added.

The sample was maintained at 25°C in a water bath, and was continuously bubbled (stripped) with air for 2 hr. Geosmin and other volatile organic compounds were removed from the air-stream using a 1.5 mg activated carbon filter, heated to 38°C to prevent condensation of water on it's surface. The airstream passing through the filter was returned to the bottle and recycled, hence forming a closed loop.

Organic compounds were eluted from the activated carbon filter using carbon disulphide (approx. 30 µL), resulting in a 33,000-fold increase in concentration from the initial 1 L sample (assuming complete recovery). The extracts were stored at -15°C in 100 µL glass micro-vial inserts placed in 2 mL glass vials sealed with teflon lined screw-cap lids.

Extracts were analysed by gas chromatography (Varian 3300) linked to a mass selective detector (Hewlett Packard 5970 Series MSD). One to five microlitre samples were injected into a Hewlett Packard fused silica capillary column (I.D. 0.2 mm) with a cross-linked methyl silicone film (0.33µm), and helium as the carrier gas (1 mL min^{-1} flow rate). The oven temperature was maintained at 30°C for 1 minute at the time of injection, and was programmed to increase to 80°C at 10°C min^{-1} and to 250°C at 5°C min^{-1}, where the final temperature was maintained for 5 minutes. The injection splitter was turned off for the first 1.5 minutes, then was switched on for the remainder of the run.

Geosmin concentration was determined using data collected by the MSD, and was based on single ion monitoring for specific molecular fragments of geosmin. These values were multiplied by a factor which was determined by stripping a series of geosmin solutions of known concentration (range 0-100,000 ng L^{-1}).

All analyses on the GC/MS system, including determination of running parameters, was conducted by Mr Wolfgang Korth.

Chapter 3

Sensory Analysis: Selection of Sensory Panel

3.1 Introduction

Sensory analysis is a method whereby the senses of taste and smell are used to measure the qualities of a product. One example is wine tasting, where taste and smell are used to indicate the quantities of sugars, alcohol, tannins and other compounds present. Other examples where sensory analysis is important include the brewing (Meilgaard *et al.*, 1982), food manufacturing (Meilgaard, 1988) and cosmetics industries, where it is used for quality control and product development.

Sensory analysis in these industries is essential because only through taste and smell can the acceptance of the product be ultimately gauged. Although chemical analysis can describe the variety of compounds present, it is unable to predict the final sensory properties of a sample.

Sensory analysis forms a vital role in the investigation of off-flavours in potable water. It is usually the first indication from consumers of water supply problems. Furthermore, it assists in identifying the sources of tainted water, focusing research in areas of need. Records of consumer complaints and of planned sensory monitoring allows recognition of seasonal or episodic trends, which in turn can be correlated to biological and chemical investigations.

There are many examples of such investigations in the literature, demonstrating the integration of sensory, chemical and biological analyses.

These include the identification of odour-causing organisms (Cyanobacteria and Actinomycetes) through field correlations with odour episodes, followed by confirmation using laboratory cultures (Krasner *et al.*, 1985; Negoro *et al.*, 1988; Yagi, 1988; and Means and McGuire, 1986). Furthermore, sensory and chemical analysis were used to identify sources of odour-causing compounds associated with aquaculture and fisheries (Martin and Suffet, 1992; Davis *et al.*, 1992; Persson, 1979,1980; Whitfield, 1988); water disinfection practices (Dietrich *et al.*, 1992; Thorell *et al.*, 1992); urban run-off (Hrudey *et al.*, 1988); and food packaging (Sato *et al.*, 1988).

Studies such as these led to the identification of geosmin (Gerber and Lechevalier, 1965) and methylisoborneol (MIB) (Gerber, 1969), two volatile organic compounds with characteristic earthy/musty odours. Further research showed that these compounds were produced by Actinomycetes, a type of filamentous bacteria, and several genera of Cyanobacteria (Safferman *et al.*, 1967; Tabachek and Yurowski, 1976; Gerber, 1979; Berglind *et al.*, 1983a, 1983b; Medsker *et al.*, 1968).

The widespread occurrence of geosmin and MIB led to further investigations on the sensory properties of these compounds, and methods of analysing tainted waters to determine their concentration. Sensory studies indicated that the sensitivity of individuals to both compounds could be as low as 4 ng L^{-1} (Krasner *et al.*, 1985; Persson, 1980). Chemical methods were developed to measure quantities of geosmin and MIB, however detection limits above that of the human nose led to a reliance on sensory analysis to measure low concentrations of these compounds. Although recent advances in chemical methods has resulted in sub-threshold detection limits, other disadvantages still remain (Korth *et al.*, 1991).

Firstly, concentrating geosmin from solution followed by quantification using gas chromatography/mass spectrometry requires 2-3 hr per sample (Korth *et al.*, 1991). This reduces the number of analyses to only a few per day, and does not allow rapid routine testing of water. A second disadvantage is the high cost associated with the necessary equipment, and the need for experienced operational personnel. This therefore confines such apparatus to larger water authorities and research institutions.

Sensory analysis overcomes many of the problems associated with chemical analysis: it is relatively inexpensive to implement; the sensory properties of samples can be determined rapidly; and sensory analysis only detects compounds that cause tastes and odours whose concentrations are high enough to be offensive.

The application of sensory analysis to monitoring water quality is therefore an attractive alternative to water managers, particularly in smaller or regional offices with very limited resources. It provides them with the opportunity to measure water quality before it enters the reticulation system, allowing time to divert tainted waters or adopt other corrective measures. In addition, sensory analysis can be used in conjunction with biological and chemical measurements to identify sources of odour causing compounds.

Although a large variety of sensory analyses are available from the literature, common to all is the need to establish a group of individuals that are responsible for assessing samples (Meilgaard, 1988). This group comprises the sensory panel.

One overall objective of this study is to establish a sensory panel to measure the levels of geosmin in water. This sensory panel forms the basis

of an alternative sensory protocol, which is presented in the following chapter. However, the selection of individuals that participate in sensory analysis is an important consideration in the success of the analysis.

It is important to select individuals that are best suited to sensory tests, and provide them with a suitable environment to optimise the overall performance of the sensory panel. This is necessary as individuals differ in their abilities to sense odours due to a number of factors which are related to their specific sensory physiology, attitude and surroundings.

3.1.1 Factors affecting the performance of sensory panels

The sensitivity of people to geosmin (and other compounds) varies greatly within a population. This ranges from those able to detect very low levels (< 4 ng L^{-1}) to others who do not respond at all. These variations within populations is one reason why the odour threshold concentration is defined on a level that is detectable by a proportion of the population, usually 50%. It is therefore preferable to select those individuals that are most sensitive to geosmin to enable the lowest concentrations to be detected and measured.

The inability to detect a specific compound, or anosmia, is effectively a form of "smell blindness" which affects a very small proportion of the population (Krasner *et al.*, 1985; Suffet *et al.*, 1988). Individuals that are anosmic to geosmin must therefore be identified and precluded from sensory assessment. However, all individuals can temporarily lose their ability to smell a compound as a result of nose fatigue (Krasner *et al.*, 1985). This results from nerve receptors within the nose becoming saturated with the stimulating compound, leading to an inability to detect it for

several minutes. Geosmin, MIB, and other musty-smelling compounds are particularly prone to causing nose fatigue. This reaction is an important consideration in sensory assessment, where repeated sampling of solutions can easily lead to a rapid loss of sensitivity, and therefore judgement between samples. Measures should therefore be adopted to minimise the likelihood of this event.

Another response which varies greatly within a population is the ability to distinguish between odoriferous solutions of differing strengths. Concentrations of solutions differing by 15-30% are indistinguishable by most people, although some individuals are able to resolve solutions differing by less than 5% (Mallevialle and Suffet (1987) citing; Zoetman (1980) and Cain (1977).

The resolving capabilities of individuals is also a function of the overall intensity of odour. That is, the perceived odour intensity difference between two samples at low concentrations is less difficult to discern than two high intensity samples differing by the same proportion. This results from a semi-logarithmic relationship between perceived odour intensity and concentration, known as the Weber-Fechner Law (Zoeteman, 1980). This relationship was demonstrated for MIB (Krasner *et al.*, 1985).

The resolving capability of a panellist is an important individual characteristic for selection. This will affect the consistency with which unknown samples are rated against standards, and therefore the variability of their response.

An overall important factor affecting sensory performance is the willingness of people to participate. Individuals contented to take part will generally put in a greater effort to reach a firm result. As this

commitment can vary with pressures from work, home, tiredness and illness, attention should be paid to selected individuals to ensure their continued participation.

Finally, other surrounding odours may affect the sensory response of individuals, as they compete for nose receptor sites. Although additional odours from water samples cannot be avoided, odours originating from people (sweat, soap, cosmetics) and from the surrounding space can be minimised.

These factors that affect the odour detecting capabilities of individuals will therefore also affect the performance of the sensory panel used to measure geosmin. Consequently, the selection of individuals is an important consideration in establishing such a panel.

3.2 Objectives

The objective of this chapter is to describe the selection process to ensure the participation of the best individuals in a sensory panel. This panel will be used to test a proposed sensory protocol for determining geosmin concentration in water samples, described in the next chapter. Therefore the aims of this chapter are to:

1. Survey a group of individuals for their sensitivity to geosmin solutions.

2. Analyse the performance of these individuals on the basis of sensitivity and consistency.

3. Select the best performing individuals to form a sensory panel.

3.3 Methods

3.3.1 Selection of individuals for proposed sensory analysis

The selection of individuals to take part in sensory analysis was based on their individual performance in assessing a series of geosmin solutions of varying concentrations. Therefore individual odour threshold concentrations (a measure of sensitivity) and consistency could be determined. As the sensory panel was to be based in Griffith, 29 individuals at the Griffith Laboratory were surveyed for their responses to geosmin. The performance of individuals from this survey was analysed to determine which persons would make up the sensory panel.

In addition to the Griffith sample, another 47 individuals were surveyed as part of a general study in Albury, NSW, at the Murray Darling Freshwater Research Centre. Due to the distance between these two centres, Albury individuals were not considered for inclusion in the sensory panel. Nevertheless, the results obtained are presented and compared to the responses of individuals from the Griffith Laboratory.

As a different methodology was used for the Albury survey, both trials will be described separately below.

Griffith Selection Trial

A total of 11 geosmin standards were prepared representing concentrations of 2, 4, 8, 14, 20, 30, 40, 60, 90, 120, and 160 ng L^{-1}. This range includes geosmin levels that are well above and below reported threshold concentrations of approximately 10 ng L^{-1} (Persson, 1980). In addition, 4 water blanks (A,B,C and D) were included as a check against participants pre-empting results, taking to 15 the number of test samples. Table 2 shows the order in which solutions were presented and assessed, and the placement of water blanks. All samples were prepared immediately before use.

Geosmin standards and blanks were presented at room temperature in 250 mL acid washed (5% HCl) and oven dried (overnight at 105°C) Erlenmeyer flasks. These were filled to a volume of 150 mL, and stoppered using inverted 100 mL beakers to prevent loss of geosmin from solution and contamination by nearby samples. Flasks were sequentially marked as shown in Table 2 with adhesive backed labels with writing in either pencil or pen - marker pens were avoided as some individuals in previous trials could detect the carrier solvent after several hours. All preparatory work was conducted in a separate room from the test area to avoid contamination of the surrounding air. Sensory evaluation was conducted in a fume-hood free of any chemicals and containers, and was pre-washed by sponging with water and alcohol.

Participants were tested on an individual basis. Prior to commencing the test, they were familiarised with geosmin odour by comparing a contaminated ampoule to a water blank. Repeated sampling was allowed until they were satisfied they could recognise the earthy/musty odour of geosmin. This is important as odour recognition is a function of both individual sensitivity and familiarity with the odour (Bartels *et al.*, 1987).

	Geosmin concentration (ng L^{-1})														
ID	A	2	4	8	B	14	20	C	30	40	60	D	90	120	160
AC			+						+	+	+		+	+	+
AH				+					+	+	+		+	+	+
CP			+			+			+	+	+	+	+	+	+
DE										+	+		+	+	+
FC		+	+		+		+	+		+	+				+
Gh			+						+	+					+
GH				+	+	+	+	+	+	+	+		+		+
GM			+		+		+		+	+	+		+	+	+
gH			+					+	+	+	+	+	+	+	+
HB			+	+	+					+					+
KR	+	+	+				+		+	+		+	+	+	+
KW						+	+	+	+	+	+		+	+	+
LS					+				+	+	+		+	+	+
Ls	+	+		+	+		+		+	+	+			+	+
MA			+		+				+	+	+		+	+	+
MK			+		+	+	+				+		+	+	+
NJ					+				+	+	+				+
NP			+	+	+	+	+	+	+	+	+	+	+	+	+
NS				+			+		+		+		+	+	
PB				+		+	+		+	+	+		+	+	+
PO	+		+			+						+	+	+	+
PP					+	+		+	+	+	+	+	+	+	+
PW							+		+	+	+		+	+	+
RF						+	+		+	+	+		+	+	+
RG	+	+		+	+	+		+	+	+	+		+	+	
RS	+	+		+	+	+	+	+	+	+	+	+	+	+	+
SC				+		+			+	+	+		+		
SO									+	+	+			+	+
WK										+	+		+	+	
Sum	5	5	12	10	13	12	13	8	23	26	25	7	23	23	25
%	17	17	41	34	45	41	45	28	79	90	86	24	79	79	86

Table 2. Summary of the sensory evaluation of geosmin solutions by individuals (identified by initials) from the Griffith Laboratory. Samples were presented in ascending order with water blanks A, B, C and D placed as shown.

Participants were required to wear disposable plastic gloves to reduce interference of sweat, soap, cosmetics, dirt etc. from their hands, and to prevent contamination of the outside of flasks for subsequent individuals. Panellists were instructed to grasp flasks near the bottom to keep any potential source of interfering odour (including sleeves, wrists and gloves) from their nose.

Panellists assessed solutions in the order presented by swirling the contents five or six times to saturate the air space within the flask, then quickly removing the cover and sniffing the contents. Repeated sampling of each solution was allowed, however, panellists were warned that nose fatigue was likely to occur where they may not be able to detect geosmin on second or successive attempts after having noticed it during the first attempt (Krasner *et al.*, 1985; Suffet *et al.*, 1988). If an individual suspected that was the case, they were instructed to rest for several minutes and smell water blanks before re-commencing.

All results were recorded on separate score sheets as either a "+" if they could detect geosmin, or a "-" for no odour recognition.

Albury Survey

The Albury survey differed from the Griffith selection trial in two respects. Firstly, based on the outcome of the Griffith survey, less samples covering a smaller range of concentrations were tested. Participants were presented with 6 geosmin solutions at room temperature at the following concentrations: 0, 4, 8, 14, 20, and 30 ng L^{-1}.

Secondly, disposable plastic cups and lids were substituted for glass flasks and beakers. Feedback from a number of participants of the Griffith trial indicated interference from the chalky/dusty odour of glassware to that of geosmin. This tended to confuse some panellists, and alternative vessels were investigated.

A variety of disposable paper and plastic cups were obtained and tested for background odour using the two most sensitive panellists from the Griffith trial. On this basis, plastic 200 mL (Lily-pack brand) polystyrene cups were chosen as they were considered to be virtually odour-free. Paper containers were not considered suitable as the waxed inside surfaces had a distinct chalky odour.

All plastic cups were filled to a constant volume of 100 mL, and were covered with clear plastic disposable petri dishes. As the Albury trial ran for an entire day, all solutions were replaced after 4 hours. All other procedures were as described for the Griffith survey.

3.4 Results

3.4.1 Griffith and Albury geosmin sensitivity surveys

The responses of individuals surveyed at the Griffith Laboratory for their sensitivity to geosmin are summarised in Table 2 and Fig. 3.1. All participants were able to detect geosmin in at least one of the geosmin solutions, and the proportion of individuals recognising geosmin increased with concentration. A maximum of 90% of those surveyed could detect geosmin at 40 ng L^{-1}, however, this proportion decreased slightly to between 80% and 90% at higher concentrations.

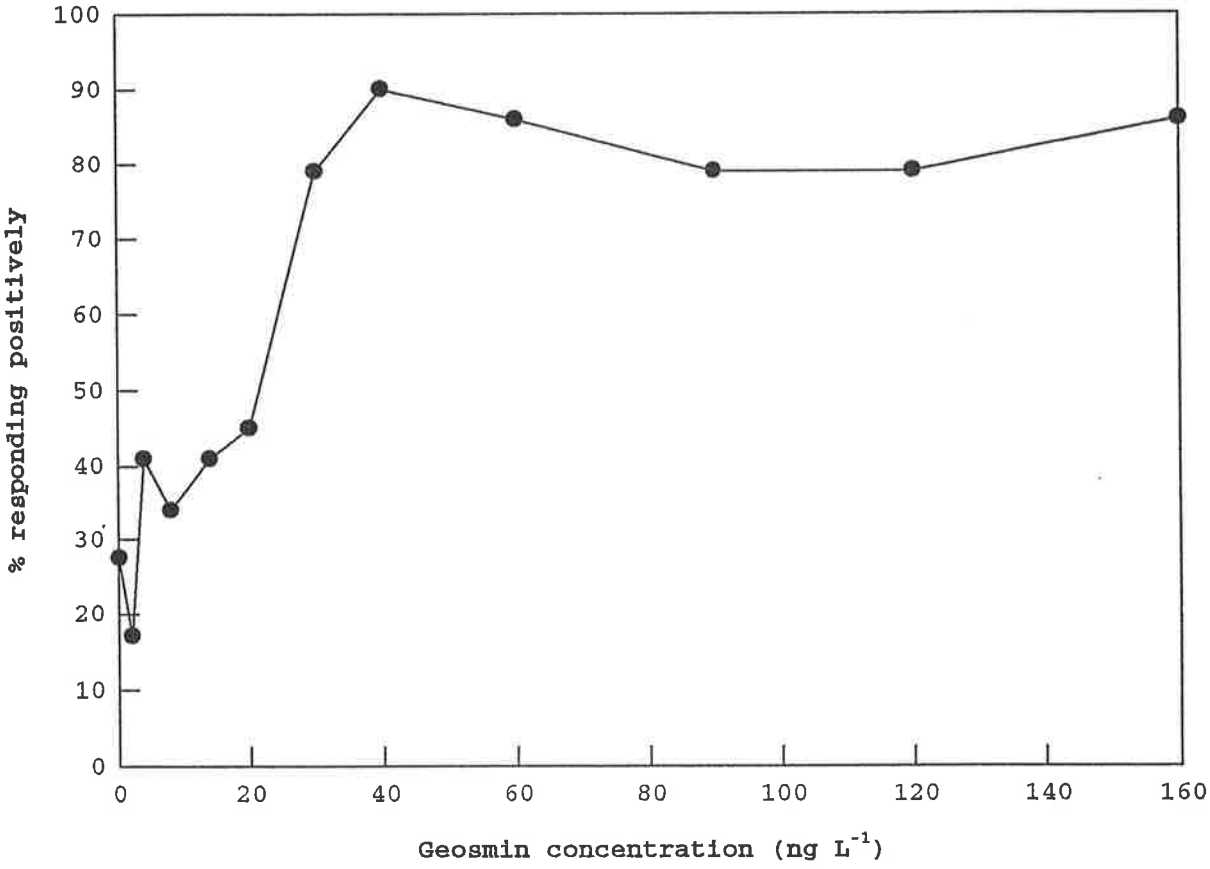

Fig. 3.1. Response of individuals from Griffith Laboratory to geosmin solutions.

The odour threshold concentration for geosmin (i.e. the 50% response) was approximately 20 ng L^{-1}, with the majority (79%) responding to 30 ng L^{-1}. At 2 ng L^{-1}, 17% of individuals indicated they could smell geosmin, as did an average of 28% for the water blanks.

A significant proportion of panellists recorded positive responses to all four water blanks (samples A to D) presented in the series. This was particularly the case for water blank B where 45% of panellists thought that geosmin was present. However, the proportion of positive responses almost halved to 28% and 24% for samples C and D, respectively (Table 2).

Corresponding to the high response for blank B, the data collected in the 2 - 20 ng L^{-1} range showed individual responses were more sporadic and less consistent than at higher concentrations. That is, in the majority of cases, a positive response to a particular concentration was not followed by another expected positive response for the next immediately higher concentration. This was far less evident for responses collected for solutions in the range 30 - 160 ng L^{-1} (Table 2).

The results of the Albury survey, covering a smaller number and range of concentrations, are shown in Fig. 3.2. The proportion of individuals responding positively to the presence of geosmin was overall similar to that of the Griffith survey in the range examined. The majority of individuals (nearly 80%) could sense geosmin at 30 ng L^{-1}, with 50% of the survey group responding to a concentration of 18 ng L^{-1}.

However, unlike in the Griffith survey, a much lower proportion of individuals responded positively to the lower concentrations. Only 5%

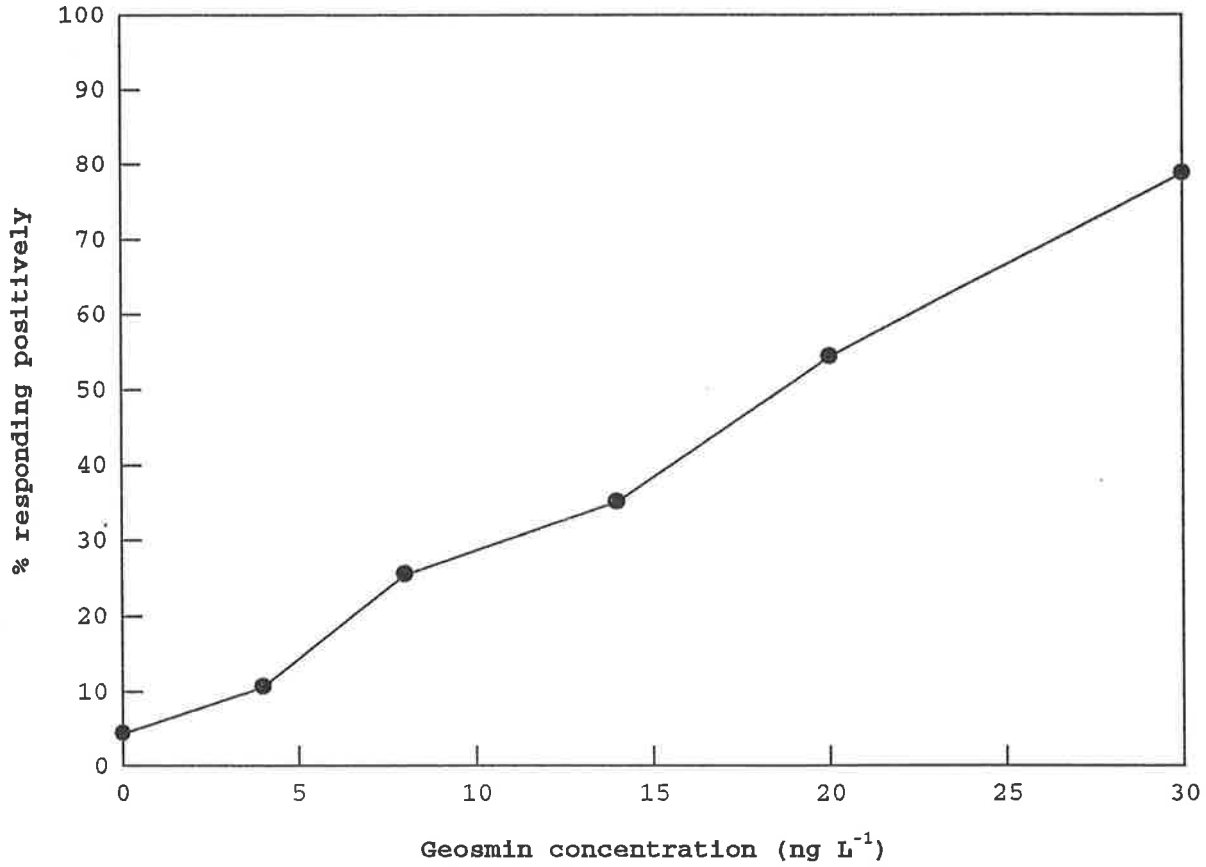

Fig. 3.2. Response of individuals from Albury survey to geosmin solutions.

registered a reaction to the water blank, and 11% (as opposed to 41% in the Griffith trial) responded positively to the 4 ng L^{-1} solution.

3.4.2 Selection of panellists

The data presented in Table 2 were used as the basis for the selection of individuals to take further part in sensory trials. Large variations in scoring consistency and sensitivity to geosmin amongst individuals is evident.

The odour threshold concentration (OTC) of most individuals was difficult to measure as responses along the series of solutions were not consistent. Only 4 panellists (DE, PB, PW, RF) recorded perfectly consistent scores i.e. they correctly identified all water blanks and detected geosmin in all solutions above their respective OTCs. The sensitivities of these individuals varied markedly, with values of 8, 14, 20 and 40 ng L^{-1} for PB, RF, PW and DE, respectively.

In the majority of cases, however, some discrepancies in individual responses were evident, with extremes ranging from most responses (including water blanks) scored as positive (e.g. RG, RS) to very irregular scoring along the series (e.g. FC, KR and PO). Other discrepancies included: positive responses for water blanks, despite all other scores being consistent (e.g. KW and NP); solitary scores assigned at low concentrations, while scoring consistently at higher concentrations (e.g. AC, GM and MA); and no responses at high concentrations despite consistent responses at lower levels (e.g. SO and WK). In many cases, individual discrepancies could be placed into more than one of the above categories, making performance evaluation difficult.

Those surveyed were nevertheless evaluated in their performance in assessing geosmin solutions. Two methods were used in this process: firstly, a descriptive grouping procedure which considers sensory assessment problems including nose fatigue and sensory interference of glassware; and secondly, an objective ranking method that rewards a correct evaluation (a "+") and penalises an incorrect evaluation of a water blank.

Descriptive Evaluation

Those surveyed were categorised into four general groups, reflecting varying degrees of consistency in the assessment of geosmin solutions and water blanks (Table 3). Group I included those individuals which either scored very consistently, or failed to recognise one standard at a higher concentration. The numbers in brackets indicate the relative sensitivities of those individuals to geosmin, with No. 1 being the most sensitive.

Group II includes those that were less consistent. Although their recognition of geosmin at higher concentrations (>30 ng L^{-1}) was accurate, they assigned a positive score for water blanks B or C, or their scoring was either isolated or scattered at lower concentrations.

Panellists that either scored positively for water blank D, or for which it was difficult to determine their threshold concentrations were placed in Group III. Finally, individuals whose responses were either very irregular or almost all positive are included in Group IV.

Descriptive Grouping	Individual	Objective Ranking
I (1)	PB	542
I (1)	RF	534
I (3)	PW	520
II	AH	508
II	AC	504
II	GM	490
II	MA	470
I (=5)	DE	470
II	LS	466
IV	MK	434
I (=4)	SO	410
III	KW	404
II	Ls	392
III	NS	328
I (=5)	WK	310
III	GH	258
II	NJ	256
II	SC	242
IV	Gh	234
III	RG	186
IV	HB	178
III	CP	148
IV	FC	122
III	KR	82
IV	NP	12
III	gH	4
IV	PO	4
IV	RS	-4
IV	PP	-20

Table 3. Sensory performance of individuals from the Griffith Laboratory using descriptive and objective ranking methods (Group I comprise the most consistent individuals, with No. 1 (in brackets) the most sensitive; rank 542 the most consistent individual).

Ranking method

This procedure rewards individuals for each geosmin solution detected, but penalises a positive scoring of a water blank. This allows the identification of those who detected the largest range of geosmin solutions, as well as those who were unclear of the odour characteristics of geosmin and were confused or unsure in the evaluation of solutions.

Ranking scores were calculated for each individual as follows:

1. For each geosmin solution correctly identified, a score is given whose value is equal to the concentration of that solution. The range of geosmin solutions used in this study therefore allows a maximum of 548 points to be assigned (2+4+8+...+160).

2. For each water blank scored as positive, points are subtracted whose value is the sum of all geosmin solutions immediately following the water blank up until the next water blank. Therefore, penalties for each water blank are as follows: A=14 (2+4+8); B=34 (14+20); C=130 (30+40+60); and D=370 (90+120+160). The total sum of these penalties equals the total number of points available for the correct evaluation of all solutions.

Increasing penalties for water blanks adjacent to higher geosmin solutions will ensure that less sensitive or less discerning individuals are identified.

Table 3 summarises the ranking of individuals and compares them to the categories assigned using the descriptive process.

3.5 Discussion

The ability of all 76 individuals assessed in the Griffith and Albury surveys to detect geosmin suggests that no individual was anosmic (smell-blind) to this compound. However, large variations in individual sensitivity is evident. This is seen by comparing the lowest concentrations detected by PB (8 ng L^{-1}) to DE (40 ng L^{-1}) in Table 2. In addition, the asymptotic increase in the proportion of those detecting geosmin at increasing levels indicates variations between individuals (Figs. 3.1 and 3.2).

The odour threshold concentration of geosmin measured by both the Griffith and Albury surveys (20 and 18 ng L^{-1} respectively) were well within the range of values reported in the literature (Persson, 1980). Although much lower estimates of OTCs are reported, these studies tended to use heated solutions to increase the volatility of compounds, enabling the detection of lower concentrations of geosmin.

The high proportion of Griffith panellists (Table 2) indicating the presence of an odour in the four water blanks was almost certainly due to interference of the ever-present chalky/dusty odour of the glass flasks used in this test. This odour was found to be very similar to the earthy/musty odour of geosmin itself. The use of plastic containers in the Albury trial resulted in a very low proportion of people assigning a positive score to the water blank. Although some panellists could sense a plastic odour associated with these cups, this odour was sufficiently distinct from geosmin to avoid any confusion.

At concentrations greater than 20 ng L^{-1} in the Griffith survey, levels of geosmin were sufficiently high to surpass the background odour of the

flasks, resulting in significantly less people assigning a positive score to blanks C and D, relative to the adjacent geosmin solutions (Table 2).

It is not clear why a higher proportion of panellists (45%) responded to blank B compared to the other water blanks (or indeed the 4 ng L^{-1} standard). One explanation may be that it is adjacent to geosmin solutions whose concentrations are close to the odour threshold levels. As individuals began to perceive an additional odour (i.e. geosmin) in the 14 and 20 ng L^{-1} solutions, they may have returned to previous samples to re-evaluate their responses (although solutions were presented in order of increasing concentration, individuals were free to return to previous samples).

The high value for blank B may therefore reflect unfamiliarity with the odour characteristics of geosmin and uncertainty in the assignment of their scores, compounded by the high background odour of the glass flasks. This also explains the almost random nature of responses to solutions 2, 4 and 8 ng L^{-1} seen in Table 2.

A similar response at low concentrations was not obtained in the Albury survey when containers with low background odours were used. These results show the importance of minimising the interference of extraneous odours which compete with geosmin and future sensory evaluations should utilise these relatively odourless plastic containers.

The inability of some panellists (Table 2) to detect high concentrations of geosmin, despite having successfully sensed lower levels, suggests they were affected by nose fatigue (Krasner *et al.*, 1985; Suffet *et al.*, 1988). Although it is a factor which should be avoided as it affects sensitivity

and consistency, it's presence is encouraging in that it suggests those surveyed were not pre-empting results.

It appears that the occurrence of nose fatigue affected the sensory performance of 9 individuals measured at the Griffith Laboratory, in the range of 60 to 160 ng L^{-1} (Table 2). When this is considered in the examination of individual performances, it is evident that an additional 2 individuals (SO, WK) gained perfectly consistent outcomes, which have also been included in Group I (Table 3).

The use of an objective ranking method to assess the performance of individuals resulted in a similar evaluation of those surveyed (Table 3). The majority of those with the highest scores were included in Groups I and II. Similarly, the majority of individuals assigned to Groups III and IV were those that were ranked the lowest.

Some discrepancies between the two methods are evident for ranked individuals lying in the middle orders. For example WK, placed in Group I using the descriptive method, was ranked lower than MK (Group IV). This anomaly is due in part to some individuals attaining a lower rank score by having assigned a negative score to a high geosmin concentration. For example, WK did not register the 160 ng L^{-1} solution; if this solution was scored as positive, WK would have performed better than MK in the ranking scores.

The discrepancies between the descriptive and objective ranking procedure is a result of the latter not considering factors that affect sensory performance such as nose fatigue and the interference of background odours caused by glassware. These factors were considered in the descriptive classification of individuals.

The assessment procedure of individuals can, however, be simplified in future trials by eliminating these sources of interference. Background odours can be minimised by using plastic disposable containers instead of glassware, and ensuring an odour-free environment in which to assess samples.

Nose fatigue may be minimised by reducing the number and concentrations of geosmin solutions presented. Most variability between individuals occurred at concentrations lower than 40 ng L^{-1} (Fig. 3.1). Solutions up to and including 60 ng L^{-1} should therefore be retained, in addition to a solution of 100 ng L^{-1}. The 90, 120 and 160 ng L^{-1} solutions can be omitted.

A reduction in the number and concentration of geosmin solutions, the removal of factors that interfere with odour assessment (nose fatigue, glassware odours) and the use of an objective ranking system to classify individual performance will simplify the process used for selecting the best individuals to take part on sensory panels. Section 4.6.1 summarises a modified procedure for the screening and selection of individuals.

The selection of the best panellists for sensory assessment was based primarily upon scoring consistency. This was considered overall the most important factor in classifying panellists, as it suggests that individuals understood the sensory characteristics of geosmin. This consequently enabled them to distinguish geosmin from other competing odours, such as the chalky aroma of glass.

Consistency in performance was also preferred when considering the minimal amount of training and exposure to geosmin individuals were subjected to in this laboratory. Bartels *et al.* (1987) noted the importance of familiarity

with the target odour. They found that with training and practice, individuals were able to fine tune their senses and distinguish lower amounts of geosmin, down to their individual threshold levels.

Although some individuals were very sensitive to geosmin and could detect it at low concentrations, the majority of people in Groups I and II had an OTC averaging 30 ng L^{-1} (Table 3). These thresholds are high compared to individuals participating in other sensory panels noted in the literature (Krasner *et al.*, 1985). This again is thought to reflect the inexperience of our sensory panel, in addition to solutions used in this study being presented at room temperature.

As individuals gain more experience from further participation in sensory assessment, their sensitivity to geosmin and discriminatory capabilities are expected to improve (Bartels *et al.*, 1987). Consequently, the OTCs measured for these individuals are expected to represent their minimum capabilities.

The classification of individuals surveyed at the Griffith Laboratory summarised in Table 3 forms the basis of selecting people to take part in future sensory panels. Individuals in Groups I and II are preferred on the basis of consistency and sensitivity, forming a pool of 14 individuals to choose from. These two groups represent 50% of all individuals originally surveyed in Griffith. However, individuals in Groups III and IV may still be used, as they are all able to detect geosmin, albeit to varying degrees.

Similarly, the ranking of individuals using a more objective method of evaluation will result in a similar but continuous grading that will assist in the selection of panellists when forming a sensory panel.

This outcome was used to select the best performing individuals to test a proposed sensory protocol that is discussed in the following chapter.

Chapter 4

Sensory Analysis: Proposed sensory protocol

4.1 Introduction

Although many types of sensory tests have been developed, one major disadvantage is that most methods are qualitative, and are suited to a specific set of samples (Meilgaard, 1988). This does not allow direct comparison of results between different samples assessed at different times.

One type of sensory test which overcomes these limitations are the Descriptive Tests (Meilgaard, 1988). These provide a description of the sensory attributes of the sample, as well as an estimate of their respective intensities i.e. they are semi-quantitative. One example of such a test refined in recent years is Flavour Profile Analysis (Bartels *et al.*, 1986). However, panellists are required to undergo thorough training to enable them to distinguish different aromas, and to attain a consistency in estimating perceived odour intensity over time. Regular training and use of all panellists is essential, as Brady *et al.* (1988) found that the estimated intensity of the same solution fluctuated greatly when assessed over 11 consecutive weeks by an untrained sensory panel.

The obvious need to train individuals, although feasible for a larger water authority, is not a viable proposition for a smaller regional office. Limitations include distance from a training centre, and lack of suitable guidance and/or supervision to carry out such analyses.

4.2 Objectives

The overall aim of this chapter is to develop a semi-quantitative sensory method to measure the concentration of geosmin in water. This method should allow geosmin levels to be estimated on a consistent basis, and therefore enables samples assessed at different times and places to be directly compared.

An important feature of this method is that individuals receive minimal training. Consequently this procedure will enable water managers that do not possess resources to extensively train personnel to adopt sensory assessment to measure geosmin.

The method adopted in this study was modelled on the Flavour Profile Method developed by Arthur Little and Co. (Cairncrass and Sjostrom, 1950), and later modified by Krasner *et al*. (1985). However, it differs in that panellists are provided with odour standards at all determinations. These standards serve to calibrate individuals intensity ratings by providing a stable bench-mark against which to compare unknown samples.

Geosmin was selected as the odour reference standard as a consequence of it's confirmed presence in this study area, and of reports of similar dominant odours in other inland waters (NSW Dept. Water Resources, pers. comm.). The unacceptable odour characteristics and low threshold concentration of geosmin, as well as the widespread occurrence of cyanobacterial blooms that are able to produce it (Hayes and Burch, 1989) makes geosmin an important compound to monitor.

Therefore the aims of this chapter are to:

1. Propose a sensory protocol to estimate geosmin concentration in water.

2. Test the performance of the proposed protocol using geosmin solutions, field and culture samples.

4.3 Methods

4.3.1 General principles

In the proposed sensory protocol, the geosmin concentration of an unknown sample was estimated by comparing it's odour intensity to that of four geosmin solutions of known concentrations. These standards, along with a water blank, were provided at each sitting. Panellists assigned the score of the standard whose odour intensity was most similar to that of the unknown. In that way, the intensity score could then be converted and expressed as geosmin concentration, rather than as a relative or indexed measure. Table 4 shows the concentrations of the standards used, and their respectively assigned scores.

Intensity scores rather than absolute concentrations were used to simplify the panellist's procedure as much as possible. A water blank was provided as a no-odour reference, and to minimise nose fatigue when sampled between standards and unknowns. A score of "-" was assigned if a panellist was certain no geosmin was present.

Intensity score	Geosmin (ng L^{-1})	Note
-	0	no odour detected
X	10	presence of odour unsure
1	21.5	definite odour detected
2	46.6	definite odour detected
3	100	definite odour detected

Table 4. Concentration of geosmin test standards presented to panellists at each assessment, and the intensity scores used to identify each test standard. The notes describes the scores panellists should assign to samples being evaluated. For example, intensity scores 1, 2 and 3 are assigned if a panellist is sure geosmin is present, which score that is assigned depending on which standard has an odour intensity closest to that of the sample being tested.

The lowest geosmin concentration (10 ng L^{-1}, intensity X) was chosen to represent the odour threshold concentration, corresponding to barely detectable levels. Although in the previous chapter the geosmin OTC of the Griffith and Albury panels was measured as 20 and 18 ng L^{-1}, respectively, a lower value of 10 ng L^{-1} was selected as 30% of individuals from the Albury panel were able to detect levels that were below 10 ng L^{-1} and as low as 4 ng L^{-1} (Fig. 3.2). A lower value of 10 ng L^{-1} as the OTC (intensity X) would allow these individuals to categorise lower concentrations of geosmin, which affect the more sensitive members of the general population.

The maximum concentration (100 ng L^{-1}, intensity 3) was selected to represent a strong odour intensity. Similar strength solutions of MIB are also described as having a strong odour by other sensory panels (Krasner *et al.*, 1985). In addition, this upper limit facilitates pre-dilutions of unknown samples prior to assessment. Raw water samples can contain geosmin levels up to three orders of magnitude beyond this range, and need to be diluted to fall within the limits of this test. An upper value of 100 ng L^{-1} allows a sufficiently broad calibration range to accommodate dilutions of raw samples without excessive trial and error.

The intermediate geosmin standards, corresponding to intensity scores of 1 and 2, were calculated based on the Weber-Fechner Law, which describes a semi-logarithmic relationship between odour intensity and concentration (Mallevialle and Suffet, 1987; Krasner *et al.*, 1985). Therefore concentrations were calculated on the basis of intensity scores increasing linearly (with score X taken as intensity zero) and geosmin concentration increasing logarithmically between 10 and 100 ng L^{-1}.

4.3.2 Procedure for proposed sensory protocol

Samples to be analysed were initially screened by two panellists to determine the number of dilutions required (if any) to bring the concentration of geosmin to within the range of the test standards. This is not intended to measure the concentration of geosmin, but to determine the number of dilutions required (if any) to prevent excessively strong solutions being presented to the main panel.

Unknown samples underwent 10 sequential three-fold dilutions. Each solution was stored in a plastic 200 mL cup filled to 100 mL, and was placed covered (plastic petri dish) in a fume hood. Two panellists were selected to assess each solution (from lowest to highest concentration) for the presence or absence of geosmin. The solution in which geosmin was first detected approximates the odour threshold concentration of 10 ng L^{-1} (score X). Consequently, a dilution three times less (i.e. a solution three times more concentrated) was expected to contain geosmin at 30 ng L^{-1}, corresponding to a score of between 1 and 2. This ensures the final concentration of geosmin in diluted unknown samples was positioned around the mid-point of the range of standards.

Samples to be analysed by the complete panel were diluted by the pre-determined amount immediately prior to testing, and were presented to panellists with standards corresponding to scores of X, 1, 2 and 3, along with a water blank. Standards were placed in an ordered row towards the back of the work area, with unknowns located towards the front. Preparation of fume hood, standards and handling of samples was as described in section 3.3.1.

The number of unknown samples presented to panellists ranged from 4 to 10. Panellists individually compared the odour intensity of each unknown to that of the standards. Repeat sampling of all solutions was allowed as many times as required, until panellists were confident with the score selected.

All individuals were reminded of the likely possibility of nose fatigue, and were encouraged to rest for at least several minutes and up to half an hour prior to recommencing, if unable to assign a score.

The problem of perceived odour intensity of an unknown sample falling between two standards was overcome by permitting half scores to be used. Consequently, this test allowed the following nine scores to be assigned: "-", X, ½, 1, 1½, 2, 2½, 3 and 3+. These additional scores were calculated to correspond to the following geosmin concentrations: ½, 14.7 ng L^{-1}; 1½, 31.6 ng L^{-1}; 2½, 68.1 ng L^{-1}; 3+, > 100 ng L^{-1}.

Panellists were encouraged to comment on any factor that may have influenced their decision including: other odours present; difficulty experienced in deciding a score; general tiredness; and if suffering from any slight illness. These results were recorded by panellists on separate score sheets, and were kept from other participants.

On the return of score sheets, immediate feedback was provided by divulging the origin of the samples assessed, how their assessment compared to those of other panellists, and if available, the actual geosmin concentration determined by chemical analysis.

4.3.3 Testing the proposed sensory protocol

The proposed sensory protocol was tested using individuals that were selected on their abilities to sense geosmin. This selection process is described in chapter 3, and individuals chosen to participate were selected from those listed in Table 3. On various occasions, a shortage of tested panellists resulted in the inclusion of other individuals that although not tested, demonstrated an ability to detect geosmin.

The performance of the sensory panel was assessed on five separate occasions over a 10 month period. Individuals taking part varied from panel to panel, as well as the number of participants, ranging from 5 to 9 per sitting.

Performance was evaluated by comparing geosmin concentrations estimated by sensory assessment to that determined by chemical analysis of the same sample. A total of 35 test solutions were analysed, including geosmin standards at various concentrations, cultures of *A. circinalis*, and river samples. Samples were obtained and identified as follows: A, B, C - *A. circinalis* laboratory cultures; M - *Anabaena* bloom in a pool alongside the Murrumbidgee River, Benerembah State Forest, NSW; T - Toonumbah Dam water following an algal bloom; Four Posts and Lawson (Lawson's siphon) - Edwards River, Deniliquin, NSW, following a bloom of *Anabaena*. These labels followed by a number indicates the number of times that sample was diluted to fall within the range of the test standards (e.g. A/100 indicates a 100 fold dilution of sample A).

4.4 Results

The results of five sensory panels conducted over a 10 month period are summarised in Tables 5 to 9. Samples presented to panellists as "unknowns" included geosmin solutions, algal cultures and field (river) samples.

These panels were comprised mainly of individuals previously tested for their abilities to sense geosmin solutions (see chapter 3). However, individuals who were not previously tested, but who demonstrated an ability to detect geosmin in solution, were included in all sensory panels.

Differences in the performance of tested and un-tested individuals was determined using the following procedure: each individual estimation was divided by the corresponding actual geosmin concentration to place all assessments on an equal par (geosmin Std 0 was omitted); a one-way non-parametric ANOVA by ranks (Kruskal-Wallis) was performed using individuals as different treatments, with the evaluation of geosmin solutions and field/culture unknowns tested separately. The outcome of this analysis is presented in Table 10, and clearly shows no statistical difference in the assessments of samples by tested and untested panellists at the 5% level.

Tables 5 to 9 nevertheless show that there is great variation in the response of different panellists to the same sample. For example, the assessment of sample E by Panel No.2 ranged from X to 2½ (Table 5). Wide ranges in responses are seen for most other samples assessed by all panels.

Individual performance also varies over time as shown by comparing the assessment of geosmin solutions by panellist GK in Panel Nos.2, 3 and 4. Despite this individuals good performance in the first two assessments, the presence of geosmin was not even noted when participating in Panel No.4.

Sample		Panellist									Geosmin conc. (ng L^{-1})	
ID	Content	GM	LS	AC	MA	KG	KS	PB	PW	GK	Perceived (mean)	Actual
A	Std ½	½	X	1	½	1	0	1½	½	½	15.9	14.7
B	Std 1½	1	1	1	1½	1½	1½	X	2	1½	27.5	31.6
C	Std X	1	½	X	1	½	X	X	X	X	13.6	10.0
D	C/1333	X	1½	2	3	2	2½	2½	2½	2	53.9	68.1
E	Std 1	½	1½	1½	2	2½	X	X	1	1	28.4	21.5
F	C/667	3	2½	3	3+	3+	3+	3+	3+	3+	96.5+	136.1
G	Std 2	0	2	2	2	2½	2	2½	1½	2½	46.8	46.4
H	Std 3	2	3	3	3	2½	1½	3	3	3	82.9	100.0
I	Std 2½	2½	2½	2½	X	2	1½	X	2½	2½	48.7	68.1

Table 5. Sensory Panel No.2. Nine panellists (GM..GK) assessed nine samples (A..I) comprised of geosmin standards and different dilutions of a culture of *A. circinalis* presented as unknowns. Perceived (mean) geosmin concentration was calculated by converting intensity scores to their corresponding geosmin concentration and averaging these values. This is compared to the samples Actual concentration as determined by chemical analysis. Panellist ranking (based on Table 3) as follows: Group I- PB, PW; Group II- GM, LS, AC, MA; Untested- KG, KS, GK).

Sample		Panellist					Geosmin conc. (ng L^{-1})	
ID	Content	KB	PB	GK	GM	LS	Perceived (mean)	Actual
A	A/4000	3	1½	1½	0	½	35.6	1.6
B	Std 0	0	0	0	½	0	2.9	0
C	B/4000	2	2	1	1½	½	32.1	24.0
D	A/2000	2	½	1½	1	1	27.1	3.2
E	Std 1	0	0	½	½	½	8.8	21.5
F	B/1000	1½	2½	2	2	1½	44.8	95.9
G	B/2000	3	3	2½	3	2	82.9	48.0
H	A/1000	1	1½	2	½	2½	36.5	6.4
I	Std 3	½	3	3	2½	3+	76.6	100.0
J	Std 2	1	1	2	1	3	42.2	46.4

Table 6. Sensory Panel No.3. Five panellists (KB..LS) assessed ten samples (A..J) comprised of geosmin standards and different dilutions of two *A. circinalis* cultures presented as unknowns. Perceived (mean) geosmin concentration was calculated by converting intensity scores to their corresponding geosmin concentration and averaging these values. This is compared to the samples Actual concentration as determined by chemical analysis. Panellist ranking (based on Table 3) as follows: Group I- PB; Group II- GM, LS; Untested- KB, GK).

Sample		Panellist						Geosmin conc. (ng L^{-1})	
ID	Content	DE	GK	LS	PB	PW	KB	Perceived (mean)	Actual
A	Std ½	½	0	½	0	0	0	4.9	14.7
B	F.Posts	2	1½	1½	3	½	1	41.0	78.0
C	Lawson	3	2½	1	1	½	1	41.2	27.0
D	Std 0	0	0	½	0	0	0	2.5	0
E	Std 1	2	0	1	2	0	1	22.6	21.5

Table 7. Sensory Panel No.4. Six panellists (DE..KB) assessed five samples (A..E) comprised of geosmin standards and two post-bloom field samples (Four Posts and Lawson) presented as unknowns. Perceived (mean) geosmin concentration was calculated by converting intensity scores to their corresponding geosmin concentration and averaging these values. This is compared to the samples Actual concentration as determined by chemical analysis. Panellist ranking (based on Table 3) as follows: Group I- DE, PB, PW; Group II- LS; Untested- GK, KB).

Sample		Panellist													Geosmin conc. (ng L^{-1})	
ID	Content	PW	LS	AC	PE	RS	WP	AH	SC	DE	WK	MA	NS	VN	Perceived (mean)	Actual
A	T/100	0	X	X	X	½	X	X	X	0	0	0	0	X	6.5	4.8
B	Std 1	½	½	1	1½	½	1	1½	1	½	X	1	½	1½	20.3	21.5
C	T/50	½	X	X	1	0	X	X	X	½	½	X	X	1	12.1	9.5
D	Std 2	1	1	2	½	1	2	2	1½	1	1	3+	3	2	41.5	46.4
E	T/25	1½	½	0	X	0	X	1	X	½	1	1	1	X	14.4	19.0
F	T/10	2	1½	1	2	0	X	2	2	2	2	2	2½	2	38.6	47.5
G	Std 3	3	1	2	3	1	2	2½	1	3	2½	2	2	2½	58.0	100.0

Table 8. Sensory Panel No.5. Thirteen panellists (PW..VN) assessed seven samples (A..G) comprised of geosmin standards and different dilutions of a post-bloom field sample (Toonumbah Dam) presented as unknowns. Perceived (mean) geosmin concentration was calculated by converting intensity scores to their corresponding geosmin concentration and averaging these values. This is compared to the samples Actual concentration as determined by chemical analysis. Panellist ranking (based on Table 3) as follows: Group I- PW, DE, WK; Group II- LS, AC, AH, SC, MA; Group III- NS; Group IV- RS; Untested; PE, WP, VN).

Sample		Panellist							Geosmin conc. (ng L^{-1})	
ID	Content	PE	LS	KB	MA	AH	VN	AC	Perceived (mean)	Actual
A	M/30,000	0	½1	0	0	0	X	0	3.5	0.9
B	M/20,000	0	1	0	X	X	0	X	7.4	1.3
C	M/10,000	0	1	X	1	1	½	X	14.2	2.6
D	M/5,000	1½	1½	1	0	1	1	1	21.3	5.1

Table 9. Sensory Panel No.6. Seven panellists (PE..AC) assessed four samples (A..D) comprised of different dilutions of a field sample (*Anabaena* bloom in a Murrumbidgee River backwater, Benerembah State Forest) presented as unknowns. Perceived (mean) geosmin concentration was calculated by converting intensity scores to their corresponding geosmin concentration and averaging these values. This is compared to the samples Actual concentration as determined by chemical analysis. Panellist ranking (based on Table 3) as follows: Group II- LS, MA, AH, AC; Untested- PE, KB, VN).

Panel No.	Geosmin standards		Field/Culture samples	
	Prob.	Sig.	Prob.	Sig.
2	0.111	no	0.224	no
3	0.112	no	0.512	no
5	0.356	no	0.356	no

Table 10. Kruskal-Wallis ANOVA by ranks comparing the sensory performance of Tested to Un-tested panellists in their assessments of geosmin standards and field/culture samples.

Panel No.	Note	Sample	Geosmin concentration (ng L^{-1})		
			SENSORY		CHEMICAL
			Perceived	Adjusted	(Actual)
2	culture	C/1333	53.9	66.8	68.1
	"	C/667	96.5+	123.4+	136.1
3	culture	A/1000	36.5	43.7	6.4
	"	A/2000	27.1	31.2	3.2
	"	A/4000	35.6	42.5	1.6
	culture	B/1000	44.8	54.7	95.9
	"	B/2000	82.9	105.4	48.0
	"	B/4000	32.1	37.9	24.0
4	field	Four Posts	41.0	49.7	78.0
	"	Lawson	41.2	50.0	27.0
5	field	T/100	6.5	3.8	4.8
	"	T/50	12.1	11.3	9.5
	"	T/25	14.4	14.3	19.0
	"	T/10	38.6	46.5	47.5
6	field	M/30,000	3.5	0.0	0.9
	"	M/20,000	7.4	5.0	1.3
	"	M/10,000	14.2	14.1	2.6
	"	M/5,000	21.3	23.5	5.1

Table 11. Summary of the sensory evaluations of field and culture samples by Panel No.2-6 showing perceived concentration (Perceived), perceived concentration after adjustment for bias (Adjusted) and the samples true concentration determined by chemical analysis (Actual).

Despite often large variations in the assessment of intensity scores by the panel as a whole, most evaluations resulted in a narrower cluster of scores representing the most common perceived intensity for that sample. The overall evaluation of a sample by a panel was determined by directly converting all intensity scores to geosmin concentration (ng L^{-1}) and averaging these values across all panellists. These estimates are summarised as perceived geosmin concentration, and are shown alongside actual concentration as determined by chemical analysis in Tables 5 to 9.

The assessment of geosmin solutions by Panel Nos.2-5 are shown plotted against actual concentration in Fig. 4.1. There was overall a direct relationship between perceived and actual geosmin concentration. Despite the assistance of reference standards during all evaluations, panellists tended to underestimate the intensity of geosmin solutions, as sensory estimates of perceived concentration are below the 45 degree line. The linear relationship between actual and perceived concentrations indicates that geosmin solutions were underestimated across the entire range tested, particularly at higher concentrations. This bias is evident amongst the different samples assessed within each panel, and in the assessment of solutions between panels as well.

The relationship between perceived and actual geosmin concentration seen in Fig. 4.1 could be approximated using linear regression, where the fitted line accounted for 89% of the variation. The uniform bias demonstrated in the sensory assessment of geosmin solutions is expected to extend through to the assessments of all field and culture samples. Therefore, the equation between actual and perceived concentration derived from Fig. 4.1 was used to adjust the sensory evaluations of these samples. The origin of the unknown samples, along with the adjustment of perceived concentration

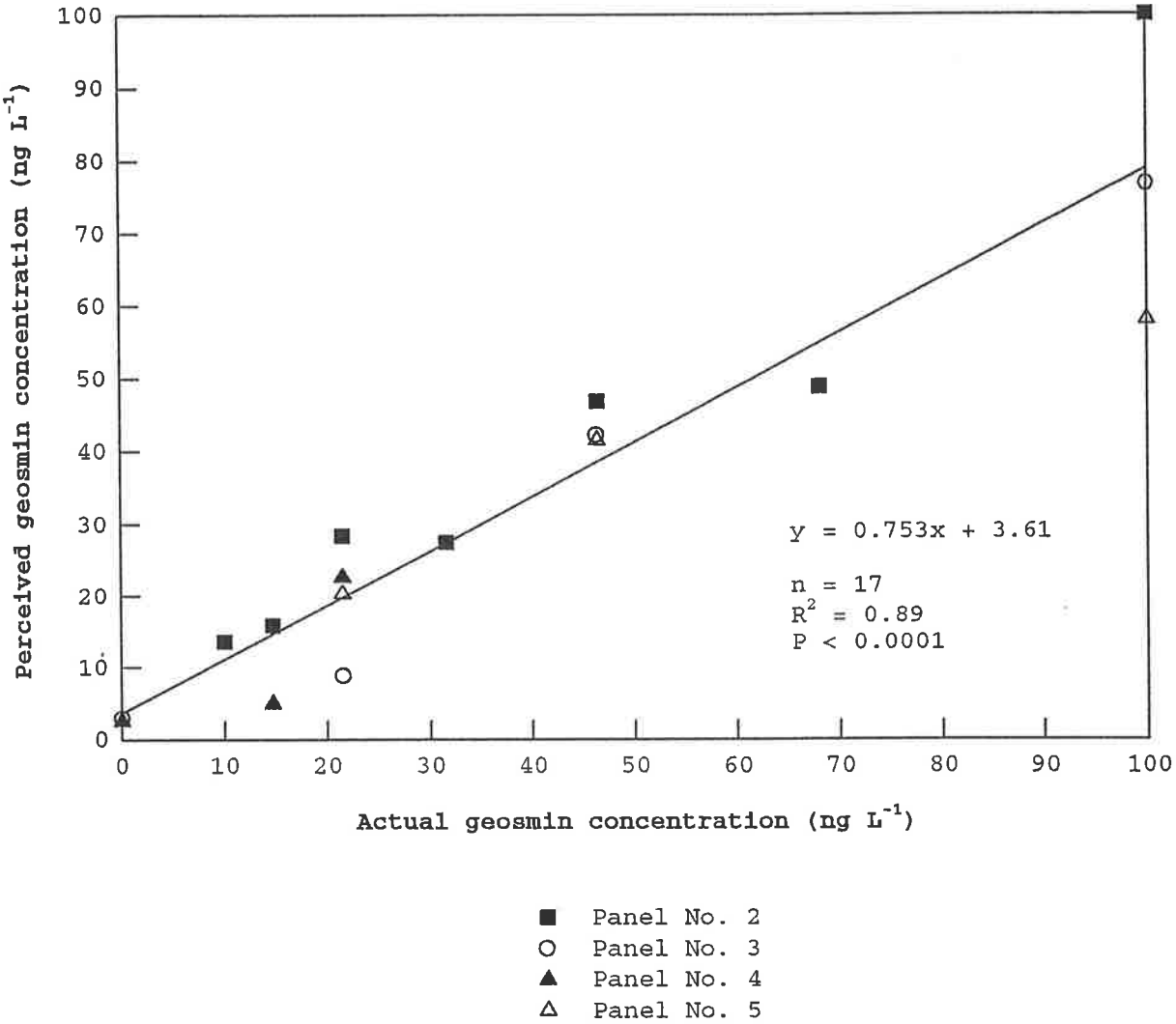

Fig. 4.1. Comparison of perceived (sensory) concentrations of geosmin standards (as unknowns) by all panels to actual (chemical) levels.

is shown in Table 11, and is plotted with the evaluations of geosmin solutions against actual concentration in Fig. 4.2.

As expected, the adjusted perceived and actual concentration for geosmin solutions resulted in a 1:1 relationship. Excellent agreement with actual concentration was obtained in the sensory evaluation of field samples by Panel No.5, and the culture samples presented to Panel No.2. The sensory evaluations by these two panels demonstrated an ability to discern between a number of dilutions, down to 5 ng L^{-1} in the case of the most diluted sample presented to Panel No.5 (Table 11). In the case of one culture sample assessed by Panel No.2, adjustment of the original sensory estimate resulted in a final value above 100 ng L^{-1}, beyond the range of reference standards presented to panellists.

The sensory estimations of geosmin concentrations well below the OTC is suggested in the evaluation of field samples by Panel No.6. The more diluted samples, with actual concentrations of less than 2 ng L^{-1}, resulted in similar estimates between sensory and chemical analysis. However, the evaluation of samples M/5,000 and M/10,000 by sensory assessment differed markedly from expected (Table 11). Nevertheless, a good relationship between perceived concentration and sample dilution is evident in all four evaluations.

The sensory evaluation of two different culture samples by Panel No. 3 and two different field samples by Panel No.4 overall resulted in poor agreement with actual geosmin concentration (Table 11). Although the true concentration of culture A assessed by Panel No.3 was less than the OTC for geosmin, sensory assessment resulted in geosmin estimates significantly higher (40 ng L^{-1}) than expected for all three dilutions. The evaluation of different dilutions of culture B was markedly varied, ranging from one good

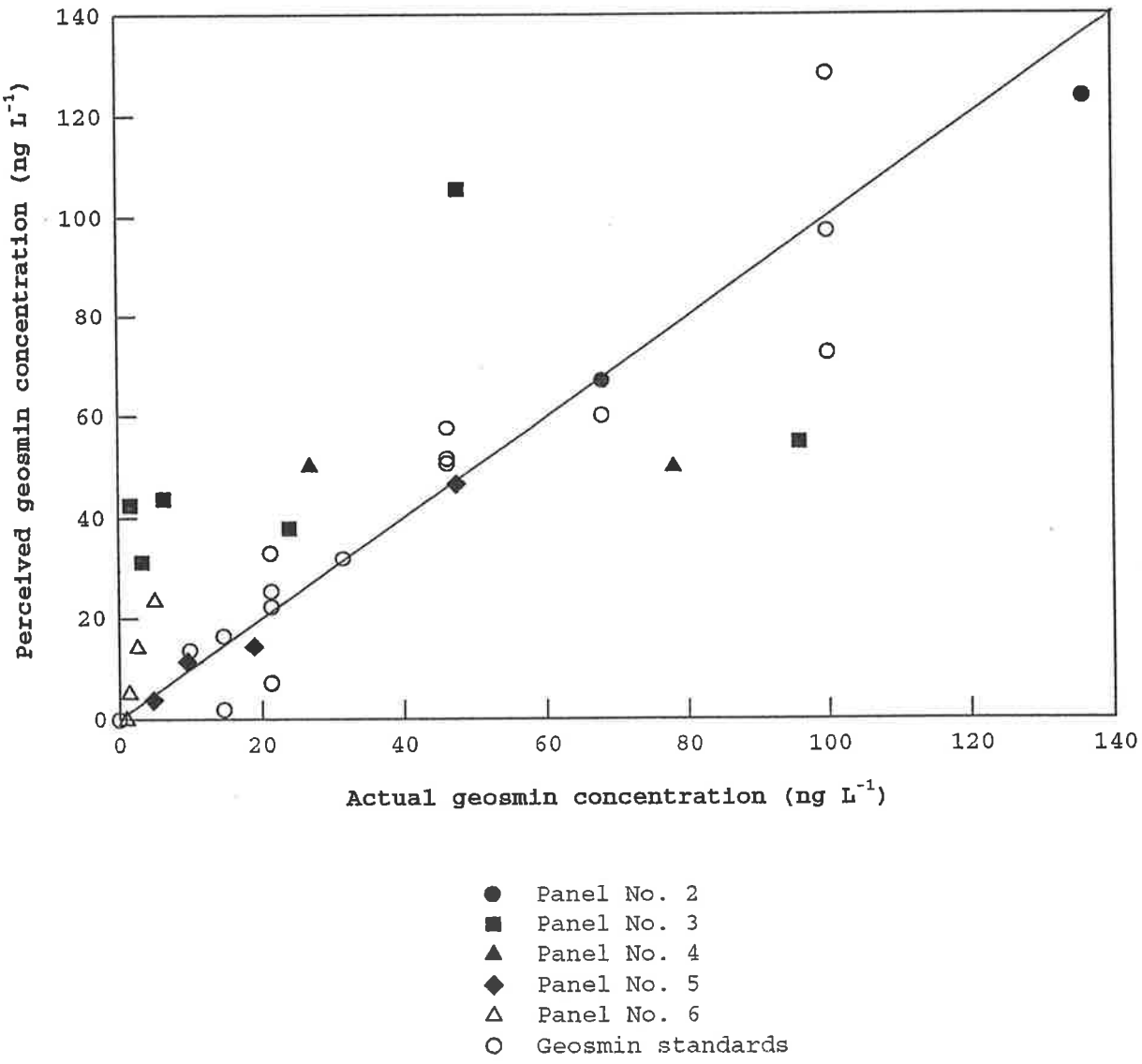

Fig. 4.2. Performance summary of all sensory panels. Comparison of perceived (adjusted) and actual geosmin concentrations of standards, *A. circinalis* cultures and field samples (fitted line is y=x).

agreement with actual concentration, and the remaining two significantly under- and over-estimated. The sensory evaluations of both algal cultures by Panel No.3 showed no relationship between dilution factor and perceived geosmin concentration.

The relationship between the error associated with the sensory assessment of samples (geosmin solutions and field/culture samples) and panel size (number of participating panellists) is shown plotted in Fig. 4.3. This relationship was determined by calculating the standard deviation of the perceived:actual ratio for geosmin solutions and field/culture samples separately. That is, the standard deviation was calculated using the perceived (adjusted) geosmin concentration divided by the corresponding actual geosmin concentration to place the different solutions presented on an equal par. All estimates between different samples could therefore be pooled into two groups of unknowns (geosmin solutions and field/culture samples) and the standard deviation determined for each group.

Fig. 4.3 shows that there is a strong inverse relationship between panel size and the standard deviation of the response ratio (perceived-(adjusted)/actual concentration) for the sensory evaluations of geosmin solutions and field/culture samples. The standard deviation of geosmin solutions was lower than the standard deviation of field/culture samples, particularly with smaller (5-7) sample sizes. The error associated with both groups of samples increased markedly when panel size was less than 9 individuals, and a smaller range of errors was associated with panel sizes of 9 and 13 individuals.

The error associated with sensory analyses by Panel Nos.2 and 5, composed of the highest number of individuals (9 and 13 respectively), had a standard deviation of 0.2 around an expected mean of unity for both geosmin

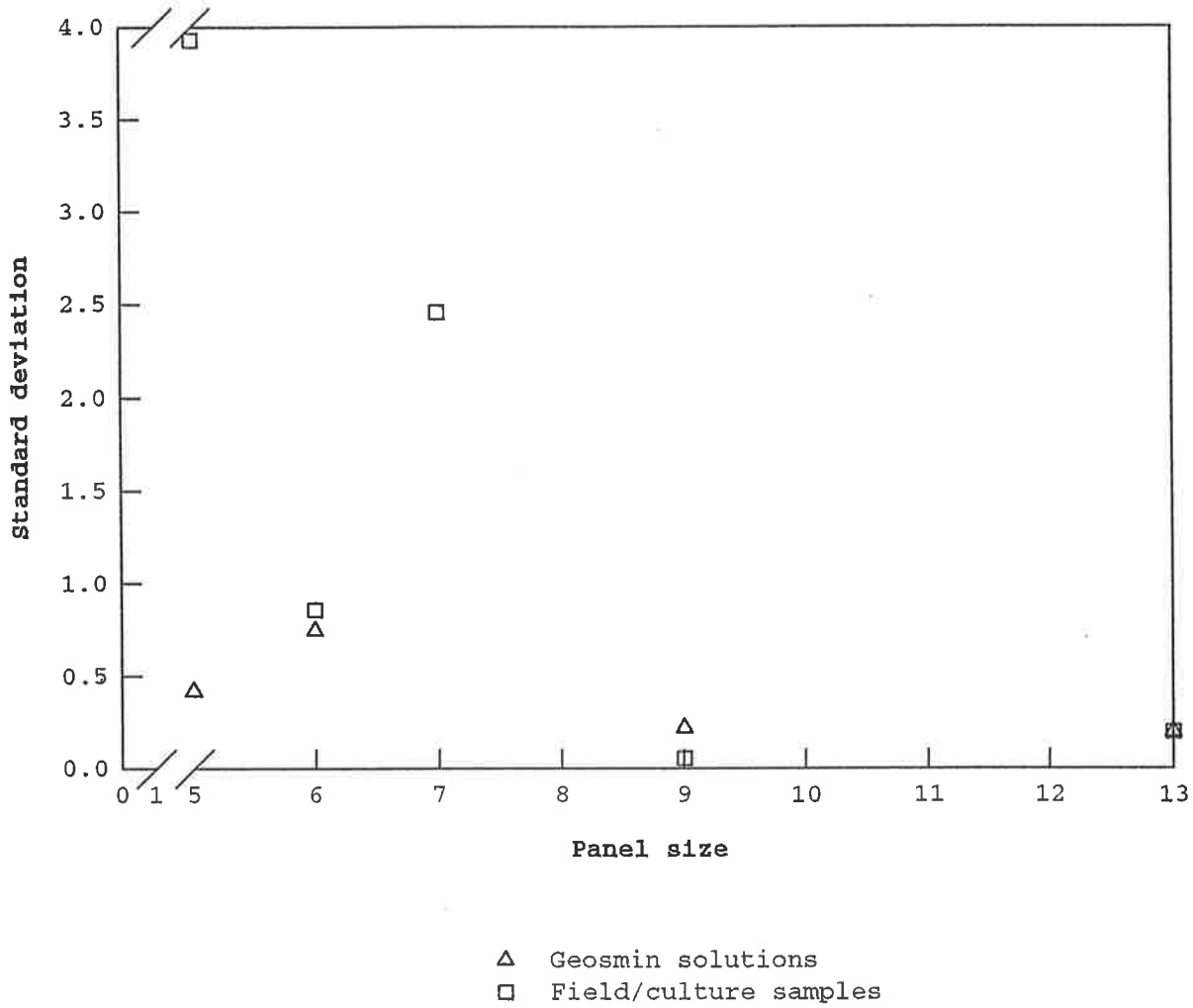

Fig. 4.3. The effect of sensory panel size on the standard deviation (around a mean of 1) of panel assessments of geosmin solutions and field/culture samples.

solutions and field/culture samples (Fig. 4.3). This corresponds to a measurement error of 40% (at the 95% level) when the panel size is at least 9 individuals. The error associated with panel sizes of less than 9 individuals ranged from 88% up to nearly 2,000%.

4.5 Discussion

Similar performances by tested and un-tested individuals in the assessment of geosmin and field/culture samples questions the need for the thorough testing of prospective panellists investigated in the previous chapter (Table 10). These results suggest that there is no advantage in testing individuals, apart from ensuring that they are not anosmic to geosmin and can recognise it's odour characteristics. However, this result may be a consequence of the high variability in panellist responses both within and between panels, masking any real differences.

Variations in individual performance between assessments nevertheless should be monitored to determine how an individual performs on different panels (i.e. over time) and how they compare to other panellists and the actual geosmin concentration. This process will allow some degree of quality control over the performance of the panel as a whole, as those individuals that are seen to be more consistent over time or more accurate in their assessments may be favoured over others.

Despite differences in panel sizes and individual composition, it was unexpected to find that different panels consistently underestimated the odour intensity of geosmin solutions, and that the magnitude of this bias was similar between panels (Fig. 4.1). With geosmin reference standards at hand to compare against unknown samples, it was expected that the perceived

geosmin concentration would be similar to the actual concentration. The constant nature of this error across all panels indicates the action of similar factors on all individuals.

One explanation may be related to individuals "remembering" the odour intensity of a reference standard coupled to varying degrees of nose fatigue. By continuously alternating between smelling samples and reference solutions, it is not unlikely that individuals were affected to some extent by nose fatigue. This would be particularly significant at higher concentrations where fatigue is more likely to occur. After smelling two reference standards to establish a range of "remembered" intensities, it is probable that individuals were temporarily de-sensitised to geosmin, resulting in the perceived odour intensity of an unknown sample immediately after being underestimated.

The consistency in the bias between panels seen in this study allowed a corrective factor to be applied to the original perceived estimates of geosmin solutions, which was extended to the assessments of field and culture samples as well. This resulted in good agreement between sensory estimates and actual concentration of geosmin solutions.

Other studies in the literature relying on individuals assessing solutions as strong or weak (using a rated scoring system) have shown marked variability in their responses over consecutive periods ranging from 11 weeks (Brady *et al.* 1988) up to one year (de Greef *et al.*, 1983). Although in the latter case variations in panel responses were correlated in part with holiday periods, it is not clear in both studies if this variation reflected the method, the panel's experience or the nature of sensory analysis itself.

Studies such as these highlight the importance of calibrating untrained panels by including a range of geosmin standards as unknowns along with real unknown samples that need to be measured. The use of internal standards allows any bias in the evaluation of samples to be identified and a corrective factor calculated and applied. As nose fatigue is accentuated by high geosmin concentrations, the four lower geosmin solutions corresponding to Stds ½, 1, 1½ and 2 should be randomly included with real unknowns. The inclusion of 4 check standards will allow regression analysis to be applied.

The accurate evaluation of 9 samples by Panel No.2 in one sitting suggests that up to 5 unknowns may be assessed together with the 4 check standards recommended. The repetitive smelling of geosmin solutions as a factor contributing to nose fatigue, and the subsequent underestimation of geosmin intensities, indicates that the total number of samples assessed should be kept to a minimum where possible. This may be achieved by dividing samples between a morning and afternoon session, with check standards included for each evaluation.

The adjustment of sensory evaluations for bias in all panels resulted in good agreement between sensory and actual concentrations for geosmin solutions (Fig. 4.2). However, the sensory evaluation of field/culture samples were on occasions markedly different to actual geosmin concentration. The variation between perceived and actual concentration is thought to result from the interaction of panel size, other competing odours, and the experience of panellists.

The relationship between the standard deviation of normalised sensory estimates and panel size in Fig. 4.3 demonstrates the markedly higher variability in sensory estimates associated with smaller panel sizes,

particularly for field/culture samples. The poorest performing panels (Nos. 3 and 4) were also comprised of the smallest groups of people.

Smaller panels are more sensitive to outlier scores that are markedly greater and/or smaller than the majority of responses, and similarly are more sensitive to variations in an individuals performance on that day. Ensuring that panels are comprised of at least 9 individuals will markedly reduce the error associated with the sensory measurement of geosmin concentration to within an accuracy of 40 per cent (Fig. 4.3).

The excellent agreement between perceived concentration and dilution factor in the assessment of a field sample by Panel No.6 indicates the consistency of this panel in measuring the magnitude of odour intensity. However, both the marked difference between perceived and actual concentration, and perceived geosmin concentrations well below the OTC (5 ng L^{-1}) suggests the interaction of other odoriferous compound(s) in addition to geosmin.

Similarly, the poor correlation between actual and perceived concentration of the two algal cultures assessed by Panel No.3 suggest the interference of other compounds to the detection of geosmin (Fig. 4.2). The presence of other odoriferous compounds in culture A resulted in estimates of geosmin levels exceeding 30 ng L^{-1}, when this culture actually contained geosmin at sub-threshold concentrations. The mixed abilities of panellists to distinguish geosmin from other compounds would have contributed to the large differences between sensory and chemical analyses, and the lack of correlation between sensory analyses and dilution factor for both cultures.

The inability of some panellists to distinguish the sensory characteristics of geosmin from those of other compounds is not unexpected. These individuals were not extensively trained to improve their resolving

capabilities as those from other centres. Through continued use, the discriminatory capabilities of the sensory panel, as well as their sensitivity to odours should improve.

Nevertheless, the ability of the panel to give an indication of odour intensity is still of value to the water manager. Although the odour characteristics of different tainted waters may differ from that of geosmin, the intensity of any musty compound may still be gauged. In addition, by referring to geosmin standards as a guide to odour intensity, a panel with minimal training is continuously kept in calibration. This ensures that panel assessments are measured against a common base and are consistent between different panels and over time.

The ability of the sensory panels assembled in this study to determine geosmin concentration of geosmin solutions, culture and field samples indicates that the proposed sensory protocol is a viable one for determining geosmin levels in water. By using the sensory protocol described here, water odour quality from different locations can be rapidly monitored by untrained sensory panels, maintaining relative comparability between different assessments.

The following section summarises the recommended procedure for the selection of a sensory panel and the measurement of geosmin using an untrained sensory panel.

4.6 Recommendations for the selection of panellists and the sensory measurement of geosmin.

4.6.1 Protocol for the Selection of Individuals for Sensory Assessment.

1. Number of individuals to assess and select

In the sensory protocol developed in this study, at least 9 individuals are needed to estimate geosmin concentration (chapter 4), however, a pool of approximately 15 screened people should be chosen from which to form a sensory panel. This number should ensure that on most occasions a minimum core of 9 individuals can be assembled (a shortage of panellists may arise due to illness, holiday periods, field work and other duties).

As the Griffith survey showed that approximately 50% of those screened performed to a satisfactory level, at least 30 individuals should be screened and the best performing 15 selected.

2. Testing area

A fume cupboard to remove lingering odcurs is the preferred work area, although a well ventilated room may be used. The test area should be well lit and free of any extraneous odours, including those from food, soaps, detergents, chemicals, dust and scented plants. Other distractions including the passage of colleagues and noises should be avoided. The immediate work area is sponged clean with water and alcohol, and is allowed to dry thoroughly.

3. Geosmin test solutions

All solutions are prepared in a room separate to and if possible away from the test area. Geosmin solutions corresponding to 2, 4, 8, 14, 20, 30, 40, 60 and 100 ng L^{-1} are prepared from serial dilutions of a stock solution using odour-free water, immediately before use. 100 mL is transferred into a 200 mL plastic disposable cup, and is covered with one half of a plastic disposable petri-dish. Four water blanks (A, B, C, and D) are also included, and are positioned in the order shown below:

A 2 4 B 8 14 C 20 30 40 D 60 100

All containers are labelled alphabetically (A to M) using adhesive labels with pencil markings, and are transferred to the test area and arranged in ascending order.

4. Testing procedure

Participants are tested on an individual basis. They are instructed not to wear perfumes or scented cosmetics on the day of the survey, and are required to wear plastic gloves or wash their hands with odour-free soap. This is to avoid interference with odours from the hands and contaminating the outside of cups for subsequent individuals. Prior to commencing the test, they are familiarised with geosmin odour by comparing a strong solution (e.g. 100 ng L^{-1}) to a water blank. This is repeated as many times as required until the individual is satisfied they can recognise the earthy/musty odour of geosmin.

Panellists assess solutions at room temperature by swirling contents five or six times, removing the cover and smelling the air space. Repeated sampling of each solution is allowed, but individuals should be constantly reminded that nose fatigue may occur. In such an event, they are instructed to rest for a few minutes and smell a separate and clearly labelled water blank before re-commencing.

All results are recorded by individuals on separate score sheets as either a "+" if geosmin is detected, or a "-" for no odour recognition.

5. Analysis of results

All score sheets are collated and individuals are ranked objectively as follows:

1. For each geosmin solution scored as positive, award points corresponding to the geosmin concentration of the solution detected.

2. For each water blank scored as positive, subtract points as follows: A=6, B=22, C=90 and D=160.

3. After calculating the total score for each participant, rank individuals and select the 15 with the highest scores.

This process assists in forming a pool of 15 screened people from which to chose at least 9 individuals to form a sensory panel.

4.6.2 Protocol for the Sensory Measurement of Geosmin in Water.

1. Geosmin Reference Standards

Prepare the following concentrations of geosmin solutions: 0, 10, 21.5, 46.6 and 100 ng L^{-1} (parts per trillion). These correspond to geosmin reference standards of 0, X, 1, 2 and 3 respectively.

100 mL of each solution is prepared immediately before use using odour-free water, in a well ventilated room separate from the area where sensory tests are to be conducted.

Each solution is placed in a labelled (0, X, 1, 2 and 3) plastic (polystyrene) 200 mL cup and covered with one half of a disposable plastic petri dish. These solutions not be used more than half a day, after which they are discarded and fresh solutions made up.

2. Preparation of Sensory Assessment Site

Either a fume-hood or a clean, well ventilated room is required for conducting sensory tests. Both should be thoroughly cleaned to ensure the removal of any odoriferous compounds that may interfere.

Remove chemicals, detergents, air fresheners, dusty files etc. from the work area, and remove any distracting items including glassware and other equipment. Wipe bench tops with a moist sponge followed by ethanol or acetone, and allow to dry thoroughly.

The test area should be separate to the preparation room to avoid contamination of the airspace from geosmin solutions and samples to be measured.

3. Selection of Panellists

The procedure described in 4.6.1 is recommended for selecting the most consistent individuals used to form the sensory panel. This procedure also assists in introducing prospective panellists to the sensory characteristics of geosmin, and to the sample handling and smelling techniques.

4. Number of Panellists Required

A minimum of 9 individuals are required for each sensory panel. A pool of 15 volunteers should be formed both to maximise the number of panellists assessing each sample, and to safeguard against a shortage of individuals at different times, reducing the available number of panellists.

5. Number of Solutions Presented

Up to 10 solutions may be presented to the panel. These are made up of 4 geosmin solutions (corresponding to reference standards ½, 1, 1½ and 2; concentrations 14.7, 21.5, 31.6 and 46.4 ng L^{-1} respectively) and up to 6 other samples to be measured. The 4 reference standards should always be included in each sitting to allow calibration of the sensory panel.

6. Sample Pre-treatment

Samples to be measured must be initially screened by two panellists to ensure they fall within the range of geosmin reference standards provided. This is not intended to measure the concentration of geosmin, but to determine the number of dilutions required (if any) to prevent excessively strong solutions being presented to the main panel.

Sequentially dilute each sample three-fold until 5 solutions of decreasing concentration are obtained. Place 100 mL of each solution in a 200 mL plastic cup and cover with a petri dish half. Transfer to the sensory assessment area.

Select two panellists to independently screen these samples. Solutions are swirled and immediately smelt in turn starting from the most dilute. Smelling continues until an odour is first detected, and the dilution of that sample recorded. If an odour is detected in the most dilute solution, sequentially dilute that solution three-fold another five times, and repeat the above procedure.

Dilutions of samples that are presented to the entire panel is the solution that is three times stronger than the solution first detected in the preliminary screening. This increases the likelihood of the odour intensity of unknown solutions falling within the range of the geosmin reference standards.

7. Sensory Procedure

a) Samples to be measured and geosmin solutions are randomly arranged and labelled alphabetically. These are placed towards the front of the work area. Place the water blank and geosmin reference standards X, 1, 2 and 3 in increasing order towards the back of the work area.

b) Sensory analysis is conducted one person at a time. Individuals assess standards and unknowns by gently swirling the contents of the cups to saturate the airspace, immediately removing the cover and smelling the airspace. Cups should be held as close to the base as possible to minimise interference with odours from the hand.

c) The odour intensity of each unknown is compared to the odour intensity of the reference standards, and is given a score corresponding to the reference standard whose intensity it most resembles. Panellists are allowed to smell each unknown and standard as many times as is needed for them to make an assessment. Panellists are again warned of the likelihood of nose fatigue, and are instructed to rest for 5 minutes if they suspect they are affected.

d) In the event of the odour intensity of an unknown falling between two geosmin standards, half scores may be assigned. For example, if a panellist cannot decide whether an unknown has an odour intensity of 1 or 2, then a score of 1½ may be used. Table 12 summarises all allowable scores that may be assigned.

e) Each individual is given a separate score sheet to record their assessments of all unknown samples. They are also requested to record

Intensity score	Geosmin (ng L^{-1})	Note
0	0	no odour detected
X	10	presence unsure
½	14.7	definite presence
1	21.5	definite presence
1½	31.6	definite presence
2	46.4	definite presence
2½	68.1	definite presence
3	100	definite presence
3+	>100	out of range

Table 12. Intensity scores that may be assigned to samples being tested. The odour intensity of each unknown is compared to the odour intensity of the reference standards (0, X, 1, 2 and 3) and is given a score corresponding to the reference standard whose intensity it most resembles. If the odour intensity of an unknown falls between two geosmin standards, half scores are assigned.

any other odours they sensed, or any problems they may have had in assessing a particular unknown.

f) As one individual finishes assessing the samples, the next is prepared to start.

When collecting completed score sheets, individuals are taken aside and are given immediate feedback on their evaluations. This may be by either comparing their evaluations to those of previous panellists, comparison with chemical analyses, or revealing the sources of the samples. Feedback is considered to be important as a way of maintaining interest, increasing confidence in their assessments (i.e. promote a positive attitude) and encouraging panellists to be available for future assessments.

8. Calculations

a) Construct a table summarising the assessments of the entire panel using rows for samples and columns for panellists. Using Table 12, convert intensity score to actual geosmin concentration.

b) Calculate an average concentration value for each sample.

c) Plot the observed versus expected values of the geosmin internal standards presented as unknowns. Fit a line using linear regression and derive an equation which is used to adjust the values of the real unknown samples.

d) Express results as geosmin equivalents after multiplying the average score of an unknown sample by the dilution factor (from step 6), if any.

Chapter 5

Geosmin production during the growth of *Anabaena circinalis* 852E in culture under high and low light intensity.

5.1 Introduction

The management of odour-producing algae (and algae in general) often involves inhibiting algal growth by the application of non-specific algicides such as copper sulphate, usually after the occurrence of water quality problems (Burch *et al.*, 1987; McGuire *et al.*, 1984). Modification of various environmental parameters such as pH, nutrients and mixed depth has also been used to control algal problems (Shapiro 1984; May 1974).

An alternative approach to managing odours may be to modify the environment to reduce or inhibit the synthesis of odoriferous metabolites by these organisms. However, this requires an understanding of the environmental and strain specific factors that effect production of these compounds. At the very least, such information may allow the prediction of odour episodes in waters where these organisms are known to occur. Although numerous examples of organisms capable of producing geosmin and MIB can be found in the literature, little is known about how the production of these metabolites is affected by either the environment or the growth characteristics of the organism.

Izaguirre *et al.* (1983) measured the production of MIB in a batch culture of *Oscillatoria curviceps*. They found synthesis occurred throughout the life of the culture, and was not related to death or decay of cells.

Continuous production of geosmin in batch cultures of *O. brevis* was observed by Naes *et al.* (1985). Furthermore, the growth of these cultures at two light intensities (4.5 and 30 $\mu M\ m^{-2}\ s^{-1}$) showed a link between geosmin and chlorophyll synthesis. This supported the findings of Bentley and Meganathan (1981) suggesting geosmin and MIB are synthesised by the isoprenoid pathway, which is known to produce the phytol component of the chlorophyll molecule.

Investigations on the production of another group of compounds by cyanobacteria, toxins, have shown it to be dependent upon several environmental and genetic factors. The production of geosmin and MIB by cyanobacteria is known to be strain specific as well (Izaguirre *et al.*, 1983; Berglind *et al.*, 1983b).

Culture studies on toxin synthesis by *Aphanizomenon flos-aquae* showed a dependence on factors such as age of the culture, temperature and light intensity (Gentile and Maloney, 1969). The effects of some of these variables on the toxicity of *Microcystis aeruginosa* and *Anabaena flos-aquae* have also been demonstrated (Collins, 1978; Watanabe and Oishi, 1985), and May (1981) reported that the toxicity of blooms of *M. aeruginosa* and *Anabaena circinalis* in Australia paralleled their growth stage. Presumably the effect of the age of the population or culture on toxicity is a reflection of the changing environment due to algal growth.

An additional factor which is known to greatly effect the toxicity of both algal cells and the growth medium is the storage and release of toxins by cells (Codd *et al.*, 1989). Izaguirre *et al.* (1983) demonstrated that the cellular pool of MIB in cultured *Oscillatoria curviceps* ranged from 0% to 25% of the total in early and late growth stage cells, respectively. This

partitioning is an important consideration in the investigation of geosmin synthesis, as any storage of geosmin prior to release into the water provides a means of anticipating increased odour problems, allowing time to adopt available control measures.

Species of the genus *Anabaena* are confirmed producers of geosmin (Izaguirre *et al.*, 1982; this study), and field correlations indicate *Aphanizomenon* to be a likely producer (Juttner *et al.*, 1986). Therefore factors that affect the production of toxins (e.g. age, light and temperature) may also influence the production of geosmin by the strain of *A. circinalis* (852E) isolated from the Murrumbidgee River.

Although all measurements to date in this study have shown that cultures of *A. circinalis* 852E were able to produce geosmin, nothing was known of the relationship between geosmin synthesis and the stages of growth. Furthermore, little to date is known about the cellular and extra-cellular pools of geosmin, and the factors (if any) which affect the release of geosmin by cells.

5.2 Objectives

The principle objective of this chapter is to:

1. Investigate whether the production of geosmin is continuous during the growth of *A. circinalis* 852E in culture.

The secondary aims are to:

2. Examine the effect of light intensity on geosmin synthesis.

3. Determine the extent of intra- and extra-cellular geosmin partitioning at different stages of growth.

One possible outcome of a growth phase trial is that geosmin could be continuously synthesised as a normal part of cell metabolism. Alternatively, production could be in response to a particular stage of growth. This includes slow growth during the initial lag phase, rapid cell division in the exponential phase, and growth under stress as cells approach senescence (due to shortage of nutrients, high pH, accumulation of toxic cellular metabolites, and limiting light due to self-shading).

5.3 Methods

In batch culture, the changing environment in response to cell growth allows a spectrum of conditions to be established, and was therefore adopted in these experiments.

A 5 L glass carboy containing 4.5 L sterile W.C. media was inoculated with a small (100-200 mL) actively growing culture of *A. circinalis* 852E. The culture was maintained at 20°C, constantly aerated and provided with continuous illumination at an intensity of 70 $\mu M\ m^{-2}\ s^{-1}$. An 8 L culture in a 10 L glass carboy was set up in a similar manner but illumination was reduced to 17 $\mu M\ m^{-2}\ s^{-1}$. Both the inoculating cultures were maintained at 20°C under constant illumination at 70-100 $\mu M\ m^{-2}\ s^{-1}$.

A greater culture volume was used at 17 $\mu M\ m^{-2}\ s^{-1}$ in anticipation of higher sample volumes being needed. Lower growth rates and levels of biomass expected at this light intensity would result in a longer running experiment where a greater number of samples would be taken. Lower levels

of biomass and geosmin would also require greater sample volumes to process to ensure detectable parameter levels. Furthermore, the examination of geosmin partitioning into intra- and extra-cellular pools required extra samples for geosmin analysis.

One growth cabinet was utilised for this experiment, with both cultures grown consecutively, commencing with that illuminated at 70 $\mu M\ m^{-2}\ s^{-1}$. Carboys were placed in the centre of the cabinet and were kept off the base of the growth chamber using a height adjustable metal grill. Illumination was provided from above, and was reflected around the chamber by the walls and base. Light intensity was measured by placing the sensor at a point that would become the centre of the carboy. The sensor was rotated to measure the source of the illumination (the roof) as well as that reflected at right angles off the walls. The light intensity recorded is an approximate mean.

The growth of both cultures were monitored at 2-3 day intervals by measuring pH and the optical density (O.D.) at 438 nm to provide an estimate of algal biomass. The same sample was filtered to give a single (unreplicated) reading of dry weight (sample volume 100-150 mL).

At selected stages of population growth, duplicate samples for chlorophyll *a* (150-250 mL) and dry weight (150-250 mL) determinations were taken. Algal growth rates were calculated as the specific growth rate (k', ln units day^{-1}). Algal biomass as cell density was not measured in this experiment as chlorophyll *a* and dry weight are more accurate to measure (as opposed to counting Anabaena clumps/filaments) and are directly relevant to factors that may affect geosmin synthesis. In the next chapter, cell densities were determined and are related to chlorophyll *a* and dry weight.

Total geosmin concentration at 17 μM m^{-2} s^{-1} was measured by treating a single sample (200-500 mL) with $CuSO_4$ (final concentration 69 ppm-Cu for 1 hr) prior to analysis to lyse cells. Extra-cellular levels were determined by analysing a separate sample (200-500 mL) without copper pre-treatment. Cellular geosmin was calculated as the difference between total and extra-cellular levels. Refer to Bowmer *et al.* (1992) for more on method development.

For the culture grown at 70 μM m^{-2} s^{-1}, a single sample (day 10, 500 mL; 250 mL for subsequent days) was immediately analysed (extra-cellular geosmin), followed by a treatment with copper and a second geosmin determination (total geosmin).

This experiment was not replicated (i.e. only one culture per light intensity) as the principle objective was to determine whether geosmin was produced throughout the growth of the culture. To achieve this end, two cultures at different light intensities were investigated. It was considered that multiple sampling of each culture at different stages of growth would be sufficient to establish any trends that may arise from the data.

5.4 Results

The two light treatments resulted in marked differences in the growth response of *A. circinalis* 852E measured as O.D. (Fig. 5.1). The culture at 70 μM m^{-2} s^{-1} demonstrated three stages of population growth: lag, exponential and decline. However, little growth occurred at 17 μM m^{-2} s^{-1} even though the growing period was twice that of the culture at

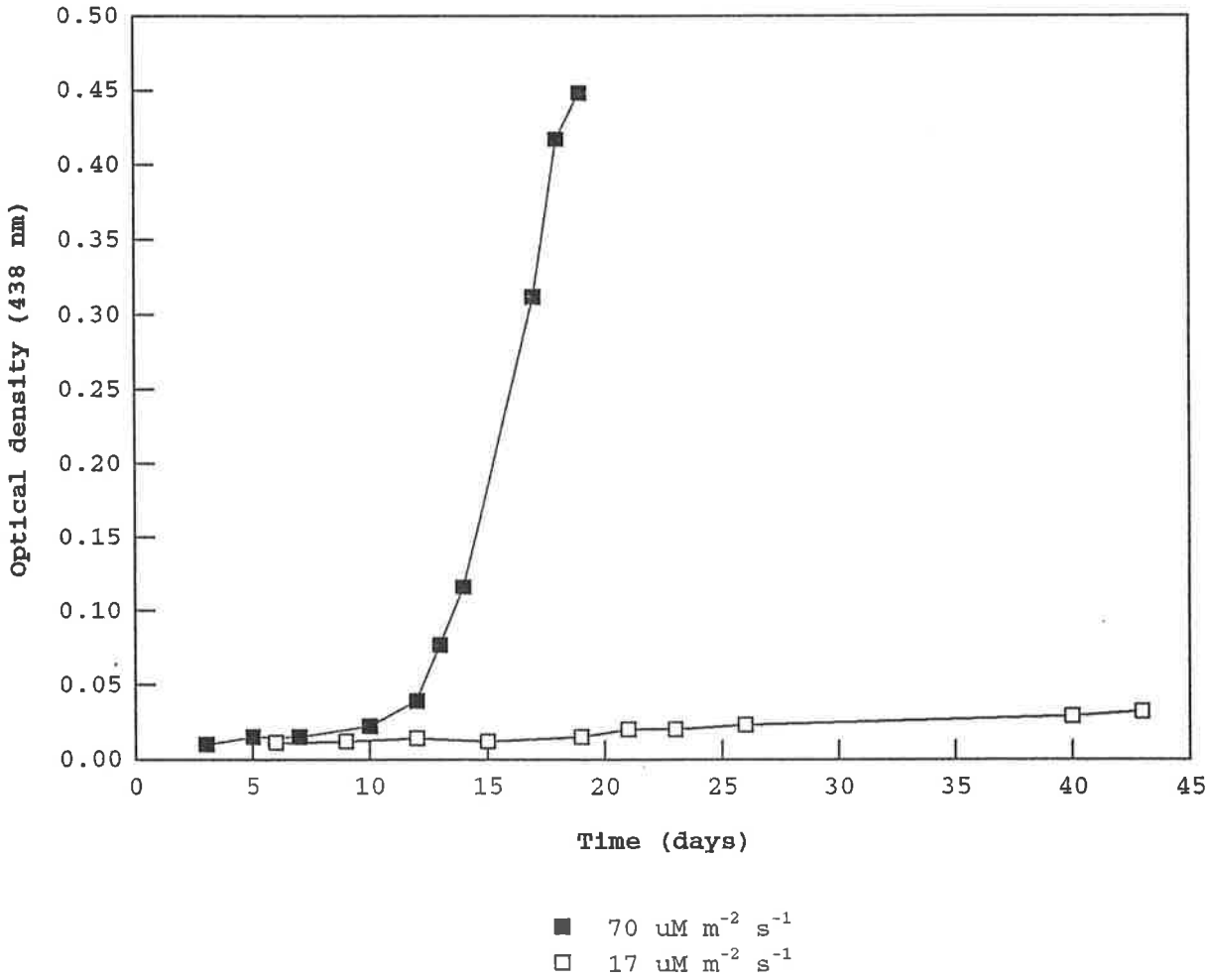

Fig. 5.1. Changes in optical density over time in two cultures of *A. circinalis* 852E.

70 μM m^{-2} s^{-1}. This difference was reflected in the pH of the media, remaining almost constant at the lower light intensity, but rising above 9 at the higher intensity (Fig. 5.2).

Algal biomass measured as dry weight and chlorophyll *a* showed marked changes over time at 17 μM m^{-2} s^{-1} (Fig. 5.3). Chlorophyll *a* concentration increased from 2.4 μg L^{-1} on day 6 to a peak of 8.7 μg L^{-1} by day 26. Similarly, dry weight increased over the course of the trial, attaining a maximum of 4.1 mg L^{-1} by day 40. Unlike chlorophyll *a*, the change in dry weight over time was irregular.

At 70 μM m^{-2} s^{-1}, both dry weight and chlorophyll *a* increased markedly over time, attaining maximum values on days 18 and 17, respectively (Fig. 5.4). Absolute estimates of cell dry weight and chlorophyll *a* are orders of magnitude greater compared to the culture at 17 μM m^{-2} s^{-1}, as was total geosmin concentration (Figs. 5.3 and 5.4).

In both cultures, geosmin was detected on every sampling day. At 17 μM m^{-2} s^{-1}, total geosmin peaked on day 26, coinciding with that of chlorophyll *a* (Fig. 5.3). This was in contrast to the culture at 70 μM m^{-2} s^{-1}, where the trend in geosmin concentration was more closely related to dry weight, both peaking on day 18 (Fig. 5.4).

Changes in the concentration of geosmin and chlorophyll *a* expressed on a dry weight basis were closely related (Figs. 5.5 and 5.6). At both light intensities, changes in the chlorophyll *a* to dry weight ratio (Chl*a*:DW) over time were associated with similar changes in the geosmin to dry weight (G:DW) ratio. However, although a similar range in the Chl*a*:DW ratio (1-7 μg mg^{-1}) was evident in both cultures, the G:DW ratio for the culture at 17 μM m^{-2} s^{-1} was up to six times greater.

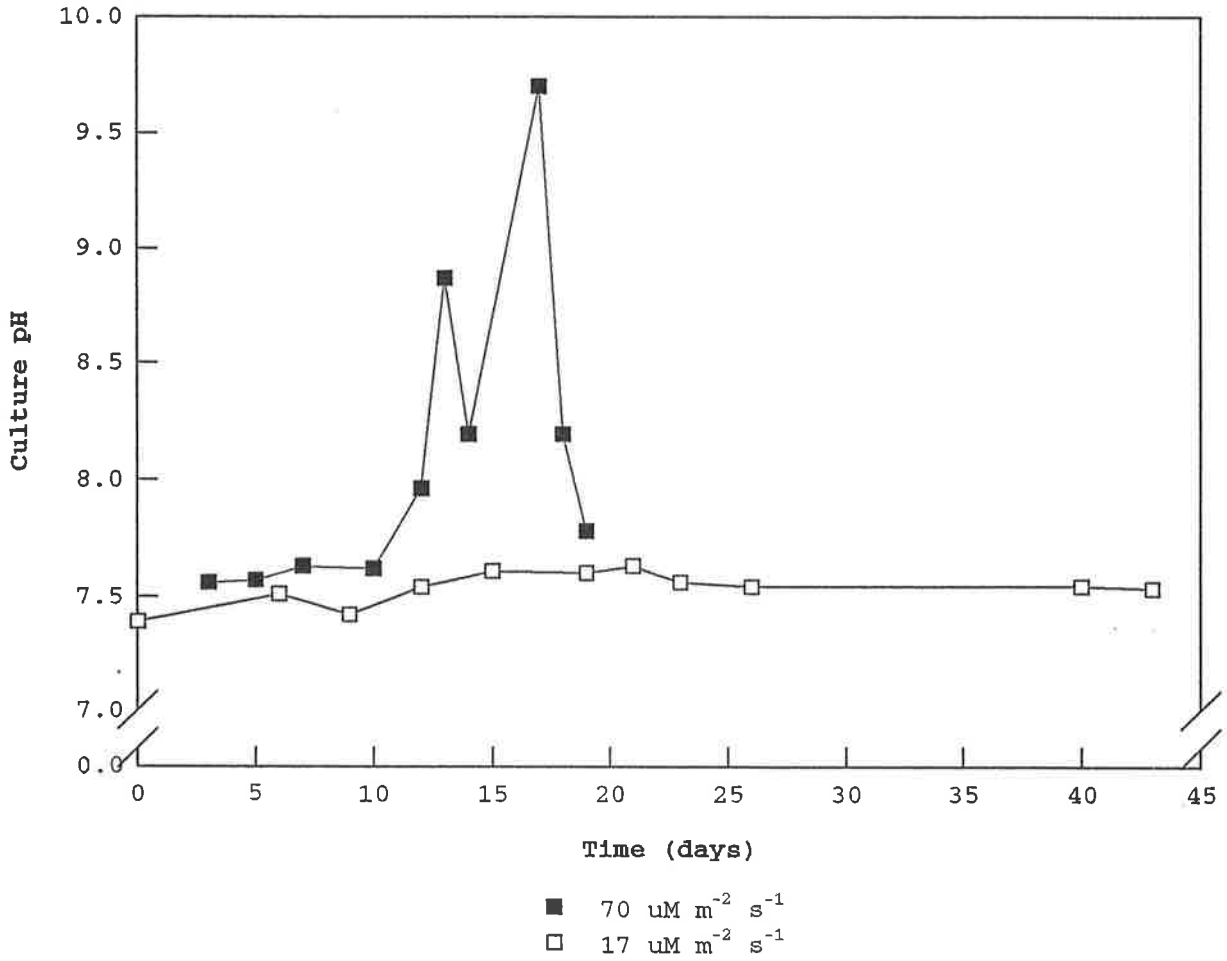

Fig. 5.2. Changes in culture pH over time in two cultures of *A. circinalis* 852E.

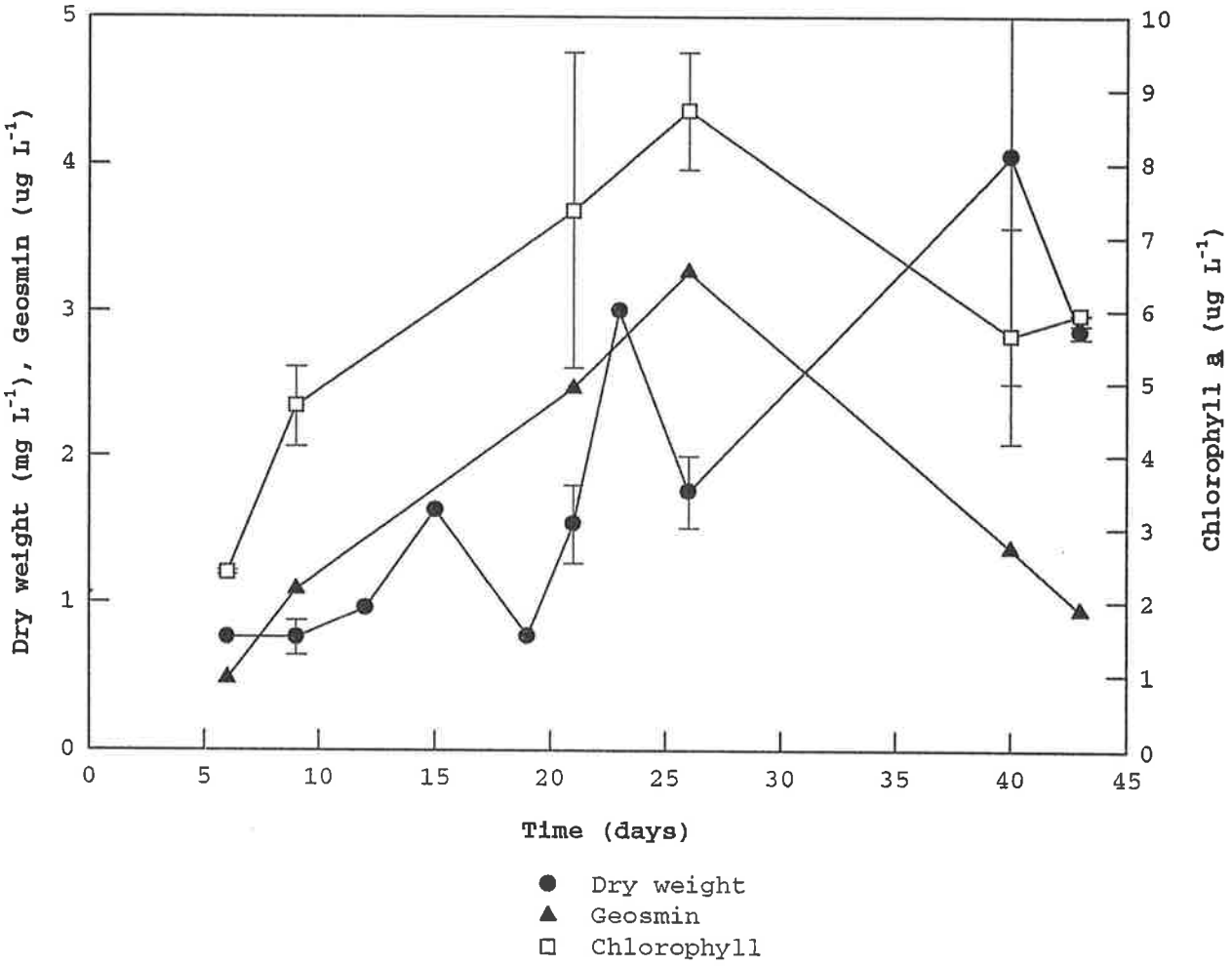

Fig. 5.3. Changes in dry weight, chlorophyll a and geosmin over time in a culture of *A. circinalis* 852E illuminated at 17 uM m^{-2} s^{-1}. Symbols are the mean of duplicate measurements whose range is indicated by a bar. No range bar indicates an un-replicated measure. Geosmin concentration is presented as ug L^{-1} and not ng L^{-1}.

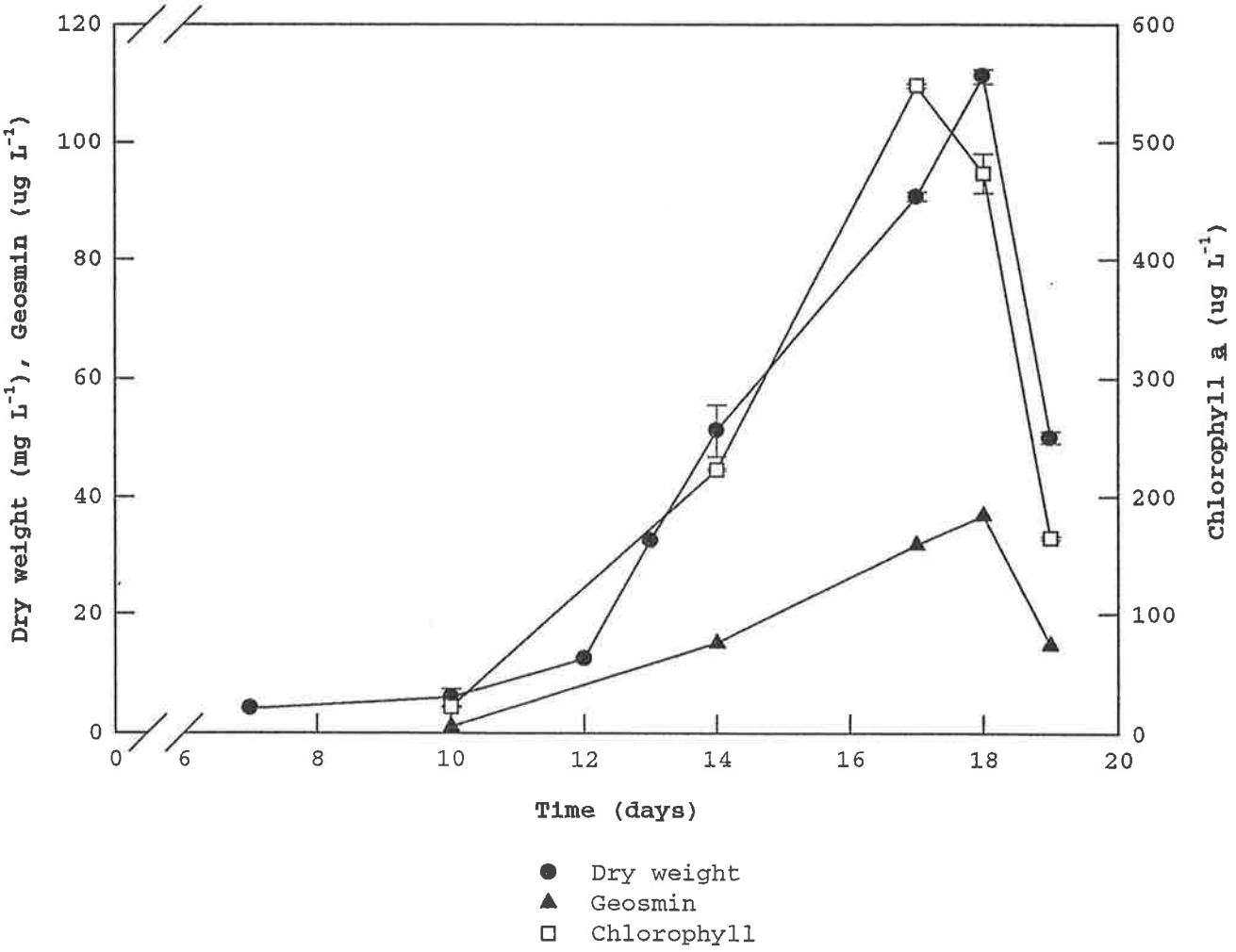

Fig. 5.4. Changes in dry weight, chlorophyll a and geosmin over time in a culture of *A. circinalis* 852E illuminated at 70 uM m^{-2} s^{-1}. Symbols are the mean of duplicate measurements whose range is indicated by a bar. No range bar indicates an un-replicated measure. Geosmin concentration is presented as ug L^{-1} and not ng L^{-1}.

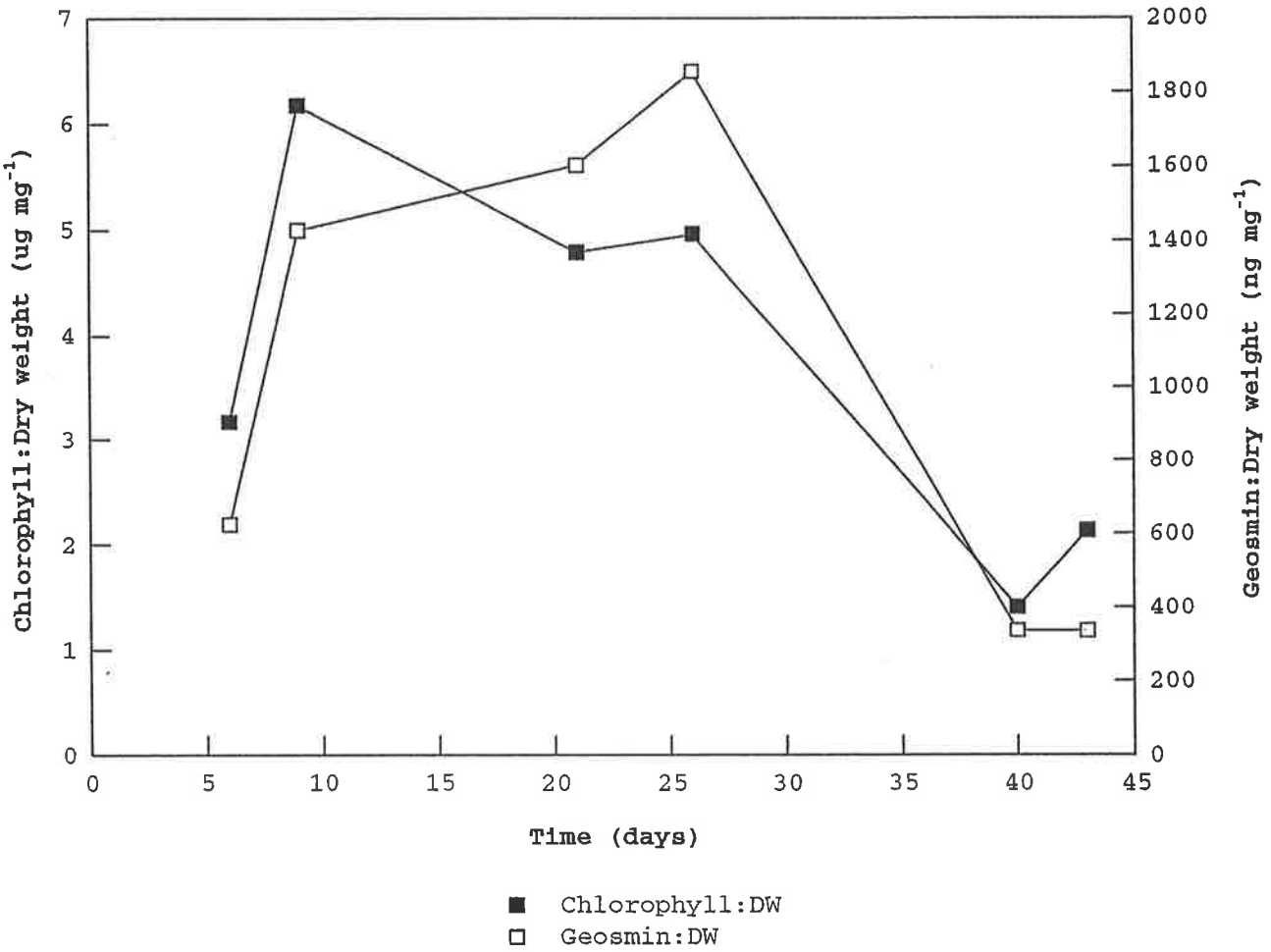

Fig. 5.5. The relationship between chlorophyll a and geosmin on a per dry weight basis over time at 17 uM m^{-2} s^{-2}.

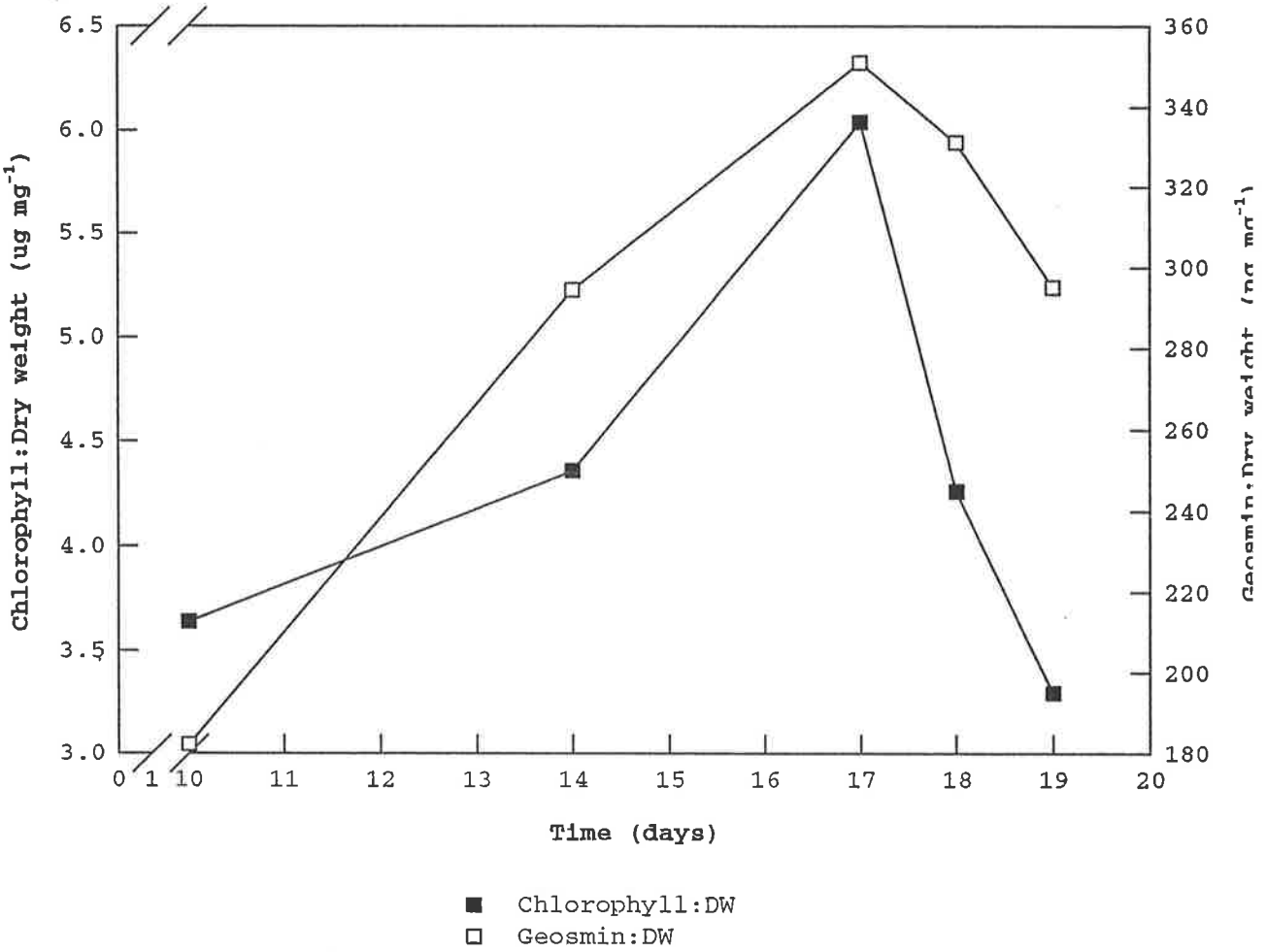

Fig. 5.6. The relationship between chlorophyll a and geosmin on a per dry weight basis over time at 70 uM m^{-2} s^{-1} (NB: axes do not commence at origin).

Similar differences in the geosmin to chlorophyll *a* ratio (G:Chl*a*) between both cultures were evident (Fig. 5.7). G:Chl*a* at 17 $\mu M\ m^{-2}\ s^{-1}$ was up to five times greater than in the other culture, and was consistently higher throughout the experiment.

The G:Chl*a* ratio at both light intensities are correlated with the specific rates of culture growth (k') calculated using changes in dry weight (negative growth rates were omitted). The highest G:Chl*a* ratios (>200 ng μg^{-1}) were obtained during periods of slowest cell growth (<0.1 ln units day^{-1}). These values were associated with the culture grown at 17 $\mu M\ m^{-2}\ s^{-1}$. The G:Chl*a* ratios for the culture at 70 $\mu M\ m^{-2}\ s^{-1}$ were all similar at around 50 ng μg^{-1} for growth rates ranging from 0.2 to 0.5 ln units day^{-1} (Fig. 5.7). These results indicate that at growth rates greater than 0.2 ln units day^{-1}, the G:Chl*a* ratio is constant, but then rapidly increases as k' declines to levels less than 0.2 ln units day^{-1}.

Absolute rates of geosmin synthesis were calculated for periods of increasing chlorophyll *a* concentration, and are summarised in Table 13. These estimates were obtained by dividing the change in geosmin concentration by the average chlorophyll *a* concentration during that period, and by the length of the period.

Rates of geosmin synthesis declined with culture age at both light intensities. Overall, higher rates of geosmin synthesis occurred at reduced light, with a maximum rate of 57 ng $\mu gChl a^{-1}\ day^{-1}$ measured on day 6 at 17 $\mu M\ m^{-2}\ s^{-1}$. The highest rate calculated for the culture at 70 $\mu M\ m^{-2}\ s^{-1}$ was half this value.

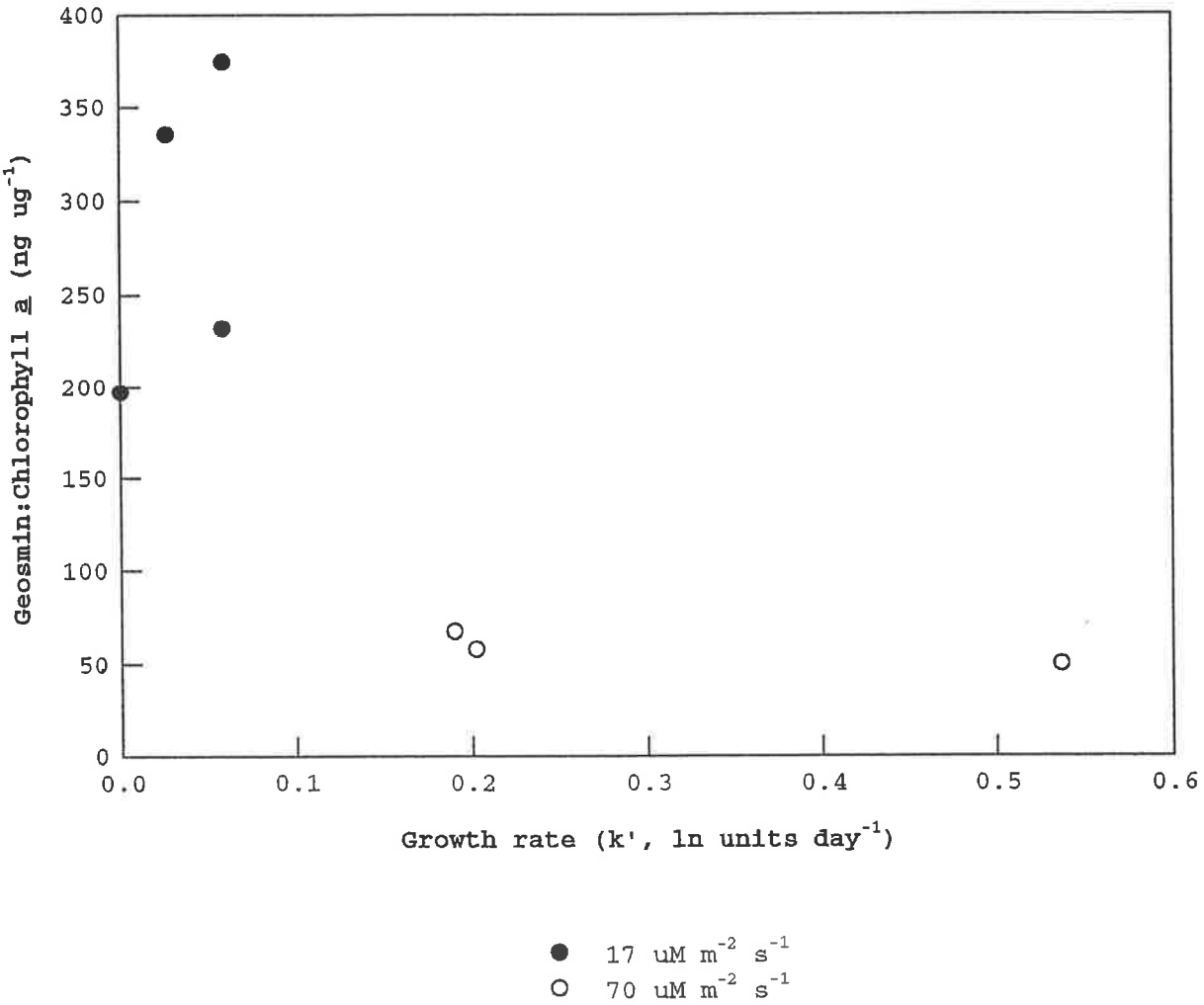

Fig. 5.7. The relationship between the geosmin:chlorophyll a ratio at different growth rates calculated as changes in dry weight.

17 µM m^{-2} s^{-1}		70 µM m^{-2} s^{-1}	
day	ng µg$Chl\underline{a}^{-1}$ day^{-1}	day	ng µg$Chl\underline{a}^{-1}$ day^{-1}
6	57	10	29
9	19	14	14
21	20	17	10

Table 13. Absolute rates of geosmin synthesis for cultures at 17 and 70 µM m^{-2} s^{-1}. Day number represents the end of the period used in calculations.

The partitioning of the total geosmin pool into intra-cellular and extra-cellular fractions for *A. circinalis* 852E grown at 17 μM m^{-2} s^{-1} is shown in Fig. 5.8. Except for day 6, extra-cellular geosmin always exceeded internal levels, ranging from 65% to almost 100% of the total. Furthermore, despite marked variation in dry weight, the intra-cellular geosmin pool varied little over the course of the experiment. An exception was on day 21 where internal geosmin was 3-4 times greater than at other times. This point coincided with a period of rapid cell growth commencing on day 19 (Fig. 5.8).

Geosmin content (ng mg^{-1} dry weight) was found to be dependent upon the age of the culture, where younger cells had a higher geosmin content than older cells (Fig. 5.9). This figure includes only days where geosmin was sampled, and note that the horizontal axis is not in relative proportion.

Quantities of geosmin ranged from over 400 ng mg^{-1} on day 6 down to less than 100 ng mg^{-1} by day 43. In one instance (day 40), cellular geosmin fell to almost zero, which coincided with the lowest chlorophyll a content per cell for this culture (Fig. 5.5). Furthermore, the highest level of cellular geosmin seen on day 21 coincides with the end of a period of rapid cell division (Fig. 5.8).

Results showing geosmin partitioning for the culture at 70 μM m^{-2} s^{-1} are not presented. The extraction procedure for geosmin was different to that used on the 17 μM m^{-2} s^{-1} culture, and the comparison of intra-cellular to extra-cellular levels of geosmin at 70 μM m^{-2} s^{-1} was not considered suitable.

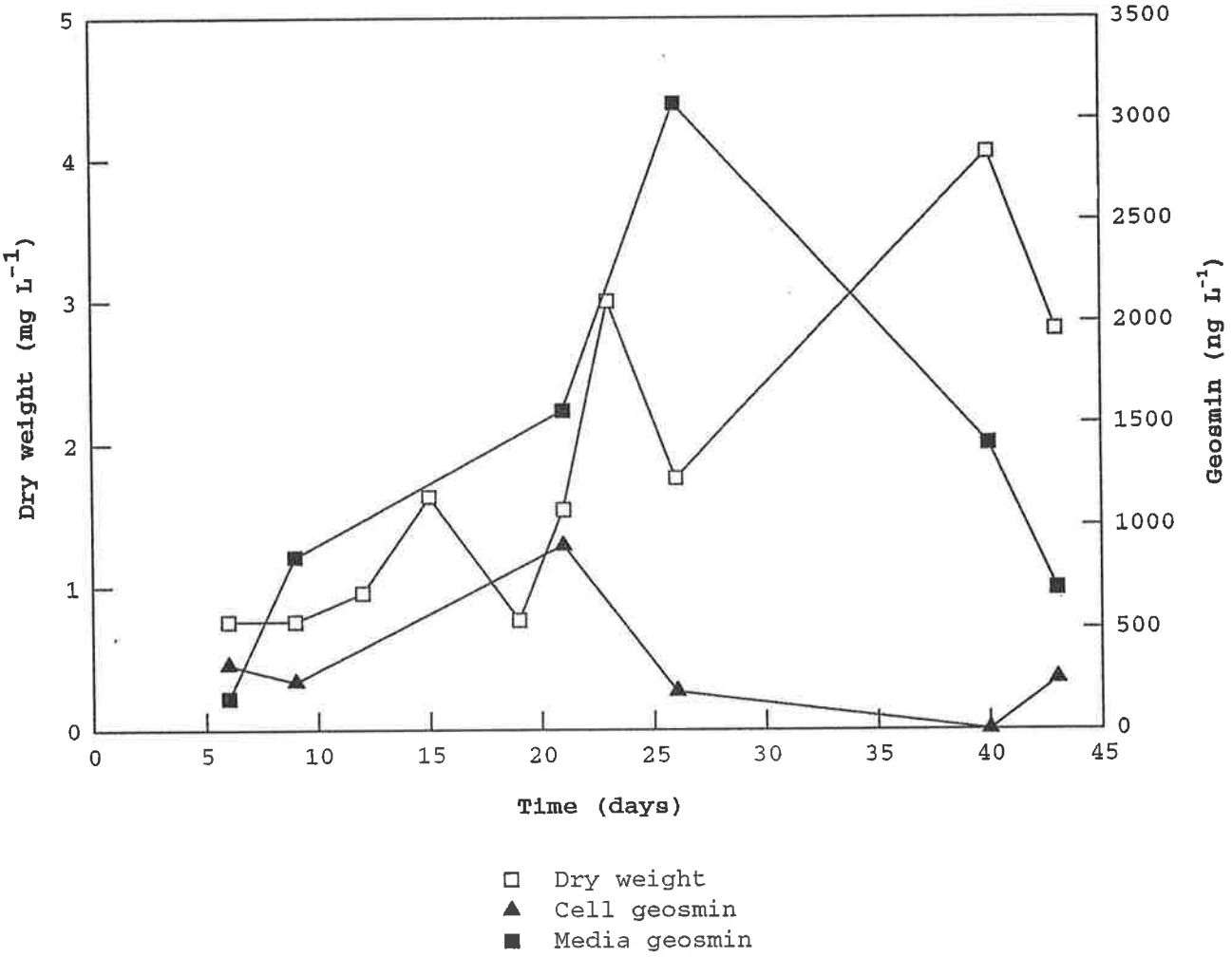

Fig. 5.8. Changes in the partitioning of geosmin into cell and media fractions over time at 17 uM m^{-2} s^{-1}.

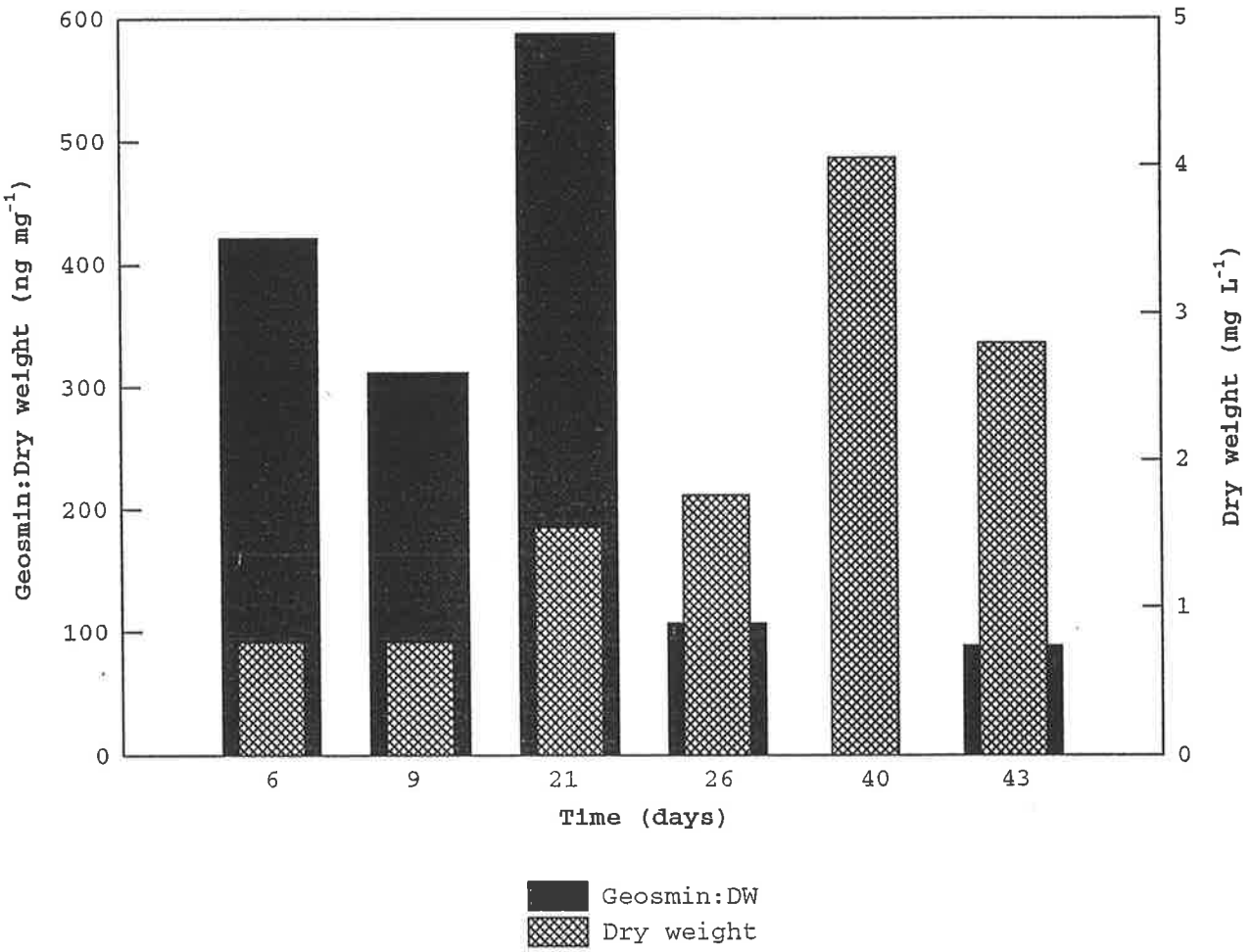

Fig. 5.9. The relationship between the geosmin:dry weight ratio and dry weight over time in *A. circinalis* 852E illuminated at 17 uM m^{-2} s^{-1}.

5.5 Discussion

The marked difference in the growth response of *A. circinalis* 852E at the two light intensities provided a wide range of growth conditions that may influence geosmin synthesis (Figs. 5.1 - 5.4). The very low levels of growth in the culture at 17 $\mu M\ m^{-2}\ s^{-1}$ over the entire 43 day period suggests that this light intensity is close to the light compensation point for this strain. This was reflected in slight increases in culture media pH, indicating very little uptake of carbon and therefore slow photosynthetic rate (Fig. 5.2).

In contrast, large fluctuations in pH (and therefore photosynthetic rate) at 70 $\mu M\ m^{-2}\ s^{-1}$ is paralleled with large changes in algal biomass. Reasons for pH trend reversals on days 13 and 14 are not known, as this does not coincide with changes in biomass expressed as either O.D., dry weight or chlorophyll *a*, and cultures were sampled early morning on every sampling day. Consequently, it is not clear whether these two values are truly representative of media pH, or are a result of errors on either day 13 or 14.

The long lag phase at 70 $\mu M\ m^{-2}\ s^{-1}$ (Fig. 5.1) may be due to light shock experienced by cells at the commencement of the 4.5 L culture. Although the inoculating culture was grown at a similar light intensity (70-100 $\mu M\ m^{-2}\ s^{-1}$) to that of the experimental conditions, high cell density in the inoculation culture may have led to self shading. This would have resulted in these cells experiencing a much lower effective light intensity, and light shock on transfer to the new conditions.

Geosmin was measured in cultures at both light intensities throughout the trial, and was detected on each day cultures were sampled (Figs. 5.3 and

5.4). These results show that under these experimental conditions, the production of geosmin by *A. circinalis* 852E occurred continuously, and was not activated or de-activated by a particular stage of growth.

Changes in total geosmin concentration over time in both cultures was generally related to algal biomass. This merely reflects the capacity of the total population to synthesise geosmin, and is further reflected in substantially higher concentrations being attained at 70 $\mu M\ m^{-2}\ s^{-1}$, where biomass was greater throughout the trial (Figs. 5.3 and 5.4).

More specifically, the synthesis of geosmin was found to be directly related to algal biomass measured as chlorophyll *a* at both light intensities (Figs. 5.5 and 5.6). This finding is similar to that of Naes *et al.* (1985) studying geosmin synthesis by *O. brevis* at two light intensities. Furthermore, these results support the hypothesis of a similar biosynthetic pathway for chlorophyll *a* and geosmin proposed by Bentley and Meganathan (1981).

The inclusion of an algal culture at 17 $\mu M\ m^{-2}\ s^{-1}$ in this study was intended to provide a wider range of conditions to test the relationship between geosmin and chlorophyll *a* synthesis. The reduced light intensity was expected to stimulate chlorophyll *a* production (i.e. increase the Chl*a*:DW ratio) as an adaptive response to low light, which would be correlated to changes in geosmin concentration.

However, a reduction in light intensity from 70 to 17 $\mu M\ m^{-2}\ s^{-1}$ had little effect on the Chl*a*:DW ratio. Levels ranged from between 1 and 6 $\mu g\ mg^{-1}$ in both cultures, usually averaging 3-5 $\mu g\ mg^{-1}$ over the whole trial (Figs. 5.5 and 5.6). Despite similar chlorophyll *a* contents, substantially greater quantities of geosmin on a per weight basis (G:DW) were measured at

17 μM m^{-2} s^{-1}. These levels were 5 to 6 time greater than at 70 μM m^{-2} s^{-1} (compare Figs. 5.5 and 5.6). This difference is also reflected in the G:Chl*a* ratios, where values were 3 to 6 times greater at the lower light intensity (Fig. 5.7).

These findings show that geosmin synthesis with respect to chlorophyll *a* (G:Chl*a* ratio) is not constant, where greater quantities of geosmin are produced per unit chlorophyll *a* under reduced light intensity. This result strongly indicates that although geosmin and chlorophyll *a* synthesis share a partly similar biosynthetic pathway, additional factors may be controlling geosmin production. Alternatively, indicators other than chlorophyll *a* may be more closely associated with geosmin synthesis.

Chlorophyll *a* is only one of many pigments synthesised by cyanobacteria. Others include phycobiliproteins, and carotenoids (including xanthophylls), of which ß-carotene is often the major pigment in the latter category (Goodwin 1974). The relative cellular concentrations of these pigments is known to be a function of taxonomic grouping, and of external factors including light intensity and quality (Fogg *et al.*, 1973; Wyman and Fay, 1987).

Although changes in light intensity in these experiments did not markedly alter levels of cellular chlorophyll *a*, it may have altered the concentrations of other pigments. For example, ß-carotene in many cyanobacteria is often synthesised as a protective measure against harmful radiation levels, especially towards the UV end of the spectrum (Paerl, 1984; Paerl *et al.*, 1983; Millie *et al.*, 1990). Furthermore, the phycobiliprotein content of cells is inversely related to light intensity (Wyman and Fay, 1987). Like geosmin, these pigments are synthesised by the

isoprenoid pathway, and it is not unlikely that a relationship between geosmin and the production of these other pigments also exists.

Naes *et al.* (1989) have demonstrated through the use of selective metabolic inhibitors, a coupling of geosmin to ß-carotene synthesis in *O. brevis*, and were able to increase the production of geosmin relative to chlorophyll a during nitrogen limited growth. Furthermore, they concluded that isoprenoid precursors are directed towards geosmin production during restricted pigment synthesis. Utkilen and Froshaug (1992) also demonstrated a strong link between geosmin and ß-carotene synthesis in two species of *Oscillatoria* over a range of light intensities. In addition, they showed that the geosmin:ß-carotene ratio remained approximately constant under these varying conditions.

Interactions between pigment and geosmin synthesis in *A. circinalis* 852E may explain the marked increase in the G:Chla ratio seen at low light. Although light reduction did not result in significant increases in cellular chlorophyll a, increases in the production of other pigments may result in isoprenoid precursors being directed towards geosmin synthesis. This would result in higher concentrations of geosmin being measured in culture relative to chlorophyll a.

Investigations on geosmin production by *Fischerella muscicola* at different temperatures and under aerobic and anaerobic conditions concluded that geosmin synthesis and biomass production (growth) were inversely related (Wu and Juttner, 1988b). A similar general conclusion could be drawn for geosmin production by *A. circinalis* 852E.

This is further highlighted by higher absolute rates of geosmin synthesis (with units of ng $\mu g Chla^{-1}$ day^{-1}) calculated at 17 μM m^{-2} s^{-1}. These rates

decrease with increasing culture age, which is analogous to less favourable growth conditions.

Changes in the rate of geosmin synthesis with growth rate and light intensity are similar to changes in the net rates of chlorophyll a and phycobiliprotein synthesis observed in several types of blue-green algae (Wyman and Fay, 1987). In severe to moderate light limited cells (<20 μM m^{-2} s^{-1}) net rates of chlorophyll a and phycobiliproteins are found to vary with the rate of growth. However at higher irradiances, growth increases at a more rapid rate than the relative rates of chlorophyll a and phycobiliprotein synthesis.

This is consistent with the changes in geosmin production by *A. circinalis* 852E observed in this study. That is, G:Chla was much greater under reduced light, and was more dependent upon the rate of growth than at the higher light intensity. Furthermore, it offers an explanation as to why G:DW was significantly lower at 70 μM m^{-2} s^{-1}, where the overall rate of growth was much greater.

Although the capacity of *A. circinalis* 852E to produce geosmin gives an indication of the potential magnitude of odour problems, it is not until geosmin is released into the environment that such problems are manifested. At 17 μM m^{-2} s^{-1}, this study showed that a large proportion of geosmin was immediately released from cells and that this release was not related to a particular stage of growth but seemed to increase with culture age (Fig. 5.8).

In addition, the total cellular geosmin pool remained relatively constant throughout the growth of the culture, despite increases in biomass over

time (Fig. 5.8). This was achieved by a corresponding decrease in the actual quantity of geosmin contained within each cell, expressed as ng mg^{-1}, hence negating the effect of increasing biomass.

The very high levels of geosmin present within cells on day 21 (Fig. 5.9) was associated with a period of rapid cell division and increasing biomass (Fig. 5.8). It is thought that this elevated measure reflects a temporary increase in cellular geosmin due to a lag in the diffusion/excretion of geosmin from the cell.

The release of geosmin from cells of *A. circinalis* 852E grown at 17 µM m^{-2} s^{-1} is in marked contrast to other reports published in the literature. With the exception of day 6 (Fig. 5.8) where 66% of geosmin was contained within the cells, the majority of geosmin for the remainder of the experiment was found dissolved in the media. However, studies on geosmin released by *O. brevis* and *O. bornetii* (Utkilen and Froshaug, 1992), *O. tenuis* (Wu and Juttner, 1988a), *Fischerella muscicola* (Wu and Juttner, 1988b), *Anabaena macrospora* (Miwa and Morizane, 1988) and *A. circinalis* (Rosen *et al.*, 1992) have all shown that at least 90% of geosmin was cell bound.

The release of geosmin from cells in these cases was associated with both the physiological stress of cells (Miwa and Morizane, 1988), and a decline in the "health" of the culture with age, ultimately leading to cell lysis and release of geosmin (Rosen *et al.*, 1992).

In the context of these findings, it is very likely that the culture of *A. circinalis* 852E grown at 17 µM m^{-2} s^{-1} was severely stressed by light limitation throughout this trial. As a result, geosmin was continuously

released from cells, and was not retained as is often the case in non-stressed cultures.

Although the determination of the intracellular geosmin pool at 70 $\mu M\ m^{-2}\ s^{-1}$ could not be completed, two *tentative* estimates on days 10 and 14 were calculated to compare geosmin retention by cells grown under favourable light conditions. Representing the commencement and mid-period of exponential growth (Fig. 5.4), 71% and 80% of geosmin was located in the cellular pool, respectively. This higher proportion during early growth supports the view that non-stressed cells retain the majority of geosmin synthesised, and it is released with the onset of stress and senescence.

The onset of stress in previous studies was either surmised through the combination of several factors as the culture approached senescence (Rosen *et al.*, 1992), or was induced by the removal of chelating agents from the media (Miwa and Morizane, 1988). This study has shown that light limitation is an additional factor that can accelerate the release of geosmin from cells.

Through this study, light has been found to interact with the production of geosmin by *A. circinalis* 852E in two important ways. Firstly, low light intensity causes a marked increase in the rate of geosmin synthesis. This has the potential to increase the quantity of geosmin synthesised by a factor of five, and therefore the magnitude of the problem i.e. higher geosmin concentrations in drinking water results in a greater proportion of the population being affected.

From a different perspective, higher rates of geosmin synthesis enables problematic concentrations of geosmin (>20 ng L^{-1}) to be produced by lower levels of algal biomass. For example, an absolute rate of geosmin

production of 10-60 ng $\mu gChla^{-1}$ day^{-1} (Table 13) requires chlorophyll *a* levels of 0.03-2 $\mu g\ L^{-1}$ to synthesise geosmin to a concentration of 20 ng L^{-1}. This corresponds to 400-2300 cells mL^{-1}.

Secondly, stressing of cells by limiting light results in the earlier release of geosmin into the environment. This outcome has very important management implications. The commencement of geosmin release at an earlier stage results in a gradual and therefore prolonged release. Consequently the period of time at which high geosmin levels are in the water is also increased, affecting a greater proportion of consumers for a longer period of time.

An additional consequence of earlier geosmin release is that water treatment plants that are able to remove algal cells (e.g. dissolved air flotation systems) will have limited impact in contributing to the abatement of odour problems. Although these systems are able to remove algal cells, they are unable to remove dissolved substances. However such systems will have a significant role in the removal of geosmin from drinking water when it is still contained within the cells.

The impact of light on the production and release of geosmin by *A. circinalis* 852E and other species will be an important consideration in all habitats where these odour-producers are found. In transparent water bodies, light limiting conditions can result from either self-shading through excessive algal growth, an increase in mixed depth due to destratification, heavily overcast periods and increases in water turbidity. These factors can either individually or in combination affect light levels to varying extents.

A characteristic feature of Australian waters is their turbidity and high colouring due to silting and dissolved organic matter. Together these result in lowered light penetration, and are therefore more likely to result in light limitation leading to cell stress. This may encourage higher rates of geosmin production, and extend the period in which water quality problems prevail.

Chapter 6

The relationship between the production of geosmin and chlorophyll a by *A. circinalis* 852E.

6.1 Introduction

The previous chapter demonstrated a relationship between geosmin production and chlorophyll a. In addition, it showed that geosmin was produced at a greater rate relative to chlorophyll a at lower growth rates and reduced light intensity, and that the absolute rate of geosmin production (ng μgChla^{-1} day^{-1}) also decreased with increasing growth rate.

This outcome was based on a limited number of points from an experiment designed to address another question, primarily, was geosmin continuously produced throughout the growth of the culture?. A wider range of growth (light) conditions is required to strengthen the case that these relationships are significant.

The primary aim of this chapter is to examine the relationship between geosmin and chlorophyll a synthesis. More specifically, the relationship between the variability in the rate of geosmin production with respect to chlorophyll a and growth rate will be examined. This will be achieved by comparing geosmin production to that of algal biomass in cultures grown under a wide range and number of light intensities. Varying light intensity will have the dual effect of altering the chlorophyll a content of cells (the chlorophyll a:dry weight ratio, Chla:DW) and varying the rates of growth of the populations.

One concern from chapter 5 is related to the experimental conditions. That is, cultures were grown and sampled for up to 43 days, and the continuous aeration with compressed air to ensure complete mixing of the culture may have removed some geosmin from solution leading to some accumulative loss. The significance of this loss is, however, unknown.

Finally, estimates of rates of geosmin production to be of practical (management) use need to be in a form that is applicable to the field. Units of dry weight and chlorophyll biomass, although of importance in laboratory studies, are not suitable field units as they do not target specific algal types in the phytoplankton community. The most applicable and specific measure of algal biomass is cell density (cells mL^{-1}). Another aim of this chapter is to allow the rates of geosmin production obtained from this laboratory study to be translated to the field.

6.2 Objectives

The aims of this chapter are to:

1. Examine the relationship between geosmin and chlorophyll *a* production by *A. circinalis* 852E under a wide range of light conditions in the laboratory.

2. To estimate the loss of geosmin from cultures due to aeration.

3. Determine the relationships between dry weight and chlorophyll *a* and cells mL^{-1} to enable transfer of laboratory estimates of geosmin production to fields conditions.

6.3 Methods

A culture of *A. circinalis* 852E (16 L) was grown in a glass carboy filled with sterile WC Media at 150 $\mu M\ m^{-2}\ s^{-1}$ under continuous illumination. The culture was mixed with compressed air and maintained at 20°C. After 5 days, the culture was divided into six 3 L Erlenmeyer flasks filled to a volume of 2 L. One flask was placed at each of the following light intensities: 20, 40, 60, 80, 100 and 150 $\mu M\ m^{-2}\ s^{-1}$. All cultures were: constantly illuminated from below; constantly agitated with air; and maintained at 20°C.

Light intensity was adjusted by placing 30 cm x 30 cm pieces of shade cloth over a transparent base supporting the cultures. Intensity was measured by holding the light sensor 10 cm above the base and pointing it directly towards the light source. This was repeated in five different places to ensure that the correct light intensity was obtained.

To measure geosmin loss due to aeration, flasks were sealed with a 7.5 cm diameter rubber bung with two holes drilled near the centre. One hole allowed access to a glass tube delivering compressed air for agitating the culture. A short (10 cm) glass tube was forced through the second hole which allowed air to escape. To the end of this tube was fitted an activated carbon filter, consisting of a 5 cm length of glass tube (ID 3 mm) containing 100 mg of activated carbon held in place with glass fibre.

Cultures were sampled at approximately two day intervals (exact times 1.75, 3.75 and 5.81 days) after commencing exposure to the six different light treatments. A 600 mL sample was removed each time for the duplicate analysis of chlorophyll *a*, dry weight and total geosmin (after pre-treating with 69 mg L^{-1} $CuSO_4$-Cu for 1 hr). In addition, a 30 mL sample was preserved with Lugol's iodine for the determination of cell density. The original 16 L culture was also measured as described above immediately prior to splitting (day 0). Analysis for dry weight, chlorophyll *a*, geosmin and cell density are as described in chapter 2.

On each sampling day, the activated carbon filters were removed and replaced with a clean re-activated filter. Used filters were eluted into 2 mL micro-vials using 1 mL carbon disulphide. Each filter was eluted three times and each elution stored in a separate vial at -15°C until analysed by GC/MS.

There was no replication in this experiment, where only one culture was grown at each of the six light intensities. Although replication would strengthen any conclusions derived from the data, it was considered that the number and range of light intensities selected, in addition to the three sampling periods, would provide a broad range of conditions and data with which to address the objectives.

6.4 Results

Algal biomass measured as dry weight and chlorophyll *a* increased exponentially from the time cultures were initiated on day 0 (Figs. 6.1 and 6.2, respectively). Between days 0-2, however, the dry weight of cultures at 20 and 40 $\mu M\ m^{-2}\ s^{-1}$ declined slightly prior to attaining exponential

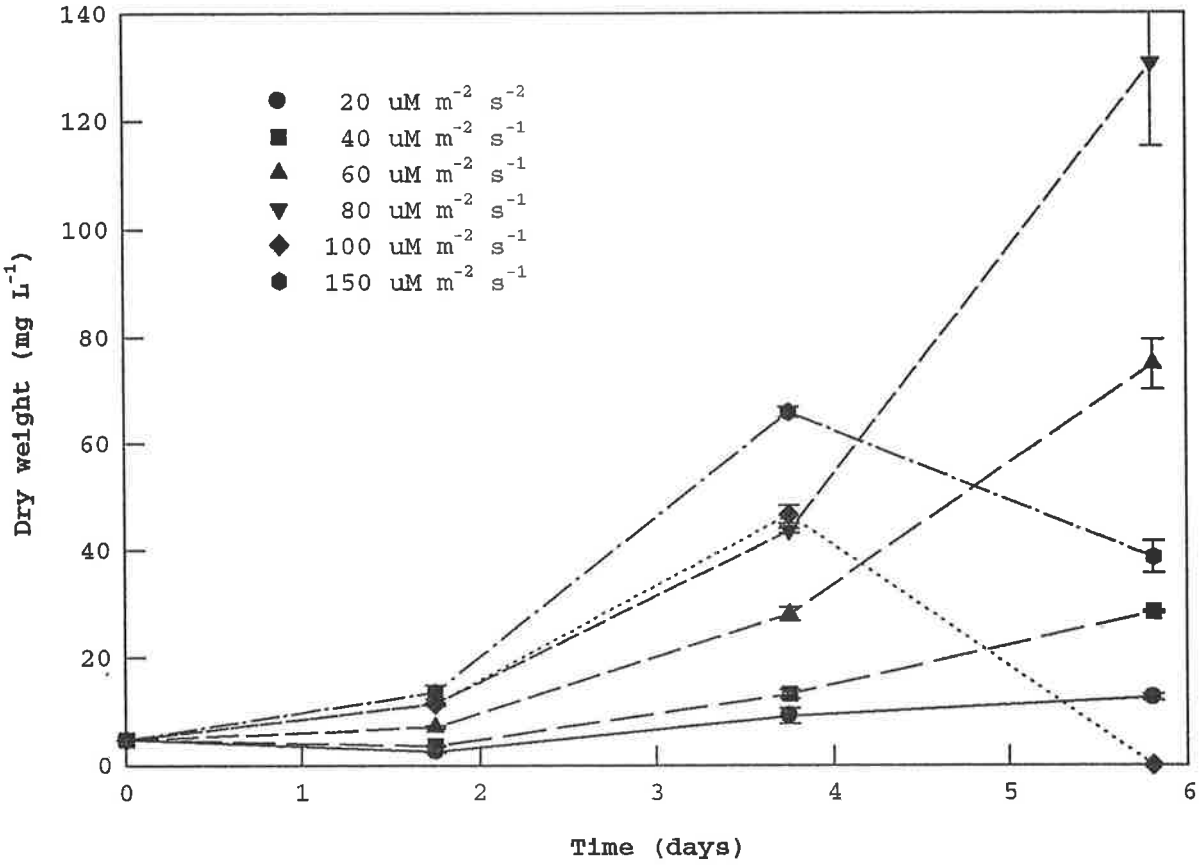

Fig. 6.1. Changes in dry weight over time in cultures of *A. circinalis* 852E illuminated at a range of light intensities. Values are the mean of duplicate measures whose range is indictated by a bar.

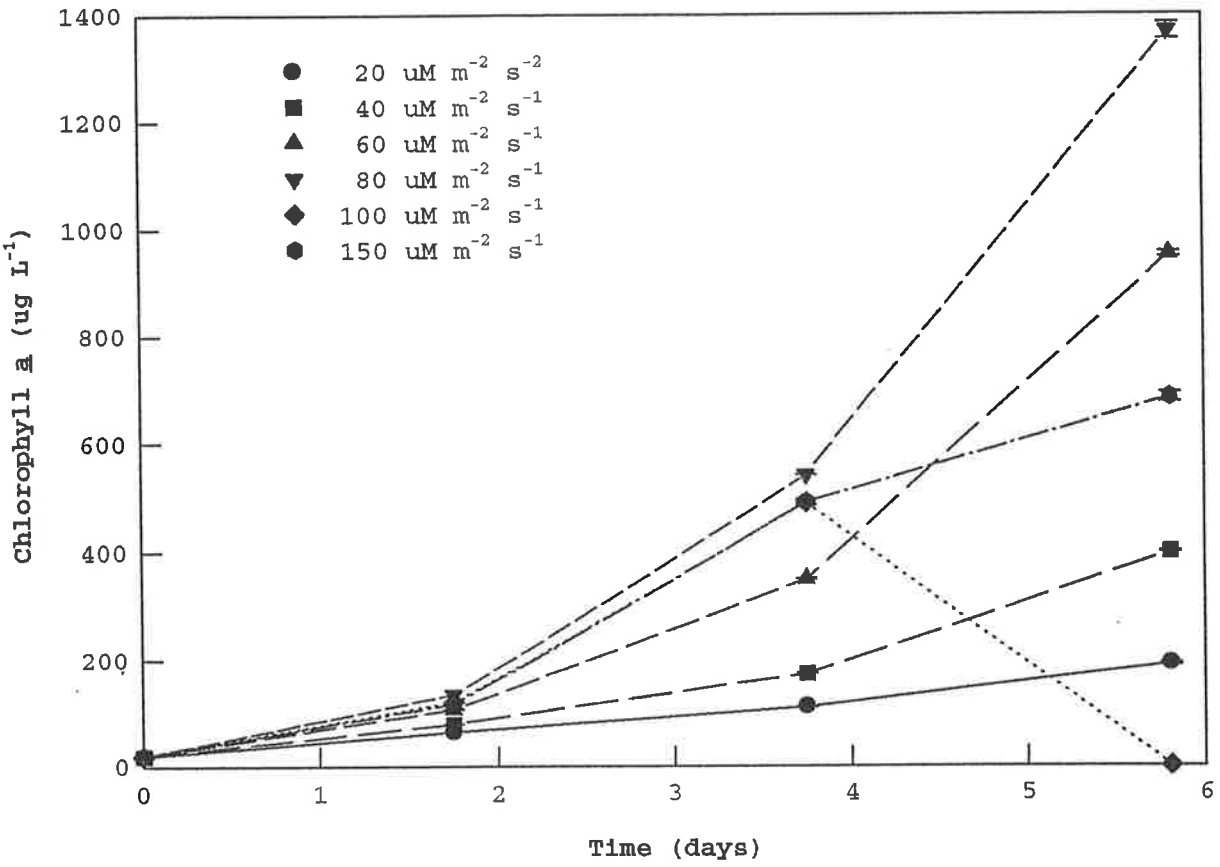

Fig. 6.2. Changes in chlorophyll a over time in cultures of *A. circinalis* 852E illuminated at a range of light intensities. Values are the mean of duplicate measures whose range is indicated by a bar.

growth. Cultures illuminated at 20, 40, 60 and 80 μM m^{-2} s^{-1} maintained exponential growth until day 6 of the experiment. By contrast, cultures at 100 and 150 μM m^{-2} s^{-1} were in a state of decline by day 6, particularly at 100 μM m^{-2} s^{-1} where total collapse of the culture did not allow the measurement of biomass (dry weight, chlorophyll a, cell density) or geosmin on day 6. The doubtful condition of the cultures at 100 and 150 μM m^{-2} s^{-1} between days 4 and 6 precluded them from further consideration over this period. However, data from these cultures between days 0 and 4 was used as their growth characteristics and general appearance did not appear abnormal over this period.

The increase in dry weight and chlorophyll a throughout the experiment was proportional to the light intensity selected (Figs. 6.1 and 6.2). Maximum biomass was obtained at 80 μM m^{-2} s^{-1} on day 6 where 131 mg L^{-1} dry weight and 1,367 μg L^{-1} chlorophyll a were measured.

The effect of light intensity on the rate of culture growth (k', ln units day^{-1}) is shown in Fig. 6.3. Growth rate increased in proportion to light intensity, ranging from 0.647 ln units day^{-1} at 20 μM m^{-2} s^{-1} to 0.793 ln units day^{-1} at 150 μM m^{-2} s^{-1}. Growth rates were calculated on the basis of dry weight using values measured on days 2 and 4, which is the period of maximum growth at all light intensities.

Algal biomass measured as cells mL^{-1} is related to dry weight and chlorophyll a as shown in Figs. 6.4 and 6.5, respectively. Cells mL^{-1} and chlorophyll a are directly and linearly related throughout the range of values measured. There is a good linear relationship between dry weight and cells mL^{-1} below 30 mg L^{-1}, however there is greater variability at higher levels of dry weight (Fig. 6.4).

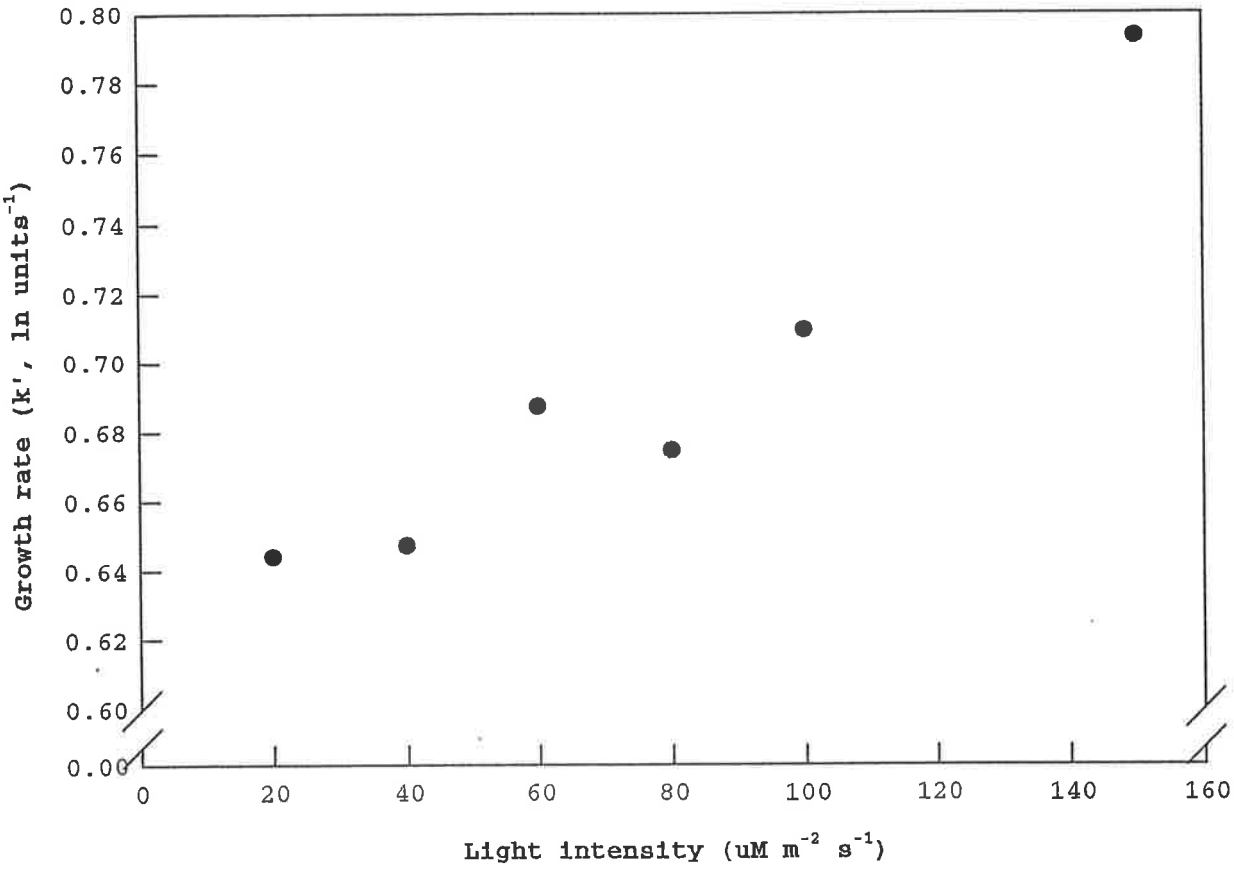

Fig. 6.3. The effect of light intensity on growth rate, calculated using the increase in dry weight between days 2 and 4 at all light intensities.

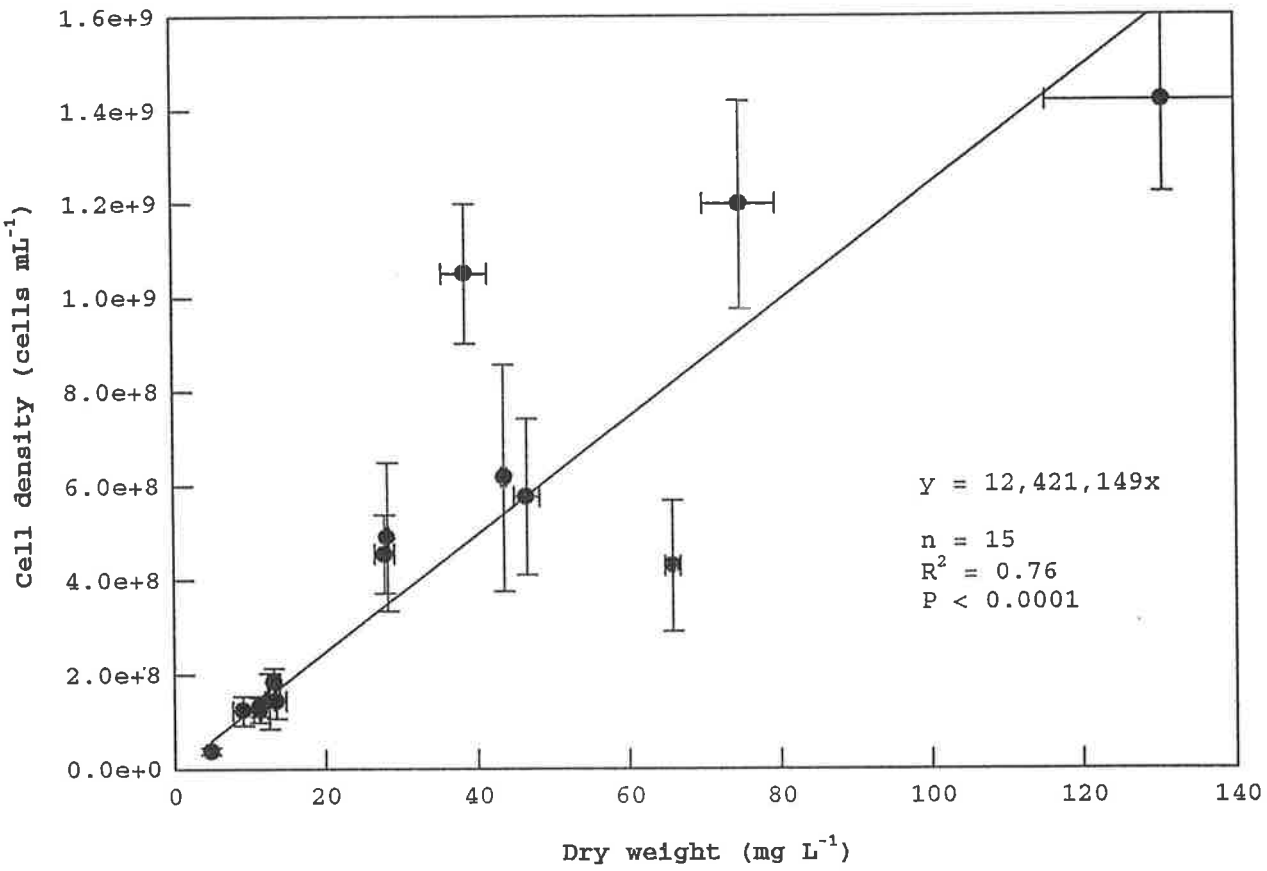

Fig. 6.4. The relationship between dry weight and cell density. Error bars for cell density represent standard deviation of five replicate counts; dry weight error bars represent the range of two replicates.

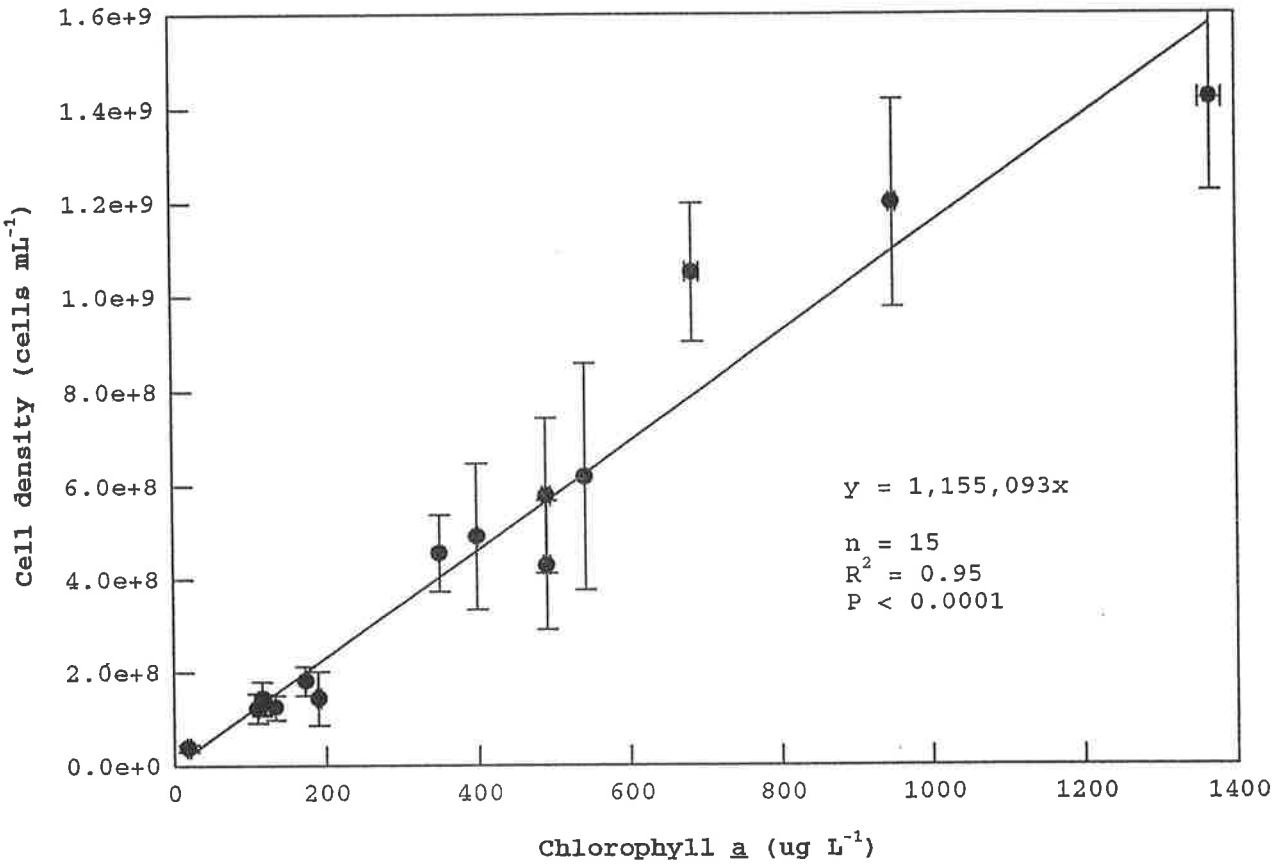

Fig. 6.5. The relationship between chlorophyll a and cell density. Error bars for cell density represent standard deviation of five replicate counts; chlorophyll error bars represent the range of two replicates.

The overall aim of growing cultures at different light intensities was to obtain a range of cell chlorophyll *a* to dry weight ratios (Chl*a*:DW) and to relate this to geosmin production. The range of light intensities selected had a marked effect on Chl*a*:DW, as did the age of the cultures (Fig. 6.6). Between days 0 and 2, Chl*a*:DW increased between 2 and 7 fold, with the greatest increase occurring at the lowest light intensity (20 $\mu M\ m^{-2}\ s^{-1}$) where 25 $\mu g\ mg^{-1}$ was measured. By day 4, the Chl*a*:DW ratio at 20, 40 and 60 $\mu M\ m^{-2}\ s^{-1}$ had declined to values similar to those at 80, 100 and 150 $\mu M\ m^{-2}\ s^{-1}$. By day 6, the Chl*a*:DW ratio had increased slightly at lower light intensities, however the variability between all light treatments was not as great at seen on day 2.

Geosmin was measured on all sampling days and at all light intensities throughout the experiment. The loss of geosmin from solution, determined by the quantity of geosmin trapped on the activated carbon filter on the air vent of each flask, was minimal (Fig. 6.7). Expressed as a percentage of the total geosmin lost to total geosmin produced by each sampling day for each culture, only 1-3% of geosmin was removed from solution due to aeration. Only in one instance was this range exceeded, when almost 12% was lost from solution. The proportion of geosmin lost was constant in all cases, despite the wide range (2,500-36,000 ng L^{-1}) of geosmin concentrations throughout the cultures. The measures of geosmin presented throughout the remainder of this chapter have been corrected for these losses.

The total concentration of geosmin in solution increased significantly over time in all cultures, attaining a maximum concentration of over 35,000 ng L^{-1} on day 6 at 80 $\mu M\ m^{-2}\ s^{-1}$ (Fig. 6.8). Changes in geosmin concentration parallelled changes in algal biomass measured as both chlorophyll *a* and dry weight (Figs. 6.1 and 6.2).

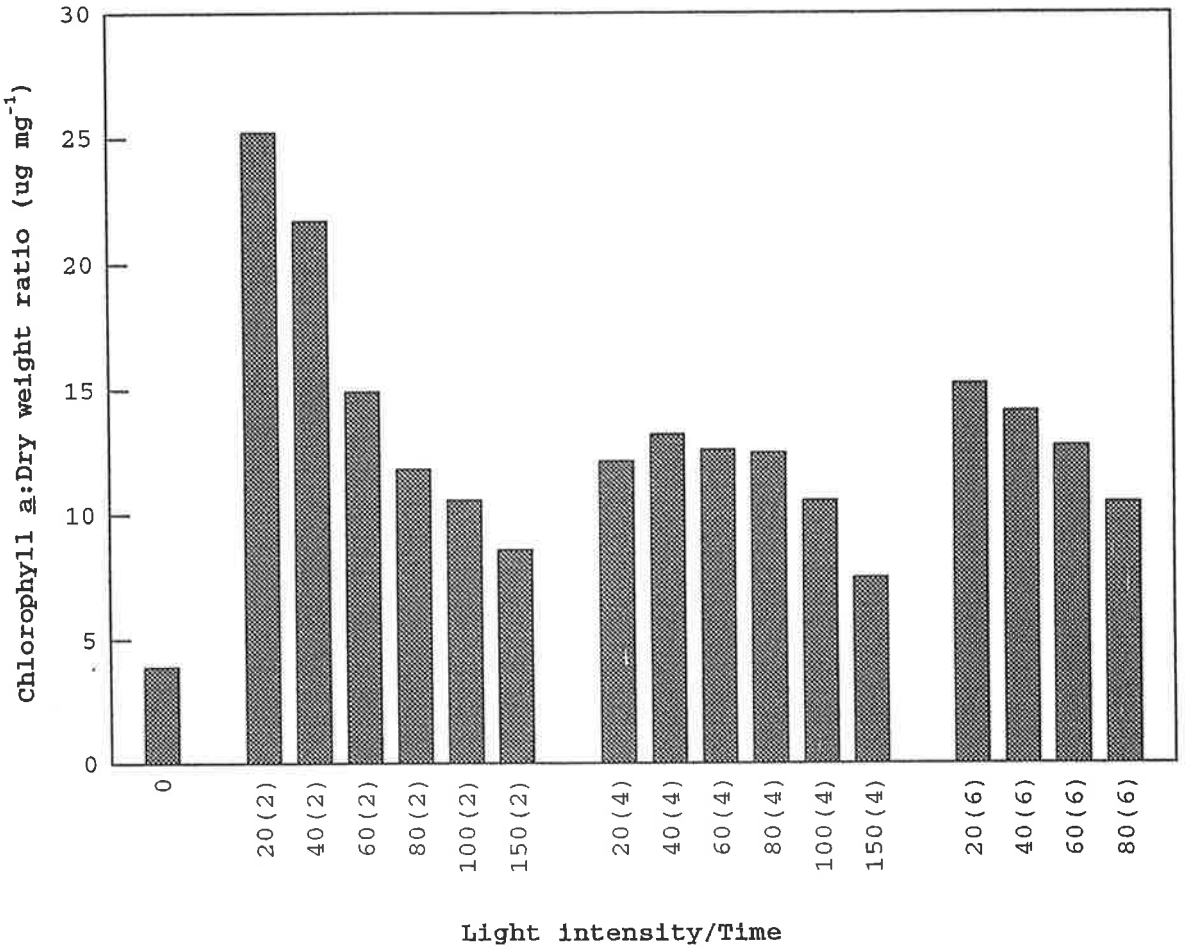

Fig 6.6. Changes in the chlorophyll a:Dry weight ratio in cultures at all light intensities and sampling times (unbracketed No. = light intensity (uM m^{-2} s^{-1}); bracketed No. = sample day; cultures at 100(6) and 150(6) senesced and were omitted).

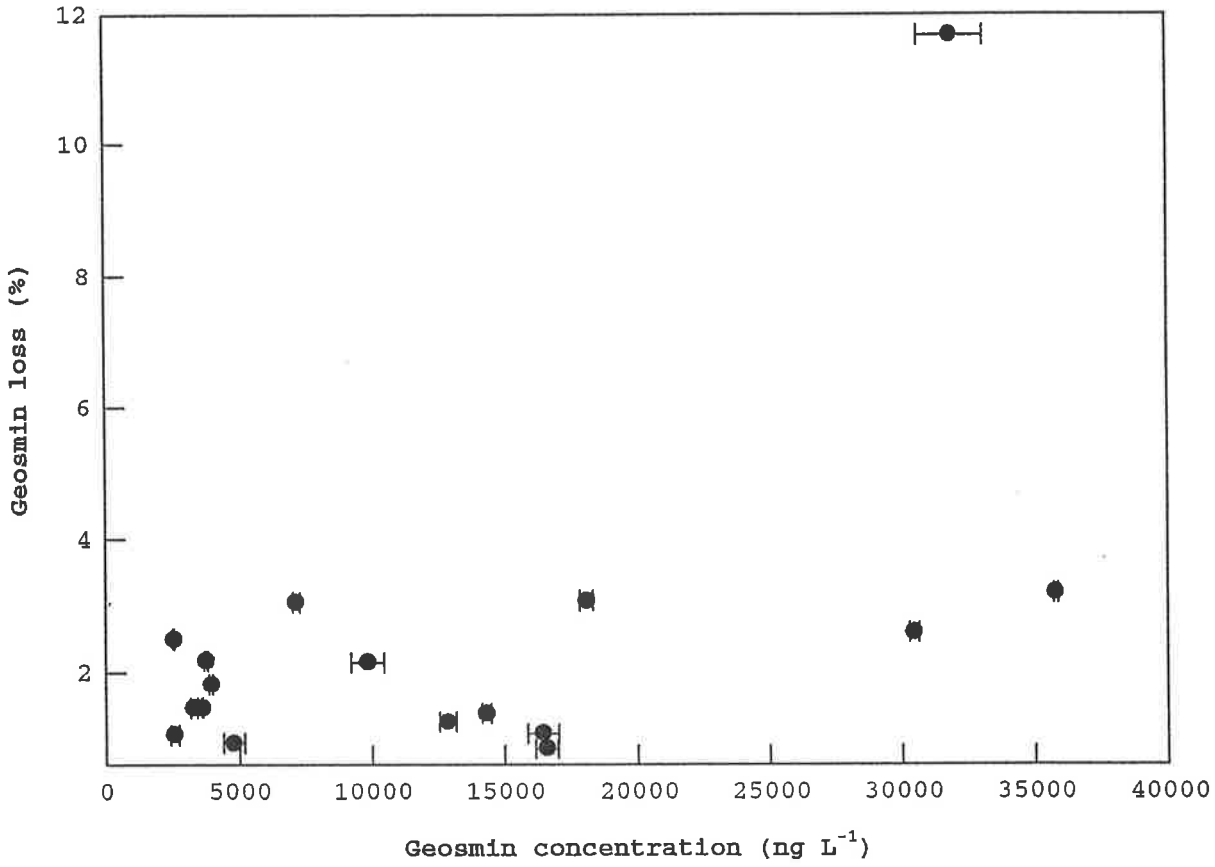

Fig. 6.7. Loss of geosmin from all cultures at the end of each sampling period (horizontal error bars represent the range of two replicate measures).

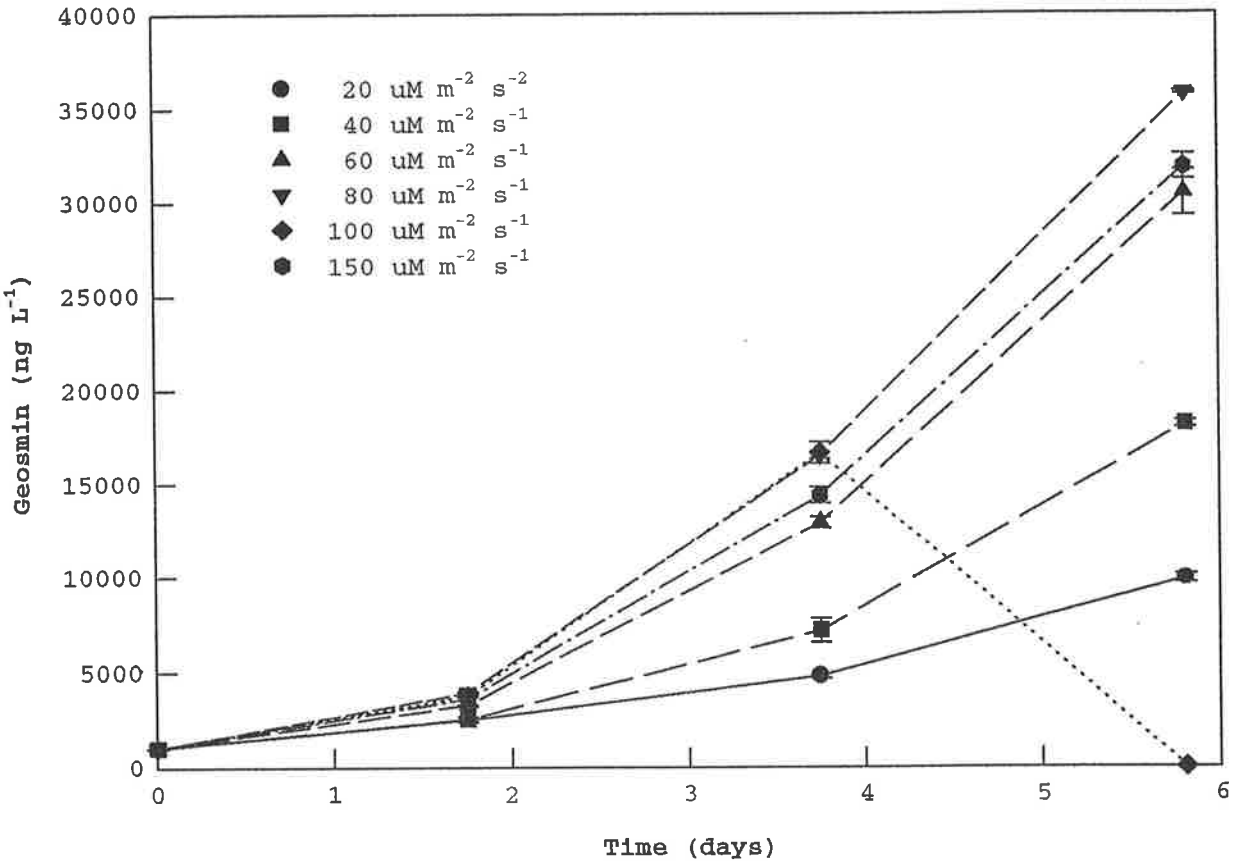

Fig. 6.8. Changes in geosmin concentration over time in cultures of *A. circinalis* 852E illuminated at a range of light intensities. Values are the means of duplicates whose range is indicated by a bar.

The relationship between geosmin and algal biomass is better compared by directly plotting geosmin concentration measured at all light treatments on all sampling days against dry weight and chlorophyll *a* concentrations, as shown in Figs. 6.9 and 6.10, respectively. At low levels of dry weight (<30 mg L^{-1}), there is a good relationship between dry weight and geosmin concentration (Fig. 6.9). Above 30 mg L^{-1}, the relationship is less convincing. By contrast, there is a better relationship between chlorophyll *a* and geosmin concentration throughout this range (Fig. 6.10). In both instances, it appears that the relationship between biomass and geosmin concentration is not linear, but asymptotic i.e. there seems to be proportionately less geosmin present at higher biomass than at lower biomass levels.

Differences in the production of geosmin to that of the corresponding biomass is seen by comparing the increase in geosmin concentration to the increase in dry weight and chlorophyll *a* between sample days (Figs. 6.11 and 6.12, respectively). The ratio of geosmin to dry weight and chlorophyll *a* is not constant throughout the trial, and varies between light intensities and sampling times. The ratio of geosmin to chlorophyll *a* ranged from 23 to 62 ng μg^{-1}, and from 200 to nearly 1,500 ng mg^{-1} (excluding negative values) on a dry weight basis. Overall however, the general trend was for a higher production of geosmin per unit biomass at lower light intensities (Figs. 6.11 and 6.12).

The relationship between the rate of culture growth and the geosmin:biomass ratio are plotted in Figs. 6.13 and 6.14 for dry weight and chlorophyll *a*, respectively. Growth rates were calculated as the natural logarithmic increase in biomass between days 0-2, 2-4 and 4-6 at all light intensities. An inverse relationship between dG:dDW and dG:dChl*a* and growth rate is

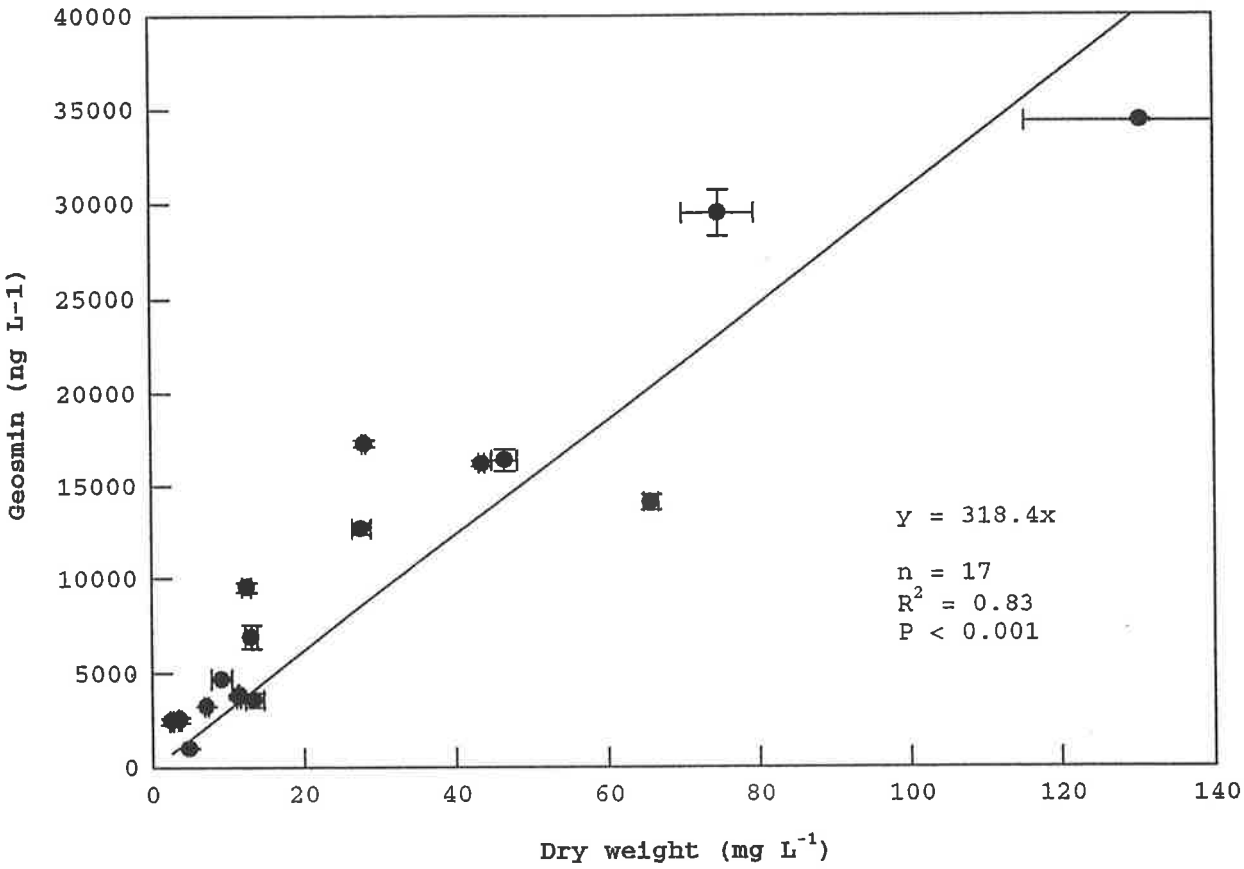

Fig. 6.9. The relationship between dry weight and geosmin concentration in all cultures on all sampling days (exluding cultures at 100 and 150 uM m^{-2} s^{-1} on day 6). Error bars indicate the range of duplicate analyses for both measures.

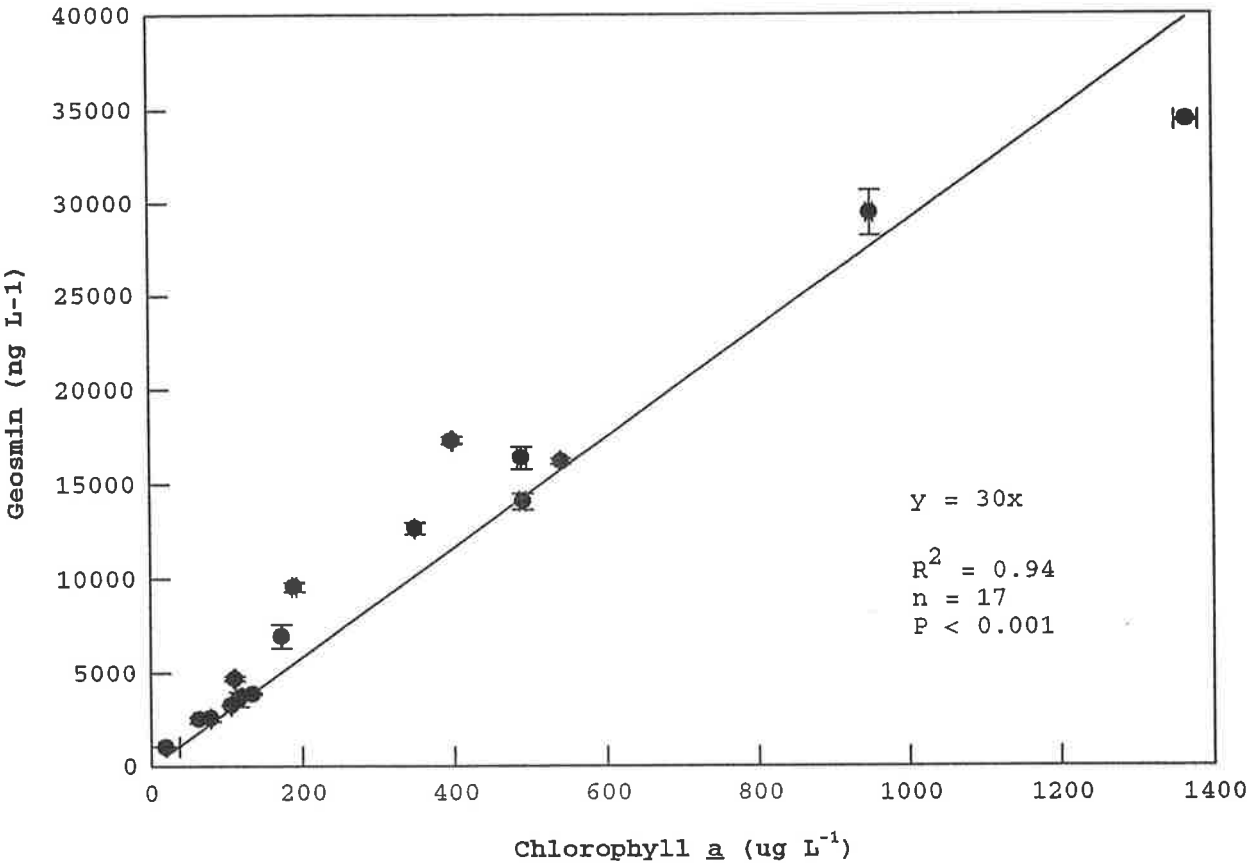

Fig. 6.10. The relationship between chlorophyll a and geosmin concentration in all cultures on all sampling days (excluding 100 and 150 uM m-2 s-1 on day 6). Error bars indicate the range of duplicate analyses for both measures.

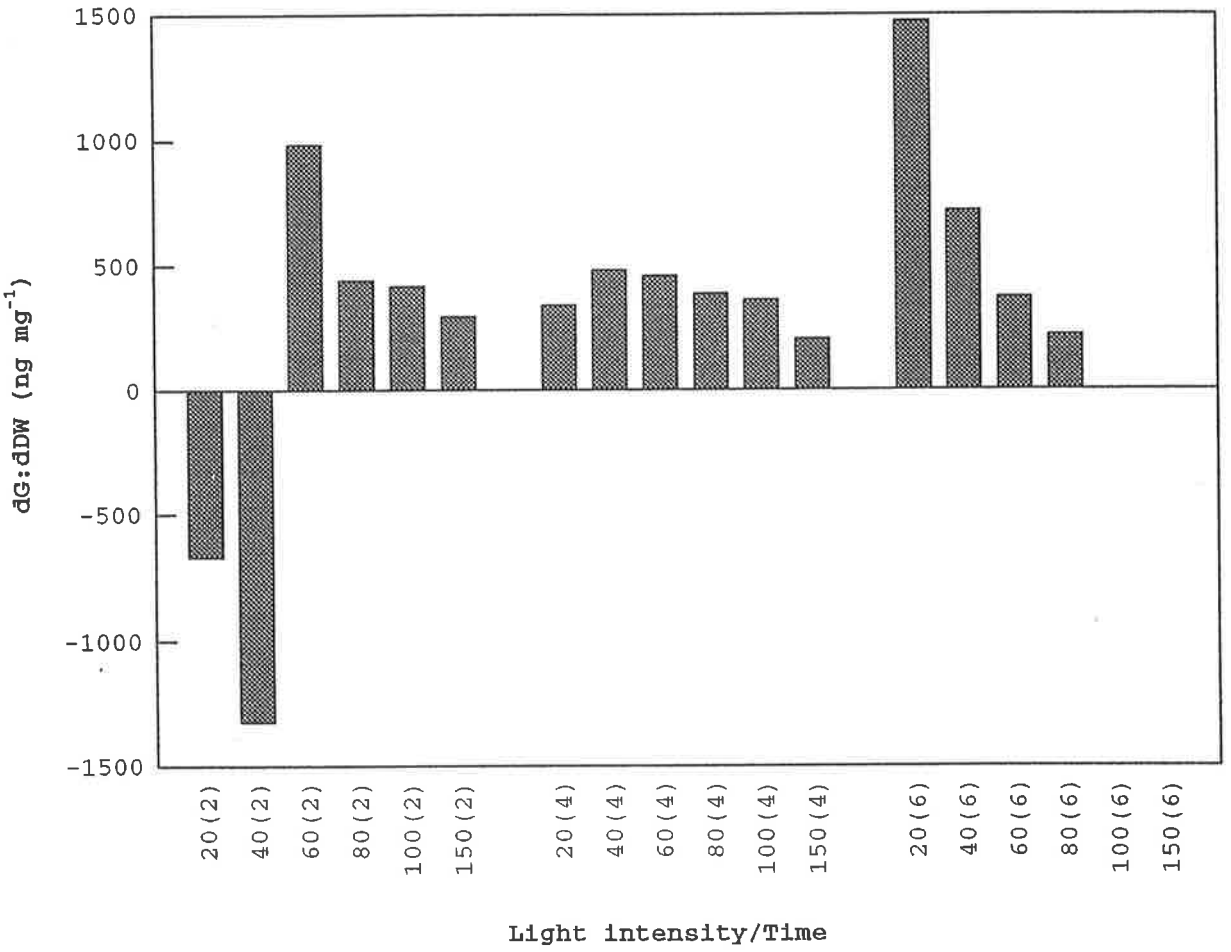

Fig 6.11. Change in geosmin to change in dry weight ratio at all light intensities and sampling times (bracketed No. = light intensity (uM m^{-2} s^{-1}); bracketed No. = sample day; cultures at 100(6) and 150(6) senesced and were omitted).

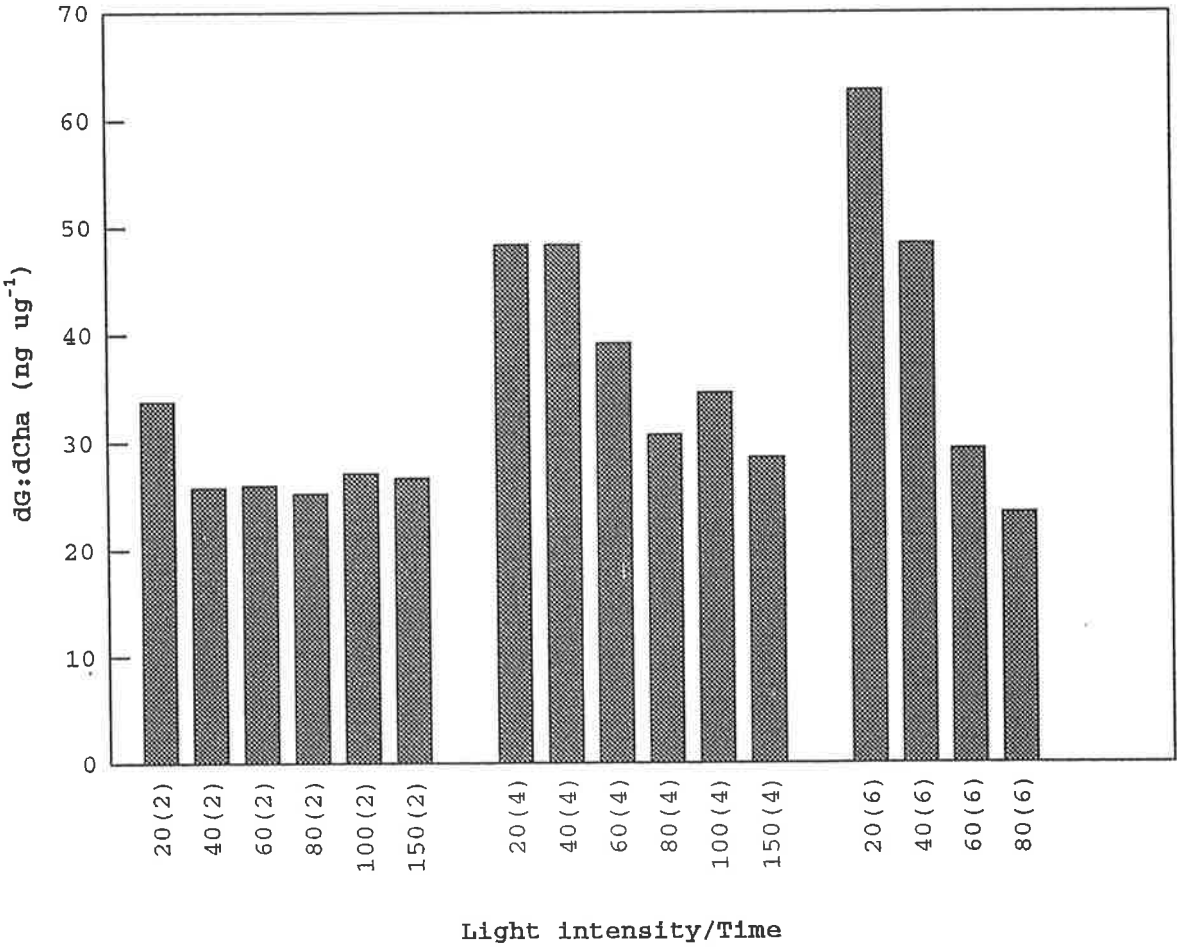

Fig 6.12. Change in geosmin to change in chlorophyll a ratio at all light intensities and sampling times (unbracketed No. = light intensity (uM m^{-2} s^{-1}); bracketed No. = sample day; cultures at 100(6) and 150(6) senesced and were omitted).

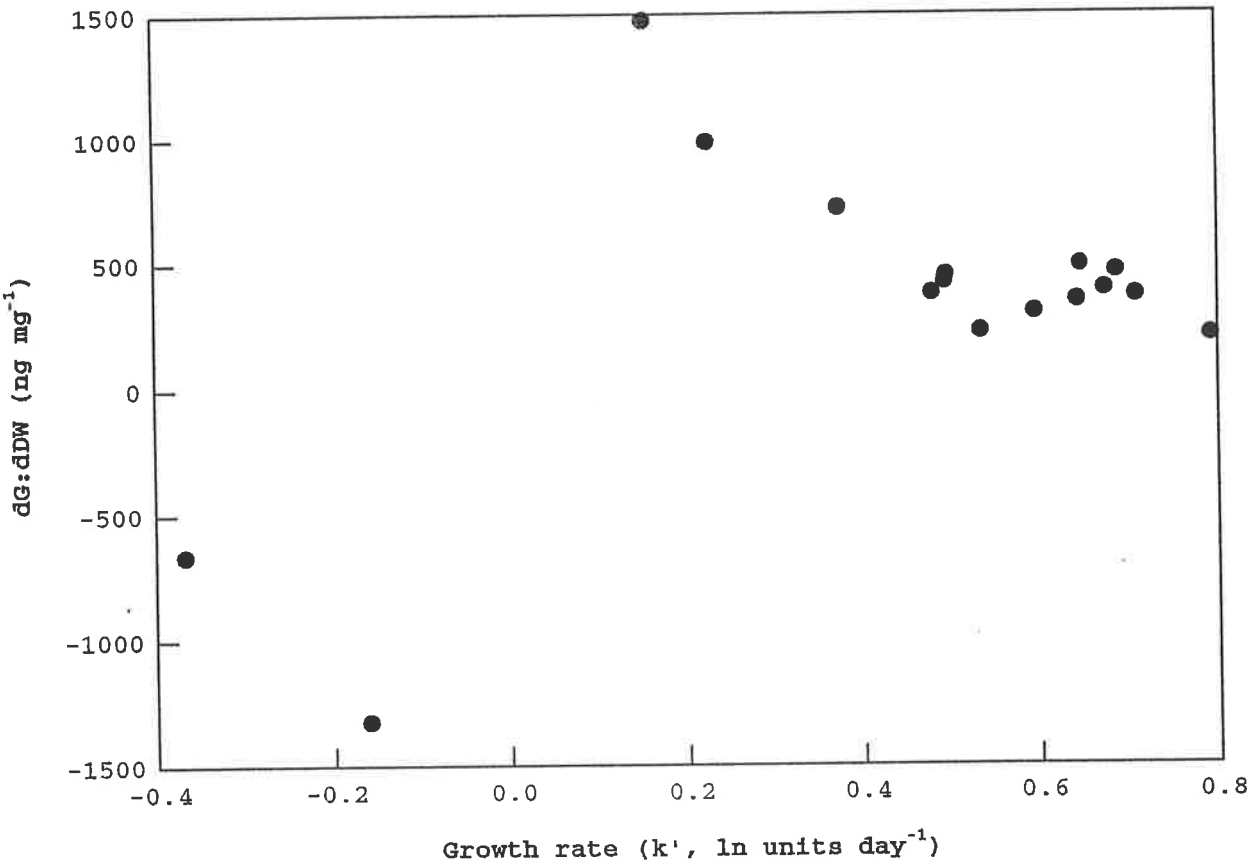

Fig. 6.13. The relationship between the change in geosmin to change in dry weight ratio at different growth rates calculated as changes in dry weight (NB: axes do not commence at origin).

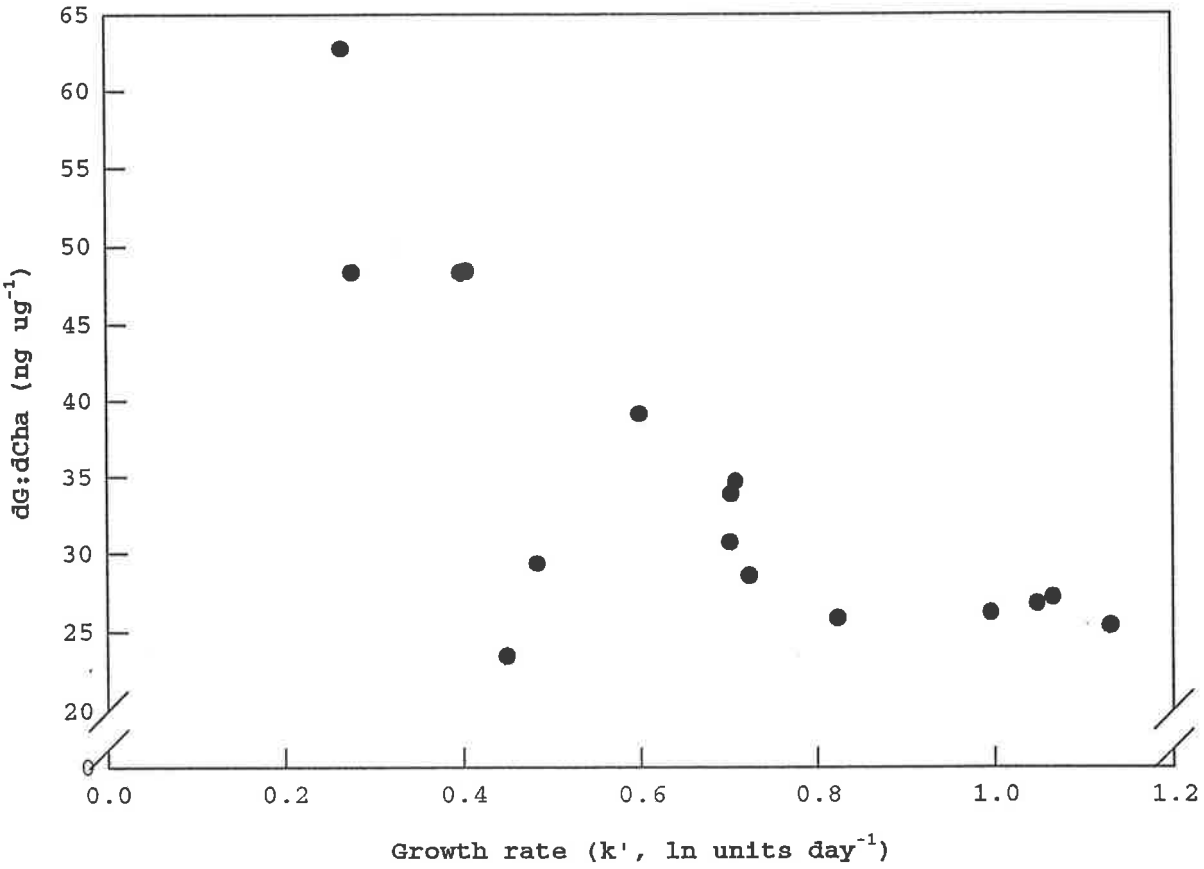

Fig. 6.14. The relationship between the change in geosmin to change in chlorophyll a ratio at different growth rates calculated as changes in chlorophyll a.

evident in both cases. A higher proportion of geosmin is produced per unit of biomass at lower rates of growth. The proportion of geosmin produced per unit biomass decreased with increasing growth rate until a stable minimum rate was reached and maintained. In the case of geosmin produced per unit dry weight, dG:dDW decreased from 1,500 ng mg^{-1} between growth rates of 0.15 and 0.5 ln units day^{-1}, then remained constant about a mean of 300 ng mg^{-1} up to 0.8 ln units day^{-1}. Similarly, dG:dChl*a* decreased from 63 ng μg^{-1} between growth rates of 0.25 and 0.8 ln units day^{-1}, then stabilised at 27 ng μg^{-1} (Figs. 6.13 and 6.14).

Absolute rates of geosmin production were calculated and compared to the corresponding rates of growth (Figs. 6.15 and 6.16). Absolute rates were calculated as the increase in geosmin concentration by the average biomass over the periods 0-2, 2-4 and 4-6 days, on a per day basis. The relationship between the rate of geosmin production per unit of chlorophyll *a* (Fig. 6.16) and k' is similar to that obtained in Fig. 6.14 i.e. a declining rate of geosmin production with increasing growth rate. An absolute rate of geosmin production ranging between 61 and 18 ng $\mu gChl a^{-1}$ day^{-1} was measured under these experimental conditions. However, no relationship between the absolute rate of geosmin production and growth rate was obtained when biomass was expressed on a dry weight basis (Fig. 6.15).

The absolute rate of geosmin synthesis on a dry weight and chlorophyll *a* basis is shown plotted against k' calculated using cell density, an independent measure of algal biomass (Figs. 6.17 and 6.18, respectively). Rates of growth were similar to those obtained using dry weight and chlorophyll *a*, ranging from 0.08 to 0.8 ln units day^{-1}.

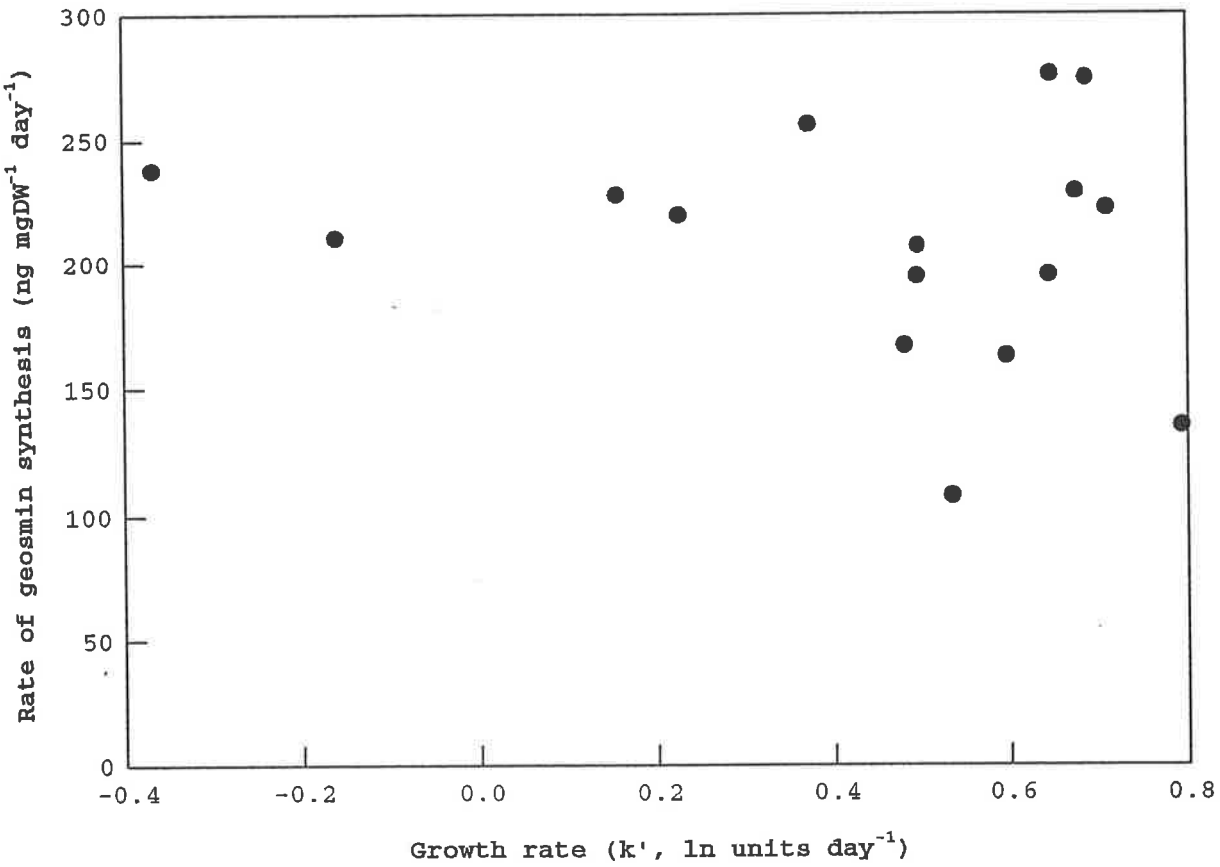

Fig. 6.15. The relationship between the absolute rate of geosmin synthesis on a per dry weight basis and rate of culture growth measured as dry weight.

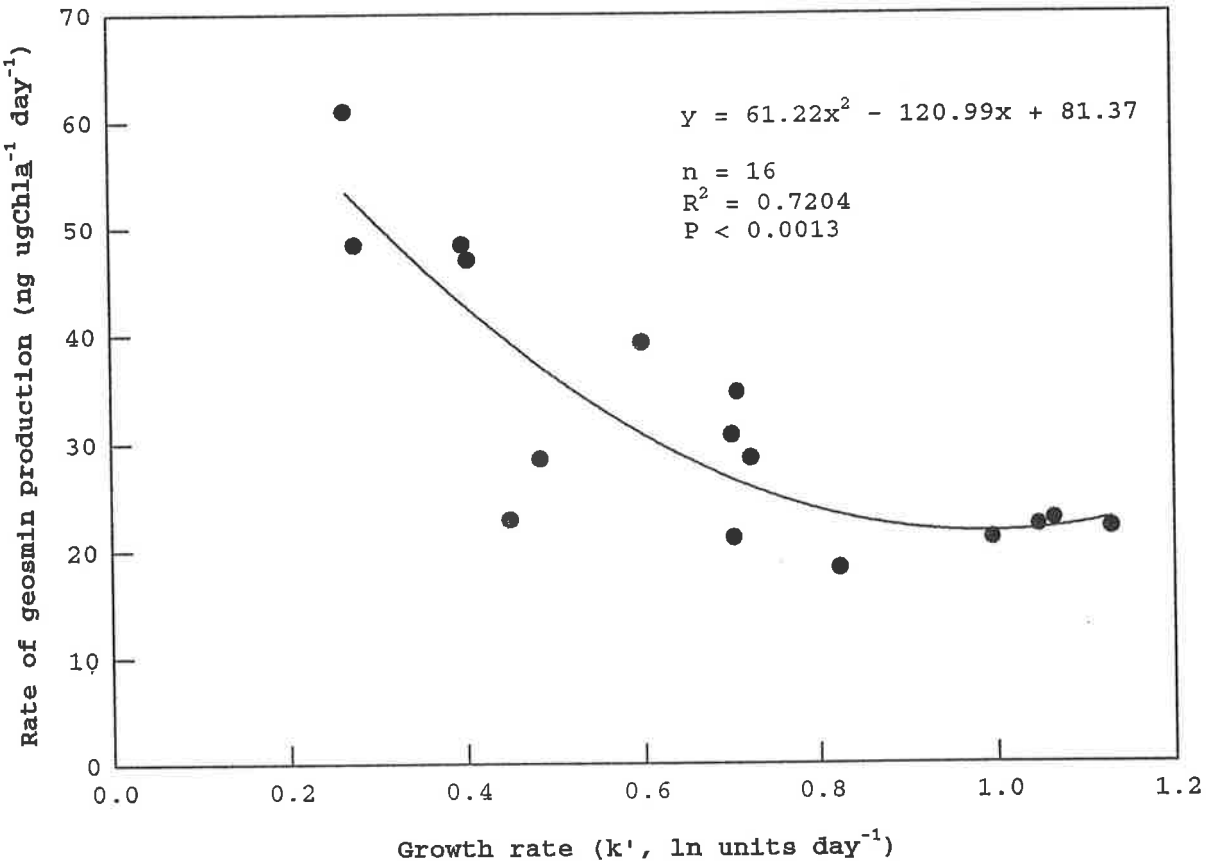

Fig. 6.16. The relationship between the absolute rate of geosmin synthesis on a per chlorophyll a basis and rate of growth measured as chlorophyll a.

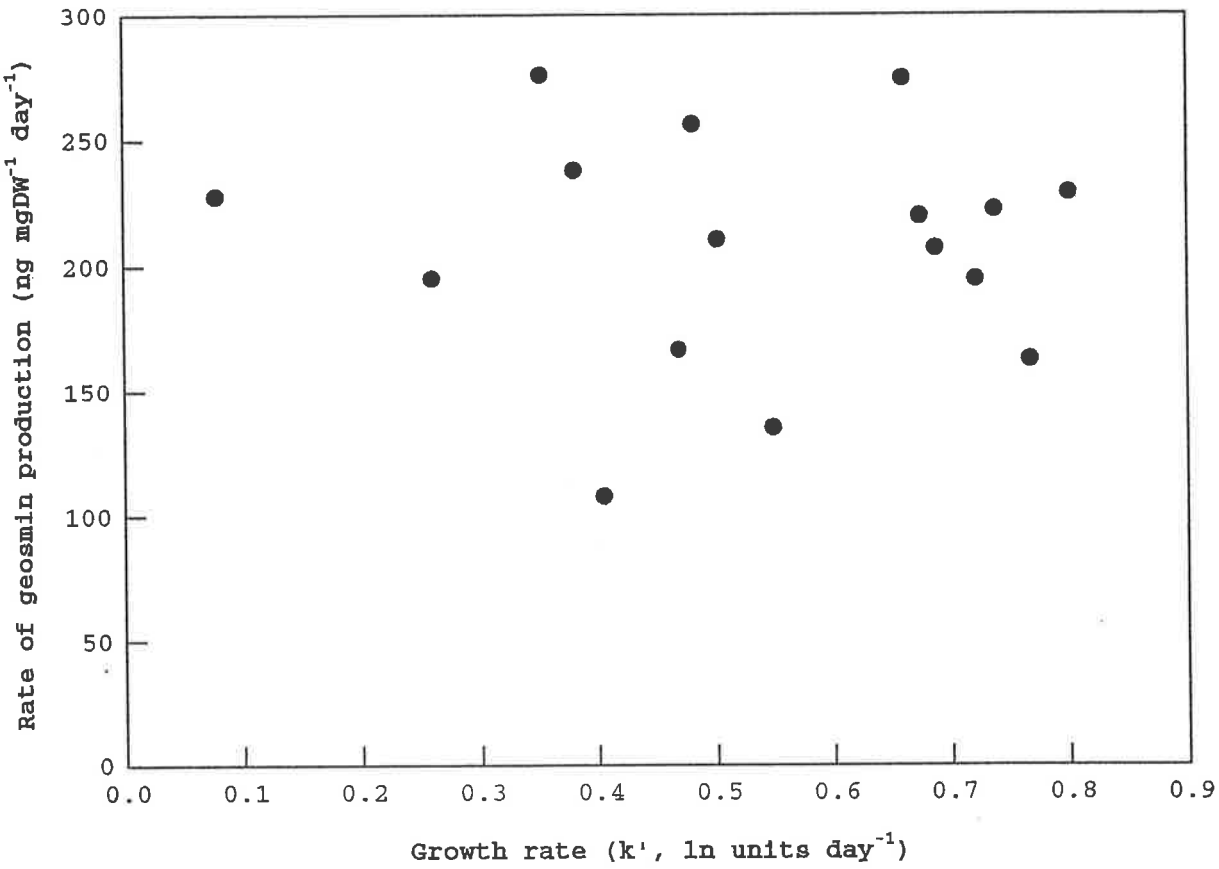

Fig. 6.17. The relationship between the absolute rate of geosmin synthesis on a per dry weight basis and rate of culture growth measured as cell density.

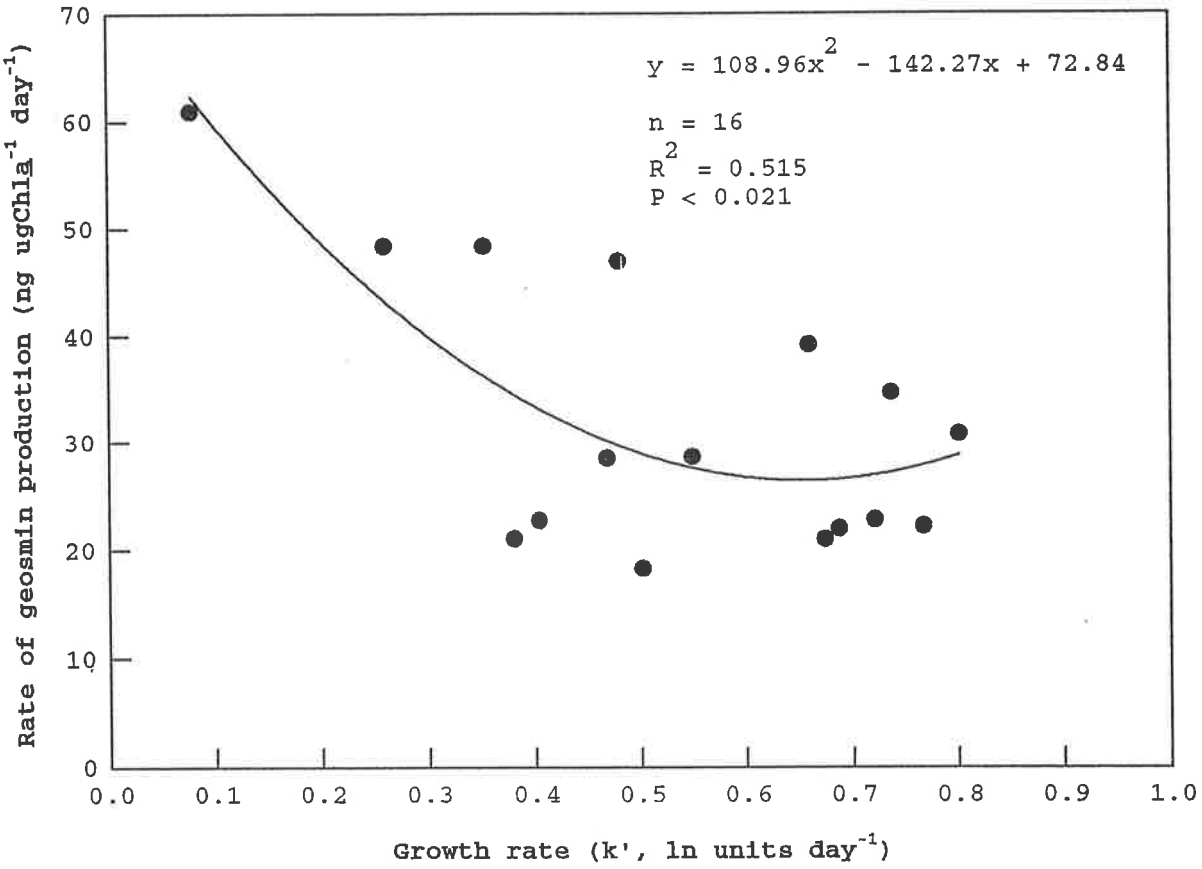

Fig. 6.18. The relationship between the absolute rate of geosmin synthesis on a per chlorophyll a basis and rate of culture growth measured as cell density.

There was no relationship between the absolute rate of geosmin synthesis on a dry weight basis and growth rate (Fig. 6.17). However, a relationship was observed between geosmin synthesis per unit chlorophyll a and growth rate (Fig. 6.18). Although exhibiting greater variability than when k' was calculated using chlorophyll a (Fig. 6.16), an inverse relationship between geosmin synthesis and k' was observed.

6.5 Discussion

The three aims of this chapter were to: a) estimate the loss of geosmin from cultures resulting from aeration, b) examine the relationship between geosmin and chlorophyll a production under a wide range of light conditions in the laboratory and c) determine the relationship between dry weight/chlorophyll a and cell density to enable the transfer of laboratory estimates of geosmin production to the field.

The loss of geosmin from solution as a result of aeration was minor, in most cases less than 3% of the total geosmin present (Fig. 6.7). This small loss across a wide range of solution concentrations could therefore not explain the decrease in geosmin concentrations measured in the culture at 70 $\mu M\ m^{-2}\ s^{-1}$ in chapter 5 (Fig. 5.3), or indeed the decrease in geosmin levels at the culture illuminated at 100 $\mu M\ m^{-2}\ s^{-1}$ between days 4 and 6 in this chapter (Fig. 6.8).

The decline in geosmin levels must therefore be related to other mechanisms. One possibility is the release of cell-bound constituents (e.g. enzymes) during cell lysis that converts geosmin into other products. Alternatively, the bacterial population present in cultures is able to

metabolise geosmin, the rate of which is markedly increased during senescence of algal cultures when bacterial numbers rapidly increase.

The effects of exposing cultures of *A. circinalis* 852E to a wide range of light intensities had the effect of producing wide variation in the Chl*a*:DW ratio between light treatments (Fig. 6.6) and variations in the rates of culture growth (Fig. 6.3). This therefore provided the scope for examining the relationship between geosmin and chlorophyll *a* production.

The results from this study show there is a direct relationship between chlorophyll *a* and geosmin production (Fig. 6.10). Furthermore, the ratio at which geosmin was produced relative to chlorophyll *a* was not constant, but varied with the rate of culture growth (Fig. 6.14).

However, the production of geosmin was not uniquely related to chlorophyll *a*, but was also related to algal biomass in general, measured as both dry weight (Figs. 6.9 and 6.13), and by inference, cell density (Fig. 6.4 and 6.5). This similar association between geosmin and biomass measured as dry weight and chlorophyll *a* is not surprising. Gross changes in geosmin, dry weight and chlorophyll *a* during the growth of all cultures parallelled one another, resulting in strong relationships between them. Furthermore, the measure of chlorophyll *a* used here is a subset of dry weight biomass, and gross relative changes in chlorophyll *a* will be reflected in gross changes in dry weight.

The expression of geosmin production on an absolute basis, however, clearly demonstrates a unique link between geosmin and chlorophyll *a* synthesis (Fig. 6.16), but not between geosmin and dry weight synthesis (Fig. 6.15). The absolute rate of geosmin production on a chlorophyll *a* basis exhibited

a relationship with the rate of chlorophyll a production that persisted across all light treatments and sampling occasions.

The use of cell density as an independent measure of algal growth is consistent in supporting a strong association between geosmin and chlorophyll a, but not between geosmin and dry weight (Figs. 6.17 and 6.18). The fact that k' (cell density) was not as good a predictor as k' (chlorophyll a) suggests that the rate of geosmin synthesis is more specifically related to processes regulating chlorophyll a synthesis. Growth rates based on cell density is a measure of production of biomass, whereas k' (chlorophyll a) is more precisely a measure of the rate of production of chlorophyll a as well as algal biomass.

Differences in the rate of geosmin production relative to the rate of chlorophyll a production explains the variations in the dG:dChla (and dG:dDW) ratios across the treatments seen in Figs. 6.11 to 6.14. Different light intensities and culture ages resulted in differing rates of chlorophyll a production, which in turn led to differing rates of geosmin synthesis, resulting in variations in dG:dChla and dG:dDW.

The rates of geosmin production measured in chapter 5 (Table 13) compare closely with those shown in Fig. 6.16. The culture grown at 17 $\mu M\ m^{-2}\ s^{-1}$ synthesised geosmin at the rate of 57, 19 and 20 ng $\mu gChla^{-1}\ day^{-1}$ when measured at three different times, and the culture at 70 $\mu M\ m^{-2}\ s^{-1}$ gave rates of 29, 14 and 10 ng $\mu gChla^{-1}\ day^{-1}$, the latter two values slightly lower than the lowest values in Fig. 6.16. Furthermore, an inverse relationship between the absolute rate of geosmin synthesis shown in Fig. 6.16 is consistent with the findings of chapter 5.

These results therefore support the conclusions of the previous chapter that there is a relationship between geosmin and chlorophyll a production. Furthermore, this relationship is not constant but is a function of the rate at which chlorophyll a is produced, where higher rates of geosmin are produced per unit chlorophyll a during slower rates of culture growth.

The existence of an inverse relationship between geosmin synthesis and the rate of growth may only be speculated. One explanation is that cells undergoing rapid rates of cell division under favourable conditions divert substrates away from metabolic processes not directly related to increasing biomass, including those reactions involving geosmin synthesis. Alternatively, the synthesis of geosmin may be more closely linked with the synthesis of other pigments produced by the isoprenoid pathway, of which chlorophyll a is an example. Differing rates of growth would also affect the rates of synthesis of other light pigments, in addition to geosmin and chlorophyll a. See section 5.5 for a more detailed discussion on variations in photosynthetic pigments with cell growth.

From a field perspective, the rates of geosmin synthesis derived from this study can be used to calculate the amount of geosmin that may be produced by a population of *A. circinalis* in a water body. This can be estimated by expressing the rate of synthesis on a per cell basis to allow results to be directly applicable to field estimates of algal biomass. The density of *Anabaena* cells at the Hay Weir Pool, from where *A. circinalis* 852E was isolated, can range from 100 to over 40,000 cells mL^{-1} in the months November to April (Jones, 1994; reproduced in Fig. 6.19). In the summer of 1990/1991 cell densities in excess of 10,000 cells mL^{-1} persisted for over 7 weeks.

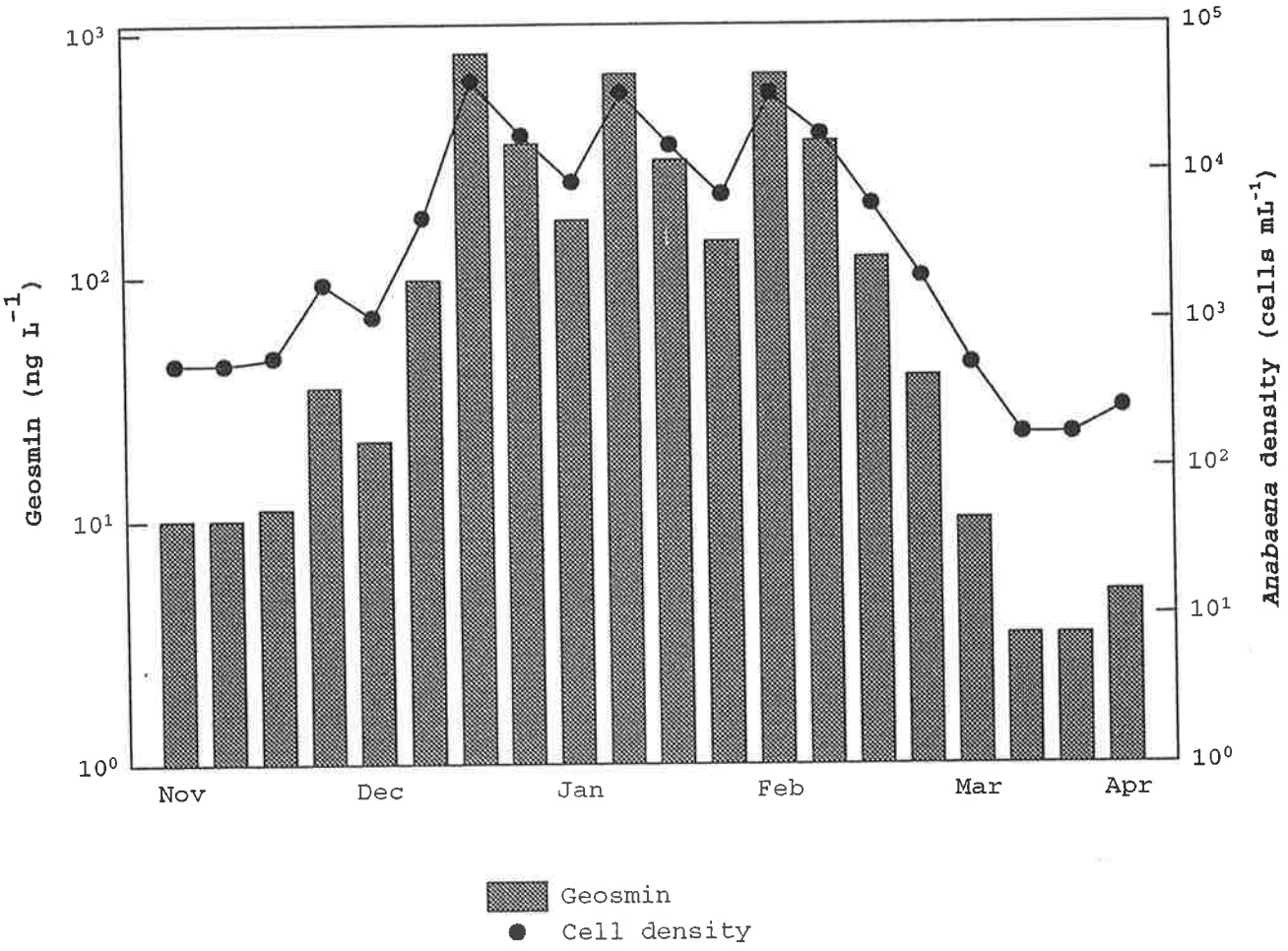

Fig. 6.19. Density of *A. circinalis* cells and predicted concentration of geosmin in the Hay Weir Pool during the summer of 1990/1991 (cell density data from Jones 1994).

These estimates of cell density were used to calculate the levels of geosmin expected in the water body. The lower rate of geosmin synthesis (20 ng $\mu g Chl\underline{a}^{-1}$ day^{-1}) shown in Fig. 6.16 was selected, and when converted from chlorophyll *a* concentration to cell density using the regression from Fig. 6.5, corresponded to a rate of 0.02 pg $cell^{-1}$ day^{-1}. Geosmin concentration was calculated on the basis of only one day's production. Fig. 6.19 summarises the predicted geosmin concentrations for the days cell density were determined.

These results show that the population of *A. circinalis* resident in the Hay Weir Pool over the 1990/1991 summer had the potential to produce quantities of geosmin that frequently exceeded its odour threshold concentration. This was achieved at a cell density of less than 1,000 cells mL^{-1} over the period of only one day. The highest concentration of geosmin expected during this period exceeded 800 ng L^{-1}, with levels over 100 ng L^{-1} sustained for a period of over 8 weeks (Fig. 6.19). This was associated with cell densities in excess of 6,000 cells mL^{-1}.

The accuracy of the predictions in Fig. 6.19 in depicting concentrations of geosmin in the Hay Weir Pool is not known as a number of assumptions were made in the calculation. These are: all cells in a population of *A. circinalis* are actively producing geosmin; other factors affecting cell growth (temperature, nutrients) do not greatly modify geosmin production; no geosmin is lost from the water body due to volatilisation, biological or chemical degradation, adsorption onto particles or flushing due to water flow; and that no accumulation of geosmin occurs from one day to another.

These concentrations of geosmin, however, are not unrealistic. Levels of geosmin in four field samples measured in this study were: 27 and 78 ng L^{-1} from the Edwards River; 475 ng L^{-1} from Toonumbah Dam; and 26,000 ng L^{-1} in

the case of an isolated pool along the Murrumbidgee River supporting a bloom of *Anabaena* (Table 11).

Nevertheless, these results show that *A. circinalis* 852E is a prolific producer of geosmin, and that if field conditions allow geosmin to accumulate, cell densities as low as 1,000 cells mL^{-1} can lead to concentrations exceeding the OTC in a matter of days. This cell density corresponds to less than 1 $\mu g\ L^{-1}$ of chlorophyll *a* which is significantly less than the value of 20 $\mu g\ L^{-1}$ that is often used as defining an algal bloom.

This highlights the importance of closely monitoring water bodies that are known to support geosmin-producing strains of *Anabaena*. Over the warmer periods of the year when algal growth is most active, regular monitoring of raw water for low levels (1,000 cells mL^{-1}; <1 $\mu g\ L^{-1}$ chlorophyll *a*) of algal biomass is essential. However, the quantities of geosmin produced will also depend on the proportion of the population that is genetically capable of synthesising geosmin.

Only the sensory evaluation of water will determine it's acceptability for human consumption. The sensory protocol developed in chapter 4 may be used by local water authorities as the basis for additional monitoring of their water supplies and as an investigative tool, to ensure that the quality of their drinking water is maintained.

APPENDIX

APPENDIX A

Literature summary of species associated with the production of geosmin, MIB and toxins.

Genus	Species	Strain	Plank./ Benthic	Culture/ Field	F'water/ Marine	Geosmin/ MIB/ Toxin	Country	Ref.
Anabaena	circinalis		P	F	F	T	Australia	1
Anabaena	circinalis		P	C	F	G		2
Anabaena	circinalis		P	C	F	G	Australia	3
Anabaena	circinalis		P	C	F	G	USA	4
Anabaena	circinalis	(Kutz.) Rabenhorst	P	C	F	T	Canada	5
Anabaena	flos-aquae		P	C	F	G	Australia	6
Anabaena	flos-aquae		P	F	F	G	Japan	7,8
Anabaena	macrospora		P	C	F	G	USA	9
Anabaena	scheremetievi	Elenkin	P	C	F	G		10
Anabaena	solitaria		?	C	?	G		11,12
Aphanizomenon	flos-aquae		P	C	F	T		13
Aphanizomenon	flos-aquae		P	C	F	G	Japan	14,15
Aphanizomenon	gracile		P	F	F	G	Germany	16
Aphanizomenon	sp.		?	C	F?	G	Japan	17
Fischerella	muscicola	ATCC 29114	?	C	F?	G		18
Lyngbya	cf. aestuarii	(Martens) Liebman	P	C	M	G	Canada	18
Lyngbya	cf. cryptovaginata	Schkorbatov	P	C	M	M	Canada	14
Lyngbya	limnetica		P	F	F	G	Germany	11
Lyngbya	majuscula		?	C	M	T		18
Lyngbya	sp.		P	C	M	G	Canada	11
Lyngbya	gracilis		?	C	M	T		19,20
Microcystis	aeruginosa		P	C	F	T	Japan	1
Microcystis	aeruginosa		P	F	F	T	Australia	20
Microcystis	viridis	(Lyngb.) de Breb.	P	C	F	T	Japan	1
Nostoc	spumigena		P	F	F	T	Australia	

Appendix A continued

Genus	Species	Strain	Plank./ Benthic	Culture/ Field	F'water/ Marine	Geosmin/ MIB/ Toxin	Country	Ref.
Oscillatoria	agardhii		?	F	M	G	Finland	21
Oscillatoria	agardhii	NIVA CYA 12 Gom.	P	C	F	G	Norway	22
Oscillatoria	agardhii	NIVA CYA 18 Gom.	P	C	F	G	Norway	22
Oscillatoria	amoena		?	C	F?	G	Japan	16
Oscillatoria	amoena		P?	C	F	G	Japan	13
Oscillatoria	animalis		?	C	F	G	Japan	8
Oscillatoria	argardhii	Gomont	P	C	M	G	Canada	18
Oscillatoria	autumnale		?	C	F	G	Japan	8
Oscillatoria	bornetii	f. tenuis NIVA CYA 58	P	C	F	G	Norway	22
Oscillatoria	bornetii	f. tenuis NIVA CYA 70	P	C	F	G	Norway	22
Oscillatoria	brevis	(Kutz.) Gom. NIVA CYA	P	C	F?	G	Norway	23,24,25
Oscillatoria	brevis	NIVA CYA 7a	?	C	F	G,M	Norway	26
Oscillatoria	cf. cortiana	Meneghini	P	C	M	G	Canada	18
Oscillatoria	cf. formosa	NIVA CYA 92	?	C	?	G		10
Oscillatoria	cf. prolifica	(Greville) Gomont	P	C	M	G	Canada	18
Oscillatoria	cf. splendida	Greville	P	C	M	G	Canada	18
Oscillatoria	cf. variabilis	Rao	P	C	M	G	Canada	18
Oscillatoria	curviceps		?	C	F	M	USA	9
Oscillatoria	curviceps		?	C	F	M	USA	27
Oscillatoria	geminata		?	C	F?	M	Japan	16
Oscillatoria	geminata		P?	C	F	M	Japan	13
Oscillatoria	limnetica		P?	C	F	M	Japan	13
Oscillatoria	nigroviridis		?	C	M	T		11
Oscillatoria	simplicissima	Gomont	P	C	F	G	USA	9
Oscillatoria	sp.		P	C	M	G	Canada	18
Oscillatoria	sp.		P	F	F	G,M	Australia	6
Oscillatoria	sp.	from aircon. tower	-	C	F	G,M	Japan	13
Oscillatoria	splendida		?	C	F?	G	Japan	16
Oscillatoria	splendida		P?	C	F	G	Japan	13
Oscillatoria	tenuis		P?	C	F?	G	USA?	28
Oscillatoria	tenuis	Ag. ex Gomont	?	C	F?	G,M	Taiwan	29
Oscillatoria	tenuis	Argardh	P	C	M	G	Canada	18
Oscillatoria	tenuis	var. Levis Gardner	?	C	F	M	USA	9
Oscillatoria	tenuis	var. Levis Gardner	?	C	F	M	USA	27

Appendix A continued

Genus	Species	Strain	Plank./ Benthic	Culture/ Field	F'water/ Marine	Geosmin/ MIB/ Toxin	Country	Ref.
Phormidium	inundatum		?	C	?	G		10
Phormidium	sp.		?	C	F	G,M	USA	30
Phormidium	tenue		P	C	F	M	Japan	8,31
S.	calcicola		?	C	M	T		11
Schizothrix	meulleri		?	C	F	G	Japan	8
Symploca	cf. muscorum	(Argardh) Gomont	P	C	M	G	Canada	18
Symploca	muscorum		P?	C	F?	G	USA?	28
Synechococcus	sp.	not axenic	?	C	?	M		10

"?" indicates information was not contained in the reference.

1. Alpin (1983); 2. Silvey *et al.* (1970); 3. This study; 4. Rosen *et al.* (1992); 5. Carmichael and Gorham (1977); 6. Hayes and Burch (1989); 7. Miwa and Morizane (1988); 8. Yagi *et al.* (1983); 9. Izaguirre *et al.* (1982); 10. Persson (1988); 11. Collins (1978); 12. Gentile and Maloney (1969); 13. Matsumoto and Tsuchiya (1988); 14. Juttner *et al.* (1986); 15. Juttner (1984); 16. Tsuchiya *et al.* (1981); 17. Wu and Juttner (1988b); 18. Tabachek and Yurowski (1976); 19. Watanabe and Oishi (1985); 20. Watanabe *et al.* (1989); 21. Persson (1979); 22. Berglind *et al.* (1983a); 23. Naes *et al.* (1985); 24. Naes and Post (1988); 25. Naes *et al.* (1989); 26. Berglind *et al.* (1983b); 27. Izaguirre *et al.* (1983); 28. Medsker *et al.* (1968); 29. Wu and Juttner (1988a); 30. Izaguirre (1992); 31. Sugiura *et al.* (1986).

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