THE VALUE OF LOCALLY ISOLATED FRESHWATER MICRO-ALGAE IN TOXICITY TESTING FOR WATER RESOURCE MANAGEMENT IN SOUTH AFRICA

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Abstract

The ecological position of micro-algae at the base of the aquatic food web makes them critical components of aquatic ecosystems. Their short generation time also makes them useful biological indicators because they respond quickly to changes in environmental condition, enabling timely identification and assessment of water quality changes. The inclusion of micro-algae as indicators in water resource regulation and management in South Africa has started recently, their more extensive use in biomonitoring and ecotoxicology programmes for water resource management would contribute to the South African policy if water resource protection.

The standard algal growth inhibition assay with the species *Pseudokirchneriella subcapitata* is currently used for monitoring toxicity of in-stream and industrial wastewater discharges to freshwater micro-algae. The relevance of the data generated by standard toxicity bioassays has been questioned, since micro-algae in particular are extremely variable in their sensitivity to a range of contaminants and these standard species used may not occur in the local aquatic environment. As a result, international regulatory agencies, have recommended algal growth inhibition tests be changed from a single standard species to tests with a number of species. One recommendation, in addition to the use of standard toxicity tests, is the use of species isolated from the local environment which may be more relevant for assessing site specific impacts.

This study investigated the value and application of locally isolated South African freshwater micro-algae in toxicity tests for water resource management and was carried out in three phases. The first phase involved isolating micro-algae from South African aquatic resources. Micro-algae suitable for toxicity testing were identified and selected using as set of criteria. Three (*Scenedesmus bicaudatus*, *Chlorella sorokiniana* and *Chlorella vulgaris*) out of eight successfully isolated species satisfied the prescribed selection criteria and these were selected as potential toxicity test species.

The second phase focused on refining and adapting the existing algal toxicity test protocol (the algal growth inhibition assay) for use on the locally isolated algal species. The refinement of the test protocol was achieved by exposing the locally isolated species to reference toxicants in order to assess and compare their growth and sensitivity to the

toxicants under the prescribed toxicity test conditions with that of the standard toxicity test species (*Pseudokirchneriella subcapitata*) and a commercial laboratory species (*Chlorella protothecoides*). During this phase, one of the three local species (*Scenedesmus bicaudatus*) was eliminated as a potential toxicity test species due to inconsistent growth.

The third phase of the study involved assessing the sensitivity of the two remaining species (*C. vulgaris* and *C. sorokiniana*) to a range of toxicants (reference toxicants, salts, effluents and a herbicide) and comparing it to that of the standard toxicity test species *P. subcapitata* and *C. protothecoides*. The toxicants were selected based on their relative importance in the South African context, as well as the practicality of using these local micro-algae to routinely determine the impact of these toxicants on local aquatic resources. The growth of the four micro-algae was stimulated by the selected effluents. The standard toxicity test species *P. subcapitata* was ranked the most sensitive and of the four species to two reference toxicants and two inorganic salts. *Chlorella sorokiniana* was ranked the most sensitive of the three *Chlorella* species to two reference toxicants and two inorganic salts. The herbicide stimulated the growth of *C. vulgaris* while inhibiting the growth of the other species. *Pseudokirchneriela subcapitata* and *C. sorokiniana* showed high intra-specific variability in growth, which made it difficult to determine the effective concentrations of the herbicide and therefore compare the sensitivity of the species. This varied response of micro-algal species to toxicants may result in the biodiversity shifts in aquatic ecosystems, and also supports the recommendation of using a battery of different species to support more informed decisions in water resource management.

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ACRONYMS

AAP	Algal assay procedure
ANOVA	Analysis of variance
ANZECC	Australian and New Zealand Environment Conservation Council
ARMCANZ	Agricultural and Resource Management Council of Australia and New Zealand
CCME	Canadian Council of Ministers of the Environment
CSIR	Council for Scientific and Industrial Research
DEEEP	Direct Estimation of the Ecological Effects Potential
DWA	Department of Water Affairs
DWS	Department of Water and Sanitation
DWAF	Department of Water Affairs and Forestry
EC	Effective concentration
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
GLP	Good laboratory practice
ISO	International Organisation for Standardisation
LOEC	Lowest observed effect concentration
NEMA	National Environmental Management Act
NEMP	National Eutrophication Monitoring Programme
NIWA	National Institute of Water and Atmospheric Research
NLA	National Laboratory Association
NOEC	No observed effect concentration
NTMP	National Toxicity Monitoring Programme
NWA	National Water Act
NWRS	National Water Resource Strategy
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
PES	Present ecological state
PTS	Proficiency testing scheme
RHP	River Health Programme
RQO	Resource quality objective
RQS	Resource Quality Services

RWQO	Resource water quality objective
SANAS	South African National Accreditation System
SSD	Species Sensitivity Distribution
TWQR	Target water quality range
UCEWQ	Unilever Centre for Environmental Water Quality
US EPA	United States Environmental Protection Agency
WET	Whole Effluent Toxicity

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DECLARATION

In accordance with the regulations for the award of the degree of Doctor of Philosophy, I declare that the work presented in this thesis is my own original research. This thesis has not been submitted to any other university.

1. THE VALUE OF LOCALLY ISOLATED FRESHWATER MICRO-ALGAE IN TOXICITY TESTING FOR WATER RESOURCE MANAGEMENT

1.1 INTRODUCTION

As primary producers and the base of the aquatic food web, algae are an ecologically important collection of organisms which play a vital role in the healthy functioning of aquatic ecosystems. They convert solar energy into biomass, and produce the oxygen and organic substances that most life forms depend on (Lurling 2006). Algae are primarily distinguished by colour: red, green, blue-green and brown. They can grow as single cells, filaments or in colonies. They may be found attached to substrates (benthic) or floating freely (planktonic) in water bodies (Bold and Wayne 1978).

Freshwater planktonic algae (phytoplankton), mainly diatoms (Bacillariophyceae), green algae (Chlorophyceae) and blue-green (Cyanophyceae) algae are the most abundant algae in aquatic habitats. Bacillariophyceae are a class of algae commonly known as diatoms. They are found in marine and freshwater habitats where they can be free-floating or attached to sediments or solid substrate. They can be single cells or colonies, usually yellow to light brown in colour. They are easily distinguished by their siliceous walls (frustules) which are often sculptured with pores and striations, whose patterns are used to identify and classify different species (Van Vuuren *et al*. 2006).

Chlorophyceae make up a large and important class of green algae. Chlorophyceans may be unicellular, colonial or filamentous. The main feature of this class is the chloroplast, with the presence of chlorophylls *a* and *b*. The chloroplast morphology varies greatly and may be used for taxonomic purposes. Cells of this group may be non-motile or swim actively by means of flagellae. Both benthic and planktonic species occur (Van Vuuren *et al.* 2006). Cyanophyceae or blue-green algae are often referred to as cyanobacteria. They are a group of photosynthetic organisms more closely related to bacteria than to other algae because they are prokaryotic (lacking membrane bound organelles such as nuclei, mitochondria, chloroplast and other specialised organelles) (Nyberg 1982, Van Vuuren *et al.* 2006). The blue-green colour is a result of of photosynthetic pigments such as chlorophyll *a* (green pigment) and phycocyanin (blue pigment); some also contain phycoerythrin (a red pigment). No flagellated stages are present but some filamentous organisms can perform gliding movements. A characteristic

feature of many blue-green algae is the presence of gas vacuoles in cells, which provides buoyancy to the organisms. Cells are often covered by a thick cell wall that is often surrounded by mucous. Cyanophyceae may proliferate under conditions of excessive nutrient availability, slow moving or stagnant water. They have the ability to produce a surface scums, with taste and odour problems, as well as toxic substances (Van Vuuren *et al*. 2006).

Micro-algae are microscopic algae which form a large group of primary producers in marine and freshwater ecosystems (Hernandes *et al*. 2009). They occur almost universally in water bodies, are sensitive to changes in water quality, and suitable for monitoring changes in aquatic systems (McCormick and Cairns 1994, Twist *et al.* 1998). Micro-algae have short generation times, enabling them to respond relatively quickly to changes in environmental and to show impacts earlier than higher-level organisms (Rioboo *et al*. 2002). Disruption of ecosystem function at lower trophic levels could impair the health of organisms at higher levels of organization, and adverse effects on primary producers could lead to biodiversity shifts or altered trophic pathways. Micro-algae are therefore effective indicator organisms, which warrant consideration for inclusion in biomonitoring and ecotoxicology programmes.

The inclusion of micro-algae in water resource regulation and management in South Africa is fairly recent. It is only in the last decade that algal research has featured prominently in national water quality biomonitoring and toxicity monitoring programmes (Murray 2005, Taylor *et al.* 2007, Jooste *et al*. 2008). Biomonitoring programmes such as the National Eutrophication Monitoring Programme (NEMP), and the River Health Programme (RHP), implemented by the Department of Water Affairs and Sanitation (DWS), include micro-algae as indicators of water quality. The NEMP is a programme designed to measure, assess and report on the trophic status and the nature and extent of eutrophication-associated problems in South African impoundments and rivers. It uses measurements of nutrients (mainly phosphates) and chlorophyll *a* as an indirect quantification of algal biomass. The programme associates the trophic status of impoundments with total phosphorus (one of the nutrients promoting algal growth) and chlorophyll *a* (a measure of phytoplankton biomass) measurements (DWAF 2002). The NEMP has been implemented nationwide with well-documented conceptual designs and data records, indicating that most South African impoundments exhibit high nutrient enrichment and eutrophication-related problems (Van Ginkel *et. al* 2000, DWAF 2002, Van Ginkel 2003, Van Niekerk 2004).

The potential for diatom indices for monitoring water quality in South Africa has also recently been explored and resulted in the inclusion of diatoms as indicators of water quality in the River Health Programme (RHP) (Bate *et al*. 2004, de la Rey *et al*. 2004, Harding *et al*. 2005, River Health Programme 2005, Taylor *et al*. 2007). Diatoms form a large component of attached flora found on various substrata in rivers and streams, and their composition is directly impacted by physico-chemical characteristics of the aquatic environment (Bate *et al*. 2004, Taylor *et al.* 2007, Blanco and Bécares 2010). The RHP uses biological indicators to assess the condition of the health of river systems. The rationale for using biological monitoring is that the integrity of biota inhabiting river ecosystems provides an integrated measure of the integrity or health of the river. The RHP is meant to be a source of information regarding the ecological state of river ecosystems in South Africa, in order to support the national management of these natural resources (DWAF 2008).

Micro-algae are also included in toxicity monitoring programmes that regulate in-stream and industrial wastewater discharges. In the past the DWS controlled wastewater discharges resulting from land based and in-stream activities by substance-specific monitoring and concentration limits which enabled the management of levels of single substances in the water (DWAF 2003). Only physico-chemical analytical methods were used to monitor and control industrial and in-stream wastewater discharges (Slabbert *et al*. 1998). Although some of these chemical detection methods were highly sophisticated, sensitive and accurate, more comprehensive and holistic approaches of dealing with toxicity hazards of complex wastewaters and effluents were needed to protect the ecological integrity of aquatic ecosystems (Slabbert *et al* 1998, DWAF 2003). Biological toxicity tests were introduced as an important complementary approach to chemical analysis in controlling and managing effects of hazardous chemicals in water, because they show potential effects of the chemicals on organisms (Palmer and Scherman 2000). As the relevance and need for toxicity-based assays to assess the effects of in-stream wastewater discharges became apparent, a suite of toxicity tests with species at different trophic levels were selected for use in toxicity monitoring programmes, such as the National Toxicity Monitoring Programme (NTMP)(Murray 2005, Jooste *et al*. 2008).

The National Toxicity Monitoring Programme (NTMP) is designed to report on the status and trends of potential toxic effects of substances on selected organisms in South African inland surface water resources (Murray 2005, Jooste *et al*. 2008). The programme uses

ecotoxicological parameters in order to monitor priority hazardous substances in the aquatic ecosystems (Jooste *et al*. 2008). The NTMP applies toxicity tests to in-stream effluent discharges and agricultural run-off to assess effects on aquatic resources (Murray 2005). A suite of standard toxicity tests from different trophic levels (**algae**, invertebrates and fish) have been selected for the NTMP to be applied nationwide, and to allow protection of ecosystem integrity because monitoring a single trophic level could give misleading results. The NTMP emphasises both toxicity testing and analysis of individual toxicants because toxicity tests are more likely to capture antagonistic, synergistic and cumulative effects of toxicants (Slabbert *et al* 1998, Murray 2005, Jooste *et al*. 2008).

Algae not only feature in the battery of tests selected for use in the NTMP, but also in the Direct Estimation of the Ecological Effects Potential (DEEEP), which is a tool proposed by DWS for assessing the ecological hazards of complex wastewater discharges before they enter the natural resources (DWAF 2003, Slabbert 2004). The National Water Act (No. 36 of 1998) requires that before a user is allowed to discharge wastewater into the natural aquatic system, the effect of that wastewater on the environment must be assessed. However, the prescribed wastewater standards are based on the maximal concentrations of a variety of limited substances allowable in the effluents entering the system (DWAF 1996, Dallas and Day 2004), rather than the effects of the whole effluent on the integrity of the ecosystem. Effluents are generally chemically complex and comprise a wide variety of chemical substances, all of which are not known. Therefore, the approach of focusing prescribed wastewater standards on concentrations of selected, albeit limited, substances and parameters does not provide sufficient information on the potential harmful effects of complex effluents on the aquatic environment. This also applies to formulations of substances where the active ingredient is delivered as a mixture, and where the carrier substances may interact to alter toxicity.

The DEEEP approach is based on applying a battery of toxicity test procedures using organisms at different trophic levels (Slabbert 2004), including the algal growth inhibition assay, to measure the potential effects of wastewater discharges on aquatic organisms. The intention of the DEEEP is to supplement assessments using single substances with assessments that measure the potential effects of complex discharges, especially when the composition of the discharge is not completely known (DWAF 2003). This approach takes into account the possible synergistic, antagonistic and additive effects that the individual

effluent components could have on the aquatic system. The DEEEP is intended for use by regulators and dischargers in order to demonstrate environmental care, and toxicity is considered to be an effluent characteristic to be monitored and controlled (DWAF 2003). The DEEEP is designed to be applied at the end-of-pipe, before the effluent is discharged into the receiving water resource, allowing the potential in-stream effects of the effluent to be determined before discharge (DWAF 2003).

Many developing countries have adopted the use of micro-algae in water resource regulation by using algal community indices for water quality biomonitoring or including algal toxicity tests in a battery of tests to test industrial and municipal effluent discharges as a requirement to ensure that such discharges do not have adverse effects on aquatic organisms (NIWA 1998, EC 2000, OECD 2006). South Africa is now following that international trend by including micro-algae in the above-mentioned monitoring programmes, affirming their importance in aquatic ecosystem health. These national programmes and approaches were initiated by the Department of Water and Sanitation (DWS) to provide information on different water quality variables. They require different techniques and skills and are at various stages of development (Van Niekerk 2004).

The South African National Water Act clearly advocates that water resources be managed sustainably and equitably so that they are available for use now and in the future. Therefore, as the public trustee of water resources in South Africa, the Minister of the Department of Water Affairs and Sanitation (DWS) has a responsibility to develop national monitoring programmes and establish national information systems resulting from these programmes, in order to ensure the protection of water resources is balanced with the use of water for social and economic development (Palmer *et al*. 2002, NWA [36 of 1998], King and Pienaar 2011). Such monitoring programmes assist in ensuring that, although water users are allowed to abstract water from, and discharge wastewater into natural water resources, the aquatic ecosystem functions are impaired at levels defined by the classification system (Murray 2005, Jooste *et al*. 2008) and not irreparably impaired. These monitoring programmes are crucial in water resource regulation, as they produce information that supports and guides water management decisions (Van Niekerk 2004).

Although using selected standard toxicity tests for regulatory and management purposes in in-stream and industrial wastewater is a positive step, there are questions of environmental

realism and the ecological relevance of the data generated by these standard toxicity bioassays. Micro-algae in particular are extremely variable in their sensitivity to a range of contaminants. No one algal species has consistently emerged as most sensitive to a variety of chemicals (Blanck *et al.* 1984). Because of the uncertainty regarding the most sensitive algal species, the OECD (1989) recommended that the algal growth inhibition test be changed from a single standard species to a battery of tests with a number of species. Over the years, species of Chlorophyceae (green algae) such as *Scenedesmus subspiatus, Chlorella vulgaris* (OECD 1984), *Chlorella kessleri* (Lukavsky 1992), *Chlorella protothecoides* (Stauber *et al*. 1994*),Chlamydomonas reinhardii*, *Chlamydomonas variabilis*, and *Scenedesmus quadricauda* (Van Coillie *et al*. 1983, Swanson *et al*. 1991) have been used in toxicity tests internationally, and recommended as toxicity test species.

Algae can be isolated from local water bodies and established in the laboratory to make useful toxicity test organisms for assessing impacts on specific sites. Toxicity assessments using local species may be more environmentally realistic and ecologically relevant than relying on standard test species organisms which may not occur in the natural aquatic system of interest (Stauber 1995, Stauber and Davies 2000). The use of indigenous algae, in addition to the standard test organism has been recommended (Hörnström 1990, Stauber 1995). The reason for this is that tests with standard species are reproducible and generate the necessary baseline data for comparison with the data from the indigenous species which may be more variable, but more relevant for assessing site specific impacts.

This study therefore poses the following research question:
Is there any value in using locally isolated freshwater micro-algae in toxicity tests for application in South African water resource management?

The objectives will address this research question and guide the study design as outlined below. The objectives of this study are as follows:

- Developing capacity to use South African freshwater algae in toxicity testing.
  Phase 1: This is the basis and most important aspect of the study. It is also the most time-consuming and labour-intensive part of the study. It will be done by isolating and selecting suitable local micro-algal species to be used in toxicity tests using the following criteria:

- Ability to grow relatively rapidly, constantly and uniformly under laboratory conditions; and
- Ease with which cells can be counted under a microscope.

- Refining the toxicity test methods for using South African taxa in toxicity tests.

  Phase 2 - This is an essential part of this study because the existing algal toxicity test methods were developed for the use of the standard species *Pseudokirchneriella subcapitata* (Korshikov) Hindák 1990, and will require adjustment and refinement if local species are to be used. This refinement will be achieved by exposing the local and standard toxicity test species to reference toxicants in order to assess:
  - Their ability to grow under the prescribed toxicity test conditions; as well as
  - The sensitivity of the local species to the reference toxicants compared to the standard toxicity test species.
- Assessing the application and value of using the local micro-algal species in toxicity testing for use in water resource management in South African.

  Phase 3 – In this phase, the sensitivity of the local micro-algae to a range of toxicants (reference toxicants, salts, effluents and a herbicide) will be assessed. Toxicants will be carefully selected based on their relative importance in the South African context, as well as the practicality of using the local micro-algae to routinely determine the impact of these toxicants on local aquatic resources.

The use of locally-isolated species is a challenge in South Africa, due to the lack of resources and capacity for culturing and maintaining laboratory cultures. Maintaining algal cultures is a time-consuming and labour-intensive exercise (Persoone and van de Vel 1988, Griffin *et al*. 2011), hence there are currently no locally-isolated algal species being used for toxicity testing in South Africa. There are culture facilities available in some laboratories, and algal cultures are available, but these are not used for the development of toxicity testing techniques. Laboratories at institutions such as the Nelson Mandela Metropolitan University, the North West University (Potchefstroom campus) and the Environmental Biotechnology Research Unit (Rhodes University) use algal cultures for other research needs, such as taxonomy and teaching.

1.2 USING LOCALLY-ISOLATED ALGAE IN TOXICITY TESTS FOR WATER RESOURCE MANAGEMENT

The requirement for an algal toxicity test species is that it is easy to maintain in culture, demonstrates relatively rapid, constant and uniform growth under laboratory conditions, and that it is representative of the ecosystem (Hart 1986, Hörnström 1990, Stauber 1995). These basic requirements will be used in this study to identify select potential algal toxicity test species. Chlorophyceae (green algae) are the easiest to isolate and keep in culture, which is why they are mostly used in bioassays (Hörnström 1990). Freshwater Cyanophyceae (blue-green algae) and Bacillariophyceae (diatoms) have not been frequently used in toxicity tests because of the difficulty in maintaining viable cultures in the laboratory and obtaining sufficient growth during the three- to four-day test period (Lewis 1995). However, it has been suggested that the test battery of species to be used in algal toxicity tests should include representatives from different taxonomic groups, e.g. Chlorophyceae, Cyanophyceae and Bacillariophyceae (Holst and Ellwanger 1982, Swanson *et al*. 1991). There are also suggestions that a test battery should consist of at least five species selected according to sensitivity and widespread distribution (Hornstrom 1990).

Sensitivity to test substances may be species-dependent, and the use of a number of species with varying affinities is recommended to achieve a sensitive test, especially when testing complex effluents with unknown components (Wangberg and Blanck 1988, Hörnström 1990, Stauber *et al*. 1994a, Rojíčková-Padrtová and Mašálek 1999, Pavlić *et al*. 2005). Tests with a variety of single species are important as a tool in algal toxicology because they are likely to cover a range of sensitivities which enhances ecological relevance in the assessment process (Schmitt-Jansen *et al.* 2008). Selection of the appropriate test species depends on the type of toxicity test, the purpose of the test, the water quality characteristics of the sample being tested and the availability of cultured organisms (Stauber *et al*. 1994).

The use of locally-isolated algal species to generate toxicity test data is an important step towards understanding the impact of contaminants on specific aquatic systems and protecting those systems. Including algal species that have been isolated from the natural environment in a species sensitivity distribution (SSD) may enhance the ecological relevance of the data generated, as these species become true representatives of the given community. Toxicity data generated from these single species may be useful for inclusion in SSDs, which are

designed to represent the sensitivity of species of a community in a given ecosystem (Schmitt-Jansen *et al.* 2008). The aim of a SSD is to use the data on the sensitivity of various species to a chemical to determine a chemical concentration that will be less harmful to most species in the environment (Wheeler *et al*. 2002), which is why it is important for these species batteries to consist of species from different taxonomic groups, as this enhances the degree to which the species are representative of the environmental community (Kefford *et al*. 2005).

Even though laboratory toxicity tests may be valuable in decision making and regulation for water resource management, there are inherent uncertainties associated with using these data and these need to be recognised. One uncertainty is that most of the data are based on single species toxicity tests, which do not adequately capture the complexity of the diverse aquatic ecosystem that these tests aim to protect (Cairns 1986, Chapman 1995a, Schmitt-Jansen *et al*. 2008). Species in the environment may interact via competition and food web interactions, which are not captured by single species tests. However, toxicity tests with a battery of single species covering a range of taxonomic groups are used in aquatic ecotoxicology for decision making because they can cover a range of endpoints, thereby becoming a useful prognostic tool (Schmitt-Jansen *et al*. 2008). The use of SSDs is one of the ways of dealing with this uncertainty. The SSD approach focuses on the distribution of sensitivities of the species exposed to a selected toxicant. The SSDs have been accepted as a tool in decision making processes as they are widely used to develop water quality guidelines for aquatic resources (ANZECC and ARMCANZ 2000, Schmitt-Jansen *et al*. 2008).

Another uncertainty is that some untested species may be more sensitive than the species whose data is available. Regulatory decisions are based on data generated mostly by tests with standard toxicity test species (US EPA 2004, OECD 2006, Slabbert 2004). Although these standard toxicity test species are the most commonly used in laboratory toxicity tests, they may not be the most sensitive or representative of the range of species in the aquatic ecosystem (Cairns 1986, Chapman 1995a). Using local species; together with the standard toxicity test species that are commonly used (Chapman 1995b), but not necessarily occurring in the field; may assist in dealing with the representativity question. It is therefore ideal to use the same species found in the field in laboratory toxicity tests as this would strengthen the environmental realisms of these tests. This by no means down plays the importance of standard species in toxicity tests; they also play an important part because they are

reproducible and generate the much needed toxicity data. Countries such as New Zealand and Australia have developed toxicity test protocols for using indigenous freshwater algae, invertebrates and fish which are included in routine testing for regulatory purposes in water resource management (ANZECC and ARMCANZ 2000). There are toxicity test protocols developed in South Africa for using indigenous freshwater invertebrates and fish, and protocols for using indigenous algae and the data generated from this study will add to that body of knowledge.

1.3 ALGAL TOXICITY TESTING IN WATER RESOURCE MANAGEMENT

Algae are as important as any other living component of the aquatic ecosystem for holistic management of natural aquatic resources due to their role and relevance in aquatic ecosystems (Hart 1986, Wangberg *et al*. 1995, Ra *et al.* 2007). As the base of the aquatic food chain, algae are ecologically important organisms, and well suited for use in assessing toxicity of chemicals and effluents that may enter aquatic systems. Their involvement as useful tools in water resource management is warranted by their changes in species composition as a reflection of not only variations in water quality variables but also physical and biotic interactions (O'Farrell 2002, Bate *et al*. 2004, de la Rey *et al*. 2004, Harding *et al*. 2005, Taylor *et al*. 2007). They are useful biological indicators because they respond relatively quickly to changes in environmental condition, therefore enabling timely assessment of water quality changes.

Algae are not better than any other organism as indicators of toxicity, although some authors have shown them to be more sensitive to several compounds and effluents than invertebrates and fish (Hart 1986, Lewis 1995, Wanberg *et al*. 1995). The use of algal toxicity data has not been as significant in regulatory processes worldwide as that of invertebrates and fish data, which have dominated the decision-making processes. Algae are a useful and essential part of a battery of toxicity testing for hazard assessment and aquatic environmental protection (Stauber 1995). Algal toxicity data have been used in conjunction with other data, for the calculation of national and site-specific water quality guidelines, pre-manufacturer notices for new chemicals, evaluating environmentally safe levels of chemicals, registration of pesticides, and assessing the environmental impact of human and animal drugs, in the United States of America, and other countries (Holst and Ellwanger 1982, Carlson *et al.* 1985, U.S. EPA 1985).

The first algal toxicity test method for freshwater algae was developed and evaluated in the mid-1960s in the United States, and published as the Algal Assay Procedure Bottle Test (AAP) (U.S. EPA 1969, 1971a, 1978). The purpose of the early standard method was not monitoring toxic effects of chemicals, but determining the limiting algal nutrients for growth potential and productivity in natural waters. A modified version of the AAP method has served as the basis of the current toxicity test methods that have been published by international societies and regulatory agencies (US EPA, 1978, OECD 1984, US EPA 1984).

Most algal bioassays are growth-inhibition tests, measuring the decrease in growth rate or cell biomass after exposure to a toxicant, compared to a control. Algae are cultured in a nutrient-enriched medium, then healthy, exponentially growing cells are added to a range of toxicant concentrations, incubated under controlled conditions, and growth is estimated daily by counting cells microscopically, or measuring other biomass parameters such as cell fluorescence, optical density, turbidity, dry weight or chlorophyll a (US EPA 1978, OECD 1984, Lewis 1995, Slabbert *et al* 1998, Slabbert 2004). The toxicity response of algal cells to contaminants depends on the chemical contaminant, the algal species and the test endpoint measured (Stauber 1995). Algae tend to be sensitive to most contaminants because they respond either by stimulation or inhibition, or both (Wangberg *et al*. 1995, Yen *et al*. 1996, OECD 2006, Saçan and Balcioğlu 2006).

The most widely used algal bioassay is the algal growth inhibition assay with the freshwater green microalga, *Pseudokirchneriella. subcapitata* (formerly *Selenastrum capricornutum*) as the standard organism (US EPA 1978, OECD 1984, Slabbert *et al* 1998, Slabbert 2004). This bioassay has been accepted as a standard procedure for ecotoxicological studies and is used internationally and locally to provide data for regulatory purposes in hazard and risk assessment of substances (OECD 1984, USEPA 1984, ISO 1989, Slabbert 2004). It is the most preferred toxicity test method because it is a simple, relatively rapid method of reasonable cost and accepted reproducibility (Clarkson *et al.* 1998, Paixão *et al.* 2008). This method relies on the use of the basic laboratory equipment and a range of concentrations of the toxicant can be tested simultaneously and dose-response curves constructed followed by statistical analysis using simple standard techniques.

Over the years, the algal growth inhibition assay has been optimized by performing it at micro-scales using cuvettes, scintillation tubes, or micro-plates, as opposed to the original Erlenmeyer flasks (Blaise 1986, Slabbert *et al.*1998, Eisentraeger *et al.* 2003, Slabbert 2004, Paixão 2008). As a miniaturized version of the Erlenmeyer flask test, the micro-plate test is a more popular choice because more samples and more concentrations can be tested with low sample volumes and less incubation room. The use of disposable micro-plates also eliminates the risk of contamination from reused test vessels (Paixão *et al.* 2008).

The algal growth inhibition assay has been simplified even further by the introduction of a toxicity test kit (AlgalToxKit F) that follows the prescribed OECD guideline 201 and ISO procedure (OECD 1984, ISO 1989, OECD 2004). The AlgalToxKit F^{TM} contains all the materials necessary to perform the standard growth inhibition assay with *Pseudokirchneriella subcapitata*. The *P. subcapitata* algal cells are immobilized in beads, from which algal cells can easily be set free. Test medium and test containers made of biologically inert material to ensure uniform exposure conditions are included in the kit. The kit has easy-to-follow instructions and detailed illustrations. The use of kits excludes the cost of maintaining live cultures associated with the conventional algal growth inhibition assay. Laboratories worldwide use toxicity test kits for routine screening of chemicals and environmental samples. The AlgalToxKit F^{TM} adheres to the prescribed standard algal growth inhibition assay protocol (OECD 2004, ISO 1989) and generates results quickly and efficiently.

The simplification of the algal growth inhibition assay is meant to improve the performance of the method and generate highly reproducible and consistent results. Reproducibility and consistency are considered essential in toxicity testing for hazard assessment and aquatic environmental protection. Some authors have expressed that reproducibility afforded by standard toxicity test organisms is more important than sensitivity if toxicity tests are used for regulatory purposes (Lin *et al*. 2005, Chapman *et al*. 2011a). The application of algal toxicity testing in water resource management is fairly recent in South Africa, and different versions of the standard algal growth inhibition assay are performed by different laboratories. Some laboratories have an ongoing culture of *P. subcapitata*, and use the micro-plate assay, while others do not have culturing facilities and opt for the toxicity test kit to eliminate the cost associated with the maintenance of the algal cultures.

1.4 THE USE OF THE ALGAL GROWTH INHIBITION ASSAY IN SOUTH AFRICA

In the late 1980s, the Council for Scientific and Industrial Research (CSIR) adjusted the Standard US EPA Algal Growth Inhibition Flask test by performing it in 24-well micro-plates, using BG-11 medium (Rippka *et al*. 1979) instead of EPA medium and applying a slightly larger inoculum (200 000 cells/mL instead of 10 000 cells/mL) (Slabbert and Hinler 1990, Slabbert *et al*. 1998, Slabbert 2004). In 2004 the establishment of an inter-laboratory evaluation of this test, with the intention of having it in the national proficiency scheme, was initiated (Slabbert and Truter 2004). Interested laboratories were invited to participate in training for the algal growth inhibition test that was provided by the CSIR. Four laboratories (University of Johannesburg, Sasol, DWAF: Resource Quality Services (RQS) and CSIR) initially participated in the inter-laboratory training. These laboratories then included the standard algal growth inhibition assay in their battery of routine toxicity test assays.

Over recent years, more laboratories and water boards have been using the algal growth inhibition assay as part of their toxicity test routine. It is now included in the proficiency testing scheme, which is a national inter-laboratory evaluation of toxicity tests to ensure that tests are adequately standardised for routine use nationally. This assay is also part of a suite of toxicity tests included in the Introduction to Environmental Water Quality course offered by the Unilever Centre of Environmental Water Quality (Institute for Water Research, Rhodes University) in association with DWAF (RQS). The course has provided a broad spectrum of individuals, including personnel from government departments, industries and water boards, with training to perform these toxicity tests.

Despite these efforts to include the algal growth inhibition assay for routine toxicity testing in South African laboratories, there are still challenges. One of the challenges is that there are not enough laboratories that are able to perform this assay, and with those that use this test routinely, there is inter- and intra-laboratory variability in the data generated (Chapman *et al*. 2011b). Most laboratories do not have the necessary materials and equipment to set up the culture facilities to grow and maintain the algal toxicity test species (Slabbert and Truter 2004), and therefore use the toxicity test kitAlgalToxKit F^{TM} which is more costly in the long term (Griffin *et al*. 2011).

1.5 ALGAL RESEARCH AND APPLICATION IN WATER RESOURCE MANAGEMENT IN SOUTH AFRICA

Algal research in South Africa initially focused on taxonomic work with freshwater diatoms (Archibald 1972). South Africa has a long history of freshwater diatom research, with the most comprehensive collection of diatom sample material in the world, dating back to the 1950s (Cholnoky 1956, 1957). The diatom research continued until diatoms were recognized as indicators of water quality in South Africa (Taylor *et al*. 2007). Diatom indices based on species abundances were used for biomonitoring in addition to macro-invertebrates, and included in the River Health Programme. The use of diatoms as biomonitoring tools in South Africa is still in the early stages of development and therefore has challenges to be addressed. One of the challenges is that the taxonomically based diatom indices used for biomonitoring in South Africa have been developed elsewhere (mainly Europe) (Taylor *et al*. 2007). Although diatoms are cosmopolitan and found almost everywhere, endemic species have been recorded in South African rivers, and these are not included on the existing reference lists. This could lead to miscalculation of the diatom indices and necessitates the development of diatom indices that will include South African endemic species and be accurate for water quality monitoring in South Africa (Taylor *et al*. 2007).

Even though within species differences may be expected in other groups of algae for example between different ecotypes, ecotypes are not currently considered in these indices. The diatom indices being referred to are based on presence/absence as well as counts of species, (Griffin, N – Personal Comm.). Ecotypes do not really exist within the diatoms at species level as they are microorganisms which lack significant dispersal barriers. Therefore chances of habitat selective ecotypes developing are slim. In terms of genus there may be some differences between tropical and more temperate waters (e.g. *Nitzschia* species being dominant in clean habitats in the DRC, whereas *Nitzschia* in South Africa is generally accepted as indicating impact) (Taylor, J – Personal Comm.). The taxonomic research has subsequently extended to other taxonomic groups of algae such as Chlorophyceae (green) and Cyanophyceae (blue-green) algae.

In the 1980s, research on the eutrophication status of South African impoundments and rivers was initiated through the Trophic Status Project, which measured variables including chlorophyll *a*, total phosphorus, and percentage of cyanobacteria (DWAF 2002). This project

was developed and improved over the years to give rise to what is now called the National Eutrophication Monitoring Programme (DWAF 2002). Eutrophication is an acute problem that seems to be escalating in many impoundments and rivers in South Africa (DWAF 2002, Van Ginkel 2011). Eutrophication tends to be a water resource problem in most industrialized and urban areas. It is attributed to the overabundance of algal growth resulting from shifts in the algal community structure (Ma *et al*. 2006). The factors driving eutrophication are high nutrient concentrations and stagnation of the water body for relatively long periods, with suitable temperature, light and oxygen concentration (van Ginkel 2011). Phytoplankton biomass is routinely used to measure algal growth to assess the degree of eutrophication.

In some instances, pollution by nitrate-containing fertilizers may result in increased nitrate concentrations entering surface waters. The nitrates may be, for example, leached out of farm lands by surface run-off and find their way to streams, rivers and dams resulting in changes in the community structure of the aquatic ecosystem because nitrates are known to support growth of certain algae. Sometimes, even subtle changes in algal population structure can cause long-term biodiversity shifts throughout the water ecosystem, and that is the fundamental reason why measurements of algal biomass and growth rates are widely used to monitor nutrient enrichment in the biomonitoring of lakes, estuaries and rivers internationally (Twist *et al.* 1998, Dorigo *et al*. 2004).

The severity of the impact of eutrophication depends on the extent of the shift in community or population structure as well as the type of species whose growth is stimulated by the changing environment. When blue-green algae (Cyanophyceae), such as *Mycrocystis*, dominate the aquatic ecosystem, they may have deleterious environmental impacts, because they produce toxins that may affect other organisms, exacerbating the negative impact on the aquatic ecosystem (Shaw and Chadwick 1998). Monitoring eutrophication is very important, and having a national programme that is dedicated to this is essential, as, under extreme conditions, eutrophication may negatively impact water resources and consequently human health.

In the 1990s toxicity testing was introduced to regulation and decision-making in water resource management in South Africa, and the algal growth inhibitions test was among the toxicity tests to feature in policies on water quality management (Slabbert *et al.* 1998). Although the standard algal growth inhibition test has been in use for about two decades in

South Africa, there are still not many laboratories nationally that are able to perform the assay. There are also issues with standardization of the test, and inter- and intra-laboratory variability in results. There are no standardised operating procedures and quality requirements pertaining to the algal growth inhibition assay in South Africa (Chapman *et al*. 2011b). The absence of quality assurance requirements could have a negative bearing on the analysis and interpretation of the data. Although the algal growth inhibition assay is based on recognised international standard toxicity test protocols (US EPA 1984, OECD 1984, ISO 1989), quality assurance requirements are essential as they would contribute to the accuracy and precision of the data generated.

It is thus necessary to revise and improve algal toxicity testing in South Africa, in order to be in line with developed countries such as Australia, Europe and the United States, which now use more than one species (Stauber 1995, ISO 2004, OECD 2006) for algal toxicity testing, in order to deal with variability in sensitivity among algal species. Therefore, the fundamental work of isolating indigenous South African algal species is necessary step toward improving and advancing the use algal toxicity testing in water resource management in South Africa. The use of South African algal species in species sensitivity distribution to compare responses to single substances, and their comparative responses to effluents in this study depended on the isolation and culturing of these local species. Testing with different species may be particularly useful for effluents, since each species may be affected differently and synergistic, antagonistic and additive effects of the different effluent components may be captured through stimulation or inhibition of growth by different algal species.

2. DEVELOPING CAPACITY TO USE SOUTH AFRICAN FRESHWATER MICRO-ALGAL SPECIES IN TOXICITY TESTING: Isolation and culturing

2.1 INTRODUCTION

This chapter addresses the first and fundamental phase of this study, which is developing capacity to use locally isolated micro-algal species in toxicity testing for application in South African water resource management. This involves identifying isolation and culturing techniques to be used in order to obtain suitable micro-algal species for use in toxicity testing. Isolating micro-algae from the natural environment is not a trivial task. Different algal species require different isolation and culturing techniques, which are not always obvious at the initial phases of isolation. The objective of this phase is to isolate freshwater micro-algae from local water resources and culture them in the laboratory. Once freshwater micro-algal mono-cultures are established, they are identified to species level and suitable species are selected for use in toxicity tests, using the following criteria:

- Ability to grow relatively rapidly, constantly and uniformly under laboratory conditions and
- Ease with which cells can be counted under a microscope.

2.2 MICRO-ALGAL ISOLATION AND CULTURING

Micro-algae have different applications, and this has led to advances in micro-algal culture technology with the development of sophisticated isolation and culture techniques (Srinivasakumar and Rajaseka 2009). These applications include using micro-algae as nutritional supplements in human and animal foods, their role in aquaculture, as well as their use in cosmetics because of their chemical composition (Spolaore *et al*. 2006). Micro-algae are often isolated and cultured for use in studies on algal growth characteristics, culture methods and taxonomy (Wehr and Sheath 2003, Srinivasakumar and Rajasekha 2009). There have also been studies on aspects of species composition, density, distribution and seasonal variation of micro-algae in freshwater (Schmitt-Jansen and Altenburger 2005, Srinivasakumar and Rajasekha 2009).

Algae are now identified as alternative sources of vitamins, fuels, chemicals, pharmaceuticals and antibiotics (Spolaore *et al*. 2006). Because of the N_2 fixing capabilities of some algae, such as some cyanobacteria (blue-green algae), their role in agriculture as biofertilizers, soil conditioners and plant growth regulators has been recognized (Metting 1988). Nitrogen fixation is known to occur in heterocyst bearing filaments of cyanobacteria such as *Anaboena* sp. Heterocysts are thick-walled cells which have unique features that facilitate nitrogen-fixation (Sah 2008). The establishment of uni-algal cultures dates back to the late 1800s (Beijerinck 1890) and mass production of micro-algae in cultures for aquaculture, studies of plant physiology, and other purposes, was achieved in the early 1900s (Allen and Nelson 1910, Warburg 1919). Green (Chlorophyceae) and blue-green algae (Cyanophyceae) were the first algae to be isolated successfully, followed by diatoms and other types of algae (Preisig and Andersen 2005).

Large-scale cultures of *Chlorella*, *Dunaliella* and *Spirulina* have been established to produce biomass for food (Borowitzka 1999). Large-scale algal cultures have also been used for wastewater treatment (Tam and Wong 1989, Talbot *et al*. 1991, Rose and Dunn 2013). Understanding of the biology of the algae and the requirements of algal culturing has advanced the establishment of algal cultures at different scales. Small-scale laboratory cultures have been used in phycological research, to advance studies towards understanding the genetic characteristics, physiology and taxonomy of the algae, as well as to use algae in toxicity tests (Prescott 1969, Tam and Wong 1989, Lindemann *et al*. 1990, MacIntyre and Cullen 2005). There have been advances in understanding the biology of algae and the requirements of algal culturing. Factors such as the light intensity, temperature, pH and the role of symbiotic microbes have been reported to affect the culturing of micro-algae (Moss 1973, Talbot *et al*. 1991, Srinivasakumar and Rajasekha 2009, Hernandes *et al*. 2009). By experimentation and by trial and error, scientists have been able to develop culture methods, ingredients of artificial media, and necessary controlled culture conditions to grow micro-algae under laboratory conditions (Prescott 1969, Andersen and Kawachi 2005).

Depending on the objective of the growth of the culture, various types of cultures can be established: an enrichment culture (mixture of algal species), a uni-algal culture (single species of algae), or an axenic culture (single species free from any other micro-organism) (Prescott 1969, Bold and Wayne 1978, Guillard 2005). There are also different culture methods used, depending on the purpose of the culture. There is continuous culture method,

where fresh medium is continuously added to the culture at a rate sufficient to maintain it (Wood *et al*. 2005). An alternative to the continuous culture method is the batch culture method, a closed system where the culture is grown in a fixed volume of culture medium under specific (Wood *et al*. 2005). The algal population cell density increases while nutrient components of the culturing medium decrease over time, causing exhaustion of a limiting factor, which then results in a decline in population density. The batch culture method is common in most small-scale laboratory cultures used for research and academic purposes, because of its simplicity and low cost. The batch culture method was used in this study as it is also adopted by most algal toxicity test protocols (US EPA 1984, OECD 1984, ISO 1989, Chen *et al*. 1997).

2.2.1 Micro-algal isolation techniques

Isolating pure algal cultures from the natural environment and maintaining them in the laboratory is a painstaking process. Understanding and mimicking the natural environmental conditions is an essential step towards successfully isolating micro-algae (Andersen and Kawachi 2005). Culture techniques and media can seldom duplicate the natural habitat. Hence it is to be expected that, in adapting to an artificial situation, organisms would exhibit morphological, physiological, and reproductive characteristics unlike those they would exhibit in the natural environment (Prescott 1969, Bold and Wayne 1978). This could lead to difficulty in identifying algal culture species.

The initial supply of algal material for isolation is usually from a natural population. Although isolation of micro-algae into culture is well established, some species are easily cultured whereas others are difficult to grow. Challenges, such as presence of contaminants, may prevent successful isolation of some species or prevent maintenance of established isolates. Algae may vary in their requirements and demand different culture media and different handling techniques (James 1978). The growth rate of different species may also vary widely and the turnover frequency in the cultivation must be adapted to this rate so that the cultures grow exponentially (Hörnström 1990). There are various methods that can be used to isolate single cells from an environmental sample or enrichment culture. A single cell is isolated in order to establish a uni-algal culture. Uni-algal cultures established from a single cell may be regarded as clones even though gene mutation may occur during long-term culture maintenance. Culture established from more than one individual include genetic

variation, and are therefore not clonal cultures (Kawai *et al*. 2005). Each organism reacts differently, therefore the techniques for isolation must be adapted to meet the individual needs of the target organisms.

2.2.1.1 *Isolation by glass Pasteur pipette washing*

This is one of the common methods where a specially prepared micropipette tip is used to pick up an algal cell from an environmental sample or enrichment culture. The cell is then repeatedly washed by depositing it in a sterile droplet until it is free from all other organisms. The cell then remains in the droplet, and can be placed into the culture medium. This method requires great skill and practice as delicate cells can easily be damaged in the process (Andersen and Kawachi 2005).

2.2.1.2 *Gravity separation*

This method uses gentle centrifugation for a short time to settle larger and heavier micro-algal forms and diatoms to a loose pellet, and smaller cells can remain in the supernatant to be decanted. The cells in the pellet are then re-suspended and the process is repeated. The centrifugation speed and time may vary. This method is effective in separating larger, heavier cells from smaller bacterial and algal cells. Excessive centrifugation can damage the cells. It is difficult to obtain uni-algal cultures with this method so it is common to use it with other isolation methods in order to obtain single cells (Andersen and Kawachi 2005).

2.2.1.3 *Dilution method*

In this method, an aliquot of a sample is placed into a test tube or well of a multi-well plate containing a sterile medium. After mixing, an aliquot is removed from there and placed into the next tube or well, and this process is repeated. Each dilution increases the probability of single algal cell isolation. Five or six repeated serial dilutions (1:10) are enough in most cases (Andersen and Kawachi 2005) for single cell isolation to obtain a uni-algal culture. Dilution can be made with growth medium, distilled water or filtered water from the sample site.

2.2.1.4 *Isolation on agar plates*

This kind of isolation is achieved by streaking the sample across the agar surface with an inoculation loop, and then incubating the agar plate in an appropriate environment until algal colonies can be observed. Individual colonies can then be picked up from the agar plate and either re-streaked onto a new agar plate or rinsed into liquid culture medium to free the cells. This method favours those algal species that are able to grow well on agar plates (Andersen and Kawachi 2005). Agar is also a good medium for bacteria and fungi, and this can contaminate the efforts of algal isolation. This method is commonly used by small-scale laboratories that isolate cultures for academic and research purposes. It was ideal for use in this study because it is simple and requires minimum technical skill.

2.2.2 Culture media and nutritional requirements

Algae are mostly photoautotrophic: they use light energy to synthesize their protoplasm from inorganic sources, and it is therefore important that sufficient concentrations of the required inorganic compounds are included in the culture medium, and the medium has the desired pH (Bold and Wayne 1978, Watanabe 2005). Some algae are heterotrophic and require organic compounds in their nutrition. Culture media whose components are known are called 'defined media'. There are also undefined culture media whose components are unknown, such as soil extracts or filtered water from the natural source of the sample (Bold and Waynne 1978). The use of defined or undefined media in cultures depends on the objectives of the culturing. Defined media are commonly used for physiological and ecotoxicological experiments.

Most defined culture media include major elements such as C, H, O, P, K, N, S, Ca, Fe and Mg as well as those elements required in trace amounts such as Zn, Mn, Mo, Cu, Co and B. These elements may be provided in the culture medium in different forms, for example, nitrogen may be supplied as NO_3, NO_2 or NH_4 (Bold and Wayne 1978). Nitrogen and phosphorus are the main limiting nutrients for algal growth (Chen *et al*. 1997). There are species differences in the efficiency of uptake of nitrates and phosphates. In batch cultures, increasing nitrate and phosphate concentrations may increase the final yield, but have little effect on growth rate (Moss 1973). Some algae require vitamins to grow, and in those instances vitamins should be part of the medium composition (Bold and Wayne 1978).

Various media have been developed and used for isolation and cultivation of micro-algae. Growth media are enriched with nutrients and allow the algae to grow under nutrient-saturated conditions (Chen *et al*. 1997). The nutrient requirements of algal species may vary widely and culture media must be composed to meet particular requirements for the growth of the desired species (Hörnström 1990). While some culture media are formulated according to nutrient requirements of species, others are derived from analysis of the water in the natural habitat of the species (Watanabe 2005). Selecting the desired culture medium and the correct nutrient concentration of the culture medium is crucial because nutrients are the most important factor affecting the growth of algae (Srinivasakumar and Rajasekha 2009).

2.2.3 Culture maintenance and culture conditions

In batch cultures, the transfer of cells from a culture to fresh medium (sub-culturing) is required for continued cell population growth and culture maintenance. It is not uncommon for the target species to grow in the initial stages of isolation but then die after being transferred to fresh culture medium. This often indicates that the culture medium lacks a particular substance or the organism may be accumulating wastes that poison its environment, therefore killing it (Lorenz *et al*. 2005).

Consideration must be given to the light and temperature requirements of organisms. When nutrients do not limit growth in the culture, the degree of light penetration and the temperature conditions are the key factors affecting growth. Light intensity and quality, as well as day/night cycles may strongly influence the growth and physiological state of algal populations (Stauber 1995). Algal growth is correlated with the amount of light usable for photosynthesis (Mayer *et al.* 1998). The quality and quantity of light delivered to micro-algal cells is significant for the cells to grow efficiently (Markl 1980). A daily rhythm with 12h light and 12h darkness or 18h light and 6h darkness is normally suitable for culture maintenance, particularly with regard to simulating natural conditions (Lorenz *et al*. 2005). Most processes of algal metabolism are highly dependent on temperature (Mayer *et al*. 1998), so thermal effects on algal growth may be significant. Temperatures of 20-30^{o}C are often favourable (Wayne and Bold 1978, Lorenz *et al*. 2005).

Light and temperature tolerances and requirements of algae depend on physiological capabilities, which are species specific and related to environmental factors. The response of cultured organisms to light availability and intensity, as well as temperature, may also be related to the geographic distribution and habitat of the corresponding natural population (Talbot *et al*. 1991).

2.2.4 The Batch Culture Growth Curve

Batch culture systems are closed systems where population growth is characterised by a constant increase in population density until the exhaustion of some limiting factor, while other nutrient components of the culture medium decrease over time. Concentrations of waste and other substances produced by cells also increase in the culture medium (Bold and Wayne 1978). There are five phases of the batch culture growth curve: the lag, exponential, declining, stationary and death phases (Table 2.1). The different phases can be measured by the duration and slope of the growth curve (Fogg and Thake 1987, Bolier and Donze 1989). In algal research, the growth rate of batch cultures is often characterised by the exponential phase: the S-shape of the growth curve, from the starting point to the point of inflection and a specific growth rate is often calculated (Schanz and Zahler 1980).

Table 2.1 Description phases of algal growth in batch culture (Fogg and Thake 1987)

No	Phase	Growth	Interpretation
1	Lag	Zero	Physiological adaptation of the algal cells to changing conditions. The condition of the inoculum has strong bearing on the lag phase.
2	Exponential	Constant	Population growth changes the environment of the cells, physiological adaptation is faster. The duration of the exponential phase is dependent on the size of the inoculum, the capacity of the growth medium and the culturing conditions that support algal growth.
3	Declining	Decreasing	Effects of changing conditions appear. A specific requirement for cell division may be limiting growth.
4	Stationary	Zero	One or more nutrients are exhausted down to the threshold level of the cells.
5	Death	Negative	The duration of the stationary phase and rate of death depend on the organism.

2.3 SELECTION OF SPECIES FOR USE IN TOXICITY TESTS

The variation in sensitivity to toxicants among algal species has resulted in uncertainty regarding the species most suitable for toxicity testing (Wangberg and Blanck 1988). Differences in species sensitivity to toxicants may result from inter-specific variability due to differences in receptors or repair mechanisms (Schmitt-Jansen and Altenburger 2005).

The use of species sensitivity distributions (SSD) has been applied in ecological risk assessment to extrapolate results from single-species toxicity test data with different species to communities in complex systems (Newman *et al*. 2000, Wheeler *et al*. 2002). The SSD approach focuses on the distribution of varying sensitivities of test species that are supposed to represent the community of an ecosystem, rather than on the most sensitive species (Schmitt-Jansen and Altenburger 2005). This approach has become a practical method for the derivation of water quality guidelines and is an accepted instrument in ecological risk assessment (ANZECC and ARMCANZ 2000).

The use of a battery of uni-algal species has been suggested by various authors to decrease the uncertainty regarding sensitivity among algal species (Blanck *et al*. 1984, Wangberg and Blanck 1988, Rojickova-Padrtova and Marsalek 1999). Researchers differ in their views of the species composition of a representative algal test battery: some have argued that morphology, ecophysiology, phylogeny and cultivation properties must be considered as criteria for selecting algal strains as test organisms (Komarek and Marvan 1979, Wangberg and Blanck 1988), without forgetting that cytology and genetics may also be toxicologically relevant. Others have suggested that test organisms should be good laboratory organisms that show constant and uniform growth rather than being sensitive or abundant in nature (Nyholm and Kallqvist 1989).

According to Hornstrom (1990), a test battery containing up to five species, selected for constant and uniform growth, sensitivity, and wide-spread distribution (representative of the ecosystem), would be sufficient. In this study, suitable test organisms will be selected according to their ability to show constant and uniform growth under controlled laboratory conditions, and they will have to be unicellular and form homogeneous suspensions in test media. It is important that the cells do not clump together in culture, such that they cannot be counted easily under a microscope.

2.4 MATERIALS AND METHODS

2.4.1 Sample collection

Clean 50 mL polyethylene bottles were used to collect water samples with phytoplankton from sites on the Palmiet and Keiskamma rivers in the Eastern Cape, South Africa (See Table 2.2). These two rivers were selected because they are relatively un-impacted, with no significant land-use practices that could result in major effects on the water quality. Seven sampling sites were selected along the Palmiet River upstream of Howisonspoort Dam, and a single sample was taken from each site. The selected sites were on the stretch of the river that runs through a nature reserve (Thomas Baines) with no expected impacts from upstream. Three sites were selected along the Keiskamma River below the small town of Middledrift and two samples were taken from each site. The sites on the Keiskamma River were on the part of the river surrounded by small rural dwellings with small-scale cattle farming. There was no major impact expected from the surrounding activities.

2.4.2 Culture media

Two defined culture media, Combo medium (Kilham *et al* 1998) and BG11 medium (Rippka *et al*. 1979) were used. Combo medium was selected to initiate enrichment cultures because it is a nutrient-rich culture medium that facilitates growth of algae from a range of phyla. It contains the major elements and micro-nutrients as well as additional components, such as vitamins and silica, to ensure the growth of a wide range of organisms from different taxonomic groups, including diatoms. The 50% Combo medium seemed to give optimum algal growth when compared to full-strength (100%) Combo medium. BG11 medium was selected for its general use in the growth and maintenance of freshwater green and blue-green algal cultures (Olvera-Ramirez *et al*. 2000, Slabbert 2004, Tripathi *et al*. 2006). BG11 medium of 10% was the medium concentration of choice for routine culture maintenance of green (Chlorophyceae) and blue-green algae (Cyanophyceae) and was therefore used to grow and maintain the green and blue-green cultures once they were established. This medium is used routinely for the algal growth inhibition assay in South Africa (Slabbert 2004). BG-11 was also used in this study as the toxicity test medium, so it served the additional purpose of acclimation of algae before they were used in the test. Algae grown in Combo medium were acclimatised in BG-11 by growing the cells in test medium for four generations before the

test. The process of acclimation allows the cells to adjust their physiological mechanisms to the new nutrient source (Voltolina *et al*. 1998).

All stock solutions and growth media were prepared in Milli-Q® water. The pH of Combo medium remained between 8 and 8.5 and that of BG11 medium between 7 and 7.6 and without adjustment. The growth medium was sterilized by autoclaving for 15 minutes at 121°C before use. Agar plates of the respective media were prepared by dissolving 1% agar in the respective media and sterilizing it by autoclaving for 15 minutes at 121°C before pouring it into sterile Petri-dishes and leaving the plates to solidify. Agar plates were stored at 4°C for a maximum period of two weeks. Nutrient agar plates were also prepared by dissolving approximately 3% of nutrient agar in Milli-Q® water and preparing the plates as described above.

2.4.3 Culture conditions

All liquid medium cultures were incubated in a growth chamber at 24±2°C under cool white fluorescent light in 16:8 hour light/dark cycle. Plate cultures were incubated in an environmentally controlled room at 24±2°C under cool white fluorescent light in 16:8 hour light/dark cycle until colonies were visible and then stored at ±4°C in the dark for a maximum period of three months (Slabbert 2004). The storing of starter cultures of *P. subcapitata* at ±4°C is also recommended by the algal growth inhibition assay protocol used by Environment Canada (EPS 1/RM/25 (EC 2007)).

2.4.4 Isolation and maintenance of species

The method of isolation was based on streaking on agar plates as described in Figure 2.1. The collected environmental samples were initially inoculated in liquid 50% Combo medium to obtain enriched enrichment cultures. This was done by inoculating approximately 1 mL of the environmental sample with a sterile glass Pasteur pipette into 10 mL of 50% Combo medium in a sterile 25 mL test tube fitted with a cap. The enrichment cultures were maintained by sub-culturing them in fresh 50% Combo medium monthly.

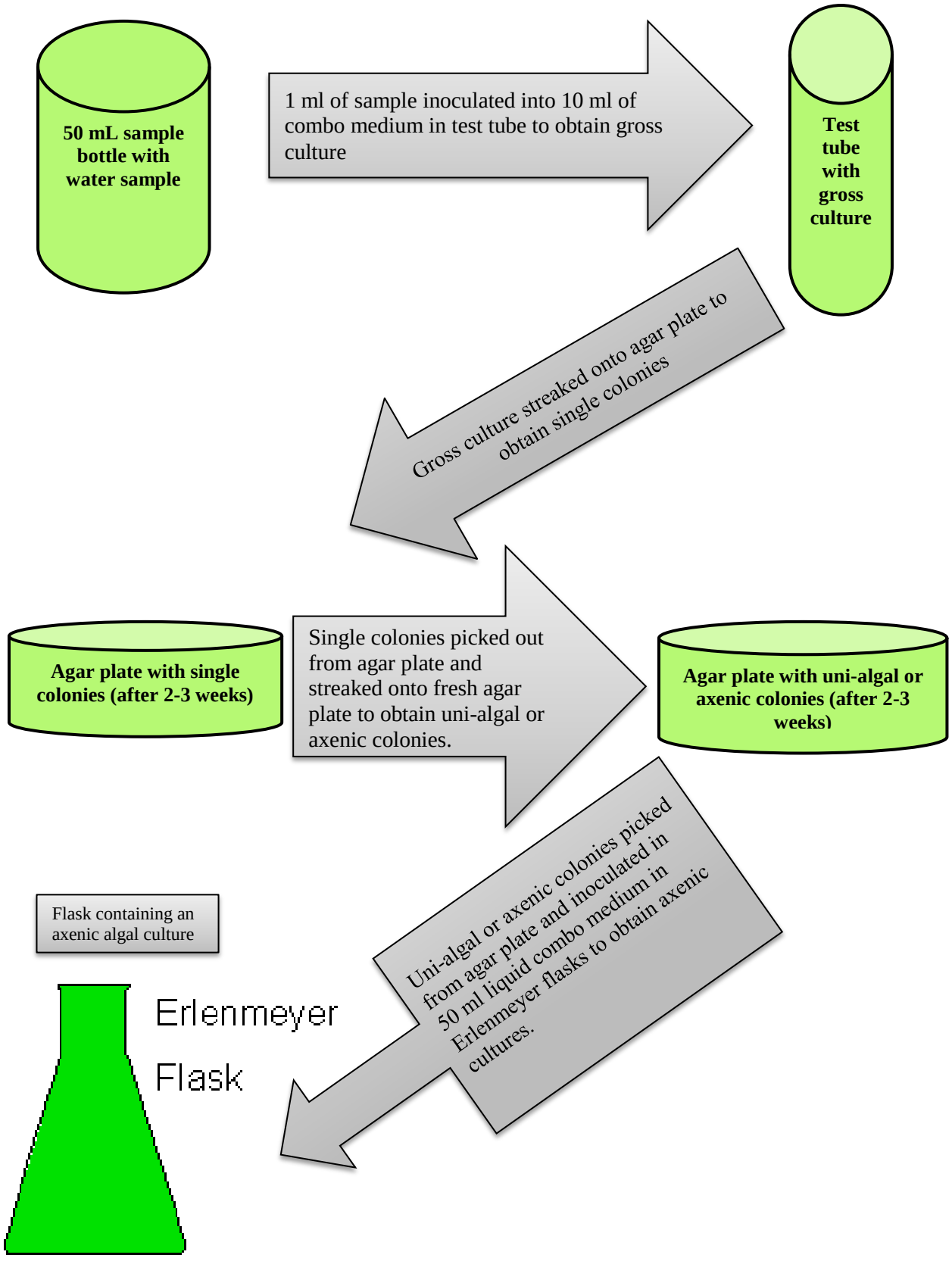

Figure 2.1 An illustration summarising the isolation method used to obtain axenic cultures in this study.

When enrichment cultures were obtained, they were streaked onto 50% Combo medium agar plates and incubated under controlled conditions for three weeks to allow algal cell colonies to develop. When colonies were visible, different colonies were individually picked with an inoculation loop and re-streaked onto new plates to ensure that uni-algal or axenic cultures were obtained. When colonies were visible after incubation with no visible bacteria or fungi on the agar plates, single algal colonies were picked with an inoculation loop and inoculated in liquid Combo medium and the liquid medium cultures were incubated for two to three weeks under controlled conditions. These liquid cultures were then observed under a light microscope at 40× magnification to check if they were uni-algal (cells observed were similar) and axenic (free of other micro-organisms). If the plates had visible algal colonies but were contaminated with bacteria or fungi, or both, the colonies were picked and re-streaked and the process repeated until axenic cultures were obtained.

In cases where culture plates were free of bacteria and fungi but had different cells when observed under the microscope, indicating the presence of more than one species in the culture, single colonies would be picked and re-streaked and the above process repeated to obtain axenic cultures. When axenic culture plates were obtained, they were stored at ±4°C in the dark as back-up cultures for up to three months (Slabbert 2004), so that they could be used to start new cultures in cases of contamination. These back-up cultures were maintained by re-streaking onto fresh plates every three months.

Fresh colonies were then picked from the plates that were uni-algal and free from bacteria, and inoculated into 50 mL of fresh Combo medium in 100 mL or 250 mL Erlenmeyer flasks to establish axenic cultures. These axenic cultures were maintained by sub-culturing in fresh Combo medium every week. Cultures were checked for contaminants once a month by observing them under the microscope for the presence of bacteria or fungi and also spreading 1 mL of the culture onto a nutrient agar plates and incubating the nutrient agar plates at 37°C in the dark for 48 hours. Contaminated cultures were discarded and new cultures were started from the back-up culture plates by picking colonies and inoculating them into fresh sterile culture medium in flasks as shown in Figure 2.1.

2.4.5 Growth, selection and identification of potential toxicity test species

Axenic algal cultures were inoculated with colonies from agar plates and grown in 100 mL of liquid BG-l1 in 250 mL Erlenmeyer flasks, under controlled conditions (as described in section 2.2.3) in a growth chamber for 30 days. Three flasks were inoculated with colonies picked from three different agar plates for each algal species. The pH of the growth medium was 7.3 and not adjusted. The flasks were shaken at 125 rpm on an orbital shaker for an hour daily to facilitate cell mixing and enhance population growth.

Cultures were sent to the Natural Resources and the Environment department of the Council for Scientific and Industrial Research (CSIR) in Stellenbosch and the Botany Department of the North-West University, Potchefstroom Campus, for identification by specialists. Identification was carried out using cell morphology and taxonomic keys.

Algae that would be potentially useful for toxicity tests were selected for their relatively rapid and constant growth under laboratory conditions, formation of homogeneous suspension in growth medium, and the ease with which cells could be counted under the microscope. Species that contained clumping cells or cells that would otherwise be difficult to count were eliminated as toxicity test species since it is essential to count cells in order to determine initial cell density for a toxicity test.

The specific growth rate of the selected algal species was determined by monitoring the cell density. Cell density was determined by counting cells under the microscope using a haemocytometer. An aliquot (40 µl) of the diluted culture (1:10) in growth medium, was placed on the counting chamber of the Neubauer haemocytometer and cells were counted on ten fields of view under an Olympus BX51 research microscope at 400 times magnification under phase contrast illumination. Cell density was expressed as number of cells counted × 10^1(10 fold dilution) × 10^4 (factor for haemocytometer) (Slabbert 2004).

The growth curves and exponential phases of the algal cultures were determined and specific growth rate was calculated as follows:

$$\mu = \frac{\ln (x_2/x_1)}{t_2 - t_1}$$

where μ is the specific growth rate and, x_1 and x_2 denote cell densities at times t_1 and t_2 (Chao and Chen 2001).

2.4.6 Statistical analysis

There were three growth flasks for each species, prepared for determining growth curves and calculating specific growth rates. Differences in specific growth rate between the three selected species were determined using a one-way analysis of variance (ANOVA) with the statistical program STATISTICA (Version8), with $p<0.05$ indicating a statistically significant difference.

2.5 RESULTS AND DISCUSSION

Table 2.2 indicates the number of species isolated from different sites of the Palmiet and Keiskamma rivers, as well as the species selected as potential toxicity test species. Algae in mixed cultures may compete for available nutrients; some may even produce substances that may inhibit the growth of other algal species (Franklin *et al*. 2004). This competition has an impact on the type of species that are successfully isolated and their ability to be cultured. All species successfully isolated and cultured axenically in this study are green algae (Chlorophyceae), with the exception of the diatom species, *Nitzschia* sp. (Table 2.2). Chlorophyceae are generally easy to isolate and keep in culture. They are mainly autotrophic, requiring only light and inorganic salts to grow, although growth can be stimulated by organic substances. Algae belonging to other taxonomic groups such as Bacillariophyceae (diatoms) and Dinophyceae (dinoflagellates) require different forms of additives e.g. vitamins, silicon and selenium (Hornstrom 1990). The Combo medium initially used for isolation contained these additives, and was capable of supporting growth of a variety of freshwater algae from different taxonomic groups (Kilham *et al.* 1998). The medium was composed of a wide range of nutrients required by a variety of algal taxonomic groups, and therefore would, in theory, be able to facilitate the isolation of species from different groups.

Four species were successfully isolated and cultured from the Palmiet River: *Chlorella vulgaris* Beijerink 1890, *Stichococcus minutissimus* Skuja 1956, *Monoraphidium minutum* (Nägeli) Komárková-Legnerová 1969 and *Scenedesmus bicaudatus* Dedusenko 1925 (Table 2.2). The *Chlorella vulgaris* culture was composed of small (±5.5 μm in diameter), spherical,

unicellular organisms. *Stichococcus minutissimus* culture had minute (±2 µm wide, ±3.5 µm long), solitary cells, which were cylindrical to ellipsoid in shape. *Scenedesmus bicaudatus* were coenobial cells of two or four which were held together linearly or alternately. Cells were small (±2 µm wide, ±6 µm long) and ovoid-cylindric in shape, with a spine at the apex of each marginal cell. *Monoraphidium minutum* organisms were small (±2 µm wide, ±7.5 µm long) crescent shaped cells with narrowing apices. They were sometimes spirally twisted around each other in pairs or larger groups. All of the above-mentioned cultures exhibited relatively rapid growth under laboratory conditions and formed homogenous suspensions in culture, although the cells of *C. vulgaris* clumped together at high densities. From this group, *Chlorella vulgaris* and *Scenedesmus bicaudatus* were identified and selected as potential toxicity test species due to their relative rapid growth under the defined laboratory conditions, the ease with which they could be counted under a microscope, and their forming homogenous suspensions in the selected culture media.

Table 2.2 Species cultured from different sites of the Palmiet and Keiskamma rivers (*-selected as toxicity test species).

Source	**No. of species**	**Species ID**	**Total no. of species /river**
Palmiet	2	**Chlorella vulgaris* Beijerink 1890	4
		Stichococcus minutissimus Skuja 1956	
	2	**Scenedesmus bicaudatus* Dedusenko 1925	
		Monoraphidium minutum (Nägeli) Komárková-Legnerová 1969	
Keiskamma	2	*Oocystis lacustris* Chodat 1897	4
		Nitzschia sp.	
	1	**Chlorella sorokiniana* Shihira, I. and R.W. Krauss 1965	
	1	*Scenedesmus arcuatus* (Lemmermann) Lemmermann 1899	

Stichococcus minutissimus and *Monoraphidium minutum* were eliminated as toxicity test species. *Stichococcus minutissimus* was sensitive to the given growth conditions and did not always grow upon sub-culturing, and therefore would be difficult to use as a toxicity test species as it would not always be available on demand. It is not uncommon for species to grow initially upon isolation, and then die upon subsequent sub-culturing (Lorenz *et al*. 2005). Because *S. minutissimus* did not grow under laboratory conditions, there would be a chance of not achieving the desired growth during a toxicity test. One of the conditions placed on a toxicity test organism is that it grows constantly under laboratory conditions (Hornstrom 1990), and *S. minutissimus* did not meet this condition. *Monoraphidium minutum* was eliminated due to its crescent-shaped cells which were sometimes spirally twisted around each other, making it difficult to count them. It is necessary to determine the initial algal concentration, by some measure of cell density, during a toxicity test. The simplest and most cost-effective way of doing this is counting cells under a microscope, therefore species such as *M. minutum*, which are not easy to count would make it difficult to determine the initial cell density, and therefore would not be good toxicity test species.

Four species were successfully cultured from the Keiskamma River: *Oocystis lacustris* Chodat 1897, *Nitzschia* species, *Scenedesmus arcuatus* (Lemmermann) Lemmermann 1899, and *Chlorella sorokiniana* Shihira, I. and R.W. Krauss 1965 (Table 2.2). *Oocystis lacustris* consisted of small ellipsoidal cells with round apices. Some of the cells in the culture tended to adhere to the walls of the culture vessel, while others were suspended in the culture medium. This made it difficult to interpret the results of cell counts with certainty, as the culture was not suspended homogeneously in the medium. Therefore this species would not make a good toxicity test species and it was thus eliminated from further testing (Table 2.2).

The *Nitzschia* culture was a diatom culture that consisted of small (±2 μm wide, ±8 μm long), elliptical, solitary cells. The culture crashed after a number of generations, and could not be revived, and therefore could not be used for toxicity testing. Diatoms reproduce asexually and sexually, with asexual reproduction as the primary mode of reproduction. Asexual reproduction results in smaller cell size, as cell size diminishes with each reproduction cycle. When cells have diminished to about 30% of their original size, sexual reproduction occurs to regenerate the large cell size. Gamete production is influenced by factors such as light availability, temperature and the concentration of nutrients (Mos 2001). In the presence of limiting factors, diatoms may fail to reproduce, and the population size may decrease, and in

case of cultures, a culture crash is possible. Although *Nitzschia* species are among the most common freshwater diatoms and have been used in algal toxicity testing (Pavlić *et al*. 2005), our culture was eliminated as a potential toxicity test species (Table 2.3) due to challenges in keeping the culture viable .

The *Scenedesmus arcuatus* culture was composed of relatively larger (±10 μm wide, ±20 μm long) oval or slightly cylindrical, solitary or coenobial cells. Although *S. arcuatus* grew well under laboratory conditions and formed a homogenous suspension under laboratory conditions, the growth of this species was difficult to interpret. There was no noticeable exponential growth phase during the 30-day period of determination of the growth curves. Cell numbers were low, although there was evidence of cell division upon observation under the microscope. There was no clear indication of increasing population growth. This would make it a difficult species to use in the algal growth inhibition assay, so it was eliminated as a toxicity test species (Table 2.2).

Chlorella sorokiniana consisted of small green cells with seemingly different life stages. Adult cells were solitary spherical cells (±2.5 μm wide, ±5 μm long), with asexual reproduction by means of autospores which are formed internally through internal cell division. The parent cell first becomes square shaped and releases four to eight smaller square-shaped autospores. This species was selected for use in toxicity testing (Table 2.2), as it grew relatively rapidly under laboratory conditions, forming a homogenous suspension in culture medium, and is unicellular, which makes it easy to count under the microscope.

Specific growth rates of the selected three species, *Chlorella vulgaris, Scenedesmus bicaudatus* and *Chlorella sorokiniana*, in BG-11 medium are shown on Table 2.3 and Figure 2.2. The choice of medium for specific growth rate determination was motivated by the fact that BG-11 is the medium used for toxicity testing in South Africa (Slabbert 2004), which then served as a means of getting the algae acclimated adapted to the medium while determining their growth potential in the medium. The specific growth rate of the *Chlorella* sp. seemed to be slightly higher than the other two species (Table 2.3), although there were no statistically significant differences ($p>0.05$) between the specific growth rates of the three species. The exponential growth phase (steepest part of the growth curve (Figure 2.2)) of *Chlorella sorokiniana* was between days 8 and 13, whereas the other two species, *Chlorella vulgaris* and *Scenedesmus bicaudatus* showed exponential growth between days 10 and 15.

The specific growth rate of different species may vary, and the frequency in sub-culturing must be adapted to the specific growth rate in order to keep the culture at the exponential growth phase for testing.

Table 2.3 Specific growth rates (average cell density (n=3) per day) of the three species (*Chlorella vulgaris*, *Scenedesmus bicaudatus* and *Chlorella sorokiniana*) cultures selected for use in toxicity tests

Species	**Specific growth rate** (Average Cell Density Per Day)
Chlorella vulgaris	0.30
Scenedesmus bicaudatus	0.32
Chlorella sorokiniana	0.43

The two *Chlorella* species showed signs of the stationary phase by day 20, whereas *Scenedesmus bicaudatus* exhibited steady growth until around day 25 (Figure 2.2). These three species were selected for further testing as potential test species in toxicity testing on the basis of their suitable morphology and growth properties in culture. No consideration was given to other factors such as physiology, genetic diversity or cytology (structure, function, division and life history of cells), although these may also be toxicologically relevant. Species of the genera *Chlorella* and *Scenedesmus* are generally easy to culture, and are found in culture collections all over the world (Borowitzka 1999, Wong *et al*. 2000, Chen and Jiang 2001). *Chlorella* species have been cultured in large-scale cultures for commercial use since the 1960s (Borowitzka 1999, Spolaore *et al*. 2006).

Scenedesmus and *Chlorella* species have also been extensively used in toxicity tests worldwide (Tam and Wong 1989, Wong *et al*. 2000, Eguchi *et al*. 2004, Lürling 2005, Palvić *et al*. 2005). These two genera are cosmopolitan which means they are fairly widely distributed in the aquatic ecosystem. This then makes them good representatives of the aquatic ecosystem, which is an important factor in selecting a toxicity test species (Hornstrom 1990).

Chlorella vulgaris is one of the more commonly used species in micro-algal toxicity tests (Muñoz *et al*. 1996, Wong *et al*. 2000, Rioboo *et al*. 2002, Cronin *et al*. 2004, Nie *et al*. 2008)

as an alternative to the standard test species *Pseudokirchneriella subcapitata* and is available in most commercial culture collections. It is a widely used and recommended species for ecotoxicological testing (OECD1993) because of its widespread distribution and the ease with which it can be cultivated and maintained under controlled conditions (Wong *et al*. 2000, Nie *et al*. 2008, Silva *et al.* 2009). *Chlorella sorokiniana* is mainly used in studies of biomass production and photosynthesis research (de Bashan *et al*. 2008).

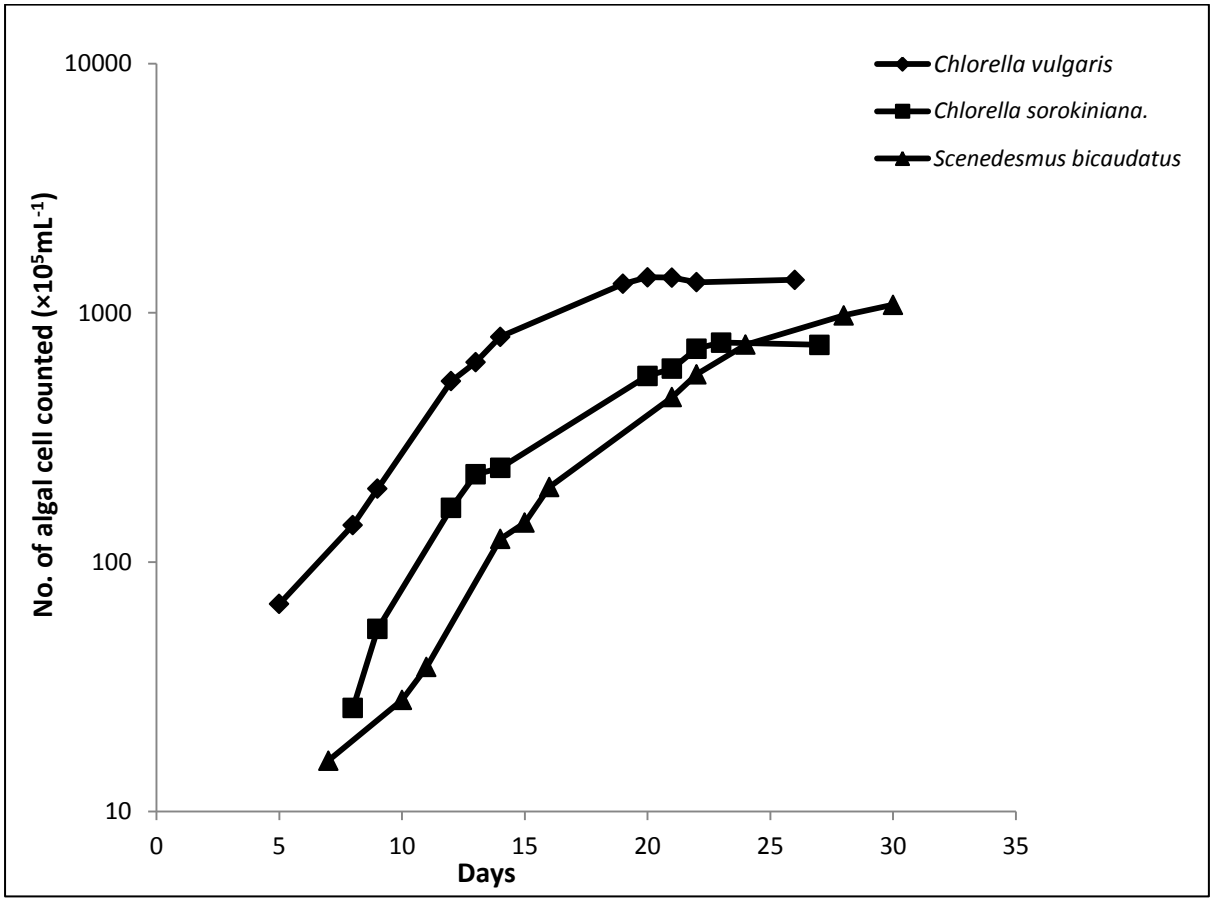

Figure 2.2 Growth curves of the three micro-algal species, *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus bicaudatus*, selected as potential toxicity test species

Most of the currently available literature and data about toxicity tests on algae has relied on *Pseudokirchneriella subcapitata* as the test species. There is limited information available on other micro-algal species (especially in South Africa), although some of them could be equally appropriate as toxicity test species (Pavlić *et al*. 2005). Using a battery of algal species from different taxonomic groups, with varying sensitivities to a range of toxicants, may provide valuable toxicity data that is environmentally realistic representative of the ecosystem and thus improve our ability to manage water quality. The challenge, however, is

isolating and maintaining cultures, as this is the time-consuming and labour-intensive process.

A further challenge is identifying the isolated species, mainly because of the lack of freshwater algal taxonomic expertise (especially of cultures) in South Africa. Identifying micro-algal cultures is challenging because many algae do not retain their original morphology in cultures; some growth forms and lifecycle stages which occur naturally cannot be induced in cultures (Brandham 1970). Algal cultures may also become genetically distinct from their original isolates with time, which further complicates identification of the cultured species (Peterson *et al*. 1997). It would have been be advantageous to supplement and validate the morphological identification from this study with molecular identification, but due to lack of expertise in our laboratory, coupled with lack of resources to outsource this task that was not possible for the duration of the study.

The next step of the study will be to expose the above-selected species to reference toxicants, $CdCl_2$ and $K_2Cr_2O_7$ to test their ability to withstand toxicity test conditions and assess their potential as toxicity test species (Chapter 4). This next step addresses the second phase of this study. Some species, for example, grow fairly rapidly in culture, but fail to grow sufficiently in the given toxicity test period of 72-96 hours for the test to be considered viable. Other species grow sufficiently but the intra-specific variability in growth is so high that it is difficult to interpret growth stimulation or inhibition results. The details of how the species isolated and cultured in this chapter exhibited the above mentioned limitations are described in Chapter 4 of this study. The following chapter (Chapter 3) describes how the standard micro-algal toxicity test method (the algal growth inhibition assay) was adapted and refined to accommodate the growth characteristics of the isolated species identified and based on the selection criteria defined in this chapter.

3. REFINING THE TOXICITY TEST METHODS FOR USE OF SOUTH AFRICAN TAXA IN TOXICITY TESTS: The Algal Growth Inhibition Assay

3.1 INTRODUCTION

This chapter forms part of the second phase of the study, which is refining the existing micro-algal toxicity test method in South Africa, such that it is suitable to use for the species identified in the previous chapter (Chapter 2). It describes the general methods used in algal toxicity tests, focussing on the most widely used algal bioassay, the algal growth inhibition assay. This is an essential part of the study, because the existing algal growth inhibition test was developed to use with the standard toxicity test species *Pseudokirchneriella subcapitata*, and therefore may not necessarily be applicable in its existing protocol to the locally isolated species, and may require adjustment and refining.

Despite the wide use of this assay internationally, there are no standardised operating procedures and quality requirements pertaining to this toxicity test method in South Africa (Chapman *et al*. 2011a). The only published South African protocol is described in the "DEEEP (Direct Estimation of Ecological Effects Potential) Methods Manual" (Slabbert 2004). As an adaptation of the US EPA standard algal growth inhibition protocol, the DEEEP Methods Manual describes the materials and methods for executing the algal toxicity test (Slabbert 2004, Chapman *et al*. 2011a), and with adjustments, these methods are used as the basis for toxicity tests employed in the following chapters (4, 5, and 6) of this study. This chapter gives the general background to the algal growth inhibition test (Section 3.2), highlighting the adaptations of the US EPA protocol described on the Methods Manual (Section 3.3), and the further adjustments of the materials and methods described in the manual to accommodate using the indigenous algae identified in this study (Section 3.4).

3.2 THE ALGAL GROWTH INHIBITION ASSAY

Growth is the most commonly used end-point in algal toxicity tests (US EPA 1978, OECD 1984, ISO 1989, Altenburger *et al*. 2008, Paixão *et al.* 2008). Algal growth assays with unicellular green algae (Chlorophyceae) were among the first bioassays established to determine the toxic effects of chemicals, wastewater and other environmental samples on algae (Altenburger *et al*. 2008). Algal growth assays are relatively sensitive because algae

respond by stimulation, inhibition, or both, to a variety of contaminants (Sbrilli *et al*. 2005). The Algal Growth Inhibition Assay is the most widely used algal assay, because of its simplicity, reasonable cost and accepted reproducibility (US EPA 1978, OECD 1984, ISO 1989, Slabbert *et al* 1998, Slabbert 2004). This assay was developed in the mid-1960s as the Algal Assay Procedure Bottle Test (U.S. EPA 1969) and has since been modified and accepted by various regulatory agencies as a standard toxicity test assay with *Pseudokirchneriella subcapitata* as the standard species (US EPA 1978, OECD 1984, Slabbert *et al* 1998, Slabbert 2004). One of the most important improvements of the efficiency of the test is the miniaturization of test vessels by substituting the traditional 250 mL flasks with 24-well or 96-well micro-plates, with no deleterious effect on the precision, reliability and reproducibility of the test (Thellen *et al*. 1989). The use of micro-plates simplifies the test because smaller sample volumes are used, incubation space is reduced and more samples can be tested at a time (Slabbert 2004, Paixão *et al*. 2008). Protocols for the growth inhibition test with green algae have been published and are routinely used in water resource management internationally and nationally (US EPA 1978, OECD 1984, ISO 1989, Slabbert 2004).

3.2.1 Summary of the standard algal growth inhibition test methodology and experimental design (ISO 1989, US EPA 1996, OECD 2006)

In summary, a culture of known algal cell density of the standard species e.g. *Pseudokirchneriella subcapitata,* in its exponential growth phase is exposed to a range of test concentrations in defined test vessels under specified static conditions in a temperature and photo-period controlled environment. An untreated control is maintained to measure the normal growth of the algal cells. The effect of the test material is evaluated by comparing the growth of the test culture to the control. Algae are exposed to a minimum of five concentrations of the test substance in a geometric series of the ratio between 1.5 and 2.0 to generate a concentration-response (US EPA 1996).

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The assay is performed in Erlenmeyer flasks of any volume between 125 and 500 mL (as long as flasks of the same size are used for each test, the volume of test substance does not exceed half of the total flask volume. Test vessels should contain an initial cell inoculum of 1×10^4 cells per mL of *P. subcapitata*. The constituents of the recommended test medium for this test are listed on Table 3.1. The experimental design should include sufficient replication

to allow statistical evaluation of results (Parrish1985). A minimum of three replicates per concentration is required for this assay (US EPA 1996).

Table 3.1: Chemical composition of the EPA assay medium (EPA 1978) and the 10% BG-11 medium (Rippka *et al*. 1979, Slabbert 2004).

MACRONUTRIENTS		
Compound	**EPA Algal Assay Medium**	**10% BG-11 Medium**
	Concentration **(mg/L)**	Concentration **(mg/L)**
$CaCl_2.2H_2O$	4.41	3 600
$NaNO_3$	25.5	15 000
K_2HPO_4	1.044	4 000
$MgSO_4.7H_2O$	14.7	7 500
$NaHCO_3$	15	2 000
$MgCl_2.6H_2O$	12.164	
MICRONUTRIENTS		
	Concentration **(mg/L)**	Concentration **(mg/L)**
H_3BO_3	0.185	286
$MnCl_2.4H_2O$	0.415	181
$Na_2MoO_4.2H_2O$	0.00726	39
$Na_2EDTA.2H_2O$	0.3	100
$CuSO_4.5H_2O$		7.9
$CuCl_2.2H_2O$	0.00012	
$ZnSO_4.7H_2O$		22.2
$ZnCl_2$	0.00327	
$Co(NO_3)_2.6H_2O$		4.94
$CoCl_2.2H_2O$	0.00143	
$FeCl_3.6H_2O$	0.16	
$Fe(NH_3)$-citrate		600
Citric acid		600

The conventional response endpoints applied in the algal growth assay include final yield (biomass or cell density), growth rate, chlorophyll a content or total biovolume (Lin *et al.* 2005). Algal growth may be recorded using a spectrophotometer to measure optical density, a

particle counter to count the algal cells, a fluorometer to measure chlorophyll fluorescence or by counting cells under the microscope. Measuring chlorophyll may be combined with particle counting to enable correlation between chlorophyll concentration and cell volume. This could be a valuable parameter since cell volume may be influenced by toxicity without chlorophyll concentration being changed. Counting cells under the microscope also allows further information on cell deformities or structural modifications to be obtained (Hornstrom 1990). The qualitative and descriptive microscopic observations are not used in endpoint calculations, but they can be useful in determining additional effects of the tested chemical (US EPA 1996).

Toxicity of a test solution is determined as reduced growth rate or reduced final biomass in relation to the control. The most common parameter to measure is the EC_{50}, that is, the effective concentration of the test substance that inhibits growth by 50%. However, the EC_{50} value alone may be unsatisfactory since the dose-response curve may vary. A substance may inhibit growth at a lower concentration but have no effect on growth at higher concentrations, or stimulate growth at lower concentrations and have no effect or inhibit growth at higher concentrations. A no observed effect concentration (NOEC) or lowest observed effect concentration (LOEC) may also be used to exhibit stimulation responses to substances, which are usually omitted while determining EC_x values (Hornstrom 1990, Slabbert 2004).

The NOEC is derived by hypothesis testing, where treatment responses are compared with control responses to test the null hypothesis that the responses are the same. Determination of NOEC is widely used as an endpoint for chronic toxicity data. However it is criticized for several reasons, the most important is that NOEC depends on the choice of test concentrations and the number of replicates (Sbrilli *et al*. 2005). Precision of the NOEC increases with the number of concentrations tested, while confidence in the NOEC value increases with the number of replicates (Kooijman *et al*. 1996). In order to overcome the difficulty in statistically deriving NOEC using hypothesis testing, a regression-based estimation process (EC_x) should also be used (Sbrilli *et al*. 2005, Chen *et al*. 2009). Most NOEC values have been found to be close to EC_{10} – EC_{30} values. It is therefore recommended that the regression-based approach using EC_x is a better tool than the hypothesis testing (NOEC) for estimating toxic effects lower than the EC_{50} (Chen *et al*. 2009).

3.2.2 Important physico-chemical parameters

It is important to control physico-chemical parameters for the duration of the toxicity test. A controlled environment room that can maintain the specified air temperature, light intensity, and photoperiod is required (US EPA 1996). Physical and chemical parameters that are not controlled adequately may be the primary cause of variability (and thus reduced confidence in either EC_{50} or NOEC/LOEC values) in algal growth during a toxicity test. Thus, homogenous and constant conditions with regard to temperature, light and nutrients are important in the growth inhibition assay to ensure test precision and test reproducibility (Altenburger *et al*. 2008).

3.2.2.1 *Temperature*

The growth rate and cell morphology of the standard species *Pseudokirchneriella subcapitata* ATCC® 22662 are temperature dependent. This species can grow at a temperature range of 6 to 33 °C, with an optimum temperature of 28 °C. The standard growth inhibition test is performed at temperatures between 23 °C and 25 °C with an allowable temperature variability of 2 °C during the test period (ISO 1989, US EPA 1996, Slabbert 2004). Most processes of algal metabolism are dependent on temperature, and effects of toxicity therefore could be temperature dependent. More homogenous temperature requirements could reduce variability in growth rate, resulting in more reproducible and precise results (Mayer *et al*. 1998).

3.2.2.2 *Light*

Light intensity is another important parameter for this assay because algal growth is correlated to the amount of light available for photosynthesis. Variability in light intensity may be less crucial at light saturation conditions of the standard growth inhibition test, than under environmental light- limiting conditions. Prescribed light intensity for the standard algal assay is within the range of 60-120 $\mu Em^{-2}s^{-1}$ with cool white fluorescent light as the source (Mayer *et al*. 1998). One of the requirements of the standard algal growth inhibition assay is continuous illumination during the test period. However, a daily rhythm of light-dark cycles may be more suitable, particularly with regard to simulating natural conditions (Hornstrom 1990). Unicellular green algae allocate their resources towards biomass

accumulation via photosynthesis during the light period, and achieve their biomass gain by cell-division in the dark period. Given this growth pattern, unicellular algae may not follow a simple exponential growth function during a toxicity test with continuous illumination. This may result in problems such as growth stimulation or variability of growth in controls during a test (Altenburger *et al*. 2008). The observation of exponential growth with relatively low variability in the control is an important validity criterion in assessing the validity of the results of the growth inhibition assay (Mayer *et al*. 1997).

3.2.2.3 *Nutrients and pH*

The bioassay is sensitive to nitrogen, phosphorus, potassium and magnesium, and growth is affected when one of these nutrients is omitted from the medium (Payne 1975). Generally, toxic effects of contaminants may be more severe under limited nutrient conditions. However, the toxic effects under nutrient limitation are species specific and depend on the degree of nutrient limitation (Interlandi 2002). Another parameter of the growth inhibition test to be considered is pH. *Pseudokirchneriella subcapitata* can tolerate a pH range of 6 to 9 (Mayer *et al*. 1998). The initial pH in the standard test medium should be buffered to values of 7 to 8.3 (Mayer *et al*. 1998, Slabbert 2004). The US EPA standard method recommends and initial pH of 7.5 (± 0.1) of the test medium for the test with *P. subcapitata* (US EPA 1996). Sample pre-treatment, surface to volume ratio, as well as amount and age of the inoculums (Payne 1975) should also be taken into account during the test as these may affect the physic-chemistry of the test medium.

3.3 THE ALGAL GROWTH INHIBITION TEST PROTOCOL IN SOUTH AFRICA: the "DEEEP Methodology" (Slabbert 2004)

The protocol for the algal growth inhibition test recommended for routine use in South Africa is published in the DEEEP Methods Manual (Slabbert 2004). The test is an adapted version of the US EPA flask test with *Pseudokirchneriella subcapitata* ATCC® 22662 as the standard test species. The assay is performed in 24-well micro-plates, using 2 mL volumes of test substance. An increased initial cell inoculum of *P. subcapitata* is used to improve algal biomass obtained at the end of the test (2×10^5 cells per mL instead of 1×10^4 cells per mL used for the US EPA test).

The recommended test medium is modified 10% BG-11 (Rippka *et al*. 1979) since it has been shown to result in more improved growth than the EPA assay medium (EPA 1978, Slabbert *et al*. 1998, Slabbert 2004). The improved growth is no surprise, given that the BG-11 medium is generally used for growth and maintenance of green and blue-green algae (Olvera-Ramirez *et al*. 2000, Wang *et al*. 2004, Slabbert 2004, Tripathi *et al*. 2006). The chemical composition of both test media is shown in Table 3.1.

Table 3.2 Summary of test conditions for the algal growth inhibition: comparing the DEEEP protocol (Slabbert 2004) to the protocol modified for the selected locally isolated organisms in this study

Parameters	**DEEEP Protocol**	**Protocol for indigenous organisms**
Test species	*P. subcapitata*	*P. subcapitata, S. bicaudatus, C. vulgaris* and *C. sorokiniana*
Test vessel	24 well micro-plates	24 well micro-plates
Temperature	24±2	25±3
Light quality	Cool white fluorescent	Cool white fluorescent
Photoperiod	Continuous	Continuous
Light intensity	80-95 µE/m^2/s	35-40 µE/m^2/s
pH	7.1-7.2 adjusted	7.0-7.6 not adjusted
Test medium	modified BG-11	modified BG-11
Volume of test sample	2 mL	2 mL
Age of algal culture	3-4 days	4-6 days
Inoculum size	200 000 cells/mL	300 000 cells/mL
No. of replicate wells	6 controls + 3 test samples	6 controls + 3 test samples
Control or dilution water	Autoclaved Milli-Q®	Autoclaved Milli-Q®
Test duration	72 hours	96 hours
measured endpoint	EC20 and EC50	EC20, EC50, NOEC

3.3.1 Summary of test protocol

The unicellular alga *P. subcapitat*a is maintained axenically in batch culture using 250 mL Erlenmeyer flasks according to Slabbert (2004), as described in Chapter 2. The culture is maintained at 24±2 °C under cool fluorescence light of 16:8 hour light/dark cycle, without agitation. Algae are kept in exponential growth phase by sub-culturing weekly in order to keep a constant supply cells for a toxicity test. A summary of test conditions for this assay is given in Table 3.2. The cultures are used for toxicity tests three to four days after sub-culturing. The medium used for culture maintenance and toxicity testing is a modified version of 10% BG-11 (Rippka *et al*. 1979, Slabbert 2004). The initial pH of the test medium should be 7.1 ±0.1 and must be adjusted to with NaOH or HCl.

The algal suspension is prepared by removing the supernatant medium from the undisturbed culture vessel and re-suspending cells in fresh medium. The fresh 10% BG-11 medium is added to the algal suspension to obtain a concentration of approximately 2×10^5 cells per mL of cell inoculum. The prepared algal inoculum is added at a ratio of 1:1 to 20-times concentrated BG-11 medium for improved nutrient saturation, thereby improving growth. A volume of 200 µl of algal suspension is added to 180 µl of test substance in each well of the 24-well micro-plate. Sterile Milli-Q® water is used for control wells and as a diluent for test treatments. A mixture of appropriate concentrations of test substance and medium is used for blank wells. Plates are covered with lids and a layer of dark insulation tape is taped around the sides of each plate (to avoid over-illumination of outer wells). The tape is cut to enable opening of the lid and to allow air circulation during incubation. Plates are incubated for 72 hours at 24±2 °C under continuous illumination of approximately 80-95 $\mu E/m^2/s$. Optical density measurements are read at 450 nm in the beginning (0 hours) and at the end (72 hours) of the test. Results are expressed as percentage growth inhibition (or stimulation). Linear regression is applied to the inhibition data within the 16-84% growth inhibition range, to determine EC_{20} and EC_{50} values. R^2 values are determined to establish how the regression line fits the measured data. Stimulation data are omitted in the EC_x calculation. A toxicity test with a reference toxicant ($CdCl_2$ or K_2Cr2O_7) must be performed parallel to every toxicity test.

3.3.2 Test validity criteria

The algal toxicity test must meet certain criteria to be considered valid. A summary of validity criteria as described on the DEEEP Methods Manual, and validity criteria for the modified method used in this study for indigenous species is shown in Table 3.3. The validity criteria for the toxicity test with indigenous species (Table 3.3) were determined in accordance with the growth characteristics of these identified species.

Table 3.3 A summary of test validity criteria for the reliability of data: the DEEEP methodology (Slabbert 2004) and the method adapted for the selected locally isolated species.

Criteria	**DEEEP methodology**	**Methodology with locally isolated species** (this study)
1. Coefficient of variation of controls	Less than 10%	Less than 10% for *P. subcapitata*, less than 20% for commercial culture collection species and indigenous species.
2. OD_{450nm} readings of the control	0.15±0.03	More than 0.100 for all species.
3. Inhibition data used in linear interpolation	Between 20% and 80%	Between 10% and 90%
4. R^2 for the regression line	More than 0.9	More than 0.8

3.4 THE ALGAL GROWTH INHIBITION ASSAY ADAPTED FOR THE LOCALLY ISOLATED ORGANISMS IDENTIFIED IN THIS STUDY

The methodology used in this study is based on the DEEEP Methods Manual (Slabbert 2004), summarized above (Section 3.3.1, Tables 3.1 and 3.2), with adaptations to accommodate species specific requirements of the selected locally isolated organisms.

3.4.1. Culture maintenance

Batch cultures of the test species *Pseudokirchneriella subcapitata* CCAP 278/4 (Appendix B) were maintained axenically under controlled conditions at 24±2 °C under cool fluorescence light of 16:8 hour light/dark cycle, without agitation. Cultures were maintained in the exponential growth phase by sub-culturing once a week in 10% BG-11 medium, and used in toxicity tests four to six days after sub-culturing. Culture medium was sterilized in an autoclave at 121 °C for 15 minutes, and toxicity test medium was filter-sterilized through a sterile 0.22 μm syringe filter membrane. The initial pH of the toxicity test and culture medium ranged between 7.0 and 7.6 without adjustment.

3.4.2. Inoculum preparation and exposure to test solutions

Test solutions were inoculated with an initial density of approximately 3×10^5 cells per mL. The initial cell density was determined by counting cells in the counting chamber of the Neubauer haemocytometer under an Olympus BX51 research light microscope at 400 times magnification in phase contrast illumination. Cell density was expressed as number of cells counted × 10^1(10 fold dilution) × 10^4 (factor for haemocytometer) (Slabbert 2004). The algal inoculum volume and the volume of BG-11 medium required for the number of treatment and control wells of six replicate 24-well microplates was determined. The determined volumes of the aforementioned respective substances were combined to form an inoculum suspension (Appendix D).

Six replicate microplates were prepared for each test substance and marked according to the plate configuration on Table 3.4. A range of eight concentrations of test substance in 1:2 serial dilutions were prepared. There were two microplates for each replicate of each contaminant to accommodate eight concentrations (see Table 3.4), and six replicates of each

Sterile (autoclaved at 121 °C for 15 minutes) Milli-Q® water was used for controls and substance dilutions. A volume of 1.8 mL of control and treatment solutions was was dispensed into each of the appropriated wells of each of the six replicate microplates (Table 3.4, Appendix D). A volume of 0.2 mL of the inoculum suspension was dispensed into each of the controls and treatment wells of the six replicate microplates (see Table 3.4 for plate configuration).

Table 3.4 Configuration for a toxicity test micro-plate with four concentration treatments

Microplate 1					
Blank	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**
Control	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Control
Control	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Control
Control	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Control
Microplate 2					
Blank	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**
Control	Conc. 5	Conc.6	Conc. 7	Conc. 8	Control
Control	Conc. 5	Conc. 6	Conc. 7	Conc. 8	Control
Control	Conc. 5	Conc. 6	Conc. 7	Conc. 8	Control

Two micro-plates, with four concentrations on each, were used for the eight concentrations of each toxicant on each species. Blanks of treatment samples without inoculum were prepared for each concentration treatment and control (Table 3.4). A volume of 2 mL of cell inoculums with test substance was added in each well of the 24-well micro-plates. There were three replicate wells for each concentration treatment and six replicate wells for the control on each 24-well micro-plate (Table 3.4).

Micro-plates were incubated for 96 hours at 25±3 °C under continuous illumination of approximately 35-40 $\mu E/m^2/s$. This low light intensity was due to increased distance between the test vessels and the light source. Although cool white fluorescent light bulbs were used, when the distance between the test vessels and the light source was decreased, the temperature on the test surface increased, so there had to be a trade-off between light intensity and temperature.

Light intensity was measured at the test surface with a Lutron LX101 lux meter, using the illumination unit, lux, which is gives an indication of illumination intensity to the human eye. Since algae and plants do not see light the ways human do, illumination intensity was converted to photosynthetic active radiation (PAR) which is expressed in units of $\mu E/m^2/s$ (Aldos *et al*. 1995, Pearcy, 2000). The exposure time was extended from 72 to 96 hours to accommodate the indigenous species that did not show sufficient growth, as specified in the DEEEP method (Table 3.3) after 72 hours.

3.4.3. Growth measurement

Growth was determined spectrophotometrically by measuring optical density on a Biotek (EL*x*800 UV) micro-plate reader at 450 nm at the beginning and the end (96 hours) of the test. Results were expressed as percentage growth inhibition (or stimulation) compared to the control, using the following formula:
$[(ODC\text{-}OD_0) - (ODT\text{-}OD0\text{-}ODB_{>0.005})]/(ODC\text{-}OD_0) \times 100$ (Slabbert 2004), where

ODC = mean optical density of the control wells at the end of the experiment
OD_0 = mean optical density of the test wells for each concentration treatment at the beginning of the experiment.
ODT = mean optical density of the test wells for each concentration treatment at the end of the experiment (96hrs)
$ODB_{>0.005}$ = the blank optical density measurements of the test well >0.005 (to compensate for precipitation and/colour interference).

The inhibitory concentration to reduce growth by 20% and 50% (EC_{20} and EC_{50} values) was determined using linear regression and graphic interpolation (using inhibition data between 10% and 90% as stated on the test validity criteria). The growth inhibition was read off the linear scale (Y axis) and the toxicant concentration on the logarithmic scale (X axis). R^2 values were determined to establish how the regression line fitted the measured data, and data that did not fit the model well (with R^2 values less than 0.8) were not used. Stimulation data were omitted in the EC_x calculation. Specific growth rate at each concentration treatment and control was also calculated for all species, using the initial (0 hours) and final (96 hours) optical density readings with the formula:

$\mu = \ln(x_2/x_1)/t_2\text{-}t_1$ where:

μ=specific growth rate, x_2= OD_{450nm} at 96 hours, x_1=OD_{450nm} at 0 hours, t_2=96 hours and t_1= 0 hrs

3.4.4. Data Analysis

The 96 hour EC_{50} values for each toxicant for each species were averaged to obtain a single EC_{50} value. Some of the replicate tests did not meet all of the test validity criteria described on Table 3.4, and therefore not all of the six replicates for each toxicity test could be used to calculate EC_{50} values. This resulted in uneven replication of exposures (unequal number of averaged EC_{50} values) of each toxicant to each species. These uneven numbers of replicates were used in statistical analyses to establish if there were any significant differences in the EC_{50} values of each toxicant between species, and also to determine significant differences in the sensitivity (EC_{50} values) of each species to the different toxicants. Data were checked for homogeneity of variance using Levene's Test. If there were no significant differences in the variance of the EC_{50} values, normality of data was determined using the Kolmogorov-Smirnov test. If data were normal, one-way-ANOVA was used, with the Unequal N HSD post-hoc test; the Kruskal-Wallis ANOVA by ranks was used for data that were not normal.

The specific growth rate response of each species to each toxicant at each concentration and control was determined, for all six toxicity test replicates. The six replicates were averaged to give one specific growth rate value for each toxicant concentration and control for each species. Statistically significant differences in specific growth rate between the control and toxicant concentrations were determined in order to determine the NOEC (No Observed Effect Concentration) of each toxicant for each species. The LOEC (Lowest Observed Effect Concentration) was determined in instances where the NOEC could not be determined. Toxicant concentrations resulting in significantly lower specific growth rate than the control (indicating inhibition) were used to determine the NOEC or the LOEC, not concentrations with specific growth rates significantly higher than the control (indicating stimulation). Specific growth rate data were checked for homogeneity of variance with Levene's Test, and normality using the Kolmogorov-Smirnov test. If there were significant differences in variance, or the data were not normal, a non-parametric Mann-Whitney U test was used to

compare control specific growth rate for each species to specific growth rate at each toxicant concentration. If there were no significant differences in homogeneity of variance, and data were normally distributed, the Dunnett's test was used to determine treatments with specific growth rates that were significantly different from the control. $P \leq 0.05$ was used as the level of significance for all analyses.

3.4.5 General Discussion

Deviations from the currently defined protocols were to accommodate the growth of the locally isolated species so that they could be compared to the standard species *Pseudokirchneriella subcapitata.* Preliminary experiments with the locally isolated species were used to define the protocol for using these species and the deviations from the standard protocols such as the EPA and OECD methods.

For example the major deviation from the protocol on the document: Environment Canada, March 2007 - EPS 1/RM/25 Second Edition, mentioned are as follows

- The use of 96 well microplate vessels instead the 24 well microplates used in this study, which means smaller volume of the test medium.
- Higher light intensity of 50-62 $\mu E/m^2/s$ as opposed to 35-40 $\mu E/m^2/s$ used in this study. This was a practical issue with the test facility that needs to be addressed. It should be borne in mind that the light intensity used in the study is still within the acceptable growth range of the species used.
- Starting inoculum of 10 000 cells/mL as opposed to 300 000 cells/mL used in this study. This was based on the preliminary experiments which showed 300 000 cells/mL to be the optimal starting inoculum given the conditions of light intensity and temperature.
- The toxicity tests in the study were extended to 96 hours instead of the conventional 72 hrs, to ensure sufficient growth of controls at the end of the experiment, particularly for the local isolates.

4. THE RESPONSE OF FRESHWATER MICRO-ALGAE TO REFERENCE TOXICANTS: CADMIUM CHLORIDE ($CdCl_2$) AND POTASSIUM DICHROMATE ($K_2Cr_2O_7$)

4.1 INTRODUCTION

Chapter 2 described the protocol used to isolate three species of freshwater micro-algae from local rivers and culture them axenically in the laboratory, and Chapter 3 focused on the adjustment and refinement of the existing algal growth inhibition assay so that it can be used in toxicity tests with the indigenous species isolated in Chapter 2. As part of the second phase of the study which addresses the objective to refine toxicity test methods for using South African taxa, this chapter follows on from Chapter 3 and describes how the three locally isolated species, *Scenedesmus bicaudatus* Dedusenko 1925, *Chlorella sorokiniana* Shihira, I. and R.W. Krauss 1965 *and Chlorella vulgaris* Beijerink 1890 (Chapter 2), respond when exposed to two reference toxicants, $CdCl_2$ (cadmium chloride) and $K_2Cr_2O_7$ (potassium dichromate).

This chapter addresses two separate parts of the study:

- As part of the second phase of this study, which is assessing the suitability of the locally isolated species for toxicity testing by looking at their ability to withstand the prescribed experimental conditions of the refined growth inhibition test.
- As part of the third phase of this study, which is assessing the application and value of using the local micro-algal species in toxicity testing for use in water resource management in South African, this chapter assesses
    - the response of these local isolates to the reference toxicants in comparison to that of *Chlorella protothecoides* Kruger 1894 obtained from a commercial culture collection, and the standard species *Pseudokirchneriella subcapitata* (Korshikov) Hindák 1990, and
    - the sensitivity of these locally isolated species to the selected reference toxicants, in comparison to a range of micro-algal species using species sensitivity distribution (SSD) curves, and toxicity data (EC_{50} values) extracted from an international database (US EPA ECOTOX).

The use of laboratory toxicity data to generate species sensitivity distributions is a commonly used approach in ecological risk assessment and derivation of water quality guidelines

(ANZECC and ARMCANZ 2000). Generally an estimate of the maximum concentration of a toxicant or chemical protective of most species (typically 95%) in the environment can be deduced from an SSD with laboratory toxicity data from a variety of species (Wheeler 2002). If the toxicant is kept below such a concentration in nature, the assumption is that most species (95%) will not be harmed by the toxicant (Kefford *et al*. 2006). The use of SSDs in the context of this study will be as a tool to compare the sensitivity of a range of species to a particular toxicant or chemical.

Cadmium chloride ($CdCl_2$) and potassium dichromate ($K_2Cr_2O_7$) are the reference toxicants most commonly used internationally, and in South Africa, for toxicity testing (Wang 1987, Slabbert *et al*. 1998, ISO 2004, Slabbert 2004). A reference toxicant is a chemical that a particular organism is known to respond to. The reference toxicant is typically used as a positive control during the toxicity test to determine if the test organism is responding to the toxicant in the expected manner (US EPA 1996). These chemicals are used in the South African toxicity proficiency test scheme, which is an inter-laboratory evaluation of toxicity tests to ensure adequate standardization for routine use. Cadmium chloride is used for the daphnia proficiency test, while potassium dichromate is used for the algal growth inhibition proficiency test (Chapman *et al*. 2011a). Cadmium chloride was initially recommended as a reference toxicant for the algal growth inhibition test and used in inter-laboratory training exercises for this assay in South Africa (Slabbert *et al*. 1998, Slabbert 2004). The use of cadmium chloride as a reference toxicant was later substituted with potassium dichromate. This was done to align South African algal toxicity testing with international trends and to enable comparison of our data with internationally generated data, since $K_2Cr_2O_7$ is the reference toxicant most widely used internationally (US EPA 1996, NIWA 1998, ISO 2004, OECD 2006).

Cadmium is found in trace concentrations in freshwater, mostly as a result of industrial discharges from mining, metal smelters, agricultural fertilizers and manufacturing of metal plated containers (Terry and Stone 2002). It is a non-essential metal, potentially toxic to marine and freshwater aquatic life (Terry and Stone 2002, Tukaj *et al*. 2007). The toxicity of cadmium in water depends on the different parameters such as hardness, chemical speciation, pH, temperature and the presence of other metal ions in the water. According to the South African water quality guidelines for aquatic ecosystems (DWAF 1996), the target water quality range (TWQR) for cadmium is 0.15, 0.25 and 0.35 µg/L for soft, medium, and hard

waters respectively. Measurements of cadmium for any particular site should be within the TWQR 90% of the time in order to comply with the guidelines. The chronic effect values that should not be exceeded at any site in order ensure protection of aquatic ecosystems in soft, medium, and hard waters are 0.3, 0.5 and 0.7 μg/L respectively (DWAF 1996).

Cadmium has been shown to have adverse effects on some vital processes of algal cells (Rebhun and Ben-Amotz 1986, Cepák 2002). Investigations on the toxicological response of micro-algae to cadmium have taken two approaches: (a) how cadmium as an environmental toxicant affects algae as primary producers in aquatic ecosystems and (b) biochemical-physiological studies determining the mode of action of toxicity to algae (Rebhun and Ben-Amotz 1986). Cadmium has been reported to impact on algal growth and reproductive processes by affecting the nuclei and chloroplasts of the cells. It may inhibit or inactivate some enzymes, thereby affecting growth, respiration or photosynthesis in algae (Tukaj *et al*. 2007). Loss of cell motility in flagellates and reduction in cell volume in some species, have also been reported (Cepák 2002). Although cadmium may be taken up and assimilated by green algae at low concentrations, it may be toxic to sensitive organisms and its effects are species specific and dependent on growth conditions (Terry and Stone 2002, Tukaj *et al*. 2007). The relevance of the use of $CdCl_2$ in toxicity tests is supported by the fact that, Cd^{2+} is more readily taken up by aquatic plants than other cadmium ions (Rebhun and Ben-Amotz 1986).

Potassium dichromate is a well-known algal toxicant and an internationally recommended reference in algal growth inhibition tests (Wang 1987, Nyholm 1990, ISO 2004, European Chemicals Bureau 2005, OECD 2006, Labra *et al*. 2007). The toxicity of potassium dichromate to some microalgae may not only be dependent on concentration, but also on the duration of exposure (Aizdaicher and Markina 2011). At elevated levels, chromium is considered a mutagenic and carcinogenic agent for living organisms (Mortuza *et al*. 2009). It is genotoxic to micro-algae and genomic damage may alter gene transcription resulting in modification of cell physiological activity (Labra *et al*. 2007, Aizdaicher and Markina 2011).
In aqueous systems, chromium exists primarily in two biologically significant oxidation states, hexavalent (Cr (VI)) and trivalent chromium (Cr (III)), which differ in their toxicity (Labra *et al*. 2007, Mortuza *et al*. 2009, Vannini *et al*. 2009). Hexavalent chromium is extensively used in a variety of commercial processes, followed by unregulated discharge of chromium containing effluent into aquatic systems, in both developing and developed

countries (Mortuza *et al*. 2009). In contrast to Cr (III), Cr (VI) may cause serious damage to living cells and induce growth inhibition to micro-algae because, not only is it more soluble but it can cross cellular membranes via non-specific anion carriers (Labra *et al*. 2007, Vignati *et al*. 2010). However, Vignati *et al.* (2010) argue that Cr (III) concentrations added to algal toxicity test media decrease over the duration of the test, due to the formation of hydrolysis products in the medium. This temporal decrease of Cr (III) may therefore underestimate its toxicity to algae during a test.

Potassium dichromate ($K_2Cr_2O_7$) is one of the most soluble Cr (VI) salts. It is used extensively in the manufacture of paints and dyes, as well as in the leather industry as a tanning agent. The target water quality range and the chronic effect value for chromium (VI) according to the South African water quality guidelines are 7 and 14 μg/L, respectively (DWAF 1996). Notably, these values are much higher than those of cadmium (mentioned earlier), which means that chromium is perceived to be less of a threat to aquatic ecosystems than cadmium.

The ability of algal cells to accumulate cadmium and chromium as a defense mechanism has been reported (Tukaj *et al*. 2007, Mortuza *et al*. 2009). These algae produce cellular structures which form complexes with these metal ions. These complexes are then accumulated in vacuoles in the algal cells (Tukaj *et al*. 2007, Mortuza *et al*. 2009). Chromium also induces changes in cell morphology, chloroplast structure and enzyme activity in some micro-algal species (Aizdaicher and Markina 2011).

Unicellular algae are good test organisms for investigating aquatic environmental pollution because they are in immediate contact with the environment, and therefore respond readily to unfavorable conditions (Mortuza *et al*. 2009). The use of single phytoplankton test species under regulated laboratory conditions to assess ecosystem toxicity induced by different compounds has been criticized because the species-specific responses of test species to individual toxicants cannot mimic those of natural phytoplankton species living under highly variable environmental conditions (Chapman 1995a). However, the single-species phytoplankton responses observed in bioassays may provide potential answers to reasons for changes in phytoplankton community structures induced by pollutants in the natural environment (Oberholster *et al*. 2010).

This chapter is aimed at assessing the potential of the selected freshwater micro-algal species isolated from local rivers (Chapter 2) as toxicity test species, and having their sensitivity compared to that of the standard test species. This was done by exposing the locally isolated species, *Scenedesmus bicaudatus*, *Chlorella sorokiniana*, and *Chlorella vulgaris* to two toxicants, potassium dichromate and cadmium chloride using growth inhibition as toxicity test endpoint. *Pseudokirchneriella subcapitata* and *Chlorella protothecoides* obtained from commercial culture collections were also exposed to these toxicants for comparison with the local species, using the same toxicity test endpoint. *Pseudokirchneriella subcapitata* is the standard toxicity test species for the algal growth inhibition test, and *C. protothecoides* has been used in toxicity tests in a number of studies (Stauber 1995, Zeng *et al*. 2009, Neil *et al*. 2009). *Chlorella protothecoides* is a widely distributed species in rivers and lakes of Australia and has been recommended as a toxicity test species in that country (Stauber *et al*. 1994). All of the above-mentioned species are unicellular or coenobial green-algae. They have been shown to grow well under the defined laboratory conditions and form homogenous suspensions in defined test medium (Chapter 2), important characteristics of an algal toxicity test species.

4.2 MATERIALS AND METHODS

4.2.1 Test organisms, conditions and chemicals

Cultures of *Scenedesmus bicaudatus* Dedusenko 1925, *Chlorella sorokiniana* Shihira, and Krauss 1965 and *Chlorella vulgaris* Beijerink 1890 were isolated from the Palmiet and Kieskamma rivers, *Pseudokirchneriella subcapitata* CCAP 278/4 (Appendix B) was obtained from a stock culture from an ISO 17025 accredited toxicity testing laboratory (Rand Water Analytical Services, Vereeniging) and *Chlorella protothecoides* ATCC® 30411™ was obtained from a commercial culture collection (Environmental Biotechnology Research Unit, Rhodes University, Grahamstown). All cultures were maintained axenically in 50 mL of 10% BG-11 medium in 250 mL Erlenmeyer flasks, under controlled conditions in a growth chamber at 24±2 °C under cool fluorescence light of 16:8 hour light/dark cycle, without agitation. The 10% BG-11 medium was prepared according to the method described Slabbert (2004), with the final pH ranging from 7.0 to 7.6 without adjustment.

The toxicity of $CdCl_2$ and $K_2Cr_2O_7$ to five species of freshwater green micro-algae was tested using the growth inhibition assay (96 hrs). The general method used in these toxicity tests is described in Chapter 3 (section 3.4). A batch method was used for the bioassay using sterile 24-well micro-plates, covered with lids as test vessels (Slabbert 2004). Cultures in exponential growth phase were diluted with 10% BG-11 growth medium to an algal density of 3×10^5 cells per mL. Initial cell density was determined microscopically by counting cells in a sub-sample of the algal culture using a haemocytometer. Each well contained 200 µL mixture of medium and algal inoculum and 1.8 mL of test substance in appropriate concentration dilution. Micro-plates were incubated at 25±3 °C under continuous cool fluorescence light (35-40 $\mu E/m^2/s$) for 96 hours. Cadmium chloride and potassium dichromate were applied at a series of eight concentrations respectively, for each species (0.007, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5 and 1 mg/L). Each exposure of one species to a toxicant was repeated six times. The six exposures were treated as replicates, since they were performed simultaneously. Algal population growth was determined spectro-photometrically by measuring optical density at 450 nm for each plate on a micro-plate reader, at the beginning and the end of the toxicity test.

Test acceptability criteria were:

- Coefficient of variation of control growth ≤10% for *P. subcapitata* and ≤20% for other species.
- An average OD_{450nm} reading of >0.10 for the controls at the end of the test
- R^2 of more than 0.8 in the linear regression for EC_x calculations
- Inhibition data between 10% and 90% growth inhibition used in the linear interpolation for EC_x calculations

4.2.2 Analysis

4.2.2.1 Growth inhibition and effective concentration (EC_x) determination

Data for all test replicates were assessed for validity using the above acceptability criteria, and those that did not meet all the criteria were not used. Percentage inhibition values were estimated for each experimental group (exposure of each species to each toxicant). EC_{50} and EC_{20} values were determined by linear regression and graphic interpolation on the percentage

inhibition data (between 10% and 90%). Data belonging to the upper-most and lower-most part of the curve (< 10% and > 90%) may negatively influence the curve-fitting; exclusion of these data could minimize that influence (Mayer *et al*. 1997, Slabbert *et al*. 2004). The experimental data that did not fit the model well (R^2<0.8) were not used in EC_{50} and EC_{20} calculations, as stated in the data acceptability criteria above (section 4.2.1). EC_{50} and EC_{20} values for all replicates of each toxicant on each species were pooled together and expressed as mean EC_x (+standard deviation). The differences in sensitivity of species to each of the two toxicants were determined by statistically comparing the EC_{50} values. Data were tested for normality and homogenous variance, and a one-way-ANOVA (Analysis of Variance), with Unequal N HSD post-hoc test was used for normally distributed data (due to unequal replicates after quality control of the data). A non-parametric Kruskal-Wallis ANOVA by ranks was used for data that were not normally distributed.

4.2.2.2 Specific growth rate, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)

All the data were used (even those with higher than acceptable variability in control growth after 96 hrs) to determine specific growth rate for the control and each toxicant concentration treatment (see Chapter 3.4). This was done in order to include and present stimulation data, which was omitted in EC_x calculations. The data were tested for normality and homogeneity, and a one-way ANOVA was used to determine which treatments differed significantly from the control, thus determining NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration) values (using treatments with significantly reduced growth compared to the control). All statistical analyses were undertaken using STATISTICA (Version 8) software package with $p \leq 0.05$ as the level of significance.

4.2.2.3 Species sensitivity distributions

Species sensitivity distribution (SSD) curves were generated in order to compare the sensitivity of different micro-algae to the selected reference toxicants ($CdCl_2$ and $K_2Cr_2O_7$). Median concentration effect (EC_{50} and LC_{50}) data of each of the two toxicants to several algal species used for the SSDs were extracted from the US EPA ECOTOX database, as well as toxicity data generated in this study. The usefulness of an SSD is dependent on the quality of data used to generate the SSD curve. Including poor data could possibly result in

misrepresentation and affect the interpretation of the SSD curve. The toxicity data extracted from the ECOTOX database for each of the two toxicants were pre-screened and filtered by only using laboratory generated toxicity data and excluding the following data:

- seawater as the medium of exposure,
- exposure duration longer than 96 hours and shorter than 24 hours, and
- no reports on fields such as endpoint, effects measurement, exposure duration and/or concentration units.

Ecotoxicology databases often contain multiple entries for a single species and chemical, because data come from different sources. Therefore, after the pre-screening, in cases where there was more than one data point (or effect concentration value) for a species, the geometric mean of the different values was used to represent the species; where there was only one data point, that single value represented the species (Warne *et. al.* 2004). Species sensitivity distribution curves were drawn using SSD generator (US EPA 2005), which is an easy-to-use tool designed to create custom SSDs. This tool creates the curves by fitting a linearized log-normal distribution to effect concentration data (LC_{50} or EC_{50}) for different species. The SSD curve enables the estimation of the proportion of species affected by the toxicant at different exposure concentrations. The SSD generator was used to construct SSD curves with single species toxicity data generated from this study, and pre-screened data extracted from the ECOTOX database.

4.3 RESULTS

4.3.1 Growth inhibition and effective concentration (EC_{50})

All of the toxicity tests with *Scenedesmus bicaudatus* ($CdCl_2$ and $K_2Cr_2O_7$ exposures) had high control variability in growth, with coefficient of variation ranging between 24% and 55% for all exposures (results not shown). This control variability in growth was outside the prescribed test acceptability limits. As a result, the data were not analyzed any further. *Scenedesmus bicaudatus* was therefore eliminated as a potential toxicity test species, due to variability in control growth during the test period. Figure 4.1 shows the effective concentrations at 50% growth inhibition (EC_{50} values) for $CdCl_2$ and $K_2Cr_2O_7$ on the standard species *Pseudokirchneriella subcapitata* at 72 and 96 hours. There were no significant differences between 72 and 96 hour EC_{50} values of both chemicals to *P. subcapitata*.

Pseudokirchneriella subcapitata was significantly more sensitive to $CdCl_2$ than $K_2Cr_2O_7$ ($p<0.05$) with mean 96 hour EC_{50} values of 0.039±0.005 mg/L and 0.17+0.06 mg/L respectively.

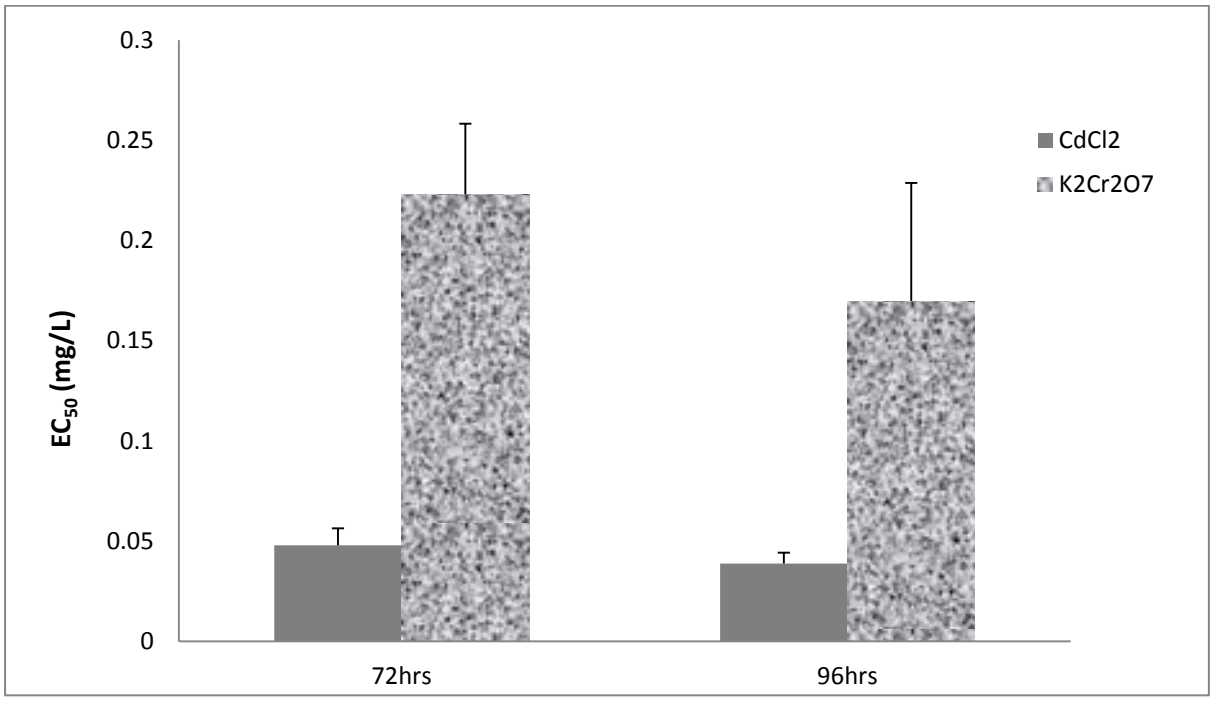


Figure 4.1 Mean 72 and 96 hours EC_{50} (+standard deviation) of $CdCl_2$ and $K_2Cr_2O_7$ for *Pseudokirchneriella subcapitata*.

The 96 hour EC_{50} values of $CdCl_2$ for *P .subcapitata*, and the three *Chlorella* species, *C. prototheco*ides, *C. sorokiniana* and *C. vulgaris* varied between 0.002+0.0003 and 0.336+0.062 mg/L and are shown in Figure 4.2. *Chlorella prototheco*ides was the most tolerant (EC_{50}, 0.336±0.062 mg/L) and *Chlorella sorokiniana* the most sensitive ($EC_{50,}$ 0.002+0.0003 mg/L) species to $CdCl_2$. The 96 hour EC_{50} values of $CdCl_2$ to the two species were significantly different from each other ($p<0.05$). The 96 hour EC_{50} values of $CdCl_2$ for *P. subcapitata* and *C. vulgaris* were 0.039+0.005 mg/L and 0.093+0.058 mg/L respectively, and not significantly different from each other. The EC_{50} values of $CdCl_2$ to *P. subcapitata* and *C. vulgaris* were also not significantly different from those of $CdCl_2$ to *C. prototheco*ides and *C. sorokiniana*. The order of increased sensitivity of the four species to $CdCl_2$ was as follows: *C. prototheco*ides, *C. vulgaris*, *P. subcapitata* and *C. sorokiniana* (Figure 4.2).

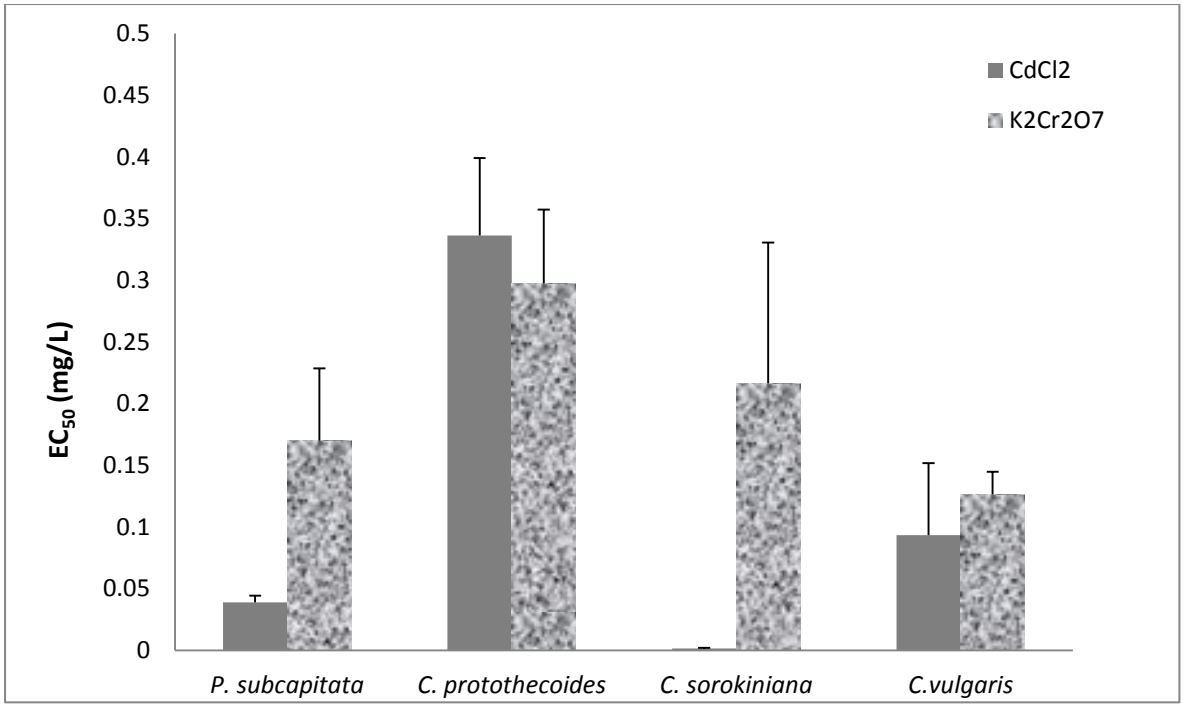


Figure 4.2 Mean 96 hour EC_{50} (+standard deviation) of $CdCl_2$ and $K_2Cr_2O_7$ to *Pseudokirchneriella subcapitata* (n^a=5, n^b=3), *Chlorella protothecoides* (n^a=4, n^b=5), *Chlorella sorokiniana* (n^a=2, n^b=2) and *Chlorella vulgaris* (n^a=2, n^b=3). (n^a is the number of replicate tests for $CdCl_2$, and n^b the number of replicate tests for $K_2Cr_2O_7$).

The algae were slightly more sensitive to $CdCl_2$ than $K_2Cr_2O_7$, except *C. protothecoides* which showed a slightly higher 96 hour EC_{50} value of $CdCl_2$ (0.336+0.062 mg/L) than $K_2Cr_2O_7$ (0.297+0.06 mg/L). However, there were no statistically significant differences in EC_{50} values of $CdCl_2$ and $K_2Cr_2O_7$ to all species except *P. subcapitata* which had a significantly higher 96 or 72 hour EC_{50} of $K_2Cr_2O_7$ than $CdCl_2$ (Figures 4.1 and 4.2). *Chlorella protothecoides* was the most tolerant species to $K_2Cr_2O_7$ with the highest $EC_{50}$of 0.297+0.06 mg/L, and *Chlorella vulgaris* the most sensitive with an EC_{50} of 0.126+0.018 mg/L.

Table 4.1 Toxicity data (72 and 96 hour EC_{50}s) for $CdCl_2$ and $K_2Cr_2O_7$ to the freshwater green alga *Pseudokirchneriella subcapitata*. Data extracted from the US EPA ECOTOX database, various sources in literature, and **the current study**

Test duration	**$CdCl_2$**		**$K_2Cr_2O_7$**	
	EC_{50} (mg/L)	**Source**	**EC_{50} (mg/L)**	**Source**
72 hrs	0.11	Vasseur *et. al*. 1988	1.8	Nyholm 1990
	0.08		1.38	Mayer *et. al*. 1998
	0.06	**This study**	1.18	Arensberg *et. al*. 1995
	0.047		0.98	Paixao *et. al*. 2008
	0.046	Vasseur *et. al*. 1988	0.96	
	0.045	**This study**	0.9	Arensberg *et. al*. 1995
	0.044	Benhra *et. al*. 1997	0.074	Benhra *et. al*. 1997
	0.042		0.67	Christensen and Nyholm 1985
	0.04	**This study**	0.66	
	0.04	Benhra *et. al*. 1997	0.66	Radetski *et. al*. 1995
	0.037		0.43	Nyholm 1990
	0.031	Vasseur *et. al*. 1988	0.37	Cerejeira *et. al*. 1998
	0.032	Benhra *et al* 1997	0.31	Comber *et. al*. 1995
	0.03	Vasseur *et. al*. 1988	0.28	
	0.028		**0.26**	**This study**
	0.014		**0.25**	
			0.24	
			0.23	
			0.21	Radetski *et. al*. 1995
			0.19	**This study**
			0.17	
			0.14	Benhra *et. al*. 1997
96hrs	0.067	Thellen *et. al*. 1989	2.73	Mayer *et. al*. 1998
	0.065		2.42	
	0.063		**0.22**	**This study**
	0.059		**0.18**	
	0.055		0.18	Richter 1982
	0.054		0.18	Call *et. al*. 1981
	0.05	Blaise *et. al*. 1986	0.17	Hickey *et. al*. 1991
	0.048	Thellen *et. al*. 1989	0.13	St Laurent *et. al*. 1992
	0.043	**This study**	**0.11**	**This study**
	0.043		0.9	Adema *et. al*. 1981
	0.041		0.084	St Laurent *et. al*. 1992
	0.035			
	0.031			
	0.023	Thellen *et. al*. 1989		

The 96 hour EC_{50}s of $K_2Cr_2O_7$ to *P. subcapitata* and *C. sorokiniana* were 0.17+0.06 and 0.216+0.114 respectively. There were no statistically significant differences in the 96 hour EC_{50} values of $K_2Cr_2O_7$ to all four species. The order of sensitivity of the four species to $K_2Cr_2O_7$ was *C. protothecoides<C. sorokiniana<P. subcapitata<C. vulgaris*.

Toxicity data (72 and 96 hour EC_{50} values) for $CdCl_2$ and $K_2Cr_2O_7$ to *Pseudokirchneriella subcapitata* presented in Table 4.1 were obtained from the US EPA ECOTOX database and general literature. This was done to compare the data obtained from this study to the data that are available in literature. Data obtained from the database were from studies done in the 80s and 90s; there are few published toxicity data (EC_{50} values) from recent studies (e.g. Paixao *et. al*. 2008) available in literature for $CdCl_2$ and $K_2Cr_2O_7$ to *P. subcapitata*, although these two chemicals are well known reference toxicants for this species. The 72 hour EC_{50} values for $CdCl_2$ to *P. subcapitata* varied between 0.014 and 0.11 mg/L, and varied between 0.14 and 1.8 mg/L for $K_2Cr_2O_7$. The range of 96 hour EC_{50} values for $CdCl_2$ to *P. subcapitata* was between 0.023 - 0.067 mg/L, and between 0.084-2.73 mg/L for $K_2Cr_2O_7$. On average, $CdCl_2$ appears to be more toxic to *P. subcapitata* than $K_2Cr_2O_7$ (Table 4.1).

4.3.2 Specific growth rate, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)

The mean specific growth rate of the four species of green algae exposed to $CdCl_2$ and $K_2Cr_2O_7$was determined for all concentration treatments and compared to the specific growth rate of the controls (Figure 4.3 and 4.4 (a-d)) in order to determine the NOEC or the LOEC (Table 4.2). The average control specific growth rate for *P. subcapitata* was not significantly different to that of the lower concentration treatments (0.007 - 0.0313 mg/L) of $CdCl_2$, but significantly higher than that of the higher concentration treatments (0.0625 - 1 mg/L). The concentration treatment of 0.0313 mg/L was determined as the NOEC of $CdCl_2$ to this standard toxicity test species (Table 4.2). Figure 4.3(c) shows that the specific growth rate of *Chlorella protothecoides* was significantly higher than that of the control at lowest concentrations of $CdCl_2$ (0.007 – 0.0313 mg/L), and significantly lower at the two highest concentrations (0.5 and 1 mg/L). The $CdCl_2$ concentrations of 0.0625 – 0.25 mg/L showed no significant differences in specific growth rate to the controls of *C. protothecoides* (Figure 4.3c).

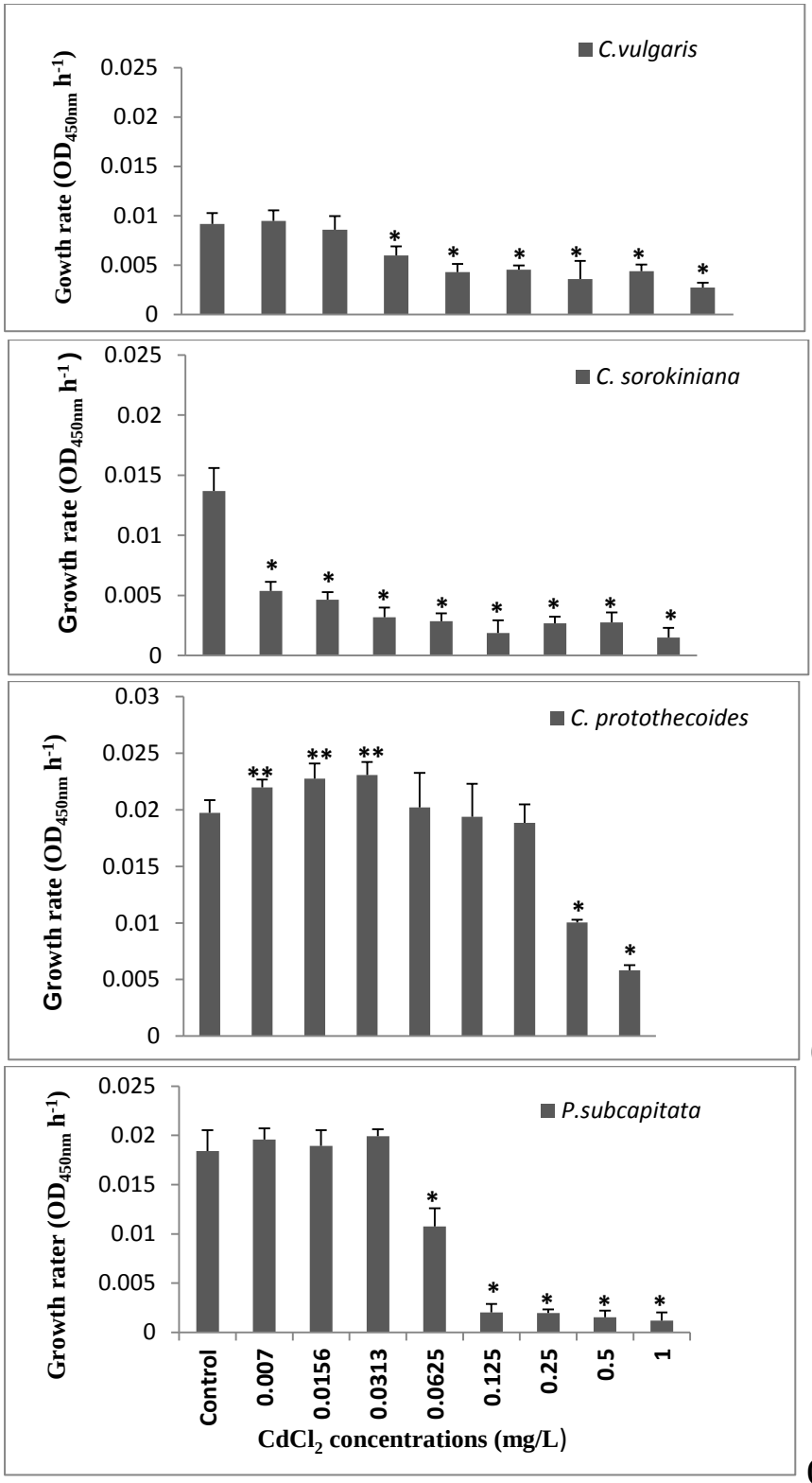


Figure 4.3 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation(n=6)) of (a)*Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to $CdCl_2$.*=significantly lower than the control, **=significantly higher than the control.

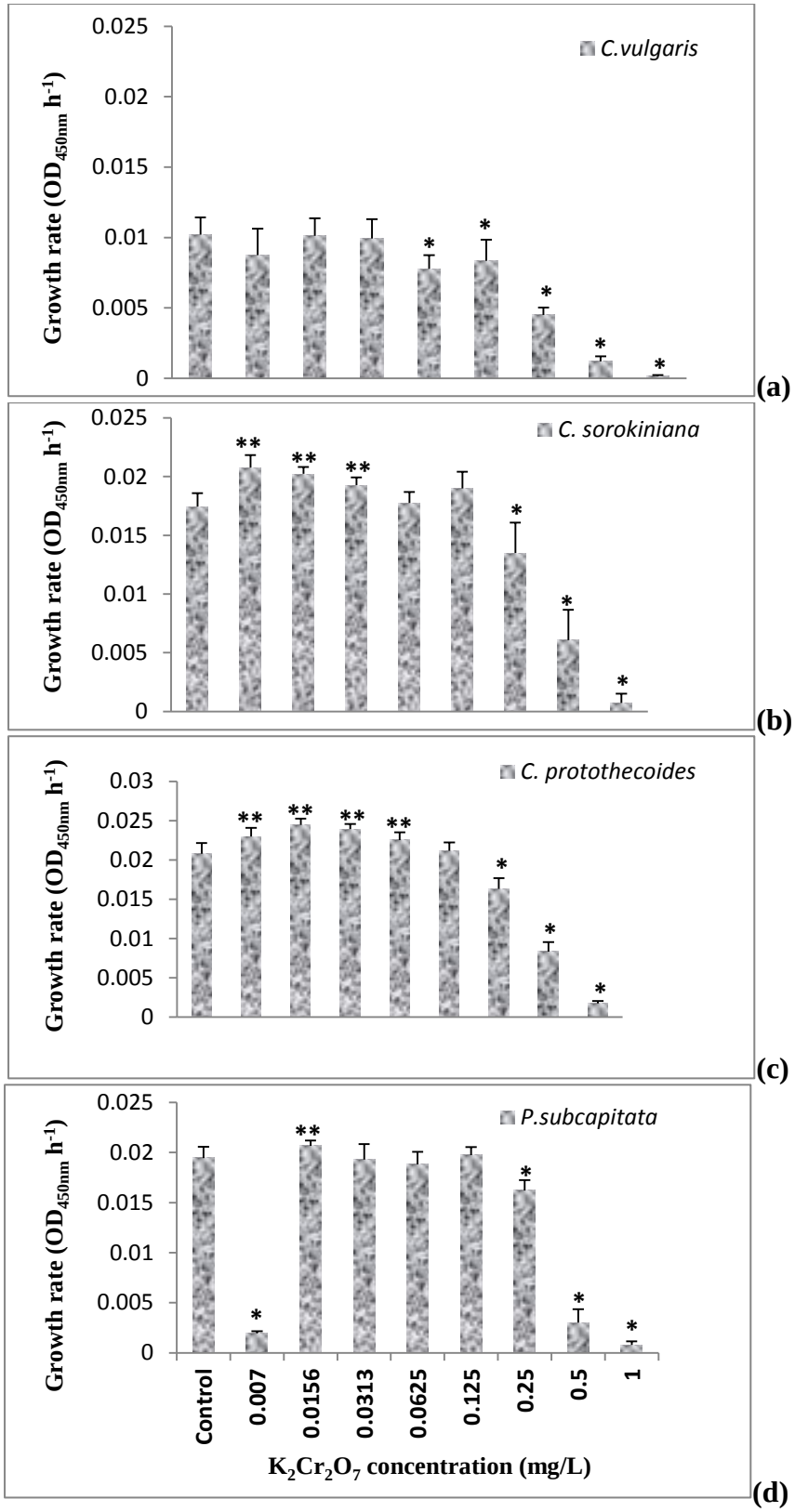


Figure 4.4 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to $K_2Cr_2O_7$. *=significantly lower than the control, **=significantly higher than the control.

Table 4.2 NOEC or LOEC and EC_{20} values (+ standard deviations) of two toxicants ($CdCl_2$ and $K_2Cr_2O_7$) for four species of freshwater algae.

Test algal species	**$CdCl_2$**		**$K_2Cr_2O_7$**	
	NOEC (mg/L)	**EC_{20}** (mg/L)	**NOEC** (mg/L)	**EC_{20}** (mg/L)
Pseudokirchneriella subcapitata	0.0313 **Inhibition**	0.02+0.003	0.125**Inhibition**	0.051+0.023
*Chlorella prototheco*ides	0.25 **Inhibition**	0.136+0.06	0.125 **Inhibition**	0.15+0.05
Chlorella sorokiniana	0.007 **Inhibition** (LOEC)	0.00003+0.000008	0.125 **Inhibition**	0.095+0.067
Chlorella vulgaris	0.0156 **Inhibition**	0.004+0.003	0.0313 **Inhibition**	0.026+0.007

The specific growth rate of control *Chlorella sorokiniana* was significantly higher than all the concentration treatments of $CdCl_2$, therefore the NOEC of $CdCl_2$ to this species could not be determined. The lowest concentration treatment (0.007 mg/L) was the LOEC of $CdCl_2$ for this species (Table 4.2). The specific growth rate of *Chlorella vulgaris* was not significantly affected by the two lowest concentration treatments of $CdCl_2$ (Figure 4.3a), but significantly affected by concentration treatments of 0.0313-1 mg/L.

The specific growth rate of the algal species appeared to be stimulated by $K_2Cr_2O_7$ at the lowest concentrations (Figure 4.4 b-c), except *P. subcapitata* which exhibited a significant decrease in specific growth rate at the lowest concentration treatment (0.007 mg/L) (Figure 4.4 d), and *C. vulgaris* which showed no effect on specific growth rate at the lowest concentration treatments (0.007, 0.0156 and 0.0313 mg/L) (Figure 4.4 a). The general specific growth rate response of *C. sorokiniana*, *C. prototheco*ides and *P. subcapitata* (Figure 4.3 b-d) to $K_2Cr_2O_7$ was stimulation at the lowest concentrations, no effect at moderate concentrations, and inhibition at the lowest concentrations. The specific growth rate of *C. sorokiniana* was significantly higher than the control at the three lowest concentrations (0.007, 0.0156 and 0.0313 mg/L), not significantly different from the control at the moderate concentrations of 0.0625 and 0.125 mg/L, and significantly lower than the control at the three highest concentrations (0.25, 0.5 and 1 mg/L). *Chlorella prototheco*ides showed a similar response trend, with the lowest concentrations (0.007, 0.0156, 0.0313 and 0.0625 mg/L)

exhibiting a significantly higher specific growth rate than the control, the moderate concentration (0.125 mg/L), no significant difference to the control, and the highest three concentrations (0.25, 0.5 and 1 mg/L), significantly lower specific growth rate compared to the control.

Pseudokirchneriella subcapitata also exhibited a more or less similar specific growth rate response trend to *C. sorokiniana* and *C. protothecoides* while *C. vulgaris* did not show significant difference in specific growth rate compared to the control at lower concentrations but a significantly lower specific growth rate at the highest concentrations (0.125, 0.25, 0.5 and 1 mg/L). The NOEC value of $K_2Cr_2O_7$ on *C. vulgaris* was 0.0313 mg/L, while the other three species shared the NOEC value of 0.125 mg/L (Table 4.2).

The control specific growth rate seemed to be consistent for all the species after 96 hours of exposure to both $CdCl_2$ and $K_2Cr_2O_7$respectively. After 96 hours of exposure during the $CdCl_2$ experiments, the average control specific growth rate of *P. subcapitata* was 0.018+0.0021(OD_{450nm} h^{-1}), and 0.019+0.0011(OD_{450nm} h^{-1}) for the $K_2Cr_2O_7$ exposure. *Chlorella protothecoides* exhibited average control specific growth rates of 0.0197+0.0011 and 0.0208+0.0014 (OD_{450nm} h^{-1}) after 96 hours of exposure to the respective toxicants. The average control specific growth rate of *C. vulgaris* after 96 hours of exposure, was 0.0092+0.0011(OD_{450nm} h^{-1}) for $CdCl_2$ and 0.01+0.0012(OD_{450nm} h^{-1}) for $K_2Cr_2O_7$. The average control specific growth rate for *C. sorokiniana* was slightly lower for the $CdCl_2$ exposure (0.014+0.0019 (OD_{450nm} h^{-1})) than the $K_2Cr_2O_7$ exposure (0.017+0.0011(OD_{450nm} h^{-1})). Consistent growth rate is an important characteristic for algae in toxicity tests and one of the requirements for consideration of algal species as toxicity test organisms.

4.3.3 Species sensitivity distributions

Toxicity data for ten algal species were used to generate a species sensitivity distribution (SSD) curve for $CdCl_2$; these included data from the ECOTOX database and this study (Table 4.3). All the species used for the SSD were green algae as these were the only data that could be used. Fifty percent of the data set (five data points) consisted of species from the genus *Chlorella*, and three of them were species used in this study. There were also two *Scenedesmus* species, one *Chlamydomonas* species, and one *Staurastrum* species. *Pseudokirchneriella subcapitata* had the highest sample size of 27 data points. The data set

for the $K_2Cr_2O_7$ SSD consisted of eleven species, with eight green-algae, one diatom and one blue-green alga (Table 4.4).*Chlorella* also dominated the $K_2Cr_2O_7$ dataset with the highest number of data points (3 data points, 27% of the dataset), and all of them were species also used in this study. *Chlorella vulgaris* consisted of seven data points for each of the two toxicants. Two out of seven data points of *C. vulgaris* for $CdCl_2$ and three out of seven data points for K2Cr2O7 were generated from this study. The $K_2Cr_2O_7$ dataset consisted of eight geni of algae, compared to the five genuses of the $CdCl_2$ dataset. *Pseudokirchnerialla subcapitata*, once again had the highest sample size of 28 data points. As the standard toxicity test species, *P. subcapitata* is expected to be the most frequently used species in toxicity tests.

Table 4.3 A summary of single algal species toxicity data for $CdCl_2$ obtained from the ECOTOX database and this study, and used to generate a species sensitivity distribution (SSD) curve.

Species Scientific Name	**Geometric mean (µg/L)**	**Taxonomic group**	**Exposure duration (hours)**	**No. of data points**
Chlamydomonas reinhardtii	397	green algae	72-96	4
Chlorella protothecoides	332	green algae	96	4
Chlorella pyrenoidosa	68	green algae	24	5
Chlorella saccharophila	110	green algae	96	1
Chlorella sorokiniana	2	green algae	96	2
Chlorella vulgaris	684	green algae	48-96	7
Pseudokirchneriella subcapitata	42	green algae	24-96	27
Scenedesmus acutus	11	green algae	96	1
Scenedesmus subspicatus	131	green algae	48-96	6
Staurastrum cristatum	11	green algae	96	1

Figure 4.5 shows the SSD curve for the ten algal species and their sensitivity to $CdCl_2$, based on 24-96 hour effects concentration data obtained from the ECOTOX database as well as this study. The position of the species on the curve represents the sensitivity pattern of the species

to the toxicant. The most sensitive species are typically positioned at the left-tail end of the curve while the most tolerant species are at the right tail-end of the curve. *Chlorella sorokiniana*, at the left tail-end of the curve appeared to be the most sensitive of the ten species to $CdCl_2$ and the second most sensitive to $K_2Cr_2O_7$ (Figure 4.5 and 4.6), while *Chlorella vulgaris* at the right tail-end was the least sensitive to $CdCl_2$. *Chlorella vulgaris* appeared to be the most sensitive of eleven species to $K_2Cr_2O_7$ (Figure 4.6). The standard toxicity test species, *P. subcapitata*, appeared in the middle of both the $CdCl_2$ and $K_2Cr_2O_7$ curves, which indicated that, compared to other algal species on the dataset, it was moderately sensitive to both reference toxicants. *Pseudokirchneriella subcapitata*, appeared to be the least sensitive of the species tested in this study to $K_2Cr_2O_7$, bearing in mind that the EC_{50} value used on the SSD represents 28 values from different studies. The blue-green alga, *Synechococcus leopoliensi* appeared at the right tail-end of the curve as the least sensitive of the eleven species to $K_2Cr_2O_7$ (Figure 4.6).

Table 4.4 A summary of single algal species toxicity data for $K_2Cr_2O_7$ obtained from the ECOTOX database and this study, and used to generate a species sensitivity distribution (SSD) curve.

Species Scientific Name	**Geometric means EC50 (µg/L)**	**Taxonomic group**	**Exposure duration (hours)**	**Number of data points**
Navicula seminulum	405	Diatom	96	18
Parachlorella kessleri	575	Green algae	72	1
Scenedesmus quadricauda	2527	Green algae	72	1
Scenedesmus subspicatus	1023	Green algae	24-96	11
Stichococcus bacillaris	1642	Green algae	72	1
Synechococcus leopoliensis	3308	Blue-green algae	96	1
Chlamydomonas reinhardtii	813	Green algae	72	1
Pseudokirchneriella subcapitata	434	Green algae	48-96	28
Chlorella vulgaris	194	Green algae	72-96	7
Chlorella protothecoides	293	Green algae	96	5
Chlorella sorokiniana	201	Green algae	96	2

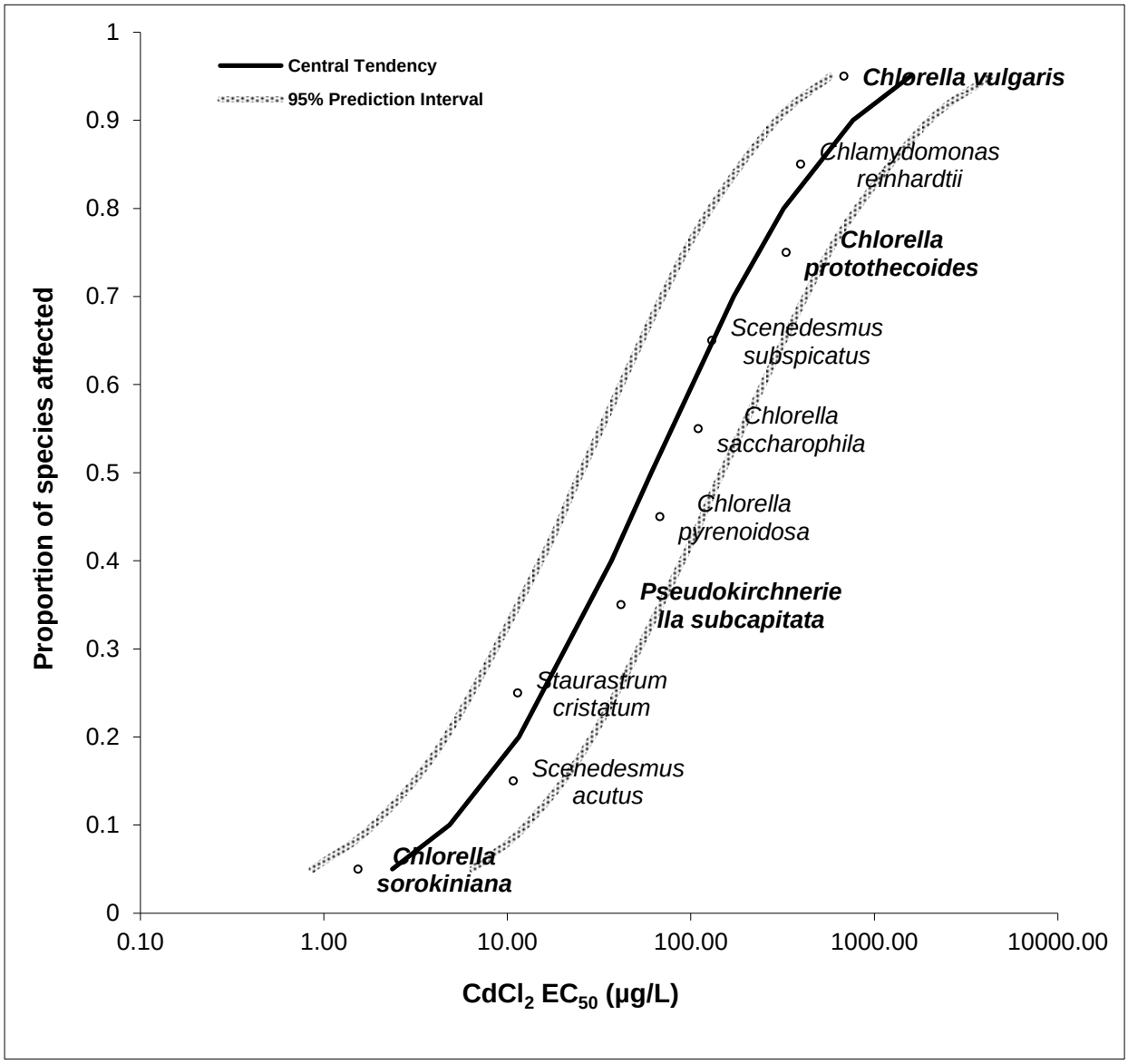


Figure 4.5 The species sensitivity distribution (SSD) curve of micro-algae based on 24-96 hour toxicity test data for $CdCl_2$ obtained from the ECOTOX database and this study. $CdCl_2$ concentrations are expressed in µg/L.

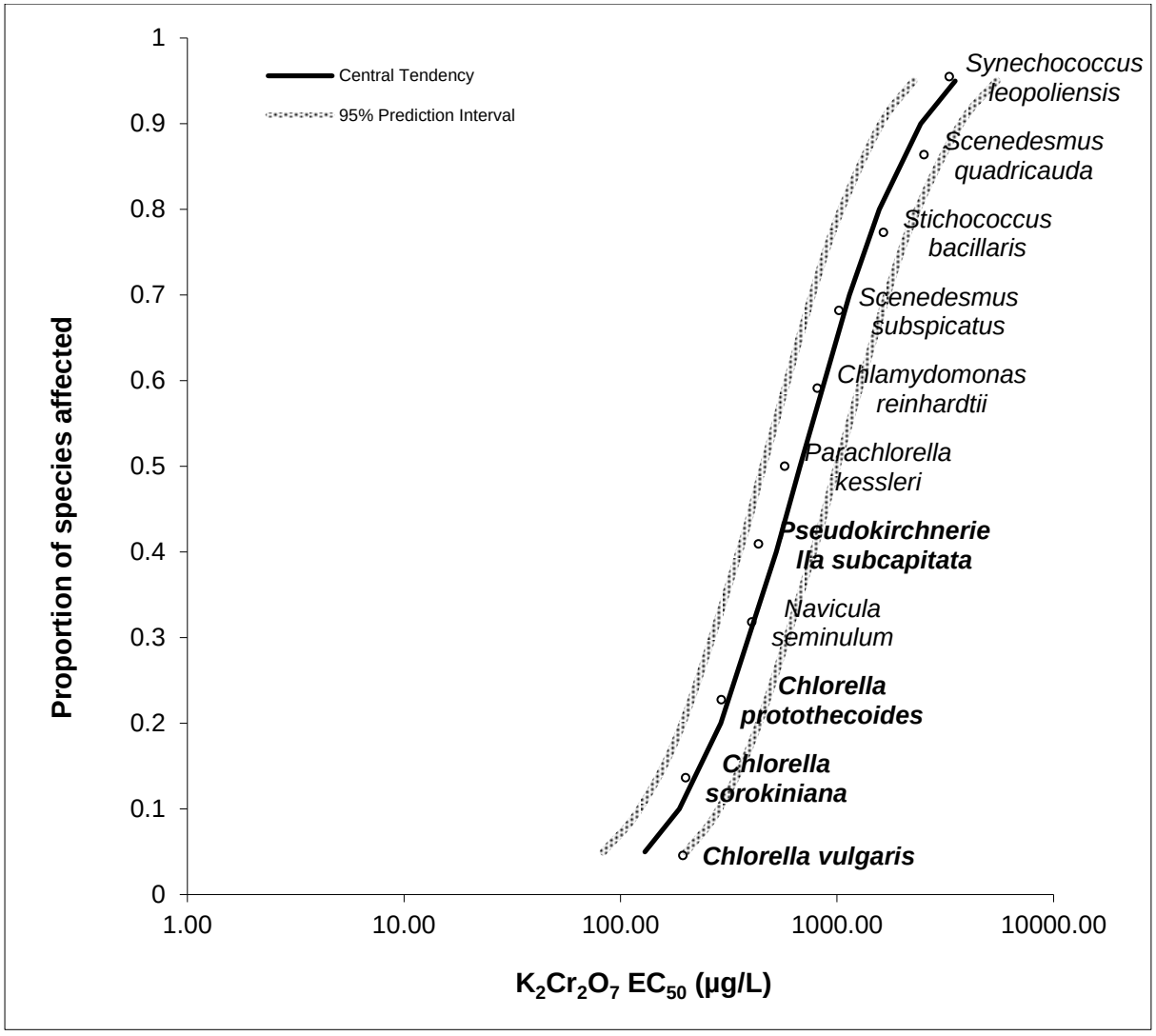


Figure 4.6 The species sensitivity distribution (SSD) curve of micro-algae based on 24-96 hour toxicity test data for $K_2Cr_2O_7$ obtained from the ECOTOX database and this study.

The micro-algae generally appeared to be more on the left end of the curve for $CdCl_2$ (Figure 4.5), compared to the curve for $K_2Cr_2O_7$ (Figure 4.6), indicating that they were more sensitive to the former. Moreover, the $CdCl_2$ curve was wider that the $K_2Cr_2O_7$ curve, which appeared to be narrow and steep. The EC_{50} values of the $CdCl_2$ data were more widely spread, indicating a more varied response of the different species to $CdCl_2$compared to $K_2Cr_2O_7$. All three *Chlorella* species used in this study appeared at the left tail-end of the $K_2Cr_2O_7$ curve, indicating their sensitivity to this toxicant, compared to other micro-algal species.

4.4 DISCUSSION

Five algal species were initially exposed to $CdCl_2$ and $K_2Cr_2O_7$. One of those five species, *Scenedesmus bicaudatus*, exhibited a high variability in control growth after the exposure period of 96 hours and was eliminated as a potential toxicity test species. The variability in control growth exceeded the acceptability limit prescribed for the validity of the toxicity test method. There may be physical and chemical experimental factors that caused this variability in control growth. The prescribed experimental conditions for this study are basic conditions adequate to support balanced growth of the standard algal species (*P. subcapitata*) during a toxicity test. The light intensity in our experiment (35-40 $\mu E/m^2/s$) was less than the light intensities specified in standard algal test protocols (60-120 $\mu E/m^2/s$). This was due to the design of our experimental facility. Despite using cool white fluorescent light bulbs, when the distance between the test surface and the light source was decreased, the temperature on the test surface increased. This required a compromise between light intensity and temperature to obtain adequate conditions for both parameters during the test. Light intensity was therefore adjusted to obtain the prescribed temperature of 25±3°C, because temperature is a major factor and needs to be carefully controlled. Temperature homogeneity is essential to obtain reproducible and precise test results. Slight differences in temperature may result in differences in growth rate for some algal species (Mayer *et. al*. 1998). Temperature increases may also result in increases in evaporation (Lorenz *et al*. 2005).

Although the light intensity in this study differed from the prescribed light intensity, it was adequate for the standard species *P. subcapitata* (and the other three species) to obtain balanced control growth and fulfil the established control growth variability requirements for test acceptability, as shown when comparing test results from this study with internationally published test results (Table 4.1). The ability of the standard species to withstand the given test conditions provided a basis for comparison of results obtained for the other four species under the same conditions. The test conditions used in this study were not outside the prescribed temperature and light culture conditions for the particular strain of *P. subcapitata* used in this study i.e. 20-25°C and 30-40 $\mu E/m^2/s$ (Appendix B). Moreover species such as *Chlorella sorokiniana* are also known to grow at light intensities of 30-60 $\mu E/m^2/s$ (de Bashan 2008).

It was on the basis of comparing growth with that of the standard species, and the perceived lack of reproducibility in tests with high control growth variability (Altenburger *et. al*. 2008), that *S. bicaudatus* was eliminated as a potential toxicity test species. Unicellular green algae typically adapt to periodic illumination: they allocate their resources towards biomass accumulation via photosynthesis during light phases and towards multiple cell-division in the dark (Altenburger *et. al*. 2008). Notably, the standard algal growth inhibition assay is performed under continuous illumination, and this might result in problems with the aforementioned growth pattern of unicellular algae, leading to high variability in growth of the controls.

Furthermore, cell division of unicellular algae seems to be linked to cell size. Upon analysing the cell size distribution of *Desmodesmus subspicatus* during a 72 hour toxicity test, Altenburger *et al*. (2008) noted that the cells of the algal population initially increased their cell volume until such time that the autospores were liberated from the dividing mother cells. At that point, the algal population had two subpopulations with different size distributions (the dividing mother cells, and the small daughter cells). In a dual subpopulation culture, such as mentioned above, the small cells may display little chlorophyll and the larger cells higher chlorophyll content. The variability in the proportions of small vs. bigger cells in a control population may contribute to the variability in control growth observed during a toxicity test, particularly if biomass measurement is based on photometric determination, as is the case with spectrophotometric analysis. Changes in light, nutrients or biological factors at the beginning or during the toxicity test may contribute to growth behaviour of algal cells. Therefore, it may be worth understanding the specificities of the growth and cell cycle of *Scenedesmus bicaudatus* in order to explain the problem of control growth variability before this species can be used in toxicity tests.

Although the standard species *P. subcapitata* was sensitive to both toxicants, it was significantly more sensitive to $CdCl_2$ than $K_2Cr_2O_7$ ($p \leq 0.05$). This finding is consistent with other studies (Table 4.1) which showed $K_2Cr_2O_7$ to be less toxic to *P. subcapitata* than $CdCl_2$. The mean of the 72 hour EC_{50} values of $CdCl_2$to *P. subcapitata* from different studies (excluding this study) as shown on Table 4.1 was 0.045 mg/L with the standard deviation of 0.023 and a coefficient of variation of 57%. The mean 72 hour EC_{50} of 0.048+0.008 mg/L obtained from this study was close to the mean (0.044 mg/L) of the data reported in literature. The 72 hour EC_{50} values of $K_2Cr_2O_7$ to *P. subcapitata* from different studies varied around

the mean of 0.64 mg/L (with 0.5 mg/L standard deviation, and 78% coefficient of variation). The mean 72 hour $EC_{50}$of 0.22+0.035 mg/L from this study was relatively lower than that of 0.64+0.5 mg/L obtained from the US EPA ECOTOX database (Table 4.1).

The difference in variability of 72 hour EC_{50} data of the two respective toxicants to *P. subcapitata* (57% vs. 78%) could be explained by the fact that the data were obtained from only two studies (Vasseur *et al*. 1988, Benhra *et al*. 1997) for $CdCl_2$ and more than 10 studies for $K_2Cr_2O_7$.The scarce data record on the growth inhibitory effect of $CdCl_2$ for *P. subcapitata* is due to the fact that studies of $CdCl_2$ on algae have focused on determining the biochemical and physiological effects as well as the mode of action, rather than growth inhibition (Rebhun and Ben Amotz 1986, Cepak *et al*. 2002, Terry and Stone 2002). Furthermore, because of its morphological and physiological properties, *P. subcapitata* may not have been suitable for these biochemical and physiological studies. Potassium dichromate on the other hand is the most commonly used reference toxicant for *P. subcapitata* (Wang 1987, US EPA 2001, ISO 2004, OECD 2006), and there is a relatively extensive data record of its growth inhibitory effects on this species (Table 4.1). The possible sources of high variability in toxicity data may be differences in test methods, test conditions and response variables selected for analysis. A combination of factors, such as illumination, temperature, medium composition and pH drifting, may cause differences in algal toxicity (Mayer *et al*. 1998). Despite this high variability in toxicity data, on average, *P. subcapitata* appears to be less sensitive to $K_2Cr_2O_7$ than $CdCl_2$.

The 72 hour EC_{50} of $K_2Cr_2O_7$ to *P. subcapitata* (0.22+0.035 mg/L) obtained in this study was in contrast to findings by Vannini *et al*. 2009, who found no effect of $K_2Cr_2O_7$on the growth of *P. subcapitata* at concentrations of 0.2 and 1 mg/L. Nyholm (1990) and Mayer *et al*. (1998) reported 72 hour EC_{50} values as high as 1.8 mg/L and 1.38 mg/L respectively for $K_2Cr_2O_7$ to *P. subcapitata*. The EC_{50} values of $K_2Cr_2O_7$ to *P. subcapitata* obtained from this study were relatively lower than most from the data reported in literature (Table 4.1). This could be due to differences in protocols and test conditions, as well as algal culturing techniques. The medium used in this study (BG-11), for example, was different from media used in other standard toxicity test methods. Furthermore, this study was performed under lower illumination than recommended by most standard toxicity test conditions. The afore-mentioned, in combination with other factors may have played a role in the low EC_{50} values obtained from this study.

Chlorella protothecoides appeared to be the least sensitive of the four species isolated from local aquatic ecosystems to both toxicants (Figure 4.2). As opposed to other tested species, *C. protothecoides* seemed slightly more sensitive to $K_2Cr_2O_7$ than $CdCl_2$ (Figure 4.2), although the mean 96 hour EC_{50} values of the two toxicants to this species were not significantly different ($p \leq 0.05$). The choice of test medium has been shown to be critical in determining metal toxicity in some *Chlorella* species (Stauber and Florence 1989). Some algal test protocols for determining metal toxicity have recommended that bioassays be performed in the same medium used to culture the algae, but in the absence of EDTA which may form complex with the metals and reduce toxicity. Stauber and Florence (1989), for example, have shown that high concentrations of nutrients and chelators (such as EDTA and iron) in test medium may decrease the toxicity of copper to *C. pyrenoidosa* (now known as *C. protothecoides*). The test medium used in this study (BG-11) is very rich in nutrients and contains EDTA and iron, which could explain the low toxicity of the two reference toxicants to *C. protothecoides*.

Chlorella sorokiniana seemed to be the most sensitive of the four species to $CdCl_2$, although the EC_{50} value of $CdCl_2$ to this species was only significantly lower than that of *C. protothecoides* and not significantly different ($p > 0.05$) to those of the other two species (*P. subcapitata* and *C. vulgaris*). This species also appeared to be the most sensitive to $CdCl_2$ of all the algal species on the species sensitivity distribution curve (Figure 4.5). Although *C. sorokiniana* appeared to be the second least sensitive to $K_2Cr_2O_7$, there were no statistically significant differences in sensitivity among the four species. The difference in EC_{50} values of the two toxicants to *C. sorokiniana* was not statistically significant ($p > 0.05$). Cadmium chloride significantly affected *C. sorokiniana* at the lowest concentration (LOEC = 0.007 mg/L), while $K_2Cr_2O_7$ only started showing a significant inhibitory effect at 0.25 mg/L (Figures 4.3 and 4.4 (c)). The variability in sensitivity of this species to the different toxicants may be attributed to different modes of action of the two chemicals to the organism. Rhebhun and Ben-Amotz (1986) suggested that cadmium uptake by algae is a membrane phenomenon, meaning that cadmium toxicity may be affected by any variable which alters the properties of the cell surface. In the case of *C. sorokiniana*, cell division is a process that involves changes in cell shape (Chapter 2). The spherical adult cells undergo asexual reproduction by means of internally formed autospores. Upon cell division, the parent cell first changes shape from spherical to square, before releasing four to eight smaller square-shaped daughter cells. These

changes in cell shape, translate to changes in cell surface, which may affect cadmium uptake and exacerbate toxicity.

Although $K_2Cr_2O_7$ may affect the population growth dynamics, cell morphology and physiological parameters in algal cells, algae tend to respond to chromium toxicity through adaptive changes in metabolic activity (Aizdaicher and Markina 2011). Figure 4.4 (c) shows that *C. sorokiniana* was significantly stimulated by $K_2Cr_2O_7$ at the lowest concentrations (0.007-0.0313 mg/L), but no significant effect at 0.0625 and 0.125 mg/L. The aforementioned stimulatory effect at the lowest concentrations was also observed on *P. subcapitata* and *C. protothecoides*, and may be due to changes in metabolic function of the algal cells as an adaptive response to $K_2Cr_2O_7$. An increase in photosynthetic pigment as an adaptive response to $K_2Cr_2O_7$ has been reported for the diatom *Attheyaussurensis* (Aizdaicher and Markina 2011). The effect of $K_2Cr_2O_7$ on photosynthetic activity was also observed on *Euglena gracilis* (Novikova *et al*. 2008). Vannini *et al*. (2009) also reported changes in photosynthetic apparatus of *P. subcapitata* as a response to low doses of $K_2Cr_2O_7$.

Other studies showed algal cells increase in size in response to chromium (Aizdaicher and Markina 2011). The effect of chromium on photosynthetic activity and cell size may explain the low sensitivity of most of the tested species to $K_2Cr_2O_7$ (with growth inhibition as an endpoint), compared to $CdCl_2$ as shown in this study. Biomass as a measure of growth was determined photometrically (with optical density) in this study, therefore, any stimulation of photosynthetic activity may manifest as increased chlorophyll content, that and/or increased cell size may result in increased optical density. This then would also explain the observed growth stimulation by $K_2Cr_2O_7$ at low concentrations (Figure 4.4 (b-d)). These metabolic adjustments and changes in cellular composition are mostly aimed at maximizing algal growth rate as a response to chemical stress (Friis *et al*. 1998).

Chlorella vulgaris appeared to be the most sensitive of the four species to $K_2Cr_2O_7$, and the second most tolerant to $CdCl_2$. However, the EC_{50} values of both toxicants to *C. vulgaris* were not significantly different ($p>0.05$) to those of other species. This species is also a recommended toxicity test species (OECD 1984), and has been used in toxicity tests with its sensitivity to toxicants being compared to that of *P. subcapitata* (Kasai and Hatakeyama 1993, Moreira-Santos *et al*. 2004, Ma *et al*. 2006). The sensitivity of *C. vulgaris* to the two reference toxicants in this study does not appear to be significantly different to that of *P.*

subcapitata. In fact the sensitivity of all three *Chlorella* species to both reference toxicants does not appear to be significantly different to that of the standard species *P. subcapitata*. However, *C. sorokiniana* was significantly more sensitive to $CdCl_2$ than C. *protothecoides* ($p>0.05$).

The 96 hour EC_{20} values and NOEC (or LOEC) values of the two reference toxicants on *P. subcapitata* and three *Chlorella* species are shown on Table 4.2. These endpoints are meant to be the determinants of low level effects of chemicals to organisms. The NOEC is a parameter that describes the highest tested toxicant concentration with no significant growth inhibition on the organism, while the EC_{20} is the concentration which decreases growth by 20%. These low toxic effect concentrations are important in ecological risk assessment of chemicals and development of environmental water quality criteria (Chen *et al.* 2009). The EC_{20} values of $CdCl_2$ to all the species were slightly lower than the NOEC values (Table 4.2). The NOEC value of $CdCl_2$to *C. sorokiniana* could not be determined because all chemical concentrations significantly affected the specific growth rate of the species. In this case the LOEC was determined and its value was higher than the EC_{20}. Similarly the NOEC values of $K_2Cr_2O_7$ were slightly higher than the EC_{20} for all the species with the exception of *C. protothecoides* which had a NOEC value slightly less than the EC_{20}. Overall though, the NOEC values were generally close to the EC_{20} values.

Although it is perceived to be easy to calculate and understand, the NOEC has been criticized for its high dependence on the values of the tested concentrations and the variation among the replicates (Kooijman *et al*. 1996, Chen *et al*. 2009). This criticism has led to the replacement of the NOEC with regression based low effect values (EC_x). Low effect values (EC_5, EC_{10}, EC_{15}, EC_{20}) have been suggested, but there has been a lack of consensus about which "low effect" value is appropriate for use in ecological risk assessment. The concern with this approach is that the smaller the effect size, the more dependent it is on the model used in calculating the results. The confidence intervals are also quite large at the lowest toxic effects (Kooijman *et al*. 1996, Chen *et al*. 2009). According to Kooijman *et al*. (1996), the model dependency of low toxic effects is not an issue for the effect sizes EC_{15} and/or EC_{20}.

Although the inadequacies of the NOEC are understood, it has the advantage of not depending on the dose-response slope, which makes it useful in interpreting stimulation data in algal toxicity tests. Since their values are close to each other, the two endpoints (NOEC

and EC_{20}) will be used inter-changeably further on in this study to determine the low toxic effects of toxicants. The EC_{20} will be employed when there is a dose-related response to a chemical, and the NOEC when the response is not dose-dependent, making it difficult to extrapolate the EC_x.

4.5 CONCLUSION

Scenedesmus bicaudatus was eliminated as a toxicity test species due to its inability to withstand the prescribed experimental conditions. One of the important validity criteria of an algal growth inhibition test is constant and homogenous growth in the controls. The variability in control growth of *S. bicaudatus* in this study was higher than the prescribed limit of 20%. More needs to be understood about growth and cell cycle specificities of this species before it can be used as a toxicity test species, and that is beyond the scope of this study.

The commercial species *Chlorella protothecoides* appeared to be the least sensitive of the four species to both toxicants. The use of high nutrient medium (BG-11) may have been more important in the bioavailability or toxicity of these chemicals to *C. protothecoides* than other species. The standard species *P. subcapitata* was not significantly more sensitive to either of the two toxicants than the other species. The two local isolates (*C. sorokiniana* and *C. vulgaris*) showed no significant differences in sensitivity to both toxicants. *Chlorella sorokiniana* was significantly more sensitive to $CdCl_2$ than *C. protothecoides*. Although *Pseudokirchneriella subcapitata* and the two locally isolated species (*C. sorokiniana* and *C. vulgaris*) appeared to be more sensitive to $CdCl_2$ than $K_2Cr_2O_7$, only *P. subcapitata* showed significant differences in sensitivity to the two toxicants. The difference in toxicity of these two toxicants is consistent with findings from other studies. The higher toxicity of cadmium is also supported by the fact that, according to the South African water guidelines, the water quality criteria for chromium (CrVI) are higher than those for cadmium (DWAF 1996), indicating that, according to existing data, cadmium is more of a threat to aquatic ecosystems than chromium.

Chlorella protothecoides on the other hand, showed a slightly higher sensitivity (although not statistically significant) to $K_2Cr_2O_7$ than $CdCl_2$which highlights the variability in response (or sensitivity) of different algal species to toxic substances. This variability in sensitivity can be

explained by differences in morphology, physiology and cell cycle specificities of the different algal species. The two locally isolated *Chlorella* species (*C. sorokiniana* and *C. vulgaris*) proved useful as toxicity test species because of their ability to withstand the prescribed experimental conditions and their relative sensitivity to the two reference toxicants. *Chlorella sorokiniana* was not only the most sensitive to $CdCl_2$ of the four tested species, but also more sensitive than most algal species as shown by the SSD. *Chlorella vulgaris* was the most sensitive species to $K_2Cr_2O_7$. These two locally isolated species will therefore be used further in toxicity tests to assess their response to other toxicants, in comparison to the standard species and the commercial species.

5. THE RESPONSE OF FRESHWATER MICRO-ALGAE TO INORGANIC SALTS: SODIUM CHLORIDE (NaCl) and SODIUM SULPHATE (Na_2SO_4)

5.1 INTRODUCTION

The previous chapters described how freshwater micro-algal species (*Scenedesmus bicaudatus*, *Chlorella vulgaris* and *Chlorella sorokiniana*) were successfully isolated from samples obtained from local rivers, cultured, and maintained axenically in the laboratory for use in toxicity tests (Chapter 2). Chapter 4 examined the responses of these species to when they were exposed to reference toxicants in order to assess their ability to withstand experimental conditions, and thus assess their potential as toxicity test species. *Scenedesmus bicaudatus* was not a good toxicity test species because it did not achieve constant, uniform growth during the experimental period (Chapter 4). The response of the remaining two species, *C. vulgaris* and *C. sorokiniana*, to reference toxicants was compared to that of the standard species *Pseudokirchneriella subcapitata* CCAP 278/4 (Appendix B) and the commercial species *Chlorella protothecoides* ATCC® 30411™. Furthermore, the sensitivity of the two species, *C. sorokiniana* and *C. vulgaris*, to the reference toxicants was compared to that of other algal species, using species distribution curves (Chapter 4).

This chapter begins to address the third and last phase of this study, namely assessing the application and value of using the local freshwater micro-algal species in toxicity testing for water resource management in South Africa. In this phase, the sensitivity of the local micro-algae to a range of carefully selected toxicants is assessed. Toxicants are carefully selected based on their relative importance in the South African context, as well as the practicality of using the local micro-algae to routinely determine the impact of these toxicants on local aquatic resources. The response of the two locally isolated species (*C. vulgaris* and *C. sorokinaina*) to the selected inorganic salts (NaCl and Na_2SO_4), in comparison to the standard toxicity test species, *Pseudokirchneriella subcapitata*, CCAP 278/4 and a commercial species, *Chlorella protothecoides* ATCC® 30411™ is assessed. Because land use in South Africa is characterized mainly by agricultural and mining activities, which potentially leach salts into the natural aquatic resources, selecting salts (NaCl and Na_2SO_4) as toxicants to be tested in this study is considered to be appropriate as representative of those land-use activities respectively.

The sensitivity of these algae to the salts will also be compared to that of freshwater macro-invertebrates using species sensitivity distribution (SSD) curves and data obtained from an international and a local database. The SSDs focus on the distribution of sensitivity of test species to selected toxicants, and these test species are supposed to be representatives of a given ecosystem (Schmitt-Jansen *et al*. 2007). Comparing the sensitivity to salts of both algae and macro-invertebrates is appropriate because, in aquatic communities, algae are an integral part of these communities and many macro-invertebrates rely on algae as their source of nutrition. Therefore, any effect on algae (chemical or otherwise) may directly or indirectly affect the macro-invertebrates in the aquatic environment. Kefford (2006) suggested that the community structure freshwater biota may be affected by salinity (the presence of dissolved solids or salts).

The process of increased salinity (salinization) in natural waters is a recognized environmental problem in some parts of the world, including Australia and southern Africa (Kefford *et al*. 2004, Kefford 2007). In many industrialized and agricultural regions, salt contamination may become a serious problem. There are aspects of land-use, in-stream and riparian habitat that are correlated with elevated salt concentrations (Kefford *et al*. 2006). Salts from agricultural and other land surface activities may be washed out from the land surface into rivers and streams and pose a risk to aquatic biota in their environments. Salts are generally not considered as toxicants, since their presence at low concentrations in aquatic environments may be beneficial to organisms (Kefford 2007). There are concerns that low salt concentrations in some rivers may have sub-lethal effects on aquatic species and ecosystems (Gross 2003). However, at elevated concentrations, salts may affect organisms directly or indirectly by causing biodiversity shifts and disrupting trophic pathways. Macro-invertebrate community structure in rivers has been shown to be related to salinity (Kefford 2000). Macro-invertebrates have a broad range of salinity tolerances and therefore salt sensitive organisms can be excluded from the community, leaving behind the more tolerant species (Gillis 2011).

The effects of dissolved solids, or salts, on algae have been studied extensively in order to synthesize growth media for culturing these algae. However, there is not much work done on the ecological effects of increased salt concentrations on freshwater algae. Salinity or elevated salt concentrations can affect the productivity of algae by increasing the osmotic pressure and subsequently inhibiting photosynthesis in these organisms (Cleave *et al*. 1981).

Osmotic pressure is dependent on the ionic strength of the algal growth medium, and therefore, changes in the salinity of the medium may cause inhibition of photosynthesis in some freshwater algae (Cleave *et al*. 1981).

There is existing literature on the physiological effects of organisms (algae and macro-invertebrates) exposed to changes in salinity when sodium chloride is present. The interest in studies of the effects of NaCl to organisms has possibly stemmed from it accounting for about four-fifths of the total salts in seawater (Batterton and Van Baalen 1971). Only recently has attention been given to the effects of sodium sulphate to organisms, despite it being a common component of saline agricultural drainage waters and some industrial effluents (Soucek 2007). Much of the information on sodium sulphate has examined the acute and sub-lethal effects on freshwater invertebrates such as crustaceans and molluscs (Soucek 2007a, b, and c). Sub-lethal effects of sodium sulphate on macro-invertebrates include reduced rates of growth, reproduction, metabolism, feeding etc. (Soucek 2007, Gillis 2011).

The inorganic salts (such as Na_2SO_4 and NaCl) are not on the list of water quality constituents of the South African Water Quality Guidelines for aquatic ecosystems (DWAF 1996). This may because the derivation of criteria was based on the available information and cause-effect data at the time (DWAF 1996). Electrical conductivity (EC) is used as a surrogate of salinity in the South African Water Quality Guidelines for aquatic ecosystem. Electrical conductivity (EC) is a measure of the ability of water to conduct an electrical current. The total dissolved salts concentration is a measure of the quantity of all dissolved compounds in water, and most dissolved salts in water carry an electrical charge, therefore total dissolved salts concentration is directly proportional to the electrical conductivity (EC). Ions such as **chloride**, **sulphate** and **sodium** carry an electrical charge when dissolved in water.

Both salts were selected for this study because they are common salts and salts of concern in terms of water quality management in South Africa (DWAF 2006a). They serve as indicators of the most prominent land-use activities of South Africa, mining and agriculture. Mining effluent is generally high in dissolved solids with sulphate as the dominant anion, and aquatic resources around some mining areas contain high sodium content (DWA 2004). Salinity generally becomes a serious water quality concern in most mining areas (DWA 2011). According to resource water quality objectives (RWQOs) in South Africa, electrical

conductivity, sulphate (SO_4^{2-}), and chloride (Cl^-) are among the parameters selected as indicators of fitness for use of water resources by certain user groups such as the mining and the agricultural sector. The RWQOs for South African catchments such as the Olifants River and the Vaal River that are heavily impacted typically include water quality standards for Cl^- and SO_4^{2-}, as these are problematic in such mining and agricultural areas. Resource Water Quality Objectives (RWQOs) form the water quality component of the Resource Quality Objectives (RQOs). The purpose of RQOs as defined by the National Water Act (Chapter 3, Section 13) is to establish clear goals relating to the quality of water resources, finding a balance between protecting and sustaining the resources, and the need to use them (DWAF 2006a). As part of compliance monitoring of the water quality standards of water resources, in-stream levels of indicator parameters are compared to RWQOs established for that particular resource (DWAF 2006a).

These two salts feature in the determination of the Present Ecological State of the EcoClassification process of South Africa's Reserve determination (DWAF 2008b). EcoClassification refers to the determination and categorization of the Present Ecological State of various biophysical attributes of rivers relative to natural reference conditions (DWAF 2007). Table 5.1 presents the Present Ecological State rating values for the two inorganic salts **(**Na_2SO_4 and NaCl) as outlined on the EcoClassification process of Reserve determination (DWAF 2008b).

Table 5.1 Present ecological state (PES) rating values for inorganic salts **(**Na_2SO_4 and NaCl) (DWAF 2008)

PES rating	**Deviation from reference condition**	**Water quality Category**	**Na_2SO_4 (mg/L)**	**NaCl (mg/L)**
0	No change	A	20	45
1	Small change	B	33	191
2	Moderate change	C	38	243
3	Large change	D	51	389
4	Serious change	E	64	535
5	Extreme change	F	>64	>535

Processes such as photosynthesis, protein synthesis and metabolic activity may be affected by salt stress in plants. A relationship between water and salt uptake, enzymatic salt removal capacity and toxicity of salts has been found in some plants (Trapp *et al*. 2008). Salt stress in plants has three main effects: it reduces water potential, causes ionic imbalance and induces toxicity (Parida and Das 2005). However, plants and algal cells may be able to develop phenotypic adjustments such as changes in biochemical and molecular mechanisms that enable them to cope with salt stress. Biochemical strategies such as change in photosynthetic pathways, induction of anti-oxidative enzymes, as well as alteration of membrane structures may assist plants and algae to cope with salt stress (Berube *et al*. 1999, Trapp *et al*. 2008). These biochemical processes may act additively or synergistically in plants to improve salt tolerance (Parida and Das 2005). Some micro-algae such as some blue-green algae have been found in some hypersaline environments indicating their tolerance to salts. This may be cause for concern in aquatic environments because some blue-greens may cause algal blooms when they are able to out-compete salt-sensitive algae from other taxonomic groups.

5.2 MATERIALS AND METHODS

5.2.1 Test organisms, conditions and chemicals

Four species of freshwater micro-algae were exposed to two inorganic salts, NaCl and Na_2SO_4. Cultures of *Chlorella sorokiniana* and *Chlorella vulgaris* were isolated from local rivers (Palmiet and Kieskamma). A stock culture of the standard toxicity test species, *Pseudokirchneriella subcapitata*, was provided by Rand Water Analytical Services (Vereeniging, South Africa) and *Chlorella protothecoides* was obtained from a commercial culture collection (Environmental Biotechnology Research Unit, Rhodes University, Grahamstown, South Africa). All cultures were maintained axenically in 10% BG-11 medium (Slabbert 2004) at 24 °C under cool fluorescence light of 16:8 hour light/dark cycle without agitation,as described in Chapters 3 and 4.

The toxicity of NaCl and Na_2SO_4 to the standard toxicity test species *P. subcapitata* and the three *Chlorella* species was assessed using the growth inhibition assay (96 hours). The toxicity experiments were conducted in sterile 24 well micro-plates with lids at 25±3 °C under 35-40 $\mu E/m^2/s$ of continuous cool fluorescence light according to Slabbert (2004). The

general method used in these toxicity tests is described in Chapter 3 (Section 3.4). Sodium chloride and sodium sulphate were applied at a series of eight concentrations respectively, for each species (0.07, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 g/L). Each exposure of one species to a toxicant was replicated six times. Algal population growth was determined spectro-photometrically by measuring optical density at 450 nm for each plate on a micro-plate reader, at the beginning and the end of the toxicity test.

Test following criteria were used to determine test validity:

- Coefficient of variation of control growth ≤10% for *P. subcapitata* and ≤20% for other species.
- An average OD_{450nm} reading of >0.10 for the controls at the end of the test
- R^2 of more than 0.8 in the linear regression for EC_x calculations
- Inhibition data between 10% and 90% growth inhibition used in the linear interpolation for EC_x calculations

5.2.2 Analysis

5.2.2.1 Growth inhibition and effective concentration (EC_x) determination

Data for all test replicates were assessed for validity using the above mentioned criteria, and those that did not meet all the criteria were not used. Percentage inhibition values were estimated for each experimental group (exposure of each species to each toxicant). EC_{50} values were determined by linear regression and graphic interpolation on the percentage inhibition data (between 10% and 90%). Data belonging to the upper-most and lower-most part of the curve (< 10% and > 90%) were excluded so as to minimize any negative influence they may have on the curve-fitting (Mayer *et al*. 1997, Slabbert *et al*. 2004). The EC_{50} values for all replicates of each toxicant on each species were pooled together and expressed as mean EC_{50} (+standard deviation). The differences in sensitivity of species to each of the two toxicants were determined by statistically comparing the EC_{50} values. Data were tested for normality and homogenous variance, and a one-way-ANOVA (Analysis of Variance), with Unequal N HSD post-hoc test was used for normally distributed data (due to unequal replicates after quality control of the data). A non-parametric Kruskal Wallis ANOVA by ranks was used for data that were not normally distributed.

5.2.2.2 Specific growth rate, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)

All the experimental data were used (even those with higher than acceptable variability in control growth after 96hrs) to determine specific growth rate for the control and each toxicant concentration treatment (see Chapter 3.4). This was done in order to include and present stimulation data, which was omitted in EC_x calculations. The data were tested for normality and homogeneity, and a one-way ANOVA was used to determine which treatments differed significantly from the control, thus determining NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration) values (using treatments with significantly reduced growth compared to the control). All statistical analyses were undertaken using STATISTICA (Version 8) software package with $p \leq 0.05$ as the level of significance.

5.2.2.3 Species sensitivity distributions

Species sensitivity distribution curves were generated in order to compare the sensitivity of macro-invertebrates and micro-algae to the selected inorganic salts (NaCl and Na_2SO_4). Median concentration effect (EC_{50} and LC_{50}) data of the two salts on algae and macro-invertebrates used for the SSDs were extracted from the US EPA ECOTOX database, the UCEWQ (Unilever Centre for Environmental Water Quality) database (Palmer *et al.* 2004), and data generated in this study. The data from the ECOTOX database were pre-screened and filtered by only using laboratory generated toxicity data and excluding the following data:

- seawater as the medium of exposure,
- exposure duration longer than 96 hours and shorter than 24 hours, and
- no reports on fields such as endpoint, effects measurement, exposure duration and/or concentration units.

Data extracted from the UCEWQ database were pre-screened by excluding data with exposure duration longer than 96 hours and shorter than 24 hours, and those with concentration units other than mass/volume (mg/L or g/L). Species sensitivity distribution curves were drawn using SSD generator (US EPA 2005), and effect concentration data (LC_{50} or EC_{50}) for different species. In cases where there was more than one effect concentration value of a species on the salt, the geometric mean of all the values was taken as a

representative value for that species. In cases where there was only one value for the species, that value represented that species on the SSD. The SSD generator was used to construct SSD curves with single species toxicity data generated from this study, and pre-screened data extracted from the ECOTOX and UCEWQ databases.

5.3 RESULTS

5.3.1. Growth inhibition and median effective concentration (EC_{50})

The 96 hour EC_{50} values of NaCl and Na_2SO_4 to *P. subcapitata*, and the three *Chlorella* species, C. *protothecoides, C. sorokiniana* and *C. vulgaris,* are presented in Figure 5.1. The EC_{50} values of the NaCl to the four species ranged from 0.24±0.1 to 1.69+0.19 g/L (Figure 5.1). *Chlorella sorokiniana* appeared to be the most sensitive to NaCl with the EC_{50} value of 0.24+0.1 g/L, and *C. protothecoides* the most tolerant with the EC_{50} of 1.69+0.19 g/L. The EC_{50} values of these two species were statistically significantly different from each other. *Chlorella sorokiniana* was also significantly more sensitive to NaCl than the other local isolate, *C. vulgaris*. Compared to the other three species, the standard species, *P. subcapitata* was also relatively sensitive to NaCl (EC_{50} = 0.54+0.12 g/L) while *C. vulgaris* was relatively tolerant (EC_{50} = 1.37 +0.49 g/L). *Pseudokirchneriella subcapitata* was significantly more sensitive to NaCl than *C. protothecoides*, although not significantly more sensitive than the two local isolates, *C. vulgaris* and *C. sorokiniana*. The order of sensitivity of the four species to NaCl was as follows: *C. protothecoides<C. vulgaris<P. subcapitata<C. sorokiniana* (Figure 5.1).

Pseudokirchneriella subcapitata appeared to be the most sensitive to Na_2SO_4 (EC_{50} = 0.35+0.06 g/L), while the two locally isolated species *C. vulgaris* (EC_{50} = 2.31+0.8 g/L) and *C. sorokiniana* (EC_{50} = 2.35+0.35 g/L) were the most tolerant. There was a statistically significant difference between the EC_{50} values of Na_2SO_4 to *P. subcapitata* and *C. vulgaris*, although the EC_{50} values of *P. subcapitata* and *C. sorokiniana* were not significantly different. The order of sensitivity of the four species to Na_2SO_4 was as follows, *P. subcapitata>C. protothecoides>C. vulgari>C. sorokiniana* (Figure 5.1).

Only *C. sorokiniana* showed a significant difference in sensitivity between NaCl and Na_2SO_4; other species were not significantly more sensitive to one salt than the other.

5.3.2. Specific growth rate, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)

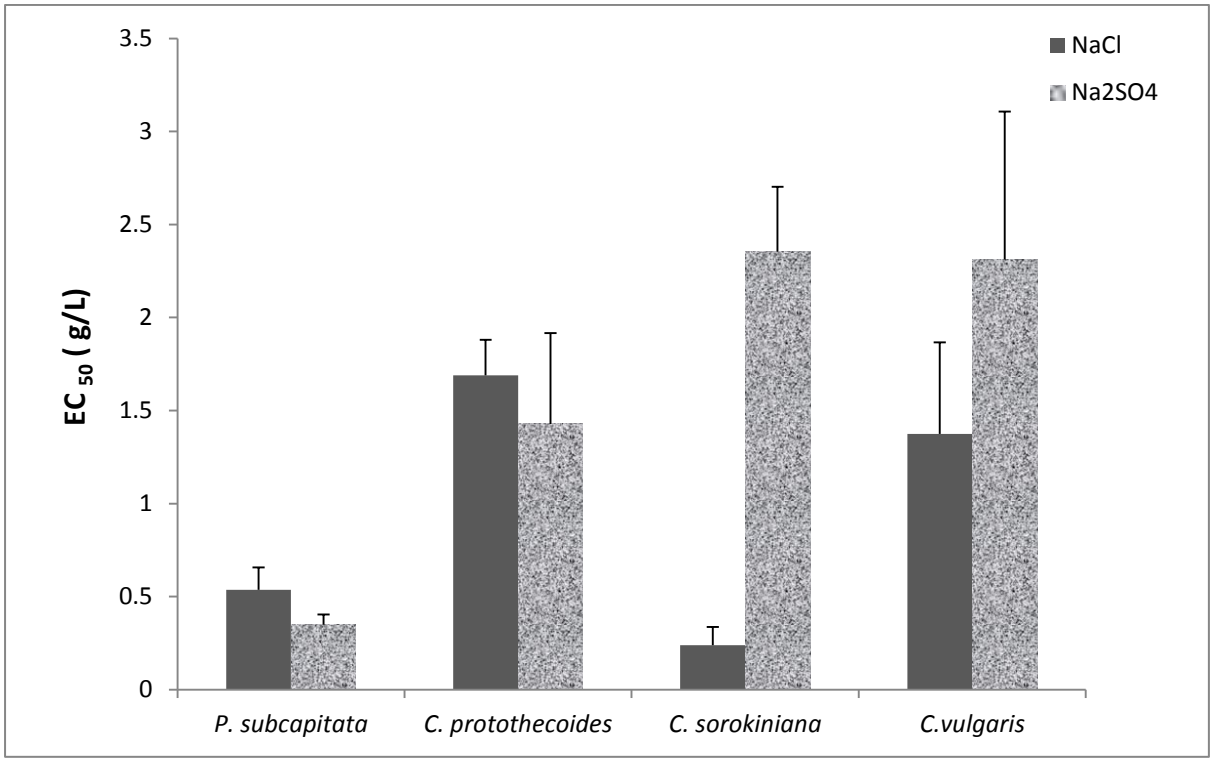


Figure 5.1 Mean 96 hour EC_{50} (+standard deviation) of NaCl and Na_2SO_4 to *Pseudokirchneriella subcapitata* (n^a=2, n^b=4), *Chlorella prototheco ides* (n^a=3, n^b=5), *Chlorella sorokiniana* (n^a=2, n^b=2) and *Chlorella vulgaris* (n^a=3, n^b=3). (n^a is the number of replicate tests for NaCl, and $n^b$the number of replicate tests for Na_2SO_4).

The mean specific growth rates of the four species of green algae exposed to NaCl and Na_2SO_4 is presented in Figures 5.2 and 5.3. The mean specific growth rate of the control was compared to that of concentration treatments for each salt and the NOEC of each salt on each species was determined (Table 5.2). In terms of specific growth rate, the three species, *P. subcapitata*, *C. prototheco ides* and *C. sorokiniana*, exhibited a similar response to NaCl, with stimulation at lower concentrations, no effect at medium concentrations and inhibition at the highest concentrations. *Chlorella vulgaris* was not stimulated by NaCl, although it also showed inhibition at the highest concentrations. *Pseudokirchneriella subcapitata* and *C. sorokiniana* were stimulated at the two lowest concentrations (0.07 and 0.156 g/L), while *C. prototheco ides* was stimulated at the lowest three concentrations (0.07 – 0.313 g/L).

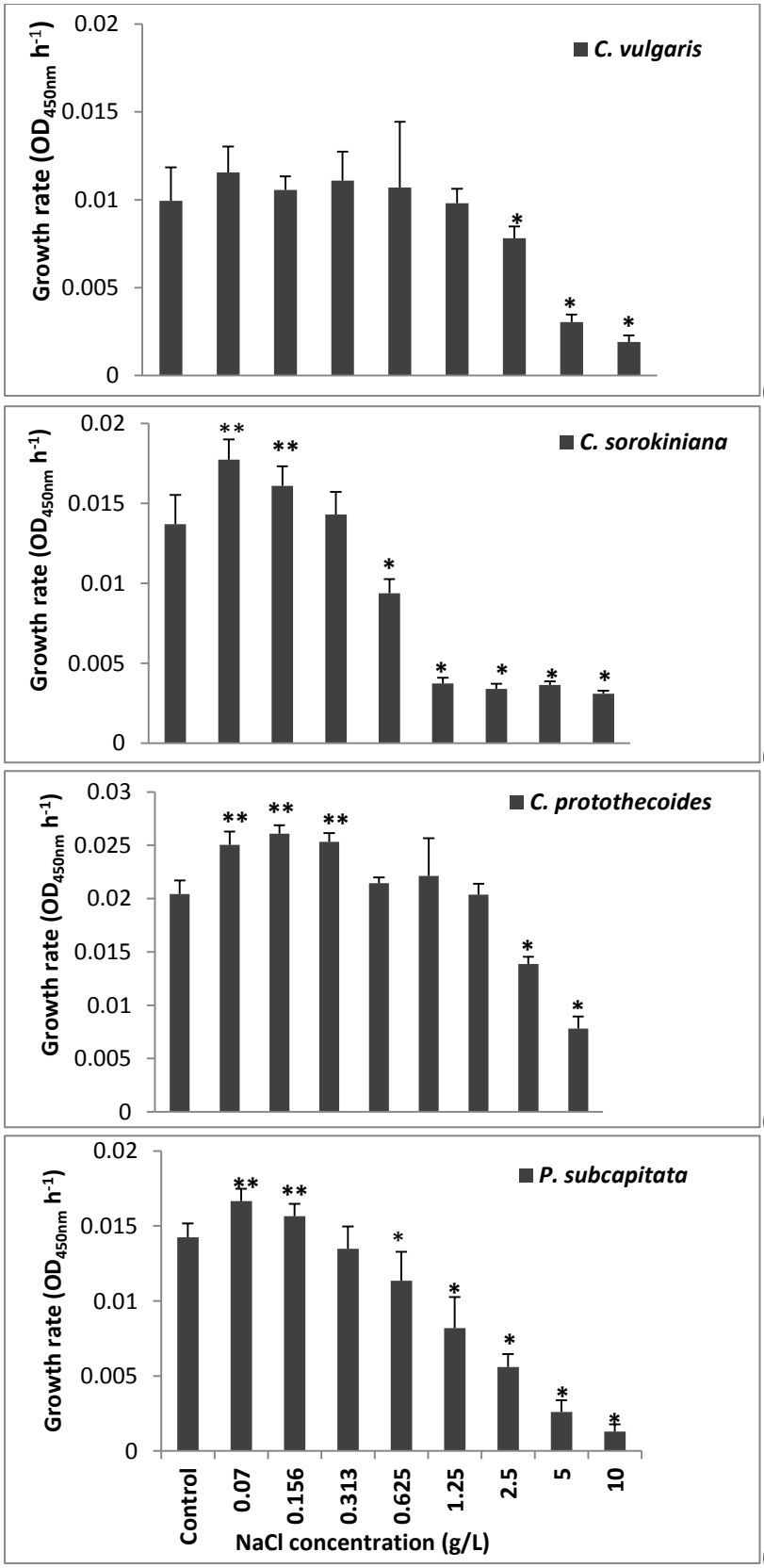


Figure 5.2 Mean specific growth rate (OD_{450nm} h^{-1}) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to NaCl. *=significantly lower than the control, **=significantly higher than the control.

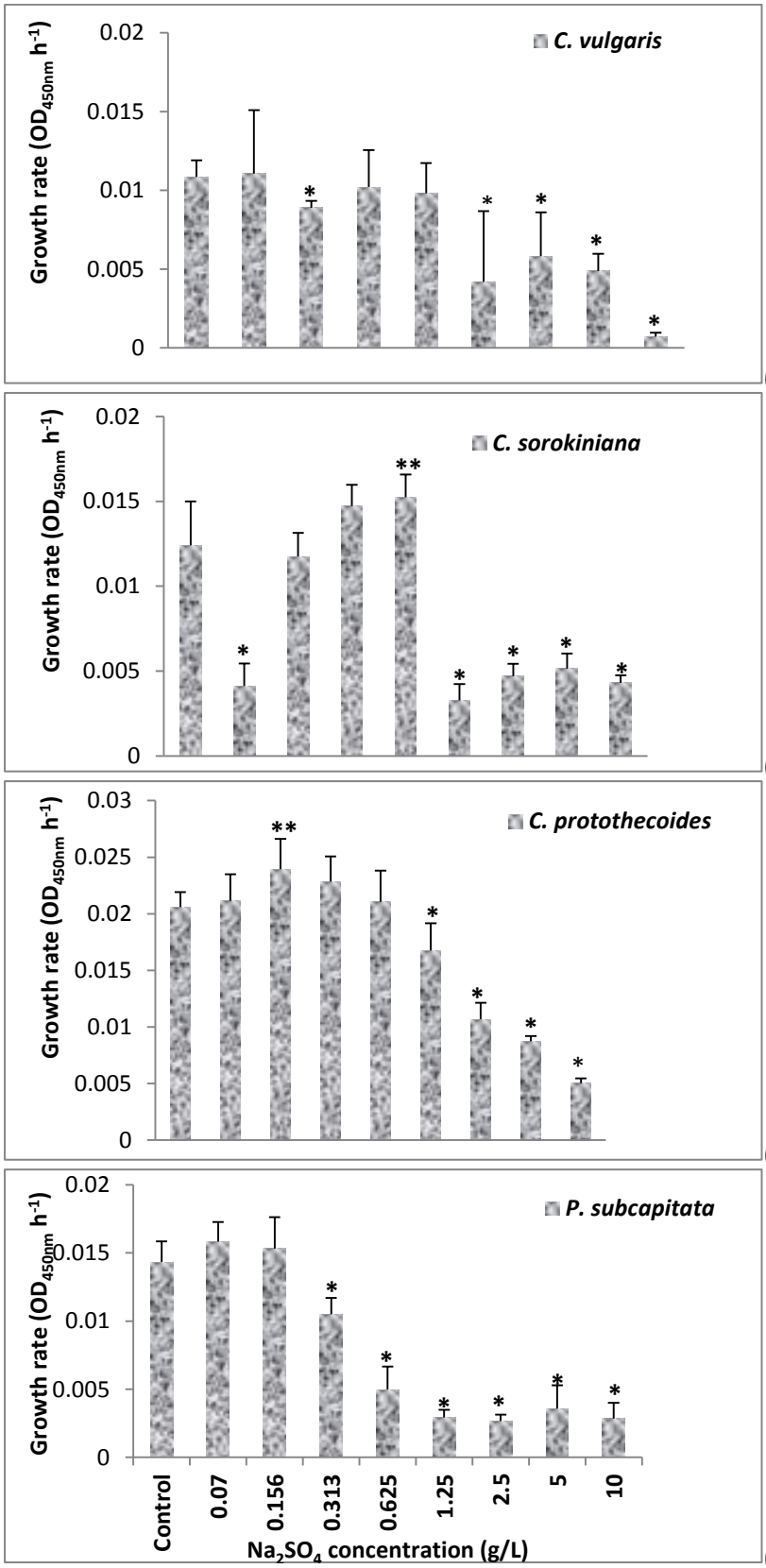


Figure 5.3 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to Na_2SO_4. *=significantly lower than the control, **=significantly higher than the control.

Table 5.2 NOEC and EC_{20} (+standard deviation) values of two toxicants (NaCl and Na_2SO_4) on four species of freshwater algae.

Test algal species	**NaCl**		**Na_2SO_4**	
	NOEC (g/L)	**EC_{20} (g/L)**	**NOEC (g/L)**	**EC_{20} (g/L)**
Pseudokirchneriella subcapitata	0.313	0.15+0.059	0.156	0.16+0.04
*Chlorella prototheco*ides	2.5	0.59+0.084	0.625	0.42+0.27
Chlorella sorokiniana	0.313	0.12+0.076	0.625	0.76+0.12
Chlorella vulgaris	1.25	0.52+0.319	0.625	0.40+0.25

Pseudokirchneriella subcapitata and *C. sorokiniana* were inhibited by NaCl at the five highest concentrations (0.625 – 10 g/L), *C. vulgaris* was inhibited at the three highest concentrations (2.5 – 10 g/L) and *C. prototheco*ides inhibited at the two lowest concentrations (5 and 10 g/L). The NOEC values for NaCl on all four species are presented on Table 5.2. *Chlorella sorokiniana* and *C. prototheco*ides showed significant stimulation by Na_2SO_4 at low concentrations. *Chlorella sorokiniana* was significantly stimulated at the concentration of 0.625 g/L while *C. prototheco*ides was stimulated at 0.156 g/L.

The three *Chlorella* species were inhibited by Na_2SO_4 at the four highest concentrations (1.25–10 g/L) and *P. subcapitata* was inhibited at the six highest concentrations (0.313-10 g/L). *Chlorella vulgaris* showed a significant inhibition in specific growth rate at the low concentration of 0.156 g/L, followed by no effect at the next high concentrations of 0.313 and 0.625 g/L. This could be associated with experimental error and the high variability in specific growth rate shown at the lowest concentration (Figure 5.3a). Similarly, *C. sorokiniana* showed a significant inhibition in specific growth rate at the lowest concentration (0.07 g/L), and although there is a high variability in control growth (Figure 5.3b), this inhibition is slightly more than that shown for *C. vulgaris*. This response may be due to a hormetic effect of Na_2SO_4 on *C. sorokiniana*, or just experimental error.

Table 5.2 presents the NOEC and EC_{20} (+standard deviation) values for Na_2SO_4 for all four species. *Pseudokirchneriella subcapitata* appeared to have the lowest NOEC value for

Na_2SO_4, while *P. subcapitata* and *C. sorokiniana* showed the lowest NOEC to NaCl. These NOEC results were in agreement with the EC_{50} results shown on Figure 5.1, where Na_2SO_4 had the lowest EC_{50} to *P. subcapitata* and the other three species showed slightly higher EC_{50} values that were not too different from each other. Similarly for NaCl (Figure 5.1) the EC_{50} values to *P. subcapitata* and *C. sorokiniana* are low and close to each other, while the other two species showed slightly higher EC_{50} values which also seemed closer to each other.

5.3.3 Species sensitivity distributions

There were few data for algae and the two selected salts on the ECOTOX database, and even those that were available did not meet the pre-screening criteria and therefore could not be used to generate SSD curves. The toxicity data for algae used to generate SSD curves for the two salts were thus from this study only (Tables 5.3 and 5.4). The NaCl dataset consisted of data for 39 species of macro-invertebrate, 26 of those were extracted from the ECOTOX database and 13 were from the UCEWQ database. As shown in Table 5.3, *Pseudo sidaramosa* had the highest number of data points (20), followed by *Daphnia pulex* (18) and *Daphnia magna* (16). The two *Daphnia* species are standard toxicity test species, and therefore it is to be expected that they should have such a high number of data points compared to other species which are not routinely used in toxicity tests.

The data used for the SSDs had exposure durations between 24 and 96 hours (Tables 5.3 and 5.4). The algal species appeared to be relatively more sensitive to NaCl than most of the macro-invertebrates. *Chlorella sorokiniana* appeared at the left tail-end of the curve, indicating that it was the most sensitive of all the species to NaCl (Figure 5.4). *Pseudokirchneriella subcapitata* was also more sensitive to NaCl than all the invertebrate species. *Chlorella vulgaris* also appeared at the left tail-end of the curve, although it was less sensitive to NaCl than *Lampsilis siliquoidea* and *Tubifex tubifex. Chlorella protothecoides* was the most tolerant of all algal species to NaCl, and more also tolerant than some of the macro-invertebrates (*Tricorythus tinctus, Pseudosida ramose*, *Daphnia magna*, *Lampsilis siliquoidea* and *Tubifex tubifex*). The Na_2SO_4 dataset used to generate the SSD consisted of toxicity data for 28 species, 24 of which were macro-invertebrate. The macro-invertebrate toxicity data consisted of 7 data points from the ECOTOX database and 17 data points from the UCEWQ database (Table 5.4).

Table 5.3 A summary of single species toxicity data of NaCl to freshwater micro-algae and macro-invertebrates obtained from the ECOTOX and UCEWQ databases as well as this study, and used to generate a species distribution (SSD) curve.

Species Scientific Name	Geometric mean (g/L)	Taxonomic group	Exposure durations (hours)	Number of data points
Pseudokirchneriella subcapitata	0.5	Algae	96	2
Chlorella protothecoides	1.7	Algae	96	3
Chlorella sorokiniana	0.2	Algae	96	2
Chlorella vulgaris	1.3	Algae	96	3
Lithobates sylvaticus	3.7	Macro-invertebrate	96	2
Ceriodaphnia dubia	1.8	Macro-invertebrate	24-96	8
Daphnia ambigua	2.0	Macro-invertebrate	48	1
Daphnia magna	3.3	Macro-invertebrate	24-96	16
Gammarus pseudolimnaeus	7.7	Macro-invertebrate	96	1
Hyalella azteca	5.0	Macro-invertebrate	96	1
Lirceus fontinalis	3.0	Macro-invertebrate	96	1
Pseudo sidaramosa	1.4	Macro-invertebrate	48	20
Streptocephalus rubricaudatus	3.1	Macro-invertebrate	24	1
Baetis tricaudatus	6.1	Macro-invertebrate	24-48	7
Acroneuria abnormis	10.0	Macro-invertebrate	96	1
Agnetina capitata	10.0	Macro-invertebrate	96	1
Callibaeti sfluctuans	5.0	Macro-invertebrate	96	1
Chaoborus americanus	5.0	Macro-invertebrate	96	1
Pycnopsyche guttifer	3.5	Macro-invertebrate	96	1
Pycnopsyche lepida	3.5	Macro-invertebrate	96	1
Tipula abdominalis	10.0	Macro-invertebrate	96	1
Elliptio complanata	2.4	Macro-invertebrate	24-48	2
Lampsili fasciola	2.3	Macro-invertebrate	24-96	3
Lampsilis siliquoidea	1.1	Macro-invertebrate	24-96	3
Villosa constricta	2.7	Macro-invertebrate	24-48	2
Villos adelumbis	4.0	Macro-invertebrate	24-48	3
Physagyrina	2.5	Macro-invertebrate	96	1
Physella integra	5.0	Macro-invertebrate	96	1
Tubifex tubifex	1.0	Macro-invertebrate	24-96	3
Caenorhabditis elegans	21.3	Macro-invertebrate	24-48	9
Tricorythu stinctus	1.7	Macro-invertebrate	96	1
Euthraulus elegans	7.6	Macro-invertebrate	96	1
Afronurus peringueyi	6.7	Macro-invertebrate	96	1
Caridina nilotica	5.9	Macro-invertebrate	48-96	5
Daphnia pulex	2.8	Macro-invertebrate	24-48	18
Burnupia stenochorias	3.9	Macro-invertebrate	96	1
Flatworm sp.	5.6	Macro-invertebrate	96	1
Plea pullula	7.6	Macro-invertebrate	96	1
Coenagrionidea sp.	22.5	Macro-invertebrate	96	3
Oligoneuridae lawrencei	4.8	Macro-invertebrate	96	1
Leptocerid sp.	6.2	Macro-invertebrate	96	2
Athericidae sp.	18.6	Macro-invertebrate	96	1
Caenidae sp.	3.6	Macro-invertebrate	96	1

Table 5.4 A summary of single species toxicity data of Na_2SO_4 on freshwater micro-algae and macro-invertebrates obtained from the ECOTOX and UCEWQ databases as well as this study, and used to generate a species distribution (SSD) curve.

Species Scientific Name	**Geometric mean (g/L)**	**Taxonomic group**	**Exposure duration (hours)**	**No. of data points**
Pseudokirchneriella subcapitata	0.7	Algae	72-96	6
Chlorella protothecoides	1.4	Algae	96	5
Chlorella sorokiniana	2.3	Algae	96	2
Chlorella vulgaris	2.2	Algae	96	3
Ceriodaphnia dubia	2.5	macro-invertebrate	48-72	63
Daphnia magna	1.6	macro-invertebrate	24-48	16
Hyalella azteca	2.0	macro-invertebrate	96	48
Chironomus tentans	14.1	macro-invertebrate	48	1
Tricorythus sp.	0.7	macro-invertebrate	96	1
Lampsilissi liquoidea	2.8	macro-invertebrate	48-96	10
Sphaerium simile	2.4	macro-invertebrate	96	7
Tricorythus tinctus	2.8	macro-invertebrate	96	1
Adenophlebia auriculata	7.2	macro-invertebrate	96	7
Afroptilumsud africanum	3.4	macro-invertebrate	96	4
Tricorythus discolor	2.7	macro-invertebrate	96	3
Euthraulus elegans	10.2	macro-invertebrate	96	1
Caridina nilotica	5.0	macro-invertebrate	48-96	6
Daphnia pulex	2.0	macro-invertebrate	48	8
Burnupia stenochorias	5.3	macro-invertebrate	96	1
Baetis harrisoni	2.9	macro-invertebrate	96	1
Afronurus barnardi	6.0	macro-invertebrate	96	1
Flatworm sp.	6.5	macro-invertebrate	96	2
Cloeon virgiliae	4.2	macro-invertebrate	96	3
Plea pullula	11.6	macro-invertebrate	96	2
Coenagrionidae sp.	27.0	macro-invertebrate	96	3
Oligoneuridae lawrencei	0.3	macro-invertebrate	96	2
Leptocerid sp.	8.8	macro-invertebrate	96	3
Belostomatidae sp.	6.9	macro-invertebrate	96	1

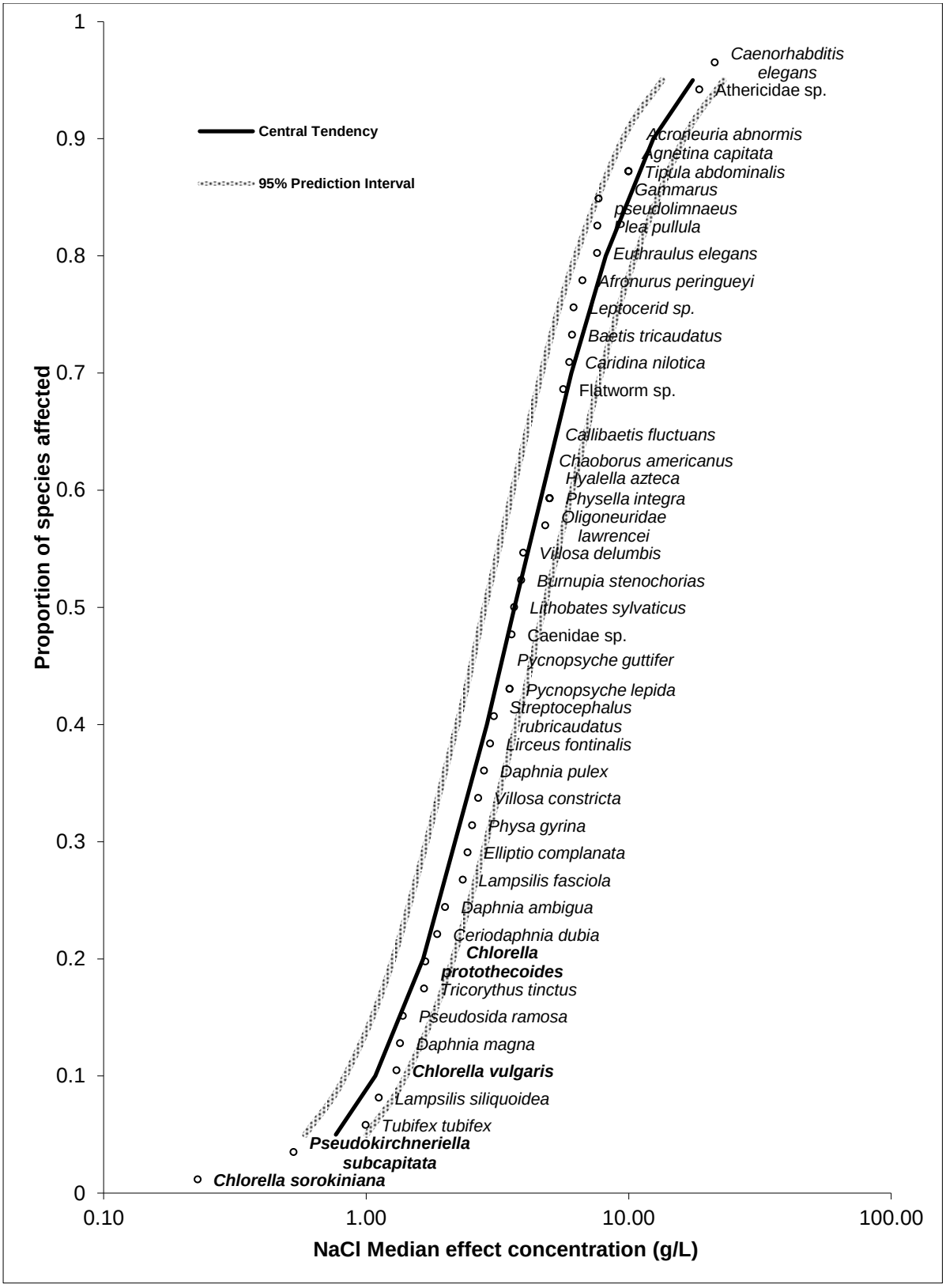


Figure 5.4 The species sensitivity distribution (SSD) curve of micro-algae and macro-invertebrates based on the geometric means of EC_{50} values of 24-96 hour toxicity test data for NaCl obtained from the ECOTOX and UCEWQ databases, and this study.

The higher number of toxicity data points for the NaCl than Na_2SO_4 on the ECOTOX re-iterates that there has generally been more work done on the effects of NaCl on organisms than Na_2SO_4 (Soucek 2007). *Ceriodaphnia dubia* had the highest number of data points (63), followed by *Hyalella azteca* (48), and then *Daphnia magna* (16). *Pseudokidokirchneriella subcapitata* and *Chlorella protothecoides* appeared to be more tolerant to Na_2SO_4 than two of the macro-invertebrates (*Tricorythus* sp. and *Oligoneuridae lawrencei*) (Figure 5.5).

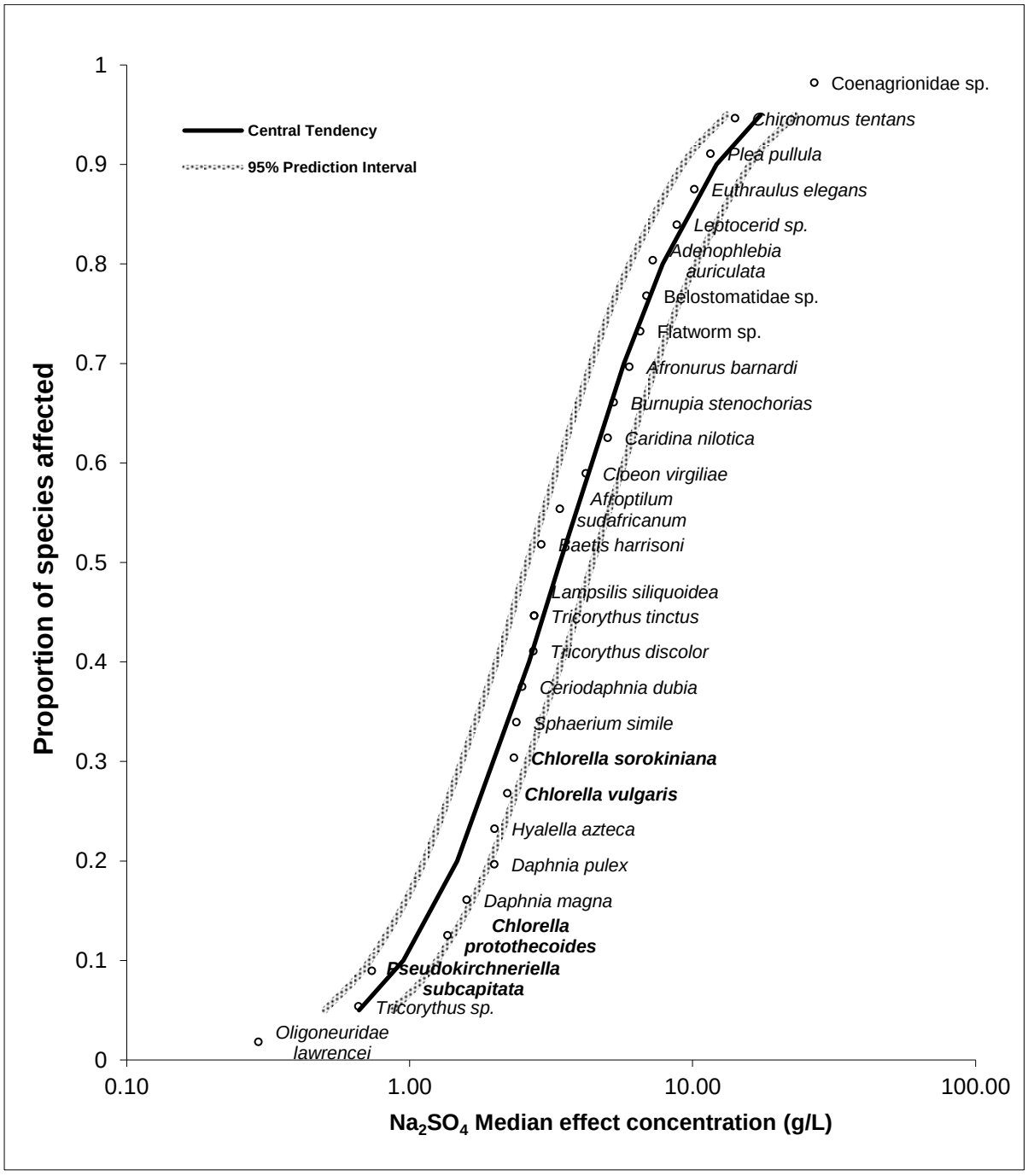


Figure 5.5 The species sensitivity distribution (SSD) curve of micro-algae and macro-invertebrates based on 24-96 hour toxicity test data for Na_2SO_4 obtained from the ECOTOX and UCEWQ databases, and this study.

The two locally isolated algal species, *C. sorokiniana* and *C. vulgaris*, appeared to be slightly more tolerant to Na_2SO_4 than the other two algal species, even more tolerant than five of the macro-invertebrates (*Tricorythus* sp., *Oligoneuridae lawrencei, Daphnia magna, Hyalella Azteca* and *Daphnia pulex*) (Figure 5.5).

5.4 DISCUSSION

The salt concentrations selected for this study were higher than the boundary values used for the Present Ecological State rating (Table 5.1) in the Reserve determination process (DWAF 2008). The lowest concentration of 78 mg/L for both salts in this study was higher than the lowest concentrations for NaCl and Na_2SO_4 (45 mg/L and 20 mg/L) outlined on the Present Ecological State rating table (Table 5.1) (DWAF 2008). The highest concentration levels in this study were much higher than the maximum boundary values for Na_2SO_4 and NaCl (0.045 mg/L and 0.535 mg/L) respectively (Table 5.1). This implies that the highest concentration of 10 000 mg/L (10 g/L) for both salts from the current study is not expected to be found in the natural environment, except in highly polluted areas where mining and agriculture are prevalent.

The growth of the algae was stimulated by the salts at lower concentrations and inhibited at higher concentrations. This is not an uncommon phenomenon, as most maintenance media of phytoplankton contain salts at low concentration to assist in growth of the algal cultures (Cleave *et al*. 1990). The stimulatory effect of NaCl to *P. subcapitata* also observed by Cleave *et al*. (1990) at a low concentration of 0.25 g/L is in agreement with the findings in this study. In this study *P. subcapitata* was stimulated at the low concentrations (0.07 and 0.156 g/L) with no effect at 0.313 g/L. It has been reported that in some instances photosynthesis may be stimulated by low salt concentration (Parida and Das 2005). This would then explain the growth stimulation of algae observed in this study. Aquatic species, including algae, have adaptive ways of dealing with salt stress. Salt stress induces phenotypic adjustments or physiological adaptations characterised by increases in metabolic activity and transport processes such as protein synthesis, in some algae. Changes in cell volume, alteration in sizes of the cell components such as starch grains, nucleus, cytoplasm, chloroplast, pyrenoid etc., have been recorded for some algal species (Berube *et al*. 1999). Changes in chloroplast ultrastructure and the presence of large starch grains as a result of salt stress have also been observed for plants (Parida and Das 2005). Some of these phenotypic

adjustments may be interpreted as growth stimulation during the growth inhibition assay. If, for example, the size of the cytoplasm or chloroplast increases as a result of salt stress, that increase in size could be interpreted as stimulation in photometric analysis.

The standard toxicity test species, *P. subcapitata,* was more sensitive to both NaCl and Na_2SO_4, compared to the other three tested algal species in this study. There were no significant differences in the response of this species to the two salts. The commercial species, *Chlorella protothecoides* was relatively tolerant to both salts with no significant differences in response to both salts, even though it was more sensitive to Na_2SO_4 than the two locally isolated species. *Chlorella vulgaris* was relatively tolerant to both salts with no significant differences in response to the two salts. *Chlorella sorokiniana* responded differently to the two salts; it was significantly more sensitive to NaCl than Na_2SO_4. This species was the most sensitive of the four algal species to NaCl and the most tolerant to Na_2SO_4. This difference in response of a species to different toxicants was also observed for *C. vulgaris* to the two reference toxicants in Chapter 4.

The use of indigenous, or locally isolated, species in toxicity tests is recommended because the results may be more applicable to site specific conditions in the natural environment. Micro-algal organisms which have evolved and adapted to the variable physical natural environment may be able to tolerate more toxic environments than organisms that are adapted to the stable laboratory environment (Cleave *et al*. 1981). The two locally isolated species were more tolerant to Na_2SO_4 than the standard species and the commercial species. The two local isolates *C. vulgaris* ($p \geq 0.05$) and *C. sorokiniana* ($p \leq 0.05$) were relatively more tolerant to Na_2SO_4 than NaCl, as opposed to the other two species *P. subcapitata* ($p \geq 0.05$) and *C. protothecoides* ($p \geq 0.05$), which were more tolerant to NaCl than Na_2SO_4. Cleave *et al*. (1981) also reported that sulphate (SO_4^{2-}) affected *P. subcapitata* more than chloride (Cl^-) did.

Compared to macro-invertebrates, micro-algae were generally more sensitive to both salts. Different sensitivities to toxicants may be expected for different taxonomic groups because these toxicants have specific modes of action in organisms (Hose and Van den Brink 2004). However, this high sensitivity of micro-algae to salts compared with macro-invertebrates should be interpreted with caution. Results of the algal growth inhibition may be influenced

by the fact that algal growth medium used in the experiments also had a salt content that is much higher than that found in natural environments (Fred Lee 1973).

There is also the concern that the macro-invertebrates were far more represented than the micro-algae on the species sensitivity distribution curves (SSDs). This is due to there being no sufficient suitable data on the toxicity of salts to algae in databases. There is not much work documented in literature on the adverse effects of these salts to freshwater algae (US EPA ECOTOX). However, the data set sizes used for the SSDs were acceptable for both salts. There have been questions around how much data is enough to generate SSDs of acceptable quality. The Organisation for Economic Cooperation and Development recommended five data points (OECD 1992), and so did the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ 2000); the European Commission guidance document recommends eight (Campbell *et al*. 2000) and, according to Wheeler *et al*. (2002), a minimum of data for 10 species would be adequate to give reliable results.

This higher sensitivity of algae to salts compared to macro-invertebrates could potentially cause problems in the natural environment. As primary producers, algae are a nutritional source that some invertebrates depend on. One of the fundamental assumptions in community ecology is that sensitive individuals may be replaced by more tolerant ones under toxicant exposure, and this may result in shifts in the biotic composition (Schmitt-Jansen *et al*. 2007). It may then follow from the afore-mentioned assumption that if algae were to be exposed to high salt concentrations in their natural environments, the more salt-tolerant species would out-compete the sensitive species, which could then impact on the biotic composition in the system, which in turn would result in the upset of the system because of changes in trophic pathways. Although data from laboratory tests cannot be directly applied to natural conditions, they remain an important basis for decision making on likely impacts of toxicants on the aquatic environment. Salinity is generally known to influence biotic communities, because some species have evolved greater tolerance to salts than others.

5.5 CONCLUSION

The algae appeared to be stimulated by the salts at low concentrations. This algal growth stimulation at low salt concentrations could result in eutrophication, which in turn could result in the disruption of ecosystem processes, shifts in biodiversity patterns under extreme

conditions. The determination of algal reaction to contaminants, whether inhibitory or stimulatory, is important background information on the physiological effects of the salts to these species. Understanding the stimulatory effect of low salt concentrations of salts on algae could assist in understanding some of the eutrophication problems associated with mining and agricultural areas in South Africa. The algae were generally more sensitive to the salts compared to macro-invertebrates, as indicated by the species sensitivity distribution curves. Although different taxonomic groups are expected to have different sensitivities to contaminants, the paucity of algal toxicity data for use in the SSDs for this study should be borne in mind when interpreting the SSD results. It would have been interesting to see how these algae (tested in this study) compare to other algal species in terms of sensitivity to these salts. The quality and quantity of toxicity data used to generate SSD curves plays a major role on how the SSD curves look.

The two indigenous species, *Chlorella vulgaris* and *Chlorella sorokiniana,* appeared to be sensitive to NaCl and tolerant to Na_2SO_4. Moreover, these two indigenous species were more tolerant to Na_2SO_4 than the standard species *P. subcapitata*. These species may come from the natural environment where the salt is present and are therefore adapted to the presence to the salt compared to the standard toxicity test species. The use of indigenous species in toxicity tests has been recommended because they are presumed to be more applicable to the natural environment.

The standard species *P. subcapitata* was sensitive to both salts, which highlights its sensitivity and therefore its benefit as a toxicity test species. However, the use of additional species in toxicity tests generates a better picture and draws attention to the varied responses of different algal species to single chemicals. The sensitivity of *P. subcapitata* to toxicants is not in question, but its presentation as the most sensitive species to most chemicals warrants further investigation, even though that is outside the scope of this study.

The use of the standard species in toxicity tests is instrumental in providing a database of chemical toxicity to this alga, and it is information on this database that may be used to generate the SSD curves used in the development of water quality guidelines and other regulatory aspects of water resource management. *Chlorella protothecoides* was moderately sensitive to both NaCl and Na_2SO_4, compared to the other three species. The micro-algae were relatively more sensitive to both salts than macro-invertebrates, as shown by the species

sensitivity distribution curves. The response of micro-algae to salts shown in this chapter is important for water resource management and protections as the stimulation of micro-algae by low salt concentration could have deleterious impacts such as eutrophication under extreme conditions. Eutrophication is an important issue in South Africa, especially in areas where mining and agriculture are prevalent. Understanding how micro-algae respond to chemicals such as salts, which are indicators of the above-mentioned land-use practices, is therefore essential for the protection and management of water resources. The use of SSDs in this context especially with micro-algae and macro-invertebrates could contribute to understanding the effect that salts may have on the food-chain interaction between the organisms. Furthermore the results from this chapter of the study may contribute additional toxicity data that could possibly be useful in generating SSDs for deriving and refining water quality guidelines for salts, especially given the paucity of toxicity data for salts and micro-algae in local and international databases. The South African water quality guidelines (DWAF 1996) and the Present Ecological State (PES) rating values for inorganic salts **(**DWAF 2008) were generated with data available at the time. These need to be refined and updated with new information so that they are more relevant and realistic for effective water resource management.

6. THE SENSITIVITY OF FRESHWATER MICRO-ALGAE TO TWO EFFLUENTS AND A HERBICIDE FORMULATION

6.1 INTRODUCTION

This chapter forms the third and last experimental part of the third phase of this study, assessing the application and value of using local micro-algal species in toxicity testing for use in water resource management in South Africa. This phase focuses on assessing the sensitivity of the local micro-algae to a range of carefully selected toxicants, namely effluents and a herbicide formulation, according to their importance in this country, as well as the practicality of using the identified species in routine toxicity. The objective of this chapter is to assess the response of two locally isolated species of freshwater micro-algae (*Chlorella sorokiniana* and *Chlorella vulgaris*), in comparison to the standard toxicity test species *Pseudokirchneriella subcapitata*, and *Chlorella protothecoides* obtained from a commercial culture, to effluents from a coal-based power plant and a coal-based petro-chemical industrial plant. The differential sensitivities of these four species of freshwater green-algae to a glyphosate-based herbicide formulation (Roundup®), will also be compared in this chapter.

Land use in South Africa is characterised mainly by mining, mineral processing and agricultural activities. As the second largest mining sector after gold in South Africa, coal mining has important social, economic and environmental impacts (Hobbs *et al*. 2008). Although the mining and agricultural industries play an important role in providing employment and income to the people of this country, they also exacerbate the already growing water demand. The water quality of surface and groundwater is also threatened by mining and agricultural activities. Mining operations may affect the environment by altering the hydrological and topographical characteristics, thereby affecting surface runoff, soil moisture and evapotranspiration (DWAF 2007, Hobbs *et al.* 2008). The quality of most water resources is continuously deteriorating due to pollution caused by industrial effluents, inflows of treated and untreated sewage, as well as deforestation and agricultural return flows. The above activities have contributed progressively to the adverse effects on water quality that result in negative impacts on aquatic environments (Oberholster *et al*. 2010).

The distribution of invasive exotic plants has also been increasing, and this has resulted in the increasing use of herbicides to control the exotic vegetation which has led to concerns about

their impact on non-target aquatic organisms such as algae. Algae have physiological properties similar to those of the intended target invasive plants or weeds, and therefore may be affected by the herbicides (Doringo *et al*. 2004). The targets of herbicides are usually photosynthesis or energy transport enzymes of plants, which are also important physiological properties of algae (Sbrilli *et al*. 2004).

Biocide spray drift, leaching and run-off are some of the major components of non-point source agricultural pollution in aquatic systems (Sbrilli *et al*. 2005). Although biocides (pesticides or herbicides) in water are generally chemically analysed, this does not provide sufficient information for hazard evaluation and ecological risk assessment (Dabrowski and Balderacchi 2013). Chemical techniques cannot assess the bioavailability of the herbicide to aquatic life (Sbrilli *et al*. 2005). Detecting ecotoxicological impacts of pesticides on aquatic organisms is an essential component of monitoring and managing the effects of pesticide contamination to water resources. The responses of aquatic organisms and communities, such as micro-algae, should be used as biological indicators to complement the chemical analysis. The input of herbicides, to surface waters may occur in pulses coinciding with spray events, resulting in low level contamination between consecutive spray episodes. Measured herbicide concentrations residues in water samples thus may not always reflect the effects on the aquatic system, whereas toxicity exposures of organisms may capture the effects of herbicide stress on these aquatic biota and thus contribute to improved understanding and management of associated risks with use of the products.

The Conservation of Agricultural Resources Act (Act No. 43 of 1983) makes provision for control over the utilisation of the country's natural agricultural resources in order to promote conservation of the soil, water resources and vegetation, and to combat weeds and invader plants. Invasive plants do not only threaten the country's biological biodiversity, but also water security and the ecological functioning of aquatic ecosystems. Working for Water, a programme administered by the Department of Water Affairs and Sanitation (DWS) is at the forefront of the fight against invasive alien species. The DWA is in partnership with other government departments, such as the Department of Agriculture, Forestry and Fisheries, to combat the invasion of aquatic ecosystems by alien plant species. One of the methods used to curb the invasion of alien species in South Africa is the use of herbicides. Glyphosate is an extensively used active ingredient in herbicides used to control aquatic and terrestrial weeds and aggressive exotic vegetation around South Africa. It reduces plant growth by inhibiting

aromatic amino acid biosynthesis. Studies assessing the effect of technical grade glyphosate to single species of micro-algae have resulted in a range of variable responses depending on the algal species and test conditions (Gardner *et al*. 1997). In this study, the Roundup® formulation of glyphosate was used, as it is one of the country's most commonly used glyphosate-formulations in weed and invasive plant control (Mensah *et al*. 2013).

Increased industrialization worldwide has led to a high demand for the production and use of different chemicals, and as a result advanced and developing countries all face increasing ecological problems associated with the release of these chemicals into the environment (Paixão *et al*. 2008). These chemicals may end up in aquatic resources by way of wastewater discharges, accidental releases and so on. This then puts at risk the natural life-supporting function and ecology of these aquatic resources, making necessary the establishment of water quality criteria for industrial and wastewater discharges for the protection and sustainable management of aquatic resources. Because effluents are complex, and chemical analysis of them for regulatory purposes is limited, most developed countries have included whole effluent toxicity (WET) tests as a requirement for surface water dischargers to ensure that they comply with quality standards and permit limits (US EPA 1996, European Community 2006, Ra *et al*. 2007). Treated effluent may exhibit low concentrations of pollutants that are within discharge limits but are still toxic to aquatic organisms (Ra *et al*. 2007, Silva *et al*. 2009). The impact of disposed effluents on aquatic organisms should be determined using toxicity testing in order to monitor and protect the biodiversity of aquatic ecosystems (Silva *et al*. 2009).

The use of whole effluent toxicity assessments is a monitoring requirement for compliance with discharge permits in most countries, and South Africa is also beginning to follow this trend. In recent years, South Africa has introduced the inclusion of toxicity tests to complement the traditional use of chemical analysis in the control and management of hazardous effects of wastewater discharges into aquatic ecosystems (Murray 2005, Jooste *et al*. 2008). The National Water Act (Act 36 of 1998) requires that an ecological effect-based approach be applied to water resource management to deal with industrial effluents and wastewaters released into water resources (DWAF 2003). The National Toxicity Monitoring Programme (NTMP) and the Direct Estimation of Ecological Effects Potential were introduced to fulfil this requirement of the NWA (DWAF 2003, Murray 2005, Jooste *et al*. 2008). The NTMP focuses on in-stream toxicity by monitoring priority hazardous substances

in aquatic resources, whereas DEEEP is intended for use by regulators and dischargers to manage effluent discharges into surface waters (DWAF 2003, DWAF 2005, Jooste *et al*. 2008).

Multiple species tests at different trophic levels (fish, invertebrates and algae) are typically used in WET testing to represent their different functional niches in the aquatic ecosystems. The advantage of this multiple-species approach is not only the representation from different trophic levels but also the possibility of capturing the differences in sensitivity to toxicants of these different functional groups. Although the use of algae in WET tests is fairly recent compared to the faunal organisms (fish and invertebrates), it broadens the understanding of the effect of complex effluents on the flora and fauna of surface water environments (Paixão *et al*. 2008, Silva *et al*. 2009). The advantage of using algae is that they respond either by inhibition or stimulation to contaminants and are likely to capture synergistic, antagonistic and additive effects of the chemical components of the complex effluents tested (Silva *et al*. 2009).

As primary producers and the basic link in the aquatic food chain, algae are of great importance in the structure and function of the aquatic ecosystem (Paixão 2008). There are several factors that make algae suitable organisms for assessing effects of effluents on aquatic organisms: they belong to the first level of the trophic chain and disturbances at this trophic level could affect the ecosystem at higher levels of organisation; because they have relatively short lifecycles, they are able to show effects over several generations in fairly short timeframes, and lastly, they are fairly sensitive to their environment (Ma 2005, Silva *et al*. 2009). Moreover, algae have been shown to be sensitive indicators for testing several compounds, including municipal and industrial effluents (McDonald *et al*. 1996, Ra *et al*. 2007, Riedl and Altenburger 2007, Paixão 2008).

Algae are a diverse assemblage of organisms and their great species-specific sensitivity to environmental conditions and high diversity in habitats provide the potential for using them to assess physical and chemical conditions that could be detrimental to the environment (Wehr and Sheath 2003). The algal bio-assessments have the potential to complement and provide corroborative evidence to the physical and chemical data that are currently being used to assess the effects of industrial effluents in South African water resources (DWAF 1996, Dallas and Day 2004).

6.2 MATERIALS AND METHODS

6.2.1 Test organisms and test conditions

Toxicity tests were carried out with four freshwater algal species, *P. subcapitata*, *C. protothecoides*, *C. sorokiniana* and *C. vulgaris*. *Pseudokirchneriella subcapitata* was obtained from Rand Water (Analytical Services, Vereeniging), *Chlorella protothecoides* was obtained from the Environmental Biotechnology Research Unit (Rhodes University, Grahamstown), *Chlorella sorokiniana* and *Chlorella vulgaris* were isolated from local rivers (Kieskamma and Palmiet respectively). All stock cultures of these algal species were maintained in the laboratory according to the method described in chapter 3 (section 3.4.1).

6.2.2 Effluent collection

The toxicity of two effluents, from a power plant and an oil-from-coal petro-chemical industrial plant, as well as a glyphosate formulation herbicide (Roundup®) to the standard toxicity test species *P. subcapitata* and the three *Chlorella* species (*C. protothecoides*, *C. sorokiniana* and *C. vulgaris*) was assessed using the growth inhibition assay (96 hours). Industrial activities on both plants are coal-based. Effluent samples were collected from the two plants in clean one liter polyethylene bottles. Both effluents were collected at end-of-pipe from the plants, after all the necessary on-site treatment of the effluent was carried out. The coal-based power plant effluent was collected from an evaporation pond, where the final effluent is held after all treatment, and the coal-to-oil based plant effluent collected was also final effluent after treatment. The effluent samples were kept at 4 – 6 °C during transportation until reception in the laboratory, where they were frozen until toxicity testing could be carried out. Effluent samples were allowed to reach laboratory temperature and filtered through a 0.45 µm membrane filter for each sample prior to toxicity testing. Water quality parameters such as pH, dissolved oxygen and electrical conductivity were determined for the effluent samples pre-and post-freezing. Liquid Roundup® (active ingredient: 360g glyphosate (glycine) acid equivalent/litre (a.e/L), which contains 480g isopropylamine salt of glyphosate per litre) registered and distributed by Monsanto South Africa (Pty) Ltd) was purchased from a local chemical store in Grahamstown South Africa. A two percent stock solution was prepared by dissolving 2 mL of the herbicide formulation (Roundup®) in 980 mL of distilled water to obtain a concentration of 7200 mg/L of the active ingredient.

6.2.3 Algal Growth Inhibition

The algal growth inhibition toxicity experiments were conducted in sterile 24 well micro-plates with lids at 25±3 °C under continuous cool fluorescence light according to Slabbert (2004). The general method used in these toxicity tests is described in Chapter 3 (Section 3.4). The two effluent samples were applied at a geometric series of eight concentrations by using a dilution factor of 0.5 to yield the following concentrations (0.78%, 1.56%, 3.13%, 6.25%, 12.5%, 25%, 50% and 100%). Appropriate dilutions of the prepared stock solution of the herbicide formulation Roundup® were made with dechlorinated tap water to obtain the following nominal test concentrations: 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 mg/L.

Each exposure of one species to a toxicant was replicated six times. Algal population growth was determined spectro-photometrically by measuring optical density at 450 nm for each plate on a micro-plate reader, at the beginning and the end of the toxicity test.

The following criteria were used to determine test validity:

- Coefficient of variation of control growth ≤10% for *P. subcapitata* and ≤20% for other species.
- An average OD_{450nm} reading of >0.10 for the controls at the end of the test
- R^2 of more than 0.8 in the linear regression for EC_x calculations
- Inhibition data between 10% and 90% growth inhibition used in the linear interpolation for EC_x calculations

6.2.4 Analysis

6.2.4.1. Growth inhibition and median effective concentration (EC_{50})

Data for all test replicates were assessed for validity using the above-mentioned criteria, and those that did not meet all the criteria were not used. Percentage inhibition values were estimated for each experimental group (exposure of each species to each toxicant). EC_{50} values were determined by linear regression and graphic interpolation on the percentage inhibition data (between 10% and 90%). Data belonging to the upper-most and lower-most part of the curve (< 10% and > 90%) were excluded so as to minimize any negative influence

they may have on the curve-fitting (Mayer *et al*. 1997, Slabbert *et al*. 2004). The EC_{50} values for all replicates of each toxicant on each species were pooled together and expressed as mean EC_{50} (+standard deviation). The differences in sensitivity of species to each of the two toxicants were determined by statistically comparing the EC_{50} values. Data were tested for normality and homogenous variance, and a one-way-ANOVA (Analysis of Variance), with Unequal N HSD post-hoc test was used for normally distributed data (due to unequal replicates after quality control of the data). A non-parametric Kruskal Wallis ANOVA by ranks was used for data that were not normally distributed.

6.2.4.2 Specific growth rate, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)

All the experimental data were used (even those with higher than acceptable variability in control growth after 96 hrs) to determine specific growth rate for the control and each toxicant concentration treatment (see Chapter 3.4). This was done in order to include and present stimulation data, which was omitted in EC_x calculations. The data were tested for normality and homogeneity, and a one-way ANOVA was used to determine which treatments differed significantly from the control, thus determining NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration) values (using treatments with significantly reduced growth compared to the control). All statistical analyses were undertaken using STATISTICA (Version 8) software package with $p \leq 0.05$ as the level of significance.

6.3 RESULTS

6.3.1 Coal-Based Power Plant Effluent

The raw effluent was slightly opaque in colour, but cleared considerably when the particles larger than 0.45 µm were removed by filtering. Table 6.1 shows the physico-chemical parameters of the effluent before and after it was frozen. The salinity (as measured by electrical conductivity) and the pH of the effluent did not seem to change as a result freezing the effluent. Both the pH and electrical conductivity of the effluent were well within the South African Standards for wastewaters and effluents (Regulation No. 991, 1984).

The electrical conductivity limit as per water quality requirements for effluents and wastewaters is 250 mS/m. The acceptable pH range for effluents according to South African effluent standards is between 5.5 and 7.5. The dissolved oxygen of the effluent increased slightly after freezing (Table 6.1).

Table 6.1 Physico-chemical characteristics of the power plant effluent, pre- and post-freezing.

	pH	**Electrical Conductivity**	**Dissolved Oxygen**
Pre-freezing	7.27	3.89 mS/m	6.25 mg/L
Post-freezing	7.28	3.74 mS/m	7.43 mg/L

All the algal species, *P. subcapitata*, *C. protothecoides*, *C. sorokiniana* and *C. vulgaris*, were stimulated by the coal-based power plant effluent, especially at the lowest concentrations (Figure 6.1). The growth of *C. vulgaris* was inhibited by the two highest concentrations (50% and 100%), with average growth inhibition percentages of 37.3±13.2 and 43.3±7.5 respectively. Because of the stimulatory effect, no EC_{50} values were determined for the effluent for any of the species.

As shown on Figure 6.1d, the growth *Pseudokirchneriella subcapitata* was significantly stimulated by the effluent at all the tested concentrations. *Chlorella protothecoides* was stimulated at all concentrations except 12.5% which was not significantly different from the control (Figure 6.1c). *Chlorella sorokiniana* and *Chlorella vulgaris* were stimulated at the three lowest concentrations (0.78%, 1.56% and 3.13%). The effect of the 6.25% effluent concentration, and the three highest concentrations (25%, 50% and 100%) were not significantly different from the control for *C. sorokiniana*, while the 12.5% concentration significantly stimulated growth (Figure 6.1b). The effluent had no effect on *C. vulgaris* (Figure 6.1a) at the three medium concentrations (6.25%, 12.5% and 25%), and significantly inhibited growth at the two highest concentrations (50% and 100%).

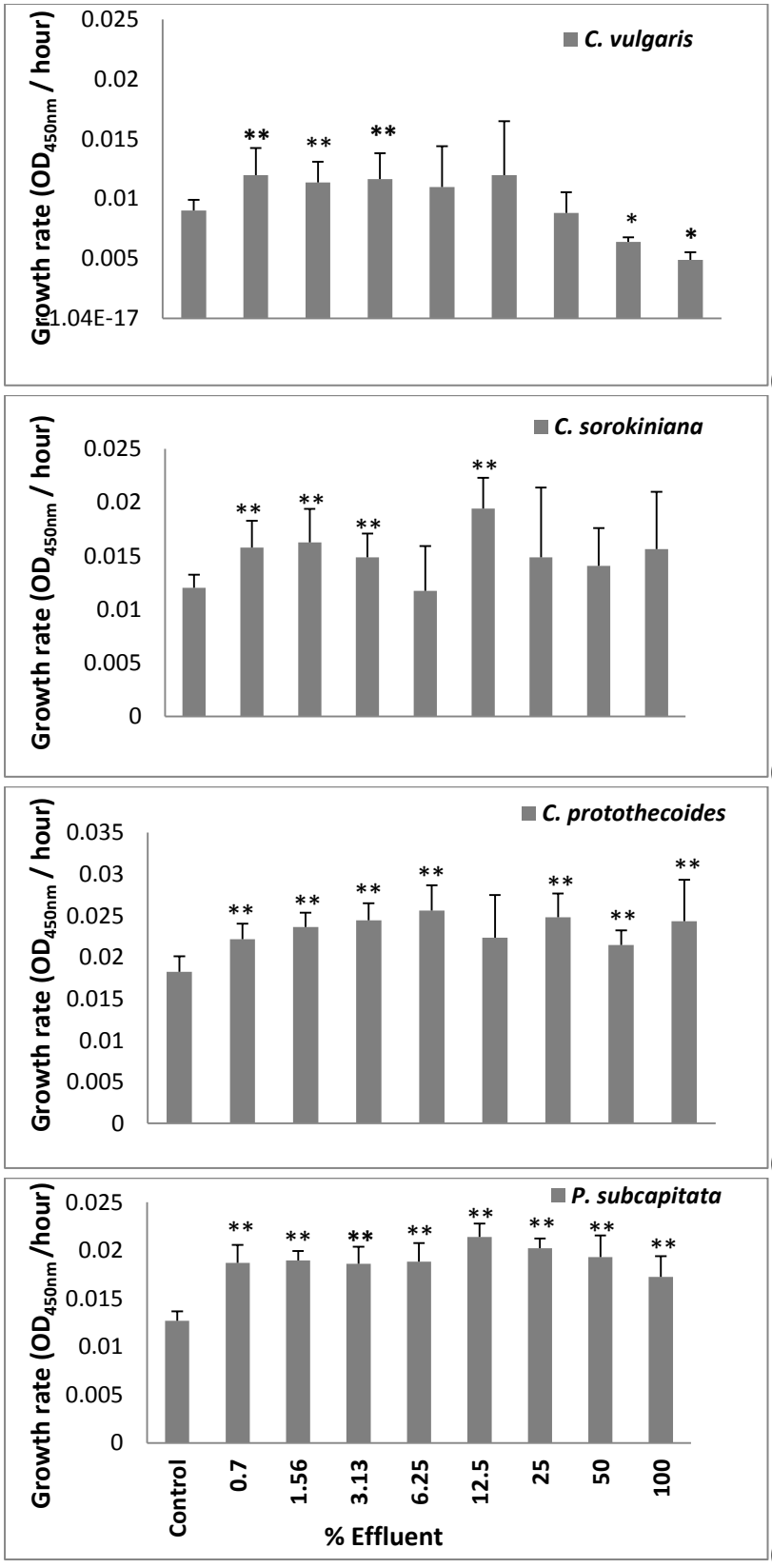


Figure 6.1 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to a coal-based power plant effluent. *=significantly lower than the control, **=significantly higher than the control.

6.3.2 Effluent from a Coal-based Petro-chemical Industrial Plant

The raw effluent was dark-bluish in colour, and the blue colour persisted even though it was much lighter and more transparent after filtration. The effluent seemed to be slightly volatile as shown by the high evaporation in the test concentrations during the test period of 96 hours, compared to the control. This volatility is also shown by the change in the effluent physico-chemical parameters after freezing (Table 6.2). The pH of the effluent was slightly acidic pre-freezing and increased slightly after freezing. However the pH was within the acceptable range as stated on the South African standard for effluents and wastewaters. Salinity, as measured by electrical conductivity, decreased by almost 50% after freezing while dissolved oxygen also decreased by about 20%. Salinity of the post-storage sample was also within the limit specified on the standards for wastewaters and effluents (250 mS/m).

Table 6.2 Physico-chemical characteristics of the petro-chemical plant effluent, pre- and post-freezing.

	pH	**Electrical Conductivity**	**Dissolved Oxygen**
Pre-freezing	6.03	4.68 mS/m	5.59 mg/L
Post-freezing	7.57	2.86 mS/m	4.47 mg/L

Effect concentration values could not be calculated for the petro-chemical industry effluent due to the stimulatory effect of the effluent on the algae. *Chlorella vulgaris* was significantly stimulated by the effluent at all concentrations (Figure 6.2 a). The growth stimulatory effect of the effluent on *C. vulgaris* increased with increasing concentration at the lower effluent concentrations (0.78% to 3.13%), decreasing at the 6.25% concentration and then increasing again in a dose-related manner at the higher concentrations (12.5% to 50%), while at full strength (100%), the growth stimulation decreased again (Figure 6.2a). At the 1.56% concentration and higher, the effluent significantly stimulated the growth of *C. sorokiniana*, with the growth at full strength (100%) not significantly different from that of the control (Figure 6.2b).

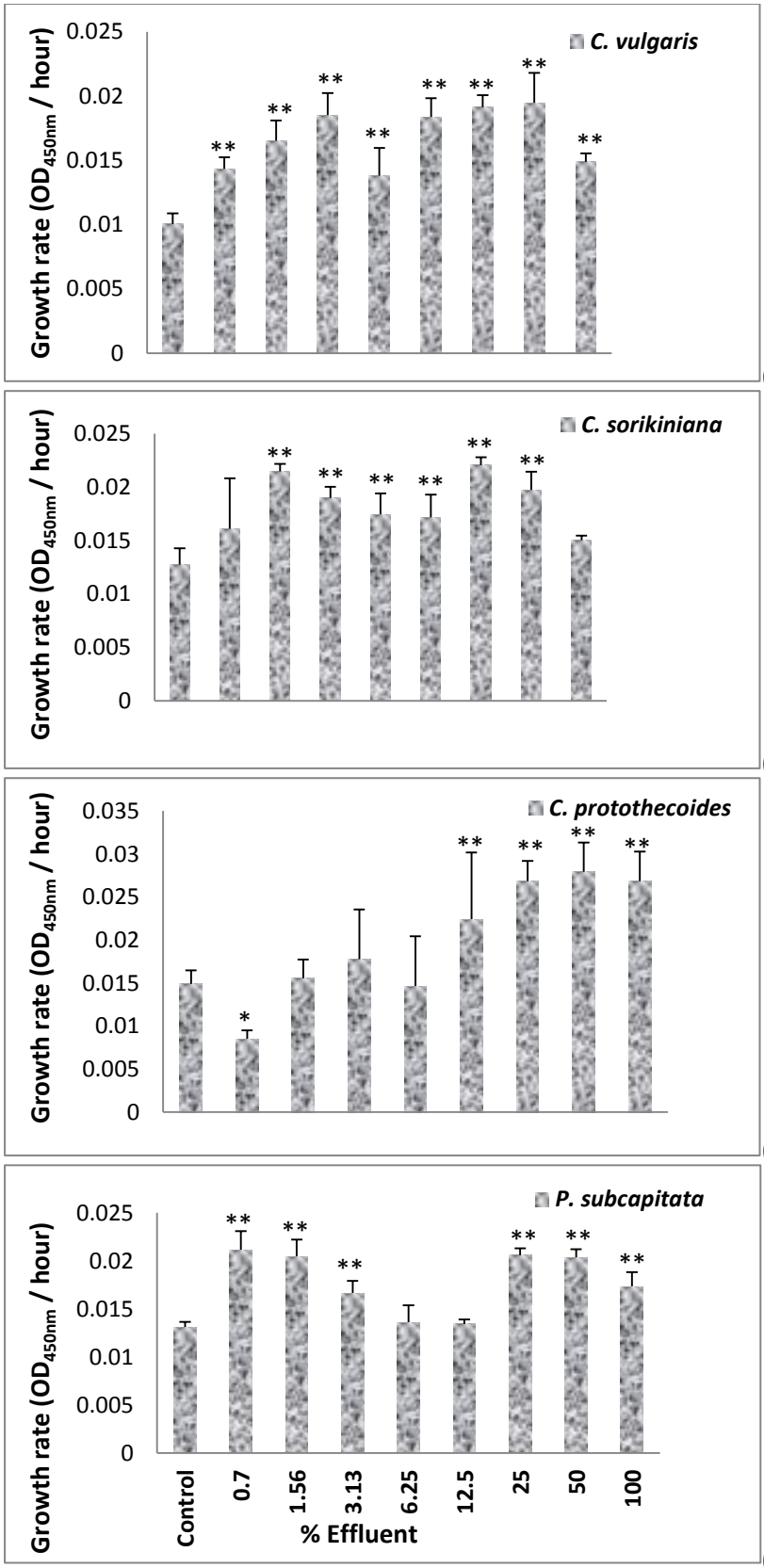


Figure 6.2 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to a petro-chemical industry plant effluent. *=significantly lower than the control, **=significantly higher than the control.

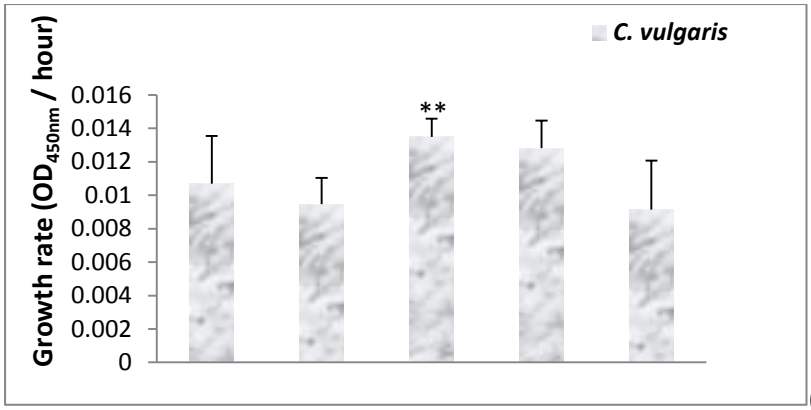
As illustrated in Figure 6.2c, the growth of *C. protothecoides* was significantly inhibited by the effluent at the lowest concentration 7%, with growth at 1.56 to 6.25% not significantly different from the control, and significant growth stimulation at the higher concentrations (12.5% to 100%). Figure 6.2d shows that the standard species, *P. subcapitata* was stimulated by the effluent at the lowest concentrations (0.78% to 3.13%), with no significant effect at the 6.25% and 12.5% concentrations and stimulation again at the highest concentrations (25% to 100%).

6.3.3 Herbicide formulation (Roundup®)

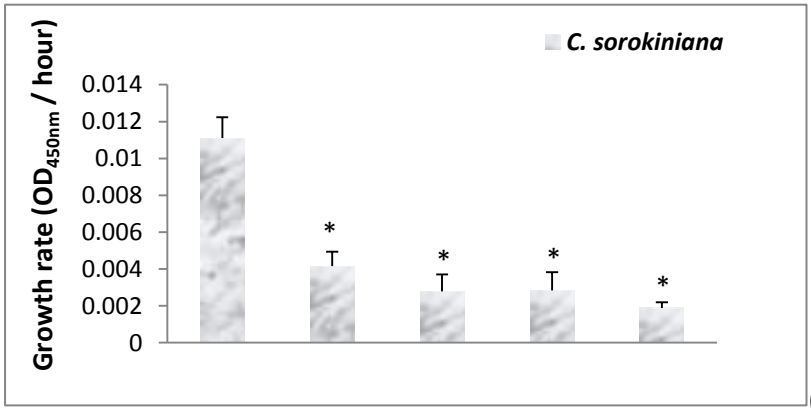
There was no growth of all the species in the Roundup® exposures at the highest concentrations (6.25 mg/L to 50 mg/L). The EC_{50} values for Roundup® on *P. subcapitata* could not be calculated because no toxicity test replicates satisfied all the validity criteria. The variability in the control growth was above the acceptable 10% for all six test replicates (with a minimum coefficient of variation of 18% and a maximum of 42%).

The pesticide highly inhibited the growth of *P. subcapitata* with mean ±standard deviation percentage growth inhibition values of 73±3%, 50±17%, 87±6 and 94±3 by the following concentrations 0.39, 0.78, 1.56, 3.13 mg/L, respectively. Figure 6.3d illustrates the effect of Roundup® on the specific growth rate of *P. subcapitata*, and indicates a significant decrease in the specific growth rate of this species at the lowest concentration (0.39 mg/L). The specific growth rate of *P. subcapitata* was highly variable for the six test replicates at the 0.78 mg/L concentration of the pesticide, with specific growth rate not significantly different from the control. The two highest concentrations (1.56 mg/L and 3.13 mg/L) of Roundup® significantly decreased the specific growth rate of *P. subcapitata* in comparison to the control (Figure 6.3d).

Only three out of the six toxicity test replicates with *C. protothecoides* were considered valid according to the prescribed criteria, and therefore only EC_{50} values for those tests could be determined. The mean EC_{50} of Roundup® on *C. protothecoides* was 1.2±0.1 mg/L. There was high variability in the response of *C. protothecoides* to the lowest concentration (0.39 mg/L) of the pesticide for the three valid test replicates. In one of the test replicates the growth inhibition was 5.7%, while the other two tests showed growth stimulation of 107% and 146% respectively.



(a)



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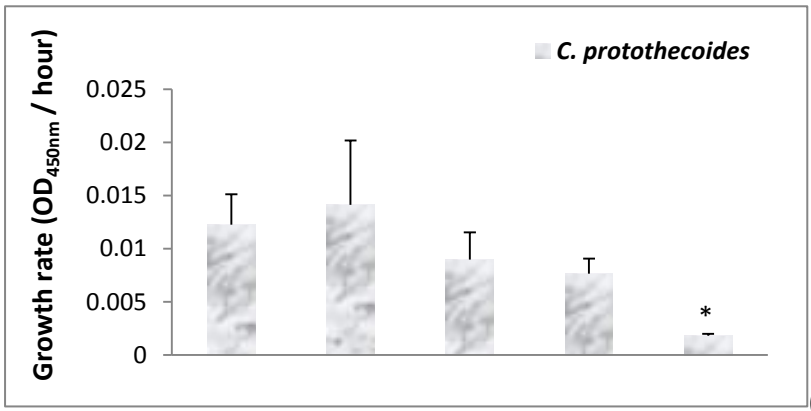
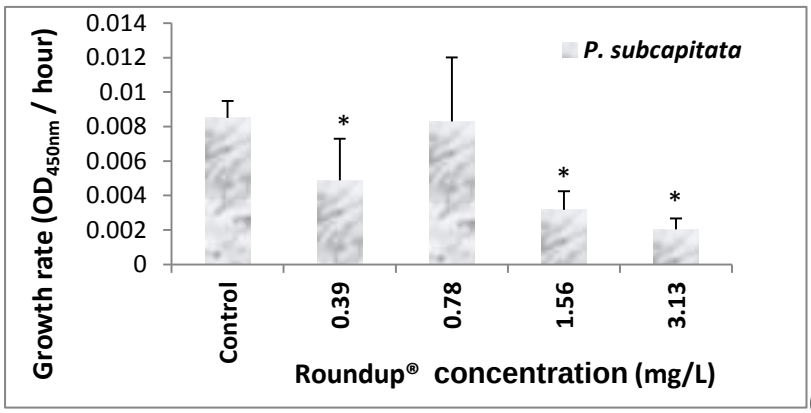


(c)



(d)

Figure 6.3 Mean specific growth rate (OD_{450nm} per hour) (+ standard deviation (n=6)) of (a) *Chlorella vulgaris*, (b) *Chlorella sorokiniana*, (c) *Chlorella protothecoides* and (d) *Pseudokirchneriella subcapitata* after 96 hours of exposure to a pesticide **(Roundup®)**. *=significantly lower than the control, **=significantly higher than the control.

The stimulation data was omitted for the EC_{50} calculations. The specific growth rate of *C. protothecoides* was slightly increased at the lowest concentration (0.39 mg/L) of Roundup® compared to the control, although this increase was not statistically significant. At the three highest concentrations (0.78 mg/L, 1.56 mg/L and 3.13 mg/L) of Roundup® , the specific growth rate of *C. protothecoides* decreased, in comparison to the control, although only growth at the highest concentration (3.13 mg/L) was significantly different from the control (Figure 6.3c).

The mean percentage growth inhibition of *C. sorokiniana* by Roundup® was high (74%, 86%, 85% and 93%) at all four concentrations (0.39, 0.78, 1.56and 3.13 mg/L) respectively. The validity criteria state that the inhibition data to be used in the linear interpolation for EC_{50} calculations must be between 10% and 90%, and the growth inhibition of *C. sorokiniana* by the herbicide formulation was between 74% and 93%, which could. This high growth inhibition, coupled with the low R^2 (less than 0.8) for the regression, led to none of the test replicates satisfying the validity criteria, and therefore EC_{50} values could not be determined. However, the specific growth rate of *C. sorokiniana* was significantly decreased at all concentrations (Figure 6.3b), compared to the control.

There was high variability in the response of *C. vulgaris* to Roundup®. At the following concentrations, 0.39, 1.56 and 3.13 mg/L, some test replicates exhibited growth inhibition, while others showed growth stimulation. Roundup® stimulated growth in *Chlorella vulgaris* at the 0.78 mg/L concentration, for all six test replicates as shown in Figure 6.3a. There was a slight decrease in the specific growth rate of *C. vulgaris* at the lowest concentration of 0.39 mg/L, compared to the control, even though this decrease was not statistically significant. The Roundup® concentration of 0.78 mg/L significantly increased the specific growth rate of *C. vulgaris*, compared to the control, and the specific growth rate at the other concentrations (0.39, 1.56 and 3.13 mg/L) was not significantly different from the control.

Of the four micro-algal species exposed to Roundup®, the growth of two species *P. subcapitata* and *C. sorokiniana* was inhibited by the pesticide. The growth of the other two species, *C. protothecoides* and *C. vulgaris,* was not greatly affected by the pesticide, except for a slight stimulation of *C. vulgaris* at a low concentration (0.78 mg/L) of the pesticide and high inhibition of *C. protothecoides* at the highest concentration (3.13 mg/L).

6.4 DISCUSSION

Algal species are useful indicators of pollution in aquatic systems because they are able to respond by stimulation, inhibition, stimulation at low concentrations and inhibition at higher concentrations, or inhibition at low concentrations and stimulation at high concentrations. Algal growth stimulation may have implications for alteration of the species composition in aquatic communities (Ma 2005), which could potentially result in eutrophication in natural water bodies (Sbrilli *et al*. 2005).The variability in response of algae to the complex effluents could be attributed to the bioactivity and the related interaction of effluent components (Sbrilli *et al.* 2005), coupled with the differences in the morphology and metabolic activities of the different algal species.

In South Africa, coal mining is the second largest mining sector after gold. Most of the active coal mining in South Africa produces coal for power generation which supports the country's power generation for export and domestic consumption (Hobbs *et al*. 2008). The two effluents from coal-based operations stimulated the growth of the algal species, especially at low concentrations. This algal growth stimulation could have serious implications for environmental water quality and in-stream ecological responses. Industries are permitted to discharge effluent into the receiving waters, and for the most part these discharges are allowed during periods of high rainfall, high run-off and increased water levels (Hobbs *et al*. 2008, Naddy *et al*. 2011). These effluent releases, although permitted and controlled by legislation, could lead to low levels of effluent present in the receiving water resources, and stimulating algal growth in those aquatic systems.

Phosphate concentrations in the effluent may be higher than in the receiving surface water (Naddy *et al.* 2011), and may thus result in the stimulation of algal growth in the receiving environment. Although phosphate and nitrate levels in the effluents were not measured, high nutrient levels in the effluents may be a possible explanation for the observed growth stimulation by the effluent at low concentration levels compared to the controls. The problem of excess phosphate is typically manifested through stimulation of algal growth, especially when it is accompanied by high nitrogen concentrations (Interlandi 2002, Naddy *et al.* 2011). The two locally isolated species *C. vulgaris* and *C. sorokiniana* were stimulated by the petro-chemical industrial effluent at all concentrations (except the highest concentration for *C. sorokiniana*).

The standard species, *P. subcapitata,* was stimulated by the power plant effluent at all concentrations, whereas the petro-chemical industrial effluent stimulated the algae at the lowest concentrations, with no effect at the medium concentrations and stimulation at the highest concentrations. A similar response of this species was also observed by Sbrilli *et al*. (2005), in their evaluation of the effects of surface and ground water samples on the growth of *P. subcapitata*. The alteration of the chemistry of the algal growth medium by the chemical components of the effluent during the toxicity test must also be taken into account (Interlandi 2002), as this could also explain the variability in response of the algal species to the different effluent concentrations. However, no chemical analysis was undertaken to corroborate this statement.

Chlorella protothecoides was stimulated by the power plant effluent at all concentrations, but responded slightly differently to the petro-chemical effluent, with growth inhibition at the lowest concentrations, no significant response at the medium concentrations and stimulation at the highest concentrations. There was concern of the petro-chemical effluent being slightly volatile, as observed during the toxicity test, because this may have led to the instability of concentrations over the exposure period and potentially resulted in the pronounced growth inhibition of *C. protothecoides* at the lowest concentration. Evaporated substances may evoke effects on adjacent wells in a micro-plate test and the evaporation loss of volatile substances may influence observation in algal growth inhibition tests (Riedl and Altenburger 2007).

The two locally isolated algae (*C. vulgaris* and *C. sorokiniana*) responded relatively similarly to both effluents, and their response was slightly different to that of the other two species *P. subcapitata* and *C. protothecoides*. The local isolates were stimulated at low concentrations and inhibited at high concentrations of the power plant effluent, whereas the other two species were stimulated at all concentrations. The two locally isolated species were stimulated by the petro-chemical industrial effluent at all concentrations, a slightly different response to that of *C. protothecoides*, which was inhibited at low concentrations and stimulated at high concentrations, and *P. subcapitata* which was stimulated a low concentrations, not affected by the medium concentrations and stimulated again by high concentrations. The algal growth stimulation could be the result of high nutrient load in the effluent coupled with the synergistic or additive interactions of the effluent components.

Because invasive exotic plants have become increasingly widely distributed, herbicides to control the exotic vegetation are also being increasingly used. Herbicide formulations with glyphosate as an active ingredient, such as Roundup® used in this study, are used extensively. Glyphosate reduces plant growth by inhibiting aromatic amino acid biosynthesis (Gardner 1997).

The pesticide Roundup® (a formulation of glyphosate) showed relatively high toxicity to the locally isolated *C. sorokiniana*, even at the relatively low concentrations used in the test. The sensitivity of *C. sorokiniana* to glyphosate was also reported by Christy *et al*. (1981), where an EC_{50} value of 0.017 mg/L was determined. The other local isolate *C. vulgaris* was not adversely affected by the pesticide at the tested concentrations, except to show slight stimulation at low concentration. The standard species *P. subcapitata* was also inhibited by the pesticide, while the growth of *C. protothecoides* was not significantly affected, compared to the control, except being significantly inhibited at the highest concentration. The EC_{50} value of Roundup® on *C. protothecoides* was 1.2±0.1 mg/L. Given that the concentrations tested were much lower than the application concentration of two – four percent (equivalent to 7200 mg/L -14400 mg/L active ingredient) recommended by the manufacturer, it can be deduced from this study that Roundup® is relatively toxic to the tested micro-algae, except *C. vulgaris*. Another *Chlorella* species (C. *pyrenoidosa*) was reported to be relatively insensitive to glyphosate (Anton *et al*. 1993).

The concern about herbicides is that they may impact non-target organisms. Micro-algae are at risk of being affected by pesticide spray-drift and runoff since they have physiological similarities with the intended target organisms (invasive plants) (Dorigo *et al*. 2004). Herbicide input into surface water generally occurs in pulses, often highly concentrated, with low levels persisting during the intervals between the pulses. As shown in this study, these low levels may have deleterious effects on non-target organisms such as micro-algae when they enter aquatic environments. The response of micro-algae to herbicides may be a useful tool to monitor herbicide impacts on aquatic resources, and using more than one species could be more appropriate since algae respond variably depending on tested concentrations, and the species tested (Dorigo *et al*. 2004). The toxicity of herbicides to micro-algae may also depend on the ability of the toxic components to permeate the algal cells. Water quality conditions may influence the stability and solubility of the pesticide, subsequently affecting toxicity (Gardner *et al*. 1997).

Until recently there have been no water quality guidelines determined to control and monitor the entry of these herbicides into water resources (Mensah *et al*. 2013), despite their extensive use in South Africa. Water quality guidelines are the determined minimum acceptable levels of a chemical to protect aquatic biota. These guidelines are generally based on toxicity data compiled in various forms to determine a nominated environmental level of the chemical required to protect the aquatic ecosystem (Chapman 1995b). Toxicity data from this study were used in SSDs that developed water quality guidelines for Roundup® in South Africa (Mensah *et al*. 2013). The contribution of a taxonomic group is one of the most important parameters for the SSD (Schmitt-Jansen *et al.* 2007), and this study was the main source of micro-algal data to the Roundup® SSDs.

Although it is understood that responses to toxicants, of single species of algae under regulated laboratory conditions, cannot adequately mimic those of natural phytoplankton communities, bioassays provide useful information on the effect of toxicants at biochemical, physiological, morphological and perhaps population levels (Oberholster*et al.* 2010).

6.5 CONCLUSION

The poor use of algal toxicity data, in particular, in whole effluent testing in this country requires urgent attention. Although government programmes such as the National Toxicity Monitoring Programme (NTMP) and the Direct Estimation of Ecological Effects Potential (DEEEP) are aimed at addressing the impact of effluent discharges on aquatic resources, the implementation of these programmes is slow because of lack of human and laboratory resource capacity. Algae are included in the battery of toxicity tests used in these programmes, and the information obtained from this study will add value to improving algal toxicity testing in this country. Using more than one micro-algal species (the standard toxicity test species, *P. subcapitata)* in toxicity testing will provide additional information on the effect of effluent discharges to aquatic flora.

The lack of information on the effect of effluents on aquatic flora could result in limited understanding of the impact of effluent discharges on aquatic ecosystems. The addition of algae to the more traditionally used invertebrates and fish in toxicity testing could broaden the understanding of toxicant effects on our water resources. The bioactivity and interaction

of components in a complex mixture is difficult to explain, but algal assessments with different species could be a sensible screening tool for monitoring the effects of effluent to aquatic ecosystems.

The ability of algae to respond by stimulation or inhibition, or stimulation at lower concentrations and inhibition at higher concentrations, is what makes them useful indicators of potential pollution. When algal growth stimulation occurs, it can result in eutrophication, which could cause problems in aquatic ecosystems. Eutrophication is attributed to overabundance of algal growth caused by the gradual shift of the algal community structure from dominance by one species to dominance by another. The reasons of these shifts in community structure may not only be pollution related, they may be attributed to physical factors such as light and temperature, nutritional factors such as nitrogen and phosphorus in the water, and biological factors such as competition and changes in the food chain. However, pollutants from effluent discharges may result in different responses by different species, stimulating growth in others while inhibiting growth in others. This may then cause these shifts in community structure, because of differential sensitivities of the algae to toxicant components of the effluent. Eutrophication is a significant water quality issue in South Africa (NWRS 2013), particularly in areas where agriculture and mining are prevalent. The results from this study could therefore contribute to understanding the influence of complex whole effluents on eutrophication as this would assist in addressing this issue.

Evaluating the impact of discharged effluents on natural aquatic system should take into account the differential sensitivities of species, and using more than one species of algae in toxicity tests could provide relevant information on the potential hazards of the complex effluents to aquatic ecosystems. These tests could be valuable as they can identify effluents that are bio-stimulatory and may cause nuisance growth of algae. The limited use of micro-algae in toxicity testing in South Africa does not correspond with their ecological importance as primary producers in aquatic ecosystems, nor has their value as indicators of aquatic environmental pollution been recognised because of their species-specific sensitivity to varying toxicants.

7. EVALUATING THE APPLICATION OF THE STUDY IN WATER RESOURCE MANAGEMENT

7.1 INTRODUCTION

Water is one of the key elements of livelihood in South Africa and hence the appropriate management of water resources is crucial for future economic growth. Water resource management occurs in a changing environment, therefore monitoring techniques and the type of data collected should be aligned with those changes in order to provide the correct assessment of the status of water resources (NWRS 2013). This is important because the South African Constitution (Act No. 108 of 1996) grants all people the right to an environment that is not harmful to their health and well-being, and imposes a duty on the State to protect the environment from ecological degradation and promote conservation through reasonable legislation and other measures. In the context of water resources, protection of our resources from pollution and degradation and the conservation of biodiversity are fundamental to maintaining a healthy functioning aquatic ecosystem. The National Water Act of 1998 (Chapter 3) is critical in promoting the protection of aquatic resources. One of the fundamentals of the NWA is balancing water resource protection with use in a sustainable manner, in order to address basic human needs, achieve ecological sustainability and enable socio-economic prosperity. This study contributes new capacity and results to South African water resource management.

As part of water resource management, regulators have to make decisions regarding the acceptable levels of potentially toxic chemicals or complex effluents that can be discharged into water resources. Toxicity testing is particularly valuable in assessing the biological effects of chemicals and effluents on aquatic ecosystems. Toxicity-based data in conjunction with chemical data can be used to derive acceptable discharge levels for regulatory control (Chapman 1995b). Toxicity testing may play a pro-active or reactive role in water resource management. Reactive tests are those that aim to assess contamination in aquatic ecosystems by confirming the effect of contaminants on species by showing similar effects in the laboratory as those in the field. The aim of pro-active tests is to protect the species in the aquatic ecosystems by assisting in predicting safe levels of contaminants and preventing hazardous effects to aquatic ecosystems (Chapman 1995c).

This study provides insight to both reactive and pro-active toxicity testing in water resource managements:

- Reactively, toxicity tests with locally isolated micro-algae may be used to assess the effects of chemical contamination by effluents, pesticides and other substances that may enter aquatic resources, thereby estimating impacts on the aquatic environment.
- Pro-actively, the data obtained from tests with locally isolated micro-algae may contribute to species sensitivity distribution (SSD) curves that may be used to determine water quality guidelines as well as predicting safe levels of effluent and pesticide levels in the environment.

Furthermore, this study demonstrates the importance of toxicity tests in water resource management through:

- Deriving water quality guidelines;
- Predicting the potential ecological hazard of chemicals and pesticides;
- Establishing dilution levels of complex effluents and chemicals into environmental water bodies; and
- Determining the cause-effect relationship in impact studies, using selected bio-indicators (Chapman 1995a).

The objective of this chapter is to reflect on the research findings presented in chapters 2 to 6 and to assess the application of the results and data generated from this study to water resource management, particularly in the South African context.

Chapters two and **three** provided the essential foundational steps for this study to be able to evaluate the role of algae as toxicity test organisms within water resource management. As a developing country, South Africa is lagging behind in implementing certain techniques and methods that have been used for a long time by developed countries. Even though South Africa has a comprehensive water resource policy (National Water Act No. 36 of 1998), implementation is still a problem due to lack of capacity to develop and apply the necessary techniques and methods. Studies such as this one add value and contribute to water resource management by providing tools (e.g. isolation and algal identification) and adding to the existing knowledge and capacity (e.g. refining and adapting the existing micro-algal toxicity test method for the use of indigenous algae).

Chapter two of this study was dedicated to developing and refining culturing protocols of locally isolated micro-algae for use in toxicity testing. Although culturing micro-algae is not a novel undertaking, in South Africa both isolating and culturing algae for use in toxicity testing is new. Micro-algae have been isolated and cultured for use in studies on growth characteristics, culture methods and taxonomy. What makes isolating and culturing micro-algae for toxicity testing different is that the focus is on specific attributes of the organisms, such as relatively rapid, constant and uniform growth under laboratory conditions, as well as ease with which cells can be counted under a microscope. These traits are essential to ensure compatibility of the culturing protocol with the toxicity test method and desirable test endpoint.

Chapter three built on this and focused on adapting the existing micro-algal toxicity test method so that it is suitable for using the micro-algae isolated and identified in Chapter two. The standard algal toxicity test method, the growth inhibition assay is an internationally recognised toxicity test method (US EPA 1978, OECD 1984, ISO 1989). This assay, developed in the mid-1960s as the Algal Assay Procedure Bottle Test, has been modified and accepted by various regulatory agencies as a standard toxicity test assay with *Pseudokirchneriella subcapitata* as the standard species (U.S. EPA 1969, US EPA 1978, OECD 1984, Slabbert *et al* 1998, Slabbert 2004). It is in the list of a battery of single species toxicity tests recommended for use in the implementation of programmes designed to be used to manage effluent discharges into surface waters and monitoring surfaces waters in South Africa (Slabbert *et al* 1998, Slabbert 2004).

Chapters four, five and **six** used these foundational steps to evaluate the newly isolated taxa and to assess their relative sensitivity to the following range of carefully selected toxicants, based on their relative importance in the South African context:

- Internationally recognised reference toxicants ($K_2Cr_2O_7$ and $CdCl_2$),
- Inorganic salts (NaCl and Na_2SO_4) selected for the important role they play in South African water resource management,
- Effluents from industries (power generation and petro-chemical) of significant function in the country, and

- A glyphosate-based herbicide (Roundup®) important for its common use in agriculture, and its role in controlling invasive alien species.

The reference toxicants ($K_2Cr_2O_7$ and $CdCl_2$) were selected for their common use, especially for test with micro-algae, particularly the standard species *P. subcapitata*. They are the most commonly used reference toxicants locally and internationally for micro-algae and have been used in inter-laboratory evaluation of toxicity tests to ensure adequate standardization for routine use (Wang 1987, Slabbert *et al*. 1998, ISO 2004, Slabbert 2004, Chapman *et al*. 2011a). Using these two reference toxicants enabled the comparison of the toxicity data generated in this study with available internationally generated data, contributing to the validity of the subsequent tests with other toxicants and mixtures. This step also enabled the comparison of the sensitivity of the locally isolated species *Chlorella sorokiniana* and *Chlorella vulgaris* with that of the standard toxicity test species *P. subcapitata*, and *Chlorella protothecoides* (from a commercial culture collection). Furthermore the sensitivity of the local isolates *C. sorokiniana* and *C. vulgaris* to the reference toxicants was compared to that of other micro-algal species using species sensitivity distribution (SSD) curves. The data used in SSDs was obtained from international and local databases to ensure that the sensitivity of these local isolates is compared to a broader list of freshwater micro-algal species and that data are more representative of the local natural communities.

The inorganic salts NaCl and Na_2SO_4 were selected for the important role they play as indicators of the country's two main land-use activities, mining and agriculture. In-stream levels of Na_2SO_4 are monitored in national programmes as an indicator of mining activity, while levels of NaCl indicate agricultural activity. The response of the locally isolated algae, *C. vulgaris* and *C. sorokiniana*, to the salts was assessed and compared to that of the standard species *P. subcapitata* as well as the commercial species *C. protothecoides*. This step also enabled the comparison of the sensitivity of the locally isolated micro-algae with that of macro-invertebrates using SSDs. This would enable an indication of the potential effect of the organisms' sensitivity to salts on the algae-herbivore relationship as an important part of the aquatic food chain. Substances that affect micro-algae at the base of the food chain could threaten the ecosystem function at the higher levels of organisation.

Effluents were selected from coal-based industries of significant importance in the country (power generation and petro-chemical). Coal mining is one of the growing industries in this

country as it plays a vital role in power generation as well as fuel and chemical production. Coal-based power generation and its effects on the environment, as well as acid mine drainage from coal mines have recently become points of concern and discussion in South Africa. The use of these effluents contributed to the applicability, site-specificity and environmental realism of the study. The response and sensitivity of these local species to the effluents was compared to that of the standard toxicity test species *P. subcap*itata and *C. protothecoides* from a culture collection in order to consider the potential of using of the selected local micro-algae in addition to the standard species in routine toxicity testing to determine industrial effluent impacts on local aquatic resources. Furthermore, the sensitivity of the locally isolated micro-algae to a commonly used herbicide Roundup was assessed. This herbicide was selected for the important role it plays in controlling weeds and alien vegetation. This herbicide is used by commercial farmers in the agricultural sector as well as in formal national programmes that are designed to control invasive alien species. The use of the herbicide in this study further contributed to the environmental realism and applicability of the study to the country's water resource management as the use of this herbicide may negatively impact on non-target organisms such as micro-algae in the aquatic ecosystem.

7.2 A CRITICAL EVALUATION OF THE STUDY

This chapter contributes a perspective of the whole study, and its developmental sequence may best be understood if described in phases as summarised in Figure 7.1. This study was designed such that it consisted of three phases.

7.2.1 Phase 1

The objective of the first phase was to develop capacity to use South African freshwater micro-algae in toxicity testing. The approach taken was that of refining existing protocols to isolate and culture micro-algae from environmental freshwater samples obtained from local rivers. It was necessary to first develop the skills and capacity required to isolate and culture micro-algae that would be later used in toxicity tests. This does not only refer to human resource capacity development but also to laboratory facilities, such as the appropriate equipment and tools required for micro-algal isolation and culturing. The entire study depended on the success of this phase.

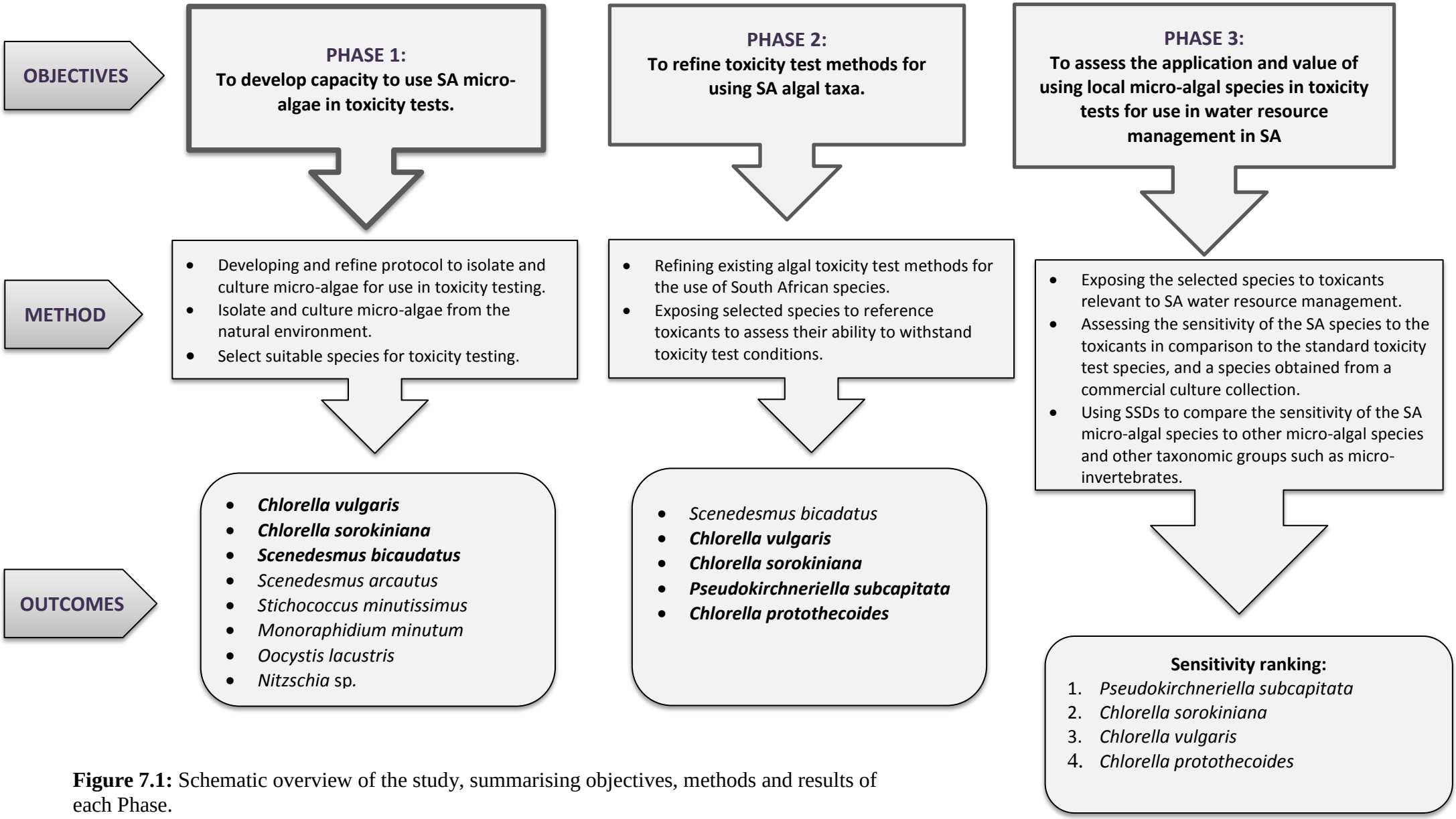


Figure 7.1: Schematic overview of the study, summarising objectives, methods and results of each Phase.

In South Africa, micro-algal cultures have typically been used to advance research towards understanding the physiological and genetic characteristics, as well as taxonomy of the organisms. Although isolating micro-algal cultures for use in toxicity tests has been used for some time in developed countries such as Australia, Canada and the United States of America, it is a fairly new phenomenon in South Africa. There are currently no local species isolated and cultured for use in toxicity test in South Africa. This is largely due to the lack of capacity and facilities to establish the cultures and maintain them.

As expected the isolation and establishing of cultures from environmental samples was a labour intensive and complicated task. Various isolation techniques were attempted but ultimately isolation on agar plates was selected as the method of choice for his study due to it being a relatively easy technique that requires minimum technical skill. It is a commonly used technique by small-scale laboratories that isolate cultures for academic and research purposes. It seemed to be the appropriate isolation and culturing method for the purpose and objective of growing the cultures.

When isolating micro-algae to use in toxicity tests, consideration must be given to the morphology, eco-physiology and growth properties of the organism. This is to ensure compatibility of the test organisms with the endpoint of the toxicity test protocol. In addition to the isolating and culturing, there is the maintenance of the cultures axenically once they are established. One of the common problems with this isolation and general maintenance of cultures with isolation on agar plates is contamination of axenic cultures by bacteria and fungi. This was encountered during this study, and periodically cleaning up the cultures was one of the setbacks of the process. However, improved laboratory facilities, such as obtaining a laminar flow system and a growth chamber remarkably reduced the rate of contamination and improved the success of the isolation and culturing process.

Given that biomass and growth rate are measures of the growth endpoint of the micro-algal toxicity test protocol, useful organisms toxicity testing are those that grow relatively well under defined laboratory conditions. Suitable organisms must also form homogenous suspensions in growth media, for easy quantitative determination of growth. The two techniques selected for quantifying micro-algal growth in this study were counting cells on a haemocytometer under the microscope and determining optical density, both of which require

organisms that form homogenous suspensions in growth media, rather than those than organisms where cells clump together.

Eight single species cultures were successfully established (Figure 7.1) and identified according to morphology. The limitation was not to support this identification with molecular identification. However, for the purpose of the study, this was adequate since molecular identification can still be done at a later stage for confirmation. It is also important to note that the isolation technique plays a role in the outcome in terms of species that are able to out-compete others and grow successfully in the defined culture conditions. Some species may find it difficult to grow on agar plates. Due to the process of elimination used in this study, only three species out of the eight species that satisfied the selection criteria for toxicity testing (***Scenedesmus bicaudatus***, ***Chlorella sorokiniana*** and ***Chlorella vulgaris***).

Even though there was a positive outcome in that three of the obtained species seemed to be suitable for toxicity testing, all of them were green algae (Chlorophyta). This was seen as a limitation of this study because the ideal scenario would have been to obtain species from different taxonomic groups (green, blue-green and diatoms). Different taxonomic groups would have contributed to improved representativity of the ecosystem (Hornstrom 1990). Green algae are generally easy to culture and they are mostly used in toxicity bioassays (Hawxby *et al*. 1977, Gardner *et al*. 1997, Rioboo *et al*. 2002, Yan *et al*. 2002). Blue-green algae and diatoms have been known to be difficult to maintain in viable cultures and they typically grow relatively slowly (Lewis 1995) compared to green-algae. Species of *Chlorella* and *Scenedesmus* are the most common, well known and widespread green algae. They are truly cosmopolitan and can be found in freshwater bodies all around the world. They are well known for being particularly easy to isolate and maintain in culture, and they are available in most commercial culture collections (Lürling 2005, Silva *et al*. 2009).

7.2.2 Phase 2

The objective here was to refine the existing algal toxicity test method for use of the South African taxa isolated in the previous phase in toxicity testing (Chapter 3). The approach adopted was that of adapting the existing algal growth inhibition assay, which is a well-recognised toxicity test protocol, to make it suitable for the use of the locally isolated. This

involved adapting the protocol to take into consideration the ideal growth conditions of the locally isolated species.

Algal toxicity testing was introduced in the 1980s in South Africa, and the only published protocol for the algal bioassay is described in the Direct Estimation of Ecological Effect Potential (DEEEP) Methods Manual (Slabbert 2004). This protocol is based on the standard US EPA Algal Growth Inhibition Test protocol (US EPA 1978), which was adjusted for use in routine toxicity testing in South Africa (Slabbert and Hinler 1990, Slabbert *et al*. 1998). The DEEEP Methods Manual describes the key practical steps involved in the execution of this bioassay. Even though the method of this bioassay described in the DEEEP Manual is the only published method for use in South Africa, there are no quality requirements pertaining to the standard operating procedures for the practical execution of the protocol (Chapman *et al*. 2011a). The limitation in terms of quality assurance is that there is no recognised national test protocol for this bioassay endorsed by the South African Bureau of Standards (SABS) that can be used nationally by all laboratories (Chapman *et al*. 2011b). South African laboratories typically use different international test protocols of the bioassay (US EPA 1978, OECD 2006, ISO 1989).

The algal toxicity test protocol described in the DEEEP Methods Manual (Slabbert 2004) was adapted and refined for the use of locally isolated species (***Scenedesmus bicaudatus***, ***Chlorella sorokiniana*** and ***Chlorella vulgaris***). Adaptations were based on the conditions for growth required by the selected locally isolated species. This refined method was then used to assess the suitability of these species for toxicity testing, by assessing their ability to withstand the experimental conditions prescribed in the refined protocol. The two most commonly used reference toxicants $K_2Cr_2O_7$ and $CdCl_2$ in South Africa and internationally for algal toxicity testing (Wang 1987, Slabbert *et al*. 1998, ISO 2004, Slabbert 2004) were selected as suitable chemicals for use in this phase of the study. There was relatively sufficient data available in literature on the toxicity of these two reference toxicants to *Pseudokirchneriella subcapitata*, to enable the comparison of the results from this study with existing data so as to validate the authenticity and feasibility of the refined method.

The tests with reference toxicants $K_2Cr_2O_7$ and $CdCl_2$ on the standard species *P. subcapitata* were used as the basis for comparison in this phase. Out of the three species, two (***Chlorella sorokiniana*** and ***Chlorella vulgaris***) adequately met the selection criteria. *Scenedesmus*

bicaudatus was eliminated as a potential toxicity test species due high growth variability in controls. Constant and uniform growth was a prescribed requirement of toxicity test species in this study. There may have been physical and/or chemical experimental factors that contributed to the variability in the growth of *S. bicaudatus*. One of the weaknesses in this phase is that these factors were not quantified due to time constraints. It is unknown whether manipulation of one or two parameters in the growth environment would have improved the growth rate and variability of growth of controls in *S. bicaudatus*.

Another factor may be that the morphology of *S. bicaudatus* was different from that of the Chlorella species tested. The cells were either bi-cellular or colonies of up to 8 cells in pairs. One observation was that the *S. bicaudatus* cells were polymorphic, being unicellular or colonial. These morphological changes depend on environmental conditions and may occur in *Scenedesmus* sp. without any effects on growth (Lürling 2005). This could mean that morphological changes may be a more relevant and sensitive endpoint for *Scenedesmus* than the traditional growth rate or biomass. The toxicity test method (algal growth inhibition assay), even though adapted and refined, was still originally based on supporting the growth of the standard species *P. subcapitata* bioassay (US EPA 1978, Slabbert 2004), and therefore could not necessarily be expected to adequately support the growth of some species.

Three *Chlorella* species (*C. protothecoides, C. vulgaris* and *C. sorokiniana*) that were also tested grew constantly and uniformly under the prescribed experimental conditions. It was therefore on the basis of comparing the growth of *S. bicaudatus* under those prescribed experimental conditions with that of the standard species *P. subcapitata*, the commercial species *C. protothecoides* and the two indigenous *Chlorella* species (*C. sorokiniana* and *C. vulgaris*) that that this species was eliminated as a potential toxicity test species. It should be clarified that although *S. bicaudatus* was eliminated as a toxicity test species for the test protocol prescribed in this study, it may be a useful toxicity test species for other test methods with different experimental conditions and endpoints.

One aspect that can be pointed out as a weakness in this phase of the study was the lower light intensity in the experimental facility than the prescribed light intensity. However, the light intensity used in this study was adequate for the standard species *P. subcapitata* (and the other three species) to obtain balanced control growth and fulfil the established control growth variability requirements for test acceptability. The growth of the standard species

provided a basis for comparison of results obtained for the other four species under the same test conditions. The test conditions used in this study were not outside the prescribed temperature and light culture conditions for the particular strain of *P. subcapitata* used in this study i.e. 20-25°C and 30-40 µE/m^2/s (Appendix C). Moreover species such as *Chlorella sorokiniana* are also known to grow at light intensities of 30-60 µE/m^2/s (de Bashan *et al*. 2008). If conditions are kept constant and within the bounds of survival, micro-algal cells will become acclimated to their environment and their growth will be balanced (MacIntyre and Cullen 2005). Standard light intensities between 10-30 µE/m^2/s are appropriate for long-term culturing of most micro-algal taxa. Localised heating may be problematic and temperature needs to be carefully controlled, because as temperature increases, evaporation also increases (Lorenz *et al*. 2005), and this could affects the results of a toxicity test particularly where small volumes such as those of wells of a 24-well microplate are used.

7.2.3 Phase 3

This phase addresses the main objective of the study, which is to assess the value and application of using the selected indigenous micro-algae in toxicity tests, for use particularly in South African water resource management. The approach taken was that of exposing the locally isolated species *Chlorella sorokiniana* and *Chlorella vulgaris* to a range of toxicants, widely used reference toxicants ($K_2Cr_2O_7$ and $CdCl_2$), inorganic salts of water quality concern in South Africa (Na_2SO_4 and NaCl) coal-based effluents from important industries in the country (power plant and petro-chemical industry) as well as a commonly used glyphosate-based herbicide (Roundup®) in order to assess the response and sensitivity of these local species to these toxicants compared to the standard toxicity test species *Pseudokirchneriella subcap*itata and a species obtained from a culture collection *Chlorella protothecoides*.

A summary of the results of the median effective concentrations (EC_{50}) of a selected range of seven toxicants ($K_2Cr_2O_7$, $CdCl_2$, Na_2SO_4, NaCl, coal-based power plant effluent, petro-chemical effluent and Roundup®); on the four micro-algal species (Chapters 4-6) is presented in Table 7.1.

Table 7.1: A summary of results of toxicity tests with a range of toxicants ($K_2Cr_2O_7$, $CdCl_2$, Na_2SO_4, NaCl, petro-chemical and power-plant effluents, as well as a glyphosate-based herbicide Roundup®) on four species of freshwater micro-algae *Chlorella vulgaris*, *Chlorella sorokiniana*, *Pseudokirchneriella subcapitata* and *Chlorella protothecoides* (Chapters 4-6)

Species		*Reference toxicants*		*Inorganic Salts*		*Effluents*		*Herbicide*
Name	**Type**	**$K_2Cr_2O_7$** (96hr EC_{50} mg/L)	**$CdCl_2$** (96hr EC_{50} mg/L)	**Na_2SO_4** (96hr EC_{50} g/L)	**NaCl** (96hr EC_{50} g/L)	**Petro-chemical** (% effluent)	**Coal-based power plant** (% effluent)	**Roundup®** (96hr EC_{50} mg/L)
Pseudokirchneriella subcapitata	Standard toxicity test species	0.17	0.039	0.349	0.537	Stimulation	Stimulation	Inhibition
Chlorella protothecoides	Culture collection	0.297	0.336	1.428	1.690	Stimulation	Stimulation	1.2
Chlorella sorokiniana	Locally isolated	0.216	0.0016	2.354	0.239	Stimulation	Stimulation	Inhibition
Chlorella vulgaris	Locally isolated	0.126	0.093	2.312	1.745	Stimulation	Stimulation	Stimulation

Species sensitivity distribution (SSD) curves were used to compare the sensitivity of the selected local algal species to that of species other than those tested in this study. This was done to in order to demonstrate the sensitivity of these local species in a context broader than only the species tested in this study. The SSDs show the distribution of sensitivities of species that are meant to represent the community of a given ecosystem, to a particular toxicant. The SSDs can be used prospectively or retrospectively as an assessment tool in decision making processes in water resource management (Schmitt-Jansen *et al*. 2007). They have been accepted as a valuable instrument in the process of derivation of water quality guidelines in some countries (CCME 1992, ANZECC and ARMCANZ 2000).

The **first part of this phase** involved assessing the sensitivity of the indigenous micro-algal species to the two reference toxicants, and comparing their sensitivity to that of *P. subcapitata* and *C. protothecoides*. The sensitivity of these species was further compared to that of other freshwater micro-algae on SSDs using existing data from an international database (ECOTOX). There was sufficient data on the database on the effect of the two reference toxicants on freshwater micro-algae to generate SSD curves.

The relative sensitivities of these four species to the selected toxicants were ranked using a simple ranking method (Rojíčková-Padrtová 1999). The most sensitive result (the lowest EC_{50} value) for each chemical was assigned the lowest number and the least sensitive result (highest EC_{50} value) assigned the highest number (1 and 4 respectively). The average of the ranks was calculated for each species to get the mean across all chemicals. Stimulation or inhibition results where EC_{50} values could not be determined were not assigned rank. The average sensitivity ranks of the four species *Chlorella vulgaris*, *Chlorella sorokiniana*, *Pseudokirchneriella subcapitata* and *Chlorella protothecoides*, to four chemicals $K_2Cr_2O_7$, $CdCl_2$, Na_2SO_4 and NaCl, are shown in Figure 7.2.

The standard species *P. subcapitata* was relatively sensitive to both reference toxicants, ranking second when compared to the other species (Tables 7.1 and 7.2). *Chlorella protothecoides* was the most tolerant of the four species to both reference toxicants. The indigenous species *C. vulgaris* was ranked as the most sensitive of the four species to the reference toxicant $K_2Cr_2O_7$ while *C. sorokiniana* was the most sensitive to $CdCl_2$ (Tables 7.1 and 7.2).

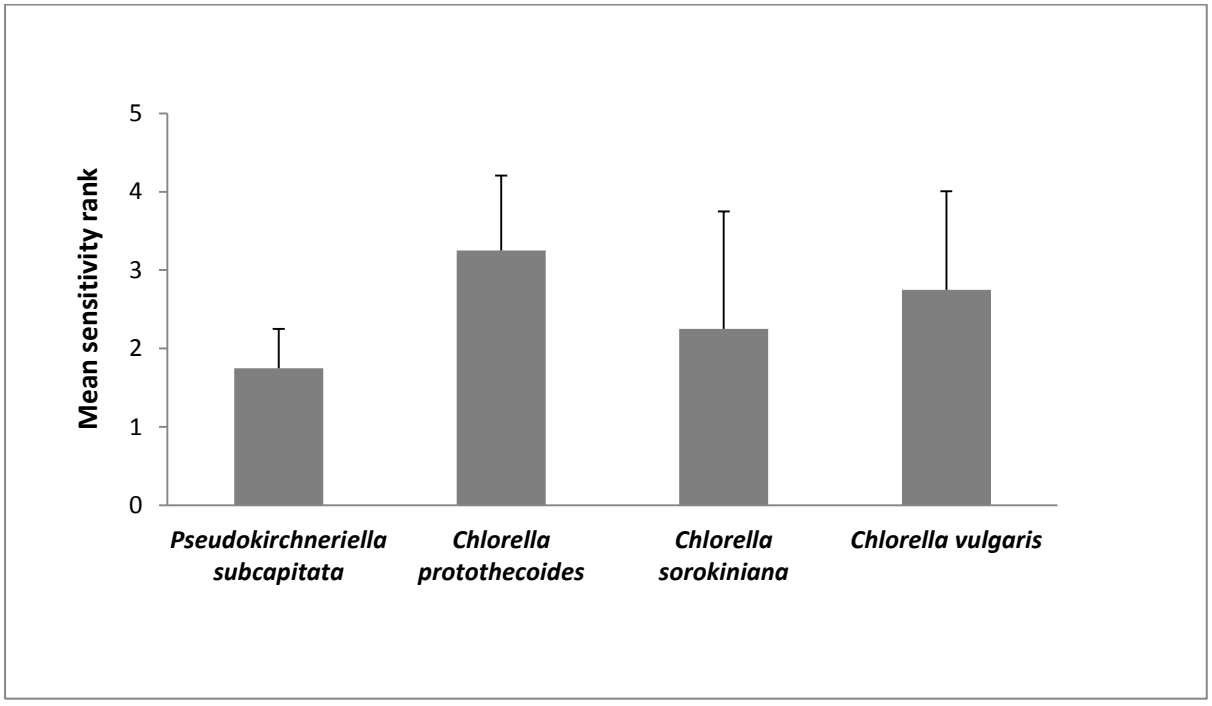


Figure 7.2: Mean sensitivity rank for four freshwater micro-algal species (*Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella protothecoides* and *Pseudokirchneriella subcapitata*) to the four selected toxicants ($K_2Cr_2O_7$, $CdCl_2$, Na_2SO_4 and NaCl)

Results from the current study showed *C. vulgaris* to be more sensitive to $CdCl_2$ than *C. protothecoides* (Tables 7.1 and 7.2). However, *Chlorella vulga*ris was the most tolerant of all the micro-algal species on the SSD curve (including *C. protothecoides*) to $CdCl_2$ (Figure 4.5). According to the SSD curve *C. vulgaris* was the most sensitive species to $K_2Cr_2O_7$ followed by *C. sorokiniana*. This study showed *C. sorokiniana* to be more tolerant to $K_2Cr_2O_7$ than *P. subcapitata* (Table 7.2) while the SSD curve showed *C. sorokiniana* to be more sensitive than *P.subscapitata* (Figure 4.6). These locally isolated species were both relatively sensitive to $K_2Cr_2O_7$ according to the SSD curve. *Chlorella sorokiniana* was the most sensitive species to $CdCl_2$ both on the SSD curve and the current study.

The slight disagreement in comparative sensitivities of species between the SSD curves and this study is due to the fact that SSDs also considered EC_{50} values from other studies. Variability in those data then contributed to the slight differences. Some studies use growth rate when determining EC_{50} values while others use biomass as a measure of growth (Nyholm 1985). Other practical considerations such as exposure times and general experimental conditions also contribute to variability in toxicity data used to generate SSDs. Even when different laboratories use the same test protocol there may be differences in EC_{50} values due to differing test conditions with respect to factors such as temperature, light intensity and perhaps test duration (Nyholm 1985).

Table 7.2: Mean sensitivity rank for the four micro-algal species *Chlorella vulgaris*, *Chlorella sorokiniana*, *Pseudokirchneriella subcapitata* and *Chlorella protothecoides*, to four chemicals $K_2Cr_2O_7$, dCl_2, Na_2SO_4 and NaCl

Species Name	*Reference toxicants*		*Inorganic Salts*		**Mean Rank**
	$K_2Cr_2O_7$	**$CdCl_2$**	**Na_2SO_4**	**NaCl**	
Pseudokirchneriella subcapitata	2	2	1	2	**1.75**
Chlorella protothecoides	4	4	2	3	**3.25**
Chlorella sorokiniana	3	1	4	1	**2.25**
Chlorella vulgaris	1	3	3	4	**2.75**

The **second part of phase three** involved assessing the sensitivity of these two indigenous algae (*C. sorokiniana* and *C. vulgaris*) to the selected inorganic salts Na_2SO_4 and NaCl. These two salts play a vital role in South African water resource management as their in-stream levels are monitored as indicators of the country's two main land-use activities, mining and agriculture.

Mining and agriculture are the most prominent land-use practices in South Africa (DWA 2011). These practices are associated with releases of salts into aquatic resources. Increased salt concentrations in South African aquatic resources result from point sources such as industrial discharges, as well as non-point sources such as agricultural run-off and spray drift. Evaporation exacerbates the problem of increased salt concentrations in aquatic resources. Salinisation is one of the major water quality concerns in this country. The South African Water Quality Guidelines are the primary source of information for determining the water quality requirements of different water uses for the protection and maintenance of aquatic ecosystems health (DWAF 1996). The water quality guidelines for aquatic ecosystems specify the water quality requirements for protection of ecosystem structure and function in surface waters. The water quality guidelines indicate the acceptable levels of specific water quality constituents to afford the aquatic ecosystem some protection.

Individual inorganic salts (such as Na_2SO_4 and NaCl) are not on the listed of water quality constituents of the South African Water Quality Guidelines for aquatic ecosystem. This may be because the derivation of criteria was based on the available information and cause-effect data at the time (DWAF 1996). However, subsequent work indicated variable toxicity for different salts (Goetsch and Palmer 1997, Scherman *et al*. 2003, Palmer *et al*. 2004). Electrical conductivity (EC) is used as a surrogate of salinity in the South African Water Quality Guidelines for aquatic ecosystem, and is a measure of the ability of water to conduct an electrical current.

The total dissolved salts concentration is a measure of the quantity of all dissolved compounds in water, and most dissolved salts in water carry an electrical charge, therefore total dissolved salts concentration is directly proportional to the electrical conductivity (EC). Ions such as **chloride**, **sulphate** and **sodium** carry an electrical charge when dissolved in water. These ions are recognised as constituents of importance in water resource protection measures such as the Resource Water Quality Objectives. They feature among the parameters

selected as indicators of fitness for use of water resources by user groups such as the mining and the agricultural sector. According to resource water quality objectives (RWQOs) in South Africa, electrical conductivity is an indicator of salinity, sulphate (SO_4^{2-}) indicates mining impacts, and chloride (Cl^-) indicates impacts from agricultural run-off and industrial effluent (DWA 2011).

This study focused on the salinity, as indicated by the response of the organisms to the total salt content rather than focusing on the individual salts in solution. Therefore nominal concentrations of salts were used rather than measured salts concentrations. The salt based experimental approach used in this study assumed that effects that can be quantified in aqueous solution where added ions have a significant impact on nutrition and/or physic-chemical nature of the solution are the mean effects of all ions in the solution in question. primary challenge with interpreting the effects of ion concentrations independent of salts is the inability to accurately calculate feasible ion mixtures using fixed proportionalities of salts, particularly for micro-algal experiments where culture media also contain mixtures of salts (Evens and Niedz 2011). Furthermore, determining exposure concentration vs nominal concentration may not necessarily explain the toxicity effects on an organism, there are bioavailability and toxicokinetic processes that may not be quantified. Chemical concentrations that trigger a response on organisms do not only depend on chemical speciation, but also on other factors such as absorption and/or distribution among cellular sites as well as metabolism (Groh *et al.* 2015). It was important for this study to first establish baseline information using nominal concentrations, before the effects of the salts could be quantified further.

The sensitivity of these species to the selected toxicants was once again compared to that of the standard species *P. subscapitata* and the commercial culture species *C. protothecoides*. The sensitivity of these species to the salts was further compared to that of freshwater macro-invertebrates in species sensitivity distribution, partially due to unavailability of sufficient toxicity data on salts and freshwater micro-algae, and also to give an indication of what effects the differential sensitivities of the taxonomy groups on food chain interactions. Both indigenous species showed no response or growth stimulation at low concentrations of both salts, and growth inhibition at high concentrations. This phenomenon was also observed in *P. subcapitata* and *C. protothecoides*. It is worth noting that the low concentrations which showed algal stimulation in this study are the concentrations likely to be found in the

environment. According to the Present Ecological State (DWAF 2008) boundary values (Figure 6.1), these concentration levels of the salts would cause moderate to no change to the aquatic environment.

Algal growth stimulation is not regarded as a toxic response in algal toxicity testing, which is ironic because eutrophication, one of the prevailing and concerning water quality issues, is a result of algal growth stimulation. The results of stimulation at low salt concentration could be very important, particularly when considering effects of in-stream industrial discharges and agricultural return flows, which could likely result in these low salt levels in the natural environment. The highest salt concentrations, which resulted in algal growth inhibition in this study are extremely high and would not likely be found in the natural environment. They would perhaps be found at the discharge points before dilution or at end-of pipe effluents before discharge. For example the highest salt concentration in this study was 10 g/L and the concentrations regarded as likely to be a result of serious change in the aquatic environment according to the PES boundary values (Table 6.1) are 0.064 g/L and 0.535 g/L for Na_2SO_4 and NaCl respectively (DWAF 2008). Perhaps the shortcoming in this part of the study was not to measure electrical conductivity at each salt concentration, so that the concentration effects could be correlated with EC values as done by Kefford *et al*. (2004). That would also take into consideration any additional salinity from the growth medium salt content.

The Na_2SO_4 seemed to have a slight hormetic effect on the locally isolated species, where there was significant growth inhibition at the lowest concentration followed by stimulation (even though not significant in the case of *C. vulgaris*) and then inhibition at the highest concentration. Organisms generally have a salinity range in which they can survive and within that broad range there is a finer range which is the optimum salinity range (Chadwick Ecological Consultants Inc. 2000). This was shown by the response of all the algae to the salts in this study, where the lower concentrations promoted algal growth.

Chlorella protothecoides was the most tolerant of the four species to both reference toxicants (Table 7.2 and Figure 7.2). The standard species, *P. subcapitata*, was relatively sensitive to both salts, ranking as the most sensitive to Na_2SO_4 and the second most sensitive to NaCl. *Chlorella sorokiniana* was the most sensitive of all four species to NaCl, and the most tolerant to Na_2SO_4 (Table 7.2). *Chlorella vulgaris* was relatively tolerant to both salts, compared to the other tested species, ranking as the most tolerant to NaCl and the second

most tolerant to Na_2SO_4. Both indigenous species were ranked as the least sensitive to Na_2SO_4, with *C. sorokiniana* as the most tolerant (Table 7.2). In contrast, *C. sorokiniana* was the most sensitive of the four tested species to NaCl, with *C. vulgaris* as the most tolerant. This means that *C. vulgaris* was relatively tolerant to both salts.

In terms of SSD curves for the salts there was insufficient data on the effects of salts on freshwater algae, so the only algal data available for SSD was from the current study. The sensitivity of algae to salts was therefore compared to that of macro-invertebrates. This was useful as it would give an indication of the potential effect of the organisms' sensitivity to salts on the algae-herbivore part of the aquatic food chain. Substances that affect this base of the food chain could threaten the ecosystem function at the higher trophic levels. According to the SSDs the algae were generally more sensitive to the salts than macro-invertebrates (Figures 5.4 and 5.5). *Chlorella sorokinian*a was the most sensitive of all the species (including invertebrates) to NaCl. *Chlorella vulgaris* was more tolerant to NaCl than *Tubifex tubifex* and *Lampsilis siliquoidea.* The two indigenous algal species were more tolerant to Na_2SO_4 than the standard species *P. subcapitata* and the commercial culture species *C. protothecoides*, and they were also more tolerant than the two *Daphinia* species *D. magna* and *D. pulex*. This sensitivity of algae to salts compared to invertebrates could have a negative impact on the algae-herbivore interaction, thereby leading to altered biodiversity and shifts in trophic pathways in the aquatic environment. Disruption of ecosystem functions at lower trophic level also has potential to affect organisms at higher trophic levels which such as fish (McDonald *et al*. 1996, Riedl and Altenburger 2007). Elevated salinity or even fluctuations in salinity levels could have adverse effects on the community structure in aquatic ecosystems. Alterations of species composition of the aquatic community may affect the overall ecosystem function (Ma *et al.* 2006).

In South African water salinity impacts are related to mining, agriculture as well as municipal and industrial waste, are prevalent (DWA 2011). In some of areas eutrophication is exacerbated because to the high salt content inhibiting some algal species and promoting or stimulation the growth of others, thereby changing the algal community structure. The observed differential sensitivities of the algae to the salts from this study could provide potential answers to the reasons for the changes in phytoplankton community structure in the aquatic ecosystems, especially in environments where salinity is also a problem. The effects of salts on freshwater micro-algae are not well understood, especially in South Africa, due to

lack of toxicity data. This kind of work needs more attention so that the possible linkages between the stimulatory and inhibitory effects of salts on micro-algae and eutrophication can be explored.

This **third part of phase three** focused on assessing the sensitivity of the indigenous micro-algal species to industrial effluents, in comparison to the standard species *P. subcapitata* and *C. protothecoides* from a commercial culture. Two effluents were selected, one from a coal-based power plant and another from a petro-chemical industry. Coal-based power generation and its effects on the environment has recently become a point of concern and discussion in South Africa. The relevance of the effluents to South African water resource management is that the discharge of effluents into the countries natural water resources could have deleterious effects on the aquatic ecosystems. Furthermore, eutrophication is one of the major water quality concerns in South African water resources, and under extreme conditions, stimulation of algal growth due to effluent discharges could lead significant ecological impacts.

This part of phase three was the extension of the second part, because even though mining and industrial waste related salinity is recognised as a problem, salts are not present in isolation in industrial and mining effluents. They are in complex mixtures with other chemicals, and therefore their environmental impacts cannot be isolated. It is not sufficient to recognise the effects of the salt components of effluents, there also needs to be an understanding of the effects of whole effluents on the aquatic ecosystems. Whole effluents may consist of complex mixtures of chemical components each of which may have a different effect on organisms. It is stated in the South African water quality standards for wastewaters and effluents that effluents should not contain any constituents at concentration levels that are deleterious to aquatic life (Regulation No. 991, 1984). The only means of sourcing information regarding these deleterious concentration levels to aquatic life with some degree of certainty is through toxicity tests with representative aquatic organisms. Toxicity tests are able to capture the synergistic, antagonistic and additive interactions of the chemical and physical components of effluents, which are manifested through changes in physiological and biological functions of the tested organisms.

The direct discharge of effluents into surface waters could result in changes not only directly to water quality, but also subsequent changes in biotic interactions and consequent ecosystem

responses (Twist *et al*. 1998, O'Farrell *et al.* 2002). It is important that toxicity tests with effluents take into account differential sensitivities of species to chemicals by using different species of organisms. Monitoring programmes that are employed to predict or assess the effects of effluents on receiving waters typically use toxicity test systems consisting of algae, macro-invertebrates and fish to represent the trophic levels of a typical aquatic system (Murray 2005, Jooste *et al*. 2008, Silva *et al*. 2009, Carbonell *et al*. 2010). South Africa has also introduced such monitoring programmes or tools (Murray 2005, Jooste *et al*. 2008, Chapman *et al.* 2011a), to provide information for use in decision making and improving techniques that are intended to reduce the potential adverse effects of effluents to the aquatic ecosystem.

Micro-algae are well suited as indicators to monitor changes in the aquatic environment because they respond fairly rapidly to changes in water quality. Micro-algal bioassays are therefore applicable for use in monitoring programmes that assess the effects of industrial and wastewater discharges into aquatic resources. The advantage of using micro-algae in whole effluent testing is their variable response to toxicants. As far as growth response to toxicants is concerned algae can stimulated, inhibited or both (Silva *et al*. 2009). The stimulation of algae was by both selected effluents was observed in this study. The two effluents used in this study (coal-based power plant and petro-chemical industry) were selected for their relevance in the South African context. Coal mining is one of the biggest industries in this country. Coal mines produce coal to increase the country's power generation capacity as well as fuel and chemical production. Although coal mining plays an important social-economical role in the country, in terms of providing employment and income to a large sector of the population, it results in increased water demand and environmental degradation that impacts directly or indirectly on aquatic fauna and flora. Extensive coal mining, and various associated industries, has resulted in poor water quality in some parts of the country (Hobbs *et al*. 2008).

The two effluents selected were within acceptable discharge standards (in terms of electrical conductivity and pH) according to the South African Wastewater and Effluent standards (Reg. No. 991, 1984). All the species were stimulated by the effluent from the petrochemical industry. *Chlorella protothecoides* was stimulated at a much higher concentration that the rest of the species. The power-plant effluent stimulated the standard species *P. subcapitata* and *C. protothecoides* at all concentrations. The effect of this effluent on the locally isolated species, however, was slightly different. The indigenous species were both stimulated at low

concentrations and inhibited at high concentrations. This phenomenon was more pronounced on *C. vulgaris* than *C. sorokiniana*. *Chlorella vulgaris* was therefore more sensitive to this effluent than *C. sorokiniana.* Micro-algal bioassays may be useful because they may identify effluents that stimulate algal growth and could possibly result in growth of nuisance algae, with concomitant ecological consequences. The growth of all tested micro-algae, indigenous, standard and commercial culture species were stimulated by both effluents, therefore it was difficult to compare sensitivities of the different species to the effluents with certainty.

In South Africa eutrophication is rightly linked to high levels of nitrates and phosphates, with wastewaters from sewage treatment works and agricultural return flows as the recognised drivers (Van Ginkel 2011, NWRS 2013). So far, no links have been established between industrial waste and eutrophication. Understanding the stimulatory effects of salts and industrial effluents that contain these salts on aquatic resources would go a long way into addressing the eutrophication problems that exist across the country but especially in the areas with extensive coal mining in the country. Micro-algal toxicity testing in monitoring programmes such as the National Eutrophication Monitoring Programme (NEMP) would assist, especially when focussing on specific impacted areas. Using locally isolated micro-algae in this process would add to the environmental realism and ecological relevance of these bioassays. The kind of data that would be generated would also contribute to understanding the specific impacts of episodic releases of effluent discharges to the aquatic environment. The algal growth stimulation by effluents as observed in this study, under extreme conditions, could lead to some algal species dominating and out-competing others in the natural environment, thereby contributing to eutrophication.

It was a limitation not to analyse nutrients in the effluent as nutrient analysis would have shed light into the growth stimulation exhibited by the effluents on the micro-algae. However, the objective of the study was to merely assess the response of the organisms to the toxicants, not to quantify the effects of the toxicants. Moreover, the salt components in the effluents may also have contributed to the growth stimulation by effluents (as exhibited by the growth stimulation exerted on the organisms at low salt concentrations shown in this study).

The **fourth part of phase three** focused on assessing the sensitivity of the indigenous micro-algae to the glyphosate formulation herbicide (Roundup®). Due to agriculture being one of the main land-use activities in South Africa, the relevance of using a herbicide as one of the

toxicants is unquestionable. Herbicides also play a more direct role in water resource management, due to their use in controlling aquatic and terrestrial weeds and alien vegetation. Glyphosate-based herbicides are used extensively in South Africa (Mensah *et al.* 2013) and internationally (Anton *et al*. 1993, Gardner *et al*. 1997) to control weeds and invading alien plant species. The relevance of this part of the study to water resource management is in evaluating the impacts of the most commonly used herbicide on freshwater ecosystem by using bioassays with indigenous algae that are likely to be found in South African aquatic environments. Glyphosate-based herbicides are used by commercial farmers in the agricultural sector as well as in formal national programmes (such as Working for Water) and are designed to control invasive aquatic alien species. Some herbicides are known to inhibit growth and metabolic processes in algae (Hawxby *et al*. 1977, Rioboo *et al*. 2002). Micro-algae are often affected non-target species by the herbicides, because photosynthesis is a primary target site for most of the commercially available herbicides (Rioboo *et al*. 2002).

The two indigenous species *C. vulgaris* and *C. sorokiniana* showed differing responses to the herbicide (Roundup®). The growth of *C. vulgaris* was slightly stimulated by Roundup® at 0.78 mg/L and not significantly affected by the herbicide at other concentrations, while the growth of *C. sorokiniana* was significantly inhibited at all the tested concentrations of the herbicide. Although in this study the EC_{50} value of Roundup® to *C. sorokiniana* could not be determined due to practical challenges with experimental conditions (Chapter 6), the significant inhibition was obvious and in agreement with a study by Christy *et al.* (1981) where growth inhibition by glyphosate was also observed, and an EC_{50} value of 17.7 µg/L was obtained. *Chlorella protothecoides* was also inhibited by the herbicides with an EC_{50} of 1.2 mg/L. The standard species *P. subcapitata* was also significantly inhibited by the herbicide although the EC_{50} value could not be determined due to practical challenges (Chapter 6) where none of the six test replicates satisfied all the criteria for a valid toxicity test to effectively calculate EC_{50} values. All four species responded differently to the herbicide.

Despite the extensive use of Roundup® in South Africa, there have been no water quality guidelines for the herbicide until recently (Mensah *et al.* 2013). This part of the study contributed to the determination of water quality guidelines for Roundup®. The contribution toxicity data (EC_{50} values and NOEC) from this study to species sensitivity distribution curves for the derivation of the water quality guidelines for the herbicide was important,

especially in the context of indigenous micro-algae that are environmentally relevant to South African aquatic resources. Herbicides enter aquatic water bodies via spray drift, run-off or leaching, and it is therefore important to have environmental limits in form of water quality guidelines so that the environmental levels of these herbicides can be monitored against the set guidelines.

Species sensitivity distribution curves based on acute toxicity data have confirmed that primary producers such as algae and macrophytes are most sensitive to glyphosate acid, an active ingredient in Roundup®, compared to fish and invertebrates (Brock *et al.* 2010, CCME 2012). Toxicity data for glyphosate are expressed either as concentration causing a specific effect such as death or growth inhibition (EC_{50} or LC_{50}) or as the highest concentration not showing an effect compared to a control (NOEC). Generally, short-term (acute) toxicity data are expressed EC_{50} or LC_{50} while long-term data are expressed as NOEC (Brock *et al.* 2010). In generating water quality guidelines SSDs are used determine the concentration at which a specified proportion of species are expected to be affected by a toxicant.

The SSDs have the advantage of making use of available toxicity data for a range of species. Statistical extrapolation is generally used to calculate the concentration of the toxicant at which a specified proportion of species (p) from different taxonomic groups is expected to be affected. This concentration is referred to as the Hazardous Concentration to the proportion of the species (HC_p). The SSD from which the HC_p is derived can be based on either acute (EC_{50} or LC_{50}) or chronic (NOEC) data (Brock *et al.* 2010, CCME 2012). The geometric mean of the available data for the same species is used to account for intra-specific variability and represent the most sensitive endpoint and lifestage (CCME 2012). It is important to note that Roundup® has been reported to be more toxic than the glyphosate (Brock *et al.* 2010) and that may be attributed to the fact that Roundup® is a formulation that contains glyphosate and other products that are meant to enhance the efficiency of the herbicide.

The **fifth and final part of phase three** focused on comparing the sensitivity of the four species, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella protothecoides* and *Pseudokirchneriella subcapitata*, species to the four toxicants ($K_2Cr_2O_7$, $CdCl_2$, Na_2SO_4 and NaCl), as shown on Figure 7.2. Tests with effluents showed stimulation and therefore EC_{50} values could not be calculated, so the sensitivity of the species to the effluents could not be ranked using EC_{50} values. Out of the toxicity tests with the herbicide Roundup® only the test

with *C. protothecoides* satisfied all the criteria for a valid toxicity test and had the EC_{50} value calculated, this EC_{50} value could not be ranked and compared to the EC_{50} values for other species because those could not be calculated. The standard toxicity test species *P. subcapitata* emerged as the most sensitive. This conclusion of *P. subcapitata* as the most sensitive species should be interpreted with caution because there were a few species and compounds tested here, and the species was not the most sensitive to all the compounds (Table 7.2). The sensitivity of the *P. subcapitata* is not discounted and this standard species is a very important part of routine toxicity testing because tests with this species are highly reproducible. The standard toxicity test with *P. subcapitata* (OECD 2004, ISO 1989) has been improved and automated in many ways, including the development of a toxicity test kit (AlgalToxKit F^{TM}) to make it cost effective and easy to perform routinely. The toxicity test kit is used by many laboratories in South Africa. It contains *P. subcapitata* in immobilised algal beads that can be easily set free when beginning the test. It generates results quickly and efficiently, eliminating the time and effort associated with culture cultivation and maintenance (Griffin *et al.* 2011).

However, although standard toxicity test with *P. subcapitata* is useful for its reproducibility, and the species itself has proven to be sensitive to a number of toxicants, the question of ecological distribution and relevance of this species in water resources management remains. The ultimate purpose of generating toxicity data is to protect the aquatic ecosystem, and with basing routine toxicity testing for water resource management solely on the standard toxicity test species there is the possibility of protecting a species that is not even a resident of the protected ecosystem (Cairns 1986).

Chlorella sorokiniana was the more sensitive of the two locally isolated species to the four chemicals. *Chlorella sorokiniana* was extremely sensitive to $CdCl_2$, and extremely tolerant to Na_2SO_4. This species was relatively sensitive to NaCl and $K_2Cr_2O_7$. The variability in sensitivity of a single species to different chemicals could be explained by the physico-chemical interaction between the organism and the toxicants (Hornstrom 1990). This species seemed to be the most sensitive to the herbicide Roundup. The demonstrated sensitivity of *C. sorokiniana* to a variety of chemicals from this study warrants its recommendation as a potential toxicity test species. The sensitivity of this indigenous species would be particularly useful in assessing site specific impacts of contaminants to the environment.

Chlorella vulgaris was found to be relatively sensitive to the tested chemicals. The advantage of using *C. vulgaris* in toxicity testing would be its widespread distribution. It is fairly easy to isolate and has been routinely cultured in many laboratories around the world. Its cosmopolitan nature assures its representativity of the aquatic environment. This species has been recommended internationally as a toxicity test species (OECD 1993) and has been used extensively in toxicity test studies (Muñoz *et al*. 1996, Wong *et al*. 2000, Rioboo *et al*. 2002, Cronin *et al*. 2004, Ma 2005, Nie *et al*. 2008). The use of *C. vulgaris* as an additional species in routine toxicity testing in South Africa would add environmental realism and ecological relevance to micro-algal toxicity testing, since it is evident from this study that this species is present in South African waters.

The species from a commercial culture *C. protothecoides* was overall ranked the most tolerant of the four species to the four toxicants. The order of increasing sensitivity was as follows; *C. protothecoides*, *C. vulgaris*, *C. sorokiniana*, *P. subcapitata*. Sensitivity of the species to the individual chemicals differed showing that the toxicity or effect of chemicals is species specific. The variability in response of the four species to a single chemical may be attributed to differences in morphology, physiology and genetics, each of which may be toxicologically relevant. This study has shown effects of toxicants to micro-algae to species specific. The reality is that no single micro-algal species has been declared the most sensitive species (Hörnström 1990), which has led to the international trend of proposing several species to be used in routine toxicity testing to represent algae (Rojíčková-Padrtová and Maršálek 1999). The advantage of using a battery of single species in micro-algal toxicity testing is that the species may have complementary sensitivity patterns which could be representative of the natural community. The inclusion of locally isolated species in the battery of toxicity test species may add value to the toxicity data generated.

The use of a battery of single species in toxicity test is therefore important for comparative purposes, especially when dealing with complex effluents which consist of different components. It has been recognised that using a single toxicity test species to predict toxic effects on algae is not the most pragmatic approach, and the variability in species sensitivity shown in this study is in agreement with this. Using a battery of single algal species in routine toxicity testing is more representative of the ecosystem and is preferred by many countries (OECD 1984, Swanson *et al*. 1991, Lukavsky 1992, Stauber *et al*. 1994). The use of a battery of micro-algae (that includes locally isolated micro-algae) for toxicity tests in programmes

that assess discharge and in-stream effluent such as the DEEEP and the NTMP in South Africa would assist in alerting operations and managers of any changes in effluent composition and in-stream water quality.

Micro-algae are useful tools to assess changes associated with eutrophication in aquatic resources. Incorporating micro-algal toxicity testing in monitoring programmes such as the NEMP in South Africa would be a positive step towards understanding and dealing with the problem of eutrophication. Single species toxicity tests have been used as the source of biological data for hazard and risk assessment internationally. Using a wide range of species in SSDs to develop water quality guidelines is a method used by some countries to ensure meaningful and representative guidelines for protection of aquatic ecosystems. The use of SSDs in in developing water quality guidelines is an attempt to take into account intra- and inter-specific variations in data. It is recognised that, because of the species richness in aquatic ecosystems, it is not possible to measure the effects of a toxicant to all species in deriving water quality guidelines and risk assessment to protect them from chemical contaminants. Species sensitivity distributions address variability and reduce uncertainty in risk assessment by making use of the available toxicity data (Kefford *et al*. 2005). The use of uncertainty/safety/assessment factors is not uncommon in the derivation of water quality guidelines (ANZECC and ARMCANZ 2000, EFSA 2005). These uncertainty/assessment factors are meant to account for the factors that contribute to uncertainty due to variation in toxicity between species, and account for species whose response to the toxicant are not measured. There are other factors contributing to uncertainty that cannot be easily quantified, and these are generally not addressed by uncertainty/assessment factors. The testing of more species reduces the uncertainty of the risk assessment attributable to interspecies differences in sensitivity and permits a reduction of the uncertainty factor that is applied (EFSA 2005, Kefford *et al*. 2005).

The South African water quality guidelines developed more than a decade ago (DWAF 1996) are the main source of information for water quality monitoring in this country. The guidelines were developed with the data that were available at the time. Given the increased industrial and economic growth since then, which has led to a growing pressure on the already constrained water resources of the country, the refinement and review of these guidelines seems inevitable. Refinement of the water quality guidelines will require

innovative and improved methods of generating data that are both relevant and in line with the altered environment associated with socio-economic changes in the country.

One of the main problems in this study was the general high variability in control growth for some experiments with some of the species. This was adequately addressed as there were more were enough controls used for each experiment. However in some instances the growth variability was still outside the acceptable values. This may be due to the fact that algal growth is a function of many factors such as nutrients, pH, salinity, temperature and light. Factors such as temperature and light intensity directly influence the photosynthetic mechanism of algal cells (Sharma *et al.* 2012). Keeping these factors absolutely constant for all algal cells in each well of the microplate is impossible, as there are issues of evaporation and shading as well as changes in pH that algal cells also respond to. There was also an issue of variation in intra-specific response to toxicants. Toxicants are known to exert different effects at different doses, with diverse pathways at each concentration (Groh *et al*. 2015). These may lead to intra-specific variations in response of species to toxicants.

7.2.4 Limitations of the study

Although the results of this work are positive, there were limitations that could be addressed. The following limitation could be investigated and explored to improve and refine the carry work forward:

- Isolated micro-algae were only from green algae (Chlorophyceae). Other taxa may be included as toxicity test species. This could be done by trying isolation techniques other than isolation on agar plates, such as isolation by glass Pasteur pipette washing and/or gravity separation.
- The identification of the species obtained in this study was only done using morphologicy. This must be validated with molecular identification in order to obtain the strain details and perhaps distribution and ecology of the species used in this study.
- It is important to gain further insight into other physiological parameters of the locally isolated species in order to understand the factors that influence their biomass production and growth rate.
- *Scenedesmus bicaudatus* was eliminated from the study based on homogenous growth under the specified conditions. The potential of *Scenedesmus bicaudatus* as a toxicity test

species could still be explored further using an endpoint other than growth. This would require insight into the morphology and cell cycle specificities of this species.

- The light and temperature conditions were not adequately balanced in the experimental environment. This could be improved by obtaining environmental conditions such as an incubator or similar experimental chamber where temperature and light can be easily adjusted.
- The inclusion of positive controls, such as tests with reference toxicant/s running parallel with every experiment could add value in terms of verifying the validity of the test protocol and results as well as interpreting the data.
- SSDs generated only used LC_{50}s and EC_{50} data, other endpoints such as LOECs and NOECs could be included so that the generated SSDs can be relevant for the derivation of water quality guidelines.
- Nutrient analysis was not included for effluents and this would have shed light into the growth stimulation exhibited by the effluents on the micro-algae.

7.3 APPLICATION TO WATER RESOURCE MANAGEMENT IN SOUTH AFRICA

7.3.1 Practical Considerations

Even though toxicity tests are now recognised as tools to be used in monitoring programmes associated with water resource management, the implementation of these tests nationwide is still a challenge (Chapman *et al*. 2011a). The implementation of toxicity tests in South Africa is limited by the unavailability of the necessary human skills as well as facilities to perform the tests (Griffin *et al*. 2011). The importance of this study is therefore in developing the necessary skills and capacity for the development and refinement of algal toxicity test protocols that will contribute to generating much needed data for use in water resource management. The developed protocols are a major research contribution that assists in advancing algal toxicity testing in this country.

The DEEEP and NTMP are the only national water quality monitoring programmes that specifically include toxicity testing in South Africa. The first step towards including toxicity testing in water resource management in South Africa was the compilation of a toxicity test methods manual for the implementation of the DEEEP. The manual describes the basic

protocols for the various standard toxicity tests with organisms at different levels of organisation, fish, macro-invertebrates and micro-algae (Slabbert 2004). The challenge, however, is that there are no standardised operating procedures and quality requirements pertaining to these toxicity test methods in South Africa (Chapman *et al*. 2011b). Internationally there is a prerequisite for quality assurance and quality control in biological assays that are routinely used for regulatory purposes (US EPA 2000, EC 2000). Although the standard toxicity test methods used in the NTMP and the DEEEP and documented in the DEEEP methods manual (Slabbert 2004) are based on recognised standard toxicity test protocols (US EPA 1984, OECD 1984, ISO 1989), quality assurance requirements are essential as they contribute to the accuracy and precision of the data generated.

The implementation of the DEEEP began as a pilot in 2005, but full scale implementation remains a challenge due to the lack of skills and capacity to perform toxicity tests in the country. The idea of the DEEEP is for toxicity tests to be applied at end-of-pipe before the industrial effluent is discharged into the receiving water, allowing the effects of the effluent to be isolated from in-stream effects (Chapman *et al* 2011a). Furthermore, toxic limits can be determined and used by water managers to supplement chemical limits in discharge licence authorisation. In order for the application of toxicity testing to be implemented in water resource management in South Africa, the generated toxicity test results have to be legally defensible, and accredited. This is a challenge given the expense of maintaining an accredited method and laboratory and the small number of toxicity testing laboratories in South Africa. The lack of quality assurance requirements could have a negative bearing on the analysis and interpretation of the data. There should be guidelines for the expression of data and handling of results. In order to include toxicity testing in regulatory procedures, South Africa needs to develop good quality assurance procedures that will ensure confidence in the data generated, for these data to be used effectively in monitoring programmes for water resources management.

The Department of Water Affairs and Sanitation, in partnership with the South African National Accreditation System (SANAS) and the National Laboratory Association (NLA) must establish a national toxicity testing accreditation programme that will ensure competence and proficiency of laboratories to perform the tests to acceptable international standards (ISO/IEC 17025). The SANAS is an independent body that assesses organisations and laboratories verifying compliance and competence to the relevant international and

national standards for the tasks and practices they undertake (Chapman *et al*. 2011b). This accreditation body was established to comply with the Accreditation and Conformity Assessment, Calibration and Good Laboratory Practice Act (Act 19 of 2006). The formal recognition of competence by an accreditation body increases confidence in the personnel performing the task and the data generated.

There are also challenges of limitations associated with the available skills base and facilities to carry out toxicity tests. There is currently inadequate human resources available to roll out routine toxicity testing at a national scale in South Africa (Chapman *et al*. 2011a, Griffin *et al*. 2011). There have been regional training courses by various institutions, but these have not borne satisfactory results in terms of adequately increasing human resource capacity in toxicity testing. The reality is that for toxicity tests to be used in the regulation of water resource management practice, personnel in government, municipalities, commercial and academic laboratories, water boards and industries must be capacitated at least to understand and interpret toxicity test data.

Another challenging aspect is availability of laboratory facilities to carry out these tests in the country. There are only 16 laboratories in South Africa which are able to conduct toxicity tests, and only four of those are SANAS accredited for aquatic toxicity testing (Chapman *et al*. 2011b). Three of these accredited laboratories are in the Gauteng region, and they include the DWA Resource Quality Services laboratory, while the other accredited laboratory is one of five toxicity test laboratories in the KwaZulu-Natal region. The Western Cape region has two laboratories (not accredited) while the Eastern Cape, Mpumalanga and the Free State regions each have one toxicity testing laboratory (Chapman *et al*. 2011b). Although these laboratories are not accredited, they are in Good Laboratory Practice (GLP) compliance and are participating in the Proficiency Testing Scheme (PTS). The PTS is the national inter-laboratory evaluation of toxicity tests to ensure comparable results of standard toxicity tests between laboratories. The PTS in South Africa has for a long time focused on the standard macro-invertebrate test with *Daphnia pulex,* and laboratories have recently expressed the need for additional testing schemes with fish (*Danio rerio*) and micro-algae (*Pseudokirchneriella subcapitata*) (Chapman *et al*. 2011a). Out of the laboratories that undertake toxicity testing in South Africa, even fewer carry out algal toxicity testing.

The laboratory at the Unilever Centre for Environmental Water Quality, Institute for Water Research is the only laboratory undertaking toxicity tests in the Eastern Cape. This highlights the lack of capacity and laboratory facilities to undertake toxicity testing in South Africa and further highlights the importance of the role that this laboratory plays in advancing micro-algal toxicity testing in South Africa. Not only does this laboratory have an established culture of the standard toxicity test species *P. subcapitata*, there are culturing facilities and capacity to maintain indigenous cultures as well. The addition of indigenous species in a suite of species for routine toxicity testing will not only advance research but will also be a practical applicable addition to water resource management.

Another further challenge to be recognised is the difficulty associated with establishing and maintaining micro-algal cultures (Griffin *et al*. 2011). This task is labour intensive but very necessary. The international trend is towards the use of battery of species in micro-algal toxicity testing and South Africa needs to adopt this if this country wants to be aligned with the rest of the world. This study has provided the ground work in terms of identifying the species that would be useful toxicity test species. The locally isolated species identified in this study are easy to isolate and maintain in culture. *Chlorella sorokiniana* is a particularly sensitive species while *C. vulgaris* has the advantage of being a true cosmopolitan species that is found in freshwater environments around the world. *Chlorella vulgaris* has also been recommended by some countries as a toxicity test species in addition to *P. subcapitata*, and doing the same in South Africa would be a great contribution to routine toxicity testing in South Africa, especially with now that it is known for certain that this species is available in our own freshwaters.

7.3.2 Aligning with South African Policy

The right to sufficient water and a clean safe environment that is not harmful to people's health and well-being is clearly specified in the Bill of Rights (Chapter Two of the South African Constitution (Act No. 108 of 1996)). Moreover the South African Constitution recognises the importance of access to sufficient water, as essential to a good quality of life. In the broader context of integrated environmental management, the National Environmental Management Act (NEMA) (Act No. 107 of 1998) focuses on environmental assessment procedures for the identification and evaluation of the likely impacts of proposed developments on the receiving environment, including aquatic resources. The National Water

Act (Act N0. 36 of 1998) is the main South African legislation that incorporated water resource protection into policy. The relationship between the NEMA and the NWA is implied in the land-based activities such as mining that have water related impacts such as effluent discharge. Controlling the impacts of such activities on the country's water resources is a requirement of the NEMA and the NWA. Chapter 6 of this study draws attention to the contribution that micro-algal toxicity testing can have in this regard. Micro-algal toxicity tests maybe appropriate and useful in assessing and regulating effluent discharges to natural aquatic systems as they are able to respond with stimulation, inhibition or both, thereby reflecting synergistic or antagonistic effects that the components of these effluents.

The NWA forms the basis of managing water resources through balancing protection and use and ensuring that water resources are developed, conserved and controlled in a sustainable and equitable manner for the benefit of all persons. The two approaches that balance water resource protection and use according to the NWA are referred to as **'resource directed measures'** (Chapter 3) and **'source directed controls'** (Chapter 4) respectively. Resource directed measures provide descriptive and quantitative goals for the state and condition of the resource and are geared towards the **protection** of water resources, while source directed controls specify the criteria for controlling impacts such as waste discharge and abstraction licences thus focusing on the **use** of the country's water resources.

The NWA recognises three resource directed measures: water resource Classification, the Reserve and Resource Quality Objectives. These three measures form the basis of protecting South Africa's water resources according to Chapter 3 of the NWA. In recent years micro-algae have been used as one of water quality assessment tools in resource directed measures in South Africa. Diatoms and periphyton (green-algae) are now a major part of water quality monitoring in Reserve and Resource Quality Objective determinations. Incorporating of toxicity testing in source directed controls as one of the conditions in wastewater and industrial discharge water licences would advance water resource management in South Africa. The addition of micro-algae as one of the recommended toxicity test organisms in this regard would be a significant contribution as they play an important role in aquatic ecosystems as the base of the aquatic food chain.

Mining and mineral processing are major land-use activity as well as contributors to the South African economy. A number of mining and mineral processing activities either require

Environmental Impact Assessments to be conducted in terms of NEMA or constitute water use requiring a licence in terms of the NWA. The Minerals and Petroleum Resources Development Act (Act No. 28 of 2002) is linked to NEMA in that it makes provision for environmental planning and management (Chapter 4, Sections 37-39) in activities associated with mining, mineral processing and fuel production. Some of the water related activities associated with the aforementioned processes are included in the broad definition of water use as stated in the NWA (Chapter 4, Section 21). Monitoring programmes and tools such as the DEEEP and the NTMP that include micro-algal toxicity testing when fully implemented could be instrumental in ensuring compliance of mines with the set effluent discharge standards and toxicity limits, in order to protect the country's aquatic resources.

As one of the major land-use practices in South Africa, agriculture impacts on the management of the country's water resources. The quality of some water resources in South Africa is deteriorating due to agricultural return flows, as well as pesticide spray-drift and surface run-off. According to the Pesticide Management Policy (Gazette No. 33899, December 2010) there need to be restrictions on users to limit the movement of harmful pesticides to water resources, under the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act No. 36 Of 1947). The Conservation of Agricultural Resources Act (Act No. 43 of 1983) makes provision for control over the utilisation of the country's natural agricultural resources in order to promote conservation of the soil, water resources and vegetation, and to combat weeds and invader plants. Invasive plants do not only threaten the country's biological biodiversity, but also water security and the ecological functioning of aquatic ecosystems. Working for Water, a programme administered by the Department of Water Affairs and Sanitation is at the forefront of the fight against invasive alien species. Chapter 6 of this study includes toxicity tests with Roundup, one of the pesticides used to combat invasive plants in South Africa, and assessing the effects of these pesticides to non-target organisms such as algae in the country's waters is an important contribution of this study to water resource management. Water quality guidelines for Roundup have recently been developed and this study (Chapter 6) has contributed to the toxicity data that was used to determine those guidelines.

Eutrophication is one of the significant water quality issues in South Africa (NWRS 2013), particularly in catchments where agriculture and mining are prevalent. This issue is exacerbated by the poor performance of some of the wastewater treatment works in the

country. Some of the wastewater treatment works lack proper maintenance and management of the infrastructure and processes, resulting in poor treatment and high nutrient loads in the discharged effluent. As shown in Chapters 5 and 6 of this study, understanding the contribution of the stimulatory effects of salts and effluents to micro-algae to the problem of eutrophication could be the key to controlling the problem in the country. Including micro-algal toxicity testing in monitoring programmes such as the National Eutrophication Monitoring Programme (NEMP) would be major step forward in dealing with eutrophication. This study has shown stimulatory effects of effluents on micro-algae, although this requires further investigation, it could be a starting point and a justification for including micro-algal toxicity testing (particularly with indigenous micro-algae) in eutrophication monitoring for water resource management.

7.4 THE GLOBAL CONTEXT

Water quality guidelines are formulated all over the world by different countries to control the concentrations of chemicals in the aquatic environment and protect aquatic organisms from these chemical contaminants (O'Hagan *et al.* 2005). The contribution of species sensitivity distribution in the derivation of water quality guidelines is valuable. They are the basis for the water quality target values which are derived using protective or hazardous concentration values (Hose and Van de Brink 2004). Species sensitivity distributions may be based on laboratory toxicity data using endpoints such as NOEC, LOEC and/or EC_x values. These data generally come from single species toxicity tests measuring effects of specific chemicals on organisms. Most of these data are from tests with standard toxicity test species which are readily available in cultures in laboratories (Hose and Van de Brink 2004, O'Hagan *et al.* 2005). This raises a question of whether these standard species based data provide sufficient and appropriate protection for the organisms in the field. In Australia for example, there has been recent emphasis on using site-specific data or data derived from using local species where available in SSDs to derive water quality guidelines (.Hose and Van de Brink 2004). This is meant to improve the environmental realism of the data. Given that the aquatic populations, communities and ecosystems are the entities to be protected (Newman 2000) from these chemical contaminants, it sounds reasonable to expand the pool of species tested so as to increase the representativity of the data.

The reality is that sufficient toxicity information is available for only a few chemicals, and there are still more toxicity data needed to ensure the reliability of the SSDs and the consequent water quality criteria derived from them. There is a need for data from more species of more taxonomic groups including the tolerant and rare species (Kefford *et al*. 2005) that are not readily available in laboratory cultures. Adding species such as *Chlorella sorokiniana*, which are not commonly used in toxicity tests to the pool of toxicity test species would not only contribute to adding environmental realism to the generated SSDs and water quality guideline but also add to the much needed toxicity data for the derivation ofthise water quality guidelines. Adding more species to the SSD reduces the uncertainty associated with interspecific variability. South Africa is in the process of refining and updating its water quality guidelines and this is an opportunity to use the species from this study to generate more toxicity data with these additional species for use in this process.

7.5 RECOMMENDATIONS AND CONCLUSION

The application of toxicity testing in water resource management is South Africa through the implementation of the DEEEP and the NTMP is a positive step. However, there is room for improvement and advancement in the application of toxicity testing in South Africa. A further step in the use of indigenous micro-algae in addition to the existing toxicity test species would make a substantive contribution. There has been an expression of interest in including toxicity testing in the conditions for water discharge licence authorisations in the country. This would require background research into deriving toxicity limits that could be used in those licence conditions. This involves generating new toxicity data in order to come up with meaningful limits that will protect aquatic fauna and flora from the adverse effects of industrial and wastewater effluent discharges. The addition of toxicity testing data with indigenous organisms would ensure that the toxicity limits and standards are realistic and applicable to South African conditions. This re-iterates the importance of using locally isolated micro-algal species in to supplement data with standard toxicity test species to that ensure relevant and realistic information is used in decisions made to manage the country's aquatic resources.

Following on from this study, further attempts must be made to isolate micro-algae from taxa other than green algae (Chlorophyceae) to include as toxicity test species. This could be done by trying isolation techniques other than isolation on agar plates, such as isolation by glass

Pasteur pipette washing and/or gravity separation. The morphological identification of the species obtained must be validated with molecular identification in order to obtain the strain details and perhaps distribution and ecology of the species used in this study. It is important to gain further insight into other physiological parameters of the locally isolated species in order to understand the factors that influence their biomass production and growth rate. The potential of of species such as *Scenedesmus bicaudatus* as a toxicity test species must be explored further using an endpoint other than growth. This would require insight into the morphology and cell cycle specificities of this species.

The South African Water Quality Guidelines (DWAF 1996) are the main source of information for water quality management in South Africa. These guidelines are outdated and based on the information that was available at the time. The water quality has changed since then, with the increase in urbanisation and industrialisation in the country. These guidelines need to be revised using more recent information, data and techniques. Some countries such as Canada and Australia rely on toxicity tests and species sensitivity distributions in the derivation of their water quality guidelines. This method of guideline development uses realistic and ecologically relevant data ensuring that the guidelines offer adequate protection to the majority of species in the environment. Adopting the use of indigenous algae routinely in toxicity testing, and using these in addition to the standard toxicity test species to test the effect of single chemicals would contribute to generating ecologically relevant data to the refinement of water quality guidelines in South Africa. It is therefore recommended that using indigenous micro-algae from this study in routine toxicity testing be considered as one of the option of generating much needed data for refining water quality guidelines. The advantage of using algae is that the test protocol is fairly easy to perform and micro-algae respond in a relatively short period of time (72-96hrs).

Future work following from this study must generate SSDs that include other endpoints such as LOECs and NOECs, not just LC_{50}s and EC_{50}s, so that they can be relevant for the derivation of water quality guidelines. The experiments with the chemicals (reference toxicants, salts and herbicide) on the species used in this study must be repeated to include refinements in order to improve the robustness of the results obtained. Experimental conditions must be improved to stabilise and balance light and temperature in the experimental environment. This could be done by obtaining an incubator or similar experimental chamber where temperature and light can be easily adjusted. The inclusion of

positive controls, such as tests with reference toxicant/s running parallel with every experiment could add value in terms of verifying the validity of the test protocol and results as well as interpreting the data.

It is recognised that isolating and maintaining micro-algal culture is a labour intensive task, and with the apparent lack of human and infrastructure resource in the country, using indigenous micro-algae routinely could be a problem. This however, may also have some solutions. There are laboratories in the country that culture micro-algal species for purposes other than toxicity testing. Academic institutions such as the University of North West (Potchefstroom campus) and the Nelson Mandela Metropolitan University for example maintain micro-algal cultures for research associated with taxonomy and physiology. These existing cultured species could be screened and their suitability as potential toxicity test species be investigating. This would then limit the time required to establish cultures, as these would be sourced from existing culturing facilities.

It is also recommended that the role that routine toxicity testing of wastewater and industrial effluents with micro-algal be investigated as an option of isolating the stimulatory effects of the effluents and the implications of that in eutrophication. The original purpose of the early standard micro-algal bioassay, the Algal Assay Procedure Bottle Test (AAP) (U.S. EPA 1969,1971a, 1978) was determining the limiting algal nutrients for growth potential and productivity in natural waters, rather than monitoring toxic effects of chemicals. It was only later that the AAP method was modified and served as the basis of the current toxicity test methods that have been published by international societies and regulatory agencies (OECD 1984,US EPA 1984, ISO 1989, US EPA 2000, EC 2000, OECD 2006). The micro-algal bioassay could still be useful in determining the productivity associated with wastewater and industrial effluents that result in eutrophication in mining and agricultural areas of South Africa. Nutrient analysis must be included for effluents as this would have shed light into the growth stimulation exhibited by the effluents on the micro-algae.

Due to similar physiology with plants, micro-algae may be useful indicators of the advert effects that pesticides have on non-target organisms. Chapter 6 of this study, with Roundup exhibited slight stimulatory effects on some of the micro-algae while inhibiting others, and this warrants further investigation as it may also have implications on the biodiversity shifts in aquatic ecosystems. The variable sensitivity of micro-algae to different toxicants is what

makes this method of using a battery of different species appealing. Using different species would give a better indication of what occurs in the environment, and lead to more informed decision making in water resource management than just using a single standard species to assess sensitivity to different toxicant.

In conclusion, this study makes a substantive contribution to advancing toxicity testing in South Africa, especially in ensuring that the implementation of toxicity tests and their application in water resource management is both aligned with international trends and can include the use of indigenous (local) taxa. The South African water law is recognised as one of the best in the world, the challenge now is ensuring that there are appropriate tools and techniques to implement the policy and adequately protect the country's water resources. Although toxicity testing in this country is lagging behind when compared to developed countries, studies such as this may support and advance toxicity testing in South Africa.

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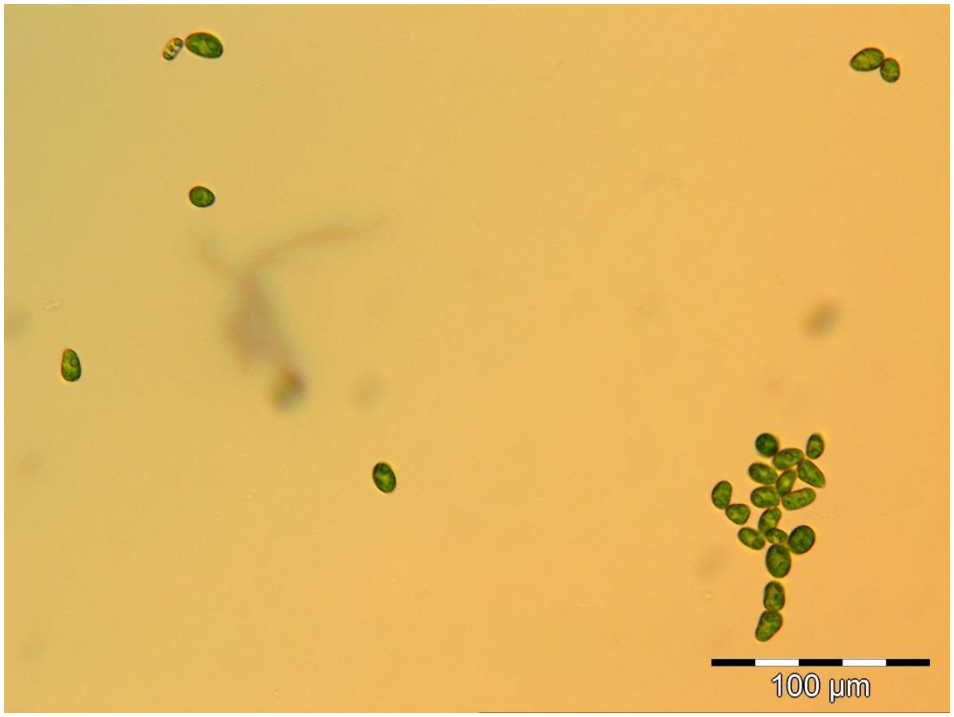
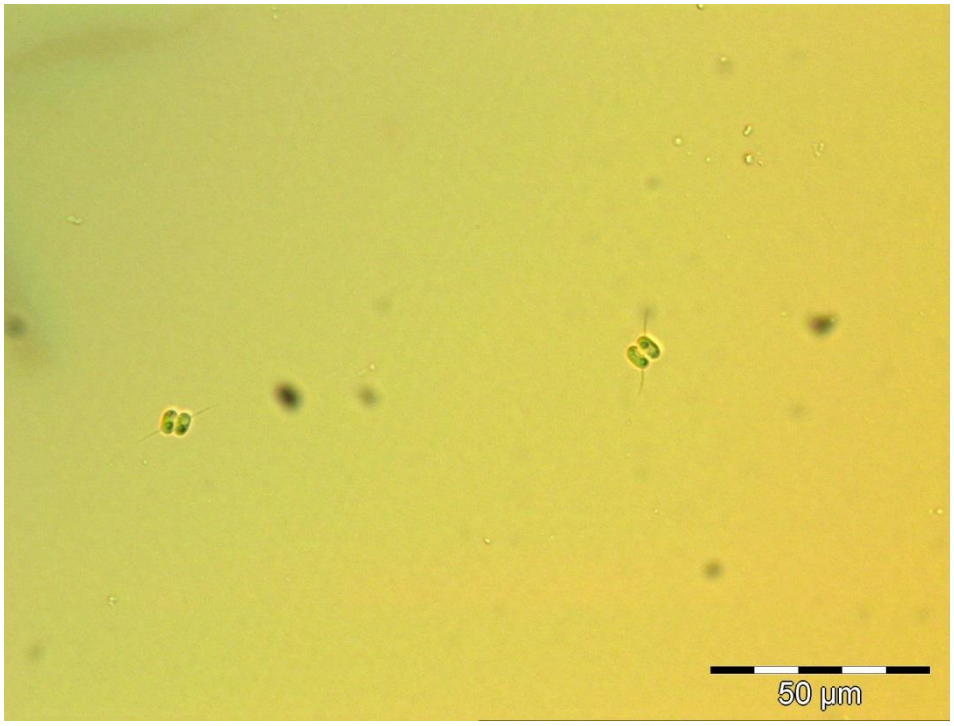
APPENDICES

APPENDIX A

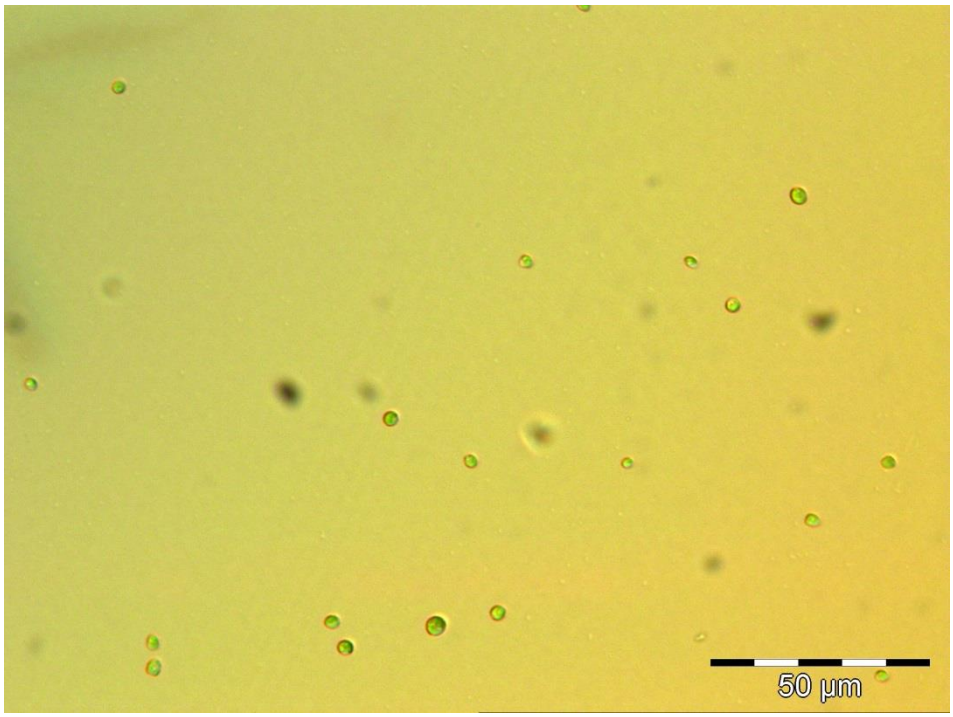
Images of some of the isolated freshwater micro-algae from this study.



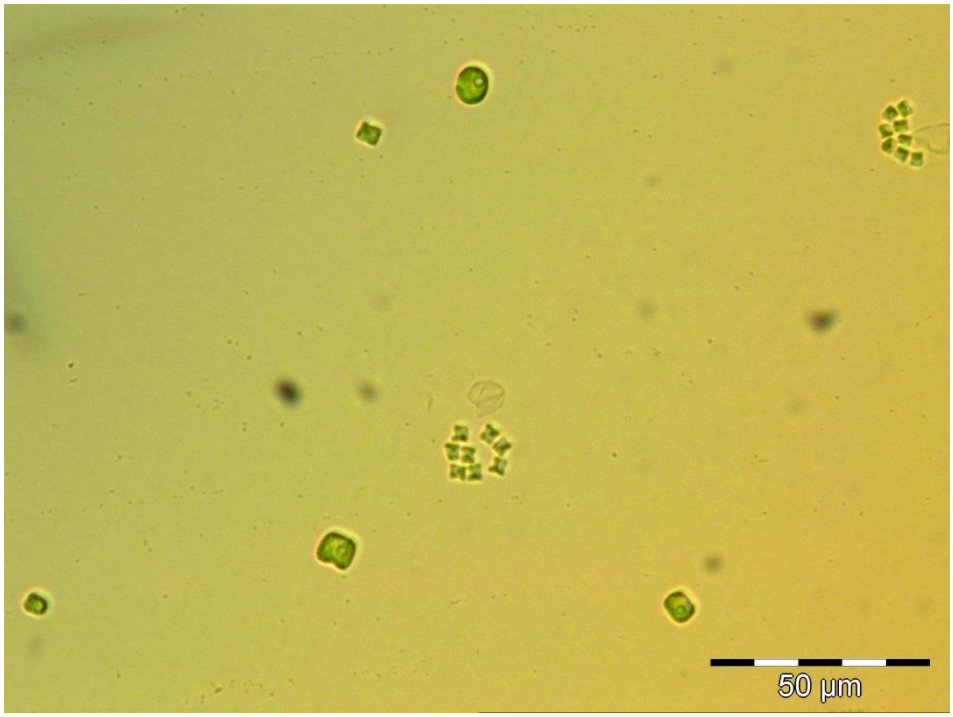
Scenedusmus arcuatus (Lemmermann) Lemmermann 1899



Scenedesmus bicaudatus Dedusenko 1925



Chlorella vulgaris Beijerink 1890



Chlorella sorokiniana Shihira, I. and R.W. Krauss 1965

APPENDIX B

DISTRIBUTION DETAILS OF SPECIES

1. Chlorella vulgaris Beyerinck [Beijerinck] 1890

General environment
This is a freshwater/terrestrial species.

Detailed distribution with sources

***Asia*:** Taiwan (Shao 2003-2014, Yamamoto & Shiah 2012), China (Hu & Wei 2006), Japan (Ettl & Gärtner 1995), Korea (Cho et al. 2013), Nepal (Ettl & Gärtner 1995)
***Arctic*:** Svalbard (Matula *et al.* 2007).
***Ireland*:** Dublin (Rindi & Guiry 2004).
***Europe*:** Austria (Ettl & Gärtner 1995), Balearic Islands (Cambra Sánchez, Álvarez Cobelas & Aboal Sanjurjo 1998), Baltic Sea (Hällfors 2004), Black Sea (BSPC Editorial Board 2014), Britain (John & Tsarenko 2002, Whitton *et al.* 2003, Müller et al 2005, John, Whitton & Brook 2011), Czech Republic (Stirk et al. 2013), Denmark (Rindi & Guiry 2004), France (Rindi & Guiry 2004, Anon. 2012), Germany (Müller et al 2005, Täuscher 2014), Netherlands (Müller et al 2005, Gustavs et al. 2011, Krienitz et al. 2012), Romania (Caraus 2002, Caraus 2012), Spain (Álvarez Cobelas 1982, Aboal & Llimona 1984a, Aboal & Llimona 1984b, Alvárez Cobelas & Gallardo 1986, Aboal 1996, Aboal 1996, Cambra Sánchez, Álvarez Cobelas & Aboal Sanjurjo 1998, Uher, Aboal & Kovacik 2004, Müller et al 2005, Uher et al. 2005, Fanés Treviño, Comas González & Sánchez Castillo 2009), Sweden (Müller et al 2005), Russia (Europe) (Stirk et al. 2013).
.***Atlantic Islands*:** Iceland (Ettl & Gärtner 1995).
***North America*:** Arkansas (Smith 2010), Great Lakes (Prescott 1962).
***South America*:** Brazil (Menezes 2010).
***South-west Asia*:** Iran (Afsharzadeh *et al.* 2003), Pakistan (Shahnaz et al. 2007, Hussain et al. 2010), Turkey (Asia) (Soylu & Gonulol 2006).
Asia Taiwan (Shao 2003-2014,).
***South-east Asia*:** Singapore (Pham *et al.* 2011).
***Australia and New Zealand*:** New Zealand (Broady *et al.* 2012), Queensland (Day *et al.* 1995), Victoria (Day *et al.* 1995).

2. Chlorella sorokiniana Shihira & R.W.Krauss 1965

General environment

This is a Freshwater/Terrestrial species.

Detailed distribution with sources

***Europe*:** Germany (Gustavs et al. 2011).
***Asia*:** China (Qiao et al. 2009), Korea (Cho et al. 2013), Taiwan (Shao 2003-2014).

3. ***Chlorella protothecoides*** Krüger 1894

General environment
This is a Terrestrial species.

Type information
Type locality: Germany: near Halle a. Salle sap of poplars and elms (Silva 1996-to date).

Detailed distribution with sources

Europe**:** Romania (Caraus 2002).
Australia and New Zealand**:** New Zealand (Broady *et al.* 2012).

4. Pseudokirchneriella subcapitata Hindák, F. (1990).

Published in: Studies on the chlorococcal algae (Chlorophyceae). V. *Biologické Práce* 36: 1-227.

General environment
This is a freshwater species.

Detailed distribution with sources
Europe**:** Spain (Cambra Sánchez, Álvarez Cobelas & Aboal Sanjurjo 1998).

REFERENCE
Guiry, M.D. & Guiry, G.M. 2015. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 23 February 2015.

APPENDIX C

Strain details and image of the culture of *Pseudokirchneriella subcapitata* (Korshikov) Hindák 1990 used in this study.

culturecollection of algae and protozoa

ccap strain data

Strain Number:	**CCAP 278/4**
Strain name:	***Selenastrum capricornutum***
Author:	Printz 1913
Synonyms:	*Kirchneriella subcapitata* Korschikov 1953; *Raphidocelis subcapitata* (Korschikov) Nygaard et al. 1986; *Kirchneria subcapitata* (Korschikov) Hindák 1988; *Pseudokirchneriella subcapitata* (Korschikov) Hindák 1990
Collection place:	Freshwater; River Nitelva, Akershus, Norway
Isolated by:	Skulberg 1959
Type material:	No
Other permanent collections:	(7)SAG 61-81 as Ankistrodesmus bibraianus; UTEX 1648; ATCC 22662; UTCC 37
Culture medium:	3N-BBM+V; EG:JM; Axenic Media recipes are available on our website: www.ccap.ac.uk
Subculture interval:	
Culture conditions:	15°C ± 2°C (for faster growth, grow at 20-25°C; cool white fluorescent tubes about 10 cm from the culture, with an intensity of 30-40 µmol m-2 s-1; 12 hr light : 12 hr dark (for faster growth, use continuous light)

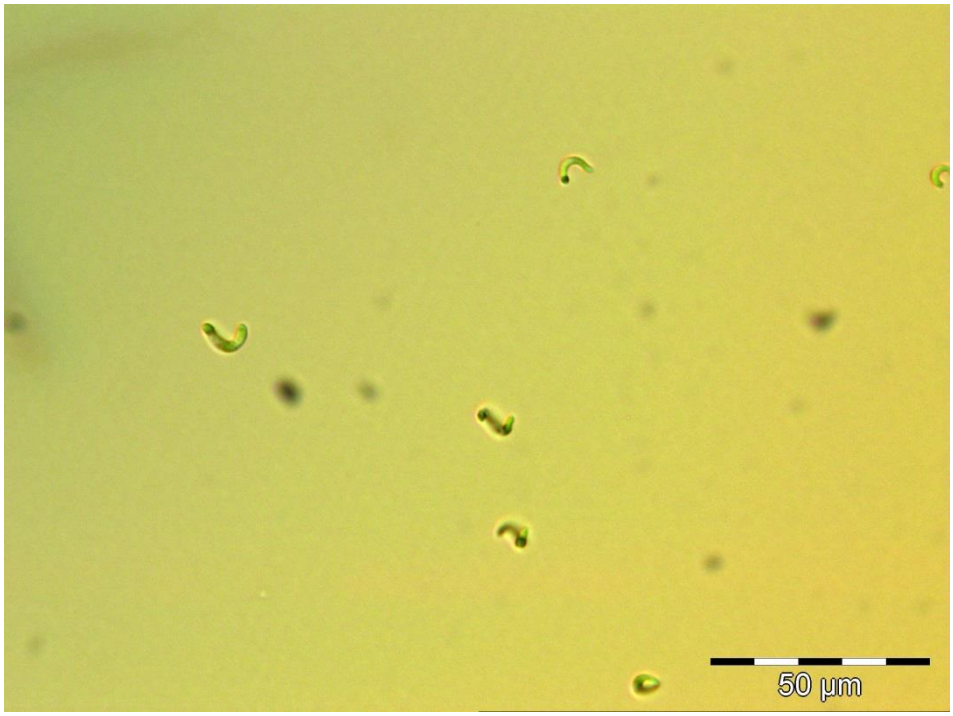
Liquid algae in tubes:
The algae should survive for at least 1-2 weeks, however it is advisable to subculture it as soon as possible. Normally, 2-5 ml of culture is inoculated into 50 ml of fresh sterile medium. Unless noted above, prior to despatch the culture will have been stored at 15°C - 20°C, cool white fluorescent lighting about 10 cm from the culture, with an intensity of approx. 40 µmol m^{-2} s^{-1} (10-15 µmol for cyanobacteria); 12 hr light : 12 hr dark. If such facilities are unavailable, placing the tube in a north facing window at room temperature should suffice.

Algae on agar slopes:
Algae grown on agar slopes are sent as single tubes. Immediately before despatch, the cultures have been kept at CCAP under the following conditions (may be different from those stated above): 15°C - 20°C, cool white fluorescent lighting about 10 cm from the culture, with an intensity of approx. 40 µmol m^{-2} s^{-1} (10-15 µmol for cyanobacteria); 12 hr light : 12 hr dark. If such facilities are unavailable, placing the tube in a north facing window at room temperature should suffice. If maintained in suitable conditions, the culture should survive for about 4 weeks before it requires subculturing.

Special features:	recommended in ecotoxicity testing guidelines OECD 201 and 87/302/CEC
Size in µm:	
Original designation:	NIVA CHL 1
Other Information:	

Please Note: There may be additional information available on certain strains which can be provided on application

CCAP (Culture Collection of Algae and Protozoa), Scottish Marine Institute, Oban, Argyll, PA37 1QA, UK
Tel: +44 (0)1631 559000 Fax: +44 (0)1631 559001 Email: ccap@sams.ac.uk Web: www.ccap.ac.uk



Pseudokirchneriella subcapitata (Korshikov) Hindák 1990

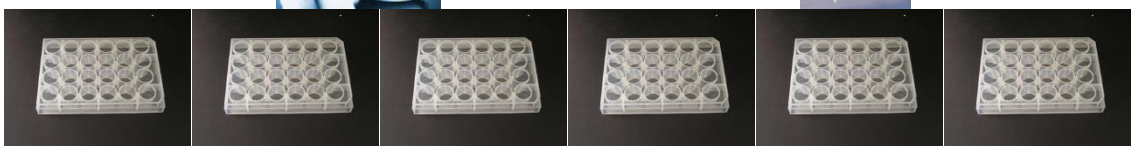
APPENDIX D

1. Prepare Test solutions

1.1. Prepare serial dilutions of the treatment solution

1.2. Prepare six replicate microplates for each test and mark them according to the configuration given on Table 3.4.

1.3. Dispense 1.8 mL of control and treatment solutions into appropriate wells of each of the six replicate microplates.



2. Prepare Algal Inoculum

Flask with 3-4 day old exponential phase algal culture

2.1. Determining the number of cells in the algal culture by counting cells on a haemocytometer under a light microscope

2.2. Calculating the algal inoculum volume required for the number of treatment and control wells of the six replicate microplates

2.3. Determining the volume of BG-11 medium required for the number of treatment and control wells of the six replicate microplates

2.4. Combine the required volume of algal inoculum with the required volume of BG-11 medium.

3. Dispense the combined algal inoculum and BG-11 medium in wells of six replicate microplates (excluding the 1st rows of Blank wells in each plate)

3.1. Dispense 0.2 mL of inoculum suspension into each of the wells of six replicate microplates.

4. Incubate microplates in the light and temperature controlled facility of 96 hours.

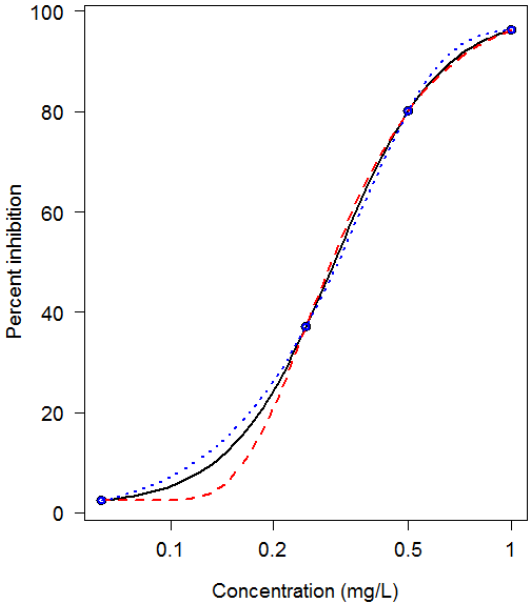
Figure : Schematic representation of the experimental steps and design of the toxicity tests.

APPENDIX E

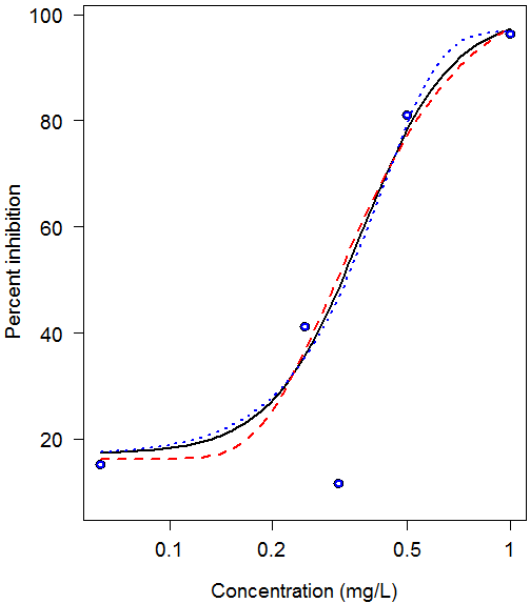
Results of the R-analysis.

$K_2Cr_2O_7$

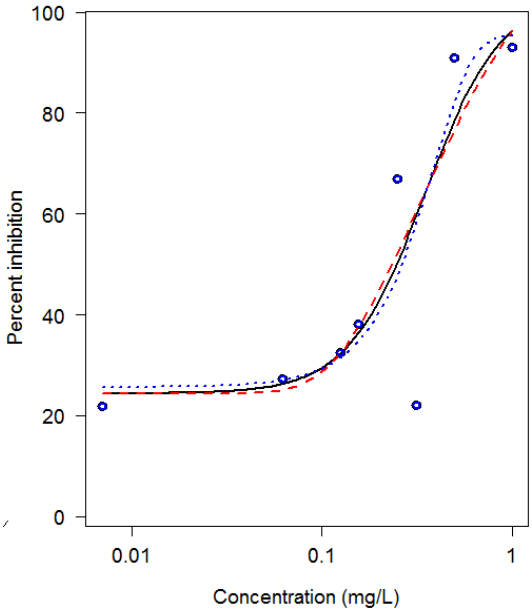
Species	Model	EC_{50}	Std. Error	Lower Confidence	Upper Confidence
Chlorella protothecoides	Log logistic	0.304	0.288	0.243	0.366
	Weibull 1	0.305	0.030	0.241	0.369
	Weibull 2	0.299	0.034	0.227	0.371
Chlorella sorokiniana	Log logistic	0.365	0.051	0.251	0.472
	Weibull 1	0.371	0.0717	0.220	0.521
	Weibull 2	0.366	0.051	0.260	0.473
Chlorella vulgaris	Log logistic	0.350	0.083	0.181	0.519
	Weibull 1	0.468	0.394	0.332	1.268
	Weibull 2	0.331	0.057	0.215	0.447
Pseudokirchneriella subcapitata	Log logistic	0.273	0.009	0.253	0.291
	Weibull 1	0.269	0.009	0.251	0.288
	Weibull 2	0.276	0.0136	0.248	0.304



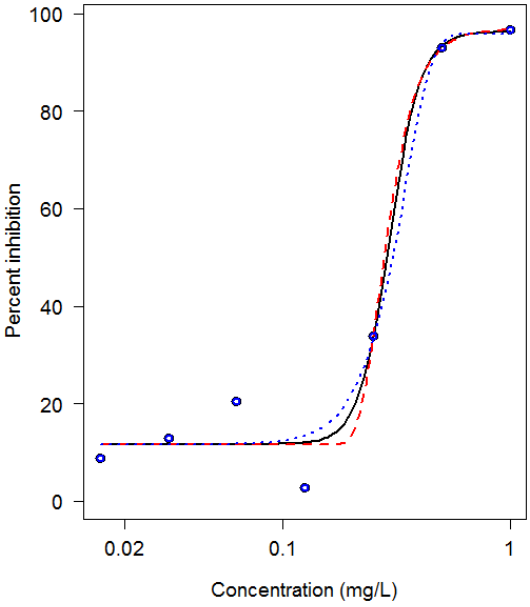
C. protothecoides



C. sorokiniana



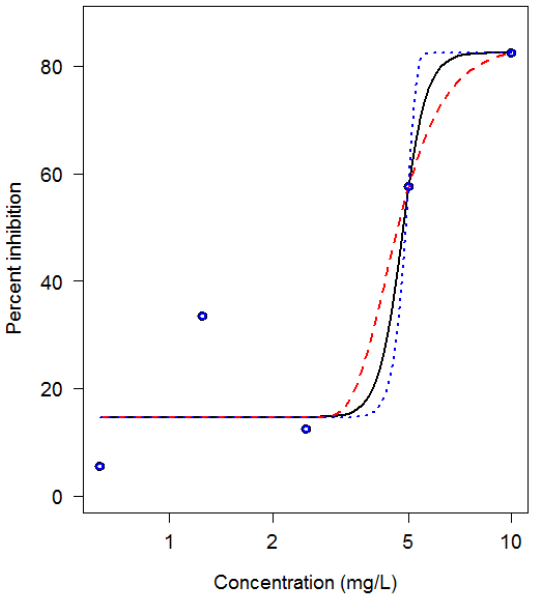
C. vulgaris



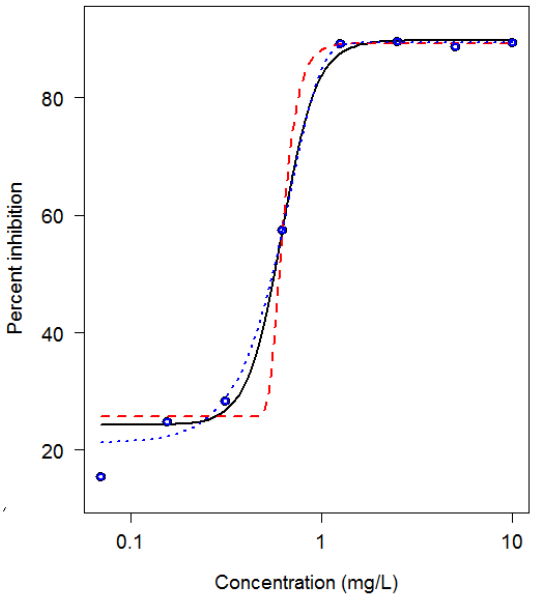
P. subcapitata

NaCl

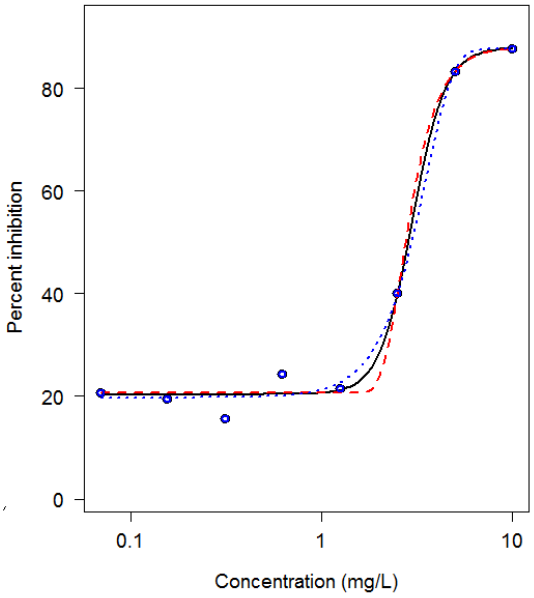
Species	**Model**	**EC_{50} mg/L**	**Std. Error**	**Lower Confidence**	**Upper Confidence**
*Chlorella prototheco*ides	Log logistic	4.786	0.669	3.380	6.191
	Weibull 1	4.613	1.462	1.541	7.684
	Weibull 2	4.896	1.763	1.191	8.600
Chlorella sorokiniana	Log logistic	0.617	0.034	0.549	0.686
	Weibull 1	0.625	0.013	0.598	0.652
	Weibull 2	0.603	0.0412	0.520	0.687
Chlorella vulgaris	Log logistic	2.984	0.327	2.317	3.650
	Weibull 1	2.882	0.302	2.267	3.498
	Weibull 2	3.141	0.341	2.445	3.836
Pseudokirchneriella subcapitata	Log logistic	0.881	0.160	0.556	1.206
	Weibull 1	0.974	0.188	0.592	1.355
	Weibull 2	0.761	0.204	0.348	1.175



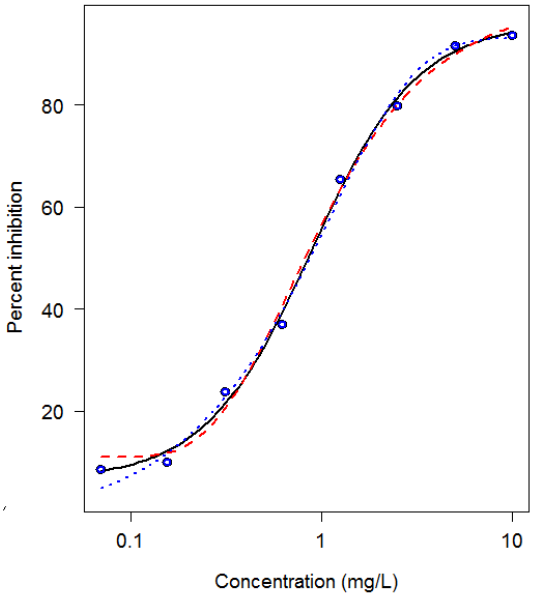
C. protothecoides



C. sorokiniana



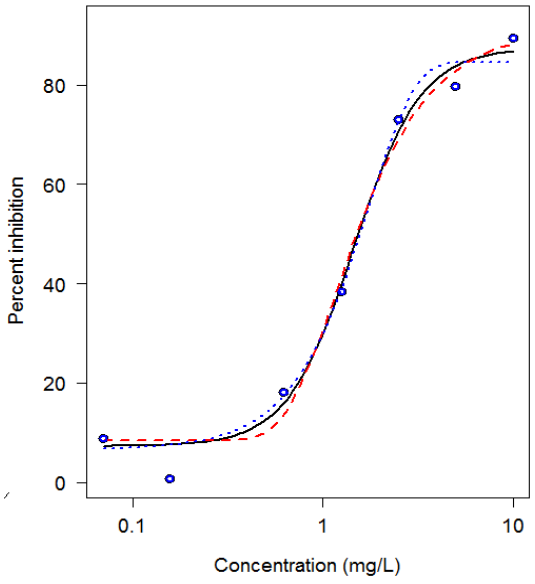
C. vulgaris



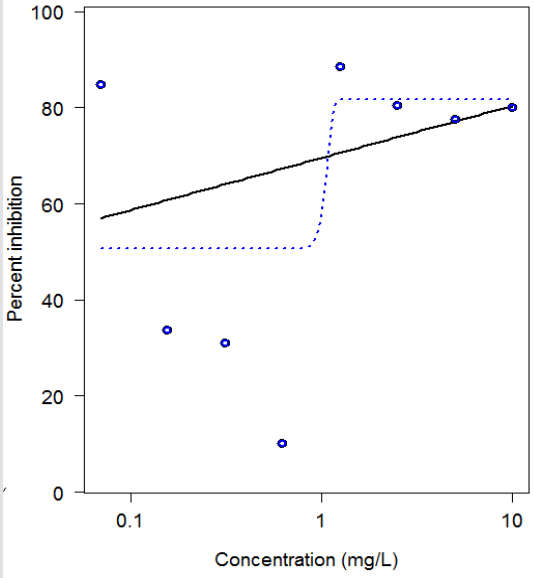
P. subcapitata

Na_2SO_4

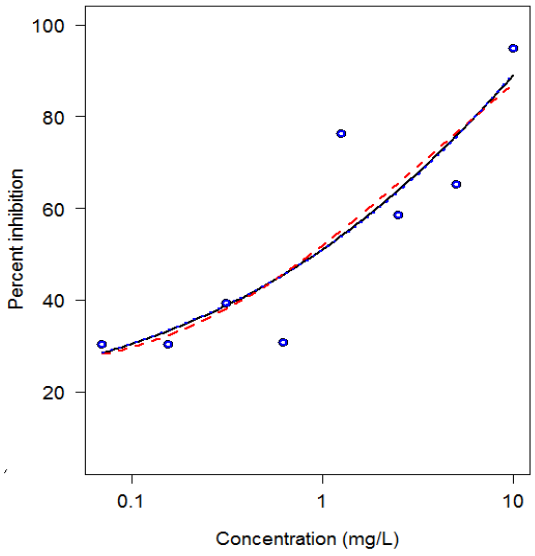
Species	**Model**	**EC_{50}**	**Std. Error**	**Lower Confidence**	**Upper Confidence**
Chlorella protothecoides	Log logistic	1.459	0.144	1.164	1.755
	Weibull 1	1.503	0.173	1.148	1.857
	Weibull 2	1.448	0.147	1.147	1.749
Chlorella sorokiniana	Log logistic	1.9045e-01			
	Weibull 1				
	Weibull 2	1.065	0.892	-0.745	2.875
Chlorella vulgaris	Log logistic	4.5029e+01	2.7879e+02	-5.2155e+02	6.1161e+02
	Weibull 1	1.2689e+01	4.5458e+01	-7.9694e+01	1.0507e+02
	Weibull 2	1.3022e+02	1.2338e+03	-2.3772e+03	2.6376e+03
Pseudokirchneriella subcapitata	Log logistic	0.328	0.023	0.281	0.376
	Weibull 1	0.344	0.023	0.296	0.392
	Weibull 2	0.312	0.033	0.243	0.381



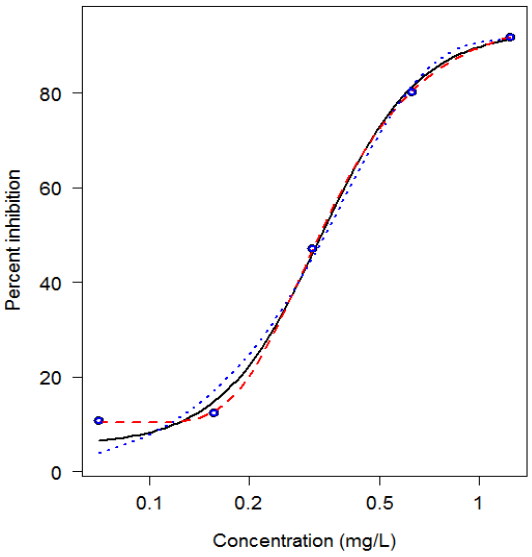
C. protothecoides



C. sorokiniana



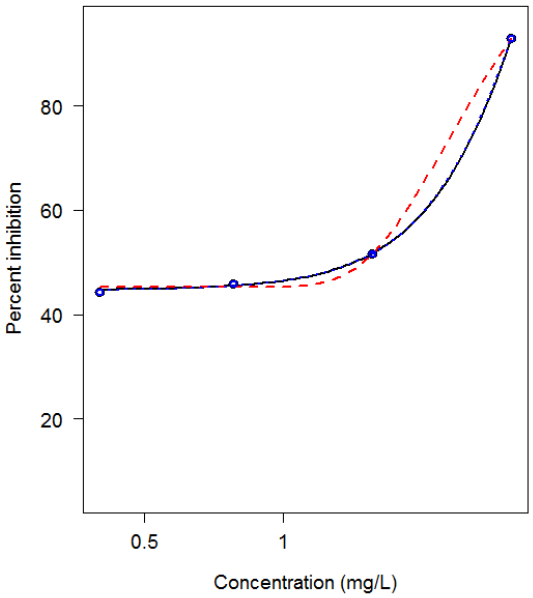
C. vulgaris



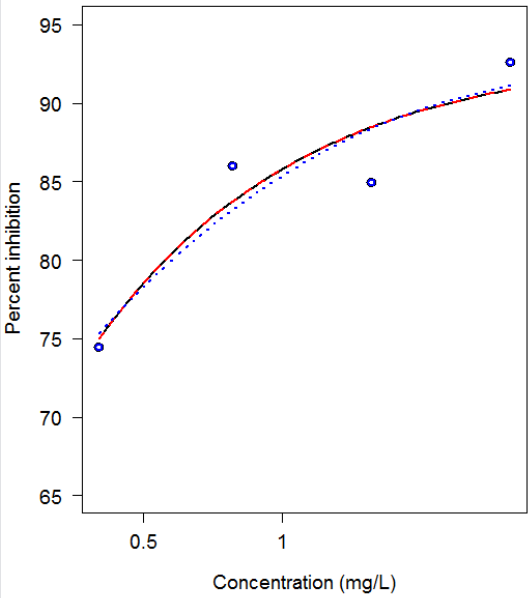
P. subcapitata

Roundup

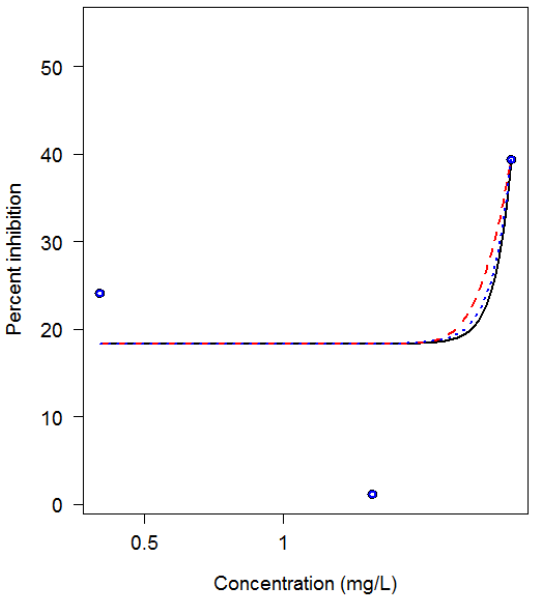
Species	**Model**	**EC_{50}**	**Std. Error**	**Lower Confidence**	**Upper Confidence**
Chlorella protothecoides	Log logistic	4.567	57.667	-117.681	126.813
	Weibull 1	2.639			
	Weibull 2	3.271	18.71	-36.392	42.934
Chlorella sorokiniana	Log logistic	0.025	0.120	-0.226	0.277
	Weibull 1	0.055	0.317	-0.606	0.717
	Weibull 2	1.2353e-02	4.9171e-02	-9.0216e-02	0.1149
Chlorella vulgaris	Log logistic	4.226			
	Weibull 1	5.34			
	Weibull 2	4.445	14.420	-35.593	44.481
Pseudokirchneriella subcapitata	Log logistic	1.429	0.591	0.214	2.644
	Weibull 1	1.322	3.975	-6.835	9.478
	Weibull 2	1.474	0.494	0.460	2.488



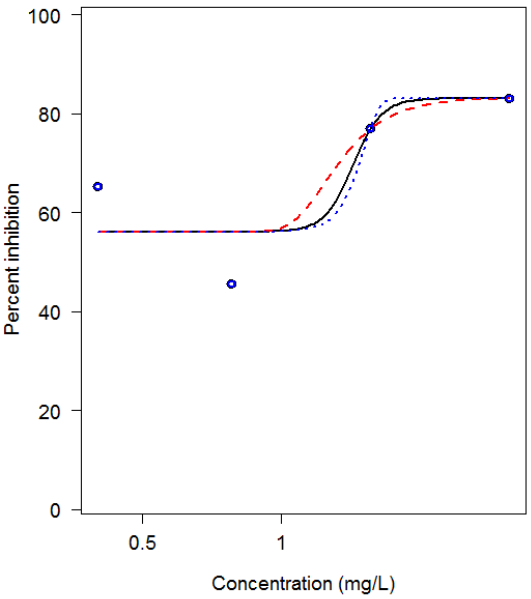
***C. prototheco**ides*



C. sorokiniana



C. vulgaris



P. subcapitata

APPENDIX F

1. Cadmium chloride – **$CdCl_2$**

Plate configuration

Blank	Blank	Blank	Blank	Blank	Blank
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
	Plate 2				
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control

1.1. OD readings at 450nm for *Pseudokirchneriela subcapiatata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 26/09/2011							*OD reading at 96hrs* – 30/09/2011					
Replicate 1							**Replicate 1**					
0.033	0.032	0.033	0.034	0.034	0.035		0.038	0.039	0.039	0.039	0.039	0.038
0.034	0.034	0.034	0.035	0.035	0.037		0.133	0.267	0.269	0.253	0.116	0.218
0.036	0.034	0.034	0.035	0.036	0.036		0.173	0.253	0.311	0.288	0.111	0.219
0.035	0.037	0.035	0.035	0.038	0.038		0.158	0.284	0.251	0.19	0.092	0.169
Mean	**0.035**	**0.034333**	**0.035**	**0.036333**			**Mean**	**0.268**	**0.277**	**0.243667**	**0.106333**	
0.032	0.034	0.033	0.034	0.035	0.033		0.038	0.041	0.039	0.04	0.04	0.038
0.034	0.033	0.034	0.034	0.035	0.036		0.195	0.039	0.044	0.037	0.038	0.183
0.034	0.034	0.034	0.035	0.036	0.036		0.18	0.041	0.04	0.038	0.038	0.197
0.036	0.035	0.034	0.035	0.038	0.037		0.208	0.042	0.041	0.039	0.039	0.187
Mean	**0.034**	**0.034**	**0.034667**	**0.036333**	**0.03575**		**Mean**	**0.040667**	**0.041667**	**0.038**	**0.038333**	**0.185**
Replicate 2							**Replicate 2**					
0.033	0.032	0.034	0.035	0.035	0.036		0.036	0.036	0.038	0.039	0.04	0.04
0.033	0.034	0.034	0.034	0.035	0.036		0.216	0.199	0.191	0.217	0.087	0.154
0.034	0.033	0.034	0.039	0.036	0.036		0.176	0.164	0.17	0.213	0.096	0.173
0.034	0.034	0.035	0.035	0.036	0.036		0.159	0.238	0.173	0.216	0.079	0.128
Mean	**0.033667**	**0.034333**	**0.036**	**0.035667**			**Mean**	**0.200333**	**0.178**	**0.215333**	**0.087333**	
0.032	0.032	0.033	0.033	0.035	0.034		0.037	0.037	0.038	0.039	0.042	0.04
0.033	0.033	0.033	0.034	0.035	0.036		0.202	0.04	0.039	0.037	0.038	0.199

0.033	0.034	0.036	0.034	0.036	0.036		0.219	0.039	0.044	0.038	0.039	0.19
0.036	0.034	0.034	0.035	0.036	0.037		0.225	0.04	0.039	0.041	0.04	0.173
Mean	**0.033667**	**0.034333**	**0.034333**	**0.035667**	**0.035**		**Mean**	**0.039667**	**0.040667**	**0.038667**	**0.039**	**0.1845**
Replicate 3							**Replicate 3**					
0.033	0.032	0.033	0.034	0.034	0.035		0.038	0.036	0.04	0.039	0.038	0.037
0.033	0.033	0.034	0.034	0.035	0.036		0.247	0.252	0.17	0.223	0.087	0.21
0.035	0.034	0.034	0.034	0.036	0.036		0.2	0.211	0.22	0.285	0.084	0.197
0.034	0.034	0.034	0.035	0.035	0.037		0.237	0.258	0.248	0.245	0.057	0.209
Mean	**0.033667**	**0.034**	**0.034333**	**0.035333**			**Mean**	**0.240333**	**0.212667**	**0.251**	**0.076**	
0.031	0.032	0.033	0.033	0.035	0.034		0.036	0.037	0.04	0.042	0.043	0.04
0.033	0.033	0.034	0.034	0.035	0.036		0.26	0.037	0.042	0.046	0.049	0.209
0.034	0.033	0.034	0.035	0.036	0.036		0.267	0.038	0.044	0.042	0.044	0.228
0.036	0.034	0.034	0.035	0.036	0.037		0.271	0.04	0.043	0.041	0.047	0.229
Mean	**0.033333**	**0.034**	**0.034667**	**0.035667**	**0.03525**		**Mean**	**0.038333**	**0.043**	**0.043**	**0.046667**	**0.230333**
Replicate 4							**Replicate 4**					
0.032	0.032	0.033	0.034	0.035	0.033		0.037	0.037	0.038	0.039	0.04	0.037
0.034	0.034	0.034	0.034	0.036	0.037		0.136	0.201	0.201	0.248	0.161	0.176
0.035	0.034	0.034	0.035	0.037	0.037		0.134	0.21	0.223	0.273	0.148	0.17
0.036	0.035	0.036	0.036	0.036	0.038		0.16	0.183	0.175	0.176	0.076	0.151
Mean	**0.034333**	**0.034667**	**0.035**	**0.036333**			**Mean**	**0.198**	**0.199667**	**0.232333**	**0.128333**	
0.033	0.032	0.033	0.034	0.035	0.036		0.037	0.038	0.039	0.04	0.041	0.04
0.033	0.033	0.033	0.034	0.036	0.037		0.139	0.038	0.038	0.037	0.039	0.202
0.033	0.033	0.034	0.035	0.036	0.036		0.145	0.038	0.038	0.038	0.038	0.187
0.034	0.034	0.034	0.035	0.036	0.037		0.151	0.039	0.039	0.04	0.039	0.147
Mean	**0.033333**	**0.033667**	**0.034667**	**0.036**	**0.035583**		**Mean**	**0.038333**	**0.038333**	**0.038333**	**0.038667**	**0.158167**
Replicate 5							**Replicate 5**					
0.034	0.032	0.034	0.034	0.036	0.053		0.039	0.037	0.037	0.041	0.041	0.038
0.033	0.033	0.033	0.034	0.034	0.036		0.216	0.175	0.18	0.244	0.125	0.226
0.034	0.034	0.035	0.036	0.037	0.036		0.228	0.244	0.222	0.247	0.108	0.261
0.035	0.036	0.034	0.035	0.034	0.039		0.228	0.272	0.253	0.209	0.096	0.189
Mean	**0.034333**	**0.034**	**0.035**	**0.035**			**Mean**	**0.230333**	**0.218333**	**0.233333**	**0.109667**	
0.032	0.032	0.033	0.034	0.035	0.034		0.037	0.037	0.039	0.042	0.041	0.039
0.03	0.033	0.033	0.034	0.035	0.036		0.22	0.039	0.039	0.038	0.038	0.275

3							3					
0.033	0.033	0.034	0.035	0.038	0.037		0.226	0.039	0.043	0.038	0.04	0.244
0.034	0.034	0.034	0.035	0.037	0.037		0.283	0.042	0.042	0.042	0.041	0.236
Mean	**0.033333**	**0.033667**	**0.034667**	**0.036667**	**0.03525**		**Mean**	**0.04**	**0.041333**	**0.039333**	**0.039667**	**0.23625**
Replicate 6							**Replicate 6**					
0.033	0.032	0.034	0.034	0.035	0.035		0.038	0.037	0.039	0.04	0.039	0.041
0.033	0.034	0.035	0.035	0.037	0.037		0.213	0.185	0.192	0.258	0.119	0.296
0.033	0.035	0.039	0.035	0.036	0.036		0.193	0.239	0.21	0.245	0.107	0.303
0.034	0.035	0.034	0.035	0.036	0.039		0.299	0.229	0.201	0.239	0.09	0.241
Mean	**0.034667**	**0.036**	**0.035**	**0.036333**			**Mean**	**0.217667**	**0.201**	**0.247333**	**0.105333**	
0.032	0.032	0.034	0.034	0.035	0.034		0.038	0.04	0.04	0.043	0.041	0.039
0.033	0.034	0.034	0.034	0.036	0.036		0.234	0.05	0.04	0.039	0.041	0.349
0.036	0.034	0.035	0.034	0.036	0.037		0.228	0.052	0.044	0.05	0.042	0.338
0.035	0.034	0.035	0.038	0.036	0.038		0.322	0.044	0.042	0.046	0.041	0.243
Mean	**0.034**	**0.034667**	**0.035333**	**0.036**	**0.035583**		**Mean**	**0.048667**	**0.042**	**0.045**	**0.041333**	**0.271583**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
$CdCl_2$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	***-45.645331***	***-9.85604***	***-8.07726***	***-48.9394***	1.830283	29.99354
0.0156	***-51.311647***	4.983389	6.496927	***-50.4545***	7.8203	36.45766
0.0313	***-30.325289***	***-19.8228***	***-13.6962***	***-80.1515***	0.332779	18.48739
0.0625	56.13851	65.22702	78.4899	14.39394	62.06323	73.56173
0.125	97.4816369	96.89922	98.33187	96.21212	96.8386	95.53975
0.25	96.8520462	96.23477	95.87357	96.21212	96.17304	98.1254
0.5	99.1605456	97.56368	95.87357	96.21212	97.17138	96.96186
1	98.9506821	97.34219	93.94205	95.90909	97.00499	98.38397
Coefficient of variation for control	*10.2084168*	*10.99915*	*7.784759*	*6.900485*	*10.43645*	***15.49712***

1.2.OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/10/2011							*OD reading at 96hrs* – 10/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.033	0.035	0.036	0.036	0.039		0.035	0.036	0.037	0.038	0.039	0.042
0.035	0.033	0.033	0.034	0.036	0.037		0.259	0.219	0.314	0.263	0.32	0.261
0.037	0.033	0.035	0.034	0.036	0.037		0.241	0.26	0.289	0.28	0.284	0.253
0.036	0.034	0.034	0.034	0.035	0.037		0.195	0.258	0.214	0.257	0.293	0.179
Mean	**0.033333**	**0.034**	**0.034**	**0.035667**				**0.245667**	**0.272333**	**0.266667**	**0.299**	
0.033	0.037	0.038	0.035	0.035	0.036		0.036	0.04	0.037	0.037	0.038	0.037
0.032	0.035	0.034	0.034	0.034	0.034		0.203	0.135	0.205	0.09	0.066	0.331
0.033	0.033	0.034	0.034	0.035	0.035		0.212	0.16	0.202	0.098	0.066	0.286
0.035	0.035	0.034	0.035	0.035	0.037		0.204	0.216	0.217	0.091	0.06	0.236
Mean	**0.034333**	**0.034**	**0.034333**	**0.034667**	**0.035417**		**Mean**	**0.170333**	**0.208**	**0.093**	**0.064**	**0.238333**
Replicate 2							**Replicate 2**					
0.032	0.033	0.033	0.034	0.034	0.041		0.058	0.037	0.037	0.037	0.037	0.038
0.032	0.033	0.033	0.033	0.034	0.037		0.197	0.224	0.294	0.278	0.336	0.23
0.033	0.033	0.033	0.034	0.036	0.037		0.242	0.297	0.276	0.3	0.482	0.285
0.035	0.034	0.034	0.034	0.035	0.036		0.249	0.271	0.301	0.285	0.317	0.238
Mean	**0.033333**	**0.033333**	**0.033667**	**0.035**			**Mean**	**0.264**	**0.290333**	**0.287667**	**0.378333**	
0.032	0.032	0.034	0.035	0.034	0.036		0.036	0.037	0.037	0.037	0.037	0.037
0.032	0.033	0.033	0.033	0.034	0.035		0.292	0.145	0.182	0.084	0.062	0.227
0.033	0.033	0.033	0.034	0.035	0.036		0.199	0.192	0.162	0.093	0.065	0.315
0.034	0.035	0.033	0.034	0.035	0.037		0.188	0.205	0.216	0.081	0.058	0.199
Mean	**0.033667**	**0.033**	**0.033667**	**0.034667**	**0.03475**		**Mean**	**0.180667**	**0.186667**	**0.086**	**0.061667**	**0.238417**
Replicate 3							**Replicate 3**					

0.032	0.033	0.034	0.035	0.034	0.036		0.036	0.036	0.039	0.039	0.039	0.038
0.033	0.033	0.033	0.034	0.035	0.037		0.209	0.296	0.205	0.292	0.233	0.254
0.035	0.033	0.033	0.034	0.035	0.039		0.182	0.26	0.259	0.42	0.138	0.261
0.034	0.035	0.034	0.034	0.035	0.036		0.165	0.266	0.267	0.356	0.124	0.151
Mean	**0.033667**	**0.033333**	**0.034**	**0.035**			**Mean**	**0.274**	**0.243667**	**0.356**	**0.165**	
0.033	0.032	0.033	0.034	0.034	0.035		0.038	0.037	0.036	0.037	0.04	0.037
0.033	0.033	0.033	0.034	0.035	0.036		0.213	0.121	0.149	0.099	0.066	0.194
0.033	0.033	0.035	0.036	0.037	0.036		0.264	0.228	0.199	0.092	0.062	0.162
0.033	0.033	0.033	0.034	0.035	0.036		0.165	0.16	0.191	0.087	0.067	0.172
Mean	**0.033**	**0.033667**	**0.034667**	**0.035667**	**0.035083**		**Mean**	**0.169667**	**0.179667**	**0.092667**	**0.065**	**0.199333**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.034	0.035	0.034		0.036	0.035	0.037	0.037	0.038	0.036
0.032	0.033	0.033	0.033	0.034	0.035		0.213	0.291	0.382	0.31	0.255	0.169
0.033	0.033	0.033	0.035	0.035	0.036		0.252	0.311	0.386	0.412	0.26	0.158
0.034	0.033	0.035	0.034	0.035	0.036		0.173	0.246	0.235	0.187	0.196	0.22
Mean	**0.033**	**0.033667**	**0.034**	**0.034667**			**Mean**	**0.282667**	**0.334333**	**0.303**	**0.237**	
0.032	0.032	0.034	0.034	0.034	0.037		0.036	0.037	0.036	0.037	0.036	0.037
0.032	0.033	0.034	0.033	0.034	0.036		0.259	0.244	0.197	0.079	0.059	0.255
0.033	0.034	0.033	0.034	0.036	0.036		0.245	0.229	0.187	0.102	0.062	0.19
0.033	0.033	0.034	0.034	0.036	0.036		0.167	0.187	0.156	0.082	0.06	0.226
Mean	**0.033333**	**0.033667**	**0.033667**	**0.035333**	**0.034333**		**Mean**	**0.22**	**0.18**	**0.087667**	**0.060333**	**0.210583**
Replicate 5							**Replicate 5**					
0.032	0.033	0.034	0.035	0.034	0.034		0.035	0.038	0.038	0.038	0.036	0.037
0.032	0.033	0.033	0.034	0.035	0.036		0.274	0.288	0.321	0.409	0.25	0.226
0.0	0.034	0.033	0.034	0.035	0.036		0.3	0.311	0.348	0.352	0.236	0.25

33												
0.035	0.034	0.033	0.034	0.034	0.036		0.32	0.278	0.262	0.288	0.237	0.229
Mean	**0.033667**	**0.033**	**0.034**	**0.034667**			**Mean**	**0.292333**	**0.310333**	**0.349667**	**0.241**	
0.034	0.032	0.033	0.034	0.035	0.033		0.038	0.037	0.037	0.037	0.038	0.036
0.033	0.032	0.033	0.034	0.035	0.037		0.422	0.206	0.282	0.091	0.056	0.204
0.033	0.034	0.034	0.035	0.035	0.036		0.374	0.263	0.208	0.089	0.061	0.213
0.034	0.033	0.034	0.034	0.035	0.036		0.227	0.3	0.173	0.089	0.06	0.183
Mean	**0.033**	**0.033667**	**0.034333**	**0.035**	**0.03475**		**Mean**	**0.256333**	**0.221**	**0.089667**	**0.059**	**0.2685**
Replicate 6							**Replicate 6**					
0.033	0.033	0.036	0.034	0.034	0.04		0.036	0.038	0.04	0.038	0.039	0.037
0.032	0.033	0.035	0.033	0.034	0.035		0.303	0.293	0.327	0.307	0.221	0.158
0.033	0.034	0.034	0.034	0.036	0.037		0.282	0.343	0.403	0.379	0.214	0.176
0.033	0.034	0.034	0.034	0.035	0.038		0.244	0.254	0.325	0.245	0.148	0.139
Mean	**0.033667**	**0.034333**	**0.033667**	**0.035**			**Mean**	**0.296667**	**0.351667**	**0.310333**	**0.194333**	
0.034	0.033	0.034	0.038	0.034	0.037		0.042	0.037	0.038	0.041	0.039	0.038
0.035	0.032	0.033	0.034	0.035	0.038		0.36	0.302	0.29	0.098	0.061	0.23
0.033	0.033	0.033	0.036	0.039	0.037		0.366	0.369	0.281	0.091	0.06	0.217
0.034	0.033	0.033	0.037	0.036	0.04		0.243	0.315	0.221	0.089	0.062	0.228
Mean	**0.032667**	**0.033**	**0.035667**	**0.036667**	**0.035417**		**Mean**	**0.328667**	**0.264**	**0.092667**	**0.061**	**0.2455**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheocoides*

$CdCl_2$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	***-14.4665***	***-7.05329***	***-64.0732***	***-35.118***	***-41.2684***	***-21.3788***
0.156	***-28.9331***	***-19.4357***	***-43.2494***	***-63.2486***	***-51.1949***	***-46.9404***
0.313	***-25.859***	***-18.1818***	***-120.366***	***-46.1887***	***-72.886***	***-27.7304***
0.0625	***-43.3996***	***-60.815***	10.75515	***-10.2541***	***-12.9596***	26.18125
0.125	26.40145	32.13166	7.551487	***-0.99819***	***-21.4154***	***-36.251***
0.25	5.96745	29.31034	0.686499	20.7804	***-1.93015***	***-6.19675***
0.5	68.35443	76.64577	60.4119	71.05263	70.49632	73.43145
1	84.0868	88.08777	79.40503	85.93466	87.40809	88.14872
Coefficient of variation for control	*11.53364*	***17.15005***	*10.05899*	***15.09523***	*10.59169*	*13.63327*

1.3.OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 11/08/2011							*OD Readings at 96 hrs* – 14/08/2011					
Replicate 1							**Replicate 1**					
0.032	0.033	0.034	0.037	0.038	0.037		0.036	0.039	0.039	0.042	0.04	0.037
0.037	0.038	0.038	0.037	0.038	0.038		0.197	0.061	0.058	0.051	0.047	0.118
0.037	0.038	0.039	0.04	0.039	0.038		0.213	0.058	0.063	0.052	0.047	0.091
0.036	0.037	0.038	0.037	0.037	0.038		0.146	0.055	0.053	0.049	0.048	0.083
Mean	**0.037667**	**0.038333**	**0.038**	**0.038**			**Mean**	**0.058**	**0.058**	**0.050667**	**0.047333**	
0.032	0.034	0.033	0.035	0.035	0.035		0.04	0.038	0.04	0.037	0.039	0.037
0.035	0.04	0.037	0.036	0.036	0.04		0.156	0.04	0.046	0.045	0.04	0.21
0.039	0.039	0.037	0.036	0.037	0.049		0.179	0.04	0.052	0.044	0.04	0.236
0.037	0.038	0.038	0.036	0.035	0.041		0.201	0.039	0.047	0.042	0.04	0.186
Mean	**0.039**	**0.037333**	**0.036**	**0.036**	**0.03875**		**Mean**	**0.039667**	**0.048333**	**0.043667**	**0.04**	**0.168**
Replicate 2							**Replicate 2**					
0.032	0.031	0.033	0.035	0.034	0.034		0.038	0.038	0.039	0.04	0.041	0.037
0.037	0.037	0.037	0.037	0.037	0.037		0.189	0.055	0.053	0.063	0.048	0.132
0.036	0.038	0.039	0.039	0.039	0.039		0.174	0.057	0.056	0.053	0.047	0.16
0.0	0.038	0.038	0.037	0.038	0.04		0.1	0.062	0.055	0.048	0.05	0.136

36							68					
Mean	**0.037667**	**0.038**	**0.037667**	**0.038**			**Mean**	**0.058**	**0.054667**	**0.054667**	**0.048333**	
0.032	0.032	0.032	0.033	0.034	0.034		0.036	0.038	0.036	0.038	0.037	0.045
0.037	0.036	0.037	0.034	0.034	0.038		0.201	0.04	0.047	0.044	0.05	0.141
0.037	0.038	0.04	0.035	0.035	0.037		0.218	0.041	0.041	0.042	0.044	0.123
0.036	0.036	0.036	0.035	0.034	0.038		0.149	0.041	0.046	0.043	0.039	0.089
Mean	**0.036667**	**0.037667**	**0.034667**	**0.034333**	**0.037333**		**Mean**	**0.040667**	**0.044667**	**0.043**	**0.044333**	**0.156667**
Replicate 3							**Replicate 3**					
0.031	0.032	0.033	0.035	0.034	0.034		0.036	0.069	0.058	0.041	0.042	0.037
0.035	0.035	0.037	0.032	0.042	0.038		0.188	0.063	0.06	0.033	0.053	0.183
0.036	0.036	0.038	0.033	0.041	0.04		0.152	0.065	0.062	0.036	0.05	0.192
0.034	0.037	0.039	0.038	0.041	0.039		0.173	0.067	0.064	0.052	0.051	0.126
Mean	**0.036**	**0.038**	**0.034333**	**0.041333**			**Mean**	**0.065**	**0.062**	**0.040333**	**0.051333**	
0.031	0.032	0.033	0.033	0.034	0.033		0.036	0.037	0.036	0.04	0.037	0.038
0.034	0.038	0.036	0.034	0.035	0.039		0.163	0.047	0.047	0.042	0.036	0.19
0.035	0.038	0.037	0.035	0.036	0.039		0.137	0.046	0.045	0.043	0.039	0.193
0.035	0.038	0.036	0.036	0.036	0.04		0.113	0.045	0.046	0.041	0.037	0.124
Mean	**0.038**	**0.036333**	**0.035**	**0.035667**	**0.037**		**Mean**	**0.046**	**0.046**	**0.042**	**0.037333**	**0.161167**
Replicate 4							**Replicate 4**					
0.032	0.033	0.033	0.034	0.035	0.036		0.038	0.039	0.042	0.044	0.043	0.04
0.035	0.038	0.036	0.038	0.04	0.036		0.116	0.069	0.055	0.055	0.06	0.079
0.036	0.037	0.037	0.039	0.041	0.038		0.138	0.072	0.054	0.052	0.051	0.147
0.036	0.04	0.043	0.039	0.039	0.037		0.115	0.068	0.066	0.052	0.049	0.102
Mean	**0.038333**	**0.038667**	**0.038667**	**0.04**			**Mean**	**0.069667**	**0.058333**	**0.053**	**0.053333**	
0.032	0.032	0.034	0.034	0.035	0.054		0.037	0.032	0.035	0.034	0.034	0.039
0.0	0.036	0.035	0.033	0.034	0.039		0.1	0.051	0.052	0.045	0.038	0.06

34						23					
0.035	0.038	0.037	0.036	0.037	0.04	0.111	0.049	0.049	0.049	0.049	0.096
0.035	0.038	0.036	0.034	0.036	0.04	0.113	0.047	0.047	0.057	0.044	0.07
Mean	**0.037333**	**0.036**	**0.034333**	**0.035667**	**0.03675**	**Mean**	**0.049**	**0.049333**	**0.050333**	**0.043667**	**0.105833**
Replicate 5						**Replicate 5**					
0.031	0.032	0.033	0.034	0.035	0.033	0.039	0.036	0.043	0.044	0.048	0.041
0.036	0.037	0.036	0.04	0.041	0.037	0.121	0.068	0.062	0.056	0.056	0.179
0.036	0.037	0.037	0.037	0.04	0.038	0.143	0.063	0.07	0.057	0.054	0.18
0.036	0.037	0.038	0.037	0.041	0.038	0.088	0.059	0.056	0.05	0.055	0.099
Mean	**0.037**	**0.037**	**0.038**	**0.040667**		**Mean**	**0.063333**	**0.062667**	**0.054333**	**0.055**	
0.033	0.032	0.033	0.034	0.034	0.059	0.036	0.032	0.034	0.034	0.034	0.038
0.045	0.037	0.036	0.033	0.034	0.038	0.09	0.049	0.05	0.046	0.037	0.104
0.035	0.037	0.036	0.035	0.038	0.038	0.099	0.046	0.047	0.045	0.043	0.097
0.035	0.038	0.037	0.035	0.035	0.039	0.082	0.047	0.044	0.046	0.037	0.09
Mean	**0.037333**	**0.036333**	**0.034333**	**0.035667**	**0.037583**	**Mean**	**0.047333**	**0.047**	**0.045667**	**0.039**	**0.114333**
Replicate 6						**Replicate 6**					
0.032	0.034	0.033	0.034	0.035	0.037	0.037	0.038	0.043	0.042	0.045	0.041
0.04	0.038	0.037	0.037	0.039	0.038	0.271	0.069	0.063	0.05	0.053	0.255
0.037	0.039	0.039	0.038	0.039	0.04	0.241	0.065	0.068	0.058	0.063	0.209
0.04	0.037	0.037	0.037	0.038	0.038	0.207	0.055	0.05	0.049	0.054	0.109
Mean	**0.038**	**0.037667**	**0.037333**	**0.038667**		**Mean**	**0.063**	**0.060333**	**0.052333**	**0.056667**	
0.032	0.032	0.034	0.035	0.035	0.046	0.036	0.033	0.037	0.034	0.035	0.038
0.034	0.037	0.036	0.033	0.034	0.037	0.078	0.053	0.052	0.053	0.043	0.099
0.055	0.038	0.038	0.038	0.037	0.038	0.075	0.047	0.05	0.049	0.043	0.11
0.035	0.041	0.036	0.034	0.035	0.038	0.058	0.051	0.048	0.043	0.039	0.073
Mean	**0.038667**	**0.036667**	**0.035**	**0.035333**	**0.039167**	**Mean**	**0.050333**	**0.05**	**0.048333**	**0.041667**	**0.14875**

96 hour % Growth Inhibition (+) on *C. sorokiniana*						
$CdCl_2$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	85.41906	85.34091	79.94825	59.0099	62.61905	76.19808
0.0156	85.41906	87.61364	82.27684	72.47525	63.57143	78.75399
0.0313	90.47072	87.61364	99.09444	78.81188	75.47619	86.42173
0.0625	92.76693	91.93182	90.55627	78.41584	74.52381	82.26837
0.125	98.04822	97.15909	94.69599	83.56436	85.47619	88.33866
0.25	92.07807	94.43182	94.69599	83.16832	85.95238	88.65815
0.5	95.29277	95.56818	97.80078	81.9802	87.85714	90.25559
1	97.8186	94.65909	101.423	89.90099	97.38095	96.64537
Coefficient of variation for control	*14.59513*	*13.47699*	***19.93826***	*8.391054*	***19.79826***	***50.58427***

1.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 11/08/2011							*OD Readings at 0 hrs* – 11/08/2011					
Replicate 1							**Replicate 1**					
0.031	0.032	0.033	0.033	0.034	0.035		0.035	0.035	0.037	0.035	0.037	0.037
0.033	0.032	0.033	0.033	0.035	0.036		0.081	0.091	0.085	0.057	0.052	0.078
0.033	0.033	0.033	0.034	0.038	0.036		0.094	0.105	0.08	0.057	0.054	0.078
0.034	0.034	0.034	0.045	0.035	0.036		0.114	0.083	0.07	0.115	0.049	0.071
Mean	**0.033**	**0.033333**	**0.037333**	**0.036**			**Mean**	**0.093**	**0.078333**	**0.076333**	**0.051667**	
0.032	0.032	0.033	0.034	0.034	0.034		0.037	0.035	0.038	0.037	0.037	0.037
0.043	0.033	0.033	0.034	0.033	0.04		0.076	0.049	0.044	0.049	0.042	0.097
0.033	0.034	0.037	0.036	0.036	0.042		0.083	0.054	0.064	0.053	0.045	0.094
0.034	0.033	0.034	0.035	0.035	0.037		0.071	0.05	0.048	0.054	0.043	0.078
Mean	**0.033333**	**0.034667**	**0.035**	**0.034667**	**0.036417**		**Mean**	**0.051**	**0.052**	**0.052**	**0.043333**	**0.084583**
Replicate 2							**Replicate 2**					
0.031	0.032	0.033	0.033	0.034	0.034		0.038	0.035	0.038	0.036	0.038	0.037
0.033	0.043	0.036	0.034	0.035	0.04		0.096	0.096	0.129	0.062	0.054	0.177
0.034	0.033	0.033	0.034	0.035	0.036		0.102	0.098	0.08	0.062	0.051	0.085
0.03	0.033	0.034	0.034	0.037	0.04		0.04	0.051	0.059	0.054	0.049	0.054

3						1					
Mean	**0.036333**	**0.034333**	**0.034**	**0.035667**		**Mean**	**0.081667**	**0.089333**	**0.059333**	**0.051333**	
0.031	0.032	0.033	0.034	0.035	0.034	0.035	0.037	0.045	0.036	0.038	0.037
0.047	0.032	0.032	0.034	0.035	0.036	0.088	0.049	0.032	0.057	0.046	0.135
0.033	0.034	0.033	0.035	0.037	0.037	0.081	0.051	0.033	0.054	0.049	0.093
0.033	0.034	0.033	0.034	0.035	0.036	0.07	0.051	0.033	0.054	0.048	0.078
Mean	**0.033333**	**0.032667**	**0.034333**	**0.035667**	**0.0365**	**Mean**	**0.050333**	**0.032667**	**0.055**	**0.047667**	**0.091667**
Replicate 3						**Replicate 3**					
0.034	0.032	0.033	0.034	0.035	0.033	0.039	0.036	0.037	0.036	0.037	0.036
0.032	0.034	0.033	0.033	0.035	0.036	0.08	0.109	0.099	0.066	0.056	0.09
0.034	0.046	0.033	0.034	0.036	0.037	0.089	0.099	0.074	0.068	0.056	0.089
0.037	0.034	0.034	0.034	0.036	0.036	0.147	0.114	0.069	0.058	0.059	0.075
Mean	**0.038**	**0.033333**	**0.033667**	**0.035667**		**Mean**	**0.107333**	**0.080667**	**0.064**	**0.057**	
0.034	0.031	0.033	0.033	0.035	0.04	0.037	0.036	0.038	0.038	0.038	0.042
0.033	0.033	0.034	0.035	0.034	0.043	0.109	0.048	0.049	0.047	0.042	0.079
0.034	0.033	0.034	0.037	0.039	0.062	0.138	0.046	0.047	0.049	0.044	0.084
0.034	0.034	0.036	0.037	0.036	0.058	0.093	0.052	0.05	0.053	0.045	0.079
Mean	**0.033333**	**0.034667**	**0.036333**	**0.036333**	**0.039667**	**Mean**	**0.048667**	**0.048667**	**0.049667**	**0.043667**	**0.096**
Replicate 4						**Replicate 4**					
0.035	0.032	0.033	0.035	0.035	0.046	0.036	0.035	0.036	0.036	0.037	0.037
0.033	0.033	0.033	0.033	0.034	0.035	0.077	0.089	0.067	0.054	0.056	0.079
0.033	0.034	0.034	0.034	0.036	0.035	0.077	0.08	0.068	0.053	0.048	0.078
0.033	0.034	0.033	0.034	0.034	0.038	0.073	0.077	0.063	0.057	0.05	0.07
Mean	**0.033667**	**0.033333**	**0.033667**	**0.034667**		**Mean**	**0.082**	**0.066**	**0.054667**	**0.051333**	
0.034	0.032	0.034	0.033	0.034	0.035	0.036	0.036	0.038	0.037	0.037	0.043
0.05	0.035	0.035	0.034	0.035	0.039	0.23	0.054	0.054	0.051	0.045	0.074

							6					
0.033	0.04	0.034	0.035	0.036	0.035		0.112	0.073	0.049	0.053	0.049	0.074
0.035	0.034	0.038	0.035	0.036	0.037		0.158	0.051	0.068	0.052	0.048	0.077
Mean	**0.036333**	**0.035667**	**0.034667**	**0.035667**	**0.036333**		**Mean**	**0.059333**	**0.057**	**0.052**	**0.047333**	**0.09875**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.038	0.035	0.058		0.038	0.036	0.036	0.037	0.043	0.044
0.032	0.032	0.033	0.033	0.034	0.042		0.045	0.082	0.124	0.061	0.082	0.094
0.032	0.033	0.033	0.034	0.036	0.035		0.045	0.093	0.075	0.058	0.051	0.051
0.033	0.033	0.033	0.034	0.034	0.035		0.044	0.052	0.051	0.05	0.046	0.052
Mean	**0.032667**	**0.033**	**0.033667**	**0.034667**			**Mean**	**0.075667**	**0.083333**	**0.056333**	**0.059667**	
0.031	0.032	0.032	0.033	0.035	0.055		0.036	0.036	0.036	0.037	0.036	0.038
0.043	0.034	0.033	0.036	0.035	0.035		0.154	0.049	0.046	0.056	0.045	0.074
0.033	0.033	0.033	0.036	0.036	0.035		0.066	0.052	0.046	0.059	0.049	0.08
0.033	0.035	0.036	0.036	0.035	0.037		0.07	0.062	0.066	0.056	0.046	0.072
Mean	**0.034**	**0.034**	**0.036**	**0.035333**	**0.035417**		**Mean**	**0.054333**	**0.052667**	**0.057**	**0.046667**	**0.070583**
Replicate 6							**Replicate 6**					
0.031	0.031	0.033	0.038	0.034	0.042		0.037	0.035	0.036	0.036	0.036	0.039
0.034	0.033	0.032	0.035	0.034	0.034		0.059	0.08	0.066	0.057	0.051	0.075
0.033	0.038	0.035	0.035	0.035	0.035		0.053	0.079	0.063	0.063	0.048	0.059
0.033	0.033	0.034	0.034	0.035	0.035		0.064	0.08	0.063	0.055	0.046	0.057
Mean	**0.034667**	**0.033667**	**0.034667**	**0.034667**			**Mean**	**0.079667**	**0.064**	**0.058333**	**0.048333**	
0.032	0.033	0.033	0.034	0.035	0.048		0.042	0.036	0.041	0.039	0.039	0.041
0.041	0.033	0.033	0.035	0.034	0.053		0.141	0.052	0.047	0.055	0.046	0.317
0.034	0.034	0.033	0.037	0.037	0.036		0.074	0.051	0.056	0.067	0.05	0.08
0.034	0.034	0.034	0.036	0.035	0.035		0.07	0.053	0.049	0.053	0.048	0.071
Mean	**0.033667**	**0.033333**	**0.036**	**0.035333**	**0.036417**		**Mean**	**0.052**	**0.050667**	**0.058333**	**0.048**	**0.093333**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
$CdCl_2$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	***-4.91249E-14***	16.36905	2.654867	***-16.5289***	***-15.1961***	***-0.76046***
0.156	25.95870206	2.678571	38.0531	23.1405	***-37.7451***	34.98099
0.313	29.49852507	56.25	60.17699	51.23967	41.66667	47.90875
0.0625	73.15634218	70.53571	69.46903	59.50413	31.86275	70.72243
0.125	74.33628319	72.32143	80.53097	39.66942	47.54902	62.35741
0.25	72.56637168	103.869	80.53097	45.45455	52.45098	65.39924
0.5	72.56637168	63.9881	79.20354	57.85124	39.70588	47.90875
1	87.90560472	77.08333	87.16814	69.42149	70.09804	71.48289
Coefficient of variation for control	13.83716812	***33.94981***	***25.15272***	2.471547	***23.56062***	***39.25548***

2. Potassium dichromate – **$K_2Cr_2O_7$**

Plate configuration

Blank	Blank	Blank	Blank	Blank	Blank
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
Control	0.007 mg/L	0.156 mg/L	0.313 mg/L	0.0625 mg/L	Control
	Plate 2				
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control
Control	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	Control

2.1. OD readings at 450nm for *Pseudokirchneriela subcapiatata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 26/09/2011							*OD Readings at 96 hrs* – 30/09/2011					
Replicate 1							**Replicate 1**					
0.032	0.035	0.034	0.034	0.034	0.05		0.037	0.04	0.039	0.039	0.04	0.039
0.033	0.034	0.034	0.034	0.035	0.036		0.2	0.041	0.323	0.328	0.337	0.293
0.034	0.034	0.035	0.035	0.037	0.037		0.238	0.041	0.241	0.218	0.232	0.306
0.03	0.035	0.038	0.035	0.036	0.038		0.23	0.043	0.236	0.183	0.148	0.197

4							3					
Mean	**0.034333**	**0.035667**	**0.034667**	**0.036**			**Mean**	**0.041667**	**0.266667**	**0.243**	**0.239**	
0.032	0.035	0.034	0.035	0.036	0.051		0.039	0.041	0.04	0.041	0.04	0.042
0.037	0.035	0.034	0.035	0.037	0.037		0.239	0.206	0.164	0.04	0.04	0.27
0.034	0.034	0.034	0.035	0.038	0.037		0.24	0.229	0.163	0.041	0.04	0.272
0.034	0.035	0.036	0.036	0.038	0.037		0.281	0.281	0.104	0.043	0.043	0.234
Mean	**0.034667**	**0.034667**	**0.035333**	**0.037667**	**0.035667**		**Mean**	**0.238667**	**0.143667**	**0.041333**	**0.041**	**0.25025**
Replicate 2							**Replicate 2**					
0.034	0.034	0.035	0.034	0.035	0.051		0.038	0.04	0.041	0.039	0.039	0.039
0.033	0.035	0.034	0.034	0.035	0.036		0.22	0.04	0.236	0.218	0.207	0.241
0.034	0.034	0.035	0.035	0.036	0.036		0.23	0.04	0.215	0.214	0.19	0.203
0.035	0.037	0.036	0.035	0.036	0.037		0.197	0.047	0.223	0.198	0.171	0.211
0.032	0.033	0.034	0.035	0.036	0.039							
0.034	0.033	0.034	0.035	0.037	0.036		***Plate 2 spilled before the reading***					
0.034	0.034	0.034	0.035	0.038	0.036							
0.033	0.034	0.035	0.036	0.038	0.038							
Replicate 3							**Replicate 3**					
0.032	0.032	0.034	0.034	0.036	0.035		0.039	0.039	0.04	0.039	0.038	0.04
0.034	0.034	0.034	0.034	0.035	0.036		0.202	0.04	0.238	0.2	0.188	0.254
0.034	0.034	0.035	0.035	0.036	0.036		0.26	0.046	0.244	0.229	0.223	0.249
0.034	0.036	0.034	0.035	0.036	0.037		0.261	0.043	0.228	0.205	0.188	0.197
Mean	**0.034667**	**0.034333**	**0.034667**	**0.035667**			**Mean**	**0.043**	**0.236667**	**0.211333**	**0.199667**	
0.033	0.033	0.034	0.035	0.036	0.043		0.042	0.042	0.04	0.04	0.043	0.047
0.033	0.034	0.034	0.035	0.038	0.038		0.231	0.226	0.167	0.042	0.042	0.238
0.034	0.034	0.035	0.035	0.038	0.037		0.251	0.24	0.186	0.045	0.044	0.234

0.035	0.038	0.035	0.038	0.039	0.037		0.333	0.269	0.215	0.044	0.045	0.251
Mean	**0.035333**	**0.034667**	**0.036**	**0.038333**	**0.035417**		**Mean**	**0.245**	**0.189333**	**0.043667**	**0.043667**	**0.24675**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.035	0.034	0.039		0.037	0.037	0.038	0.04	0.04	0.039
0.034	0.034	0.034	0.034	0.035	0.036		0.237	0.04	0.298	0.3	0.273	0.285
0.034	0.034	0.034	0.035	0.036	0.037		0.199	0.039	0.281	0.291	0.264	0.33
0.037	0.034	0.035	0.035	0.036	0.038		0.231	0.042	0.225	0.225	0.233	0.252
Mean	**0.034**	**0.034333**	**0.034667**	**0.035667**			**Mean**	**0.040333**	**0.268**	**0.272**	**0.256667**	
0.037	0.034	0.034	0.035	0.037	0.033		0.041	0.042	0.038	0.04	0.04	0.039
0.033	0.034	0.035	0.035	0.038	0.036		0.198	0.249	0.185	0.044	0.04	0.238
0.034	0.036	0.035	0.035	0.038	0.037		0.174	0.249	0.168	0.042	0.041	0.322
0.035	0.035	0.035	0.036	0.038	0.037		0.217	0.254	0.154	0.055	0.04	0.281
Mean	**0.035**	**0.035**	**0.035333**	**0.038**	**0.035667**		**Mean**	**0.250667**	**0.169**	**0.047**	**0.040333**	**0.247**
Replicate 5							**Replicate 5**					
0.032	0.032	0.034	0.035	0.035	0.052		0.037	0.037	0.037	0.039	0.038	0.04
0.033	0.034	0.035	0.036	0.035	0.036		0.184	0.039	0.258	0.185	0.214	0.162
0.034	0.034	0.034	0.035	0.036	0.037		0.203	0.04	0.25	0.193	0.208	0.202
0.034	0.034	0.035	0.037	0.036	0.037		0.204	0.041	0.23	0.209	0.191	0.179
Mean	**0.034**	**0.034667**	**0.036**	**0.035667**			**Mean**	**0.04**	**0.246**	**0.195667**	**0.204333**	
0.032	0.032	0.034	0.035	0.036	0.035		0.039	0.037	0.039	0.04	0.04	0.042
0.033	0.034	0.035	0.035	0.037	0.036		0.185	0.207	0.165	0.042	0.039	0.207
0.034	0.034	0.036	0.037	0.038	0.037		0.225	0.228	0.186	0.048	0.04	0.227
0.035	0.035	0.035	0.035	0.038	0.039		0.221	0.23	0.158	0.058	0.04	0.147
Mean	**0.034333**	**0.035333**	**0.035667**	**0.037667**	**0.035417**		**Mean**	**0.221667**	**0.169667**	**0.049333**	**0.039667**	**0.1955**
Replicate 6							**Replicate 6**					

0.034	0.034	0.037	0.034	0.034	0.035		0.039	0.038	0.041	0.04	0.039	0.04
0.034	0.033	0.034	0.035	0.035	0.036		0.217	0.039	0.213	0.222	0.205	0.202
0.034	0.034	0.034	0.035	0.037	0.036		0.245	0.042	0.259	0.181	0.217	0.212
0.034	0.035	0.034	0.035	0.036	0.041		0.213	0.042	0.254	0.199	0.169	0.193
Mean	**0.034**	**0.034**	**0.035**	**0.036**			**Mean**	**0.041**	**0.242**	**0.200667**	**0.197**	
0.032	0.033	0.035	0.036	0.035	0.036		0.039	0.039	0.042	0.042	0.041	0.041
0.034	0.035	0.034	0.036	0.037	0.036		0.221	0.217	0.151	0.045	0.039	0.223
0.034	0.047	0.035	0.039	0.04	0.037		0.256	0.25	0.166	0.081	0.042	0.206
0.034	0.035	0.036	0.035	0.039	0.039		0.209	0.221	0.163	0.053	0.041	0.211
Mean	**0.039**	**0.035**	**0.036667**	**0.038667**	**0.03575**		**Mean**	**0.229333**	**0.16**	**0.059667**	**0.040667**	**0.217333**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*					
$K_2Cr_2O_7$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*
0.0156	***-13.7959***	8.845577	***-7.73395***	***-24.9264***	***-7.86517***
0.0313	***-2.20408***	20.23988	***-9.5901***	4.7105	13.56958
0.0625	***-0.2449***	25.48726	***-2.47486***	***-0.39254***	15.47105
0.125	***-0.08163***	5.097451	0.309358	***-10.5986***	***-1.29646***
0.25	46.44898	30.13493	38.20572	20.01963	34.6586
0.5	96.57143	95.65217	94.81825	90.87341	86.68971
1	96.73469	95.65217	97.91183	96.56526	96.54278
Coefficient of variation for control	10.80672	***17.0364***	***17.26014***	8.479737	8.412851

2.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/10/2011							*OD Readings at 96 hrs* – 10/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.035	0.036	0.035	0.045		0.036	0.036	0.037	0.039	0.037	0.041
0.036	0.033	0.033	0.034	0.035	0.035		0.372	0.35	0.348	0.316	0.338	0.307
0.034	0.033	0.034	0.035	0.038	0.038		0.251	0.385	0.392	0.386	0.35	0.345
0.04	0.034	0.039	0.034	0.034	0.036		0.255	0.251	0.311	0.249	0.345	0.351
0.035333	**0.033333**	**0.035333**	**0.034333**	**0.035667**	**Mean**		**Mean**	**0.328667**	**0.350333**	**0.317**	**0.344333**	

0.033	0.034	0.034	0.035	0.035	0.04		0.037	0.037	0.038	0.037	0.039	0.037
0.032	0.034	0.034	0.034	0.036	0.034		0.291	0.335	0.142	0.097	0.047	0.304
0.034	0.034	0.034	0.035	0.038	0.036		0.211	0.266	0.185	0.093	0.041	0.392
0.033	0.034	0.036	0.035	0.038	0.036		0.274	0.265	0.157	0.079	0.049	0.317
Mean	**0.034**	**0.034667**	**0.034667**	**0.037333**			Mean	0.288667	0.161333	0.089667	0.045667	**0.305833**
Replicate 2							**Replicate 2**					
0.034	0.033	0.034	0.035	0.035	0.049		0.037	0.036	0.036	0.038	0.037	0.039
0.033	0.033	0.033	0.034	0.034	0.037		0.273	0.424	0.409	0.331	0.371	0.296
0.036	0.038	0.034	0.035	0.037	0.036		0.26	0.423	0.398	0.388	0.29	0.3
0.035	0.034	0.034	0.034	0.036	0.037		0.175	0.277	0.352	0.392	0.288	0.311
0.035167	**0.035**	**0.033667**	**0.034333**	**0.035667**	**Mean**		**Mean**	**0.374667**	**0.386333**	**0.370333**	**0.316333**	
0.032	0.035	0.033	0.035	0.036	0.034		0.036	0.037	0.036	0.038	0.037	0.038
0.034	0.034	0.033	0.035	0.036	0.035		0.285	0.3	0.168	0.08	0.044	0.341
0.033	0.034	0.034	0.035	0.037	0.036		0.239	0.336	0.159	0.091	0.045	0.251
0.033	0.034	0.034	0.035	0.037	0.037		0.193	0.253	0.185	0.077	0.043	0.264
Mean	**0.034**	**0.033667**	**0.035**	**0.036667**			**Mean**	**0.296333**	**0.170667**	**0.082667**	**0.044**	**0.265667**
Replicate 3							**Replicate 3**					
0.032	0.032	0.034	0.034	0.034	0.047		0.036	0.035	0.036	0.036	0.036	0.039
0.035	0.035	0.033	0.034	0.034	0.035		0.189	0.274	0.414	0.317	0.28	0.232
0.034	0.034	0.034	0.035	0.036	0.037		0.244	0.315	0.393	0.351	0.449	0.274
0.035	0.035	0.034	0.034	0.036	0.036		0.191	0.256	0.351	0.318	0.299	0.247
0.034917	**0.034667**	**0.033667**	**0.034333**	**0.035333**	**Mean**		**Mean**	**0.281667**	**0.386**	**0.328667**	**0.342667**	
0.032	0.032	0.034	0.035	0.037	0.034		0.036	0.036	0.037	0.037	0.038	0.038
0.034	0.033	0.033	0.034	0.037	0.035		0.209	0.298	0.168	0.069	0.044	0.264
0.033	0.033	0.034	0.036	0.037	0.036		0.253	0.262	0.194	0.088	0.044	0.245
0.033	0.03	0.035	0.035	0.037	0.0		0.1	0.184	0.122	0.07	0.043	0.219

	4				36		6					
Mean	0.033333	0.034	0.035	0.037			**Mean**	0.248	0.161333	0.075667	0.043667	**0.22725**
Replicate 4							**Replicate 4**					
0.038	0.034	0.035	0.036	0.034	0.035		0.036	0.036	0.037	0.04	0.037	0.038
0.035	0.035	0.034	0.033	0.034	0.035		0.239	0.396	0.397	0.322	0.277	0.247
0.033	0.034	0.035	0.036	0.039	0.038		0.328	0.247	0.375	0.385	0.274	0.306
0.035	0.034	0.036	0.034	0.035	0.036		0.167	0.239	0.287	0.281	0.283	0.277
0.035083	**0.034333**	**0.035**	**0.034333**	**0.036**	**Mean**		**Mean**	**0.294**	**0.353**	**0.329333**	**0.278**	
0.036	0.033	0.034	0.038	0.037	0.034		0.041	0.036	0.037	0.04	0.039	0.038
0.035	0.036	0.033	0.034	0.04	0.035		0.329	0.29	0.211	0.094	0.048	0.212
0.033	0.035	0.034	0.035	0.037	0.036		0.197	0.306	0.21	0.079	0.044	0.247
0.033	0.033	0.034	0.035	0.037	0.037		0.11	0.17	0.182	0.077	0.044	0.17
Mean	**0.034667**	**0.033667**	**0.034667**	**0.038**			**Mean**	**0.255333**	**0.201**	**0.083333**	**0.045333**	**0.23575**
Replicate 5							**Replicate 5**					
0.032	0.033	0.035	0.034	0.034	0.035		0.035	0.035	0.037	0.037	0.036	0.037
0.033	0.034	0.033	0.033	0.034	0.035		0.242	0.283	0.404	0.379	0.345	0.173
0.034	0.034	0.034	0.035	0.036	0.036		0.216	0.406	0.397	0.346	0.29	0.181
0.033	0.035	0.035	0.034	0.036	0.036		0.128	0.239	0.269	0.278	0.233	0.213
0.034833	**0.034333**	**0.034**	**0.034**	**0.035333**	**Mean**		**Mean**	**0.309333**	**0.356667**	**0.334333**	**0.289333**	
0.032	0.032	0.036	0.036	0.036	0.035		0.037	0.035	0.038	0.037	0.037	0.039
0.034	0.034	0.037	0.034	0.036	0.036		0.417	0.276	0.147	0.088	0.041	0.301
0.035	0.036	0.034	0.036	0.037	0.036		0.234	0.249	0.18	0.065	0.04	0.274
0.033	0.033	0.034	0.035	0.037	0.037		0.165	0.219	0.106	0.061	0.042	0.184
Mean	**0.034333**	**0.035**	**0.035**	**0.036667**			**Mean**	**0.248**	**0.144333**	**0.071333**	**0.041**	**0.227333**
Replicate 6							**Replicate 6**					
0.033	0.03	0.035	0.035	0.037	0.0		0.0	0.036	0.036	0.038	0.038	0.038

	3				44		36					
0.037	0.035	0.033	0.034	0.034	0.035		0.352	0.246	0.363	0.303	0.237	0.278
0.034	0.034	0.035	0.035	0.036	0.036		0.264	0.293	0.321	0.405	0.262	0.26
0.034	0.035	0.037	0.036	0.038	0.037		0.392	0.348	0.316	0.415	0.388	0.209
0.035333	**0.034667**	**0.035**	**0.035**	**0.036**	**Mean**		**Mean**	**0.295667**	**0.333333**	**0.374333**	**0.295667**	
0.035	0.033	0.034	0.035	0.037	0.033		0.039	0.036	0.036	0.037	0.037	0.037
0.033	0.037	0.034	0.036	0.036	0.037		0.41	0.274	0.171	0.069	0.041	0.306
0.034	0.034	0.035	0.036	0.038	0.036		0.342	0.204	0.14	0.07	0.043	0.253
0.034	0.039	0.036	0.036	0.036	0.037		0.286	0.259	0.154	0.064	0.045	0.245
Mean	**0.036667**	**0.035**	**0.036**	**0.036667**			**Mean**	**0.245667**	**0.155**	**0.067667**	**0.043**	**0.29975**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheocoides*						
$K_2Cr_2O_7$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	***-22.0069***	***-50.5917***	***-29.6231***	***-24.5572***	***-47.7477***	2.379461
0.0156	***-31.0035***	***-55.7692***	***-84.4873***	***-53.0596***	***-73.3333***	***-11.7721***
0.0313	***-17.1626***	***-48.6686***	***-54.3383***	***-41.6264***	***-61.2613***	***-27.176***
0.0625	***-28.5121***	***-24.7041***	***-61.7003***	***-16.8277***	***-36.9369***	2.379461
0.125	***-5.39792***	***-15.8284***	***-11.9194***	***-5.87762***	***-14.5946***	21.16468
0.25	47.47405	39.94083	33.65469	20.37037	41.44144	55.22855
0.5	77.23183	78.99408	78.70289	77.21417	80.9009	88.04008
1	95.50173	96.15385	95.53024	95.57166	97.2973	97.30745
Coefficient of variation for control	***19.69295***	***20.33562***	13.70624	***19.67676***	***24.40588***	***19.70686***

2.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs –16/08/2011							*OD Readings at 96 hrs* –20/08/2011					
Replicate 1							**Replicate 1**					
0.034	0.032	0.036	0.035	0.036	0.045		0.037	0.035	0.039	0.037	0.038	0.041
0.041	0.035	0.035	0.035	0.037	0.037		0.239	0.252	0.254	0.214	0.2	0.186
0.038	0.036	0.036	0.036	0.037	0.039		0.167	0.266	0.271	0.266	0.224	0.223
0.036	0.035	0.036	0.036	0.042	0.038		0.275	0.171	0.197	0.176	0.254	0.162
0.038333	**0.035333**	**0.035667**	**0.035667**	**0.038667**	**Mean**		**Mean**	**0.229667**	**0.240667**	**0.218667**	**0.226**	

0.036	0.032	0.034	0.036	0.037	0.039		0.039	0.037	0.037	0.038	0.037	0.044
0.035	0.036	0.036	0.037	0.039	0.037		0.236	0.196	0.145	0.123	0.041	0.162
0.037	0.036	0.036	0.037	0.04	0.038		0.17	0.165	0.146	0.056	0.04	0.185
0.039	0.037	0.037	0.037	0.041	0.045		0.168	0.19	0.126	0.064	0.043	0.152
Mean	**0.036333**	**0.036333**	**0.037**	**0.04**			**Mean**	**0.183667**	**0.139**	**0.081**	0.041333	**0.19375**
Replicate 2							**Replicate 2**					
0.033	0.032	0.033	0.034	0.035	0.033		0.038	0.038	0.037	0.038	0.036	0.041
0.034	0.034	0.034	0.035	0.036	0.037		0.225	0.241	0.239	0.213	0.2	0.191
0.034	0.034	0.035	0.035	0.037	0.037		0.08	0.227	0.265	0.211	0.21	0.169
0.035	0.035	0.035	0.035	0.037	0.041		0.09	0.213	0.175	0.225	0.206	0.157
0.036	**0.034333**	**0.034667**	**0.035**	**0.036667**	**Mean**		**Mean**	**0.227**	**0.226333**	**0.216333**	**0.205333**	
0.032	0.032	0.035	0.035	0.037	0.034		0.039	0.036	0.037	0.039	0.036	0.04
0.034	0.035	0.035	0.038	0.04	0.037		0.224	0.24	0.123	0.079	0.041	0.197
0.034	0.035	0.036	0.038	0.039	0.037		0.182	0.232	0.168	0.051	0.065	0.188
0.034	0.035	0.036	0.037	0.039	0.038		0.244	0.203	0.122	0.044	0.041	0.175
Mean	**0.035**	**0.035667**	**0.037667**	**0.039333**			**Mean**	**0.225**	**0.137667**	**0.058**	**0.049**	**0.176833**
Replicate 3							**Replicate 3**					
0.034	0.032	0.034	0.034	0.034	0.037		0.037	0.037	0.037	0.037	0.038	0.043
0.034	0.034	0.035	0.035	0.036	0.038		0.187	0.291	0.259	0.255	0.232	0.225
0.035	0.034	0.036	0.036	0.037	0.039		0.293	0.271	0.258	0.262	0.173	0.181
0.035	0.035	0.035	0.036	0.037	0.04		0.144	0.267	0.289	0.227	0.262	0.12
0.036833	**0.034333**	**0.035333**	**0.035667**	**0.036667**	**Mean**		**Mean**	**0.276333**	**0.268667**	**0.248**	**0.222333**	
0.032	0.033	0.033	0.034	0.037	0.034		0.038	0.035	0.038	0.036	0.036	0.04
0.034	0.034	0.035	0.036	0.038	0.039		0.27	0.271	0.184	0.108	0.04	0.185
0.037	0.034	0.035	0.037	0.038	0.038		0.259	0.251	0.169	0.072	0.041	0.211
0.035	0.035	0.035	0.036	0.038	0.03		0.12	0.231	0.114	0.048	0.04	0.175

					8		4					
Mean	**0.034333**	**0.035**	**0.036333**	**0.038**			**Mean**	**0.251**	**0.155667**	**0.076**	**0.040333**	**0.197833**
Replicate 4							**Replicate 4**					
0.032	0.032	0.033	0.035	0.034	0.035		0.038	0.036	0.038	0.039	0.039	0.04
0.038	0.034	0.034	0.035	0.037	0.037		0.259	0.28	0.245	0.239	0.242	0.203
0.035	0.034	0.034	0.035	0.036	0.038		0.274	0.282	0.236	0.209	0.184	0.203
0.035	0.034	0.035	0.036	0.037	0.04		0.245	0.275	0.22	0.176	0.164	0.179
0.037	**0.034**	**0.034333**	**0.035333**	**0.036667**	**Mean**		**Mean**	**0.279**	**0.233667**	**0.208**	**0.196667**	
0.04	0.032	0.034	0.034	0.038	0.034		0.037	0.036	0.037	0.037	0.036	0.041
0.035	0.035	0.035	0.042	0.039	0.041		0.275	0.038	0.044	0.043	0.04	0.214
0.035	0.035	0.036	0.037	0.039	0.037		0.275	0.049	0.164	0.052	0.04	0.193
0.035	0.036	0.04	0.041	0.04	0.038		0.194	0.043	0.04	0.051	0.043	0.197
Mean	**0.035333**	**0.037**	**0.04**	**0.039333**			**Mean**	**0.043333**	**0.082667**	**0.048667**	**0.041**	**0.225917**
Replicate 5							**Replicate 5**					
0.032	0.033	0.034	0.035	0.035	0.047		0.038	0.035	0.036	0.039	0.038	0.042
0.035	0.034	0.035	0.035	0.036	0.037		0.25	0.274	0.257	0.243	0.281	0.148
0.035	0.035	0.035	0.035	0.037	0.037		0.2	0.277	0.27	0.27	0.21	0.147
0.035	0.036	0.035	0.036	0.037	0.038		0.291	0.27	0.195	0.203	0.119	0.2
0.036225	**0.035**	**0.035**	**0.035333**	**0.036667**	**Mean**		**Mean**	**0.273667**	**0.240667**	**0.238667**	**0.203333**	
0.038	0.032	0.034	0.036	0.036	0.034		0.043	0.035	0.037	0.038	0.036	0.041
0.035	0.036	0.035	0.036	0.038	0.039		0.277	0.036	0.141	0.116	0.042	0.256
0.034	0.034	0.035	0.036	0.041	0.038		0.268	0.249	0.143	0.088	0.041	0.243
0.034	0.035	0.036	0.036	0.039	0.038		0.272	0.202	0.118	0.045	0.04	0.071
Mean	**0.035**	**0.035333**	**0.036**	**0.039333**			**Mean**	**0.2255**	**0.134**	**0.083**	**0.041**	**0.218583**
Replicate 6							**Replicate 6**					
0.033	0.032	0.034	0.036	0.035	0.05		0.03	0.036	0.036	0.038	0.038	0.041

					3		9					
0.035	0.034	0.037	0.037	0.037	0.037		0.155	0.22	0.266	0.257	0.209	0.145
0.038	0.035	0.035	0.036	0.038	0.038		0.207	0.289	0.259	0.224	0.141	0.179
0.036	0.035	0.035	0.035	0.037	0.041		0.133	0.225	0.257	0.2	0.172	0.11
0.037255	**0.034667**	**0.035667**	**0.036**	**0.037333**	**Mean**		**Mean**	**0.244667**	**0.260667**	**0.227**	**0.174**	
0.034	0.035	0.035	0.036	0.038	0.033		0.046	0.039	0.038	0.04	0.039	0.043
0.035	0.037	0.035	0.037	0.039	0.04		0.315	0.215	0.172	0.091	0.041	0.165
0.035	0.036	0.039	0.037	0.04	0.038		0.197	0.217	0.152	0.054	0.042	0.117
0.035	0.036	0.036	0.038	0.039	0.039		0.24	0.045	0.143	0.043	0.037	0.138
Mean	**0.036333**	**0.036667**	**0.037333**	**0.039333**			**Mean**	**0.216**	**0.155667**	**0.062667**	**0.04**	**0.175083**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
$K_2Cr_2O_7$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	***-11.9534***	***-37.7381***	***-47.4385***	***-25.2159***	***-30.3391***	***-21.394***
0.156	***-18.3673***	***-37.2619***	***-42.7254***	***-1.72712***	***-12.1907***	***-30.6873***
0.313	***-5.53936***	***-30.119***	***-30.0205***	11.57168	***-11.0907***	***-11.1326***
0.0625	***-9.81535***	***-22.2619***	***-14.2418***	17.44387	8.340972	19.6515
0.125	14.8688	***-36.3095***	***-31.8648***	100	***-3.84968***	***-4.74347***
0.25	40.91351	26.07143	26.7418	76.51123	46.47113	30.3001
0.5	74.73275	82.97619	75.71721	94.12781	74.51879	84.31752
1	97.862	89.40476	97.64344	98.10017	97.61687	97.48306
Coefficient of variation for control	***22.37413***	***41.36908***	***25.5237***	14.67841	***35.6589***	***31.20379***

2.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 16/08/2011							*OD Readings at 0 hrs* – 20/08/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.033	0.034	0.035	0.053		0.037	0.037	0.039	0.037	0.038	0.039
0.034	0.033	0.034	0.034	0.035	0.037		0.096	0.075	0.067	0.073	0.072	0.094
0.033	0.035	0.034	0.034	0.035	0.036		0.076	0.072	0.123	0.108	0.066	0.071
0.036	0.034	0.034	0.034	0.035	0.037		0.067	0.065	0.071	0.067	0.056	0.063
0.035333	**0.034**	**0.034**	**0.034**	**0.035**	**Mean**		**Mean**	**0.070667**	**0.087**	**0.082667**	**0.064667**	

0.032	0.033	0.034	0.036	0.037	0.035		0.04	0.036	0.036	0.039	0.037	0.043
0.034	0.033	0.033	0.034	0.037	0.036		0.12	0.082	0.054	0.04	0.037	0.163
0.033	0.035	0.035	0.035	0.038	0.037		0.089	0.068	0.05	0.039	0.038	0.112
0.034	0.033	0.034	0.036	0.037	0.037		0.063	0.065	0.049	0.043	0.038	0.084
Mean	**0.033667**	**0.034**	**0.035**	**0.037333**			**Mean**	**0.071667**	**0.051**	**0.040667**	**0.037667**	**0.0915**
Replicate 2							**Replicate 2**					
0.034	0.032	0.033	0.035	0.034	0.048		0.04	0.037	0.039	0.038	0.037	0.04
0.035	0.038	0.033	0.034	0.035	0.036		0.09	0.143	0.071	0.103	0.087	0.091
0.033	0.033	0.033	0.034	0.035	0.036		0.101	0.074	0.118	0.083	0.094	0.141
0.033	0.033	0.033	0.035	0.035	0.039		0.106	0.073	0.06	0.069	0.071	0.069
0.035255	**0.034667**	**0.033**	**0.034333**	**0.035**	**Mean**		**Mean**	**0.096667**	**0.083**	**0.085**	**0.084**	
0.034	0.032	0.034	0.035	0.037	0.034		0.042	0.035	0.037	0.038	0.038	0.043
0.033	0.033	0.033	0.034	0.037	0.036		0.073	0.106	0.054	0.045	0.039	0.092
0.035	0.035	0.034	0.035	0.037	0.036		0.165	0.073	0.049	0.04	0.037	0.133
0.034	0.034	0.034	0.035	0.038	0.037		0.084	0.074	0.057	0.038	0.038	0.085
Mean	**0.034**	**0.033667**	**0.034667**	**0.037333**			**Mean**	**0.084333**	**0.053333**	**0.041**	**0.038**	**0.1025**
Replicate 3							**Replicate 3**					
0.032	0.032	0.035	0.035	0.036	0.035		0.042	0.039	0.039	0.039	0.038	0.041
0.033	0.035	0.038	0.035	0.039	0.036		0.125	0.059	0.065	0.09	0.097	0.083
0.033	0.033	0.038	0.034	0.036	0.038		0.178	0.064	0.069	0.078	0.07	0.101
0.033	0.034	0.035	0.034	0.035	0.038		0.067	0.057	0.2	0.072	0.067	0.089
0.035333	**0.034**	**0.037**	**0.034333**	**0.036667**	**Mean**		**Mean**	**0.06**	**0.111333**	**0.08**	**0.078**	
0.035	0.032	0.034	0.035	0.036	0.043		0.047	0.038	0.039	0.039	0.036	0.04
0.033	0.033	0.037	0.035	0.037	0.037		0.076	0.066	0.052	0.038	0.037	0.091
0.034	0.036	0.034	0.035	0.038	0.037		0.134	0.063	0.053	0.039	0.039	0.104
0.034	0.036	0.034	0.036	0.038	0.03		0.07	0.068	0.064	0.04	0.039	0.077

					8		4					
Mean	**0.035**	**0.035**	**0.035333**	**0.037667**			**Mean**	**0.065667**	**0.056333**	**0.039**	**0.038333**	**0.099917**
Replicate 4							**Replicate 4**					
0.031	0.032	0.034	0.035	0.035	0.036		0.04	0.036	0.038	0.041	0.038	0.043
0.032	0.032	0.033	0.034	0.035	0.037		0.096	0.083	0.069	0.072	0.064	0.075
0.033	0.033	0.033	0.035	0.036	0.038		0.087	0.106	0.085	0.086	0.071	0.076
0.034	0.033	0.034	0.035	0.036	0.038		0.068	0.081	0.062	0.084	0.08	0.067
0.036417	**0.032667**	**0.033333**	**0.034667**	**0.035667**	**Mean**		**Mean**	**0.09**	**0.072**	**0.080667**	**0.071667**	
0.033	0.033	0.034	0.035	0.037	0.033		0.044	0.037	0.039	0.039	0.038	0.041
0.038	0.037	0.034	0.034	0.037	0.036		0.088	0.071	0.056	0.037	0.037	0.166
0.034	0.035	0.034	0.036	0.038	0.04		0.109	0.067	0.049	0.038	0.038	0.156
0.034	0.036	0.037	0.036	0.038	0.043		0.199	0.094	0.055	0.04	0.04	0.072
Mean	**0.036**	**0.035**	**0.035333**	**0.037667**			**Mean**	**0.077333**	**0.053333**	**0.038333**	**0.038333**	**0.104917**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.034	0.034	0.037		0.039	0.038	0.037	0.038	0.039	0.042
0.033	0.034	0.033	0.034	0.036	0.038		0.072	0.075	0.078	0.094	0.082	0.064
0.034	0.033	0.033	0.034	0.036	0.042		0.069	0.08	0.07	0.091	0.07	0.069
0.035	0.035	0.035	0.035	0.035	0.038		0.062	0.063	0.135	0.124	0.066	0.064
0.037083	**0.034**	**0.033667**	**0.034333**	**0.035667**	**Mean**		**Mean**	**0.072667**	**0.094333**	**0.103**	**0.072667**	
0.032	0.032	0.034	0.035	0.037	0.034		0.041	0.036	0.037	0.04	0.036	0.043
0.037	0.033	0.033	0.035	0.037	0.036		0.091	0.133	0.057	0.037	0.039	0.106
0.035	0.033	0.035	0.035	0.041	0.037		0.098	0.081	0.053	0.038	0.041	0.103
0.033	0.034	0.034	0.035	0.037	0.047		0.078	0.066	0.055	0.04	0.038	0.072
Mean	**0.033333**	**0.034**	**0.035**	**0.038333**			**Mean**	**0.093333**	**0.055**	**0.038333**	**0.039333**	**0.079**
Replicate 6							**Replicate 6**					
0.033	0.032	0.034	0.034	0.036	0.03		0.03	0.037	0.038	0.038	0.038	0.044

					4		8					
0.034	0.033	0.033	0.034	0.035	0.037		0.088	0.091	0.099	0.099	0.075	0.216
0.033	0.033	0.034	0.034	0.035	0.037		0.074	0.09	0.09	0.117	0.094	0.096
0.034	0.034	0.034	0.034	0.036	0.038		0.07	0.074	0.103	0.103	0.073	0.07
0.035417	**0.033333**	**0.033667**	**0.034**	**0.035333**	**Mean**		**Mean**	**0.085**	**0.097333**	**0.106333**	**0.080667**	
0.034	0.032	0.034	0.035	0.037	0.033		0.042	0.036	0.043	0.038	0.036	0.041
0.033	0.034	0.033	0.035	0.037	0.037		0.114	0.074	0.047	0.039	0.037	0.106
0.034	0.034	0.036	0.036	0.038	0.037		0.079	0.072	0.05	0.04	0.038	0.082
0.033	0.034	0.034	0.036	0.038	0.038		0.091	0.063	0.049	0.04	0.039	0.076
Mean	**0.034**	**0.034333**	**0.035667**	**0.037667**			**Mean**	**0.069667**	**0.048667**	**0.039667**	**0.038**	**0.096833**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
$K_2Cr_2O_7$ Concentration (mg/l)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.007	28.33876	6.801008	57.26744	24.20814	12.92776	1.904762
0.156	***-3.58306***	27.45592	***-32.2674***	48.64253	***-36.5019***	***-21.5873***
0.313	4.885993	24.43325	22.38372	36.87783	***-56.2738***	***-38.7302***
0.0625	40.06515	25.94458	25.87209	49.09502	12.92776	10.15873
0.125	26.38436	25.44081	47.38372	100	***-34.2205***	31.11111
0.25	66.77524	72.29219	63.66279	73.9819	53.23194	71.11111
0.5	86.97068	90.93199	93.89535	94.34389	91.25475	88.25397
1	92.83388	95.46599	95.05814	94.34389	88.97338	91.42857
Coefficient of variation for control	***24.91248***	***17.15972***	***24.13904***	***43.23281***	***17.51621***	***18.47337***

3. Sodium chloride – **NaCl**

Plate configuration

Plate 1					
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Plate 2					
Blank	Blank	Blank	Blank	Blank	Blank
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control

3.1. OD readings at 450nm for *Pseudokirchneriella subcapitata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 30/09/2011							*OD Readings at 96 hrs* – 04/10/2011					
Replicate 1							**Replicate 1**					
0.033	0.032	0.033	0.034	0.035	0.033		0.037	0.036	0.037	0.039	0.043	0.036
0.035	0.035	0.033	0.034	0.035	0.036		0.127	0.174	0.157	0.098	0.091	0.126
0.035	0.033	0.033	0.034	0.035	0.036		0.16	0.171	0.126	0.109	0.11	0.129
0.034	0.034	0.034	0.035	0.035	0.037		0.15	0.194	0.12	0.099	0.105	0.114
Mean	**0.034**	**0.033333**	**0.034333**	**0.035**			**Mean**	**0.179667**	**0.134333**	**0.102**	**0.102**	
0.035	0.033	0.033	0.034	0.036	0.033		0.038	0.039	0.04	0.046	0.041	0.038
0.033	0.034	0.033	0.034	0.038	0.036		0.122	0.086	0.054	0.041	0.041	0.113
0.035	0.034	0.034	0.034	0.036	0.037		0.153	0.099	0.061	0.039	0.039	0.108
0.033	0.034	0.034	0.035	0.036	0.038		0.138	0.091	0.049	0.042	0.04	0.098
Mean	**0.034**	**0.033667**	**0.034333**	**0.036667**	**0.035417**		**Mean**	**0.092**	**0.054667**	**0.040667**	**0.04**	**0.128167**
Replicate 2							**Replicate 2**					
0.032	0.033	0.034	0.035	0.035	0.047		0.036	0.036	0.038	0.043	0.042	0.037
0.034	0.033	0.033	0.033	0.035	0.035		0.148	0.162	0.15	0.118	0.137	0.13
0.037	0.033	0.034	0.034	0.036	0.036		0.125	0.137	0.139	0.128	0.125	0.149
0.035	0.034	0.035	0.034	0.035	0.036		0.13	0.143	0.148	0.11	0.099	0.103
Mean	**0.033333**	**0.034**	**0.033667**	**0.035333**			**Mean**	**0.147333**	**0.145667**	**0.118667**	**0.120333**	
0.032	0.032	0.033	0.035	0.036	0.034		0.036	0.043	0.041	0.042	0.042	0.038
0.033	0.033	0.034	0.034	0.036	0.037		0.144	0.094	0.054	0.043	0.043	0.148
0.033	0.034	0.033	0.036	0.038	0.036		0.138	0.063	0.065	0.044	0.044	0.144
0.033	0.035	0.037	0.034	0.037	0.037		0.117	0.047	0.05	0.042	0.042	0.184
Mean	**0.034**	**0.034667**	**0.034667**	**0.037**	**0.035167**		**Mean**	**0.068**	**0.056333**	**0.043**	**0.043**	**0.138333**
Replicate 3							**Replicate 3**					

0.033	0.033	0.034	0.034	0.034	0.053		0.036	0.037	0.037	0.039	0.046	0.038
0.034	0.034	0.034	0.034	0.035	0.036		0.195	0.187	0.189	0.123	0.149	0.13
0.034	0.034	0.034	0.035	0.036	0.036		0.196	0.161	0.157	0.163	0.157	0.138
0.033	0.034	0.035	0.034	0.035	0.036		0.097	0.157	0.112	0.156	0.119	0.199
Mean	**0.034**	**0.034333**	**0.034333**	**0.035333**			**Mean**	**0.168333**	**0.152667**	**0.147333**	**0.141667**	
0.032	0.034	0.036	0.035	0.036	0.035		0.037	0.049	0.046	0.044	0.043	0.039
0.035	0.036	0.033	0.033	0.035	0.036		0.152	0.054	0.061	0.04	0.048	0.162
0.035	0.033	0.034	0.034	0.036	0.036		0.166	0.067	0.05	0.04	0.042	0.161
0.033	0.033	0.033	0.034	0.037	0.036		0.149	0.046	0.057	0.04	0.041	0.142
Mean	**0.034**	**0.033333**	**0.033667**	**0.036**	**0.035**		**Mean**	**0.055667**	**0.056**	**0.04**	**0.043667**	**0.15725**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.035	0.034	0.051		0.035	0.035	0.036	0.04	0.039	0.037
0.035	0.033	0.033	0.033	0.038	0.049		0.111	0.189	0.141	0.161	0.084	0.116
0.034	0.034	0.035	0.034	0.038	0.039		0.127	0.161	0.166	0.131	0.106	0.129
0.034	0.043	0.035	0.034	0.037	0.039		0.166	0.205	0.152	0.125	0.119	0.096
Mean	**0.036667**	**0.034333**	**0.033667**	**0.037667**			**Mean**	**0.185**	**0.153**	**0.139**	**0.103**	
0.032	0.034	0.033	0.035	0.036	0.035		0.036	0.04	0.042	0.042	0.043	0.038
0.033	0.033	0.033	0.034	0.035	0.038		0.186	0.044	0.067	0.044	0.041	0.138
0.034	0.033	0.033	0.034	0.036	0.037		0.164	0.067	0.064	0.047	0.04	0.135
0.033	0.034	0.034	0.035	0.036	0.037		0.106	0.086	0.068	0.046	0.04	0.12
Mean	**0.033333**	**0.033333**	**0.034333**	**0.035667**	**0.036833**		**Mean**	**0.065667**	**0.066333**	**0.045667**	**0.040333**	**0.132833**
Replicate 5							**Replicate 5**					
0.033	0.032	0.034	0.035	0.034	0.05		0.036	0.035	0.036	0.04	0.043	0.036
0.033	0.033	0.033	0.033	0.035	0.036		0.212	0.121	0.136	0.124	0.089	0.1
0.034	0.033	0.034	0.035	0.035	0.036		0.174	0.2	0.186	0.12	0.086	0.09
0.03	0.034	0.034	0.034	0.036	0.036		0.24	0.225	0.176	0.153	0.082	0.079

3												
Mean	**0.033333**	**0.033667**	**0.034**	**0.035333**			**Mean**	**0.182**	**0.166**	**0.132333**	**0.085667**	
0.032	0.032	0.034	0.035	0.035	0.037		0.036	0.045	0.041	0.042	0.042	0.038
0.032	0.033	0.033	0.034	0.036	0.037		0.201	0.127	0.067	0.046	0.042	0.126
0.037	0.035	0.034	0.035	0.037	0.038		0.148	0.06	0.051	0.047	0.042	0.12
0.034	0.035	0.034	0.034	0.036	0.037		0.199	0.063	0.044	0.048	0.04	0.099
Mean	**0.034333**	**0.033667**	**0.034333**	**0.036333**	**0.03525**		**Mean**	**0.083333**	**0.054**	**0.047**	**0.041333**	**0.149**
Replicate 6							**Replicate 6**					
0.034	0.033	0.034	0.035	0.034	0.036		0.037	0.19	0.037	0.039	0.045	0.037
0.037	0.037	0.036	0.034	0.035	0.036		0.135	0.174	0.178	0.113	0.089	0.113
0.034	0.034	0.036	0.035	0.036	0.037		0.12	0.169	0.173	0.118	0.092	0.111
0.034	0.038	0.034	0.034	0.035	0.036		0.211	0.158	0.169	0.106	0.097	0.076
Mean	**0.036333**	**0.035333**	**0.034333**	**0.035333**			**Mean**	**0.167**	**0.173333**	**0.112333**	**0.092667**	
0.032	0.032	0.035	0.037	0.035	0.05		0.036	0.043	0.045	0.043	0.041	0.038
0.033	0.033	0.033	0.034	0.037	0.037		0.164	0.077	0.057	0.049	0.04	0.116
0.033	0.033	0.033	0.036	0.043	0.036		0.207	0.111	0.065	0.051	0.043	0.124
0.033	0.034	0.034	0.034	0.036	0.038		0.123	0.078	0.056	0.047	0.041	0.094
Mean	**0.033333**	**0.033333**	**0.034667**	**0.038667**	**0.035333**		**Mean**	**0.088667**	**0.059333**	**0.049**	**0.041333**	**0.132833**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
NaCl Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	**-35.3488**	**-13.7353**	**-7.66129**	**-38.0518**	8.444902	***-5.55556***
0.156	6.821705	***-12.0603***	4.973118	***-8.82801***	18.33162	***-10.582***
0.313	36.89922	15.07538	9.274194	3.957382	39.13491	37.83069
0.625	36.89922	13.40034	13.84409	36.83409	67.97116	53.43915
1.25	46.20155	65.99665	83.19892	70.92846	69.41298	56.61376
2.5	80.93023	77.72194	82.93011	70.31963	87.53862	79.89418
5	93.95349	91.12228	95.83333	89.1933	91.86406	88.09524
10	94.57364	91.12228	92.87634	94.06393	95.3656	94.17989
Coefficient of variation for control	10.70213	8.835113	***23.23665***	***23.07042***	***16.18309***	***25.64786***

3.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 10/10/2011							*OD Readings at 96 hrs* – 14/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.033	0.034	0.034	0.035	0.034		0.034	0.035	0.037	0.038	0.039	0.038
0.032	0.032	0.033	0.033	0.034	0.035		0.247	0.356	0.449	0.367	0.226	0.242
0.033	0.033	0.033	0.034	0.035	0.036		0.272	0.351	0.345	0.337	0.253	0.25
0.037	0.033	0.034	0.034	0.035	0.039		0.243	0.289	0.319	0.351	0.34	0.186
Mean	**0.032667**	**0.033333**	**0.033667**	**0.034667**			**Mean**	**0.332**	**0.371**	**0.351667**	**0.273**	
0.032	0.032	0.034	0.034	0.034	0.037		0.034	0.035	0.033	0.037	0.038	0.036
0.033	0.033	0.034	0.033	0.034	0.035		0.271	0.287	0.293	0.12	0.062	0.218
0.033	0.033	0.033	0.034	0.035	0.036		0.244	0.353	0.205	0.107	0.063	0.209
0.033	0.033	0.033	0.034	0.035	0.038		0.179	0.331	0.217	0.12	0.063	0.195
Mean	**0.033**	**0.033333**	**0.033667**	**0.034667**	**0.035**		**Mean**	**0.323667**	**0.238333**	**0.115667**	**0.062667**	**0.229667**
Replicate 2							**Replicate 2**					
0.033	0.032	0.034	0.034	0.035	0.034		0.035	0.034	0.036	0.037	0.037	0.036
0.032	0.034	0.033	0.033	0.034	0.036		0.3	0.399	0.421	0.341	0.244	0.268
0.033	0.033	0.033	0.034	0.035	0.036		0.273	0.421	0.412	0.366	0.3	0.365
0.033	0.033	0.033	0.034	0.035	0.036		0.224	0.299	0.401	0.362	0.291	0.231
Mean	**0.033333**	**0.033**	**0.033667**	**0.034667**			**Mean**	**0.373**	**0.411333**	**0.356333**	**0.278333**	
0.032	0.032	0.033	0.034	0.034	0.033		0.034	0.035	0.036	0.037	0.034	0.036
0.032	0.033	0.033	0.033	0.035	0.035		0.32	0.35	0.255	0.123	0.109	0.228
0.033	0.033	0.034	0.034	0.035	0.036		0.294	0.365	0.246	0.11	0.078	0.234
0.033	0.033	0.033	0.034	0.035	0.036		0.26	0.342	0.201	0.13	0.075	0.224
Mean	**0.033**	**0.033333**	**0.033667**	**0.035**	**0.034225**		**Mean**	**0.352333**	**0.234**	**0.121**	**0.087333**	**0.268417**
Replicate 3							**Replicate 3**					
0.03	0.032	0.033	0.036	0.035	0.033		0.03	0.035	0.036	0.037	0.039	0.037

2							4					
0.033	0.032	0.033	0.033	0.034	0.035		0.33	0.341	0.435	0.338	0.25	0.264
0.033	0.032	0.033	0.033	0.035	0.036		0.299	0.422	0.364	0.371	0.274	0.255
0.033	0.033	0.033	0.034	0.035	0.036		0.241	0.377	0.351	0.385	0.245	0.19
Mean	**0.032333**	**0.033**	**0.033333**	**0.034667**			**Mean**	**0.38**	**0.383333**	**0.364667**	**0.256333**	
0.032	0.032	0.034	0.035	0.035	0.034		0.034	0.035	0.037	0.038	0.038	0.036
0.033	0.033	0.033	0.034	0.034	0.035		0.286	0.426	0.253	0.033	0.079	0.232
0.033	0.033	0.033	0.034	0.035	0.036		0.307	0.298	0.277	0.13	0.071	0.278
0.034	0.033	0.034	0.034	0.035	0.036		0.201	0.334	0.299	0.137	0.076	0.193
Mean	**0.033**	**0.033333**	**0.034**	**0.034667**	**0.034417**		**Mean**	**0.352667**	**0.276333**	**0.1335**	**0.075333**	**0.256333**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.034	0.034	0.035		0.034	0.034	0.036	0.038	0.038	0.036
0.032	0.032	0.033	0.033	0.035	0.036		0.216	0.447	0.488	0.481	0.257	0.228
0.033	0.033	0.034	0.034	0.036	0.036		0.221	0.413	0.51	0.483	0.314	0.234
0.035	0.033	0.034	0.034	0.036	0.037		0.212	0.417	0.392	0.339	0.211	0.173
Mean	**0.032667**	**0.033667**	**0.033667**	**0.035667**			**Mean**	**0.425667**	**0.463333**	**0.434333**	**0.260667**	
0.032	0.033	0.034	0.034	0.034	0.034		0.034	0.036	0.037	0.037	0.037	0.035
0.032	0.032	0.033	0.033	0.034	0.035		0.215	0.047	0.044	0.134	0.07	0.212
0.033	0.033	0.034	0.034	0.035	0.036		0.233	0.302	0.259	0.151	0.069	0.224
0.033	0.033	0.033	0.034	0.034	0.038		0.077	0.057	0.194	0.131	0.074	0.158
Mean	**0.032667**	**0.033333**	**0.033667**	**0.034333**	**0.034667**		**Mean**	**0.302**	**0.2265**	**0.138667**	**0.071**	**0.20025**
Replicate 5							**Replicate 5**					
0.034	0.032	0.034	0.034	0.034	0.036		0.037	0.035	0.037	0.038	0.039	0.036
0.033	0.033	0.033	0.033	0.034	0.035		0.284	0.364	0.429	0.462	0.306	0.369
0.045	0.033	0.033	0.034	0.035	0.036		0.22	0.304	0.446	0.421	0.295	0.331
0.033	0.035	0.034	0.034	0.035	0.036		0.176	0.257	0.307	0.314	0.27	0.244

Mean	**0.033667**	**0.033333**	**0.033667**	**0.034667**			**Mean**	**0.308333**	**0.394**	**0.399**	**0.290333**	
0.033	0.033	0.034	0.034	0.036	0.034		0.043	0.053	0.037	0.038	0.038	0.037
0.034	0.033	0.033	0.033	0.034	0.035		0.307	0.24	0.032	0.105	0.083	0.271
0.033	0.033	0.033	0.034	0.035	0.036		0.314	0.114	0.383	0.123	0.036	0.319
0.033	0.033	0.033	0.034	0.035	0.036		0.231	0.391	0.194	0.154	0.086	0.266
Mean	**0.033**	**0.033**	**0.033667**	**0.034667**	**0.035417**		**Mean**	**0.248333**	**0.203**	**0.127333**	**0.068333**	**0.277667**
Replicate 6							**Replicate 6**					
0.033	0.033	0.034	0.034	0.034	0.037		0.035	0.034	0.037	0.039	0.038	0.036
0.076	0.034	0.033	0.033	0.034	0.036		0.392	0.379	0.463	0.347	0.209	0.275
0.033	0.033	0.033	0.034	0.035	0.036		0.289	0.416	0.41	0.442	0.301	0.285
0.033	0.034	0.033	0.035	0.035	0.038		0.261	0.359	0.399	0.398	0.33	0.244
Mean	**0.033667**	**0.033**	**0.034**	**0.034667**			**Mean**	**0.384667**	**0.424**	**0.395667**	**0.28**	
0.032	0.032	0.034	0.034	0.035	0.034		0.035	0.036	0.036	0.04	0.038	0.038
0.034	0.033	0.033	0.034	0.034	0.035		0.274	0.111	0.22	0.113	0.069	0.245
0.033	0.033	0.033	0.033	0.035	0.036		0.277	0.246	0.234	0.137	0.077	0.225
0.033	0.033	0.034	0.034	0.035	0.036		0.174	0.079	0.267	0.141	0.086	0.187
Mean	**0.033**	**0.033333**	**0.033667**	**0.034667**	**0.034818**		**Mean**	**0.145333**	**0.240333**	**0.130333**	**0.077333**	**0.260667**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. protothecoides*						
NaCl Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	***-42.7092***	***-32.6371***	***-42.0478***	***-126.789***	***-3.15259***	***-68.0868***
0.156	***-61.3546***	***-47.6501***	***-43.413***	***-148.646***	***-35.5612***	***-87.0579***
0.313	***-52.1116***	***-26.1097***	***-35.7679***	***-131.818***	***-37.4527***	***-73.3923***
0.625	***-14.502***	4.438642	8.600683	***-31.0445***	3.656999	***-17.6045***
1.25	***-38.7251***	***-24.5431***	***-30.8532***	***-55.029***	19.54603	47.34727
2.5	2.071713	21.80157	0.409556	***-11.2186***	36.69609	1.527331
5	60.71713	66.05744	58.90785	39.74855	65.32156	54.58199
10	86.05578	79.24282	82.73038	79.01354	87.64187	80.14469
Coefficient of variation for control	13.95918	***16.48809***	***17.17598***	11.85689	***15.78748***	14.52139

3.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 24/08/2011							*OD Readings at 96 hrs* – 28/08/2011					
Replicate 1							**Replicate 1**					
0.033	0.033	0.033	0.035	0.035	0.051		0.032	0.036	0.038	0.04	0.044	0.035
0.034	0.034	0.034	0.035	0.036	0.039		0.316	0.16	0.154	0.144	0.103	0.046
0.035	0.035	0.035	0.035	0.037	0.038		0.159	0.199	0.165	0.185	0.106	0.048
0.034	0.035	0.036	0.036	0.037	0.037		0.086	0.136	0.081	0.132	0.069	0.045
Mean	**0.034667**	**0.035**	**0.035333**	**0.036667**			**Mean**	**0.165**	**0.133333**	**0.153667**	**0.092667**	
0.032	0.032	0.034	0.034	0.035	0.033		0.032	0.042	0.039	0.038	0.041	0.036
0.033	0.033	0.034	0.034	0.036	0.036		0.229	0.048	0.045	0.05	0.049	0.047
0.033	0.035	0.035	0.035	0.036	0.037		0.214	0.048	0.046	0.05	0.048	0.048
0.034	0.034	0.035	0.035	0.036	0.038		0.13	0.047	0.046	0.046	0.046	0.052
Mean	**0.034**	**0.034667**	**0.034667**	**0.036**	**0.035667**		**Mean**	**0.047667**	**0.045667**	**0.048667**	**0.047667**	**0.118333**
Replicate 2							**Replicate 2**					
0.033	0.033	0.033	0.034	0.039	0.036		0.033	0.037	0.037	0.038	0.05	0.038
0.035	0.036	0.034	0.034	0.036	0.036		0.096	0.196	0.193	0.119	0.081	0.076
0.034	0.033	0.034	0.036	0.036	0.039		0.215	0.156	0.177	0.127	0.094	0.058
0.034	0.034	0.034	0.035	0.036	0.037		0.044	0.188	0.102	0.082	0.066	0.048
Mean	**0.034333**	**0.034**	**0.035**	**0.036**			**Mean**	**0.18**	**0.157333**	**0.109333**	**0.080333**	
0.032	0.032	0.034	0.034	0.035	0.033		0.032	0.041	0.04	0.039	0.039	0.047
0.033	0.035	0.034	0.034	0.035	0.036		0.165	0.046	0.048	0.05	0.051	0.065
0.033	0.034	0.034	0.035	0.036	0.036		0.246	0.047	0.045	0.049	0.049	0.067
0.034	0.034	0.035	0.035	0.037	0.039		0.176	0.048	0.048	0.048	0.046	0.055
Mean	**0.034333**	**0.034333**	**0.034667**	**0.036**	**0.0355**		**Mean**	**0.047**	**0.047**	**0.049**	**0.048667**	**0.10925**
Replicate 3							**Replicate 3**					
0.03	0.032	0.034	0.035	0.035	0.036		0.03	0.035	0.039	0.041	0.042	0.037

2							2					
0.033	0.033	0.034	0.035	0.036	0.036		0.204	0.185	0.143	0.136	0.084	0.144
0.034	0.034	0.034	0.036	0.036	0.037		0.131	0.212	0.222	0.133	0.104	0.082
0.034	0.036	0.034	0.035	0.036	0.039		0.109	0.146	0.117	0.165	0.094	0.052
Mean	**0.034333**	**0.034**	**0.035333**	**0.036**			**Mean**	**0.181**	**0.160667**	**0.144667**	**0.094**	
0.032	0.032	0.034	0.034	0.035	0.033		0.032	0.04	0.041	0.042	0.04	0.045
0.033	0.033	0.034	0.034	0.036	0.036		0.175	0.047	0.049	0.051	0.05	0.156
0.033	0.034	0.035	0.036	0.036	0.036		0.199	0.045	0.048	0.049	0.052	0.137
0.034	0.034	0.034	0.035	0.036	0.038		0.146	0.052	0.052	0.049	0.048	0.122
Mean	**0.033667**	**0.034333**	**0.035**	**0.036**	**0.03525**		**Mean**	**0.048**	**0.049667**	**0.049667**	**0.05**	**0.138083**
Replicate 4							**Replicate 4**					
0.032	0.033	0.033	0.034	0.035	0.033		0.032	0.037	0.037	0.039	0.041	0.037
0.033	0.033	0.035	0.035	0.036	0.036		0.146	0.198	0.161	0.143	0.094	0.135
0.034	0.034	0.034	0.035	0.036	0.037		0.161	0.172	0.192	0.148	0.104	0.169
0.034	0.034	0.034	0.035	0.036	0.037		0.109	0.183	0.137	0.118	0.089	0.18
Mean	**0.033667**	**0.034333**	**0.035**	**0.036**			**Mean**	**0.184333**	**0.163333**	**0.136333**	**0.095667**	
0.032	0.033	0.033	0.034	0.035	0.034		0.032	0.041	0.04	0.039	0.042	0.039
0.033	0.033	0.034	0.035	0.036	0.036		0.16	0.048	0.046	0.047	0.046	0.15
0.034	0.034	0.038	0.035	0.036	0.036		0.151	0.051	0.05	0.053	0.049	0.117
0.036	0.034	0.035	0.035	0.036	0.037		0.131	0.051	0.054	0.051	0.049	0.107
Mean	**0.033667**	**0.035667**	**0.035**	**0.036**	**0.03525**		**Mean**	**0.05**	**0.05**	**0.050333**	**0.048**	**0.143**
Replicate 5							**Replicate 5**					
0.033	0.032	0.034	0.034	0.035	0.035		0.033	0.038	0.04	0.041	0.043	0.037
0.034	0.034	0.034	0.034	0.036	0.036		0.14	0.192	0.189	0.15	0.093	0.146
0.034	0.034	0.035	0.035	0.037	0.037		0.074	0.175	0.177	0.138	0.101	0.124
0.035	0.036	0.034	0.035	0.036	0.038		0.044	0.213	0.198	0.194	0.089	0.065

Mean	**0.034667**	**0.034333**	**0.034667**	**0.036333**			**Mean**	**0.193333**	**0.188**	**0.160667**	**0.094333**	
0.032	0.032	0.033	0.034	0.036	0.043		0.032	0.04	0.04	0.041	0.042	0.05
0.033	0.034	0.034	0.035	0.036	0.036		0.201	0.047	0.044	0.046	0.048	0.145
0.034	0.034	0.034	0.035	0.036	0.037		0.182	0.046	0.047	0.048	0.048	0.13
0.033	0.034	0.034	0.035	0.036	0.037		0.085	0.051	0.051	0.05	0.047	0.068
Mean	**0.034**	**0.034**	**0.035**	**0.036**	**0.035333**		**Mean**	**0.048**	**0.047333**	**0.048**	**0.047667**	**0.117**
Replicate 6							**Replicate 6**					
0.032	0.032	0.034	0.034	0.035	0.033		0.032	0.037	0.038	0.039	0.044	0.047
0.034	0.034	0.034	0.035	0.036	0.037		0.201	0.183	0.159	0.131	0.076	0.147
0.034	0.034	0.034	0.035	0.038	0.037		0.207	0.241	0.186	0.135	0.08	0.146
0.038	0.035	0.035	0.036	0.037	0.066		0.207	0.28	0.157	0.132	0.087	0.136
Mean	**0.034333**	**0.034333**	**0.035333**	**0.037**			**Mean**	**0.234667**	**0.167333**	**0.132667**	**0.081**	
0.035	0.032	0.034	0.034	0.035	0.047		0.036	0.042	0.041	0.04	0.044	0.053
0.033	0.034	0.037	0.034	0.036	0.037		0.202	0.047	0.051	0.047	0.05	0.142
0.036	0.035	0.035	0.035	0.036	0.038		0.222	0.053	0.049	0.051	0.048	0.157
0.034	0.036	0.034	0.037	0.036	0.037		0.207	0.057	0.047	0.057	0.048	0.161
Mean	**0.035**	**0.035333**	**0.035333**	**0.036**	**0.035909**		**Mean**	**0.052333**	**0.049**	**0.051667**	**0.048667**	**0.177917**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
NaCl Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	15.46724	***-0.27435***	***-15.98951507***	***-25.2454***	***-60.3041***	***-15.622***
0.156	35.8754	15.27206	***2.18261E-14***	***-7.57363***	***-54.8986***	23.33655
0.313	22.77121	48.19387	12.5819135	15.14727	***-27.1959***	43.39441
0.625	62.08378	68.08413	52.42463958	49.36886	40.03378	73.28833
1.25	91.08485	90.9465	88.59764089	87.79804	86.99324	89.87464
2.5	92.37379	90.9465	87.2870249	87.79804	87.66892	91.80328
5	90.44039	89.57476	87.2870249	87.51753	86.99324	90.26037
10	91.08485	89.80338	87.0249017	89.48107	87.33108	91.99614
Coefficient of variation for control	***43.20779***	***31.5918***	***23.81034203***	12.41403	39.81778	3.624908

3.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 24/08/2011							*OD Readings at 96 hrs* – 28/08/2011					
Replicate 1							**Replicate 1**					
0.033	0.032	0.034	0.037	0.034	0.037		0.036	0.037	0.041	0.039	0.041	0.039
0.033	0.033	0.033	0.034	0.035	0.034		0.093	0.164	0.089	0.181	0.073	0.109
0.033	0.034	0.034	0.034	0.035	0.036		0.112	0.126	0.085	0.104	0.084	0.106
0.035	0.034	0.034	0.034	0.037	0.035		0.07	0.082	0.088	0.072	0.081	0.08
Mean	**0.033667**	**0.033667**	**0.034**	**0.035667**			**Mean**	**0.124**	**0.087333**	**0.119**	**0.079333**	
0.033	0.033	0.034	0.034	0.039	0.035		0.036	0.041	0.04	0.039	0.043	0.041
0.035	0.034	0.035	0.035	0.036	0.036		0.089	0.076	0.067	0.043	0.041	0.098
0.034	0.034	0.034	0.034	0.036	0.037		0.088	0.092	0.068	0.044	0.046	0.087
0.034	0.035	0.034	0.035	0.036	0.039		0.077	0.133	0.077	0.044	0.044	0.081
Mean	**0.034333**	**0.034333**	**0.034667**	**0.036**	**0.035083**		**Mean**	**0.100333**	**0.070667**	**0.043667**	**0.043667**	**0.090833**
Replicate 2							**Replicate 2**					
0.032	0.032	0.034	0.034	0.035	0.038		0.035	0.037	0.04	0.039	0.042	0.04
0.035	0.033	0.034	0.034	0.035	0.035		0.099	0.105	0.114	0.118	0.089	0.09
0.034	0.034	0.034	0.034	0.035	0.036		0.081	0.081	0.125	0.079	0.078	0.09
0.033	0.034	0.034	0.034	0.035	0.037		0.064	0.069	0.079	0.073	0.078	0.067
Mean	**0.033667**	**0.034**	**0.034**	**0.035**			**Mean**	**0.085**	**0.106**	**0.09**	**0.081667**	
0.049	0.032	0.034	0.035	0.035	0.036		0.044	0.043	0.041	0.041	0.04	0.035
0.032	0.033	0.033	0.034	0.035	0.035		0.08	0.085	0.074	0.047	0.04	0.091
0.033	0.036	0.034	0.034	0.036	0.039		0.068	0.089	0.08	0.047	0.044	0.09
0.035	0.034	0.034	0.034	0.037	0.037		0.063	0.08	0.077	0.045	0.044	0.064
Mean	**0.034333**	**0.033667**	**0.034**	**0.036**	**0.035083**		**Mean**	**0.084667**	**0.077**	**0.046333**	**0.042667**	**0.078917**
Replicate 3							**Replicate 3**					
0.033	0.032	0.034	0.034	0.034	0.037		0.036	0.037	0.04	0.039	0.042	0.041

0.033	0.033	0.033	0.034	0.035	0.036		0.089	0.104	0.109	0.098	0.082	0.096
0.033	0.033	0.034	0.034	0.035	0.036		0.065	0.11	0.109	0.119	0.134	0.113
0.033	0.034	0.034	0.034	0.035	0.036		0.079	0.072	0.07	0.073	0.156	0.084
Mean	**0.033333**	**0.033667**	**0.034**	**0.035**			**Mean**	**0.095333**	**0.096**	**0.096667**	**0.124**	
0.034	0.032	0.035	0.034	0.035	0.035		0.034	0.042	0.041	0.042	0.043	0.034
0.033	0.034	0.033	0.034	0.035	0.035		0.07	0.087	0.079	0.045	0.042	0.066
0.033	0.033	0.034	0.034	0.036	0.039		0.075	0.081	0.072	0.044	0.051	0.094
0.033	0.035	0.034	0.035	0.037	0.041		0.062	0.077	0.08	0.051	0.044	0.082
Mean	**0.034**	**0.033667**	**0.034333**	**0.036**	**0.035083**		**Mean**	**0.081667**	**0.077**	**0.046667**	**0.045667**	**0.08125**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.035	0.035	0.037		0.04	0.038	0.04	0.042	0.045	0.039
0.033	0.033	0.033	0.034	0.035	0.035		0.102	0.095	0.115	0.102	0.081	0.277
0.033	0.033	0.034	0.034	0.035	0.036		0.192	0.086	0.092	0.177	0.253	0.059
0.034	0.034	0.034	0.034	0.036	0.036		0.096	0.094	0.076	0.068	0.221	0.17
Mean	**0.033333**	**0.033667**	**0.034**	**0.035333**			**Mean**	**0.091667**	**0.094333**	**0.115667**	**0.185**	
0.033	0.032	0.034	0.036	0.035	0.034		0.033	0.041	0.04	0.043	0.041	0.047
0.033	0.033	0.034	0.034	0.035	0.036		0.098	0.085	0.075	0.043	0.041	0.123
0.033	0.033	0.034	0.034	0.036	0.036		0.211	0.091	0.067	0.05	0.045	0.075
0.033	0.034	0.034	0.035	0.037	0.037		0.07	0.088	0.071	0.045	0.043	0.069
Mean	**0.033333**	**0.034**	**0.034333**	**0.036**	**0.034583**		**Mean**	**0.088**	**0.071**	**0.046**	**0.043**	**0.1285**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.035	0.035	0.04		0.041	0.038	0.038	0.039	0.043	0.037
0.033	0.033	0.034	0.034	0.035	0.035		0.1	0.135	0.091	0.077	0.076	0.078
0.033	0.033	0.034	0.034	0.035	0.035		0.136	0.109	0.094	0.085	0.077	0.086
0.034	0.034	0.034	0.034	0.035	0.036		0.075	0.065	0.085	0.072	0.076	0.066
Me	**0.0333**	**0.034**	**0.034**	**0.035**			**Me**	**0.103**	**0.09**	**0.078**	**0.0763**	

an	**33**						**an**				**33**	
0.033	0.032	0.033	0.034	0.035	0.038		0.034	0.04	0.039	0.039	0.042	0.037
0.034	0.033	0.034	0.034	0.036	0.035		0.072	0.077	0.063	0.044	0.04	0.069
0.033	0.034	0.033	0.034	0.036	0.036		0.098	0.08	0.06	0.044	0.04	0.07
0.033	0.034	0.034	0.035	0.036	0.037		0.109	0.081	0.073	0.046	0.043	0.069
Mean	**0.033667**	**0.033667**	**0.034333**	**0.036**	**0.0345**		**Mean**	**0.079333**	**0.065333**	**0.044667**	**0.041**	**0.085667**
Replicate 6							**Replicate 6**					
0.032	0.033	0.034	0.035	0.035	0.043		0.037	0.038	0.04	0.04	0.042	0.039
0.034	0.033	0.034	0.034	0.035	0.036		0.083	0.186	0.092	0.079	0.078	0.096
0.033	0.034	0.035	0.034	0.036	0.036		0.08	0.098	0.093	0.086	0.085	0.12
0.033	0.034	0.035	0.035	0.035	0.038		0.067	0.063	0.082	0.133	0.077	0.085
Mean	**0.033667**	**0.034667**	**0.034333**	**0.035333**			**Mean**	**0.115667**	**0.089**	**0.099333**	**0.08**	
0.032	0.032	0.034	0.034	0.036	0.034		0.033	0.045	0.043	0.042	0.042	0.038
0.035	0.033	0.033	0.034	0.035	0.036		0.099	0.098	0.075	0.048	0.045	0.066
0.033	0.034	0.034	0.034	0.036	0.036		0.137	0.084	0.068	0.055	0.044	0.07
0.034	0.034	0.035	0.035	0.036	0.037		0.064	0.083	0.067	0.043	0.041	0.072
Mean	**0.033667**	**0.034**	**0.034333**	**0.035667**	**0.035083**		**Mean**	**0.088333**	**0.07**	**0.048667**	**0.043333**	**0.086583**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
NaCl Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	***-53.1977***	1.219512	***-11.859***	40.03591	***-7.17949***	***-23.9075***
0.156	10.75581	*-41.4634*	*-13.141*	37.16338	12.82051	17.22365
0.313	***-44.4767***	***-8.94309***	***-14.4231***	14.18312	31.28205	1.285347
0.625	24.7093	7.99458	***-66.9872***	***-60.5027***	33.84615	31.1054
1.25	***-11.9186***	1.897019	14.42308	43.98564	29.23077	18.25193
2.5	39.82558	17.47967	23.39744	62.29803	50.76923	46.52956
5	86.9186	79.8103	81.73077	89.22801	82.5641	79.43445
10	86.9186	87.26287	83.65385	92.45961	88.20513	87.66067
Coefficient of variation for control	13.50731	12.15739	***17.75248***	***64.97543***	***23.95054***	***25.67426***

4. Sodium sulphate– Na_2SO_4

Plate configuration

Plate 1					
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Control	0.07 g/L	0.156 g/L	0.313 g/L	0.625 g/L	Control
Plate 2					
Blank	Blank	Blank	Blank	Blank	Blank
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control
Control	1.25 g/L	2.5 g/L	5 g/L	10 g/L	Control

4.1. OD readings at 450nm for *Pseudokirchneriella subcapitata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 30/09/2011							*OD Readings at 96 hrs* – 04/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.034	0.034	0.035	0.042		0.032	0.035	0.034	0.035	0.034	0.049
0.034	0.033	0.033	0.034	0.035	0.037		0.171	0.196	0.2	0.104	0.065	0.231
0.033	0.033	0.033	0.034	0.036	0.036		0.133	0.174	0.183	0.102	0.055	0.23
0.034	0.033	0.033	0.034	0.035	0.037		0.153	0.152	0.178	0.137	0.113	0.203
Mean	**0.033**	**0.033**	**0.034**	**0.035333**			**Mean**	**0.174**	**0.187**	**0.114333**	**0.077667**	
0.032	0.034	0.034	0.034	0.036	0.034		0.035	0.039	0.042	0.042	0.041	0.038
0.033	0.033	0.033	0.034	0.035	0.035		0.118	0.041	0.041	0.05	0.049	0.143
0.035	0.033	0.033	0.034	0.036	0.037		0.13	0.043	0.046	0.052	0.054	0.198
0.034	0.034	0.034	0.034	0.036	0.036		0.113	0.042	0.043	0.043	0.044	0.211
Mean	**0.033333**	**0.033333**	**0.034**	**0.035667**	**0.035083**		**Mean**	**0.042**	**0.043333**	**0.048333**	**0.049**	**0.1695**
Replicate 2							**Replicate 2**					
0.033	0.032	0.033	0.034	0.035	0.034		0.039	0.038	0.037	0.039	0.041	0.038
0.033	0.033	0.034	0.034	0.035	0.036		0.111	0.162	0.167	0.099	0.048	0.127
0.034	0.033	0.033	0.034	0.035	0.036		0.121	0.149	0.149	0.098	0.053	0.126
0.03	0.034	0.033	0.034	0.035	0.037		0.08	0.137	0.133	0.072	0.057	0.133

3						9					
Mean	**0.033333**	**0.033333**	**0.034**	**0.035**		**Mean**	**0.149333**	**0.149667**	**0.089667**	**0.052667**	
0.032	0.034	0.033	0.034	0.036	0.033	0.036	0.041	0.053	0.043	0.041	0.038
0.034	0.033	0.033	0.034	0.036	0.036	0.098	0.042	0.048	0.051	0.071	0.127
0.033	0.034	0.034	0.035	0.036	0.036	0.106	0.042	0.048	0.089	0.051	0.123
0.034	0.036	0.034	0.035	0.036	0.036	0.081	0.045	0.041	0.046	0.041	0.126
Mean	**0.034333**	**0.033667**	**0.034667**	**0.036**	**0.034833**	**Mean**	**0.043**	**0.045667**	**0.062**	**0.054333**	**0.114**
Replicate 3						**Replicate 3**					
0.033	0.032	0.034	0.034	0.035	0.044	0.036	0.036	0.038	0.04	0.043	0.038
0.033	0.036	0.033	0.034	0.035	0.035	0.1	0.155	0.139	0.095	0.058	0.16
0.036	0.033	0.033	0.034	0.035	0.036	0.123	0.148	0.132	0.091	0.06	0.151
0.034	0.033	0.033	0.034	0.035	0.037	0.086	0.126	0.128	0.08	0.056	0.177
Mean	**0.034**	**0.033**	**0.034**	**0.035**		**Mean**	**0.143**	**0.133**	**0.088667**	**0.058**	
0.032	0.032	0.033	0.035	0.037	0.033	0.035	0.041	0.043	0.042	0.042	0.037
0.033	0.033	0.033	0.035	0.036	0.036	0.101	0.049	0.043	0.048	0.059	0.122
0.033	0.033	0.034	0.034	0.036	0.038	0.109	0.043	0.041	0.066	0.052	0.17
0.034	0.034	0.034	0.035	0.036	0.036	0.093	0.042	0.042	0.056	0.046	0.156
Mean	**0.033333**	**0.033667**	**0.034667**	**0.036**	**0.035083**	**Mean**	**0.044667**	**0.042**	**0.056667**	**0.052333**	**0.129**
Replicate 4						**Replicate 4**					
0.033	0.038	0.034	0.035	0.035	0.035	0.035	0.041	0.039	0.04	0.043	0.038
0.033	0.033	0.034	0.033	0.036	0.036	0.178	0.199	0.194	0.103	0.057	0.182
0.033	0.033	0.033	0.034	0.035	0.037	0.249	0.196	0.184	0.099	0.051	0.191
0.033	0.035	0.035	0.034	0.035	0.036	0.119	0.149	0.186	0.081	0.054	0.095
Mean	**0.033667**	**0.034**	**0.033667**	**0.035333**		**Mean**	**0.181333**	**0.188**	**0.094333**	**0.054**	
0.033	0.032	0.034	0.034	0.036	0.034	0.037	0.042	0.042	0.042	0.04	0.037
0.03	0.035	0.033	0.036	0.035	0.036	0.11	0.044	0.04	0.043	0.04	0.173

7						2					
0.034	0.034	0.034	0.034	0.036	0.037	0.129	0.055	0.041	0.043	0.042	0.154
0.034	0.033	0.034	0.037	0.036	0.037	0.147	0.042	0.041	0.045	0.043	0.196
Mean	0.034	0.033667	0.035667	0.035667	**0.03525**	Mean	0.047	0.040667	0.043667	0.041667	**0.160417**
Replicate 5						**Replicate 5**					
0.033	0.034	0.033	0.034	0.034	0.051	0.037	0.039	0.038	0.039	0.04	0.036
0.034	0.033	0.033	0.034	0.035	0.035	0.139	0.161	0.135	0.087	0.052	0.153
0.033	0.033	0.034	0.034	0.035	0.036	0.125	0.144	0.102	0.087	0.052	0.139
0.033	0.034	0.037	0.034	0.036	0.036	0.129	0.15	0.142	0.107	0.055	0.163
Mean	**0.033333**	**0.034667**	**0.034**	**0.035333**		**Mean**	**0.151667**	**0.126333**	**0.093667**	**0.053**	
0.032	0.032	0.034	0.034	0.036	0.033	0.035	0.04	0.042	0.041	0.04	0.036
0.035	0.033	0.033	0.034	0.035	0.036	0.144	0.047	0.045	0.04	0.039	0.122
0.033	0.033	0.034	0.034	0.036	0.036	0.148	0.046	0.04	0.041	0.045	0.153
0.033	0.033	0.033	0.034	0.036	0.039	0.094	0.049	0.043	0.041	0.04	0.106
Mean	**0.033**	**0.033333**	**0.034**	**0.035667**	**0.034917**	**Mean**	**0.047333**	**0.042667**	**0.040667**	**0.041333**	**0.134583**
Replicate 6						**Replicate 6**					
0.033	0.033	0.035	0.035	0.035	0.051	0.036	0.037	0.037	0.039	0.04	0.037
0.036	0.034	0.034	0.035	0.038	0.036	0.114	0.147	0.15	0.086	0.054	0.14
0.035	0.04	0.035	0.035	0.039	0.038	0.105	0.131	0.1	0.095	0.056	0.125
0.034	0.035	0.038	0.037	0.036	0.039	0.099	0.131	0.102	0.074	0.047	0.126
Mean	**0.036333**	**0.035667**	**0.035667**	**0.037667**		**Mean**	**0.136333**	**0.117333**	**0.085**	**0.052333**	
0.032	0.032	0.034	0.035	0.036	0.034	0.037	0.043	0.043	0.042	0.041	0.037
0.034	0.033	0.033	0.034	0.037	0.037	0.139	0.045	0.056	0.047	0.057	0.208
0.033	0.033	0.035	0.034	0.036	0.037	0.137	0.041	0.042	0.044	0.042	0.117
0.033	0.033	0.035	0.035	0.04	0.036	0.126	0.042	0.042	0.042	0.043	0.188
Mean	**0.033**	**0.034333**	**0.034333**	**0.037667**	**0.035667**	**Mean**	**0.042667**	**0.046667**	**0.044333**	**0.047333**	**0.135333**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
Na_2SO_4 Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	***-4.375***	***-24.5962***	10.86351	***-2.2093***	***-24.1563***	***-4.31034***
0.156	***-14.125***	***-24.9633***	19.22006	***-6.86047***	2.841918	15.34483
0.313	40.375	41.11601	56.26741	58.48837	37.65542	48.7931
0.625	67.875	81.8649	81.89415	86.62791	80.99467	82.58621
1.25	94.625	92.51101	93.03621	91.51163	87.03375	92.58621
2.5	93.625	89.57416	95.26462	95.93023	92.0071	88.44828
5	89.875	71.5859	83.00836	93.83721	94.13854	90.86207
10	89.375	80.02937	86.62953	95.23256	93.42806	87.75862
Coefficient of variation for control	***18.48741***	2.587666	12.27694	***24.39673***	12.35456	5.443813

4.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 10/10/2011							*OD Readings at 96hrs* – 14/10/2011					
Replicate 1							**Replicate 1**					
0.033	0.032	0.034	0.034	0.035	0.033		0.035	0.035	0.036	0.038	0.038	0.036
0.034	0.035	0.033	0.033	0.034	0.035		0.299	0.335	0.376	0.31	0.281	0.31
0.034	0.033	0.033	0.034	0.035	0.036		0.19	0.29	0.342	0.29	0.252	0.276
0.033	0.033	0.033	0.034	0.035	0.036		0.209	0.24	0.267	0.213	0.273	0.271
Mean	**0.033667**	**0.033**	**0.033667**	**0.034667**			**Mean**	**0.288333**	**0.328333**	**0.271**	**0.268667**	
0.032	0.032	0.033	0.034	0.035	0.038		0.034	0.035	0.038	0.038	0.046	0.037
0.032	0.032	0.033	0.033	0.035	0.035		0.236	0.164	0.079	0.082	0.059	0.21
0.033	0.033	0.033	0.034	0.036	0.036		0.212	0.147	0.093	0.081	0.06	0.211
0.033	0.033	0.033	0.034	0.036	0.037		0.214	0.132	0.084	0.082	0.064	0.22
Mean	**0.032667**	**0.033**	**0.033667**	**0.035667**	**0.0345**		**Mean**	**0.147667**	**0.085333**	**0.081667**	**0.061**	**0.238167**
Replicate 2							**Replicate 2**					
0.032	0.033	0.034	0.034	0.034	0.052		0.034	0.034	0.036	0.037	0.036	0.037
0.033	0.033	0.033	0.033	0.034	0.035		0.222	0.297	0.258	0.256	0.304	0.257
0.033	0.034	0.034	0.034	0.035	0.036		0.263	0.261	0.257	0.232	0.306	0.255
0.03	0.033	0.033	0.034	0.035	0.037		0.20	0.23	0.243	0.291	0.234	0.234

4						8					
Mean	**0.033333**	**0.033333**	**0.033667**	**0.034667**		**Mean**	**0.262667**	**0.252667**	**0.259667**	**0.281333**	
0.033	0.032	0.034	0.035	0.037	0.034	0.038	0.035	0.037	0.038	0.04	0.036
0.032	0.032	0.033	0.034	0.035	0.035	0.2	0.155	0.087	0.073	0.06	0.248
0.033	0.032	0.033	0.034	0.035	0.035	0.229	0.176	0.089	0.076	0.058	0.281
0.033	0.033	0.033	0.034	0.035	0.038	0.214	0.142	0.081	0.082	0.058	0.249
Mean	**0.032333**	**0.033**	**0.034**	**0.035**	**0.0345**	**Mean**	**0.157667**	**0.085667**	**0.077**	**0.058667**	**0.238333**
Replicate 3						**Replicate 3**					
0.032	0.032	0.033	0.035	0.035	0.034	0.034	0.034	0.036	0.037	0.037	0.036
0.034	0.033	0.033	0.033	0.034	0.035	0.226	0.233	0.255	0.318	0.45	0.284
0.034	0.033	0.033	0.034	0.035	0.036	0.182	0.132	0.269	0.248	0.349	0.333
0.034	0.034	0.034	0.034	0.035	0.041	0.195	0.191	0.189	0.197	0.336	0.224
Mean	**0.033333**	**0.033333**	**0.033667**	**0.034667**		**Mean**	**0.185333**	**0.237667**	**0.254333**	**0.378333**	
0.032	0.032	0.033	0.035	0.035	0.035	0.035	0.035	0.038	0.039	0.04	0.035
0.032	0.033	0.033	0.033	0.035	0.035	0.223	0.129	0.079	0.078	0.056	0.189
0.033	0.033	0.033	0.034	0.035	0.036	0.216	0.128	0.079	0.074	0.059	0.245
0.034	0.033	0.033	0.034	0.036	0.036	0.174	0.107	0.077	0.079	0.055	0.181
Mean	**0.033**	**0.033**	**0.033667**	**0.035333**	**0.035**	**Mean**	**0.121333**	**0.078333**	**0.077**	**0.056667**	**0.222667**
Replicate 4						**Replicate 4**					
0.033	0.033	0.034	0.034	0.034	0.037	0.035	0.036	0.036	0.038	0.037	0.037
0.033	0.034	0.033	0.033	0.034	0.035	0.384	0.344	0.476	0.42	0.353	0.369
0.033	0.033	0.033	0.034	0.035	0.037	0.395	0.376	0.397	0.467	0.312	0.347
0.033	0.034	0.034	0.035	0.035	0.037	0.239	0.339	0.452	0.419	0.241	0.26
Mean	**0.033667**	**0.033333**	**0.034**	**0.034667**		**Mean**	**0.353**	**0.441667**	**0.435333**	**0.302**	
0.032	0.033	0.034	0.034	0.035	0.043	0.034	0.035	0.037	0.037	0.039	0.035
0.03	0.033	0.033	0.033	0.035	0.035	0.33	0.169	0.095	0.077	0.054	0.309

2							1					
0.033	0.033	0.033	0.034	0.035	0.036		0.331	0.239	0.109	0.079	0.061	0.315
0.033	0.034	0.034	0.034	0.035	0.036		0.275	0.222	0.141	0.089	0.055	0.225
Mean	**0.033333**	**0.033333**	**0.033667**	**0.035**	**0.034417**		**Mean**	**0.21**	**0.115**	**0.081667**	**0.056667**	**0.315**
Replicate 5							**Replicate 5**					
0.032	0.033	0.034	0.035	0.033	0.046		0.033	0.035	0.038	0.038	0.037	0.036
0.033	0.033	0.033	0.033	0.034	0.036		0.279	0.04	0.393	0.316	0.23	0.268
0.035	0.033	0.035	0.034	0.035	0.036		0.251	0.332	0.477	0.311	0.184	0.321
0.033	0.033	0.034	0.034	0.035	0.036		0.17	0.25	0.239	0.203	0.188	0.197
Mean	**0.033**	**0.034**	**0.033667**	**0.034667**			**Mean**	**0.207333**	**0.369667**	**0.276667**	**0.200667**	
0.033	0.032	0.034	0.034	0.035	0.035		0.035	0.035	0.036	0.038	0.04	0.036
0.032	0.032	0.033	0.033	0.036	0.035		0.221	0.197	0.109	0.07	0.053	0.2
0.033	0.033	0.034	0.034	0.035	0.036		0.232	0.256	0.101	0.071	0.055	0.294
0.033	0.033	0.033	0.034	0.036	0.036		0.204	0.212	0.103	0.078	0.055	0.195
Mean	**0.032667**	**0.033333**	**0.033667**	**0.035667**	**0.0345**		**Mean**	**0.221667**	**0.104333**	**0.073**	**0.054333**	**0.236**
Replicate 6							**Replicate 6**					
0.032	0.033	0.038	0.035	0.034	0.052		0.034	0.036	0.035	0.037	0.037	0.036
0.033	0.033	0.033	0.033	0.035	0.036		0.28	0.289	0.493	0.406	0.208	0.231
0.034	0.033	0.033	0.035	0.035	0.036		0.27	0.22	0.4	0.335	0.178	0.256
0.034	0.033	0.034	0.034	0.035	0.036		0.262	0.267	0.368	0.34	0.179	0.215
Mean	**0.033**	**0.033333**	**0.034**	**0.035**			**Mean**	**0.258667**	**0.420333**	**0.360333**	**0.188333**	
0.038	0.033	0.035	0.035	0.036	0.035		0.035	0.035	0.037	0.038	0.045	0.036
0.033	0.035	0.033	0.033	0.035	0.036		0.292	0.202	0.092	0.074	0.053	0.266
0.034	0.034	0.034	0.034	0.035	0.037		0.284	0.16	0.091	0.078	0.057	0.288
0.033	0.034	0.034	0.035	0.035	0.036		0.249	0.104	0.094	0.087	0.057	0.245
Mean	**0.034333**	**0.033667**	**0.034**	**0.035**	**0.034833**		**Mean**	**0.155333**	**0.092333**	**0.079667**	**0.055667**	**0.2615**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheco*ides						
Na_2SO_4 Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	***-16.0578***	***-3.96825***	10.50545	***-12.0164***	9.758204	5.91922
0.156	***-34.3227***	***0.610501***	***-20.6145***	***-43.2005***	***-74.3523***	***-61.6295***
0.313	***-8.14307***	***-2.59463***	***-30.5253***	***-40.973***	***-26.1658***	***-36.5599***
0.625	***-7.07763***	***-12.5153***	***-104.262***	5.920281	13.21244	35.30641
1.25	48.17352	44.10867	48.56293	38.27667	2.331606	49.09471
2.5	76.63623	77.0757	74.1328	71.68816	63.12608	75.41783
5	78.3105	81.04396	74.92567	83.41149	79.36097	80.71031
10	87.74734	89.43834	87.01685	92.20399	89.03282	90.73816
Coefficient of variation for control	***15.39254***	6.099186	12.99223	13.29851	***16.67895***	5.764653

4.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 30/08/2011							*OD Readings at 96 hrs* – 03/09/2011					
Replicate 1							**Replicate 1**					
0.032	0.031	0.035	0.036	0.037	0.055		0.035	0.035	0.037	0.039	0.041	0.039
0.035	0.034	0.035	0.032	0.035	0.037		0.122	0.047	0.133	0.198	0.156	0.114
0.035	0.034	0.036	0.035	0.037	0.037		0.116	0.052	0.103	0.144	0.146	0.068
0.035	0.036	0.035	0.036	0.04	0.038		0.079	0.042	0.065	0.115	0.129	0.055
Mean	**0.034667**	**0.035333**	**0.034333**	**0.037333**			**Mean**	**0.047**	**0.100333**	**0.152333**	**0.143667**	
0.032	0.034	0.034	0.034	0.036	0.033		0.036	0.037	0.037	0.039	0.039	0.037
0.033	0.034	0.034	0.035	0.036	0.036		0.107	0.044	0.054	0.06	0.061	0.127
0.034	0.034	0.035	0.035	0.037	0.038		0.111	0.044	0.053	0.066	0.063	0.086
0.035	0.035	0.036	0.037	0.037	0.037		0.082	0.043	0.051	0.056	0.051	0.06
Mean	**0.034333**	**0.035**	**0.035667**	**0.036667**	**0.035833**		**Mean**	**0.043667**	**0.052667**	**0.060667**	**0.058333**	**0.093917**
Replicate 2							**Replicate 2**					
0.033	0.032	0.034	0.036	0.037	0.037		0.035	0.035	0.036	0.038	0.038	0.037
0.033	0.033	0.035	0.034	0.037	0.038		0.059	0.039	0.075	0.13	0.186	0.199
0.034	0.034	0.035	0.036	0.036	0.038		0.084	0.042	0.082	0.155	0.145	0.134
0.03	0.034	0.037	0.035	0.038	0.039		0.05	0.043	0.108	0.148	0.152	0.108

4							6					
Mean	**0.033667**	**0.035667**	**0.035**	**0.037**			**Mean**	**0.041333**	**0.088333**	**0.144333**	**0.161**	
0.032	0.032	0.036	0.034	0.036	0.035		0.036	0.036	0.04	0.039	0.038	0.04
0.033	0.034	0.037	0.035	0.036	0.037		0.057	0.041	0.051	0.065	0.064	0.193
0.036	0.034	0.034	0.035	0.037	0.036		0.066	0.042	0.053	0.062	0.057	0.204
0.036	0.035	0.034	0.035	0.037	0.038		0.065	0.043	0.053	0.055	0.049	0.186
Mean	**0.034333**	**0.035**	**0.035**	**0.036667**	**0.036**		**Mean**	**0.042**	**0.052333**	**0.060667**	**0.056667**	**0.117583**
Replicate 3							**Replicate 3**					
0.032	0.036	0.033	0.034	0.035	0.037		0.035	0.038	0.035	0.037	0.036	0.038
0.034	0.033	0.034	0.035	0.036	0.039		0.074	0.051	0.117	0.122	0.183	0.217
0.035	0.034	0.034	0.035	0.036	0.037		0.155	0.05	0.121	0.121	0.182	0.231
0.035	0.035	0.035	0.035	0.036	0.038		0.171	0.046	0.088	0.148	0.17	0.149
Mean	**0.034**	**0.034333**	**0.035**	**0.036**			**Mean**	**0.049**	**0.108667**	**0.130333**	**0.178333**	
0.032	0.032	0.034	0.034	0.037	0.033		0.036	0.036	0.038	0.039	0.039	0.038
0.033	0.033	0.034	0.035	0.037	0.037		0.26	0.047	0.056	0.057	0.059	0.188
0.034	0.034	0.035	0.036	0.037	0.037		0.204	0.05	0.054	0.059	0.055	0.213
0.035	0.035	0.035	0.035	0.037	0.037		0.138	0.049	0.05	0.053	0.049	0.122
Mean	**0.034**	**0.034667**	**0.035333**	**0.037**	**0.035917**		**Mean**	**0.048667**	**0.053333**	**0.056333**	**0.054333**	**0.176833**
Replicate 4							**Replicate 4**					
0.032	0.032	0.033	0.034	0.034	0.035		0.035	0.038	0.037	0.036	0.039	0.037
0.034	0.033	0.034	0.034	0.035	0.036		0.165	0.048	0.116	0.179	0.249	0.081
0.035	0.034	0.034	0.035	0.036	0.037		0.205	0.057	0.115	0.226	0.195	0.146
0.034	0.034	0.034	0.035	0.036	0.037		0.115	0.061	0.12	0.116	0.103	0.166
Mean	**0.033667**	**0.034**	**0.034667**	**0.035667**			**Mean**	**0.055333**	**0.117**	**0.173667**	**0.182333**	
0.032	0.032	0.034	0.034	0.038	0.034		0.037	0.036	0.037	0.038	0.04	0.037
0.03	0.034	0.035	0.035	0.036	0.036		0.18	0.045	0.06	0.071	0.057	0.047

3						7					
0.034	0.035	0.034	0.036	0.037	0.037	0.16	0.045	0.07	0.067	0.063	0.07
0.035	0.035	0.035	0.036	0.037	0.037	0.114	0.046	0.058	0.056	0.053	0.056
Mean	**0.034667**	**0.034667**	**0.035667**	**0.036667**	**0.035417**	**Mean**	**0.045333**	**0.062667**	**0.064667**	**0.057667**	**0.126**
Replicate 5						**Replicate 5**					
0.033	0.033	0.033	0.033	0.035	0.036	0.046	0.036	0.036	0.036	0.037	0.036
0.033	0.033	0.033	0.034	0.036	0.037	0.139	0.054	0.101	0.157	0.17	0.15
0.034	0.033	0.034	0.035	0.036	0.036	0.229	0.053	0.12	0.139	0.145	0.151
0.035	0.034	0.034	0.035	0.037	0.037	0.093	0.059	0.098	0.097	0.126	0.135
Mean	**0.033333**	**0.033667**	**0.034667**	**0.036333**		**Mean**	**0.055333**	**0.106333**	**0.131**	**0.147**	
0.034	0.035	0.033	0.034	0.036	0.033	0.04	0.035	0.036	0.038	0.037	0.039
0.034	0.033	0.034	0.035	0.036	0.037	0.148	0.047	0.057	0.049	0.055	0.099
0.034	0.033	0.034	0.035	0.036	0.037	0.084	0.055	0.052	0.049	0.054	0.107
0.034	0.034	0.035	0.035	0.037	0.037	0.061	0.052	0.051	0.051	0.049	0.068
Mean	**0.033333**	**0.034333**	**0.035**	**0.036333**	**0.035417**	**Mean**	**0.051333**	**0.053333**	**0.049667**	**0.052667**	**0.122**
Replicate 6						**Replicate 6**					
0.035	0.032	0.033	0.034	0.034	0.035	0.041	0.035	0.036	0.036	0.037	0.041
0.034	0.033	0.034	0.034	0.035	0.037	0.062	0.054	0.11	0.125	0.16	0.142
0.035	0.034	0.034	0.034	0.036	0.037	0.079	0.053	0.136	0.131	0.132	0.122
0.035	0.034	0.034	0.035	0.036	0.038	0.059	0.06	0.119	0.129	0.115	0.087
Mean	**0.033667**	**0.034**	**0.034333**	**0.035667**		**Mean**	**0.055667**	**0.121667**	**0.128333**	**0.135667**	
0.032	0.034	0.034	0.034	0.036	0.035	0.041	0.038	0.038	0.039	0.038	0.04
0.034	0.034	0.04	0.035	0.036	0.037	0.063	0.048	0.059	0.058	0.055	0.127
0.035	0.035	0.036	0.037	0.037	0.038	0.057	0.054	0.058	0.064	0.056	0.118
0.036	0.036	0.036	0.037	0.038	0.039	0.046	0.053	0.056	0.052	0.05	0.108
Mean	**0.035**	**0.037333**	**0.036333**	**0.037**	**0.03625**	**Mean**	**0.051667**	**0.057667**	**0.058**	**0.053667**	**0.089167**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
Na_2SO_4 Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	81.70732	97.19439	92.06511	82.861	77.30061	77.40586
0.156	3.658537	61.87375	55.64598	32.92848	14.72393	***-5.43933***
0.313	***-72.439***	19.78958	42.42116	***-12.9555***	***-15.5419***	***-13.8075***
0.625	***-59.7561***	7.264529	13.12309	***-19.973***	***-35.1738***	***-23.0126***
1.25	86.58537	96.69339	92.26857	90.95816	82.20859	82.42678
2.5	73.41463	88.92786	89.42014	76.92308	79.7546	74.8954
5	61.70732	82.66533	87.58901	75.30364	84.25358	74.47699
10	65.12195	85.67134	88.80977	80.97166	80.5726	79.91632
Coefficient of variation for control	***17.54093***	***23.31904***	***23.80095***	***23.53969***	***27.72896***	***15.8593***

4.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 30/08/2011							*OD Readings at 96 hrs* – 03/09/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.033	0.033	0.034	0.035		0.035	0.035	0.036	0.036	0.037	0.037
0.033	0.033	0.033	0.034	0.035	0.038		0.119	0.158	0.086	0.094	0.094	0.076
0.033	0.033	0.034	0.034	0.035	0.036		0.202	0.137	0.086	0.098	0.085	0.08
0.036	0.033	0.034	0.034	0.036	0.037		0.079	0.121	0.076	0.071	0.078	0.074
Mean	**0.033**	**0.033667**	**0.034**	**0.035333**			**Mean**	**0.138667**	**0.082667**	**0.087667**	**0.085667**	
0.031	0.032	0.034	0.034	0.034	0.036		0.036	0.037	0.039	0.039	0.037	0.038
0.035	0.032	0.033	0.034	0.035	0.036		0.086	0.071	0.063	0.052	0.037	0.075
0.035	0.033	0.034	0.034	0.036	0.036		0.092	0.074	0.062	0.052	0.038	0.087
0.035	0.034	0.034	0.036	0.036	0.037		0.085	0.036	0.069	0.054	0.038	0.066
Mean	**0.033**	**0.033667**	**0.034667**	**0.035667**	**0.035583**		**Mean**	**0.060333**	**0.064667**	**0.052667**	**0.037667**	**0.093417**
Replicate 2							**Replicate 2**					
0.032	0.033	0.033	0.033	0.035	0.037		0.034	0.035	0.035	0.035	0.038	0.037
0.032	0.033	0.033	0.034	0.035	0.036		0.105	0.072	0.074	0.079	0.082	0.081
0.034	0.033	0.033	0.034	0.035	0.036		0.192	0.072	0.077	0.081	0.092	0.197
0.033	0.033	0.033	0.034	0.035	0.036		0.103	0.074	0.068	0.073	0.079	0.08

Mean	**0.033**	**0.033**	**0.034**	**0.035**			**Mean**	**0.072667**	**0.073**	**0.077667**	**0.084333**	
0.033	0.032	0.034	0.036	0.036	0.035		0.036	0.036	0.039	0.04	0.037	0.039
0.033	0.033	0.034	0.035	0.035	0.036		0.098	0.101	0.058	0.048	0.037	0.074
0.033	0.033	0.034	0.036	0.038	0.036		0.091	0.072	0.067	0.049	0.04	0.088
0.034	0.034	0.034	0.035	0.036	0.038		0.115	0.077	0.065	0.05	0.038	0.143
Mean	**0.033333**	**0.034**	**0.035333**	**0.036333**	**0.03475**		**Mean**	**0.083333**	**0.063333**	**0.049**	**0.038333**	**0.113917**
Replicate 3							**Replicate 3**					
0.032	0.032	0.033	0.034	0.035	0.034		0.035	0.035	0.037	0.038	0.038	0.036
0.033	0.032	0.033	0.033	0.035	0.036		0.089	0.079	0.083	0.12	0.093	0.293
0.035	0.033	0.033	0.034	0.035	0.036		0.115	0.091	0.077	0.205	0.078	0.088
0.034	0.035	0.036	0.035	0.037	0.036		0.081	0.078	0.092	0.102	0.074	0.067
Mean	**0.033333**	**0.034**	**0.034**	**0.035667**			**Mean**	**0.082667**	**0.084**	**0.142333**	**0.081667**	
0.032	0.036	0.033	0.034	0.037	0.033		0.037	0.038	0.042	0.042	0.039	0.036
0.033	0.033	0.033	0.034	0.035	0.035		0.092	0.034	0.073	0.066	0.038	0.083
0.033	0.034	0.033	0.034	0.036	0.036		0.102	0.035	0.075	0.069	0.04	0.093
0.033	0.035	0.034	0.034	0.036	0.037		0.068	0.037	0.081	0.054	0.039	0.08
Mean	**0.034**	**0.033333**	**0.034**	**0.035667**	**0.03475**		**Mean**	**0.035333**	**0.076333**	**0.063**	**0.039**	**0.104225**
Replicate 4							**Replicate 4**					
0.034	0.032	0.032	0.033	0.035	0.033		0.037	0.035	0.035	0.036	0.038	0.037
0.032	0.032	0.033	0.033	0.035	0.037		0.079	0.071	0.086	0.081	0.094	0.073
0.034	0.033	0.033	0.034	0.035	0.04		0.096	0.069	0.075	0.087	0.238	0.159
0.034	0.034	0.034	0.034	0.037	0.038		0.095	0.076	0.069	0.076	0.068	0.078
Mean	**0.033**	**0.033333**	**0.033667**	**0.035667**			**Mean**	**0.072**	**0.076667**	**0.081333**	**0.133333**	
0.032	0.033	0.035	0.034	0.034	0.036		0.035	0.035	0.035	0.039	0.038	0.036
0.034	0.039	0.034	0.034	0.035	0.035		0.133	0.037	0.068	0.065	0.039	0.077

0.034	0.035	0.034	0.035	0.037	0.036		0.079	0.035	0.062	0.054	0.038	0.086
0.036	0.035	0.034	0.035	0.036	0.036		0.062	0.037	0.062	0.054	0.038	0.059
Mean	**0.036333**	**0.034**	**0.034667**	**0.036**	**0.0355**		**Mean**	**0.036333**	**0.064**	**0.057667**	**0.038333**	**0.089667**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.033	0.034	0.036		0.034	0.035	0.035	0.036	0.037	0.036
0.033	0.032	0.033	0.034	0.036	0.037		0.125	0.245	0.078	0.09	0.095	0.092
0.034	0.033	0.033	0.035	0.036	0.036		0.116	0.208	0.079	0.077	0.085	0.091
0.038	0.034	0.034	0.034	0.036	0.036		0.091	0.068	0.078	0.075	0.078	0.077
Mean	**0.033**	**0.033333**	**0.034333**	**0.036**			**Mean**	**0.173667**	**0.078333**	**0.080667**	**0.086**	
0.032	0.035	0.033	0.033	0.035	0.033		0.036	0.034	0.041	0.04	0.036	0.036
0.032	0.033	0.033	0.034	0.035	0.036		0.125	0.034	0.061	0.05	0.037	0.081
0.033	0.034	0.033	0.034	0.036	0.036		0.204	0.034	0.057	0.05	0.039	0.101
0.033	0.035	0.034	0.034	0.037	0.037		0.087	0.036	0.06	0.053	0.037	0.071
Mean	**0.034**	**0.033333**	**0.034**	**0.036**	**0.035083**		**Mean**	**0.034667**	**0.059333**	**0.051**	**0.037667**	**0.105083**
Replicate 6							**Replicate 6**					
0.032	0.032	0.033	0.033	0.035	0.033		0.035	0.035	0.036	0.036	0.036	0.035
0.033	0.033	0.033	0.034	0.035	0.036		0.187	0.071	0.082	0.077	0.085	0.088
0.035	0.033	0.034	0.034	0.035	0.037		0.156	0.081	0.079	0.101	0.075	0.079
0.035	0.034	0.035	0.034	0.036	0.037		0.078	0.071	0.076	0.083	0.092	0.075
Mean	**0.033333**	**0.034**	**0.034**	**0.035333**			**Mean**	**0.074333**	**0.079**	**0.087**	**0.084**	
0.032	0.033	0.034	0.035	0.035	0.037		0.036	0.036	0.036	0.041	0.037	0.039
0.034	0.034	0.034	0.034	0.035	0.035		0.086	0.09	0.035	0.071	0.04	0.086
0.037	0.035	0.035	0.034	0.036	0.036		0.084	0.085	0.036	0.054	0.04	0.089
0.046	0.042	0.035	0.034	0.036	0.036		0.078	0.081	0.038	0.05	0.039	0.073
Mean	**0.037**	**0.034667**	**0.034**	**0.035667**	**0.036417**		**Mean**	**0.085333**	**0.036333**	**0.058333**	**0.039667**	**0.096583**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
Na_2SO_4 Concentration (g/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.07	***-122.383***	49.86761	13.59517	41.64384	-53.945	16.11722
0.156	***-1.08303***	49.4263	11.17825	33.9726	51.00917	5.860806
0.313	***-11.9134***	43.24801	***-94.5619***	26.30137	48.44037	***-11.7216***
0.625	***-7.58123***	34.42189	15.40785	-59.1781	42.56881	***-5.12821***
1.25	47.29242	35.74581	99.39577	100.274	99.08257	***-8.05861***
2.5	37.90614	62.22418	25.07553	54.79452	71.92661	99.6337
5	63.89892	81.20035	49.24471	65.20548	81.10092	51.28205
10	96.38989	95.32215	92.74924	96.9863	95.77982	92.30769
Coefficient of variation for control	8.233395	***40.20602***	14.58597	***40.12202***	***33.88282***	8.471723

5. Coal power plant effluent

Plate configuration

Plate 1					
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Plate 2					
Blank	Blank	Blank	Blank	Blank	Blank
Control	12.5 %	25 %	50 %	100 %	Control
Control	12.5 %	25 %	50 %	100 %	Control
Control	12.5 %	25 %	50 %	100 %	Control

5.1. OD readings at 450nm for *Pseudokirchneriella subcapitata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 21/10/2011							*OD Readings at 96 hrs* – 25/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.034	0.035	0.036	0.037		0.033	0.035	0.037	0.038	0.044	0.059
0.036	0.034	0.034	0.034	0.036	0.037		0.18	0.194	0.257	0.207	0.231	0.153
0.034	0.034	0.034	0.035	0.037	0.038		0.124	0.172	0.21	0.204	0.224	0.157
0.034	0.034	0.034	0.035	0.037	0.038		0.085	0.179	0.226	0.171	0.231	0.074
Mean	**0.034**	**0.034**	**0.034667**	**0.036667**			**Mean**	**0.181667**	**0.231**	**0.194**	**0.228667**	

0.033	0.034	0.037	0.038	0.041	0.033		0.032	0.038	0.041	0.042	0.041	0.036
0.033	0.035	0.036	0.038	0.042	0.036		0.137	0.353	0.273	0.249	0.25	0.123
0.034	0.035	0.037	0.04	0.042	0.037		0.095	0.327	0.224	0.236	0.229	0.127
0.034	0.036	0.037	0.038	0.042	0.037		0.082	0.221	0.169	0.137	0.138	0.118
Mean	**0.035333**	**0.036667**	**0.038667**	**0.042**	**0.035667**		**Mean**	**0.300333**	**0.222**	**0.207333**	**0.205667**	**0.126167**
Replicate 2							**Replicate 2**					
0.033	0.033	0.034	0.034	0.035	0.033		0.034	0.036	0.037	0.038	0.04	0.046
0.035	0.034	0.036	0.035	0.036	0.036		0.15	0.291	0.279	0.163	0.187	0.119
0.034	0.034	0.034	0.035	0.037	0.038		0.099	0.247	0.231	0.188	0.181	0.131
0.036	0.035	0.036	0.035	0.036	0.037		0.051	0.253	0.19	0.164	0.16	0.128
Mean	**0.034143**	**0.035143**	**0.034857**	**0.036143**			**Mean**	**0.263667**	**0.233333**	**0.171667**	**0.176**	
0.032	0.035	0.037	0.038	0.041	0.034		0.032	0.038	0.038	0.04	0.043	0.036
0.033	0.035	0.036	0.038	0.042	0.036		0.039	0.36	0.265	0.229	0.207	0.138
0.034	0.035	0.037	0.039	0.042	0.037		0.036	0.367	0.376	0.244	0.221	0.167
0.034	0.036	0.038	0.038	0.043	0.037		0.035	0.299	0.166	0.174	0.157	0.161
Mean	**0.035333**	**0.037**	**0.038333**	**0.042333**	**0.036833**		**Mean**	**0.342**	**0.269**	**0.215667**	**0.195**	**0.140667**
Replicate 3							**Replicate 3**					
0.034	0.033	0.034	0.034	0.036	0.034		0.034	0.035	0.037	0.037	0.044	0.052
0.033	0.035	0.034	0.035	0.036	0.036		0.128	0.248	0.215	0.162	0.195	0.134
0.034	0.034	0.034	0.035	0.036	0.037		0.11	0.267	0.23	0.185	0.189	0.135
0.034	0.034	0.036	0.035	0.036	0.037		0.039	0.241	0.251	0.197	0.172	0.176
Mean	**0.034333**	**0.034667**	**0.035**	**0.036**			**Mean**	**0.252**	**0.232**	**0.181333**	**0.185333**	
0.032	0.034	0.037	0.038	0.041	0.035		0.032	0.039	0.04	0.041	0.041	0.044
0.034	0.035	0.036	0.038	0.042	0.036		0.157	0.257	0.204	0.205	0.181	0.147
0.034	0.035	0.038	0.039	0.042	0.037		0.202	0.274	0.235	0.232	0.217	0.128

0.035	0.035	0.036	0.038	0.042	0.037		0.106	0.309	0.254	0.139	0.161	0.127
Mean	**0.035**	**0.036667**	**0.038333**	**0.042**	**0.036667**		**Mean**	**0.28**	**0.231**	**0.192**	**0.186333**	**0.141167**
Replicate 4							**Replicate 4**					
0.033	0.034	0.034	0.035	0.034	0.034		0.034	0.038	0.037	0.038	0.039	0.044
0.034	0.034	0.034	0.034	0.036	0.037		0.148	0.225	0.224	0.201	0.228	0.16
0.034	0.034	0.035	0.035	0.037	0.037		0.163	0.184	0.214	0.22	0.244	0.192
0.035	0.035	0.035	0.035	0.036	0.04		0.091	0.203	0.181	0.211	0.209	0.066
Mean	**0.034333**	**0.034667**	**0.034667**	**0.036333**			**Mean**	**0.204**	**0.206333**	**0.210667**	**0.227**	
0.032	0.034	0.037	0.038	0.041	0.034		0.032	0.039	0.039	0.041	0.045	0.036
0.033	0.035	0.036	0.038	0.042	0.036		0.141	0.29	0.26	0.261	0.198	0.116
0.034	0.035	0.037	0.039	0.042	0.037		0.155	0.279	0.311	0.299	0.243	0.14
0.035	0.037	0.036	0.038	0.043	0.038		0.144	0.203	0.269	0.203	0.131	0.065
Mean	**0.035667**	**0.036333**	**0.038333**	**0.042333**	**0.034167**		**Mean**	**0.257333**	**0.28**	**0.254333**	**0.190667**	**0.140333**
Replicate 5							**Replicate 5**					
0.036	0.033	0.034	0.035	0.034	0.055		0.036	0.037	0.036	0.038	0.037	0.048
0.034	0.034	0.035	0.034	0.036	0.037		0.189	0.166	0.187	0.234	0.221	0.149
0.034	0.034	0.044	0.035	0.037	0.037		0.18	0.169	0.254	0.288	0.207	0.123
0.034	0.035	0.036	0.035	0.036	0.037		0.08	0.212	0.218	0.282	0.281	0.077
Mean	**0.034333**	**0.038333**	**0.034667**	**0.036333**			**Mean**	**0.182333**	**0.219667**	**0.268**	**0.236333**	
0.032	0.033	0.037	0.038	0.042	0.033		0.033	0.038	0.039	0.041	0.042	0.036
0.033	0.036	0.036	0.038	0.042	0.036		0.157	0.248	0.32	0.283	0.303	0.064
0.034	0.035	0.037	0.039	0.042	0.037		0.207	0.227	0.284	0.317	0.351	0.063
0.034	0.036	0.036	0.038	0.042	0.037		0.138	0.273	0.187	0.347	0.261	0.056
Mean	**0.035667**	**0.036333**	**0.038333**	**0.042**	**0.033833**		**Mean**	**0.249333**	**0.263667**	**0.315667**	**0.305**	**0.1585**

Replicate 6							**Replicate 6**					
0.032	0.033	0.035	0.035	0.036	0.034		0.032	0.035	0.037	0.038	0.038	0.049
0.035	0.036	0.035	0.036	0.039	0.039		0.161	0.171	0.201	0.254	0.325	0.107
0.036	0.036	0.039	0.037	0.038	0.041		0.155	0.185	0.182	0.241	0.351	0.101
0.036	0.037	0.036	0.037	0.041	0.038		0.173	0.196	0.212	0.249	0.287	0.087
Mean	**0.036333**	**0.036667**	**0.036667**	**0.039333**			**Mean**	**0.184**	**0.198333**	**0.248**	**0.321**	
0.034	0.035	0.037	0.039	0.042	0.033		0.034	0.04	0.04	0.04	0.043	0.046
0.034	0.035	0.036	0.038	0.042	0.036		0.109	0.249	0.269	0.374	0.27	0.067
0.034	0.035	0.037	0.038	0.044	0.038		0.138	0.27	0.326	0.338	0.306	0.086
0.04	0.036	0.036	0.038	0.044	0.037		0.142	0.206	0.21	0.222	0.244	0.046
Mean	**0.035333**	**0.036333**	**0.038**	**0.043333**	**0.035833**		**Mean**	**0.241667**	**0.268333**	**0.311333**	**0.273333**	**0.146333**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-61.326***	***-118.459***	***-106.061***	***-59.9686***	***-19.1176***	***-34.0875***
1.56	***-115.838***	***-89.2456***	***-86.9219***	***-62.1664***	***-49.0642***	***-47.0588***
3.13	***-74.954***	***-29.8555***	***-38.437***	***-66.248***	***-87.8342***	***-92.006***
6.25	***-113.26***	***-34.0289***	***-42.2648***	***-81.6327***	***-62.4332***	***-158.069***
12.5	***-192.449***	***-193.9***	***-132.855***	***-110.204***	***-72.861***	***-86.2745***
25	***-105.893***	***-123.596***	***-85.9649***	***-131.554***	***-84.3583***	***-110.407***
50	***-89.6869***	***-72.2311***	***-48.6443***	***-107.378***	***-126.07***	***-149.321***
100	***-87.8453***	***-52.3274***	***-43.2217***	***-47.4097***	***-117.513***	***-114.932***
Coefficient of variation for control	***24.23558***	13.62755	13.10348	***18.12649***	***28.67645***	***15.22963***

5.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 01/11/2011							*OD Readings at 96 hrs* – 05/11/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.034	0.035	0.034	0.046		0.035	0.034	0.036	0.04	0.038	0.037
0.034	0.032	0.033	0.036	0.035	0.036		0.149	0.204	0.361	0.371	0.443	0.27
0.034	0.033	0.034	0.034	0.036	0.036		0.185	0.267	0.331	0.346	0.456	0.257

0.033	0.034	0.034	0.035	0.038	0.037		0.14	0.284	0.311	0.319	0.461	0.165
Mean	**0.033**	**0.033667**	**0.035**	**0.036333**			**Mean**	**0.251667**	**0.334333**	**0.345333**	**0.453333**	
0.031	0.033	0.035	0.039	0.042	0.032		0.036	0.039	0.04	0.042	0.042	0.036
0.032	0.034	0.035	0.037	0.042	0.035		0.146	0.087	0.258	0.335	0.592	0.208
0.032	0.034	0.035	0.038	0.044	0.036		0.141	0.129	0.353	0.112	0.349	0.207
0.033	0.034	0.035	0.038	0.042	0.035		0.123	0.208	0.105	0.225	0.171	0.13
Mean	0.034	0.035	0.037667	0.042667	**0.035833**		Mean	0.141333	0.238667	0.224	0.370667	**0.206167**
Replicate 2							**Replicate 2**					
0.032	0.032	0.033	0.034	0.035	0.034		0.034	0.034	0.035	0.038	0.038	0.036
0.032	0.032	0.033	0.034	0.035	0.036		0.195	0.292	0.271	0.336	0.485	0.149
0.033	0.033	0.033	0.034	0.036	0.036		0.212	0.284	0.265	0.286	0.466	0.189
0.033	0.033	0.033	0.035	0.036	0.036		0.139	0.194	0.203	0.281	0.256	0.143
Mean	**0.032667**	**0.033**	**0.034333**	**0.035667**			**Mean**	**0.256667**	**0.246333**	**0.301**	**0.402333**	
0.031	0.033	0.035	0.038	0.042	0.043		0.035	0.036	0.039	0.041	0.042	0.038
0.032	0.033	0.035	0.037	0.042	0.035		0.233	0.536	0.509	0.145	0.818	0.045
0.033	0.034	0.035	0.04	0.043	0.037		0.252	0.274	0.421	0.759	0.701	0.249
0.033	0.034	0.035	0.038	0.042	0.036		0.147	0.069	0.363	0.126	0.52	0.153
Mean	**0.033667**	**0.035**	**0.038333**	**0.042333**	**0.032667**		**Mean**	**0.293**	**0.431**	**0.343333**	**0.679667**	**0.196333**
Replicate 3							**Replicate 3**					
0.032	0.032	0.032	0.034	0.035	0.034		0.034	0.034	0.035	0.038	0.037	0.036
0.032	0.032	0.033	0.033	0.036	0.035		0.206	0.329	0.346	0.299	0.401	0.133
0.033	0.033	0.033	0.034	0.035	0.036		0.243	0.344	0.32	0.289	0.203	0.117
0.033	0.033	0.033	0.034	0.035	0.036		0.143	0.308	0.286	0.268	0.112	0.11
Mean	**0.032667**	**0.033**	**0.033667**	**0.035333**			**Mean**	**0.327**	**0.317333**	**0.285333**	**0.238667**	

0.032	0.034	0.035	0.039	0.042	0.033		0.035	0.037	0.039	0.041	0.044	0.038
0.032	0.033	0.035	0.038	0.046	0.037		0.279	0.615	0.591	0.266	0.191	0.116
0.033	0.034	0.035	0.038	0.043	0.036		0.248	0.651	0.604	0.371	0.326	0.129
0.033	0.034	0.035	0.038	0.043	0.039		0.133	0.267	0.431	0.243	0.126	0.106
Mean	**0.033667**	**0.035**	**0.038**	**0.044**	**0.032667**		**Mean**	**0.511**	**0.542**	**0.293333**	**0.214333**	**0.208667**
Replicate 4							**Replicate 4**					
0.031	0.033	0.033	0.034	0.036	0.035		0.035	0.036	0.037	0.038	0.039	0.038
0.032	0.033	0.034	0.034	0.035	0.036		0.282	0.262	0.299	0.441	0.466	0.287
0.032	0.032	0.033	0.034	0.036	0.036		0.199	0.19	0.4	0.546	0.489	0.351
0.033	0.033	0.034	0.034	0.036	0.036		0.14	0.206	0.304	0.146	0.367	0.171
Mean	**0.032667**	**0.033667**	**0.034**	**0.035667**			**Mean**	**0.219333**	**0.334333**	**0.377667**	**0.440667**	
0.031	0.033	0.035	0.04	0.042	0.033		0.035	0.037	0.039	0.043	0.043	0.036
0.032	0.033	0.035	0.038	0.042	0.035		0.29	0.141	0.315	0.531	0.649	0.207
0.032	0.034	0.035	0.038	0.043	0.036		0.271	0.299	0.278	0.222	0.879	0.239
0.033	0.034	0.035	0.038	0.043	0.037		0.164	0.155	0.439	0.128	0.598	0.153
Mean	**0.033667**	**0.035**	**0.038**	**0.042667**	**0.032333**		**Mean**	**0.198333**	**0.344**	**0.293667**	**0.708667**	**0.224333**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.034	0.035	0.048		0.034	0.034	0.037	0.04	0.038	0.036
0.033	0.033	0.033	0.034	0.035	0.035		0.263	0.307	0.268	0.388	0.51	0.215
0.034	0.033	0.033	0.034	0.036	0.036		0.241	0.271	0.33	0.416	0.594	0.192
0.033	0.034	0.034	0.034	0.035	0.036		0.195	0.231	0.321	0.314	0.58	0.108
Mean	**0.033333**	**0.033333**	**0.034**	**0.035333**			**Mean**	**0.269667**	**0.306333**	**0.372667**	**0.561333**	
0.032	0.033	0.034	0.039	0.041	0.034		0.034	0.036	0.038	0.04	0.042	0.037
0.035	0.033	0.035	0.038	0.042	0.035		0.264	0.51	0.568	0.074	0.54	0.206
0.033	0.034	0.036	0.038	0.043	0.035		0.214	0.297	0.353	0.426	0.603	0.251

0.033	0.035	0.035	0.038	0.042	0.036		0.121	0.096	0.205	0.364	0.558	0.176
Mean	**0.034**	**0.035333**	**0.038**	**0.042333**	**0.034333**		**Mean**	**0.301**	**0.375333**	**0.288**	**0.567**	**0.222**
Replicate 6							**Replicate 6**					
0.032	0.033	0.034	0.034	0.034	0.048		0.034	0.035	0.035	0.037	0.037	0.036
0.033	0.033	0.033	0.035	0.035	0.036		0.368	0.52	0.397	0.55	0.435	0.267
0.033	0.033	0.034	0.034	0.035	0.035		0.344	0.455	0.439	0.496	0.528	0.316
0.035	0.035	0.033	0.034	0.037	0.035		0.215	0.115	0.418	0.457	0.495	0.241
Mean	**0.033667**	**0.033333**	**0.034333**	**0.035667**			**Mean**	**0.363333**	**0.418**	**0.501**	**0.486**	
0.031	0.033	0.035	0.039	0.042	0.035		0.036	0.037	0.038	0.041	0.042	0.038
0.033	0.033	0.035	0.039	0.042	0.037		0.305	0.449	0.807	0.651	0.736	0.234
0.034	0.037	0.035	0.038	0.043	0.036		0.254	0.684	0.336	0.294	0.145	0.236
0.033	0.034	0.036	0.038	0.043	0.036		0.153	0.29	0.116	0.172	0.138	0.168
Mean	0.034667	0.035333	0.038333	0.042667	**0.034333**		Mean	0.474333	0.419667	0.372333	0.339667	**0.256**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheocoides*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	**-26.7123**	**-36.8635**	**-67.2348**	2.604167	***-25.3996***	***-48.4211***
1.56	**-75.2446**	**-30.5499**	**-61.7424**	***-57.2917***	***-44.9378***	***-73.0827***
3.13	**-81.7025**	**-63.9511**	**-43.5606**	***-79.8611***	***-80.2842***	***-110.526***
6.25	**-145.108**	**-125.866**	**-17.0455**	***-112.674***	***-180.817***	***-103.759***
12.5	38.06262	***-59.0631***	***-171.78***	13.54167	***-42.0959***	***-98.4962***
25	***-19.0802***	***-143.381***	***-189.394***	***-62.3264***	***-81.7052***	***-73.8346***
50	***-10.4697***	***-89.8167***	***-48.1061***	***-36.1111***	***-35.1687***	***-52.4812***
100	***-96.5753***	***-295.316***	***-3.2197***	***-252.257***	***-183.837***	***-37.7444***
Coefficient of variation for control	***25.81132***	***23.24203***	***28.52733***	***29.03252***	***15.58324***	***22.75034***

5.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 01/11/2011							*OD Readings at 96 hrs* – 05/11/2011					
Replicate 1							**Replicate 1**					

0.032	0.032	0.033	0.034	0.035	0.043		0.034	0.036	0.036	0.037	0.039	0.037
0.033	0.033	0.033	0.034	0.036	0.036		0.14	0.199	0.222	0.161	0.11	0.076
0.035	0.034	0.034	0.035	0.036	0.037		0.162	0.229	0.262	0.188	0.123	0.082
0.034	0.036	0.035	0.035	0.038	0.039		0.136	0.098	0.155	0.159	0.11	0.072
Mean	**0.034333**	**0.034**	**0.034667**	**0.036667**			**Mean**	**0.175333**	**0.213**	**0.169333**	**0.114333**	
0.032	0.033	0.035	0.038	0.041	0.034		0.035	0.036	0.038	0.041	0.042	0.036
0.033	0.035	0.036	0.037	0.043	0.036		0.127	0.168	0.352	0.298	0.151	0.071
0.034	0.034	0.036	0.038	0.043	0.037		0.219	0.276	0.311	0.129	0.127	0.065
0.034	0.035	0.036	0.038	0.043	0.037		0.154	0.171	0.266	0.1	0.099	0.064
Mean	**0.034667**	**0.036**	**0.037667**	**0.043**	**0.033833**		**Mean**	**0.205**	**0.309667**	**0.175667**	**0.125667**	**0.156333**
Replicate 2							**Replicate 2**					
0.031	0.033	0.033	0.034	0.035	0.051		0.034	0.036	0.037	0.037	0.038	0.036
0.033	0.033	0.033	0.034	0.036	0.035		0.137	0.203	0.235	0.154	0.069	0.076
0.034	0.034	0.034	0.035	0.036	0.037		0.139	0.242	0.196	0.135	0.059	0.054
0.04	0.034	0.034	0.035	0.037	0.037		0.131	0.154	0.202	0.137	0.051	0.057
Mean	**0.033667**	**0.033667**	**0.034667**	**0.036333**			**Mean**	**0.199667**	**0.211**	**0.142**	**0.059667**	
0.031	0.033	0.035	0.039	0.042	0.035		0.034	0.036	0.037	0.04	0.044	0.036
0.033	0.034	0.036	0.038	0.043	0.036		0.177	0.375	0.382	0.512	0.141	0.072
0.034	0.035	0.036	0.039	0.043	0.037		0.15	0.275	0.442	0.125	0.119	0.07
0.034	0.035	0.036	0.038	0.043	0.037		0.075	0.263	0.196	0.113	0.103	0.058
Mean	**0.034667**	**0.036**	**0.038333**	**0.043**	**0.034667**		**Mean**	**0.304333**	**0.34**	**0.25**	**0.121**	**0.134833**
Replicate 3							**Replicate 3**					
0.032	0.032	0.033	0.035	0.035	0.035		0.034	0.034	0.037	0.039	0.039	0.037
0.035	0.033	0.037	0.035	0.036	0.037		0.146	0.185	0.228	0.144	0.088	0.065
0.034	0.034	0.034	0.035	0.036	0.037		0.152	0.182	0.184	0.15	0.107	0.069

0.034	0.035	0.034	0.035	0.036	0.037		0.117	0.051	0.18	0.126	0.059	0.067
Mean	**0.034**	**0.035**	**0.035**	**0.036**			**Mean**	**0.139333**	**0.197333**	**0.14**	**0.084667**	
0.032	0.033	0.036	0.038	0.042	0.039		0.034	0.036	0.037	0.04	0.043	0.041
0.034	0.034	0.036	0.038	0.043	0.036		0.125	0.241	0.126	0.102	0.129	0.067
0.034	0.034	0.036	0.039	0.043	0.037		0.102	0.169	0.125	0.103	0.116	0.069
0.034	0.035	0.036	0.038	0.043	0.037		0.056	0.122	0.129	0.065	0.123	0.114
Mean	**0.034333**	**0.036**	**0.038333**	**0.043**	**0.034167**		**Mean**	**0.177333**	**0.126667**	**0.09**	**0.122667**	**0.116333**
Replicate 4							**Replicate 4**					
0.032	0.032	0.033	0.035	0.034	0.041		0.034	0.036	0.035	0.038	0.037	0.038
0.033	0.034	0.035	0.034	0.036	0.036		0.126	0.137	0.143	0.172	0.137	0.158
0.034	0.034	0.034	0.035	0.036	0.036		0.122	0.131	0.17	0.18	0.186	0.108
0.034	0.034	0.035	0.035	0.036	0.037		0.107	0.084	0.093	0.083	0.073	0.07
Mean	**0.034**	**0.034667**	**0.034667**	**0.036**			**Mean**	**0.117333**	**0.135333**	**0.145**	**0.132**	
0.031	0.033	0.036	0.039	0.042	0.037		0.034	0.036	0.038	0.041	0.042	0.037
0.033	0.034	0.036	0.038	0.043	0.036		0.128	0.036	0.051	0.193	0.2	0.146
0.033	0.034	0.036	0.039	0.043	0.037		0.109	0.037	0.12	0.139	0.199	0.158
0.034	0.035	0.036	0.038	0.043	0.037		0.107	0.041	0.062	0.085	0.508	0.103
Mean	**0.034333**	**0.036**	**0.038333**	**0.043**	**0.0335**		**Mean**	**0.038**	**0.077667**	**0.139**	**0.302333**	**0.1165**
Replicate 5							**Replicate 5**					
0.032	0.032	0.034	0.035	0.034	0.058		0.034	0.038	0.037	0.039	0.038	0.035
0.033	0.033	0.034	0.034	0.036	0.037		0.115	0.046	0.088	0.274	0.137	0.131
0.034	0.034	0.034	0.035	0.036	0.037		0.128	0.056	0.088	0.131	0.181	0.12
0.034	0.037	0.034	0.035	0.036	0.036		0.079	0.054	0.124	0.155	0.089	0.103
Mean	**0.034667**	**0.034**	**0.034667**	**0.036**			**Mean**	**0.052**	**0.1**	**0.186667**	**0.135667**	

0.032	0.033	0.035	0.038	0.042	0.036		0.035	0.036	0.037	0.04	0.042	0.038
0.033	0.034	0.035	0.038	0.043	0.036		0.113	0.037	0.107	0.175	0.408	0.175
0.033	0.034	0.036	0.039	0.043	0.036		0.109	0.036	0.117	0.156	0.267	0.134
0.035	0.035	0.037	0.039	0.043	0.037		0.094	0.041	0.045	0.08	0.479	0.105
Mean	**0.034333**	**0.036**	**0.038667**	**0.043**	**0.035167**		**Mean**	**0.038**	**0.089667**	**0.137**	**0.384667**	**0.111667**
Replicate 6							**Replicate 6**					
0.032	0.033	0.034	0.035	0.035	0.054		0.034	0.039	0.04	0.039	0.038	0.037
0.034	0.034	0.034	0.034	0.039	0.035		0.105	0.064	0.182	0.115	0.256	0.135
0.033	0.036	0.035	0.036	0.041	0.038		0.127	0.071	0.195	0.084	0.2	0.136
0.033	0.035	0.036	0.035	0.036	0.04		0.094	0.069	0.106	0.105	0.143	0.102
Mean	**0.035**	**0.035**	**0.035**	**0.038667**			**Mean**	**0.068**	**0.161**	**0.101333**	**0.199667**	
0.032	0.033	0.036	0.038	0.043	0.035		0.035	0.038	0.04	0.042	0.044	0.037
0.033	0.034	0.036	0.038	0.043	0.037		0.143	0.041	0.163	0.163	0.168	0.161
0.034	0.035	0.036	0.039	0.043	0.038		0.135	0.039	0.141	0.142	0.393	0.161
0.037	0.035	0.037	0.039	0.043	0.038		0.108	0.039	0.068	0.112	0.147	0.1
Mean	**0.034667**	**0.036333**	**0.038667**	**0.043**	**0.036167**		**Mean**	**0.039667**	**0.124**	**0.139**	**0.236**	**0.1265**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-15.5102***	***-64.7255***	***-27.9919***	***-1.00402***	145.9695	140.0369
1.56	***-46.2585***	***-76.0399***	***-98.5801***	***-22.6908***	15.25054	***-38.1919***
3.13	***-10.6122***	***-7.15474***	***-28.8032***	***-34.3373***	***-98.0392***	27.85978
6.25	***34.28571***	***75.0416***	38.53955	***-18.6747***	***-31.3725***	***-80.9963***
12.5	***-39.7279***	***-169.218***	***-74.2394***	140.3614	***145.9695***	***140.0369***
25	***-125.17***	***-204.825***	***-12.5761***	46.78715	28.75817	2.767528
50	***-15.7823***	***-114.975***	32.04868	***-27.1084***	***-33.1155***	***-13.8376***
100	25.03401	13.81032	***-7.70791***	***-223.896***	***-356.863***	***-121.218***
Coefficient of variation for control	***21.22537***	***24.87554***	***29.9588***	8.493084	11.60269	13.45964

5.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 21/10/2011							*OD Readings at 96 hrs* – 25/10/2011					
Replicate 1							**Replicate 1**					
0.032	0.032	0.033	0.035	0.035	0.034		0.032	0.038	0.035	0.037	0.039	0.033
0.033	0.033	0.033	0.035	0.035	0.036		0.098	0.188	0.111	0.081	0.078	0.068
0.033	0.033	0.034	0.034	0.036	0.036		0.08	0.085	0.099	0.096	0.076	0.075
0.033	0.034	0.033	0.034	0.035	0.036		0.068	0.094	0.07	0.079	0.073	0.061
Mean	**0.033333**	**0.033333**	**0.034333**	**0.035333**			**Mean**	**0.122333**	**0.093333**	**0.085333**	**0.075667**	
0.032	0.033	0.037	0.038	0.041	0.034		0.032	0.036	0.039	0.04	0.041	0.036
0.034	0.033	0.035	0.037	0.041	0.035		0.088	0.236	0.077	0.077	0.063	0.091
0.034	0.034	0.035	0.038	0.041	0.036		0.089	0.199	0.074	0.072	0.066	0.067
0.033	0.034	0.035	0.038	0.042	0.036		0.076	0.069	0.067	0.062	0.062	0.065
Mean	**0.033667**	**0.035**	**0.037667**	**0.041333**	**0.033333**		**Mean**	**0.168**	**0.072667**	**0.070333**	**0.063667**	**0.083167**
Replicate 2							**Replicate 2**					
0.032	0.032	0.034	0.035	0.035	0.033		0.032	0.035	0.036	0.037	0.038	0.048
0.034	0.033	0.033	0.034	0.035	0.036		0.117	0.081	0.093	0.105	0.092	0.075
0.033	0.033	0.033	0.034	0.039	0.036		0.11	0.109	0.09	0.083	0.095	0.076
0.034	0.035	0.033	0.034	0.036	0.036		0.081	0.095	0.08	0.081	0.076	0.077
Mean	**0.033667**	**0.033**	**0.034**	**0.036667**			**Mean**	**0.095**	**0.087667**	**0.089667**	**0.087667**	
0.032	0.034	0.038	0.038	0.041	0.033		0.032	0.039	0.04	0.039	0.041	0.035
0.033	0.034	0.035	0.037	0.042	0.036		0.103	0.16	0.088	0.068	0.075	0.059
0.034	0.034	0.037	0.038	0.042	0.036		0.077	0.221	0.081	0.072	0.067	0.071
0.034	0.035	0.035	0.038	0.043	0.036		0.068	0.115	0.07	0.061	0.065	0.062
Me	**0.034333**	**0.035667**	**0.037667**	**0.042333**	**0.033667**		**Me**	**0.165333**	**0.079667**	**0.067**	**0.069**	**0.092667**

an							**an**					
Replicate 3							**Replicate 3**					
0.031	0.032	0.033	0.034	0.034	0.052		0.032	0.036	0.034	0.035	0.037	0.051
0.032	0.032	0.033	0.034	0.035	0.035		0.068	0.128	0.097	0.09	0.083	0.072
0.034	0.033	0.033	0.034	0.036	0.036		0.096	0.174	0.109	0.11	0.088	0.078
0.033	0.034	0.034	0.034	0.036	0.036		0.078	0.144	0.071	0.067	0.067	0.071
Mean	**0.033**	**0.033333**	**0.034**	**0.035667**			**Mean**	**0.148667**	**0.092333**	**0.089**	**0.079333**	
0.032	0.033	0.036	0.038	0.041	0.034		0.032	0.038	0.037	0.04	0.04	0.036
0.032	0.034	0.035	0.037	0.042	0.035		0.071	0.184	0.072	0.072	0.063	0.063
0.033	0.034	0.036	0.038	0.042	0.036		0.086	0.175	0.109	0.074	0.067	0.062
0.033	0.034	0.035	0.038	0.042	0.037		0.066	0.071	0.068	0.063	0.058	0.063
Mean	**0.034**	**0.035333**	**0.037667**	**0.042**	**0.032833**		**Mean**	**0.143333**	**0.083**	**0.069667**	**0.062667**	**0.0775**
Replicate 4							**Replicate 4**					
0.032	0.033	0.034	0.034	0.035	0.038		0.037	0.038	0.036	0.038	0.038	0.037
0.033	0.034	0.034	0.034	0.035	0.035		0.091	0.098	0.224	0.126	0.237	0.139
0.034	0.037	0.034	0.035	0.036	0.036		0.073	0.091	0.099	0.168	0.118	0.11
0.034	0.034	0.034	0.035	0.036	0.037		0.075	0.098	0.102	0.083	0.08	0.081
Mean	**0.035**	**0.034**	**0.034667**	**0.035667**			**Mean**	**0.095667**	**0.141667**	**0.125667**	**0.145**	
0.031	0.033	0.036	0.038	0.04	0.043		0.032	0.037	0.038	0.041	0.041	0.04
0.033	0.034	0.035	0.037	0.041	0.035		0.068	0.069	0.134	0.082	0.067	0.103
0.033	0.034	0.036	0.038	0.041	0.036		0.076	0.071	0.149	0.072	0.068	0.112
0.033	0.034	0.035	0.037	0.041	0.036		0.069	0.059	0.059	0.067	0.066	0.082
Mean	**0.034**	**0.035333**	**0.037333**	**0.041**	**0.035833**		**Mean**	**0.066333**	**0.114**	**0.073667**	**0.067**	**0.1045**

Replicate 5							**Replicate 5**					
0.033	0.033	0.036	0.035	0.035	0.053		0.033	0.036	0.039	0.039	0.038	0.037
0.034	0.034	0.034	0.034	0.034	0.035		0.081	0.083	0.106	0.134	0.098	0.098
0.033	0.034	0.034	0.035	0.036	0.037		0.073	0.091	0.111	0.105	0.099	0.084
0.034	0.033	0.035	0.035	0.036	0.035		0.078	0.078	0.084	0.09	0.081	0.083
Mean	**0.033667**	**0.034333**	**0.034667**	**0.035333**			**Mean**	**0.084**	**0.100333**	**0.109667**	**0.092667**	
0.033	0.033	0.036	0.038	0.041	0.041		0.033	0.037	0.037	0.04	0.041	0.047
0.032	0.034	0.035	0.037	0.041	0.035		0.073	0.084	0.081	0.066	0.066	0.105
0.033	0.034	0.036	0.037	0.041	0.036		0.072	0.072	0.086	0.067	0.063	0.086
0.033	0.035	0.035	0.038	0.042	0.036		0.074	0.067	0.063	0.072	0.061	0.08
Mean	**0.034333**	**0.035333**	**0.037333**	**0.041333**	**0.035667**		**Mean**	**0.074333**	**0.076667**	**0.068333**	**0.063333**	**0.089333**
Replicate 6							**Replicate 6**					
0.032	0.032	0.034	0.035	0.035	0.036		0.032	0.035	0.037	0.039	0.038	0.048
0.033	0.033	0.034	0.034	0.035	0.036		0.079	0.081	0.118	0.237	0.139	0.112
0.033	0.033	0.034	0.036	0.036	0.036		0.086	0.16	0.093	0.117	0.267	0.145
0.035	0.034	0.034	0.035	0.036	0.037		0.075	0.071	0.072	0.084	0.087	0.087
Mean	**0.033333**	**0.034**	**0.035**	**0.035667**			**Mean**	**0.104**	**0.094333**	**0.146**	**0.164333**	
0.031	0.033	0.035	0.038	0.04	0.033		0.033	0.037	0.037	0.04	0.045	0.035
0.032	0.034	0.035	0.038	0.042	0.035		0.096	0.093	0.08	0.072	0.063	0.093
0.033	0.034	0.036	0.038	0.041	0.036		0.068	0.072	0.079	0.07	0.098	0.103
0.033	0.034	0.035	0.038	0.042	0.036		0.068	0.071	0.063	0.062	0.061	0.085
Mean	**0.034**	**0.035333**	**0.038**	**0.041667**	**0.036**		**Mean**	**0.078667**	**0.074**	**0.068**	**0.074**	**0.104167**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-78.5953***	**-3.9548**	**-159.328**	12.86408	9.937888	0.244499
1.56	***-20.4013***	8.474576	**-33.209**	**-54.1262**	**-20.4969**	14.42543
3.13	***-4.34783***	5.084746	**-25.7463**	**-30.8252**	**-37.8882**	***-61.3692***
6.25	15.05017	8.474576	**-4.10448**	**-58.9806**	**-6.21118**	***-88.2641***
12.5	***-170.234***	***-123.164***	***-147.388***	55.58252	27.95031	37.40831
25	21.07023	22.0339	***-12.3134***	***-13.835***	23.60248	44.25428
50	25.75251	43.50282	17.53731	44.90291	39.13043	53.05623
100	39.13043	40.11299	33.20896	54.61165	48.4472	44.25428
Coefficient of variation for control	12.82503	***21.5251***	***15.04208***	***20.69067***	11.05133	***21.5384***

6. Petrochemical industry effluent

Plate configuration

Plate 1					
Blank	Blank	Blank	Blank	Blank	Blank
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Control	0.78 %	1.56 %	3.13 %	6.25 %	Control
Plate 2					
Blank	Blank	Blank	Blank	Blank	Blank
Control	12.5 %	25 %	50 %	100 %	Control
Control	12.5 %	25 %	50 %	100 %	Control
Control	12.5 %	25 %	50 %	100 %	Control

6.1. OD readings at 450nm for *Pseudokirchneriella subcapitata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 24/01/2012							*OD Readings at 96 hrs* – 28/01/2012					
Replicate 1							**Replicate 1**					
0.034	0.032	0.033	0.035	0.037	0.05		0.034	0.043	0.052	0.086	0.09	0.048
0.034	0.035	0.034	0.036	0.036	0.036		0.099	0.34	0.28	0.218	0.165	0.13
0.034	0.035	0.035	0.036	0.038	0.036		0.104	0.362	0.339	0.216	0.151	0.126
0.035	0.036	0.035	0.036	0.037	0.037		0.102	0.344	0.266	0.16	0.084	0.118
Mean	**0.035333**	**0.034667**	**0.036**	**0.037**			**Mean**	**0.348667**	**0.295**	**0.198**	**0.133333**	

0.032	0.036	0.041	0.051	0.068	0.033		0.034	0.196	0.32	0.358	0.06	0.045
0.035	0.036	0.041	0.051	0.069	0.036		0.137	0.142	0.293	0.406	0.323	0.14
0.034	0.039	0.041	0.051	0.073	0.036		0.144	0.119	0.283	0.33	0.296	0.164
0.034	0.038	0.042	0.052	0.07	0.037		0.119	0.131	0.274	0.368	0.359	0.13
Mean	**0.037667**	**0.041333**	**0.051333**	**0.070667**	**0.035333**		**Mean**	**0.130667**	**0.283333**	**0.368**	**0.326**	**0.126083**
Replicate 2							**Replicate 2**					
0.032	0.032	0.033	0.035	0.036	0.035		0.032	0.043	0.057	0.066	0.09	0.041
0.034	0.033	0.034	0.035	0.036	0.036		0.12	0.316	0.228	0.181	0.158	0.105
0.034	0.034	0.035	0.036	0.038	0.036		0.105	0.34	0.335	0.197	0.15	0.108
0.034	0.034	0.035	0.036	0.036	0.035		0.106	0.242	0.273	0.168	0.122	0.084
Mean	**0.033667**	**0.034667**	**0.035667**	**0.036667**			**Mean**	**0.299333**	**0.278667**	**0.182**	**0.143333**	
0.032	0.036	0.041	0.051	0.068	0.039		0.032	0.19	0.286	0.292	0.061	0.052
0.034	0.036	0.042	0.052	0.069	0.034		0.149	0.118	0.313	0.381	0.332	0.141
0.033	0.037	0.041	0.051	0.07	0.036		0.13	0.149	0.312	0.331	0.295	0.176
0.033	0.037	0.042	0.051	0.07	0.036		0.124	0.112	0.268	0.376	0.333	0.113
Mean	**0.036667**	**0.041667**	**0.051333**	**0.069667**	**0.034583**		**Mean**	**0.126333**	**0.297667**	**0.362667**	**0.32**	**0.12175**
Replicate 3							**Replicate 3**					
0.033	0.032	0.033	0.035	0.036	0.033		0.033	0.043	0.051	0.067	0.092	0.054
0.034	0.034	0.034	0.035	0.037	0.036		0.095	0.273	0.221	0.16	0.166	0.116
0.033	0.033	0.034	0.036	0.038	0.036		0.104	0.294	0.34	0.192	0.148	0.106
0.034	0.034	0.034	0.035	0.036	0.035		0.07	0.223	0.23	0.169	0.129	0.079
Mean	**0.033667**	**0.034**	**0.035333**	**0.037**			**Mean**	**0.263333**	**0.263667**	**0.173667**	**0.147667**	
0.032	0.036	0.041	0.051	0.068	0.035		0.032	0.145	0.304	0.386	0.061	0.052
0.033	0.038	0.041	0.051	0.069	0.036		0.155	0.148	0.337	0.322	0.455	0.142
0.033	0.038	0.042	0.051	0.069	0.036		0.148	0.148	0.35	0.387	0.313	0.146

0.034	0.037	0.042	0.051	0.071	0.035		0.09	0.126	0.25	0.306	0.295	0.123
Mean	**0.037667**	**0.041667**	**0.051**	**0.069667**	**0.034583**		**Mean**	**0.140667**	**0.312333**	**0.338333**	**0.354333**	**0.1145**
Replicate 4							**Replicate 4**					
0.032	0.033	0.034	0.035	0.037	0.045		0.032	0.044	0.065	0.068	0.095	0.049
0.033	0.035	0.034	0.036	0.036	0.036		0.092	0.273	0.245	0.157	0.106	0.108
0.034	0.034	0.034	0.035	0.038	0.036		0.091	0.3	0.265	0.15	0.086	0.067
0.034	0.035	0.035	0.036	0.038	0.037		0.091	0.194	0.145	0.111	0.106	0.055
Mean	**0.034667**	**0.034333**	**0.035667**	**0.037333**			**Mean**	**0.255667**	**0.218333**	**0.139333**	**0.099333**	
0.036	0.037	0.041	0.051	0.069	0.035		0.037	0.203	0.279	0.295	0.061	0.038
0.034	0.037	0.041	0.051	0.07	0.036		0.148	0.149	0.315	0.329	0.328	0.186
0.034	0.037	0.042	0.051	0.069	0.036		0.13	0.159	0.349	0.397	0.36	0.167
0.033	0.037	0.042	0.051	0.07	0.036		0.144	0.12	0.269	0.366	0.385	0.111
Mean	**0.037**	**0.041667**	**0.051**	**0.069667**	**0.034917**		**Mean**	**0.142667**	**0.311**	**0.364**	**0.357667**	**0.115833**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.035	0.036	0.033		0.032	0.045	0.071	0.081	0.092	0.044
0.033	0.033	0.034	0.035	0.037	0.036		0.103	0.267	0.224	0.233	0.221	0.125
0.033	0.034	0.034	0.035	0.037	0.036		0.102	0.216	0.204	0.178	0.154	0.103
0.033	0.035	0.034	0.035	0.037	0.036		0.069	0.151	0.129	0.124	0.094	0.064
Mean	**0.034**	**0.034**	**0.035**	**0.037**			**Mean**	**0.211333**	**0.185667**	**0.178333**	**0.156333**	
0.032	0.036	0.041	0.051	0.069	0.033		0.032	0.218	0.272	0.351	0.061	0.035
0.034	0.036	0.041	0.051	0.07	0.036		0.139	0.157	0.334	0.397	0.499	0.19
0.034	0.037	0.041	0.051	0.07	0.036		0.162	0.13	0.38	0.43	0.489	0.164
0.034	0.038	0.042	0.051	0.071	0.036		0.149	0.114	0.268	0.413	0.389	0.149
Mean	**0.037**	**0.041333**	**0.051**	**0.070333**	**0.03475**		**Mean**	**0.133667**	**0.327333**	**0.413333**	**0.459**	**0.126583**

Replicate 6							**Replicate 6**					
0.032	0.033	0.034	0.037	0.037	0.053		0.033	0.049	0.068	0.072	0.087	0.046
0.034	0.033	0.035	0.035	0.036	0.035		0.104	0.234	0.337	0.252	0.179	0.134
0.034	0.034	0.034	0.035	0.037	0.036		0.103	0.214	0.237	0.174	0.154	0.112
0.033	0.035	0.035	0.035	0.036	0.036		0.117	0.181	0.178	0.135	0.106	0.061
Mean	**0.034**	**0.034667**	**0.035**	**0.036333**			**Mean**	**0.209667**	**0.250667**	**0.187**	**0.146333**	
0.032	0.036	0.042	0.051	0.069	0.036		0.032	0.228	0.313	0.321	0.061	0.062
0.032	0.036	0.041	0.051	0.069	0.036		0.131	0.133	0.374	0.404	0.448	0.229
0.034	0.037	0.042	0.051	0.071	0.036		0.122	0.144	0.275	0.315	0.484	0.207
0.034	0.04	0.044	0.051	0.07	0.037		0.099	0.14	0.183	0.26	0.338	0.138
Mean	**0.037667**	**0.042333**	**0.051**	**0.07**	**0.03475**		**Mean**	**0.139**	**0.277333**	**0.326333**	**0.423333**	**0.12975**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-234.52***	***-199.624***	***-181.93***	***-169.636***	***-98.8722***	***-122.526***
1.56	***-177.224***	***-176.316***	***-182.341***	***-124.291***	***-69.9248***	***-174.316***
3.13	***-73.6655***	***-67.2932***	***-71.4579***	***-28.3401***	***-61.6541***	***-93.8947***
6.25	***-4.62633***	***-23.6842***	***-39.4251***	***20.24291***	***-36.8421***	***-42.5263***
12.5	***-1.77936***	***-4.51128***	***-30.8008***	***-32.3887***	***-11.2782***	***-33.2632***
25	***-164.769***	***-197.744***	***-242.3***	***-236.842***	***-229.699***	***-208***
50	***-255.16***	***-271.053***	***-274.333***	***-301.215***	***-326.692***	***-269.895***
100	***-210.32***	***-222.932***	***-294.045***	***-293.522***	***-378.195***	***-392.421***
Coefficient of variation for control	7.905457	13.40865	***26.47661***	***23.85923***	***28.78373***	11.20829

6.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 24/01/2012							*OD Readings at 96 hrs* – 28/01/2012					
Replicate 1							**Replicate 1**					
0.032	0.033	0.035	0.036	0.037	0.056		0.034	0.042	0.055	0.068	0.1	0.053
0.034	0.033	0.034	0.034	0.035	0.035		0.24	0.107	0.161	0.204	0.201	0.05
0.03	0.034	0.034	0.036	0.038	0.036		0.16	0.084	0.306	0.312	0.445	0.297

4						2					
0.034	0.033	0.035	0.036	0.036	0.037	0.053	0.077	0.121	0.143	0.138	0.047
Mean	**0.033333**	**0.034333**	**0.035333**	**0.036333**		**Mean**	**0.089333**	**0.196**	**0.219667**	**0.261333**	
0.032	0.037	0.042	0.052	0.069	0.041	0.032	0.198	0.26	0.257	0.061	0.035
0.033	0.037	0.042	0.05	0.07	0.035	0.187	0.952	0.783	0.888	1.017	0.299
0.033	0.037	0.043	0.051	0.069	0.036	0.228	0.907	0.495	1.207	1.248	0.219
0.034	0.037	0.043	0.051	0.069	0.036	0.101	0.222	0.319	0.457	0.815	0.178
Mean	0.033333	0.037	0.042667	0.050667	**0.03475**	Mean	0.693667	0.532333	0.850667	1.026667	**0.171675**
Replicate 2						**Replicate 2**					
0.032	0.033	0.034	0.036	0.037	0.049	0.034	0.045	0.05	0.066	0.082	0.055
0.033	0.033	0.034	0.034	0.035	0.035	0.173	0.081	0.232	0.272	0.382	0.172
0.035	0.034	0.034	0.036	0.037	0.037	0.121	0.083	0.252	0.326	0.341	0.171
0.033	0.035	0.035	0.035	0.036	0.037	0.059	0.064	0.121	0.178	0.085	0.047
Mean	**0.034**	**0.034333**	**0.035**	**0.036**		**Mean**	**0.076**	**0.201667**	**0.258667**	**0.269333**	
0.034	0.037	0.042	0.051	0.069	0.036	0.034	0.191	0.275	0.232	0.061	0.042
0.033	0.037	0.042	0.051	0.069	0.036	0.18	0.268	0.709	1.149	1.168	0.271
0.033	0.037	0.042	0.051	0.07	0.037	0.189	1.078	0.697	0.997	1.132	0.28
0.033	0.038	0.042	0.051	0.07	0.037	0.107	0.17	0.489	0.52	0.96	0.208
Mean	**0.037333**	**0.042**	**0.051**	**0.069667**	**0.034917**	**Mean**	**0.505333**	**0.631667**	**0.888667**	**1.086667**	**0.164833**
Replicate 3						**Replicate 3**					
0.032	0.032	0.035	0.035	0.036	0.045	0.033	0.043	0.053	0.075	0.091	0.054
0.033	0.033	0.034	0.035	0.036	0.036	0.097	0.077	0.122	0.295	0.137	0.21
0.033	0.033	0.034	0.035	0.037	0.036	0.148	0.071	0.179	0.287	0.365	0.399
0.034	0.034	0.034	0.035	0.036	0.036	0.054	0.069	0.118	0.175	0.102	0.067
Mean	**0.033333**	**0.034**	**0.035**	**0.036333**		**Mean**	**0.072333**	**0.139667**	**0.252333**	**0.201333**	

0.033	0.036	0.042	0.051	0.069	0.037		0.033	0.131	0.254	0.278	0.062	0.038
0.033	0.037	0.041	0.051	0.069	0.036		0.101	0.15	0.331	0.294	1.322	0.264
0.033	0.037	0.042	0.052	0.069	0.036		0.11	0.261	0.699	1.012	0.981	0.222
0.034	0.037	0.042	0.051	0.069	0.036		0.079	0.164	0.585	0.372	1.038	0.217
Mean	**0.037**	**0.041667**	**0.051333**	**0.069**	**0.034667**		**Mean**	**0.191667**	**0.538333**	**0.559333**	**1.113667**	**0.164**
Replicate 4							**Replicate 4**					
0.032	0.033	0.034	0.036	0.037	0.035		0.032	0.042	0.063	0.067	0.094	0.034
0.033	0.033	0.033	0.034	0.036	0.036		0.153	0.081	0.11	0.247	0.104	0.083
0.034	0.034	0.035	0.036	0.038	0.037		0.131	0.083	0.157	0.298	0.085	0.067
0.034	0.034	0.034	0.035	0.036	0.036		0.062	0.064	0.144	0.201	0.085	0.077
Mean	**0.033667**	**0.034**	**0.035**	**0.036667**			**Mean**	**0.076**	**0.137**	**0.248667**	**0.091333**	
0.033	0.036	0.041	0.051	0.068	0.041		0.033	0.209	0.262	0.33	0.061	0.042
0.033	0.037	0.042	0.051	0.07	0.037		0.182	0.637	0.835	0.983	0.747	0.175
0.034	0.037	0.041	0.051	0.069	0.036		0.118	1.008	0.704	0.912	0.597	0.194
0.033	0.037	0.043	0.051	0.07	0.035		0.124	0.182	0.543	0.638	0.537	0.135
Mean	**0.037**	**0.042**	**0.051**	**0.069667**	**0.034833**		**Mean**	**0.609**	**0.694**	**0.844333**	**0.627**	**0.125083**
Replicate 5							**Replicate 5**					
0.033	0.033	0.034	0.036	0.039	0.054		0.033	0.048	0.051	0.067	0.096	0.048
0.033	0.033	0.034	0.035	0.036	0.036		0.169	0.079	0.141	0.267	0.096	0.059
0.033	0.034	0.035	0.036	0.038	0.036		0.157	0.084	0.131	0.215	0.113	0.071
0.035	0.034	0.034	0.035	0.036	0.036		0.132	0.069	0.131	0.218	0.097	0.061
Mean	**0.033667**	**0.034333**	**0.035333**	**0.036667**			**Mean**	**0.077333**	**0.134333**	**0.233333**	**0.102**	
0.035	0.036	0.041	0.051	0.07	0.036		0.035	0.224	0.272	0.291	0.062	0.037
0.034	0.036	0.041	0.051	0.07	0.035		0.11	0.162	0.675	1.25	0.755	0.225

0.034	0.037	0.041	0.051	0.069	0.037		0.11	0.143	0.627	1.072	0.563	0.193
0.033	0.037	0.043	0.051	0.07	0.039		0.104	0.169	0.32	0.402	0.573	0.153
Mean	**0.036667**	**0.041667**	**0.051**	**0.069667**	**0.035083**		**Mean**	**0.158**	**0.540667**	**0.908**	**0.630333**	**0.128667**
Replicate 6							**Replicate 6**					
0.035	0.033	0.035	0.037	0.038	0.055		0.034	0.042	0.052	0.07	0.092	0.055
0.035	0.033	0.034	0.034	0.035	0.035		0.07	0.057	0.11	0.06	0.078	0.073
0.034	0.034	0.035	0.035	0.037	0.037		0.091	0.077	0.142	0.065	0.079	0.085
0.033	0.034	0.034	0.035	0.036	0.037		0.068	0.069	0.119	0.06	0.083	0.061
Mean	**0.033667**	**0.034333**	**0.034667**	**0.036**			**Mean**	**0.067667**	**0.123667**	**0.061667**	**0.08**	
0.035	0.036	0.041	0.051	0.069	0.043		0.033	0.146	0.299	0.372	0.062	0.053
0.035	0.037	0.042	0.051	0.07	0.036		0.12	0.135	0.432	0.312	0.776	0.193
0.034	0.037	0.041	0.051	0.07	0.036		0.132	0.143	0.459	0.677	0.935	0.21
0.035	0.038	0.042	0.051	0.07	0.035		0.124	0.161	0.192	0.347	0.881	0.162
Mean	**0.037333**	**0.041667**	**0.051**	**0.07**	**0.035167**		**Mean**	**0.146333**	**0.361**	**0.445333**	**0.864**	**0.11575**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheco*ides						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	62.84444	66.41671	83.95171	60.62847	69.15545	49.87469
1.56	***-8.26667***	***-34.5474***	54.21084	4.251386	27.29498	***-34.3358***
3.13	***-24.0444***	***-80.3428***	4.446408	***-98.9526***	***-45.41***	58.89724
6.25	***-51.8222***	***-88.9127***	26.97291	46.45718	51.04039	31.32832
12.5	***-340.044***	***-278.522***	31.24264	***-431.978***	9.914321	***-68.4211***
25	***-232.489***	***-380.021***	***-121.879***	***-510.536***	***-271.114***	***-391.228***
50	***-444.711***	***-586.502***	***-131.154***	***-649.476***	***-540.881***	***-518.045***
100	***-562.044***	***-745.581***	***-376.001***	***-448.614***	***-336.965***	***-1147.62***
Coefficient of variation for control	***30.3905***	***28.34266***	***30.18933***	***18.48745***	***19.23933***	***28.05344***

6.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 05/03/2012							*OD Readings at 96 hrs* – 09/03/2012					
Replicate 1							**Replicate 1**					
0.034	0.033	0.034	0.036	0.036	0.054		0.034	0.047	0.056	0.082	0.099	0.051
0.037	0.035	0.035	0.036	0.038	0.037		0.188	0.398	0.407	0.183	0.141	0.14
0.035	0.035	0.036	0.038	0.039	0.038		0.191	0.284	0.382	0.335	0.207	0.124
0.035	0.036	0.036	0.036	0.038	0.038		0.089	0.149	0.126	0.128	0.137	0.093
Mean	**0.035333**	**0.035667**	**0.036667**	**0.038333**			**Mean**	**0.277**	**0.305**	**0.215333**	**0.161667**	
0.032	0.037	0.043	0.053	0.067	0.045		0.033	0.144	0.152	0.201	0.23	0.048
0.034	0.038	0.044	0.054	0.068	0.036		0.193	0.166	0.38	0.316	0.267	0.124
0.035	0.039	0.044	0.054	0.068	0.037		0.17	0.173	0.332	0.29	0.275	0.112
0.035	0.039	0.044	0.054	0.071	0.04		0.117	0.176	0.39	0.316	0.285	0.109
Mean	**0.038667**	**0.044**	**0.054**	**0.069**	**0.03641667**		**Mean**	**0.171667**	**0.367333**	**0.307333**	**0.275667**	**0.1375**
Replicate 2							**Replicate 2**					
0.033	0.033	0.035	0.036	0.039	0.041		0.033	0.045	0.055	0.074	0.12	0.054
0.035	0.034	0.035	0.036	0.038	0.038		0.194	0.458	0.461	0.254	0.225	0.163
0.035	0.035	0.035	0.037	0.039	0.038		0.159	0.233	0.244	0.272	0.233	0.146
0.035	0.036	0.036	0.037	0.038	0.038		0.11	0.217	0.153	0.169	0.174	0.11
Mean	**0.035**	**0.035333**	**0.036667**	**0.038333**			**Mean**	**0.302667**	**0.286**	**0.231667**	**0.210667**	
0.033	0.036	0.043	0.052	0.066	0.035		0.034	0.153	0.309	0.216	0.264	0.099
0.034	0.038	0.043	0.053	0.071	0.037		0.186	0.203	0.37	0.321	0.31	0.141
0.035	0.039	0.044	0.054	0.074	0.038		0.163	0.242	0.434	0.302	0.339	0.165
0.035	0.039	0.044	0.053	0.075	0.038		0.13	0.178	0.337	0.345	0.309	0.122
Mean	**0.038667**	**0.043667**	**0.053333**	**0.073333**	**0.03618182**		**Mean**	**0.207667**	**0.380333**	**0.322667**	**0.319333**	**0.149083**
Replicate 3							**Replicate 3**					

0.037	0.033	0.034	0.036	0.037	0.036		0.035	0.045	0.052	0.067	0.097	0.038
0.035	0.034	0.035	0.036	0.038	0.038		0.058	0.087	0.228	0.211	0.242	0.136
0.035	0.035	0.036	0.037	0.039	0.038		0.073	0.12	0.323	0.206	0.163	0.133
0.035	0.035	0.036	0.037	0.038	0.038		0.065	0.107	0.209	0.16	0.149	0.108
Mean	**0.034667**	**0.035667**	**0.036667**	**0.038333**			**Mean**	**0.104667**	**0.253333**	**0.192333**	**0.184667**	
0.033	0.038	0.043	0.052	0.07	0.042		0.032	0.124	0.364	0.204	0.252	0.043
0.034	0.038	0.043	0.053	0.073	0.037		0.066	0.305	0.385	0.383	0.29	0.129
0.035	0.039	0.043	0.053	0.073	0.037		0.082	0.226	0.404	0.428	0.325	0.149
0.037	0.039	0.05	0.052	0.071	0.038		0.081	0.367	0.338	0.433	0.349	0.116
Mean	**0.038667**	**0.045333**	**0.052667**	**0.072333**	**0.03627273**		**Mean**	**0.299333**	**0.375667**	**0.414667**	**0.321333**	**0.098182**
Replicate 4							**Replicate 4**					
0.034	0.033	0.035	0.036	0.036	0.042		0.033	0.047	0.055	0.071	0.13	0.036
0.034	0.035	0.036	0.036	0.039	0.037		0.094	0.138	0.255	0.35	0.252	0.184
0.036	0.036	0.036	0.037	0.041	0.038		0.105	0.139	0.46	0.241	0.191	0.159
0.035	0.035	0.036	0.04	0.038	0.038		0.075	0.132	0.174	0.151	0.222	0.184
Mean	**0.035333**	**0.036**	**0.037667**	**0.039333**			**Mean**	**0.136333**	**0.296333**	**0.247333**	**0.221667**	
0.032	0.037	0.042	0.051	0.072	0.035		0.032	0.158	0.209	0.304	0.254	0.039
0.034	0.039	0.044	0.052	0.074	0.038		0.102	0.178	0.344	0.317	0.3	0.112
0.035	0.039	0.045	0.054	0.073	0.038		0.112	0.187	0.293	0.597	0.311	0.135
0.035	0.039	0.044	0.054	0.071	0.038		0.143	0.187	0.33	0.434	0.325	0.161
Mean	**0.039**	**0.044333**	**0.053333**	**0.072667**	**0.03633333**		**Mean**	**0.184**	**0.322333**	**0.449333**	**0.312**	**0.127727**
Replicate 5							**Replicate 5**					
0.032	0.034	0.036	0.037	0.037	0.046		0.032	0.048	0.06	0.083	0.101	0.053
0.036	0.035	0.036	0.037	0.038	0.036		0.084	0.126	0.397	0.27	0.214	0.132
0.035	0.036	0.036	0.037	0.039	0.038		0.098	0.126	0.3	0.32	0.205	0.192

0.035	0.036	0.036	0.037	0.039	0.038		0.079	0.092	0.146	0.18	0.164	0.143
Mean	**0.035667**	**0.036**	**0.037**	**0.038667**			**Mean**	**0.114667**	**0.281**	**0.256667**	**0.194333**	
0.033	0.037	0.041	0.052	0.067	0.04		0.033	0.123	0.201	0.17	0.246	0.039
0.035	0.039	0.042	0.054	0.076	0.038		0.104	0.197	0.36	0.362	0.326	0.153
0.036	0.039	0.044	0.056	0.074	0.038		0.113	0.154	0.391	0.382	0.315	0.132
0.035	0.039	0.044	0.054	0.074	0.039		0.119	0.199	0.377	0.329	0.32	0.105
Mean	**0.039**	**0.043333**	**0.054667**	**0.074667**	**0.03658333**		**Mean**	**0.183333**	**0.376**	**0.357667**	**0.320333**	**0.121167**
Replicate 6							**Replicate 6**					
0.033	0.033	0.034	0.036	0.037	0.043		0.032	0.046	0.055	0.074	0.104	0.049
0.035	0.035	0.036	0.036	0.038	0.036		0.103	0.171	0.41	0.252	0.467	0.175
0.035	0.036	0.037	0.038	0.04	0.038		0.116	0.208	0.255	0.294	0.252	0.16
0.035	0.038	0.036	0.036	0.039	0.041		0.073	0.085	0.147	0.16	0.144	0.102
Mean	**0.036333**	**0.036333**	**0.036667**	**0.039**			**Mean**	**0.154667**	**0.270667**	**0.235333**	**0.287667**	
0.032	0.037	0.042	0.053	0.07	0.048		0.032	0.15	0.292	0.218	0.248	0.047
0.034	0.039	0.043	0.054	0.074	0.037		0.097	0.165	0.421	0.292	0.297	0.144
0.035	0.04	0.044	0.055	0.074	0.038		0.092	0.212	0.465	0.318	0.306	0.133
0.037	0.039	0.043	0.055	0.072	0.037		0.076	0.198	0.256	0.346	0.286	0.108
Mean	**0.039333**	**0.043333**	**0.054667**	**0.073333**	**0.0365**		**Mean**	**0.191667**	**0.380667**	**0.318667**	**0.296333**	**0.114917**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-201.681***	***-119.236***	26.23853	16.52542	26.8254	***-17.8151***
1.56	***-236.975***	***-105.593***	***-137.431***	***-119.068***	***-131.587***	***-134.79***
3.13	***-123.95***	***-61.1187***	***-70.2752***	***-77.5424***	***-108.413***	***-99.1597***
6.25	***-56.3025***	***-43.9291***	***-61.8349***	***-55.791***	***-49.0476***	***-151.933***
12.5	***-68.9076***	***-41.4734***	***-188.073***	***-23.8701***	***-38.5714***	***-55.1261***
25	***-315.546***	***-182.81***	***-272.11***	***-141.102***	***-222.063***	***-245.714***

50	***-239.916***	***-135.607***	***-315.046***	***-248.729***	***-204.603***	***-183.193***
100	***-200***	***-132.879***	***-212.294***	***-132.345***	***-169.048***	***-160.672***
Coefficient of variation for control	13.73917	***20.51707***	11.40284	***18.10225***	***20.25948***	***20.91212***

6.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD readings at 0 hrs - 05/03/2012							***OD readings at 96 hrs - 09/03/2012***					
Replicate 1							**Replicate 1**					
0.033	0.033	0.036	0.036	0.037	0.037		0.035	0.046	0.054	0.076	0.076	0.052
0.034	0.034	0.034	0.035	0.038	0.036		0.115	0.149	0.179	0.342	0.146	0.108
0.034	0.034	0.035	0.036	0.038	0.037		0.124	0.147	0.101	0.315	0.11	0.115
0.035	0.035	0.035	0.036	0.038	0.039		0.11	0.136	0.139	0.145	0.139	0.094
Mean	**0.034333**	**0.034667**	**0.035667**	**0.038**			**Mean**	**0.144**	**0.139667**	**0.267333**	**0.131667**	
0.032	0.036	0.042	0.051	0.067	0.041		0.032	0.124	0.237	0.216	0.28	0.038
0.035	0.038	0.043	0.05	0.068	0.037		0.086	0.216	0.268	0.314	0.261	0.11
0.034	0.038	0.043	0.051	0.068	0.037		0.084	0.177	0.272	0.289	0.264	0.086
0.037	0.039	0.046	0.053	0.068	0.038		0.101	0.191	0.29	0.317	0.275	0.083
Mean	**0.038333**	**0.044**	**0.051333**	**0.068**	**0.036083**		**Mean**	**0.194667**	**0.276667**	**0.306667**	**0.266667**	**0.101333**
Replicate 2							**Replicate 2**					
0.032	0.033	0.034	0.036	0.037	0.039		0.032	0.045	0.051	0.071	0.101	0.05
0.034	0.034	0.034	0.035	0.037	0.037		0.115	0.164	0.191	0.275	0.208	0.114
0.035	0.035	0.035	0.037	0.038	0.037		0.123	0.144	0.223	0.275	0.104	0.122
0.034	0.035	0.035	0.037	0.038	0.039		0.105	0.125	0.161	0.152	0.124	0.104
Mean	**0.034667**	**0.034667**	**0.036333**	**0.037667**			**Mean**	**0.144333**	**0.191667**	**0.234**	**0.145333**	
0.032	0.039	0.042	0.05	0.067	0.034		0.032	0.136	0.225	0.196	0.249	0.036
0.038	0.038	0.042	0.051	0.068	0.037		0.067	0.207	0.264	0.282	0.245	0.114

0.035	0.038	0.043	0.051	0.07	0.037		0.068	0.202	0.286	0.293	0.377	0.097
0.035	0.039	0.043	0.052	0.068	0.037		0.1	0.21	0.179	0.295	0.287	0.077
Mean	**0.038333**	**0.042667**	**0.051333**	**0.068667**	**0.03625**		**Mean**	**0.206333**	**0.243**	**0.29**	**0.303**	**0.1005**
Replicate 3							**Replicate 3**					
0.033	0.033	0.034	0.035	0.036	0.053		0.032	0.045	0.051	0.071	0.101	0.05
0.037	0.037	0.035	0.037	0.037	0.036		0.115	0.164	0.191	0.275	0.208	0.114
0.034	0.036	0.035	0.036	0.039	0.037		0.123	0.144	0.223	0.275	0.104	0.122
0.035	0.035	0.035	0.036	0.038	0.037		0.105	0.125	0.161	0.152	0.124	0.104
Mean	**0.036**	**0.035**	**0.036333**	**0.038**			**Mean**	**0.144333**	**0.191667**	**0.234**	**0.145333**	
0.032	0.036	0.041	0.05	0.066	0.035		0.032	0.136	0.225	0.196	0.249	0.036
0.034	0.037	0.042	0.05	0.068	0.037		0.067	0.207	0.264	0.282	0.245	0.114
0.035	0.038	0.043	0.051	0.068	0.037		0.068	0.202	0.286	0.293	0.377	0.097
0.034	0.039	0.043	0.051	0.068	0.037		0.1	0.21	0.179	0.295	0.287	0.077
Mean	**0.038**	**0.042667**	**0.050667**	**0.068**	**0.035833**		**Mean**	**0.206333**	**0.243**	**0.29**	**0.303**	**0.1005**
Replicate 4							**Replicate 4**					
0.033	0.033	0.035	0.036	0.036	0.057		0.032	0.049	0.053	0.067	0.084	0.045
0.035	0.034	0.035	0.035	0.037	0.037		0.103	0.148	0.223	0.166	0.097	0.085
0.034	0.035	0.035	0.036	0.038	0.037		0.106	0.157	0.216	0.23	0.099	0.084
0.035	0.035	0.035	0.036	0.038	0.038		0.092	0.115	0.154	0.201	0.103	0.077
Mean	**0.034667**	**0.035**	**0.035667**	**0.037667**			**Mean**	**0.14**	**0.197667**	**0.199**	**0.099667**	
0.032	0.039	0.042	0.05	0.066	0.034		0.032	0.247	0.246	0.186	0.222	0.037
0.035	0.038	0.043	0.05	0.07	0.038		0.076	0.184	0.301	0.361	0.288	0.069
0.035	0.038	0.043	0.052	0.069	0.037		0.074	0.221	0.296	0.335	0.251	0.092
0.034	0.039	0.044	0.051	0.073	0.038		0.085	0.228	0.284	0.741	0.351	0.069
Me	**0.0383**	**0.0433**	**0.051**	**0.0706**	**0.0360**		**Me**	**0.211**	**0.2936**	**0.479**	**0.2966**	**0.0843**

an	**33**	**33**		**67**	**83**		**an**		**67**		**67**	**33**
Replicate 5							**Replicate 5**					
0.034	0.034	0.034	0.036	0.036	0.037		0.035	0.057	0.053	0.071	0.083	0.052
0.034	0.035	0.035	0.036	0.037	0.035		0.119	0.14	0.142	0.161	0.224	0.085
0.037	0.037	0.035	0.037	0.038	0.037		0.117	0.189	0.17	0.23	0.133	0.087
0.036	0.035	0.036	0.036	0.038	0.037		0.093	0.109	0.129	0.157	0.154	0.138
Mean	**0.035667**	**0.035333**	**0.036333**	**0.037667**			**Mean**	**0.146**	**0.147**	**0.182667**	**0.170333**	
0.032	0.042	0.042	0.05	0.067	0.035		0.032	0.161	0.112	0.199	0.253	0.069
0.035	0.038	0.042	0.05	0.067	0.037		0.098	0.379	0.282	0.275	0.247	0.084
0.036	0.038	0.043	0.052	0.069	0.038		0.1	0.239	0.263	0.633	0.242	0.063
0.034	0.04	0.044	0.051	0.067	0.036		0.093	0.22	0.253	0.278	0.285	0.064
Mean	**0.038667**	**0.043**	**0.051**	**0.067667**	**0.036**		**Mean**	**0.279333**	**0.266**	**0.395333**	**0.258**	**0.095083**
Replicate 6							**Replicate 6**					
0.032	0.033	0.033	0.035	0.036	0.053		0.032	0.046	0.051	0.067	0.086	0.045
0.034	0.035	0.035	0.036	0.037	0.036		0.106	0.113	0.195	0.199	0.148	0.086
0.037	0.034	0.035	0.036	0.041	0.037		0.103	0.121	0.192	0.183	0.236	0.09
0.035	0.035	0.036	0.036	0.038	0.037		0.095	0.114	0.115	0.148	0.158	0.091
Mean	**0.034667**	**0.035333**	**0.036**	**0.038667**			**Mean**	**0.116**	**0.167333**	**0.176667**	**0.180667**	
0.032	0.037	0.044	0.05	0.067	0.034		0.033	0.161	0.26	0.309	0.307	0.043
0.034	0.038	0.043	0.051	0.074	0.037		0.088	0.334	0.404	0.294	0.31	0.066
0.035	0.041	0.043	0.053	0.068	0.037		0.092	0.237	0.296	0.265	0.286	0.065
0.034	0.038	0.045	0.053	0.07	0.036		0.099	0.211	0.232	0.275	0.307	0.062
Mean	**0.039**	**0.043667**	**0.052333**	**0.070667**	**0.03575**		**Mean**	**0.260667**	**0.310667**	**0.278**	**0.301**	**0.086917**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
Effluent concentration (%)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
0.78	***-76.4543***	***-58.9109***	***-57.0388***	***-98.4076***	***-64.4836***	***-31.2155***
1.56	***-69.2521***	***-129.208***	***-125.971***	***-208.599***	***-65.995***	***-116.298***
3.13	***-281.44***	***-192.079***	***-187.621***	***-211.146***	***-119.899***	***-131.768***
6.25	***-55.9557***	***-60.396***	***-58.4951***	***-21.3376***	***-101.259***	***-138.398***
12.5	***-160.665***	***-150.99***	***-147.33***	***-234.076***	***-265.995***	***-270.994***
25	***-296.953***	***-205.446***	***-200.728***	***-392.038***	***-245.844***	***-353.867***
50	***-346.814***	***-275.248***	***-269.175***	***-746.178***	***-441.31***	***-299.724***
100	***-280.332***	***-294.554***	***-288.107***	***-397.771***	***-233.753***	***-337.845***
Coefficient of variation for control	12.53068	***15.40164***	***15.6408***	***18.2303***	11.32988	6.992733

7. Pesticides - **Roundup®**

Plate configuration

Plate 1					
Control	0.4 mg/L	0.78 mg/L	1.56 mg/L	3.13 mg/L	Control
Control	0.4 mg/L	0.78 mg/L	1.56 mg/L	3.13 mg/L	Control
Control	0.4 mg/L	0.78 mg/L	1.56 mg/L	3.13 mg/L	Control
Blank	Blank	Blank	Blank	Blank	Blank

7.1. OD readings at 450nm for *Pseudokirchneriella subcapitata* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/02/2012							*OD Readings at 0 hrs* – 10/02/2012					
Replicate 1							**Replicate 1**					
0.032	0.037	0.034	0.035	0.035	0.033		0.053	0.036	0.042	0.045	0.047	0.061
0.033	0.034	0.034	0.034	0.036	0.036		0.06	0.044	0.039	0.077	0.059	0.095
0.033	0.034	0.034	0.035	0.035	0.036		0.065	0.037	0.04	0.079	0.045	0.11
0.034	0.036	0.034	0.036	0.035	0.038		0.034	0.038	0.043	0.045	0.045	0.037
Mean	**0.035**	**0.034**	**0.034667**	**0.035333**	**0.033833**		**Mean**	**0.039**	**0.040333**	**0.067**	**0.050333**	**0.074**
Replicate 2							**Replicate 2**					
0.032	0.032	0.033	0.034	0.034	0.036		0.055	0.039	0.041	0.053	0.054	0.049
0.035	0.034	0.035	0.035	0.035	0.036		0.061	0.039	0.049	0.063	0.042	0.107

0.035	0.034	0.034	0.035	0.035	0.036		0.062	0.04	0.04	0.045	0.043	0.068
0.034	0.035	0.035	0.036	0.035	0.037		0.033	0.042	0.042	0.042	0.044	0.036
Mean	**0.033333**	**0.034**	**0.034667**	**0.034667**	**0.035**		**Mean**	**0.039333**	**0.043333**	**0.053667**	**0.046333**	**0.067**
Replicate 3							**Replicate 3**					
0.033	0.033	0.034	0.035	0.034	0.049		0.063	0.042	0.045	0.054	0.046	0.073
0.033	0.034	0.034	0.035	0.035	0.036		0.064	0.042	0.051	0.066	0.044	0.121
0.035	0.034	0.034	0.035	0.035	0.036		0.067	0.042	0.042	0.041	0.042	0.11
0.035	0.035	0.035	0.036	0.035	0.036		0.033	0.042	0.043	0.047	0.045	0.036
Mean	**0.033667**	**0.034**	**0.035**	**0.034667**	**0.037**		**Mean**	**0.042**	**0.046**	**0.053667**	**0.044**	**0.083**
Replicate 4							**Replicate 4**					
0.034	0.033	0.034	0.034	0.034	0.035		0.056	0.041	0.044	0.071	0.046	0.098
0.035	0.033	0.034	0.035	0.035	0.037		0.055	0.044	0.049	0.131	0.067	0.119
0.035	0.034	0.034	0.035	0.035	0.036		0.046	0.044	0.045	0.056	0.05	0.142
0.034	0.035	0.034	0.036	0.035	0.038		0.034	0.041	0.041	0.045	0.043	0.037
Mean	**0.033333**	**0.034**	**0.034667**	**0.034667**	**0.035333**		**Mean**	**0.043**	**0.046**	**0.086**	**0.054333**	**0.086**
Replicate 5							**Replicate 5**					
0.033	0.032	0.033	0.035	0.034	0.035		0.047	0.038	0.044	0.076	0.056	0.073
0.033	0.034	0.034	0.034	0.035	0.036		0.055	0.043	0.051	0.145	0.07	0.143
0.034	0.034	0.034	0.035	0.036	0.037		0.039	0.036	0.068	0.174	0.071	0.141
0.035	0.035	0.034	0.036	0.035	0.037		0.033	0.042	0.043	0.04	0.045	0.037
Mean	**0.033333**	**0.033667**	**0.034667**	**0.035**	**0.034667**		**Mean**	**0.039**	**0.054333**	**0.131667**	**0.065667**	**0.083**
Replicate 6							**Replicate 6**					
0.033	0.034	0.034	0.034	0.034	0.055		0.052	0.047	0.044	0.054	0.043	0.102
0.034	0.034	0.034	0.034	0.035	0.036		0.061	0.047	0.058	0.191	0.156	0.191

0.034	0.034	0.034	0.035	0.035	0.036		0.061	0.039	0.04	0.04	0.044	0.093
0.034	0.035	0.034	0.035	0.038	0.037		0.034	0.041	0.042	0.044	0.043	0.036
Mean	**0.034**	**0.034**	**0.034333**	**0.034667**	**0.038**		**Mean**	**0.044333**	**0.047333**	**0.095**	**0.081**	**0.093333**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *P. subcapitata*						
Roundup concentration (mg/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
3.13	92.54658	91.37931	97.26776	91.63347	96.38554	97.6834
1.56	90.06211	81.03448	90.71038	88.04781	77.91165	94.20849
0.78	40.37267	54.31034	78.14208	40.23904	***-15.261***	38.99614
0.4	71.42857	73.27586	93.98907	78.08765	64.25703	55.21236
Coefficient of variation for controls	***28.31553***	***39.60151***	***24.81537***	***18.39073***	***33.48716***	***42.10064***

7.2. OD readings at 450nm for *Chlorella protothecoides* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/02/2012							*OD Readings at 0 hrs* – 10/02/2012					
Replicate 1							**Replicate 1**					
0.033	0.033	0.033	0.035	0.034	0.057		0.056	0.045	0.056	0.096	0.089	0.044
0.033	0.034	0.034	0.034	0.035	0.036		0.127	0.04	0.06	0.139	0.181	0.482
0.034	0.036	0.033	0.036	0.035	0.036		0.111	0.04	0.055	0.121	0.148	0.199
0.034	0.036	0.033	0.036	0.04	0.037		0.033	0.038	0.04	0.041	0.043	0.036
Mean	**0.034333**	**0.033333**	**0.035**	**0.034667**	**0.038167**		**Mean**	**0.041667**	**0.057**	**0.118667**	**0.139333**	**0.169833**
Replicate 2							**Replicate 2**					
0.033	0.033	0.033	0.035	0.034	0.037		0.045	0.041	0.057	0.091	0.041	0.041
0.033	0.033	0.033	0.034	0.035	0.036		0.088	0.039	0.063	0.109	0.387	0.476
0.034	0.033	0.034	0.035	0.035	0.037		0.081	0.037	0.068	0.084	0.269	0.049
0.034	0.034	0.034	0.035	0.035	0.037		0.033	0.039	0.04	0.04	0.044	0.037
Mean	**0.033**	**0.033333**	**0.034667**	**0.034667**	**0.035**		**Mean**	**0.039**	**0.062667**	**0.094667**	**0.232333**	**0.13**
Replicate 3							**Replicate 3**					
0.032	0.032	0.033	0.034	0.034	0.042		0.069	0.038	0.069	0.079	0.152	0.048

0.037	0.033	0.033	0.034	0.035	0.036		0.11	0.039	0.088	0.081	0.185	0.204
0.034	0.033	0.033	0.035	0.035	0.036		0.111	0.04	0.071	0.089	0.217	0.102
0.033	0.035	0.034	0.035	0.035	0.037		0.033	0.041	0.041	0.041	0.045	0.037
Mean	**0.032667**	**0.033**	**0.034333**	**0.034667**	**0.036167**		**Mean**	**0.039**	**0.076**	**0.083**	**0.184667**	**0.107333**
Replicate 4							**Replicate 4**					
0.032	0.032	0.034	0.035	0.034	0.035		0.075	0.04	0.066	0.064	0.078	0.042
0.033	0.033	0.033	0.034	0.035	0.036		0.155	0.039	0.083	0.09	0.234	0.108
0.033	0.034	0.033	0.035	0.035	0.036		0.058	0.039	0.091	0.091	0.372	0.379
0.034	0.034	0.033	0.035	0.035	0.035		0.033	0.043	0.042	0.042	0.046	0.036
Mean	**0.033**	**0.033333**	**0.034667**	**0.034667**	**0.034167**		**Mean**	**0.039333**	**0.08**	**0.081667**	**0.228**	**0.136167**
Replicate 5							**Replicate 5**					
0.035	0.032	0.033	0.034	0.034	0.035		0.076	0.045	0.062	0.06	0.077	0.04
0.033	0.033	0.033	0.034	0.034	0.035		0.115	0.037	0.062	0.068	0.077	0.042
0.034	0.034	0.033	0.035	0.035	0.036		0.125	0.038	0.076	0.078	0.054	0.039
0.033	0.034	0.034	0.035	0.035	0.036		0.035	0.04	0.04	0.041	0.046	0.036
Mean	**0.033**	**0.033**	**0.034333**	**0.034333**	**0.034667**		**Mean**	**0.04**	**0.066667**	**0.068667**	**0.069333**	**0.072833**
Replicate 6							**Replicate 6**					
0.032	0.033	0.034	0.035	0.034	0.037		0.137	0.038	0.074	0.056	0.064	0.041
0.033	0.033	0.033	0.033	0.034	0.036		0.216	0.039	0.091	0.059	0.061	0.041
0.033	0.033	0.033	0.035	0.035	0.036		0.118	0.038	0.065	0.057	0.06	0.041
0.033	0.033	0.033	0.035	0.035	0.035		0.033	0.041	0.041	0.045	0.045	0.037
Mean	**0.033**	**0.033333**	**0.034333**	**0.034333**	**0.0345**		**Mean**	**0.038333**	**0.076667**	**0.057333**	**0.061667**	**0.099**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. prototheocoides*						
Roundup concentration (mg/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
3.13	93.41317	88.59649	95.37037	92.827	91.58879	95.44236
1.56	79.64072	26.31579	43.98148	41.35021	54.20561	64.61126
0.78	24.2515	***-57.8947***	34.25926	39.24051	51.40187	80.16086
0.4	5.688623	***-420.175***	***-106.944***	***-145.992***	50.46729	76.6756
Coefficient of variation for controls	***32.18013***	***28.61371***	4.581625	***35.68371***	***24.57987***	***33.10264***

7.3. OD readings at 450nm for *Chlorella sorokiniana* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/02/2012							*OD Readings at 0 hrs* – 10/02/2012					
Replicate 1							**Replicate 1**					
0.034	0.033	0.033	0.034	0.035	0.043		0.124	0.047	0.049	0.046	0.053	0.097
0.034	0.035	0.035	0.035	0.037	0.036		0.116	0.038	0.049	0.052	0.054	0.093
0.034	0.035	0.035	0.035	0.038	0.037		0.132	0.039	0.046	0.05	0.059	0.083
0.034	0.035	0.036	0.035	0.039	0.038		0.033	0.042	0.04	0.041	0.045	0.036
Mean	**0.034333**	**0.034333**	**0.034667**	**0.036667**	**0.036333**		**Mean**	**0.041333**	**0.048**	**0.049333**	**0.055333**	**0.107533**
Replicate 2							**Replicate 2**					
0.032	0.032	0.034	0.034	0.034	0.047		0.088	0.044	0.047	0.043	0.048	0.075
0.034	0.035	0.036	0.035	0.036	0.037		0.077	0.039	0.053	0.048	0.049	0.101
0.034	0.034	0.035	0.035	0.036	0.037		0.095	0.038	0.047	0.043	0.052	0.096
0.034	0.034	0.035	0.035	0.036	0.038		0.033	0.039	0.04	0.041	0.044	0.036
Mean	**0.033667**	**0.035**	**0.034667**	**0.035333**	**0.036833**		**Mean**	**0.040333**	**0.049**	**0.044667**	**0.049667**	**0.088667**
Replicate 3							**Replicate 3**					
0.033	0.033	0.034	0.034	0.034	0.048		0.105	0.041	0.04	0.042	0.048	0.087
0.033	0.034	0.035	0.034	0.036	0.037		0.116	0.041	0.04	0.042	0.05	0.124
0.034	0.034	0.035	0.035	0.037	0.037		0.1	0.041	0.041	0.042	0.047	0.088
0.034	0.035	0.035	0.035	0.037	0.037		0.033	0.043	0.039	0.041	0.043	0.036
Me	**0.0336**	**0.0346**	**0.0343**	**0.0356**	**0.037**		**Me**	**0.041**	**0.0403**	**0.042**	**0.0483**	**0.1033**

an	**67**	**67**	**33**	**67**			**an**		**33**		**33**	**33**
Replicate 4							**Replicate 4**					
0.032	0.033	0.034	0.034	0.034	0.037		0.08	0.04	0.042	0.042	0.048	0.084
0.034	0.034	0.035	0.034	0.036	0.036		0.109	0.041	0.045	0.043	0.053	0.103
0.035	0.034	0.035	0.035	0.036	0.037		0.11	0.042	0.043	0.042	0.059	0.143
0.034	0.035	0.037	0.035	0.037	0.037		0.033	0.042	0.041	0.041	0.043	0.037
Mean	**0.033667**	**0.034667**	**0.034333**	**0.035333**	**0.035167**		**Mean**	**0.041**	**0.043333**	**0.042333**	**0.053333**	**0.104833**
Replicate 5							**Replicate 5**					
0.032	0.033	0.034	0.034	0.034	0.035		0.079	0.043	0.043	0.045	0.057	0.103
0.034	0.034	0.035	0.035	0.036	0.036		0.1	0.041	0.046	0.048	0.066	0.108
0.034	0.034	0.035	0.035	0.036	0.037		0.126	0.04	0.065	0.062	0.056	0.122
0.034	0.034	0.036	0.035	0.036	0.037		0.034	0.04	0.041	0.042	0.048	0.036
Mean	**0.033667**	**0.034667**	**0.034667**	**0.035333**	**0.034667**		**Mean**	**0.041333**	**0.051333**	**0.051667**	**0.059667**	**0.106333**
Replicate 6							**Replicate 6**					
0.032	0.035	0.034	0.034	0.035	0.035		0.077	0.042	0.041	0.04	0.052	0.119
0.033	0.034	0.035	0.034	0.036	0.036		0.109	0.038	0.044	0.042	0.053	0.154
0.034	0.037	0.036	0.035	0.036	0.037		0.085	0.04	0.043	0.043	0.055	0.141
0.038	0.036	0.036	0.036	0.036	0.038		0.033	0.042	0.041	0.042	0.045	0.036
Mean	**0.035333**	**0.035**	**0.034333**	**0.035667**	**0.0345**		**Mean**	**0.04**	**0.042667**	**0.041667**	**0.053333**	**0.114167**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. sorokiniana*						
Roundup concentration (mg/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
3.13	92.97424	93.24759	93.96985	91.62679	90.69767	93.09623
1.56	83.60656	76.52733	94.97487	88.27751	76.74419	89.74895
0.78	81.73302	84.88746	92.46231	89.71292	76.27907	91.00418
0.4	73.30211	75.24116	82.91457	73.92344	65.11628	76.35983
Coefficient of variation for controls	***17.96818***	12.03477	14.37566	***21.56239***	***15.92279***	***26.53547***

7.4. OD readings at 450nm for *Chlorella vulgaris* at the beginning of the experiment and at 96hrs

OD Readings at 0 hrs – 06/02/2012							*OD Readings at 0 hrs* – 10/02/2012					
Replicate 1							**Replicate 1**					
0.033	0.032	0.034	0.034	0.034	0.046		0.092	0.075	0.105	0.144	0.075	0.082
0.034	0.034	0.034	0.035	0.035	0.037		0.129	0.09	0.119	0.157	0.1	0.104
0.035	0.035	0.035	0.035	0.035	0.038		0.099	0.084	0.155	0.147	0.125	0.087
0.035	0.034	0.035	0.034	0.036	0.038		0.033	0.041	0.04	0.048	0.045	0.036
Mean	**0.033667**	**0.034333**	**0.034667**	**0.034667**	**0.037167**		**Mean**	**0.083**	**0.126333**	**0.149333**	**0.1**	**0.098833**
Replicate 2							**Replicate 2**					
0.032	0.033	0.073	0.035	0.034	0.053		0.113	0.063	0.095	0.101	0.064	0.06
0.034	0.034	0.034	0.034	0.035	0.036		0.108	0.171	0.119	0.129	0.06	0.091
0.034	0.495	0.034	0.035	0.036	0.036		0.121	0.065	0.132	0.125	0.086	0.095
0.035	0.034	0.034	0.035	0.036	0.037		0.033	0.039	0.04	0.061	0.043	0.037
Mean	**0.0335**	**0.034**	**0.034667**	**0.035**	**0.0375**		**Mean**	**0.099667**	**0.115333**	**0.118333**	**0.07**	**0.098**
Replicate 3							**Replicate 3**					
0.033	0.032	0.033	0.034	0.034	0.051		0.089	0.071	0.091	0.099	0.06	0.072
0.034	0.034	0.034	0.035	0.036	0.035		0.098	0.067	0.198	0.154	0.078	0.074
0.034	0.034	0.035	0.035	0.036	0.036		0.1	0.059	0.11	0.144	0.089	0.074
0.034	0.034	0.035	0.035	0.036	0.037		0.033	0.058	0.039	0.04	0.045	0.036
Mean	**0.033333**	**0.034**	**0.034667**	**0.035333**	**0.037167**		**Mean**	**0.065667**	**0.133**	**0.132333**	**0.075667**	**0.0845**
Replicate 4							**Replicate 4**					
0.032	0.033	0.034	0.034	0.034	0.039		0.098	0.061	0.108	0.12	0.069	0.077
0.033	0.034	0.034	0.034	0.035	0.035		0.41	0.221	0.186	0.128	0.086	0.088
0.034	0.034	0.035	0.035	0.036	0.037		0.229	0.096	0.127	0.128	0.104	0.098
0.034	0.035	0.035	0.035	0.037	0.037		0.033	0.04	0.041	0.041	0.043	0.037

Mean	**0.033667**	**0.034333**	**0.034333**	**0.035**	**0.035**		**Mean**	**0.126**	**0.140333**	**0.125333**	**0.086333**	**0.166667**
Replicate 5							**Replicate 5**					
0.032	0.032	0.033	0.034	0.034	0.041		0.107	0.063	0.085	0.104	0.094	0.073
0.033	0.034	0.035	0.035	0.035	0.035		0.127	0.064	0.115	0.109	0.11	0.078
0.034	0.035	0.036	0.035	0.036	0.037		0.115	0.063	0.089	0.115	0.094	0.082
0.038	0.036	0.038	0.035	0.037	0.039		0.034	0.039	0.039	0.041	0.042	0.036
Mean	**0.033667**	**0.034667**	**0.034667**	**0.035**	**0.035333**		**Mean**	**0.063333**	**0.096333**	**0.109333**	**0.099333**	**0.097**
Replicate 6							**Replicate 6**					
0.032	0.033	0.033	0.034	0.034	0.044		0.08	0.062	0.086	0.117	0.101	0.069
0.033	0.034	0.034	0.034	0.035	0.036		0.088	0.07	0.092	0.122	0.091	0.068
0.034	0.034	0.034	0.035	0.037	0.037		0.122	0.061	0.114	0.139	0.093	0.066
0.036	0.037	0.034	0.035	0.036	0.037		0.033	0.041	0.042	0.041	0.043	0.036
Mean	**0.033667**	**0.033667**	**0.034333**	**0.035333**	**0.036**		**Mean**	**0.064333**	**0.097333**	**0.126**	**0.095**	**0.082167**

96 hour % Growth Inhibition (+) or *Stimulation (-)*on *C. vulgaris*						
Roundup concentration (mg/L)	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*	*Replicate 5*	*Replicate 6*
3.13	24.42159	***-2.75482***	39.78873	***-65.5963***	54.59459	38.62816
1.56	***-42.4165***	***-28.6501***	***-102.465***	***-91.896***	1.081081	***-32.852***
0.78	***-77.892***	***-33.6088***	***-101.056***	***-64.3731***	***-20***	***-94.9458***
0.4	***-1.79949***	46.28099	18.66197	7.186544	***-3.78378***	***-27.7978***
Coefficient of variation for controls	***16.97199***	***22.15014***	15.1507	11.09417	***22.98761***	***25.87936***