The utility of *Trichilia dregeana* leaves as a bioindicator of air pollution within selected industrial areas in the eThekwini Municipality, South Africa

MINOLI APPALASAMY

Submitted in fulfilment of the academic

Requirements for the degree of

Master of Science in the

School of Biological &

Conservation Sciences,

University of KwaZulu-Natal

Durban

January 2016

As the candidates supervisor I have/have not approved this thesis/dissertation for

Submission

Signed: Name: Date:

ABSTRACT

Increased anthropogenic activities worldwide have led to dangerously high levels of gaseous emissions. Air pollution levels within industrial areas in South Africa, such as South Durban Basin (SDB), are monitored daily at a few monitoring stations; however, limited coverage of, and data generated by, these stations necessitates alternative strategies such as biomonitoring. In this regard, the use of tree leaves as bioindicators of air pollution can generate valuable data on environmental health and pollutant levels. The present study investigated the utility of *Trichilia dregeana* Sond. leaves as a bioindicator of air pollution within selected industrial areas in SDB. The first part of the study focussed on effects of SO_2 pollution on leaf morphological (leaf area), physiological (leaf chlorophyll content and leaf fluorescence) and biochemical (intracellular superoxide and hydrogen peroxide production, total aqueous antioxidant activity, and electrolyte leakage) biomarkers of environmental stress. Leaves were sampled over four seasons from (four) trees growing at three industrial (treatment) sites (Prospecton, Ganges and Southern Works) within SDB and from greenhouse-located trees, which served as an *ex situ* control. Results indicated annual SO_2 concentrations ($[SO_2]$) were high by global standards and significantly different ($p<0.001$) across sites, with levels being highest at Southern Works. All biomarkers, except leaf chlorophyll fluorescence, could discriminate between SO_2-exposed and -unexposed leaves. Seasonal data for many of these biomarkers were significantly ($p<0.001$) correlated with seasonal $[SO_2]$; however, none of them reflected differences in $[SO_2]$ across treatment sites. The second part of the study, partial least squares regression (PLSR) was used to quantify the relationship between two air pollution biomarkers (chlorophyll content and leaf area) and hyperspectral data. *Trichilia dregeana* leaves (n=28) were sampled in spring and summer only. Spectral reflectance data were able to distinguish between SO_2-exposed and -unexposed leaves and PLSR was able to relate the hyperspectral dataset to both biomarkers. However, the interaction between biomarkers suggests simultaneous prediction of these, using an algorithm such as PLS-2, may be more suitable. The variable importance in projection method identified wavebands within the red-edge region of the electromagnetic spectrum that showed promise in identifying stress in the leaves of *T. dregeana*. Collectively, the results provide ample motivation for the establishment of *T. dregeana* leaves as a bioindicator of air pollution.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Durban, from February 2014 to January 2016, under the supervision of Dr Sershen Naidoo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1 – PLAGIARISM

I, Minoli Appalasamy, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain any persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain any persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the Reference sections.

Signed:

DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experiment work and writing of each publication)

Publication 1: Appalasamy, M., Varghese, B., Ismail, R. and Sershen (in preparation). An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in tree leaves: a case study on *Trichilia dregeana* Sond.

Publication 2: Appalasamy, M., Ismail, R., Varghese, B. and Sershen (in preparation). Examining the utility of hyperspectral remote sensing and partial least squares to predict plant stress responses to SO_2 pollution: A case study of *Trichilia dregeana* Sond.

Signed:

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following for supporting me throughout this research:

- First to God Almighty for the guidance and countless blessings throughout my endeavours.

- The National Research Foundation for providing me with the necessary funding and to the eThekwini Municipality for providing the air quality data used in this study.

- My supervisor, Dr Sershen Naidoo, co-supervisor Dr Riyad Ismail and advisor, Dr Boby Varghese. I am truly indebted to you all for your technical and editorial advice, and the time you put into my work.

- To my parents, Veona and Elton Appalasamy, thank you for your constant love and support throughout my academic career. It would not have been possible without your encouragement and belief in me.

- To Candyce Areington and Prelina Munien, I thank you for the sacrifices, hard work, laughs and tears throughout this MSc. Candyce, your love, assistance, singing and support towards me will never be forgotten.

- Furthermore, my sincere gratitude to Wynston Woodenberg for your insightful advice, impeccable editorial input and your willingness to assist me in times of need.

- To the numerous research assistants who took the time to help me either in the lab and out in the field, I thank you.

Lastly, to my Angel from above, thank you for watching over me. I dedicate this to you! Nothing is Impossible with God

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABTS	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
Trolox	2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ANOVA	Analysis of variance
ASD	Analytical spectral device
CO_2	Carbon dioxide
CO	Carbon monoxide
CAT	Catalase
CV	Cross validation
DMOSS	Durban municipal open space system
E	East
FW	Fresh weight
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl radicals

LN	Liquid nitrogen
F_m	Maximum fluorescence
CH_4	Methane
NIR	Near infrared
NO_2	Nitrogen dioxide
N_2O	Nitrous oxide
NDIR	Non-dispersive infrared
N	North
NE	North east
OECD	Organisation for economic cooperation and development
O_3	Ozone
PLS	Partial least squares
PLS-2	Partial least squares 2
PLSR	Partial least squares regression
PM	Particulate matter
Ppb	Parts per billion

Ppm	Parts per million
POX	Peroxidases
PAR	Photosynthetically active radiation
PVP	Polyvinylpyrrolidone
PCA	Principle component analysis
ROS	Reactive oxygen species
RMSEP	Root mean error of prediction
S	South
SANAS	South African national accreditation service
SDB	South Durban Basin
SW	South west
SD	Standard deviation
SO_2	Sulphur dioxide
$[SO_2]$	Sulphur dioxide concentration
$\cdot O_2^-$	Superoxide
SOD	Superoxide dismutase

TAA	Total aqueous antioxidants
UN	United Nations
USA	United States of America
USEPA	United States environmental protection agency
UNEP	Unproductive environmental protocols
F_v	Variable fluorescence
VIP	Variable importance in projection
W	West
WHO	World health organisation

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Preamble

Air pollution has been the focus of many geo-political, social and scientific discussions since the start of industrialisation in the eighteenth century and has gained enormous attention in the past century. The main source of atmospheric pollution is human-induced emissions (viz. mobile or stationary sources in cities) of harmful gases (Karl and Trenberth, 2003) associated with unregulated energy use, which has resulted in unprecedented changes in the atmosphere (Silver and DeFries, 1990; Chang and Terwilliger, 2000). There is much uncertainty around the rates of change in atmospheric properties due to pollution; however, what is certain is that these changes will be experienced by all natural life (Karl and Trenberth, 2003).

Air pollution levels are higher in developing countries due to increasing urbanisation and industrialisation compared with developed countries (Diab and Motha, 2007). This, combined with a casual approach towards the environment, corruption, and the lack of political will (Diab and Motha, 2007) makes the effects of air pollution in developing countries significantly greater (Matooane *et al*., 2004). Cities of developed and developing countries are dominated by anthropogenic activities making them the main contributors to air pollution as well as the prime victims of it (Fenger, 1999; Gurjar *et al.,* 2008). In many of these cities, urbanisation encourages unsustainable practices with regard to natural resource management, and greenhouse gas emissions (Breheny, 1992; Marquez and Smith, 1999). Hence, urban air quality is a major environmental concern in most developing countries (Mage *et al.,* 1996).

Air quality data in most cities around the world is usually based on one or a few monitoring stations placed at critical sites (micro-environments), making regional averages not entirely representative (Fenger, 1999). Many cities that are part of the industrialised world have participated in programmes to ensure sustainable environmental development after the introduction of Agenda 21 (a product of the Earth Summit, United Nations (UN) Conference on Environment and Development, 1992;

Fenger, 1999). The main focus of this programme is to reduce energy use and harmful emissions in order to improve air quality. In South Africa, increased industrialisation, poor land-use planning, increased urbanisation and poverty have led to rising levels of air pollution (Matooane *et al*., 2004). Poor land-use planning often leads to the development of industries within close proximity to residential areas (Wright *et al*., 2000) and these areas are often occupied by low income and rural groups (Matooane *et al*., 2004). Studies have revealed that many residents living in close proximity to these air pollution sources have health problems such as respiratory diseases due to the inhalation of particulates suspended in the atmosphere (Matooane and Diab, 2003). This, together with threats to environmental health and limited financial resources for air quality monitoring has led to poor air quality in many cities in South Africa, including Durban, which served as the location for the present study.

1.2 Problem identification

Since the beginning of the industrial era, the greenhouse effect has intensified due to the increase in atmospheric greenhouse gas emissions from anthropogenic activities and the growing demand for energy produced via the use of fossil fuels such as coal and oil (Jain and Hayhoe, 2008). Air pollution arising from these anthropogenic activities occurs on multiple geographical and temporal scales and threatens human, plant and animal health, and structural integrity of the built environment (Matooane and Diab, 2003). Trends of declining ambient air quality have been reported for many cities across the world, and if pollution levels are not stemmed, these threats are likely to escalate in the centuries ahead (Fenger, 1999; Gurjar *et al.,* 2008). According to Marquez and Smith (1999), if cities are the sources of air pollution, then they should be the areas of solutions too. In this regard, a number of countries have made attempts to monitor air quality (Tiwari *et al.,* 2006; Tripathi and Gautam, 2007) or at least plan to do so in the near future.

Developing countries depend on biomass fuel such as wood, plant material and cow dung (Bruce *et al.,* 2000) for their basic tasks (Matooane *et al.*, 2004) and even when provided with alternatives, rural communities stick to their way of fuelling fires as they are convenient and the material is easy to obtain (Bruce *et al.*, 2000). This phenomenon leads to high indoor pollution concentrations, exceeding those experienced in the

developed world (Brunekreef and Holgate, 2002). Developing countries are also exposed to degrading air quality in urban areas due to various factors, including increased industrialisation, improper environmental protocols and inadequate legislation (UNEP, 1999; Agrawal *et al.,* 2003). To decrease or prevent damage caused by air pollution, monitoring systems should therefore be put in place to detect and quantify sources, and the extent of pollution (Raju *et al.* 2012). Monitoring of poor air quality is also required due to negative effects of air pollution on crops and wild plants (Thomas, 1961; Taylor *et al.,* 1994; Swart *et al.,* 2004; Bytnerowicz *et al.,* 2007; Oksanen *et al.,* 2013; Areington *et al.,* 2015). However, monitoring options within developing countries, such as instrumental monitoring stations, are not always financially feasible and are labour-intensive in terms of maintenance (Conti and Conchetti, 2001; Emberson *et al.,* 2001, 2003).

Various factors contribute to increased levels of pollutants in the atmosphere (Brunekreef and Holgate, 2002). For example, vegetation fires, whether natural (lightning-induced), prescribed (set periods of burning) or of human origin, are common in South Africa (Van Wilgen *et al.,* 2000) and lead to high amounts of carbon being released into the atmosphere along with other gases and particulate matter (PM) (Langmann *et al.,* 2009). Carbon dioxide (CO_2) is the main product emitted into the atmosphere after the burning of vegetation, whereas carbon monoxide (CO) along with various other products are emitted into the atmosphere when vegetation fires are incomplete (Langmann *et al.,* 2009). Furthermore, anthropogenic activities such as transportation also emit pollutants into the atmosphere. Gases, fumes and a combination of other pollutants are emitted from public and private vehicle exhausts, adding to ground-level pollution (Agrawal *et al.,* 2003). Even the dust particles that are raised off the road surface due to movement of vehicles contribute to air pollution in urban areas (Bignal *et al.,* 2007; Jha *et al.,* 2011). Tall chimney stacks are used in various industries to carry harmful gases and particles to higher altitudes in an effort to reduce pollution at a local level; however, due to dispersal mechanisms such as wind, these pollutants still find their way back to ground levels at great distances away from the source of emission (Gheorghe and Ion, 2011).

The types of pollutants that are emitted into the atmosphere from various sources, which can be mobile or stationary, include nitrous and sulphur oxides, ozone and particulate matter, amongst others (Tiwari *et al.,* 2006). Oxides of nitrogen are released into the atmosphere by anthropogenic activities such as the burning of fossil fuels for heating or power generation (Brunekreef and Holgate, 2002). Nitric oxides are changed into nitrogen dioxide (NO_2) due to oxidants in the atmosphere (Brunekreef and Holgate, 2002). Most developing countries are becoming rapid emitters of oxides of nitrogen due to the rapid increase in urban populations and the unstructured growth of industries to support rising demands (Phoenix *et al.,* 2006). Tropospheric ozone is produced by a number of processes that involve nitrogen dioxide, hydrocarbons and sunlight (Brunekreef and Holgate, 2002). According to Jaffe (1967), ozone is the main oxidant compound that is formed during the process of photochemical smog production, which is seasonally dependent as sunlight aids in its formation (Agrawal *et al.,* 2003). Sulphur compounds are also found in the atmosphere and originate from both the natural environment and air pollution emissions (Robinson and Robbins, 1970). The main way in which sulphur oxides are released into the atmosphere is through the combustion of fossil fuels, and once released, this gas can travel great distances in the atmosphere (Lovett *et al.,* 2009). Of the total amount of sulphur dioxide (SO_2) emitted annually worldwide, 70% arises from burning of coal, while burning of petroleum products accounts for 16% (Robinson and Robbins, 1970). Particulate matter can be solid or liquid particulates which vary in size, with the largest particles being formed involuntarily by the erosion of larger particles, whilst smaller particles are formed by condensation and chemical reactions (Brunekreef and Holgate, 2002).

Globally, industries are the main contributors of air pollution (Lamego, 2000) and consume the most energy. The same can be said for Durban, South Africa (Thambiran and Diab, 2011), where industrial zones such as the South Durban Basin (SDB) exhibit extremely poor air quality as a result of emissions from industrial (mainly petrochemical) activities (Matooane and Diab, 2001; Diab *et al*., 2002). These air pollution emissions have been reported to be the highest within Africa (Batterman *et al*., 2008) and the SDB is constantly at risk of high pollution concentrations due to the lack of effective emission controls and its typical coastal topography, which promotes the recirculation of air (Batterman *et al*., 2008). Industrial activities in Durban contribute

22% to the Gross Domestic Product of the country and include various operations such as petroleum refineries; chemical, paper and pulp industries; and vehicle manufacturing (Thambiran and Diab, 2011). Air quality monitoring within the area is limited to a few real-time monitoring stations (Diab *et al.,* 2002), which monitor ground-level SO_2, NO_2, CO and PM. Atmospheric SO_2 concentrations, which have been reported to be exceptionally high (15.28 ppb) in this area (Diab and Motha, 2007), is often used as an indicator of air quality within the SDB, since it represents one of the main pollutants emitted via industrial combustion processes (Diab and Motha, 2007).

Apart from air pollution impacting on human health (Brunekreef and Holgate, 2002) there are also direct and indirect adverse effects on vegetation (Sanders *et al*., 1995). Land, in general, is seen as the reaction surface for certain gaseous air pollutants, and therefore vegetation is considered as a sink for air pollutants (such as CO_2) as it covers vast areas of land worldwide (Hill, 1971). Plants are responsible for removing CO_2 from the atmosphere for the process of photosynthesis while at the same time lowering the levels of global warming (Gheorghe and Ion, 2011). Vegetation and other land cover can in fact determine the amount of radiation absorbed and emitted by the earth's surface (Jain and Hayhoe, 2008). Hence, changes in the atmosphere due to pollution may alter the biogeography of plant species by impacting on their physiology and population dynamics (Chang and Terwilliger, 2000). The most crucial impacts of air pollution on natural vegetation may not relate directly to growth or visible injury but rather to changes in species and genetic composition (Sanders, 1995). The impacts of air pollution on plants are, however, species-specific and pollutant-specific and plants are generally exposed to many types of air pollutants at the same time (Lovett *et al.,* 2009). This makes it difficult to assess the general influence of air pollution on plants in an ecosystem (Lovett *et al.,* 2009). Over extended periods of time, the evolution of tolerance to air pollutants may become an important factor within natural communities (Bell *et al*., 1991). Plant communities also display dynamic inter-species competitive interactions and species-specific responses to air pollution, which may influence these competitive interactions and hence, species composition (Sanders, 1995). For example, high levels of atmospheric pollutants such as nitrogen dioxide can affect species diversity by leading to soil acidification (Bobbink, 1998). The damage inflicted by air

pollution on vegetation may have a positive or negative effect on a particular species, depending on the level of exposure and sensitivity of the species (Bobbink, 1998).

In this regard, impacts of pollution on vegetation may be caused by direct or indirect exposure to air pollution: directly from the atmosphere to vegetation, or indirectly from the atmosphere into the soil and then to vegetation growing in that soil (Chang and Terwilliger, 2000; El-Kilani and Belal, 2010). Sulphur and nitrogen gases, for example, may be deposited directly onto vegetation and the surfaces of soil by a process known as "dry deposition", or may find its way into droplets of water in the atmosphere which increases the possibility of acid rain (Lovett *et al.,* 2009). The measurable/observable effects on plants are also wide ranging. Acid rain, for instance, causes foliar injury to plants (Hogan, 1998; Fan and Wang, 2000; Ramlall *et al.,* 2015) and brings about biochemical and physiological changes (Verlikova *et al.,* 2000). Studies report visible (chlorosis and/or necrosis) and invisible (decrease in photosynthetic activity, a loss of leaf nutrients and various enzyme activities) symptoms of injury due to acid rain (Ferenbaugh, 1976; Evans, 1982; Ramlall *et al.,* 2015). These wide-ranging effects of air pollution on vegetation are challenging to measure and understand, but they grant us the opportunity to use plants as potential bioindicators (organisms used in identification and quantification of the effects of anthropogenic factors) of air pollution (Tripathi and Gautam, 2007), which forms the focus of the present study.

1.3 Rationale and motivation for this study

Plants are exposed to varying levels of air pollution within the SDB, depending on the time of day (Guastella and Knudsen, 2007) and stomatal behaviour (Darrall, 1989). Wind speed is higher during the day due to north-easterly sea breezes, which allow for the dispersion of air pollution (Guastella and Knudsen, 2007). High levels of air pollution are associated with low wind speed occurring at night when temperature inversions are frequent and act as a "lid" trapping air pollution close to the ground (Guastella and Knudsen, 2007). The opening of stomatal pores on leaves allow for the diffusion of gases and water into and out of the leaves; these open stomatal pores are what lead to the physiological damage inflicted on plants by air pollution (Thomas, 1961).

Tall trees that are exposed to high daytime levels of air pollution act as filters by providing canopy cover to various plants and vegetation on the ground (Bytnerowicz, 1996). Agrawal *et al.* (2003) stated that canopies absorb the pollutants in their leaves and therefore levels of air pollution are said to be lower in areas made up of tall trees. Even though tall trees protect other forms of vegetation through their canopy covering, according to many forest monitoring programmes, air pollution affects trees in terms of defoliation (Percy and Ferretti, 2004), growth reduction, leaf necrosis and leaf discoloration (Maatoug *et al.,* 2012). In urban areas too, vegetation has varying degrees of damage due to air pollution, which is explained in detail by Vike (1999) and Areington *et al.* (2015), who state that leaf tips and leaf margins from trees in such areas are subject to both chlorosis and necrosis. Studies have shown though that gaseous pollutants such as fluoride compounds only damage vegetation when they are at high concentrations (Thomas, 1961; Vike, 1999). Vegetation therefore plays a vital role in the purification of the atmosphere/ reduction of air pollution; leaves from trees not only retain particulate matter and absorb gaseous pollutants (Nowak *et al*., 1994), but also help in creating microclimates for other organisms to survive in (Gheorghe and Ion, 2011).

Urban forests are therefore of great importance to neutralise the severe air pollution associated with urban and industrialised areas. The removal of pollutants in the atmosphere by urban vegetation and trees is recognised as a regulating ecosystem service (Setala *et al*., 2013). However, as alluded to above, this service is threatened by the fact that air pollution can lead to either acute or chronic injury in plants: acute injury referring to rapid amounts of pollutants being absorbed therefore killing tissue; and chronic injury referring to sub-lethal pollutants being absorbed over a long period - such injuries are long lasting (Marquez and Smith, 1999). For example, sulphur dioxide diffuses through the stomatal pores of leaves and into the mesophyll layers where its high concentrations lead to inactive cells and may eventually cause the death of these cells (Thomas, 1961). Once vast amounts of cellular area are killed within a leaf, tissues collapse and dry up leading to various internal injuries (Thomas, 1961). The surface of a leaf is therefore of crucial importance as it is able to remove and absorb atmospheric pollution (Spedding, 1969) and in turn reflect the damage inflicted. This makes it

possible for many tree species from such areas to be used as bioindicators of pollution, metal toxicity, or climate change scenarios.

Bioindicators are organisms (or parts thereof) used in identification and quantification of the effects of anthropogenic factors such as air pollution (Conti and Cecchetti, 2001). They have the ability to store contaminants in their tissue, allowing for quantification of these contaminants (Conti and Cecchetti, 2001). Lichens, for example, are considered to be reliable bioindicators since they lack cuticles and therefore absorb pollutants (from air) over their entire surface (Hale, 1969, 1983; Balasooriya *et al.,* 2009; Sawidis *et al.,* 2011; Maatoug *et al.,* 2012). Lichens have been used in air pollution research for the determination of spatial patterns of atmospheric deposition through the content of elements found in them (Berryman, 2009). Pollutants are deposited directly or indirectly by precipitation, and indirect deposits have high nutrient and contaminant levels when conditions in the atmosphere are stable (Conti and Cecchetti, 2001). The spectral reflectance from lichen-dominated vegetation types together with the sensitivity of lichens to air pollution (such as SO_2 and heavy metals) allow for their use in surveying and monitoring of lichen-covered forests and mountains in both high- and low-pollution areas (Tommervik *et al*., 1998).

Similarly, trees and their leaves can be used as effective bioindicators of air pollution (Chapin, 1991; Sawidis *et al.,* 2011). Physiological, morphological and biochemical responses of tree leaves to air pollution have been used as biomarkers (an indicator of biological conditions or processes) of air pollution-related stress (Areington *et al.,* 2015). Leaf chlorophyll content, for example, is seen as a useful biomarker of air pollution in many areas since air pollutants damage plant membranes and chlorophyll pigments (Agrawal *et al.,* 2003). Chlorophyll fluorescence, which is a measure of light that is re-emitted after being absorbed by chlorophyll molecules of plants, can also be used as a biomarker since it reflects plant health (Misra *et al.,* 2012). Light energy absorbed by the plant is degenerated from excited chlorophyll via a process known as photosynthetic quantum conversion (Lichtenthaler, 1996). A smaller portion of light energy is de-excited via the emission of heat or red chlorophyll fluorescence and when there is a decrease in the photosynthetic quantum efficiency, there is an increase in chlorophyll fluorescence (Lichtenthaler, 1996). Hence, stress-induced changes in the photosynthetic quantum conversion, along with damage to the photosynthetic

machinery, are easily identified by measuring chlorophyll fluorescence (Lichtenthaler, 1996; Maxwell and Johnson, 2000). Chlorophyll fluorescence and photosynthesis are also physiologically linked (Naidoo and Chirkoot, 2004), and since the amount of photosynthesis that occurs depends on the area of the leaf and its chlorophyll content, all four of these parameters represent potential biomarkers of plant stress (Burton *et al.* 1991; Seyyednejad and Koochak, 2011).

Environmental stressors such as air pollution also lead to oxidative stress within plants (Jaleel *et al.,* 2009), which precedes physiological and morphological stress responses (Tripathi and Gautam, 2007; Assadi *et al.,* 2011; Bermudez and Pignata, 2011; Minibayeva *et al.,* 2012; Tanee and Albert, 2013). High levels of reactive oxygen species (ROS) produces free radicals, such as superoxide ($^{\cdot}O_2^-$), hydroxyl radicals (OH^-) and non-radicals such as hydrogen peroxide (H_2O_2), which if uncontrolled can lead to biomolecule oxidation and membrane damage (measured as electrolyte leakage) in plants (Fridovich, 1986; Davies, 1987; Dionisio-Sese and Tobita, 1998; Verlikova *et al.,* 2000; Jaleel *et al.,* 2009). For protection against oxidative stress, plants produce two types of antioxidants: enzymatic, which includes superoxide dismutase (SOD), peroxidases (POX), enzymes of the glutathione-ascorbate cycle and catalase (CAT); and non-enzymatic compounds, such as glutathione, ascorbate and tocopherol (Jaleel *et al.,* 2009). When plant tissues produce ROS, in excess under conditions of stress, antioxidants serve to quench these ROS but excessive levels of stress can lead to failure of the antioxidant system (Scandalios, 1997).

Prior to the injury of plants, symptoms of stress can be effectively detected by remote sensing (Smith *et al*., 2004). Remote sensing applications have therefore become popular in detecting changes in vegetation due to gaseous emissions (Sanches *et al.,* 2013). Hyperspectral remote sensing can be applied to detect the effects of various types of environmental stress, such as air pollution (Sanches *et al.,* 2013), by acquiring images in many contiguous narrow spectral bands (< 10 nm) (Hansen and Schjoerring, 2003). Numerous narrow wavelength bands in hyperspectral data provide spectral sensitivity needed to detect subtle changes in reflectance caused by environmental factors (Delalieux *et al.,* 2007). Spectral reflectance analyses have proven to be useful in many studies, due to its ability of detecting plant stress caused by the change in the

amount of incident light absorbed in the visible and near infrared regions of the electromagnetic spectrum (Mohammed *et al.,* 2000; Lawrence, 2003; Zhang *et al.,* 2003; Apan *et al.,* 2005; Smith *et al.,* 2005). Differences between healthy and stressed leaves are detectable within wavelength bands 1350–1750 nm and 2200–2500 nm, having the ability to identify abnormalities in plant processes at early stages (Delalieux *et al.,* 2007). Reference to the literature suggests that the type and degree of pollution damage can be effectively assessed using remote sensing technology (Rosso *et al.,* 2005).

The measureable responses of tree leaves to air pollution (i.e. proven biomarkers of air pollution) described above motivated the present study on the use of tree leaves as a bioindicator of air pollution within selected industrial areas in the eThekwini Municipality, South Africa. The selection of the SDB as the study site was ideal since this area is widely recognised for its poor air quality (Matooane and Diab, 2001; Diab *et al*., 2002). Ground-level SO_2 was selected as the proxy for air pollution in this study since its concentrations are exceptionally high within the SDB (Diab and Motha, 2007) and it represents one of the main pollutants emitted via industrial combustion processes (Diab and Motha, 2007). Many industrial zones in Durban harbour a number of threatened vegetation types, e.g. KwaZulu-Natal Sandstone Sourveld (Scott-Shaw and Escott, 2011) and Coastal Forest (van Aarde *et al*., 1996). However, the impact of air pollution on natural vegetation surrounding the SDB is largely unknown, while the number of functional monitoring stations is limited. Tree leaves have previously been suggested as a non-destructive and cost effective method of monitoring air pollution within the SDB (Areington *et al*., 2015), hence the present study was conducted on the leaves of *Trichilia dregeana. Trichilia dregeana* Sond. (Meliaceae), which is an evergreen coastal forest tree, grows naturally in South Africa (specifically in the Eastern Cape and in KwaZulu-Natal), Swaziland and Zimbabwe (Pooley, 1993). According to Pooley (1993), *T. dregeana* is found in high rainfall areas and grows quickly from fresh seed into a tree that can be 10 to 35 m tall. It is a large (Vander-Willigen *et al.,* 2000), long-lived tree with dark green, compound leaves (Fig. 1) (Pooley, 1993) and according to Siversten *et al.* (1995), is found in close proximity to industrial and urban areas that have high levels of atmospheric pollution.

Figure 1. Study species: *Trichilia dregeana* Sond. Inset: Dark green compound leaves.

1.4 Aim and objectives

With rationale and motivation for the study in place, the broad aim of the study was to assess the utility of *Trichilia dregeana* leaves as a bioindicator of air pollution within selected industrial areas in the eThekwini Municipality, South Africa. Specific objectives included:

i. Measuring leaf chlorophyll content, chlorophyll fluorescence and leaf area (all indicators of plant productivity and vigour) in leaves from trees growing at polluted industrial sites, in comparison with those grown under *ex situ* (greenhouse) conditions.
ii. Measuring oxidative stress levels in terms of intracellular superoxide levels, intracellular hydrogen peroxide production, total aqueous antioxidant capacity and electrolyte leakage in leaves from trees growing at polluted industrial sites, in comparison with those grown under *ex situ* (greenhouse) conditions.
iii. Assessing the strength of the relationship, if any, between the various biomarkers and SO_2 levels, to determine the accuracy with which they reflected actual air pollution levels.

iv. Assessing whether hyperspectral remote sensing can quantify the effect of air pollution on the chlorophyll content and leaf area in leaves from trees growing at polluted industrial sites, in comparison with those grown under *ex situ* (greenhouse) conditions.

1.5 Outline of thesis

This thesis is presented in four chapters and structured primarily around two major chapters (chapters two and three) that have been designed for submission to peer-reviewed journals. Both chapters have detailed sections relating to the study area, literature review, and methodology, therefore these sections are not covered in the introductory section of the thesis in order to avoid repetition.

Chapter two assesses whether or not *Trichilia dregeana* is a suitable bioindicator of air pollution within the SDB, eThekwini Municipality. Various physiological, morphological and biochemical markers (n=24) were used to identify whether or not these parameters are able to be used as biomarkers, and if there are relationships between SO_2 concentrations and these parameters, at treatment (industrial) sites and the *ex situ* control.

Chapter three assesses the capabilities of hyperspectral remotely sensed data to determine the effects of air pollution on leaves of *Trichilia dregeana.* Partial least squares regression (PLSR) was used to obtain an optimal number of components, and the variable importance in projection (VIP) method was used to identify the most important wavebands for selective parameters (n=28). Relationships between the measured parameters and hyperspectral data were determined to examine the extent of SO_2 pollution on leaves of *T. dregeana.*

Chapter four provides a summary to the study. The aim and objectives of the research are discussed, highlighting major findings from the study. Additionally, the chapter examines the limitations of this study and presents recommendations for future research.

References

Agrawal, M., Singh, B., Ragput, M., Marshall, F. and Bell, J.N.B. 2003. Effect of air pollution on peri- urban agriculture: a case study. *Environmental Pollution*, 126, pp. 323 - 329

Apan, A., Held, A., Phinn, S. and Markley, J. 2004. Detecting sugarcane 'orange rust' disease using EO-1 Hyperion hyperspectral imagery. *International Journal of Remote Sensing*, 25(2), pp. 489 - 498

Areington, C.A., Varghese, B., Ramdhani, S. and Naidoo, S.E. 2015. An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in the leaves of *Brachylaena discolor*. *Water, Air and Soil Pollution*, 226(9), pp. 1 - 14

Assadi, A., Pirbalouti, A. G., Malekpoor, F., Teimori, N. and Assadi, L. 2011. Impact of air pollution on physiological and morphological characteristics of *Eucalyptus camaldulensis* Den. *Journal of Food, Agriculture and Environment*, 9(2), pp. 676 - 679

Balasooriya, B.L.W.K., Samson, R., Mbikwa, F., Boeckx, P. and Van Meirvenne, M. 2009. Biomonitoring of urban habitat quality by anatomical and chemical leaf characteristics. *Environmental and Experimental Botany*, 65(2), pp. 386 - 394

Batterman, S.A., Chernyak, S.M., Gounden, Y., Matooane, M. and Naidoo, R.N. 2008. Organochlorine pesticides in ambient air in Durban, South Africa. *Science of the Total Environment*, 397, pp. 119 - 130

Bell, J.N.B., Ashmore, M.R. and Wilson, G.B. 1991. Ecological genetics and chemical modifications of the atmosphere. *In Ecological Genetics and Air Pollution*, pp. 33 - 59

Bermudez, G.M. and Pignata, M.L. 2011. Antioxidant response of three *Tillandsia* species transplanted to urban, agricultural, and industrial areas. *Archives of Environmental Contamination and Toxicology*, 61(3), pp. 401 - 413

Berryman, S., Straker, J. and Straker, D. 2009. Using lichens as bio indicators of air pollution deposition near remote mining operations. Available at https://open.library.ubc.ca/cIRcle/collections/britishcolumbiaminereclamationsy/20212/items/1.0042570.

Bignal, K.L., Ashmore, M.R., Headley, A.D., Stewart, K. and Weigert, K. 2007. Ecological impacts of air pollution from road transport on local vegetation. *Applied Geochemistry*, 22(6), pp. 1265 - 1271

Bobbink, R., Hornung, M. and Roelofs, J. G. 1998. The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. *Journal of Ecology*, 86(5), pp. 717 - 738

Breheney, M.J. 1992. Sustainable development and urban form. In *Regional Science Association*. London: Pion.

Bruce, N., Perez- Padilla, R. and Albalak, R. 2000. Indoor air pollution in developing countries: a major environmental and public health challenge. *World Health Organisation*, 78(9), pp. 1078 - 1092

Brunekreef, B and Holgate, S.T. 2002. Air pollution and health. *The Lancet*, 360, pp. 1233 - 1242

Burton, A. J., Pregitzer, K.S. and Reed, D.D. 1991. Leaf area and foliar biomass relationships in northern hardwood forests located along an 800 km acid deposition gradient. *Forest Science*, 37(4), pp. 1041 - 1059

Bytnerowicz, A. and Fenn, M.E. 1996. Nitrogen deposition in California forests: a review. *Environmental Pollution*, 92(2), pp. 127 - 146

Bytnerowicz, A., Omasa, K. and Paoletti, E. 2007. Integrated effects of air pollution and climate change on forests: a northern hemisphere perspective. *Environmental Pollution*, 147(3), pp. 438 - 445

Chang, E. and Terwilliger, V.J. 2000. The effects of air pollution on vegetation from a geographic perspective. *Progress in Physical Geography*, 24(1), pp. 53 - 74

Chapin, F.S. 1991. Integrated responses of plants to stress. *BioScience*, 41, pp. 29 - 36

Conti, M.E. and Cechetti, G. 2001. Biological mointoring: lichens as bioindicators of air pollution assessment – a review. *Environmental Pollution*, 114, pp. 471 - 492

Davies, K. J. 1987. Protein damage and degradation by oxygen radicals. *Journal of Biological Chemistry*, 262(20), pp. 9895 - 9901

Delalieux, S., van Aardt, J., Keulemans, W., Schrevens, E. and Coppin, P. 2007. Detection of Biotic Stress (*Venturia inaequalis*) in Apple Trees Using Hyperspectral Data: Non-Parametric Statistical Approaches and Physiological Implications. *European Journal of Agronomy*, 27, pp. 130 - 143

Diab, R.D. and Motha, A. 2007. An analysis of key institutional factors influencing air quality in South Durban basin using the DPSIR framework. *South African Geographical Journal,* 89(1), pp. 22 - 33

Diab, R., Prause, A. and Bencherif, H. 2002. Analysis of SO_2 pollution in the South Durban Industrial Basin. *South African Journal of Science*, 98, pp. 543 - 546

Dionisio-Sese, M.L. and Tobita, S. 1998. Antioxidant responses of rice seedlings to salinity stress. *Plant Science*, 135(1), pp. 1 - 9

El-Kilani, R.M. and Belal, M.H. 2010. Modelling an environmental pollutant transport from the stacks to and through the soil. *Journal of Advanced Research*, 1(3), pp. 243 - 253

Emberson, L., Ashmore, M. and Murray, F. 2003. Air pollution impacts on crops and forests: An introduction. In Emberson, L., Ashmore, M. and Murray, F. ed. Air pollution impacts on crops and forests: A global assessment. London: Imperial College Press, pp. 3 - 34

Emberson, L.D., Ashmore, M.R., Murray, F., Kuylenstierna, J.C.I., Percy, K.E., Izuta, T., Zheng, Y., Shimizu, H., Sheu, B.H., Liu, C.P. and Agrawal, M. 2001. Impacts of air pollutants on vegetation in developing countries. *Water, Air, and Soil Pollution*, 130(1-4), pp. 107 - 118

Evans, L.S. 1982. Biological effects of acidity in precipitation on vegetation: a review. *Environmental and Experimental Botany*, 22(2), pp. 155 - 169

Fan, H.B. and Wang, Y.H. 2000. Effects of simulated rain on germination, foliar damage, chlorophyll contents and seedling growth of five hardwood species growing in China. *Forest Ecology and Management*, 126, pp. 321 - 329

Fenger, J. 1999. Urban air quality. *Atmospheric Environment*, 33, pp. 4877 - 4900

Ferenbaugh, R.W. 1976. Effects of simulated acid rain on *Phaseolus vulgaris* L. (Fabaceae). *American Journal of Botany*, pp. 283 - 288

Fridovich, I. 1986. Biological effects of the superoxide radical. *Archives of Biochemistry and Biophysics*, 247(1), pp. 1 - 11

Gheorghe, I.F. and Ion, B. 2011. The effects of air pollutants on vegetation and the role of vegetation in reducing atmospheric pollution. In Khallaf, M.K. ed. *The Impact of Air Pollution on Health, Economy, Environment and Agricultural Sources*. Europe: *INTECH* Open Access Publisher, pp. 241 - 280

Guastella, L. and Knudsen, S. 2007. South Durban basin multi-point plan case study report. Air quality act implementation: Air quality management planning. Book 12, series C, pp. 1 - 34

Gurjar, B.R., Butler, T.M., Lawrence, M.G. and Lelieveld, J. 2008. Evaluation of emissions and air quality in megacities. *Atmospheric Environment*, 42(7), pp. 1593 - 1606

Hale, M.E. 1969. How to know the lichens. In Wm. C. ed. Iowa: Brown Company publishers

Hansen, P. M. and Schjoerring, J. K. 2003. Reflectance measurement of canopy biomass and nitrogen status in wheat crops using normalized difference vegetation indices and partial least squares regression. *Remote Sensing of Environment*, 86, pp. 542 - 553

Hill, A.C., 1971. Vegetation: a sink for atmospheric pollutants. *Journal of the Air Pollution Control Association*, 21(6), pp. 341 - 346

Hogan, G. D. 1998. Effect of simulated acid rain on physiology, growth and foliar nutrient concentrations of sugar maple. *Chemosphere*, 36(4), pp. 633 - 638

Jaffe, L.S. 1967. The biological effects of ozone on man and animals. *American Industrial Hygiene Association Journal*, 28(3), pp. 267 - 277

Jain, A.K. and Hayhoe, K.A.S. 2008. Handbook of atmospheric sciences 2. *Problems, Tools and Applications*, pp. 2 - 61

Jaleel, C.A., Riadh, K., Gopi, R., Manivannan, P., Inès, J., Al-Juburi, H.J., Chang-Xing, Z., Hong-Bo, S. and Panneerselvam, R. 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. *Acta Physiologiae Plantarum*, 31(3), pp. 427 - 436

Jha, D.K., Sabesan, M., Das, A., Vinithkumar, N.V. and Kirubagaran, R. 2011. Evaluation of Interpolation Technique for Air Quality Parameters in Port Blair, India. *Universal Journal of Environmental Research and Technology*, 1(3), pp. 301 - 310

Karl, T.R. and Trenberth, K.E. 2003. Modern global climate change. *Science*, 302, pp. 1719 - 1723

Lamego, M.M., de Souza Jr, P.A., Ferriera, E.P. and Garg, V.K. 2000. Quantification of air pollution sources. *Revista Controle and Automacao*, 11(2), pp. 77 - 81

Langmann, B., Duncan, B., Textor, C., Trentmann, J. and van der Werf, G.R. 2009. Vegetation fire emissions and their impact on air pollution and climate. *Atmospheric Environment*, 43(1), pp. 107 - 116

Lawrence, R. and Labus, M. 2003. Early detection of Douglas-fir beetle infestation with subcanopy resolution hyperspectral imagery. *Western Journal of Applied Forestry*, 18(3), pp. 202 - 206

Lichtenthaler, H.K. 1996. Vegetation stress: an introduction to the stress concept in plants. *Journal of Plant Physiology*, 148(1), pp. 4 - 14

Lovett, G. M., Tear, T. H., Evers, D. C., Findlay, S. E., Cosby, B. J., Dunscomb, J. K. and Weathers, K. C. 2009. Effects of air pollution on ecosystems and biological diversity in the eastern United States. *Annals of the New York Academy of Sciences*, 1162(1), pp. 99 - 135

Maatoug, M., Akermi, A., Taïbi, K., Achir, M. and Mestrari, M. 2012. Bio-Monitoring of Air Quality Using Leaves of Tree and Lichens in Urban Environments. In Khare, M. ed. *Monitoring, Modelling and Health.* Europe: *INTECH* Open Access Publisher, pp. 223 - 244

Mage, D., Ozolins, G., Peterson, P., Webster, A., Orthofer, R., Vandeweerd, V. and Gwynne, M. 1996. Urban air pollution in megacities of the world. *Atmospheric Environment*, 30(5), pp. 681 - 686

Marquez, L.O. and Smith, N.C. 1999. A framework for linking urban form and air quality. *Environmental Modelling and Software*, 14, pp. 541 - 548

Matooane, L. and Diab, R.D. 2001. Air pollution carrying capacity in the South Durban Industrial Basin: research in action. *South African Journal of Science*, 97(11 and 12), pp. 450 - 453

Matooane, M. and Diab, R. 2003. Health risk for sulphur dioxide pollution in South Durban, South Africa. *Archives of Environmental Health: An International Journal*, 58(12), pp. 763 - 770

Matooane, M., Oosthuizen, J.R. and Binedell, M. 2004. Vulnerability of South African communities to air pollution. *8th World Congress on Environmental Health*, pp. 1 - 8

Maxwell, K. and Johnson, G.N. 2002. Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, 51(345), pp. 659 - 668

Minibayeva, F., Dmitrieva, S., Ponomareva, A. and Ryabovol, V. 2012. Oxidative stress-induced autophagy in plants: the role of mitochondria. *Plant Physiology and Biochemistry*, 59, pp. 11 - 19

Misra, A.N., Misra, M. and Singh, R. 2012. Chlorophyll Fluorescence in Plant Biology. *Biophysics*, pp. 171 - 192

Mohammed, G.H., Noland, T.L., Irving, P.H., Sampson, P.H., Zarco-Tejada, P.J. and Miller, J.R. 2000. Natural and stress-induced effects on leaf spectral reflectance in *Ontario* species. *Forest Research Report*, 156, pp. 1 - 34

Naidoo, G. and Chirkoot, D. 2004. The effects of coal dust on photosynthetic performance of the mangrove, *Avicennia marina* in Richards Bay, South Africa. *Environmental Pollution*, 127(3), pp. 359 - 366

Nowak, D.J. 1994. Air pollution removal by Chicago's urban forest. Chicago's urban forest ecosystem: Results of the Chicago urban forest climate project, Air pollution

removal by Chicago's urban forest. In: McPherson, E.G., Nowak, D.J., Rowntree, R.A. eds. *Chicago's Urban Forest Ecosystem: Results of the Chicago Urban Forest Climate Project. USDA Forest Service General Technical Report NE-186*. Radnor: PA, pp. 63 - 81

Oksanen, E., Pandey, V., Pandey, A.K., Keski-Saari, S., Kontunen-Soppela, S. and Sharma, C. 2013. Impacts of increasing ozone on Indian plants. *Environmental Pollution*, 177, pp. 189 - 200

Percy, K.E. and Ferretti, M. 2004. Air pollution and forest health: toward new monitoring concepts. *Environmental Pollution*, 130(1), pp. 113 - 126

Phoenix, G.K., Hicks, W.K., Cinderby, S., Kuylenstierna, J.C.I., Stock, W.D., Dentener, F.J., Giller, K.E., Austin, A.T., Lefroy, R.D.B., Gimeno, B.S., Ashmore, M.R. and Ineson, P. 2006. Atmospheric nitrogen deposition in world biodiversity hotspots: the need for a greater global perspective in assessing N deposition impacts. *Global Change Biology* 12, pp. 470 - 476

Pooley, E. 1993. The complete field guide to trees of Natal, Zululand and Transkei, 3rd Edition. Durban, Natal flora publications trust, pp. 204 - 205

Raju, H.P., Partheeban, P. and Hemamalini, R.R. 2012. Urban mobile air quality monitoring using GIS, GPS, sensors and internet. *International Journal of Environmental Science and Development*, 3(4), pp. 323

Ramlall, C., Varghese, B., Ramdhani, S., Pammenter, N.W., Bhatt, A. and Berjak, P. 2015. Effects of simulated acid rain on germination, seedling growth and oxidative metabolism of recalcitrant-seeded *Trichilia dregeana* grown in its natural seed bank. *Physiologia Plantarum*, 153(1), pp. 149 - 160

Robinson, E. and Robbins, R.C. 1970. Gaseous atmospheric pollutants from urban and natural sources. In Singer, S.F. ed. *Global Effects of Environmental Pollution*. New York: Springer, pp. 50 - 64

Rosso, P.H., Pushnik, J.C., Lay, M. and Ustin, S.L. 2005. Reflectance properties and physiological responses of *Salicornia virginica* to heavy metal and petroleum contamination. *Environmental Pollution*, 137(2), pp. 241 - 252

Sanches, I.D., Souza Filho, C.R., Magalhaes, L.A., Quitério, G.C.M., Alves, M.N. and Oliveira, W.J. 2013. Assessing the impact of hydrocarbon leakages on vegetation using reflectance spectroscopy. *Journal of Photogrammetry and Remote Sensing*, 78, pp. 85 - 101

Sanders, G.E., Skärby, L., Ashmore, M.R. and Fuhrer, J. 1995. Establishing critical levels for the effects of air pollution on vegetation. *Water, Air, and Soil Pollution*, 85(1), pp. 189 - 200

Sawidis, T., Breuste, T., Mitrovic, M., Pavlovic, P. and Tsigaridas, K. 2011. Trees as bioindicators of heavy metal pollution in three European countries. *Environmental Pollution*, 159, pp. 3560 - 3570

Scandalios, J.G. 1997. Oxidative stress and the molecular biology of antioxidant defenses. In. Scandalios, J.G. ed. *Oxidative stress and the molecular biology of antioxidant defences*. New York: Cold Spring Harbor Laboratory Press, pp. 1 - 890

Scott-Shaw, C.R. and Escott, B.J. 2011. KwaZulu-Natal Provincial Pre-Transformation Vegetation Type Map–2011. Unpublished GIS Coverage [kznveg05v2_1_11_wll. zip], Biodiversity Conservation Planning Division, Ezemvelo KZN Wildlife, PO Box, 13053

Setälä, H., Viippola, V., Rantalainen, A.L., Pennanen, A. and Yli-Pelkonen, V. 2013. Does urban vegetation mitigate air pollution in northern conditions? *Environmental Pollution*, 183, pp. 104 - 112

Seyyednejad, S.M., Niknejad, M. and Koochak, H. 2011. A review of some different effects of air pollution on plants. *Research Journal of Environmental Sciences*, 5(4), pp. 302

Silver, C.S. and DeFries, R.S. 1990. One Earth: Our Changing Global Environment; One Future. In Silver, C.S. and DeFries. ed. Washington Dc: National Academy Press, pp. 319 - 325

Sivertsen, B., Matale, C. and Pereira, L.M.R. 1995. Sulphur emissions and transfrontier air pollution in southern Africa. In. Sivertsen, B., Matale, C. and Pereira, L.M.R. ed. Sulphur emissions and transfrontier air pollution in southern Africa *Report (SADC*

Environment and Land Management Sector. Coordination Unit) Pretoria: SADC Environment and Land Management Sector Coordination Unit, pp. 1 - 117

Smith, K.L., Steven, M.D. and Colls, J.J. 2004. Use of hyperspectral derivative ratios in the red-edge region to identify plant stress responses to gas leaks. *Remote Sensing of Environment*, 92(2), pp. 207 - 217

Smith, K.L., Steven, M.D. and Colls, J.J. 2005. Plant spectral responses to gas leaks and other stresses. *International Journal of Remote Sensing*, 26(18), pp. 4067 - 4081

Spedding, D. J. 1969. Uptake of sulphur dioxide by barley leaves at low sulphur dioxide concentrations. *Nature*, 224, pp. 1229 - 1231

Swart, R.J., Raskin, P. and Robinson, J. 2004. The problem of the future: sustainability science and scenario analysis. *Global Environmental Change*, 14(2), pp. 137 - 146

Tanee, F. B. G. and Albert, E. 2013. Air pollution tolerance indices of plants growing around Umuebulu Gas Flare Station in Rivers State, Nigeria. *African Journal of Environmental Science and Technology*, 7(1), pp. 1 - 8

Taylor, G.E., Johnson, D.W. and Andersen, C.P. 1994. Air pollution and forest ecosystem: A regional to global perspective. *Ecological Applications*, 4, pp. 662 - 689

Thambiran, T. and Diab, R.D. 2011. Air quality and climate change co-benefits for the industrial sector in Durban, South Africa. *Energy Policy*, 39, pp. 6658 - 6666

Thomas, R. 1961. Studies in the biosynthesis of fungal metabolites. 3. The biosynthesis of fungal perinaphthenones. *Biochemical Journal*, 78(4), pp. 807

Tiwari, S., Agrawal, M. and Marshall, F.M. 2006. Evaluation of ambient air pollution impact on carrot plants at a sub urban site using open top chambers. *Environmental Monitoring and Assessment*, 119, pp. 15 - 30

Tømmervik, H., Johansen, M.E., Pedersen, J.P. and Guneriussen, T. 1998. Integration of remote sensed and in-situ data in an analysis of the air pollution effects on terrestrial ecosystems in the border areas between Norway and Russia. *Environmental Monitoring and Assessment*, 49(1), pp. 51 - 85

Tripathi, A.K. and Gautam, M. 2007. Biochemical parameters of plants as indicators of air pollution. *Journal of Environmental Biology*, 28, pp. 127 - 132

UN Conference on Environment and Development, 1992. https://sustainabledevelopment.un.org/content/documents/Agenda21.pdf

UNEP, 1999. www.unep.org/eou/Portals/52/.../AnnualEvalReportEnglish1999.pdf

Van Aarde, R.J., Ferreira, S.M. and Kritzinger, J.J. 1996. Successional changes in rehabilitating coastal dune communities in northern KwaZulu/Natal, South Africa. *Landscape and Urban Planning*, 34(3), pp. 277 - 286

Van Wilgen, B.W., Biggs, H., O'regan, S.P. and Mare, N. 2000. Fire history of the savanna ecosystems in the Kruger National Park, South Africa, between 1941 and 1996. *South African Journal of Science*, 96, pp. 167 - 178

Vander Willigen, C., Sherwin, H.W. and Pammenter, N.W., 2000. Xylem hydraulic characteristics of subtropical trees from contrasting habitats grown under identical environmental conditions. *New Phytologist*, 145(1), pp. 51 - 59

Velikova, V., Yordanov, I. and Edreva, A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*, 151(1), pp. 59 - 66

Vike, E. 1999. Air-pollutant dispersal patterns and vegetation damage in the vicinity of three aluminium smelters in Norway. *Science of the Total Environment*, 236(1), pp. 75 - 90

Wright, S.F. and Anderson, R.L. 2000. Aggregate stability and glomalin in alternative crop rotations for the central Great Plains. *Biology and Fertility of Soils*, 31(3-4), pp. 249 - 253

Zhang, M., Qin, Z., Liu, X. and Ustin, S. L. 2003. Detection of stress in tomatoes induced by late blight disease in California, USA, using hyperspectral remote sensing. *International Journal of Applied Earth Observation and Geoinformation*, 4(4), pp. 295 - 310

CHAPTER TWO

An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in tree leaves: a case study on *Trichilia dregeana* Sond.

2.1 Abstract

Air pollution from various sources impacts negatively (either directly or indirectly) on plants. Industrial zones in eThekwini, South Africa, such as the South Durban Basin (SDB) are often characterised by extremely poor air quality owing to industrial emissions. In order to establish whether or not the leaves of *Trichilia dregeana* Sond. could be used as a bioindicator of air pollution, this study investigated the effects of SO_2 pollution on leaf morphological (leaf area), physiological (leaf chlorophyll content and leaf fluorescence) and a range of biochemical (intracellular superoxide and hydrogen peroxide production, total aqueous antioxidant activity and electrolyte leakage) biomarkers of environmental stress. Leaves were sampled from (four) trees growing at three industrial (treatment) sites (Prospecton, Ganges and Southern Works) within the SDB and from greenhouse-located trees which served as an *ex situ* control. The sampling strategy accommodated for directional and seasonal effects and yielded a sample size of n=24 for each of the four seasons. Ground-level SO_2 concentrations measured at each site throughout the sampling period, were relatively high by world standards (4.57-6.59 ppb). Values for the various biomarkers did not differ significantly for leaves from different cardinal points within sites but seasonal variations were evident (particularly for chlorophyll content, leaf chlorophyll fluorescence, intracellular $\cdot O_2^-$ and electrolyte leakage). Except for leaf chlorophyll fluorescence, all biomarkers could discriminate between SO_2 exposed and unexposed (*ex situ* control) leaves. Though seasonal data for a number of these biomarkers (viz. leaf area, leaf chlorophyll content and H_2O_2) were significantly correlated with seasonal [SO_2], none of the biomarkers appeared to be sensitive enough to reflect differences in [SO_2] across the treatment sites. The results also suggested a physiological link between leaf area and chlorophyll content, as well as between intracellular H_2O_2 production and electrolyte leakage; these biomarkers should thus be measured in combination with each other. All

biomarkers, except for chlorophyll fluorescence and intracellular $\cdot O_2^-$ production (the data for which were highly variable), can therefore be used to establish *T. dregeana* leaves as a bioindicator of air (specifically, SO_2) pollution.

Keywords: Air pollution, bioindicator, biomarker, SO_2, tree leaves

2.2 Introduction

Global climate change is largely a consequence of anthropogenic activities that emit greenhouse gases and aerosols into the atmosphere (Fenger, 1999; Jain and Hayhoe, 2008). Common atmospheric pollutants include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and carbon monoxide (CO) (Karl and Trenberth, 2003; Jain and Hayhoe, 2008). These gases accumulate and have long lifespans in the atmosphere (Karl and Trenberth, 2003). Atmospheric CO_2 concentrations, in particular, have increased from 280 ppm in pre-industrial times to 365 ppm currently (Tubiello *et al*., 2000). Changes to the atmosphere induced by these pollutants have led to damage of crop and wild plants, largely due to environmental phenomena such as acid rain (Fan and Wang, 2000), depletion of the ozone layer and global warming (Marquez and Smith, 1999; Jain and Hayhoe, 2008). Air pollution is therefore a major concern in the face of increasing industrialisation, urbanisation, and human population size (Bickerstaff and Walker, 2000). South Africa is no exception, with increased industrialisation, poor land-use planning, increased urbanisation and wide-spread poverty all contributing to rising levels of air pollution (Bruce *et al.,* 2000; Matooane *et al*., 2004).

In light of the effects of industrial pollutants such as SO_2, NO_2, particulate matter (PM) and ozone (O_3) on human health and the environment (Marquez and Smith, 1999; Matooane and Diab, 2003), it has become increasingly important to monitor air quality in rapidly urbanising cities in developing countries such as South Africa. In most of the industrialised world, air pollution is monitored on a regular basis with pollutant concentrations being reported annually (The Organisation for Economic Cooperation and Development [OECD], 1997). However, since air quality data are usually based on one or a few monitoring stations at strategic sites across cities, average pollution levels may not be representative of specific regions (Fenger, 1999). This is often unavoidable since the installation of multiple air quality monitoring stations is not always financially

feasible, particularly in countries within the developing world. An alternate approach is to supplement instrumental air quality monitoring with data collected using bioindicators, i.e. organisms that can be used to identify and determine the effect that biotic and abiotic stresses have on the environment (Conti and Cechetti, 2001). In this regard, Novak *et al.* (2003) argued that it is best to use indigenous species as bioindicators of air pollution, provided that these species are able to withstand high pollution levels, have a wide geographical distribution, are abundant and easily accessible, and are negatively impacted on by atmospheric air pollution (Conti and Cechetti, 2001).

The use of plants and trees in particular, as bioindicators of air pollution is a widespread strategy (Markert, 1995; Mičieta and Murin, 1998; Moraes *et al*., 2002; Aksoy, 2003; Madejon *et al.,* 2004) and the identification of reliable bioindicators of air quality may be useful in many countries across the developing world (Areington *et al*., 2015). *Plantago lanceolata* L., for example, is often used as a bioindicator of urban air pollution in the city of Gent (Belgium) as it is easy to identify, common to urban environments, and has large leaves which allow for multiple parameters or biomarkers (an indicator of a biological condition or process) to be measured (Bakker *et al*., 1999). Several authors report the value of using morphological and biochemical characteristics of leaves as biomarkers of environmental pollution (Bakker *et al*., 1999). This motivated the present study, which investigated the utility of leaves of the indigenous tree species, *Trichilia dregeana* Sond. as a bioindicator of air pollution within selected industrial areas in Durban (in eThekwini), South Africa. The sites at which trees were sampled all occur within industrial areas in the South Durban Basin (SDB), which is located within an urban matrix. Industries are the largest contributors to air pollution within urban areas (Lamego *et al*., 2000) and this is particularly true for the SDB, in which many industries rely heavily on fossil fuel based energy (Thambiran and Diab, 2011). The selection of *T. dregeana* leaves is based on the fact that this evergreen coastal forest tree grows naturally and is planted as a street tree in KwaZulu-Natal and parts of the Eastern Cape (both in South Africa), Swaziland and Zimbabwe (Pooley, 1993).

Vegetation represents passive sampling in biomonitoring and has the advantage of high spatial and temporal resolution due to widespread distribution of plants and low

sampling costs (Sawidis *et al.,* 1997). The reasoning behind sampling the leaves of *T. dregeana* trees is based on a number of studies (reviewed by Madejon *et al*. 2004) that have shown leaves to be reliable bioindicators of air pollution. One of the prerequisites of using tree leaves as bioindicators is that they have to be susceptible to the pollutants investigated but be tolerant enough to avoid mortality (Madejon *et al*., 2004). Literature suggests that one can measure a number of physiological, biochemical and morphological biomarkers of air pollution in tree leaves (Sawidis *et al.,* 2011). For instance, as a consequence of air pollution and other environmental stressors, plants increase the production of reactive oxygen species (ROS) in their tissues (Vranova *et al*., 2002). The stress experienced by the plants and damage incurred is determined by the equilibrium between oxidative stress and antioxidant activity (Arora *et al*., 2002; Gill and Tuteja, 2010). During non-stressful periods, antioxidant defence systems are able to protect the organism from ROS (Gill and Tuteja, 2010); however, excessive production of ROS can lead to uncontrolled, harmful levels of toxic oxygen by-products (Arora *et al*., 2002). According to those authors, this increases antioxidant activity, but if this increase is insufficient, then secondary damage such as lipid peroxidation, protein oxidation and oxidative damage to nucleic acids can occur. In this regard, damage to plant cell membranes can be measured in terms of leaf electrolyte leakage (Santamaria and Martin, 1997). One must also acknowledge though, that ROS is produced naturally in many metabolic pathways (mainly within the chloroplast and mitochondria) and are also involved in signalling processes (Gill and Tuteja, 2010). Other biomarkers such as biomass (Kancheva and Borisova, 2007), chlorophyll content (Kancheva and Borisova, 2007) and leaf area (Assadi *et al.,* 2011; Tanee and Albert, 2013; Areington *et al.,* 2015) have also proven to be useful biomarkers of environmental stress (Kancheva and Borisova, 2007). Leaf chlorophyll fluorescence, though rarely applied in pollution research is also a valuable, non-destructive physiological parameter that measures the efficiency of photosystem II (PSII) (Branquinho *et al.,* 1999). Chlorophyll fluorescence has, however, been used as an indicator of stress-induced damage in a number of plant species (Lichtenthaler, 1996; Maxwell and Johnson, 2000).

In light of the above, the present study assessed the utility of morphological, physiological and biochemical parameters as biomarkers of industrial air pollution in *T. dregeana* tree leaves. Morphological (leaf area), physiological (leaf chlorophyll content

and fluorescence) and a range of biochemical (intracellular superoxide and hydrogen peroxide production, total aqueous antioxidants activity and electrolyte leakage) biomarkers of environmental stress were compared in terms of their ability to reflect the effects of exposure to different levels of atmospheric SO_2. Leaves were sampled from trees growing at three industrial sites (referred to as treatment sites hereafter) within the SDB, and based on a recent study by Areington *et al*. (2015), the control trees were housed in a greenhouse in which SO_2 levels were negligibly low. The sampling strategy accommodated for directional and temporal effects and ground-level SO_2 concentrations were measured daily at each treatment site throughout the sampling period. The findings of this study contribute towards the establishment of *T. dregeana* tree leaves as a bioindicator of industrial air pollution within the SDB and supplement the broader body of knowledge on the use of plants as bioindicators of air pollution.

2.3 Materials and methods

2.3.1 Study Area: polluted (treatment) sites, *ex situ* control site, location of trees and geography of area

The geographic location of the three industrial treatment sites, all of which occur within the SDB, in KwaZulu-Natal (South Africa) is shown in Figure 2. For the purposes of this study they are labelled as follows: 'Southern Works' (located in Wentworth); 'Ganges High School' (located in Merebank); and 'Prospecton' (located in Isipingo). At each site, four *T. dregeana* trees were selected for investigation, with each tree occurring within 750 m of an air pollution monitoring station (the locations of which are also indicated in Fig. 2). Sampling sites were selected on the basis of the presence of air pollution monitoring stations (owned and operated by the eThekwini Municipality) that measured ground-level SO_2 levels on an hourly basis. Trees located in a greenhouse on the grounds of the University of KwaZulu-Natal (Westville, Durban), a month prior to and for the duration of the study, served as the *ex situ* control. Levels of SO_2 in the greenhouse were negligibly low (method of measurement discussed below), and other studies (Areington *et al.,* 2015) have shown the utility of using greenhouse-located control trees in studies of this nature.

The SDB is situated in a shallow basin on the east coast of South Africa and has a coastal strip of approximately 96 km^2. The area is referred to as a "flat alluvial corridor"

(Batterman *et al*., 2008) surrounded by a border of sand dunes (seaward) and hills (landward). Wind within the SDB flows mainly from the south-west (SW) and north-east (NE) in equal proportions, with wind from the SW being associated with a low pressure and harsh weather conditions, and wind from the NE being associated with high pressure and fine weather conditions (Guastella and Knudsen, 2007). A combination of these predominant wind types and the topography of the area lead to the channelling of pollutants within the basin. The basin receives approximately 1009 mm of rain annually, which removes dust and gas from the atmosphere (Guastella and Knudsen, 2007). Within the eThekwini Municipality and the areas surrounding the SDB, land-use types include cemeteries, golf courses, informal settlements, urban areas, nature reserves, paper mills, landfill sites, reservoirs, open areas, wastewater treatment works, refineries and many others. Refineries within the SDB contribute to over 80% of the SO_2 emissions in the area, along with various transport-related sources (eThekwini Health and Norwegian Institute for Air Research, 2007).

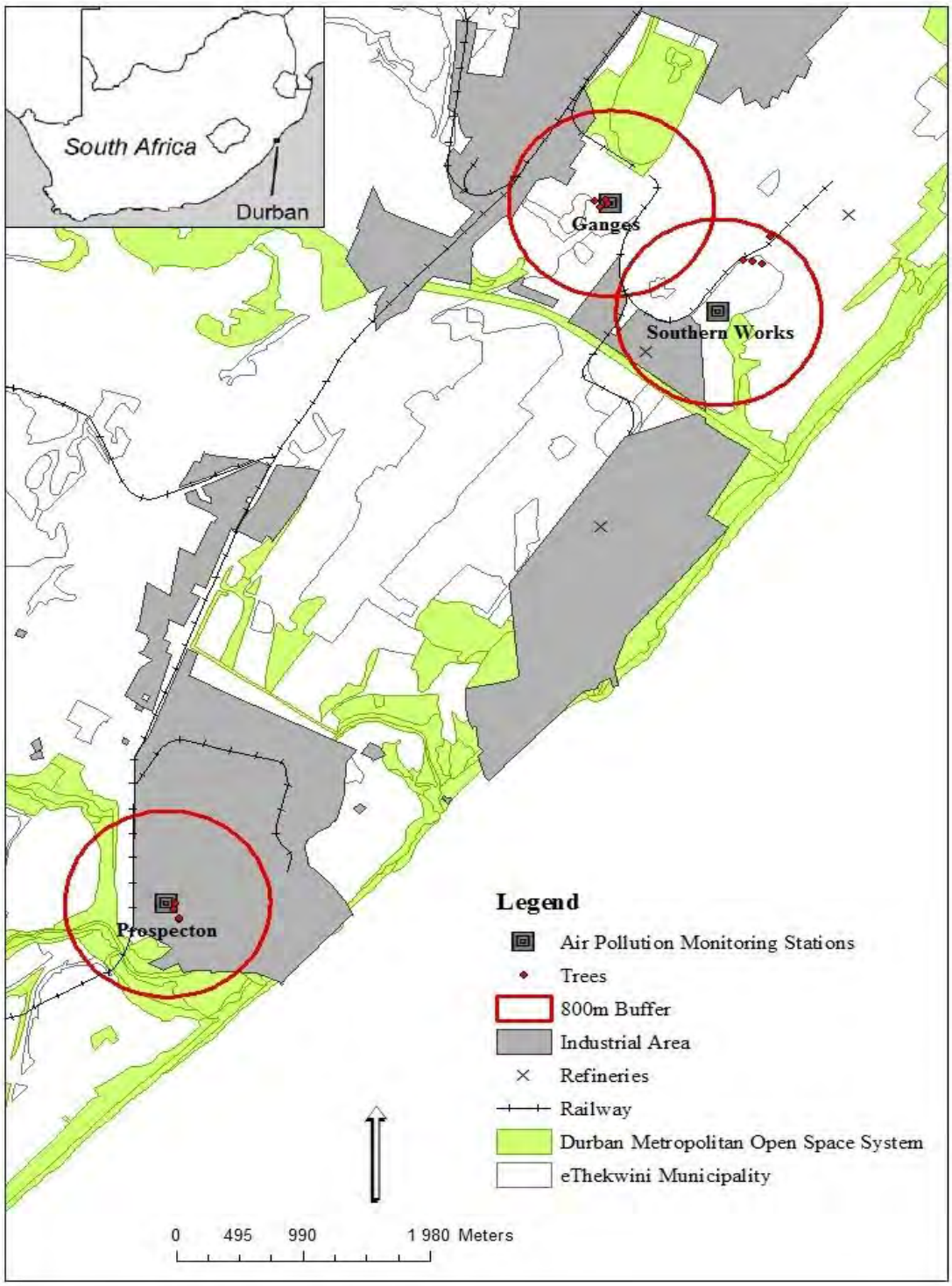

Figure 2. Study area showing industrial sites (Ganges, Southern Works and Prospecton) at which sampling was conducted. All sites are located within the South Durban Basin in eThekwini, South Africa.

2.3.2 Measurement of ground-level SO_2 concentrations ([SO_2])

Ground-level [SO_2] (in parts per billion [ppb]) were measured hourly (by the eThekwini Municipality) at each of the monitoring stations (Fig. 3) and this was made available for use in this study. The data used were collected in each of the four sampling seasons, between 2014 and 2015. Prior to any analyses, the data were cleaned (i.e. erroneous, zero, negative and blank values were deleted) and a yearly average [SO_2] was generated for each monitoring station. The air pollution monitoring stations that provided the data used in this study have been set up across the eThekwini Municipality to measure the ground-level concentrations of SO_2, NO_2 and particulate matter (PM) (amongst others) via continuous monitoring using United States Environmental Protection Agency (USEPA) designated analysers. The analysers (Fig. 3) are calibrated on an annual basis by a South African National Accreditation Service (SANAS) consulting company. Weekly quality checks are done on the instruments and invalid data are removed on a continuous basis.

Sulphur dioxide levels were also measured on eight days within the greenhouse and at three random points (on the university campus) less than 1 km from where the greenhouse control site is located. These measurements were carried out using a HORIBA PG-350E (HORIBA Europe GmbH, Julius-Kronenberg-Strasse 9, Leichlingen, Germany) atmospheric monitoring system. The SO_2 detector functions with a cross-flow modulation, non-dispersive infrared (NDIR) absorption method (according to the European standard, DIN EN 15267-3, DIN EN 14181).

Data for NO_2 and PM were only available for two sites, viz. Ganges and Southern Works, and furthermore only collected at the monitoring stations in spring and summer. Nevertheless, this limited dataset was also analysed here in the interest of gaining some insight into the levels of pollutants other than SO_2 at the treatment sites. The NO_2 and PM results are not related to the biomarkers directly but rather used to interpret some of the trends observed at specific treatment sites.

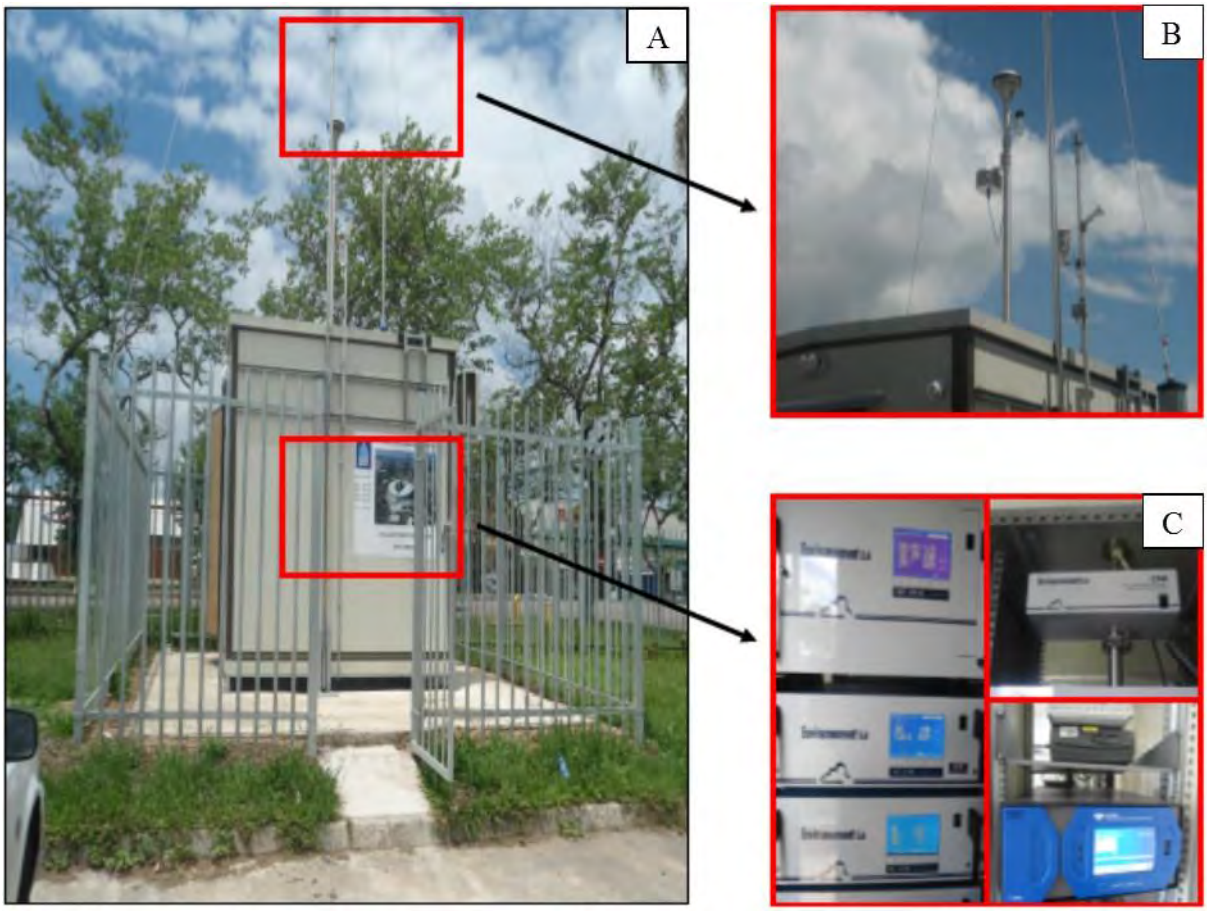

Figure 3. An example of an eThekwini Municipality air quality monitoring station (A) equipped with the different pollutant analysers (B and C).

2.3.3 Leaf sampling strategy

Four trees at each of the three treatment sites and the control were sampled as per Sawidis *et al*. (2011), with slight modifications. On any sampling day, which was always at least 24 h after the last rain event, branches were removed from selected trees at all the sites; leaves which showed signs of recent insect damage were not selected. Trees were sampled randomly across all sites and in each of the four seasons: autumn (1 March to 31 May), winter (1 June to 31 August), spring (1 September to 30 November) and summer (1 December to 28 February), between 2014 and 2015. On any particular sampling day, a maximum of two trees were sampled at each site (treatment and control). No individual tree was sampled more than once a week, and within any particular season the trees were sampled until the required number of replicates (n=24) was achieved for all the biomarkers (as shown in Figure 4). A random branch located at

each cardinal point (north, east, south and west) was collected to accommodate for the effects of wind direction (Klumpp *et al*., 2003). Control trees were subjected to the same sampling regime.

Once collected, the branches were immediately conveyed to the laboratory with the cut ends immersed in deionised water. Upon arrival, the abaxial and adaxial surfaces were rinsed with deionised water to remove bird droppings and PM and then the fresh weight (FW) of individual leaves was recorded prior to any further analyses, which are shown in Figure 4 and detailed below.

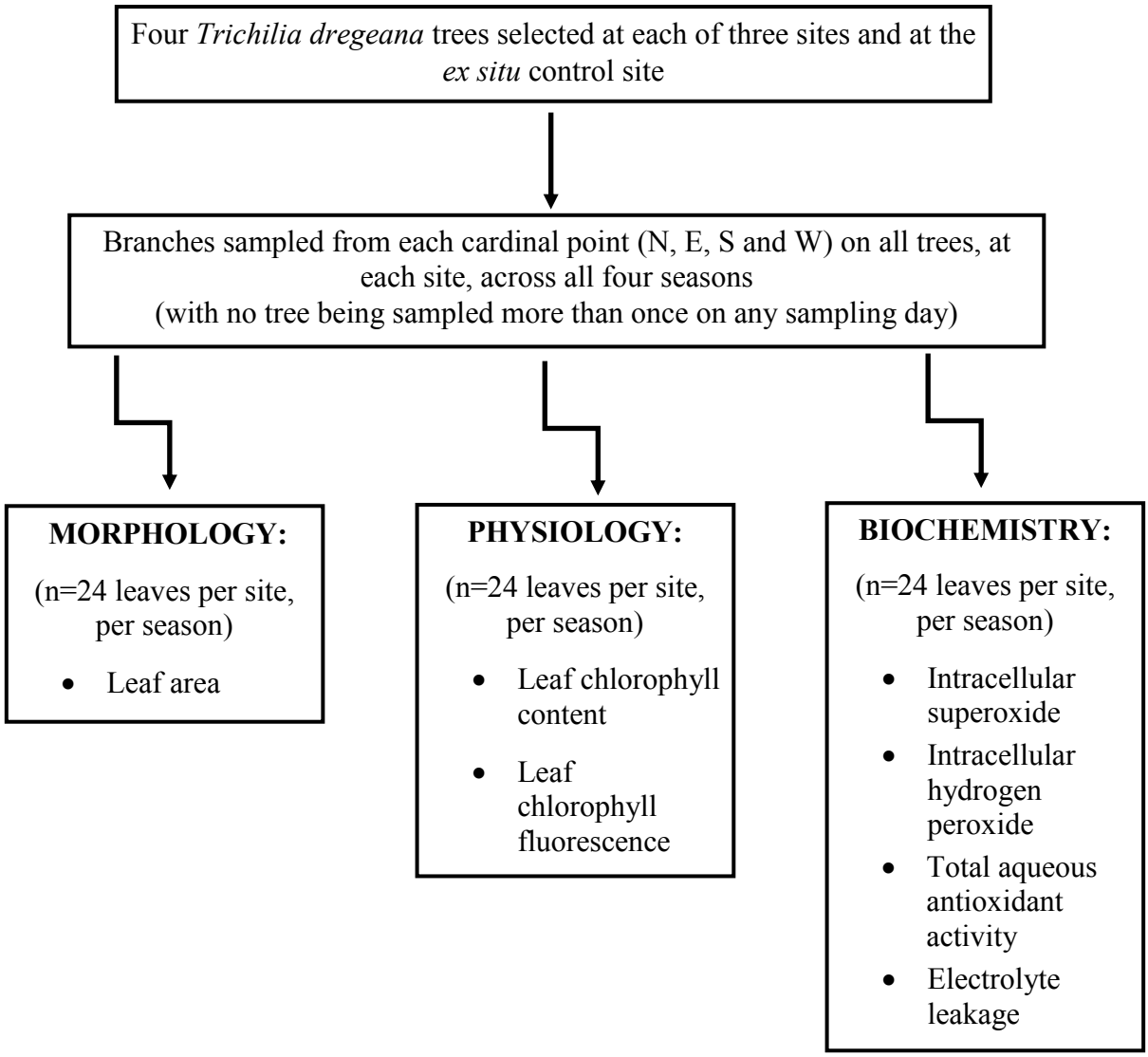

Figure 4. Leaf sampling strategy used in this study and biomarkers measured. Replication is given in each case and biomarkers are listed according to the aspect of plant performance they are related to.

2.3.3.1. *Ex situ* control and validation studies

When [SO_2] was measured at the *ex situ* control site, levels within the greenhouse in which the control trees were housed were below the detectable limits of the instrument. The greenhouse is constructed from clear 5 mm thick polycarbonate sheeting with a light transmittance of ±90%. There was, however, a slight difference in photosynthetically active radiation between the greenhouse (1938.65±100.28 µm of photons $m^{-2}s^{-1}$) and the treatment sites (2027.65±124.24 µm of photons $m^{-2}s^{-1}$) (n=20, $p<0.05$, ANOVA) when measured using a portable photosynthesis system (Li-6400, Li-COR, Lincoln, Nebraska, USA) at midday on four clear sunny days. So, in order to investigate whether this difference in light intensity had any confounding effects on the results obtained for light-dependent biomarkers measured in this study (e.g. chlorophyll content and leaf area, described below), these were also measured for four trees located within 1 km of the greenhouse in which the control trees were housed. These data were in turn related to the SO_2 measurements carried out on the university campus within 1 km of the greenhouse (as described above) in order to validate the trends observed for chlorophyll content and leaf area.

2.3.4 Morphological markers

2.3.4.1. Leaf area

Leaf samples (n=24 per site, per season) were detached at the petiole for leaf area (cm^2) measurements. Individual leaves were measured using a Leaf Area meter (Licor CI-202 Area Meter, Lincoln, Nebraska, USA) as described by Tiwari *et al*. (2006); three measurements were taken for each leaf and these were averaged for each leaf before further data analyses.

2.3.5 Physiological markers

2.3.5.1. Chlorophyll content

Leaves (n=24 per site, per season) were measured for chlorophyll content (in SPAD units), while still attached to the branch, using a handheld Konica Minolta SPAD-505 Plus (Konica Minolta Inc., Japan) as described by Tiwari *et al*. (2006). Readings were taken at three random points for each leaf and these were averaged for each leaf before further data analyses.

2.3.5.2. Leaf chlorophyll fluorescence

Leaves (n=24 per site, per season) were dark-adapted, while still attached to branches, for 30 min in a dark room with a green light before readings were taken (after Netto *et al*., 2005). Leaf chlorophyll fluorescence was measured using a Leaf Chamber Fluorometer (Li-Cor 6400-40, XT model, Li-Cor, Lincoln, Neb.), as described by Pitterman and Sage (2000). Individual leaves were measured for optimum quantum efficiency (F_v/F_m), which is the ratio of maximum fluorescence (F_m) to variable fluorescence (F_v) (Maxwell and Johnson, 2000).

2.3.6 Biochemical markers

All biochemical parameters described below were measured for 24 leaves, per site, per season (see Fig. 4) and all fine chemicals were supplied by Sigma-Aldrich (Germany) unless otherwise stated.

2.3.6.1. Intracellular superoxide

Intracellular superoxide ($\cdot O_2^-$) levels were measured according to Elstner and Heupel (1976). Leaves were ground using liquid nitrogen (LN) in a pre-chilled mortar and pestle, with 0.1 g of insoluble polyvinylpyrrolidone (PVP) and 4 ml of chilled 65 mM sodium phosphate buffer (pH 7.8). The homogenate was centrifuged at 4400 rpm for 30 min; thereafter, 1 ml of supernatant was removed and added to 1 ml of 10 mM hydroxylamine HCl (HCl dissolved in 65 mM sodium phosphate buffer, pH 7.8). After incubating the mixture for 30 min in the dark, 0.5 ml of the mixture was added to 0.5 ml of 17 mM sulphanilamide (dissolved in 65 mM sodium phosphate buffer, pH 7.8). A further 0.5 ml of 7 mM 2-naphthylamine (2-naphthylamine dissolved in 100% ethanol and made up to the required volume with 65 mM sodium phosphate buffer, pH 7.8) and 30 µl of 5 N HCl were added to the mixture, followed by 30 min incubation in the dark. Finally, the solution was centrifuged at 4ºC for 5 min at 13 000 rpm before absorbance was read at 530 nm using a UV-2600 UV-VIS Spectrophotometer (Shimadzu, Japan). The amount of intracellular $\cdot O_2^-$ produced was calculated using a standard curve, constructed using 0.1 µm to 50 µm sodium nitrite in 65 mM sodium phosphate buffer and expressed as n mol g^{-1} FW.

2.3.6.2. Intracellular hydrogen peroxide

Intracellular hydrogen peroxide (H_2O_2) levels were measured according to Hung *et al*. (2008). Leaves were ground using LN in a pre-chilled mortar and pestle, with 0.1 g of insoluble PVP and 4 ml of chilled 50 mM sodium phosphate buffer (pH 6.5) with 1 mM hydroxylamine (to inhibit enzyme catalase). The homogenate was centrifuged at 4400 rpm for 30 min, after which the supernatant was decanted and added to 1 ml of titanium chloride (0.1% titanium [III] chloride in 20% sulphuric acid). After 15 min incubation in the dark, the mixture was centrifuged at 4400 rpm for 30 min before absorbance was read at 410 nm using a UV-2600 UV-VIS Spectrophotometer (Shimadzu, Japan). The amount of intracellular H_2O_2 produced was calculated using the extinction coefficient 0.28 $\mu mol^{-1}g^{-1}$ and expressed as $\mu mol\ g^{-1}$ FW.

2.3.6.3. Electrolyte leakage

Electrical conductivity was measured according to Santamaria and Martin (1997). Fresh leaves were cut into 1 cm^2 discs, weighing approximately 0.1 g. Leaf discs were then placed into test tubes containing 20 ml of distilled water and then incubated in a water bath (Grant, Duxford Cambridge, England) held at 30ºC for 2 h. Thereafter, 1.5 ml of leachate was pipetted into cells of a conductivity tray and electrolyte leakage was measured using a multi-cell electrical conductivity meter (CM 100-2 Conductivity Meter, Reid and Associates, South Africa). Electrolyte leakage was expressed as $S\ m^{-1}\ g^{-1}$ FW.

2.3.6.4. Total aqueous antioxidants activity

Total aqueous antioxidant activity (TAA) was measured according to Re *et al*. (1999). Leaves were ground using LN in a pre-chilled mortar and pestle, with 0.1 g of insoluble PVP and 4 ml of chilled extraction buffer (50 mM sodium potassium phosphate buffer containing 1 mM $CaCl_2$, 1 mM KCl and 1 mM EDTA, pH 7.0). Prior to centrifugation for 30 min at 4400 rpm, the homogenate was transferred into 15 ml tubes and vortexed for 15 min at 5 min intervals for extraction of total antioxidants. The supernatant was split equally into Eppendorf® tubes and centrifuged for 30 min at 13000 rpm at 4ºC, after which the supernatant was decanted into fresh Eppendorf® tubes and placed on ice for the TAA assay. Before the estimation of TAA, the extract was diluted 1:1 to enable

estimation in the absorbance range recommended by Re *et al*. (1999). Total aqueous antioxidant activity was measured in terms of the depletion of 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical, in the presence of the antioxidant extract. Decolourisation of the working solution (800 µl of ABTS dissolved in 50 ml of potassium phosphate buffer) was measured at 0 s and 120 s after addition of 10 µl of the antioxidant extract. A standard curve was constructed using 0.1–1.5 M (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}, Sigma-Aldrich), dissolved in extraction buffer, and used to express TAA in terms of Trolox equivalents g^{-1} FW.

2.4 Statistical Analysis

The data were analysed using Predictive Analytics Software (PASW) version 21 (SPSS Inc., Chicago, IL). All data were tested for normality using the Shapiro-Wilks/ Kolmogorov-Smirnov test before deciding on how means would be compared. There were no significant differences across cardinal points for all sites and seasons ($p>0.05$, Analysis of Variance [ANOVA]) so data for the different cardinal points were pooled within each site for the subsequent analyses. Analyses of Variance were used to test for significant differences in chlorophyll content, leaf fluorescence, leaf area, intracellular $\cdot O_2^-$, intracellular H_2O_2, TAA and electrolyte leakage. Differences were tested for at the following levels: season main effects (comparisons within sites across seasons); site main effects (comparisons within seasons across sites); interaction between site and season (labelled 'site × season', henceforth); and across sites with data for different seasons pooled (labelled 'annual data', henceforth). Analyses of Variance was also used to test for differences in [SO_2] within seasons, across sites and across sites, with seasonal data pooled (referred to as 'annual data' henceforth). Data for NO_2 and PM were compared within spring and summer, between Southern Works and Ganges only as data for other seasons and sites were unavailable. Means were thereafter separated using a Bonferroni post-hoc test at the 0.05 level of significance. A Pearson's correlation test was used to determine whether there was a relationship between average seasonal SO_2 concentrations and seasonal averages for individual biomarkers.

2.5 Results

The results presented below represent a comparison of the effects of differing levels of ground-level SO_2 exposure on selected biomarkers of plant stress in *T. dregeana* leaves. Leaves were sampled at three industrial (treatment) sites within the SDB. In all cases, data for individual biomarkers are compared across treatment sites and the *ex situ* control.

2.5.1 Ground-level sulphur dioxide levels

Ground-level SO_2 concentrations differed significantly ($p<0.001$) when compared across treatment sites, within seasons. Sulphur dioxide levels for Southern Works were significantly higher than Ganges and Prospecton in autumn, spring and summer. Except for spring, levels at Ganges were significantly higher than Prospecton (Fig. 5A). In winter, SO_2 levels were significantly higher at Ganges, followed by Prospecton and Southern Works, while in spring, levels were comparable between Ganges and Prospecton. However, given the wide range in SO_2 concentrations recorded (1–25 ppb), and the lack of data for some days within specific seasons, seasonal data were pooled to generate an annual average for individual sites (Fig. 5B). Comparisons based on these averages revealed significant differences ($p<0.001$) across sites: SO_2 levels at Southern Works were significantly higher than Ganges and Prospecton, both of which exhibited statistically comparable SO_2 levels. When [SO_2] was measured at the *ex situ* control site, levels were below the detectable limits of the instrument within the greenhouse, while levels at three random points within 1 km of the greenhouse (located on a university campus), averaged 2.73±0.56 ppb. This meant that *T. dregeana* trees growing outside the greenhouse on the university campus were exposed to significantly lower ($p<0.05$, ANOVA) SO_2 levels than the treatment sites, but higher than the control.

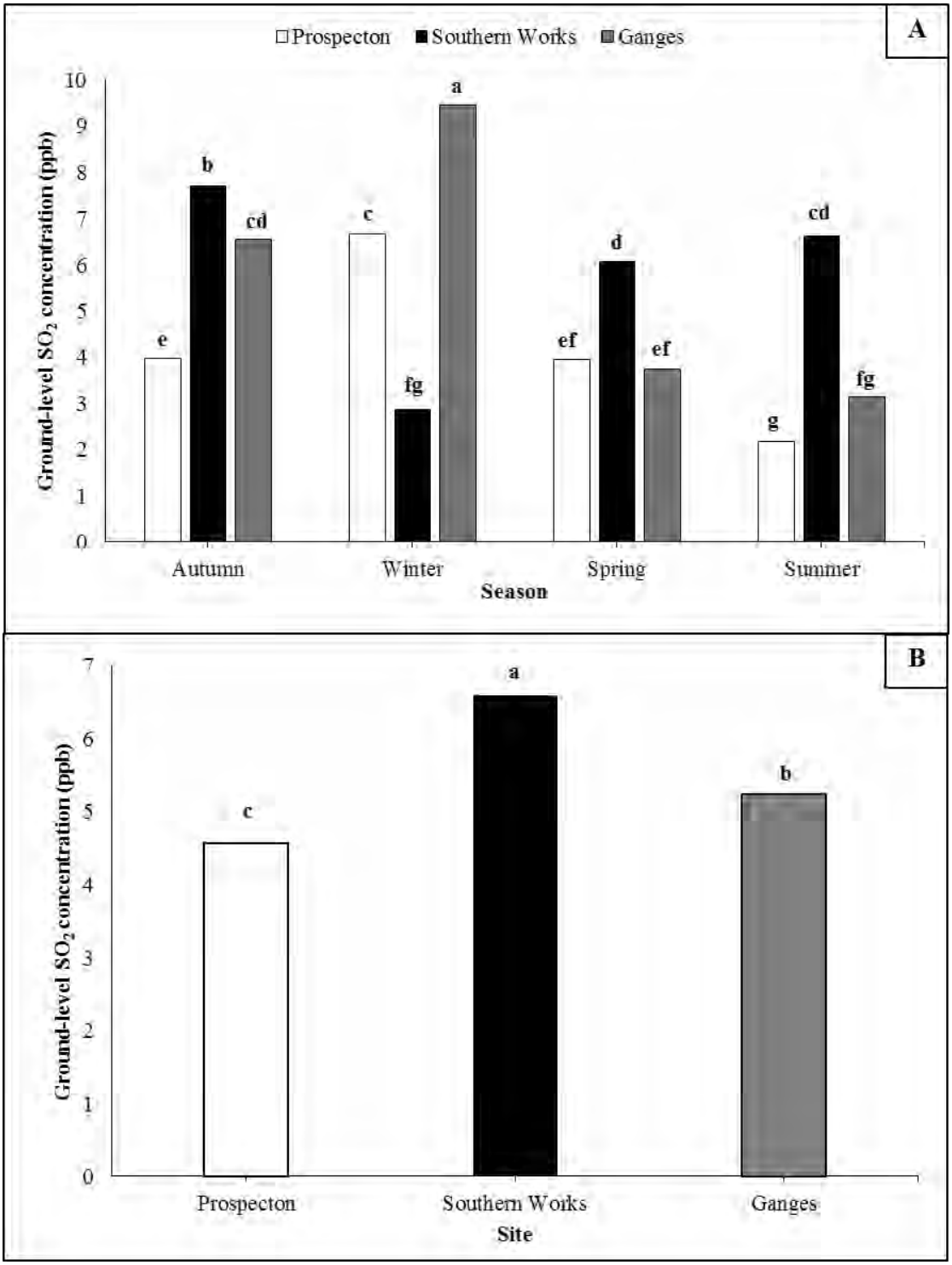

Figure 5. Ground-level SO_2 concentration (ppb) measured at three industrial (treatment) sites within the SDB between March 2014 and February 2015: (A) Mean seasonal [SO_2]; (B) Mean annual [SO_2]. Values represent means, ranging from n=725 to 1752 for seasonal data and n=3982 to 4962 for annual data. Bars labelled with different letters are significantly different ($p<0.001$, ANOVA). Standard deviations ranged from 2.24 to 6.65 for seasonal data and from 4.09 to 5.29 for annual data.

Additionally, from the limited NO_2 and PM data available at the time of this study it was evident that the levels of both these pollutants at Ganges were significantly higher than those at Southern Works in summer and spring (Table 1).

Table 1: Spring and summer ground-level nitrogen dioxide (NO_2) and particulate matter (PM) concentrations at two industrial (treatment) sites at which sampling was conducted within the SDB between September 2014 and February 2015.

		Southern Works	Ganges
spring	**NO_2 (ppb)**	10.46±6.31[b]	17.23±5.20[a]
	PM (ug/m^3)	11.07±5.90[a]	17.12±5.23[a]
summer	**NO_2 (ppb)**	8.48±5.22[b]	16.13±4.79[a]
	PM (ug/m^3)	9.30±4.79[a]	13.88±5.63[a]

Values represent mean±SD (n=4313 for PM and 8391 for NO_2 for spring, and n=4274 for PM and 6527 for NO_2 in summer); values followed by different letters are significantly different ($p<0.001$, ANOVA) when compared within pollutants, between sites.

2.5.2 Morphological, physiological and biochemical markers

As mentioned earlier, when data for the various biomarkers investigated were compared within sites, there were no significant differences ($p>0.05$, ANOVA) across cardinal points, so data for the different cardinal points were pooled within sites (including the control) for all subsequent statistical analyses.

2.5.2.1. Leaf area

Site and season had a significant ($p<0.001$) effect on leaf area, both independently and in combination (i.e. there was a significant interaction between site and season) (Fig. 6). Except for spring, in which leaf area was significantly lower at Ganges, this parameter did not differ significantly when compared within sites (treatments and control) across seasons. Values for the treatment sites were significantly lower than the control, when compared within seasons and in terms of pooled annual data. Across the treatment sites,

values at Ganges were significantly higher in winter compared with Prospecton, but significantly lower in spring. When annual data were compared across the treatment sites, leaf area was significantly higher at Southern Works and statistically comparable between Prospecton and Ganges.

Annual leaf area data reflected differences in [SO_2] between the control and the treatment sites, but did not appear to reflect differences in annual [SO_2] across the treatment sites (e.g. the highest annual leaf area was found at the site with the highest annual [SO_2]). Seasonal leaf area levels were, however, significantly negatively correlated with seasonal [SO_2] ($r=-0.716$, $p<0.05$).

Based on reasons discussed in Section 2.3.3.1, leaf area was also measured for trees growing within 1 km of the *ex situ* control. These trees, which were exposed to significantly lower SO_2 levels than those at the treatment sites (but higher than the control), exhibited significantly ($p<0.001$) higher leaf areas than trees at all the treatment sites (but lower than the control) in winter (25.24 ± 8.02 cm^2) and spring (24.13 ± 4.99 cm^2) (data not shown; only measured in two seasons).

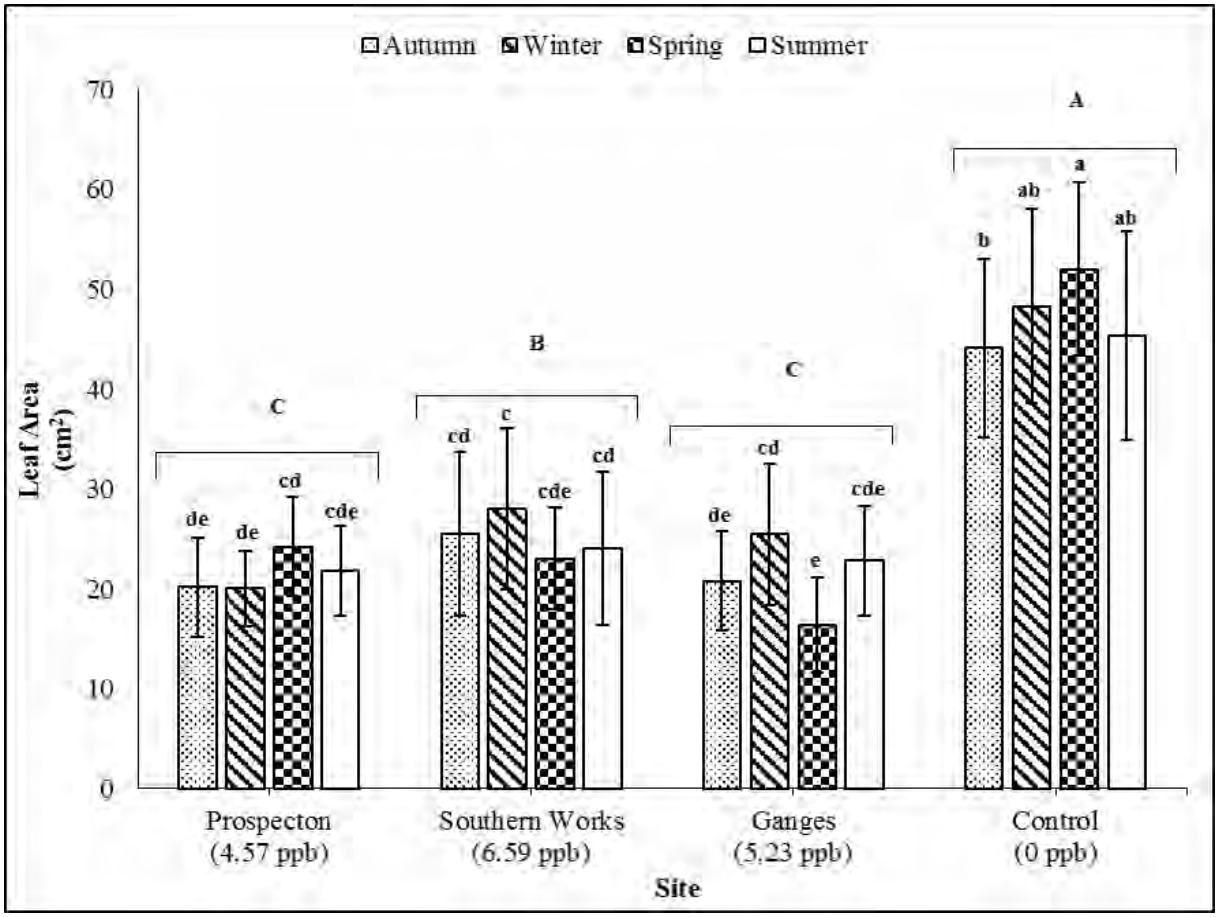

Figure 6. Leaf area for *T. dregeana* trees exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.2. Leaf chlorophyll content

Site and season had a significant ($p<0.001$) effect on leaf chlorophyll content, both independently and in combination (Fig. 7). Values for the treatment sites were significantly higher than the control, when compared within seasons and when compared in terms of annual data. Across the treatment sites, values at Ganges were significantly higher in autumn and winter, and when annual data were compared, values were significantly highest at Ganges and statistically comparable between Prospecton

and Southern Works. Annual leaf chlorophyll content could therefore reflect differences in [SO_2] between the control and the treatment sites, but this was not true for differences in annual [SO_2] across the treatment sites. Seasonal leaf chlorophyll content was significantly related to seasonal [SO_2] ($r=0.723$, $p<0.05$).

Additionally, based on reasons discussed in Section 2.3.3.1, leaf chlorophyll content was also measured for trees growing within 1 km of the *ex situ* control. Values measured for these trees were significantly ($p<0.001$) higher than Prospecton, Southern Works and the control in winter (67.36±6.14 SPAD units) and spring (66.02±3.41 SPAD units) (data not shown; only measured in two seasons).

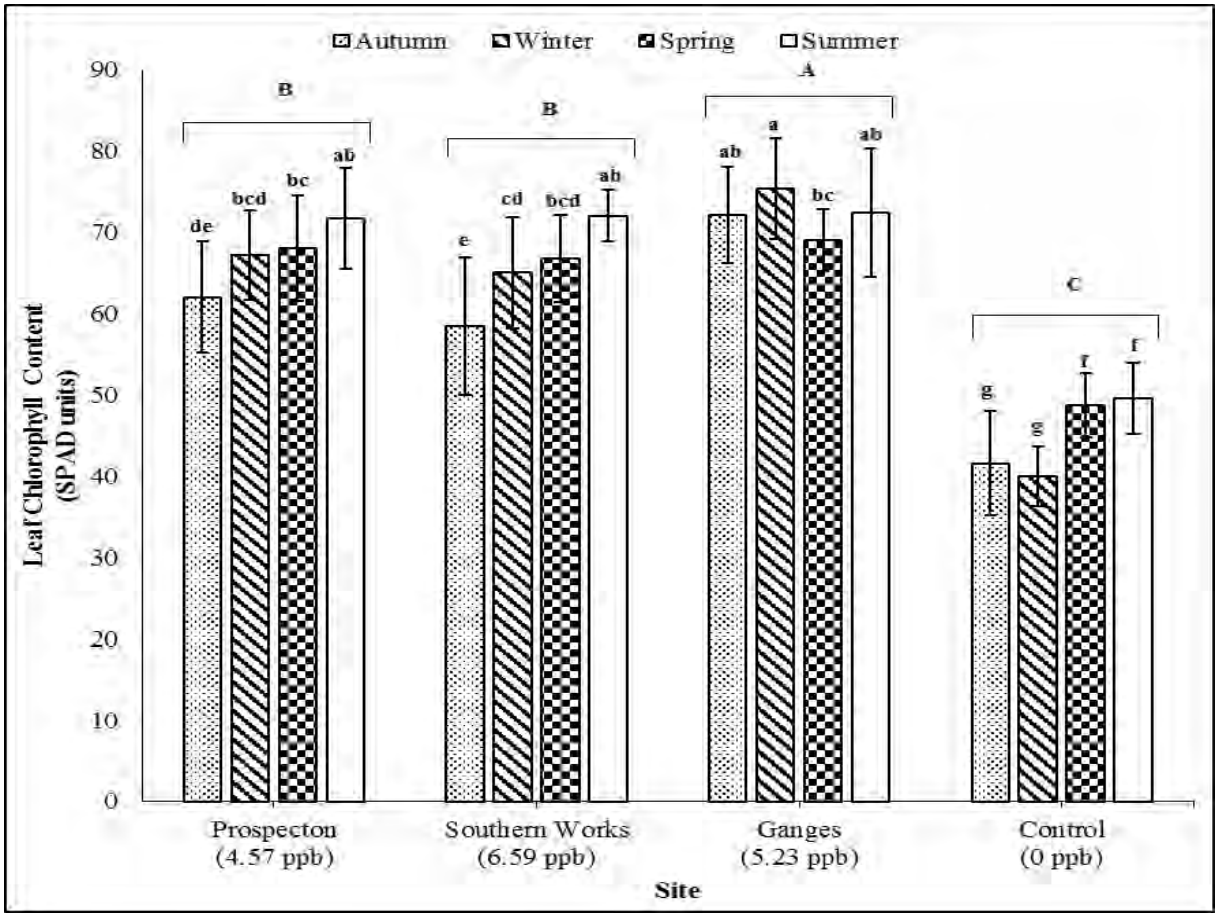

Figure 7. Leaf chlorophyll content for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.3. Chlorophyll fluorescence

Site (in terms of seasonal data) and season had a significant ($p<0.001$) effect on leaf chlorophyll fluorescence, both independently and in combination (Fig. 8). Except for summer, in which values for Prospecton were significantly lower than Ganges and the control, chlorophyll fluorescence did not differ significantly across sites when compared within seasons, and in terms of annual data. Annual chlorophyll fluorescence levels did not reflect differences in [SO_2] between the control and the treatment sites, nor across

the treatment sites. Seasonal chlorophyll fluorescence levels were also not significantly correlated with seasonal [SO_2] (r=0.334, p=0.206).

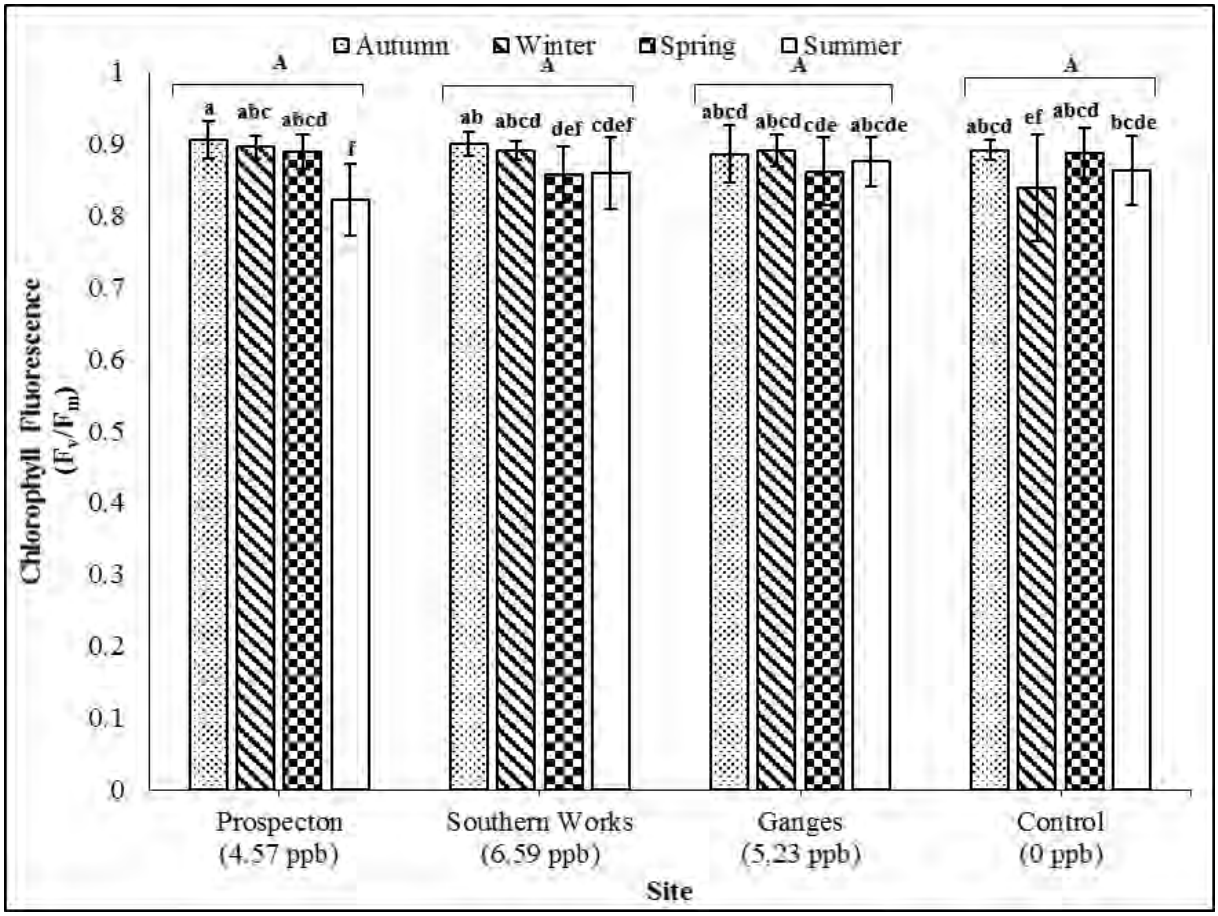

Figure 8. Chlorophyll fluorescence for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.4. Intracellular superoxide ($\cdot O_2^-$) production

Based on the fact that intracellular $\cdot O_2^-$ data collected for winter and spring were highly variable within sites, sampling was discontinued for the remaining two seasons (summer and autumn). Nevertheless, site and season had a significant ($p<0.001$) effect

on intracellular $\cdot O_2^-$ production, both independently and in combination (Fig. 9). Across sites, intracellular $\cdot O_2^-$ was significantly lower in the control during spring. Values for the treatment sites were also significantly higher than the control in terms of annual data. Across the treatment sites, there was a trend for values at Prospecton to be lower in winter, but these differences were not significant. There were also no significant differences across treatment sites in terms of annual data.

Annual $\cdot O_2^-$ levels could reflect differences in $[SO_2]$ between the control and the treatment sites, but this was not true for differences in annual $[SO_2]$ across the treatment sites. Seasonal $\cdot O_2^-$ levels were not significantly correlated with seasonal $[SO_2]$ ($r=0.283$, $p=0.435$).

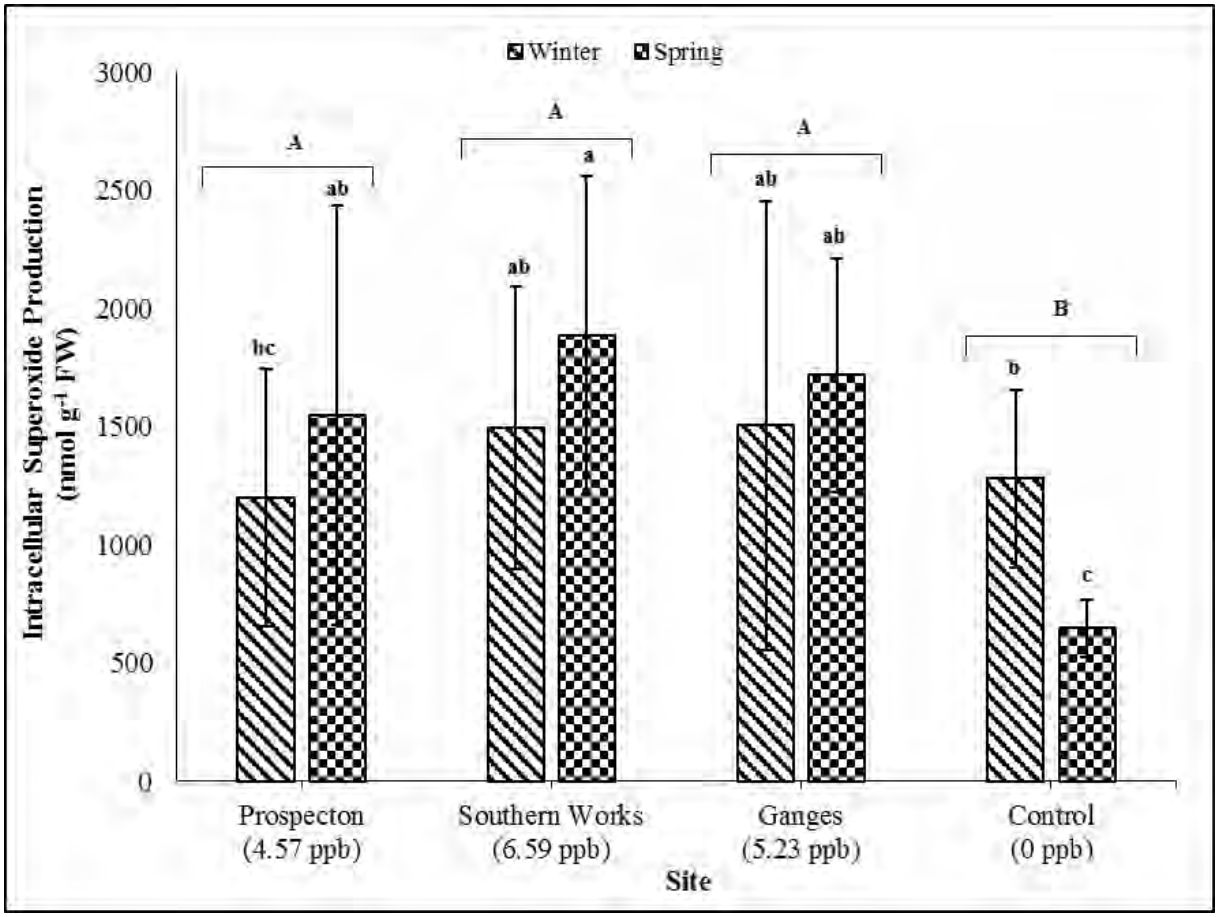

Figure 9. Intracellular superoxide production for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over two seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.5. Intracellular hydrogen peroxide (H_2O_2) production

Site and season had a significant ($p<0.001$) effect on intracellular H_2O_2 production, both independently and in combination (Fig. 10). Values for treatment sites were often higher than the control in autumn, winter and spring, and in some cases these differences were significant. For example, H_2O_2 levels at Prospecton were significantly higher than the control in winter. When annual H_2O_2 data were compared across sites, values for the control were significantly lower than the treatment sites, while across the

treatment sites values were significantly higher at Ganges and statistically comparable between Prospecton and Southern Works. Annual H_2O_2 levels reflected differences in [SO_2] between the control and the treatment sites, but this was not true for differences in annual [SO_2] across the treatment sites. Seasonal H_2O_2 production levels were significantly correlated with seasonal [SO_2] ($r=0.575$, $p<0.05$).

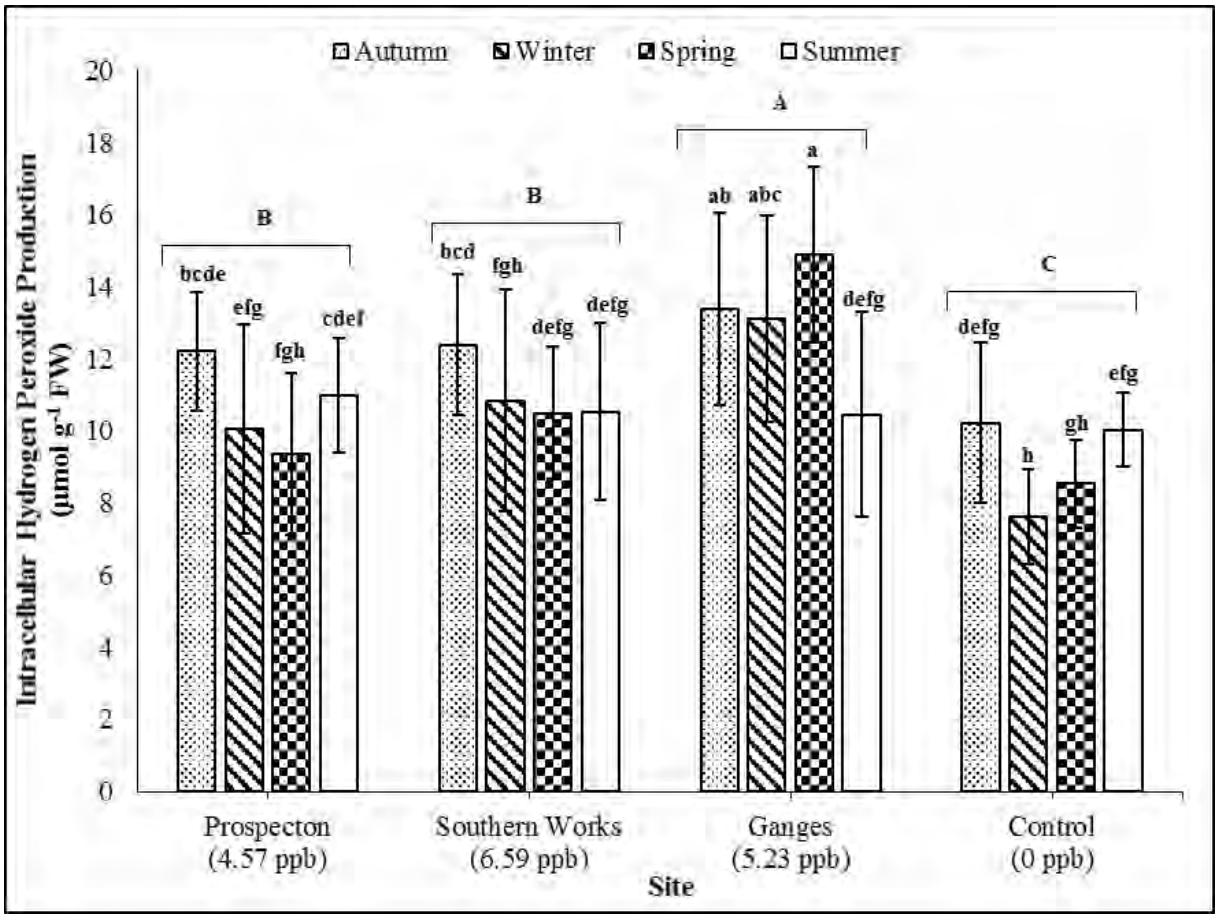

Figure 10. Intracellular hydrogen peroxide production for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.6. Electrolyte leakage

Site and season had a significant ($p<0.001$) effect on electrolyte leakage, both independently and in combination (Fig. 11). In autumn and summer, values at Southern Works and Ganges were significantly higher than the control; values at Ganges were also higher than Prospecton in both these seasons. When annual data were compared, values at Southern Works and Ganges were significantly higher than the control, but still statistically comparable across the treatment sites.

Annual electrolyte leakage levels reflected differences in annual [SO_2] between the control and the treatment sites, but this did not apply to differences in annual [SO_2] across the treatment sites. Seasonal electrolyte leakage levels were not significantly related to seasonal [SO_2] ($r=0.183$, $p=0.269$).

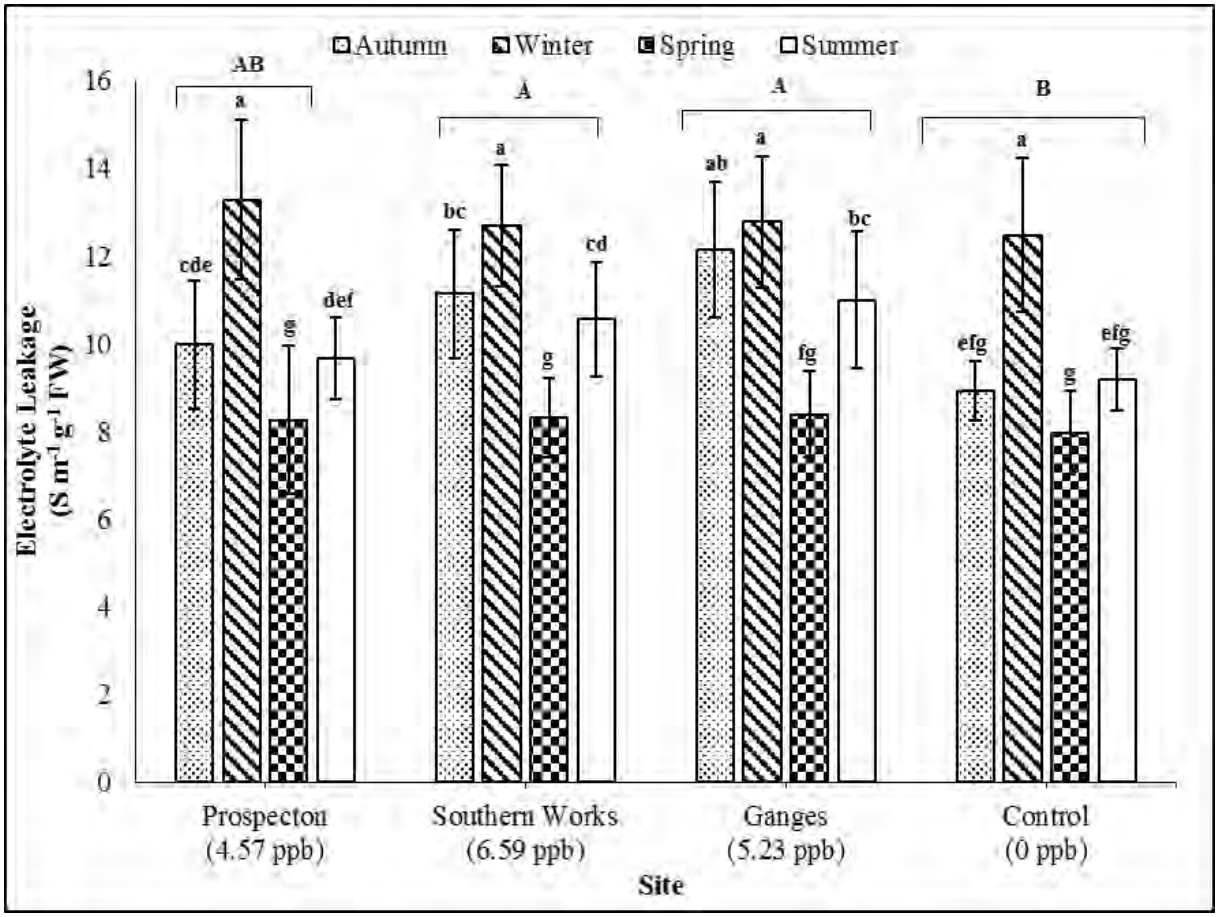

Figure 11. Electrolyte leakage for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.5.2.7. Total aqueous antioxidant (TAA) activity

Site and season had a significant ($p<0.001$) effect on TAA, both independently and in combination (Fig. 12). Within the control, values were significantly lower in spring and summer. Values at the treatment sites were significantly lower than the control in autumn and winter and significantly higher than the control in the cases of Prospecton and Southern Works in summer. When annual TAA data were compared, values were significantly highest in the control and lowest at Ganges; values were, however,

statistically comparable between Prospecton and Southern Works. Annual TAA levels reflected differences in [SO_2] between the control and the treatment sites, but this did not apply to differences in annual [SO_2] across the treatment sites. Seasonal TAA values were not significantly correlated with seasonal [SO_2] (r=-0.324, p=0.221).

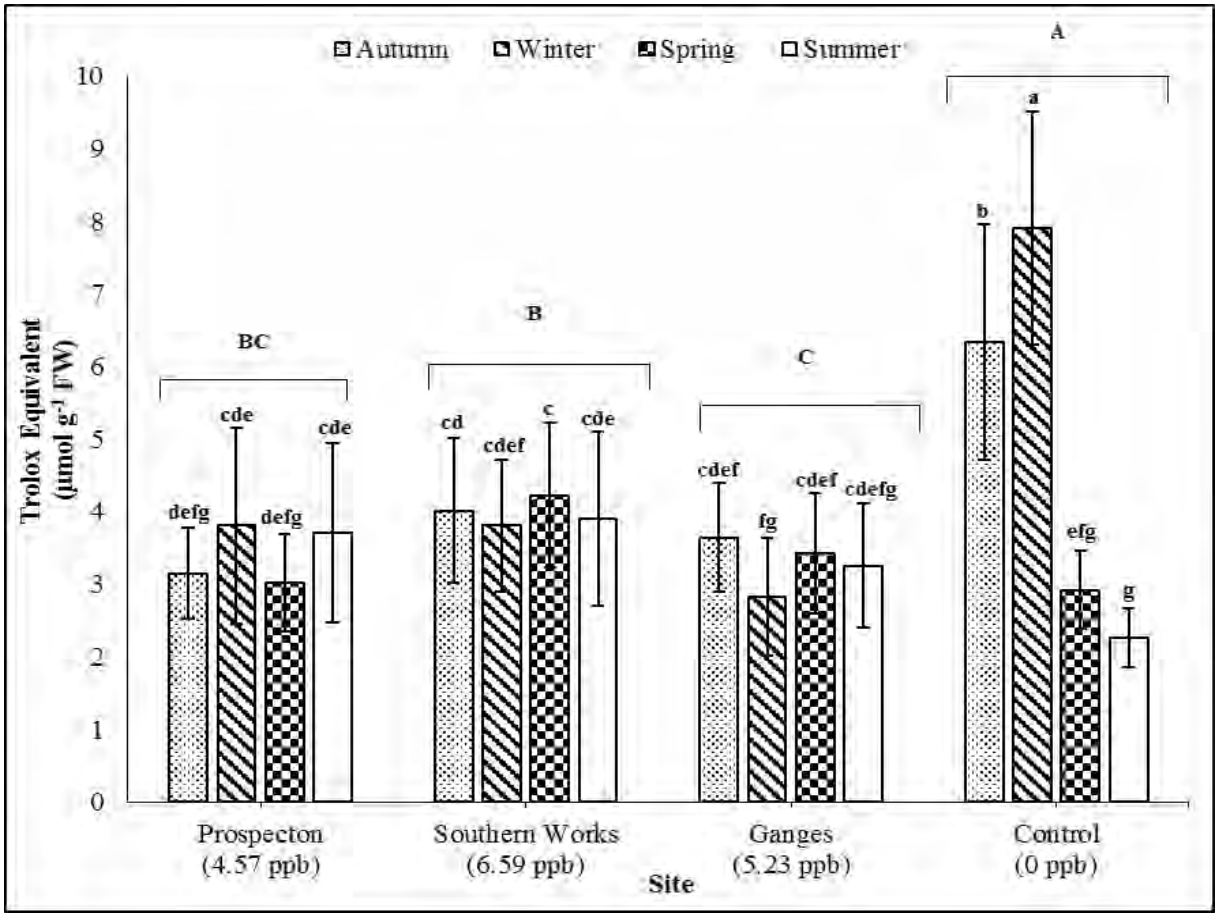

Figure 12. Total aqueous antioxidant capacity for *T. dregeana* leaves exposed to differing levels of SO_2 at the treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over four seasons. Values represent the mean±SD (n=24). Bars labelled with different lowercase letters are significantly different ($p<0.001$, ANOVA) when compared across various site × season combinations. Uppercase letters indicate significant differences ($p<0.001$, ANOVA) across sites for annual data.

2.6 Discussion

Biomonitoring of air quality using plant material has been widely employed to detect the effects of pollution on ecosystems (Sawidis *et al*., 2011). Trees in particular are efficient at taking in and thereby reducing harmful pollutants in the atmosphere (Sawidis *et al*., 2011). Individual pollutants have unique effects on plants, and even though the present study focussed on [SO_2] as a proxy for air pollution, it should be noted that pollutants such as NO_2, O_3, and SO_2 act together to compromise plant growth and performance (Tiwari *et al*., 2006).

SO_2 pollution levels

Sulphur and nitrogen oxides are released into the atmosphere by the burning of coal and can travel great distances in the atmosphere (Lovett *et al*., 2009). It is one of the major air pollutants focussed on in a number of studies as it is the pollutant for which most monitoring records exist, particularly for the SDB (Matooane and Diab, 2003). Ground-level SO_2 concentrations ranged from 2.18–9.45 ppb at the treatments sites across the four seasons, and except for winter, was highest at Southern Works, followed by Ganges and Prospecton (Fig. 5A). Within individual sites, the highest SO_2 levels were recorded in either autumn or winter which may be explained by the fact that wind speed is generally higher in spring and lower from autumn to winter (Guastella and Knudsen, 2007). Lower wind speeds lead to poor vertical mixing and low horizontal transportation of pollution out of the source area (Guastella and Knudsen, 2007). Other studies have also attributed seasonal variation in SO_2 levels (Carmichael, 2003) to seasonal differences in wind patterns (Diab *et al.,* 2002). Winter SO_2 results from the present study do not follow seasonal trends described by Guastella and Knudsen (2007), but as reported by Diab *et al.* (2002) [SO_2] varied across the SDB with annual averages being significantly highest at Southern Works, followed by Ganges and Prospecton (Fig. 5B). Annual [SO_2] across all three sites ranged from 4.57–6.59 ppb, which is relatively high by global standards and within the global SO_2 threshold range (3.8–11.4 ppb) in which irreversible damage can be inflicted on agricultural crops and natural vegetation (Carmichael *et al.* 2003; Josipovic *et al.* 2010). These data also suggest that very little has changed within the SDB with regards to SO_2 since Diab *et al.*'s 2002 study, which showed SO_2 levels to be >3.5 within the SDB. Diab *et al.* (2002) also

showed Southern Works, which is located at the centre of the SDB, to have relatively higher SO_2 levels than other monitoring stations and attributed this to strong NE and SW winds (Diab *et al.,* 2002). Stack down-drifting together with strong wind lead to high [SO_2] at ground-level, while differences among sites is also due to the location of monitoring stations in relation to air pollution sources within the SDB (Diab *et al.,* 2002).

NO_2 and PM pollution levels

As mentioned earlier, limited data on NO_2 and PM levels were available for two of the three treatment sites. Data for NO_2 ranged from 10.46–17.23 ppb in spring and 8.48–16.13 ppb in summer, with a significant difference between Ganges and Southern Works in spring and summer (Table 1). Data for PM ranged from 11.07–17.12 µg/m^3 in spring to 9.30–13.88 µg/m^3 in summer, but unlike NO_2, there were no significant differences between treatment sites for PM (Table 1). Levels of NO_2 were not as high as other countries around the world (Emberson *et al.,* 2001); however, values for spring and summer at Ganges were above the European threshold for vegetation protection (15.96 ppb) (Baldasano *et al.,* 2003; Josipovic *et al.,* 2010). *Trichilia dregeana* was therefore most likely affected by both SO_2 and NO_2 pollution at the treatment sites investigated here. According to Josipovic *et al.* (2010), high levels of NO_2 are found at sites close to industrial hubs with much seasonal variation; [NO_2] peaks in spring and is lower in summer. The range of PM values recorded in the present study is below the European threshold (40 µg/m^3) (Baldasano *et al.,* 2003), but it should be noted that even though PM is monitored worldwide, the World Health Organisation (WHO) does not have a guideline threshold value for this pollutant (WHO, 1999; Baldasano *et al.,* 2003).

Comparison of biomarkers

Changes in leaf morphology are the first visible and most easily identifiable stress biomarkers (Hijano *et al*., 2005), as the surface of a leaf is in direct contact with air pollution (Assadi *et al.,* 2011; Tanee and Albert, 2013). According to Pandey (2005), high levels of SO_2 can have adverse effects on plant growth such as reduced leaf area, but the effects of air pollution on leaf area are 'species-dependent' and are based on a plant's ability to protect itself or adapt to its surrounding environment (Wuytack *et al*.,

2011). In this study, *T. dregeana* leaf area was significantly reduced relative to the control with exposure to SO_2 irrespective of the season (Fig. 6), and was significantly negatively correlated with [SO_2] (in terms of seasonal data), but this biomarker was not sensitive enough to reflect differences in [SO_2] across the treatment sites. A number of authors have reported trees in unpolluted locations to have higher leaf areas (Dineva, 2004; Pandey, 2005; Tiwari *et al.*, 2006; Assadi *et al.*, 2011; Areington *et al.*, 2015) and in species like white willow (*Salix alba* L.) a decrease in leaf area is said to minimise the uptake of air pollution (Wen *et al*., 2004; Wuytack *et al.,* 2011).

In the present study, the decrease in leaf area across all the treatment sites relative to the control was accompanied by a significant increase in leaf chlorophyll content (Fig. 7). The exposure of plants to high levels of SO_2 can cause chlorosis and necrosis, reducing a plant's overall growth (Kropff, 1987). Increases and decreases in leaf chlorophyll content have been reported for trees exposed to high levels of air pollution; while an increase suggests the tree's ability to tolerate air pollution, a decrease indicates the opposite (Assadi *et al.,* 2011; Tanee and Albert, 2013). Based on the reasoning developed in more recent reports (Assadi *et al.*, 2011; Areington *et al.,* 2015), the physiological link between leaf area and chlorophyll content may have resulted in *T. dregeana* trees decreasing leaf area to reduce the surface area exposed to the pollution, while increasing chlorophyll content to compensate for the reduction in the leaf area available for harvesting photosynthetically active radiation (PAR).

Leaf chlorophyll content was significantly positively correlated with [SO_2] (in terms of seasonal data), but like leaf area, was not sensitive enough as a biomarker to reflect differences in [SO_2] across the treatment sites. What is noteworthy though, is that trees growing within 1 km of the *ex situ* control, exposed to significantly lower levels of SO_2 than those at the treatments sites, exhibited significantly higher leaf areas than the treatment sites, but lower than the control. Furthermore, chlorophyll contents in trees growing within 1 km of the *ex situ* control were higher than the control and comparable to the Ganges site. This suggests that the slightly lower PAR within the greenhouse was unlikely to have had a confounding effect on the trends in leaf area and chlorophyll content reported here. Furthermore, the results for leaf area and chlorophyll content support previous suggestions by Areington *et al.* (2015) and Assadi *et al.* (2011) that

chlorophyll content should be measured in conjunction with leaf area to accommodate for the interactive responses of these two biomarkers to air pollution.

However, the question still exists as to why trees at Southern Works, which were exposed to the highest SO_2 levels, exhibited higher leaf areas than Ganges and Prospecton (Fig. 6). Reference to the NO_2 and PM levels at Southern Works and Ganges may offer an explanation: Ganges exhibited significantly higher levels of NO_2 and PM than Southern Works (Table 1). The accumulation of PM on leaves can block the opening of stomata, reducing the gas exchange and consequentially leaf growth (Cui *et al*., 2006), while increased NO_2 levels, as alluded to earlier, can affect plant growth negatively (Tiwari *et al.,* 2006). Assadi *et al.* (2011) stress that the effects of atmospheric pollutants on plants is cumulative. This suggests that while SO_2 is a good proxy for pollution within the SDB, levels of other pollutants (such as NO_2 and PM) should also be considered when using tree leaves as bioindicators of pollution, since the effects of SO_2 have been reported to be more severe when combined with other atmospheric pollutants (Varshney *et al.,* 1979).

Injury to plants is a common method used to determine the effects of air pollution; however, physiological and biochemical responses of plants to air pollution may occur before visible injury is seen (Bytnerowicz, 1996). Chlorophyll fluorescence (Maxwell and Johnson, 2000), for example, can give insight into the ability of a plant to tolerate an environmental stress or the extent of damage caused by the stress on photosynthetic system (Maxwell and Johnson, 2000). Whilst there were isolated indications that chlorophyll fluorescence (assessed in terms of photochemical efficiency [F_v/F_m]) in the trees exposed to SO_2 were lower than the control (e.g. at Prospecton in summer [Fig. 8]), annual values did not differ significantly across sites. Furthermore, differences within treatment sites, across seasons, were more likely due to weather-induced changes in photosynthetic performance (Naidoo and Chirkoot, 2004) than seasonal differences in [SO_2].

Air pollution can also have significant effects on the biochemistry of plants, often leading to increased production of ROS and associated oxidative stress in leaves (Dat *et al.,* 2000). Air pollutants such as SO_2, for example, enter a leaf through stomata causing damage to one of the main sources of ROS in leaves, viz. chloroplasts (Dat *et al*., 2000).

High levels of air pollution excite chloroplasts to abnormally high energy levels, thereby increasing the production of ROS and inducing oxidative stress (Seyyednejad *et al*., 2013). Superoxide ($\cdot O_2^-$) is one of the ROS that is most often produced in surplus when a plant is exposed to air pollution (Tiwari *et al.,* 2006; Bermudez and Pignata, 2011; Areington *et al.,* 2015) and is the precursor to other ROS (Gill and Tuteja, 2010). Levels of $\cdot O_2^-$ production in SO_2-exposed leaves were significantly higher than the control in spring (Fig. 9). However, high levels of variation within sites, the inability of $\cdot O_2^-$ production to reflect differences in [SO_2] across the treatment sites, and the lack of a significant correlation between $\cdot O_2^-$ production and [SO_2] support findings in other species (e.g. *Brachylaena discolor* [Areington *et al.*, 2015]) that the parameter is not a suitable biomarker for air pollution-related stress. This is not surprising, given superoxide's sensitivity to change and short-lived nature in plant tissues (Gill and Tuteja, 2010).

Chloroplasts are also a major producer of hydrogen peroxide (Quan *et al*., 2008), which plays a key role in plants during environmental stress (Dat *et al*., 2000). This is because H_2O_2 is a key regulator of physiological processes such as senescence, photosynthesis, stomatal movement as well as growth and development (Noctor and Foyer, 1998; Foreman *et al*., 2003; Peng *et al*., 2005 and Bright *et al*., 2006), but is also more harmful than $\cdot O_2^-$ when produced in excess since it has a relatively longer lifespan and a high permeability across membranes (Hung *et al*., 2002). Studies have shown that exposure to high levels of air pollution can lead to harmful levels of H_2O_2 in plants (Valavanidis *et al.,* 2006; Areington *et al.,* 2015). Except for summer, H_2O_2 levels in *T. dregeana* leaves exposed to SO_2 were often higher than the control (Fig. 10), and H_2O_2 production was significantly correlated with [SO_2]. However, H_2O_2 production was not sensitive enough as a biomarker to reflect differences in [SO_2] across the treatment sites. This may be partly due to the widely reported dual role (i.e. harmful versus beneficial) of H_2O_2 in plant tissues (Dat *et al*., 2000). Apart from inflicting damage in plant tissues, H_2O_2 can also be part of the plant's overall adaptation to changes in the environment (Neill *et al.,* 2002), as it participates in activating defence responses to stress (Quan *et al.,* 2008).

Electrolyte leakage is a popular measure of damage to cell membranes in plants (Ismail *et al.,* 1997; Garty *et al.,* 2000; Verlikova *et al.,* 2000; Bajji *et al.,* 2001; Al-Jebory,

2013; Areington *et al.,* 2015). Damage to plant tissues caused by excess levels of ROS can be measured in terms of an increase in electrolyte leakage (Santamaria and Martin, 1997; Blokhina *et al.,* 2003). Given the fact that SO_2 exposure led to heightened levels of ROS in *T. dregeana* leaves (Figs 9 and 10), it was not surprising that annual electrolyte leakage levels at the treatment sites with the two highest levels of SO_2 (Southern Works and Ganges) were significantly higher than the control (Fig. 11). However, when one looks at the seasonal values this was only true for autumn and summer, which may explain why electrolyte leakage levels were not significantly correlated with [SO_2]. A positive correlation between ROS and electrolyte leakage in tree leaves at polluted sites has been reported in a number of studies (Santamaría and Martín, 1997; Valavanidis *et al*., 2006; Bermudez and Pignata, 2011; Areington *et al.,* 2015). However, while electrolyte leakage in the present study could be used to distinguish between the control and the two sites with the highest SO_2 levels, it was not sensitive enough as a biomarker to reflect differences in [SO_2] across the treatment sites. This may be due to the fact that electrolyte leakage is influenced by plant and leaf age as well as the sampling position of the leaf (Bandurska and Gniazdowska-Skoczek, 1995; Bajji *et al.,* 2001). While these factors were considered when sampling in the present study, slight differences in tree age and sampling position may have masked differences in electrolyte leakage across the treatment sites and/or led to the differences observed across seasons.

Plants have developed antioxidant (enzymic and non-enzymic) defence mechanisms to combat oxidative stress (Jaleel *et al.,* 2009). However, when plants are exposed to high levels of environmental stress, the balance between the ROS produced and the quenching activity of antioxidants is disturbed, leading to oxidative damage (Spychalla and Desborough, 1990; Ahmad *et al*., 2008). According to Tiwari *et al.* (2006), antioxidant levels in plant tissues increase with an increase in pollution stress. However, in the present study this was only true in summer, and the difference between the control and treatment sites (Prospecton and Southern Works) were significant (Fig. 12). Interestingly, leaves from the *ex situ* control had significantly higher antioxidant levels than the treatment sites in autumn and winter. This may be an indication that control leaves (in autumn and winter, at least) were healthier than those at the treatment sites; Novak *et al.* (2003) suggest that high antioxidant levels are indicative of healthy

vigorous plants. In this regard, control leaves exhibited exceptionally high TAA levels in autumn and winter when the highest SO_2 values were recorded across sites (Fig. 5A). However, TAA was not significantly correlated with [SO_2] and the seasonal variability in TAA responses to SO_2 exposure suggests that this parameter may not be a suitable biomarker of air pollution in *T. dregeana*.

2.7 Conclusions

The use of plants as bioindicators of environmental stress represents an easy and inexpensive way of monitoring environmental changes, including air pollution (Tripathi and Gautam, 2007). The present study set out to compare the effects of industrial SO_2 pollution on selected morphological, physiological and biochemical biomarkers of stress in *T. dregeana* leaves. These data were in turn used to assess whether or not *T.* dregeana tree leaves could be used as a bioindicator of air pollution in the SDB. The results suggest that except for leaf chlorophyll fluorescence, all biomarkers assessed (viz. leaf area, chlorophyll content, intracellular $\cdot O_2^-$ and H_2O_2 production, electrolyte leakage and TAA) can to some extent discriminate between SO_2 polluted and unpolluted (*ex situ* control) sites. However, though seasonal data for a number of these biomarkers (viz. leaf area, leaf chlorophyll content and H_2O_2 production) were significantly correlated with seasonal [SO_2], all the biomarkers were not sensitive enough to reflect differences in [SO_2] across the industrial sites investigated. Additionally, the study reinforced previous reports of the physiological link between leaf area and chlorophyll content (Assadi *et al.,* 2011; Areington *et al.,* 2015), as well as hydrogen peroxide and electrolyte leakage (Areington *et al.,* 2015), which suggests that these should be measured in conjunction with each other. In light of the above, all biomarkers, except for chlorophyll fluorescence and intracellular superoxide production (the data for which were highly variable even within sites), can be used to establish *T. dregeana* leaves as a bioindicator of air pollution.

References

Ahmad, P., Sarwat, M. and Sharma, S. 2008. Reactive oxygen species, antioxidants and signalling in plants. *Journal of Plant Biology* 51(3), pp. 167 - 173

Aksoy, A., Sahin, U. and Duman, F. 2000. *Robinia pseudo-acacia* L. as a possible bio-monitor of heavy metal pollution in Kayseri, Turkey. *Journal of Botany*, 24, pp. 279 - 284

Al-Jebory, E.I. 2013. Changes in cellular membrane tolerance due to heat stress during *Triticum sativum* L. seeds germination. *Al-Kufa Journal for Biology*, 5(2), pp. 1 - 9

Areington, C.A., Varghese, B., Ramdhani, S. and Naidoo, S.E. 2015. An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in the leaves of *Brachylaena discolor*. *Water, Air and Soil Pollution*, 226(9), pp. 1 - 14

Arora, A., Sairam, R.K. and Srivastava, G.C. 2002. Oxidative stress and antioxidative system in plants. *Current Science*, 82(10), pp. 1227 - 1238

Assadi, A., Pirbalouti, A. G., Malekpoor, F., Teimori, N. and Assadi, L. 2011. Impact of air pollution on physiological and morphological characteristics of *Eucalyptus camaldulensis* Den. *Journal of Food, Agriculture and Environment*, 9(2), pp. 676 - 679

Bajji, M., Kinet, J. M. and Lutts, S. 2002. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regulation*, 36(1), pp. 61 - 70

Bakker, M.I., Vorenhout, M., Sijm, D.T.H.M. and Kolloffel, C. 1999. Dry deposition atmospheric polycyclic aromatic hydrocarbons in three *Plantago* species. *Environmental Toxicology and Chemistry*, 18, pp. 2289 - 2294

Baldasano, J.M., Valera, E. and Jimenez, P. 2003. Air quality data from large cities. *Science of the Total Environment*, 307(1), pp. 141 - 165

Bandurska, H. and Gniazdowska-Skoczek, H. 1995. Cell membrane stability in two barley genotypes under water stress conditions. *Acta Societatis Botanicorum Poloniae*, 64(1), pp. 29 - 32

Batterman, S.A., Chernyak, S.M., Gounden, Y., Matooane, M. and Naidoo, R.N. 2008. Organochlorine pesticides in ambient air in Durban, South Africa. *Science of the Total Environment*, 397, pp. 119 - 130

Bermudez, G. M. and Pignata, M. L. 2011. Antioxidant response of three *Tillandsia* species transplanted to urban, agricultural, and industrial areas. *Archives of Environmental Contamination and Toxicology*, 61(3), pp. 401 - 413

Bickerstaff, K. and Walker, G. 2000. Public understandings of air pollution: the 'localisation' of environmental risk. *Global Environmental Change*, 11, pp. 133 - 145

Blokhina, O., Virolainen, E. and Fagerstedt, K. V. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of botany*, 91(2), pp. 179 - 194

Branquinho, C., Catarino, F., Brown, D.H., Pereira, M.J. and Soares, M. 1999. Improving the use of lichens as biomonitors of atmospheric metal pollution. *The Science of the Total Environment*, 232, pp. 67 - 77

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S. and Neill, S.J. 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *The Plant Journal*, 45(1), pp. 113 - 122

Bruce, N., Perez- Padilla, R. and Albalak, R. 2000. Indoor air pollution in developing countries: a major environmental and public health challenge. *World Health Organisation*, 78(9), pp. 1078 - 1092

Bytnerowicz, A. and Fenn, M.E. 1996. Nitrogen deposition in California forests: a review. *Environmental Pollution*, 92(2), pp. 127 - 146

Carmichael, G.R., Ferm, M., Thongboonchoo, N., Woo, J.H., Chan, L.Y., Murano, K., Viet, P.H., Mossberg, C., Bala, R., Boonjawat, J. and Upatum, P. 2003. Measurements of sulfur dioxide, ozone and ammonia concentrations in Asia, Africa, and South America using passive samplers. *Atmospheric Environment*, 37(9), pp. 1293 - 1308

Conti, M.E. and Cechetti, G. 2001. Biological mointoring: lichens as bioindicators of air pollution assessment - a review. *Environmental Pollution*, 114, pp. 471 - 492

Cui, H.X., Jiang, G.M., Niu, S.L., Jiang, C.D., Liu, M.Z., Yu, S.L. and Gao, L.M. 2006. Ecophysiological Response of Plants to Combined Pollution from Heavy-duty Vehicles and Industrial Emissions in Higher Humidity. *Journal of Integrative Plant Biology*, 48(12), pp. 1391 - 1400

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D. and Van Breusegem, F. 2000. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences* CMLS, 57(5), pp. 779 - 795

Diab, R., Prause, A. and Bencherif, H., 2002. Analysis of SO_2 pollution in the South Durban Industrial Basin. *South African Journal of Science*, 98, pp. 543 - 546

Dineva, S. B. 2004. Comparative studies of the leaf morphology and structure of white ash *Fraxinus americana* L. and London plane tree *Platanus acerifolia* Willd growing in polluted area. *Dendrobiology*, 52, pp. 3 - 8

Elstner, E.F. and Heupel, A. 1976. Inhition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismatase. *Analytical Biochemistry*, 70, pp. 616 - 620

Emberson, L.D., Ashmore, M.R., Murray, F., Kuylenstierna, J.C.I., Percy, K.E., Izuta, T., Zheng, Y., Shimizu, H., Sheu, B.H., Liu, C.P. and Agrawal, M. 2001. Impacts of air pollutants on vegetation in developing countries. *Water, Air, and Soil Pollution*, 130 (1 - 4), pp. 107 - 118

eThekwini Health and Norwegian Institute for Air Research. 2007. Air quality management plan for eThekwini Municipality, South Africa.

Fan, H.B. and Wang, Y.H. 2000. Effects of simulated rain on germination, foliar damage, chlorophyll contents and seedling growth of five hardwood species growing in China. *Forest Ecology and Management*, 126, pp. 321 - 329

Fenger, J. 1999. Urban air quality. *Atmospheric Environment*, 33, pp. 4877 - 4900

Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A. and Dolan, L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature*, 422(6930), pp. 442 - 446

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A. and Orlovsky, L. 2000. Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiologia Plantarum*, 109(4), pp. 410 - 418

Gill, S.S. and Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, 48, pp. 909 - 930

Guastella, L. and Knudsen, S. 2007. South Durban basin multi-point plan case study report. Air quality act implementation: Air quality management planning. Book 12, series C, pp. 1 - 34

Hijano, C.F., Domínguez, M.D.P., Gimínez, R.G., Sínchez, P.H. and García, I.S. 2005. Higher plants as bioindicators of sulphur dioxide emissions in urban environments. *Environmental Monitoring and Assessment*, 111(1), pp. 75 - 88

Hung, K.T., Cheng, D.G., Hsu, Y.T. and Kao, C.H. 2008. Abscisic acid-induced hydrogen peroxide is required for antho cyanin accumulation in leaves of rice seedlings. *Journal of Plant Physiology*, 165, pp. 1280 - 1287

Ismail, A.M., Hall, A.E. and Close, T.J. 1997. Chilling tolerance during emergence of cowpea associated with a dehydrin and slow electrolyte leakage. *Crop Science*, 37(4), pp. 1270 - 1277

Jain, A.K. and Hayhoe, K.A.S. 2008. Handbook of atmospheric sciences 2: Problems, Tools and Applications, pp. 2 - 61

Jaleel, C.A., Riadh, K., Gopi, R., Manivannan, P., Inès, J., Al-Juburi, H.J., Chang-Xing, Z., Hong-Bo, S. and Panneerselvam, R. 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. *Acta Physiologiae Plantarum*, 31(3), pp. 427 - 436

Josipovic, M., Annegarn, H.J., Kneen, M.A., Pienaar, J.J. and Piketh, S.J. 2010. Concentrations, distributions and critical level exceedance assessment of SO_2, NO_2 and O_3 in South Africa. *Environmental Monitoring and Assessment*, 171(1-4), pp. 181 - 196

Kancheva, R. and Borisova, D. 2007. Vegetation stress indicators derived from multispectral and multitemporal data. *Space Technology*, 26, pp. 1 - 8

Karl, T.R. and Trenberth, K.E. 2003. Modern global climate change. *Science*, 302, pp. 1719 - 1723

Klumpp, A., Hintemann, T., Lima, J.S. and Kandeler, E. 2003. Bioindication of air pollution effects near a copper smelter in Brazil using mango trees and soil microbiological properties. *Environmental Pollution*, 126, pp. 313 - 321

Kropff, M.J. 1987. Physiological effects of sulphur dioxide. 1. The effect of SO_2 on photosynthesis and stomatal regulation of *Vicia faba* L. *Plant, Cell and Environment*, 10(9), pp. 753 - 760

Lamego, M.M., de Souza Jr, P.A., Ferriera, E.P. and Garg, V.K. 2000. Quantification of air pollution sources. *Revista Controle and Automacao*, 11(2), pp. 77 - 81

Lovett, G.M., Tear, T.H., Evers, D.C., Findlay, S.E., Cosby, B.J., Dunscomb, J.K. and Weathers, K.C. 2009. Effects of air pollution on ecosystems and biological diversity in the eastern United States. *Annals of the New York Academy of Sciences*, 1162(1), pp. 99 - 135

Madejon, P., Maranon, T., Murillo, J.M. and Robinson, B. 2004. White poplar (*Populus alba*) as a biomonitors of trace elements in contaminated riparian forests. *Environmental Pollution*, 132, pp. 145 - 155

Markert, B. 1995. Sample preparation (cleaning, drying, homogenization) for trace element analysis in plant matrices. *Total Environments*, 176, pp. 45 - 61

Marquez, L.O. and Smith, N.C. 1999. A framework for linking urban form and air quality. *Environmental Modelling and Software*, 14, pp. 541 - 548

Matooane, M. and Diab, R. 2003. Health risk for sulphur dioxide pollution in South Durban, South Africa. *Archives of Environmental Health: An International Journal*, 58(12), pp. 763 - 770

Matooane, M., Oosthuizen, J.R. and Binedell, M. 2004. Vulnerability of South African communities to air pollution. *8th World Congress on Environmental Health*, pp. 1 - 8

Maxwell, K. and Johnson, G.N. 2002. Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, 51(345), pp. 659 - 668

Mičieta, K. and Murin, G. 1998. Three species of genus Pinus suitable as a bioindicator of polluted environment. *Water, Air and Soil Pollution*, 104, pp. 413 - 422

Moraes, R.M., Klumpp, A., Furlan, C.M., Klumpp, G., Domingos, M., Rinaldi, M.C.S. and Modesto, I.F. 2002. Tropical fruit trees as bioindicators of industrial air pollution in southeast Brazil. *Environment International*, 28(5), pp. 367 - 374

Naidoo, G. and Chirkoot, D. 2004. The effects of coal dust on photosynthetic performance of the mangrove, *Avicennia marina* in Richards Bay, South Africa. *Environmental Pollution*, 127(3), pp. 359 - 366

Neill, S., Desikan, R. and Hancock, J. 2002. Hydrogen peroxide signalling. *Current Opinion in Plant Biology*, 5(5), pp. 388 - 395

Netto, A.T., Campostrini, E., de Oliveira, J.G. and Bressan-Smith, R.E. 2005. Photosynthetic pigments, nitrogen, chlorophyll a fluorescence and SPAD-502 readings in coffee leaves. *Scientia Horticulturae*, 104, pp. 199 - 209

Noctor, G. and Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology*, 49(1), pp. 249 - 279

Novak, K., Skelly, J.M., Schaub, M., Kräuchi, N., Hug, C., Landolt, W. and Bleuler, P. 2003. Ozone air pollution and foliar injury development on native plants of Switzerland. *Environmental Pollution*, 125(1), pp. 41 - 52

OECD. 1997. OECD Environmental Data Compendium 1997. Organisation for Economic Co-operation and Development, Paris

Pandey, J. 2005. Evaluation of air pollution phytotoxicity downwind of a phosphate fertilizer factory in India. *Environmental Monitoring and Assessment*, 100(1-3), pp. 249 - 266

Peng, L.T., Jiang, Y.M., Yang, S.Z. and Pan, S.Y. 2005. Accelerated senescence of fresh-cut Chinese water chestnut tissues in relation to hydrogen peroxide accumulation. *Journal of Plant Physiology and Molecular Biology*, 31(5), pp. 527 - 532

Pitterman, J. and Sage, R.F. 2000. The response of high altitude C4 grass *Muhlenbergia Montana* (Nutt.) A.S. Hitchc. to long- and short- term chilling. *Journal of Experimental Botany*, 52, pp. 829 - 838

Pooley, E. 1993. The complete field guide to trees of Natal, Zululand and Transkei, 3rd Edition. Durban: Natal flora publications trust, pp. 204 - 205

Quan, L. J., Zhang, B., Shi, W. W. and Li, H. Y. 2008. Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *Journal of Integrative Plant Biology*, 50(1), pp. 2 - 18

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Andrice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26, pp. 1231 - 1237

Santamaria, J.M. and Martin, A. 1997. Tree bark as a bioindicator of air pollution in Navarra, Spain. *Water, Air and Soil Pollution*, 98, pp. 381 - 387

Sawidis, T., Breuste, T., Mitrovic, M., Pavlovic, P. and Tsigaridas, K. 2011. Trees as bioindicators of heavy metal pollution in three European countries. *Environmental Pollution*, 159, pp. 3560 - 3570

Sawidis, T., Heinrich, G. and Chettri, M.K. 1997. Cesium-137 monitoring using lichens from Macedonia, northern Greece. *Canadian Journal of Botany*, 75(12), pp. 2216 - 2223

Seyyednejad, S.M., Koochak, H. and Vaezi, J. 2013. Changes in anti-oxidative enzymes activity, protein content and ascorbic acid level in *Prosopis juliflora* exposed to industrial air pollution. *Journal of Biology*, 2(10), pp. 482 - 492

Spychalla, J.P. and Desborough, S.L. 1990. Superoxide dismutase, catalase, and α-tocopherol content of stored potato tubers. *Plant Physiology*, 94(3), pp. 1214 - 1218

Tanee, F. B. G. and Albert, E. 2013. Air pollution tolerance indices of plants growing around Umuebulu Gas Flare Station in Rivers State, Nigeria. *African Journal of Environmental Science and Technology*, 7(1), pp. 1 - 8

Thambiran, T. and Diab, R. D. 2011. Air quality and climate change co-benefits for the industrial sector in Durban, South Africa. *Energy Policy*, 39, pp. 6658 - 6666

Tiwari, S., Agrawal, M. and Marshall, F.M. 2006. Evaluation of ambient air pollution impact on carrot plants at a sub urban site using open top chambers. *Environmental Monitoring and Assessment*, 119, pp. 15 - 30

Tripathi, A.K. and Gautam, M. 2007. Biochemical parameters of plants as indicators of air pollution. *Journal of Environmental Biology*, 28, pp. 127 - 132

Tubiello, F.N., Donatelli, M., Rosenzweig, C. and Stockle, C.O., 2000. Effects of climate change and elevated CO_2 on cropping systems: model predictions at two Italian locations. *European Journal of Agronomy*, *13*(2), pp.179 - 189

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, 64(2), pp. 178 - 189

Varshney, C.K., Garg, J.K., Lauenroth, W.K. and Heitschmidt, R.K. 1979. Plant responses to sulfur dioxide pollution. *Critical Reviews in Environmental Science and Technology*, 9(1), pp. 27 - 49

Velikova, V., Yordanov, I. and Edreva, A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*, 151(1), pp. 59 - 66

Vranova, E., Inze, D. and Van Breusegem, F. 2002. Signal transduction during oxidative stress. *Journal of Experimental Biology*, 53(372), pp. 1227 - 1236

Wen, D., Kuang, Y., Zhou, G. 2004. Sensitivity analyses of woody species exposed to air pollution based on ecophysiological measurements. *Environmental Science and Pollution Research,* 11(3), 165 - 170

World Health Organisation (WHO). 1999. Principles for the Assessment of Risks to Human Health from Exposure to Chemicals. *Environmental Health Criteria 210*, Geneva, Switzerland: WHO

Wuytack, T., Wuyts, K., Van Dongen, S., Baeten, L., Kardel, F., Verheyen, K. and Samson, R. 2011. The effect of air pollution and other environmental stressors on leaf fluctuating asymmetry and specific leaf area of *Salix alba* L. *Environmental Pollution*, 159, pp. 2405 - 2411

CHAPTER THREE

Examining the utility of hyperspectral remote sensing and partial least squares to predict plant stress responses to SO_2 pollution: A case study of *Trichilia dregeana* Sond.

3.1 Abstract

The use of air quality monitoring stations is expensive, with much of the data on various types of pollutants being either unavailable or inaccessible. For this reason, the effects of ground-level sulphur dioxide (SO_2) levels on various biomarkers related to environmental stress were investigated for *Trichilia dregeana* tree leaves, in order to assess their bioindicator potential. Leaves were sampled randomly from trees at three industrial (treatment) sites within the South Durban Basin and an *ex situ* control, across two seasons (n=28, per season). Ground-level SO_2 concentrations were measured daily and ranged between 1 to 25 ppb. Leaf chlorophyll content, leaf area and hyperspectral reflectance were examined for SO_2-exposed and -unexposed leaves, to determine the effect of SO_2 concentrations on leaves of *T. dregeana*. There were significant ($p<0.001$) differences across sites and seasons for leaf area and leaf chlorophyll content, with leaf area at the treatment sites being lower than the control, and chlorophyll content being higher than the control. Partial least squares regression (PLSR) was then used to quantify the relationship between the air pollution biomarkers and the hyperspectral data. For leaf chlorophyll content across seasons, r^2 values ranged from 0.32 and 0.475, with the root mean square error of prediction (RMSEP) ranging between from 8.75 to 8.98 SPAD units. Leaf area across seasons had r^2 values ranging between 0.429 and 0.586, with RMSEP ranging from 9.20 to 12.52 cm^3, with. Based on these results, the variable importance in projection (VIP) method was utilised to identify significant hyperspectral wavebands. Important wavebands were identified at 552 nm and 704 nm for the spring dataset, and at 552 nm and 708 nm for the summer dataset. Partial least squares regression was therefore able to relate the hyperspectral dataset to both a physiological and morphological stress biomarker. However, the interaction between leaf chlorophyll content and leaf area suggests that a simultaneous prediction of these

biomarkers, using an algorithm such as partial least squares 2 (PLS-2), may be more suitable. Importantly, the VIP method identified important wavebands within the red-edge region of the electromagnetic spectrum, which showed promise in identifying stress in the leaves of *T. dregeana*.

Keywords: Air pollution, biomarker, hyperspectral, partial least squares regression, reflectance, variable importance in projection

3.2 Introduction

Industries are the main contributors of air pollution and consume the most energy (Thambiran and Diab, 2011) within urban and rural settings. The use of fossil fuels, such as coal and oil, have therefore increased to meet these energy demands worldwide (Jain and Hayhoe, 2008; Jha *et al*., 2011). Consequentially, industrial zones such as the South Durban Basin (SDB), in South Africa, are widely recognised as having extremely poor air quality owing to emissions from industrial (e.g. petrochemical) activities (Diab *et al*., 2002). These industrial activities are associated with some of the highest air pollution emission levels within Africa (Batterman *et al*., 2008). Atmospheric sulphur dioxide concentrations (hereafter referred to as [SO_2]), for instance, are exceptionally high in this area (Diab and Motha, 2007) and are used as a proxy for air pollution, since SO_2 represents one of the main pollutants emitted by industrial combustion processes (Diab and Motha, 2007). The SDB, in particular, is at risk of potentially harmful air pollution levels due to the lack of effective emission controls and its coastal topography, which promotes the recirculation of air (Batterman *et al*., 2008). Industrial air pollution emissions threaten environmental (Winner, 1994) and human health (Matooane and Diab, 2003), necessitating air quality monitoring.

Understanding the physiological and ecological responses of organisms (bioindicators) to stresses, such as air pollution within these industrial areas, can be useful for the development of suitable monitoring and mitigation strategies (Chapin, 1991; Taylor *et al*., 1994; Rai *et al*., 2011). Biological monitoring has been used to assess urban air quality in many parts of the world (Falla *et al.,* 2000; Balasooriya *et al.,* 2009) and the advantages are widely reported (Martin and Coughtrey, 1982; Wittig, 1993). The most frequently used bioindicator of air pollution are lichens (Hale, 1969, 1983; Balasooriya *et al.,* 2009; Sawidis *et al.,* 2011; Maatoug *et al.,* 2012) due to their ability to

accumulate trace elements and alter their physiology, owing to poor air quality (Bennett and Wetmore, 1999; Ra *et al.,* 2005). However, their use in industrial and populated areas is often limited by slow regeneration, low availability, irregular distribution and taxonomic uncertainty (Berlizov *et al.,* 2007). Along with lichens, plants can also be used as bioindicators of air pollution (Maatoug *et al.,* 2012). Plants are in fact seen as ideal indicators of localised conditions as they are immobile, and physiologically and/or morphologically sensitive to air pollution, when compared with humans and animals (Wuytack *et al*., 2011). An alternative to lower plants as bioindicators of air quality are higher plants, with trees being the preferred choice in a number of studies (Berlizov *et al.,* 2007; Sawidis *et al*., 2011) as they (i) have a wide distribution (Sawidis *et al.,* 1995; Celik *et al.,* 2005), (ii) are the major plant life-form in urban areas (Sawidis *et al.,* 1995; Celik *et al.,* 2005), (iii) are effective at indicating low levels of pollution (Berlizov *et al.,* 2007), and (iv) are easy to identify (Berlizov *et al.,* 2007). Trees such as *Quercus ilex* (Alfani *et al.,* 2000) and *Pinus* spp (Jensen *et al.,* 1992), amongst others, have also shown substantial potential for storing trace elements associated with air pollution (Balasooriya *et al.,* 2009). According to Berlizov *et al.* (2007), trees display a greater tolerance to changes in the environment and consequently, monitoring the morphological, physiological and biochemical responses of trees to air pollution has proven to be useful for air quality monitoring purposes (Bernhardt-Romermann *et al.,* 2006; Tripathi and Gautam, 2007; Areington *et al.,* 2015).

Trees are able to respond to air pollution-related changes in their environment by altering their leaf physiology and morphology (Areington *et al.,* 2015). Monitoring pollution-affected vegetation is of major importance (Lin and Mendelssohn, 2012; Mishra *et al.,* 2012) with most studies focusing on the effects of air pollution on either water, soil, air, or plants directly (Manzo *et al.,* 2013; Nascimbene *et al.,* 2014). Ground-based monitoring methods may, however, be time-consuming and expensive, especially over vast areas; therefore, remote sensing represents an affordable alternative (Wu *et al.,* 2005; Yi *et al.,* 2007; Fauzi *et al.,* 2013). Airborne and space-borne remote sensing can monitor areas quickly without causing damage to vegetation, and can be combined with field-based methods (Hunter *et al.,* 2009; Ayanu et al., 2012; Zhu *et al.,* 2014). Remote sensing has been used to detect environmental stress-induced changes in trees (Smith *et al.,* 2004), and various applications have also shown that hyperspectral

data can be related to physiological (e.g. leaf chlorophyll content [Agrawal *et al.,* 2003]) and morphological (e.g. leaf area [Barber *et al.,* 2004; Balasooriya *et al.,* 2009]) indicators of plant stress or health (Lillesand and Kiefer, 2001; Hansen and Schjoerring, 2003; Borengasser *et al.,* 2008; Nansen *et al.,* 2009; Abdel-Rahman *et al.,* 2013).

Vegetation stress can be characterised using remote sensing by recording the spectral signals of plants in polluted areas (Kooistra *et al.,* 2004; Zhu *et al.,* 2013; Zhu *et al.,* 2014). Manzo *et al.* (2013) used spectral reflectance analyses to assess environmental pollution at geothermal sites, and results indicated that the spectral reflectance of vegetation is sensitive to the distance from pollution sources. The closer samples were to power plants (with high hydrogen sulphide air concentrations), the lower the spectral reflectance; reflectance effects were also seen to be more significant within the near-infrared region (NIR) compared with the visible region of the electromagnetic spectrum (Manzo *et al.,* 2013). Similarly, Splajt *et al.* (2003) used reflectance spectroscopy to monitor landfill leachate dispersion, and Newete *et al.* (2014) used hyperspectral reflectance to detect stress of water hyacinth growing under herbivory and heavy metal pollution. According to Ullah *et al.* (2012), at leaf level, factors such as water content, morphological, and anatomical properties can explain changes in spectral signals and may control the variation in the overall leaf reflectance. Additionally, changes in spectral reflectance may differ due to the type of pollutant, the extent of pollution, the plants overall tolerance, and the period after exposure (Zhu *et al.,* 2014). Various factors thus affect the reflectance of vegetation, implying that many mechanisms may link environmental pollution, plant responses, and spectral variation (Zhu *et al.,* 2014). This study focuses on two biomarkers, viz. leaf chlorophyll content and leaf area, and predicts plant stress in *Trichilia dregeana* leaves due to industrial SO_2 pollution using hyperspectral data and the partial least squares regression (PLSR) algorithm.

3.3 Materials and methods

A description of *Trichilia dregeana* Sond.

The choice of *T. dregeana* is based on the fact that this naturally occurring evergreen forest tree is found in many parts of South Africa (mainly in the Eastern Cape and KwaZulu-Natal), Swaziland, and Zimbabwe (Pooley, 1993). Within Durban (South

Africa), the study area, this tree species has been planted along streets for the past 20 to 25 years.

3.3.1 Study area

The South Durban Basin (SDB) is an industrial hub situated in a shallow basin in Kwazulu-Natal, South Africa. The SDB comprises of a coastal strip of approximately 96 km^2 (Chapter 2, Fig. 2). The geography of the SDB, the polluted (treatment) sites and *ex situ* control site used in this study, and the location of the trees sampled have already been described in Chapter 2, Section 2.3.1. Nevertheless, it is worth reinforcing that the refineries within the SDB contribute to over 80% of the SO_2 emissions in the area (eThekwini Health and Norwegian Institute for Air Research, 2007).

The three industrial (referred to as treatment sites hereafter) sites at which the trees were sampled are shown in Figure 13. For the purposes of this study, the sites are labelled as follows: 'Prospecton' (located in the Isipingo suburb at 21 m above sea level); 'Southern Works' (located in the Wentworth suburb at 10 m above sea level) and 'Ganges' (located in the Merebank suburb at 17 m above sea level). These sites were selected based on the presence of an air pollution monitoring station (owned and operated by the eThekwini Municipality, KwaZulu-Natal, South Africa) that measured ground-level [SO_2] on an hourly basis. At each site, four *T. dregeana* trees were selected, with each tree occurring within 800 m of an air pollution monitoring station (as shown in Chapter 2, Fig. 2). For the control, trees were placed *ex situ* in a greenhouse (on the Westville campus of the University of KwaZulu-Natal, Durban, South Africa) for a month prior to (and for the duration of) the study. The utility of this approach of interpreting *in situ* tree responses to air pollution relative to an *ex situ* greenhouse-based control has been established previously by Areington *et al.* (2015).

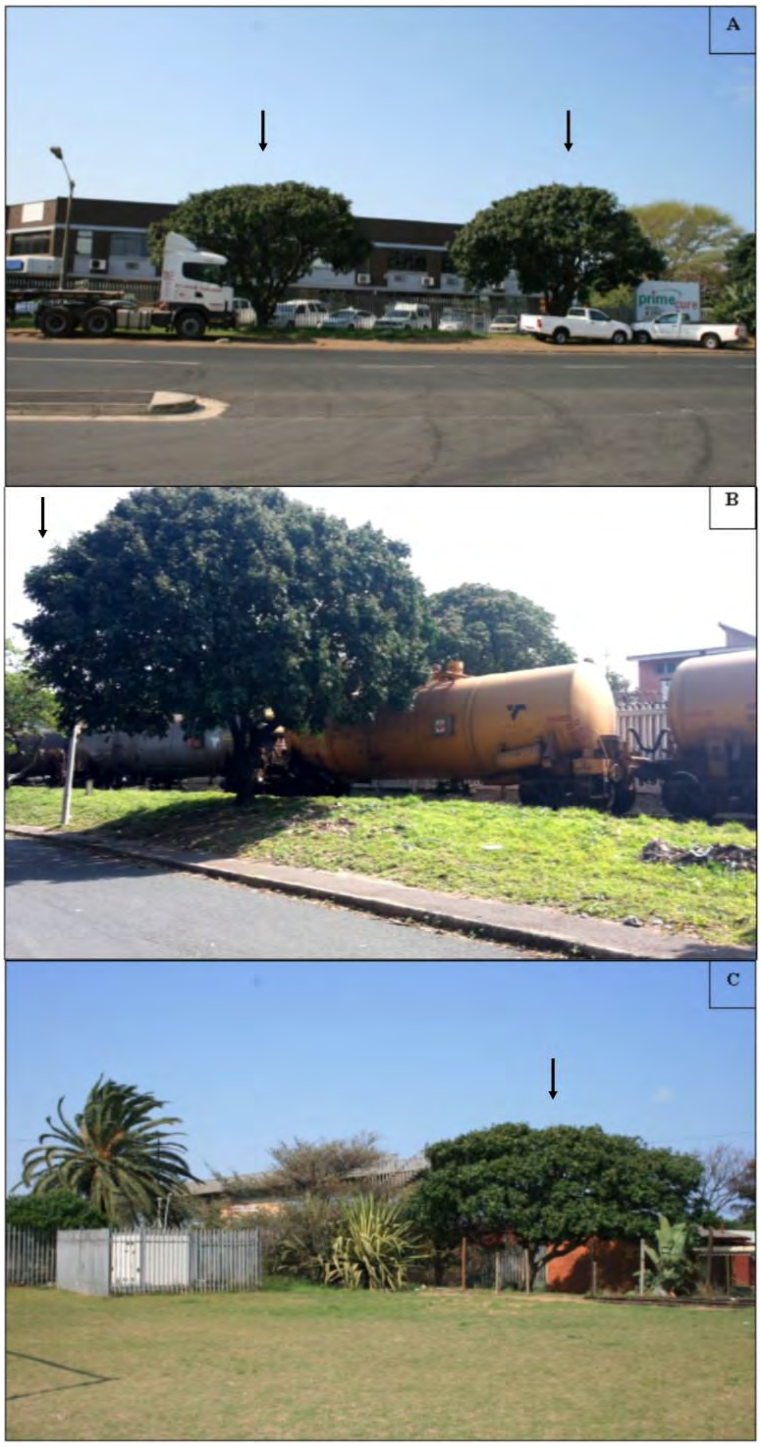

Figure 13. The three study sites (A: Prospecton, B: Southern Works, C: Ganges) located within the South Durban Basin. Black arrows indicate some of the trees samples at each site.

3.3.2 Measurement of ground-level [SO_2]

The air pollution monitoring stations (shown in Chapter 2, Fig. 3), located at each of the three study sites (Prospecton, Southern Works and Ganges), are equipped with US Environmental Protection Agency (USEPA) designated analysers (Fig. 3), and provided the SO_2 data used in this study. The analysers provided hourly ground-level [SO_2] (in ppb) for the duration of the study, and SO_2 sampling was conducted during spring (1 September, 2014 to 30 November, 2014) and summer (1 December, 2014 to 28 February, 2015). Prior to any analyses, the data were cleaned (i.e. erroneous, zero, negative and blank values were deleted), and seasonal and average [SO_2] were generated for each site.

Sulphur dioxide levels were also measured within the greenhouse (located on the Westville campus of the University of KwaZulu-Natal, Durban, South Africa), in which the control trees were housed for one month before, and for the duration of the study (after Areington *et al*., 2015). Additionally, [SO_2] levels were measured for eight days at three random points within 1 km of the greenhouse, in which the control trees were housed. Measurements were carried out as described in Chapter 2, Section 2.3.2.

3.3.3 *Ex situ* study

The greenhouse is constructed using clear 5 mm thick polycarbonate sheeting with a light transmittance of ±90%. Light intensity was measured at twenty random points, at treatment sites and *ex situ*, using a portable photosynthesis system (Li-6400, Li-COR, Lincoln, Nebraska, USA), at midday on four clear sunny days. Additionally, in order to investigate whether or not differences in light intensity had confounding effects on the results obtained, for the light-dependent biomarkers measured in this study, chlorophyll content and leaf area (described below) were also measured for four trees located within 1 km of the greenhouse in which the control trees were housed. These data were in turn related to the SO_2 measurements undertaken within 1 km of the greenhouse (as described above), in order to validate the trends observed for chlorophyll content and leaf area.

3.3.4 Leaf sampling

Four trees located at the treatment and the control sites were sampled (always at least 24 hours after the last rain event), as per the recommendations of Sawidis *et al*. (2011). The sampling regime was designed as follows:

i. Trees were sampled randomly across all sites during spring and summer.

ii. A maximum of two trees were sampled at each site (treatment and control) per sampling day.

iii. No tree was sampled more than once a week and within any particular season, the trees were sampled until the required number of samples (n=28, per season, per parameter) were collected.

iv. Short lengths of branches from each cardinal point (Klumpp *et al*., 2003) of the sampled tree were collected.

v. Once collected, the branch segments with leaves attached were rinsed with distilled water to remove bird droppings and particulate matter and immediately conveyed to the laboratory with the cut end immersed in distilled water.

vi. The leaves were dabbed dry with paper towel and were detached shortly after physiological and morphological readings were done.

vii. Hyperspectral measurements were then acquired and finally, leaves were weighed (i.e. fresh weight) individually.

3.3.5 Morphological markers

3.3.5.1. Leaf area

Leaf samples (n=28 per site, per season) were detached at the petiole for leaf area (cm^2) measurements, which were conducted on individual leaves using a Leaf Area meter (Licor CI-202 Area Meter, USA.), as described by Tiwari *et al*. (2006). Three measurements were taken for each leaf and these were averaged for each leaf before data analyses. To assess whether or not differences in light intensity between the greenhouse and the sites could have influenced the results of the leaf area studies, leaf

area was also measured for trees growing within 1 km of the greenhouse, as mentioned in section 3.3.3.

3.3.6 Physiological markers

3.3.6.1. Chlorophyll content

Leaf chlorophyll content (in SPAD units) was measured for individual leaves (n=28 per site, per season) while still attached to the branch. Leaf chlorophyll content was measured using a handheld Konica Minolta SPAD-505 Plus (Konica Minolta Inc., Japan) as described by Tiwari *et al*. (2006). Three readings were taken at three random points for each leaf, and these readings were then averaged for each leaf before data analysis. As discussed earlier, to assess whether or not differences in light intensity between the greenhouse and the sites could have influenced the results of the chlorophyll content studies, leaf chlorophyll content was also measured for trees growing within 1 km of the greenhouse, as mentioned in section 3.3.3.

3.3.7 Hyperspectral analysis

3.3.7.1. Spectral reflectance data acquisition

The spectral reflectance of individual leaves (n=28, per site, per season) was measured under laboratory conditions using an Analytical Spectral Device (ASD) FieldSpec FR spectroradiometer (ASD, USA). Leaves were placed on a black surface, at a height of 70 cm from the ground, with a halogen lamp emitting white light to capture spectra. Above the leaf sample, a fibre optic probe with a 1º field of view was placed onto a pistol grip, attached to a tripod stand (SUNPAK 6600 UT) at a height of 148 cm, and fitted to the spectroradiometer. Measurements were recorded over a range of 350–2500 nm in a dark room. Three scans were performed for each leaf and subsequently averaged, to produce a single spectral measurement. The resulting spectra were interpolated with the use of ASD software, which produced readings at every 1 nm (Smith *et al*., 2004).

3.3.8 Data analyses

Data for all parameters were tested for normality using the Shapiro-Wilk test. For the Analysis of Variance (ANOVA) analyses, biomarkers (chlorophyll content and leaf

area) were compared across the treatment and control sites, within seasons. Thereafter, data for both seasons were pooled to compare biomarkers across sites, irrespective of seasons. These analyses of variance tested for the main effects of site and season and their interaction. Means were separated using a Bonferroni post-hoc test and differences were considered significant at the 0.05 level. To test for significant relationships between parameters and [SO_2], a Pearson Correlation test was used.

3.3.8.1. Partial least squares regression (PLSR)

The PLSR algorithm was used to relate the spectral reflectance (wavebands) to the morphological and physiological parameters. Partial least squares regression was chosen over stepwise regression since the former is recommended when there are a large number of predictor variables (Mitchell *et al*., 2012). Partial least squares regression (PLSR) transforms the predictor variables to a set of latent components, which were then used for prediction purposes (Delalieux *et al.,* 2007; Mehmood *et al.,* 2012). Partial least squares regression can be compared with principle component analysis (PCA); however, PLSR is driven by a small number of latent variables (Wold *et al.,* 2001) and explains the covariance between the explanatory variables (X) and response variables (Y) (Wold *et al.,* 2001; Delalieux *et al.,* 2007; Yamamoto *et al.,* 2009). To determine the best number of latent components to be used for prediction, a tenfold cross validation (CV) technique was used (Boulesteix, 2004). Variable importance in projection (VIP) was also calculated to determine the importance of wavebands (Pérez-Enciso and Tenenhaus, 2003; Mehmood *et al.,* 2012, Peerbhay *et al.,* 2013). Variable importance in projection scores are believed to be more sensitive and specific than regression coefficients across a wide spectral data range (Chong and Jun, 2005) and was therefore chosen as the preferred method. Predictor variables (wavebands) were identified as important if their VIP score was greater than 1 (Chong and Jun, 2005; Akarachantachote *et al.,* 2014).

3.4 Results

3.4.1 Ground-level sulphur dioxide levels at treatment and control sites

When ground-level [SO_2] were compared across sites, within seasons, Southern Works possessed the highest levels of SO_2, in spring and summer, with Prospecton having significantly lower levels of SO_2 in summer, compared with Southern Works and Ganges (Fig. 14A). When seasonal data were pooled to generate a combined average for individual sites (Fig. 14B), comparisons across sites revealed SO_2 levels at Southern Works to be significantly higher than Ganges and Prospecton, both of which were statistically comparable in terms of [SO_2].

3.4.2 *Ex situ* measurements

When [SO_2] was measured at the *ex situ* control site, levels were below the detectable limits of the instrument within the greenhouse, in which the control trees were housed. Levels outside, however, measured at three random points within 1 km of the greenhouse (located on Westville campus of the University of KwaZulu-Natal), averaged 2.73±0.56 ppb. This meant that the trees growing outside the greenhouse, on the university campus, were exposed to significantly ($p<0.05$, ANOVA) lower SO_2 levels than the treatment sites, but higher than the control. There was also a slight difference in photosynthetically active radiation (PAR) between the greenhouse (1938.65±100.28 µm of photons $m^{-2}s^{-1}$) and the treatment sites (2027.65±124.24 µm of photons $m^{-2}s^{-1}$) ($n=20$, $p<0.05$, ANOVA).

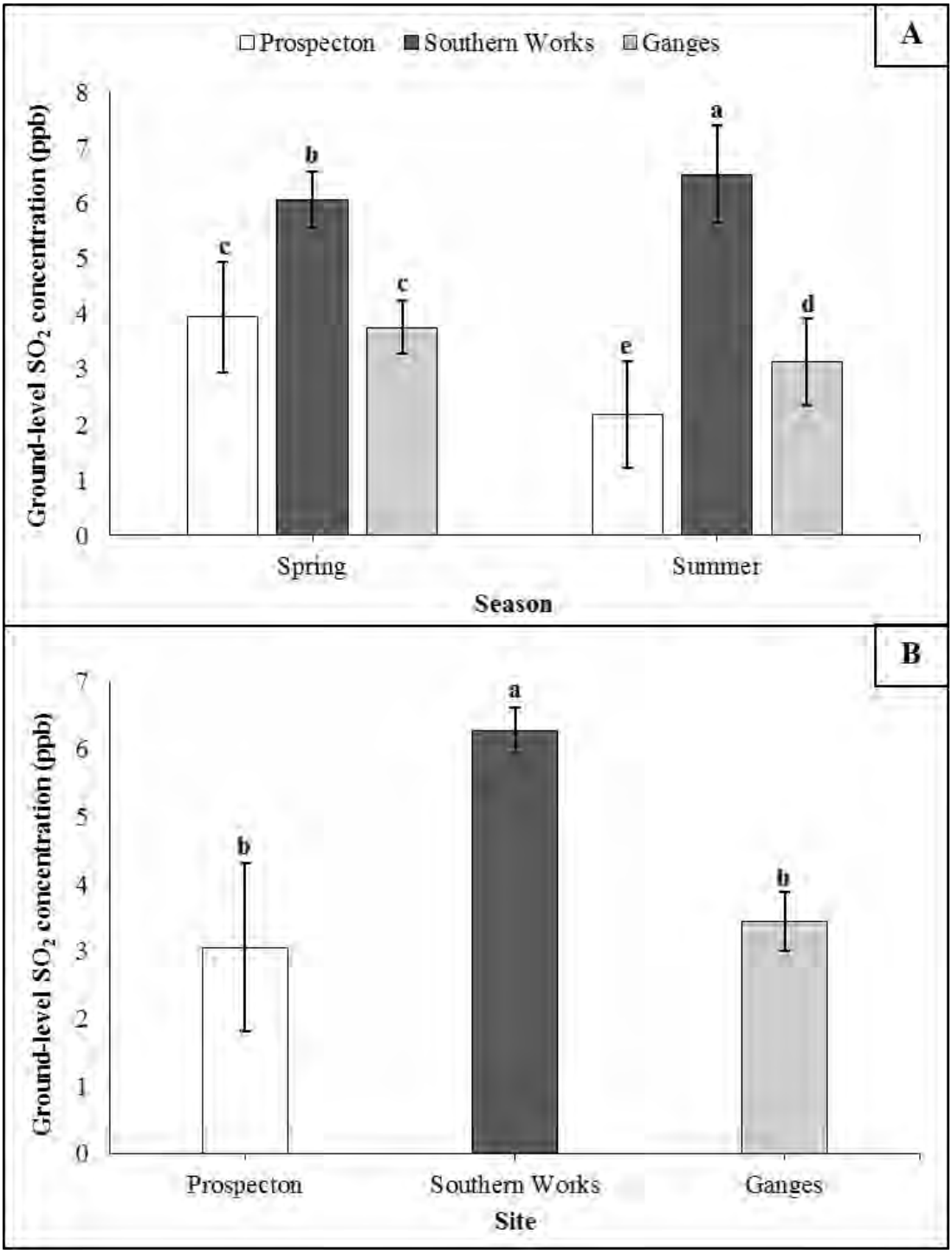

Figure 14. Ground-level [SO_2] measured at three industrial sites at which sampling was conducted within the Durban South Basin between September 2014 and February 2015 (i.e. in spring and summer): (A) Seasonal [SO_2]; (B) Combined [SO_2]. Bars labelled with different letters are significantly different ($p<0.001$). For the seasonal dataset, the standard deviation ranged from 2.24–5.23, and for the combined dataset the standard deviation ranged from 2.77–5.15.

3.4.3 Physiological and morphological markers

There were no statistically significant ($p>0.05$) differences within sites, across cardinal points, in both seasons, for all physiological and morphological biomarkers, therefore, data for the different cardinal points were pooled for all subsequent analyses.

3.4.3.1. Leaf area and sulphur dioxide levels

Leaf area values for the treatment sites were significantly lower than the control, when compared within seasons (Fig. 15). There was also a significant ($p<0.001$) interaction between site and season, suggesting that the extent to which sites differ from each other was based on the season. However, across the treatment sites (excluding the control), values did not differ significantly when compared within seasons. Leaf area data therefore, did reflect differences in [SO_2] between the control and the treatment sites, and was significantly negatively correlated with [SO_2] ($r=-0.77$, $p<0.05$). Based on reasons discussed in section 3.3.3, leaf area was also measured for trees growing within 1 km of the greenhouse, in which the control trees were housed. These trees, which were exposed to significantly lower SO_2 levels than the treatment sites, but higher than the control, exhibited significantly ($p<0.001$) higher leaf areas (24.13 ± 4.99 cm^2) [data not shown] than Southern Works and Ganges, but lower than those of the control and Prospecton.

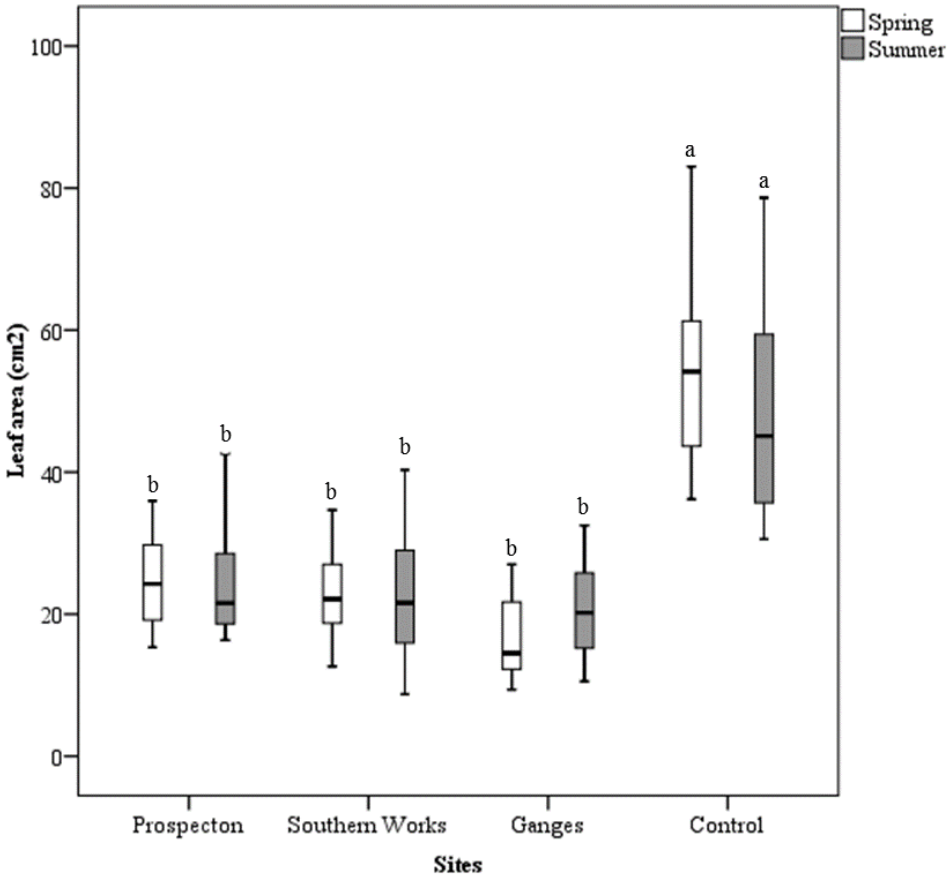

Figure 15. Leaf area for *T. dregeana* leaves exposed to differing levels of SO_2 across three treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over two seasons. Values are based on a sample size of n=28 and whiskers represent standard deviation. Boxplots labelled with different letters are significantly different when compared across different site×season combinations ($p<0.001$, ANOVA).

3.4.3.2. Leaf chlorophyll content and sulphur dioxide levels

Leaf chlorophyll values for the treatment sites were significantly higher than the control (Fig. 16). There was also a significant ($p<0.001$) interaction between site and season, suggesting that the extent to which sites differ from each other was based on the season. However, within the treatment sites (excluding the control), values did not differ significantly when compared within each season independently. Leaf chlorophyll content could therefore reflect differences in [SO_2] between the control and the treatment sites, and furthermore leaf chlorophyll was also significantly positively correlated to [SO_2] ($r=0.774$, $p<0.05$). Additionally, based on reasons discussed in

section 3.3.3, leaf chlorophyll content values were measured for trees growing within 1 km of the greenhouse. These trees, which were exposed to significantly lower SO_2 levels than the treatment sites, but higher than the control, exhibited significantly ($p<0.001$) lower levels of chlorophyll (66.02±3.41 SPAD units) [data not shown] than the treatment sites, but higher than the control.

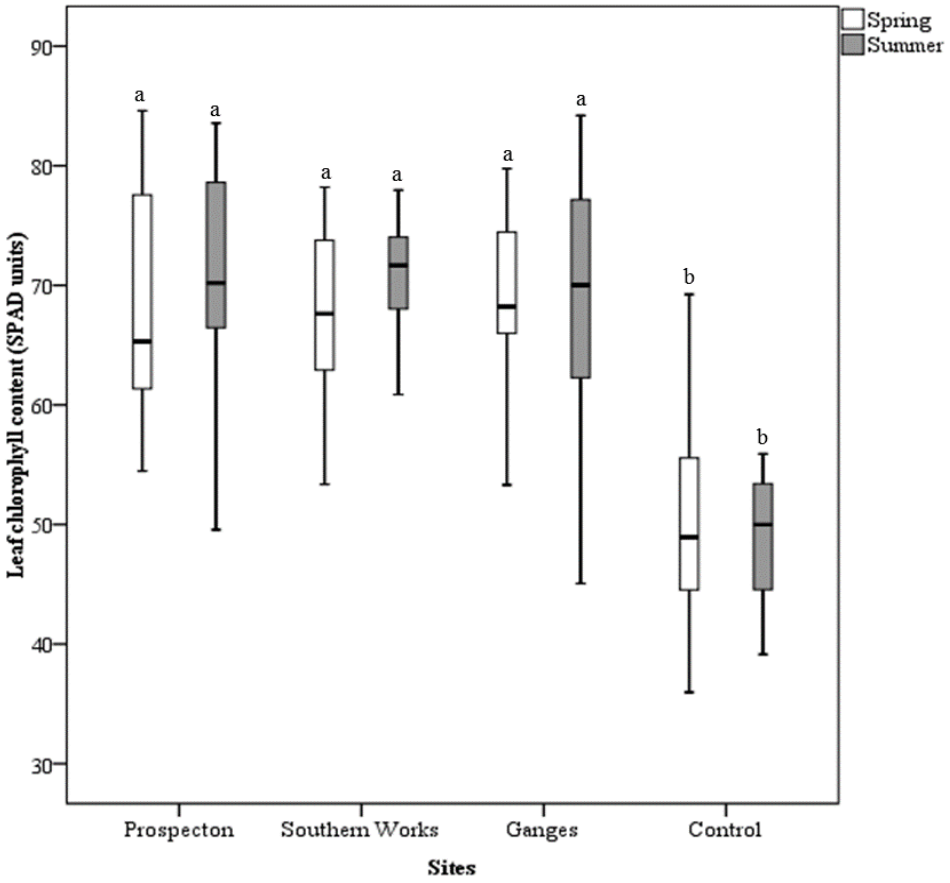

Figure 16. Leaf chlorophyll content for *T. dregeana* leaves across three treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control measured over two seasons. Values are based on a sample size of n=28 and whiskers represent standard deviation. Boxplots labelled with different letters are significantly different when compared across different site×season combinations ($p<0.001$, ANOVA).

3.4.4 Hyperspectral analysis

3.4.4.1. Spectral data

When hyperspectral reflectance curves were compared for spring and summer across the treatments and the control, it was evident that average spectral reflectance (%) (in the control) was consistently higher than the treatment sites for both seasons at 350–1880 nm. Additionally, average spectral reflectance in the control was consistently lower than the treatment sites for both seasons at 1880–2135 nm. Differences in average spectral reflectance across the treatment sites were not apparent in summer (Fig. 17B); however, in spring there were indications that average spectral reflectance at Southern Works, the site with the highest [SO_2] (Fig. 14A), was higher than the other treatment sites at 350–1242 nm (Fig. 17A). Prospecton, the site with the lowest [SO_2] (Fig. 14A), had lower reflectance values from 1115–2130 nm when compared with the other treatment sites during spring.

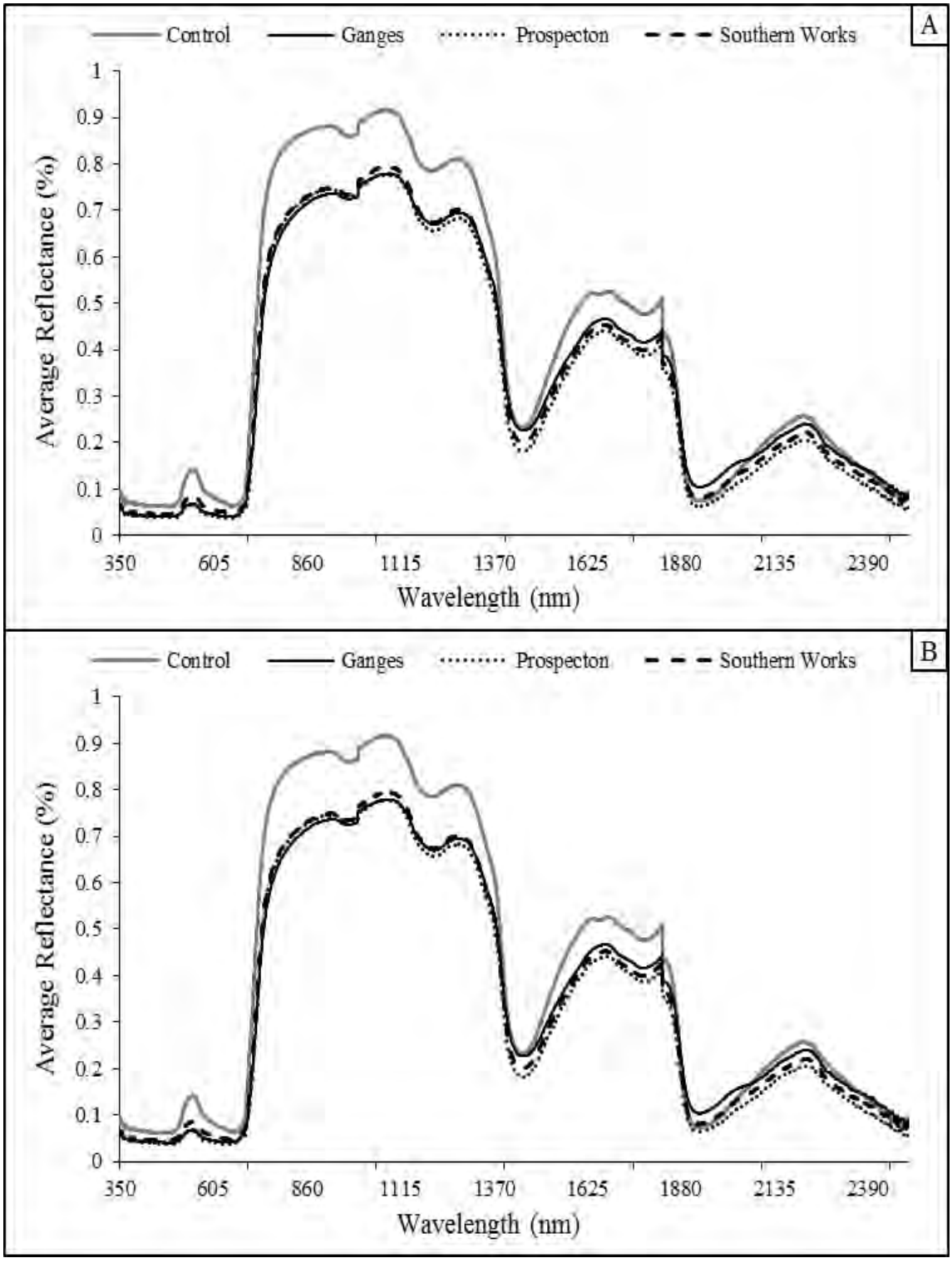

Figure 17. Average spectral reflectance curves for *T. dregeana* leaves exposed to SO_2 across the three treatment sites (Prospecton, Southern Works and Ganges) and an *ex situ* control, over two seasons (A: spring and B: summer). Values represent the mean reflectance values (n=28).

3.4.4.2. Partial least squares regression (PLSR)

For spring, leaf chlorophyll content could be predicted using 14 latent components (Fig. 18A). Furthermore, the best model produced an r^2 value of 0.325 and a RMSEP value of 8.98 (Fig. 18B). Leaf area for the same season could be predicted using 4 latent components (Fig. 18C), and the best model produced an r^2 value of 0.429 and a RMSEP value of 12.52 (Fig. 18D).

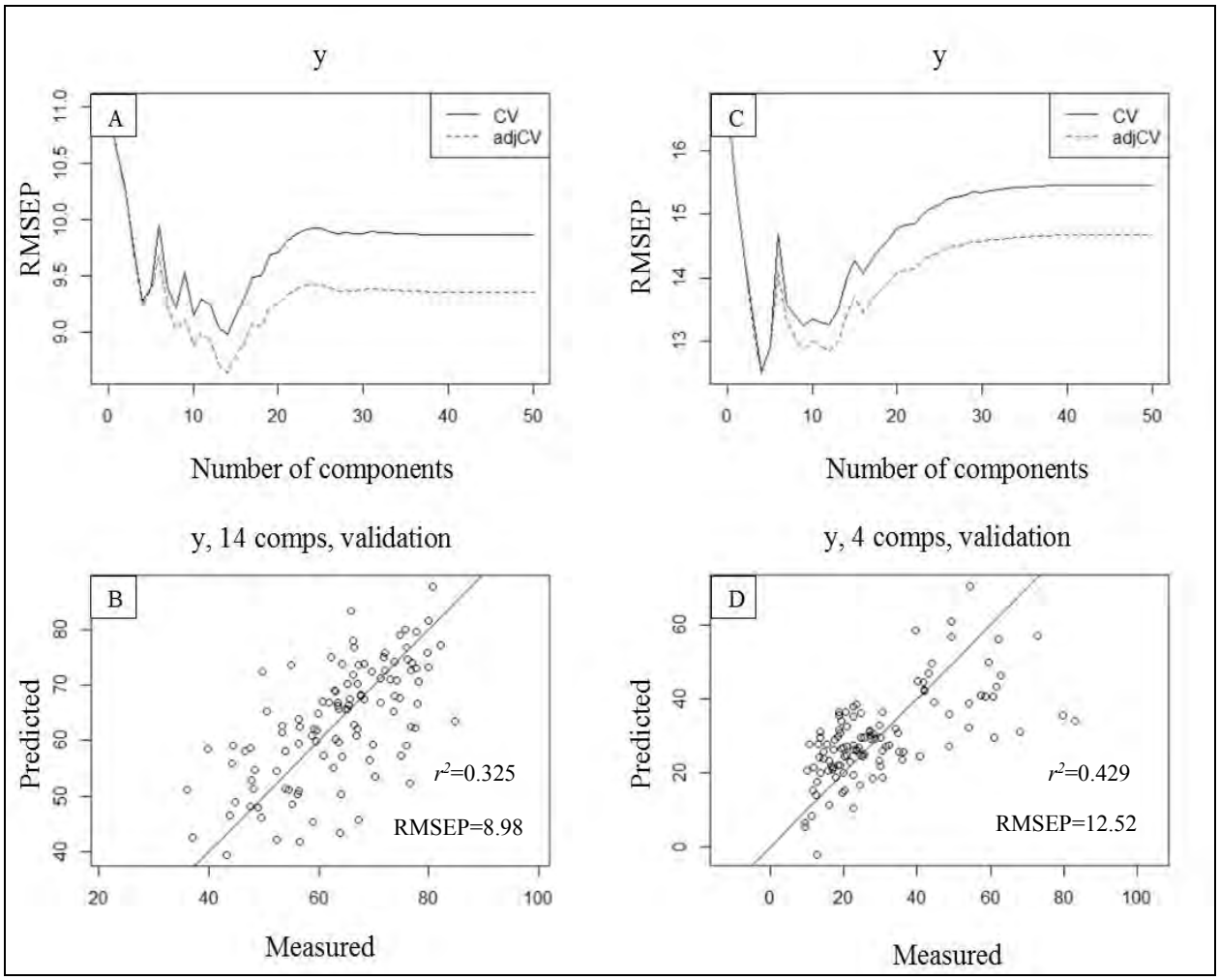

Figure 18. Results of tenfold cross validation (CV) to determine the lowest component value and predicted values for leaf chlorophyll content (A and B) and leaf area (C and D) for spring (n=28).

For summer, leaf chlorophyll content could be predicted using 11 latent components (Fig. 19A), with the best model producing an r^2 value of 0.475 and a RMSEP value of 8.75 (Fig. 19B). Leaf area for the same season could be predicted using 15 latent components (Fig. 19C), and the best model produced an r^2 value of 0.586 and a RMSEP value of 9.20 (Fig. 19D).

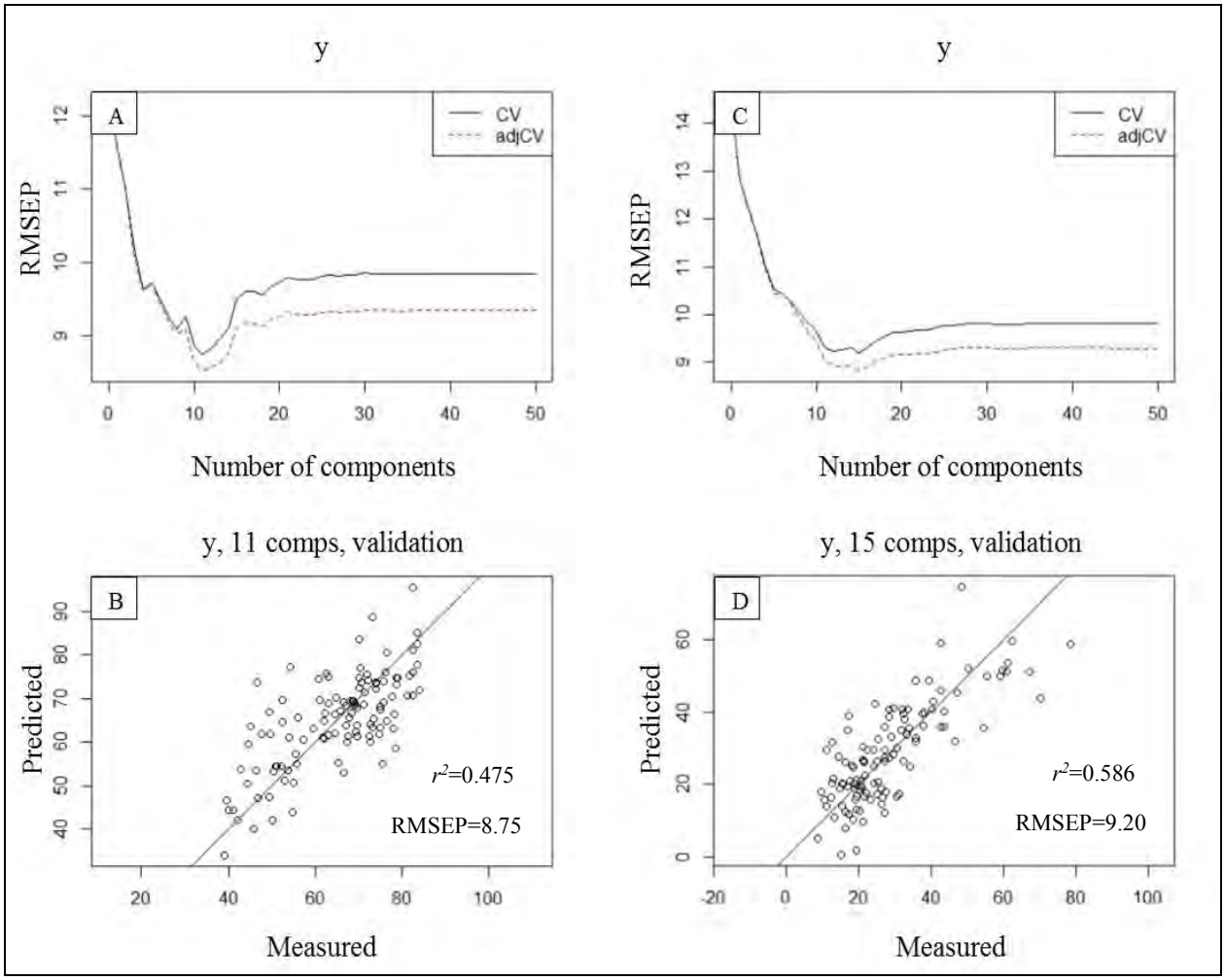

Figure 19. Results of tenfold cross validation (CV) to determine the lowest component value and predicted values for leaf chlorophyll content (A and B) and leaf area (C and D) for summer (n=28).

3.4.4.3. Variable importance in projections (VIP)

Wavebands were classified as important if they obtained a variable importance in projection (VIP) score of greater than 1. Bands with the highest VIP scores are indicated with black arrows in Figure 20. The VIP method identified important bands within the visible region (390–723 nm) of the electromagnetic spectrum (Fig. 20). Important bands were found primarily within the blue-green (390–580 nm) and red-edge (690–710 nm) regions. For leaf chlorophyll content in spring (Fig. 20A), a total of 563 bands exhibited VIP scores above 1, and wavebands with the highest VIP scores were found at 552 nm (VIP score = 1.45) and 704 nm (VIP score = 2.02). For leaf area in spring (Fig. 20B), a total of 685 bands exhibited VIP scores above 1, and wavebands with the highest VIP scores were found at 553 nm (VIP score = 1.89) and 710 nm (VIP score = 2.38). For leaf chlorophyll content in summer (Fig. 20C), a total of 441 bands exhibited VIP scores above 1, and wavebands with the highest VIP scores were found at 552 nm (VIP score = 1.97) and 708 nm (VIP score = 2.73). For leaf area in summer (Fig. 20D), a total of 600 bands had VIP scores above 1, and wavebands with the highest VIP scores were found at 554 nm (VIP score = 1.35) and 707 nm (VIP score = 1.81).

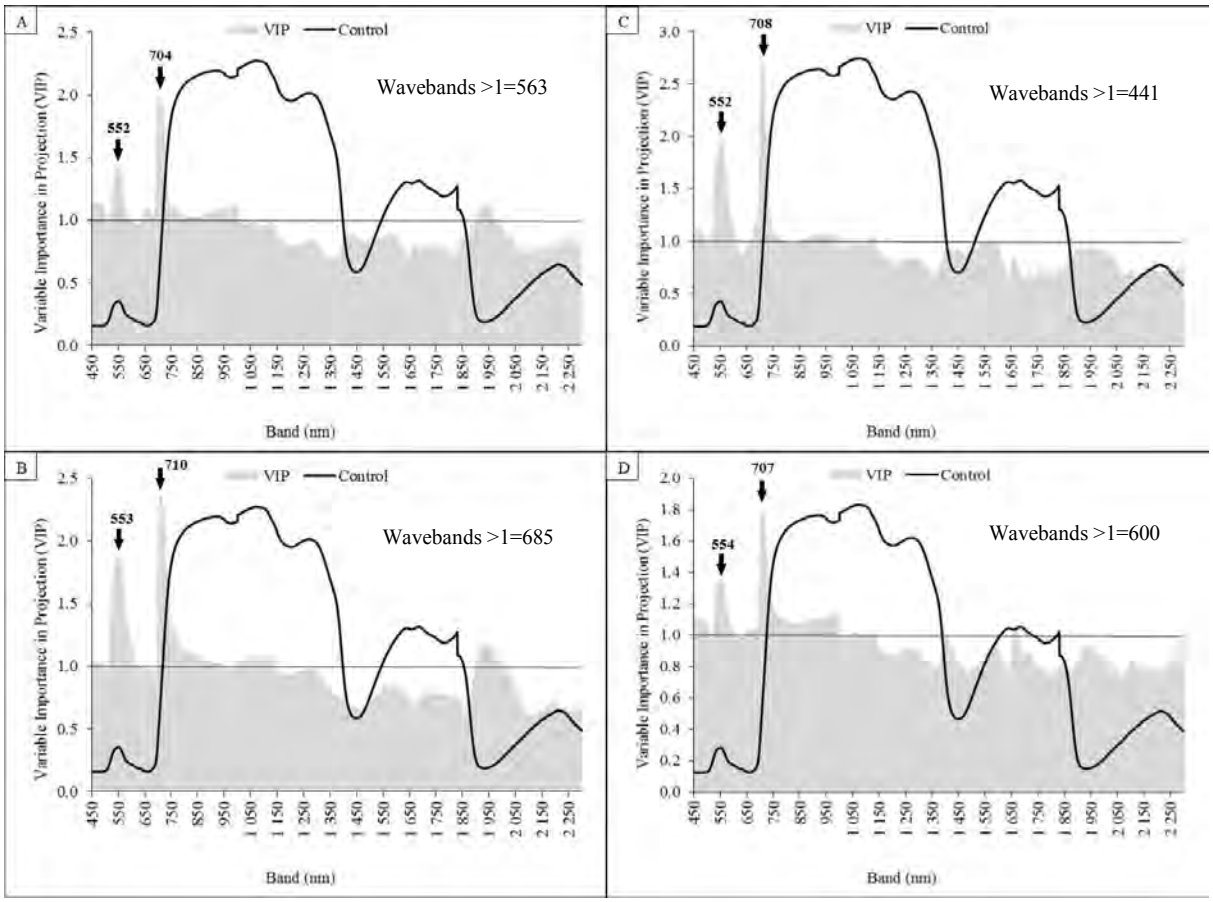

Figure 20. Waveband importance as determined by the variable importance in the projection (VIP) method for chlorophyll content (A) and leaf area (B) in spring; chlorophyll content (C) and leaf area (D) in summer. The horizontal line is the cut-off threshold and shows important wavebands with scores greater than 1, which are indicated by black arrows.

3.5 Discussion

Industrial emissions are of concern within industrial areas in South Africa, such as the SDB (Matooane and Diab, 2001; Diab *et al.,* 2002), with SO_2 representing one of the major pollutants (Matooane and Diab, 2001; Diab and Motha, 2007). There are, however, various other pollutants (e.g. NO_2, PM and O_3) (Tiwari *et al.,* 2006) that act together with SO_2 to threaten human (Matooane and Diab, 2003) and environmental health (Winner, 1994). It is therefore of major importance to monitor the quality of air, and given the substantial financial investment required for installing mechanised

monitoring stations, the use of bioindicators (such as trees) to monitor air pollution is gaining popularity across the world (Sawidis *et al.,* 2011). This study focussed on predicting *T. dregeana* (an indigenous tree) leaf stress responses to industrial air pollution, using hyperspectral reflectance and partial least squares.

SO_2 pollution levels

Sulphur dioxide levels differed within seasons and there was a difference across treatment sites, with Southern Works having the highest levels compared with Prospecton and Ganges (Fig. 14A). Results of the present study support findings by Carmichael (2003), who reported seasonal variation in ambient air quality and differences across regions. According to that author, seasonal air pollution levels are associated with changing wind patterns. Diab *et al.* (2002) conducted a study on [SO_2] within the SDB, and focussed on hourly averages by season, as well as mean annual conditions; results indicated a variation in [SO_2] across stations, which supports findings of the present study.

Ground-level SO_2 was measured across the three sampling sites and ranged between 3.48–6.30 ppb (Fig. 14B), which falls within the global SO_2 range of 3.8–11.4 ppb where irreversible damage is imposed on plants (Carmichael *et al.,* 2003; Josipovic *et al.,* 2010; Areington *et al.,* 2015). Sulphur dioxide concentrations in this range can affect plants either directly or indirectly, and these effects include: reduced photosynthesis; altered stomatal opening patterns, which influences transpiration; and reduced plant yield (Varshney *et al.,* 1979).

Annual [SO_2] was not measured, as the study focussed on two seasons only, but when [SO_2] values for summer and spring were combined to generate an average, the highest levels of SO_2 were found at Southern Works, while levels at Prospecton and Ganges were comparable. These results are comparable to Diab *et al.* (2002), who also reported Southern Works to have the highest mean annual levels of SO_2 in the SDB. It should be noted that SO_2 levels recorded at Southern Works in this study (3.06–6.28 ppb) were relatively low when compared with countries like China, which has an annual daily average of 129.77 ppb (Emberson *et al.,* 2001), but comparable with countries like India which has an annual average of 3.82–45.80 ppb (Emberson *et al.,* 2001).

Comparison of selected air pollution biomarkers

The effects of pollutants bring about species-specific (Veselkin *et al.,* 2004) changes in plants at different levels: morphological; physiological; and/or biochemical (Cui *et al.,* 2006). Individuals from the same species can also show signs of varying degrees of tolerance to the same air pollutant (Dineva, 2004). In the present study the responses of two stress biomarkers, viz. leaf area and chlorophyll content, to varying levels of SO_2 pollution was assessed for the leaves of an indigenous tree species.

Studies have shown that visible symptoms are easy to identify under field conditions, since the surface of leaves come into direct contact with atmospheric pollutants (Dineva, 2004). In the present study, leaf area at the treatment sites was significantly lower than the control, when compared within seasons across sites (Fig. 15). A number of species decrease leaf area at polluted sites, thereby reducing their level of contact with the air pollution, which is assumed to improve their tolerance of its effects (Assadi *et al.,* 2011). As in other studies (Seyyednejad et al., 2009; Assadi *et al.*, 2011), leaf area was significantly negatively correlated with [SO_2]. Furthermore, the high leaf area of trees in the greenhouse (*ex situ*) were more likely due to below detectable SO_2 levels than exposure to slightly lower levels of PAR. This reasoning is based on the fact that trees growing in full sun within 1 km of the greenhouse, in which the control trees were housed, followed similar trends to trees at the treatment sites, in terms of leaf area.

Although morphological changes to the plant may be seen first, it is believed that physiological damage precedes visible/morphological damage (Bytnerowicz, 1996; Assadi *et al.,* 2011). Changes in the leaf chlorophyll levels can therefore provide valuable information on the physiological state of a plant exposed to pollution (Assadi *et al.*, 2011). In the present study, leaf chlorophyll content was significantly higher at treatment sites compared with the *ex situ* control (Fig. 16). When compared within seasons across sites, leaf chlorophyll content was also significantly correlated with [SO_2]. In contrast to the current study, other studies have shown pollution to result in a decrease in leaf chlorophyll content (Tiwari *et al.,* 2006; Joshi and Swami, 2007).

However, physiological links between leaf chlorophyll content and leaf area have also been reported for trees exposed to air pollution (Assadi *et al.,* 2011; Areington *et al.,* 2015). *Brachylaena discolor* trees, exposed to SO_2 pollution within the SDB for

example, showed a decrease in leaf area and an increase in chlorophyll content compared with trees growing *ex situ* (Areington *et al.,* 2015). The results of this study therefore support suggestions of the interactive responses of chlorophyll content and leaf area to air pollution (Assadi *et al.,* 2011; Areington *et al.,* 2015). The low chlorophyll content in leaves of the greenhouse (*ex situ*) trees were more likely due to below detectable SO_2 levels than exposure to slightly lower levels of PAR. This reasoning arises from the fact that trees growing in full sun within 1 km of the greenhouse, in which the control trees were housed, followed similar trends to trees at the treatment sites, in terms of leaf chlorophyll content.

Hyperspectral and partial least squares regression analysis

Reflectance within the visible region of the electromagnetic spectrum increases as a response to stress (Carter, 1993; Carter and Miller, 1994; Carter *et al.,* 1996; Carter and Knapp, 2001; Smith *et al.,* 2004*;* Smith *et al.,* 2005). The leaves from trees in the *ex situ* control in this study have exhibited higher spectral reflectance in the visible region and majority of the NIR than leaves from SO_2-exposed (treatment) trees (Fig. 17). However, as alluded to above, trees at Southern Works, which were exposed to the highest [SO_2] and should therefore have relatively lower reflectance across the treatment sites, often exhibited higher reflectance values than the other two treatment sites. This contradiction can be explained in terms of the relationship between chlorophyll content and leaf area observed in this study. Exposure to SO_2 led to a decrease in leaf area (Fig. 15) and an accompanying increase in chlorophyll content (Fig. 16). Similarly, Gitelson *et al.* (2003) observed that a decrease in leaf thickness may cause an increase in chlorophyll concentration, which can lead to a decrease in reflectance in the green and red-edge region. This implies that for species in which leaf responses to air pollution involve an interaction between leaf chlorophyll content and leaf area, these biomarkers should ideally be related to hyperspectral data in an integrated manner, rather than independently. Reference to literature suggests that this may be achieved by simultaneously predicting leaf chlorophyll content and leaf area, using an algorithm such as partial least square 2 analyses (PLS2) (Martens, 1991; Gendrin *et al.,* 2007).

Partial least squares regression (PLSR) was used to predict chlorophyll and leaf area (i.e. air pollution biomarkers) using hyperspectral data. This method, PLSR, was able to

handle the complete reflectance spectrum along with many collinear wavebands (Wold *et al.,* 2001; Axelsson *et al.,* 2013) and proved to be successful in assessing the performance of the predictive models using the cross validation technique. For leaf chlorophyll content across seasons, cross validated r^2 values ranged from 0.32 and 0.475, with the RMSEP ranging between 8.75 to 8.98 (Fig. 18 and 19). Leaf area across seasons had cross validated r^2 values ranging between 0.429 and 0.586, with RMSEP ranging from 9.20 to 12.52 (Fig. 18 and 19). While the results of the study show promise, a follow-up study will attempt to improve the predictive models by examining the utility of spectral transformations (such as derivatives), and band selection methodologies (Abdel-Rahman *et al*., 2014).

Variable importance in projection (VIP) was used to identify important wavebands across the reflectance spectrum (Peerbhay *et al.,* 2013) for leaf chlorophyll content and leaf area across seasons. The VIP method proved to be most successful in identifying important wavebands with VIP scores above 1. Important bands were identified within the blue-green region (390–550 nm) and the red-edge region (690–710 nm). The red region (600–700 nm) includes the red-edge region, which has been identified as an important region in terms of identifying stress (Carter and Knapp, 2001; Smith *et al.,* 2004). Within these regions, leaf chlorophyll content related VIP bands were identified at 552 nm and 710 nm in spring, and 552 nm and 708 nm in summer (Fig. 20). For leaf area, VIP bands were found at 553 nm and 710 nm in spring, and at 554 nm and 707 nm in summer (Fig. 20). The VIP bands identified in this study are within the red-edge region, which refers to the region that indicates stress. Within this region, there is a change in the reflectance between 690 nm and 750 nm, which has been reported by Smith *et al.* 2004 and Manzo *et al*. 2013.

3.6 Conclusions

The results suggest that *T. dregeana* leaves may be a suitable bioindicator of air pollution within the South Durban Basin, with leaf chlorophyll content and leaf area showing signs of being successful biomarkers of air quality for this species. Prior to visible injury on plants due to air pollution, physiological parameters can be used, along with hyperspectral remote sensing, to identify plant stress using the red-edge region of the electromagnetic spectrum. The important findings of this study are (i) significant

differences between polluted and *ex situ* trees occur for both chlorophyll content and leaf area, (ii) partial least square regression was able to relate the hyperspectral dataset to both a physiological and morphological stress biomarker, (iii) the interaction between leaf chlorophyll content and leaf area suggests that simultaneous prediction of these biomarkers, using an algorithm such as partial least squares 2, may be more suitable, and (iv) the variable importance in projection (VIP) method was able to identify important wavebands within the red-edge region of the electromagnetic spectrum, that showed promise in identifying stress in the leaves of *T. dregeana.*

References

Abdel-Rahman, E.M., Ahmed, F.B. and Ismail, R. 2013. Random forest regression and spectral band selection for estimating sugarcane leaf nitrogen concentration using EO-1 Hyperion hyperspectral data. *International Journal of Remote Sensing*, 34(2), pp. 712 - 728

Abdel-Rahman, E.M., Mutanga, O., Odindo, A. and Ismail, R. 2014. A comparison of partial least squares (PLS) and sparse PLS regressions for predicting yield of Swiss chard grown under different irrigation water sources using hyperspectral data. *Computers and Electronics in Agriculture*, 106, pp. 11 - 19

Agrawal, M., Singh, B., Ragput, M., Marshall, F. and Bell, J.N.B. 2003. Effect of air pollution on peri- urban agriculture: a case study. *Environmental Pollution*, 126, pp. 323 - 329

Akarachantachote, N., Chadcham, S. and Saithanu, K. 2014. Cutoff threshold of variable importance in projection for variable selection. *International Journal of Pure and Applied Mathematics*, 94(3), pp. 307 - 322

Alfani, A., Baldantoni, D., Maisto, G., Bartoli, G. and De Santo, A. V. 2000. Temporal and spatial variation in C, N, S and trace element contents in the leaves of *Quercus ilex* within the urban area of Naples. *Environmental Pollution*, 109(1), pp. 119 - 129

Areington, C.A., Varghese, B., Ramdhani, S. and Naidoo, S.E. 2015. An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in the leaves of *Brachylaena discolor*. *Water, Air and Soil Pollution*, 226(9), pp. 1 - 14

Assadi, A., Pirbalouti, A. G., Malekpoor, F., Teimori, N., and Assadi, L. 2011. Impact of air pollution on physiological and morphological characteristics of *Eucalyptus camaldulensis* Den. *Journal of Food, Agriculture and Environment*, 9(2), pp. 676 - 679

Axelsson, C., Skidmore, A.K., Schlerf, M., Fauzi, A. and Verhoef, W. 2013. Hyperspectral analysis of mangrove foliar chemistry using PLSR and support vector regression. *International Journal of Remote Sensing*, 34(5), pp. 1724 - 1743

Ayanu, Y.Z., Conrad, C., Nauss, T., Wegmann, M. and Koellner, T. 2012. Quantifying and mapping ecosystem services supplies and demands: a review of remote sensing applications. *Environmental Science and Technology*, 46(16), pp. 8529 - 8541

Balasooriya, B.L.W.K., Samson, R., Mbikwa, F., Boeckx, P. and Van Meirvenne, M. 2009. Biomonitoring of urban habitat quality by anatomical and chemical leaf characteristics. *Environmental and Experimental Botany*, 65(2), pp. 386 - 394

Barber, J.L., Thomas, G.O., Kerstiens, G. and Jones, K.C. 2004. Current issues and uncertainties in the measurement and modelling of air–vegetation exchange and within-plant processing of POPs. *Environmental Pollution*, 128(1), pp. 99 - 138

Batterman, S.A., Chernyak, S.M., Gounden, Y., Matooane, M. and Naidoo, R.N. 2008. Organochlorine pesticides in ambient air in Durban, South Africa. *Science of the Total Environment*, 397, pp. 119 - 130

Bennett, J. and Wetmore, C. 1999. Changes in element contents of selected lichens over 11 years in northern Minnesota, USA. *Environmental and Experimental Botany*, 41(1), pp. 75-82

Berlizov, A.N., Blum, O.B., Filby, R.H., Malyuk, I.A. and Tryshyn, V.V. 2007. Testing applicability of black poplar (*Populus nigra* L.) bark to heavy metal air pollution monitoring in urban and industrial regions. *Science of the Total Environment*, 372(2), pp. 693 - 706

Bernhardt-Römermann, M., Kirchner, M., Kudernatsch, T., Jakobi, G. and Fischer, A. 2006. Changed vegetation composition in coniferous forests near to motorways in Southern Germany: the effects of traffic-born pollution. *Environmental Pollution*, 143(3), pp. 572 - 581

Borengasser, M., Hungate, W.S. and Watkins, R. 2007. Hyperspectral remote sensing: principles and applications. In Weng, Q. ed. *Remote Sensing Applications*. London: Crc Press, pp. 42

Boulesteix, A.L. 2004. PLS dimension reduction for classification with microarray data. *Statistical Applications in Genetics and Molecular Biology*, 3(1), pp. 1 - 30

Bytnerowicz, A. and Fenn, M.E. 1996. Nitrogen deposition in California forests: a review. *Environmental Pollution*, 92(2), pp. 127 - 146

Carmichael, G.R., Ferm, M., Thongboonchoo, N., Woo, J.H., Chan, L.Y., Murano, K., Viet, P.H., Mossberg, C., Bala, R., Boonjawat, J. and Upatum, P. 2003. Measurements of sulfur dioxide, ozone and ammonia concentrations in Asia, Africa, and South America using passive samplers. *Atmospheric Environment*, 37(9), pp. 1293 - 1308

Carter, G.A. 1993. Ratios of leaf reflectances in narrow wavebands as indicators of plant stress. *Remote Sensing*, 15(3), pp. 697 - 703

Carter, G.A., Cibula, W.G. and Miller, R. L. 1996. Narrow-band Reflectance Imagery Compared with Thermal Imagery for Early Detection of Plant Stress. *Journal of Plant Physiology*, 148(5), pp. 515 - 522

Carter, G.A. and Knapp, A.K. 2001. Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, 88(4), pp. 677 - 684

Carter, G.A. and Miller, R.L. 1994. Early detection of plant stress by digital imaging within narrow stress-sensitive wavebands. *Remote Sensing of Environment*, 50(3), pp. 295 - 302

Çelik, A., Kartal, A.A., Akdoğan, A. and Kaska, Y. 2005. Determining the heavy metal pollution in Denizli (Turkey) by using *Robinio pseudo-acacia* L. *Environment International*, 31(1), pp. 105 - 112

Chapin, F.S. 1991. Integrated responses of plants to stress. *BioScience*, 41, pp. 29 - 36

Chong, I.G. and Jun, C.H. 2005. Performance of some variable selection methods when multicollinearity is present. *Chemometrics and Intelligent Laboratory Systems*, 78(1), pp. 103 - 112

Cui, H.X., Jiang, G.M., Niu, S.L., Jiang, C.D., Liu, M.Z., Yu, S.L. and Gao, L.M. 2006. Ecophysiological Response of Plants to Combined Pollution from Heavy-duty Vehicles and Industrial Emissions in Higher Humidity. *Journal of Integrative Plant Biology*, 48(12), pp. 1391 - 1400

Delalieux, S., van Aardt, J., Keulemans, W., Schrevens, E. and Coppin, P. 2007. Detection of Biotic Stress (*Venturia inaequalis*) in Apple Trees Using Hyperspectral Data: Non-Parametric Statistical Approaches and Physiological Implications. *European Journal of Agronomy*, 27, pp. 130 - 143

Diab, R.D. and Motha, A. 2007. An analysis of key institutional factors influencing air quality in South Durban basin using the DPSIR framework. *South African Geographical Journal*, 89(1), pp. 22 - 33

Diab, R., Prause, A. and Bencherif, H. 2002. Analysis of SO_2 pollution in the South Durban Industrial Basin. *South African Journal of Science*, 98, pp. 543 - 546

Dineva, S. B. 2004. Comparative studies of the leaf morphology and structure of white ash *Fraxinus americana* L. and London plane tree *Platanus acerifolia* Willd growing in polluted area. *Dendrobiology*, 52, pp. 3 - 8

Emberson, L.D., Ashmore, M.R., Murray, F., Kuylenstierna, J.C.I., Percy, K.E., Izuta, T., Zheng, Y., Shimizu, H., Sheu, B.H., Liu, C.P. and Agrawal, M. 2001. Impacts of air pollutants on vegetation in developing countries. *Water, Air, and Soil Pollution*, 130(1-4), pp. 107 - 118

eThekwini Health and Norwegian Institute for Air Research. 2007. Air quality management plan for eThekwini Municipality, South Africa.

Fauzi, A., Skidmore, A.K., van Gils, H., Schlerf, M. and Heitkönig, I.M. 2013. Shrimp pond effluent dominates foliar nitrogen in disturbed mangroves as mapped using hyperspectral imagery. *Marine Pollution Bulletin*, 76(1), pp. 42 - 51

Fella, J., Laval-Gilly, P., Henryon, M., Morlot, D. and Ferard, J.F. 2000. Biological air quality monitoring: a review. *Environmental Monitoring and Assessment*, 64, pp. 627 - 644

Gendrin, C., Roggo, Y. and Collet, C., 2007. Content uniformity of pharmaceutical solid dosage forms by near infrared hyperspectral imaging: a feasibility study. *Talanta*, *73*(4), pp. 733 - 741

Gitelson, A.A., Gritz, Y. and Merzlyak, M.N. 2003. Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. *Journal of Plant Physiology,* 160, pp. 271 - 282

Guastella, L. and Knudsen, S. 2007. South Durban basin multi-point plan case study report. Air quality act implementation: Air quality management planning. Book 12, series C, pp. 1 - 34

Hale, M.E. 1969. How to know the lichens. In Wm. C. ed. Iowa: Brown Company publishers

Hale, M.E. 1983. The biology of lichens In Edward Arnold. London

Hansen, P.M., Schjoerring, J.K. 2003. Reflectance measurement of canopy biomass and nitrogen status in wheat crops using normalized difference vegetation indices and partial least squares regression. *Remote Sensing of Environment*, 86, pp. 542 - 553

Hunter, P.D., Tyler, A.N., Gilvear, D.J. and Willby, N.J. 2009. Using remote sensing to aid the assessment of human health risks from blooms of potentially toxic cyanobacteria. *Environmental Science and Technology*, 43(7), pp. 2627 - 2633

Jain, A.K. and Hayhoe, K.A.S. 2008. Handbook of atmospheric sciences 2: Problems, Tools and Applications, pp. 2 - 61

Jensen, S., Eriksson, G., Kylin, H., Strachan, W.M.J. 1992. Atmospheric pollution by persistent organic compounds: monitoring with pine needles. *Chemosphere*, 24, 229 - 245

Jha, D.K., Sabesan, M., Das, A., Vinithkumar, N.V. and Kirubagaran, R. 2011. Evaluation of Interpolation Technique for Air Quality Parameters in Port Blair, India. *Universal Journal of Environmental Research and Technology*, 1(3), pp. 301 - 310

Joshi, P.C. and Swami, A. 2007. Physiological responses of some tree species under roadside automobile pollution stress around city of Haridwar, India. *The Environmentalist*, 27(3), pp. 365 - 374

Josipovic, M., Annegarn, H.J., Kneen, M.A., Pienaar, J.J. and Piketh, S.J. 2010. Concentrations, distributions and critical level exceedance assessment of SO_2, NO_2 and O_3 in South Africa. *Environmental Monitoring and Assessment*, 171(1-4), pp. 181 - 196

Kooistra, L., Salas, E.A.L., Clevers, J.G.P.W., Wehrens, R., Leuven, R.S.E.W., Nienhuis, P.H. and Buydens, L.M.C. 2004. Exploring field vegetation reflectance as an indicator of soil contamination in river floodplains. *Environmental Pollution*, 127(2), pp. 281 - 290

Lillesand, T., Kiefer, R.W. and Chipman, J. 2014. Basic Principals of Photogrammetry. In *Remote Sensing and Image Interpretation*. New York: John Wiley & Sons

Lin, Q. and Mendelssohn, I.A. 2012. Impacts and recovery of the Deepwater Horizon oil spill on vegetation structure and function of coastal salt marshes in the northern Gulf of Mexico. *Environmental Science and Technology*, 46(7), pp. 3737 - 3743

Maatoug, M., Akermi, A., Taïbi, K., Achir, M. and Mestrari, M. 2012. Bio-Monitoring of Air Quality Using Leaves of Tree and Lichens in Urban Environments. In Khare, M. ed. *Monitoring, Modelling and Health.* Europe: *INTECH* Open Access Publisher, pp. 223 - 244

Manzo, C., Salvini, R., Guastaldi, E., Nicolardi, V. and Protano, G. 2013. Reflectance spectral analyses for the assessment of environmental pollution in the geothermal site of Mt. Amiata (Italy). *Atmospheric Environment*, 79, pp. 650 - 665

Martens, H. and Stark, E., 1991. Extended multiplicative signal correction and spectral interference subtraction: new preprocessing methods for near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, 9(8), pp.625 - 635

Martin, M.H., Duncan, E.M. and Coughtrey, P.J. 1982. The distribution of heavy metals in a contaminated woodland ecosystem. *Environmental Pollution Series B, Chemical and Physical*, 3(2), pp. 147 - 157

Matooane, L. and Diab, R.D. 2001. Air pollution carrying capacity in the South Durban Industrial Basin: research in action. *South African Journal of Science*, 97(11 and 12), pp. 450 - 453

Matooane, M. and Diab, R. 2003. Health risk for sulphur dioxide pollution in South Durban, South Africa. *Archives of Environmental Health: An International Journal*, 58(12), pp. 763 - 770

Mehmood, T., Liland, K.H., Snipen, L. and Sæbø, S. 2012. A review of variable selection methods in partial least squares regression. *Chemometrics and Intelligent Laboratory Systems*, 118, pp. 62 - 69

Mishra, D.R., Cho, H.J., Ghosh, S., Fox, A., Downs, C., Merani, P.B., Kirui, P., Jackson, N. and Mishra, S. 2012. Post-spill state of the marsh: Remote estimation of the ecological impact of the Gulf of Mexico oil spill on Louisiana salt marshes. *Remote Sensing of Environment*, 118, pp. 176 - 185

Mitchell, J.J., Glenn, N.F., Sankey, T.T., Derryberry, D.R. and Germino, M.J. 2012. Remote sensing of sagebrush canopy nitrogen. *Remote Sensing of Environment*, 124, pp. 217 - 223

Nansen, C., Macedo, T., Swanson, R. and Weaver, D.K. 2009. Use of spatial structure analysis of hyperspectral data cubes for detection of insect-induced stress in wheat plants. *International Journal of Remote Sensing*, 30(10), pp. 2447 - 2464

Nascimbene, J., Tretiach, M., Corana, F., Schiavo, F.L., Kodnik, D., Dainese, M. and Mannucci, B. 2014. Patterns of traffic polycyclic aromatic hydrocarbon pollution in mountain areas can be revealed by lichen biomonitoring: a case study in the Dolomites (Eastern Italian Alps). *Science of the Total Environment*, 475, pp. 90 - 96

Newete, S.W., Erasmus, B.F., Weiersbye, I.M., Cho, M.A. and Byrne, M.J. 2014. Hyperspectral reflectance features of water hyacinth growing under feeding stresses of *Neochetina* spp. and different heavy metal pollutants. *International Journal of Remote Sensing*, 35(3), pp. 799 - 817

Peerbhay, K.Y., Mutanga, O. and Ismail, R. 2013. Commercial tree species discrimination using airborne AISA Eagle hyperspectral imagery and partial least squares discriminant analysis (PLS-DA) in KwaZulu–Natal, South Africa. *Journal of Photogrammetry and Remote Sensing*, 79, pp. 19 - 28

Pérez-Enciso, M., Tenenhaus, M. 2003. Prediction of Clinical Outcome with Microarray Data: A Partial Least Squares Discriminant Analysis (PLS-DA) Approach. *Human Genetics*, 112, pp. 581 - 592

Pooley, E. 1993. The complete field guide to trees of Natal, Zululand and Transkei, 3rd Edition. Durban: Natal flora publications trust, pp. 204 - 205

Ra, H.S.Y., Geiser, L.H. and Crang, R.F.E. 2005. Effects of season and low-level air pollution on physiology and element content of lichens from the US Pacific Northwest. *Science of the Total Environment*, 343(1), pp. 155 - 167

Rai, R., Rajput, M., Agrawal, M. and Agrawal, S.B. 2011. Gaseous air pollutants: A review on current and future trends of emissions and impact on agriculture. *Journal of Scientific Research*, 55, pp. 77 - 102

Sawidis, T., Breuste, T., Mitrovic, M., Pavlovic, P. and Tsigaridas, K. 2011. Trees as bioindicators of heavy metal pollution in three European countries. *Environmental Pollution*, 159, pp. 3560 - 3570

Seyyednejad, S.M., Niknejad, M. and Yusefi, M. 2009. The effect of air pollution on some morphological and biochemical factors of *Callistemon citrinus* in petrochemical zone in South of Iran. *Asian Journal of Plant Sciences*, 8(8), pp. 562 - 565

Smith, K.L., Steven, M.D. and Colls, J.J. 2004. Use of hyperspectral derivative ratios in the red-edge region to identify plant stress responses to gas leaks. *Remote Sensing of Environment*, 92(2), pp. 207 - 217

Smith, K.L., Steven, M.D. and Colls, J.J. 2005. Plant spectral responses to gas leaks and other stresses. *International Journal of Remote Sensing*, 26(18), pp. 4067 - 4081

Splajt, T., Ferrier, G. and Frostick, L.E. 2003. Monitoring of landfill leachate dispersion using reflectance spectroscopy and ground-penetrating radar. *Environmental Science and Technology*, 37(18), pp. 4293 - 4298

Taylor, G.E., Johnson, D.W. and Andersen, C.P. 1994. Air pollution and forest ecosystem: A regional to global perspective. *Ecological Applications*, 4, pp. 662 - 689

Thambiran, T. and Diab, R. D. 2011. Air quality and climate change co-benefits for the industrial sector in Durban, South Africa. *Energy Policy*, 39, pp. 6658 - 6666

Tiwari, S., Agrawal, M. and Marshall, F.M. 2006. Evaluation of ambient air pollution impact on carrot plants at a sub urban site using open top chambers. *Environmental Monitoring and Assessment*, 119, pp. 15 - 30

Tripathi, A.K. and Gautam, M. 2007. Biochemical parameters of plants as indicators of air pollution. *Journal of Environmental Biology*, 28, pp. 127 - 132

Ullah, S., Skidmore, A.K., Naeem, M. and Schlerf, M. 2012. An accurate retrieval of leaf water content from mid to thermal infrared spectra using continuous wavelet analysis. *Science of the Total Environment*, 437, pp. 145 - 152

Varshney, C.K., Garg, J.K., Lauenroth, W.K. and Heitschmidt, R.K. 1979. Plant responses to sulfur dioxide pollution. *Critical Reviews in Environmental Science and Technology*, 9(1), pp. 27 - 49

Veselkin, D.V. 2004. Anatomical structure of ectomycorrhiza in *Abies sibirica* Ledeb. and *Picea obovata* Ledeb. under conditions of forest ecosystems polluted with emissions from copper-smelting works. *Russian Journal of Ecology*, 35(2), pp. 71 - 78

Winner, W.E. 1994. Mechanistic Analysis of Plant responses to air pollution. *Ecological Application*, 4, 471- 491

Wittig, R., 1993. General aspects of biomonitoring heavy metals by plants. *Plants as Biomonitors*, pp. 3 - 27

Wold, S., Sjostrom, M., Eriksson, L. 2001. PLS-Regression: A Basic Tool of Chemometrics. *Chemometrics and Intelligent Laboratory Systems*, 58, pp. 109 - 130

Wu, Y.Z., Chen, J., Ji, J.F., Tian, Q.J. and Wu, X.M. 2005. Feasibility of reflectance spectroscopy for the assessment of soil mercury contamination. *Environmental Science and Technology*, 39(3), pp. 873 - 878

Wuytack, T., Wuyts, K., Van Dongen, S., Baeten, L., Kardel, F., Verheyen, K. and Samson, R. 2011. The effect of air pollution and other environmental stressors on leaf

fluctuating asymmetry and specific leaf area of *Salix alba* L. *Environmental Pollution*, 159, pp. 2405 - 2411

Yamamoto, H., Yamaji, H., Abe, Y., Harada, K., Waluyo, D., Fukusaki, E., Kondo, A., Ohno, H. and Fukuda, H. 2009. Dimensionality reduction for metabolome data using PCA, PLS, OPLS, and RFDA with differential penalties to latent variables. *Chemometrics and Intelligent Laboratory Systems*, 98(2), pp. 136 - 142

Yi, Q.X., Huang, J.F., Wang, F.M., Wang, X.Z. and Liu, Z.Y. 2007. Monitoring rice nitrogen status using hyperspectral reflectance and artificial neural network. *Environmental Science and Technology*, 41(19), pp. 6770 - 6775

Zhu, L., Zhao, X., Lai, L., Wang, J., Jiang, L., Ding, J., Liu, N., Yu, Y., Li, J., Xiao, N. and Zheng, Y. 2013. Soil TPH concentration estimation using vegetation indices in an oil polluted area of eastern China. *PloS one*, 8(1), p.e54028

Zhu, L., Chen, Z., Wang, J., Ding, J., Yu, Y., Li, J., Xiao, N., Jiang, L., Zheng, Y. and Rimmington, G.M. 2014. Monitoring plant response to phenanthrene using the red edge of canopy hyperspectral reflectance. *Marine Pollution Bulletin*, 86(1), pp. 332 - 341

CHAPTER FOUR

CONCLUDING REMARKS AND RECOMMENDATIONS

4.1 Major findings

Sulphur dioxide (SO_2) has previously been used as a proxy for industrial air pollution within the South Durban basin (SDB) (Diab and Motha, 2007), and in other parts of the world (Emberson *et al.,* 2001; Carmichael *et al.,* 2003). Results of the present study showed sulphur dioxide concentration ([SO_2]) within the SDB to be relatively high by global standards (Carmichael *et al.* 2003; Josipovic *et al.* 2010); with the Southern Works study site exhibiting the highest SO_2 levels (in terms of annual averages). However, this data suggest that [SO_2] vary, both temporally and spatially within the SDB, as reported by other authors (Matooane and Diab, 2001; Diab *et al.,* 2002; Matooane and Diab, 2003). It should also be noted that from the limited data available for other pollutants, areas of high [SO_2] may not necessarily overlap with areas of high NO_2 and PM pollution.

From the seven biomarkers compared in this study, five biomarkers (viz. leaf area, chlorophyll content, intracellular superoxide, intracellular hydrogen peroxide, and total aqueous antioxidants) were able to discriminate between SO_2 exposed and unexposed leaves. However, of these five biomarkers, only three were significantly correlated with seasonal SO_2 levels (viz. leaf area, chlorophyll content, and intracellular hydrogen peroxide). These three biomarkers were also sensitive enough to reflect differences in [SO_2] across the treatment sites. The results suggest that biomarker responses were strongly influenced by seasonal variations in [SO_2], which may have also been influenced by site topography.

Having shown in the first part of the study that leaf area and chlorophyll content may be reliable biomarkers of air pollution for *T. dregeana* leaves, these biomarkers were then related to hyperspectral data collected for SO_2 exposed and unexposed leaves of the same species. The results of the partial least squares regression (PLSR) analyses

indicated that both biomarkers could be reasonably well predicted using hyperspectral wavebands located in key regions of the electromagnetic spectrum. More specifically, the variable importance in projection (VIP) method was able to identify important wavebands within the red-edge region of the electromagnetic spectrum. This region has previously been depicted as a reliable indicator/predictor of plant stress (Carter and Knapp, 2001; Smith *et al.,* 2004; Manzo *et al*., 2013; Peerbhay *et al.,* 2013), and may thus serve as reliable indicators of pollution-induced stress in *T. dregeana* leaves.

4.2 Challenges and shortcomings

A major challenge faced during the study was the lack of data for pollutants other than SO_2, and furthermore, where SO_2 data were available, there were a number of either missing or erroneous data points. This limited the data available for this study to just three of the twelve monitoring stations (Prospecton, Ganges, and Southern Works) located in Durban. Whilst data for NO_2 and PM were available for some sites, these data were not available for all the sites selected in this study.

As a consequence, SO_2 was used as the sole proxy of industrial air pollution in the present study, and since pollutants almost always act on ecosystems in combination, this was not ideal. This may also explain why trees at sites such as Ganges, which did not exhibit the highest SO_2 levels, appeared to be more stressed than those at the site that did (viz. Southern Works), in terms of biomarkers such as leaf chlorophyll content and intracellular hydrogen peroxide.

Additionally, when sampling leaves, careful attention was given to avoid leaves with visible signs of deposition (bird droppings and dust) and damage (chlorosis or necrosis) (as recommended by Sawidis *et al*., 2011). However, this was unavoidable at certain treatment sites and could have influenced the overall results.

4.3 Recommendations for future studies

The results from this study highlight the need for screening various biomarkers (physiological, biochemical and morphological) when trying to establish a particular species as a bioindicator of air pollution (Lovett *et al.,* 2009). Based on the results, the use of hyperspectral data in monitoring air pollution levels, focussing on certain key

bioindicators, may be particularly useful in developing parts of the world, where instruments and labour-intensive air quality monitoring are not feasible or available. In this regard, the red-edge region may be useful in predicting pollution-induced plant stress, and hence, monitoring air pollution levels. The physiological links between leaf chlorophyll content and leaf area identified in this study and elsewhere (Assadi *et al.*, 2011; Areington *et al.,* 2015) should also be explored in the context of hyperspectral studies. This can be achieved by simultaneously predicting these biomarkers using the PLS-2 algorithm (Martens, 1991; Gendrin *et al.,* 2007) and derivative-based transformations (Sanches *et al.,* 2013). Future studies should also consider measuring chlorophyll fluorescence in combination with photosynthetic rates, as both parameters are intimately linked (Naidoo and Chirkoot, 2004). Biomarkers that should also be considered include: stomatal density (Ramlall *et al.,* 2015); heavy metal content (Sawidis *et al.,* 2001); and element/mineral concentration (Areington *et al.,* 2015) in leaves, which are also sensitive to pollution stress.

Inconsistencies in the relationships between specific biomarkers and [SO_2], across the treatment sites, does suggest further experiments are required. For this purpose, monitoring stations presently in place within eThekwini and surrounding municipalities need to be equipped to monitor more than just [SO_2]. Given the high levels of light and heavy vehicular traffic in areas like the SDB, carbon monoxide represents a priority pollutant.

Finally, the present study provides ample motivation for the establishment of *T. dregeana* leaves as a bioindicator of air pollution within the eThekwini Municipality, further refinement is needed in terms of the exact combination of biomarkers to be used. It is evident that hyperspectral remote sensing of biomarkers, such as leaf area and chlorophyll content, may provide valuable information on air pollution levels, within industrial areas such as the SDB.

References

Areington, C.A., Varghese, B., Ramdhani, S. and Naidoo, S.E. 2015. An assessment of morphological, physiological and biochemical biomarkers of industrial air pollution in the leaves of *Brachylaena discolor*. *Water, Air and Soil Pollution*, 226(9), pp. 1 - 14

Assadi, A., Pirbalouti, A.G., Malekpoor, F., Teimori, N. and Assadi, L. 2011. Impact of air pollution on physiological and morphological characteristics of *Eucalyptus camaldulensis* Den. *Journal of Food, Agriculture and Environment*, 9(2), pp. 676 - 679

Carmichael, G.R., Ferm, M., Thongboonchoo, N., Woo, J.H., Chan, L.Y., Murano, K., Viet, P.H., Mossberg, C., Bala, R., Boonjawat, J. and Upatum, P. 2003. Measurements of sulfur dioxide, ozone and ammonia concentrations in Asia, Africa, and South America using passive samplers. *Atmospheric Environment*, 37(9), pp. 1293 - 1308

Carter, G.A. and Knapp, A.K. 2001. Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany*, 88(4), pp. 677 - 684

Diab, R.D. and Motha, A., 2007. An analysis of key institutional factors influencing air quality in South Durban basin using the DPSIR framework. *South African Geographical* Journal, 89(1), pp. 22 - 33

Diab, R., Prause, A. and Bencherif, H. 2002. Analysis of SO_2 pollution in the South Durban Industrial Basin. *South African Journal of Science*, 98, pp. 543 - 546

Emberson, L.D., Ashmore, M.R., Murray, F., Kuylenstierna, J.C.I., Percy, K.E., Izuta, T., Zheng, Y., Shimizu, H., Sheu, B.H., Liu, C.P. and Agrawal, M., 2001. Impacts of air pollutants on vegetation in developing countries. *Water, Air, and Soil Pollution*, 130(1 - 4), pp. 107 - 118

Gendrin, C., Roggo, Y. and Collet, C. 2007. Content uniformity of pharmaceutical solid dosage forms by near infrared hyperspectral imaging: a feasibility study. *Talanta*, 73(4), pp. 733 - 741

Josipovic, M., Annegarn, H.J., Kneen, M.A., Pienaar, J.J. and Piketh, S.J. 2010. Concentrations, distributions and critical level exceedance assessment of SO_2, NO_2 and O_3 in South Africa. *Environmental Monitoring and Assessment*, 171(1-4), pp. 181 - 196

Lovett, G.M., Tear, T.H., Evers, D.C., Findlay, S.E., Cosby, B.J., Dunscomb, J.K. and Weathers, K.C. 2009. Effects of air pollution on ecosystems and biological diversity in the eastern United States. *Annals of the New York Academy of Sciences*, 1162(1), pp. 99 - 135

Manzo, C., Salvini, R., Guastaldi, E., Nicolardi, V. and Protano, G., 2013. Reflectance spectral analyses for the assessment of environmental pollution in the geothermal site of Mt. Amiata (Italy). *Atmospheric Environment*, 79, pp. 650 - 665

Martens, H. and Stark, E. 1991. Extended multiplicative signal correction and spectral interference subtraction: new preprocessing methods for near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, 9(8), pp. 625 - 635

Matooane, L. and Diab, R.D., 2001. Air pollution carrying capacity in the South Durban Industrial Basin: research in action. South African Journal of Science, 97(11 and 12), pp. 450 - 453

Matooane, M. and Diab, R. 2003. Health risk for sulphur dioxide pollution in South Durban, South Africa. *Archives of Environmental Health: An International Journal*, 58(12), pp. 763 - 770

Naidoo, G. and Chirkoot, D. 2004. The effects of coal dust on photosynthetic performance of the mangrove, *Avicennia marina* in Richards Bay, South Africa. *Environmental Pollution*, 127(3), pp. 359 - 366

Peerbhay, K.Y., Mutanga, O. and Ismail, R., 2013. Commercial tree species discrimination using airborne AISA Eagle hyperspectral imagery and partial least squares discriminant analysis (PLS-DA) in KwaZulu–Natal, South Africa. *Journal of Photogrammetry and Remote Sensing*, 79, pp. 19 - 28

Ramlall, C., Varghese, B., Ramdhani, S., Pammenter, N.W., Bhatt, A. and Berjak, P., 2015. Effects of simulated acid rain on germination, seedling growth and oxidative

metabolism of recalcitrant-seeded *Trichilia dregeana* grown in its natural seed bank. *Physiologia plantarum*, 153(1), pp. 149 - 160

Sanches, I.D., Souza Filho, C.R., Magalhaes, L.A., Quitério, G.C.M., Alves, M.N. and Oliveira, W.J. 2013. Assessing the impact of hydrocarbon leakages on vegetation using reflectance spectroscopy. *Journal of Photogrammetry and Remote Sensing*, 78, pp. 85 - 101

Sawidis, T., Breuste, T., Mitrovic, M., Pavlovic, P. and Tsigaridas, K. 2011. Trees as bioindicators of heavy metal pollution in three European countries. *Environmental Pollution*, 159, pp. 3560 - 3570

Sawidis, T., Chettri, M.K., Papaioannou, A., Zachariadis, G. and Stratis, J. 2001. A study of metal distribution from lignite fuels using trees as biological monitors. *Ecotoxicology and Environmental Safety*, 48(1), pp. 27 - 35

Smith, K.L., Steven, M.D. and Colls, J.J. 2004. Use of hyperspectral derivative ratios in the red-edge region to identify plant stress responses to gas leaks. *Remote Sensing of Environment*, 92(2), pp. 207 - 217