Exploring the fertiliser potential

of biosolids from

algae integrated wastewater treatment

systems

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ABSTRACT

High rate algae oxidation ponds (HRAOP) for domestic wastewater treatment generate biosolids that are predominantly microalgae. Consequently, HRAOP biosolids are enriched with minerals, amino acids, nutrients and possibly contain plant growth regulator (PGR)-like substances, which makes HRAOP biosolids attractive as fertiliser or PGR. This study investigated HRAOP biosolids as a starting material for a natural, cost-effective and readily-available eco-friendly organic fertiliser and/or PGRs. Various HRAOP extract formulations were prepared and their effect on plant growth and development was evaluated using selected bioassays. Initial screening included assessing the effect on change in specific leaf area, radish cotyledon expansion as an indicator of PGR-like activity, and seed germination index (GI). More detailed studies on fertiliser efficacy and PGR-like activity utilised bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants. Combined effects of sonicated (S) and 40 % v/v methanol (M) extract (5:1 SM) had impressive plant responses, comparable to Hoagland solution (HS). Other potentially fertiliser formulations included 0.5 % M, 1% M, 2.5% S and 5% S formulations. The 5:1 SM and 5% S showed greater PGR-like activity, promoting cotyledon expansion by 459 ± 0.02 % and 362 ± 0.01 %, respectively. GI data showed that none of the formulations negatively impacted germination. Further investigation showed that the 5 % S formulation increased leaf length, width and area by 6.69 ± 0.24, 6.21 ± 0.2 mm and 41.55 ± 0.2 mm^2. All formulated fertiliser extracts had no adverse effect on chlorophyll content and plant nutrient balance as indicated by C:N (8-10:1) ratio. In addition, plants appeared to actively mobilise nutrients to regions where needed as evidenced by a shift in shoot: root ratio depending on C, N and water availability. Furthermore, 5 % S caused a 75 % increase in tomato productivity and had no effect on bean productivity. Whereas, 5:1 SM and 1% M formulation improved bean pod production by 33.3 % and 11 %, respectively but did not affect tomato production. Harvest index (HI) however indicated a 3 % reduction in tomato productivity with 5:1 SM and little or no enhancement in bean productivity with both 5:1 SM and 5 % S treatments. Bean plants treated with 5:1 SM and 5 % S produced larger fruits, which could be an indication of the presence of a PGR effect. Overall, HRAOP biosolids extracts prepared and investigated in this study demonstrated both fertiliser characteristics and PGR-like activity with performances comparable and in some cases exceeding that of commercial products. However additional research is needed to confirm presence of PGR-like activities and fertiliser efficacy.

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"As much as I would like to believe this is the end of this, the inner me knows it is only the beginning"
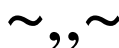
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List of abbreviations

°C	Degrees Celsius
A	Area
ASP	Algae settling pond
c.v.	Cultivar name
CE	Controlled Environment
CO_2	Carbon dioxide
d	days
DG	Anaerobic digestate
DW	Dry weight
DWA	Department of Water Affairs
E	Ethanol sonicated extract
EBRU	Environmental Biotechnology Research Institute Rhodes University
EC	Electric conductivity
et al	And others
EPA	European environment agency
F	Freeze thawed extract
GHG	Greenhouse gases
GI	Germination index
h	Hour
HI	Harvest index
HRAOP	High Rate Algae Oxidation Pond
HS	Hoagland solution
IAPS	Integrated Algae Pond System
L	Length
M	Methanol sonicated extract
mS/m	Milli Siemens per meter
PFP	Primary Facultative Pond
PGPR	Plant growth rhizobacteria
PGR	Plant growth regulator
RSA	Republic of South Africa
S	Sonicated Extract (using deionised water)
SLA	Change in specific leaf area
spp	Species
TOC	Total Organic Carbon
W	Width
WWTW	Wastewater treatment works

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Chapter 1

General introduction

1.1. Introduction

Food demands have increased drastically due to a substantial population growth; as a consequence there is a need for year round successful horticultural harvests (Shaviv & Mikkelsen, 1993; Tilman *et al*., 2002; Good & Beatty, 2011). This adds strain to crop production and pressure to produce more food per unit area of land (Tilman *et al*., 2002). Food demand needs to be accomplished even though there is an increasing scarcity of water resources, deteriorating arable lands, global climate change, changing food habits, and the use of crop biomass for the production of biofuels (Tilman *et al*., 2002; Pender *et al*., 2006; Bouman, 2007; Nahman, *et al*., 2009; Vitousek *et al*., 2009; Yang & Zhang 2010). Food security addresses the essential basic needs for health wellbeing and is influenced by natural resources and agricultural practices. For example, unfavourable environmental conditions during cultivation results in a lowered yield and higher food costs. Food production and costs are also based on resources, such as fossil fuel and mineral. Food security is in serious crisis in Sub-Saharan Africa and has manifested itself through the high numbers of chronically malnourished children (Vitousek *et al*., 2009). The challenge in sustainable food supply is linked to natural resources constrains, soil fertility, water availability and biodiversity (Tilman *et al*., 2002; Pender *et al*., 2006) and this threatens the socio economics in regions that rely heavily on horticulture (Tilman *et al*., 2002). Innovative ideas and research are needed to increase and sustain food supply and productivity (Tilman *et al*., 2002; Vitousek *et al*., 2009). In an effort to meet increasing food demands, crop production activities rely on fertiliser and manure applications which have resulted in a high fertiliser demand (Tilman *et al*., 2002; Wierderholt & Johnson, 2005). There has been a several fold increase in fertiliser production and consumption over the years which drastically influence the high pricing of fertilisers (Gelling & Parmenter, 2004). Therefore causing an increase in production costs and high priced food (Orhan *et al*., 2006; Gelling & Parmenter, 2004).

Increased demand in fertiliser application causes a serious strain and exhaustion of fertiliser resources (Vitousek *et al*., 2009, Good & Beatty, 2011). The demand is mainly driven by the

need for food, which in turn is driven by population size, declining availability of arable land and increasing wealth of emerging economies (Good & Beatty, 2011). However, most commercial fertilisers are derived either directly from petroleum (including natural gas), chemicals and through intensive fossil fuel consuming production processes. This practice poses a risk to the sustainability of agricultural development, food supply and the overall ecosystem (Tilman *et al*., 2002). Dependence on inorganic and fossil derived fertilisers cause soil to gradually lose its organic matter, alters microbial activity and eventually the soil structure deteriorates and it becomes compact, lifeless and less able to hold water and nutrients hence unsuitable for vegetation (Wani *et al*., 1995; Garcia *et al*., 2000; Gellings & Partmenter, 2004; Lalfakzuala, 2008). Prolong use of inorganic fertilisers increase soil acidity and disturb physio-chemical properties of soil. Excessive applications of chemical and inorganic fertilisers leads to salt accumulation, which then leach as runoffs, thereby contaminating surface and ground water (Garcia *et al*., 2000; Mohammadi *et al*, 2011). This practice is counter-productive and threatens the environment.

Fertiliser nutrients enter the ecosystem and water streams either by leaching and volatilisation (Tilman *et al*., 2002; Vitousek *et al*., 2009). This causes eutrophication (N and P major contributors), ammonia toxicity, nitrate contamination and microbial contamination and oxygen depletion in water bodies (Tilman *et al*., 2002; Wierderholt & Johnson, 2005). Intensive agriculture that is dependent on chemical and fossil derived fertilisers and synthetic plant growth regulators causes environmental harm, such as soil degradation, vulnerability to pests, pollution run-off and significantly contributes to greenhouse gases (GHG) emissions (European Environment Agency; EPA, 2010). Most commercial fertiliser production requires intensive fuel consumption; the energy may be derived direct from petroleum including natural gases or from fossil. These inevitably contribute to GHG emissions and influence the rising prices of fertiliser due to increasing fuel prices (Tilman *et al*., 2002).

Environmental impacts, intensive work, high energy requirement as well as high cost associated with chemical and fossil derived fertiliser production and application, necessitate investigation on alternative cost-effective renewable fertiliser resources. More research is needed to explore alternative natural cost-effective, easily accessible, eco-friendly resources to meet part of the fertiliser demand (Tilman *et al*., 2002; Mulbry *et al*, 2005; Vitousek *et al*., 2009) and to relieve the pressure on the inorganic fertilisers dependent (Abd El-Motty *et al*.,

2010). Meanwhile to minimise negative impacts perpetuated by inorganic fertilisers high demands, benefits of organic fertiliser application should be emphasised.

1.2. Organic fertilisers

Organic fertilisers are naturally occurring fertilisers and they differ from inorganic fertilisers in many aspects. Organic fertilisers contain complete nutrient essential for plant growth and development including other organic compounds that are beneficial to plants, such as humic acid and fulvic acid. Organic fertilisers are highly enriched with organic mineral which promotes healthier plant root development by allowing gaseous exchange (Egamberdiyeva, 2007; Lalfakzuala, 2008). Biofertilisers are categorised as organic fertiliser comprising a biological active microbial inoculants of bacteria, fungi or algae (Buresh *et al.*, 1997; Egamberdiyeva, 2007). These microbes colonise rhizosphere and/ or plants interior to promote growth by increasing availability of primary nutrients to the host plant. Biofertiliser stimulates plant growth through synthesis of plant growth and promotion of substances, such as plant hormones and vitamins. Symbiotic interactions occur between the biofertiliser and plant roots.

Therefore the use of biofertilisers containing beneficial microbes, instead of synthetic chemical, improves plant growth through the supply of plant nutrients and may help to sustain environmental health and soil productivity (O'Connell, 1992). This is achieved by the ability of these microbes to fix atmospheric N, solubilises phosphorus (P), decompose organic material or oxidise sulphur (S) in soil. Grouping of plant beneficial microbes is based on their nature and function and includes symbiotic nitrogen fixers (*Rhizobium* spp., Cyanobacteria Algae); asymbiotic free nitrogen fixers (*Azotobacter*, *Azospirillum,* etc.); mycorrhizae; plant growth rhizobacteria (PGPR); and phosphate solubilising microbes (Esitken *et al.*, 2005; Adholeya & Pant, 2007).

The need for converting to organic fertiliser has increased primarily because of the damages in the soil structure and environmental threats associated with chemical and fossil derived fertilisers. Organic fertilisers including biofertilisers are a better option to avoid pollution; they are eco-friendly, safe and cost-effective compared to chemical inorganic fertilisers and could be a partial replacement to reduce stress in fossil derived fertiliser production

(Egamberdiyeva, 2007, Lalfakzuala, 2008). Organic fertiliser provides protection to crops against diseases and drought which aids in achieving an increased crop yield (Lalfakzuala, 2008). Organic fertilisers are therefore viewed as economical and environment friendly. Traditional organic fertilisers are regarded as slow-release fertiliser which gradually releases nutrients as they slowly decompose (Wani *et al*., 1995; Gelling & Parmenter, 2004). The application of organic fertiliser for quicker planting to harvesting period may not be feasible which is one of the reasons the trend in chemical inorganic fertilisers applications have drastically increased worldwide (Wani *et al*., 1995; Gelling & Parmenter, 2004). In an effort to enhance the efficacy of organic fertilisers, the development of a stable plant nutrient supply involving a combination of organic fertiliser and chemical fertilisers has been taken into consideration (Abd El Moniem, *et al*., 2008; Chouliaras, *et al*., 2009). Organic fertiliser provides organic matter and minerals in the soil which increases moisture holding capacity and allows gaseous exchange through increased porosity. This promotes healthy root development and maximal nutrient absorption. Organic fertiliser helps to improve soil fertility and productivity and, to some extent, restores the natural soil structure. High organic matter in organic fertiliser increases soil moisture and nutrient holding capacity (Lalfakzuala, 2008, Egamberdiyeva, 2007). However, organic fertilisers have a low nutrient content per volume mass. Consequently, large quantities need to be applied in soils in order to obtain effective plant production.

1.3. Plant growth regulators

Plant growth regulators (PGRs) are synthetic hormonal substances which promote, inhibit or modify growth and development in plants by regulating seed growth effects, leaf formation, stem growth and overall plant growth (Saharan & Vehra, 2011). Plant naturally produce hormones in minute quantities which display their effects either at cellular, tissue or organism stage (Saharan &Vehra, 2011). These substances are an essential component for any plant growth as they mediate with internal and external signals regulating plant growth and development (Provasoli & Carlucci, 1974; Tarakhovkaya *et al*., 2007). Although PGRs are necessary for many aspects of plant growth and development, they however do not substitute plant nutrients as plants need nutrients for general growth and survival. PGRs enhance and manipulate plant productions. For examples, they are used to promote early flowering, fasten fruit ripening and in the production of seedless fruits.

The effect of PGR in a plant is dependent on the sensitivity of the targeted tissue and amount of substance (hormone) present. For example, in a seedling low auxins concentration may stimulate growth while an opposite effect is possible at higher concentration (Arshad & Frankenberger, 1991; Saharan & Vehra, 2011). Generally, auxins are applied at a high concentration to control weed, prevent immature fruit drop, leaf abscission, initiate flowering and fruit development. This indicates that PGR can be applied as a stimulant or inhibitor of specified plant physiology and/ or developmental stage (Saharan & Vehra, 2011). Tarakhovskaya *et al*. (2007) highlighted that there are 10 groups of hormone-like substances in higher plants and further reported the presence of auxin, ctyokinins and abscisic acid in green microalgae. Of the 10 hormones, auxin, cytokinin, gibberellin, ethylene and abscisic acid are recognised as major plant hormones (Table 1.1) (Tarakhoyskaya *et al*., 2007; Saharan & Vehra, 2011). Gibberellins are named after the fungus *Gibberella fujikuroi* that causes rice to grow abnormally tall. Hormone-like substances are present in algae, playing the same role as in higher plants (Provasoli & Carlucci, 1974; Stirk *et al*., 2002; Ördög *et al*., 2004; Tarakhovkaya *et al*., 2007). Arthur *et al.* (2003) reported that auxins and gibberellins found in algae extracts effectively increased fruit set and size in tomato, cucumber, aubergine and pepper plants.

Table 1.1: Five major plant hormone, biological pathway, synthetic location and physiological functions (modified from Tarakhoskaya *et al.,* 2007).

Name	Biological pathway	Synthetic location	Basic physiological action
Auxins (cell elongation)	Indole or trypophan	Leaf primordial, young leaves, developing fruits	Increase plasticity of plant cell wall, play a role in fruit development. Promotes initiation of lateral roots and new leaves, regulates elongation of stem and root cells. Induces cell elongation. Promote formation of lateral roots. Stimulate adventitious root development in a cut shoot, or shoot elongation or apical dominance, or differentiation of vascular tissue. Enhance root formation
Cytokinins (cell division + inhibits senescence)	Adenine modification (biochemically)	Root tips, young leaves, developing seeds	Promote cell division, morphogenesis, lateral buds development, delay senescence. Together with auxin: it stimulate cell division and differentiation, inhibit formation of lateral root. Play a role in root development. Help control and regulate, seeds germination, root development, nutrition uptake, plant tissue composition and seed and fruit set
Gibberellins (cell elongation + cell division - translated into growth)	Glyceraldehydes-3-phosphate	Young shoot tissues, developing seeds, epical portion of stem and roots	Induces cell division, cell elongation, breaks dormancy in buds and seeds, fasten (initiate) seed germination, have important effect on stem elongation, promotes flowering
Ethylene (promotes senescence, epinasty, and fruit ripening)	Methionine	Maturing fruit and stressed tissues	Fruit ripening, initiates stem elongation and bud development, inhibits flowering in most plants (promotes it in few plants), buds sex expressing, promotes senescence and abscission.
Abscisic Acid (abscission of leaves and fruits + dormancy induction of buds and seeds)	Carotenoids	Roots, expanding leaves, matured leaves and fruits	Slows growth, suppresses bud growth and promote leave senescence. Controls closing and opening of stomata, Together with auxin: it restrains root growth.

1.4. Arable soil and sustainable agriculture

For sustainable crop production, understanding of soil structure informs on land degradation which directly links to soil fertility and concomitant plant production levels (Tilman *et al.*, 2002). Good soil structure is characterised by fertility and organic matter content. Organic matter such as carbon (C) content, are easily degradable and subsequently increase soil microbial community and microbial activities in the soil (Marschner *et al.*, 2003; Mohammadi *et al.*, 2011). This encourages the biological processes and interactions in soil that favours crop production. Healthy soil structure has good water retention, which reduces the need for soil irrigation during plantation. Intensive frequent tillage, monoculture of grains, limited crop rotation, and excessive fertilisation escalate degradation of soil (Tilman *et al.*, 2002). Soil amendments and fertilisers are used in deteriorated soil to restore the soil's natural structure, improve its physical properties, fertility and productivity. Soil amendments could be synthetic, such as water soluble polymers and hydrogels, or natural, such as clay minerals, algae, green manure and organic manure (Falatah *et al.,* 1996; Mohammadi *et al.*, 2011). Natural amendments are generally the cost-effective options with lower environmental impacts. However, the large scale use of synthetic soil amendments is not feasible due to associated high costs and related environmental constrains (Falatah *et al.,* 1996).

1.4.1. Plant essential nutrients

Table 1.2 lists the non-mineral carbon (C), hydrogen (H) and oxygen (O) as well as macro nutrients Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) which are all required by plants in large amounts and micro nutrients are required by plants in small amounts (Uchida, 2009). About 95% of a plant weight is C, H, and O, and the remaining 5% is mineral nutrients. Plants take C, H, and O from the atmosphere, soil and water and the remainder from the soil solution. The N, P and K are primary nutrients for plant, ensuring plant survival, healthy growth, development and higher yields (Stockdale *et al.,* 1995; Vercesi, 2000; Chisti, 2006, Egamberdiyeva, 2007). Adequate supply of these elements is crucial for successful plant growth and increased yield. These elements can be modified to manipulate plant growth and production (Uchida, 2000). Nutrient bioavailability to plant is highly influenced by the physicochemical properties of growth media, e.g. soil, compost, vermiculite or aqueous matrix. Often a plant's biological process endpoints, such as germination, dry matter content, tissue nutrient and mineral content as well as shoot and root ratio, are used to measure nutrient bioavailability and toxicity.

Table 1.2: Shows 16 elements that are known to be important for plant growth and survival. N, P and K are primary with Ca, Mg and S secondary essential macro-nutrient.

IMPORTANT ELEMENTS FOR PLANT GROWTH AND SURVIVAL		
Mineral nutrient		**Non mineral nutrients**
Macro-	**Micro-**	
N ⎫	Iron (Fe)	Carbon (C)
P ⎬	Copper (Cu)	Hydrogen (H)
K ⎭	Chloride (Cl)	Oxygen (O)
Calcium (Ca)	Manganese (Mn)	
Magnesium (Mg)	Molybdenum (Mo)	
Sulphur (S)	Zinc (Zn) and Boron (B)	

1.4.1.1. Plant macronutrient role and production

Nitrogen is considered a prime essential plant nutrient for plant growth and accounts for approximately 1 – 5% dry weight of plants (Haque *et al*., 2001; Vance, 2001). For example in corn, 1.46% of the dry weight of the leaf is represented by N and 92.81% is represented by C, O and H and the remaining 5.73% of dry matter is composed of the other macronutrients (Bould, 1975). N is an important constituent of protoplasm and is also responsible for the production of enzymes, nucleoproteins, amino acid, amines, amino sugars, polypeptides, chlorophyll and promotes cell division (Barker & Bryson, 2007; Fageria & Moreira, 2011). Inadequate N supply or bioavailability during plants growth season retards growth (Haque *et al*., 2001; Fageria & Baligar, 2005). Consequently, N deficiency in crop plants significantly affects plant growth, development, and yield. Hence, N is regarded as a primary limiting nutrient in plant growth and yield (Stockdale *et al*., 1995). Adequate N enables rapid plant growth, leaf and forage crops quality improvements as well as fruits and seeds production increase. Primary plant N deficiency symptoms include growth and yellowing of older leaves (Stockdale *et al*., 1995; Haque *et al*., 2001). Less than 50% N applied as chemical fertiliser is recovered for most annual crops. The low N recovery is associated with loss of N compounds by leaching, denitrification, volatilisation, soil erosion and microbial mitigation (Fageria & Baligar, 2005). Nitrate ions are highly soluble in soil solution and can be easily lost by leaching or bacterial denitrification (Tilman *et al*., 2002; Vance, 2001), which causes ecosystem functions disturbances.

Nitrogen fertiliser production is energy intensive and involves Haber-Bosch process which produces synthetic ammonia. This requires high pressure and temperature to facilitate a reaction of N with H and an ammonia synthesising catalyst is used. The produced ammonia may be directly applied as fertiliser or refined to nitrite/ nitrate ions. Alternatively ammonia may react with oxygen to forms nitric acid, which may react with ammonia to produce ammonium nitrate fertiliser. During N-fertiliser production, a GHG Nitrous oxide (N_2O) is produced as a by-product (USEPA, 2010)

Phosphorus is another essential element in plants. Like N, P is an essential part of the process of plant photosynthesis and respiration (Vance, 2001). P is a component of nucleoproteins which are involved in the cell reproduction and division. P is mostly involved in plant physiological processes occurring from development to maturing phases which includes being component of compounds involved in metabolism of carbohydrates and enzymatic processes such as fruit ripening (Williamson *et al*., 2001; Abel *et al*., 2002; Lo´pez-Bucio *et al*., 2005). P enables transformation of solar energy into chemical energy and formation of sugars for proper plant maturation. Hence, P strengthens plant structure an increase plants resistance to diseases (Abel *et al*., 2002; Lo´pez-Bucio *et al*., 2005; Gouider *et al*., 2010,). Key benefits of P as a fertiliser includes; rapid growth, root development improvement, early plant maturity and ultimately higher crop yields. P deficiency is seen by purple young leaves, stunted growth and poor root development (Uchida, 2000; Lo´pez-Bucio *et al*., 2005). P is accessible to plant in form of orthophosphate ions (HPO_4^{2-}; H_2PO^{4-}). P bioavailability in soil and plant is greatly enhanced by phosphate solubilising bacteria. During the process bacteria produces metabolites which lowers the pH, which leads to phosphate release from organic and inorganic phosphorus compounds. Production of P-fertiliser is costly and energy demanding. Excessive amount of sulphuric acid (H_2SO_4) is used to convert rock phosphate into P-fertiliser known as superphosphate. Consequently, effluent from P-fertiliser producing industries is highly acidic (Gouider *et al*., 2010), and this has serious environmental implications in the ecosystems. Bacterial degradation of plant biomass and plant waste contributes to P level in the environment and this phosphate needs to be reclaimed for good use to miminise recurring P contamination to natural resources.

Although K is one of primary essential for plant survival, it does not form any vital organic compounds in the plant as done by N and P. However, K is vital for plant growth as an

enzyme activator for metabolism processes (Uchida, 2000). Therefore, K plays a role in enhancing biological enzymes activities for plant growth and survival. K also plays a role in plant photosynthesis, respiration, protein synthesis, fruits quality and resistance to diseases. K is critical in plant water usage regulation by controlling the opening and closing of leaf stomata as water is released to cool the plant (Uchida, 2000). K is mobile in plants and therefore it is absorbed in larger amounts than any other mineral element with exception of N and Ca. K is accessible to plants as K^+ (potash) which is readily soluble in water. Insufficient K supply in plants result in yellowing of leaves and dead spots on leaf lamina. Low K availability reduces the size and quality of seed and fruits produced. The most common manifestation of K deficient is chlorosis along the edges of leaves (leaf margin scorching), particularly older leaves. Efficient K uptake increases chlorophyll content which positively reflects in plant growth. Potassium is naturally sourced from soil minerals and organic materials, however fertiliser application are often the main supplier of K in plants during vegetative (Uchida, 2000). Importance of N, P and K in plant development has influenced the demand for their production and subsequently fertiliser pricing. Increased food demand as a result of population growth increase has also contributed to higher N and P-fertilisers costs. The use of N-fertilisers between 1960 and 1995 increased by seven-fold while P-fertilisers applications increased by 3.5 folds and are expected to increase by threefold by 2050 (Tilman *et al*., 2001). Since production and use of these fertilisers have been shown to contribute to serious environmental health implication, there is a need to shift a focus towards development of alternate low-cost, eco-friendly N and P sources. Algae biomass is one source that has not been fully exploited, particularly microalgae biomass.

1.5. Algae application in agriculture

Algae have biologically fixed nitrogen and secrete plant growth promoting substances and some secondary metabolites. Algae add organic matter in the soils, solubilise the insoluble phosphates and improve the physicochemical nature of the soil (Goyal, 1993). The use of algae biomass as an edible product is well known and recorded. However, more studies on wastewater treatment processes derived algae biomass are needed.

Agricultural application of macroalgae and seaweed is well established. Some seaweed and brown algae, such as Ecklonia maxima and *Ascophyllum nodosum* are now available commercially (Thirumaran *et al*., 2009, Craigie, 2010). Thirumaran *et al* (2009) reported

faster germination and plant development from seeds pre-treated with macroalgae extract. A good example is Kelpak, a plant growth regulator extracted from seaweed *Ecklonia maxima* containing natural plant hormones, such as auxin and cytokines (www.kelpak.co.za; Masny *et al*., 2004). In Canada the Acadian Seaplants Limited has been operational since 1981 producing biofertiliser from *Ascophyllum nodosum*. Nitrozyme is another commercial plant stimulant derived from *Ascophyllum nodosum* and has proven to effectively relieve stress, provide a more vigorous and healthy plant, which increases yields and profits. The active ingredient in Nitrozyme is cytokinins which enhances all stages of plant growth process (http://www. Newenglandturf.com/product/.nitrozyme.php). Application of microalgae as a fertiliser has not yet been fully explored (Begum *et al*., 2011). A great deal of research has focused on the potential of microalgae as raw material for biodiesel and methane production (Chisti, 2007; Deng *et al*., 2009; Mata *et al*., 2009). This is evident by the under development and applications of microalgae fertiliser in large agriculture regions or continents (Sangeetha & Thevantha, 2010). The use of both micro and macroalgae in edible products is well established (Chaumont, 1993; Borowitzka, 1999; Pulz & Gross, 2004; Spolaore, *et al*., 2006). Nevertheless some microalgae have been recognised as a renewable source of fertiliser and soil conditioner. Cyanobacteria (blue green algae) gained attention as fertiliser primarily because of their N-fixing nature (Vaishampayan *et al*., 2001; Moreno *et al*., 2003; Chisti, 2006). Application of cyanobacteria fertiliser, particularly in rice production, has been practiced for years in India, China and Ghana (Begum *et al*., 2011). Green microalgae have also been considered due to their plant essential nutrients composition, presence of amino acids, vitamins and plant hormones (Spoehr & Milner, 1949; Provasoli & Carlucci, 1974; El Fouly *et al*., 1992). A typical nutrient composition of *Chlorella vulgaris* is shown on Table 1.3 (El Fouly *et al*., 1992).

Table 1.3: Typical nutrient composition of green microalgae; *Chlorella Vulgaris* cell (source: El Fouly *et al*., 1992).

MAJOR COMPONENTS, AMINO ACIDS AND ELEMENT COMPOSITION OF MICROALGAE CELL EXTRACT			
Major components		**Macro elements (%)**	
Proteins	44.6%	N	7.10
Carbohydrates	12.8%	P	0.66
Fats	7.3%	K	2.15
		Ca	0.18
Amino acid (g/100 g protein)*	6.9	Mg	0.34
Arginine	2.0		
Histidine	3.2	**Micro elements (ppm)**	
Isoleucine	9.5	Fe	245.00
Leucine	6.4	Mn	131.20
Lysine	1.3	Zn	111.50
Methionine	5.5	Cu	28.00
Phenylalanine	5.3		
Threonine	1.5		
Tryptophan	7.0		
Valine			

In addition to N-fixing, cyanobacteria provide plants' partial tolerance to pesticides and fungicides and enable saline and alkaline soils reclamation (Booth, 1969; Adam, 1999). Booth (1969) observed that the value of seaweeds as fertilisers was not only due to their macronutrient content but also to the presence of trace elements (Fe, Cu, Zn, Co, Mo, Mn and Ni), growth promoting hormones, cytokinins, vitamins and amino acids. Rao (1991) reported an increased fruits' quality and yield when *Sargassum wightii* (seaweed) extract was applied as a foliar spray on *Zizyphus mauritiana*. Both seaweed and microalgae have high levels of organic matter which increases moisture and mineral holding capacity in rhizosphere. The application of seaweed as a soil conditioner in farmyard manure is an ancient practice; it has been commercialised since 1949 in Britain, France, Spain, Japan and China (Thirumaran *et al*., 2009).

As evident from the literature, most researchers have applied both seaweed and microalgae fertiliser extracts as a foliar feed (Bokil *et al*., 1974, Rao, 1991; Shabaan, 2001, Abd El-Moniem *et al*., 2004; Faheed & Abd-El Fattah, 2008; Craigie, 2010). Foliar feeding has some

disadvantages especially if the intention is to revitalise the nutrient depleted soil. Foliar feeding does not supply organic matter nor does it build up degraded soil (Tejada & Gonzalez, 2003; Davis, 2004). Foliar feeding is practically expensive particularly for intensive vegetation, due to the technicality involved with application; more input could be required per unit area or plant for nutrient sufficiency. There is also a possibility of an insufficient supply of macro essential nutrients and this necessitates the need to apply supplementary soil inputs (Davis, 2004). Application is limited by leaf surface area; several applications are therefore necessary to meet the required quantities (Tejada & Gonzalez, 2003). However, despite efficient nutrient absorption being possible, efficiency is not always achieved (Davis, 2004).

1.6. Project background

A pilot scale integrated algae pond system (IAPS) was constructed at the Institute for Environmental Biotechnology (EBRU) at Rhodes University for treating domestic wastewater (Rose *et al*., 2002; Wells, 2005). Wastewater treatment in the IAPS is light and temperature dependant and these influence algae activity (Oswald, 1960; Benemann & Oswald, 1988). Algae integrated systems are an advanced wastewater treatment system which effectively produces effluent acceptable for discharge (Oswald, 1988; Benemann & Oswald, 1996, Rose, 2002; Rose *et al*., 2002). Algae integrated systems enable the removal of organic matter, bacteria, nitrogen, phosphorus and potassium (Garcia *et al*., 2000; Rose *et al*., 2002; Horan *et* al., 2005; Brennan & Owende, 2009; Kumar *et al*., 2010) and are suitable for extreme growth conditions such as elevated pH and salinity (Rose *et al*., 2002). However, algal based treatment systems generate excessive amount of algae biomass. Dealing with biomass is often overlooked as an additional cost in this kind of wastewater treatment works (WWTW) (Horan, 1996; Mohale, 2003; Keirungi, 2006). This biomass requires proper disposal. Biomass is usually sent off site for disposal in landfills, where it may be needed to accelerate the microbiological, physical and chemical attenuation mechanisms responsible for decomposition of waste by providing moisture and methanogenic bacteria (Ross *et al*., 1992) or is recycled back to the treatment process. The latter being the loss of organic matter resource that can be harvested for valuable biotechnological beneficiation processes.

The EBRU IAPS consists of four ponds in series (Appendix A) (Rose *et al*., 2002; Wells, 2005). The system has the primary facultative pond (PFP) with an anaerobic fermentation pit

below ground, followed by two shallow high rate algae oxidation ponds (HRAOP) which are continuously mixed by a paddle wheel, and finally an algae settling pond (ASP) used to separate suspended algae prior to discharge of the treated water. The HRAOP is a polishing step in the water treatment process that has a high microalgae biomass concentration which comprises local species that feed on nutrients in wastewater decanted from PFP. Dominant algae species include *Chlorella* spp., *Pediastrum* spp., *Scenedesmus* spp. and *Micractinium* spp., which assimilate N and P from wastewater using solar energy and CO_2 to generate biomass (Rose *et al*., 2002; Horan *et al*., 2005; Wells, 2005; Brennan & Owende, 2009; Kumar *et al*., 2010). The IAPS is eco-friendly and cost-effective system that requires no addition of chemicals. It uses less energy when compared to conventional activated sludge systems and is relatively simple to operate (Rose *et al*., 2002; Wells, 2005). The CO_2 released from the system is counter balanced by the amount of CO_2 assimilated by algae during photosynthesis (Rose *et al*., 2002; Wells, 2005). Biosolids generated from the IAPS can beneficiate local communities in agriculture, as a soil amendment, broadcast fertiliser or as a foliar feed (Rose *et al*., 2002; Horan *et al*., 2005). Produced biosolids are pathogenic free due to extreme alkaline (pH = 9.5 ± 0.5) conditions present in the HRAOP (Rose *et al*., 2002; Wells, 2005). Apart from treated water which can be used for irrigation or industrial processes, the system generates other end-products such as, biogas (methane), fermentation feedstock, and material for use in brick making (Horan *et al*., 2005).

Possible useful applications of microalgae biomass are well documented in the literature. For example, as animal feed (poultry, fish and cattle), fertiliser, soil amendment and production of valuable pigments, amino acids, vitamins, polysaccharides and sugars as well as enzymes and bio-flocculants (Aaronson *et al*., 1980; Borowitzka, 1988; Pulz & Gross, 2004; Spolaore *et al*., 2006). Its use as compost or fertiliser remains attractive and can beneficiate local crop production, agricultural projects, nursery and municipal facilities (such as sport field). Such valorisation is feasible as the raw material is readily available. Local crop production projects could fulfil a multitude of roles and functions and could contribute to food security and a fight against poverty. One of these roles includes socio-economic stability through job creation, income generation, food provision and environmental improvement. Valorisation of the HRAOP biomass by-product could also afford proper wastewater biosolids disposal while providing a more environmentally friendly fertiliser thereby reducing the demand of fossil derived inorganic fertiliser.

However, use of microalgae generated from WWTW as raw material for fertiliser has some disadvantages which include high decomposition rate, metal content, pathogens and colonisation of soil surfaces by algae, impacts to human health, sanitary and toxicity. It is therefore clear that the use of algae biomass grown on domestic wastewater for human consumption needs to be looked at closely, although it is believed that no pathogens can survive rising pH in HRAOP

Normally, the recovery of nutrients from wastewater treatment is a very problematic exercise especially if these nutrients were to be used for something else. Typically in WWTW, P is precipitated, ammonia is volatilised, nitrite quickly denitrified and nitrogen recovery remains a problem (Benemann & Oswald, 1996). Seasonal variations affect metabolisms of wastewater microalgae and subsequently biomass nutrient concentrations (Tseng 1991; Johnson, 2010). Lower microalgae growth occurs during darker winter months. The amount of N and P immobilised by microalgae tends to be lower in late spring and greater in summer due to seasonal environmental changes, specifically light and heat (Tseng, 1991; Anderson, 2005; Johson, 2010). Wastewater influent also affects nutrient content and the composition of the microalgae.

Hypothesis

Biosolids generated during wastewater treatment in HRAOPs are enriched with minerals, macro and micro nutrients, and contain plant growth regulator-like substances and can therefore be effectively used either as a source of fertiliser or as plant growth regulators.

Aims and objectives

The aim of this study was to determine whether biosolids generated in a HRAOP during domestic wastewater treatment can be used as a starting material for the production of organic fertilisers and plant growth regulators.

Specific objectives included;

1. The preparation of extracts from biosolids harvested from the HRAOP of an IAPS treating domestic wastewater for use as either an organic fertiliser or organic plant growth regulator

2. The determination of the fertiliser and plant growth regulator potential of each preparation by bioassay

3. The development of a concept process for the production and formulation of the most successful organic fertiliser and/or plant growth regulator.

CHAPTER 2

Preparation and screening of microalgae extracts for fertiliser and plant growth regulatory activity

2.1 Introduction

Finding a fertiliser and/or plant growth regulator (PGR) that effectively supports growth and development of plants is a complex technical exercise. Chemicals, minerals and nutrient levels affect plant development, especially in the early developmental stages, i.e. germination and root development (Cheng & Chu, 2007) and in the later stages particularly during reproductive development: flowering and fruit/seed formation (Shaahan et al., 1999; James & Iersel, 2001). For many crops it is the successful completion of reproductive development that determines overall productivity and yield. Fertiliser is primarily used to provide the nutrients essential for plant growth and development and these are usually supplied at concentrations that do not exceed plant needs (Scoggins, 2005). Germination and sprouting are inhibited by toxins and the presence of excessive nutrient loads while certain heavy metals can adversely affect seedling establishment and plant vigour. Hence, germination index and seedling root length can be used as indicators of the presence of toxins. Organic fertilisers are typically derived from organic natural materials, like manure, compost, vermicompost, peat, microalgae, guano and natural mineral resources and are formulated primarily based on levels of essential macro elements, namely N, P, and/or K content as well as levels of organic compounds such as humic and fulvic acids to support plant growth and development (Egamberdiyeva, 2007; Lalfakzuala, 2008).

Plant hormones are naturally produced by plants in minute quantities and display their effects at either the cellular, tissue or organism level (Saharan &Vehra, 2011). These substances mediate internal and external signals regulating plant growth and development (Provasoli & Carlucci, 1974; Tarakhovkaya et al., 2007). Plant growth regulators (PGRs) include synthetic chemicals and pesticides used to manage plant production processes. Thus, while all plant hormones are regarded as PGRs, not all PGRs display hormonal effects. Nevertheless, PGRs do not supply nutrients for growth and survival and therefore do not substitute for fertilisers. Kelpak (Cape Town, RSA), and Nitrozyme (NewEnglandTurf, New Kingston USA) are one of commercial organic PGRs because of their inherent auxin and cytokinin like activities.

This chapter describes the harvesting of a biosolids from the high rate algae oxidation ponds (HRAOP) of an Integrated Algae Pond System (IAPS) treating domestic sewage. The preparation of various extracts for evaluation as potential fertilisers and/or for the delivery of PGR-like effects using several well-known bioassays.

2.2 Materials and methods

2.2.1 Production of biomass

Biosolids were harvested from the HRAOP, a component of the IAPS located at the Institute for Environmental Biotechnology, Rhodes University (EBRU), South Africa experimental field station, adjacent to the Belmont Valley Wastewater Treatment Works, Grahamstown (WWTW; 33° 19' 07" South, 26° 33' 25" East). The IAPS used in this study treats raw domestic wastewater which is taken from the WWTW. Two HRAOPs operate in series and are mixed continuously by paddle wheels and are populated by local microalgae species which include *Chlorella* spp., *Pediastrum* spp., *Scenedesmus* spp., *Micractinium* spp. as well as associated bacteria. The HRAOPs generate biosolids in the range 0.10 − 0.20 g/L dry weight (Johnson, 2010). Samples were collected from a point immediately after the paddle wheel of HRAOP B, just before the inlet. This point was found to be ideal for sampling of the highly concentrated diverse species of microalgae which are also well illuminated and actively growing. The HRAOP biosolids were harvested in April 2011, the average temperature was 28 ± 4 ℃ when microalgae productivity was averaged 0.16 g/L dry weight. Grab samples were collected in 50 L settling funnels and allowed to settle for 6 − 7 hours (h), thereafter; the settled slurry was decanted into a container and taken to the laboratory for further concentration. Cell integrity of the collected slurry was established by light microscopy. The slurry was further concentrated by centrifugation at $8\,000 \times g$ for 15 min (Beckman Coulter Avante J-E centrifuge, AJ rotor) and the pellet frozen and vacuum dried using a freeze drier (Vir-Tis Benchtop SLC) to preserve microalgae biomass according to Schumann *et al.* (2005). Dried HRAOP biosolids were stored in air-tight plastic bags at -20 ℃ until required.

2.2.2. Analysis of biomass

The harvested HRAOP biosolids were analysed for elemental content and composition, pH, electrical conductivity (EC), and ash content (total inorganic content). Macro elemental (C,

H, N and S) analysis of the dried HRAOP biosolids was performed using an Elementar CHNS analyser (Vario MICRO Cube). Dried HRAOP biosolids was crushed using a mortar and pestle and 5 – 10 mg was mixed with the oxidiser vanadium pentoxide (V_2O_5) and combusted at 1000 °C. The analyser separated and detected combustion gas products CO_2, H_2O, N_2, and SO_2 using helium as a carrier gas. The CHNS element content was reported as weight percentage (%). The pH and EC were determined by re-suspending 20 g dried HRAOP biosolids in 100 mL deionised water. The mixture was shaken for 2.5 h at 125 rpm and the pH and EC measured using a portable pH 330 meter (WTW 82362, Germany) and EC meter (OAKTON EC Testr 11, Eutech Instruments). Ash content was determined by combustion of 1g of dried HRAOP biosolids at 600 °C for 6 h in the Carbolite muffle furnace and expressed as (% DW). Results are presented in Table 2.1.

Table 2.1: Characteristics of HRAOP biosolids harvested when microalgae productivity was of 0.016 g/L. pH and EC were measured from 20 g dried biomass suspended in 100 mL deionised water, ash and organic content were derived from 1 g dried biomass.

	pH	EC (mS.m^{-1})	Ash Content (%)	Organic Content (%)	CHNS (wt %)	Species composition
HRAOP biosolids	8.2	3.6	23.00	77.00	C (35.29) H (6.12) N (5.65) S (0.55)	*Chlorella* spp. *Pediastrum* spp. *Scenedesmus* spp. *Micractinium* spp. *Pyrobotrys* spp. Diatoms (*cyclotera*) *Closterium* spp Unknown bacterial spp.

2.2.3. Preparations of extracts

2.2.3.1. Passive freeze-thaw

Freeze thaw has widely been used to passively rupture cells of various microorganisms (Shaaban, 2001; Horan *et al.*, 2005; Abd El Moniem & Abd-Allah, 2008). Passive rapture method used in this study was adopted from that described by Abd El Moniem & Abd-Allah (2008). Dried HRAOP biosolids (10 g) were re-suspended in 100 mL deionised water and shaken at 125 rpm for 2 h at room temperature. The suspension was then frozen at -20 °C overnight and subsequently thawed to room temperature. To remove sediments and particulate material, extracts were centrifuged at 22 000 × g for 15 min (Beckman Coulter Avante J-E centrifuge, AJ rotor) and filtered through GF/A filter paper. The pellets were re-extracted using 100 mL deionised water and the combined extracts obtained were pooled to constitute the freeze thawed extract (F), and stored at 4 °C.

2.2.3.2. Sonication

Cell poration, lysis and shear of biological macromolecules and tissues were carried out using a sonication technique (Show *et al.*, 2007; Zhang *et al.*, 2008). A metal probe sonicator was used for active disruption of biosolids. A 10g dried HRAOP biosolids sample was re-suspended in 100 mL deionised water and shaken for 2 h at 125 rpm prior to sonication. Cells were ruptured using the ultrasonic dis-membrator (Fischer Scientific model 500) at 75 % amplitude for 40 min. Sonication is an active thermo-generating process (Zhang *et al.*, 2008). Therefore to avoid heating of samples which can potentially affect properties of the extract, the samples were placed in the glass basin filled with ice cold water. The slurry was centrifuged at 22 000 × g for 15 min (Beckman Coulter Avante J-E centrifuge, AJ rotor) and the supernatant filtered using GF/A filter paper. Pellets were re-suspended and the process of extraction repeated and the combined extracts obtained were pooled to constitute the sonicated extract (S), and stored at 4 °C.

2.2.3.3.   Solvent extraction

Dried HRAOP biosolids (10 g) were re-suspended in 100 mL of either 40 % ethanol or 40 % methanol. The extracts were prepared using the same procedure as described in section 2.2.3.2. After solvent evaporation (autoclaving at 121°C for 15 min), constituted the ethanol and methanol extracts referred to as (E) and (M) respectively. These extracts were also stored at 4 °C.

2.2.4. Characteristics of prepared extracts

Prepared extracts were analysed for pH, EC and ash content before and after diluting to desirable concentrations. Shelf-life of the undiluted extracts was also noted to establish how long the extracts can remain viable when stored at 4 °C. This was achieved by looking at the colour and odour change of the prepared extracts.

2.2.5. Bioassays

2.2.5.1. Specific leaf area

Phaseolus vulgaris L. cv. contender seeds were purchased from the local seed supplier. Even sized seeds were selected and then surface sterilised with 5 % sodium hypochlorite (NaOCl) for 30 min and rinsed thoroughly with deionised water. The seeds were then blotted with paper towel and immediately planted in seedling trays containing 500 g vermiculite which was moistened with 500 mL of half strength Hoagland solution (see Appendix B). Seeds were germinated under controlled environment conditions at 24 ± 2 ℃ and 230 $\mu mol.m^{-2}.s^{-1}$ of cool white light (12 h light cycle) and irrigated daily with Hoagland solution until they provided a desirable size for use to conduct the bioassay experiment.

Mature seedlings (14 days) were transplanted into 100 mL flasks containing 60 mL deionised water and 5 g vermiculite. A three day plant adaptation period was allowed to ensure that the plants were in good physiological state before addition of any treatment. On day 4 the water level was adjusted to the initial level in all flasks with deionised water. This was followed by the addition of 30 mL of treatment solutions per flask. Treatment solutions included different dilutions of the prepared extracts, Hoagland solution (HS), commercial organic PGR (Kelpak; KP) and deionised water (no treatment or 0% treatment) as a control. The KP was used as a known PGR to compare with each extract. Since HS is a scientific tested plant nutrient solution, it was used as a benchmark to evaluate fertiliser potential by providing balanced nutritional requirement for all treatments (Hoagland & Arnon, 1938). Preliminary tests were performed to determine the appropriate sensitivity range for the bioassay. The F and S dilutions ranged between 25 and 0.5 % while E and M dilutions ranged from 5 to 0.25 % range. Each treatment comprised three flasks, each with one seedling. Length (L) and width (W) of the first two true leaves was measured as the initial reading (Y_o). This measurement was repeated on days 3, 4, 5, 6, and 7 and the readings were recorded as Y_x. Mean values of the percentage change (% Δ) in L, W and area (A) were used to extrapolate

the relative change in plant growth according to equation 1. [Y_x = L, W or A on a specific day and Yo = L, W or A on day zero]

$$\% \Delta = \frac{Yx - Yo}{Yo} \times 100 \qquad\qquad \text{Eq. 1}$$

2.2.5.2. Radish cotyledon expansion

To establish whether prepared extracts have potential PGR activity, the radish cotyledon expansion bioassay modified from Letham (1971) and Hong *et al.* (2009) was used. Radish has a high sensitivity to heavy metals and is widely used as a model plant for toxicity of various contaminants (Forbes *et al.*, 2006). Even sized radish seeds (*Raphanus sativa* L. cv. Cherry Belle) were surface sterilised with 0.5 % NaOCl for 5 min. Seeds were thoroughly rinsed with deionised water and incubated at 26 ± 1 °C for 44 h in total darkness in sterile petri dishes containing 8 mL deionised water absorbed onto a Whatman No 2 filter paper as detailed by Letham (1971).

After 44 h, the inner cotyledon was carefully excised using a sterile blade. Thereafter, it was weighed and three inner cotyledons were placed in sterile petri dishes containing 8 mL of HS and treatment solution (1:1 v/v). This was duplicated for every treatment including controls (HS and deionised water). Anaerobic digestate (DG) used in this study was from brown marine algae *Ascophyllum nodosum* residuals. *Ascophyllum nodosum* had been studied for its effects on plant growth regulation due to its high content of cytokinins thus making it an attractive plant growth stimulant (Masny *et al.*, 2004; Norrie & Keathley, 2006). All petri dishes were incubated in controlled environment at 27 ± 2 ºC with continuous illumination of 636 µmol m^{-2}s^{-1}. After 72 h, the cotyledons were blotted and weighed. Percentage change in fresh weight was calculated according to equation 2.

$$\Delta \textbf{ fresh weight } (\%) = \frac{final\ fresh\ weight - initial\ fresh\ weight}{initial\ fresh\ weight} \times 100 \qquad \text{Eq. 2}$$

2.2.5.3. Seed germination

Seed germination was monitored according to a modified method from that described by Zucconi *et al*., (1981). Germination Index (GI) is considered a sensitive measurement of plant survival as it combines germination and root growth, (Zucconi *et al*., 1981; Tam & Tiquia, 1994). Tomato seeds (*Solanum lycopersicum* Mill. cv. Moneymaker), and radish (*Raphanus sativa* L. cv. Cherry Belle) were purchased from a local seed supplier. Seeds were surface sterilised with 0.5 % NaOCl for 10 and 5 min respectively. Seeds were then rinsed thoroughly with deionised water, blotted with paper towel, and 10 seeds of even size were placed in sterilised petri dishes containing 8 mL of deionised water and treatment solution (1:1 v/v). HS (Appendix B) and deionised water were used as control treatments. This was duplicated for each treatment. The petri dishes were covered with aluminium foil and incubated at 26 ± 1 °C in total darkness for 7 days as described by Zucconi *et al*. (1981). Petri dishes were examined daily from day 2 of incubation to record the number of germinated seeds and thereafter immediately sealed and returned to the incubation chamber. On day 7, the length of the radicle (roots length excluding shoot) was measured. The GI was calculated by determining the number of germinated seeds and the average length of roots for the treatment against that of untreated controls (deionised water) according to equation 3 (Zucconi *et al*., 1981).

$$GI = \frac{number\ of\ seeds\ germinated\ in\ sample}{number\ of\ seeds\ germinated\ in\ control} \times \frac{average\ root\ length\ in\ sample}{average\ root\ length\ in\ control} \times 100 \qquad \textbf{Eq 3}$$

2.2.6. Data and statistical analysis

Data is presented as the mean value $\pm$ standard deviations (SD). Where possible, experiments were repeated more than once in a randomised design. Data were analysed using Microsoft Office Excel 2010 where mean values and SD were calculated. Statistical analyses for this study were performed using Statistica 9.0 (StatSoft Inc, 2010) where *t*-tests were carried out to determine significant differences ($p < 0.05$) between treatments. Line and bar graphs were created using Microsoft Office Excel 2010 to establish the effect of treatments on bean plant specific leaf area and radish cotyledon expansion. Relationship and linkage between formulations were established using a cluster analysis (Primer 6 (PRIMER-E Ltd, Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, United Kingdom), whereby data were pre-treated by normalising. This was done to minimise data variability,

thereby making it possible to derive sensible distances between samples (Clarke & Warwick, 2001).

2.2. Results

2.3.1. Characteristics of the IAPS biomass and prepared extracts

Harvested biosolids were processed further to obtain refined extracts rich in nutrients. Freeze thaw and sonication processes were used to disrupt cells. Sonication generated extracts that were more effective and promising for use as fertiliser preparations. Organic solvents were added to dry HRAOP biosolid prior to sonication to enhance extraction of alcohol-soluble PGR-like compounds. All prepared extracts had close to neutral pH, low EC and ash content (Table 2.2). Average pH was 7.5 and 7.8 for F and S, respectively. The F extract recorded higher EC value of 17.2 ± 0.14 mS.m^{-1} whilst S extract had EC of 3.65 ± 0.07 mS.m^{-1}. As expected, E and M had an average pH of 6.8, EC of 6.23 ± 0.11 mS.m^{-1} and comparable ash content. Dilutions were prepared by adding either 5 mL, 2.5 mL, or 1 mL of extract to an amount of deionised water to give 100 mL and the mixture had 5:1 (v/v) and 1:1 (v/v) of S and E/M respectively. Shelf-life of undiluted extracts at 4 °C was approximately 65 days. Shelf-life measurements for the diluted formulations were not captured as these were prepared immediately before use.

Table 2.2: Characterisation of extract preparations, showing pH, EC, ash content (mean values ± SD) and shelf-life [n/a = not applicable].

Extract preparations	pH	EC (mS.m^{-1})	Ash Content (%)	Shelf life at 8℃ (weeks)
F	7.50 ± 0.33	17.2 ± 0.14	0.55 ± 0.00	± 5
S	7.72.0 ± 0.63	3.65 ± 0.07	1.65 ± 0.01	± 5
E	6.81 ± 0.02	6.30 ± 0.00	1.29 ± 0.01	± 5
M	6.77 ± 0.24	6.15 ±0.07	1.10 ± 0.01	± 5
5% F	6.97 ± 0.03	-	-	n/a
2.5 % F	6.91 ± 0.06	-	-	n/a
5 % S	6.89 ±0.03	0.13 ± 0.00	0.67 ± 0.01	n/a
2.5 % S	6.28 ± 0.06	-	-	n/a
1% E	6.57 ± 0.29	-	-	n/a
0.5 % E	6.36 ± 0.22	-	-	n/a
0.25 % E	6.15 ± 0.07	-	-	n/a
1% M	6.58 ±0.16	0.02 ± 5.77	0.33 ± 0.01	n/a
0.5 % M	6.41 ± 0.15	-	-	n/a
0.25 % M	6.01 ± 0.12	-	-	n/a
5:1 SE	6.62 ± 0.35	-	-	n/a
1:1 SE	6.66 ± 0.08	-	-	n/a
5:1 SM	6.83 ±0.05	0.16 ± 0.00	0.00	n/a
1:1 SM	6.55 ± 0.08	-	-	n/a

2.3.2. Effect of extracts on specific leaf area

Prepared extracts were tested for their fertiliser potential and PGR activities. The investigation however focused on fertiliser potential as the first line screening and selection of effective formulation. In this regards, change in specific leaf area (SLA) of bean plants was used as a rapid bioassay to demonstrate the ability of the extract to improve growth. Attained plant growth results were used as a model to simulate plant relative growth rate. Figure 2.1 shows bean plants response to HRAOP biosolids extracts in comparison with HS, KP and untreated controls. Response was measured as percentage change in leaf area for the

first true plant leaves on days 3, 4, 5, 6 and 7. Figure 2.1 represents the effect of each extract (F, S, E, M, S: E, and S: M) at different ratios on bean plant change in specific leaf area (SLA), labelled as A, B, C, D, E and F respectively.

Leaf lamina surface area was greatly increased when S, M and E extracts were used. As shown in Figure 2.1 B, highest percentage increase in leaf area was obtained with the 5 % S and 2.5 % S extracts and these results were comparable to KP and for the first 5 days of HS treatment. However significant increase ($p < 0.05$) in leaf area was noted from day 6 of HS treatments when compared with both 5% S and 2.5% S (Figure 2.1.B). In comparison to KP and HS, no increase in leaf area was recorded when a mixture of E and S at either 1:1 (v/v) or 5:1 (v/v) was used. However, significant increase ($p < 0.05$) in leaf area of SE (1:1) treatment on day 5, 6 and 7 when compared with non-treated (0%) plants (Figure 2.1 E). Overall performance of the M extract was better when compared to E. As shown in Figure 2.1 D, plants treated with 1 % M exhibited a 52.9 % change on leaf area on day 4. A good performance was observed with the mixture of 5:1 SM showing results closely comparable to HS and KP on day 5 which were 70.6, 60.1 and 64.4 % respectively (Figure 2.1 F).
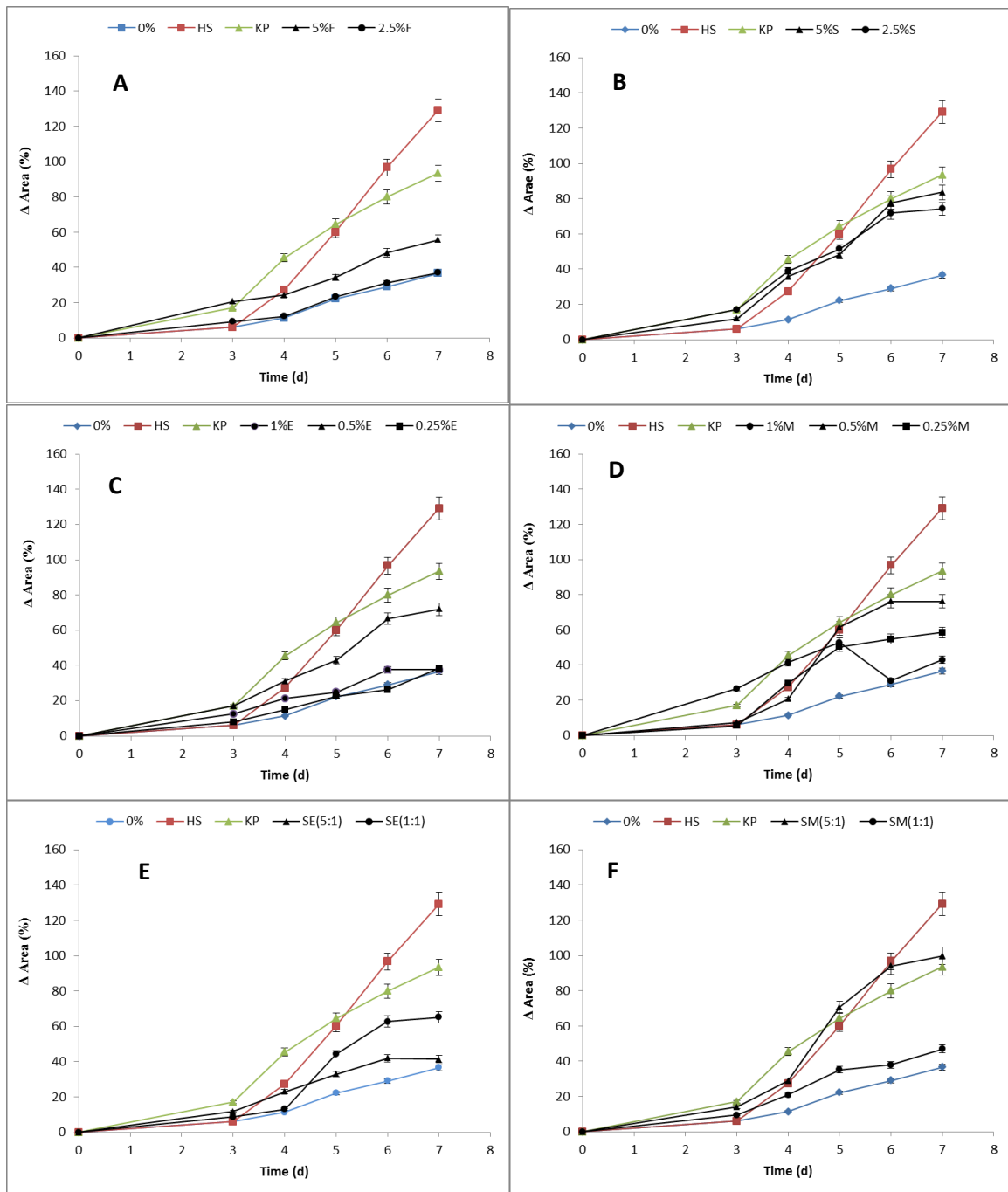
Figure 2.1: Change in specific leaf area [Δ Area (%)] of the linear surface for the first true leaves of bean plants on day 3, 4, 5, 6, 7 treated with extracts of HRAOP biosolids compared to non treated (0 %), Hoagland solution (HS) and Kelpak (KP) controls.

Multivariance cluster analysis was used to logically select best performing extract formulations to be studied further. Extracts were grouped according to their performances in effectively improving SLA of bean plants (Figure 2.2). Cluster analysis assesses data and classifies a sample of subjects according to a set of measured variables into a number of different groups. During this analysis treatments start in their own separate cluster. Then,

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most similar clusters are combined and this is done perennially until all subjects are in one cluster. This leads to groupings of treatments with similar effects being placed close together in a cluster. In Figure 2.2, KP seemed to be an outlier even though cluster analyses showed a similarity level of about 87 % with 0.5 % E, 5 % S and 2.5 % S. Analysis showed closely similar performance of other E formulations to H_2O (deionised water). Overall all formulations showed 77.5 % similarity levels which showed that all prepared extracts are potentially useful as organic fertiliser. However, several are better and this is emphasised by the observations of the close relation between HS and 5:1 SM as well as 0.5 % M. Extract formulation 5:1 SM showed some positive results on SLA with performance similarly resembled HS and was therefore considered for further study. According to the cluster in Figure 2.2, 1% M had no close similarity with either HS and/ or KP and only showed a very close association of 93. 9 % with 5 % F extract.
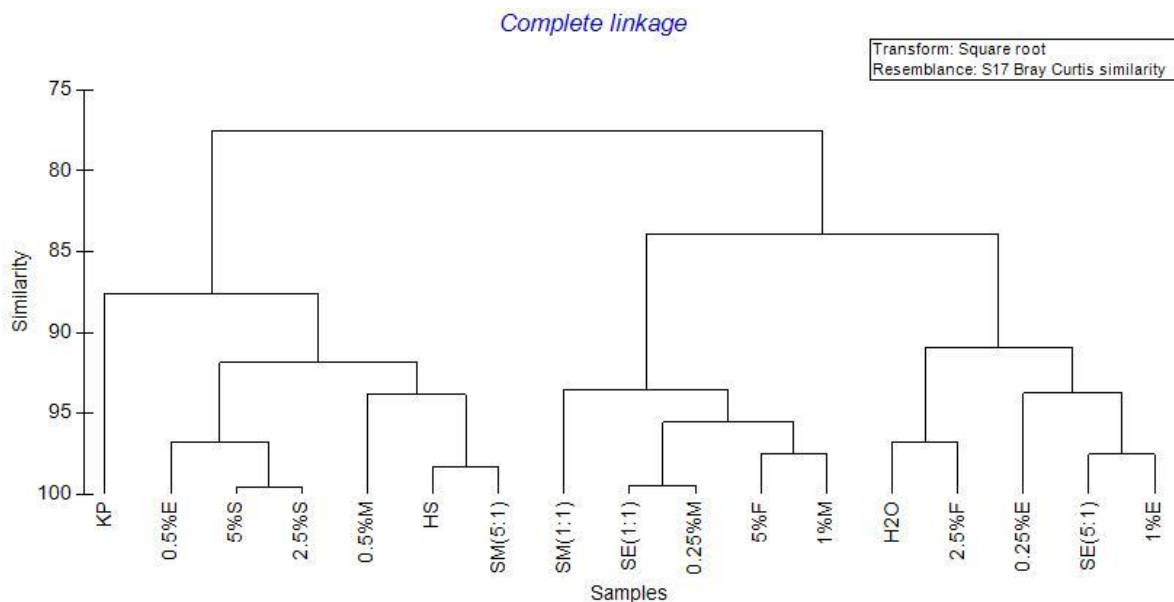
Figure 2.2: A complete group linkage cluster of the HRAOP biosolids extract formulations evaluated using specific leaf area.

2.3.3. Effect of extracts on radish cotyledon expansion growth

Figure 2.3 shows percentage change in the fresh weight of radish cotyledon as a function of PGR activity of HRAOP biosolids extracts. Extract 1 % M had an insignificant ($p > 0.05$) effect on fresh weight gain compared to KP, HS, DG and deionised water treated cotyledons. Significant differences ($p < 0.05$) were noted when 5 % S and 5:1 SM were compared to the H_2O control. Higher weight gains of 459 ± 0.02 % and 362 ± 0.009 % were obtained with 5:1 SM and 5 % S respectively. Near equal responses in increase in fresh weights of 236 ± 0.003 % and 244 ± 0.004 % were obtained for HS and KP. However, the differences were

insignificant ($p > 0.05$) between these treatments. According to this bioassay outcome only two preparations exhibited potential to be used as plant growth regulators: 5:1 SM and 5% S.
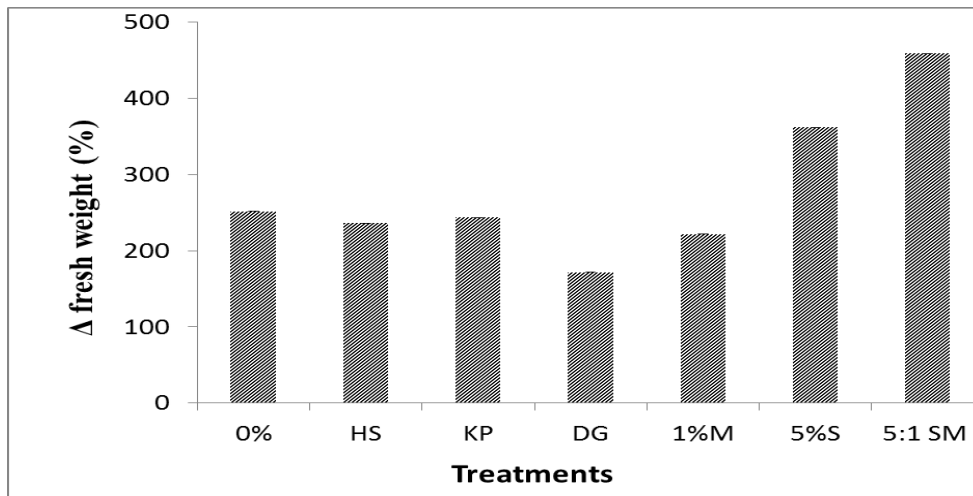
Figure 2.3: Change in fresh weight (%) of the inner cotyledon after 72 h of incubation under continuous light supply in Petri dishes containing prepared HRAOP biosolids extracts (1% M, 5% S and SM), commercial stimulants (KP and DG), deionised water (0 %) with Hoagland solution (HS) as controls.

2.3.4. Effect of extracts on seed germination

The GI for tomato and radish are given in Table 2.3. A seed was considered germinated when its root (radicle) length exceeded 5 mm. According to Keeling *et al*. (1994) GI of 100 % indicates a stimulation of plants growth and development. There was generally no inhibition of germination for the two crop plants studied. This shows that the formulated extracts have an ability to support seed germination (Table 2.3) but did not necessarily increase this ontogenic event. Tomato seeds treated with biosolids extracts showed GI nearly similar to that of deionised water (control) except for 5 % S which had lower GI of 87.00 ± 1.24 compared to 100 ± 1.17 of deionised water. For 1 % M and 5:1 SM, GI were 100 ± 0.98 and 106.95 ± 1.38 respectively. Although results were insignificant different ($p > 0.05$) 5:1 SM had the highest GI when compared to the HS and deionised water (0%) controls. Low GI presented by 5 % S was influenced by the noted delay in radicle elongation suggesting that this preparation either inhibits or delays germination. Average radicle length was 5.79 ± 2.46 for 5 % S, 6.39 ± 3.66 cm for 5:1 SM and 6.76 ± 1.39 cm for 1 % M. This was however not significantly different when compared to radicle length obtained for controls. On the other hand, when radish seeds were treated with biosolids extract, the results far exceeded those of the control with HS having lower GI of 95.73 % ± 0.68. The obtained GI for all 3 biosolids

extracts were statistically insignificant ($p > 0.05$) indicating similar effects of the extracts on radish seeds germination

Table 2.3: Showing the germination index of tomato and radish that were germinated in petri dishes containing formulated HRAOP biosolids extracts (5% S, 1% M, 5:1 SM) against deionised water control (0 %). Data are presented as mean ± SD.

Germination index (%)					
Seeds	**Controls**		**Treatments**		
	0 %	**HS**	**1 % M**	**5 % S**	**5:1 SM**
Tomato	100 ± 1.17	103.4 ± 2.38	100 ± 0.98	87.00 ± 1.24	106.95 ± 1.38
Radish	100 ± 0.97	95.73 ± 0.68	139.27 ± 0.67	135.56 ± 1.47	136.00 ± 0.93

2.3. Discussion

A source of biosolids was obtained from the HRAOP component of IAPS treating domestic sewage, which was used to derive extract solutions for determination of fertiliser and PGR potential. Fertiliser potential was used as a primary screening requirement. Thereafter formulations that demonstrated potential fertiliser effects were tested for PGR-like activity. Sterilisation by autoclaving (121 ℃ for 15 min) was routinely used to ensure a longer storage life of the extracts after it was noticed that from about 4 - 5 weeks substantial colour and odour changes were evident in non-autoclaved preparations. Autoclaving the extracts did not negatively affect performance. Rizvi & Sharma (1994) supported this method by indicating that there was no difference in effects of autoclaved and non-autoclaved microalgae filtrates as fertiliser substitutes.

From the results presented in this chapter, it is evident that the fertiliser preparation labelled 5:1 SM out-performed all other formulations and most closely resembled the response obtained when using HS. Other potentially good fertiliser formulations included 0.5 % M and both 2.5 % S and 5% S. From a PGR-like perspective, 5:1 SM and 5 % S were the most active at promoting expansion growth in the radish cotyledon bioassay. Analysis of GI using seeds of tomato and radish revealed that none of these formulations derived from HRAOP biosolids negatively impacted germination suggesting that these were without phytotoxic

effects and therefore suitable for whole plant evaluation. All other preparations described in this chapter were thus not investigated further.

In investigating the fertiliser potential, the response of *Phaseolus vulgaris* plants was evaluated using a SLA bioassay. Leaf area is an important and critical determinant of plant survival and growth and positively correlates with seedling relative growth rate (Poorter & Remkes, 1990) and leaf net photosynthetic rate (Shipley & Lechowicz, 2000). Leaf size determines absorption of light, photosynthesis and consequently plant productivity (Goudriaan & Van Laar, 1994; Demirsoy *et al*., 2007). Hence SLA has gained attention as a non-destructive, easy and quick measurement of plant performance (Shipley, 2006; Mokhtarpour, 2010). In the present study HS was used as a benchmark to evaluate fertiliser potential as it provides every nutrient necessary for plant growth and is appropriate for the growth of a large variety of plant species (Hoagland & Arnon, 1938). Increased SLA in bean plants treated with S arose largely by an increase in leaf length whereas M increased SLA by increasing leaf width. This indicated that some extracts influenced longitudinal growth (i.e. plant height) whereas others appeared to influence lateral growth (i.e. leaf width). Enhanced lateral growth is associated with an increase in plant biomass or foliage growth which could increase crop production, whereas height growth means faster plant growth and is attractive in growing crops whose maturity is determined by height rather than foliage. The HRAOP biosolid derived fertiliser extract treated plants were flourishing with no premature fall of leaves or any signs of stress and had a relative increased SLA. This suggests that these fertiliser preparations derived from HRAOP biosolids can successfully sustain plant growth and development. Although 0.5 % M fertiliser extract presented an increased in SLA, the overall performance of 0.5 % M treatment in terms of plant growth visual responses was not satisfactory in relation to those shown by 1 % M treated plants.

In addition to screening for fertiliser potential, the three prepared HRAOP biosolids extracts (shown on appendix D) were investigated for plant growth regulator-like potential. Radish (*Raphanus sativus*) cotyledon expansion growth was used for this purpose because of its sensitivity to toxins and due to its wide use to assess phytotoxicity (Han *et al*., 2000; Forbes *et al*., 2006) and plant growth promoting chemicals (Antoun *et al*., 1998; Hong *et al*., 2009; Saharan & Vehra, 2011). An unimpaired ability of radish cotyledons to expand and develop into photosynthetically active cotyledon leaves on day 3 suggested that 5 % S and 5:1 SM treatment promoted this developmental process. These bioassay tests for PGR-like activity

and HS were used as baseline nutrient or fertiliser to account for any mineral induced response because plant growth regulator activities are not necessarily nutrient dependant. Response achieved was certainly due to the PGR-like activity of the HRAOP biosolids extracts and not nutrient load. According to radish cotyledon bioassay outcomes, only two preparations exhibited potential to be used as plant growth regulators: 5:1 SM and 5% S. This was in agreement with cluster which clearly presented that 5 % S and 5:1 SM has 87 % and 66.2 % association with Kelpak (KP). Hence it would be interesting to further investigate the presence of PGR- activity on the formulated extracts.

2.4. Conclusions

Screening and preparation of various HRAOP biosolids extracts was successfully achieved primarily based on an ability to support growth of bean plants measured using SLA. Three HRAOP derived extracts evaluated presented encouraging results that were comparable and in some cases exceeded that of a known plant nutrient solution (e.g. HS) and commercially available plant growth stimulants (e.g. KP and DG). As fertiliser 5 % S, 5:1 SM and 1 % M enhanced relative plant growth illustrated by an increase in SLA. It was noted that 5 % S resulted in increased plant height whereas 1 % M and 5: 1 SM equally affects both length and width growth. The three formulations enhanced root development in GI assays. Thus based on accumulated data 5 % S was selected as fertiliser preparation, followed by 5:1 SM. These two extracts also exhibited plant growth regulator-like activities, and were therefore studied further. In addition 1 % M was selected for further study based on its overall performance in the respective bioassays and as it was derived using methanol in the extraction/preparation process and may therefore contain activity that might emerge in more detailed study.

CHAPTER 3

Fertiliser potential of extracts prepared from HRAOP biosolids

3.1. Introduction

Microalgae are a major biological component and catalyst responsible for nutrient removal in HRAOP of IAPS and are as a consequence highly enriched with minerals, trace elements and amino acids essential for plant growth (Spoehr & Milner, 1949; El Fouly *et al*., 1992; Mahmoud, 2001; Rose *et al*., 2002; Abd El-Migeed *et al*., 2004; Brady & Weil, 2007; Abd El-Moniem *et al*., 2008). They also contain chelating agents which are important in nutrient absorption by plants (El-Fouly *et al*., 1992). Microalgae also contain plant hormones, such as auxins and cytokinins (Provasoli & Carlucci, 1974; Arthur *et al*., 2003; Abd El-Migeed *et al*., 2004; Takhovskaya *et al*., 2007; Abd El-Moniem *et al*., 2008) and produce compounds that increase soil fertility through enhanced water retention (Pulz & Gross, 2004). This makes microalgae attractive in horticulture as a plant growth and development stimulant. Dry algae biomass makes a good soil supplement from which nutrients are slowly released and the effect is comparable to that of inorganic fertilisers in terms of dry weight production and plant nutrient composition (Mulbry *et al*., 2005).

Microalgae have previously been investigated as potential feedstock for biodiesel and methane production following their success in wastewater treatment (Chisti, 2007; Deng *et al*., 2009; Mata *et al*., 2009). In horticulture microalgae biomass from HRAOPs can be used as a fertiliser, soil conditioner and/or plant growth regulator (Mahmoud, 2001; Arthur *et al*., 2003; Pulz & Gross, 2004; Mulbry *et al*., 2005; Faheed & Abd-El Fattah, 2008; Iyovo *et al*., 2010). However, these potential uses have not yet been fully explored and exploited (Begum *et al*., 2011). Application of microalgae as a fertiliser in particular is only recently gaining attention: investigations of *Chlorella vulgaris* as fertiliser, for example (El Moniem *et al*., 2008; Faheed & Abd-El Fattah, 2008; Iyovo *et al*., 2010). Application of microalgae as a fertiliser has been shown to produce soil improvements that are comparable to, and in some cases better, than inorganic fertiliser (Mulbry *et al*., 2005; El Moniem *et al*., 2008; Faheed & Abd-El Fattah, 2008; Iyovo *et al*., 2010). Horan *et al*. (2008) reported similar observations from the application of IAPS microalgae when administered as a soil amendment and as a foliar feed. However, results on the use of extracted microalgae as foliar feed were inconclusive (Horan *et al*., 2008).

In this chapter the results of investigations into the use of three formulated extracts of HRAOP biosolids for use as fertiliser using bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) as test plants are presented. Bioassays were used to evaluate the effect of the various fertiliser preparations on growth and development. Beans were selected for their ease and accuracy for change in specific leaf area (SLA) determination. Tomato plants were selected due to their complexity, specificity in terms of growth conditions, and nutrient supply needs. Bean plants are N-fixers and require no addition of N-fertiliser whilst tomato plants are high N demanders.

3.2. Materials and methods

3.2.1. Plant cultivation

Bean seeds (*Phaseolus vulgaris* L. cv. Contender) and tomato seeds (*Solanum lycopersicum* Mill. cv. Money-Maker) were purchased from a local seed supplier. Their surface was sterilised with 5 % NaOCl for 30 and 10 min respectively and rinsed thoroughly with deionised water at room temperature. The seeds were then blotted dry and immediately planted in seedling trays containing vermiculite, which was adequately moistened with half strength of HS (Appendix B). Seeds were germinated under controlled environment conditions at 24 ± 2 ℃ (bean) and 28 ± 3 ℃ (tomato) with average light of 230 $\mu mole.m^{-2}.s^{-1}$ cool white light illumination at a 12 h light cycle. Seedlings were irrigated daily with HS until they provided visually desirable sizes for bioassay experiments, usually 12 – 14 days for beans and 30 – 35 days for tomatoes.

3.2.2. Effect of HRAOP biosolids fertiliser extracts on growth of bean and tomato plants

Contender bush beans (*Phaseolus vulgaris*) seedlings that were 14 days old were transplanted into 100 mL Erlenmeyer flasks containing 60 mL deionised water and 5 g vermiculite. While moneymaker tomato (*Solanum lycopersicum*) seedlings that were 33 days old and approximately 8 to 10 cm tall were transplanted into 16 cm diameter pots filled with 500 g vermiculite moistened with 500 mL half strength HS. To avoid nutrient wash off and leachate in pots, the water holding capacity for vermiculite was determined to establish the amount of nutrient solution and frequency required for irrigation. Plant adaptation period of 3 days for bean and 8 days for tomato was allowed to ensure that plants were in good physiological state before addition of any treatment and well adapted to new growth medium presented.

Following the 3 day adaptation, strong and healthy looking uniform plants were retained for the experiment, which was carried out in triplicate. On day 4, water in bean plant flasks was adjusted to the initial level in all flasks and 30 mL of desirable treatments were added in each flask. Each treatment included three flasks with one seedling arranged in a randomised block design (Figure 3.1 A). Tomato plants were considered ready for experiment when the seedlings were visually healthy and fully anchored. Vermiculite has a very high water holding capacity thus 200 mL irrigation three times per week with tap water was considered sufficient. After 8 days, tomato plants were thinned to four plants per pot followed by the addition of 200 mL of treatment and deionised water (1:1 v/v). Three pots were set for each treatment, each with four plants per pot arranged in a complete randomised block design (Figure 3.1 B). All treatments were added in the morning (during the first hours of light) when plants are said to be more receptive. Treatments used included three HRAOP biosolids formulations (1 %M, 5 % S, and 5:1 SM) prepared as described in chapter 2, organic stimulants (KP &, DG) HS and deionised water (with no treatment, 0%). Both flasks and pot plants were placed in a constant environment room which was monitored and maintained as described in section 3.2.1.

Figure 3.1: Randomised distribution of; A= 100 mL flasks showing bean seedlings on day 7 of SLA experiment; B = pots plants showing tomato seedlings on day 0 treatment (transplant day). In a CE room with temperature of at 24 ± 2 °C (bean) and 28 ± 3 °C (tomato) and average light of 230 µmole.m^{-2}.s^{-1} cool white light illumination at a 12 h light cycle during the investigation of fertiliser potential of HRAOP biosolids extract.

Length (L) and width (W) of the first two true leaves of bean plants were measured initially (Y_o). Y_x measurements were taken on days 3, 4, 5, 6, and 7. Mean values of the percentage change (% Δ) in L, W and area (A) were used to extrapolate the relative change in plant

growth according to equation 1. Bean plant height was measured on day of harvest to confirm the SLA effects of the extract in terms of leaf lamina longitudinal growth. Tomato plant height was recorded on day 37 post-treatment, plants were harvested, separated into root and shoot fractions and biomass of each was determined.

$$\% \Delta = \frac{Y_x - Y_o}{Y_o} \times 100 \qquad \qquad \textbf{Eq. 1}$$

[Y_x = L, W or A on a specific day and Y_o = L, W or A on day zero].

A sterile blade was used to separate the shoot and root. Shoots were placed in pre-weighed 500 mL beakers, fresh weights was recorded followed by immediately freezing in liquid nitrogen and freeze dried (Vir-Tis Benchtop SLC). Plant roots were carefully uprooted, rinsed with distilled water, blotted dry with paper towel and oven dried at 70 ± 5 °C until a constant weight was obtained. Mass of dried shoots and roots was recorded and reported as shoot or root dry weight (DW) in g. The difference between fresh and dry weights was used to determine relative plant water contents (%). Shoot: root ratio was then established for each treatment.

3.2.4. Determination of chlorophyll content on bean and tomato plants

Chlorophyll was extracted from fresh leaf tissues discs weighing ± 2 mg with 10 mL of 90 % (v/v) acetone solution according to method of Mackinneys (1941) under subdue light environment (Geider & Osborne, 1992; Akparobi, 2009). Leaf tissue was grinded with mortar and pestle with 5 mL (90 % v/v) acetone added for effective chlorophyll extraction. Extractant was transferred to a test tube and the mortar and pestle were rinsed with 90 % acetone and the resultant added to the test tube. The tubes were covered with aluminium foil for complete darkness and kept in a fridge at 8 °C overnight (16 − 18 hours). After which the extractant (chlorophyll) was syringe filtered through 0.45 μm pore size into a 10 mL volumetric flask and diluted to a mark with the 90 % acetone. To retard chlorophyll degradation to phaeophytin; 0.15 mL of 1 % (w/v) $MgCO_3$ was added immediately after filtration and vortexed (Hegazi *et al*., 1998). This inactivates chlorophyll degrading enzymes such as chlorophyllase (Hegazi *et al*., 1998; Cubas *et al*., 2008). Chlorophyll absorbance was measured using a Thermo Spectronic Aquamate Spectrophotometer (ThermoFisher Scientific, Waltham, MA) at 664nm and 647 nm as Chlorophyll *a* and Chlorophyll *b* (mg/mL) respectively against 90 % acetone blank (Cubas *et al*., 2008). Concentration (mg/L)

of chlorophyll *a*, *b* was obtained according to Geider and Osborne (1992) co-efficiencies. [Chl $a = 12.7$ $A_{664} - 2.79$ A_{647} and Chl $b = 20.7$ $A_{647} - 4.62$ A_{664}]

3.2.5. Effects of HRAOP biosolid fertiliser preparation in nutrient translocation

Macro-element (C, N, and S) of analysis of plant biomass post treatment was used to determine whether the formulated extract in any way impacted on nutrient uptake by the test plants. This was performed using an Elementar CHNS analyser (Vario MICRO Cube). Dried plant shoots were milled and composite samples were prepared by taking 0.1 g from each replicate to a total of 1.5 g. Mortar and pestle were used to crush $5 - 10$ mg plant biomass and mixed with oxidising agent vanadium pentoxide (V_2O_5) and combusted at 1000 °C. The analyser separated and detected combustion gas products CO_2, N_2 and SO_2 using helium as a carrier gas. The CNS element contents were reported as weight percentages (%).

3.3. Data and statistical analysis

Data presented are the mean value $\pm$ standard deviations (SD) and where possible experiments were repeated more than once in a randomised design. Data were analysed using Microsoft Office Excel 2010 where mean values and SD were calculated. Statistical analyses for this study were performed using Statistica 9.0 (StatSoft Inc, 2010). *T*-tests were carried out to determine significant difference ($p < 0.05$) between samples and treatments. Line graphs were created using Microsoft Office Excel 2010 to establish the effect of treatments on bean plant specific leaf area.

3.4. Results

Figure 3.2 shows the results of change in length (L), width (W) and area (A) of the first true leaves of bean seedlings following treatment with formulations of extracts from biosolids i.e. 5 % S, 1 % M and 5:1 SM when compared to deionised water (no treatement 0%) and HS controls. As indicated in Figure 3.2 the prepared formulation 5 % S was most effective at increasing leaf length (Figure 3.2 A), width (Figure 3.2 B) and change in leaf area (Figure 3.2 C) of bean seedling. No significant difference ($p > 0.05$) was observed on leaf length from plants treated with other remaining treatments to assay for fertiliser efficacy. By comparison the leaf length growth rate was low in KP; similarly to 1 % M extract treated seedlings. However, for leaf width, KP and HS enhanced the width growth rate whereas 1% M and anaerobic digestate reduced width growth rate (Figure 3.2 B). Formulation 5:1 SM appeared to exert no effects on leaf width of bean seedlings. These results indicated that the change in growth rate of leaves horizontal is more sensitive than growth rate in the longitudinal. Thus leaf expansion, rather than elongation, is impacted by this exogenous application. Nutrient rich formulation such as HS and anaerobic digestate enhance leaf expansion process whereas KP and 1 % M retard or delay this growth process.
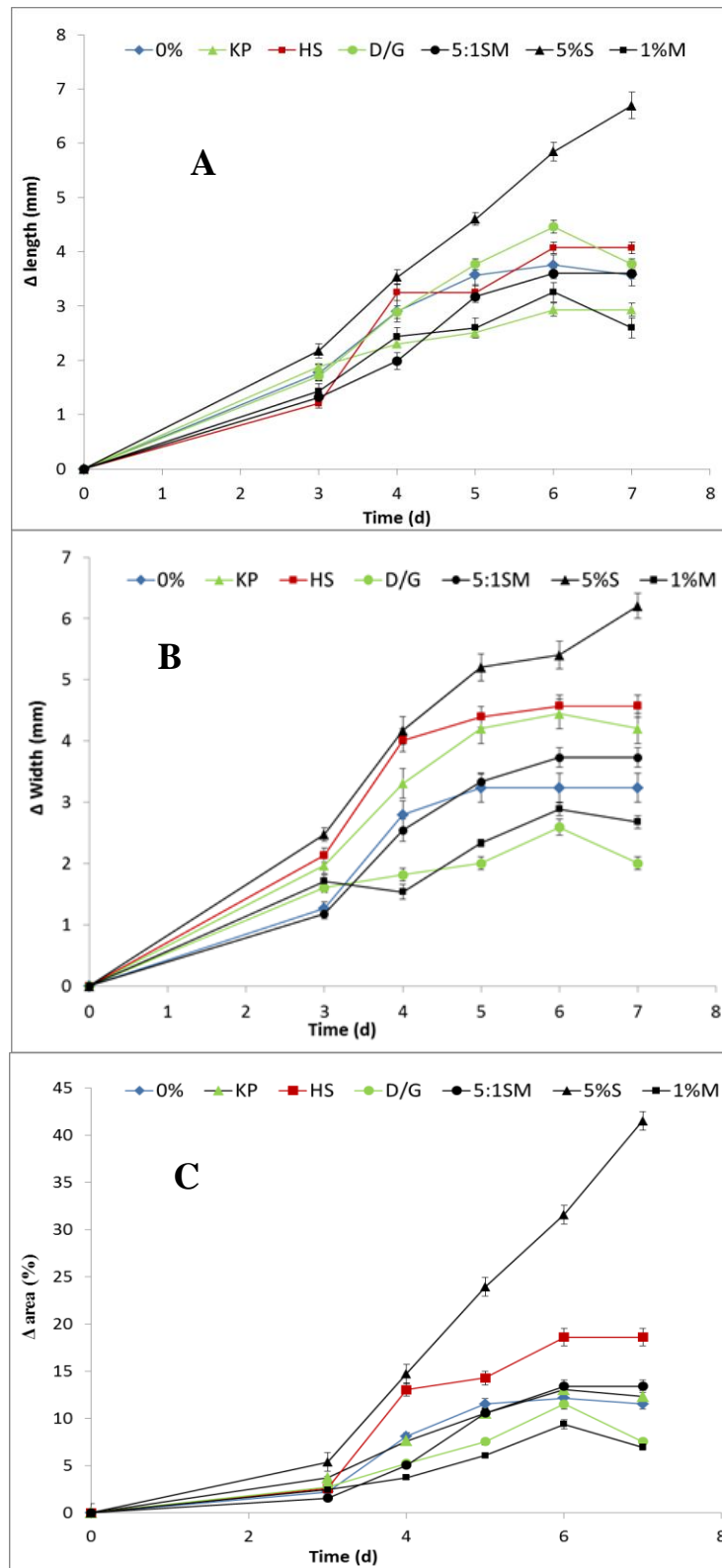
Figure 3.2. Change in length (A), width (B) and specific leaf area (C) of the linear surface for the first true leaves of bean seedlings following treatment with the three HRAOP biosolids derived extract. The 14 day old seedlings were transplanted into hydroponic culture and after 3 days of adaptation plants were supplied with treatment solution via root system. Measurements were taken as indicated and data are mean ± SD (n=6).

Effect of biosolid fertiliser extracts on bean and tomato plant height, biomass, chlorophyll content and shoot: root ratio is shown on Table 3.1 and Table 3.2. Although formulated fertiliser preparations showed no significant effect on plant height relative to deionised water treated seedlings, KP, DG and SM appeared to reduce the bean plant height. Whereas all treatments enhanced plant height of tomato with KP, DG, 5 % S, 1 % M and HS causing remarkably greater than 14 % increase (Table 3.2). In bean and tomato plants the biosolids derived fertiliser treatment measured as dry weight did not enhanced plant biomass (Table 3.1 and Table 3.2). However, the HS and commercial products produced variable results. The HS and DG reduced bean and tomato plant biomass while KP reduced bean biomass but increased biomass of tomato. In bean plants the biomass of plant treated with 5 % S appeared not to correlate with SLA results. Chlorophyll and relative water content of the whole plant for both bean and tomato were not impacted by the treatments; the shoot: root ratio was affected. Nevertheless increase in chlorophyll concentration was obtained on leaves treated with 5:1 SM, KP, DG and 1 % M. Shoot: root ratio of bean plant treated with HS was increased indicating an increase translocation of assimilated nutrient to the shoot. The decreased shoot: root ratio presented by KP treatment, however, indicated reduction in shoot development or an increase in root growth. Biosolid fertiliser extracts displayed intermediate effects on bean shoot: root ratio. However, in tomato only HS treated plants exhibited an increase in shoot: root ratio with DG showing a reduced shoot: root ratio (Table 3.2). Increase shoot: root correlated with an increase in shoot or decrease in root mass which is influenced by availability of mineral nutrients, carbon (C) and water (Ericsson, 1995; Göran & Oskar, 2003). Results presented in Table 3.1 and Table 3.2 indicates that fertiliser preparations from biosolids were consistently comparable with HS, KP and DG in performance in relation to the measured parameters for bean and tomato cultivation.

Table 3.1: Effect of formulated fertiliser preparation derived from HRAOP on plant height, root: shoot ratio, dry biomass, water content and chlorophyll content of 25 day old bean plants. Data are presented as the mean ± SD.

Bean	Height (cm)	Plant biomass DW (g)	Chlorophyll content (mg/mL)	Relative water content (%)	Shoot: root Ratio
0 %	40.9±8.93	1.58±0.42	42.19±0.01	90.15	1.87
HS	42.8±10.8	0.95±0.19	42.24±0.01	93.79	2.65
KP	33.6±2.63	0.60±0.09	47.49±0.05	92.42	1.07
DG	34.0±5.50	1.01±0.11	46.86±0.03	93.09	1.81
5 % S	39.4±7.53	1.06±0.11	42.43±0.02	92.92	1.72
1 % M	36.4±5.13	1.15±0.16	45.70±0.02	95.04	1.30
5:1SM	33.4±5.07	1.22±0.17	47.35±0.05	94.81	1.44

Table 3.2: Effect of formulated fertiliser preparations derived from HRAOP biosolids on plant height, root: shoot ratio, dry biomass, water and chlorophyll content of 78 day old tomato plants. Data are presented as mean ± SD.

Tomato	Height (cm)	Plant biomass DW (g)	Chlorophyll content (mg/mL)	Relative water content (%)	Shoot: Root: Ratio
0 %	62.2±7.49	3.01± 0.20	36.43±0.01	92.56	19.55
HS	72.5±9.76	2.86±0.14	33.68±0.02	94.39	27.63
KP	80.3±9.83	3.21±0.38	34.39±0.03	93.82	21.95
DG	74.2±3.52	1.99±0.44	26.03±0.01	95.85	16.56
5% S	75.7±14.01	2.40±0.38	34.59±0.01	94.25	18.49
1% M	74.3±9.93	2.67±0.17	39.36±0.02	94.37	20.62
5:1SM	68.5±7.37	2.46±0.19	40.87±0.01	94.09	19.47

Increase in shoot: root ratio is influenced by either increase in shoot mass or decrease root mass. When nutrients supply is high, plants translocate less to root (Göran & Oskar, 2003). Decreases in shoot: root ratio is affected by an increased in mineral and water retention in roots (Ericsson, 1995; Göran & Oskar, 2003). Fertiliser preparations used in this study had no effect on nutrient composition as evidenced by no significant difference ($p > 0.05$) in CNS content of the plant biomass (Table 3.3). Average CNS content of bean biomass was 31.16 % (C), 3.76 % (N) and 0.39 % (S) while tomato plant biomass presented similar trends with an average of 31.84 % (C), 3.95 % (N) and 0.79 % (S). Results were insignificantly different ($p > 0.05$) in all treatments both in bean and tomato plants. Formulated fertiliser extracts

resulted in a slightly increased C: N ratio. However, the balance in ratio was not altered confirming that the fertiliser preparations did not adversely affects the nutrient balance of the plant.

Table 3.3: Effects of the HRAOP derived fertiliser extract on nutrient balance in tomato and bean plants, determined by macro elements of dried plant biomass post-treatment. Data are presented as mean ± SD.

	C (%)		N (%)		S (%)		C:N ratio	
	Tomato	**Bean**	**Tomato**	**Bean**	**Tomato**	**Bean**	**Tomato**	**Bean**
Untreated	30.7±0.01	29.3±0.06	3.7±0.08	3.5±0.01	0.8±0.06	0.5±0.10	8:1	8:1
HS	30.5±0.02	30.6±0.00	4.2±0.01	4.2±0.12	0.8±0.00	0.3±0.04	7:1	7:1
KP	31.9±0.16	28.6±0.05	3.2±0.03	3.9±0.07	0.8±0.05	0.4±0.04	10:1	7:1
DG	32.5±0.04	32.9±0.02	3.2±0.02	2.9±0.11	0.8±0.01	0.4±0.03	10:1	11:1
5%S	31.6±0.10	30.5±0.05	3.8±0.04	3.6±0.03	0.7±0.05	0.1±0.03	8:1	9:1
1%M	32.1±0.07	29.7±0.01	3.5±0.00	3.3±0.09	0.8±0.08	0.3±0.01	9:1	9:1
SM	31.8±0.13	30.9±0.04	3.4±0.01	3.2±0.10	0.8±0.04	0.3±0.02	9:1	10:1

3.5. Discussion

The HRAOP biosolids derived fertiliser preparations that were developed and screened by bioassay, as described in Chapter 2 were assessed for potential to support or enhance plant growth. Bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants were used for this purpose where nutrients were added in a hydroponic culture for bean experiment and potting vermiculite for tomato experiments. Hydroponic culture allowed direct interaction of roots and nutrients. This increased nutrient accessibility to all plant tissues. Also, a mixture of hydroponic culture and vermiculite provides solid culture for roots and isolates factors affecting nutrient translocation, such as soil acidity, microbial activities and interactions of ions with soil particles (Poorter & Nagel, 2012; Simon *et al.*, 2013).

Plant growth responses are the combination of plants ability to effectively use available nutrient, water and harvest light for photosynthesis. Photosynthesis provides C which is

assimilated to sugars and used to drive plant growth; a process dependent upon N availability (Zheng, 2009). Effective C assimilation requires active functioning of the chloroplasts, and N content in leaves. Conversely, elevated C concentrations cause a reduction in N concentration on the leaf tissue which negatively impacts photosynthesis (Zheng 2009; Kant *et al*., 2011). Interactions between CO_2 and nitrate (NO_3^-) assimilation are of key importance for plant growth and production. Root system takes up $N-NO_3^-$ and/ or $N-NH_4^+$, the N assimilates from roots translocate to the leaves where C assimilation and metabolism primarily occurs. The C and N assimilates produced are exported to the growing regions of the plant to promote plant biomass growth and reproductive activities (Foyer *et al*., 2001; Kant *et al*., 2011). Thus a balanced C: N ratio plays an important role in plant metabolism and impacts overall plant performance. The N limitation in the nutrient medium inhibits shoot growth resulting in an altered C: N ratio whereas sufficient N stimulates leaf growth via cell growth and division thereby elevating photosynthesis activities (Stockdale *et al*., 1995; Haque *et al*., 2001; Lawlor, 2002). The C: N ratio is an important measure of a plant response mechanism (Zheng, 2009). Formulated fertiliser extract derived from HRAOP biosolids resulted in plant biomass with C: N ratio of 8-10:1 similar to the C: N ratio obtained with commercially available KP and HS treatments. The obtained C:N ratio suggested that, application of the formulated fertiliser extracts did not alter plant physiological functioning as well as critical metabolic processes. Evident by no adverse effect on C, N and S mineral nutrient content thereby indicating that HRAOP derived extract causes no damage on plant nutrient translocation processes. No burns marks, wilting, root system damages or any other form of plant stress were observed.

Plant growth is a quantitative physical process which is measured in relation to time and could be influenced by various internal and external factors. However, this phenomenon is highly dependent on mineral nutrient uptake (Clarkson, 1980; Sinclair, 1992), which comprises essential elements for plant growth, survival and productivity (Uchida, 2000; López-Bucio *et al*., 2003). Plant growth could be measured as an increased length or growth in the case of shoots and roots or as an increased area, volume or weight in the case of leaves and fruits. Formulated 5 % S fertiliser extract stimulated increases in leaf area. Leaf horizontal expansion was more sensitive than longitudinal growth, consequently bean seedling SLA treated with 5 % S and HS exceeded those of other treatments. The leaf is one of the plants critical components that greatly influences relative growth and development. Leaf area directly relates to light interception, mineral nutrient response and consequently

photosynthesis, transpiration (Goudriaan & Van Laar, 1994; Demirsoy *et al*., 2007) and is considered the most crucial determinant of plant growth and productivity (Poorter & Remkes, 1990; Blanco & Folegatti, 2005). Increase in leaf area is crucial for plant functionality and it determines the efficiency of light absorption, photosynthetic activities and relative plant growth. In this study HRAOP biosolids fertiliser extract effects on SLA indicated that plants responded positively to mineral nutrient assimilation and the extracts' performances were comparable to those of commercial products like KP, HS and DG.

Plant biomass was not significantly enhanced by treatment with fertiliser extracts and the extract had no adverse effects on chlorophyll content as well as plant crucial nutrient composition (C: N). This suggested that an application of these fertiliser extracts has no negative impact on the photosynthetic processes. In addition, no chlorosis or any plant stress was observed on fertiliser extract treated plants as well as with other controls. Shoot: root ratio was marginally enhanced especially with 5 % S fertiliser extract.

Plant distribution or partitioning is affected by amount of C and N availability as well as water (Ericsson, 1995; Göran & Oskar, 2003; Kant *et al*., 2011). Root mass is dependent on N and water availability whereas shoot mass responds to C availabilities (Ericsson, 1995). Nitrate is not only the predominant source of N supply to plants. It also acts as an important signal for several developmental processes (Crawford & Forde, 2002; Kant *et al*., 2011). N affects root growth and development and the resulting shoot: root ratio. Partitioning of the fertiliser preparations treated plants was intermediate which emphasizes the balance in nutrient displayed by the fertiliser extracts. At higher nutrient supply, plant growth allocates relatively less to their roots; the shoots increase which enlarges the surface for maximum absorption of light (Ericsson, 1995; Göran & Oskar, 2003). In bean plants, a decline in biomass accumulation was manifested which led to a reduced shoot: root ratio in response to extract treatment. However, in tomato cultivated in vermiculite shoot: root ratio was not affected. Reduction in shoot: root ratio is associated with retention of mineral and water within the roots (Ericsson, 1995; Göran & Oskar, 2003). In this study nutrients were supplied hydroponically to bean plants and this method might have impacted root growth. Water logging or nutrient ion accumulation in the root zone may have deprived the plant of nutrients which resulted in reduced biomass accumulation and a reduced shoot: root ratio. However, this observation was not evident in tomato plants cultivated in vermiculite. Nevertheless, the

control fertiliser treatment, HS, increased the shoot: root ratio in both the hydroponic and vermiculite culture.

Interestingly, the response of plants to 1 % M and 5:1 SM was similar to that exhibited by plants treated with KP, suggesting that these formulations might have PGR-like activity (Temple & Bomelle, 1989; Nelson & Van Staden, 2004; Papenfus *et al*., 2013). A positive response to HRAOP biosolids extract treatment was noted in both bean and tomato plant growth which means the extract is suitable for crop survival and growth. Insignificant difference in shoot: root ratio of plants treated with fertiliser extracts, DG, KP and HS indicated that the extracts have an effect comparable to known commercially available plant nutrients. Hence HRAOP biosolids extracts may be used as an organic fertiliser. Interestingly, plants treated with 5 % S continued to show strength in longitudinal growth as evidenced by increased plant height suggesting a strong effect of 5% S in longitudinal growth of the plant.

Overall, the fertiliser extracts derived from HRAOP biosolids showed results that were comparable and sometimes exceeded those of the controls. By comparison, the response of bean and tomato plants to all three formulated fertilisers was consistent indicating no difference in effect between the three preparations. As mentioned, the C: N ratio of plants treated with these fertiliser extracts was similar and the response in biomass and shoot: root ratio was also similar, indicating little difference in effect between the three preparation as fertiliser applications. For commercial purposes, preparation of 1% M requires the use of methanol which has cost implications. It is also considered a risk. Similarly 5:1 SM contains a fraction of the methanol extract and therefore it is obvious that the 5 % S fertiliser extract is the most feasible cost-effective preparation.

Results of this study have demonstrated the successful use of the HRAOP biosolid as a source of fertiliser raw material. This fertiliser can be easily prepared by sonicating the biosolid and harvesting the extract. Produced fertiliser extract is diluted accordingly and its effects as a plant fertiliser will not be substantially different to those of commercial products and/ or the scientific HS nutrient.

3.6. Conclusion

Fertiliser extract derived from HRAOP can be produced as an easy to manufacture and environmental friendly fertiliser. Prepared fertiliser extracts in this study presented encouraging results to pursue valorisation of biosolids from IAPS as a cost effective renewable resource of fertiliser. Formulated extracts were not phytotoxic and were able to support plant growth. Outcomes included increased plant height; leaf area and shoot: root ratio. Chlorophyll, relative water content and nutrient balance were not negatively affected. Plant growth, was however affected by internal factors which include substances such as plant growth hormones and alternative plant growth regulators. Improving productivity and yields is one of the reasons to apply fertiliser nutrient. Hence, the next chapter covers the ability of the extract to enhance productivity on the plant as well as plant growth regulator activities presence in the extract.

Chapter 4

Investigation of plant growth–like activities of the formulated HRAOP biosolids fertiliser extract

4.1. Introduction

The HRAOP biosolids are dominated by microalgae; therefore the HRAOP fertiliser extracts potentially have plant growth-like activities. Algae have been shown to contain all five of the major plant hormones which serve as plant growth regulators. These include auxins, cytokinins, gibberellins, ethylene and abscisic acid (Provasoli & Carlucci, 1974; Arthur *et al*., 2003). Tarakhovskaya *et al*., (2007) reported the presence of auxins, cytokinin and abscisic acid in green microalgae while agricultural benefits of cyanobacteria (Moreno *et al*., 2003, Vaishampayan *et al*., 2001) and plant growth promoting rhizobacteria (Saharan & Nehra, 2011) are also recognised. Plant growth regulators (PGRs) are essential for control of plant functioning, growth, movement and development at every stage in the life history of higher plants (Provasoli & Carlucci, 1974; Tarakhovkaya *et al*., 2007). Consequently many naturally occurring and synthetic PGR substances have found a place in commercial agriculture where they are used for crop production management to ensure yields of high quality. Generally, auxins, cytokinins, and gibberellins are growth promoters whereas abscisic acid is a growth inhibitor (Saharan & Nehra, 2011).

Over the years, studies have shown little interest in the application of microalgae as a plant growth stimulant when compared to macroalgae (Sunita, 2009). The use of macroalgae to enhance plant production is well documented since it has been practised for decades at different commercial scales of food productions (http://www.algaewheel.com; http://www.kelpak.co.za; Thirumaran, 2009; Abd El-Motty *et al*., 2010; Craigie, 2010; Saneetha & Thavantha, 2010). Most research in agriculture has focused on the application of macroalgae in high-value horticulture production. The easiness in harvest of seaweeds could be a reason why numerous seaweed extracts exist in the market instead of microalgae extracts, despite all the apparent benefits of the microalgae.

There is growing evidence that microalgae produce plant hormones or demonstrate plant hormone-like activity, which suggests that microalgae extracts contain compounds beneficial for plant production (Stirk *et al*., 2002; Molnár & Ördög, 2005). These benefits include;

increased seed germination rate, enhanced root and shoot development, decreased plant senescence and transpiration, increased pod set and leaf chlorophyll content (Stirk *et al.*, 2002; Molnár & Ördög, 2005; Abd El-Moniem & Abd-Allah, 2008). Molnár & Ördög (2005) and Abd El-Moniem & Abd-Allah (2008) showed that microalgae extracts could improve horticulture production as demonstrated by increases in plant fresh weight and shoot regeneration. Such evidence has sparked attention towards use of microalgae application in horticulture and interest in investigating its potential for PGR-like effects. This chapter presents investigations of PGR-like activity on the three formulated HRAOP biosolids fertiliser extracts using bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants. Flowering response, fruit fresh and dry weight, production and harvest index were used to measure PGR- like activities possessed by the extract.

4.2. Materials and methods

4.2.1. Seed germination and plant hydroponic cultivation

Seeds of bush bean (*Phaseolus vulgaris* L. cv. Contender) and tomato (*Solanum lycopersicum* cv. Moneymaker) were purchased from a local seed supplier and germinated as described in 3.2.1. In order to transplant 14 days old bean plants and 35 days old tomato plants from seedling trays with vermiculite to hydroponic culture, plastic plant growing containers were constructed for plants to grow in nutrient solution, mimicking non-agitated hydroponic systems. The container design was modified from "Wiscosin fast plants: Farming in Space, WFP071698 design for portable plant light house" (http://www.fastplants.org). This included use of 2 L transparent soda bottles which were cut at 15 cm height from the bottom. The bottom part served as the nutrient solution reservoir (Appendix C). Four pores were drilled along the edge of the reservoir to allow ventilation. The top part of the bottle was inverted and inserted into the reservoir, serving as a funnel that allowed plant roots to be immersed in the solution while supporting aerial plant parts for upward growth. The reservoir was covered with aluminium foil to avoid algae growth in the solution.

4.2.2. Effects of HRAOP biosolids extract on growth and development of selected plants

Seedlings of contender bush bean (14 days old) and tomato (35 days old) were transplanted in hydroponic system containing 400 mL of nutrient solution. Nutrient solution consisted of (1:1 v/v) ratio of treatment to Hoagland solution (HS). Each container had three plants and

treatments conducted in triplicates. The HS was applied twice a week to keep the solution level. After the hydroponic systems had been started, 200 mL of treatment only was applied at vegetative, flowering and fruiting stages, respectively. The PGR-like activities depend on hormonal compounds as opposed to nutrient content and availability (Clarkson, 1980; Sinclair, 1992). Therefore, HS was added in all treatment as a basic nutrient supplier in order to avoid nutrient deficiency dependent effects. The plants were placed in randomised blocks at constant environment condition as described in section 3.2.1 (Figure 4.1).

Figure 4.1: Randomised distribution of tomato plants growing in a hydroponic system in a CE room with temperature of 28 ± 3 ℃ and average light of 230 µmole.m^{-2}.s^{-1} cool white light illumination at a 12 h light cycle during the investigation of PGR-like activities of HRAOP biosolids extract.

4.2.2.1. Effects of HRAOP biosolids extract on selected plants flowering and fruiting

Plant flowering response was monitored by recording a number of emerging flowers. This was done daily for the bean plants and weekly for tomato plants. Buds were considered to be flowers when they had fully blossomed. The time taken for each plant to flower was recorded. This was done until flowers senesce and little pods/fruits were produced. Bean pods were counted as fruits when they were approximately 1.5 cm long whilst tomato fruits were counted as fruit once they were approximate 12 mm to 15 mm in diameter. Matured fruits were harvested on day 60 for beans and day 150 tomato post-treatments. The average number of produced flowers and fruits were reported per each treatment.

On the harvest day, plant height, fruit number, fruits fresh weight and plant fresh biomass were recorded. Respective plants, fruit and biomass were placed in pre-weighed 250 mL flasks and oven dried at 70 ± 5 °C until constant dry weight was obtained. Harvest Index (HI) was used to determine production yield per each treatment. The HI is a variable factor to determine crop production (Yang & Zhang, 2010) and defined as a fraction of usable plant part (which could be a seed, fruit or vegetative part) in proportion to shoot total dry weight (Donald & Hamblin, 1976; Ghafoor *et al*., 1993). The HI measures the success in partitioning of the plant from vegetative to reproductive phase of the plant (Ghafoor *et al*., 1993). The HI was expressed according to equation:

$$ HI = \frac{\text{fruit dry weight (g)}}{\text{shoot dry weight (g)}} \qquad \qquad \textbf{Eq. 4} $$

4.3. Data and statistical analysis

The data presented are the mean value $\pm$ standard deviations (SD), and where possible experiments were repeated more than once in a randomised design. The data were analysed using Microsoft Office Excel 2010 where mean values and SD were calculated. Statistical analyses for this study were performed using Statistica 9.0 (StatSoft Inc, 2010), where *t*-tests were carried out to determine significant differences ($p < 0.05$) between samples and treatments. Line graphs were created using Microsoft Office Excel 2010 to establish flowering rate in response to various treatment.

4.4. Results

4.4.1. Flowering response of bean and tomato plants on HRAOP biosolids extract application

Flowering response was used as a measure of plant growth-like activity of HRAOP biosolids extract. The bean flowers were observed on day 16 following treatment where KP treated plants had an average of 7 flowers followed by 5% S which had 2.67 flowers. Other extracts treatments had an average of 1.67 flowers (5:1SM) and 1.33 flowers (1% M). Plants treated with DG, HS and deionised water treated plants had an average of 3.00, 2.33 and 0.67 flowers, respectively (Figure 4.2). Flower number increased gradually until day 19. On day 22 the flowers were shed while others developed into pods.
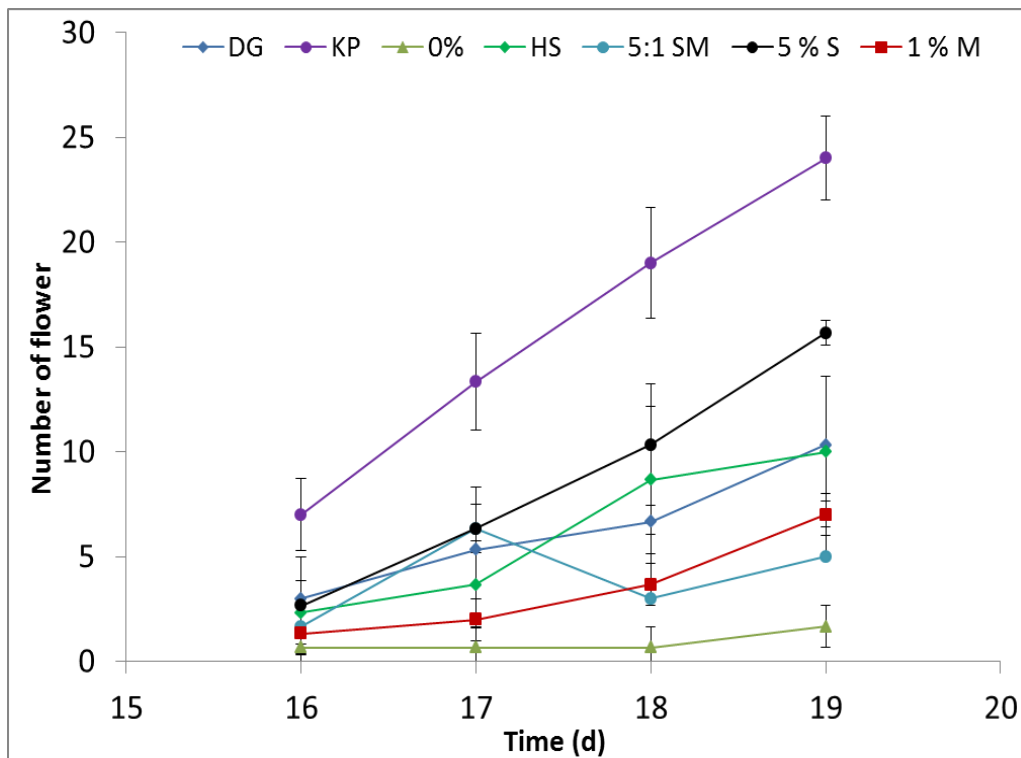
Figure 4.2: Flowering response of bean plants to formulated fertiliser extract (5% S, 1% M. 5:1 SM) and controls. Flowers started emerging from day 16 post-treatment and were counted daily until they developed to bean. Data are mean values ± SD.

Whereas in tomato plants flowers started to show on day 73 in plants treated with 5% S (Figure 4.3). Flowering was delayed by 7 and 12 days on the 1% M and 5:1 SM formulated extracts, respectively. No flowers were observed in tomato plants treated with KP until day 80 indicating a delayed response in terms of flower development. Highly variable plant response were noted as a result of KP and 5:1 SM treatments.
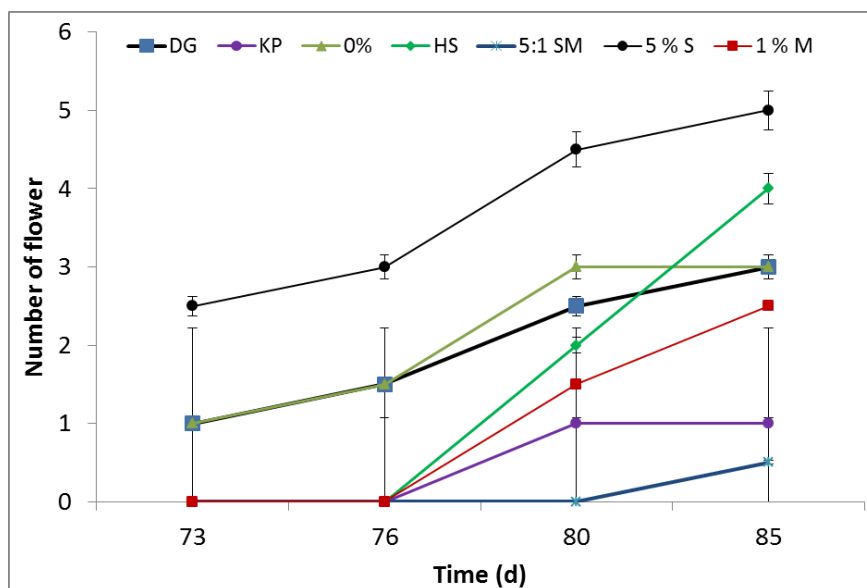
Figure 4.3: Flowering response of tomato plants to formulated fertiliser extract (5 % S, 1% M, 5:1 SM) and controls. Flowers started emerging from day 73 post-treatment and were counted once a week until they developed to tomato fruit. Data are mean values ± SD.

4.4.2. Plant productivity

The effect of formulated fertiliser extracts on fruit production and development was measured using number of fruits, fruit weight, and harvest index (HI) of bean and tomato plant, results are shown in Table 4.1.

Table 4.1: Shows growth and production of bean and tomato plants post treatment with formulated fertiliser extract for the investigation of PGR-like activities. [FW = Fresh weight, DW = dry weight, HI = Harvest index and na= not applicable].

	Fruits number		Fruit FW (g)		Fruit DW (g)		HI	
	Bean	Tomato	Bean	Tomato	Bean	Tomato	Bean	Tomato
Untreated	6.0±1.25	1.00±1.00	10.46±0.22	10.36±7.58	1.13±0.12	1.12±0.52	0.39	0.08
HS	6.0±0.82	1.33±1.15	11.68±0.88	7.44±6.82	1.13±0.10	0.54±0.50	0.40	0.03
KP	6.0±1.41	None	8.99±0.43	na	0.77±0.21	na	0.32	0.00
DG	7.7±1.63	0.33±0.58	10.39±1.33	4.26±2.46	0.90±0.13	0.16±0.28	0.36	0.01
5% S	6.0±0.82	1.33±0.58	10.38±2.15	11.10±3.91	0.99±0.39	1.04±0.33	0.39	0.08
1% M	8.0±0.47	1.00±1.73	11.34±2.48	1.21±0.70	0.94±0.29	0.02±0.04	0.35	0.00
5:1SM	6.7±1.41	1.00±1.00	9.90±3.08	12.96±7.48	0.95±0.18	0.39±0.67	0.36	0.02

In summary; the formulated fertiliser biosolid extract 1% M and 5:1 SM showed improved bean pod production and no effects on tomato plants. Whereas, 5% S treatment showed no improvement in bean production and 75% increase in tomato production. Overall, bean pod production was not enhanced by HS, KP and 5% S treatments. No productive response was shown by tomato plant treated with KP treatment (Table 4.1). Fruit dry weight as a measure of fruit development was lower for both bean and tomato plants treated with 5 % S, 1% M and 5:1 SM. However, this inhibition was slight in bean pods and no effects were noted on 5 % S treated tomato fruits. Notable, HS treatment had no effect on bean pod development as measured by fruit dry weight. The HI however indicates reduced productivity in both the bean and tomato plants that were treated with 5:1 SM whereas, 5 % S treatment presented no enhanced productivity in both crop plants. A slightly reduced HI was obtained in bean plants treated with 1 % M whereas no harvestable produces were obtained in tomato plant treated with 1 % M. By comparison, in both bean and tomato plants, KP, DG showed low productivity with HS showing effects that were similar to those presented by 5 % S.

4.5. Discussion

In view of the effective potential fertiliser, the three formulated HRAOP biosolids extract were tested for their plant growth regulator-like activities. The PGR-like activities were evaluated by recording the number of flowers, fruit production and quality, as well as HI of bean and tomato plants respectively. Successful plant production or increased yield is characterised as a function of a plants adaptation to its environment, its ability to effectively use mineral nutrients and harvest light for effective photosynthetic activities and its ability to partition and translocate photosynthesis assimilates to the reproductive organs (Foyer *et al.*, 2001; Zheng, 2009; Kant *et al.*, 2011). In this study the plants reproductive response was measured by recording flowering rate, number of fruit set and consequence the time for the plants to reach these stages. Fruit number, fruit size and HI measures nutrient assimilates distribution and successive translocation to reproductive organs where needed the most.

In both plants 5 % S extract stimulated early flowering with performance exceeding that of DG and HS. This effect suggests a possible presence of PGR-like activities in 5 % S extract. In bean plant 5% S extract performance in promoting flowering was comparable to that of Kelpak (KP). The time taken for the plants to reach maximal flowering was shorter with 5 % S treatment, measured by emergence of tiny pods. This short time frame is important for

production of even sized fruits set round about the same time which decreases the harvest period. Whereas in the tomato plants; KP treatment appeared not to induce any reproductive activities. Nevertheless, the results discussed in this chapter showed that a number of flowers was not the determinant of fruit production or final yield. This was however attributable to that plants generally shed off some flowers during pollination or fruit set. As noticeable in 5 % S treated plants which showed no improvement in productivity despite high rate response shown during flowering. And again with 1% M and 5:1 SM treated plants which both had delayed flowering but enhanced bean plant productivity although the HI indicated otherwise. In bean plants, KP and DG decreased the productivity while HS had no effects in enhancing productivity. In tomato plants, prolonged vegetation stage was noted. However higher flowering rate was recorded with 5% S treatment and this increase was significant different ($p < 0.05$) to untreated plants (0%). Whereas 5:1 SM treated plants presented very low or delayed flowering response. Tomato production was increased with 5% S and HS treatments; however the HI indicated no improvement in production on tomato treated with 5% S and reduction in HS treatments. Interestingly, tomato plants treated with 5:1 SM exhibited slow flowering rate yet the fruit production exceeded those of DG treated plants and equal to the treatment that initially had a higher number of flowers. This indicated that more flowers developed to fruits and in addition the fruit produced had higher water content or rather was fresher than other treatments. By comparison the tomato plants treated with 5:1 SM and 5 % S produced fruit that were bigger size. This indicated a possible presence of the plant hormones substances. Similar results were obtained by Arthur *et al*. (2003) where auxins and gibberellins found in microalgae extract increased the fruit set and size in tomatoes, cucumbers, aubergines and peppers. However, tomato final yield as indicated with HI was not enhanced by 5% S whereas it was reduced by 5:1 SM and no harvestable products were recorded as a result of 1% M treatment although flowering was remarkably increased. The delayed reproductive stage in tomato plants could be attributed to poor nutrient translocation. Tomatoes are known N-demanders thus it is possible that as the plant grows bigger it needed more frequency in nutrient replacement to account for the plants development functions. Hence, basic understanding of the plant needs are important as any change, or inadequate nutrient supply, may alter or prolong maturity, flowering, fruiting and the quality of fruit yield as well as physiological changes during fruit storage (Uddain *et al*., 2009). Both C and N nutrient compounds are essential for plants to perform fundamental cellular functions. Therefore the availability of these two nutrients is critical for plant growth, development and

response to any encountered conditions and for the completion of plant life cycle and the production (Zheng, 2009).

In addition, delayed leaf senescence could be a reason for low yield in tomato. Leaf senescence is characterised by a reduction in chlorophyll content and photosynthetic activities manifested by yellowing leaves (Wingler *et al*., 2006). According to Wingler *et al*., (2006), delayed leaf senescence interferes with transportation of nutrients assimilated during the vegetative stage to reproductive organs where they are needed the most. This compromises photosynthetic activity in the young leaves and subsequently the reproductive success. On the contrary, too early senescence reduces a plants ability to maximally assimilate CO_2 resulting in a decrease in plant biomass. On the other hand, early senescence can be favourable when the availability of photosynthesis assimilates are high or when the supply of mineral nutrients, such as N, is low (Ono *et al*., 1996). In this study the vegetative stage was prolonged indicating failure of the plant to partition nutrients to productive organs. Although the fertiliser extract stimulated early flowering and managed to produce the same number of flowers, or sometimes exceeding those of commercially available products, the nutrients were not evenly distributed according to areas of need in the plant. This suggested inactivated signalling in the plant life cycle.

The quality of a plant growing in containers is dependent on the size of the container, as the size restricts root growth and distribution (Mathers *et al*., 2007). Therefore, for this study the container might have significantly impacted plant growth. Root growth was noticeably limited in the plant containers; hence the normal functioning of the roots could have been adversely affected. The design of the hydroponic-like container could have infringed nutrient availability due to poor circulation within the system. In future, the design of plant growing containers could therefore be improved by continuous agitation which increases gaseous exchange, maximises nutrient circulation and availability. Air and water availability are critical factors in a root system and its functioning (Bassirirad, 2000). However, from this it was deduced that HRAOP biosolid fertiliser extracts support and enhance underground growth. This was shown by the remarkable new development of root and by the reduced shoot: root ratio discussed in Chapter 3.

Overall, the regulation of photosynthesis and plant development appears to depend on the C: N ratio rather than carbohydrates or C content (Ono *et al*., 1996; Zheng, 2009). Low N could

be the result of increased C accumulation as the demand for C-skeleton for protein synthesis declines (Ono *et al*., 1996). It was established in Chapter 3 that the HRAOP biosolid extract had no effect in plant nutrient balance and therefore had no adverse effects in the nutrient acquiring and translocation process. The plants treated with this extract demonstrated the capability of actively mobilising nutrients to the regions where needed. However, this was not true for tomato treated with 1 % M. Both 5 % S and 5:1 SM showed great potential as fertilisers and demonstrated PGR-like activities indicative of possible presence of PGR substances within the extract. However, commercial preparation of 5:1 SM formulation is restricted by additional cost and environmental hazards associated with organic solvent methanol, which is required during the preparation procedure. Economically and environmentally, this makes 5 % S formulation the most feasible, cost-effective and eco-friendly preference.

4.6. Conclusion

It can be concluded that the three formulated fertiliser extracts derived from HRAOP biosolids enhance plant growth and development. Although the presence of PGR substances was not analytically verified, induced flowering rate, fruit production, fruit development as well as results obtained at screening stage, namely improved germination index and regulated growth which was achieved during radish cotyledon expansion bioassay, suggest the presence of PGR activities in the formulated extract. However, more work is needed to verify this. Findings in this chapter suggest that formulation 5 % S and 5:1 SM are effective fertilisers and had PGR-like activities. Whereas 1 % M formulation potential as a fertiliser with PGR-like activities was not determined; the results were inconclusive. In many areas 1% M treated plants had responses similar to that of KP. However, flowering rate, fruit production and HI did not show any of PGR-like effects. Developed HRAOP derived fertiliser extracts treatments might have effects not distinct to untreated and commercial products. Its benefits in native soil structure, environment and cost-effectiveness still make the product worthwhile. The extract can still be beneficiary in areas such as fields, lawns and gardens, where yield is not a goal. Unexpected low productivity could be due to slow nutrient release and degradation, therefore the long term effects of fertiliser extract needs to be investigated.

Chapter 5

General discussion, conclusion and recommendations

5.1. Concluding discussions

This study was conducted to determine whether biosolids generated in a HRAOP as by-products of domestic wastewater treatment can be used as a starting material for the production of organic fertilisers and/or plant growth regulators. HRAOP, a component of IAPS generates an estimated 400 – 830 kg biosolids daily which are predominantly microalgae. Microalgae work as a major biological catalyst responsible for nutrient removal during the wastewater treatment process. HRAOP biosolids are therefore presumed as being highly enriched with minerals, trace elements and amino acids essential for plant growth (Mahmoud, 2001; Rose *et al*., 2002; Abd El-Moniem *et al*., 2008) and possibly contain plant hormones, such as auxins and cytokinins found in microalgae (Provasoli & Carlucci, 1974; Takhovskaya *et al*., 2007). Three fertiliser extracts derived from HRAOP were successfully developed and subjected to different tests to determine their efficacy as a fertiliser and/or plant growth regulator. This was achieved by comparing HRAOP biosolids derived fertiliser extract performances with a scientific plant nutrient, namely Hoagland solution, and the commercially available plant growth regulator; Kelpak. HRAOP biosolid remains a potential nutrient resource that is not fully exploited (Rose *et al*., 2002; Horan *et al*., 2005). Exploitation of this by-product can produce products, such as compost and/or fertiliser, which can benefit local crop production projects, nurseries and municipal facilities (e.g. sport fields). Valorisation of this biosolids is cost-effective since the raw material is readily accessible and it requires no transportation.

Generated data indicated that the HRAOP biosolids have a potential to be used as a fertiliser and contain PGR-like activities. HRAOP biosolids fertiliser extracts supported growth and development of the plant based on an evaluation that included specific leaf area bioassay, measurements of plant biomass, plant height and shoot: root ratio. Plants treated with the formulated extracts showed positive responses; the leaf area was increased, chlorophyll content was not affected and results were comparable to the controls. The C: N ratio was within normal ranges, indicating that the extracts have no adverse effects on plant critical nutrient balances. Balanced C: N ratio is critical for normal functioning of the plant. Both C

and N are interactive in modulating plant signals during the vegetative and reproductive stages of the plant (Zheng, 2009). In addition the formulated fertiliser extracts promoted root growth and development.

No effects on chlorophyll content indicated a plant positive response to the formulated extracts. Chlorophyll content directly relates to photosynthesis activities and hence chlorophyll content is used as measure of photosynthetic activities (Shaahan *et al*., 1999; Abd El-Baky *et al*., 2008). Both chlorophyll and photosynthetic activity increase during the vegetative and early flowering stages but decrease during fruit setting (Shaahan *et al*., 1999; Gauba *et al*., 2007; Jarillo *et al*., 2008). Depleted chlorophyll content can be caused by N deficiency. Chlorophyll content is a sensitive indicator of plant nutritional status, particular N contents (Wu *et al*., 2008). Apart from N deficiency, chlorosis or decrease in chlorophyll content could be due to various stress conditions, such as drought, too much light and salinity (Fanizza *et al*., 1991; Abd El-Baky *et al*., 2008; Atlassi *et al*., 2009; Rosyara *et al*., 2010). Nevertheless, some plants respond to shade by increasing leaf chlorophyll content (Nemali & Van Iersel, 2004). No adverse effects on chlorophyll content during the current study meant that there was no disruption in photosynthesis activities and this was supported by enhanced vegetative growth or plant biomass. This could be related to the ability of microalgae to increase absorption of magnesium (backbone of chlorophyll molecule) and sodium (El-Sheekh & El-Saied, 2000). Thus HRAOP biosolids derived fertiliser extracts increase the vegetative growth criteria and potential crop yield on treated plants.

In addition, formulated fertiliser extracts demonstrated PGR-like activities. Early flowering was notably induced with a potentially high productivity in plants treated with the formulated fertiliser extracts. This was attributed to related PGR substances that are possibly contained in the formulated extracts (Ördög *et al*., 2004; Molnar & Ördög, 2005; Abd El Moniem & Abd-Allah, 2008; Abd El Moniem *et al*., 2008). Some microalgae have an ability to fix nitrogen and produce bioactive compounds which influence plant growth by promoting germination, leaf or stem growth, and flowering (Pulz & Gross, 2004). As reported earlier, formulated fertiliser extract promotes germination as demonstrated by an increased germination index (GI) of radish and tomato seeds. The GI of 5:1 SM and 5 % S was remarkably higher than any other investigated treatments. Studies have shown that pre-treatment of seeds with algae extract increased the GI (Adam, 1999; Abd El Moniem & Abd-Allah, 2008; Faheed & Abd-El Fattah, 2008; Thirumaran, 2009). Metting and Pyne (1986) reported that PGR in *Chlorella*

stimulates seeds germination. The increased GI was attributed to the presence of PGR substances and the ability of the formulated extracts to increase nutrient uptake (Ördög *et al.*, 2004; Molnar & Ördög, 2005; Abd El-Moniem & Abd-Allah 2008). Increased GI indicated suitability of the HRAOP biosolids fertiliser extracts to enhance plant growth and development. Although PGR-like activities were demonstrated by the formulated fertiliser extracts, the presence of plant hormones or stimulants in the extract still needs to be verified. Extensive investigations on isolation, characterisation, evaluation and classification of PGRs on the formulated extract are needed. Lack of this information limits the ascertainment whether the stimulations were caused by a particular PGR or were solely due to the nutrients value of HRAOP biosolids extracts.

Overall, data illustrated that the response of Moneymaker tomato and Contender bean plants to the three biosolids fertiliser formulations was consistent with little or no difference to commercially available products and plants showed no stress signs. Data suggest that 5 % S extract influences plant height growth, increases plant biomass, increases leaf area, promotes germination, enhances radish cotyledon weight and induces higher flowering rate. The mixed formulation 5:1 SM extract increases plant biomass, increases leaf area, enhances GI for both tomato and radish seeds, increases the radish cotyledon fresh weight, elevates shoot: root ratio and productivity. The 1 % M extract increases plant height, plant biomass, enhances GI of radish seeds, elevates shoot: root ratio and enhances bean pod production. Both 5:1 SM and 5 % S showed efficiency as a fertiliser and demonstrated the potential presence of PGR-like activities. Data suggest that the 5 % S extract could make a better fertiliser; 5:1 SM demonstrated stronger effects as PGR while 1 % M extracts most strong points are not certain. However, for cost-effectiveness the use of methanol during the preparation of 5:1 SM restricts industrial preparation of this extract. Hence, 5 % S formulation is the more feasible, cost-effective and eco-friendly preference. The concept process for the production of 5 % S fertiliser extract formulation requires sonication of harvested HRAOP biosolids and appropriate collection of the extract. For example, preparation of a (1 L) 5 % S fertiliser extract derived from HRAOP involves harvest of HRAOP biosolids, pellet by centrifugation at 8000 *g* and drying. This is followed by suspension of 10 g of biosolids with 0.1 L deionised water, sonicating, pelleting and collecting the supernatant by passing through a filter. After which 0.05 L of the obtained concentrated extract is diluted to a 1 L using deionised water.

Presented data revealed the beneficial uses of biosolids generated in the wastewater treatment process. It is recommended that future studies should focus in, for example, carrying out a comprehensive chemical characterisation of the formulated fertiliser extracts. This should include plant essential macro, micronutrients and metals likely to be found in domestic sewage which could have an influence in plant response to the extract. To avoid potential environmental and human health concerns, fertiliser toxicity investigations should be undertaken. In terms of environmental sustainability, appropriate application rates, including effects of formulated fertiliser over-application and potential nutrient leachate (e.g. nitrate and phosphate) should be investigated. Effects of cation and anion exchange capacity, effects in substrate organic matter as well as possible effects on microbial diversity and interactions also need to be considered. This would indicate the success of the formulated HRAOP biosolids fertiliser extracts (Shaviv & Mikkelsen, 1993; Marschner *et al*., 2003; Citak & Sonmez, 2010; Mohammadi *et al*., 2011). In addition determination of nutrient values, particularly primary macro-nutrients concentration, is equally important. Reason being each crop has its unique demand and tolerance of certain elements, such information could also assist to avoid over-applications of nutrient. The ratio of ions and their counter ions such as Na/K, Cl/Br could be useful in assessing environmental impact upon applications of these extracts (Hochmuth *et al*., 2000).

Application of HRAOP biosolids, as granulated dry biomass, liquid fertiliser, pelletised or incorporated with plant beneficial microbes, such as fungi or bacteria, could also be investigated in future studies. The latter would be done as effort to enhance and possibly produce the super-active PGRs or fertiliser (Abdel Moniem *et al*., 2008; Addel Motty *et al*., 2010). Added microbes would improve the effectiveness of the extract and maintain natural interactions in the rhizosphere. This could form a fast and more effective biofertiliser which readily converts complex organic material into a simple compound for bioavailabilty of plants. Research has shown that algae cells (N-fixers) are less effective for higher yield. Therefore to increase the yield algae could be coupled with other microbes, yeast or *Azolla* (Abd el Moniem *et al.,* 2008; Abd El Motty *et al.,* 2010). The effect of combined algae extract and yeast on mango plants was examined by Abd El-Motty *et al*. (2010) who reported more effectiveness of microalgae and yeast combination and production of high quality mangos. Exploitation of the HRAOP biosolids as a soil conditioner through production of compost or direct application of dried biomass also needs attention in order to optimise valorisation of the IAPS biosolids. A composting process could improve bioavailability of N

and P due to bacterial degradation prior to applications (Preusch *et al*., 2002). Dried biomass could revitalise soil as slow nutrient release a fertiliser or soil additive. According to El Fouly *et al*. (1992), dried microalgae contain high nutrient levels and a considerable amount of amino acids.

Long term impact on the soil and environment needs to be assessed (Marschner *et al*., 2003). Variables, such as soil type, soil pH, nutrients content and water affect nutrients uptake and ultimately productivity (Marschner *et al*., 2003). Hence it is important to know properties of growth media to foresee possible growth limiting factors. Growth media, properties such as porosity status, drainage and water holding capacity play a major role in root growth. Therefore good aeration with maximal gaseous exchange and nutrient mobility for efficiency is needed (Allaire *et al*., 1996). Unless otherwise stated, in this study vermiculite was used because it was a readily available substrate with fairly stable properties. Vermiculite provides aeration, retains plant nutrients and moisture and releases them later based on the plants demand. Vermiculite is sterile and free from diseases, non-toxic, safe to use with a fairly neutral pH and relatively inexpensive. However, nutrient retention of vermiculite based on its cation exchange properties also needs to be verified.

Based on the observed PGR-like activities, it would be useful to compare the performance of formulated extracts and that of authentic plant hormones, which might be present in these extracts. The question of how much hormone, if any, is present is also one component that needs to be looked at simply because the effects of PGR on a plant is dependent on the amount of hormone present as well as sensitivity of the targeted tissue. For instance a low concentration of auxins on plant seedlings may stimulate growth while a high concentration may have inhibitory effects (Arshad & Frankenberger, 1991; Saharan & Vehra, 2011). Auxins are generally applied to initiate flowering which plays a key role in fruit development, and to prevent immature fruit drop, leaf abscission. At higher concentrations auxins are used to control weeds (Arshad & Frankenberger, 1991; Saharan & Vehra, 2011). Large volume harvesting, drying and processing of HRAOP biosolids also need to be addressed to make the production process cheaper and the product price competitive.

In conclusion HRAOP biosolids fertiliser extract demonstrated fertiliser characteristics and PGR-like activities. Such benefits could assist in overcoming problems associated with processing and disposal of wastewater treatment works (WWTW) generated biosolids and

reduce the pressure on chemical and fossil derived fertilisers. Formulated fertiliser extracts are therefore recommended as a renewable natural fertiliser which is an alternative to industrial chemical and fossil derived fertilisers, synthetic PGR and manures. Although potential use as a PGR still needs more work, some qualities of the presence of PGR substances and PGR-like activities were demonstrated in this study. Overall, it seems that with additional research the wastewater derived HRAOP biosolids (microalgae biomass) can be processed to produce an effective, environment friendly and price competitive fertilisers.

One of distinguishing feature of HRAOP biosolids is; its dominant algae biomass with significant amounts of organic carbon which most commercial fertilisers, especially inorganic fertilisers lack. Hence application of HRAOP biosolids fertilisers will increase organic matter percentage in soil over time. Furthermore, microalgae contribute to soil fertility by producing substances which assist with particle adherence and water storage (Pulz & Gross, 2004). Microalgae contain significant levels of nutrients and amino acids which add to their value as a fertiliser. Moreover, mineralisation of N slowly occurs in microalgae biomass, noted by slow release of N in microalgae treatment, as reported by Mulbry *et al*. (2005). This is important in areas where deteriorations in organic matter are of concern. When compared to traditional manures, HRAOP biosolid is less bulky, more porous with less moisture content and contains a considerably higher percentage of N-content. These qualities are attractive objectives in terms of increasing soil fertility, restoring depleted nutrients, increasing yield and sustainable food production. Microalgae generated in HRAOP hold some advantaged over industrial algae biomass productions. Generally, production of microalgae biosolids fertiliser requires specific nutrient and supplements to manipulate productions while microalgae biomass sourced from HRAOP feeds on nutrient and mineral contained in wastewater, this exclude cost for biomass generation (Oswald, 1988; Rose *et al*., 2005). An integrated system for algae production is one of the promising cost-effective tools for sustainability (Horan *et al*., 2005). Iyovo *et al*. (2010) demonstrated the possibility of integrating biomethane, biofertiliser and biodiesel production from poultry WWTW. In the study, *Chlorella vulgaris* cultures were fed on residuals from biomethane production. Ideal algae from wastewater have C: N ratio of 9:1 and are rapidly degradable (Brady & Weil 2007).

Exploitation and valorisation of HRAOP biosolids as fertiliser products is promising. The used raw material is organic, renewable and cost-effective. Overall, useful applications of

IAPS by-products could make this system more attractive. However, for commercial reasons the use of organic solvent during fertiliser preparation procedure is not recommended due to associated cost and possible environmental impacts. Incorporating wastewater treatment, particular IAPS and eco-friendly sustainable agriculture, would result in a remarkable contribution towards sustainable agriculture development, less dependence on chemical and fossil derived fertiliser and/or PGR, as well as unintended environmental threats associated with productions and applications of these products. While at the same time responding to food security, depleted water reservoir security and socio-economic growth.

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Appendices

Appendix A

Schematic diagram of the principal unit operations of the IAPS constructed on site at the Institute for Environmental Biotechnology, Rhodes University (Rose *et al*., 2002).
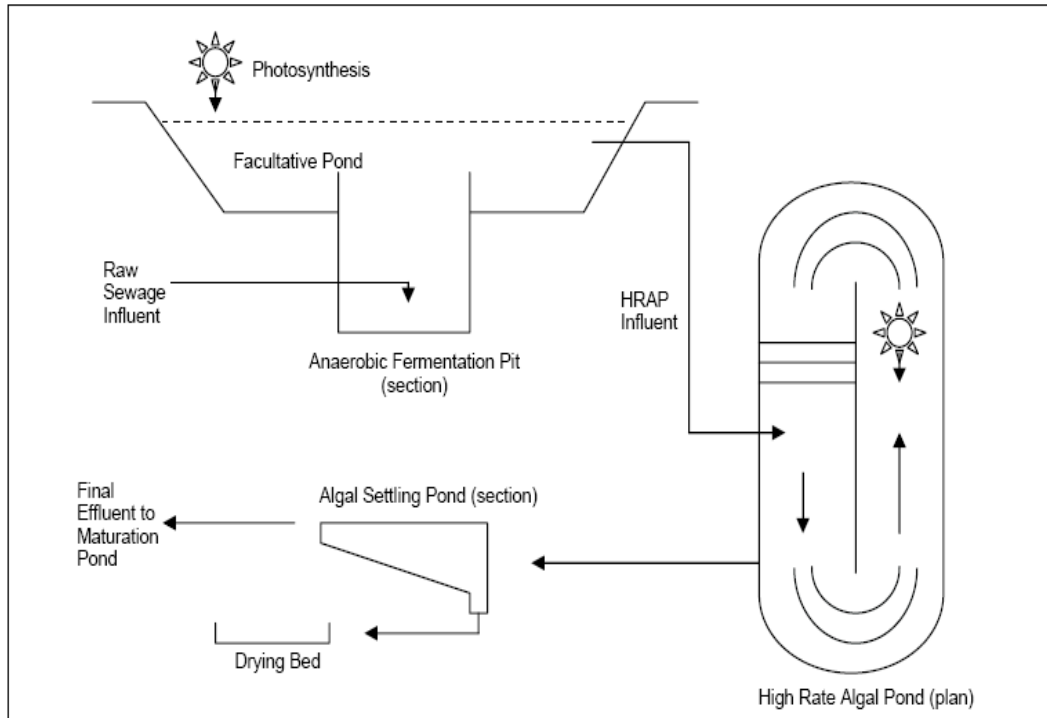
Appendix C

Plant growing container designed using 2L plastic soda bottles which was cut at 15 cm height. The bottom part serving as nutrient resevoir and the top part inverted as funnel.
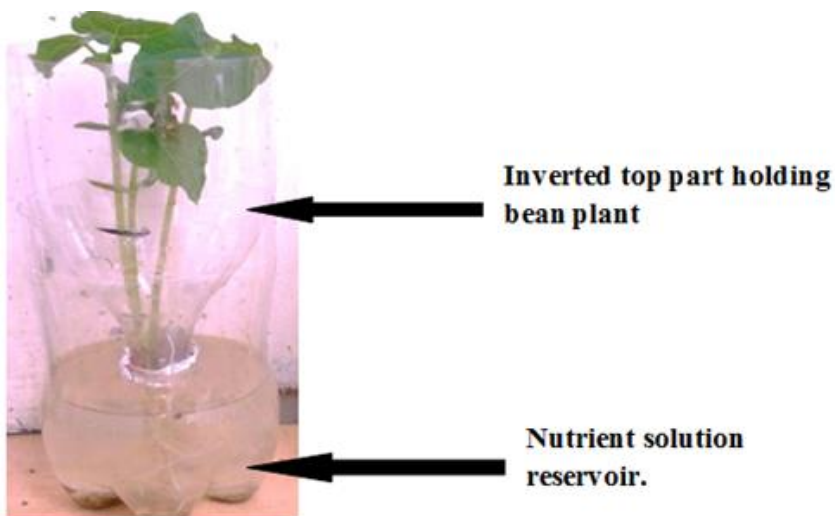
Appendix B

Detailed recipe of Hoagland solution (plant nutrient) preparation

To make 1 L Hoagland's solution from these stocks

1) Make up stock solutions and store in separate bottles with appropriate label.

2) Add each component to 800mL deionized water then fill to 1L.

3) After the solution is mixed, it is ready to water plants.

4) Keep at cool dark environment.

#	Component	Stock solution	mL Stock Solution/1L
1	2M KNO_3	202g/L	2.5
2	2M $Ca(NO_3)_2$ x $4H_2O$	236g/0.5L	2.5
3	2M $MgSO_4$ x $7H_2O$	493g/L	1
4	1M NH_4NO_3	80 g/L	1
5	1M KH_2PO_4 (pH to 6.0 with 3M KOH)	136g/L	0.5
6	Trace elements (Add up in one 1L bottle) 6.1. H_3BO_3 6.2. $MnCl_2$ x $4H_2O$ 6.3. $ZnSO_4$ x $7H_2O$ 6.4. $CuSO_4$ x 5 H2O 6.5. Na_2MoO_4 x $2H_2O$	 2.86g/L 1.81 g/L 0.22 g/L 0.1 g/L 0.12 g/L	1
7	FeEDTA solution (Add up in one 1L bottle) Make up 1 L of KOH: Dissolve 56.1 g KOH in 1 L; adjust pH to ~5.5 using H_2SO_4. Then add 10.4 g EDTA.2Na and 7.8 g $FeSO_4.7H_2O$.		1.5

Appendix D

Images showing fertiliser extracts obtained after sonication of HRAOP biosolids. A: Undiluted fertiliser extract as obtained after extracting with 40 % v/v methanol (M) and deionised water (S). B: Prepared HRAOP fertiliser extracts (3, 4 and 5) against used controls Kelp (2) and brown algae anaerobic digestate (1).