African Journal of Biotechnology

Volume 15 Number 23, 8 June 2016

ISSN 1684-5315

Academic Journals

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African Journal of Biotechnology

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Vol. 15(23), pp. 1078-1082, 8 June, 2016
DOI: 10.5897/AJB2015.15023
Article Number: D337E2E58781
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

An improved plating assay for determination of phage titer

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Received 4 October, 2015; Accepted 18 May, 2016

Phage is a virus that is parasitic on bacteria. It is very important to determine the titer of test sample in the study of phage. In this study, an improved plating assay was developed for detection of the number of recombinant phage Cap-T7 present in a test solution at a certain dilution point by counting the plaque forming units. The data demonstrated that the improved plating assay is fast, useful, and convenient for the determination of the phage titer in a sample.

Key words: Phage Cap-T7, detection method, plaque forming units.

INTRODUCTION

Bacteriophage is a virus that infects and replicates within a bacterium; it was discovered independently by Frederick Twort and Félix d'Hérelle, respectively, in 1915 and 1917 (Kaur et al., 2012). There are a wide variety of phages and they are among the most common and diverse entities in the biosphere (Clokie et al., 2011). Every bacterium is likely to have their own specific phage viruses (Flores et al., 2011; Örmälä and Jalasvuori, 2013), and phages play an important role in bacterial evolution (Labrie et al., 2010).

Phages infect bacteria very selectively; their growth and proliferation are in a specific host without harming commensal bacterial flora (Loc-Carrillo and Abedon, 2011). Phages have significant potential in the resistance to bacterial infection; they have been used for many years as the alternative to antibiotics in the former Soviet Union and Central Europe, as well as in France (Clark and March, 2006). While the role of phage anti-bacterial infection has been ignored for many years due to all kinds of reasons, including the discovery of the antibiotics (Cui, 2015). In recent years, antibiotic-resistant bacterial infection has become a global concern (Zhang et al., 2015); more and more countries presently ban the use of antibiotics to control bacterial infections in swine (Thacker, 2014). Phage therapy is re-valued by researchers to combat the growing menace of antibiotic-resistant infections (Torres-Barceló and Hochberg, 2016).

Determination of phage titer in a sample is a key step in the study of the phage involved. It is very important to select suitable dosage in the phage therapy for bacterial infection, to analyze the efficiency of packaging reactions *in vitro*, to detect the number of primary recombinant

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phages in a constructed library, and to monitor the enrichment of phage during biopanning. As a common method of detection, a plating assay at present is widely used to detect phage number present in the test samples at various diluted points (Sambrook and Russell, 2001); the results are then further used to calculate phage titer. According to the plating assay, the test sample from every one dilution at least needs one agarose plate. This may lead to the need of a lot of plates, waste large amount of reagent and consumptive materials, increase the experimental funds and labor. The aim of the current study was to develop an improved plating assay for the determination of phage titer in a fast, useful and effective manner.

MATERIALS AND METHODS

Phage and host strain

The purified recombinant phage Cap-T7, conserved in Chongqing Academy of Animal Science Veterinary Research Institute, was used in the present investigation. *Escherichia coli* BLT5403, the host strain of phage Cap-T7, was bought from Novagen, Inc. (Darmstadt, Germany).

The plating assay of phage Cap-T7 by the fixed method

The plating assay is used to calculate the number of phage Cap-T7 present in samples at various points according to the fixed protocol described briefly as follows: a series of dilutions of the Cap-T7 were prepared by adding 100 µl of the sample to 900 µl of sterile Luria-Bertani (LB) medium. 250 µl of the host cells (OD_{600} = 0.8) were pipetted into 4 ml sterile tubes. Starting with the highest dilution, 100 µl of the Cap-T7 dilution was added into each tube. After that, 3 ml of autoclaved and cooled to 45°C top agarose (Bacto tryptone 1 g, yeast extract 0.5 g, NaCl 0.5 g, agarose 0.6 g, per 100 ml) was added to the tubes. The contents were mixed and then poured onto a pre-warmed LB/carbenicillin agar plate. On the agarose spreading evenly and hardened, the plates were inverted and incubated at 37°C for 4 h. Phage number in test sample was calculated by counting the plaque forming units (pfus) on the plate.

Improved plating assay of phage Cap-T7

Preparation of the improved agarose plate

LB liquid culture (4 ml) of *E. coli* BLT5403 (OD_{600} = 0.8) were added into 100 ml triangular flask containing 50 ml autoclaved and cooled to 45°C top agarose as described earlier. The contents were immediately mixed and then 4 ml were taken out from the flask, and poured in a standard 100 × 15 mm Petri dish containing 20 ml of hardened LB/carbenicillin agar pre-warmed at 37°C. The plate was swirled gently to spread the agarose evenly. The plate was allowed to sit undisturbed until the top agarose was hardened, and then it was inverted and stored at 4°C.

Phage incubation and its titer

A series of the phage Cap-T7 dilutions were carried out as ten-fold dilution described earlier. After that, 5 µl solutions were taken out from every phage dilution and 4 different dilution samples were uniformly put in separate spots onto the surface of the improved agarose plate. The plate was allowed to sit undisturbed for more than 30 min until the 5 µl solutions become dry. Then, it was inverted and incubated at 37°C. Phage number in test solutions were calculated as described earlier.

Statistical analysis

The data was expressed as mean ± standard deviation (SD). Statistical significance of the difference was analyzed using Student's T-Test. P-value less than 0.05 ($P < 0.05$) was considered as statistically significant.

Plaque polymerase chain reaction (PCR)

The pfus were randomly selected out to be used for subsequent PCR assay according to the protocol of T7Selected® System Manual (Novagen, Darmstadt, Germany). A set of specific primers were designed using software GeneTool, the forward primer is 5'-ggc tgc agg aat tca tga ctt-3', reverse primer is 5'-tcg ata agct tgt acg ggt t c-3'. Amplifications were performed with 0.5 µM of each primer, 0.2 mM of each dNTP and 2.0 U *Taq* DNA polymerase (TaKaRa, Dalian, China) with the following cycling parameters: denaturation at 94°C, 4 min; 35 cycles (94°C, 45 s; annealing at 55°C, 40 s; 72°C, 60 s); final elongation at 72°C, 10 min. PCR products were then electrophoresed and the sequences were identified (Sangon Biotech, Shanghai, China).

RESULTS AND DISCUSSION

Plaque assay

T7 phage, one of the best-studies bacterial viruses (Häuser et al., 2012), is a lytic phage. Its phage particle assembly takes place in *E. coli* cytoplasm and mature phages are released by cell lysis (Dunn and Studier, 1983). Therefore, when the Cap-T7 was present in the test sample, pfu (clear area) will be observed in the lawn of its host *E. coli* BLT5403 which corresponds to individual phage infection event produced by single Cap-T7. In the experiments, ten-fold dilutions of the Cap-T7 ranging from 1×10^{-16} to 1×10^{-20} solutions were used to affect bacteria for pfus counting. With the extension of culture time, pfus appeared to become larger and larger. A series of plaques were observed on the plates incubated at 37°C for ~4 h. The pfus in the improved agarose plates vary from mutual fusion to clearly distinguishable and numerable single unit with the increase of gradient dilution up to no pfus appearing at last. More specifically, some pfus can be found at 10^{-17}, 10^{-18}, and 10^{-19} dilution points, with 3 clearly distinguishable pfus that can be counted at the 10^{-19} dilution point (Figure 1a). In contrast, the pfus from one of the plates of the fixed plating assay reflected the phage number of the test solution at only one dilution point. In this case, there were only 73 pfus in the 100 µl solution at the 10^{-19} dilution point (Figure 1b). Besides, some additional tests were also conducted to perform the plaque assay using the same methods described earlier,

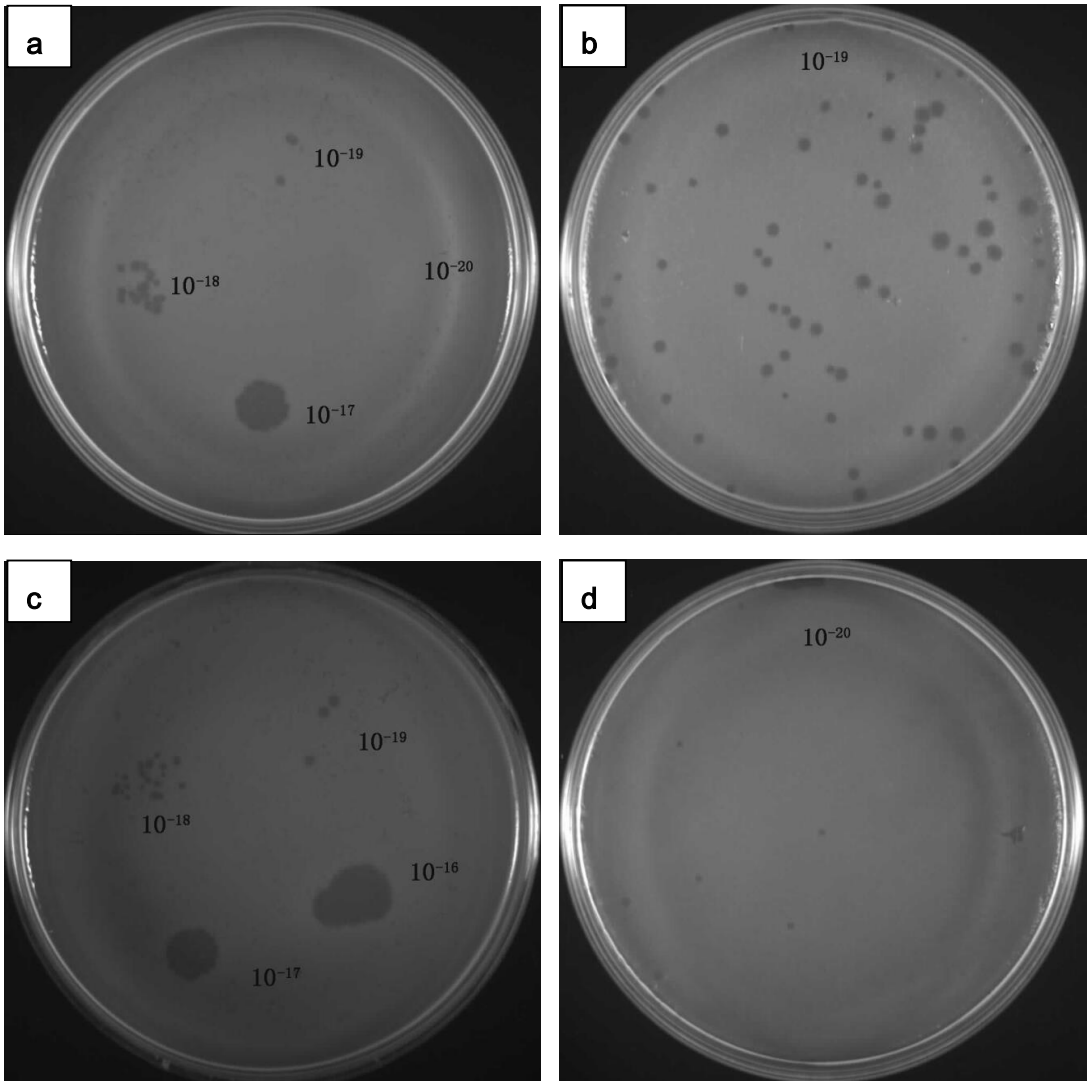

Figure 1. The pfus in the agar plates incubated at 37°C for ~4 h at different dilutions. Ten-fold dilutions of the test sample Cap-T7 ranging from 1×10^{-16} to 1×10^{-20} solutions were used for pfus counting. As indicated in the agar plates, a and c showed the plaques in the improved plate at 10^{-17}, 10^{-18}, 10^{-19}, 10^{-20} and 10^{-16} 10^{-17}, 10^{-18}, 10^{-19} dilutions, respectively, and 3 single plaques were observed in the 5 µl at 10^{-19} dilutions. b and d showed the plaques in the plate of fixed method at 10^{-19} and 10^{-20} dilutions, 73 and 7 single countable plaques can be observed in the 100 µl solutions, respectively.

and similar results were obtained. For instance, they also have a series of plaques at different dilutions including the 3 single pfus at the 10^{-19} dilution point in the improved plate (Figure 1c) and 7 plaques at the 10^{-20} dilution point in the control plate (Figure 1d). Obviously, it is more convenient for the plaque assay using the improved method than that using the fixed method.

Determination of phage titer

According to the number of the single countable plaques on the plate, the volume of the detection, and the dilution of the test sample, phage Cap-T7 titer can be easily calculated. For example, there were 3 plaques on a plate from a 10^{-19} dilution in 5 µl volume (Figure 1), then the titer of the sample was $3\times200\times10^{19} = 6\times10^{21}$ pfu/ml based on the improved method. Through three separate experiments, the titer of the phage Cap-T7 in the test sample was finally determined (Table 1). The phage titer values obtained from the improved plating assay and the fixed plating assay were not identical, showing certain degrees of variation. However, the two values ($6.67 \pm 1.15 \times 10^{21}$ and $6.93 \pm 0.40 \times 10^{21}$) have the same order of magnitude, and there was no statistically significant difference ($P > 0.05$) for the two test methods to determine

Table 1. Statistical analysis of the phage titer assays.

Group	Dilution	Spotting solutions (µl)	Phage titer (pfus/ml)	*P*-value
The improved plating assay	10^{-19}	5	$6.67\pm1.15 \times 10^{21}$	$P > 0.05$
The plating assay	10^{-19}	100	$6.93\pm0.40 \times 10^{21}$	

The values of phage titer are the average values of three determinations with standard deviation. P < 0.05, significantly different after Student's T-Test analysis.

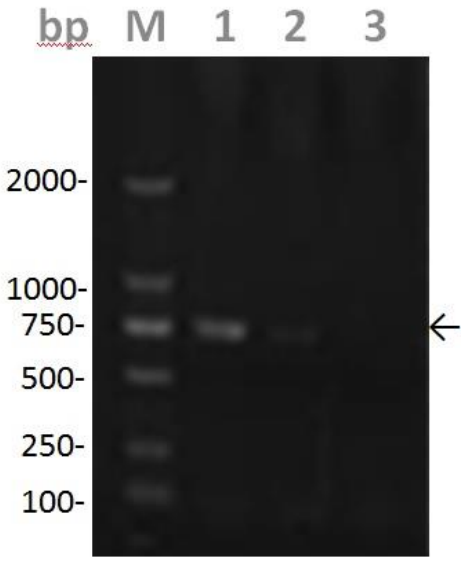

Figure 2. The electrophoresis results of the plaque PCR production. Product length was 727bp. M. Stand for DNA Marker, 1. PCR production of plaque from the plate of the improved plating assay, 2. PCR production of plaque from the plate of the common plating assay, 3. Negative control.

the phage titer. At the same time, the statistical analysis also proved that it was credible using the improved plating assay to determine phage titers.

Identification of plaque forming units

In order to determine the plaque forming unit authenticity of the recombinant phage particle, some plaques were selected out from the plates randomly and PCR analysis was conducted. The agarose gel electrophoresis map of the 2 plaques' PCR production is as shown in Figure 2. The result showed that there was a clear DNA band located at about 750 bp in the gel and the molecular weight (MW) of PCR production of the plaques from the improved plating assay group and the fixed plating assay group was consistent with the expected length size. In addition, sequence identified results also verified that the DNA bands had the same DNA sequence (Sequence 1). All proved that the phage plaques obtained from the improved method were the target plaques rather than others.

Conclusions

In this paper, recombinant phage Cap-T7 was taken as the research target to improve the common plating assay for the detection of the number of phages present in a test solution by counting pfus at a certain dilution and easily determined the titer of the phage Cap-T7. When compared with the common plating assay, the improved plating assay showed some advantages in the determination of the phage titer in a sample, such as using less agarose plates, saving labor and time. In a word, improved plating assay is feasible and worth spreading. Finally, it should be emphasized that in order to obtain the clear, distinguishable and countable pfus

Sequence 1. The DNA sequence corrosponding to the PCR production for identifying plaques.

GGCTGCAGGAATTCATGACTTATCCGCGTCGTCGCTATCGTCGTCGCCGTCACCGCCCA
CGTTCCCATCTGGGTCAGATCCTGCGTCGTCGTCCGTGGCTGGTACATCCGCGCCACCG
CTACCGTTGGCGCCGTAAAAACGGTATTTTCAACACCCGTCTGTCTCGTACCTTCGGTT
ACACTATCAAACGTACCACCGTGAAGACCCCTTCTTGGGCAGTTGACATGATGCGCTTC
AACATCAACGACTTCCTGCCGCCAGGCGGTGGTTCCAACCCGCGTTCCGTTCCGTTTGA
ATACTACCGCATTCGTAAAGTGAAAGTTGAGTTCTGGCCGTGCTCTCCGATTACGCAGG
GCGACCGTGGTGTTGGCTCTTCTGCGGTTATCCTGGATGATAATTTCGTAACGAAAGCT
ACTGCTCTGACCTACGACCCGTACGTCAATTACTCTAGCCGCCACACCATTACCCAACC
GTTCTCTTACCACAGCCGTTACTTCACGCCGAAACCGGTACTGGATTCCACCATCGACT
ATTTTCAGCCGAACAATAAGCGTAACCAGCTGTGGCTGCGTCTGCAAACTGCCGGTAAC
GTGGATCACGTTGGCCTGGGCACTGCGTTTGAGAACAGCATCTATGACCAGGAATATAA
CATCCGTGTGACCATGTACGTCCAGTTCCGCGAATTTAACCTGAAAGATCCTCCACTGA
ACCCGTACAAGCTTATCGA

using the improved plating assay, more attention should be given to well distribute the mixture of host cells and melt top agarose, select appropriate volume amount of spotting solutions, control the incubation time, and be sure to replace the pipette tips between samples of different phage dilution to avoid cross contamination.

Conflict of Interests

The authors declare that they have no conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by Chongqing Basic and Advanced Research (No. cstc2014jcyjA80027) and Chongqing Basal Research Funds (No.15439). The authors appreciate the assistance of Guo-Yang Xu in improving the manuscript. They also thank Ke-Fei Shen and Rui Yang for their generous help and valuable discussions.

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Vol. 15(23), pp. 1083-1100, 8 June, 2016
DOI: 10.5897/AJB2015.14789
Article Number: B2F21C458788
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Improving the optimum yield and growth of *Chlamydomonas reinhardtii* CC125 and CW15 using various carbon sources and growth regimes

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Received 10 June, 2015; Accepted 10 May, 2016

Chlamydomonas reinhardtii **CC125 (wild type) and CW15 (cell wall mutants) were feed up on solid and liquid Tris phosphate (TP) media with various concentrations of acetate, glycerol(10-100 mM) or methanol (0.01-718 mM) and cultivated under phototrophic, mixotrophic and heterotrophic conditions. Use of 10 and 35 mM acetate and 10 and 50 mM glycerol under constant 38 µE m^{-2} s^{-1} light illumination (mixotrophically) was the optimum condition for both strains to have a higher biomass and growth compared to other carbon sources and concentrations. Both strains had a quicker growth rate in just 35 mM of acetate and 10 mM glycerol although feed of algal cells on 35 mM acetate produced more and quicker biomass. In use of 10 mM acetate in micro plate and tissue culture flasks, CW15 had a maximum growth rate of 5.3×10^4 and 1.3×10^4 cells/hour; while on use of 35 mM acetate, the growth rate was 8.8×10^4 (micro plate) and 4.0×10^4 cells/hour (tissue culture flasks). Wild type had a maximum 2.7×10^4 (micro plate) and 4×10^3 (tissue culture) cells/hour in use of 10 mM acetate. In feed of CC125 with 35 mM acetate, growth rate correspondingly for micro plate and tissue culture flasks was 2.5×10^4 and 2.6×10^4 cells/ hour. Among the two strains, CW15 with specific growth rate of 8.8×10^4 cells/hour (in micro plates) and 4.0×10^4 cells/hour (in tissue culture flasks) on 35 mM acetate also grew quicker than CC125. Susceptibility to bacterial contamination was checked on both strains and we also found that, just as the absence of a cell wall in CW15 accelerated the growth, it also appeared to increase the chance of contamination by about twofold compared to the wild type but this can be minimized by the use of antibiotics in the growth media.**

Key words: Heterotrophic growth, mixotrophic growth, acetate, glycerol methanol *Chlamydomonas,* CC125, CW15.

INTRODUCTION

Reducing the cost of bio-products processes from its earlier stage to the final one is a vital factor that has been carried out throughout the world. Although discovery of some bacteria such as *Escherichia coli*

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reduces bio-process applications for many decades, it is time to substitute an alternative organism in which to mount the yield of biomass with minimal inputs and costs. Microalgae are a kind of organism that seems to have this functionality in addition to other advantages ranging from (1) their accessibility from fresh water and soil, (2) photosynthetic ability, and, (3) well-annotated genomes such as *Chlamydomonas reinhardtii, Chlorella vulgaris, Volvox carteri, Chlorokybus atmophyticus,* and *Dunaliella salina* to just name few.

The green algae *C. reinhardtii* among other micro algae specious has been utilized as a model species in many bio-applications (Roacha et al., 2013; Fischer et al. 2006; Ledford et al., 2007; Peers et al., 2009). Generally, it can grow phototrophically and this means it uses incident light and CO_2 and converts it to sugar and O_2. Some strains of *C. reinhardtii* have the capability to grow mixotrophically and/ or heterotrophically (Brennan and Owende, 2010; Dragone et al., 2010). Despite heterotrophic feed of microalgae that let algal cell consume solely the external organic carbon source, microalgae which can grow under mixotrophic growth condition can form synthesized carbon source and has both photosynthetic and heterotrophic characteristic. However, in mixotrophic growth regime microalgae consume simultaneously light and external organic carbon source for its growth (Dragone et al., 2010).

Production of microalgae in large-scale is somehow considered as a key parameter for industrialization and commercialization of microalgae and has been studied for decades (Becker, 1994). The ease of its, well annotated genomes and cells behavioral make it as a remarkable laboratory tool to be used in wide range variety of either practical or potential products ranging from lipid, enzymes, isoprenoids, polysaccharides cultivation as well as the production of biofuels as a green and substitute energy source to traditional fossil fuels (Brennan and Owende, 2010).

The achievement of above mentioned product (enzymes, biofuel, lipid and polysaccharides cultivation), mostly was occurred by cultivating the microalgae on varied mineral media, organic substrates, and synthetic or real wastewaters (Pulz, 2001; De-Bashan et al., 2002; De-Bashan et al., 2004; Pulz, 2004; Lebeau et al., 2006; Harun et al., 2010).

Today, growth of micro algae under phototrophic condition is the most common method compared to mixotrophic and heterotrophic conditions. However, the most common one does not necessarily mean the best one, as literatures has shown that the use of both light and external carbon source in mixotrophic condition not only causes a higher yield but also reduces the cost of cultivation compared to phototrophically growth condition (Bhatnagar et al., 2011; Cerón García et al., 2005) and this is commercially quite vital (Abad and Turon, 2012; Liang et al., 2009).

Also, Yu et al. (2009) (Shi et al., 1999) has shown that growing algae via heterotrophic and mixotrophic conditions can overcome such disadvantages of phototrophic growth (low cell densities and longtime cultivation) and the culture conditions for optimum growth and yield rate have not been suggested therefore a think of suitable organic carbon among several carbon sources can be challenging and crucial. To our knowledge, the most common used organic carbon sources for heterotrophic and mixotrophic algal growth are glucose, acetate, sucrose, lactate, lactose, ethanol and glycerol (Shi et al., 1999; Chen and Johns, 1996; Octavio et al., 2010; Zhang et al., 1999).

Despite the importance of right carbon sources and right growth conditions, several factors such as algal strains with various characteristic and behavior, pH, oxygen level, association with or without bacteria or fungi, temperature and introduction of adequate light intensity (Ras et al., 2013; Spreitzer et al., 1998; Falk et al., 2006) to permit a faster algae growth are key objectives that may alter the biomass yielding (Mandalam and Palsson, 1998; Yang et al., 2000; Suh and Lee, 2003). Here our aim is to examine the wild type (CC125) and the cell wall mutants (CW15) of *C. reinhardtii* algae grown on different types of carbon sources in particular (acetate, glycerol and methanol) at various concentration in both dark and light conditions to suggest an optimal culture condition for achieving a higher growth yield and biomass.

Acetate (or acetic acid) is one of well-known carbon sources that its use has been generally accepted for variety of both microbial species and microalgae (Droop, 1974; Moon et al., 2013). Generally, acetate can be metabolized through two routes when it has been carried by coenzyme A. The first pathway will occur from glyoxylate cycle in order to produce malat and the second pathway that can provide the energy (ATP and NADH) and carbon skeletons through the tricarboxylic acid (TCA) to citrate cycle. Solely, those micro algae species that has the first metabolic pathway can grow as the two crucial enzymes isocitrate lyase and malate synthetase (which themselves are necessary for operating the glyoxylate cycle) later will be stimulated in algal cells when they are in acetate media (Neilson and Lewin, 1974; Boyle and Morgan, 2009).

Albeit solely some types of micro algae such as *Phaeodactylum tricornutum, Nannochloropsis* sp., *Rhodomonas reticulate, and Cyclotella cryptica* can consume glycerol as a carbon source, optimizing the use of glycerol for a faster growth and higher yield in mixotrophic condition has been done for decades (Choi et al., 2011; Cero´n Garcı´a et al., 2000). It is a by-product of biofuel production and a good source for preservation of microorganism including microalgae at low temperatures (Moon et al., 2013). Despite acetate that in high concentration will caused cells degradation due to its acidity and toxicity characteristic, glycerol

has not have any toxicity effect on cells even at higher concentration (Kaplan et al., 1986) and so far several efforts have been done to utilize this non-toxic organic carbon source in microalgae growth.

Methanol is the most basic form of alcohol and in its use in biofuel processing it plays a key role to convert triglycerides (TGA) into biodiesel through a mechanism called transesterification. Introducing the methanol into algal system for instance (0.01-718 mM) compared to other carbon sources (glucose, acetate glycerol) has an advantage as it can sterilize the media and therefore minimize bacteria and fungi contamination in open ponds (Choi et al., 2011). Methanol frequently has been used in commercial purposes due to its low cost and the fact that is a biofuel by-product. In a study by Choi et al. (2011), it was shown that growth of *Chlorella* in 1% (v/v) methanol can increase the lipid yield and growth rate (Moon et al., 2013). However, the use of methanol either mixotrophically or heterotrophically has not been reported as an organic carbon source has been reported a level of flocculation in *C. reinhardtii* algal cells (cite methanol algae article). By consideration of merit and demerit points of each mentioned organic carbon source in this study we will conclude the most efficient carbon source for faster growth and yield.

MATERIALS AND METHODS

Solid cultures

In a 500 ml bottle, Tris-Phosphate in absence of acetic acid medium (TP) (Gorman and Levine, 1965) was mixed with 2% Difco agar and autoclaved for 15 minute at 121°C. Then acetate and glycerol with various concentrations (10, 20, 35, 50 and 100 mM) were individually added in a separate 500 ml TP + 2 % agar media and they also autoclaved for 15 min at 121°C.

Methanol (0.01, 25, 123, 245 and 718 mM) was added to the sterilized TP media. Each of the above prepared media (TP, Acetate, Glycerol and Methanol) was poured in 8 Petri dishes (two for each strain one for light and one for dark condition). Each axenic algal strain which was initially grown on TAP media + 2% agar and was taken from Professor Colin Robinson's lab, inoculated and streaked into the above solid prepared TP, TP-Acetate, TP-Glycerol, TP-Methanol media and was grown for 7 days in light with intensity of 38 µE m^{-2} s^{-1} and dark condition.

Liquid culture

Micro-plate

Into two 24 wells micro plates (Cellstar company) 1.5 ml of TP (as phototrophic control), Acetate or Glycerol (10, 20, 35, 50 and 100mM) and (0.01, 25, 123, 245 and 718 mM) of methanol independently was pipetted twice. Algal cells were inoculated and mixed in a separate 1.5 ml Eppendorf tube containing sterilized TP media and then 50 µL of each strain individually was pipetted into each well of the micro- plate with specific mentioned acetate, glycerol and methanol concentration. For mixotrotrophic growth one of the two microplates was put under light intensity of with 38 µE m^{-2} s^{-1} and the other one covered with aluminum foil for heterotrophic purposes. Both micro-plates then were put in the algae room on a separate *Panasonic* double orbital shaker with speed of 170 rpm for 7 days. Once cells were grown for one week, growth rate were visualized and only acetate and glycerol mixotrophically seemed to be utilized by algal cells with certain concentrations seemingly be used more efficiently, that is, 10 and 35mM of acetate and 10 and 50mM of glycerol. Consequently, each strain once more was grown at that efficient concentration of carbon sources in triplicate in a light intensity of 38 µE m^{-2} s^{-1} in both 24 well micro plate. Daily biomass rate was checked by a *Panasonic* micro plate reader in 600 nm wave length. Sample also was taken from each micro plate well and cells counted by *Neubaur* hemocytometer (Figure 5, part C and D, illustrate the callibration of cell numbers with absorption in 600nm).

Tissue culture flasks

To find out a possible role of different shaking speed in algal respiration system, the experiment was repeated at half the speed of 24 wells micro plate (70 rpm) but in tissue culture flasks. A set of three of Sigma-Aldrich 25 cm^3 tissue culture flasks independently was used for each concentration of chosen carbon source. Each set then separately filled with 25 ml of prepared TP with (10 and 35 mM) acetate and (10 and 50 mM) glycerol. In each flask, 100 µl algae cells from each strain was pipetted. Flasks were thereafter put on a 70 rpm *Panasonic* double orbital shaker for 7 days. Cell counting, optical density at 600 nm (Figure 5, part A and B, display the callibration of cell numbers with absorption in 600nm) and chlorophyll measurement was measured daily.

Chlorophyll extraction and measurement

Chlorophyll extraction from tissue culture flasks

One milliliter from each tissue culture flasks containing algal cells was pipetted into individual 1.5 ml centrifuge Eppendorfs and centrifuged for 5 min at 6000 rpm. Supernatant was removed and algal cells vortexed with 1 ml 95% ethanol (v/v) for about 45 s. Eppendorfs were again centrifuged for 4 min at 13,000 rpm. The remaining supernatant containing extracted algal chlorophyll was transferred in 1 ml plastic cuvette. The spectrophotometer was blanked against 95% ethanol before each absorption at 649, 665 and 750 nm.

Chlorophyll a counting

The rate of produced chlorophyll *a* calculated via the below equation (Bergmann and Peters, 1980):

$$\textbf{Chl } \textit{a} \textbf{ (µg/L)} = \frac{(13.7(A665 - A750) - 5.76(A649 - A750))\ V}{v\,*/}$$

Where, V is the volume of extract in ml; V is the volume of sample filtered in; l is the length of cuvette in cm.

Algal susceptibility to bacterial contamination

Algae strains from solid TAP media were inoculated and mixed with 1.5 ml fresh liquid TP media and vortexed for 2 min. Algae cells were diluted 100 times and cell density was measured by counting the cells in a hemocytometer (CW15 and CC125 respectively had 1.25×10^6 cells/ml and 5×10^5 cells/ml). Our aim was

to observe 3000 bacterial colonies in use of 1ml algal culture meaning in 0.1 ml of algae culture we would expect to see 300 bacteria colonies. Samples were diluted appropriately to give 300 colonies per plate. Two percent agar from *Diefco* Company was poured individually in a 100 ml of TP, TAP and TSB (Tryptone-Soya Broth) and autoclaved as before.

The autoclaved media independently poured in 4 individual sterilized petri dishes. 100 µl diluted sample were pipetted into 4 individual solid TP, TAP and TSB petri dishes and distributed perfectly in all petri dishes. While TSB petri dishes were kept in a 30°C incubator, TP and TAP Petri dishes were kept in 27°C algae growth room with 38 µE m^{-2} s^{-1} light intensity.

Physical morphology

Both algal strain which grown without additional of any carbon source in solid and liquid TP, TAP were visualized under the *Leica DMR* (Digital Module R) microscope with 1000 magnification and compared to the grown algae cells in various concentration of acetate, glycerol (10, 20, 35, 50 and 100 mM).

RESULTS AND DISCUSSION

Physical morphology

Visualization of both strains under light microscope with 1000X magnification showed the existence of cell wall and flagella respectively in CC125 and its absence in CW15. Despite having an approximate same size (50 µm) and circular shape of both strains, cells in CC125 mostly joined together as a colony in a capsulated environment by their cell wall while the CW15 cells remained separate from each other.

Moreover, further microscopy of the grown CC125 algal cells either mixotrophically or heterotrophically with different concentrations of acetate (10, 20, 35, 50 and 100 mM) indicated some valuable results. For instance, in dark growth conditions using 50 and 100 mM acetate the color of existed chlorophyll in CC125's cells mostly was bright yellow rather than green (Figure 7, part C and D clearly show the differentiate color of chlorophyll).

The reason that chlorophyll looks green is that it absorbs other colors existed in light (red and blue) except green. Also, in low light intensity more rate of chlorophyll will expect to produce. And the more chlorophyll means more green color (Lange et al., 1981). Therefore, this may suggest an existence of a deficient in CC125 chloroplast pigments (chlorophyll a, b or xanthophyll) in which the absence of light and high rate of concentration (50 and 100 mM) inhibits algal cells to either absorb the light or consume high concentrated acetate for growth. Another possibility which also was seen in 24 wells micro plate can be cell degradation and death due to the high toxicity of 50 and 100 mM acetate. Figure 7A shows cell degradation and death while part B display steady cell aggregation). We also found that in heterotrophic use of 50 and 100 mM acetate most of CW15 algal cells lost their circular shapes and instead formed some unstructured shapes. In Figure 1, a noticeable difference in shape of CW15 in use of low acetate concentration (B) and high acetate concentration (D) was observed.

The optimum condition and concentration for acetate in solid and media culture

From the data gathered after a period of seven days we found that mixotrophic growth of both strains was much higher than growing under either phototrophic or heterotrophic conditions. Moreover, mixotrophic growth of algal cells on acetate, glycerol and methanol revealed that acetate was the carbon source that was consumed better compared to the others. Our experiment also showed that the optimum concentration of acetate for improving the yield was 35 Mm. Figure 2A indicates a higher mixotrophic growth compared with other carbon sources (C&E)) and higher concentration of acetate (100 mM) caused cell death and degradation specifically in CC125 which might be due to the toxic effect of 100 mM acetate on algal cells. A rate of flocculation also was seen on CW15 when a range of methanol concentration (0.01, 25, 123, 245 and 718 mM) was used mixotrophically (Scholz et al., 2011).

The maximum growth rate of CW15 grown on micro plates in feed of 10 and 35 mM acetate correspondingly was 5.3×10^4 and 8.8×10^4 cells/hour. The wild type (CC125) showed less growth rate, being 2.7×10^4 (10 mM) and 2.5×10^4 (35 mM) cells/hour. Figure 3A and B show algal cell growth in 10 and 35 mM acetate.

Results given from tissue culture flask also has shown that regardless of shaking speed and its possible effect on algal respiration system, CW15 grew quicker on 35 mM acetate with maximum rate of 4.0×10^4 cells/hour than CC125 with maximum rate of 2.6×10^4 cells/hour. Figure 4C and D reflect the faster growth of CW15 on 35 mM acetate feed. The CW15 also had a faster chlorophyll production rate of 0.046 $\mu g.L^{-1}.h^{-1}$ compared to CC125 with a rate of 0.008 $\mu g.L^{-1}.h^{-1}$ (Figure 6).

We also found that in mixotrophical use of 35 mM acetate both strains produced a higher biomass than other concentrations and conditions. The maximum rate of extracted chlorophyll a, for instance, in 35 mM acetate was 17.89 $\mu g.L^{-1}.h^{-1}$ (CC125) and 12.516 $\mu g.L^{-1}.h^{-1}$ (CW15) which was noticeably higher than the phototrophic yield with rates of 0.467 $\mu g.L^{-1}.h^{-1}$ (CC125) and 0.103 $\mu g.L^{-1}.h^{-1}$ (CW15). Utilization of 10 mM acetate however produced lower chlorophyll a than 35 mM acetate but higher than phototrophic growth as CC125 had 10.95 $\mu g.L^{-1}.h^{-1}$ and CW15 had solely 4.758 $\mu g.L^{-1}.h^{-1}$.

The optimum condition and concentration for glycerol

In mixotrophic utilization of 10 and 50 mM glycerol

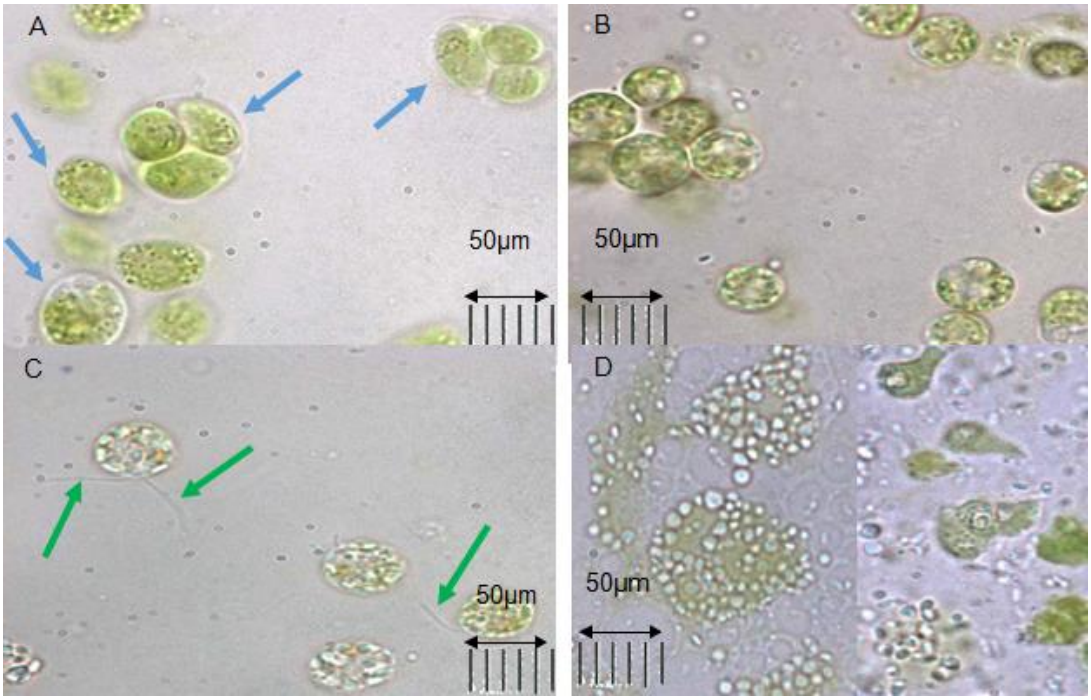


Figure 1. Physical morphology of both strains in magnification of 1000x. The blue and green arrows in A and C respectively show the existence of cell wall and flagella in CC125. B shows the CW15 that lacks both cell wall and flagella. The pale color of algae in picture C is because of the use of 20mM acetate in dark condition which may suggest the defect in its chloroplast system in dark condition. Although the size of each cell in both strains approximately is the same, the wild type (CC125) usually makes a colony of three or more cells which are capsulated by its surrounded cell wall (Picture A). Picture D shows unstructured shape of CW15 in use of 50 mM acetate in heterotrophic condition acetate. The last green column in C can show the lowest cell growth due to high toxicity of acetate). The blue and green arrows in A and C respectively illustrate cell wall and flagella in CC125 while B shows the lack of them in CW15.

however the outcomes from micro plates and tissue culture flasks were considerably different. In micro plates the maximum growth rate of CW15 respectively for 10 and 50 mM glycerol was 5.2×10^4 and 7.5×10^4 cells/hour while in tissue culture flasks cells were grown better on 10mM than 50mM as 2.4×10^4 and 1.3×10^4 cells/hour respectively were generated

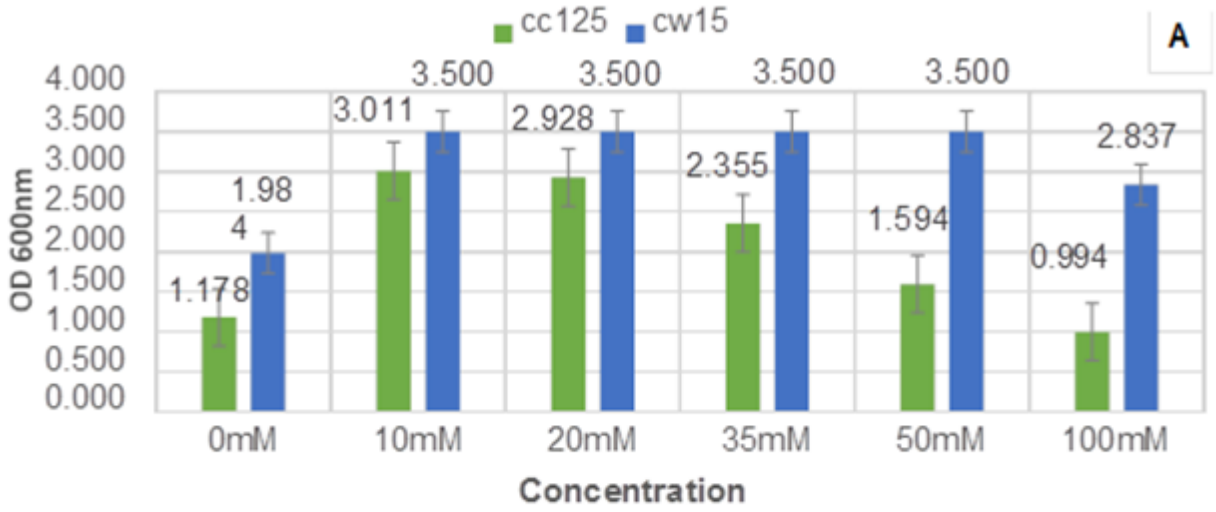
The CC125 we had a higher growth in 10 mM glycerol in both micro plates and tissue culture flasks. The maximum growth rate in micro plate and tissue culture flasks was 2.0×10^4 and 7.8×10^4 cells/hour respectively. In 50 mM glycerol media results were 1.2×10^4 cells/hour in micro plate and 2.8×10^4 cells/hour in tissue culture flasks. This is how the maximum phototrophic growth rate in CC125 and CW15 in two times replicate respectively was 3.4×10^4 and 5×10^3 cells/hour and 4.9×10^4 and 6.5×10^4 and 2.3×10^4 and 1.1×10^4 cells/hour (Figures 3 and 4.).

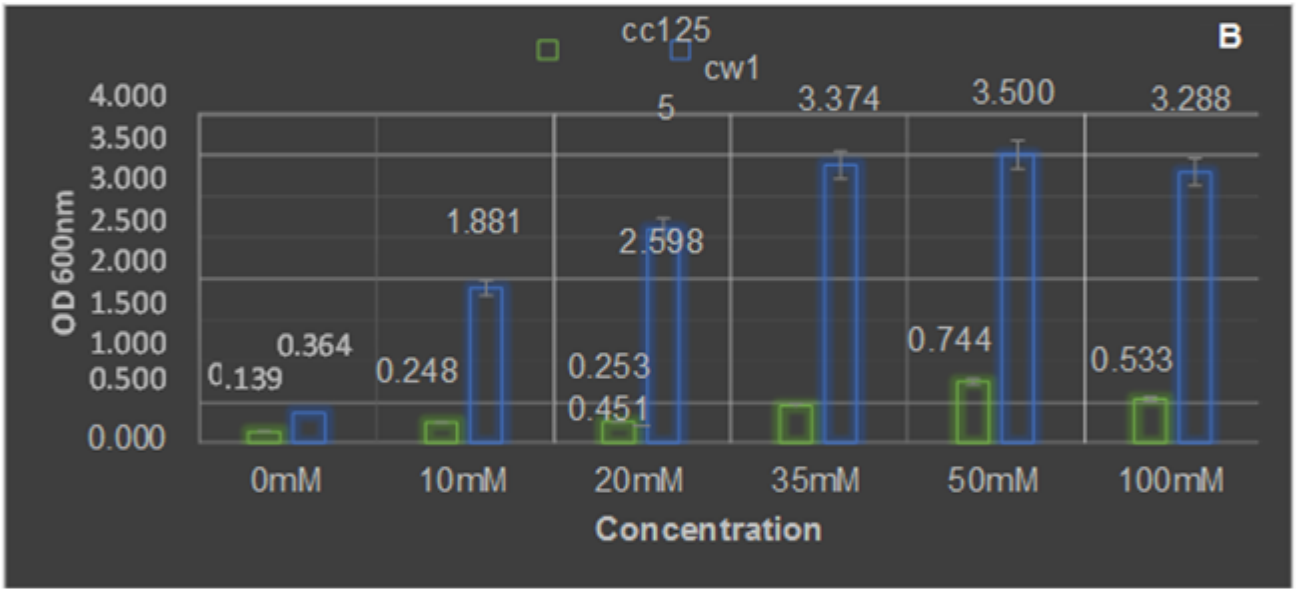
Extraction of chlorophyll from algal cells were grown on tissue culture flask also showed that CW15 and CC125 both had a faster chlorophyll production growth rate in 10 mM glycerol with corresponding rate of 0.037 (CW15) and 0.020 (CC125) $\mu g.L.^{1}\ h^{1}$ than feed on 50 mM glycerol with maximum production growth rate of 0.022(CW15) and 0.016 $\mu g.L.^{1}\ h^{1}$ (CC125) (Figure 6C and D). However, the rate of produced biomass (chlorophyll a amount) in use of 50 mM glycerol was higher in CC125 than 10 mM (0.769 and 0.458 $\mu g.L.^{1}\ h^{1}$, respectively). The cell wall mutants produced a same amount of chlorophyll a in use of 10 and 50mM glycerol (0.168 $\mu g.L.^{1}\ h^{1}$). Likely to use of acetate, feed of algal cells on glycerol culture either in 10 or 50 mM concentration, showed a higher yield than phototrophic condition with rate of 0.467 $\mu g.L.^{1}\ h^{1}$ (CC125) and 0.103 $\mu g.L.^{1}\ h^{1}$ (CW15).

The data suggests that growing CC125 on small sized culture such as micro plate may not be industrial while the growth rate of phototrophic was higher than 10 and 50 mM glycerol however in bigger culture (tissue culture flasks) the use of just 10 mM glycerol can be quite interesting.

Economics matter in use of 10 or 50 mM glycerol

An important point in yield and growth of algae is economic matters in which to reduce the cost of


cc125
cw15
A
OD 600nm
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
1.178
1.984
3.011
3.500
2.928
3.500
2.355
3.500
1.594
3.500
0.994
2.837
0mM
10mM
20mM
35mM
50mM
100mM
Concentration


cc125
cw15
B
OD 600nm
4.000
3.500
3.000
2.500
2.000
1.500
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0.500
0.000
0.139
0.364
0.248
1.881
0.253
0.451
2.598
3.374
0.744
3.500
0.533
3.288
0mM
10mM
20mM
35mM
50mM
100mM
Concentration

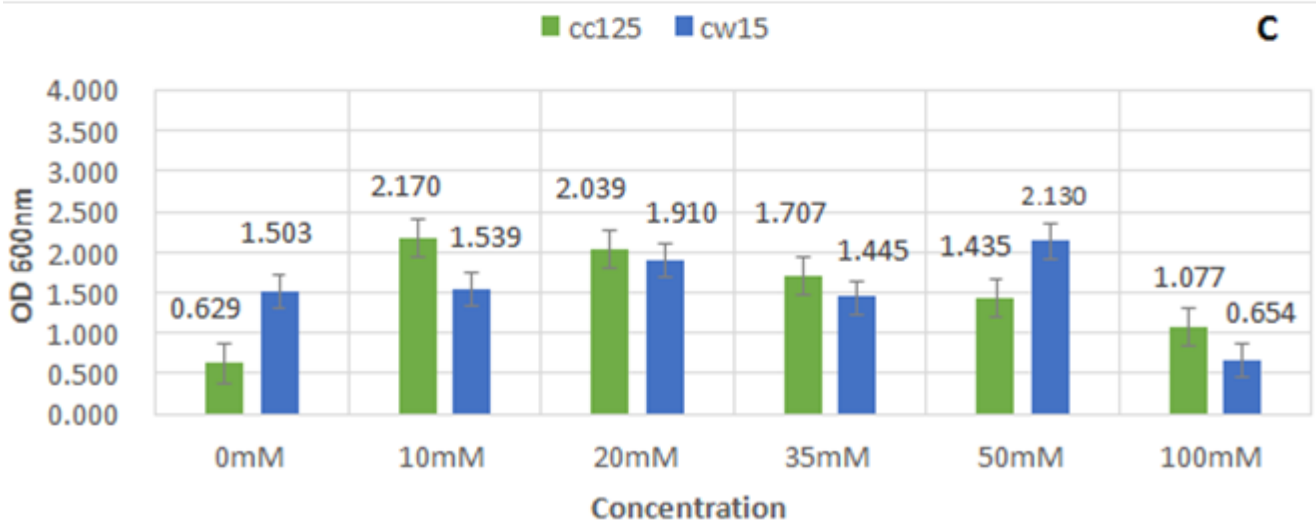
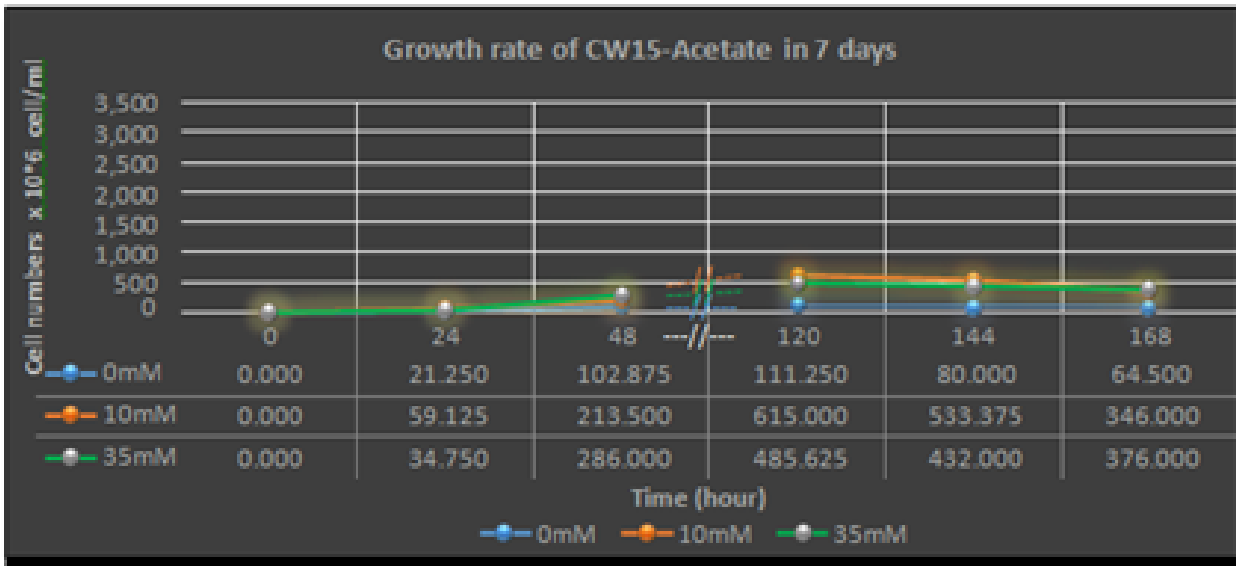
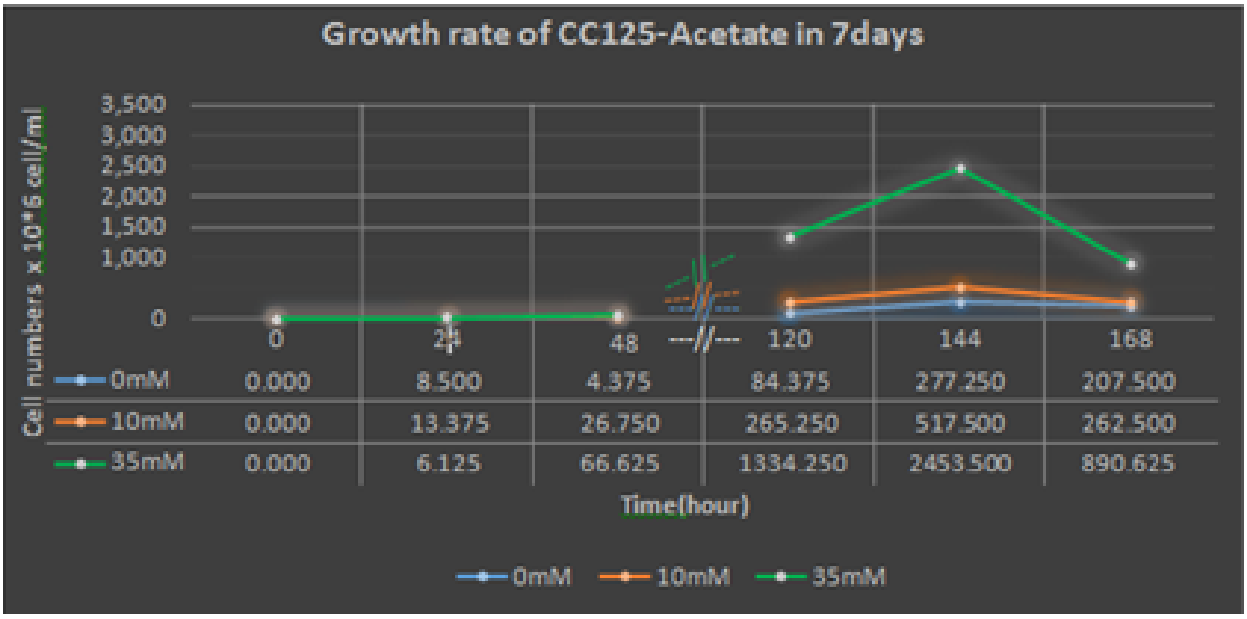

cc125
cw15
C
OD 600nm
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.629
1.503
2.170
1.539
2.039
1.910
1.707
1.445
1.435
2.130
1.077
0.654
0mM
10mM
20mM
35mM
50mM
100mM
Concentration

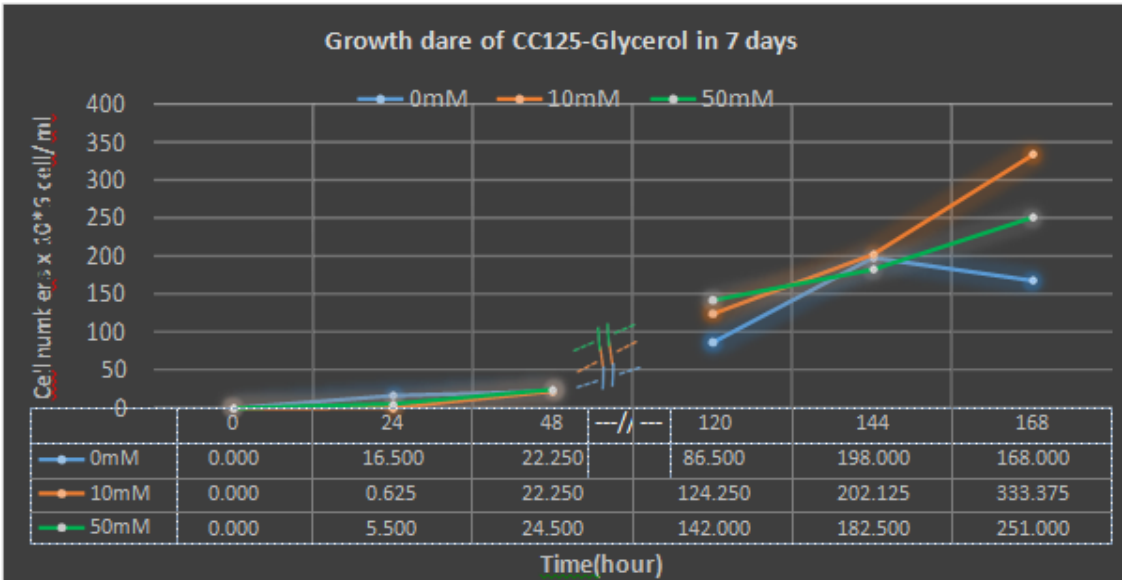
Figure 2. Various concentrations of different C-sources. Graphs illustrate the growth of microalgae (CC125 and CW15) in acetate (A-B), glycerol (C-D) and methanol (E-F) media. The graphs A, C, E represent the light and B, D, F show dark condition.



Concentration 12	Growth rate cells x10⁴ per hour	Divisions per hour	Generation time
0mM	6.5	0.094	10.63
10mM	5.3	0.076	13.15
50mM	8.8	0.127	7.87

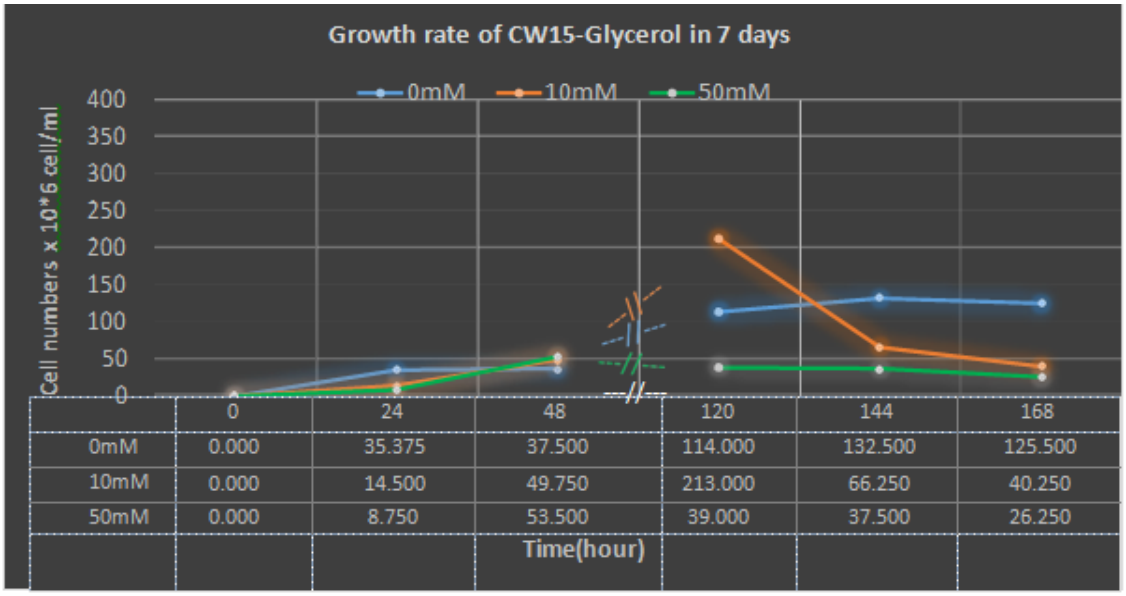


Concentration	Growth rate cells x10⁴ per hour	Divisions per hour	Generation time
0mM	4.9	0.071	14.08
10mM	2.7	0.039	25.64
35mM	2.5	0.036	27.77



	0	24	48	120	144	168
0mM	0.000	16.500	22.250	86.500	198.000	168.000
10mM	0.000	0.625	22.250	124.250	202.125	333.375
50mM	0.000	5.500	24.500	142.000	182.500	251.000

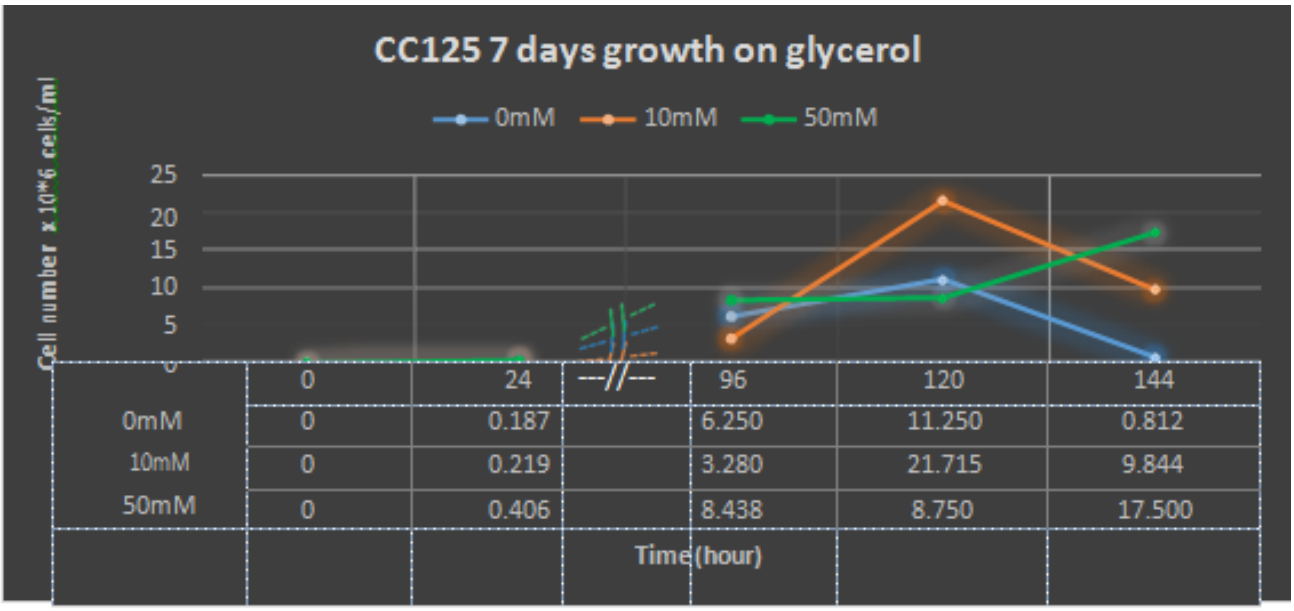
Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	3.4	0.049	20.40
10mM	2.0	0.028	35.71
50mM	1.2	0.017	58.82



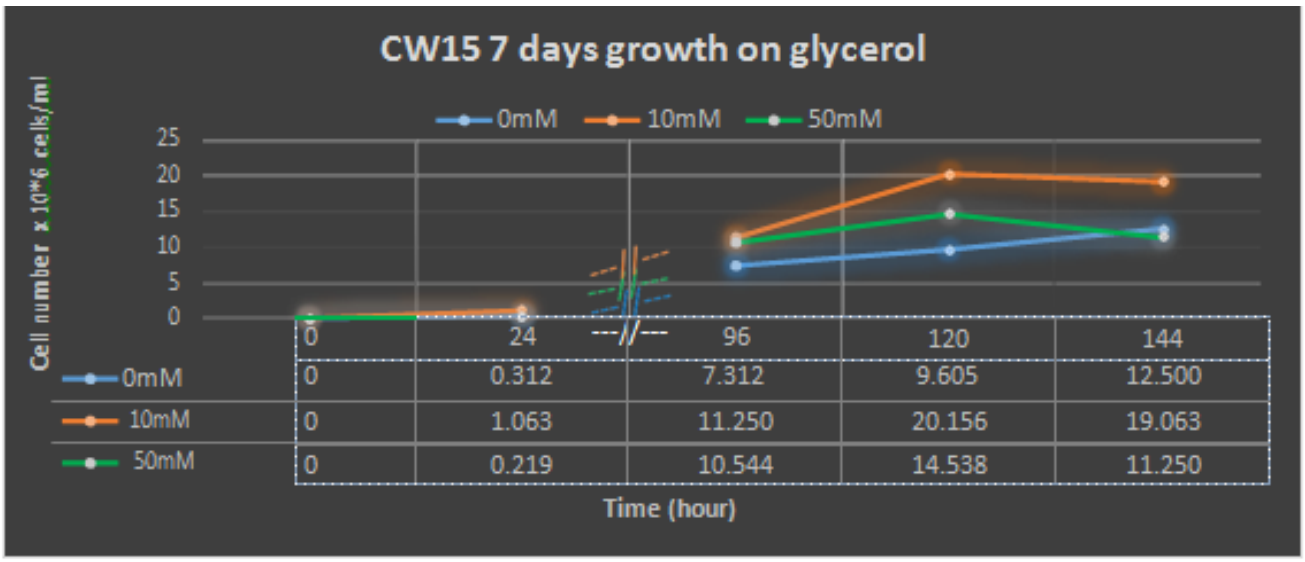
	0	24	48	120	144	168
0mM	0.000	35.375	37.500	114.000	132.500	125.500
10mM	0.000	14.500	49.750	213.000	66.250	40.250
50mM	0.000	8.750	53.500	39.000	37.500	26.250

Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	0.5	0.007	143
10mM	5.2	0.075	13.3
50mM	7.5	0.108	9.2

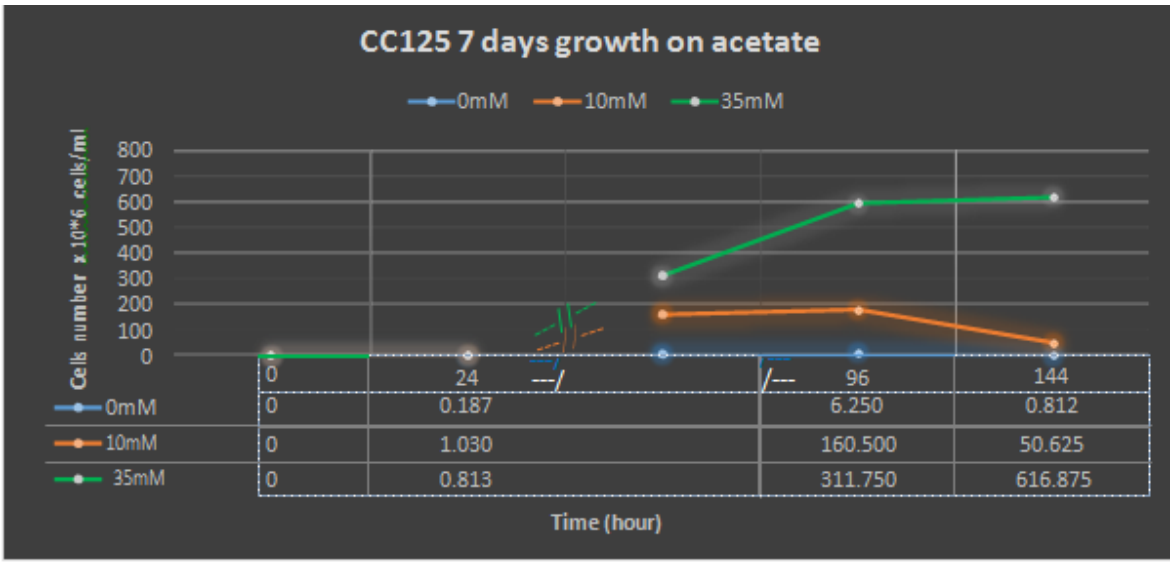
Figure 3. Comparison of 7 days CC125 and CW15 growth on 24 wells micro plate in light condition (38 µE $m^{-2}s^{-1}$) on three Acetate and Glycerol concentration (0, 10, 35 mM) and (0, 10, 50 mM) respectively. The sign (--//- - -) indicates a data gap between day 2 (48 h) and day 5 (120 h). Blue liner graph indicates phototrophic growth. The orange and green liner graph respectively shows use of 10 and 50 mM glycerol in Figure 3D).



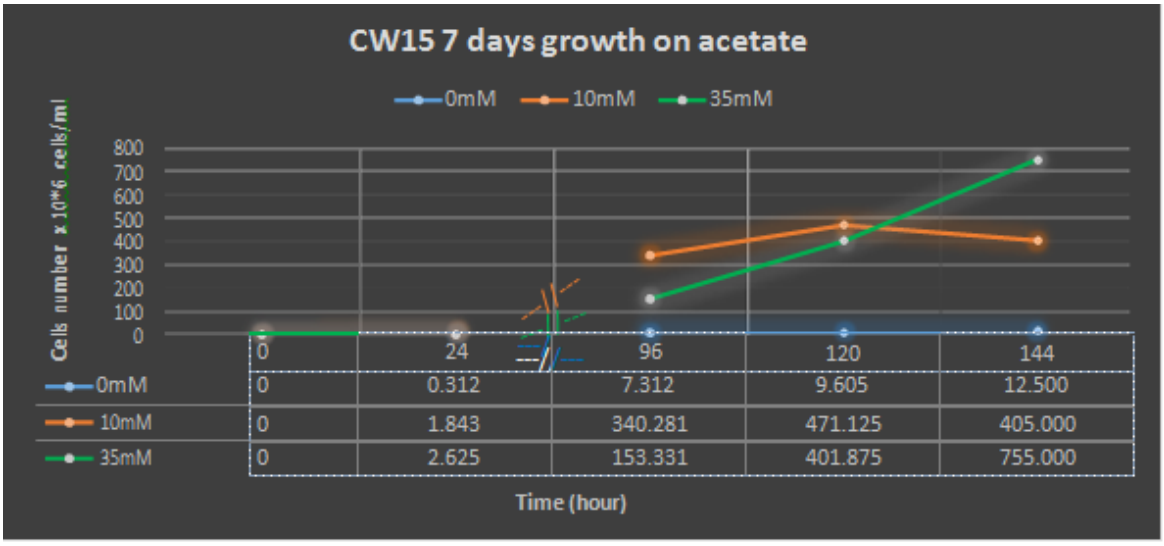
Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	2.3	0.033	30
10mM	7.8	0.113	8.85
50mM	2.8	0.040	25



Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	1.1	0.017	58.823
10mM	2.4	0.034	29.411
50mM	1.3	0.018	55.555



Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	2.3	0.033	30
10mM	0.4	0.005	200
35mM	2.6	0.037	27



Concentration	Growth rate cells x10^4 per hour	Divisions per hour	Generation time
0mM	1.1	0.017	59
10mM	1.3	0.018	55
35mM	4.0	0.057	17.5

Figure 4. Comparison of 7 days CC125 and CW15 growth in tissue culture flasks in 38 µE $m^{-2}s^{-1}$ light condition on three Acetate and Glycerol concentration (0, 10, 35 mM) and (0, 10, 50 mM) respectively. The sign (- - -//--) indicates a data gap between day 1 (24 h) and day 4 (96 h). The low growth rate of CC125 on 10mM acetate (0.004) is probably because the lack of data in exponential phase (between day 1 and 4) and the 0.004cells/hour possibly is a growth rate when cells are entering to the beginning of the stationary phase. Blue liner graph indicates phototrophic growth (The orange and green liner graph respectively show use of 10 and 50 mM glycerol in Figure 4B).

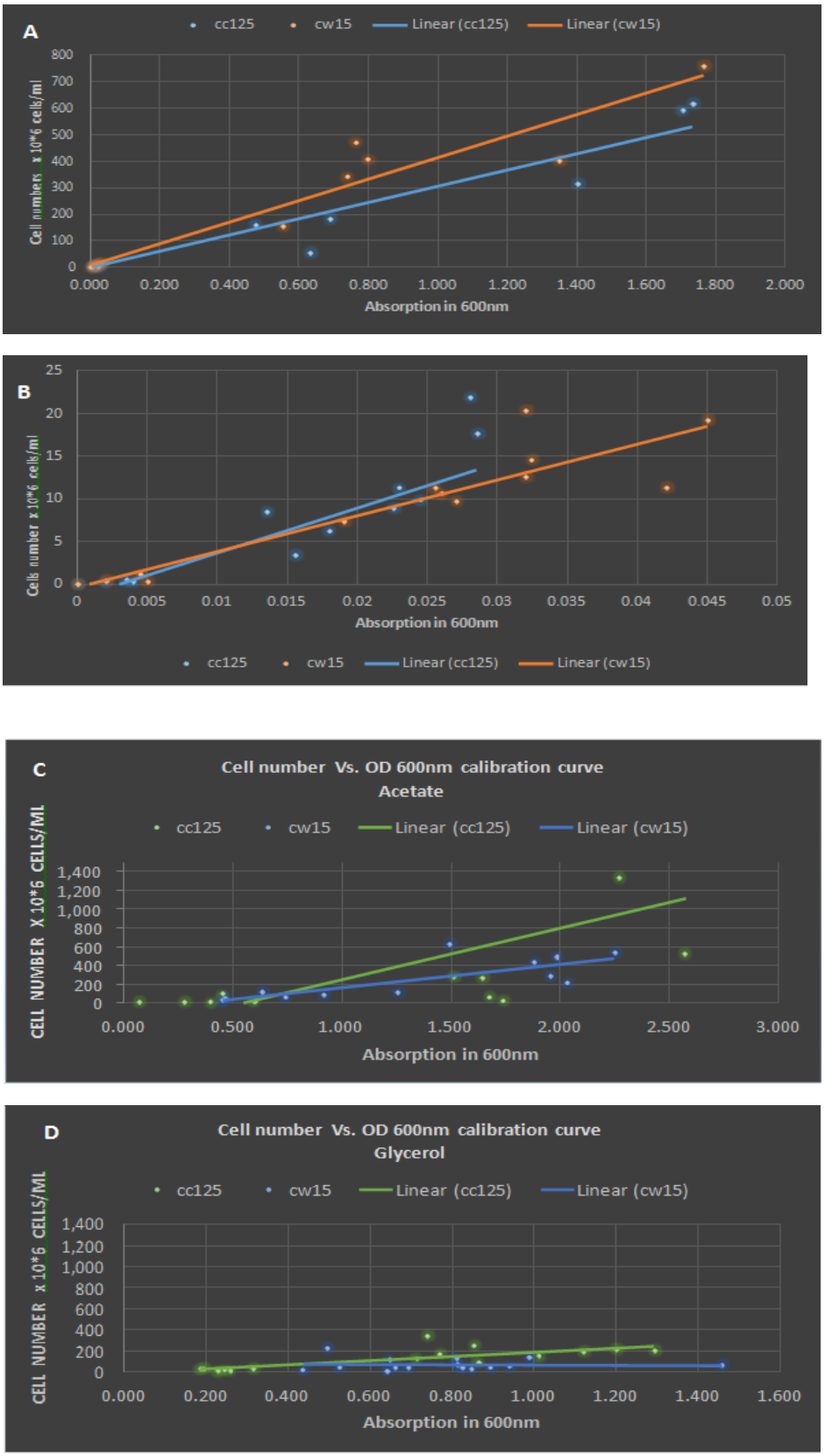
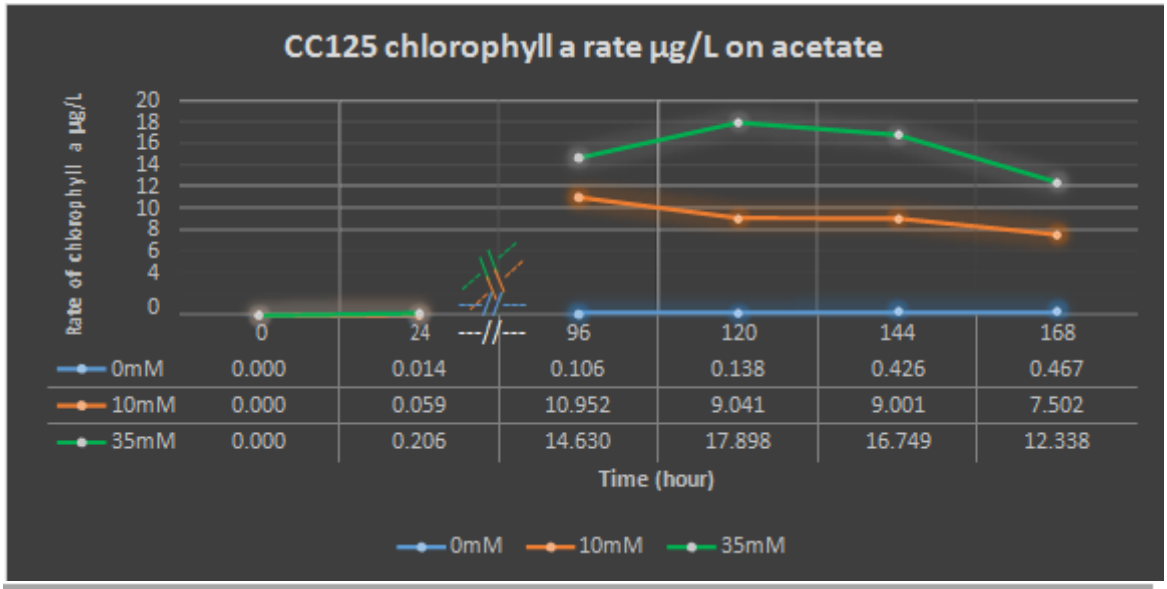
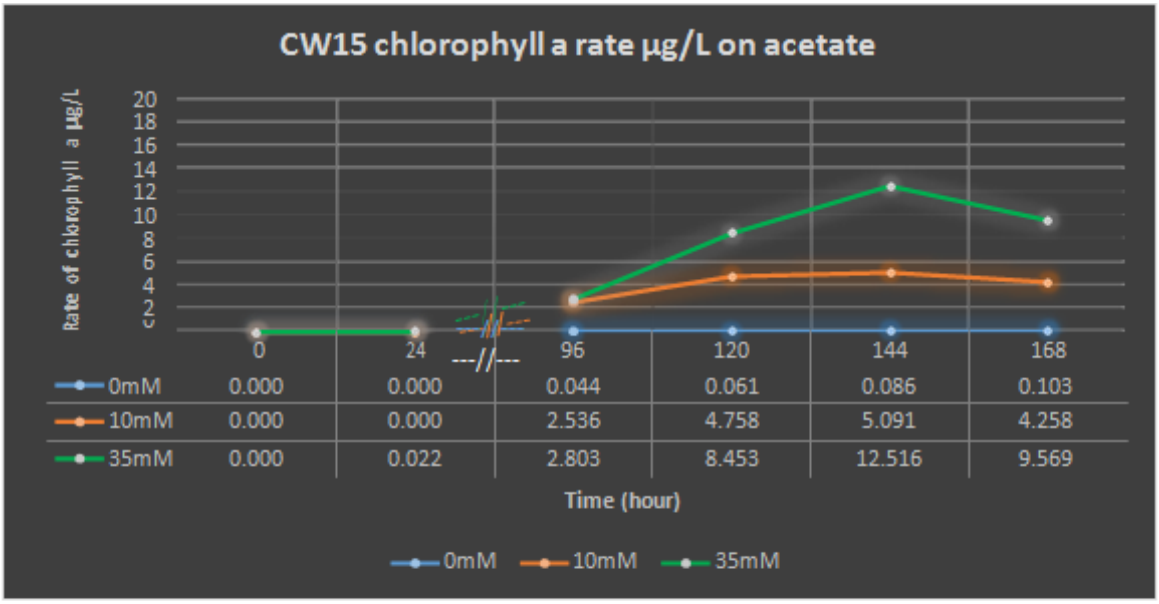


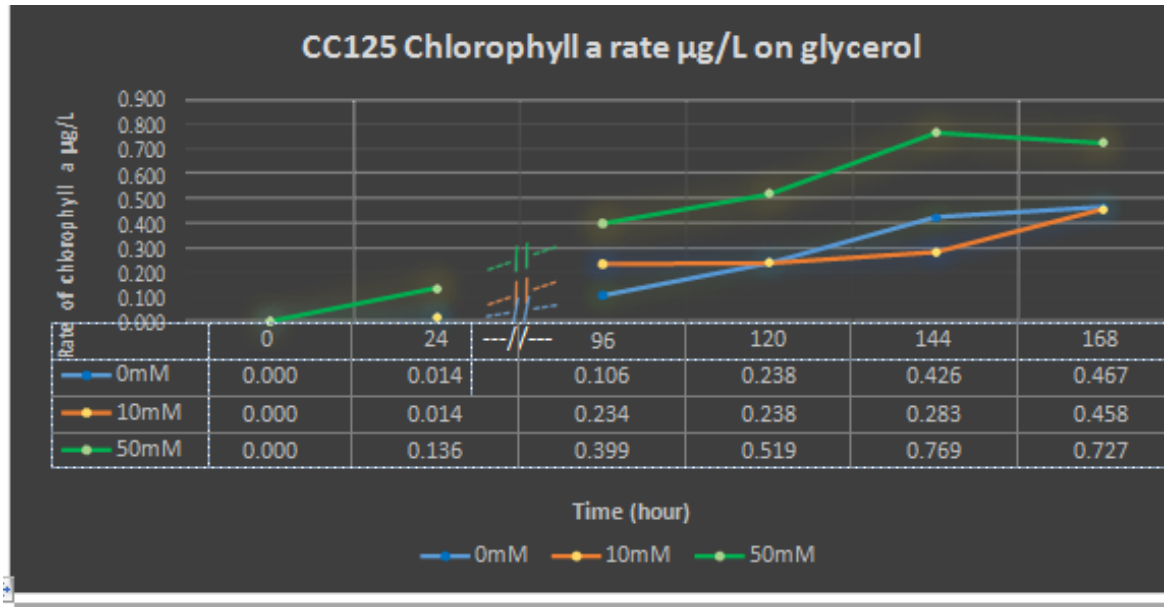
Figure 5. Calibration curve between Absorption n 600 nm and cells number. A –B represent the data for tissue culture flasks, C-D represent the data for micro plate culture. A and C are calibration in acetate. B and D are in glycerol.



Concentration	Growth rate per hour µg.L^{-1}.h^{-1}
0mM	0.046
10mM *	0.151*
35mM	0.008

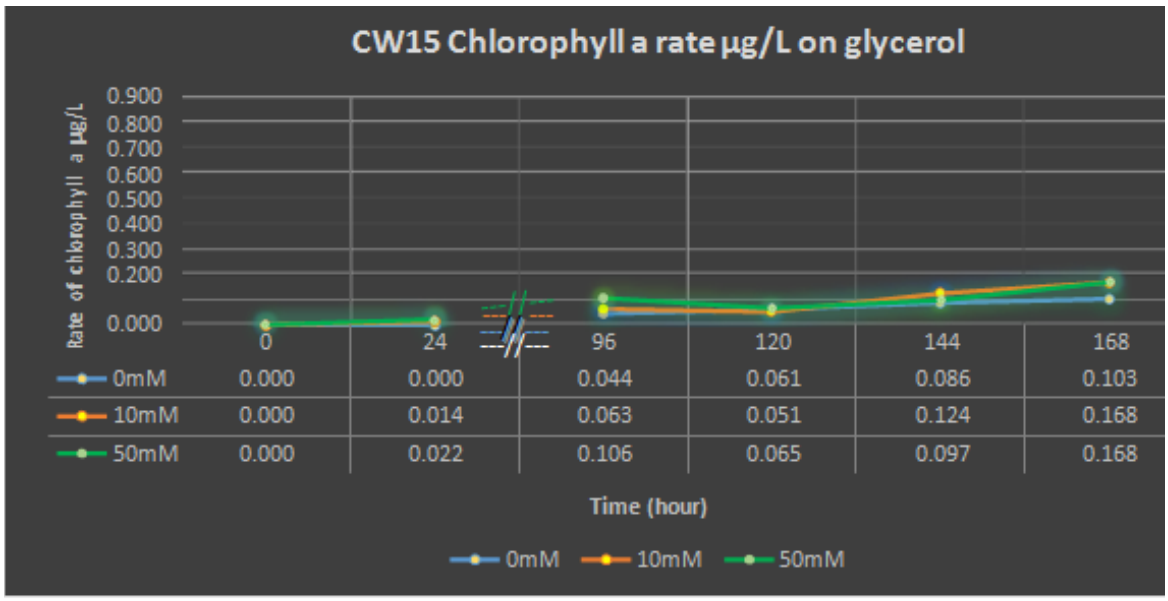


Concentration	Growth rate per hour µg.L^{-1}.h^{-1}
0mM	0.014
10mM	0.026
35mM	0.046



	0	24	96	120	144	168
0mM	0.000	0.014	0.106	0.238	0.426	0.467
10mM	0.000	0.014	0.234	0.238	0.283	0.458
50mM	0.000	0.136	0.399	0.519	0.769	0.727

Concentration	Growth rate per hour µg.L^{-1}.h^{-1}
0mM	0.046
10mM	0.020
50mM	0.016



	0	24	96	120	144	168
0mM	0.000	0.000	0.044	0.061	0.086	0.103
10mM	0.000	0.014	0.063	0.051	0.124	0.168
50mM	0.000	0.022	0.106	0.065	0.097	0.168

Concentration	Growth rate per hour µg.L^{-1}.h^{-1}
0mM	0.103
10mM	0.037
50mM	0.022

Figure 6. Chlorophyll a production rate in CC125 and CW15 on various concentrations of acetate and glycerol feed in tissue culture flasks. The sign (- - -//- - -) indicates a data gap between day 1 (24 h) and day 4 (96 h). Liner green graph in part A and B show the rate chlorophyll production in use of 35 mM.

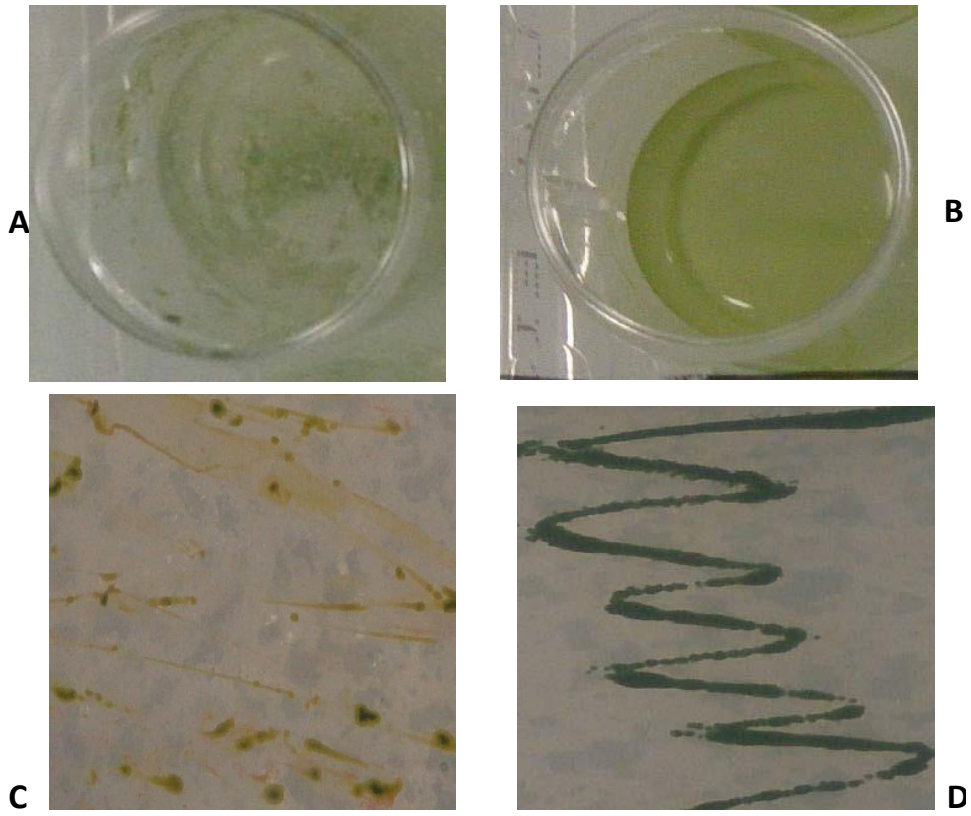


Figure 7. Chlorophyll deficient and cell aggregation in CC125. Picture A and B respectively indicate cells degradation in 100 mM acetate liquid culture under light illumination of 38 µE $m^{-2}s^{-1}$ and normal cells in phototrophic growth condition. Pictures C illustrates a possible deficient in Chloroplast system in utilizing of 100 mM acetate in solid culture under dark condition compare to the phototrophic growth (picture D).

Table 1. Comparison the growth rate of both strains (CC125 and CW15) on acetate and glycerol in TP solid culture under various concentrations and light conditions.

	Acetate	**10 mM**	**20 mM**	**35 mM**	**50 mM**	**100 mM**
CC125	Light	++	++	++	++	++
	Dark	-	-	-	+-	+-
CW15	Light	+	+	+	+	+
	Dark	+	+	+	+	+
	Glycerol	**10 mM**	**20 mM**	**35mM**	**50mM**	**100mM**
CC125	Light	++	++	++	++	++
	Dark	-	-	-	-	-
CW15	Light	+	+	+-	+-	+-
	Dark	-	-	-	-	-

The ++ is consider as highest growth;– is the lowest rate.

experiment as much as possible and introduce the most optimum industrialized system. Although CW15 had a higher growth rate in micro plate on feed of 50 mM glycerol, as commercial fermenters are much larger than micro plate and as we got a higher growth rate in use of 10 mM glycerol in tissue culture flasks as an example of bigger culture, so utilizing the 10 mM glycerol commercially and economically is more beneficial as its cost is 1/5 of 50

mM glycerol. The cost of at least minimum volume of 1000 L 10 and 50 mM glycerol solution will be, $360 and $1802, respectively.

Susceptibility to bacterial contamination

The rates of algal mixotrophical growth on solid (TPA) and liquid TP cultures were different. On solid TP plates, the growth ratio of CC125 was higher compared to CW15 in both acetate and glycerol (Table 1). In the susceptibility to bacterial contamination experiment that was done on both strains, we found that on TP media CW15 had 28 out of 300 bacterial colonies (10 %) and CC125 had 20 out of 300 colonies (6.6%) whereas on TAP media CW15 and CC125 got 23 out of 300 (7.6%) and 9 out of 300 (3%) bacteria colonies, respectively. On TSB plates, however, solely 2 out of 300 (0.6%) and 1 out of 300 (0.3%) of bacteria colonies were seen on CW15 and CC125 in orderly fashion. This can suggest that the lack of cell wall would increase the chance of bacterial contamination almost 2 times more than the wild type with its cell wall as a barrier.

Moreover, the rate of fungal contamination of CW15 on TSB plates was much higher than CC125 compared to other TP and TAP media cultures.

Thus, one possibility could be that it was as a result of the negative effect of bacteria on growth of CW15 on solid culture. Hence, when cells are introduced in a soluble medium, researchers should not always expect to see the same behavior as to when such cells were in solid media. In shaking liquid culture, there is a possibility that micro gradients are introduced in a watery atmosphere and therefore a lower potential for neighbor effects and bacteria interactions will occur compared to solid media.

Further discussion and future applications

The potential of micro algae as laboratory tool in cosmetic industries (Potvin and Zhang, 2010), drug designing (Bumbak et al., 2011; Milledge, 2010) and recombinant proteins or as a renewable energy source specifically in biofuel production make it an interesting microorganism to invest more attention and effort on its growth and yield (Chisti, 2007; Stephens et al., 2010; Wijffels and Barbosa, 2010; Hempel et al., 2010). So far we showed that mixotrophically growth of CC125 and CW15 on 35mM acetate was the optimum way for faster growth and biomass rate. By knowing that each algal strain behave differently in different media and conditions and there was not any fixed pathway so far therefore a need for further researches and experiments is vital to confidently propose a system with highest and fastest biomass production.

In theme of biofuel one possible application for future purposes can be use of organic (acetate) and inorganic carbon (bicarbonate) in TP media to see its effect on both growth and lipid production.

As it is in general acceptance that for commercial purposes inorganic carbons are needed and as (Gardner et al., 2012; Gardner et al., 2013) have shown that bicarbonate as a lipid trigger can increase the yield of lipid production such as triacylglycerol (TAG) in some microalgae specious(Gardner et al., 2012; White et al., 2012) thus it can worth to determine the outcomes of our experiment (use of 35 mM acetate for faster growth) to other literatures results as a new application to find out the possible rate of lipid production specifically in CW15 that lacks cell wall. The absent of cell wall in CW15 not only has shown a faster growth but also can speed up the cultivation process. What also we really interested in to examine in our future experiment is correlating the effect of various light intensity and temperature (Ras et al., 2013; Spreitzer et al., 1998; Falk et al., 2006) on growth and lipid (TAG) production of our used algal cells (CW15 and CC125).

Moreover, as the rate of bacterial contamination was higher in CW15 than CC125 due to the absence of its cell wall, one interesting application could be the use of bacterial 16srRNA probes to investigate a possible interaction between CW15 and bacteria and the role of bacteria in rate of biomass. The use of this small ribosomal ribonucleic acids subunit (16 srRNA) still is one of the precise method for identification of bacteria as they are conserve molecules and present in all bacteria thus for identification of different bacteria still the recognition system will remain fixed (Ludwig et al., 1998).

Conclusion

The potentiality of microalgae to substitute as renewable energy source to some conventional ways made it interesting. So far micro algae has been cultivated in various conditions (phototrophic, mixotrophic and heterotrophic), and nutritious feed, but each has some merit and demerit points. Introducing algal cells in phototrophic condition minimizes the growth rate, but its advantage is to also decrease the possibility of bacterial contamination. Mixotrophic condition can boost the algal growth and yield due to use of addition of external carbon source and light simultaneously, but also can encourage bacteria into the system due to the existed carbon source. On the other hand, in heterotrophic conditions although the cost of experiment clearly is less than both mixotrophic and phototrophic conditions, because the created dark atmosphere in addition of external carbon is strongly a suitable condition for bacterial and fungus growth the chance of algal yield and growth also will decrease. Moreover, solely some specious of algae have capability to grow on dark condition. Despite the advantage and disadvantage of each growth condition our results suggested that acetate was the most effective organic carbon source among here tested carbon sources and the optimal concentration

for *C. reinhardtii* CC125 (wild type) and CW15 (cell wall mutants) to grow quicker was use of 35 mM acetate mixotrophically.

Also, another recent study done by Moon et al. in 2013 showed that among acetate, glucose, glycerol or sucrose, mixotrophically growth of *C. reinhardtii* on acetate was the optimum way in terms of growth and lipid yield. Also, use of 35 mM acetate in 1000 L fermenter is economically even cheaper than use of the alternative carbon source (10 mM glycerol), being $327 and 363, respectively.

Even though in our experiment a minimum growth rate was seen in feed of both *C. reinhardtii* strains (CC125 and CW15) on glycerol culture (Cerón García et al., 2000) has shown that the use of 0.1M glycerol with light intensity of 165 µE $m^{-2}s^{-1}$ let *Phaeodactylum tricornutum* grow 74% faster than phototrophic condition.

Comparatively to the wild type (CC125), our experiment suggests that the lack of cell wall in CW15 possibly decrease the digesting process time and this can be the reason for a faster growth rate (cells or chlorophyll a) per hour in liquid culture. However, in absence of antibiotics in media the chance of bacterial contamination is approximately 2 times higher in CW15 compare to CC125 that has cell wall as a barrier.

Though the cells morphology data shows that a single algae cell in both CC125 and CW15 has a ring like shape with an approximate same size of 50 µ, biotechnologically growing and cultivating CW15 can be preferred since cells in CW15 cells remain separately from each other while in CC125 mostly join together as a colony in a capsulated environment by their cell wall and this means more small sized cells are better and offer a possible higher cultivation product than a massive cell in less numbers.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The author appreciates all lab technicians and specifically Professor Mark Smales and Dr. Dan Liyod who patiently guided and leaded him to increase his knowledge.

Abbreviations

TAP, Tris-acetate phosphate; **TP,** Tris-phosphate; **TPA,** TP+2% agar; **DMR,** digital module R; **TGA,** triglyceride.

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Vol. 15(23), pp. 1101-1135, 8 June, 2016
DOI: 10.5897/AJB12.1723
Article Number: 6BC23B358794
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Variation in levels and removal efficiency of heavy and trace metals from wastewater treatment plant effluents in Cape Town and Stellenbosch, South Africa

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Received 1 November, 2012; Accepted 25 September, 2013

This study focused on one year monitoring campaign to monitor the occurrence and removal of Endocrine Disruptive Metals (EDMs) and trace metals from selected wastewater treatment plants (WWTPs) in Stellenbosch and Cape Town. Composite water samples were collected from the WWTPs from January 2010 to December 2010 on a quarterly basis and concentrations determined using inductively coupled plasma-mass spectrometry (ICP-MS) after open beaker digestion. A total of 432 water samples consisting of raw, primary effluent, secondary effluent and final effluents were collected and analyzed. The general abundance distribution pattern for metals was Zn > Cu > Pb > Cr > Ni > As > Co > Cd > Hg. The removal efficiency ranged from 1.5% for Hg at Zandvliet WWTP plant during winter to 98.27% for Cu at Athlone WWTP treatment plant during summer. The final effluent concentration for most of the metals were within South African water quality guidelines while As, Hg, Cd and Pb concentration were higher than maximum limits set by the Canadian Council of Ministers of the Environment. Potsdam WWTP showed to be the most effective at heavy metals removal as compared with the other five treatment plants investigated in this study. The effluent metal concentration over time could pose health risk if used for agricultural irrigation.

Key words: Seasonal variation, endocrine disrupting metals, wastewater treatment plants, effluents, coupled plasma-mass spectrometry (ICP-MS), Cape Town.

INTRODUCTION

The presence of metals in wastewater is one of the main causes of water and soil pollution (Chanpiwat et al., 2008, 2010). The accumulation of these metals in wastewater depends upon several local factors (Oliveira

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et al., 2007; Ogunfowokan et al., 2008). Metal composition in urban wastewater in Brazil, China, Iran and Poland had been reported to be both complex and highly varying and according to the prevailing economic activities and the living pattern (Kulbat et al., 2003; Oliveira et al., 2007; Rajbanshi, 2008; Chanpiwat et al., 2010). It has been stated that metal input in wastewater treatment systems is so variable that even down to an hour-by-hour scale it remains unpredictable (Ogunfowokan et al., 2008). Major sources of sewage wastewater include households, drainage water, businesses, atmospheric deposition, pipe sediment, building materials and traffic (Sorme and Largerkrist, 2002). Moreover, metal concentrations in wastewater can be affected by people's lifestyles and their awareness of the impacts on the environment. Metal removal efficiency depends on the metal concentration, its speciation, the reactivity of the available biopolymers or biomass, and the composition of other wastewater components (Wang et al., 1999). With emphasis on the improvement of stream and river water quality, the treatment plants must achieve greater efficiency in the treatment process. No study has reported on the concentrations of heavy metals most especially metals classified as Endocrine Disrupting Metals (EDMs) (that is, cadmium, arsenic, lead and mercury) in the influent and effluent wastewater from wastewater treatment plants in Western Cape Province, South Africa.

Previous studies have largely concentrated on the water, sediment and plant samples from Diep and Berg rivers (Jackson et al., 2007, 2009; Shuping, 2008; Ayeni et al., 2010). Though a nationwide survey was carried out in 1989 and 2002 to assess levels of heavy metals in sewage sludge from 77 wastewater treatment plants, the country's population had increased and there has been rural-urban migration, thus, pressure on wastewater treatment plants (WWTPs) facilities has increased over the last decade (Jaganyi et al., 2005). Paucity of information on the available endocrine disrupting metals, other trace metals and public outcry on poor performance of WWTPs facilities necessitated the need for this study to establish: 1) The occurrence and distribution pattern of endocrine disrupting metals and other trace elements; and 2) to access the impact of seasonal changes on EDMs availability and removal from wastewater effluents.

This study follows the preliminary investigation into the possible impact of wastewater treatment plant effluents on freshwater systems in Cape Town (Olujimi et al., 2012).

MATERIALS AND METHODS

All the determinations were carried out by inductively coupled plasma mass spectrometry (ICP-MS) located at the Geology Department, University of Stellenbosch. The Agilent 7700 instrument was used with a Meinhardt nebulizer and silica cyclonic spray chamber with continuous nebulization. The operation parameters were Plasma RF power: 1550 W; Sample depth: 8.0 mm; Carrier gas: 1.08 L/min; Nebulizer pump: 0.10 rps; Helium gas: 5.3 $mlmin^{-1}$ for ICPMS. The isotopes of the elements determined were ^{111}Cd, ^{75}As, ^{208}Pb, ^{52}Cr, ^{59}Co, ^{60}Ni, ^{63}Cu and ^{66}Zn.

Reagents

Water (resistivity 18.2 MΩ cm) was de-ionized by the use of a Milli-Q system (Millipore, Bedford, MA, USA). Certified standard of all the metal (As, Cd, Cu, Co, Cr, Hg, Ni, Pb and Zn) were obtained to check for instrument performances from Merck, South Germany. $AuCl_3$, ultrapure nitric acid (65%) and 32% hydrogen peroxide were obtained from Fluka Kamika, Switzerland.

Study area and sampling protocol

Six wastewater treatment plants were investigated for the occurrence of heavy metals and for the effectiveness of the WWTPs in removing them from waste stream. Five of these WWTPs were located in the City of Cape Town, while one is located in Stellenbosch. Geographical locations and design properties of the investigated treatment plants are presented in Table 1. 24 h composite water samples were collected from the wastewater treatment on quarterly basis to observe the possible impact of seasonal variation on heavy metals in wastewater treatment plants. Sampling for heavy metals analysis commenced in January 2010 and ended in December 2010.

Wastewater digestion

Water samples for the heavy metals analysis were collected in 1 L plastic containers which were initially washed with detergent and rinsed with distilled water. The containers were finally soaked in 10% nitric acid overnight and rinsed with Milli-Q water prior to use. The samples were preserved by adding a few drops of concentrated HNO_3 to each sample bottle and pH adjusted to 2.0 by the use of pH meter. The samples were stored in a refrigerator at about 4°C, before subsequent analysis. As samples may contain particulate or organic materials, pretreatment in the form of digestion is required before analysis. Nitric acid digestion was employed in accordance with Akan et al. (2008). A few drops of $AuCl_3$ were added to the water samples to keep Hg ions in solution.

Treatment plants removal efficiency

Unfortunately, none of the WWTPs were monitored for both influent and effluent flow rates. The removal efficiency (ε) of each metal was calculated based on influent and effluent concentrations, on the assumption of steady-state conditions and that precipitation or evapotranspiration had minimal impact on the water storage as compared to inflow and outflow:

$$\varepsilon(\%) = \left(\frac{EDCi/Mi - EDCe/Me}{Mi}\right) \times 100\% = \frac{QiCi - QeCe}{QiCi} \times 100\% = \frac{Ci - Ce}{Ci} \times 100\%$$

Table 1. Description of the six waste water treatment plants investigated.

WWTP ID	Geographical Location of plant	People Equivalent	Source	Treatment process	Associated River
A	S 33.5709° E 18.3048°	900,000	Domestic Industrial	S + G + Sed + AS (BNR) + Sed + Chl + AD + Dew -	Vygekraal River
B	S 33.5923° E 18.4332°	591,000	Domestic Industrial	S + G + EAAS (N) + Sed + UVdis + Dew -	Kuils River
C	S 33.82539° E 18.70442°	133,000	Domestic	S + G + Sed + AS (N) + Sed + Chl + AD + Dew	Mosselbank River
D	S 33.5070° E 18.3108°	385,000	Domestic Industrial	S + G + Sed + AS (BNR) + Sed + Chl + AD + Dew	Diep River
E	S 33.94345° E 18.82492°	N/K	Domestic Industrial	S + G + Sed + FB + AS (BNR) + Sed + Chl + AD + Dew	Veldwachters River
F	S 34.0312° E 18.4259°	400,000	Domestic Industrial	S + G + EAAS (N) + Sed + UVdis + Dew	Kuils River

With *EDCi/e* or *Mi/e* = the metal flux in influent/effluent (mgd^{-1}); *Ci/e* = the metal concentration in influent/effluent (mgl^{-1}); *Qi/e* = the mean flow rate of influent/effluent ($l\ d^{-1}$).

Statistical analysis

Statistical analysis was performed using SPSS 19.0. Normality of the distribution was tested by means of the Kolmogorov-Smirnov test of normality (α = 0.05). As metal concentrations in the water were not normally distributed, significance of difference between raw wastewater, settling tank and effluents were assessed by means of non-parametric Wilcoxon tests (α = 0.05). Seasonal effects were analyzed by means of the non-parametric Kruskal-Wallis rank test (α = 0.05).

RESULTS AND DISCUSSION

Occurrence, distribution and removal pattern of heavy metals in wastewater treatment plants

Arsenic

Seasonal variation of arsenic at the Athlone WWTP is shown in Table 2, while Figure 1a presents the annual distribution pattern in the WWTP. The percentage removal of arsenic in treatment plants ranged from 22.14 to 68.44%. The annual mean removal efficiency of the plant for arsenic was 43.78% (Figure 2). Statistical analysis showed no significant difference (P>0.05) in the level of arsenic received at the plant during the studied period. However, it is noteworthy that the plant was not functioning optimally during the second and fourth sampling seasons. For the studied period, the removal efficiency of the plant could be adjudged ineffective as less than 50% of the total arsenic influx was removed from the waste stream. The annual distribution trend showed that about 20% of arsenic was removed at the primary settling tank, while the secondary settling tank accounted for about 60% (Figure 1). The ineffective removal of arsenic from the Athlone WWTP could be attributed to plant overload and frequent breakdown of the treatment plant. The arsenic concentrations in old and new Bellville plants (old Bellville plant received wastewater from domestic sources, while new plant received wastewater from both domestic and industrial sources) ranged from 4.62 to 9.2 μgL^{-1} and 6.01 to 43.76 μgL^{-1}. Effluent concentrations ranged from 2.57 to 4.69 μgL^{-1} and 1.12 to 5.10 $\mu g\ L^{-1}$ in new and old plants, respectively (Table 2). For the two plants, there was significant differences (P<0.05) in seasonal arsenic concentrations. Also, there was significant differences in arsenic concentrations within the plant during summer and autumn seasons due to different plant treatment processes for old and new plants. The annual distribution pattern of arsenic in the WWTPs is presented in Figure 1b and c.

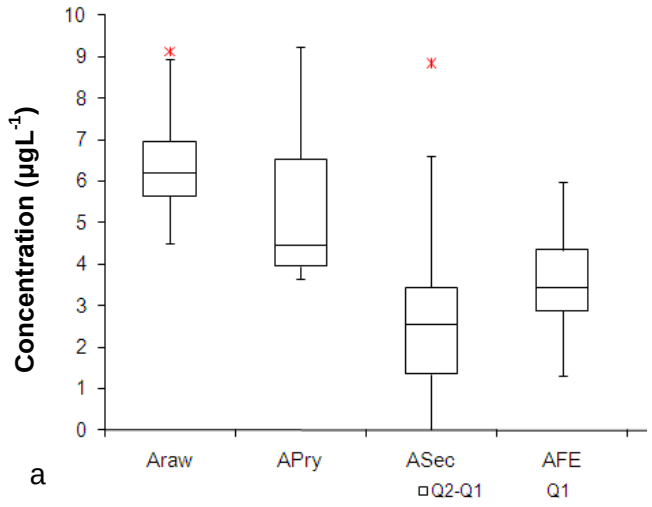
The seasonal removal efficiency of the plants ranged from 39.08 to 75.95% (old plant) and 40.31 to 94.12% (new plant) (Table 2). In the old plant, the primary settling tank accounted for about ¼% removal of arsenic on an annual basis, while the secondary settling tank removed

Table 2. Mean concentration (±SD) of As in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgl^{-1}) with associated total removal efficiency.

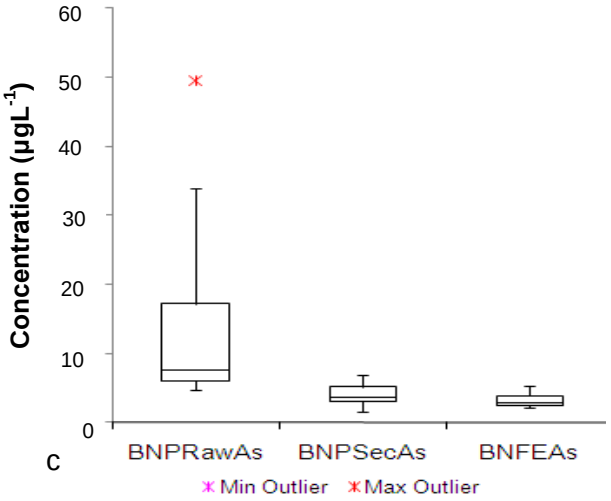
WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	6.21 ± 0.53	4.07 ± 0.42	2.02 ± 0.38	1.91 ± 1.03	*	68.44
	Autum '10	8.24 ± 0.83	7.99 ± 1.21	6.09 ± 2.42	5.69 ± 0.47		30.93
	Winter '10	6.14 ± 0.33	5.72 ± 1.17	2.83 ± 0.29	2.86 ± 0.29		53.42
	Spring '10	3.95 ± 0.31	3.95 ± 0.31	N/A	3.89 ± 0.19	*	22.14
	α_{season}	*					
Bellville old	Summer '10	4.62 ± 0.19	4.12 ± 0.48	3.71 ± 0.10	1.12 ± 0.06	*	75.67
	Autum '10	9.35 ± 0.54	3.62 ± 0.02	2.71 ± 0.81	2.25 ± 0.22	*	75.97
	Winter '10	8.38 ± 0.22	8.00 ± 0.34	6.04 ± 0.41	5.10 ± 0.48		39.08
	Spring '10	6.16 ± 1.28	5.97 ± 0.60	4.62 ± 0.79	2.63 ± 0.17		57.35
	α_{season}	*					
Bellville new	Summer '10	6.77 ± 2.36	NPST	4.60 ± 0.77	3.14 ± 1.13		53.67
	Autum '10	43.76 ± 5.06	NPST	3.52 ± 0.20	2.57 ± 0.14	*	94.12
	Winter '10	7.86 ± 0.74	NPST	5.91 ± 0.81	4.69 ±0.90		40.31
	Spring '10	6.01 ± 0.27	NPST	2.19 ± 0.45	2.63 ±0.17		56.33
	α_{season}	*					
Kraaifontein	Summer '10	4.27 ± 0.27	3.32 ± 0.16	2.38 ± 0.16	2.38 ± 0.15	*	44.28
	Autum '10	5.27 ± 0.15	3.93 ± 0.22	2.98 ± 0.65	2.38 ± 0.10		54.87
	Winter '10	8.88 ± 1.10	6.03 ± 0.47	5.16 ± 1.11	3.71 ± 0.31		58.27
	Spring '10	5.27 ± 0.09	4.48 ± 0.47	4.44 ± 0.57	1.78 ± 0.21		66.27
	α_{season}						
Potsdam	Summer '10	4.23 ± 0.16	3.11 ± 0.15	2.23 ± 0.21	1.20 ± 0.12	*	71.52
	Autum '10	7.38 ± 0.10	5.53 ± 0.49	4.24 ±1.08	2.09 ± 0.31	*	71.64
	Winter '10	6.59 ± 0.31	5.08 ± 0.54	3.27 ± 0.23	2.64 ± 0.07		59.96
	Spring '10	5.28 ± 0.21	3.25 ± 0.31	2.00 ± 0.08	3.10 ± 0.09		41.18
	α_{season}	*		*			
Stellenbosch	Summer '10	28.20 ± 3.43	4.20 ± 0.44	2.03 ± 0.07	2.75 ± 0.20	*	90.25
	Autum '10	5.33 ± 2.17	3.26 ± 0.26	3.04 ± 0.21	2.91 ± 0.49		45.37
	Winter '10	6.71 ± 0.47	6.49 ± 0.47	2.34 ± 0.12	2.98 ± 0.05		55.54
	Spring '10	5.07 ± 0.47	2.60 ± 0.55	2.01 ± 0.29	2.56 ± 0.16		49.48
	α_{season}	*					
Zandvliet	Summer '10	4.04 ± 0.38	NPST	3.3 ± 0.1	2.75 ± 0.20	*	42.63
	Autum '10	4.07 ± 0.45	NPST	2.8 ± 0.1	2.6 ± 0.1		35.54
	Winter '10	4.53 ± 0.24	NPST	2.3 ± 0.3	1.6 ± 0.8		62.77
	Spring '10	7.36 ± 0.49	NPST	5.8 ± 1.0	2.56 ± 0.16		66.58

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$.

about 40% of the total arsenic concentration. The new Bellville plant uses University of Cape Town design (UCT) tank accounted for about ¼% removal of arsenic on an annual basis, while the secondary settling tank removed about 40% of the total arsenic concentration. The new Bellville plant uses University of Cape Town design


Concentration (µgL-1)
10
9
8
7
6
5
4
3
2
1
0
Araw
APry
ASec
AFE
Q2-Q1
Q1

a


Concentration (µgL-1)
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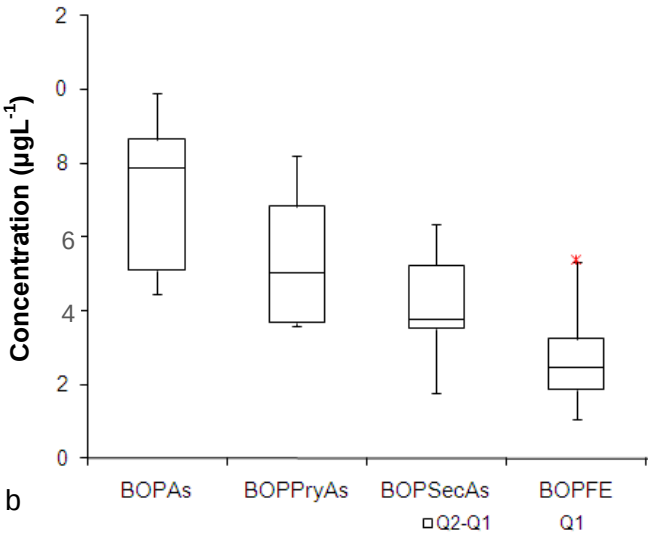
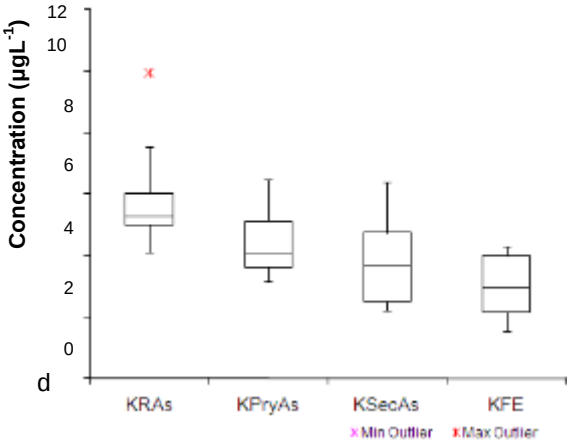
50
40
30
20
10
0
BNPRawAs
BNPSecAs
BNFEAs
Min Outlier
Max Outlier

c


Concentration (µgL-1)
2
0
8
6
4
2
0
BOPAs
BOPPryAs
BOPSecAs
BOPFE
Q2-Q1
Q1

b


Concentration (µgL-1)
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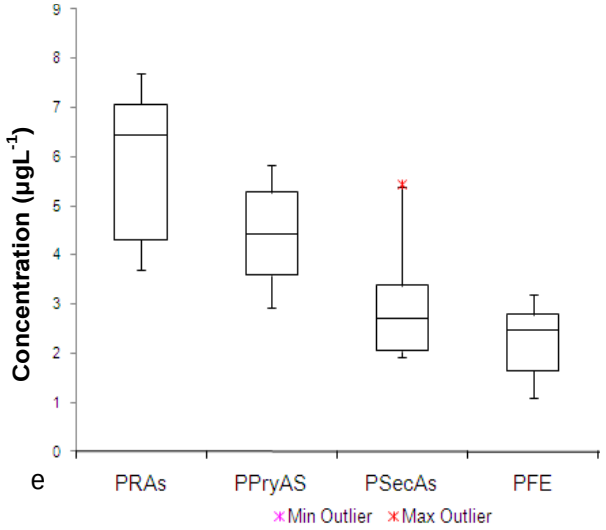
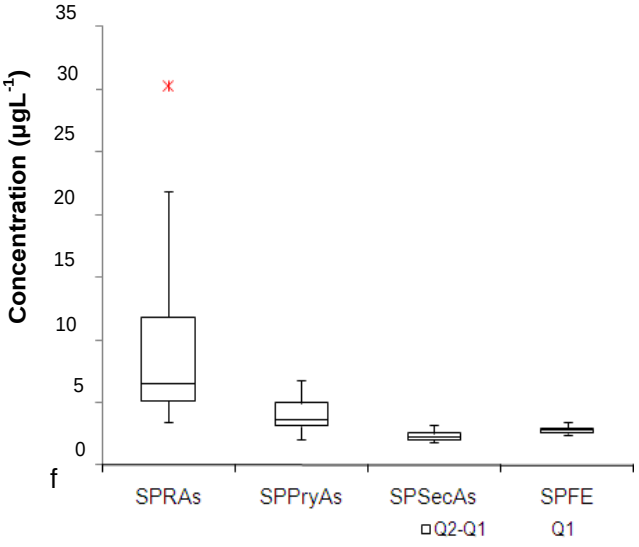
10
8
6
4
2
0
KRAs
KPryAs
KSecAs
KFE
Min Outlier
Max Outlier

d


Concentration (µgL-1)
9
8
7
6
5
4
3
2
1
0
PRAs
PPryAS
PSecAs
PFE
Min Outlier
Max Outlier

e


Concentration (µgL-1)
35
30
25
20
15
10
5
0
SPRAs
SPPryAs
SPSecAs
SPFE
Q2-Q1
Q1

f

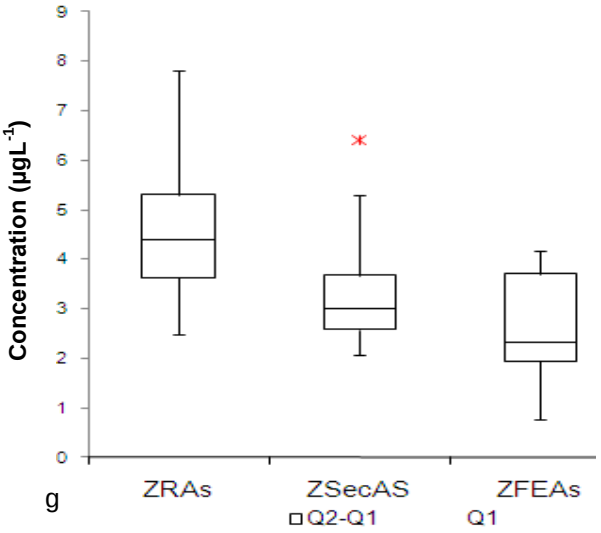


Figure 1. Box and whisker plot for annual spread of As concentration in WWTPs. a) Athlone b) Bellville old c) Bellville new d) Kraaifontein e) Potsdam f) Stellenbosch g) Zandvliet.

(UCT) system with returned activated sludge, raw effluent were pumped straight into the bioreactor with no primary settling tank. Secondary settling tank removed about 75% of the total influx of arsenic. The annual mean removal efficiency of this treatment plant (old and new systems combined) was about 62% and thus, could be rated above average in performance (Figure 2). Arsenic concentration at the Kraaifontein WWTP ranged from 4.27 to 8.8 μgL^{-1} in the influent waste and 1.78 to 3.71 μgL^{-1} in the final effluent (Table 2). The removal efficiency of the plant increased by about 22% over the sampling period (Table 2). The annual mean distribution pattern revealed that 25% of arsenic was removed at the primary settling tank, while 36.8% was removed at the secondary settling tank (Figure 1d). The annual mean removal efficiency of the plant was 55.92% (Figure 2). There was a significant difference in the concentration over the study period due to the treatment process.

The Potsdam WWTP arsenic concentrations varied between 4.23 to 7.38 μgL^{-1} in influent waste and 1.21 to 3.10 μgL^{-1} in the final effluent (Table 2). The annual mean with distribution pattern of arsenic is presented in Figure 1e. There was a significant difference ($P<0.05$) in arsenic level over the sampling period. The distribution and removal pattern showed that about 28% of arsenic in the wastewater was removed at the primary settling tank, while about 50% was removed at the secondary settling tank. The annual mean removal efficiency of the plant was 61.07% (Figure 2). This showed that the treatment plant was more effective at arsenic removal compared to Athlone and Bellville plants. The Stellenbosch WWTP received a concentration range of 5.07 to 28.20 μgL^{-1} for fourth and first quarter, respectively, in the influent wastewater while the final effluent had a concentration range of 2.56 to 2.98 μgL^{-1} (Table 2). The seasonal removal efficiency of the plants varied between 45.37 to 90.25% with an annual mean removal efficiency of 60.16%. There was significant difference in the arsenic concentration in the influent into the plant over the sampling period. An increase in concentration of arsenic in the Zandvliet WWTP was also observed from 4.04 to 7.36 μgL^{-1} in the raw effluent, while the final effluent concentrations ranged from 1.69 to 2.62 μgL^{-1}. This plant seasonal removal efficiency varied between 35.54 to 66.58% (Table 2). About 30% of arsenic concentration was removed at the secondary settling tank, while the remaining can be accounted for in the wastewater sludge.

The annual distribution pattern is presented in Figure 1g. Though, over 35% of arsenic concentration that enters the plant was removed, there was no significant difference ($P>0.05$) due to plant treatment processes except for summer season.

Cadmium

Cadmium concentration ranged from 2.21 to 3.38 μgL^{-1} and 0.52 to 2.31 μgL^{-1} in the influent and effluent wastewater of Athlone plant, respectively (Table 3). Generally, 65% of heavy metals in raw influent are believed to be removed at the primary settling tank (Chanpiwat et al., 2008). This assumption could not hold for cadmium in this plant as overall annual mean removal efficiency for the plant revealed that 25% of the total Cd concentration was removed at the primary settling tank,

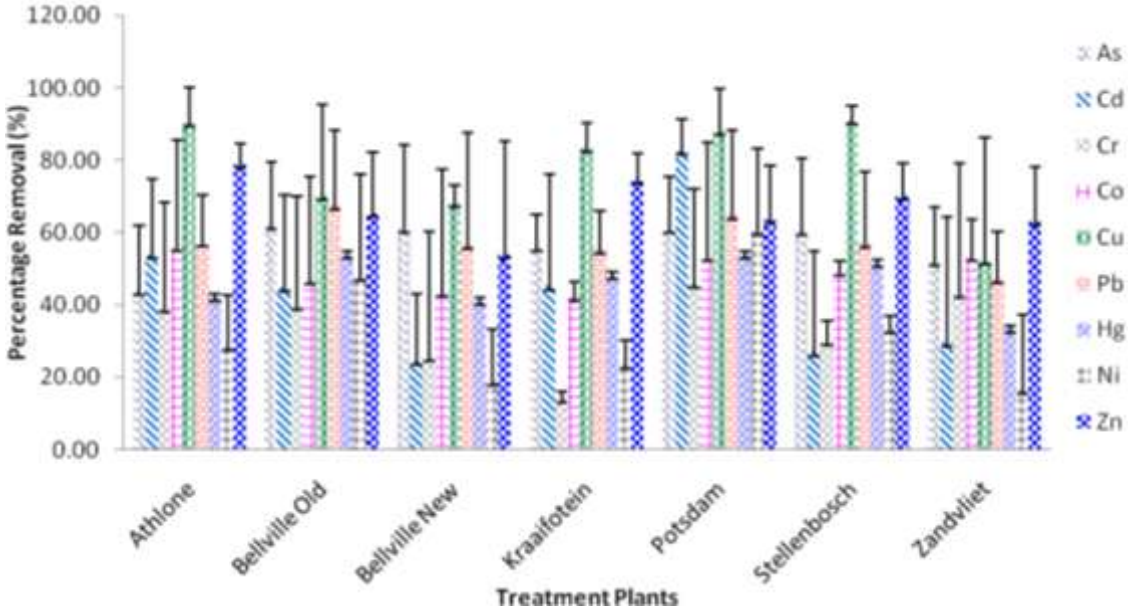


Figure 2. Comparison of annual removal efficiency for trace metals in WWTPs.

while about 35% was taken off the waste stream at the secondary settling tank. The annual distribution pattern of cadmium in the treatment plant and the annual removal efficiency are presented in Figure 3a and Table 3. Based on the removal efficiency, the plant could be rated average for cadmium removal. There was no significant difference in the influent cadmium concentrations in the plant over the study period. The influent concentrations of cadmium at the old Bellville plant varied between 1.53 and 5.52 μgL^{-1}, while the new plant concentrations varied between 1.64 and 3.55 μgL^{-1}. The final effluent concentrations ranged from 1.42 to 2.24 μgL^{-1} and 1.07 to 1.36 μgL^{-1} for the new and old plants, respectively (Table 3). The annual distribution pattern of cadmium in the two plants presented in Figure 3b and c indicated that 18.8% of cadmium concentration was removed at the Bellville old plant primary settling tank, while 28.9 and 25.22% were removed at the secondary settling tanks of the old and new plant, respectively. The annual mean percentage removal was 44.88 and 24.61% for the old and new plants, respectively (Figure 2). For the new plant, with the exception of the summer season, no significant difference in cadmium concentrations occurred over the study period or between the influent and effluent concentration.

The Kraaifontein plant received an influent concentration range of 1.85 to 8.88 μgL^{-1} and released effluent with a concentration range of 1.25 to 2.29 μgL^{-1} (Table 3). The removal efficiency of this treatment plant varied between 7.68 to 74.97% with an annual mean of 45.22% (Figure 2). The annual distribution pattern presented in Figure 3d revealed that 36.2 and 30.1% of total cadmium concentration were removed at primary and secondary settling tanks, respectively. The annual mean removal efficiency showed that the plant was below average for cadmium removal. Cadmium concentration in the influent of the Potsdam plant varied between 8.67 and 17.39 μgL^{-1} with an annual mean of 12.62 μgL^{-1}, while the effluent concentrations varied between 1.33 to 2.85 μgl^{-1} with annual mean of 2.02 μgL^{-1} (Table 3). The distribution of cadmium in the plant (Figure 3e) showed that 45.2 and 36.02% cadmium was removed from the waste stream at the primary and secondary settling tanks, respectively. The annual mean removal efficiency was 82.58% (Figure 2). The high concentration of cadmium in the influent could be due to high industrial effluent received at the plant. There were significant differences in cadmium concentration over the sampling period due to treatment processes during summer. The Stellenbosch plant's cadmium concentrations in the influent ranged from 1.68 to 2.96 μgL^{-1} with annual mean concentration of 2.45 μgL^{-1}, while cadmium concentration in the final effluent ranged from 1.29 to 2.23 μgL^{-1} with an annual mean of 1.65 μgL^{-1} (Table 3). The annual distribution pattern in Stellenbosch presented in Figure 3f shows that about 25% of the cadmium was removed at the primary settling tank, while 4.9% was removed at the secondary settling tank.

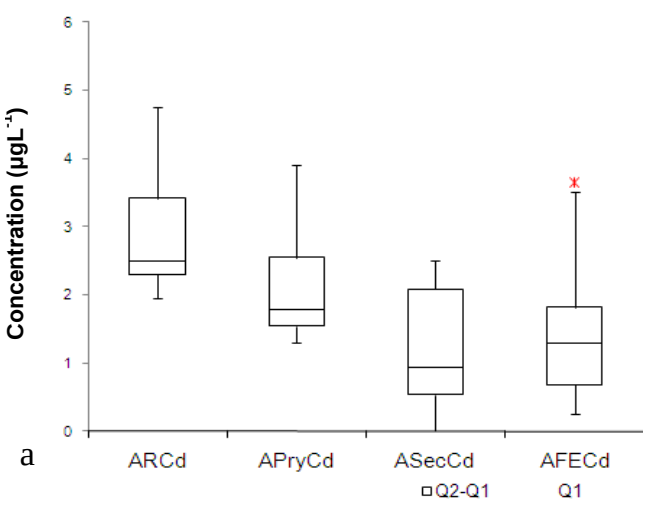
The annual mean removal efficiency was 29.17% (Figure 2). There was no significant difference due to seasonal change. However, there was significant difference due to treatment plant processes during the summer as the plant was constantly breaking down during the summer sampling protocol.

Table 3. Mean concentration (±SD) of Cd in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgl^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	2.94 ± 0.31	1.35 ± 0.04	0.86 ± 0.15	1.01 ± 0.51		65.61
	Autum '10	3.09 ± 0.69	2.64 ± 1.01	2.06 ± 0.5	1.80 ± 0.13		32.43
	Winter '10	2.21 ± 1.10	1.86 ± 0.42	1.71 ± 0.76	0.52 ± 0.23		74.97
	Spring '10	3.38 ± 0.1	2.91 ± 1.16	N/A	2.31 ± 1.27		7.68
	α_{season}						
Bellville old	Summer '10	2.40 ± 0.26	1.74 ± 0.04	1.07 ± 0.15	1.07 ± 0.5		55.44
	Autum '10	5.52 ± 0.69	4.10 ± 1.01	2.45 ± 0.49	1.36 ± 0.13		41.86
	Winter '10	1.53 ± 0.24	1.50 ± 0.43	0.69 ± 0.76	1.25 ± 0.24		18.08
	Spring '10	2.05 ± 0.54	1.97 ± 0.51	1.91 ± 0.38	1.42 ± 0.37		30.59
	α_{season}						
Bellville new	Summer '10	1.64 ± 0.05	NPST	1.33 ± 0.25	1.18 ± 0.17	*	28.16
	Autum '10	3.55 ± 0.87	NPST	3.36 ± 1.69	2.24 ± 0.23		36.81
	Winter '10	1.68 ± 0.33	NPST	0.74 ± 0.24	1.64 ± 0.96		2.38
	Spring '10	2.23 ± 0.98	NPST	1.31 ± 0.18	1.42 ± 0.38		36.11
	α_{season}			*			
Kraaifontein	Summer '10	4.57 ± 0.31	2.69 ± 0.19	N/A	1.56 ± 0.9	*	65.78
	Autum '10	1.85 ± 0.05	1.54 ± 0.23	1.27 ± 0.24	1.25 ± 0.10		32.43
	Winter '10	8.88 ± 1.10	6.03 ± 0.47	1.71 ± 0.46	1.28 ± 0.72		74.97
	Spring '10	2.48 ± 0.1	2.43 ± 0.41	1.65 ± 0.25	2.29 ± 0.49		7.68
	α_{season}	*	*				
Potsdam	Summer '10	17.39± 0.55	4.49 ± 0.24	1.64 ± 0.91	1.33 ± 0.9	*	92.33
	Autum '10	14.53± 5.10	12.14 ± 0.37	3.08 ± 1.19	2.57 ± 0.59		82.29
	Winter '10	8.67± 1.62	4.91 ± 0.12	1.97 ± 0.62	1.35 ± 0.18		84.47
	Spring '10	9.89± 1.35	6.15 ± 1.50	2.44 ± 0.15	2.85 ± 0.53		71.24
	α_{season}	*					
Stellenbosch	Summer '10	2.96 ± 0.46	1.31 ± 0.29	0.96 ± 0.11	1.29 ± 0.36	*	
	Autum '10	2.54 ± 0.57	1.57 ± 0.26	2.46 ± 0.62	2.23 ± 1.16		12.34
	Winter '10	1.68 ± 0.52	2.33 ± 1.35	1.61 ± 0.31	1.61 ± 1.16		4.17
	Spring '10	2.62 ± 1.33	2.42 ± 0.58	2.13 ± 0.58	1.47 ± 0.51		43.86
	α_{season}						
Zandvliet	Summer '10	2.32 ± 0.23	NPST	0.84 ± 0.20	1.17 ±0.15		49.62
	Autum '10	3.10 ± 0.54	NPST	2.17 ± 0.59	2.53 ± 0.43		18.39
	Winter '10	1.07 ± 0.17	NPST	1.05 ± 0.45	0.53 ± 0.08		50.78
	Spring '10	3.22 ± 0.38	NPST	1.96 ± 0.45	1.95 ± 0.65		39.62
	α_{season}						

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at α = 0.05; NA, not analysed; NPST, no primary settling tank.

The Zandvliet plant's influent cadmium concentrations were in the range of 1.07 to 3.10 μgL^{-1} with an annual mean influent concentration of 2.43 μgL^{-1}. The final effluent concentration ranged from 0.53 to 2.53 μgL^{-1} (Table 3). The annual distribution pattern for cadmium in the treatment plant revealed that 34 and 7% of cadmium in the wastewater was removed into primary and secondary sludge, respectively (Figure 3g). The annual mean removal efficiency for the plant shows that less than 40% of total cadmium concentration was removed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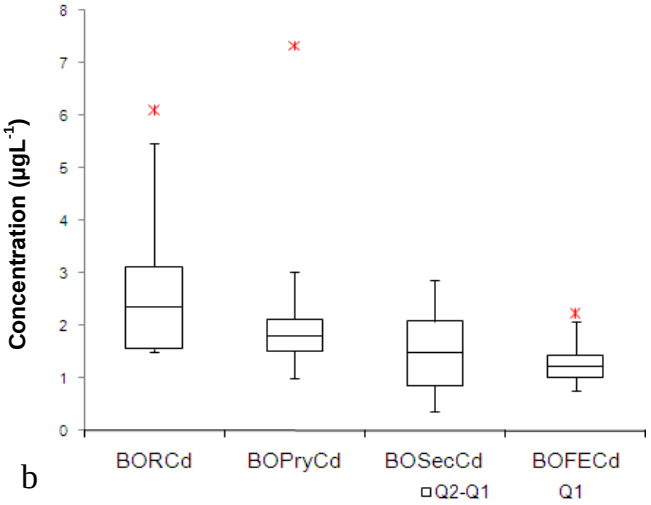
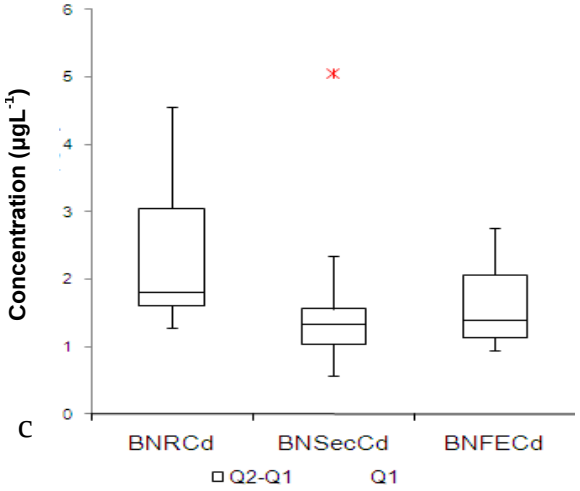
ARCd
APryCd
ASecCd
AFECd
Q2-Q1
Q1

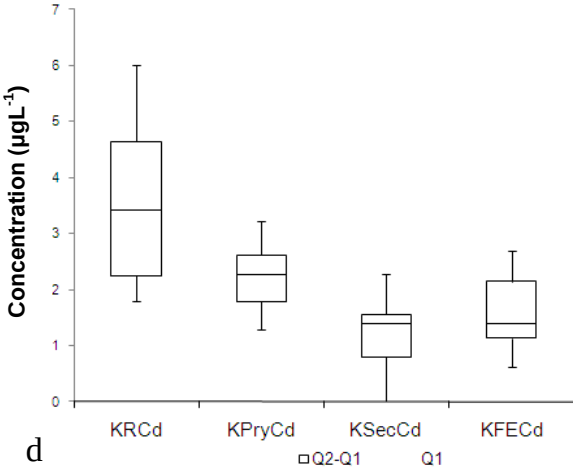
a


Concentration (µgL-1)
BORCd
BOPryCd
BOSecCd
BOFECd
Q2-Q1
Q1

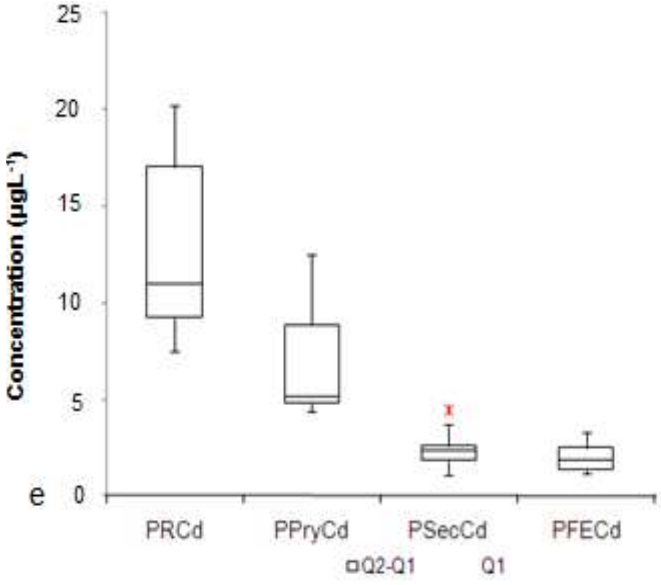
b


Concentration (µgL-1)
BNRCd
BNSecCd
BNFECd
Q2-Q1
Q1

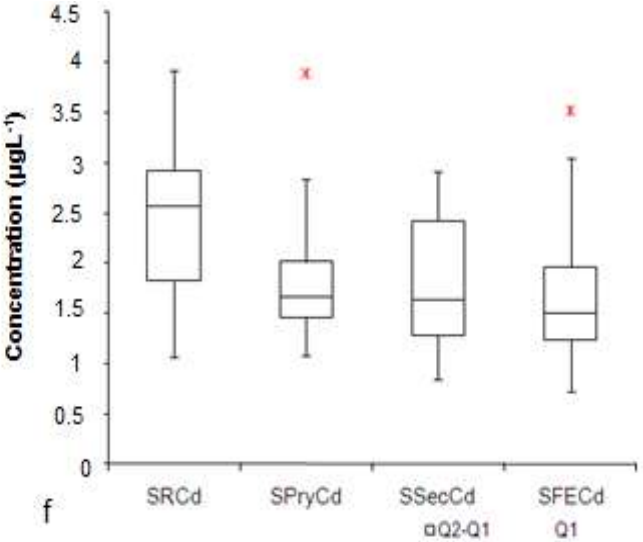
c


Concentration (µgL-1)
KRCd
KPryCd
KSecCd
KFECd
Q2-Q1
Q1

d


Concentration (µgL-1)
PRCd
PPryCd
PSecCd
PFECd
Q2-Q1
Q1

e


Concentration (µgL-1)
SRCd
SPryCd
SSecCd
SFECd
Q2-Q1
Q1

f

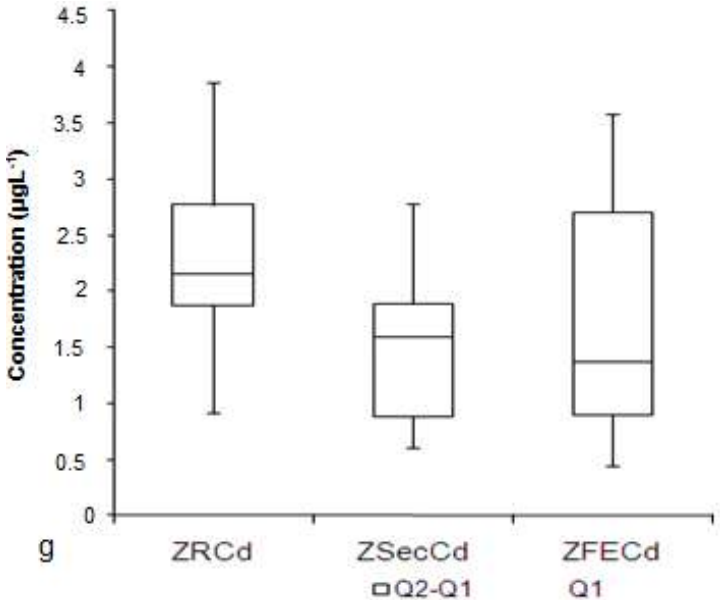


Figure 3. Box and whisker plot for annual spread of Cd concentration in WWTPs. a) Athlone b) Bellville old c) Bellville new d) Kraaifontein e) Potsdam f) Stellenbosch g) Zandvliet.

from the waste stream (Figure 2).

Cobalt

Cobalt influent concentrations into the Athlone plant ranged from 3.29 to 11.65 μgL^{-1}, while the final effluent concentration varied between 1.12 and 2.62 μgL^{-1} (Table 4). The annual distribution pattern of cobalt in the plant as depicted in Figure 4a showed that only 9.2% of cobalt concentration into the plant was removed at the primary settling tank while 77.5% removal took place at the secondary settling tank. The annual mean removal efficiency of the plant was 55.98% (Figure 2). There was significant difference in the seasonal concentration of cobalt received at the plant, also, significant difference due to treatment processes was noticeable during the summer season. The old and new Bellville plants received influent with 3.30 to 6.29 μgL^{-1} and 3.44 to 13.86 μgL^{-1} and released effluent containing between 1.13 to 3.23 μgL^{-1} and 1.54 to 3.23 μgL^{-1} of cobalt, respectively. The annual distribution patterns of cobalt in the two Bellville plants are presented in Figure 4b and c. 32.62% of the total concentration into the old plant was removed at the primary settling tank with a corresponding concentration of about 11% removed at the secondary tank. The new plant using the UCT system with only a secondary sedimentation tank removed about 55%. The mean annual removal efficiency was 46.74 and 43.5% for old and new plants, respectively (Figure 2). No significant difference was observed for the seasonal influent concentration at old plant. However, a significant difference due to the plant treatment processes was noticeable during the summer season. For the new plant, significant difference due to plant treatment processes and season was recorded. The Kraaifontein treatment plant received influent cobalt concentrations ranging between 0.34 and 3.98 μgL^{-1} and the final effluent concentration ranged from 0.18 to 2.14 μgL^{-1} (Table 4). The annual Co distribution pattern and removal efficiency are presented in Figure 4d. The distribution pattern shows that 27.45% was eliminated through the primary sedimentation tank while secondary sedimentation tank accounted for 32.35%. The annual mean removal efficiency of the plant was 42.07%. There was significant difference in the plant treatment processes.

The concentration range between 2.23 and 5.02 μgL^{-1} was received in the influent during the sampling seasons at the Potsdam treatment plant. The effluent concentration varied between 0.65 and 4.73 μgL^{-1} (Table 4). The annual mean influent concentration was 3.68 μgL^{-1} while the annual mean effluent concentration was 1.94 μgL^{-1}. The distribution pattern of cobalt in the plant (Figure 4e) indicated that 20.65% of total annual concentration was removed at the primary settling tank while about 17% was trapped into secondary sludge through the secondary sedimentation tank. The annual mean removal efficiency of the plant was 53.19%. A significant difference due to season and plant treatment processes was observed.

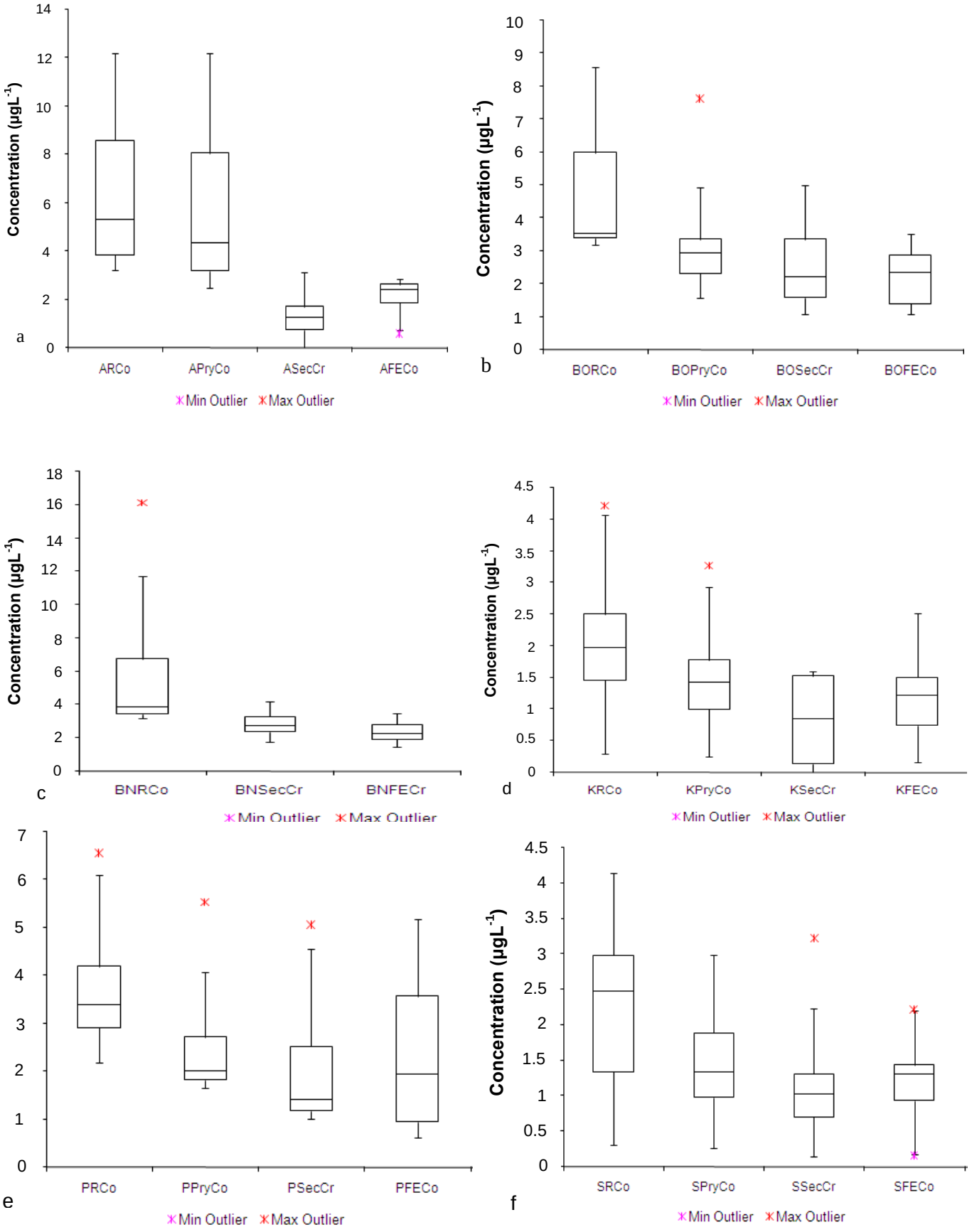
Stellenbosch and Zandvliet treatment plants influent concentration ranged from 0.35 to 3.17 μgL^{-1} and 0.34 to

Table 4. Mean concentration (±SD) of Co in the influent, primary, secondary and final effluent of WWTPs during the different seasons ($\mu g l^{-1}$) with associated total removal efficiency.

WWTP	Season	Concentration ($\mu g\ l^{-1}$)				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	11.7 ± 0.49	11.9 ± 0.25	1.4 ± 0.07	1.9 ± 1.12	*	84.01
	Autum '10	7.06 ± 0.75	5.75 ± 0.99	1.13 ± 0.14	1.52 ± 0.72		78.53
	Winter '10	4.18 ± 0.19	3.06 ± 0.50	2.92 ± 0.28	2.62 ± 0.10		37.27
	Spring '10	3.29 ± 0.11	3.26 ± 0.41	N/A	2.50 ± 0.38		24.10
	α_{season}		*	*			
Bellville old	Summer '10	3.50 ± 0.09	2.59 ± 0.15	1.72 ± 0.2	1.71 ± 0.3	*	51.04
	Autum '10	6.29 ± 0.38	1.66 ± 0.15	1.41 ± 0.3	1.13 ± 0.1		82.03
	Winter '10	3.30 ± 0.11	3.1 7 ± 0.07	2.89 ± 0.3	2.90 ± 0.2		12.22
	Spring '10	5.54 ± 2.72	5.14 ± 2.2	4.54 ± 0.4	3.23 ± 0.4		41.68
	α_{season}						
Bellville new	Summer '10	3.44 ± 0.12	NPST	3.43 ± 0.23	2.15 ± 0.08	*	37.55
	Autum '10	13.86 ± 1.97	NPST	1.91 ± 0.13	1.54 ± 0.05	*	88.89
	Winter '10	4.51 ± 0.47	NPST	2.81 ± 0.10	2.66 ± 0.34		41.05
	Spring '10	3.46 ± 0.32	NPST	3.08 ± 0.97	3.23 ± 0.41		6.50
	α_{season}		*				
Kraaifontein	Summer '10	1.94 ± 0.15	1.33 ± 0.09	N/A	1.23 ± 0.30	*	36.94
	Autum '10	1.91 ± 0.08	1.53 ± 0.06	1.53 ± 0.04	1.15 ± 0.12		39.63
	Winter '10	0.34 ± 0.04	0.28 ± 0.03	0.19 ± 0.01	0.18 ± 0.03		45.48
	Spring '10	3.98 ± 0.27	2.78 ± 0.45	1.55 ± 0.04	2.14 ± 0.55		46.21
	α_{season}		*	*			
Potsdam	Summer '10	2.23 ± 0.05	1.75 ± 0.07	1.19 ± 0.08	0.65 ± 0.04		70.62
	Autum '10	3.31 ± 0.15	2.14 ± 0.15	1.62 ± 0.37	1.06 ± 0.03		67.94
	Winter '10	4.16 ± 0.09	3.07 ± 0.10	1.94 ± 0.50	1.31 ± 0.24	*	68.43
	Spring '10	5.02 ± 1.62	4.73 ± 0.75	4.47 ± 0.55	4.73 ± 0.38		5.79
	α_{season}		*				
Stellenbosch	Summer '10	2.45 ± 0.13	1.29 ± 0.09	0.95 ± 0.08	1.27 ± 0.07	*	48.18
	Autum '10	2.77 ± 1.27	1.48 ± 0.26	1.17 ± 0.13	1.34 ± 0.13		51.74
	Winter '10	0.35 ± 0.04	0.28 ± 0.01	0.16 ± 0.01	0.17 ± 0.00		51.36
	Spring '10	3.17 ± 0.26	2.76 ± 0.30	2.31 ± 0.90	1.76 ± 0.39		44.66
	α_{season}		*	*		*	
Zandvliet	Summer '10	1.69 ± 0.36	NPST	1.66 ± 0.25	0.55 ± 0.07	*	67.59
	Autum '10	0.89 ± 0.09	NPST	0.52 ± 0.07	0.44 ± 0.02		50.75
	Winter '10	0.34 ± 0.02	NPST	0.22 ± 0.07	0.17 ± 0.0		51.36
	Spring '10	3.23 ± 1.4	NPST	2.91 ± 0.4	1.86 ± 1.67		42.30
	α_{season}		*				

$\alpha_{concentration}$ denotes the significance of difference between the stages of WWTPs; α_{season} denotes the significance of difference of seasonal differences; *: difference is significant at $\alpha = 0.05$; NA = not analysed; NPST = no primary settling tank.

3.23 μgL^{-1}, respectively (Table 4). The final effluent concentration ranged from 0.17 to 176 μgL^{-1} for Stellenbosch and 0.17 to 1.86 μgL^{-1} for Zandvliet. The annual distribution spread for the two plants are presented in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APryCo
ASecCr
AFECo
Min Outlier
Max Outlier
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Concentration (µgL-1)
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BOPryCo
BOSecCr
BOFECo
Min Outlier
Max Outlier
c
Concentration (µgL-1)
BNRCo
BNSecCr
BNFECr
Min Outlier
Max Outlier
d
Concentration (µgL-1)
KRCo
KPryCo
KSecCr
KFECo
Min Outlier
Max Outlier
e
PRCo
PPryCo
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Concentration (µgL-1)
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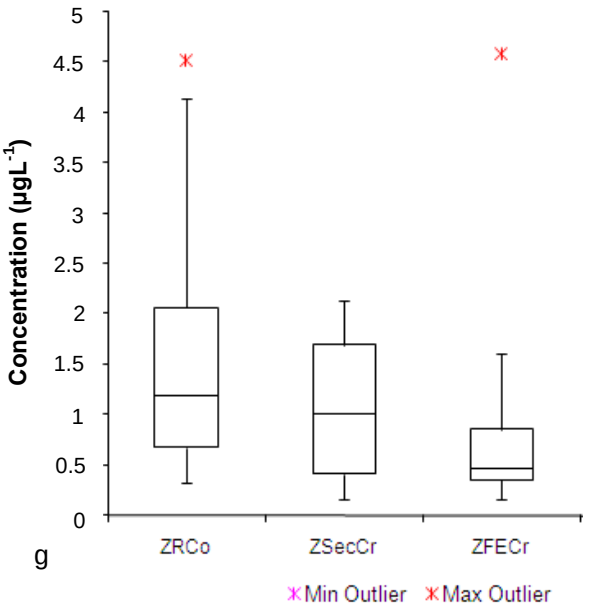


Figure 4. Box and whisker plot for annual Co concentration in WWTPs. a) Athlone b) Bellville old c) Bellville new d) Kraaifontein e) Potsdam f) Stellenbosch g) Zandvliet.

Figure 4f and g. The annual mean removal efficiency for the plants was 48.99% for Stellenbosch and 53.08% for settling tank of Stellenbosch, while 13.69 and 13.63% were removed at the secondary tanks of Stellenbosch and Zandvliet, respectively. Significant difference due to season and treatment process was observed for the plant over the study period.

Chromium

Chromium concentration in the influent waste into Athlone plant varied between 116.96 and 199.53 μgL^{-1} with an annual mean concentration of 144.82 μgL^{-1} (Table 5). The final effluent concentration ranged between 25.15 and 132.61 μgl^{-1} with an annual effluent mean concentration of 86.32 μgL^{-1}. The annual distribution pattern of chromium in the plant revealed that 37.04 and 19.62% were removed at the primary and secondary settling tanks (Figure 5a). The annual mean removal efficiency of the treatment plant fell below 40% (Figure 2). Significant difference due to treatment process was noticeable during summer season. The influent concentration of chromium into the Bellville old plant varied between 108.36 and 207.68 μgL^{-1}, while the new plant received between 106.4 and 159.61 μgL^{-1}. The effluent concentration ranged between 26.18 to 135.8 μgL^{-1} for old plant and from 30.25 to 153.44 μgL^{-1} for the new plant (Table 5). The distribution pattern (Figure 5b and c) showed that chromium was poorly removed from the waste effluent from the two plants as less than 26 and Zandvliet (Figure 2). The annual distribution of Co revealed that 33% was removed at the primary 40% were removed from old and new plants, respectively (Figure 2). There was a significant difference ($P<0.05$) during autumn due to plant treatment processes. The total influent concentration into the Kraaifontein plant ranged from 31.15 to 154.62 μgL^{-1} with an annual mean of 111.01 μgL^{-1} and the final effluent concentration varied between 26.14 and 130.5 μgl^{-1} with an annual mean effluent of 96.03 μgL^{-1} (Table 5). The percentage removal of Cr was below 20% over the study. The distribution pattern of total Cr in the plant presented in Figure 5d shows that 13.9% and about 4% was removed through primary and secondary settling tanks. The annual mean efficiency of the plant is presented in Figure 5. There was significant difference in the influent concentration due to seasonal change and plant treatment process.

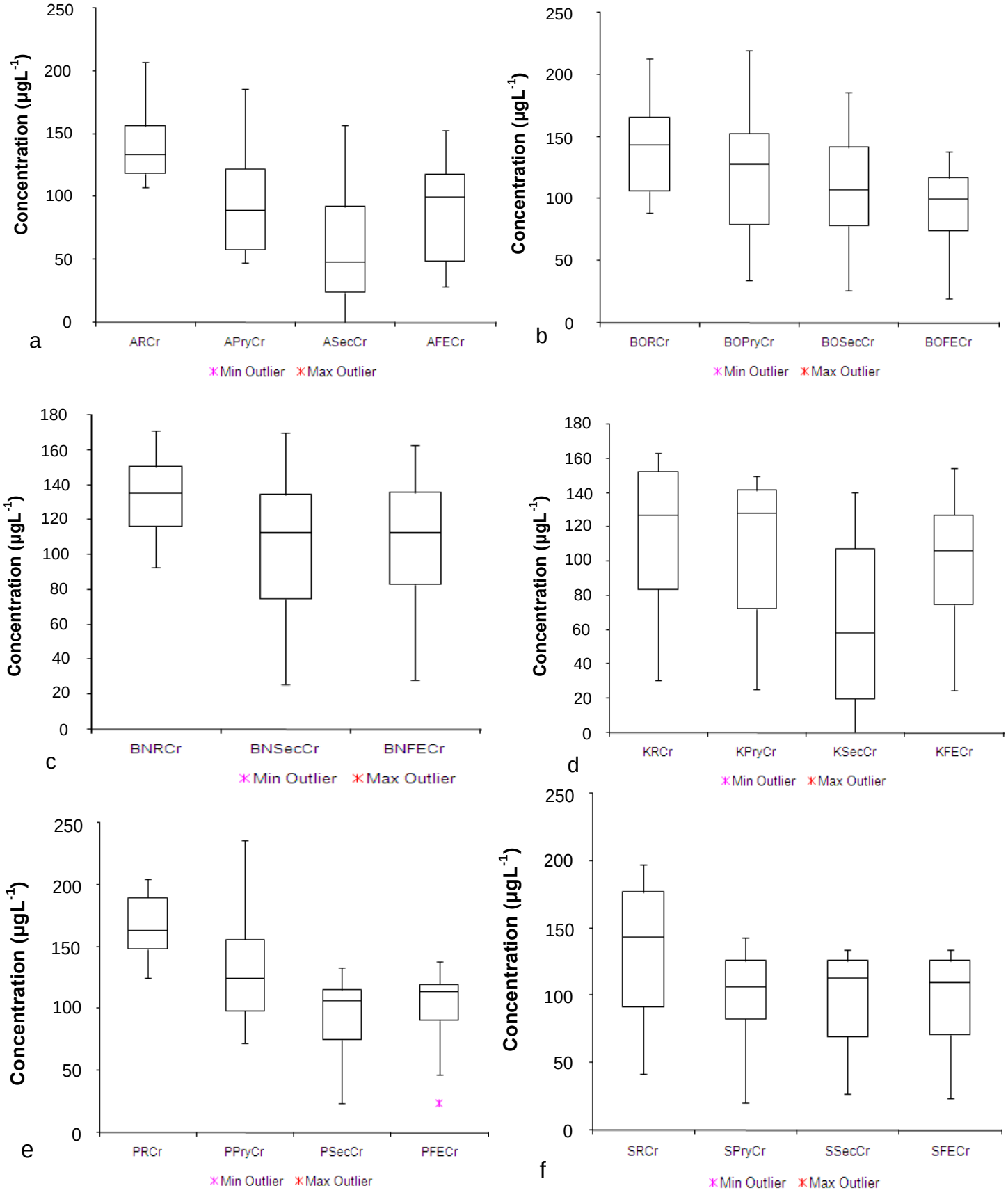
The Potsdam treatment plant received a concentration range of 146.94 to 223 μgL^{-1} in the influent and concentration in the effluent released ranged from 25.47 to 127.78 μgL^{-1} (Table 5). The annual mean concentration in the influent was 174.67 μgL^{-1}, while the annual mean concentration of chromium in the final effluent was 95.79 μgL^{-1}. The distribution pattern in the plant is presented in Figure 5e. The plant distribution pattern for chromium showed that about 29 and 25% of chromium in wastewater was removed at the primary and secondary settling tanks, respectively. The annual mean removal efficiency of the plant is presented in Figure 2. There was significant difference due to treatment process during summer season between influent and effluent

Table 5. Mean concentration (±SD) of Cr in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgl^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	135.8 ± 3.6	57.8 ± 2.4	35.9 ± 3.56	29.2 ± 1.2	*	78.54
	Autum '10	126.9 ± 14.7	56.5 ± 15.4	65.71± 9.3	80.3 ± 36.9		36.80
	Winter '10	199.5 ± 7.2	137 ± 30.0	149.3 ± 7.5	132.6 ± 24.0		33.54
	Spring '10	116.9 ± 15.1	113.8 ± 7.8	N/A	108.2 ± 12.8		7.48
	α_{season}						
Bellville old	Summer '10	207.68 ± 5.9	190.6 ± 26.1	161.3 ± 21.5	95.8 ± 5.3		53.89
	Autum '10	108.5 ± 9.1	39.6 ± 4.4	35.47 ± 10.7	26.2 ± 5.5	*	75.87
	Winter '10	152.1 ± 1.6	146.5 ± 1.3	132.0 ± 9.8	131.9 ± 9.3		42.86
	Spring '10	108.36 ± 24.5	106.4 ± 9.2	100.85 ± 2.4	112.8 ± 1.7		15.70
	α_{season}						
Bellville new	Summer '10	159.6 ± 12.7	NPST	157.9 ± 11.8	153.4 ± 8.0		3.86
	Autum '10	134.2 ± 17.2	NPST	29.09 ± 2.6	30.25 ± 1.9	*	77.47
	Winter '10	138.99 ± 13.0	NPST	127.1 ± 3.2	123.3 ± 11.7		11.33
	Spring '10	106.4 ± 4.3	NPST	97.6 ± 6.4	96.6 ± 8.3		9.19
	α_{season}		*				
Kraaifontein	Summer '10	154.62 ± 8.1	144.2± 7.7	N/A	135.8 ± 17.5	*	12.17
	Autum '10	31.15 ± 0.9	28.04± 2.4	26.94 ± 0.2	26.14 ± 0.9		16.06
	Winter '10	154.1 ± 3.9	141.6± 1.9	134.0 ± 5.5	130.5 ± 9.5		15.36
	Spring '10	104.2 ± 2.9	100.2± 18.4	95.72 ± 5.6	91.73 ±1.4		11.95
	α_{season}		*	*		*	*
Potsdam	Summer '10	177.32 ±16.3	114.54 ± 9.72	99.9 ± 9.9	99.9 ± 9.9	*	33.96
	Autum '10	150.81± 4.3	77.76 ± 4.9	26.31 ± 3.2	25.47 ± 1.9		83.11
	Winter '10	223.62 ± 8.2	198.85 ± 20.6	129.5 ± 5.8	127.8 ± 9.0		42.86
	Spring '10	146.94 ± 21.3	133.32 ± 7.9	108.5 ± 3.7	112.8 ± 1.7		23.22
	α_{season}						
Stellenbosch	Summer '10	172.40 ± 11.2	120.7 ± 2.9	116.36 ± 7.7	126.52 ± 2.50	*	26.6
	Autum '10	44.58 ± 3.7	35.9 ± 16.5	30.5 ± 5.1	28 ± 4.4	*	36.5
	Winter '10	187.89 ± 12.1	138.9 ± 3.6	132.4 ± 1.61	126.6 ± 6.9		32.6
	Spring '10	116.5 ± 9.1	93.7 ± 1.7[9]	98.3 ± 23.1	89.1 ± 9.6		23.5
	α_{season}		*	*		*	*
Zandvliet	Summer '10	98.4 ± 7.8	NPST	68.0 ± 7.6	24.6 ± 3.3	*	74.92
	Autum '10	87.24 ± 3.9	NPST	23.9 ± 0.5	23.8 ± 0.5		72.72
	Winter '10	136.62 ± 2.6	NPST	133.5 ± 2.7	130.86 ± 3.9		4.22
	Spring '10	105.5 ± 21.6	NPST	98.97 ± 5.1	89.1 ± 9.6		20.13
	α_{season}					*	

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$; NA, not analysed; NPST, no primary settling tank.

concentration. The influent chromium concentrations at the Stellenbosch treatment plant varied between 44.58 and 187.89 μgL^{-1}, while the final effluent concentration ranged from 28.31 to 126.63 μgL^{-1} (Table 5). The annual spread for chromium is presented in Figure 5f while the annual mean percentage removal efficiency of the plant was below 40%. Based on this efficiency, the plant could be rated ineffective in chromium removal. Though the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0
ARCr
APryCr
ASecCr
AFECr
Min Outlier
Max Outlier
b
Concentration (µgL-1)
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200
150
100
50
0
BORCr
BOPryCr
BOSecCr
BOFECr
Min Outlier
Max Outlier
c
Concentration (µgL-1)
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160
140
120
100
80
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20
0
BNRCr
BNSecCr
BNFECr
Min Outlier
Max Outlier
d
Concentration (µgL-1)
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100
80
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KSecCr
KFECr
Min Outlier
Max Outlier
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Concentration (µgL-1)
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PRCr
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PSecCr
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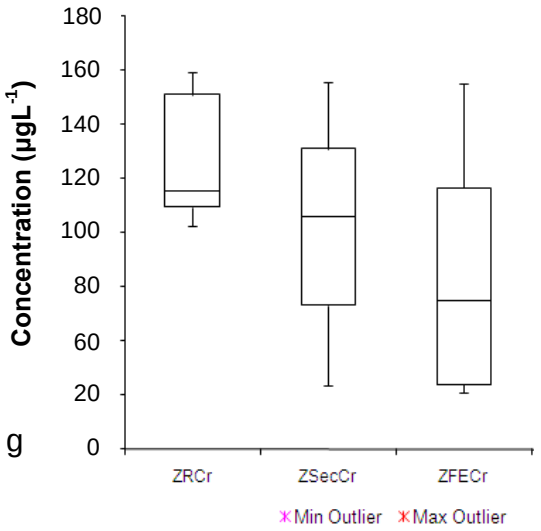


Figure 5. Box and whisker plot for annual Cr concentration in WWTPs: a, Athlone; b, Bellville old WWTP; c, Bellville new; d, Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet.

trapped in the secondary sludge. This was not the case for the new plant as it operates on UCT. 48.41% of the total copper was retained in the secondary sludge, while the balance was assumed to be present in the re-circulated activated sludge. The annual mean percentage removal efficiencies of the plants were 70.02 and 68.10% for old and new plants, respectively (Figure 2). With the exception of the spring season in the old plant, there was significant difference in the influent concentration and in concentration due to plant treatment process for all the seasons under study for the two plants.

Concentration of copper in wastewater received at the Kraaifontein varied between 96.56 and 135.76 μgL^{-1}, while the final effluent concentration ranged from 7.66 to 25.16 μgL^{-1} (Table 6). The annual distribution for copper in the plant is depicted in Figure 6d. The annual distribution pattern shows that 45.36% of the total copper concentration was removed at the primary sedimentation tank, while 35.15% was removed at the secondary settling tank which is often returned to the activated sludge. The annual mean removal efficiency was 83.28% (Figure 2). No significant difference in influent concentration was observed due to seasonal change but there was significant difference due to plant treatment process on influent concentration over the seasons. The Potsdam wastewater treatment plant exhibited significant difference in both the influent copper concentration (seasonal) and due to plant treatment process on the influent concentration. The plant copper concentration was the second highest after the Athlone plant (the second largest plant in Cape Town). The annual mean influent concentration was 484.04 μgL^{-1} while the annual mean final effluent was 30.25 μgL^{-1}. The seasonal variation is shown in Table 6 and the annual distribution in the plant is presented in Figure 6e. The plant distribution pattern showed that 50.19% of total influent concentration was removed at the primary settling tank while about 45% was removed at the secondary settling tank into their respective sludge. The annual removal efficiency was 87.99% (Figure 2).

Stellenbosch and Zandvliet plants received annual mean concentration of 221.57 and 54.33 μgL^{-1}, respectively. The seasonal influent and effluent concentrations in the two plants are presented in Table 6. The annual mean removal efficiencies were 91 and 55.26% for Stellenbosch and Zandvliet, respectively (Figure 6). The annual distribution pattern in the plants (Figure 6f and g) shows that 46.3 and 40.66% of total copper concentration was removed at the Stellenbosch primary and secondary sedimentation tanks while 58.1% was taken at the Zandvliet secondary tank. For the two plants, no significant difference due to seasonal change was noticeable; however, for Stellenbosch plant, there was significant difference between the influent and effluent concentration due to treatment process. Significant difference in Zandvliet was during summer and winter sampling seasons.

Lead

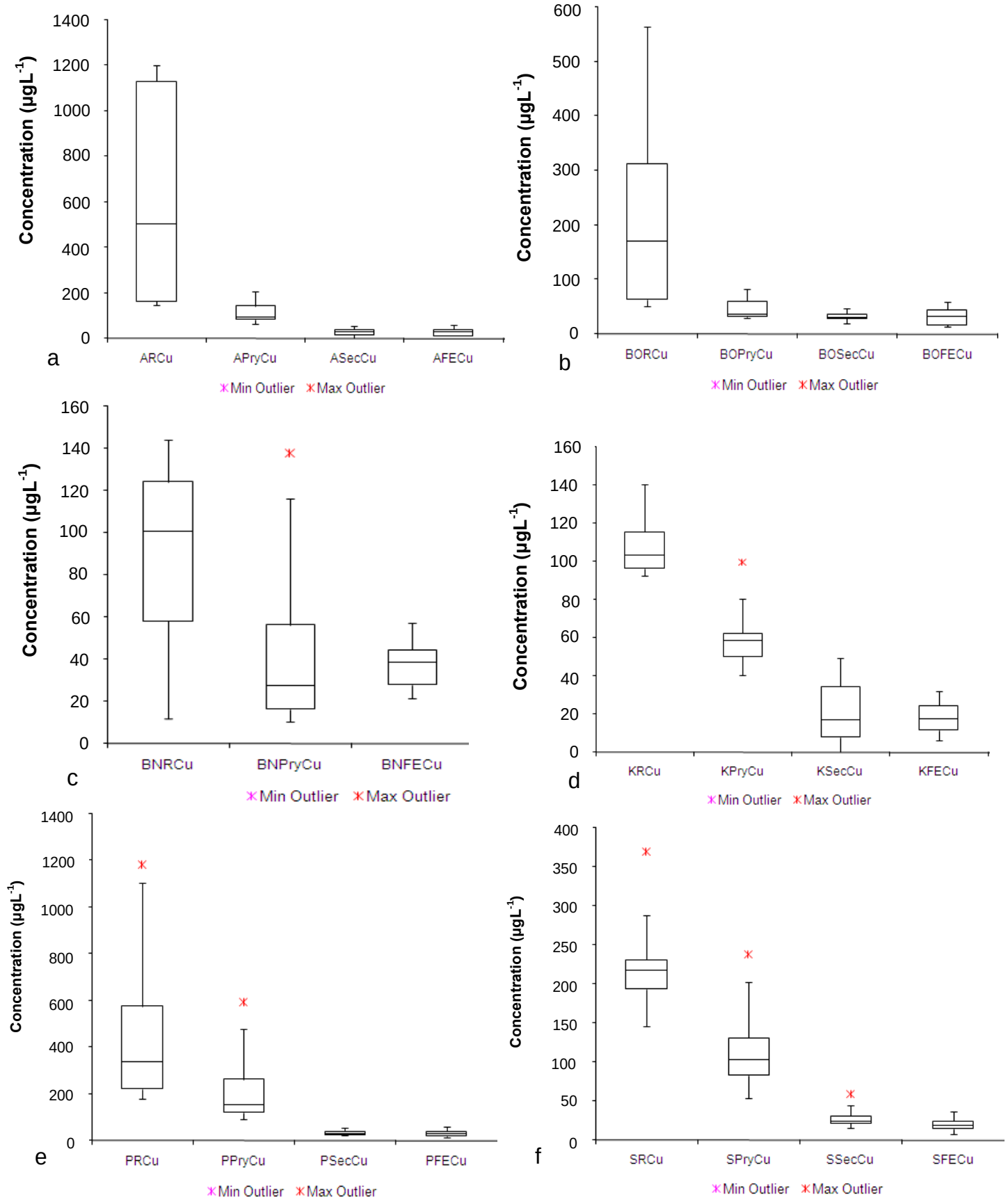
The Athlone lead influent concentration ranged from 49.61 to 81.89 μgL^{-1} and 20.40 to 30.31 μgL^{-1} in the final effluent (Table 7). Lead removal in the influent waste was above average for all the seasons except for the spring. Influent annual mean was 64.46% while annual mean in final effluent was 26.57 μgL^{-1}. The annual distribution pattern of Pb in the plant is presented in Figure 7a.

Table 6. Mean concentration (±SD) of Cu in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgL^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	1189.3 ± 11.1	195.7 ± 9.5	49.3 ± 6.5	20.6 ± 9.5	*	98.27
	Autum '10	986.9 ± 149.4	108.3 ± 18.0	24.61 ± 2.8	48.1 ± 9.9	*	95.12
	Winter '10	154.34 ± 10.6	81.15 ± 17.9	36.2 ± 1.9	12.86 ± 0.9		91.67
	Spring '10	172.4 ± 15.5	81.15 ± 17.9	N/A	40.9 ± 15.1	*	76.29
	α_{season}	*	*				
Bellville old	Summer '10	267.5 ± 12.3	76.6 ± 6.0	32.8 ± 3.8	55.4 ± 3.6	*	79.29
	Autum '10	464.9 ± 86.1	31.6 ± 1.5	29.5 ± 8.5	16.9 ± 0.9	*	96.36
	Winter '10	71.1 ± 10.5	43.6 ± 12.3	41.6 ± 8.7	22.3 ± 7.3	*	68.68
	Spring '10	55.74 ± 6.7	38.1 ± 10.3	28.7 ± 5.3	35.8 ± 2.9		35.77
	α_{season}	*					
Bellville new	Summer '10	139.26 ± 7.9	NPST	125.9 ± 11.5	53.9 ± 5.2	*	61.2
	Autum '10	112.7 ± 10.3	NPST	31.9 ± 6.1	30.8 ± 6.3	*	72.6
	Winter '10	87.2 ± 15.0	NPST	28.7 ± 9.1	28.7 ± 9.1	*	67.1
	Spring '10	40.5 ± 5.1	NPST	15.7 ± 1.9	11.6 ± 2.9	*	71.5
	α_{season}	*					
Kraaifontein	Summer '10	104.4 ± 6.6	61.1 ± 1.7	N/A	17.3 ± 0.3	*	83.4
	Autum '10	98.3 ± 6.3	73.3 ± 22.9	40.5 ± 8.3	25.16 ± 4.2	*	74.4
	Winter '10	135.76 ± 4.2	56.8 ± 8.5	31.5 ± 13.6	22.8 ± 9.9	*	83.2
	Spring '10	96.56 ± 4.5	46.7 ± 5.6	12.8 ± 2.7	7.66 ± 1.9	*	92.1
	α_{season}						
Potsdam	Summer '10	228.7 ± 11.6	94.6 ± 2.4	22.1 ± 1.0	17.3 ± 4.8	*	92.4
	Autum '10	1181.9 ± 5.2	567.4 ± 37.5	37.5 ± 11.1	23.0 ± 8.4	*	98.1
	Winter '10	367.9 ± 11.9	162.7 ± 11.9	34.5 ±12.8	34.9 ± 12.9	*	90.5
	Spring '10	157.6 ± 75.6	139.7 ± 12.6	41.5 ± 10.8	45.7 ± 10.7	*	70.9
	α_{season}	*	*				
Stellenbosch	Summer '10	190.0 ± 8.3	101 ± 4.3	25.2 ± 2.9	18.1 ± 0.9	*	90.5
	Autum '10	244.9 ± 114.1	54.9 ± 1.4	49.0 ± 10.3	33.6 ± 3.8	*	86.3
	Winter '10	236.7 ± 17.4	218.6 ± 20.2	19.3 ± 5.0	20.6 ± 3.7	*	91.3
	Spring '10	214.7 ± 17.6	101.9 ± 8.6	22.0 ± 1.9	8.7 ± 0.3	*	95.9
	α_{season}						
Zandvliet	Summer '10	95.3 ± 4.3	NPST	13.1 ± 0.7	12.7 ± 1.9	*	86.71
	Autum '10	38.1 ± 3.9	NPST	34.1 ± 3.7	32.1 ± 1.6		15.83
	Winter '10	62.9 ± 3.2	NPST	24.3 ± 9.1	15.9 ± 7.1	*	74.76
	Spring '10	21.0 ± 5.7	NPST	19.6 ± 2.0	14.4 ± 1.0		31.75
	α_{season}						

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$; NA, not analysed; NPST, no primary settling tank.

47.07% of total Pb conecntration was removed at the primary settling tank into the primary sludge, while about 22% was trapped into the secondary sludge through the secondary sedimentation tank. The annual mean removal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ARCu
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ASecCu
AFECu
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Concentration (µgL-1)
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BORCu
BOPryCu
BOSecCu
BOFECu
Min Outlier
Max Outlier
b
Concentration (µgL-1)
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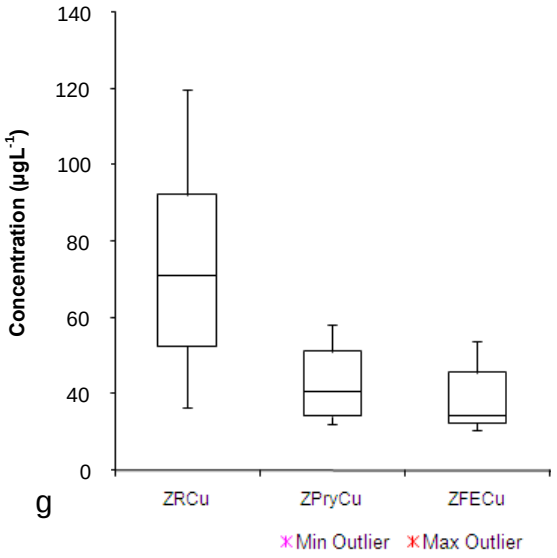


Figure 6. Box and whisker plot for annual Cu concentration in WWTPs. A, Athlone; b, Bellville old; c, Bellville new; d, Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet WWTP.

efficiency of the plant was 57.19% (Figure 2). There was no significant difference due to influent concentration over the study period, but there was significant difference during the summer sampling season due to plant treatment process between the influent and effluent concentration. The lead concentrations into old Bellville plant ranged between 29.88 and 138.35 μgL^{-1} with annual mean of 86.32 μgL^{-1}, while the effluent concentration varied between 15.03 to 37.29 μgL^{-1} with annual mean of 22.84 μgL^{-1} (Table 7). The seasonal removal efficiency ranged from 36.45 to 84.23%. The annual spread of the Pb revealed that 58% of total annual concentration was removed at the primary settling tank and about 20% was removed at the secondary sedimentation tank. The annual removal efficiency of the plant was 67.30% (Figure 2). For the new plant, influent concentration ranged from 23.66 to 282.59 μgL^{-1} with annual mean of 102.20 μgL^{-1} (Table 7). The final effluent concentration ranged between 18.99 and 20.94 μgL^{-1}, while about 77.29% of total influx was trapped into the secondary sludge (Figure 7). The annual mean removal efficiency of the plant was 57% (Figure 2). There was seasonal significant difference due to influent concentration into the two plants. However, significant difference was only observed during summer for old plant and during summer and winter for the new plant due to the plants treatment processes.

Concentrations of Pb into Kraaifontein ranged from 31.5 to 78.6 μgL^{-1} with a final effluent concentration range of 9.49 to 38.66 μgL^{-1} (Table 7). The annual distribution pattern of Pb in the plant is presented in Figure 7d. 42.69% of Pb was removed through the primary sludge, while 23.45% was eliminated in the waste stream through sludge re-circulation. The annual plant removal efficiency was 55% (Figure 2). There was significant difference during the summer due to plant treatment process. There was no significant difference in the influent concentration but there was significant difference between the influent and effluent concentration due to plant treatment processes. The Potsdam WWTP received lead concentration in the range of 36.5 to 77.2 μgL^{-1} and released a concentration range of 7.5 to 27.4 μgL^{-1} (Table 7). The annual mean of influent concentration was 60.58 μgL^{-1}, while the annual mean final effluent concentration was 19.27 μgL^{-1}. The annual distribution in the plant shows that 42.69 and 23.13% of total annual influx was removed at the primary and secondary settling tanks, respectively. There was no significant difference in the influent concentration but there was significant difference between the influent and effluent concentration due to plant treatment processes.

The annual mean concentration into the Stellenbosch and Zandvliet plants was 64.88 and 34.93 μgL^{-1}, respectively. The annual spread pattern for Stellenbosch and Zandvliet (Figure 7) showed that 34.28 and 22.30% of Pb was removed at the primary and secondary tanks of Stellenbosch while 25.02% was trapped into secondary sludge and the un-trapped was retuned in the re-circulated sludge. The annual percentage removals of Pb at the plants were 57.03 and 47% for Stellenbosch and Zandvliet plant (Figure 2). There was significant difference in the influent concentration at Stellenbosch plant but there was no significant difference for Zandvliet over the study period. The two plants had significant difference between the influent and effluent concentration due to plant treatment processes during the summer

Table 7. Mean concentration (±SD) of Pb in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgL^{-1}) with associated total removal efficiency.

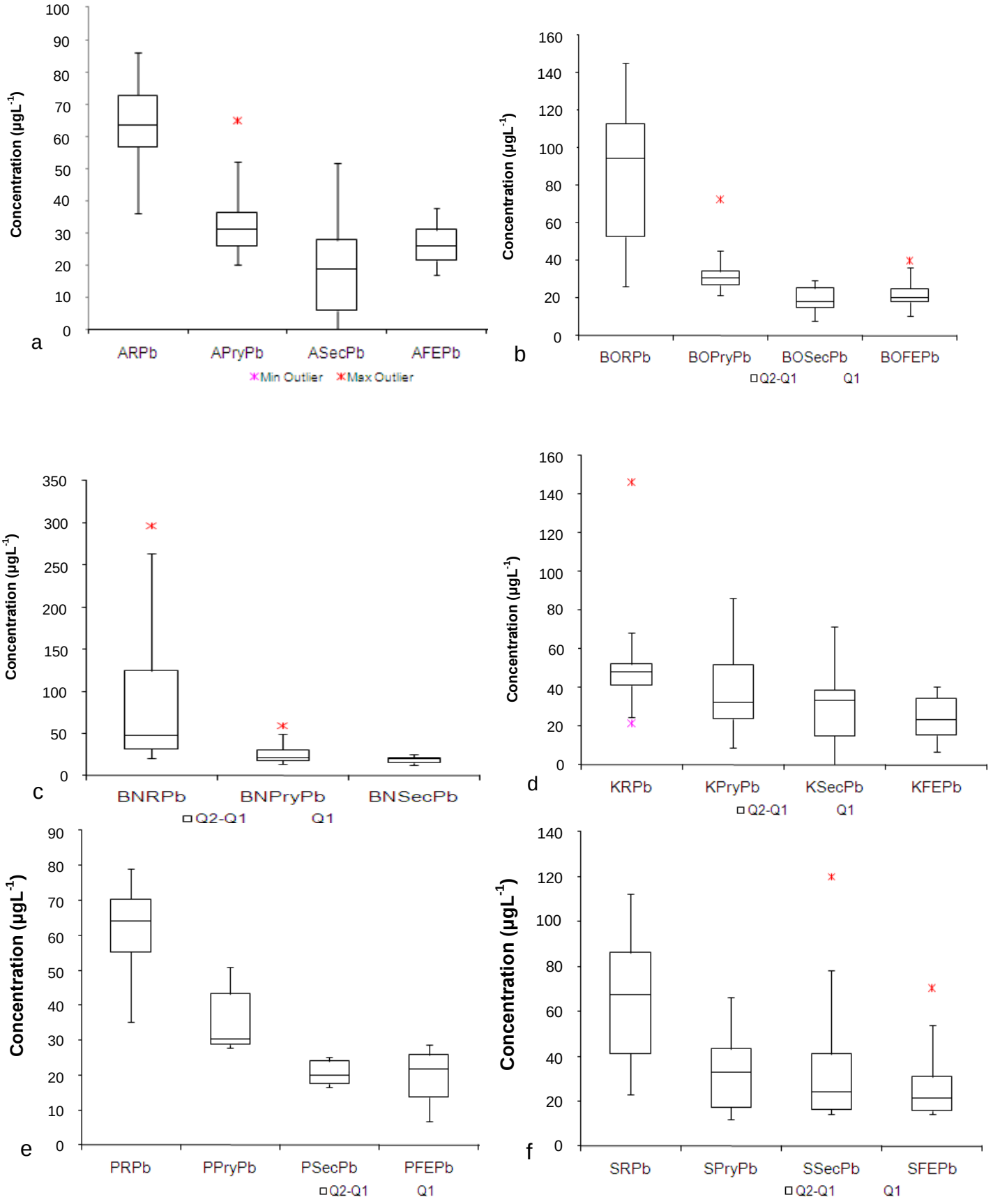
WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	81.89 ± 4.2	22.7 ± 3.7	10.3 ± 4.0	28.9 ± 5.9	*	64.75
	Autum '10	61.5 ± 8.8	28.9 ± 4.8	23.9 ± 1.1	26.7 ± 9.2		56.58
	Winter '10	64.8 ± 5.4	52.2 ± 15.0	43.2 ± 7.6	20.4 ± 1.4		68.52
	Spring '10	49.6 ± 11.6	32.6 ± 5.9	N/A	30.3 ± 7.2		38.89
	α_{season}						
Bellville old	Summer '10	138.35 ± 5.8	30.0 ± 1.6	14.9 ± 6.7	37.29 ± 2.4	*	73.1
	Autum '10	81.8 ± 23.4	57.9 ± 16.4	21.3 ± 5.1	20.0 ± 1.9		75.5
	Winter '10	95.3 ± 2.2	26.1 ± 5.1	19.2 ± 8.9	15.03 ± 5.0		84.2
	Spring '10	29.88 ± 4.3	27.9 ± 4.3	23.5 ± 7.4	18.9 ± 1.4		36.5
	α_{season}		*				
Bellville new	Summer '10	65.9 ± 12.7	NPST	48.1 ± 13.2	20.7 ± 4.7	*	68.7
	Autum '10	282.59 ± 21.6	NPST	21.4 ± 7.8	20.9 ± 0.2	*	92.6
	Winter '10	36.7 ± 3.2	NPST	12.6 ± 4.6	20.2 ± 0.4		44.9
	Spring '10	23.66 ± 2.7	NPST	18.9 ± 2.3	18.9 ±1.4		19.7
	α_{season}		*				
Kraaifontein	Summer '10	31.5 ± 15.2	12.0 ± 2.6	N/A	9.49 ± 2.5	*	69.8
	Autum '10	78.6 ± 59.0	73.4 ± 15.0	51.1 ± 17.9	38.66 ± 2.9		50.8
	Winter '10	52.8 ± 4.6	36.3 ± 12.3	35.1 ± 3.4	29.2 ± 5.3		44.7
	Spring '10	44.5 ± 3.8	32.8 ± 4.3	25.9 ± 9.3	19.6 ± 2.9		55.9
	α_{season}						
Potsdam	Summer '10	62.9 ± 1.7	28.8 ± 0.5	17.7 ± 1.6	7.5 ± 0.6	*	88.1
	Autum '10	77.2 ± 1.8	46.2 ± 4.3	24.4 ± 0.4	27.4 ± 2.2		64.5
	Winter '10	65.6 ± 2.6	37.8 ± 7.7	21.5 ± 3.9	17.2 ± 2.7		73.8
	Spring '10	36.5 ± 1.2	26.1 ± 0.8	19.2 ± 1.4	24.9 ± 1.5		31.8
	α_{season}						
Stellenbosch	Summer '10	72.5 ± 8.9	13.7 ± 1.7	14.6 ± 0.4	14.3 ± 0.1	*	80.3
	Autum '10	102.9 ± 8.2	83.4 ± 4.5	49.5 ± 32.4	40.8 ± 18.2		60.4
	Winter '10	56.8 ± 15.6	51.5 ± 12.8	29.9 ± 4.8	25.5 ± 4.7		55.2
	Spring '10	27.2 ± 4.8	21.9 ± 5.4	18.7 ± 2.6	18.5 ± 3.6		32.2
	α_{season}		*				
Zandvliet	Summer '10	45.7 ± 2.3	NPST	33.9 ± 6.2	15.4 ± 3.0	*	66.4
	Autum '10	42.7 ± 1.6	NPST	31.3 ± 19.1	25.7 ± 4.9		39.9
	Winter '10	18.3 ± 2.0	NPST	13.9 ± 1.3	11.5 ± 3.9		37.1
	Spring '10	32.9 ± 16.9	NPST	11.5 ± 3.9	17.9 ± 3.3		45.5
	α_{season}						

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$.

season.

Mercury

Mercury concentrations in raw water into Athlone ranged from 2.20 to 3.34 μgL^{-1}, while in final effluent, its concentration ranged from 0.19 to 2.57 μgL^{-1} (Table 8). Percentage removal of mercury in the treatment plant ranged from 23.01 to 91.46%, while the annual mean influent, annual mean effluent and percentage removal


a
Concentration (µgL-1)
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90
80
70
60
50
40
30
20
10
0
ARPb
APryPb
ASecPb
AFEPb
Min Outlier
Max Outlier
b
Concentration (µgL-1)
160
140
120
100
80
60
40
20
0
BORPb
BOPryPb
BOSecPb
BOFEPb
Q2-Q1
Q1
c
Concentration (µgL-1)
350
300
250
200
150
100
50
0
BNRPb
BNPryPb
BNSecPb
Q2-Q1
Q1
d
Concentration (µgL-1)
160
140
120
100
80
60
40
20
0
KRPb
KPryPb
KSecPb
KFEPb
Q2-Q1
Q1
e
Concentration (µgL-1)
90
80
70
60
50
40
30
20
10
0
PRPb
PPryPb
PSecPb
PFEPb
Q2-Q1
Q1
f
Concentration (µgL-1)
140
120
100
80
60
40
20
0
SRPb
SPryPb
SSecPb
SFEPb
Q2-Q1
Q1

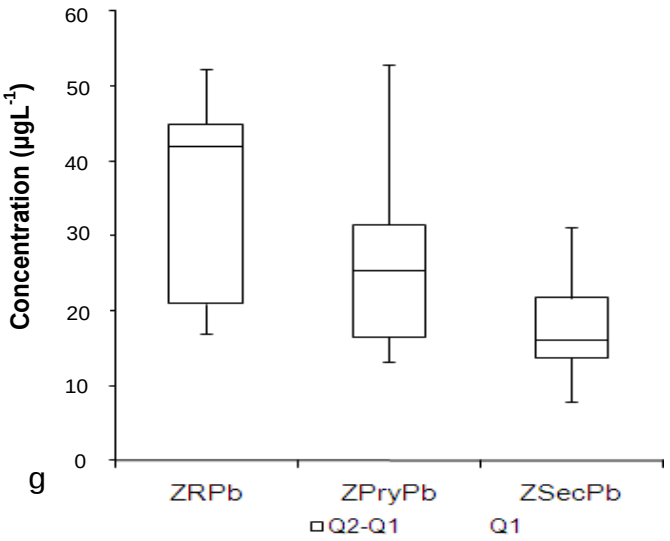


Figure 7. Box and whisker plot for annual spread of Pb concentration in WWTPs. a, Athlone; b, Bellville old; c, Bellville new; d, Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet.

were 2.82 μgL^{-1}, 1.74 μgL^{-1} and 41.93%, respectively. 19.86 and 34.04% of Hg was removed at the plant during the primary and secondary settling tank (Figure 8a) with annual removal efficiency of 41.93% (Figure 2). There was no significant at the plant either due to seasonal difference or due to plant treatment process. Concentration into the Bellville treatment plants ranged from 1.77 to 3.74 μgL^{-1} for the old plant and 0.84 to 2.92 μgL^{-1} for the new plant. The effluent concentration varied between 0.38 and 1.90 μgL^{-1} in old plant and from 0.17 to 2.61 μgL^{-1} in the new plant. The annual mean influent concentration was 2.69 and 2.01 μgL^{-1}, while mean annual effluent concentration was 1.22 and 1.40 μgL^{-1} for the old and new plants, respectively (Table 8). The annual distribution pattern and removal efficiency are presented in Figures 8b and c. 17.91% of the total Hg concentration was removed at the secondary settling tank into secondary sludge in the new plant while 59.65 and 16.72% were trapped into primary and secondary sludge of the old treatment plant. Significant difference observed was due to concentration change arising from the treatment process.

Mercury concentration at the Kraaifontein plant ranged from 0.64 to 4.07 μgL^{-1} with annual mean of 2.22 μgL^{-1} while the final effluent concentration ranged from 0.08 to 3.17 μgL^{-1} with annual mean of 1.80 μgL^{-1} (Table 8). The seasonal removal efficiency of the plant ranged between 20.96 and 88.05%. Annual spread (Figure 8d) shows that 14.65% was removed at the primary settling tank, while about 26% was taken off at the secondary sedimentation tank. The annual mean removal efficiency of the plant was 48.13% (Figure 2). There was significant difference due to seasonal change in influent concentration and due to concentration change from plant treatment process. Potsdam received the highest mercury concentration. The annual mean concentration was 5.53 μgL^{-1} with corresponding effluent concentration of 1.80 μgL^{-1}. The percentage removal varied from 7.87 to 90.04% (Table 8). The plant distribution trend shows that 13.56 and 44.3% was removed at the primary and secondary sedimentation, respectively (Figure 8e). The annual mean removal efficiency was 53.38%. There was significant difference due to seasonal change in influent concentration into the plant. Stellenbosch and Zandvleit received Hg concentration range of 0.64 to 4.26 μgL^{-1} and 0.69 to 3.99 μgL^{-1} respectively (Table 8).

The final effluent in the two plants ranged from 0.25 to 3.59 μgL^{-1} for Stellenbosch and 0.15 to 2.99 μgL^{-1} for Zandvliet, 31.06 and 8.87% of total mercury in the waste influent was removed at the primary and secondary tanks of Stellenbosch, while 22.39% was removed at the secondary tank of Zandvliet plant (Figure 8f and g). The annual removal efficiency of the plant was 51.38% for Stellenbosch and 33.41% for Zandvliet (Figure 2). There was significant difference due to influent concentration and treatment process at Stellenbosch; however, Zandvliet plant showed only significant difference in mercury influent over the study period.

Nickel

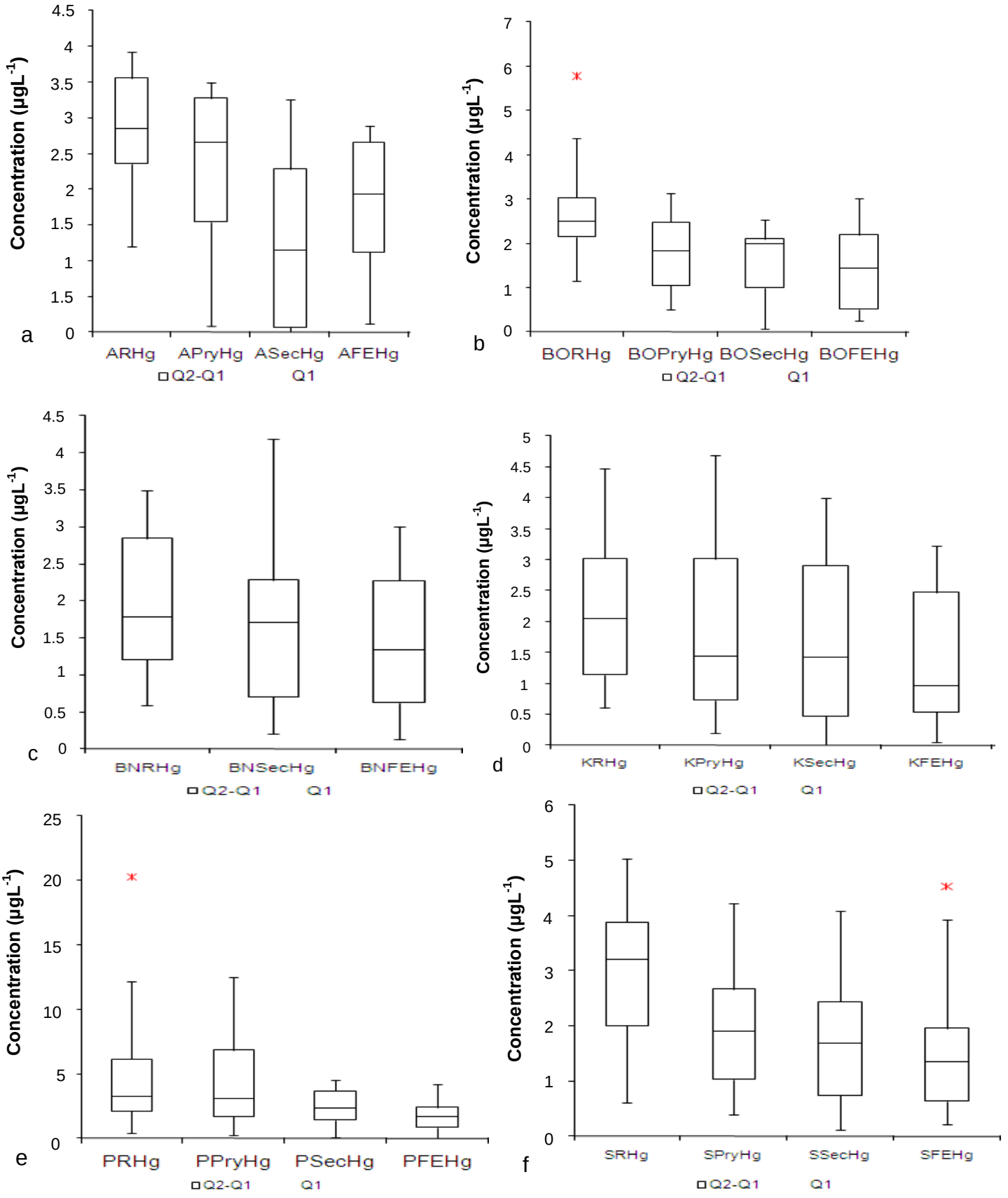
Athlone nickel concentration ranged from 50.32 to 118.72 μgL^{-1} in the raw influent. The effluent concentration varied between 30.42 and 91.68 μgl^{-1} (Table 9). The annual mean influent for the plant was 74.57 μgL^{-1} while the

Table 8. Mean concentration (±SD) of Hg in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgL^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	2.2 ± 1.2	0.4 ± 0.3	0.3 ± 0.2	0.19 ± 0.1		91.5
	Autum '10	3.2 ± 0.7	3.2 ± 0.3	2.9 ± 0.4	2.3 ± 0.8		26.5
	Winter '10	2.6 ± 0.4	2.2 ± 0.4	1.9 ± 0.2	1.9 ± 0.1		26.7
	Spring '10	3.3 ± 0.5	3.3± 0.3	N/A	2.27 ± 0.2		23.0
	α_{season}						
Bellville old	Summer '10	1.77 ± 0.9	0.8 ± 0.3	0.1 ± 0.1	0.38 ± 0.1		78.9
	Autum '10	3.74 ± 2.0	1.4 ± 0.3	1.1 ± 0.1	0.7 ± 0.3		81.5
	Winter '10	2.6 ± 0.2	2.5 ± 0.3	2.2 ± 0.2	1.9 ± 0.1		25.4
	Spring '10	2.7 ± 0.6	2.7 ± 0.6	2.5 ± 0.3	2.2 ± 0.1		29.9
	α_{season}			*			
Bellville new	Summer '10	0.84 ± 0.3	NPST	0.3 ± 0.2	0.17		80.05
	Autum '10	1.5 ± 0.4	NPST	1.4 ± 0.6	0.8		47.8
	Winter '10	2.7 ± 0.3	NPST	2.2 ± 0.3	2.0 ± 0.2		26.3
	Spring '10	2.92 ± 0.9	NPST	2.6 ± 1.4	2.6 ± 0.1		10.6
	α_{season}	*					
Kraaifontein	Summer '10	0.6± 0.03	0.3± 0.1	N/A	0.1± 0.03		88.1
	Autum '10	1.5± 0.2	0.9± 0.1	0.8± 0.2	0.7± 0.1		50.4
	Winter '10	2.7± 0.3	2.4± 0.5	2.2± 0.7	1.8± 0.6		33.1
	Spring '10	4.0± 0.7	3.9± 0.7	3.9± 0.7	3.2± 0.1		20.9
	α_{season}	*	*	*		*	
Potsdam	Summer '10	0.8 ± 0.3	0.3 ± 0.1	0.1 ± 0.04	0.1 ± 0.1		87.3
	Autum '10	14.5 ± 5.1	12.1 ± 0.4	3.1 ± 1.2	1.5 ± 0.5		90.0
	Winter '10	2.6 ± 0.1	2.2 ± 0.1	2.1 ± 0.3	1.8 ± 0.2		30.7
	Spring '10	4.2 ± 0.5	4.4 ± 0.7	4.1 ± 0.6	3.9 ± 0.3		7.9
	α_{season}	*	*	*		*	
Stellenbosch	Summer '10	0.6 ± 0.02	0.5 ± 0.1	0.1 ± 0.03	0.3 ± 0.03		61.3
	Autum '10	3.0 ± 0.7	1.4 ± 0.2	1.4 ± 0.5	1.0 ± 0.5		65.2
	Winter '10	3.8 ± 1.1	2.2 ± 0.1	1.8 ± 0.3	1.4 ± 0.1		63.3
	Spring '10	4.3 ± 0.7	4.0 ± 0.2	3.7 ± 0.3	3.6 ± 0.8		15.7
	α_{season}						
Zandvliet	Summer '10	0.7 ± 0.1	NPST	0.3 ± 0.1	0.2 ± 0.02		77.60
	Autum '10	1.8 ± 0.3	NPST	1.4 ± 0.03	1.3 ± 0.2		29.48
	Winter '10	1.57 ± 0.1	NPST	1.56 ± 0.1	1.55 ± 0.03		1.5
	Spring '10	3.9 ± 1.2	NPST	3.0 ± 0.4	2.9 ± 0.7		25.1
	α_{season}	*		*		*	

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$; NA, not analysed; NPST, no primary settling tank.

annual effluent mean was 55.49 μgL^{-1}. The distribution pattern of nickel in the plant is presented in Figure 9a. 16.54 and 27.17% of total nickel concentration into the plant was removed at the primary and secondary


a
Concentration (µgL-1)
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4
3.5
3
2.5
2
1.5
1
1.5
0
ARHg
APryHg
ASecHg
AFEHg
Q2-Q1
Q1
b
Concentration (µgL-1)
7
6
5
4
3
2
1
0
BORHg
BOPryHg
BOSecHg
BOFEHg
Q2-Q1
Q1
c
Concentration (µgL-1)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
BNRHg
BNSecHg
BNFEHg
Q2-Q1
Q1
d
Concentration (µgL-1)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
KRHg
KPryHg
KSecHg
KFEHg
Q2-Q1
Q1
e
Concentration (µgL-1)
25
20
15
10
5
0
PRHg
PPryHg
PSecHg
PFEHg
Q2-Q1
Q1
f
Concentration (µgL-1)
6
5
4
3
2
1
0
SRHg
SPryHg
SSecHg
SFEHg
Q2-Q1
Q1

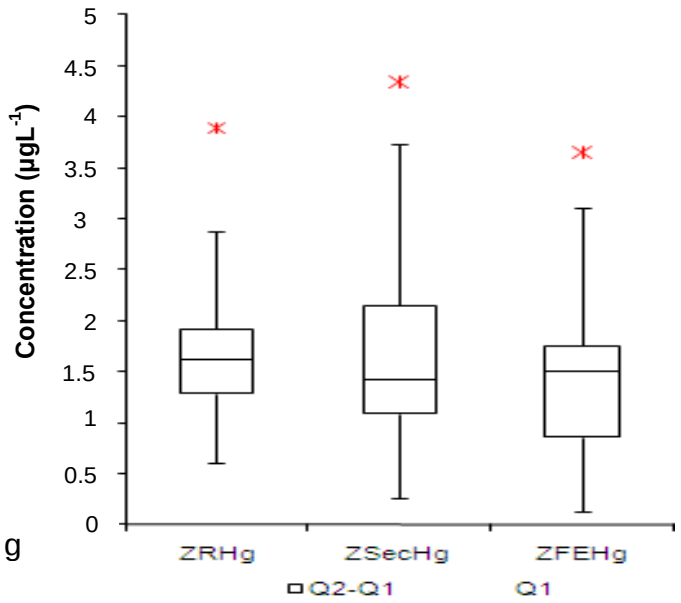


Figure 8. Box and whisker plot for annual Hg concentration in WWTP. a, Athlone; b, Bellville old; c, Bellville new; d, Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet.

sedimentation tanks respectively for the study period. The removal efficiency of the plant ranged from 10.89 to 40.52% with annual mean of 28.43% (Figure 9). Concentration difference was significant due to seasonal influent concentration during winter. The old and new Bellville plants concentrations ranged from 59.59 to 176.76 μgL^{-1} and 42.76 to 95.16 μgL^{-1}, respectively, while their final effluent ranged from 19.21 to 91.91 μgL^{-1} and 25.51 to 85.47 μgL^{-1} (Table 9). The seasonal removal efficiency varied between 7.54 and 67.77% for the old and between 9.35 to 40.35% for the new plant. The annual influent mean was 104.56 and 74.26 μgL^{-1} for the old and for the new plant while the corresponding annual effluent mean concentration was 54.43 and 62.60 μgl^{-1}. The annual distribution spread for nickel is presented in Figure 9b and c. The annual removal efficiency for the plants was 47.72 and 18.69% for old and new plant, respectively (Figure 2). There was significant difference in the old plant due to seasonal and treatment process while in the new plant, significant difference was solely due to seasonal variation in the influent concentration.

The annual mean influent into Kraaifontein plant was 47.59 ± 31.17 μgL^{-1} with corresponding effluent concentration of 37.55 ± 26.51 μgL^{-1} (Table 9). The seasonal removal efficiency of the plant varied between 13.79 and 28.25% (Table 9). The distribution pattern in the plant shows that 11.3 and 35.5% nickel was removed at the primary and secondary tanks. The mean annual removal efficinecy of the plant was 23.64% (Figure 2). There was significant difference due to influent concentration change and concentration change as a result of the treatment process. Postdam received the highest annual mean concentration of 429.01 μgL^{-1} among all the plants investigated. The corresponding annual mean effluent cocnetration was 107.02 μgL^{-1}. The annual removal efficiency was 60.42% (Figure 9). 48.4 and 25.12% of total nickel concentration was removed at the primary and secondary sedimentation tanks over the study period (Figure 2). There was significant difference in the plant due to seasonal and treatment process during summer, winter and spring. Stellenbosch and Zandvliet plant received annual influent concentration of 49.23 and 37.57 μgL^{-1} (Table 9). Zandvliet raw influent cocentration was the least of all the investigated plants. The annual effluent mean were 33.56 and 30.76 μgL^{-1} for Stellenbosch and Zandvliet, respectively. The distribution pattern revealed that 16.47 and 7.58% was removed at the primary and secondary sedimentation tanks of Stellenbosch, while 11.45% was removed at the secondary settling tanks of Zandvliet plant.

The annual mean of plant removal efficiency was 33.25 and 16.39% for Stellenbosch and Zandvliet, respectively (Figure 2). There was significant difference in the plant due to seasonal variation and treatment process.

Zinc

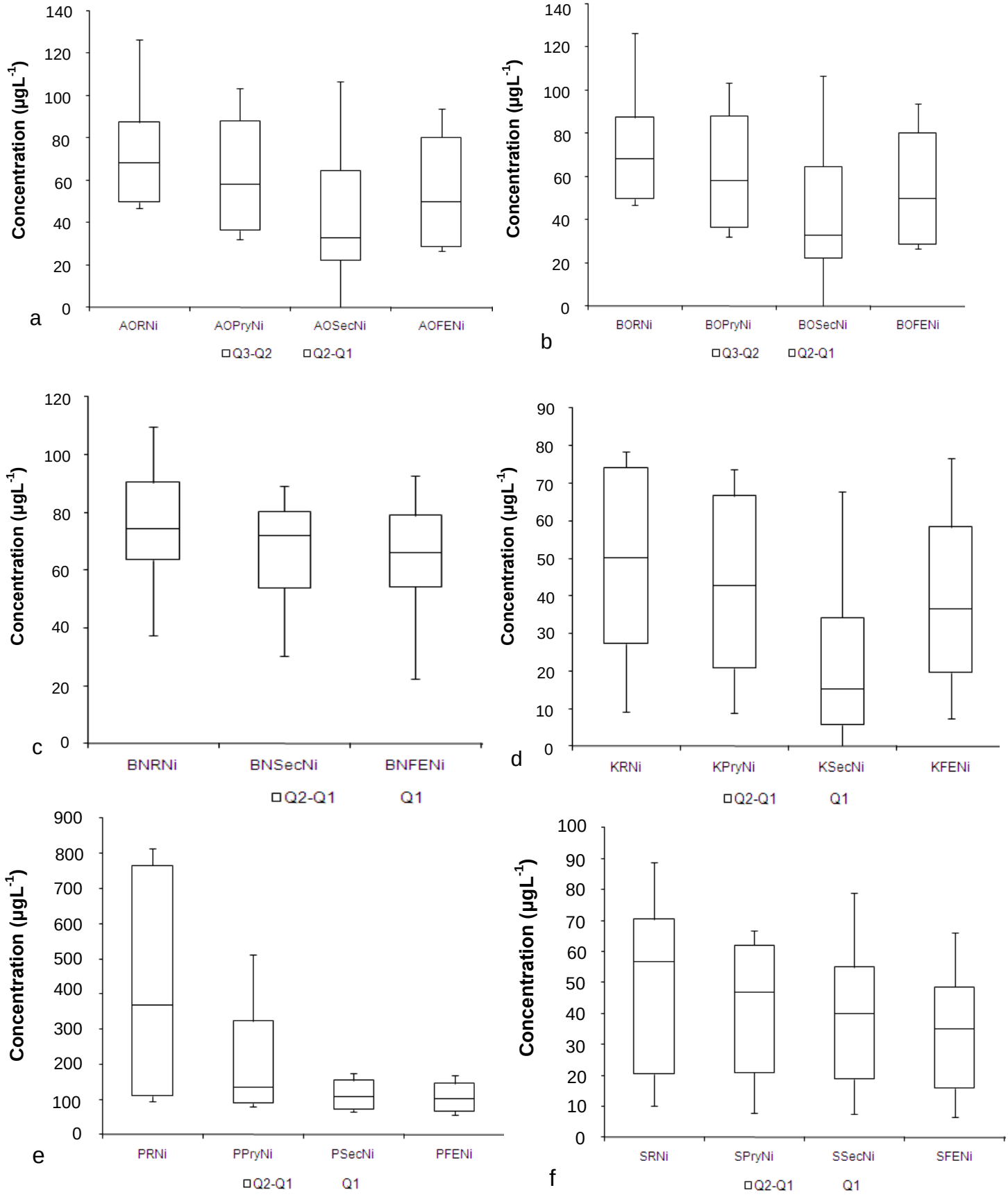
Zinc was generally the highest trace metals in all the WWTPs investigated. The Athlone influent concentration ranged from 961.367 to 1431.95 μgL^{-1} with annual mean of 1236.71 μgL^{-1} (Table 10). The final effluent

Table 9. Mean concentration (±SD) of Ni in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgL^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	50.3 ± 2.9	33.4 ± 1.2	32.8 ± 1.5	30.4 ± 2.5		39.5
	Autum '10	51.6 ± 7.8	41.4 ± 6.7	41.6 ± 17.7	30.7 ± 7.1		40.5
	Winter '10	118.7 ± 8.0	99.9 ± 3.9	93.5 ± 17.6	91.7 ± 2.2		22.8
	Spring '10	77.6 ± 2.2	74.2 ± 10.2	N/A	69.1 ± 7.9		10.9
	α_{season}	*	*		*		
Bellville old	Summer '10	99.5 ± 8.9	87.7 ± 3.0	62.7 ± 2.4	32.3 ± 10.1	*	67.6
	Autum '10	59.6 ± 3.3	29.4 ± 5.4	23.3 ± 5.1	19.2 ± 1.0		67.8
	Winter '10	176.8 ± 17.1	98.8 ± 2.9	97.2 ± 8.4	91.9 ± 6.9	*	48.0
	Spring '10	80.4 ± 19.0	77.0 ± 4.8	68.1 ± 1.7	74.3 ± 11		7.5
	α_{season}	*	*	*	*		
Bellville new	Summer '10	71.8 ± 3.8	NPST	66.9 ± 5.4	65.1 ± 2.0		9.4
	Autum '10	42.8 ± 8.3	NPST	31.0 ± 0.5	25.5 ± 3.4		40.4
	Winter '10	95.2 ± 6.1	NPST	87.1 ± 3.6	85.5 ± 8.3		10.2
	Spring '10	87.3 ± 19.6	NPST	76.2 ± 3.9	74.3 ± 11.4		14.9
	α_{season}	*		*	*		
Kraaifontein	Summer '10	70.2 ± 4.5	61.7 ± 3.8	N/A	52.7 ± 4.9	*	24.9
	Autum '10	33.2 ± 1.5	25.7± 1.8	24.1 ± 1.3	24.1 ± 0.4		27.6
	Winter '10	10.6 ± 2.2	9.8± 0.9	7.7 ± 0.1	7.6 ± 0.1		28.3
	Spring '10	76.3 ± 2.2	71.6± 2.0	65.5 ± 3.6	65.8 ± 9.4		13.8
	α_{season}	*	*	*	*		
Potsdam	Summer '10	116.9 ± 12.2	83.9 ± 6.2	71.7 ± 0.9	67.9 ± 2.4	*	41.9
	Autum '10	102.6 ± 9.9	89.3 ± 0.8	72.1 ± 7.5	61.8 ± 5.4		39.8
	Winter '10	719.8 ± 91.6	490.6 ± 26.6	166.4 ± 9	159.1 ± 7.4	*	77.9
	Spring '10	776.7 ± 35.9	220.0 ± 51.4	142.6 ± 9	139.3 ± 4.1	*	82.1
	α_{season}	*					
Stellenbosch	Summer '10	65.1 ± 3.5	61.5 ± 1.0	45.6 ± 1.4	45.6 ± 1.4	*	30.0
	Autum '10	36.7 ± 14.3	30.1 ± 4.4	25.1 ± 3.6	22.9 ± 3.8		37.4
	Winter '10	10.9 ± 0.8	8.1 ± 0.3	7.5 ± 0.1	7.1 ± 0.5	*	35.2
	Spring '10	84.2 ± 7.7	64.8 ± 12.6	64.5 ± 12.6	58.6 ± 6.6		30.4
	α_{season}	*	*	*	*		
Zandvliet	Summer '10	46.2 ± 0.6	NPST	31.5 ± 3.4	24.4 ± 1.4	*	47.2
	Autum '10	25.0 ± 0.9	NPST	23.9 ± 0.5	22.1 ± 0.6		11.9
	Winter '10	7.9 ± 0.3	NPST	7.8 ± 0.1	7.7 ± 0.5		3.3
	Spring '10	71.1 ± 14.0	NPST	69.9 ± 4.4	68.9 ± 7.6		3.1
	α_{season}	*		*	*		

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$; NA, not analysed; NPST, no primary settling tank.

concentration varied between 222.68 and 298.44 μgL^{-1} with annual mean concentration of 251.47 μgL^{-1}. The seasonal percentage removal of the plant varied from 68.96 to 84.45%. The annual distribution pattern in the


a
Concentration (µgL-1)
140
120
100
80
60
40
20
0
AORNi
AOPryNi
AOSecNi
AOFENi
Q3-Q2
Q2-Q1
b
Concentration (µgL-1)
140
120
100
80
60
40
20
0
BORNi
BOPryNi
BOSecNi
BOFENi
Q3-Q2
Q2-Q1
c
Concentration (µgL-1)
120
100
80
60
40
20
0
BNRNi
BNSecNi
BNFENi
Q2-Q1
Q1
d
Concentration (µgL-1)
90
80
70
60
50
40
30
20
10
0
KRNi
KPryNi
KSecNi
KFENi
Q2-Q1
Q1
e
Concentration (µgL-1)
900
800
700
600
500
400
300
200
100
0
PRNi
PPryNi
PSecNi
PFENi
Q2-Q1
Q1
f
Concentration (µgL-1)
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80
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50
40
30
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10
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SRNi
SPryNi
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SFENi
Q2-Q1
Q1

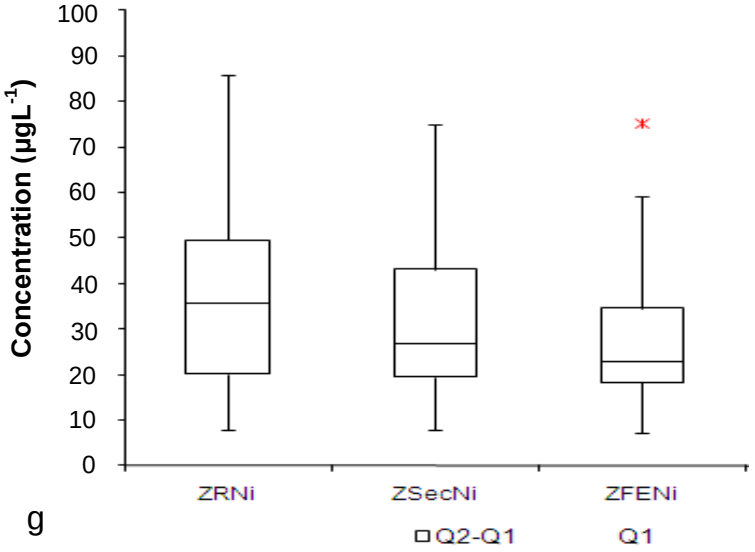


Figure 9. Box and whisker plot for annual Ni concentration in WWTP. a, Athlone; b, Bellville old; c, Bellville new; d; Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet.

plants showed that 44.99 and 31.24% of total annual concentration was removed through primary and secondary sedimentation tanks (Figure 10a). The annual mean removal efficiency of the plant was 78.78% (Figure 2). There was significant difference in the plant due to seasonal variation and treatment process. The old Bellville plant influent concentration ranged from 766.6 to 2079.12 μgL^{-1} while the final effluent varied between 332.53 to 533.77 μgL^{-1} (Table 10). The annual spread is presented in Figure 10b. For the period investigated, 44.67% of Zn concentration into the plant was removed at the primary and 18.51% was taken off at the secondary sedimentation tank. The seasonal removal efficiency ranged from 46.88 to 84.01%, while the annual mean removal efficiency was 65.45% (Figure 2). There was significant difference in the plant due to seasonal variation and treatment process. However, the new Bellville plant influent concentration ranged from 400.94 to 1472.70 μgL^{-1} with annual mean concentration of948.19 μgL^{-1} and the effluent concentration varied from 248.33 to 468.05 μgL^{-1} with annual mean of 351.86 μgL^{-1}. Seasonal plant removal efficiency ranged from 6.69 to 72.40%. 34.16% was trapped into the secondary sludge while the balance was returned in the activated sludge (Figure 2). There was significant difference in the plant due to seasonal variation and treatment process. The influent concentration at Kraaifontein ranged from 638.43 to 1206 μgL^{-1} with annual mean of 933.21 μgL^{-1} and effluent concentration varied from 208.29 to 24.30 μgL^{-1} with annual mean of 222.80 μgL^{-1} (Table 10). The plant shows that 37.59 and 36.89% of Zn influx into the plant was removed at the primary and secondary sedimentation tanks (Figure 10d) for the studied period. The seasonal removal efficiency varied between 67.17 and 82.74% while the annual mean removal efficiency was 74.47% (Figure 2).

Potsdam influent concentration ranged from 822.99 to 1065.72 μgL^{-1} with annual influent mean of 887.14 μgL^{-1} while effluent concentration ranged from 183.79 to 410.82 μgL^{-1} with annual mean of 310.56 μgL^{-1} (Table 10). The distribution of Zn in the plants shows that 21.29 and 39.42% was removed into primary and secondary sludge, respectively. The seasonal removal efficiency ranged from 50.54 to 82.74% with annual mean of 63.74% (Figure 2). There was significant difference in the plant due to seasonal variation and treatment process. Stellenbosch and Zandvliet plants received concentration range of 582.09 to 925.48 μgL^{-1} and 380.19 to 521.8 μgL^{-1}, respectively (Tables 10).

39.57 and 27.14% of total zinc concentration into Stellenbosch plant was removed at the primary and settling tanks while 56.89% was removed at the secondary tank of Zandvliet (Figure 10f and g). Removal efficiency was 70.1% for Stellenbosch and 62.83% for Zandvliet. In the two plants, there was significant difference in the plant due to seasonal variation and treatment process.

Seasonal variability and percentage removal of metals from wastewater treatment plants investigated

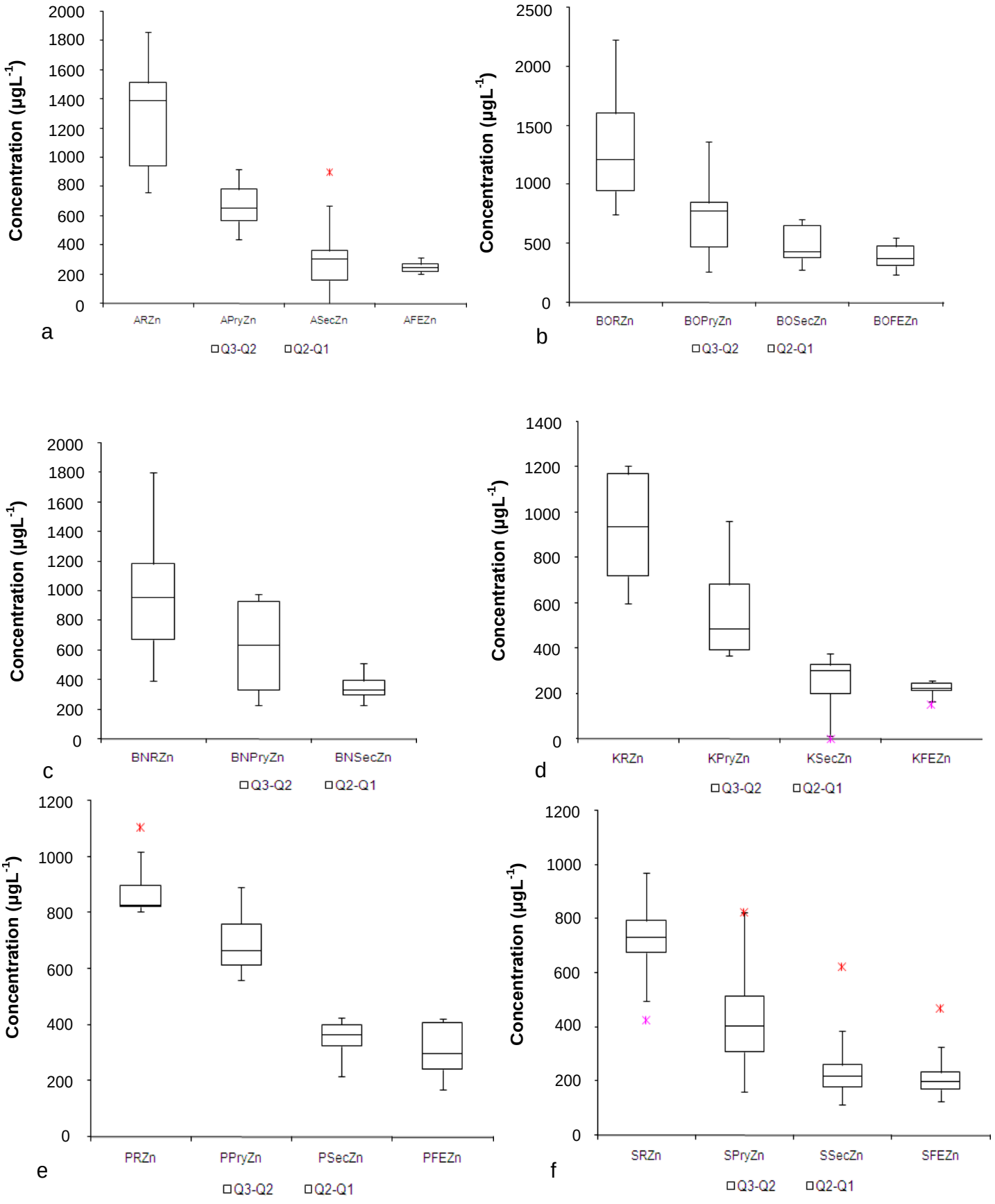
The activated sludge process are generally designed for organic matter removal by microorganisms, while heavy

Table 10. Mean concentration (±SD) of Zn in the influent, primary, secondary and final effluent of WWTPs during the different seasons (μgL^{-1}) with associated total removal efficiency.

WWTP	Season	Concentration (μgL^{-1})				$\alpha_{concentration}$	Removal efficiency
		Influent	Primary effluent	Secondary effluent	Final effluent		
Athlone	Summer '10	1431.9 ± 16.8	912.5 ± 9.6	527.1 ± 324	222.7 ± 22	*	84.5
	Autum '10	1411.9 ± 252.3	517.0 ± 69	303.6 ± 121.8	255.1 ± 14	*	82.2
	Winter '10	1121.6 ± 611.1	644.5 ± 124	344.9 ± 74.6	229.7 ± 18	*	79.5
	Spring '10	961.4 ± 24.9	647.2 ± 12.1	N/A	298.4 ± 13.5	*	68.9
	α_{season}	*					
Bellville old	Summer '10	1004.8 ± 0.8	883.5 ± 124.6	699.6 ± 8.5	533.8 ± 15.2	*	46.9
	Autum '10	2079.1 ± 134.4	832.3 ± 548.8	540.5 ± 115.2	332.5 ± 117	*	84.0
	Winter '10	766.7 ± 17.6	460.9 ± 18.7	295.6 ± 16.9	332.7 ± 36	*	56.61
	Spring '10	1455.5 ± 32.9	758.8 ± 37.1	417.7 ± 25.7	374.1 ± 22	*	74.3
	α_{season}	*					
Bellville new	Summer '10	1148.2 ± 10.7	NPST	932 ± 10.2	316.9±3.6	*	72.4
	Autum '10	1472.7 ± 288	NPST	929 ± 55	468 ± 53.9	*	68.2
	Winter '10	770.9 ± 9.3	NPST	253 ± 22.5	248 ± 16.9	*	67.8
	Spring '10	400.9 ± 5.1[fij]	NPST	383 ± 23.1	374 ± 22.4	*	6.7
	α_{season}	*					
Kraaifontein	Summer '10	1206.9 ±1.2	391.5 ± 4.6	N/A	208.3 ± 17.3	*	82.7
	Autum '10	638.4 ± 47.5	425.0 ± 51	356.5 ± 27.6	209.6 ± 53.6	*	67.2
	Winter '10	756.1 ± 35.9	560.1 ± 47	319.1 ±13.0	232.0 ± 13.3	*	69.3
	Spring '10	1131.5 ± 46.1	952.9 ±12	277.1 ± 10.0	241.3 ± 20.1	*	78.7
	α_{season}	*	*				
Potsdam	Summer '10	822.9 ± 1.5	626.3 ± 10.3	341.3 ± 7.6	260.1 ± 1.6	*	68.4
	Autum '10	1065.7 ± 47.8	846.3 ±45.9	250.1 ± 39.9	183.8 ± 10.8	*	82.8
	Winter '10	829.3 ± 29.2	727.8 ± 29.6	384.2 ± 11.0	387.6 ± 48.4	*	53.3
	Spring '10	830.6 ±13.9	592.8 ± 28.8	418.6 ± 5.9	410.8 ± 10.3	*	50.5
	α_{season}						
Stellenbosch	Summer '10	684.9 ± 16.6	351.2 ± 22.4	128.5 ± 13.9	133.7 ± 13.4	*	80.5
	Autum '10	581.1 ± 167.2	209.7 ± 43.2	247.9 ± 8.4	215.2 ± 2.9	*	62.9
	Winter '10	925.5 ± 39.1	754.2 ± 78.2	402.4 ± 191.8	353.8 ± 100.5	*	61.8
	Spring '10	734.2 ± 3.8	452.9 ± 15.1	195.2 ± 3.2	182.1 ± 4.8	*	75.2
	α_{season}						
Zandvliet	Summer '10	5128.3 ± 10.2	NPST	2119.9 ± 11.8	909.4 ± 23.1	*	82.3
	Autum '10	395.2 ± 19.8	NPST	277.2 ± 18.2	191.8 ± 10.9	*	51.5
	Winter '10	380.2 ± 14.8	NPST	223.9 ± 17.6	190.0 ± 5.2	*	50.0
	Spring '10	965.8 ± 19.8	NPST	340.6 ± 5.2	313.2 ± 1.5	*	67.6
	α_{season}						

$\alpha_{concentration}$, Significant difference between the stages of WWTPs; α_{season}, significant difference of seasonal differences; *, difference is significant at $\alpha = 0.05$; NA, not analysed; NPST, no primary settling tank.

metals removal is considered as side benefit, and has been quite variable (Busetti et al., 2005; Ustun, 2009; Chanpiwat et al., 2010). Metal removal efficiency is not only affected by metal influent concentration, but also by


Concentration (µgL-1)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
ARZn
APryZn
ASecZn
AFEZn
Q3-Q2
Q2-Q1
a
Concentration (µgL-1)
2500
2000
1500
1000
500
0
BORZn
BOPryZn
BOSecZn
BOFEZn
Q3-Q2
Q2-Q1
b
Concentration (µgL-1)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
BNRZn
BNPryZn
BNSecZn
Q3-Q2
Q2-Q1
c
Concentration (µgL-1)
1400
1200
1000
800
600
400
200
0
KRZn
KPryZn
KSecZn
KFEZn
Q3-Q2
Q2-Q1
d
Concentration (µgL-1)
1200
1000
800
600
400
200
0
PRZn
PPryZn
PSecZn
PFEZn
Q3-Q2
Q2-Q1
e
Concentration (µgL-1)
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1000
800
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400
200
0
SRZn
SPryZn
SSecZn
SFEZn
Q3-Q2
Q2-Q1
f

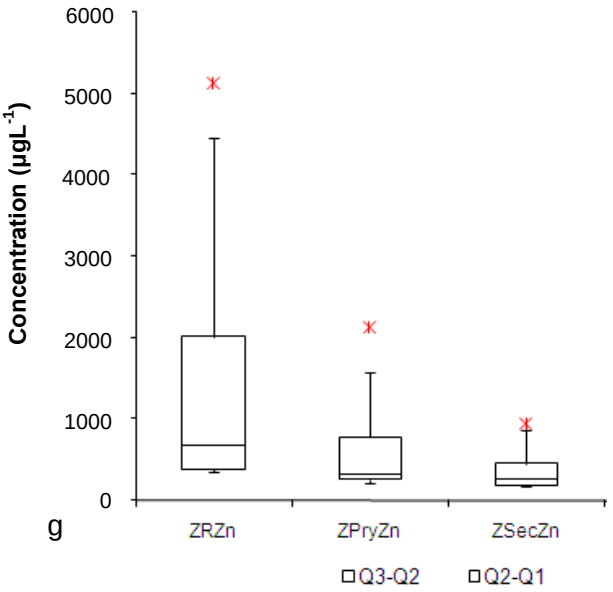

Figure 10. Box and whisker plot for annual Zn concentration in WWTPs. a, Athlone; b, Bellville old; c, Bellville new; d, Kraaifontein; e, Potsdam; f, Stellenbosch; g, Zandvliet.

other conditions such as the operating parameters, for example, the retention time in the treatment plants, flow rate, physical, chemical and biological factors (Wang et al., 1999). Metals removal is known to be dependent on dissolved organic matter (Oliveira et al., 2007) and pH (Cheng et al., 1975; Wang et al., 1999), as removal efficiency increases with pH until they precipitate as hydroxides. Wastewater treatment plants are usually operated at pH 7 to 9. Thus, because of differing metal solubilities at these pH values, retention time, flow rate, and since wastewater composition is always complex, removal is attributed to these factors (Wang et al., 1999). In this study, the pH values for untreated influent and treated effluent ranged from 6.5 to 7.7 at a temperature range of 17 to 19°C. This caused variation in removal efficiency for metals in the WWTPs investigated. Generally, the level of metal removal from the treatment plants remained unpredictable for the period investigated (Figure 2).

The long-term dataset obtained from the WWTPs showed that the investigated WWTPs received varying concentrations of heavy metals in the raw wastewater, of which Cu and Zn were the most abundant. The seasonal variations in the metals analyzed from all the treatment plants are presented in Tables 1 to 9. The results illustrated that the wastewater metals composition is complex and quite variable. The concentrations of heavy metals in the raw wastewater were generally similar in WWTPs under study. This could be attributed to the fact that all the WWTPs received a mixture of domestic wastewater, storm water and industrial effluent. Generally, the abundance distribution pattern of heavy metals in terms of concentration is Zn > Cu > Pb > Cr > Ni > As > Co > Cd > Hg. The variation in wastewater metal content can further be attributed to diversity in economic activities and the living pattern in the province. The Athlone, Potsdam, Bellville, Stellenbosch and Zandvliet plants are known to receive high industrial waste when compared to Kraaifontein (Moeletsi et al., 2004). There are many catering, restaurants, sawmills, Ni-Cd and carwash industries in the Western Cape Province that release their waste for further treatment by the municipality. Generally, the influent values are higher than effluent values. The average removal efficiency for the plants could be rated effective on an annual basis as the effluent values are always lower than the influent values for all the metals in all the measurement. As shown in Figure 2, metals removal occurs both in the primary (where portion of metals adsorb to the particles) and in the secondary biological treatment (where metals are removed by biosorption) (Ustun, 2009). The relationship between influent and removal efficiency agreed with previous research findings (Kulbat et al., 2003; Shomar et al., 2004; Oliveira et al., 2007) where it was observed that the removal of heavy metals in the wastewater is directly proportional to the metal concentration in the influent.

From this study, the Potsdam treatment plant was the most effective at heavy metal removal (Figure 2). On average, the Potsdam treatment plant like every plant, received industrial, domestic and storm water except for Kraaifontein with about 90% domestic influent, the plant effectiveness at metal removal can be attributed to the

living pattern and the installation of new treatment plant at Potsdam to complement the old plant. The annual abundance pattern for arsenic in all treatment plants can be rated as new Bellville > Stellenbosch > old Bellville > Athlone > Kraaifontein ≥ Potsdam > Zandvliet. The removal efficiency for arsenic was best at Potsdam plant for all the seasons except during the spring. Arsenic compounds are extensively used in the wood processing industries to protect the timbers. Two wood processing industries are functioning in the vicinity of the Stellenbosch WWTP. These industries may use arsenic compounds to protect timbers, the uses of which are subsequently released in their waste. Generally, arsenic concentration in effluent from all the treatment plants investigated fell below the South African water quality guideline of 10 μgL^{-1} (DWAF, 1996). However, it was above the CCME (1999) recommendation. All the treatment plants could be rated high except for Athlone during the Autum and Spring due to malfunctioning of the plants. The cadmium annual abundance pattern by plant could be rated as Potsdam > Kraaifontein > Athone ≥ old Bellville > Stellenbosch ≥ Zandvliet ≥ new Bellville. The possible sources of cadmium into water ways are launderettes, electroplating workshops, plastic manufacturing, pigments, enamels, paints among others. Cadmium was well removed from Athlone, Bellville old, Kraaifontein and Potsdam, while the Bellville new and Stellenbosch plants were not very effective.

The influent and effluent concentrations were within the reported values elsewhere (Table 11). No significant difference was observed for raw effluent except for Kraaifontein and Potsdam during the winter season. The reported concentration fell below SWQG of 10 ugl^{-1} limit for irrigation and livestock (DWAF, 1996; CCME, 1999) but higher than 0.017 and 0.02 μgL^{-1} for human consumption (CCME, 1999). The general abundance pattern for cobalt for the treatment plants could be rated as Potsdam > Kraaifontein > Athlone ≥ old Bellville > Stellenbosch ≥ Zandvliet ≥ new Bellville. The removal efficiency is presented in Figure 2. Cobalt was well removed from all the plants in this study except at Potsdam during the spring season (Table 4). Statistical analysis showed no significant difference ($P<0.05$) in the influent concentration during the sample period except for Kraaifontein and Potsdam during the winter season. Cobalt concentrations in the final effluent were within the Department of Water Affairs recommended values for freshwater (DWAF, 1996). In terms of abundance in the WWTPs, all the investigated plants could be rated as Potsdam > Athlone > old Bellville > new Bellville > Stellenbosch > Kraaifontein > Zandvliet. When compared the influent and effluent concentration with other plants in the developed countries, the range fit within the limits was reported (Table 11). However, Kraaifontein treatment plants perfomed poorly at total chromium removal from waste stream as less than 20%. This is similar to the finding in countries like Greece, Brazil and Poland (Kulbat et al., 2003; Firfilionis et al., 2004; Oliveira et al., 2007). No significant difference ($P < 0.05$) was observed between the influent and effluent concentration of the investigated plants.

Copper was the second dominant metal as zinc in the treatment plant investigated. The abundance pattern revealed that Athlone > Potsdam > Stellenbosch > Bellville old > Kraaifontein > Bellville new > Zandvliet. Athlone, Potsdam, Bellville old, and Stellenbosch received the high concentrations of copper in the influent waste; this could be attributed high industrial influx to these treatment plants (Moeletsi et al., 2004). Other possible sources of heavy metals into these plants are the leachate from landfill sites that are often pumped into the plants for treatment especially at Bellville and Stellenbosch. Generally, copper was well removed from all the treatment plants except at Zandvliet during Autum where less than 20% was removed. This is similar to the finding of Firfilins et al. (2004).

The range of copper concentration reported in this study was also within the studies reported in most countries and was within the freshwater limit set by the department of water affairs for irrigation, aquatic life and livestock management (DWAF, 1996). Pb removal efficiency in the plants could be rated effective as between 40 to 95% of the total influx was removed from the waste stream. The reported concentration range for the investigated treatment plants were collaborated by previous studies elsewhere (Table 11), while the plants abundance patter could be rated as new Bellville > old Bellville > Stellenbosch ≥ Athlone > Potsdam > Kraaifontein > Zandvliet. This abundance distribution pattern can largely be attributed to the industrial effluent being received at each of the treatment plant and the living pattern of the residents in the study area. There was significant difference between the influent and effluent concentration for most of the plants as the final effluent concentration was generally lower than the influent. The final effluent concentration fell below the SA waste quality guidelines for aquatic life, irrigation and livestock production purposes. However, the concentration was far above the CCME guidelines (1999).

Sources of mercury to the environment include dental practices, clinical thermometers, glass mirrors among others. Mercury is known to be highly toxic and can affect human health at the lowest concentration of possible exposure. For the WWTPs, no significant difference was noticeable between the influent and effluent concentration. However, all the wastewater treatment plants could be rated effective with the exception of Athlone and Zandvliet where percentage removal fell below 30% over the study period. Potsdam was highly

Table 11. Heavy metal concentrations in influent and effluents from other countries and investigated treatment plants in Cape Town.

Metal	Country	Untreated influent (μgL^{-1})	Treated effluent (μgL^{-1})	References
As	Spain	2.2	-	European Communities (2001)
	Italy	0.3-31	0.5-9.2	Busetti et al. (2005)
	Israel	5.6	5.1	Shomar et al. (2004)
	South Africa	5-43.76	1.12-5.69	Present study
Cd	Austria	<20-60	<20-60	European Communities (2001)
	Poland	<0.01	<0.01	Kulbat et al. (2003)
	France	6-85	-	European Communities (2001)
	Germany	0.4	-	European Communities (2001)
	Greece	<1-44	<1	Karvelas et al. (2003)
	Greece	0.56	0.34	Firfilionis et al. (2004)
	Israel	0.6	0.8	Shomar et al. (2004)
	Italy	0.2-1.8	0.1-1.6	Busetti et al. (2005)
	Spain	0.06 – 1.19	0.04 – 0.11	Oliveira et al. (2007)
	Turkey	0-137	4-5	Ustun (2009)
	South Africa	1.07-17.39	0.52-2.58	Present study
Hg	Austria	<10	<10	European Communities (2001)
	Spain	0-0.5	0-0.24	Oliveira et al. (2007)
	France	1-8	-	European Communities (2001)
	Italy	<1	-	European Communities (2001)
	Germany	0.6	0.1	European Communities (2001)
	Italy	0.2-147	0.1-9.5	Busetti et al. (2005)
	South Africa	0.6-14.5	0.1-3.2	Present study
Zn	Poland	270-800	-	Chipasa (2003)
	Poland	270-300	90-120	Kulbat et al. (2003)
	Austria	<20-3700	20-500	European Communities (2001)
	Greece	330-3200	20-900	Karvelas et al. (2003)
	Greece	456	268	Firfilionis et al. (2004)
	Israel	75	54	Shomar et al. (2004)
	Italy	100-900	-	European Communities (2001)
	Italy	61-833	24-238	Busetti et al. (2005)
	South Africa	400.9-5128.3		Present study
Cr	Greece	102.1	56.9	Firfilionis et al. (2004)
	Austria	6200-7900	<900-5600	Firfilionis et al. (2004)
	Italy	0.5-18.4	0.4-8.2	Busetti et al. (2005)
	Greece	28-52	0.1-16	Karvelas et al. (2003)
	Turkey	174-2120	132-423	Ustun (2009)
	Poland	20	10	Kulbat et al. (2003)
	Spain	6.87	5.74	Oliveira et al. (2007)
	South Africa	31.2-223.62	23.8-153.4	Present study
Co	South Africa	0.34-11.65	0.17-4.73	Present study
Cu	Poland	52.2	26.0	Firfilionis et al. (2004)
	Spain	17.31	9.66	Oliveira et al. (2007)

Table 11. Contd.

	Poland	70	10	Kulbat et al. (2003)
	Turkey	0-137	4-5	Ustun (2009)
	Germany	-	-	European Communities (2001)
	Italy	-	-	Busetti et al. (2005)
	South Africa	21.0-1189.3	7.7-55.4	Present study
	Poland	37-148	-	Chipasa (2003)
	Poland	15	<10	Kulbat et al. (2003)
	Austria	<20-60	<20-60	European Communities (2001)
	Greece	28.6	13.1	Firfilionis et al. (2004)
Pb	Republic of Korea	2.93-79.33	0.70-17.45	Chanpiwat et al. (2010)
	Spain	37.42	22.57	Oliveira et al. (2007)
	Turkey	6-358	22-30	Ustun (2009)
	Italy	10-61	1.0-11	Busetti et al. (2005)
	South Africa	10-61	1.0-11	Present study
	Greece	32.2	32.2	Firfilionis et al. (2004)
	Poland	30	10	Kulbat et al. (2003)
Ni	Republic of Korea	4.88-116.6	3.36-51.53	Chanpiwat et al. (2010)
	Turkey	59-202	24-53	Ustun (2009)
	South Africa	7.9-776.7	7.6-159.1	Present study

effective at Hg removal as for other metals except for the spring season. The poor performance of the plant for Hg removal could be attributed to plant overload which subsequently affected the retention time of water in the plant. The concentration reported in this study for mercury was higher than values reported in Austria, France, Spain and Germany (Oliveira et al., 2007; European Communities, 2001); however, it was lower than the finding of Busetti et al. (2005). The possible sources of nickel into wastewater or other environmental components includes alloys, electroplating, nickel-cadmium batteries, launderettes and paints productions. Ni was not well removed from all the investigated plants except for Potsdam during the winter and spring seasons. Nickel removal in Bellville new and Kraaifontein WWTPs were least as annual removal for these plants were less than 30% (Figure 2). Findings from this study were similar to values reported in influent and effluent waste from other studies as presented in Table 11. No significant difference was noticeable between the influent and effluent water; however, WWTPs like Athlone, Potsdam and Stellenbosch show some seasonal variation in the influent Ni concentration.

Sources of zinc include domestic wastes, galvanizing, batteries, paints, fungicides, textiles, cosmetics, pulp, paper mills and pharmaceutics. In this study, Zn was the most dominant metal in all the WWTPs investigated. The annual plant rating can be rated as Zandvliet > Bellville old > Athlone > Bellville new ≥ Kraaifontein > Potsdam > Stellenbosch. The range of zinc in this study was generally higher than findings in other studies (Table 11). In terms of removal efficiency, Athlone treatment plant had the highest and can be rated above other plants. There was significant difference between the influent and effluent concentration over the study period. Considering the high concentration of Zn received at these plants, the final effluent concentration was within the national water act waste discharge standards (DWAF, 2010).

Conclusion

The results revealed that differences in metal concentrations in the influent were site-specific and varied by the period of sample collection. Metal variations could be related to the diversified industrial activities, especially from a multitude of smaller sized companies. A significant difference in metal concentrations between influent and effluent was found, except for Hg. Metal concentrations in the influent to the biological treatment of the WWTP's and the removal efficiencies that have been found in this study are within the ranges reported in the current literature. However, using the final effluent for irrigation purposes as it were found for some treatment plants could pose serious health risks in the future considering plant overload and intermittent breakdown of

some these treatment plants.

Conflict of Interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors want to thank the Department of Geology, University of Stellenbosch for sample analysis. This study was financially supported by NRF and Cape Peninsula University of Technology grant given to Mr. O. O. Olujimi for part of his doctoral studies.

Abbreviations

EDMs, Endocrine disrupting metals; **WWTPs,** wastewater treatment plants; **ICP-MS,** inductively coupled plasma mass spectrometry.

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academicJournals

Vol. 15(23), pp. 1136-1146, 8 June, 2016
DOI: 10.5897/AJB2015.15169
Article Number: 6D7C76E58796
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity studies on selected rice varieties grown in Africa based on aroma, cooking and eating quality

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Received 14 December, 2015; Accepted 6 May, 2016

Rice grain quality is an important factor that has a great influence on its market value and consumer acceptance. It is determined by three parameters controlling the cooking and eating qualities of rice (amylose content, gelatinization temperature and gel consistency) and by the aroma, which becomes a criterion increasingly preferred by consumers. Molecular characterization of specific genomic regions of rice genotypes by trait specific markers can help in the development of suitable breeding program. This study was conducted at AfricaRice Regional station, Saint-Louis, Senegal. 30 rice genotypes commonly used in Africa were evaluated using eight simple sequence repeat (SSR) markers linked to the cooking, eating properties, and the aroma. The total number of alleles was 45 with an average of 5.63 allele per locus. The number of alleles per marker varied from three for RM204 to eight for RM190 and RM342A and the effective number of alleles varied from 1.66 for RM204 to 6.16 for RM342A. The polymorphic information content (PIC) varied from 0.39 to 0.83 and the allele frequency ranged from 0.015 to 0.75. A maximum genetic similarity of 1 was observed between Gambiaka Kokoum and Gambiaka Burkina Faso, Basmati 270 and Basmati 370, Sahel 108 and Sahel 201, Sahel 108 and Sahel 208, Sahel 201 and Sahel 208, Sahel 202 and Sahel 209, and Sahel 305 and Sahel 317. The Sahel varieties found with maximum genetic similarity have the same amylose content, but different gelatinization temperature except Sahel 305 and Sahel 317 which have the same cooking and eating properties. Therefore, more markers are needed to discriminate those varieties. Minimum genetic similarity was observed between traditional aromatic rice Basmati 370 and the landrace Gambiaka Nigeria. The unweighted pair-groups method using arithmetic averages (UPGMA) cluster analysis of these cultivars enabled the classification of our varieties in five major groups with additional sub-clusters in groups 2, 3 and 4. Groups 1 and 2 composed of aromatics varieties, group 3 gathered the three improved Sahel aromatic varieties, group 4 was the most diversified group with three sub-clusters and group 5 corresponded to the traditional varieties Gambiaka. The results of this study indicated that the use of trait specific SSR markers enabled to group the varieties according to their cooking and eating quality and the aroma and therefore can be very useful in breeding rice varieties harboring good cooking and eating quality traits and aroma in rice breeding program.

Key words: Grain quality, cooking and eating properties, aroma, cluster analysis, simple sequence repeat (SSR), rice.

INTRODUCTION

The genus *Oryza* of the Graminae family has 22 species among which only two *Oryza glaberrima* domesticated in Africa and *Oryza sativa* domesticated in South Asia are cultivated around the world (Bounphanousay et al., 2008). *O. sativa* L. is considered as one of the major cereal crops with agronomic and nutritional importance. It is a staple food for more than a half of the world's population and accounts for 21% of global human per capita energy, 15% of per capita protein (Maclean et al., 2002) and 21% fat supply (Kennedy and Burlingame, 2003).

The grain quality of rice is an important factor that has a great influence on its market value and consumer acceptance (Demont et al., 2012); it includes a range of parameters such as appearance, milling quality and physico-chemical properties. The latter ones are reported to be very important for consumer and market place (Juliano and Perez, 1988). It is determined by three physicochemical properties, that is, the amylose content (AC), the gelatinization temperature (GT) and the gel consistency (GC). Aromatic rice are very popular in Africa, because of their flavor and texture, which makes aroma a criterion increasingly preferred by consumers.

Rice grain quality is difficult to define, because of quality varying preferences depending on the cooking culture. For example, in Japonica rice eating countries, rice with short grains and low amylose content are preferred, because they become soft and sticky after cooking (Danbaba et al., 2011). However, in *indica* consuming countries including most of the African countries and Pakistan, long grain rice with intermediate AC and GT are preferred because they become soft and fluffy after cooking. Rice grain is mainly composed of starch which consists of two forms of glucose polymers, namely, amylose and amylopectin and therefore its cooking and eating characteristics are mainly assessed using its AC, GT and GC (McKenzie and Rutger, 1983). AC is considered as the most important criteria influencing the behavior of rice during cooking and processing (Juliano, 1979; Webb et al., 1985). It is correlated with the increase in volume, water absorption during cooking and the hardness and whiteness of cooked rice (Juliano, 1985). Based on AC, rice is classified as: Waxy rice (0 to 2% AC) and non-waxy rice which are divided into very low AC (3 to 9%), rice with low AC (10 to 20%), intermediary AC (20 to 25%) and high AC (above 25%) (Bao et al., 2006; Wani et al., 2012). Rice with high AC becomes hard after cooking (Rao et al., 1952; Williams et al., 1958). A gene named waxy gene on the short arm of chromosome 6 explained most of the variations observed in AC among cultivars and many markers present on this short arm have been mapped to quantitative trait loci (QTLs) with major effects controlling AC (Septiningsih et al., 2003; Aluko et al., 2004; Fan et al., 2005; Shu et al., 2006). The GT reflects the ease or difficulty to cook the rice (Bao et al., 2007); it is related to the chain length distribution of amylopectin (Bao et al., 2009; Noda et al., 2003). It is defined by the alkali spreading value (ASV) based on the degree of diffusion of six grains of rice in a solution of KOH at 1.7%. Rice grains with a high GT remain unchanged, while those with low GT are completely disintegrated and the ones with medium GT are partially affected. Alkali spreading value corresponds to GT as follows: ASV of 1 to 2 for high GT (74.5 to 80°C), 3 for high intermediate GT, 4 to 5 for intermediate GT (70 to 74°C), and 6 to 7 for low GT (<70°C). GT is controlled by the *ALK* gene responsible for the synthesis of the enzyme soluble starch synthase sub-type IIa (SSIIa) (Umemoto et al., 2002). A major QTL controlling GT was located in the alk locus region near the waxy gene (3.93 cM) (Tan et al., 1999). This locus was also detected by Shu et al. (2006) in addition to a second QTL detected in the *alk* locus region linked with the marker RM276. The GC is a measure of firmness of the rice after cooking and is performed to classify rice with the same amylose content, particularly those with high AC into hard, medium or soft texture (Sabouri et al., 2012). Two QTLs controlling GC were detected on chromosome 6 (Lanceras et al., 2000; Tian et al., 2005). GC QTLs have been reported to be linked to *wx* locus (Fan et al., 2005; Fitzgerald et al., 2008; Tan et al., 1999). Some research works on loci controlling rice physico-chemical properties have reported that the three parameters are under the control of the waxy locus or a genomic region closely related to this locus (Tan et al., 1999)

Aroma also called fragrance has become an important criterion in the selection of rice due to high consumer preference for aromatic rice. It plays an important role in rice price. In Africa, aromatic rice from Pakistan (Basmati) has a high market price. High milling returns and good cooking quality are often associated with aromatic rice (Tripathi and Rao, 1979; Sattari et al., 2015). Fragrance is mainly due to the presence of a compound named 2-acetyl-1-pyrroline (2AP) (Lorieux et al., 1996). 2Ap is produced by a deletion of 8 bp of exon 7 on rice chromosome 8 leading to a recessive allele *fgr* coding for a nonfunctional betaine aldehyde dehydrogenase (badh2) (Bradbury et al., 2005). Many inexpensive, simple and rapid polymerase chain reaction (PCR)-based markers, such as SNP, SSRs markers linked to aroma have been developed (Cordeiro et al., 2002). RM 515,

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RM 223 and RM 342A are located on chromosome 8 and explained 41.78, 28.38, and 22.46% of the phenotypic variation, respectively (Kibria et al., 2008).

Afirca Rice Center has created and released number of varieties in Senegal, Mauritania, Mali and Gambia. The released varieties included interspecific and aromatic with variable cooking and eating properties. However, breeding programs aiming to develop rice varieties with good cooking, eating qualities and aroma are difficult, due to the polygenic inheritance and the environmental interactions (Lapitan et al., 2007; Lestari et al., 2009). Therefore, precise information on genetic diversity of the rice genome regions controlling these parameters will be useful for efficient breeding programs. These information can be provided by SSR markers which have been used successfully for genetic diversity studies due to their multiallelic nature, high reproductibility, co-dominant inheritance, abundance and extensive genome coverage (Sivaranjani et al., 2010). In this study 30 varieties including Africa rice released varieties and some others commonly grown rice varieties in Africa were evaluated for their genetic diversity using SSR markers that are genetically linked to aroma and QTLs controlling rice cooking and eating properties.

MATERIALS AND METHODS

Plant

The research was undertaken in 2012 at the AfricaRice Regional station of Sahel (Saint-louis-Senegal). Thirty varieties (Table 1) were investigated, including 4 interspecific NERICA varieties created by AfricaRice Sahel station, 15 improved Sahel varieties of different origins mostly released and cultivated in Senegal, Mauritania and Gambia, 5 traditional aromatic varieties including, 3 Basmati genotypes, Dom-Seophid, and KDM105 having a high market value, 4 landraces Gambiaka well spread in Africa, TS2 a Taiwanese variety released and cultivated in Burkina Faso and Wab 638-1 bred in Ivory Coast at ADRAO center (former name of AfricaRice); the seeds for all the varieties were provided by AfricaRice center. Ten seeds of each variety were pre-germinated in Petri dishes and then transferred in pots containing clay as substrate and placed in the screen house 3 weeks later. Leaves were collected from 45 days old seedlings for DNA extraction.

DNA extraction

DNA was extracted from the leaves of 45-day-old seedlings using the Dellaporta protocol with some modifications (Dellaporta et al., 1983). The obtained DNA was dissolved in 200 µl of 1X TE buffer and stored at -20°C, and then diluted to 25 ng/µl using double-distilled water to obtain the working solution. DNA quality was checked using 1% agarose gel.

PCR amplification and gel electrophoresis

PCR was performed in 10 µl reaction mixture volume containing 2 µl of DNA, 4.2 µl of ddH_2O, 1 µl of 10X buffer, 1 µl of dNTPs (1 mM), 0.3 µl of $MgCl_2$ (25 mM), 0.5 µl of Taq polymerase, 0.5 µl of the Forward primer (10 mM) and 0.5 µl of the Reverse primer (10 mM). Mineral oil was added to the reaction mixture to prevent evaporation. The PCR reaction was performed using a G-Storm thermal cycler system (384 well alpha-unit). The PCR program used consisted of an initial denaturation at 95°C for 5 min followed by a series of 35 cycles, each of which consisted of a denaturation at 94°C for 4 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The program ends with a final elongation of 72°C for 7 min. The amplification products were run on an 8% non-denaturing polyacrylamide gel electrophoresis (PAGE) following a modified procedure by AfricaRice biotechnology laboratory in Senegal (Thomson et al., 2010) with 1X TBE buffer at 100 V for 150 min. Three microliters of blue dye were added to the PCR products and 4µl of the final volume were loaded into the gel well with 50 bp ladder. After electrophoresis, the gels were soaked in a solution of Ethidium bromide (3.5%) for 15 min and visualized under UV using Gel Documentation Systems.

SSR markers

Aroma SSR markers

Four markers (RM223, RM210, RM342A, RM152) mapped on chromosome 8 (Temnykh et al., 2000), linked to the region controlling the aroma were used (Table 2).

SSR markers for cooking and eating parameters

Four SSRs including RM170, RM190, RM204, and RM253 located near *Wx* and *Alk* genes were used. These markers were previously mapped to the short arm of chromosome 6 and were reported to be linked to loci controlling rice cooking and eating characteristics (Septiningsih et al., 2003; Aluko et al., 2004; Fan et al., 2005; Shu et al., 2006) (Table 3).

Data analysis

Clearly resolved bands of the genotypes were manually scored using the binary coding system, '1' for presence of band and '0' for absence of band. The resultant matrix was used to calculate genetic similarities among the accessions according to Jaccard's coefficient (Jaccard, 1908) using NTSYS-pc software package version 2.02e (Rohlf, 2004). Using pairwise similarity matrix of Jaccard's coefficient, a phylogenetic tree was constructed by the Unweighted Pair-Group Method of Arithmetic average (UPGMA)) module of the NTSYS-pc. Polymorphism information content (PIC) was calculated using the formula:

$$\mathbf{PIC} = \mathbf{1} - \sum_{\mathbf{j=1}}^{\mathbf{n}} \mathbf{P_{ij}}^{\mathbf{2}}$$

where P_{ij} is the frequency of the allele i at locus j. It is calculated for allele. The effective number of alleles was calculated using the formula:

$NE = 1/Pij^2$

The software package SPSS20.0 (SPSS, Chicago, IL, USA) was used for correlation analysis.

RESULTS AND DISCUSSION

Overall allelic diversity

All the SSR markers used in this study were polymorphic.

Table 1. Origin and physico-chemical properties of studied varieties.

Varieties	Parents	Origin	AC (%)	ASV	GT	Aroma
Gambiaka Bénin	-	Benin	-	-		No Aroma
Gambiaka Burkina Faso	-	Burkina Faso	-	-		No Aroma
Gambiaka KK	-	Malia	24.70	5,2	Intermediary	No Aroma
Gambiaka Nigeria	-	Nigeria	-	-		No Aroma
Basmati Punjab	-	India	21.24	7	Low	Aroma
Basmati 270	-	India	-	-		Aroma
Basmati 370	-	India	22.80	4.2	Intermediary	Aroma
Dom Seophid	-	Iran	19.9	3.5	Intermediary	Aroma
WAB 638-1	DR2/ DR2	WARDA (Ivory Coast)	24	2.3	High	Aroma
KDM 105	-	Thaïlande	15	6.8	Low	Aroma
TS 2	-	Taiwan	22	5	Intermediary	Not Aroma
Sahel 108	IR30(BHP)/BABAWE//IR 36	IRRI (Philippines)	27	2	High	No Aroma
Sahel 134	IR 1791-5-4-3-3/IR 9129-209-2-2-2-1	IRRI (Philippines)	25	2	High	No Aroma
Sahel 159	IR 13240-108-2-2-3/IR 9129-209-2-2-2-1	IRRI (Philippines)	24	2	High	No Aroma
Sahel 177	Sahel 134/IR66231-37-1-2	AfricaRice (Saint Louis)	30	2	High	Aroma
Sahel 201	IR 2071-586/ BG 400-1	Sri Lanka	28.10	6	Low	No Aroma
Sahel 202	TOX 494-3696/TOX 711/BG6812	Nigeria	27.70	2	High	No Aroma
Sahel 208	ITA 212/ UPL RI 7	ITA (Nigeria)	29	3	Intermediary	No Aroma
Sahel 209	TSY/MOROBERKAN//ITA306	ITA (Nigeria)	28	5	Intermediary	No Aroma
Sahel 210	-	Latin America	20	2	High	No Aroma
Sahel 217	Sahel 201/ 4456	AfricaRice (Saint Louis)	30.90	4	Intermediary	No Aroma
Sahel 222	Sahel 201/ 4456	AfricaRice (Saint Louis)	31.90	3	Intermediary	No Aroma
Sahel 305	IR64/4456	AfricaRice (Saint Louis)	27.80	7	Low	No Aroma
Sahel 317	IR64/4456	AfricaRice (Saint Louis)	27.25	7	Low	No Aroma
Sahel 328	Sahel 134/ IR66231-37-1-2	AfricaRice (Saint Louis)	31.50	7	Low	Aroma
Sahel 329	Jaya/Basmati 370	AfricaRice (Saint Louis)	30.90	7	Low	Aroma
Nerica-S-19	Tog5681/2*IR64/IR31785	AfricaRice (Saint Louis)	32	2	High	No Aroma
Nerica-S-21	Tog5681/2*IR64/IR31785	AfricaRice (Saint Louis)	32	4	Intermediary	No Aroma
Nerica-S-36	Tog5681/2*IR1529//IR1529	AfricaRice (Saint Louis)	31.60	2	High	No Aroma
Nerica-S-44	IR64/ Tog5681/4*IR64	AfricaRice (Saint Louis)	28.80	3	Intermediary	No Aroma

References of quality traits data (ASV, AC, Aroma) (Traore et al., 2015; Tabkhkar et al., 2012).

The eight markers produced 45 alleles with an average of 5.63 alleles per locus among the 30 rice genotypes. The number of alleles per primer ranged from three for RM204 to eight for RM190 and RM342A (Table 4). This number is similar to the average value of 5.89 alleles per locus obtained in a study of Philippian rice cultivars using a set of 151 polymorphic SSR markers (Lapitan et al., 2007). But it is higher than the

Table 2. SSR markers used for aroma in this study

Markers	Chromosome	Repeat types		References
RM152	8	Fwd. Rev.	GAAACCACCACACCTCACCG CCGTAGACCTTCTTGAAGTAG	Temnykh et al. (2000)
RM210	8	Fwd. Rev.	TCACA T TCGGTGGCATTG CGAGGATGGTTGTTCACTTG	Temnykh et al. (2000)
RM223	8	Fwd. Rev.	GAGTGAGCTTGGGCTGAAAC GAAGGCAAGTCTTGGCACTG	Temnykh et al. (2000)
RM342A	8	Fwd. Rev.	CCATCCTCCTACTTCAATGAAG ACTATGCAGTGGTGTCACCC	Temnykh et al. (2000)

Table 3. SSR linked to QTLs detected on chromosome 6 responsible of eating and cooking properties of rice (Tabkhkar et al. 2012).

Marker	Sequence	Studied traits	QTLs	Parents	References
RM 170	Fwd. TCGCGCTTCTTCCTCGTCGACG Rev. CCCGCTTGCCGTTCATCCCTCC	AC	*ac6a*	Zhenshan97/H94	Fan et al. (2005)
		GC	-	IR64/IRGC105491	Septiningsih et al. (2003)
RM 190	Fwd. CTTTGTCTATCTCAAGACAC Rev. TTGCAGATGTTCTTCCTGATG	AC	*ac6a*	Zhenshan97/H94	Fan et al. (2005)
		GC	*gc6b*	Zhenshan97/H94	Fan et al. (2005)
		GT	*asv6a*	Zhenshan97/H94	Fan et al. (2005)
		AC	*amy6*	Caiapo/IRGC103544	Aluko et al. (2004)
		GT	*Alk 6-1*	Caiapo/IRGC103544	Aluko et al. (2004)
RM 204	Fwd. GTGACTGACTTGGTCATAGGG Rev. GCTAGCCATGCTCTCGTACC	GT	-	-	He et al. (1999)
RM 253	Fwd. TCCTTCAAGAGTGCAAAACC Rev. GCATTGTCATGTCGAAGCC	AC	*ac6b*	Zhenshan97/H94	Fan et al. (2005)
		GC	*gc6b*	Zhenshan97/H94	Fan et al. (2005)
		GT	*asv6b*	Zhenshan97/H94	Fan et al. (2005)
		AC	-	IR64/IRGC105491	Septiningsih et al. (2003)
		GC	-	IR64/IRGC105491	Septiningsih et al. (2003)
		GT	-	Huangyu B/II32 B	Shu et al. (2006)
		GT	*alk6-2*	Caiapo/IRGC103544	Aluko et al. (2004)

Table 4. Characteristics of SSR markers in all the studied genotypes.

Markers	Chromosome	Studied Traits	Motifs	Number of alleles	Number of effective alleles	PIC
RM170	6	AC, GC	(CCT)7	4	3.80	0.74
RM190	6	AC, GC, GT	(CT)11	8	4.62	0.78
RM204	6	GT	(CT)44	3	1.63	0.39
RM253	6	AC, GC, GT	(GA)25	6	4.35	0.77
RM152	8	Aroma	(GGC)10	4	2.15	0.53
RM210	8	Aroma	(CT)23	5	3.15	0.67
RM223	8	Aroma	(CT)25	7	3.79	0.74
RM342A	8	Aroma	(CAT)12	8	6.29	0.84

average value of 4.3 alleles per locus ranging from 2 to 9 alleles reported in a Venezuelan rice cultivars genetic diversity assessment (Herrera et al., 2008) and the value of 3.33 alleles across 9 polymorphic loci obtained using a set of 24 SSR markers for the characterization and discrimination of 12 elite aromatic rice genotypes (Sajib et al., 2012). This study average alleles number is also higher than the value (3.13) reported in a study using a set of 8 markers linked to aroma and cooked kernel elongation covering chromosomes 3, 4, 8 and 9 to assess the genetic diversity among thirteen rice varieties from Kenya and Tanzania (Kioko et al., 2015). Although the number of polymorphic SSR markers in Lapitan et al. (2007) was higher than the number used in this study, almost similar average values were noticed, this can be a reflection of a high level of diversity among our cultivars. However, the allele's average number reported in this study was lower than the value of 9.3 reported in a study using a larger scale of accessions (238) representing both *japonica* and *indica* cultivated rice (Yang et al., 1994) or the value of 7.8 reported in a genetic analysis of 69 Indian aromatic rice cultivars using 30 fluorescently labeled rice SSR markers (Jain et al., 2004). Furthermore, this study's average number is less than the value of 6.33 alleles per locus recorded in a DNA fingerprinting study of 34 rice genotypes using a small set of three SSR marker for a diversity (Rahman et al., 2009). This difference observed with those reports might be due to the use of diverse germplasm and higher number of rice accessions used in the aforementioned studies. The SSR markers linked to aroma produced 26 alleles with an average of six loci per marker, which is slightly higher than the average number (5.25) produced by the marker linked to QTLs controlling cooking and eating quality. The average number of alleles linked to aroma (6) is higher than the average value of 4.7 alleles per locus reported in a Basmati rice genetic diversity analysis using 26 SSR marker associated with aroma and cooked kernel elongation (Jain et al., 2006). The average value of SSR markers linked to the cooking and eating quality is almost similar to the value of 5.86 reported in a genetic diversity assessment of 48 rice cultivars using markers linked to the cooking and eating quality of rice (Tabkhkar et al., 2012).

Rare alleles were also observed in this study. Alleles observed in less than 5% of all the rice varieties (commonly termed as rare) were investigated and a total of four (8.9% of the total number of alleles) were identified at three loci RM190 (2), RM253 (1) and RM223 (1). Gambiaka Burkina Faso, Nerica-S-36, Sahel 159 and Sahel 329 had each one rare allele. RM190 revealed the highest number of rare allele and might be useful in the creation of fingerprints of the varieties used in this study. The effective number of alleles varied from 1.63 for RM204 to 6.29 for RM342A with an average of 3.72 which was very much similar to the value of 3.74 recorded by Tabkhkar et al. (2012) and higher than the 2.19 reported by Kibria et al. (2009).

PIC value

The varying PIC values generated by the markers reflect the discriminating power of a particular marker by taking into account the number of alleles at each locus and their relative frequencies among the tested varieties. In our study, PIC values varied from 0.39 (RM204) to 0.84 for RM342 with an overall average of 0.68. The highest PIC value for markers linked to QTLs controlling cooking and eating quality is recorded for RM190 (0.78). The overall average value is similar to the PIC value reported by Lapitan et al. (2007) and the one reported by Jain et al. (2004), but it is higher than the value reported by Kioko et al. (2015) and the one recorded by Sajib et al. (2012). There is no correlation between the repeat number of SSR motifs and the PIC value and between the repeat number of the SSR motifs and the number of alleles. The later observed pattern was consistent with Kioko et al. (2015) report but not in concordance with the results of Herrera et al. (2008) who found that the maximum number of repeats within the SSRs was significantly correlated with the number of alleles at a locus (r = 0.505, $P < 0.01$). However, a significant correlation was observed between the PIC value and the number of detected alleles (r=0.816, p-value less than 0.05): the higher the allele number, the higher the observed value

of the PIC (Table 4). The same observation was also made by Lapitan et al. (2007) and in a genetic diversity study of 193 parental lines of different origins using 101 well-distributed SSR markers (Yu et al., 2003). The level of polymorphism observed in this study is relatively high and can be explained by the presence in our studies of varieties from different origins.

Cluster analysis and genetic relationships

Genetic similarity based on Jaccard coefficient off similarity implemented in the NTSYS-pc software ver.2.02e was used to assess the level of relatedness among the studied cultivars (Table 5). The pairwise genetic similarity ranged from 0.07 between the traditional aromatic rice Basmati 370 and the landrace Gambiaka Nigeria widely spread in Nigeria to a maximum similarity of 1 between Gambiaka Burkina Faso and Gambiaka Kokoum, Basmati 370 and Basmati 270, Sahel 108 bred in IRRI (Philippines) and Sahel 208 bred in ITA (Nigeria), Sahel 208 and Sahel 201 bred in Sri Lanka, Sahel 108 and Sahel 201, Sahel 209 and Sahel 202 both bred in ITA, as well as Sahel 305 and Sahel 317. Most of the Sahel varieties showing maximum similarity coefficient of 1 have the same AC, but differ in term of GT except Sahel 317 and Sahel 305, two varieties derived from the same breeding program and having the same AC and GT. This result was expected giving that among the four markers used for the cooking and eating parameters, only one (RM204) was reported specifically linked to QTLs controlling GT. Therefore, more markers linked to Loci controlling GT is needed for a more precise discrimination of the varieties used in this study. A high coefficient of similarity was observed within the two groups of traditional varieties present in our study. Within the Gambiaka group, the similarity coefficient varied from 0.70 between Gambiaka Benin and Gambiaka Nigeria to a maximum similarity of 1 between Gambiaka Kokoum and GambiakaBurkina Faso. In the Basmati group, the similarity coefficient varied from 0.94 between Basmati Punjab and the two others Basmati varieties (Basmati 370 and Basmati 270) to a maximum similarity of one between Basmati 270 and Basmati 3710. This reflects an intra-group homogeneity inside these two traditional groups. Furthermore, the coefficient of similarity is low between the Basmati varieties and the other groups. For example, the maximum coefficient of similarity value observed between the Basmati group and the interspecific NERICA (value observed between Nerica-s-44 and Basmati 370) was 0.29 and a maximum value of 0.23 was observed between the Gambiaka and the Basmati group. This observation confirmed that the traditional Basmati varieties have an intra-group homogeneity and are different from *indica* and interspecific types (Jain et al., 2004; Das et al., 2013). In the case of the improved varieties Sahel, the maximum values of the similarity coefficient with Basmati rice was observed with the three bred aromatic varieties Sahel 177, Sahel 328 and Sahel 329. The coefficient was 0.29 between Sahel 329 and Basmati 370, which is one of the parents used in the Sahel 329 breeding program parent. As expected, observed genetic similarity between aromatic varieties were high and improved varieties having the same AC have a high level of similarity.

Genetic similarity values obtained among the cultivars led to the construction of an UPGMA-based dendrogram as shown in Figure 1. At 63% level of similarity, the UPGMA diagram showed five major groups. Group 1 corresponded to two aromatic varieties; the Iranian traditional aromatic variety Domseophid and the variety Wab 638-1 created by mass selection at the ADRAO (former name of AfricaRice) in Bouake (Ivory Coast). The two varieties showed a 71% Level of similarity. Group 2 consisted of four traditional aromatic varieties: the three Basmati rice and KDM 105 originated from Thailand. As expected, the tree Basmati formed a sub-cluster (2B) separated from KDM 105 which formed a sub-cluster of its own (v2A). This is explained by the homogeneity of the basmati group mentioned in previous paragraph. Group 3 consisted of the three improved aromatic varieties; Sahel 177, Sahel 328 and Sahel 329 all bred at Africa Rice (Saint-Louis) and released in Senegal. This group could be further divided at about 66% level of similarity in two sub-groups 3A and 3B. Sub-group 3A contained the two aromatic lines Sahel 328 (AC=31.50, ASV=7) and Sahel 329 (AC=30.90, ASV =7) having both a high AC associated with a low GTre, while sub-group 3B only contained Sahel 177 having a high AC associated with a high GT (AC=30 and GT=2). Group 4 was the most diversified group and clustered 17 out of 30 varieties. This group could be separated into three sub-groups based on the origin and the cooking and eating characteristics of the varieties. The first subs-group 4A at about 70% level of similarity contained three varieties TS2 (AC=22, ASV=5), Sahel 159 (AC=24, ASV=2) and Sahel 210 (AC=20, ASV=2) with intermediate AC and high GT except TS2 which has an intermediate GT. Sahel 210 originated from Latin American and is released in Senegal, while TS2 originated from Taiwan is released in Burkina Faso. The sub-group 4C at a similarity level of about 75% is comprised of the four interspecific NERICA which have a high content of amylose. The last sub-group 4B contained 10 Sahel varieties and can be further divided in two small clusters $4B_1$ and $4B_2$. Cluster $4B_1$ contained two sister lines; Sahel 305 (AC = 27.80; ASV= 7) and Sahel 317 (AC = 27.25; ASV=7) which have a high AC associated with a low GT. The second small cluster gathered the remaining ten Sahel varieties. All the varieties present in that group have in common a high level of AC but belong to different classes of GT (from low to high) and are from different origins. The high GT class contained Sahel 108 (AC=27, ASV=2), Sahel 134 (AC=25, ASV=2), Sahel 202 (AC=27.70, ASV=2), and

Table 5. Pairwise genetic similarities obtained among the 30 rice varieties using eight SSR markers.

Parameter	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29
V2	0.85																												
V3	0.85	1.00																											
V4	0.70	0.86	0.86																										
V5	0.54	0.62	0.62	0.60																									
V6	0.55	0.56	0.56	0.56	0.95																								
V7	0.59	0.60	0.60	0.52	0.74	0.73																							
V8	0.61	0.68	0.68	0.63	0.91	0.86	0.82																						
V9	0.07	0.20	0.20	0.19	0.23	0.20	0.21	0.24																					
V10	0.09	0.20	0.20	0.19	0.24	0.21	0.21	0.27	0.94																				
V11	0.07	0.20	0.20	0.23	0.24	0.21	0.21	0.29	0.94	1.00																			
V12	0.19	0.27	0.27	0.27	0.27	0.33	0.33	0.31	0.59	0.62	0.58																		
V13	0.24	0.33	0.33	0.32	0.29	0.36	0.36	0.33	0.43	0.44	0.39	0.71																	
V14	0.30	0.42	0.42	0.37	0.42	0.45	0.45	0.47	0.65	0.63	0.73	0.63	0.71																
V15	0.50	0.52	0.52	0.42	0.58	0.59	0.57	0.65	0.19	0.23	0.23	0.36	0.63	0.52															
V16	0.58	0.55	0.55	0.46	0.75	0.79	0.65	0.70	0.14	0.13	0.12	0.21	0.26	0.37	0.67														
V17	0.59	0.56	0.56	0.44	0.70	0.71	0.61	0.70	0.14	0.15	0.14	0.26	0.27	0.37	0.67	0.94													
V18	0.44	0.43	0.43	0.37	0.60	0.61	0.46	0.60	0.17	0.21	0.21	0.25	0.31	0.40	0.71	0.68	0.68												
V19	0.48	0.56	0.56	0.46	0.56	0.56	0.48	0.56	0.30	0.27	0.29	0.35	0.38	0.52	0.52	0.65	0.63	0.48											
V20	0.56	0.52	0.52	0.43	0.74	0.78	0.63	0.68	0.11	0.10	0.08	0.19	0.23	0.35	0.65	1.00	0.93	0.67	0.63										
V21	0.59	0.56	0.56	0.44	0.77	0.80	0.68	0.77	0.18	0.18	0.18	0.26	0.27	0.41	0.67	0.94	0.89	0.68	0.63	0.93									
V22	0.60	0.57	0.57	0.43	0.71	0.74	0.62	0.71	0.11	0.13	0.11	0.20	0.25	0.36	0.68	1.00	0.94	0.70	0.64	1.00	0.94								
V23	0.59	0.56	0.56	0.44	0.77	0.80	0.68	0.77	0.18	0.18	0.18	0.26	0.27	0.41	0.67	0.94	0.89	0.68	0.63	0.93	1.00	0.94							
V24	0.50	0.42	0.42	0.31	0.61	0.62	0.59	0.61	0.15	0.16	0.15	0.32	0.35	0.39	0.74	0.71	0.70	0.75	0.48	0.69	0.79	0.72	0.79						
V25	0.57	0.60	0.60	0.48	0.74	0.76	0.65	0.74	0.21	0.21	0.21	0.29	0.31	0.45	0.64	0.88	0.85	0.65	0.67	0.88	0.95	0.89	0.95	0.75					
V26	0.62	0.58	0.58	0.46	0.65	0.67	0.64	0.65	0.14	0.15	0.14	0.27	0.33	0.38	0.62	0.88	0.84	0.64	0.65	0.87	0.84	0.88	0.84	0.74	0.89				
V27	0.65	0.68	0.68	0.55	0.73	0.75	0.64	0.73	0.19	0.19	0.19	0.28	0.35	0.44	0.70	0.88	0.84	0.64	0.76	0.87	0.84	0.88	0.84	0.65	0.84	0.83			
V28	0.62	0.70	0.70	0.57	0.70	0.71	0.61	0.70	0.22	0.22	0.22	0.30	0.38	0.46	0.67	0.82	0.80	0.61	0.77	0.81	0.80	0.83	0.80	0.62	0.85	0.84	1.00		
V29	0.48	0.56	0.56	0.46	0.56	0.56	0.48	0.56	0.30	0.27	0.29	0.35	0.38	0.52	0.52	0.65	0.63	0.68	0.68	0.63	0.63	0.64	0.63	0.48	0.67	0.65	0.76	0.77	
V30	0.36	0.35	0.35	0.30	0.50	0.54	0.48	0.50	0.29	0.27	0.29	0.35	0.33	0.52	0.52	0.62	0.56	0.54	0.62	0.60	0.63	0.57	0.63	0.61	0.60	0.52	0.52	0.50	0.91

V1=Gambiaka Nigeria, V2=Gambiaka Kokoum, V3=Gambiaka Burkina Faso, V4=Gambaiaka Benin, V5=Nerica-S-19, V6=Nerica-S-21, V7=Nerica-S-36, V8=Nerica-S-44, V9=Basmati Punjab, V10=Basmati 270, V11=Basmati 370, V12=Domseophid, V13=Wab638-1, V14=KDM105, V15=TS2, V16=Sahel 108, V17= Sahel 134, V18= Sahel 159, V19=Sahel 177, V20=Sahel 201, V21=Sahel 202, V22=Sahel 208, V23=Sahel 209, V24=Sahel 210, V25=Sahel 217, V26=Sahel 222, V27=Sahel 305, V28=Sahel 317, V29=Sahel 328, V30=Sahel 329.

Sahel 208 (AC = 29, ASV= 3). This group contained varieties released in some African countries (Sahel 208 from bred at International Institute for Tropical Agriculture in Nigeria is released in Senegal, while Sahel 208 bred originated from Nigeria is only released in Senegal. Sahel 209 (AC = 28; ASV= 5) released in Senegal and bred at the International Institute for Tropical Agriculture (IITA, Nigeria), Sahel 217 (AC = 30.90; ASV= 4) and Sahel 222 (AC= 31.90; ASV= 5) bred at AfricaRice (Saint-Louis, Senegal) belong to intermediary GT class, while Sahel 201 (AC = 28.10; ASV=6) originated from SriLanka and released in Senegal, belong to the low GT class. Group 5 corresponded to the traditional varieties Gambiaka group with high AC and high ASV (low GT) well spread in West Africa countries.

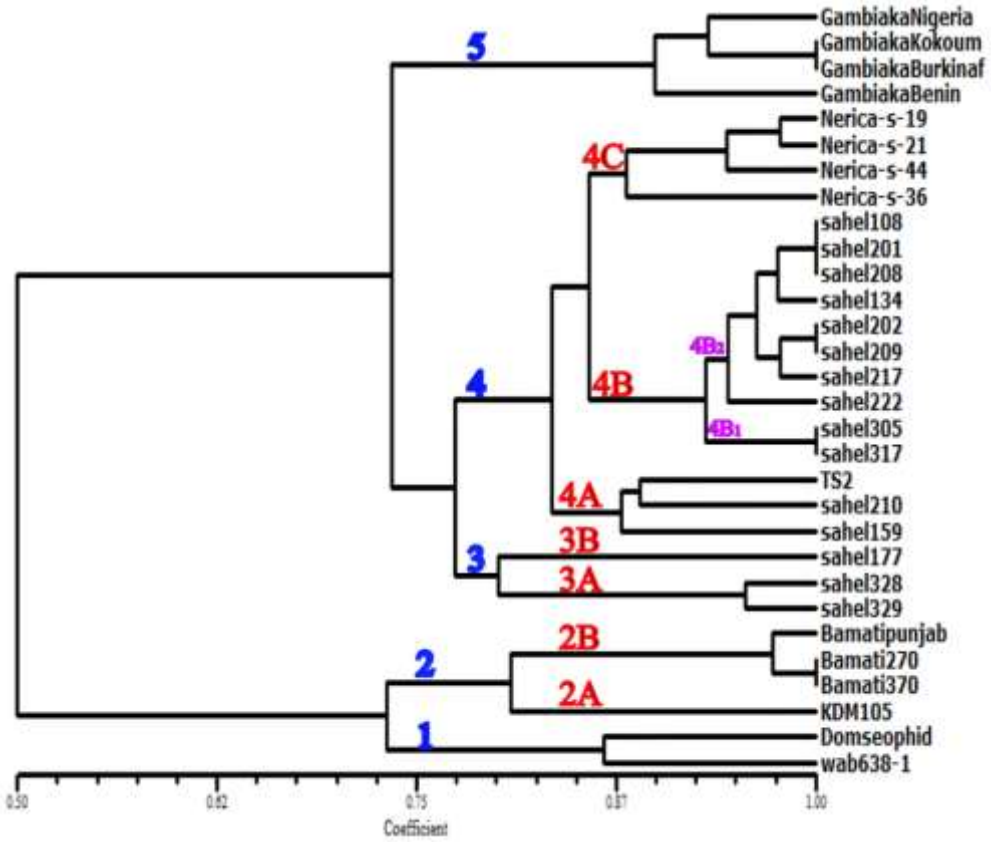

Figure 1. Dendrogram derived from UPGMA cluster analysis baed on Jaccard similarity coefficient showing genetic diversity and relatedness among the 30 rice varieties.

Conclusion

The present study provides an insight on the genetic diversity of the rice genome regions controlling aroma, cooking and eating parameters of some rice varieties commonly grown in Africa. Aroma, cooking and eating quality are the main quality traits that determine the rice market value in Africa and around the world. The results provided by SSR markers can be useful for efficient breeding programs since we were able to group the varieties based on their AC and aroma. However, the discrimination was not accurate when it comes to the GT of the varieties underlining the fact that more markers linked to the GT are required for a more precise discrimination. RM 342 has the highest PIC value and can be used to differentiate aromatic varieties from non-aromatic ones; it is followed by RM 190, which was very informative on the cooking and eating parameters of our varieties. Based on the clustering data, different breeding programs can be designed using genetically distant varieties in order to produce varieties carrying aroma and interesting cooking and eating quality. For example, an interspecific cross can be made between the varieties of the traditional aromatic group and the landraces Gambiaka or between the two groups and the improved Sahel varieties in order to get hybrids carrying more interesting cooking and eating properties and aroma. These results confirmed also the powerfulness of SSR markers to assess the genetic diversity of different cultivars reported by previous studies and strengthen the fact that SSR markers could be used to save time during the characterization of breeding materials at AfricaRice center. However, more markers linked to the cooking and eating properties or covering can be used for further studies in order to come up with more conclusive results for the fingerprinting and the discrimination of varieties used in this study based on physico-chemical parameters.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

RM, Rice microsatellite; **SSR,** simple sequence repeats; **UPGMA,** unweighted pair group method with arithmetic averages.

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Vol. 15(23), pp. 1147-1158, 8 June, 2016
DOI: 5897/AJB2015.14604
Article Number: 557D40C58798
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity and population structure among sorghum (*Sorghum bicolor*, L.) germplasm collections from Western Ethiopia

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Received 29 March, 2015; Accepted 13 May, 2016

The Western Ethiopian region harbors a unique set of sorghum germplasm adapted to conditions not conventional to sorghums grown in other parts of the world. Accessions from the region possess unique resistance to multiple leaf and grain diseases. This study is aimed at exploring the extent of genetic variation and population structure among accessions of this region. A total of 123 accessions comprising 111 from Western Ethiopia (62 from Asosa and 49 from Pawe) and 12 U.S. adapted lines were genotyped using 30 sorghum simple sequence repeat markers (SSR). Genetic diversity and population structure were analyzed using PowerMarker and STRUCTURE software, respectively, based on 23 polymorphic SSR markers. Principal component analysis (PCA) was performed to view the variability in multi-dimensional space. Population structure analysis revealed considerable admixtures between Pawe and Asosa accessions, while the PowerMarker analysis grouped the accessions into three distinct clusters largely based on collection regions. The PCA did not clearly differentiate Asosa and Pawe accessions, but U.S. adapted lines were clearly separated from the rest. The study indicated the presence of marked genetic variability among accessions from Western Ethiopia and also provided clues on shared genetic events among accessions adapted to the two areas in Western Ethiopia.

Key words: Sorghum, genetic diversity, population structure, SSR, Ethiopia.

INTRODUCTION

As a result of disruptive selection, isolation and recombination following the spread from its center of origin, sorghum amassed tremendous genetic variability for numerous traits (Doggett, 1970; Harlan and Stemer, 1976; Frederiksen, 2000; Little et al., 2012). In Ethiopia, part of the vast Northeastern Africa region where the crop

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is believed to have originated (de Wet and Harlan, 1971; de Wet, 1977; Jennings and Cock, 1977), sorghum grows in diverse agroecologies ranging from the hot and dry regions in the eastern and northern lowlands to the mild mid- and high-altitude regions in the central part and to the hot and humid lowlands in western parts of the country. Through time, sorghum may have developed adaptive traits that confer fitness to specific challenges faced in different regions of the world.

The Western Ethiopia region offers a unique environment to sorghum. High rainfall, soils of various degrees of weathering and nutrient content, warm temperature and high humidity are major characteristics of the region (Nageri, 1984; Abebe, 2007). The region extends from Wanbara-Matakkal zone (Pawe) far north of the Blue Nile River to an expansive area south of the Blue Nile which includes Asosa and further south to the Gambella region. All of these areas experience warm temperature, high rainfall and a near 100% humidity during crop seasons, a unique environment to sorghums grown worldwide. Though the region is conducive for growing variety of crops, sorghum is the primary choice for communities living in these areas and is widely grown by diverse groups of people. Over the years, variants of sorghum specifically adapted to these conditions have emerged and become unique sources of germplasm of global interest, such as the Zera-Zera sorghums (Prasada Rao and Mengesha, 1981; Gebeyehu, 1993; Rai et al., 1999).

Efforts to adapt improved sorghum varieties grown in other parts of the country to these areas repeatedly failed due to extreme disease pressure, particularly grain mold and various leaf diseases. However, local sorghums from the region consistently endure the pressure and produce reasonable yields. These diseases are critical in that they seriously undermine grain yield either through reduced grain filling caused by limited photosynthesis in crops damaged by leaf diseases or due to physical damage caused to the grains (grain mold) which together result in massive loss in grain yield as well as grain and stover quality (Thakur et al., 2006; Ibrahim et al., 1985; Somani and Indira, 1999), storability (Hodges et al. 1999) and germinability (Maiti et al., 1985). Both grain mold and leaf diseases become severe when post-flowering temperature exceeds 25°C and relative humidity surpasses 85% (Garud et al., 2000; Navi et al., 2005) which are typical condition in Western Ethiopia.

The Ethiopian national sorghum research program assembled series of accessions from Pawe and Asosa regions of Western Ethiopia to initiate a new breeding program for the region. Despite similar weather characteristics in the two regions, preliminary results revealed remarkable variability among the accessions for various plant characteristics including resistance to grain mold and leaf diseases, grain yield and a range of agronomic, and morphological features. A number of previous studies on sorghums of Eastern Africa region (Ghebru et al., 2002; Mutegi et al., 2011; Ng'uni et al., 2011) including, a recent study on *in situ* diversity and population genetic structure of several Ethiopian accessions (Adugna, 2014) revealed significant genetic variation. However, there was no information whether sorghums evolved under more or less similar agroecology express the level of diversity that is of significant interest. Thus, the objectives of this study were to estimate the extent of genotypic variability among representative accessions from Pawe and Asosa regions of western Ethiopia, and assess the impact, if any, of geographic isolation on the pattern of genetic diversity and population structure among the accessions.

MATERIALS AND METHODS

Genetic

A total of 111 accessions, 49 accessions from Pawe and 62 accessions from Asosa regions in Western Ethiopia (Figure 1) were used in this study. The accessions were collected during the 2004 collection mission. In addition, 12 genotypes from the U.S. sorghum breeding programs were also included. There were no specific criteria for selecting these lines except that seven of them are public parent line (B and R) releases that are among the most widely used tester parents in U.S. sorghum improvement programs. The remaining five represent a range of sorghum types developed by different breeding programs in the U.S. Complete list of the accessions is presented in supplementary Table 1.

The Pawe and Asosa regions are part of a geographic stretch in Western Ethiopia that extends from 9°N latitude in the south to 12°N latitude in the north. The region is subdivided into Pawe in the north and Asosa in the south by the Blue Nile River and the surrounding valley is as wide as 40 to 60 km.

The entire 123 accessions were re-coded following the original order of accession number by using a two letter prefix denoting their geographic regions followed by numeric order from 1 to 123. Accordingly, Pawe accessions were coded as PW1 to PW49, Asosa accessions as AS50 to AS111 in the order they were recorded on collection book. Materials from the U.S. were arbitrarily coded US112 to US123. The accessions from Pawe and Asosa represent most of the major races of sorghum including durra, caudatum, guinea and bicolor. The U.S. public lines are results of complex crosses and may combine pedigrees from two or more of these races.

DNA extraction and SSR genotyping

The accessions were planted in the greenhouse at Melkassa Research Center of the Ethiopian Institute of Agricultural Research, Ethiopia. Ten to fifteen seedlings were raised for each accession using small nursery pots of 15 cm diameter and 10 cm height. At 20 days after emergence, tissue samples from 10 seedlings were pooled into a plastic container and the samples were immediately desiccated using silica gel and then vacuum sealed. The desiccated tissues were then shipped to Kansas State University (KSU). At KSU, the tissues were further lyopholized using a freeze dryer (Thermo Savant®, ModulyoD-115) for 3 days. For the U.S. materials, seeds of the 12 genotypes were planted in a 10 cm wide plastic pots filled with pot mix soil. At about 10 days after planting, the tissue were harvested and immediately lypholysed. About 3 g tissue samples from each of the 111 accession and 12 U.S.

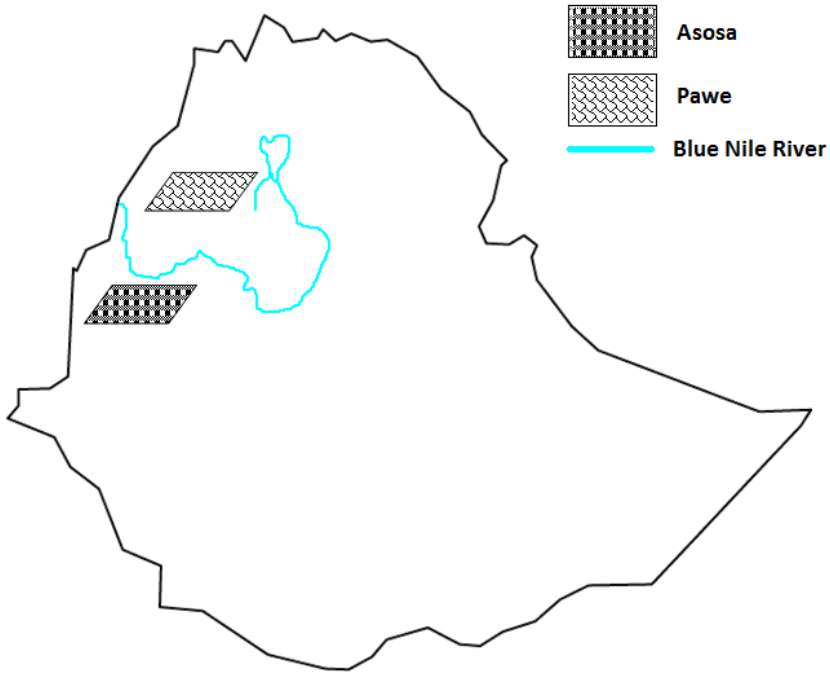

Figure 1. Geographic locations of origin of accessions in Western Ethiopia.

genotypes were pooled into plastic tubes, and the samples ground using tissue grinder, Restch Mixer mill (Retsch® MM 400) for 6 min. The DNA extraction was performed using a modified CTAB protocol (Saghai-Maroof et al., 1984). The DNA pellets were dissolved in 1x TE (10 mM Tris, 1 mM EDTA, pH 8.0) and the final DNA concentration was quantified using a spectrophotometer (NanoDrop®, ND -1000). The working DNA concentration was adjusted to 5 ng/µl for PCR analysis through further dilution with TE buffer.

Thirty simple sequence repeat (SSR) markers were used to genotype the accessions. The markers were selected from published literature based on amplicon sizes, linkage groups, and map positions (Tao et al., 1998; Bhattramakki et al., 2000; Kong et al., 2000). The markers were evenly distributed across all sorghum chromosomes except chromosome 9 (Table 1) and have varying amplicon sizes with the smallest being 145 bp based on Tx623. PCR reactions were performed in MJ Research, PTC-200 PCR thermal cycler with 4 × 96-well plate format. Twenty five nanograms of genomic DNA were used in a 20 µl total volume with a final concentration of 1.5×NH_4 buffer, 0.4 µM each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U/µl Taq DNA polymerase (Bioline, Taunton, Massachusetts). PCR amplification was performed using a touchdown protocol that consisted of one denaturation cycle at 94°C for 4 min, 8 subsequent cycles at 94°C for 1 min with annealing temperatures of 63, 61, 59, and 57°C (2 cycles each) and primer extension at 72°C for 1 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and then a final 7 min extension at 72°C. PCR products were resolved on 3% Metaphore agarose gel using a horizontal electrophoresis apparatus in a TBE buffer loaded with 3% ethidium bromide run at 5 V/cm for 6 h along with a standard DNA ladder (Bioline, Taunton, Massachusetts). A CCD Camera was used to capture the real time gel images under UV illumination projected using Bio-Rad Gel Doc™ XR imaging system. The images were optimized and allele calling was performed using Quantity One software version 4.2.1 (Bio-Rad laboratories, Hercules, CA) using a standard 100 bp molecular weight ladder as a reference. All band sizes were compared and examined for accurate allele calling and non-specific bands were removed. This helped minimizing the ambiguity due to relatively low resolution of agarose gels.

Analyses of population structure and genetic diversity

The data from 23 polymorphic SSR markers were analyzed using specific statistical software to determine population structure and genetic diversity. SRUCTURE software version 2.2.3 (Pritchard et al., 2000a) was used to analyze population structure and group the accessions into different sub-populations. The program performs a model-based quantitative cluster analysis procedure using Bayesian approach which clusters genotypes based on their posterior membership coefficients generated via a maximum likelihood function. Thus, it allows computation of the proportion of the genome of an individual originating from each inferred population (Pritchard et al., 2000b). The number of sub-populations were assumed to be between 2 and 10 (k=2-10), and STRUCTURE was run with 10 iterations for each sub-population (k). The admixture model considering correlated allele frequencies was used with 10,000 replicates for burn-in and 10,000 replicates during analysis. The number of actual sub-populations was decided on the basis of stability of grouping patterns across 10 runs. The optimum k value was chosen to be the k at which Ln (P)D showed the least variation with respect to the number of runs reaching a stable state. Thus, based on the information generated on the simulation summary of the analysis, the optimum sub-population number was determined to be k=3. Out of 10 runs performed for k=3, the results generated for the run with the highest likelihood value was selected to determine the population structure. A composite plot was generated to show the grouping pattern of the whole sample and an expanded bar plot was generated to show the posterior membership coefficients. Individuals were assigned to subpopulations based on sub-population membership coefficient where accessions with

Table 1. Summary of 23 SSR markers used in the study.

Locus	LG[1]	Repeat motifs	Primer sequence (5'to 3')	References[3]	No. of alleles	Major allele freq.	PIC[2]	Allele size (bp)
Xtxp12	4	(CT)22	F: AGA TCT GGC GGC AAC G R:AGT CAC CCA TCG ATC ATC	a	6	0.34	0.72	159-234
Xtxp18	8	(AG)21	F: ACT GTC TAG AAC AAG CTG CG R: TTG CTC TAG CTA GGC ATT TC	a	7	0.39	0.72	233-324
Xtxp32	1	(AG)16	F: AGA AAT TCA CCA TGC TGC AG R: ACC TCA CAG GCC ATG TCG	a	5	0.45	0.55	97-161
Xtxp60	4	(GT)4GC (GT)5	F: GCT AGC TGA CGC ACG TCT CTG R: TGC AAC CGA GCG GTG ACT A	b	4	0.53	0.42	222-252
Xtxp75	1	(TG)10	F: CGA TGC CTC GAA AAA AAA ACG R: CCG ATC AGA GCG TGG CAG G	b	5	0.58	0.55	153-213
Xtxp88	1	(AG)31	F: CGT GAA TCA GCG AGT GTT GG R: TGC GTA ATG TTC CTG CTC	b	4	0.51	0.55	126-170
Xtxp96	2	(GA)24	F: GCT GAT GTC ATG TTC CCT CAC R: CAT TCG TGG ACT CTG TCG G	b	7	0.29	0.71	155-251
Xtxp159	7	(CT)21	F: ACC CAA AGC CCA AAT CAG R: GGG GGA GAA ACG GTG AG	b	5	0.52	0.51	138-208
Xtxp176	6	(AG)4AAC (GA)4	F: TGG CGG ACA TCC TAT T R: GGA GAG CCC GTC ACT T	b	5	0.58	0.45	133-188
Xtxp201	2	(GA)36	F: GCG TTT ATG GAA GCA AAA T R: CTC ATA AGG CAG GAC CAA C	b	7	0.41	0.73	191-284
Xtxp217	10	(GA)23	F: GGC CTC GAC TAC GGA GTT R: TCG GCA TAT TGA TTT GGT TT	b	7	0.63	0.47	136-233
Xtxp218	3	(CA)10	F: CCG GAA AAC CTG CTA CTG R: ACG CCG GAA GGA GAA G	b	5	0.46	0.53	203-254

Table 1. Contd.

Xtxp248	1	(AG)5(GA)28	F: GGG TGT CCA ATG TTG TCT GC R: GGC CGT TAC TGT CCC TTA CTC A	b	6	0.54	0.58	214-295
Xtxp297	2	(AAG)24	F: GAC CCA TAT GTG GTT TAG TCG CAA AG R: GCA CAA TCT TCG CCT AAA TCA ACA AT	b	9	0.46	0.54	183-286
Xtxp303	5	(GT)13	F: AAT GAG GAA AAT ATG AAA CAA GTA CCA A R: AAT AAC AAG CGC AAC TAT ATG AAC AAT AAA	b	4	0.47	0.57	139-192
Xtxp304	2	(TCT)42	F: ACA TAA AAG CCC CTC TTC R: CTT TCA CAC CCT TTA TTC A	b	11	0.6	0.59	141-306
Xtxp312	7	(CAA)26	F: CAG GAA AAT ACG ATC CGT GCC AAG T R: GTG AAC TAT TCG GAA GAA GTT TGG AGG AAA	b	7	0.39	0.62	137-230
Xtxp319	1	(TC)17	F: TAG ACA TCT GAA TTA AGG AGC R: CAT GCC CCT GAA AGA GA	b	6	0.47	0.54	145-239
Xtxp327	4	(TAG)3+ (GA)22	F: ACC ACT GCT CAC GCT CAC R: GCG GTG TAC AGC TTC GTC	b	8	0.74	0.42	132-216
Xtxp340	1	(TAC)15	F: AGA ACT GTG CAT GTA TTC GTC A R: AGA AAC TCC AAT TAT CAT CCA TCA	b	4	0.58	0.46	191-231
Xtxp354	8	(GA)21+ (AAG)3	F: TGG GCA GGG TAT CTA ACT GA R: GCC TTT TTC TGA GCC TTG A	b	5	0.68	0.46	148-196
Xgap1	10	(AG)16	F: TCC TGT TTG ACA AGC GCT TAT A R: AAA CAT CAT ACG AGC TCA TCA ATG	c	6	0.3	0.75	217-300
Xgap42	1	(AG)26	F: TTT TCC TCT TTC AGA TAA CCG TA R: CCC ACC AAG GGC ATC	b	5	0.48	0.57	138-215
Average					6	0.5	0.67	-

LG[1], Linkage group; PIC[2], polymorphic information content. References[3] a= Kong et al. (2000), b= Bhattramakki et al. (2000), c= Tao et al. (1998).

membership coefficient less than 0.8 for all k were considered to result from admixture, hence classified as "admixed".

Further genetic analyses was performed using Power-Marker software version 3.25 (Liu and Muse, 2005). Gene diversity, genetic distance, allelic variability, number of alleles per locus, major allele frequency and polymorphic information (PIC) of markers were determined. The gene diversity analysis provides an unbiased estimation of genetic variation at any given locus. Genetic distance matrix was generated from the marker data set using the method described by Nei (1972) in PowerMarker. Relationship between accessions was then displayed by constructing a Neighbor Joining (NJ) tree using Interactive Tree Of Life v2 (iTOL) (Letunic and Bork, 2011) through

Table 2. Summary statistics for the three major clusters (C1 through C3) detected through neighbor joining tree analysis based on 23 SSR markers.

Population/Marker parameters	C1 (Pawe)	C2 (Asosa)	C3 (USA)	Total
Sub-population size	49	62	12	123
Allele number	130	127	97	144
Number of alleles per locus	5.65	5.52	4.22	6.00
Major allele frequency	0.45	0.44	0.50	0.50
Gene diversity	0.67	0.66	0.63	0.71
Polymorphic information content	0.62	0.61	0.58	0.67
Genetic distance between clusters		C1 and C2		0.69
		C1 and C3		0.72
		C2 and C3		0.70

employing a model-free hierarchical clustering procedure. The results generated through STRUCTURE were used to color-code the neighbor-joining tree. Based on the clustering pattern displayed by the NJ tree, separate genetic analyses within and between clusters was performed using PopGen32 software (Yeh et al., 1997). Principal Component Analysis (PCA) was conducted using XLSTAT (Addinsoft™ version 2012.1, 2009) and the three dimensional plot showing association between accessions was generated.

RESULTS

Genetic variability among accessions

Of the total of 30 SSR markers used to genotype the accessions, 23 markers showed polymorphism and were used in all analyses performed. The remaining 7 markers were not polymorphic and hence were excluded. The 23 markers generated a total of 144 distinct alleles across 123 accessions with an average of 6 alleles per locus. The average major allele frequency, PIC and mean genetic diversity were 0.50, 0.67, and 0.71, respectively (Table 2). The NJ tree analysis grouped the accessions into three broad sub-clusters primarily based on geographic patterns. Majority of the Pawe accessions were separated and pooled into one large cluster (C1), while accessions from Asosa grouped into a different cluster (C2). Two accessions each from Pawe (PW48 and PW49) and Asosa (AS73 and AS97) showed swapping of group members with PW48 and PW49 included in Asosa cluster, and AS73 and AS97 included in Pawe cluster (Figure 2). All of the seven U.S. breeding lines and five adapted genotypes were distinctly sorted and grouped into a separate cluster (C3). However, both Pawe (C1) and Asosa (C2) clusters were further split into smaller sub-clusters. The Asosa cluster was further divided into C2-1, C2-2, and C2-3 sub-clusters consisting of 26, 16, and 19 individuals, respectively, with one of the Pawe accessions PW48 sitting in the middle of C2-1 and C2-2. Similarly, Pawe accessions were apparently split into two sub-clusters C1-1 and C1-2 consisting of 26 and 23 accessions, respectively. Although no geographical coordinates were available for the collections, the pattern of numbering of the accessions which indicates the relative proximity of the accessions within a given geography also provides a clue. Accordingly, the tip branches of C2-1 sub-cluster primarily consisted of accessions AS50 to AS60 along with AS92 and 93 and the next main branch carried AS61-AS67 with few other accessions (AS85&88, AS92&93, PW48&49, AS54, 68, and 69) occurring as smaller branches or scattered in the sub-cluster. The next sub-cluster C2-2 consisted primarily of accessions AS74 to AS87 and C2-3 consisted of accessions AS94 to AS111 with few other accessions outside these number orders occasionally grouping with these sub-clusters. Likewise, the C1 cluster consisted of two sub-clusters. All of the 26 accessions grouped in this sub-cluster (C1-1) are from Pawe. Consecutive accessions PW1 to PW24 were grouped in this sub-cluster along with PW46&47 that are outside this code range. Sub-cluster C1-2 has two main branches with the ordering of accessions on these sub-branches again providing evidence on the importance of geography. Accessions PW25-34 occupied the first branch and the remaining PW35- 45 converged on the second branch except the first branch also consisted of two Asosa accessions (AS73&AS97) that were pulled off C2 (Figure 2).

Within region diversity was relatively high with number of alleles per locus for the Pawe, Asosa, and U.S. clusters being 5.65, 5.52, and 4.22, respectively, but were lower than 6, the record obtained among the entire group of accessions (Table 2). Diversity within region was higher for Pawe and Asosa accessions (0.67 and 0.66) as compared to 0.63 among the U.S. materials while major allele frequency was higher for the U.S. cluster (0.5) and lower for Pawe (0.45) and Asosa (0.44) (Table 2). Genetic distance among clusters was the highest between the U.S. cluster and Pawe (0.72) and the smallest between Asosa and Pawe clusters (0.69). The

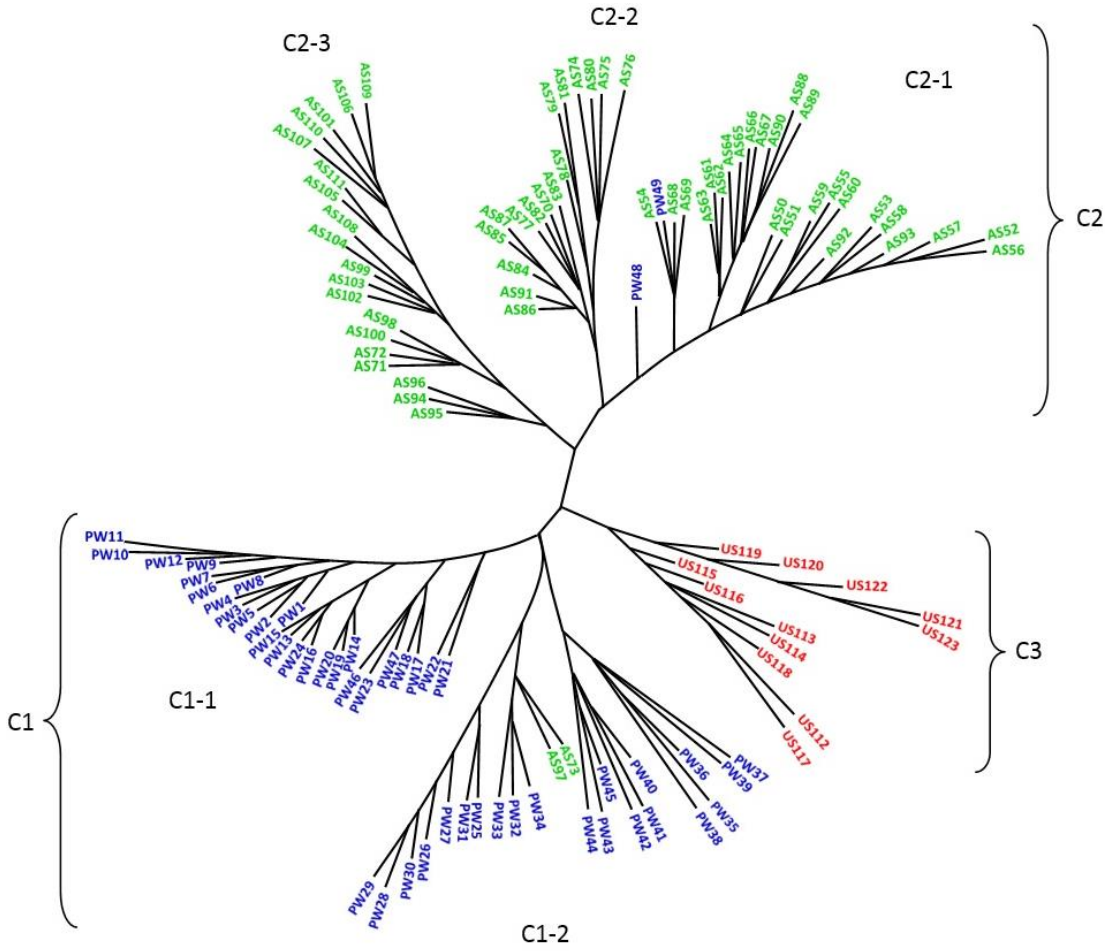

Figure 2. Neighbor-joining tree showing clustering pattern of accessions into three geographies. The accessions are color coded based on collection region, Pawe (Blue), Asosa (Green) and United States (Red).

distance between the U.S. cluster and Asosa was 0.70 (Table 2). The average PIC values for C1, C2, and C3 (0.62, 0.61, and 0.58, respectively) were in agreement with the within cluster diversity.

Population structure and PCA

The population structure analysis sorted the U.S. materials from the Ethiopian accessions (Figure 3). Although the accessions were grouped into three different sub-populations (SP1 through SP3) represented in red, green, and blue, respectively (Figure 3), over 70% of the accessions from Pawe and Asosa were admixed. This differs from the results from NJ tree analysis, except for sub-population 3 (SP3) from the STRUCTURE matched C3 from NJ analysis which included all U.S. materials and one accession from Pawe that was grouped with SP3.

The PCA transformed the genotypic information to reduce the markers to a set of principal components (axis) with the first three principal components (PC1, PC2, and PC3) jointly accounting for 47.78% of the total variation (Figure 4). In agreement with both the STRUCTURE and PowerMarker analysis, all of the twelve breeding lines from United States were adequately separated from the rest of the accessions in the population when viewed in the three dimensional space. The remaining population was aggregated as one large cluster with genotypes from the two regions (Pawe and Asosa) showing an overall pattern of dispersal towards opposite directions, but with large overlaps due to considerable number of admixed individuals. This is consistent with the results of STRUCTURE analysis where accessions from Pawe and Asosa showed marked admixture based on the membership coefficients on the y-axis of Figure 3 which presents the percentage representation of accession in each sub-population.

DISCUSSION

The advent of molecular marker technology has introduced a powerful tool for analysis of genetic variability.

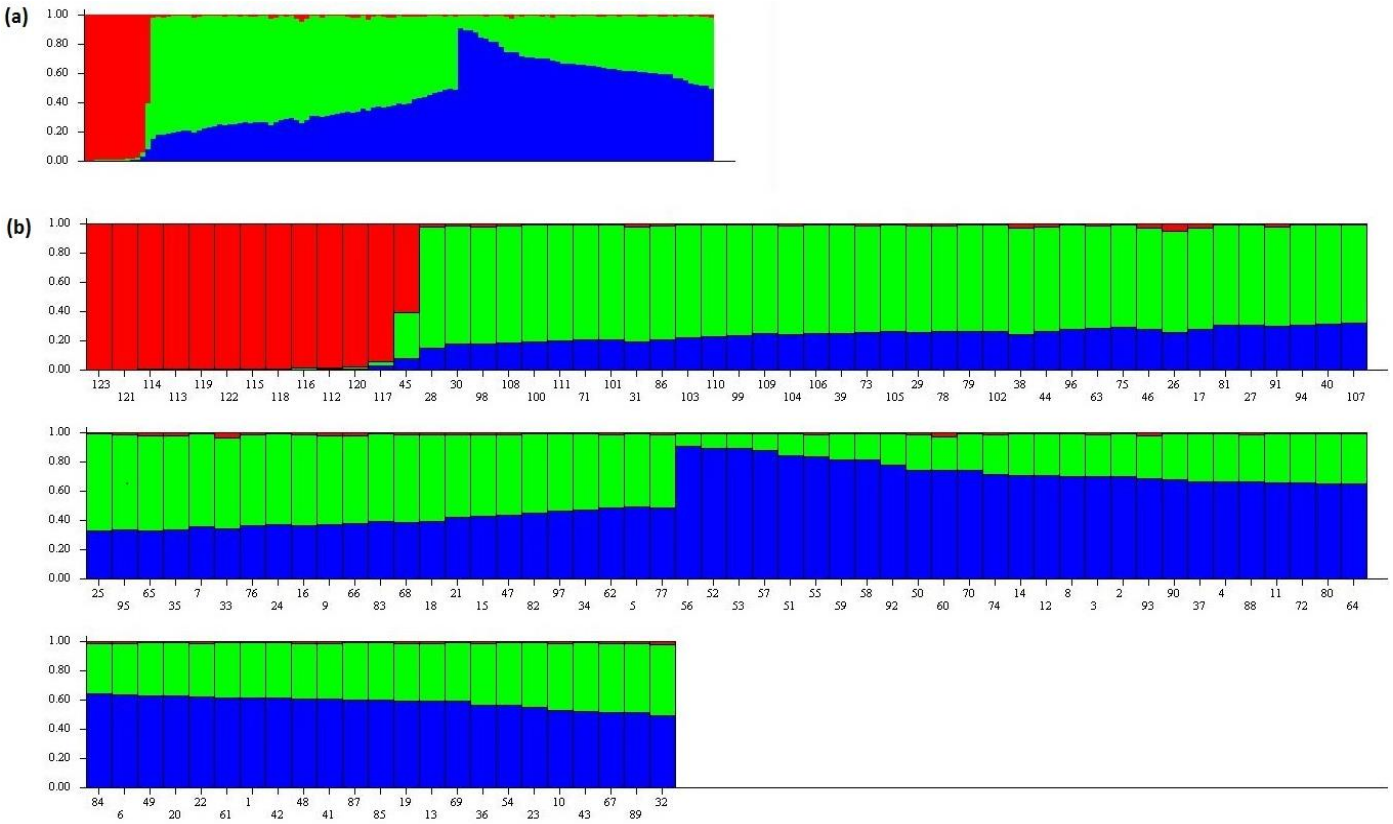

Figure 3. Graphic outputs of the STRUCTURE analysis depicting three sub-populations: (a) Composite display of sub-populations with probability of accessions assigned to a given group shown by the membership coefficients on the Y-axis; (b) Extended display of grouping of accessions into three sub-populations with the X-axis representing the accessions and the Y-axis showing membership coefficients. Red bars represent sub-population group exclusively made up of U.S. materials (SP1); Green (SP2) and blue (SP3) bars are mixture of accessions from Pawe and Asosa showing significant admixture.

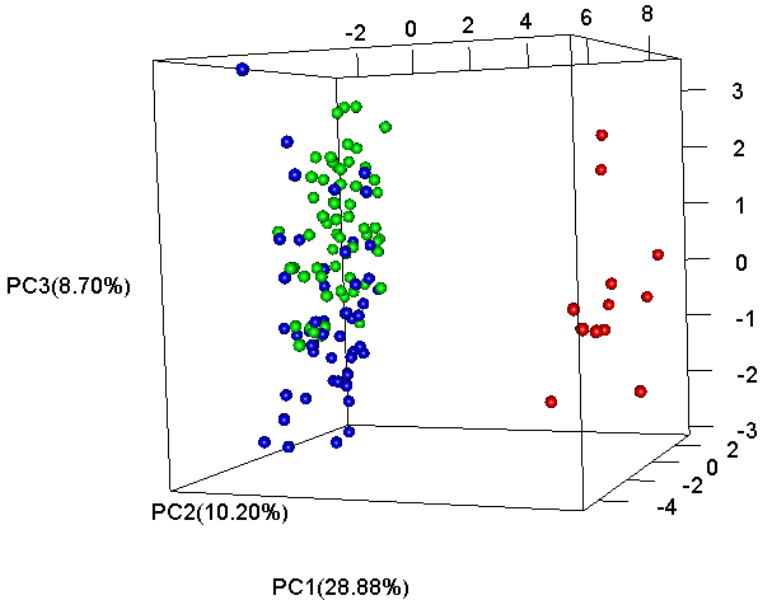

Figure 4. The principal component analysis scatter plot showing aggregation of accessions as color coded by geographical origins, Pawe (Blue), Asosa (Green) and United States (Red).

Variations that are obscured from human eyes can now be resolved, and genotypes can be differentiated based on variations present at genome level. The SSR markers are among the first group of marker systems invented that were based on PCR amplification. Though SNP markers are becoming more popular due to lower cost per data point and larger genome coverage, SSR markers are still widely utilized because of their abundance, co-dominant nature and ease of use as they require less sophisticated equipment and computation methods (Röder et al., 1995; Gupta and Varshney, 2000; Menz et al., 2002; Ellis and Burke, 2007; Varshney et al., 2013). SSR markers also reveal more diversity than most marker systems (Van Inghelandt et al., 2010). In sorghum, numerous SSR markers have been discovered that are linked to functional genes affecting economically important traits such as drought tolerance, *Striga* resistance, and cold tolerance (Crasta et al., 1999; Xu et al., 2000; Knoll and Ejeta, 2008; Burow et al., 2010; Satish et al., 2012) and thus have immediate application in marker based or marker assisted breeding.

In the current study, a total of 30 SSR markers were used to genotype 111 sorghum accessions collected from Western Ethiopia and another 12 U.S. adapted sorghum genotypes. A total of 144 alleles were resolved by 23 polymorphic markers with an average of 6 alleles per locus. Depending on the number of markers used and diversity of the population, previous studies in sorghum have reported 3.2 to 10.4 alleles per locus (Wang et al., 2006; Ali et al., 2008; Wang et al., 2009). In view of this, the allele diversity observed in the current study is intermediate and an average genetic diversity of 0.71 is comparable to many similar studies in sorghum and other species. Given the ecological similarity between the two regions (Asosa and Pawe) where the accessions come from the level of differentiation observed was significant.

Pawe and Asosa are the major sorghum producing areas in the far western part of Ethiopia. The two areas have similar weather patterns (high rainfall, warm temperature, and high humidity) and biotic and abiotic elements. But the areas are geographically disjointed by the 40 to 60 km wide Blue Nile river valley which interrupts sorghum production continuum in the region. This and the diversity in ethno-culture of the people between the two regions, differences in food habits and limited opportunity for interaction due to geographical and infrastructural barriers, the two areas remain fairly separated perhaps limiting movement of germplasm between the two regions. Thus accessions growing in the regions shaped by both natural and man-made circumstances peculiar to the specific regions may have resulted in some degree of genetic differentiation between accessions from the two regions. The present study seems to have shed light on these differences. The neighbor joining tree developed based on allelic variation among the accessions clearly sorted the population into three major clusters (Figure 2) with the U.S. adapted materials (C3) distinctly grouped in a separate cluster and accessions from Western Ethiopia sorted into two major clusters, C1 and C2, largely according to their geographic region with C2 consisting of mainly Asosa collections and C1 harboring that of Pawe. The two sub-groups seem to have further split into smaller sub-units perhaps based on within region geographic proximity or differences in ethnography between people in the two regions.

Population structure analysis run on arbitrary sub-population groupings of k=2 to10 also identified three sub-populations (k=3) as the optimal number of groups. However, sub-populations representing Pawe and Asosa accessions show significant admixture (green and blue bars in Figure 3), while U.S. adapted materials were clearly sorted as shown by red bar in Figure 3. It appears that despite presumed isolation, environmental factors such as rainfall, temperature, and biotic stress factors common to the two regions have played significant role in shaping natural selection, particularly toward well-adapted traits as is evident from significant admixture of alleles between the two regions. At the same time, selection for different plant attributes driven by differences in culture and food habits exercised by communities in these regions and limited movement of people between the two regions may have influenced the population structure to a certain degree. This was particularly evident from the NJ tree analysis where Pawe and Asosa accessions were clearly sorted into different groups with each having their own sub-groups. Nevertheless, accessions from the two regions were closely related to each other. This was evident from the high degree of admixture between accessions from the two regions (Figure 3a and b). It is also clear from genetic distances estimated among the clusters where both C1 (Pawe) and C2 (Asosa) clusters were found to be more distant from C3 (U.S. materials) than from each other. This shows that despite the presumed disruptive selection, accessions from the two regions tend to resemble each other more than they do to the U.S. materials, perhaps due to a common natural selection pressure. This implies that accessions from Pawe and Asosa may harbor shared alleles (admixed) that are perhaps responsible for adaptation to factors common to both environments including resistance to grain mold and leaf diseases.

Past efforts by the international sorghum research community working to improve sorghum production in Africa have successfully generated elite varieties and germplasm into which numerous desirable traits have been integrated. Many of the materials were proven useful in several countries in Africa while many others either completely failed to adapt to certain environments such as Western Ethiopia or not accepted by communities, because they do not fit to local production conditions or are not suitable for local food processing. The presence of significant genetic variability among collections representing such isolated geography such as

those in the current study, however, has significant value in that it can be effectively exploited to develop new varieties that are adapted to such specific environments or possess specific attributes needed by local communities. But these materials can also be used as sources of new alleles and can be equally useful to the global sorghum improvement efforts. Though this study did not include materials from other regions of the country, repeated failure of varieties and landraces from outside this region and the clear segregation of the accessions from the U.S. adapted genotypes observed in this study provide hints about the peculiarity of these materials. Moreover, although not widely represented in the global sorghum germplasm, materials from these regions have been long recognized as useful sources of disease resistance and grain quality such as the Zera-Zera sorghums of Gambella (Prasada Rao and Mengesha, 1981; Reddy et al., 2000; Thakur et al., 2006).

Although, there is no racial classification data for the accessions, field observations indicate that they are of multiple racial groups. However, unlike other parts of the country that are dominated by the durra race, sorghums of this region appear to be largely made up of caudatum and caudatum-guinea hybrids and their derivatives (Prasada Rao and Mengesha, 1981). But the grouping of the populations into clusters and sub-clusters seem to be largely influenced by geographic origin, while earlier studies have implicated racial ancestry as the major factor influencing the pattern of genetic diversity (Tao et al., 1993; Cui et al., 1995; de Oliveira et al., 1996). The lack of racial differentiation in this study may be due to absence of association between genes controlling panicle morphology that are often used to classify genotypes into racial groups (Snowden, 1936) and the markers used in this study. Nevertheless, several other studies conducted on sample sets representing global sorghum germplasm also grouped genotypes according to geographic origin or breeding history rather than race (Tesso et al., 2005; Billot et al., 2013; Lekgari and Dweikat, 2014). Moreover, studies conducted in other species also agree with our results. Genetic structure among common self-pollinated field crops including wheat (Zhang et al., 2009; Vanzetti et al., 2013), barley (Naeem et al., 2011), and horticultural crops such as grape (Bacilieri et al., 2013) as well as pathogen species of economically important crops such as oil-seed brassica (Strehlow et al., 2014) have been shown to be strongly influenced by geographic adaptation.

Owing to the extreme variation in ecological habitats under which the crop is cultivated, sorghum genetic diversity in Ethiopia appears to be compartmentalized according to regional climatic variables. The Western Ethiopia region harbors sorghum types that are resistant to leaf and panicle diseases and produce high grain quality crop despite the warm weather and very high humidity. But in contrast to the common view that materials from the region were derived from narrow genetic base, at least based on the polymorphic loci examined in this study, the accessions represent broad genetic pool similar to those collected from other parts of the country. In view of the failed attempts to adapt varieties developed elsewhere to the Western Ethiopia region, the diversity in the local collection observed in this study can serve as primary resource for developing new varieties that suit the region or as sources of new alleles for global sorghum breeding programs. Nevertheless, the recent introduction of large multi-national agricultural companies into the region is risking displacement of the traditional crops including sorghum by export crops such as rice which eventually will pose threat to this unique genetic resource. Urgent action is needed to collect and protect the materials from certain loss.

Conflict of Interests

The authors have not declared any conflict of interests

ACKNOWLEDGEMENTS

The authors are thankful to the Global Crop Diversity Trust for financial support and the Ethiopian Institute of Agricultural Research, Oromia Agricultural Research Institute, and Kansas State University for providing field and laboratory facilities used for conducting the study. This work is contributed by Kansas Agricultural Experiment Station, contribution No. 2015-188-J.

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Vol. 15(23), pp. 1159 -1167, 8 June, 2016
DOI: 10.5897/AJB2015.15011
Article Number: DF8CBA358800
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimal concentration of selective agents for inhibiting *in vitro* growth of *Urochloa brizantha* embryogenic calli

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Received 29 September, 2015; Accepted 8 April, 2016

The ability to distinguish transgenic cells of untransformed cell mass is a key step for the production of transgenic plants. Thus, the use of selection marker genes for identification of genetically modified plants is necessary. The aim of this study was to determine the optimal concentration of four selective agents (kanamycin, hygromycin, phosphinothricin and mannose) to inhibit *in vitro* growth of *Urochloa brizantha* cv. Marandu calli. Embryogenic calli were obtained from mature seeds inoculated in MS medium supplemented with 30 g/L sucrose, 3 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 300 mg/L hydrolysate casein and their growth rate was monitored for 74 days by measuring calli fresh weight. It was demonstrated that *U. brizantha* calli are more sensitive to low concentrations of hygromycin than kanamycin (25 and 50 mg/L, respectively). For the herbicide phosphinothricin, 5 mg/L was enough to prevent the calli growth, but allowed escape. Mannose should be used as the only carbon source on the plant tissue culture medium. All selective agents tested here, in the appropriate concentration, could be used in experiments aiming to produce transgenic signal grass. However, mannose selection might reduce environmental concerns about gene flow and development of herbicide resistance in escaped *Urochloa* populations.

Key words: Signal grass, transformation, marker genes, selection.

INTRODUCTION

A number of steps are required for producing transgenic plants, such as the introduction of DNA into cells, identification or selection of cells that have the exogenous DNA integrated into the plant genome and regeneration of the transformed plant cells. Due to the low efficiency of transgene integration, selectable marker genes (SMGs) are routinely used to differentiate transformed cells from a population of untransformed cells, and are typically co-transformed with the gene of interest. Among the commonly used SMGs are those that confer tolerance to antibiotics or herbicides (Ji et al., 2013). Genes providing resistance to these compounds are known as negative selectable markers and have been used to kill or reduce the population of non-transgenic cells (Puchta, 2003).

The SMGs that confer resistance to antibiotics are involved in bacterial detoxification systems and are distinct enough from plant processes, so the interactions

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between the SMGs and the co-processing genes are unlikely (Miki and McHugh, 2004). Aminoglycoside antibiotics include a number of molecules that are very toxic to plant, animal and fungal cells by binding to the ribosomal subunits and inhibiting protein synthesis in eukaryote plastids and mitochondria. The bacterial neomycin phosphotransferase II (NPTII) has been shown to be very effective as a selectable marker in mammalian and yeast cells, and in plants (Miki and McHugh, 2004; Padilha and Burgos, 2010). Currently, 45 events of genetically modified plants have been approved for commercial release containing the *nptII* gene including: oilseed rape, corn, potato, tomato, flax, chicory, papaya, melon, plum, zucchini, sugar beet, rose, tobacco and cotton (CERA, 2015). No risk for humans, animals or on the environment has been related to using NPTII or the *nptII* gene (Fuchs et al., 1993).

Hygromycin is also an antibiotic inhibitor of protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes. The *Escherichia coli* gene aphIV (*hph*, *hpt*), coding for hygromycin B phosphotransferase, confers resistance on bacteria, fungi, animal cells and plant cells by detoxifying hygromycin (Waldron et al., 1985). In plants, this antibiotic is very toxic and has been applied in transformation procedures for various monocot tissues (Sharma et al., 2005).

Amino acid biosynthesis pathways are also a target for selective agents in distinguishing transgenic from non-transgenic events. An example of marker genes used for monocotyledons transformation comprises, respectively, of the *bar* and *pat* genes from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, which confer resistance to the herbicide phosphinothricin (PPT), also known as ammonium glufosinate (Thompson et al., 1987; Strauch et al., 1988). This herbicide inhibits glutamine synthetase, a key enzyme in nitrogen assimilation, causing ammonia accumulation, damage of cell membranes and inhibition of photosynthesis and, eventually, plant death.

Among the alternative methods to produce transgenic plants without the use of antibiotic or herbicide marker genes are the so-called positive selection systems, which are defined as those that allow the growth of transformed tissues (Joersbo et al., 1998; Miki and McHugh, 2004). In this system, substances that are not normally metabolized by plants are used as selective agent– for example, the carbohydrate mannose. The *manA* gene from *E. coli*, which codes the phosphomannose isomerase enzyme (PMI, E.C. 5.3.1.8) converts mannose-6-phosphate into fructose-6-phosphate, so transformed plant cells can assimilate mannose via glycolysis while non-transformed cells cannot metabolize this carbohydrate (Reed et al., 2001). The selective mode of action of this system has been suggested to be mediated by the Pi sequestration by phosphorylating mannose into mannose-P (Brouquisse et al., 2001) and/or by the inability of the plant cell to utilize mannose as a carbon source (Stoykova and Stoeva-Popova, 2011). It has been demonstrated that the use of the *manA* gene enhanced the efficiency of transformation of monocots as compared to traditional selection on herbicide containing medium (Wright et al., 2001). The *manA* gene has successfully been applied as a selectable marker in plant transformation for several dicot and monocot plants including wheat and maize (Wright et al., 2001), sorghum (Gurel et al., 2009), oil palm (Bahariah et al., 2013), sugarcane (Zhang et al., 2014) and rice (Gui et al., 2014), among others.

In addition to the choice of the appropriate SMG, the establishment of its correct concentration on the culture media is a very important step in the transformation process. Low concentrations of SMGs may allow escapes to regenerate, whereas too high concentrations impose a stringent process capable of killing the transformed plants expressing moderate levels of resistance (Ijaz et al., 2012). Therefore, the optimum concentration of selective agents has to be determined a priori by testing a variety of concentrations in the laboratory.

The *Urochloa* genus belongs to the Poaceae family, which also covers important species such as rice, wheat and maize, that together account for about half the world's food production (Bennetzen and Freeling, 1993). Although significant research progress has been made concerning *in vitro* plant regeneration and genetic transformation in grasses (Giri and Praveena, 2015), this has not been the case for *Urochloa* species. Transient expression of glucuronidase gene (*gus*) under several heterologous promoters has been first reported in *U. brizantha* (signal grass), however, no transgenic plant was regenerated (Silveira et al., 2003). The only report that describes the regeneration of *Urochloa* transgenic plants used a genotype of *U. ruziziensis* (congo grass). In that study, a vector containing the *bar* and *gus* genes, the former conferring resistance to phosphinothricin was introduced into embryogenic callus by particle bombardment, but only two transformed plants were regenerated (Ishigaki et al., 2012).

Recently, our group published a paper that described an improvement of the protocol for *in vitro* regeneration of different *Urochloa* species (Takamori et al., 2015). Here, we reported results of the optimal concentrations of four selective agents (kanamycin, hygromycin, phosphinothricin and mannose) to restrict the *in vitro* growth of *Urochloa brizantha* cv. Marandu embryogenic callus as a part of the establishment of a transformation system for a recalcitrant species like signal grass.

MATERIALS AND METHODS

Plant material and callus induction media

Mature seeds of *U. brizantha* cv. Marandu were used as initial explants for callus induction. First, the seeds were scarified by immersion in concentrated sulfuric acid in a glass Becker and mixed with glass rod for 15 min. The seeds were then rinsed in running water to remove the acid and dried at room temperature. The scarified seeds were manually peeled and sterilized by immersion

in 70% ethanol (v/v) for 5 min and in sodium hypochlorite 5% (v/v) containing 3 drops of Tween 80™ per 20 min, followed by 5 rinses in autoclaved double distilled water.

The medium for inducing callus (MIC) was composed of MS salts (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 3 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 300 mg/L hydrolysate casein, and solidified with 8 g/L agar. The media pH was adjusted to 5.8 ± 0.1 and autoclaved for 20 min at 121 ± 1°C. Ten seeds were inoculated per Petri dishes and the plates were kept in the dark at 25 ± 1°C. The calli were subcultured into fresh medium every 14 days and maintained under the same conditions.

Determination of the optimal concentration of selective agents

After 35 days of seed inoculation, the pro-embryogenic calli were transferred to the MIC medium containing different concentration of the selective agents as follows: 0, 25, 50 and 100 mg/L for the aminoglycoside antibiotics kanamycin and hygromycin, and 0, 5, 10, 20 and 40 mg/L for the herbicide phosphinothricin. In the case of mannose as the selective agent, the calli were cultivated in the MIC media containing various concentrations of mannose as the sole carbon source, or in combination with sucrose, in the following mixtures: 0:30; 10: 20; 15:15; 20:10; 30:0 g/L of mannose: sucrose. The Petri dishes were kept in the dark at 25 ±1°C.

After 30 days, the calli were weighed and transferred to MS media without 2,4-D and casein, supplemented with 30 g/L sucrose and the corresponding concentrations of kanamycin, hygromycin, phosphinothricin and Mannose:sucrose. All petri dishes were kept under light (30 µmol/m^2/s^1) with photoperiod 16/8 (light/dark) for 14 days (44 days under selection). After this period, they were weighed again and subcultured into half strength MS salts supplemented with 2 mg/L benzyladenine and kept under the same light conditions for 30 days (74 days under selection), when the last weighing was done on a precision scale. Callus relative growth rate was determined on a fresh weight basis according to the formula: [(initial weight - final weight / initial weight)] (Dennehey et al., 1994).

Statistical analysis

All the experiments were composed of a control (without selective agent) and different concentrations of the selective agents. The treatments were arranged in a completely randomized design with six replicates, each replicate consist of a Petri dish with six calli (150 mg each). The experiments were repeated three times.

Raw data were subjected to analysis of variance (ANOVA) to detect significant differences between means. Mean separation was conducted by Tukey's test ($p < 0.05$) using the statistics software SISVAR Version 5.3 (Ferreira, 2011).

RESULTS AND DISCUSSION

Calli of *U. brizantha* cv. Marandu were induced from scarified mature seeds. After 35 days in induction medium (MIC), the calli were weighed and subcultured onto media containing different concentrations of the selective agents (kanamycin, hygromycin, phosphinothricin or mannose: sucrose combinations).

Mature seeds of *U. brizantha* are known to be good explant source for callus induction. Explants cultured on modified MS medium containing 2,4-D produced embryogenic callus, characterized by whitish globular structures surrounded by friable calli (Takamori et al., 2015). Calli maintained in medium without the addition of the selective agents (control treatment) increased their mean fresh weight as much as 5 times (724 mg fresh weight) over that of the initial value at the time of inoculation in the MIC medium at the end of the experiment.

Antibiotics

In order to determine the optimal inhibitory concentration of the aminoglycoside antibiotics, kanamycin and hygromycin, the growth of *U. brizantha* cv. Marandu calli was assessed at 30, 44 and 74 days after inoculation in the different media (Figure 1A and B).

In medium with kanamycin, there was a progressive restriction in callus growth up to the concentration of 75 mg/L. After 74 days under selective conditions, the growth reduction caused by kanamycin was 37, 57 and 66% relative to the control at the concentrations of 25, 50 and 75 mg/L, respectively (Figure 1A). There were no significant difference in the magnitude of calli growth reduction between the higher concentrations (75 and 100 mg/L) of kanamycin. Treatment of *U. brizantha* callus with increasing concentrations of kanamycin produced a progressive darkening in color from white to pale-yellow. After 44 days under selection at the concentration of 25 mg/L kanamycin, some albino shoots with purple pigmentation on the leaves were visible, yet any of these chlorotic shoots were able to further elongate and regenerate into plants. There was no shoot formation at any other kanamycin concentration tested (Figure 4A).

In contrast, the presence of hygromycin in the medium caused a dramatic reduction on growth of *U. brizantha* calli already at 30 days of selection. Even at the lowest concentration of hygromycin (25 mg/L), a severe callus growth reduction (66.5%) was observed as compared to the control cultures. There was no significant differences in callus growth among all hygromycin treatments (Figure 2B), as no further increase in the concentration elicited any greater response. There was no visual morphology of calli among the hygromycin concentrations (Figure 4).

Antibiotics are extensively used as a selection agent from the beginning of plant transformation. The popularity of these selection systems is reflected on the efficiency, availability and applicability of their use across a wide range of plant species and its regenerative efficacy in plant tissue culture systems (Sundar and Sakthivel, 2008).

The susceptibility to antibiotics varies among species, genotypes and explant source (Padilha and Burgos, 2010). Generally, dicotyledonous plants are most sensitive to kanamycin than monocots. For example, a low concentration of kanamycin (50 mg/L) allowed the regeneration of transgenic adventitious buds from epicotyl sections of the citrus rootstock, Swingle citrumelo

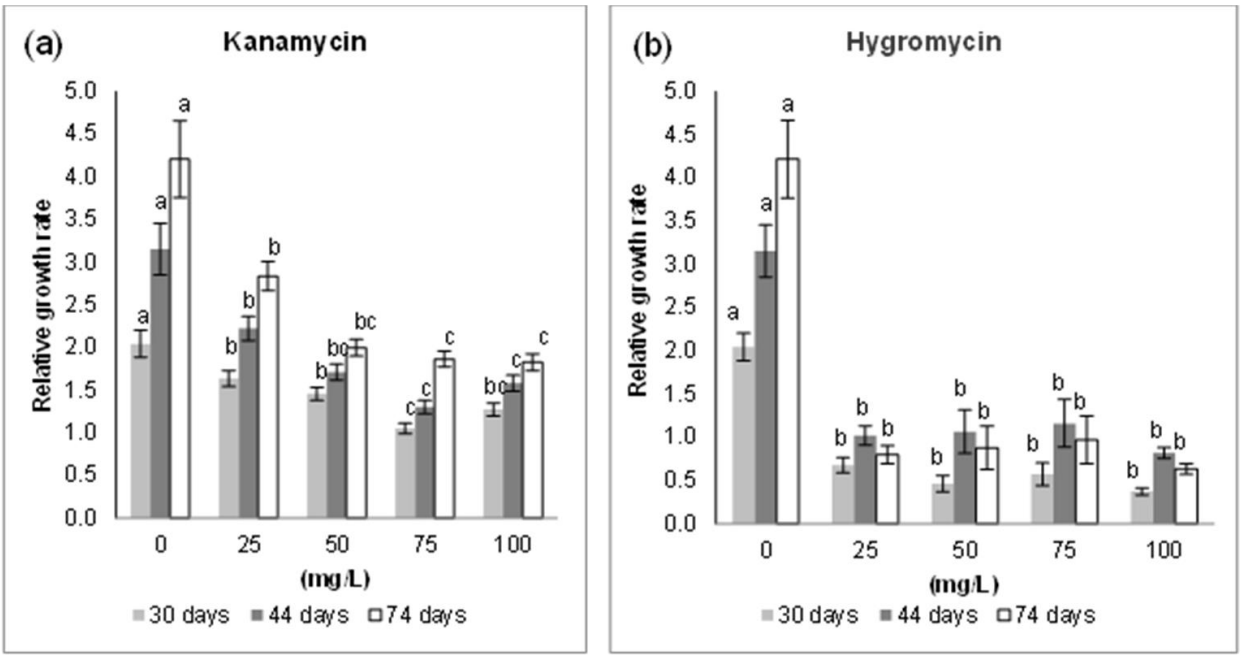

Figure 1. Relative growth rate of *U. brizantha* Marandu calli in medium containing different concentrations of kanamycin (A) and Hygromycin (B) over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

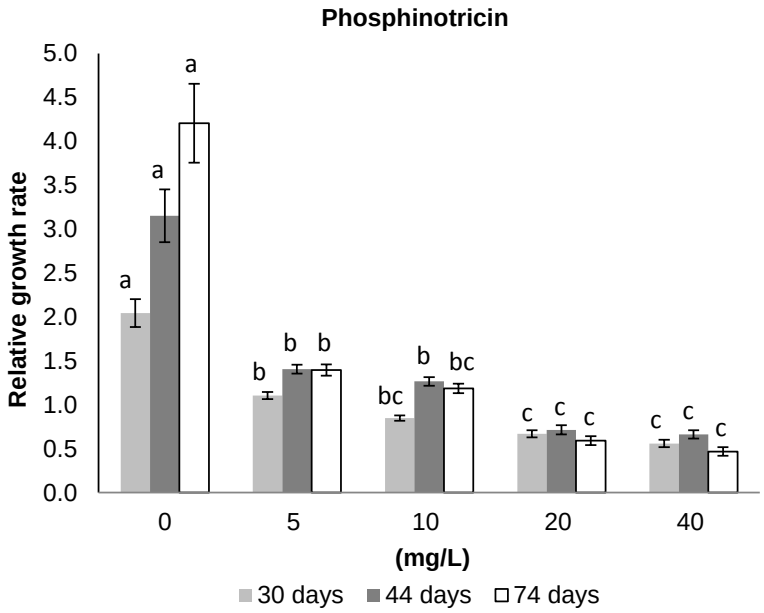

Figure 2. Relative growth rate of *U. brizantha* cv. Marandu calli in medium containing different concentrations of glufosinate ammonium over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

transformed via *Agrobacterium* (Molinari et al., 2004). Even lower concentration of kanamycin (20 mg/L) was used to recover transgenic shoots of *Jatropha curcas* (Pan et al., 2010).

In contrast, some monocots such as *Triticum monococcum*, *Panicum maximum*, *Pennisetum americanum* and a hybrid between *Pennisetum americanum*, *Pennisetum purpureum* and *Pennisetum*

squamulatum have been shown to be resistant to kanamycin selection requiring high antibiotic concentrations (800 mg/L) to inhibit 30% of growth as compared to the control (Hauptmann et al., 1988). Despite that, the antibiotic kanamycin, together with the selective marker gene neomycin phosphotransferase (*nptII*), has allowed high frequency recovering of transgenic monocots (Cheng et al., 2003; Liu et al., 2007; Gasparis et al., 2008; Liu and Goodwin, 2012). In this work, kanamycin at 50 mg/L appears to be sufficient to restrict the calli growth in *U. brizantha* cv. Marandu. This result was similar to those obtained for Caucasian bluestem (*Bothriochloa ischaemum*), a warm-season perennial grass in which calli growth was not completely suppressed but considerably reduced at the concentration of 50 mg/L kanamycin (Franklin et al., 1990).

The selectable marker gene hygromycin phosphotransferase (*htp*) is also reported to be suitable for selection of monocot transformants (Hiei and Komari 2008; Ozawa 2009) and are commonly used when *nptII* is ineffective (Miki and McHugh 2004). The growth rates of *U. brizantha* cv. Marandu calli in medium containing different concentrations of hygromycin were dramatically reduced. The concentration of 25 mg/L hygromycin was sufficient to restrict the calli growth. Similar data were reported by Ramamoorthy and Kumar (2012), who demonstrated that low concentrations of hygromycin (25 and 50 mg/L) were sufficient to restrict cell proliferation of calli of *Panicum virgatum* as compared to the control. On the other hand, another report showed that all *P. virgatum* plants regenerated on selective medium containing 25 mg/L of hygromycin escaped. Only a concentration of 75 mg/L was able to select transgenic events with the *hptII* gene (Xi et al., 2009). In maize, hygromycin was shown to be a better selective agent as compared to kanamycin, inhibiting cellular growth and proliferation at 30 mg/L (Ishida et al., 2007). In this study, it should be noted that *U. brizantha* cv. Marandu calli are more sensitive to hygromycin than kanamycin. With this latter antibiotic, some escapes occurred at the concentration of 50 mg/L kanamycin, despite the fact that the small plant shoots became chlorotic and died within few weeks.

Phosphinothricin (PPT)

The herbicide Finale® (Bayer CropScience SG), which contains in its commercial formulation, 20% of the phosphinothricin, was used at 0, 5, 10, 20 and 40 mg/L of the active ingredient in this experiment. Within the first 30 days in the selective medium, there was a cessation of active growth and slight browning of the callus surface in all concentrations tested (Figure 2). The longer the callus remained in medium containing PPT, the darker they became (Figure 5).

At 5 and 10 mg/L of PPT, the callus growth rate decreased by circa 70% as compared to the control after 74 days in the selective medium. However, the lowest concentration of the herbicide allowed the few escapes. With higher concentrations of PPT (20 and 40 mg/L), the growth rate was reduced by a factor of 4 at the end of the experimental period.

In monocots, principally, a herbicide phosphinothricin was used as the selection system to distinguish transgenic from non-transgenic events (Ishida et al., 2007; Molinari et al., 2007; Sandhu and Alpeter, 2008; Han et al., 2009). In *Paspalum notatum*, the concentration of 1.0 mg/L increased the recovery of transgenic plants and minimized the amount of escapes (approximately 10%). This result validated the use of phosphinothricin as a robust and effective selective method to obtain a transformation frequency of 64.2% in this species (Mancini et al., 2014). Three selective agents (phosphinothricin, hygromycin and paromomycin) were tested for transformation of tall fescue (Long et al., 2011). Growth of non-transformed calli was completely inhibited on callus induction medium supplemented with 100 mg/L paromomycin without any signs of newly developing callus structures, while non-transformed calli cultured on 2 mg/L PPT or 100 mg/L hygromycin grew normally for the first 1-2 weeks. After 2 weeks of selection, only transgenic calli continued to grow, while non-transformed calli displayed progressive necrosis. The *bar* gene with PPT was considered the most efficient combination for selecting transformed cells. Similar conclusions have been reported for other monocot transformation protocols (Somleva et al., 2002; Luo et al., 2004; Gondo et al., 2005).

Presently, the only transformed plant in *Urochloa* genus was achieved in *U. ruziziensis* using phosphinothricin as a selective agent. After 8-9 weeks in selective media containing 10 mg/L PPT, most of the calli were killed, and only four resistant calli (1.4% efficiency) showed strong GUS expression and remained highly embryogenic (Ishigaki et al., 2012). In this study, a complete inhibition of regeneration of *U. brizantha* cv. Marandu calli with 10 mg/L of PPT was also observed in the medium. However, as shown in *U ruziziensis,* this concentration of the herbicide can affect the development of the plants, which tend to be sterile (Ishigaki et al., 2012). Thus, it may be preferable to use a concentration of 5 mg/L PPT even if some escapes occur.

Mannose

In all three sampling periods, calli cultivated only in sucrose (30 g/L) or in combinations of mannose: sucrose (10:20, 15:15, 20:10) continued to grow and produced shoot initials. At the end of the experiment (74 days), the highest growth rate was recorded for the combination 10:20 g/L mannose: sucrose with about 3 times the initial weight (Figure 3).

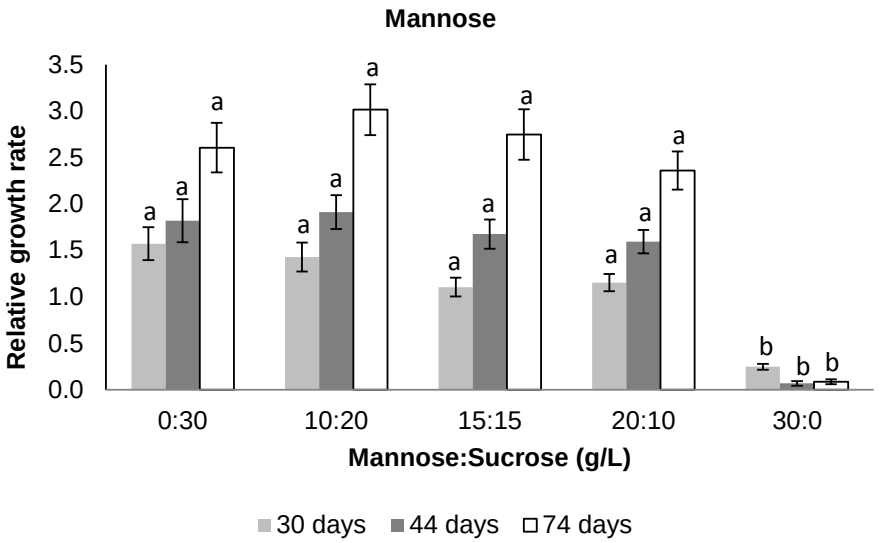

Figure 3. Relative growth rate of *U. brizantha* cv. Marandu calli cultured in medium supplemented with different combinations of sucrose and mannose over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

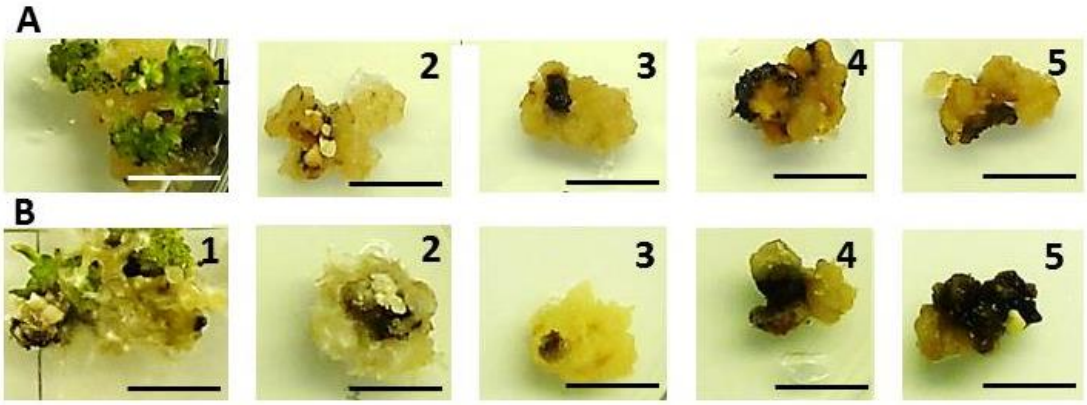

Figure 4. Morphology of *U. brizantha* cv. Marandu calli grown on media containing the antibiotics kanamycin are show in the upper row (A) while calli grown on medium containing hygromycin are shown in lower row (B) after 74 days of cultivation. The calli treated with different concentrations of antibiotics are shown in the photos marked 1 (0 mg/L – control), 2 (25 mg/L), 3 (50 mg/L), 4 (75 mg/L) and 5 (100 mg/L). Bars = 1 cm.

Independently of the sampling period, a significant difference was only detected when the calli were cultivated with mannose as the solely carbon source (Figure 3). The calli cultivated on medium containing only mannose (30 g/L) grew very poorly since the beginning of the selection procedure. A reduction in weight was observed thereafter, probably due to water loss and cells shrinkage. Regarding the morphology of the calli growing only on mannose, there was a change in color from cream to brown and gelatinous consistency with the increasing permanence of the calli in a selective media. Interestingly, root proliferation occurred at combination of 0:30, 20:10, 10:20 and 15:15 g/L mannose: sucrose, but they were not observed on the media containing mannose only (30:0) (Figure 6).

The positive selection system using phosphomannose isomerase gene (*manA*) and its correspondent selectable agent mannose have been widely used for identification and selection of transgenic cells/tissues in several monocot species (Giri and Praveena, 2015). Using mannose as selective agent demands preliminary studies to determine the best concentration of a selective agent and the need for supplemental carbon source. The toxic effect of mannose to plant cells increases with a

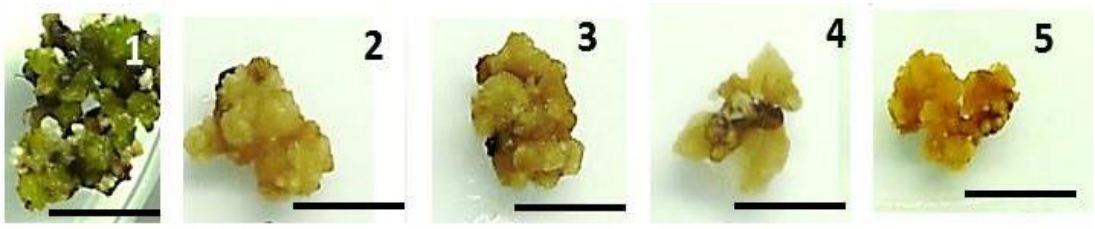

Figure 5. Morphology of *U. brizantha* cv. Maradu calli on medium containing phosphinothricin after 74 days of cultivation. A- without herbicide control, B– 5 mg/L, C– 10 mg/L, D– 20 mg/L and E– 40 mg/L. Bars = 1 cm.

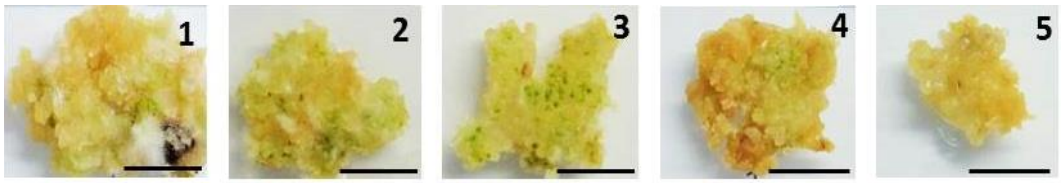

Figure 6. Morphology *U. brizantha* calli on media supplemented with different concentrations (g/L) of the mannose: sucrose A- 0:30, B – 10:20, C – 15:15, D – 20:10, E –30:0. Bars = 1 cm.

decreasing concentration of sucrose in the medium, indicating that there is an interaction between these carbohydrates (Joersbo et al., 1998). Furthermore, it has been reported that high sucrose concentrations have an additive effect in inhibiting the formation of shoots when combined with high levels of mannose (Kim et al., 2002). The addition of sucrose to selective medium containing mannose seems to have a positive effect on the recovery of transgenic corn and wheat and reduced escapes. Transformation frequency was three times higher when sucrose was added to the medium during selection (Reed et al., 2001; Wright et al., 2001).

In the first attempt to prevent *U. brizantha* calli formation using mannose as selective agent, it was demonstrated that 5 g/L mannose greatly inhibited callus formation and development of embryos even when sucrose (15 g/L) was added to the media (Silveira et al., 2003). In this study, we confirm that the use of mannose as the sole source of carbohydrate severely restricted the growth of calli and no shoots were regenerated. The present data suggest that *U. brizantha* cv. Marandu do not have the capability to metabolize mannose, which is different from the findings of Bahariah et al. (2012) who observed that palm cells are partially able to use mannose as a carbon source as indicated by the ability to form shoots. The inhibitory effect of mannose was alleviated by adding sucrose to the medium. In all other combinations of mannose and sucrose, it was observed that, the emergence of shoots, showing that medium containing only mannose should be used when *manA* is chosen as a selective marker gene for *U. brizantha* transformation.

Conclusion

In this study, the authors determined the optimal concentration of four selective agents- kanamycin, hygromycin, glufosinate ammonium and mannose– for inhibiting the *in vitro* growth of *U. brizanha* cv. Marandu embryogenic calli. All selective agents tested here, in the appropriate concentration, could be applied in experiments aiming to produce transgenic signal grass.

Although, the use of antibiotic marker genes have already been proven to be safe and very effective for transgenic plant selection on a variety of species, such SMGs from microbial origin may still cause public concerns (Breyer et al., 2014). In the case of genes conferring resistance to herbicides, as the *pat* and *bar* genes used in combination with phosphinothricin for selecting transformed plants, the main concerns are related to the introgression of the transgene in wild populations. Despite being an apomictic forage grass, the observation that *Urochloa* species exist in nature in the form of agamic complex and are cross-compatible with related species (Renvoize et al., 1996), gene flow can lead to the development of resistant volunteer plants, which may present management challenges for producers in different agricultural systems. In addition, with the transgenic trait for phosphinothricin resistance, management of volunteer signal grass could become costly. In this way, the use of mannose as a selective agent in *Urochloa* seems to be more appropriate for the development of transgenic plants for commercial purposes. The *manA* gene is considered a biosafe selectable marker due to its absence in plant genomes

and because its product, ManA, is not toxic (Stoykova and Stoeva-Popova, 2011).

Finally, this work provides information on the choice of the proper concentrations of selective agents for the establishment of more efficient transformation protocols for *U. brizantha* since no transgenic plant of this species has been regenerated so far.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study (no. 2013/04819-4) was supported by FAPESP (São Paulo State Research Foundation). The first author is grateful for TT3 scholarship from FAPESP (no. 2014/07830-1).

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Vol. 15(23), pp. 1168 -1176, 8 June, 2016
DOI: 10.5897/AJB2015.15113
Article Number: 5BFF1F058802
ISSN 1684-5315

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African Journal of Biotechnology

Full Length Research Paper

Bioactivity of mangrove humic materials on *Rizophora mangle* and *Laguncularia racemosa* seedlings, Brazil

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Received 20 November, 2015; Accepted 13 May, 2016

The mangrove sediments are rich in organic matter and humic substances, responsible for important functions such as reducing the toxicity of heavy metals, nutrient stabilization, serving as drain to atmospheric carbon and increasing the plant growth. In this study, we observed the effect of humic substances, humic acids and fulvic acids isolated from sediments of a mangrove forest, sampled from the Municipal Ecological Station Ilha do Lameirão (EEMIL, located in Vitória, Espírito Santo State, Brazil), on the growth and root acidification of *Rizophora mangle* and *Laguncularia racemosa* seedlings. For this, *R. mangle* and *L. racemosa* propagules were transferred to pots and then treated with different humic materials. The evaluation in root architecture change was performed by analyzing the main root axis length, lateral root length, density of lateral roots, fresh and dry mass weight of roots and estimated H^+-ATPase activity by specific root acidification measurement. The results indicate that all humic materials extracted from mangrove organic matter were able to modify the root architecture systems of the studied plants. In addition to inducing an increase in the number of lateral roots and root branching, it also stimulated specific root acidification when compared to control groups. There is a good potential in developing technologies for the production of seedlings of mangrove plant species treated with biostimulants based in humic materials isolated from the mangrove ecosystem itself.

Key words: Humic substances, humic acids, fulvic acids, mangrove.

INTRODUCTION

Periodical flooding from fluvial or marine origin that may occur in mangroves generates accumulation of organic matter ecosystem, leading to a carbon fixation amount, around 0.57 Mg per ha per year (Ferreira et al., 2007),

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Table 1. Chemical and physical composition of mangrove sediment located in the Escravos Canal Region used for the extraction and fractionation of organic matter.

Sediment	Chemical composition (fertility)															Physical composition (granulometry)		
	pH	P	K	Ca	Mg	Al	H+Al	Na	C	MO	Fe	Cu	Zn	Mn	S	B	Areia	Silte	Argila
		ppm		$cmol_c\ dm^{-3}$					%		ppm						%		
Escravos Canal region	4.3	6.0	754.0	23.1	27.5	1.0	20.7	60.3	13.0	22.3	1989.0	0.6	19.6	23.3	3561.0	18.1	32	49	19

which highlights the mangrove's importance in the cycling of atmospheric C. The biggest amount of the organic matter from soils, water and sediments is composed of humic substances (HS) (Baldotto, et al., 2013). According to Stevenson (1994), the HS covers heterogeneous organic compounds with no clear biochemical classification, produced as by-products of microbial metabolism. Despite the long period of study and experimentation, only recently we came to an understanding of what would these substances. Piccolo (2002) showed that HS are aggregates of organic compounds that are held together by weak interactions in a supramolecular conformation of apparently high mass. These substances promote direct effects on plant metabolism, stimulating plant growth as a whole, stimulating flowering and by consequence improving plantation yield (Vaz and Gonçalves, 2002; Rocha et al., 2004; Benites et al., 2006). They are also able to indirectly enhance soil resistance to water and nutritional deficiencies, providing better fertility and improving the system's physical and biological conditions (Guminski, 1968; Busato et al., 2009).

Many studies support the benefit generated by the use of products based on humic substances (HS) as biostimulant to different plant species incrementing, especially, root growth (Canellas et al., 2002; Dobbss et al., 2010; Aguiar et al., 2013; Canellas et al. 2012; Amorim et al., 2015; Silva et al., 2015; Ramos et al., 2015). However, further studies to quantify and characterize the specific amounts to be applied in certain species of mangrove as *Rizophora mangle* and *Laguncularia racemosa* remain scarce.

The fraction of HS originates from three products, classified according to their solubility in acid or alkaline, they are: Humin, fulvic acid (FA) and humic acid (HA) (Kononova, 1966; Canellas and Santos, 2005). The first fraction, humin, is less evolved and more stable being strongly linked to the mineral fraction of the soil, and thus is the insoluble portion of the organic matter. The second fraction (FA) constitutes the water-soluble portion, consisting of low apparent molecular mass molecules and higher amount of acid functional groups. The last, fraction (HA) is composed by molecules soluble only in alkaline medium with high apparent molecular mass (Schnitzer, 1982; Canellas et al., 2001).

According to Façanha et al. (2002), better plant development promoted by treatment with HS can be attributed to increased permeability of the plasmatic membrane, and activation of the transmembrane enzyme H^+-ATPase, which pumps H^+ into intracellular space. The acidification of the cell environment increases the plasticity of the membrane, allowing the cell to stretch wider and thus promoting the mechanism know as "acid growth" (Hager, 2003).

The aim of this study is to evaluate the effect of different humic fractions extracted from mangrove sediment on the root system, and specific root acidification in seedlings of *Rizophora mangle* and *Laguncularia racemosa*.

MATERIALS AND METHODS

Sampling area, chemical and physical composition of the sediments, location and history

Sediment samples from a mangrove ecosystem were collected at the surface of an area with very few anthropic impacts (Escravos canal region) in the EEMIL, Vitória, Espírito Santo State. The chemical and physical composition of the sediment used to extract the humic materials is shown in Table 1. The station is located between the latitudes 20° 14' S to 20° 17' S and longitudes 40° 16' W to 40° 20' W (Tulli, 2007), and covers an area of 891.8 ha, where 92.7% are represented by the mangrove. Such area was, initially, made into a preservation unit of Vitória-ES as a Municipal Biological Reserve, by publishing of the Municipal Law No. 3326 of May 27, 1986. It was later transformed into the EEMIL by the Municipal Law No. 3377 of September 12, 1986 (Tulli, 2007).

Humic substance extraction from organic matter of mangrove sediment and elemental composition of humic materials utilized

The extraction of HS, to obtain the FA and HA fractions were made in accordance to the classical methodology adopted by the International Humic Substances Society (IHSS) (Schnitzer, 1982). Explaining it briefly, 200 g of sediment samples (in triplicate) were air dried and sieved

Table 2. Dose-response model, correlation coefficient (R^2), standard deviation of regression (SD), number of units included in the sample (n), the regression significance level (p-value) and tipping point (optimal concentration) for the average root length of seedlings in *Laguncularia racemosa* and *Rizophora mangle* seedlings after treatment with humic substances, humic acids and fulvic acids.

Humic materials	Equation ($y = b2x^2 + b1x + b0$)	R^2	Standard deviation	n	*p*	Optimal concentration (dx/dy): b1 + 2(b2)x = 0
Laguncularia racemosa						
HS	$y = -0.1132x^2 + 1.2165x + 5.4525$	0.99	4.51	18	0.0023	5.37
HA	$y = -0.1776x^2 + 1.7704x + 9.6191$	0.99	3.21	18	0.0017	4.98
FA	$y = -0.0275x^2 + 0.3774x + 4.0172$	0.93	5.17	18	0.0034	6.86
Rizophora mangle						
HS	$y = -0.0956x^2 + 1.0642x + 8.2262$	0.98	6.12	18	0.0041	5.57
HA	$y = -0.1449x^2 + 1.64x + 10.483$	0.95	4.92	18	0.0026	5.66
FA	$y = -0.0931x^2 + 0.9875x + 6.0585$	0.92	3.76	18	0.0018	5.30

(2 mm mesh sieve), extraction of HS was done using NaOH 0.5 mol L^{-1}, in a sediment: solvent ratio of 1:10 (m:v). The separation of HA was achieved by lowering the solution's pH to 1.0 to 1.5 with HCl 6.0 mol L^{-1} followed by centrifugation (890 rcf / 20 min). Dissolutions and precipitations were repeated three times; all humic materials were adjusted to pH 7.0 utilizing NaOH 0.5 mol L^{-1} or HCl 6.0 mol L^{-1}, properly purified according to Canellas et al. (2005).

After purification and subsequent lyophilization, the humic materials (HS, FA and HA) had their elemental composition analyzed (CHNO) through an elemental analyzer device (CHNS - 932, Leco, Germany) with 4.0 mg samples (in triplicate). Oxygen content was determined by oxygen-difference and ash from incineration of 50 mg of the humic materials at 700°C during 8 h. The CHNO analysis is often used to relate chemical properties of humic substances with the genesis or properties of the extraction origin and this present work was basically used for the calculations of doses (in mmol C L^{-1}) were used in preliminary experiments concentration response. The values obtained for CHNO humic materials were as follows: HS (C: 21.61% H: 2.02% N: 2.06% and O: 74.31%); HA (C: 48.93% H: 5.76% N: 4.09% and O: 41.21%) and AF (C: 34.30% H: 4.63% N: 6, 35% and O: 65.53%).

Concentration response test with humic materials used on treatment of *Laguncularia racemosa* and *Rizophora mangle* seedlings and experiment with the best concentration

Seedlings *Laguncularia* and *Rizophora* were exposed to all the humic materials (HS, FA and HA) extracted from the mangrove ecosystem. In order to obtain the best doses, at witch plant growth is greatly improved, the following concentrations were used for each humic material: 0.0 (control); 1.5; 3.0; 6.0 and 12.0 mmol C L^{-1}. Following a regression analysis, a new experiment was performed, this time using the optimal dose of each humic material (Table 2), for comparing various treatments and the control.

Propagules of *L. racemosa* and *R. mangle* brought from the study area (Escravos channel region) developed during 30 days were treated with the best concentrations of the humic materials found (Table 1). Following exposure to the treatments, the number of lateral roots was assessed using a computer program to analyze digital images (ImageJ® v.1.45) and the root main axis length. Also, fresh and dry root mass were measured using a scale with analytical precision, dry weight was measured after 72 h on a stove at 60ºC. Each treatment with the best concentrations of humic material had 8 pots and each pot had 2 propagules, giving a total sample size of 16 plants per treatment for each humic material (HS, FA and HA).

The experiment was completely randomized, with 3 replicates per treatment, whose mathematical model can be described as: Yij = μ + ti + eij. As it follows: Yij = experimental response, measured in the experimental unit j, submitted to the treatment i; μ = overall average; ti = relative effect by the treatment i; eij = random error. An analysis of variance was performed and means compared by the Tukey test ($P < 0.05$) using the software SAEG v.9.1.

Measure of acidity in the growing solution - estimate of the activity H^+ -ATPase

Estimation of H^+-ATPase activity was assessed by measuring the acidity in a solution containing seedlings of *L. racemosa* and *R. mangle* treated with the best concentration of HS obtained in preliminary test concentration response test. In this experiment, only minimal medium ($CaCl_2$ 2 mmol) was used to avoid any influence of nutrients which could act in synergy with the HS, stimulating root growth and/or overall plant metabolism. A total of 5 plants (in triplicate) with similar age and height were treated with HS (along with a control) for 48 h and after this period, they were transferred to a recipient containing 50 mL of a $CaCl_2$ 2.0 mmol solution at pH 7.0. The solution's pH was measured using a potentiometer able to measure pH with a glass electrode, during 140 minutes. The roots were digitalized for latter analysis and then dried in a forced air stove, after that the amount of H^+ was expressed in moles of H^+ per gram of dry roots (mmol H^+ g of roots^{1}) (Aguiar et al., 2013).

RESULTS AND DISCUSSION

Effects of the humic materials on root growth

Changes in root system architecture can be variable in response to hormonal and environmental stimulations (Torrey, 1986; López-Bucio et al., 2003; Sorin, 2005). All the humic products (HS, HA and FA) isolated from the mangrove ecosystem sediment showed the ability to induce these changes in rooting pattern in seedlings of *L. racemosa* and *R. mangle* (Figures 2 and 3).

The quadratic regression curves for the mean root length of *L. racemosa* and *R. mangle* seedlings treated with the different humic materials are illustrated in Figure 1.

Table 2 and Figure 1 show the concentration-response

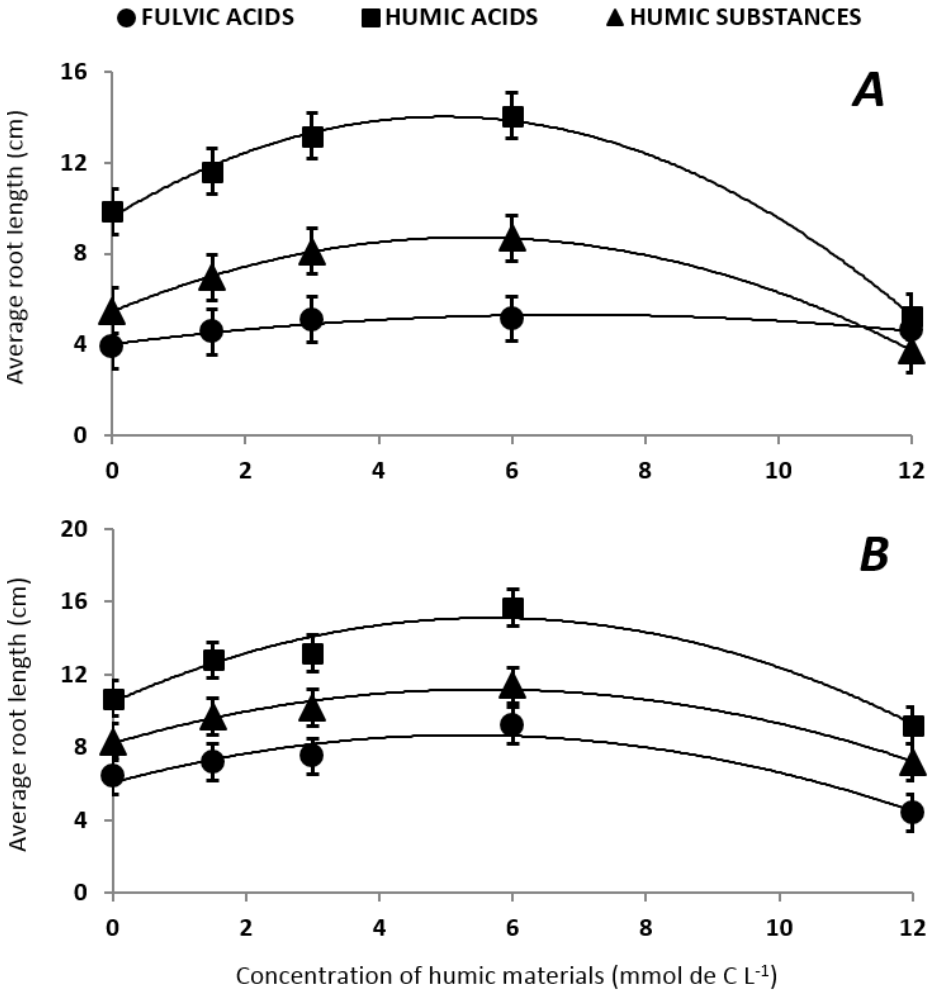

Figure 1. Quadratic regression curves for the mean root length of *L. racemosa* **(A)** and *R. mangle* **(B)** seedlings treated with humic substances (triangle); humic acids (square) and fulvic acids (circle).

model and the optimum concentrations for each humic material for seedlings of *L. racemosa* and *R. mangle*. The results of the average increase in root length, obtained by the first derivative of a quadratic regression, for the effect of concentration in seedlings of *L. racemosa* and *R. mangle* respectively, the results were 5.37 and 5.57 mmol C L^{-1} for HS; 4.98 and 5.66 mmol C L^{-1} for HA; 6.86 and 5.30 mmol C L^{-1} for FA (Table 1).

Roots architecture of the seedlings from both species were significantly altered by the addition of the HS to the culture, which results are shown in Figures 2 and 3. Also, the density of lateral roots of both species was significantly altered by the presence of humic substances. Similar results were observed by Dobbss et al. (2007, 2010), Aguiar et al. (2009), Aguiar et al. (2013). However, by comparing the two species, it was observed that seedlings of *R. mangle* showed a greater number of lateral roots per unit of primary root length (lateral roots density). Seedlings of *L. racemosa* also showed statistically higher values compared to the control plants (Figure 2A). Such results are probably related to the specific development characteristics of each studied species.

Regarding the length of the main roots, results showed a shortening caused by treatment with HS. This result is very similar to the behavior of plants treated with high concentrations of auxin (Peres et al., 2009), as auxin has already been found in the supramolecular structure of HS (Muscolo et al., 1998). However this “auxinic” effect was significant for seedlings of *R. mangle* and not significant for seedlings of *L. racemosa* (Figure 2B).

The increase in length of emerged lateral roots was 48 and 72% higher for *L. racemosa* and *R. mangle* respectively for seedlings treated with HS when compared to control seedlings (Figure 2C). Induction in lateral roots growth has been observed in other plant species as reported, indicated, highlighted by Canellas et al. (2002, 2008, 2009 and 2010), Dobbss et al. (2007,

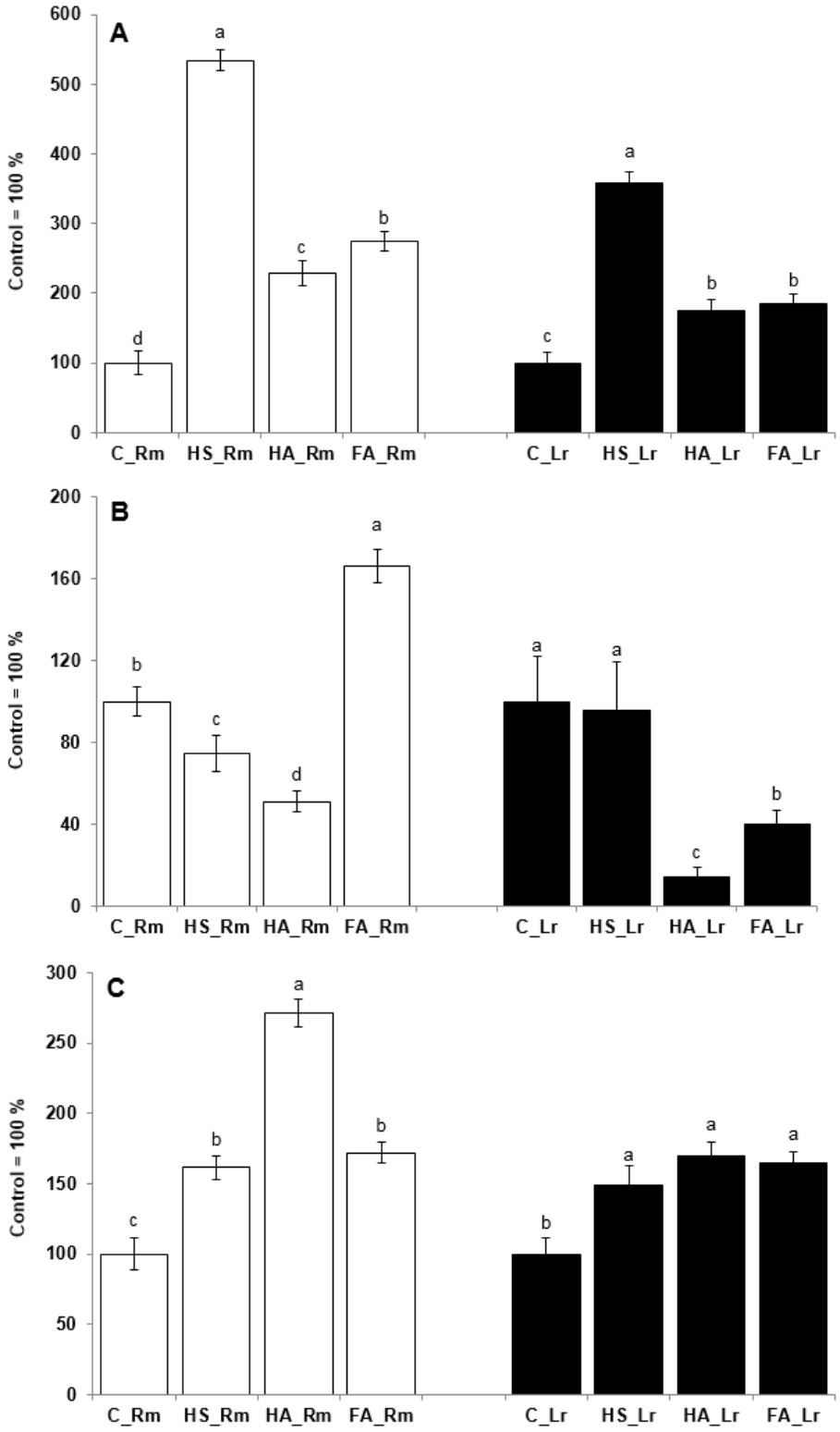

Figure 2. Effect of humic substances (5.37 and 5.57 mmol L C^{-1}), humic acids (4.98 and 5.66 mmol L C^{-1}) and fulvic acids (6.86 and 5.30 mmol C L^{-1}) isolated from the sediment mangrove ecosystem studied on the **(A)** density of lateral roots; **(B)** length of main roots and **(C)** Length of lateral roots of *L. racemosa* (Lr) and *R. mangle* (Rm). The values represent the average of 15 seedlings ± standard deviation and were normalized relative to the control (control = 100%). Means followed by different letters, in white or black columns are statistically different by Tukey test ($P < 0.05$).

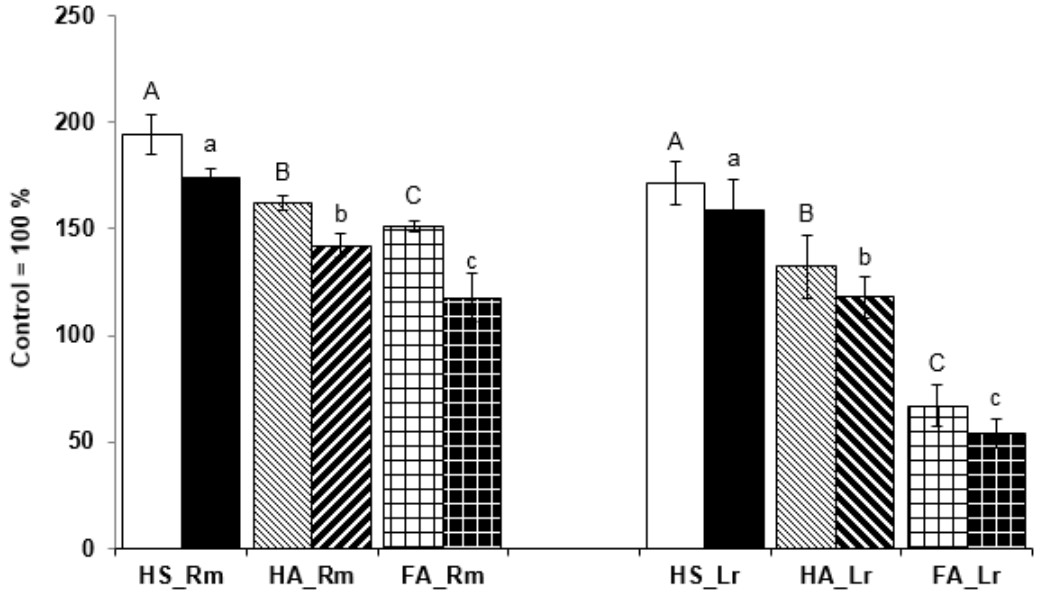

Figure 3. Effect of humic substances (5.37 and 5.57 mmol L C^{-1}), humic acid (4.98 and 5.66 mmol L C^{-1}) and fulvic acid (6.86 and 5.30 mmol C L^{-1}) isolated from the sediment mangrove ecosystem studied on fresh root (left column) and dry (right columns) mass of *L. racemosa* (Lr) and *R. mangle* (Rm). The values represent the average of 15 seedlings ± standard deviation and were normalized relative to the control (control = 100%). Means followed by different capital letters in the left column and lower the right columns are statistically different by Tukey test (P <0.05).

2010), Baldotto et al. (2011), Amorim et al. (2015), Silva et al. (2015) and Ramos et al. (2015), suggesting an occupation and soil exploitation strategy by plants treated with HS. Lateral roots explore more soil around them and compete less with each other due to the distance between them (Silva and Delatorre, 2009).

The results of fresh and dry root mass (Figure 3) were significantly higher in treatments with HS for both plant species, when compared to their respective control treatment, which corroborates similar to the findings of Canellas et al. (2009), where the observed increases in root mass were 100% higher in seedlings treated with HS.

The HA stimulated lateral rooting significantly for both species, reflecting in increases to root density, fresh and dry masses (Figures 2A and 3). The growth of the main roots was strongly inhibited by the HA in a behavior typical to plants treated with auxins (Dobbss et al., 2007; Zandonadi et al., 2007) (Figure 2B). Auxin is a key hormone for the regulation of lateral root emission in plants (Blakely et al., 1982). As expected for effects of exogenously applied hormones, auxin action on the root development is dependent on its concentration as reported by Mulkey et al. (1982). Casimiro et al. (2001) demonstrated that polar transport of auxin in both directions, basipetal (from the tip towards the base of the root) and acropetal (from the base towards the tip of the root) is necessary for the initiation and emergence of lateral roots. Stimulation of lateral roots emission and shortening of the main root, at the same time, resulted in an increase in the average density of lateral roots of 75 and 111% for *L. racemosa* and *R. mangle* respectively, when compared to their control groups (Figure 2A).

The greater effect of HA on fresh and dry weight of roots was observed in seedlings *R. mangle* (Figure 3). The promoted HA increases in the length of lateral roots in the order of 69 and 183% in *L. racemosa* and *R. mangle* respectively, when compared to their control groups (Figure 2C). According to Conceição et al. (2008), root growth occurs in two stages: Meristematic growth and vacuolated growth; the latter occurs in the stretching zone and is characterized by rapid expansion of cells, sustained by increased water uptake by the vacuoles through turgid pressure. Moreover, Cosgrove (1998) and Zandonadi et al. (2007) found that HA isolated from various sources of organic matter strongly stimulates the pumping of H^+ by V-ATPase and H^+-pirofosfatase. Also, Canellas et al. (2010) and Dobbss et al. (2010) observed that different humified materials are capable of promoting growth and various changes in the geometry of the root system, improving uptake of water and nutrients and resulting, as observed, in higher fresh plant mass and root growth.

The FA, extracted from the HS obtained from the mangrove sediment, also stimulated plant rooting significantly, markedly changing the root architecture of *L. racemosa* and *R. mangle* seedlings (Figures 2 and 3). Dobbss et al. (2007) observed similar results, where the

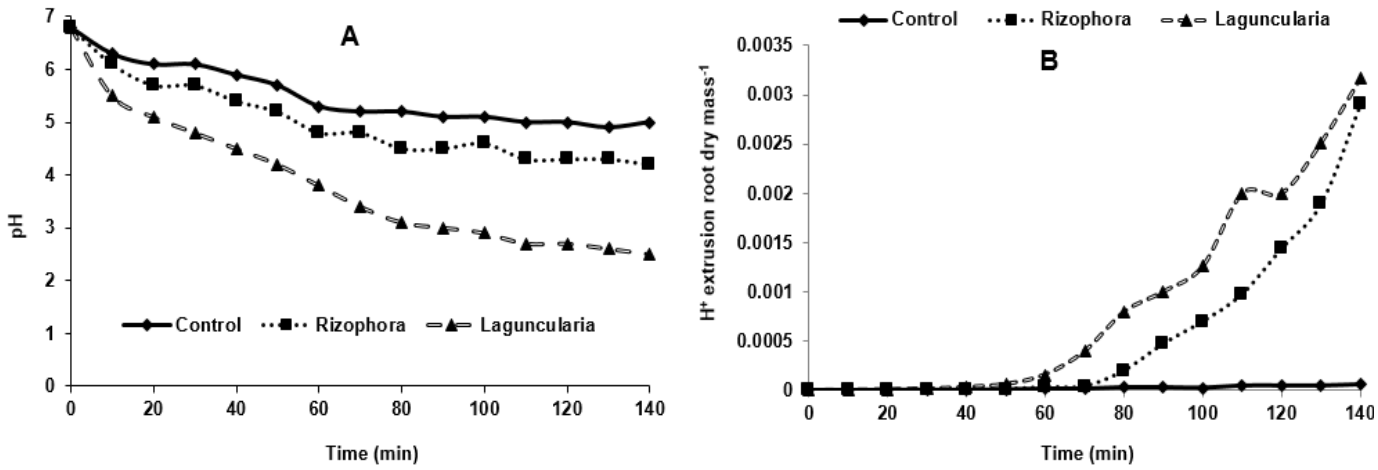

Figure 4. (A) pH monitoring of the solution containing Rizophora and Laguncularia seedlings after treatment with the humic substances best concentrations obtained in the preliminary assay dose-response (5.57 and 5.37 mmol L^{-1} C respectively). (B) Extrusion of H^+ (in mol of H^+ per gram of dried roots) by dry mass roots of Rizophora and Laguncularia after treatment with the humic substances best concentrations obtained in the preliminary assay dose-response (5.57 and 5.37 mmol C L^{-1} respectively).

authors found significant stimuli of the fraction FA to rooting Arabidopsis seedlings both in number and in length of lateral roots. The FA (that corresponds to the soluble humic fraction of the HS), at any pH value, caused an increase in lateral roots density of 196% in *R. mangle* seedlings and 84% increase in *L. racemosa* seedlings, when compared to their respective control groups (Figure 2A).

Results obtained regarding main root length were quite contrasting. An increase in the length of the main root in *R. mangle* and a reduction of main root length in *L. racemosa* seedlings after treatment with FA (Figure 2B) was observed. According to Zandonadi (2010), this difference between species is due to the fact that, the regulation of root architecture is quite complex and varies, both, in different species and within the same species.

Regarding dry and fresh root masses, it could be observed the same contrasting tendency as in main root length, an increase in plant mass to *R. mangle* and a decrease in plant mass to *L. racemosa* (Figure 3). In relation to lateral root length, significant increases were observed in both species when treated with FA (Figure 2C). According to the traditional view of the chemical structure of HS, it was postulated that "in the rhizosphere, the interaction between roots and the organic matter is possible when the humic molecules present in the soil solution are small enough to flow through the apoplast and reach the plasmatic membrane" (Muscolo et al., 2007). Thus, other works described in literature (Nardi et al., 2002 and Quaggiotti et al., 2004) consider that only the FA, known as the fraction of organic matter with lowest molecular mass, could promote increments to growth and changes in the energetic metabolism of plants. However, according to our results, we can reinforce the idea that regardless of molecular size (Canellas et al., 2010) different humidified fractions can stimulate plant growth in various ways and for different plant species. The findings of this study also collaborate with the data previously obtained by Aguiar et al. (2009) using exclusion chromatography on sephadex gel, that did not found any relationship between the molecular mass distribution and the bioactivity of HS.

Measure of acidity in the growing solution - estimate of the activity H^+ -ATPase

Figure 4 shows the pH measures of the medium containing *L. racemosa* and *R. mangle* seedlings treated with and without (control) HS extracted from the mangrove ecosystem. The increase in acidity of the solution was observed in treatments in which HS exposed the plants for 48 h. These results corroborate with those obtained by Dobbss et al. (2008) and Silva et al. (2015) which also observed increases in acidity of solutions containing *Arabidopsis thaliana* seedlings treated with humic materials extracted from the Paraiba do Sul River and solutions with seedlings of *Cattleya warneri* treated with HA from vermicompost, respectively. Zandonadi et al. (2010) directly related root acidification with specific activity of H^+-ATPase in seedlings roots treated with HA and recently Aguiar et al. (2013) positively correlated proton extrusion in seedlings roots of corn plants treated with the HA isolated from many vermicomposts with the activity of H^+-ATPase of plasmatic membrane. According to Aguiar (2011) increased acidity of the medium may be primarily associated with two main factors: (i) Production of CO_2 by root respiration, as the breathing process produces CO_2,

that dissolves in to the medium and causes a reduction of pH; and (ii) An increase in H^+ extrusion, possibly associated with the activity of HS on H^+-ATPase.

As recently reported by Aguiar et al. (2013), despite the acidity alterations provided by exposing the seedlings to HS not being exclusively related to stimulation of H^+ pumps, it may be suggested that this simplified method may be used in the study of physiologically active HS. These results were expected since the cell growth promoting mechanism is mediated by the H^+-ATPases in a process known as "acid growth theory." The process of H^+-ATPases activation that culminates in cell expansion starts with the generation of the H^+ gradient and lowering of the pH, provided by the accumulation of H^+ in the extracellular side (Hager, 2003).

Therefore, based on the data obtained one can estimate that, at least in part, the measures of acidity in solutions containing plants treated HS is probably related to the activity of H^+-ATPase (increase in H^+ extrusion) in the plasmatic membrane.

Conclusions

Different humic materials (HS, HA and FA) isolated from mangrove forest sediment, from the EEMIL region, presented the ability to modify the architecture of root systems in *R. mangle* and *L. racemosa*, leading to increases in the number and length of lateral roots, and also the fresh and dry mass weight of roots. The humic substances, when in solution, acted as regulators of plant growth, since, possibly, were able to stimulate the activity of H^+-ATPase by acidity measurements in solution assays.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Nacional de Desenvolvimento do Ensino Superior Particular (FUNADESP) for financial support. The Secretaria Municipal de Meio Ambiente (SEMMAM) from Prefeitura Municipal de Vitória (PMV) is also appreciated for the fundamental assistance towards the development of the work within the Estação Ecológica Municipal Ilha do Lameirão (EEMIL).

Abbreviations

EEMIL, Estação Ecológica Municipal Ilha do Lameirão (Municipal Ecological Station Ilha do Lameirão); **HS,** humic substances; **HA,** humic acids; **FA,** fulvic acids.

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