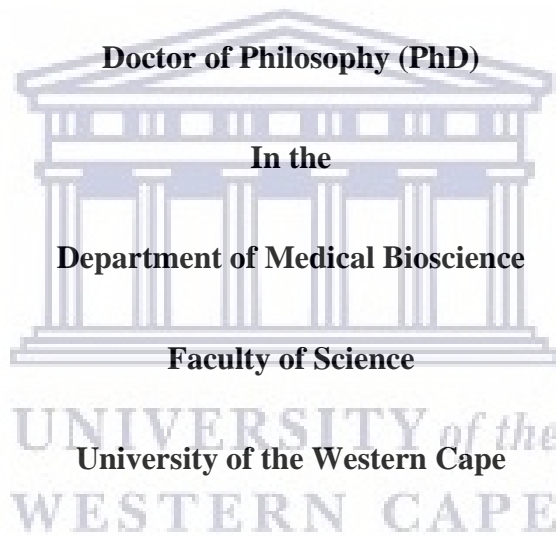# Cell culture biomarkers for monitoring of wastewater pollutants

By

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Department of Medical Bioscience

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Supervisor:

Professor Edmund J. Pool

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DECLARATION

I declare that the thesis entitled "Cell culture biomarkers for monitoring of wastewater pollutants" is my work and has not been submitted for any degree or examination at any other University and that all sources of my information have been quoted as indicated in the text and list of reference.

Vedastus Wilfred Makene

March, 2021

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ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

ANOVA - One way variance analysis

AOPs - Advanced oxidation processes

ARs - Androgen receptors

ARs - Androgenic receptors

ATP - Adenosine triphosphate

BADGE - bisphenol A diglycidyl ether

BOD - Biochemical oxygen demand

BPA - Bisphenol A

Cfu/ml - colony forming unit per milliliter

CO_2 - Carbon dioxide

COD - chemical oxygen demand

DAS ELISA - double antibody sandwich enzyme linked immunosorbent assay

DDT - Dichloro Diphenyl Trichloroethane

DES - Diethylstilbestrol

DHT - 5α-dihydrotestosterone

DMEM - Dulbecco's Modified Eagle's medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DO - Dissolved Oxygen

E_2 - Estradiol

EC - electrical conductivity

EDCs - endocrine-disrupting chemicals

EE2 - 17α-Ethnylestradiol

EHD   -   electrohydraulic discharge

ELISA   -   Enzyme linked immunosorbent assay

eNOS   -   endothelial nitric oxide synthase

ERs   -   Estrogenic receptors

FBS   -   Foetal bovine serum

H_2O_2 -   hydrogen peroxide

H_2SO_4 -   Sulfuric acid

HRP   -   Horse radish peroxidise

IL-6   -   Interleukin 6

iNOS   -   inducible nitric oxide synthase

JNKs   -   Jan N-Terminal Kinases

LPS   -   lipopolysaccharides

M1macrophages - classically activated macrophages

M2 Macrophages - alternatively activated macrophages

mg L^{-1} -   milligram per liter

mg/L   -   Milligram per liter

ml   -   Millilitre

NFκB -   Nuclear factor kappa B

ng/L   -   Nanogram per liter

ng/ml   -   Nanogram per milliliter

nm   -   Nanometres

nNOS   -   neuronal nitric oxide synthase

NO   -   Nitric oxide

NOS   -   Nitric oxide synthase

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NSAIDs - Non-steroidal anti-inflammatory drugs

NTU - Nephelometric Turbidity Units

ºC - Celsius degree

OC - Organochlorinated

OD - Optical density

OP - Organophosphorus

PAHs - Polycyclic Aromatic Hydrocarbons

PCBs - Polychlorinated biphenyls

pg/ml - Picogram per milliter

POPs - persistent organic pollutants

PRRs - germline-encoded pattern recognition receptors

RLRs - Retinoic acid-inducible gene-I-like receptors

RNS - Reactive nitrogen species

ROS - Reactive oxidative species

rpm - Revolutions per minute

SD - Standard deviation

SPE - Solid phase extraction

TDS - Soluble compound dissolved

TLRs - Toll-like receptors

TMB - 3,3′,5,5′-Tetramethylbenzidine

TNFα - Tumor necrosis factor alpha

TOC - Total organic carbon

TSS - Total suspended solids

WST-1 - (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

WWTPs   -   Wastewater treatment plants

XTT   -   (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-
              carboxanilide)

µg/ml   -   microgram per milliliter

µM   -   micrometre

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ABSTRACT

Wastewater is normally composed of a mixture of pollutants. The type and composition of pollutants in a particular wastewater depend on the source of origin. The source and characteristics of a particular wastewater determine the ideal method of sewage treatment. Specific treatment techniques are effective in the removal of certain types of pollutants and may have no impact on the levels of other types of pollutants. Therefore, a combination of treatments and assessment of the quality of effluent before release into the environment is normally recommended. The assessment of effluent can be achieved by various techniques including chemical analysis and biological assays. Chemical analyses are commonly employed; however, they often pose detection problems and are considered to be uneconomical. As a result increased efforts are being made to develop biological assays for pollutants that are easy to perform, have a broader pollutant detection range and are that are relatively cheap.

The aim of this study was to use the mouse macrophage RAW264.7 cell line as a cell culture bioassay model for monitoring aquatic pollutants and quality of wastewater effluent. The RAW264.7 cells are mouse ascites leukaemia induced cells, which once stimulated secrete inflammatory mediators and pro-inflammatory cytokines. In this study the effects of selected common endocrine distrupting chemical (EDC) pollutants and effluent samples were evaluated using cytotoxicity and secretion of inflammatory mediators (NO) and pro-inflammatory cytokines (IL-6) in RAW264.7 cell culture. Cytotoxicity was evaluated by determination of cell viability. Inflammatory responses were evaluated by determination concentrations of NO and IL-6 in supernatant of

RAW264.7 cell culture as biomarkers of inflammation. NO and IL-6 were determined in culture supernatant using Griess reaction assay and double-antibody sandwich enzyme-linked immunoassay (DAS ELISA), respectively.

The first specific objective of this study was to evaluate effects of endocrine disrupting chemicals on biomarkers of inflammation produced by lipopolysaccharide stimulated RAW264.7 macrophages. Endocrine disrupting chemicals (EDCs) are common pollutants in the environment and can induce disruption of the endocrine and immune systems. The EDCs investigated were Estradiol (E2), 5α-dihydrotestosterone (DHT) and Bisphenol A (BPA). To evaluate if the effects caused by EDCs were modulated by steroid hormone receptors, antagonists of estrogen and androgen receptors were used. The steroid receptor antagonists used were Tamoxifen, an estrogen receptor antagonist, and Flutamide an androgen receptor antagonist. The lipopolysaccharide (LPS) stimulated RAW264.7 cells were exposed to DHT, E2 and BPA alone or in combination with flutamide and tamoxifen. Secretion of biomarkers of inflammation, namely nitric oxide (NO) and interleukin 6 (IL-6), were monitored. RAW264.7 cell culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays. The results show that DHT, E2 and BPA at concentration of 5µg/ml had no cytotoxic effects to RAW264.7 cell culture. However, the same treatments significantly (P <0.001) induced suppression of both NO and IL-6 secretion in stimulated RAW264.7 cells. The suppression of NO and IL-6 indicates that DHT, E2 and BPA can induce anti-inflammatory activities in stimulated RAW264.7 cells. The anti-inflammatory effects were induced via their respective steroid receptors, because it was reversed by their respective antagonist compounds. DHT combined with flutamide reversed the anti-inflammatory effects of

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DHT, while a combination of an estrogenic E2 or BPA with tamoxifen reversed the effects of E2 and BPA respectively. The results show that stimulated RAW264.7 cells culture can be a useful bioassay model for monitoring androgenic and estrogenic pollutants.

The second specific objective of this study was to assess toxicity and inflammatory activity of municipal wastewater samples using RAW264.7 cell culture model. The wastewater samples analysed were influent, post bio-filtration, post activated sludge treatment and final effluent collected from a wastewater treatment plant. RAW264.7 cell cultures were exposed to sterile filtered water samples from the treatment plant. RAW264.7 cell culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays. The results show that none of wastewater samples tested induced cell toxicity when compared to negative control. The results also show that all wastewater samples significantly (P<0.001) induced NO and IL-6 production in RAW264.7 cells. The highest inflammatory activities were induced by post bio-filtration wastewater sample. Final effluent sample induced the lowest inflammatory response. The lower inflammatory activity in final effluent indicates effective removal of pollutants upon sewage treatment. The findings of this study show that sewage samples can induce inflammatory responses in RAW264.7 cells. The results also give evidence that RAW264.7 cells can be used as a model for monitoring the quality of treated municipal sewage.

The third specific objective of this study was to evaluate cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by

coagulation-flocculation methods, using RAW264.7 cell culture model. RAW264.7 cell cultures were exposed to sterile filtered water samples from raw effluent and effluent treated with various coagulation-flocculation methods. RAW264.7 cell culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays. The results show that raw effluent induced cytotoxicity by reducing cell viability significantly (P<0.001) compared to the negative control. The results on inflammatory activities show that the raw effluent and effluent treated with 1.6g/L of Fe-Mn oxide induced significantly (P<0.001) higher NO production than the negative control. The inflammatory results further show that the raw effluent induced significantly (P<0.001) higher production of IL-6 than the negative control. Among the coagulants/flocculants evaluated $Al_2(SO_4)_3.14H_2O$ at a dosage of 1.6 g/L was the most effective to remove both toxic and inflammatory pollutants from the textile effluent used in this study. Therefore, the inflammatory responses in RAW264.7 cells can be used as sensitive biomarkers for monitoring the effectiveness of coagulation/flocculation processes used for textile effluent treatment.

The last specific objective of this study was to evaluate toxicity and inflammatory activities of a textile effluent treated with electrohydraulic discharge and coagulant/flocculants. A combination of coagulant/flocculants and electrohydraulic discharge (EHD) are tested for treatment efficiency of textile wastewater. Pre- and post-treatment samples were used to evaluate process efficiencies. Process efficiencies were evaluated using physicochemical characteristics, and cytotoxicity and inflammatory activities induced in macrophage RAW264.7 cell culture model. RAW264.7 cell cultures were exposed to sterile filtered water samples from raw textile industrial effluent and

effluent after treatment with EHD in absence and presence of coagulant/flocculants. RAW264.7 cell culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays. The results show that raw effluent was characterized by high physicochemical parameters and intense colour. Treatment of textile wastewater with EHD alone successfully reduced the chemical oxygen demand (COD) and total organic carbon (TOC) values by 95.3 and 96.23 % respectively. The results show further that a single treatment approach is not effective in removing all pollutants. However, a combined treatment approach effectively removed complex organic pollutants, toxic and inflammatory pollutants based on the results of NO and IL-6 in RAW264.7 cell cultures. The study confirms that induction of NO and IL-6 secretions in macrophage RAW264.7 cells is a very sensitive model system to monitor the efficiency of textile effluent treated with electrohydraulic discharge and /or combined with other processes.
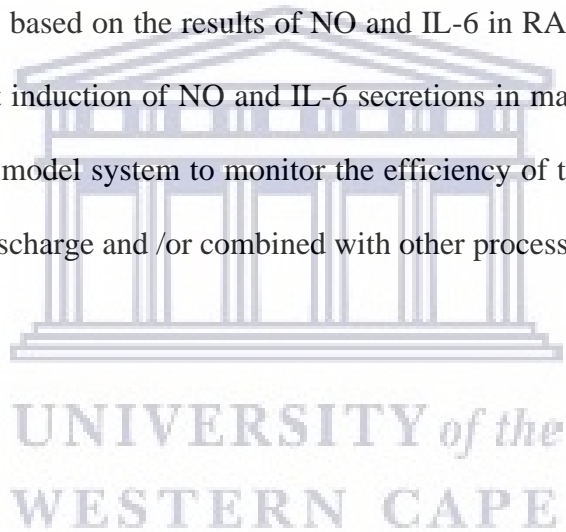
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(i) Makene, V.W. and Pool, E.J. (2019). The Effects of Endocrine Disrupting Chemicals on Biomarkers of Inflammation Produced by Lipopolysaccharide Stimulated RAW264.7 Macrophages. *International Journal of Environmental Research and Public Health 16:* 2914.

(ii) Makene, V.W. and Pool, E.J. (2015). The assessment of inflammatory activity and toxicity of treated sewage using RAW264. 7 cells. *Water and Environment Journal, 29*(3), pp.353-359.

(iii) Makene, V.W., Tijani, J.O., Petrik, L.F. and Pool, E.J. (2016). Evaluation of cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by coagulation-flocculation methods. *Environmental monitoring and assessment, 188*(8), 471.

(iv) Makene, V.W., Tijani, J.O., Massima E., Petrik, L.F. and Pool, E.J. (2019). Toxicity study of a textile effluent treated with electrohydraulic discharge and coagulant/flocculants. *Water, Air, & Soil Pollution, 230*: 167.

Chapter 1: Introduction and Objectives

1.1 Introduction

Global population growth and increasing economic activities have been associated with increased production of wastewater. Wastewater is a mixture of used water that may carry different types of pollutants depending on sources of origin. The main sources are domestic, commercial establishments, storm water, agricultural farm runoff and industrial effluents. Wastewater from domestic sources carries pollutants such as nutrients (Nitrogen and Phosphorus), suspended solids, heavy metals, pathogens and organic compounds (Ratolab et al. 2012; Akpor et al. 2014). The main organic compounds reported in domestic wastewater are steroids, pesticides, industrial chemicals, pharmaceuticals and personal care products (Navalon, et al. 2011; Ratolab et al. 2012). On the other hand, types of pollutant in industrial wastewater depend on the nature of a particular industry. For instance, textile industries produce wastewater that contains many pollutants including synthetic dyes characterized by reactive groups (Patel and Patel, 2011).

Regardless of source of origin, raw wastewater is normally made up of a mixture of many pollutants. Therefore, discharging of raw effluent continues to be a public environmental concern worldwide. This is because a mixture of pollutants in wastewater may cause various serious adverse environmental and health effects (Drury et al. 2013; Naidoo and Olaniran, 2013; Li et al. 2015). Environmental effects posed by pollutants include eutrophication and toxicity to ecosystem (Smith, 2003; Englert, et al. 2013). Eutrophication is caused by increased nutrient loading from agricultural run-off, municipal sewage and industrial effluent.

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Adverse health effects posed by wastewater are outbreaks of waterborne disease, toxicity, endocrine disruption, genotoxicity and immunotoxicity (Liney, et al. 2005). These effects can be acute, chronic and/or bioaccumulative. Furthermore, pollutants present in textile wastewater such as dyes are known to cause adverse biological effects like carcinogecity, mutagenicity and allergic reactions (Puvaneswari et al. 2006; Malinauskiene et al. 2012; Nygaard et al. 2013; Leme et al. 2014).

In order to minimize adverse effects of pollutants, treatment of wastewater before discharge is always indispensable. To achieve efficient removal of pollutants, different types of treatment techniques are being developed and implemented. The most common techniques include converntional methods which apply physical, chemical and biological processes, advanced processes and combinations of more than one technique.

Converntional wastewater treatment methods include coagulation, flocculation, biological treatment, precipitation, adsorption, chlorination, among others (Kasprzyk-Hordern et al. 2009; Maletz et al. 2013). They are commonly applied in most of wastewater treatment plants (WWTPs) worldwide. These techniques are credited for effectively removing of suspended solids and organic matters, pathogens and some organic pollutants (Oller et al. 2011; Enjarlis, 2013). However, efficiencies of these methods are low in terms of decomposition of non-biodegradable contaminants. For instance, pollutants which are more persistent such as endocrine disrupting chemicals (EDCs), textile dyes and other emerging pollutants may be resistant to these treatment processes. Besides, treatment methods such as chlorination have disadvantages due to the production of toxic disinfection by-products (DBP) that are genotoxic, mutagenic and

carcinogenic to humans (Gultekin and Ince 2007). Furthermore, chemical oxidation is hindered by economics and wastewater characteristics (Malik 2010). In fact, all the treatment processes have different shortcomings and challenges, therefore in order to comply with the stringent environmental regulatory framework with regard to ensuring a balanced relationship between economic growth and human health, sustainable and technically feasible water treatment systems are desired.

In order to achieve effective removal of persistent pollutants, the use of a combination of methods or more advanced techniques is normally recommended. An example of the advanced technique is the electrohydraulic discharge (EHD) system. This technology involves the passage of a high-voltage electrical discharge between two electrodes immersed in an aqueous solution to form plasma. The plasma can initiate both physical and chemical processes and subsequently generates active species such as hydroxyl radical ($\cdot$OH), ozone (O_3), perhydroxyl radical ($\cdot$OOH), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), ultraviolet light and shock waves depending on the solution pH, conductivity and the discharge magnitude (Bruggeman and Locke 2013). These radicals are very reactive and non-selective, especially hydroxyl radicals, which can degrade some of the complex organic substances into innocuous compounds (Malik 2010; Jiang et al. 2014). This technology is widely regarded as a promising and effective treatment techniques particularly in the decomposition of biologically active and persistent organic pollutants present in wastewater (Sharma et al. 2011; Olivier et al. 2013). However, the presence of radical scavengers in textile effluent can affect the efficiency of EHD systems. Besides, the high cost of energy consumption in the course of

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wastewater treatment limits the industrial application of this technique for wastewater treatment.

To achieve complete removal of persistent organic pollutants, with overall minimum cost, a combination of different physical and advanced oxidation treatment technologies are desired. This not only allows the exploitation of individual process strengths but also reduce the economic constraints of a single advanced oxidation technology. Evidences from research have demonstrated that combinations of processes are feasible and practical tools for improving treatment efficiencies (Enjarlis 2013). Unfortunately, none of the individual treatment techniques such as biological, physico-chemical or advanced oxidation processes is capable of the complete elimination of organic pollutants in wastewater; thus a combined approach is preferable (Nawaz and Ahsan 2014). Furthermore, based on previous studies, combined techniques are considered to be more efficient than most of conventional methods (Azbar et al. 2004; Nawaz and Ahsan 2014). As a result combined techniques have been widely used for treatment of industrial and municipal wastewater for removal of dyes, endocrine disruptors and other organic pollutants (Stasinakis 2008; Gerrity et al. 2010). The combined technology has also received increasing application and is reported for efficient removal of pathogens in river water (Izdebski et al. 2011).

To ascertain the performance efficiency of any of the wastewater treatment methods, evaluation of effluent quality is very important. In most cases, the quality of wastewater effluent has been evaluated based on physicochemical parameters only. The physicochemical parameters which are commonly used are pH, Biochemical Oxygen

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Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS) and microbial characteristics (Sonune et al. 2015; Osuolale and Okoh, 2015). These quality parameters are extensively applied to evaluate performance of WWTPs and quality of effluent before discharge, as well as quality of receiving water bodies (Popa et al. 2012; Rahmanian et al. 2015)

Another common approach of assessing the performance of wastewater treatment facility is use of chemical analysis of specific pollutants present in effluent. Several pollutants can be analysed in wastewater and water resources using chemical analysis methods. For example, specific organic compounds and inorganic pollutants like heavy metals are analyzed using potentiometric and atomic absorption spectrophotometer methods (Popa et al. 2012; Islam et al. 2016). Other organic compounds such as hormones are analyzed by quantitative assays like enzyme linked immunosorbent assay (ELISA) (Swart and Pool, 2007; Faul et al. 2013; Manickum and John, 2015).

Although chemical analysis methods are considered to be precise and reliable, identification of a specific compound in a complex matrix of wastewater may pose many challenges. One example of such challenge is detection problems. This is a challenge because concentration of most pollutants in wastewater may be at a very low concentration making it undetectable by most of analytical instruments. To overcome the low concentration problem, extraction methods such as solid phase extraction are normally used to concentrate the pollutants present in trace levels (Swart and Pool, 2007). Unfortunately, concentration processes may have adverse effects to some analytical procedures. Given the diversity of pollutants in wastewater, identification of single

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specific compound in a complex matrix of wastewater may need performing several assays which can be uneconomical. In fact some pollutants in wastewater mixture are unknown, hence difficult to determine.

Furthermore, physicochemical parameters and analysis of specific pollutants in wastewater effluent may not give information regarding the efficiency of removal of adverse biological effects (Sacan and Balcioglu, 2006). This is because pollutants present in wastewater can cause many biological effects such as cytotoxicity, endocrine disruption and immunotoxicity (Casanova-Nakayama et al. 2011; Nygaard et al. 2013; Leme et al. 2014). In order to ascertain the efficient removal of pollutants in a mixture of wastewater with adverse biological effects, the use of biomarkers of effects is considered ideal (Silins and Högberg, 2011).

A biomarker is any measurable biological change in an organism and cells that relate to the exposure to pollutants. Biological changes in organisms and cells can be assessed by bioassays. There are several bioassays that have been developed and applied to evaluate biological effects of immunotoxicity of pollutants in wastewater. The most common bioassays implemented for assessment of immunotixicity of pollutants in water include the use of whole animal (*in vivo*) (Gust et al. 2013; Baršienė et al. 2009) and whole human blood cultures (i*n vitro)* studies (Pool et al. 2000; Pool and Magcwebeba, 2009; Wichmamn et al. 2004 and Adebayo et al. 2014). The use of *in vivo* animal studies is usually done by exposing aquatic living organisms to effluent and water. The living organisms preferred in these *in vivo* studies are either aquatic invertebrates or vertebrates. For examples, Gust et al. (2013) used *Lymnaea stagnalis* snail to examine the

immunotoxic effects of surface waters contaminated by municipal effluent. The results showed changes in hemocyte number, viability, reactive oxygen species (ROS) production and gene expression of several biomarkers of immunotoxicity including nitric oxide synthases (NOS). In another study, Baršienė, et al. (2009) assayed cytotoxicity and immunotoxicity in peripheral blood cells of rainbow trout (*Oncorhynchus mykiss*) exposed to treated wastewater effluent. The results revealed that although effluent quality satisfied current criteria for effluent discharge, exposure for 12 days induced cytotoxicity and significant decrease of immune cells.

Alternative to animal studies, use of human whole blood culture has been developed and implemented to assess inflammatory activities in effluent and water bodies (Pool et al. 2000; Pool and Magcwebeba, 2009). Instead of using whole blood culture, other studies have used isolated peripheral human mononuclear cell culture to assess immunotoxicity of wastewater (Wichmann et al. 2004 and Adebayo et al. 2014). The use of whole blood culture and isolated peripheral human mononuclear cell culture are therefore recommended for assessment inflammatory activities due to their precision, simplicity and low cost (Damsgaard et al. 2009; Silva et al. 2013). Although these techniques are recommended, the routine application of whole animals and whole blood culture for assessment of effluent quality may be hindered by ethical issues.

In order to avoid stern ethical requirements, the use of established cell lines that can be induced to display typical immune system responses such as inflammation can be implemented as strategies towards to replacement of *in vivo* and whole blood cultures. One example of such established cell lines, which is commonly used to evaluate

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inflammatory responses, is the mouse macrophage RAW264.7 cell line. The RAW264.7 cells are mouse ascites leukaemia induced cells, which when activated secrete inflammatory mediators and pro-inflammatory cytokines such as nitric oxide (NO) and interleukin 6 (IL-6), respectively.

The RAW264.7 cell line has been used extensively for assessment of inflammatory responses caused by environmental pathogens. For example, Hirvonen et al., (2005) used macrophage RAW264.7 cells to evaluate cytotoxicity and inflammatory effects of environmental bacterial and fungal strains from damaged buildings. The environmental bacterial and fungal strains induced cytotoxicity and increased production of pro-inflammatory cytokines like IL-6 in RAW264.7 cell culture. Similarly, RAW264.7 cells line has been commonly used to assess anti-inflammatory effects of natural products. For instance, Lee and Park, (2011) used stimulated RAW264.7 cells to investigate the anti-inflammatory effects of myristicin, an aromatic compound extracted from seeds of nutmeg *Myristica fragrans*. Myristicin inhibited production of NO and secretion of several cytokines including IL-6 in stimulated RAW264.7 cell culture. In a similar study, Chen et al. (2016) used RAW264.7 cells to evaluate the anti-inflammatory activities of Eupafolin extracted from *Artemisia princeps*. Treatment of stimulated RAW264.7 cell culture with Eupafolin inhibited production of inflammatory mediators like NO, and secretion of pro-inflammatory cytokines including IL-6.

Although macrophage RAW264.7 cell line has been extensively applied for assessment of various inflammatory responses, very few studies have reported use of this same cell line to evaluate inflammatory effects of water pollutants and quality of effluent.

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Therefore, the aim of this study was to use mouse macrophage RAW264.7 cell line as a cell culture model for monitoring of environmental pollutants in wastewater.

1.2   Objectives

1. To evaluate effects of endocrine disrupting chemicals on biomarkers of inflammation produced by lipopolysaccharide stimulated RAW264.7 macrophages.

2. To assess toxicity and inflammatory activity of sewage samples using RAW264.7 cell culture model.

3. To evaluate cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by coagulation-flocculation methods using RAW264.7 macrophages.

4. To evaluate toxicity and inflammatory activities of a textile effluent treated with electrohydraulic discharge and coagulant/flocculants using RAW264.7 macrophages.

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Chapter 2: Literature review

2.1 Introduction

Wastewater is a mixture of used water that may carry different types of pollutants depending on sources of origin. The predominant sources of wastewater are domestic, storm water, agricultural and farm runoff. Other common sources of wastewater include commercial establishments like hospitals, restaurants and industrial effluents. The source of wastewater determines contents or type of pollutants and characteristics of a particular wastewater (Popa et al. 2012). In most situations, wastewater contains high levels of nutrients, pathogens, heavy metals, and various other inorganic and organic compounds. However, the type and amount of wastewater generated by industries depend on the type of production involved in the particular industry. The release of wastewater from municipal and industrial effluents is considered as one of the main point sources of pollution of surface water bodies worldwide (Katsoyiannis and Samara, 2007; Igbinosa and Okoh, 2009; Olugbuyiro, 2011).

The composition and characteristics of wastewater determine the type of treatment method that is ideal for the particular type of wastewater (Katsoyiannis and Samara, 2007). Based on the diverse nature, sources, composition, and characteristics of wastewater, various wastewater treatment techniques are required to render treated waste water suitable for discharge into water bodies. Furthermore, performance efficiency of each treatment technique differs depending on the type and nature of a particular wastewater. Release of raw or inadequately treated effluent is the main cause of various adverse environmental and health effects (Drury et al. 2013; Naidoo and Olaniran, 2013; Li et al. 2015).

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In order to avoid adverse effects associated with discharging of partially treated wastewater, there is an increasing need to assess the performance efficiency of individual treatment techniques used, as well as the quality of the final effluent before discharge. Therefore, the focus of this review is mainly on sources, composition, characteristics, and treatment techniques of municipal and textile industry wastewater. In addition, the review also focus on common adverse effects of wastewater pollutants and macrophages immunotoxicity biomarkers that can potentially be used to evaluate performance of different wastewater treatment techniques, as well as quality of effluent.

2.2 Sources of wastewater

2.2.1 Sources of municipal wastewater

Municipal wastewater, commonly known as sewage, receives wastewater from diverse sources. The main sources are domestic and commercial establishments, storm water, agricultural farm runoff and industrial effluents. Wastewater from domestic sources carries many pollutants discharged from black and grey water, migration from or corrosion of infrastructures, and anthropogenic activities (Tjandraatmadja and Diaper, 2006; Tjandraatmadja et al. 2010). Black water is wastewater originating from toilets made up of feces and urine. Grey water, on the other hand, is water used in showers, laundry and kitchen sinks. Both black and grey water are the main contributors to municipal sewage. However, the contribution of individual black and grey water to municipal sewage is variable depending on local condition, infrastructure, and household characteristics like water usage and habits (Tjandraatmadja and Diaper, 2006). Nevertheless, it is generally agreed that the contribution of black and grey water to the organic and inorganic pollutants in municipal sewage is very significant (Palmquist and

Harneus, 2005; Zeng and Mitch, 2015). In fact, both black and grey water are the main sources of nutrients (Nitrogen and Phosphorus), pathogens, and other excreted organic compound like hormones, antibiotics, pharmaceuticals in sewage, while contributing much lower levels of heavy metals (Tjandraatmadja and Burn, 2005). In addition, grey water carries other pollutants from corrosion of infrastructure and various household anthropogenic activities (Friedler, 2004; Donner et al., 2010). Migration of pollutants and corrosion of plumbing infrastructure are the main sources of heavy metals. Domestic anthropogenic activities in sewage are due to laundry products like detergents, disinfectants, bleaches, and personal care products (Donner et al. 2010; Leonard et al. 2016).

Apart from pollutants from domestic sources, sewage also receives wastewater from storm water, agricultural farm runoff and industrial effluents. Storm water is well known as an important source of wastewater to sewage and water bodies. For example, Wilkison et al. (2001) analyzed at least 10 storm flow events and their effects on water quality of Blue River in Kansas, USA. It was concluded that quality of water in the receiving river was continuously affected by continuous discharge of treated sewage effluent and storm water flows.

Agricultural farm runoff is also a source of wastewater that carries sediments, fertilizers, manure, heavy metals and various chemicals (Brodie et al. 2012). Fertilizer is also a source of nutrients like nitrogen in form of nitrate and phosphorus as phosphate. Chemicals used on farms to control weeds, insects, and pathogens are sources of organic pollutants like herbicides, pesticides, fungicides and antibiotics are common component

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of farm runoff. Manure is a source of nutrients in form of organic materials, pathogens and hormones excreted by farm animals. Therefore, increased modern farming characterized by extensive use of fertilizers and disease control strategies like use of pesticides has resulted in the accumulation of pollutants in soils. Once the soil receive excess infiltration of water from heavy irrigation and/or precipitation it results in diffused source of pollution to receiving sewage and surface water bodies. Some studies have linked agricultural runoff and the levels of environmental water pollution. Bowmer (2013) reviewed agriculture pollutants that affect the aquatic environment in Australia. The review showed that the agricultural chemicals are the main sources of nitrogen and phosphorus from fertilizer, herbicides, insecticides and fungicides to surface water bodies.

Another important source of wastewater to municipal sewage and environment is industrial effluent. Industrial effluent is normally classified into inorganic and organic wastewater (Hanchang, 2009). Inorganic industrial wastewater is produced mainly from metal processing industries, mining and nonmetallic mineral industries. Wastewater from these industries is the main source of heavy metals. On the other hand, organic industrial wastewater is produced from chemical industries which use organic compounds in their production processes. Examples of industries producing organic industrial wastewater are breweries, tanneries, paper mills, oil refineries, and pharmaceuticals and textiles industries (Hanchang, 2009). Effluent from any of these industries contributes significantly to total municipal sewage and environmental pollution. However, composition and characteristics of industrial wastewater varies depending on the type of production processes used in a particular industry.

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2.2.2 Sources of textile industry wastewater

The main sources of wastewater from textile industries are due to cooling, sanitary and processing operations. Processing in the textile industry uses a large amount of water and generates a large amount of wastewater. Processing in textile industries involve three main steps namely; preparation, dyeing and finishing (Le Marechal et al. 2012). Preparation is the first step aiming at removing impurities from fabrics by using various chemicals. The chemicals used for cleaning fabrics are many and include alkalis and detergents. After cleaning, fabrics are bleached using hydrogen peroxide and chlorine compounds.

The second step is dyeing process which is the application of color to textile materials using synthetic organic dyes. The dyeing process includes pre-treatment dyeing, printing, finishing, and other technologies. This process uses large volumes of water containing chemicals such as acids, electrolytes, surfactants, chelating agents, emulsifying oils etc (Carmen and Daniela, 2012; Ghaly et al. 2014).

The final process in textile dyeing is washing that removes excess amount of dyes and other chemicals. The washing process can release up to 10-25% of dyes used (Ahmed et al. 2012). Washing generates a large amount of wastewater containing dyes and other chemicals including fixing agents, bleaching agents and organic softeners (Le Marechal et al. 2012; Kent, 2012). The contribution of textile wastewater to total pollutant discharges is well documented (Tsuzuki, 2012; Ahmed et al. 2012). Consequently, discharging of textile effluent is one of the main sources of pollutants to receiving water bodies. In summary, textile effluent, due to the large volume and high concentrations of processing additives, changes the physico-chemical parameters of water bodies and could

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lead to various adverse environmental and health effects (Awomeso et al. 2010; Ahmed et al. 2012; Khan and Malik, 2014).

2.3 Characteristics of wastewater

In most cases, wastewater is made up of water (99%), with variable physical, chemical and biological characteristics (Saha et al. 2012). Physically, wastewater is characterized by its color, odor, hydrogen ion concentration, and temperature among many others. Chemically, wastewater is characterized based on the levels of organic, inorganic as well as gas contents. Biologically, wastewater is characterized based on its microbial contents. These characteristics differ from place to place depending on the sources of wastewater. Understanding characteristics of wastewater determines the performance of wastewater treatment for a particular type of wastewater. Therefore, characterization of wastewater is important for making choice of treatment technique and extent of treatment. Furthermore, characteristics of wastewater are useful for assessment of efficiency of treatment and quality of effluent (Muserere et al. 2014). The following is an overview of some important characteristics of wastewater that are commonly used for evaluation of quality of effluent and hence performance of treatment facilities.

2.3.1 pH of effluent

This is a measure of hydrogen ion concentration expressed as pH. It is an indicator of acidity or alkalinity of wastewater. The pH of municipal sewage is usually above 7. However, this can be affected by presence of industrial wastewater. While pH of raw sewage ranges between 5.5 –8.0, that of textile wastewater is usually alkaline and in the range of 9.22 to 11.60 (Uwidia and Ejeomo, 2014). Therefore, discharging effluent with

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extreme pH values can adversely affect quality of receiving water bodies and pose risks to aquatic living organisms.

2.3.2 Temperature of effluent

Temperature of wastewater is an important qualitative parameter that varies with season of the year and source of effluent origin. For instance, temperature of wastewater from processing industries is usually slightly higher than domestic sewage. Generally in most tropical areas, temperature of municipal sewage ranges from 15 to 35°C in different seasons. Temperature is an important parameter that affects oxygen solubility and microbial activities, hence wastewater treatment processes.

2.3.3 Color of effluent

Color of wastewater depends on the nature and concentration of pollutants in it. Color is a qualitative characteristic which is useful for evaluation of quality of effluent. Compared to municipal sewage, textile wastewater is usually characterized with extremely strong color (Dey and Islam, 2015). Strong color of wastewater from textile industry is due to the concentration of dyes used in a particular industry.

2.3.4 Odor of effluent

Odor of wastewater is a result of chemical content and decomposition processes of organic matter. The decomposition process produces different types of odors depending on compounds produced. For example, ammonia produces pungent smell. Therefore, odor is a qualitative characteristic which is useful for evaluation of quality of wastewater. Compared to municipal sewage, textile wastewater is usually characterized with extremely strong odor (Dey and Islam, 2015).

2.3.5 Turbidity of effluent

Turbidity is a measure of extent of light transmission properties of wastewater. It is influenced by the content of suspended matter. Therefore, concentrated wastewater is normally characterized by greater turbidity. High turbidity can adversely affect primary producer organisms in a wastewater treatment facility and receiving water bodies.

2.3.6 Solid contents of effluent

Total solid content in wastewater consist of the suspended solids (TSS) and dissolved solids (TDS). TSS is a measure of the total amount of solids that include non-filterable organic and inorganic solids in wastewater. On the other hand, TDS measures filterable organic and inorganic matter in wastewater. The solid content is used to determine strength of wastewater and pollutants contents in the wastewater effluent. Therefore, estimation of solid content is important for determination of wastewater treatment methods as well as the quality of effluent before discharged to the environment.

2.3.7 Organic materials in effluent

Organic matter is the main pollutant in most types of wastewater. Organic matters present in wastewater are mixture of carbohydrates, lipids, proteinous materials. Gross content of organic matter is indirectly determined based on the oxidation of organic materials in wastewater. The oxidation of organic materials is determined by indirect methods for estimating the levels of biodegradable organic matter using biochemical oxygen demand (BOD) and chemical oxygen demand (COD). BOD is a measure of organic content of wastewater as determined by the amount of dissolved oxygen that is required to decompose biodegradable organic matter in wastewater. It is used extensively to determine quality of effluent and water. The BOD of raw municipal sewage ranges from

100 to 400mg/L. Textile industry wastewater contains a high concentration of organic materials hence high BOD values (Dey and Islam, 2015).

Next to BOD is COD which is a measure of dissolved oxygen required for chemically oxidation of organic matter in wastewater. It is widely used for evaluation of quality of water and performance of wastewater treatment facilities. The general range of COD of raw municipal sewage is 200 – 700 mg/L. COD is also extensively used to evaluate the quality of effluent before it is discharged. Popa et al., (2012) analyzed urban wastewater before discharged into a river; and their results revealed that domestic waste water had higher COD values than some industrial wastewater. However, COD of raw textile wastewater is usually high due to high content of organic materials (Hussaini et al. 2013).

2.3.8 Microbial characteristics of effluent

Microbial characteristics of wastewater are determined by its microbe contents. Important microorganisms in wastewater are bacteria, viruses, fungi, helminthes and protozoans. Presence of these microorganisms in wastewater has both advantages and disadvantages. The main disadvantage of microbes' preseny in wastewater is the association of microbes with outbreak of water borne diseases (Cabral, 2010; Gall et al. 2015). Examples of common water borne diseases are typhoid, dysentery and gastritis caused by bacteria (Rodrigues and Cunha, 2017). Gastritis is also caused by viruses such as enterovirus, rotavirus and norovirus which are common in sewage (Rodrigues and Cunha, 2017).

Apart from disease causing effects, some microbes present in wastewater have many known advantages. In wastewater treatment plants (WWTPs), microbial metabolism is used to facilitate biological removal of organic pollutants during secondary treatment

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processes. Thus, microbes are used in biological processes like aerated lagoons, aerated tanks, oxidation ponds trickling filters and activated sludge (Topare et al. 2011). The other benefit of microbes in wastewater is their use as indicator organisms of the quality of sewage, effluent and water resources. Due to difficulties in the identification of individual microbes in water, the use of indicator organisms are usually implemented. For example, to evaluate quality of effluent and water sources, enteric bacteria such as coliforms, faecal coliforms and faecal streptococci, which are excreted by both human and animals are commonly used as indicator organisms of fecal contamination (Okereke et al., 2016). The most commonly used microbial indicators are total coliform, fecal coliforms and *Escherichia coli* determined as colony forming unit per milliliter (Cfu/ml) (Montazeri et al. 2015).

2.4 Types of pollutants in wastewater

Types of pollutants in wastewater depend on the source of origin. In most cases, types of pollutants in wastewater are nutrients, pathogens, heavy metals and organic compounds like pesticides, industrial chemicals, pharmaceuticals and natural hormones (Ratolab et al. 2012; Akpor et al. 2014). Common pollutants in wastewater and their main sources are summarized in table 2.1.

Table 2.1 Examples of common types of pollutants in wastewater

Class pollutant	Examples of pollutants	Main source to wastewater
Nutrients	Nitrogen (N)	Domestic waste, natural decaying organic materials, manure, human feces, industrial wastewater
	Phosphorus (P)	Food residuals, synthetic detergents, fertilizer, industrial wastewater
Pathogens	Bacteria –e.g *E.Coli, Salmonella Spp, Vibrio Cholera;* Virus e.g Enterovirus; and protozoa e.g *Crytosporidium spp*	Alimentary canal of human and animals
Heavy metals	Chromium (Cr), Copper (Cu), Lead (Pb), Nickel (Ni), Mercury (Hg), Arsenic (As) and Cadmium (Cd)	Mining activities, Landfill leachates and industrial effluents
Pesticides	DDT, Endosulfan, Diedrin, Methoxychlor	Agricultural farm run-off, household insecticide application
Industrial chemicals	Polychlorinated biphenyls (PCBs) Bisphenol A (BPA)	Electric transformers and capacitors Polycarbonate and Epoxy
	Textile dyes (Azo dyes)	Textile industrial effluent
Pharmaceuticals	Antibiotics, Nonsteroidal anti-inflammatory drugs-NSAID (Diclofenac, Ibuprofen); Contraceptives e.g 17α-Ethnylestradiol (EE2), and Diethylestradiol (DES)	Hospital waste effluent, Medical and veterinary medicine disposals, animal and human excretion
Natural hormones	Androgens (Testosterone), Estrogens (Estradiol, Estrone)	Animal and human excretion

2.4.1 Nutrients

Nutrients are essential elements for animals and plants metabolism. They include

Nitrogen (N) and Phosphorous (P), whose occurrence in wastewater is more common in

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municipal sewage than in textile wastewater. In municipal sewage nitrogen and phosphorus originate from diverse sources such as human feces, manure, urine, fertilizers, garbage dumps and landfill leachate (Tjandraatmadja and Burn, 2005). Other common sources of nutrients include food residues, synthetic detergents and some industrial wastewater (Mtshali et al. 2014). In textile wastewater N is normally from dyes and textile raw materials, and P is from detergents (Kent, 2012).

Although nitrogen and phosphorus are vital nutrients for both animal and plants, discharge of untreated wastewater with excessive amount of nutrients causes pollution to receiving water bodies. High levels of nutrients in water bodies are the main causes of excessive growth of aquatic weeds and algae leading to algal bloom and eutrophication (Wang et al. 2010). Eutrophication of surface water and water bodies is a common concern of water pollution in both developed and developing countries (Dorgham, 2010; Schindler, 2012). Eutrophication is associated with blooms of toxic *Cyanobacteria microsystis* in water bodies (Xu et al. 2010). Furthermore, excessive nitrogen during eutrophication can be converted to nitrate that may cause toxicity to aquatic animals (Cargo and Alonso, 2006). Fortunately, nutrients are effectively removed from wastewater by most of the converntional techniques that employs physical, chemical and biological processes and more advanced techniques (Jiménez et al. 2010).

2.4.2 Pathogens
Pathogens are disease causing microorganisms. The most common and important sources of pathogens to wastewater are excretions from animals and humans. Therefore, raw municipal wastewater is typically characterized by high pathogens content due to domestic wastewater and farm runoff. Pathogenic microbes are rarely found in textile

wastewater. Common pathogens in wastewater are bacteria, viruses, fungi, helminthes and protozoans.

Bacteria are the major pathogens in municipal sewage. The most common bacteria in sewage are *Vibrio spp*, *Salmonella spp*, *Shigella spp* and *Eschericia coli* (Naidoo and Olaniran, 2013; Xu et al. 2014). These pathogenic bacteria are well known to contaminate water resources. The contamination of surface water resource is by release of raw or inadequately treated sewage containing bacteria. For example, Khouadja et al., (2014) investigated the occurrence of *V. parahaemolyticus* and *V. cholerae* strains in effluent samples from a WWTP in Tunisia. Some *V. parahaemolyticus* and *V. cholerae* strains identified had several virulence genes. Identification of *Vibrio* spp with several virulence genes revealed the potential public health risks associated with treated wastewater. In South Africa, Nongogo and Okoh (2014) evaluated the occurrence of *Vibrio* spp in effluent samples from five WWTPs in Eastern Cape. The final effluent samples contained mainly *V. parahaemolyticus, V. fluvialis, and V. vulnificus*. Therefore, exposure to effluent and water resources contaminated with pathogenic bacteria is the main cause of waterborne and food borne infections (Pandey et al. 2014). Common waterborne and food borne diseases caused by pathogenic bacteria in wastewater are cholera, typhoid fever, and bacillary dysentery (Cabral, 2010).

On the other hand, common pathogenic viruses in water are enteroviruses and cytomegaloviruses (Cannon et al. 2011; WHO 2011). Exposure to wastewater and water resources contaminated with pathogenic viruses is also a main cause of waterborne and food borne infections (Pandey et al. 2014). The commonest waterborne disease caused by

exposure to wastewater and water resources contaminated with pathogenic virus is gastroenteritis characterized by abdominal pain, vomiting and fever (Gall et al. 2015).

Apart from bacteria and viruses, other important pathogens found in wastewater are protozoa, fungi and helminthes. The common pathogenic protozoans reported in WWTP are *Giardia* spp and *Cryptosporidium* spp. For example, Sroka et al. (2013) reported high prevalence of *Cryptosporidium* oocysts and Giardia cysts in effluent from selected WWTPs in Poland. Hence, contamination of water bodies with *Cryptosporidium* spp is a main cause of both water and food borne diseases characterized by outbreaks of diarrhea (Gertler et al. 2015).

Pathogenic fungi found in wastewater can also contaminate water resources. Fungi are multicellular aerobic hetertropihic pathogen important in biological decomposition processes in WWTP. As a result they are abundant in effluent from most of WWTP. Presence of fungi in WWTP and effluent can also be source of environmental contamination (Korzeniewska, 2011).

2.4.3 Heavy Metals

Heavy metals are naturally occurring elements found ubiquitously in the earth crust. The most important heavy metal pollutants in municipal sewage are chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), mercury (Hg), arsenic (As) and cadmium (Cd). The main sources of heavy metals to sewage are mining activities, landfill leachates and industrial effluents (Akpor et al. 2014). Heavy metals are also common in textile wastewater. The most common metals in textile wastewater are Pb, zinc (Zn), As, Cd, Ni, Cr, and Cu (Das et al. 2011).

Heavy metals are persistent in wastewater, since it cannot be degraded. Therefore, high levels of heavy metals in effluent are potential sources of contamination of water resources. As a result heavy metal concentrations exceeding drinking water standards are commonly reported in some places (Li et al. 2014; El-Kowrany et al. 2016).

Furthermore, heavy metals can be bioaccumuled along the food chain. Therefore, water resources contaminated with heavy metals are potential sources of many adverse health effects including toxicity, carcinogenicity, neurological and psychological disturbances, endocrine disruption and immunotoxicity (El-Kowrany et al. 2016). In order to avoid these adverse effects, efficient removal of heavy metals from wastewater is very important. Efficient removal of heavy metals can be achieved by most of conversional methods such as chemical adsorption, precipitation, ion exchange, electrochemical deposition and constructed wetlands (Barakat, 2011; Roy et al. 2014; Shibambu et al. 2017).

2.4.4 Pesticides

Pesticides are chemical compounds with wide range of applications. They are extensively used in agriculture for crop protection, food and food product preservation, and prevention of vector-borne diseases in both livestock and humans. After application, the residual chemicals are released into municipal wastewater from agricultural farm run-off, industrial effluents and from household. Unfortunately, most of these pesticides are non-degradable in nature hence are persistent in environment. Some important pesticides common in wastewater are Organophosphorus (OP) and Organochlorinated (OC) pesticides; herbicides and insecticides (Jayaraj et al. 2016). The list of pesticides that are the most common and persistent in wastewater is very long, including Dichloro Diphenyl

Trichloroethane (DDT); Endosulfan; Aldrin and Dieldrin and Methoxychlor (Jayaraj et al. 2016).

Although the use of organochlorinated pesticides has been banned in developed countries, they are still widely used in tropical countries to control mosquitoes causing malaria. As a result of extensive uses, pesticides are commonly reported in wastewater and water bodies in many countries. Syakti et al. (2012) measured levels of OC in marine sediments from sites receiving discharged wastewater in Marseille, France. The sediments contained high levels of DDT ranging from 0.7 to 114.3 ng/g. The high levels of DDT in sediments were associated with long term contamination. Recently, in South Africa, Buah-Kwofie and Humphries (2017) determined the distribution of OC pesticides in a lake and wetland parks. The findings showed that high concentrations of OC pesticides were widely distributed in sediment samples. The high contents and distribution were attributed to wastewater received from agricultural activities and household application of pesticides.

2.4.5 Industrial Chemicals

Industrial chemicals form part of persistent organic pollutants (POPs) that are widely used in industrial processes. They are resistant to degradation and hence persistent in the environment. As a result of persistence, they are characterized by bioaccumulation in environment and biota. There are a large number of currently used industrial POPs. However the common examples discussed in the following sections are the polychlorinated biphenyls (PCBs), Bisphenol A (BPA) and textile dyes.

2.4.5.1 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are organic chlorides, which are widely used in various industrial manufacturing. They are extensively used in manufacturing capacitors, in electric transformers as hydraulic exchange fluid and as an additive in paints and lubricants. Factories involved in production of such products are the main sources of PCBs contamination of wastewater and the environment. Other sources of PCBs to the environment are combustion processes such as waste incineration, fossil fuel burning and many other incomplete combustion processes (Samara et al. 2006).

Given the diverse sources, the possibility of environmental contamination with PCBs is also high. For instance, Syakti et al. (2012) measured levels of PCBs and their congeners in marine sediments from sites receiving discharged wastewater in Marseille, France. The results showed high levels of PCBs and their congeners, which were associated with continuous inputs from industrial effluent and resistance to degradation processes. Yao et al. (2014) investigated the occurrence and fate of PCBs in the conversional WWTP located in a dyeing chemical industry zone in Zhejieng, China. The analysis of raw wastewater revealed high total PCBs contents with various congeners. The removal efficiency of the converntional treatment processes was only 23.2%, indicating possible environmental risks of the effluent.

2.4.5.2 Bisphenol A

Bisphenol A (BPA) is another industrial chemical used widely with adverse environmental and health effects. BPA is a synthetic organic chemical with a wide range of industrial uses. It is an important component in production of polycarbonate plastics, epoxy resins and other plastics (CEH, 2010). Polycarbonates are used in production of

food and drinks packaging materials, electronic and media appliances, and some medical devices. Epoxy resins which contain a derivative of BPA, bisphenol A diglycidyl ether (BADGE or DGEBA) is also commonly used for coating interior of food and beverage cans (CEH, 2010). Therefore food contents, beverage and water can be contaminated with BPA due to leachate from containers (Li et al. 2010). For example analysis of infant formula recorded the concentration of BPA at a range of 0.55 - 5.4 ug/kg (Ackerman et al. 2010). Thus, consumption of canned food is one of the main sources of human exposure that may lead to BPA content in the body (Milke, 2009). The BPA in the body is excreted through urine, which is one of the sources to sewage (Ye, et al. 2009).

Other uses of BPA include production of flame retardants, Polyvinyl Chloride (PVC) stabilizers and thermal papers (CEH, 2010). Thus effluent from industries involved in such productions are also potential sources of BPA to sewage. Other common sources of BPA to sewage are leachate from plastic materials and landfills (Viecelli et al. 2014). As a result of these diverse sources, BPA is commonly reported in many WWTP, both in influent and effluent because it can escape most of the traditional wastewater treatment techniques. Pookpoosa et al. (2015) investigated the occurrence and fate of BPA in five municipal WWTP in Bangkok. The concentration of BPA in influents ranged between 128.5ng/L and 606.5ng/L. After treatment in WWTPs using variants of the activated sludge technique, the concentration of BPA in effluent ranged between 57.5ng/L and 257.0ng/L, the amount which can still pose environmental and health threats to the receiving water bodies.

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2.4.5.3 Textile dyes

Textile dyes are compounds used to add color to textile fabric and fibers. They are extensively used in textile industry processes. There are several types of textile dyes in use including synthetic dyes which are more commonly used currently. There are two main classes of synthetic dyes namely the azo dyes and anthraquinone dyes. The azo dyes are characterized by reactive groups, and are the most commonly used textile colorant constituting 70% of commercial textile dyes (Chanquer et al. 2013). The second most used textile dyes are the anthraquinone dyes which are used for attaining violet, blue and green colors.

During the dying processes, a large amount of residual dyes are released in wastewater. The high amount of dyes released contributes to the typical characteristics of textile wastewater namely strong color, BOD and COD (Chequer et al. 2013). Therefore, contamination of water resources with textile dyes increases water turbidity, which interferes with light penetration and photosynthesis leading to poor water quality. The synthetic dyes also have many toxic effects due to its direct action, or due to its derivatives generated during biotransformation (Rajaguru et al. 1999). For example, when azo dyes are ingested, they can be metabolized to aromatic amines by azoreductase and nitroreductase enzymes of intestinal microorganisms. Similarly, azo dyes can be metabolized by liver enzymes of mammals by catalyzing reductive cleavage and nitroreduction of azo bond and nitro group respectively. The N-hydroxylamines formed in both metabolic pathways are known to induce DNA damage (Rajaguru et al. 1999; Marthur et al. 2005; Umbuzeiro, et al. 2005). Further toxicity can be induced by continuous exposure to synthetic dyes that may result to various toxic effects including

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acute toxicity (Suryavathi et al. 2005), immunotoxicity (Verma et al. 2012) and contact allergies (Ryberg et al. 2006; Malinauskiene et al. 2011).

2.4.6 Pharmaceuticals

Pharmaceuticals are chemicals used extensively for the treatment of animal and human diseases. Therefore, the main sources of pharmaceuticals in wastewater are excretion from human and animals, hospital medical waste and pharmaceutical industrial effluents (Sim et al. 2011). Because of the diverse sources of pharmaceuticals, they are also frequently reported in sewage and effluents (Sim et al. 2011; Bahera et al. 2011; Ngumba et al. 2016). Once in sewage, most of the pharmaceuticals are resistant to removal by most of the conventional treatment techniques, and hence passes through effluent to water resources. The most common classes of pharmaceuticals reported in effluent and water resources are antibiotics, antivirals, analgesics, antineoplastics and contraceptives.

Antibiotics are pharmaceuticals that are commonly used for treatment of bacterial infections in animals and humans. Antibiotics are numerous and include sulfonamide, sulfamethoxazole, trimethoprim, tetracycline, erythromycin, tylosin and ciprofloxacin. Some antibiotics are persistent in nature and escape most of the traditional wastewater treatment methods. For example, Ngumba et al. (2016) investigated the occurrence of antibiotic in the Nairobi River Basin, Kenya. The results showed the presence of sulfamethoxazole, trimethoprim and ciprofloxacin in river water. Similarly, Li et al. (2014) investigated the occurrence and levels of antibiotics in WWTP and in receiving rivers in Beijing, China. The results showed that some antibiotics were difficult to remove from wastewater by conversional treatment methods, and were detected in effluent and river water.

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Analgesics, also known as non-steroidal anti-inflammatory drugs (NSAIDs), are the most prescribed and extensively used drugs in the world. They are used as antipyretic, analgesic and anti-inflammation drugs. Some of the most popular NSAIDs and commonly reported in WWTPs are diclofenac, ibuprofen, phenozone, and acetaminophen. Martin et al. (2012) reported the presence of various NSAIDs in influent and effluent wastewater, indicating that ibuprofen posed the highest ecological risks. Therefore discharging effluent could be one of the main sources of NSAIDs to water resources. Similarly, Valcárcel et al. (2011) determined analgesics/NSAIDs drugs in river water samples from Madrid, Spain. The findings showed that river water contained high levels of diclofenac and ibuprofen.

Antineoplastics are anti-cancer drugs used for treatment of different types of cancers. Examples of anti-cancer drugs which are commonly reported in WWTPs are cisplatin, cyclophosphamide, ifosfamide, methotrexate, tamoxifen and flutamide among many others (Xie, 2012). Several studies have attempted to evaluate the presence of anti-cancer drugs in WWTPs as well as in surface water bodies. Buerge et al. (2006) determined the occurrence and fate of cyclophosphamide, ifosfamide in WWTP and surface water bodies in Switzerland. The concentration of cyclophosphamide and ifosfamide ranged between <0.3 to 11ng/L and <50 to 170pg/L in effluent and surface water bodies respectively. In a similar study, Ferrando-Climent, et al. (2014) studied the presence of anticancer drugs in WWTPs effluent and in receiving water bodies. The study reported the presence of cyclophosphamide and tamoxifen in both WWTP effluent and in the receiving river.

Another class of pharmaceutical of concern in wastewater is contraceptives. Contraceptives of concern are groups of synthetic oral steroids which are extensively used by women for birth control. The most common synthetic steroid reported in WWTP and water resources is 17α-Ethnylestradiol (EE₂). 17α-Ethnylestradiol is an EDCs that can mimic natural estrogens leading to disruption of the endocrine system. Like many other steroids, synthetic steroids contraceptives have been reported in WWTP and surface water bodies. Mohagheghian et al. (2014) determined EE2 in influent and effluent of several municipal WWTPs in Tehran, Iran. The results showed mean concentration of EE2 as high as 4.18 – 11.76 ng/L and 0.5 – 2.58 ng/L in influent and effluent respectively. In a similar study, Zhou et al. (2012) reported the occurrence of 17α-Ethnylestradiol (EE₂) in WWTP in Beijing, China. The concentration of EE₂ in influent ranged from no detection to 3.3×10^2 ng/L with mean concentration of 48.0 ng/L. In the effluent the mean concentration was 2.0 ng/L.

2.4.7 Natural steroid hormones
The main class of natural hormones of concern in wastewater is steroid. Steroids of concern includes androgenic, estrogenic and glucocorticoid steroids. They are secreted by both vertebrate animals and humans through feces and urine.

2.4.7.1 Glucocorticoid steroids
The glucocorticoid steroids secreted by animals and humans are cortisol, prednisone, dexamethasone, prednisolone and cortisone. Their main physiological roles in the body are to regulate development, stress adaptation and control of energy metabolism through gluconeogenesis (Wilson et al. 2013; Zhao et al. 2016). Because of their role in energy metabolism, sometimes they are extensively used as growth promoters, and fattening in

animals. Glucocorticoids also suppress many inflammatory responses. This anti-inflammatory property has promoted the extensive uses of steroids to manage several inflammatory diseases in animals and humans. The extensive use of glucocorticoids has led to increased release of a large amount of glucocorticoids into sewage. As a result of extensive uses and release to wastewater, steroids have been reported in WWTPs and receiving water bodies (Schriks, et al. 2010; Chang et al. 2007; Liu et al. (2012). Schriks et al. (2010) determined various glucocorticoids in hospital wastewater influent with total concentration ranging from 13 to 1900 ng/L. The concentration of cortisol and cortisone were 27.5-300 ng/L and 381-472 ng/L respectively. Liu et al. (2012) investigated the occurrence of glucocorticoids and other steroids in WWTPs in Guangdong, China. The influent samples contained total glucocorticoids with concentration ranging from 171 - 192 ng/L. The concentration of cortisol and cortisone in influent ranged from 48.2 to 130ng/L.

2.4.7.2 Estrogenic steroids

Estrogenic steroids are predominantly female hormones which includes 17β-Estradiol, estrone and estriol. The 17β-Estradiol hormone is synthesized in ovaries and secreted by women and female vertebrate animals. While estrone is a peripherally produced metabolite of 17β-Estradiol, estriol is a metabolic product of both 17β-Estradiol and Estrone. Estrogenic steroids' main physiological roles are the maintainance of reproductive functions such as sexual development and differentiation. These physiological effects are induced through estrogenic receptors (ERs), known as ERα and ERβ (Marino et al. 2006).

All estrogenic hormones are metabolized in the liver and are excreted in urine and feces. The amount of estrogen excreted varies markedly between individuals depending on reproductive status. Menstruating woman can excrete 2-12µg of 17β-Estradiol and 3-20 µg of estrone daily, while a pregnant woman excretes as much as 260 µg of 17β-Estradiol and 600 µg of estrone per day (Johnson et al. 2000). Therefore estrogenic hormones excreted by women and female animals are considered as the main sources of estrogens to municipal wastewater (Ting and Praveena, 2017). Other common sources include hospital and pharmaceutical industrial effluent (Ting and Praveena, 2017). As a result of continuous release of excretion and industrial effluent, estrogenic hormones have been frequently detected in sewage effluents and surface water bodies (Zhou et al. 2012; Manickum and John, 2014). For instance, Zhou et al. (2012) investigated the occurrence of estrogenic hormones in WWTP in Beijing, China. The mean concentrations of estrogenic hormones in influent were 66.8, 44.7, and 2.6×10^2ng/L for estrone, 17β-Estradiol and estriol respectively. The mean concentrations in effluent were 12.7, 1.7, and 1.9 ng/L for estrone, 17β-Estradiol and estriol respectively. In South Africa, Manickum and John (2014) determined estrogenic steroids in the Pietermaritzburg wastewater treatment plant and surface water. The total mean concentrations of estrogenic steroids in influent samples were 84.0ng/L, 119.0ng/L and 5.0ng/L for estrone, 17β-Estradiol and estriol respectively. In the effluent samples the mean concentrations were 23.0ng/L, 20.0ng/L and <1ng/L for estrone, 17β-Estradiol and estriol respectively. The overall removal efficiency of steroids by activated sludge processes employed at the WWTP was 98%. As a result the presence of steroids in effluent is considered as an important source of estrogens to surface water resources. Manickum and John, (2014) reported levels of

estrone, 17β-Estradiol and estriol in a stream river receiving effluent as high as 8ng/L, 10ng/L and 6ng/L respectively. These levels of steroids can pose many adverse environmental and health risks.

2.4.7.3 Androgenic steroid

The main androgenic steroid is testosterone, which is secreted by Leydig cells of male testis. The main physiological roles of testosterone are sexual development and reproduction in male. Testosterone can also be metabolized by enzymatic action of 5α-reductace to a more biological active form known as 5α-dihydrotestosterone (DHT) (Pham and Ziboh 2002; Wu et al. 2013). Both testosterone and DHT induce their physiological effects through androgenic receptors (ARs), and are excreted through feces and urine. Therefore, androgens are common in sewage, effluent and surface water bodies. For example, Liu et al. (2012) investigated the occurrence of androgens among other steroids in WWTPs in China. The concentrations of total androgen were in range of 1554-1778 ng/L in influent and 13.3-47.8 ng/L in effluent.

High levels of androgens in effluent can contaminate water resources when WWTP processes are inefficient. For example, Manickum and John (2014) determined levels of testosterone in the Pietermaritzburg wastewater treatment plant and surface water, in South Africa. While on average the influent samples contained as high as 343ng/L of testosterone, the effluent level was 11ng/L. The level of testosterone in the treated effluent receiving water body was 10ng/L.

2.5 Wastewater treatment techniques

Wastewater treatment techniques involve several processes that take place in a wastewater treatment plant (WWTP). The processes aim at removing suspended solids,

decomposition of organic materials and reduction of pathogens. The removal and decomposition of contaminants are achieved by several processes that include physical, chemical and biological processes (ESCWA, 2003; Topare et al. 2011). In order to improve treatment performance, several individual methods can be combined to improve treatment efficiency. The treatment operations are broadly classified into preliminary, primary, secondary and tertiary treatment stages as summarized in figure 2.1 (ESCWA, 2003).
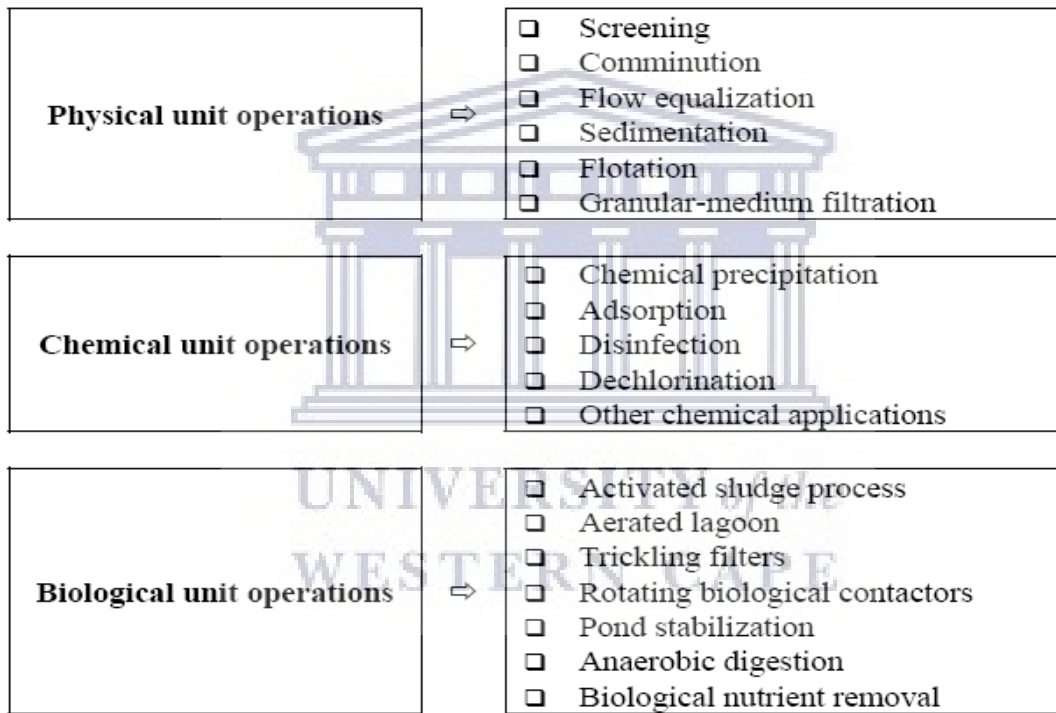
Figure 2.1 Examples of physical, chemical and biological processes of treating wastewater (ESCOWA, 2003)

2.5.1 Wastewater treatment stages

2.5.1.1 Preliminary treatments

Preliminary wastewater treatment is an initial stage of preparing raw wastewater by removing course materials that may interfere with subsequent treatment processes. The

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removal of course materials in preliminary treatment stage depends mainly on use of physical processes like using screen/sieve filters, comminution, sedimentation and floatation (ESCWA, 2003). These processes aim at removing debris, coarse materials and removal of oil and grease (Sonune and Ghate, 2004). After the preliminary processes, wastewater is allowed to the next stage of treatment processes.

2.5.1.2 Primary treatment

Primary treatment of wastewater involves mainly physical processes aiming further separation of gross materials. The common methods employed in separation process are screening, mixing, floatation, sedimentation and flocculation which remove suspended solids organic matters. In most cases physical processes in the primary treatment unit alone do not achieve effective wastewater treatment. As a result effluent from primary treatment is normally characterized by poor quality with high BOD that can be source of pollutants (Sonune and Ghate, 2004). Therefore, effluent from primary treatment requires further treatment by processes in secondary treatment stage.

2.5.1.3 Secondary treatment

Secondary treatment stage aims at removing of suspended solids and biodegradable organic matters. The main processes employed are biological processes under either aerobic or anaerobic conditions. Examples of common processes in secondary treatments are trickling filters and activated sludge processes.

2.5.1.4 Tertiary treatment

This is normally considered as the final stage in most of the wastewater treatment plants. Processes involved are physical, chemical and biological processes that aim at removal of

dissolved solids and most of organic pollutants. In some WWTPs advanced techniques such as activated charcoal, filtration and chlorination are also used (Topare et al., 2011).

2.5.2 Physical treatment processes
The most common physical processes employed in wastewater treatment are screening, comminuation, sedimentation and flotation (ESCOWA, 2003).

2.5.2.1 Screening
Screening is a preliminary treatment physical process that aims at removal of course materials that would damage the WWTP facilities. The process is achieved by use of screens that ranges from coarse screen, fine screen, very fine and micro screens (ESCWA, 2003).

2.5.2.2 Comminution
Comminution is a physical process of reducing solid materials floating in wastewater into small sizes. The process is achieved by installation of communutors with blades that crush, chop or grind floating materials into small sizes. As a result the process decreases adour, flies and unsightliness. The remaining grits, sand and gravel are removed in the grit removal chambers (ESCWA, 2003).

2.5.2.3 Sedimentation
Sedimentation is the process of settling down of suspended materials by gravitation force. The process occurs in clarifiers or settling tanks to achieve removal of grit and settled particulate materials. Sedimentation is a very common process in many wastewater treatment plants.

2.5.2.4 Flow equalization

Flow equalization is a temporary storage of wastewater in an equalization basin. Therefore, equalization entail to temporary retaining of wastewater to stabilize fluctuation parameters that can interfere with subsequent chemical and biological processes. Proper equalization facility can efficiently stabilize parameters such as temperature, pH and BOD of wastewater (ECOWAS, 2003). This process is more applicable in treatment of industrial wastewater than municipal wastewater.

2.5.3 Biological processes

Biological processes use microorganisms such bacteria, under aerobic or anaerobic condition, to remove biodegradable pollutants. Under aerobic conditions, bacteria oxidize biodegradable organic materials in wastewater into gases. Biological processes are more common in secondary treatment of wastewater aiming at removing organic substances and nutrients. Examples of biological processes are aerated lagoons, aerated tanks, oxidation ponds trickling filters and activated sludge (Topare et al., 2011).

2.5.3.1 Activated sludge

The activated sludge process depends on bacterial population suspended in wastewater under aerobic conditions to decompose organic matters. The decomposition of organic matter is achieved in several interdependent compartments. The main components of activated sludge process are aeration (bioreactor), settling or clarification and sludge return. The activated sludge is a common biological process implemented in many WWTPs. It is widely used for treatment of municipal sewage as well as industrial wastewater (Kasprzyk-Hordern et al. 2009; Rashed et al. 2014). This is an effective method of treatment that can achieve 80% - 95% efficient removal of BOD from wastewater. For example use of activated sludge process for treatment of municipal

sewage characterized with high levels of several pharmaceuticals, personal care products endocrine disruptors resulted in over 85% efficiency removal of BOD (Kasprzyk-Hordern et al. 2009). However, activated sludge process has the disadvantage of producing large quantities of sewage sludge, which requires further management before discharge.

2.5.3.2 Trickling filters

Tickling filter technology is used to remove organic materials in wastewater using aerobic bacteria attached to a medium. The bacteria population in wastewater gradually attach to a medium to form a slime biological film. As the wastewater flows over the formed film, organic materials adsorb to the film and are removed by aerobic degradation (Samer, 2015). Tickling filter processes are generally considered to be simple and reliable biological process suitable for treating of municipal sewages. However, the process requires necessitate additional treatment to remove microbial activites in the final effluent.

2.5.4 Chemical processes

Chemical reactions are very common in wastewater treatment processes. In some situations chemical reactions can be combined with physical processes hence physicochemical processes. Chemical reactions are applied in tertiary treatment process of wastewater treatment plants. Common examples of chemical processes are precipitation, adsorption and disinfection.

2.5.4.1 Precipitation

Precipitation is a physicochemical process that uses chemicals to form first coagulation and then flocculation. Coagulation and flocculation are important treatment processes in

treating industrial wastewater and drinking water. Coagulation is achieved by addition of coagulant chemicals with rapid mixing to promote coagulation process. Common coagulants are iron and aluminium compounds, synthetic organic polymers and lime (Bolto and Gregory 2007; Verma et al. 2012). The rapid mixing in a coagulation process is followed by a slow mixing stage that allows flocculation. The coagulation-flocculation processes help to sediment flocs formed during chemical coagulation of wastewater. The coagulation-flocculation can efficiently achieve removal of biodegradable organic pollutants, and nutrients, suspended matters and COD (Oller et al. 2011; Verma et al. 2012). The coagulation-flocculation has been reported to effectively achieve reduction of COD in textile industry wastewater (Sabur et al. 2012; Muukhilish et al. 2013).

2.5.4.2 Disinfection

Disinfection is the inactivation/destruction of pathogens in water or wastewater using disinfectants. Disinfection is a chemical reaction process that employs chemical agents such as chlorine, ozone and bromine to destroy structural components required for pathogen function. The most common and widely used disinfectant is chlorine and its derivatives. Chlorine is a strong oxidant that is capable of oxidizing and it efficiently removes of most of pathogenic microorganisms that are not removed by the previous techniques derung sewage treatment. Chlorination is considered one of the most economical techniques of treating wastewater and water resources (Netshidaulu, 2016). However, the process may need further dechlorination to remove harmful by-products formed during the inactivation procedure (Samer, 2015).

2.5.5 Advanced wastewater treatment processes

Advanced wastewater treatment processes are relatively new techniques intended for achieving higher quality effluent than what is achieved by secondary treatment processes. These techniques are required for further removal of residual organic pollutants remaining after secondary treatments processes. Examples of advanced wastewater treatment processes are membrane filtration; activated charcoal and advanced oxidation processes (Zhou and Smith, 2002).

2.5.5.1 Membrane filtration

Membrane filtration process applies membrane technology to separate pollutants from wastewater. The membrane processes are driven by hydraulic pressure. The technology uses different types of membrane materials of different pore size. The pore size of membrane ranges from 0.01 to 10uM. Based on the pore size, the membrane filtration processes are classified into microfiltration, ultra-filtration and nanofiltration. When the membrane filtration is applied concurrently with biological methods like activated sludge, the process is referred to as membrane bioreactor technology. The technology can selectively remove suspended solids, organic materials and microorganisms (Hai et al. 2014).

2.5.5.2 Activated charcoal

Activated charcoal is an adsorbent that is a black solid, powdered or granulated material produced from wood, bones and coconut shell. Activated charcoal is characterized by a micro-porous structure and has a large surface area that provides reactive and adsorptive properties (Mohan and Pitmann Jr. 2006). These characteristics enable activated charcoal to effectively adsorb and eliminate several pollutants from wastewater. The technique is commonly used to treat industrial wastewater. It is a very effective treatment method for

removing various inorganic and organic pollutants including textile dyes (Wang et al. 2011). As a result activated charcoal technology is one of the most suitable and widely used methods for the treatment of textile industry wastewater. Syafalni et al. (2012) applied the technology and reported 59.46% effective removal of COD and 54.4% efficient removal of color in textile wastewater.

2.5.5.3 Advanced oxidation processes

Advanced oxidation processes (AOPs) are wastewater treatment processes that are based on generation of highly reactive radicals such as hydroxyl or sulfate to remove organic pollutants (Deng and Zhao, 2015). Reactive radicals are strong oxidants formed from primary oxidants like ozone (O_3) and hydrogen peroxide (H_2O_2). As a result most of biologically active and persistent organic pollutants are oxidized and converted to non-toxic materials by one or a combination of the processes. Examples of advanced oxidation processes are ozonation, Fenton-reaction, electrochemical oxidation-reduction reactions and electrohydraulic discharge (EHD) treatment (Holender et al. 2009).

The electrohydraulic discharge (EHD) technique applies strong electric field to eliminate micro contaminants in wastewater. This technology involves the passage of a high-voltage electrical discharge between two electrodes immersed in an aqueous solution to form plasma. The formed plasma initiates both physical and chemical processes that lead to generation of free reactive species. Depending on the solution pH, conductivity and the discharge magnitude, the free reactive radicals formed are ozone (O_3), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), perhydroxyl radical ($\bullet OOH$), hydrogen peroxide (H_2O_2), ultraviolet light and shock waves (Bruggeman and Locke 2013). The generated free radicals are very reactive and non-selective, which can degrade some of the complex

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organic substances into harmless compounds (Malik 2010; Jiang et al. 2014). The reactive radicals degrade and eliminate many organic compounds which cannot be removed by traditional techniques or degraded by biological processes. The main disadvantage of this, similar to other advanced oxidation processes (AOPs), is the formation of oxidative by-products that can induce toxicity.

2.6 Adverse effects of pollutants in wastewater

Discharge of raw wastewater or inadequately treated effluent into the environment and receiving water bodies may cause serious adverse effects. In most cases, the effects are categorized as environmental and health effects (Arpor and Muchie 2011; Mazumber 2011). Health effects of exposing living organisms to water resources contaminated with effluent containing high levels of pollutants are normally associated with various health conditions. Some common health conditions associated with exposure to wastewater pollutants are due to waterborne diseases (Arpor and Muchie 2011), endocrine disruption (Carr and Patino, 2010; Mann et al., 2009), genotoxicty and immunotoxicity (Nygaard et al. 2013; Leme et al. 2014).

2.6.1 Environmental effects

Wastewater is the main contributor to the degradation of the water quality of water bodies like rivers, lakes, etc. In most cases, degradation of water quality is due to increased nutrient load, decreased levels of dissolved oxygen (DO) and the presence of toxic pollutants (Arpor and Muchie, 2011; Sibanda et al. 2015). Another environmental effect of increased nutrients in water is algal blooms commonly known as eutrophication. The main consequence of eutrophication is increased oxygen consumption leading to decreased dissolved oxygen (DO) in water. The resulting low level of DO in water

creates hypoxic condition that hinders breathing of aquatic living organisms. Furthermore, during eutrophication condition, there is increased death and decomposition of algae that also consumes dissolved oxygen in water bodies.

In addition to hypoxic condition, eutrophication also creates favourable conditions for overgrowth of toxic cyanobacteria. The favorable condition for toxic cyanobacteria overgrowth is the availability of excessive nutrients which is the main cause of blooms of toxic cyanobacteria like *Microsystis spp* in water bodies (Xu et al. 2010). Similarly, excessive nitrogen in water bodies is normally converted to nitrite that can be one of the causes of toxicity to aquatic animals (Camargo and Alonso, 2016). Further toxic effects of wastewater could be as a result of biological or biochemical stress in aquatic living organisms induced by various pollutants. For instance, Cazenave et al. (2014) analyzed toxic responses in ray-finned fish exposed to untreated sewage effluent. The results showed that fishes exposed to untreated effluent had a distinct physiological profile including changes in morphological indices, hematological and biochemical parameters.

Similar to raw wastewater, effluent and contaminated surface water bodies can induce various adverse effects. Park et al. (2014) evaluated effects of treated sewage effluent on the development of *Bombina orientalis* embryos. The results showed that malformations were more pronounced in wastewater compared to the control water. Furthermore, some pollutants contaminating water resources can induce alteration of reproduction functioning in aquatic living organisms. For example, Paschke et al. (2014) assessed toxic effects of water streams affected by wastewater on aquatic gammarid reproduction. The results showed decreased reproduction performance and changes in the sex ratio of

gammarids towards females. These effects of wastewater could be due to a single pollutant or combined effects of several pollutants in wastewater. Generally, the ecotoxicological risks associated with wastewater pollutants are many and could extend even into distant water bodies as well as drinking water sources (Hoerger et al. 2014).

Similar to sewage effluent, experimental oral exposure of textile dye wastewater in mammals has been also associated with many toxic effects. For instance, Suryavathi et al. (2005) studied the toxicity of raw and treated textile dye wastewater on male rat reproduction. The short term exposure decreased weights of reproductive organs and altered spermatogenesis characterized by increased sperm abnormalities and decreased sperm count and motility. Relatively similar but milder effects were observed in animals exposed to textile dye wastewater treated by biological treatment techniques.

2.6.2 Waterborne diseases

Waterborne diseases are caused by exposure to or consumption of water contaminated with pathogenic microorganism. Pathogenic microorganisms are very common in wastewater, therefore effluent is an important source of various waterborne disease outbreaks (Arpor and Muchie 2011; Naidoo and Olaniran, 2013; Pandey et al. 2014; Beer et al. 2015). The main pathogenic microorganisms known to contaminate water resources are bacteria, viruses, protozoans and helminths (Rodrigues and Cunha, 2017). These microorganisms are the main causes of different types of disease outbreaks. Examples of common pathogenic bacteria in wastewater are *Salmonella spp*, *E.Coli*, *Vibrio spp*, and *Campylobacter spp*. While *Salmonella spp* causes typhoid, *E. Coli* is the main cause of bacillary dysentery. Exposure to water resources contaminated with pathogenic *Vibrio*

spp and *Campylobacter spp* are the main cause of gastroenteritis (Rodrigues and Cunha, 2017).

Gastroenteritis outbreaks can also be caused by viruses like enterovirus, rotavirus and norovirus which are common in sewage (Rodrigues and Cunha, 2017). That is why an extensive gastroenteritis outbreak has been associated with drinking tap water contaminated with sewage effluent in Finland (Laine et al. 2011).

Other common diseases associated with water contamination with wastewater effluent are Cryptosporidiosis, Giardiasis, respiratory diseases and skin allergies (Rodrigues and Cunha, 2017). Cryptosporidiosis is a disease caused by a protozoan called *Cryptosporidium*. *Cryptosporidium* infects both animals and humans. In human the disease is caused by *C. parvum* and *C. hominis*, with symptoms of abdominal cramps, diarrhea, headache and vomiting (Chalmers, 2012). Pathogenic *C. parvum* and *C. hominis* are commonly reported in sewage (Chalmers, 2012; Widerström et al. 2014). Therefore, sewage effluent is considered a common source of these pathogens. Widerström, et al. (2014) investigated a large outbreak of cryptosporidiosis in Europe, caused by *C. hominis*. The study attributed the disease outbreak to *C. hominis* which was identified in clinical samples, wastewater and water supply.

Similar to Cryptosporidiosis, Giardiasis is also a protozoan waterborne disease of both animals and humans. Giardiasis is caused by *Giardia lamblia*, and *Giardia duodenalis* parasites of the gastrointestinal tract. The infection is acquired through drinking contaminated water and exposure to contaminated water during swimming. The disease is characterized by abdominal cramps, prevalent worldwide (Rodrigues and Cunha, 2017).

Another health condition associated with pollutants present in wastewater is contact dermatitis due to skin allergy. The allergic contact dermatitis is caused by repeated exposure to allergen, characterized by hypersensitive reactions (Martin, 2012). It is a chronic skin inflammatory disease which in most cases is associated with environmental factors and occupational origin. For example, a cross-sectional study associated the use of wastewater for irrigation with high prevalence of dermatitis among farmers (Anh et al. 2007). Dermatitis can be induced by many pollutants common in wastewater such as heavy metals, ingredients of personal care products and textile dyes (Kim 2015; Chung et al. 2016). Heavy metals which are considered to elicit contact allergic reactions are cobalt, chromium and nickel (Basko-Plluska et al. 2011). In cases of textile dyes, the main causes of contact allergic dermatitis reactions are disperse dyes that belong to Azo or athraquinone group of dyes (Malinauskiene et al. 2012).

2.6.3 Endocrine disruption

Many pollutants in wastewater are known to induce adverse health effects by disruption of the endocrine system. The endocrine disruption is caused by alteration of endocrine functions through various mechanisms (Kavlock et al. 1996). Some compounds mimick endogenous hormones by binding to their respective receptors. Other chemicals disrupt hormonal function by blocking receptors preventing binding of hormones. There are also compounds that alter synthesis and elimination of hormones in the body. Given the diverse nature of pollutants and their mode of action, their adverse effects are also many. Many chemical used in daily human life are released into the sewage and eventually end up in water bodies. Some of these chemicals are oestrogenic and can affect aquatic wildlife and consumers of the water. They cause effects like hermaphroditism, impaired

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testicular developments, decreased sperm counts and increased incidence of testicular cancer as well as other reproductive disorders associated with infertility (Christiansen et al. 2010). Endocrine disruption has been associated with many reproductive, developmental and a variety of detrimental health effects in wildlife and human. For example, Song et al. (2011) exposed goldfish to river water and assessed the estrogenic effects of river water. The results of early life exposure showed adverse effects on developmental, reproductive, conditioning factors and vitellogenesis. Other effects reported in association with exposure to reproductive disrupting pollutants include sex reversal, disruption of gonadal (testis and ovary) tissues and morphological changes of the male breeding nuptial gland of frogs (van Wyk et al. 2003; Kloas and Lutz, 2006).

Apart from disruption of the reproductive system, other endocrine organs may also be disrupted. For example disruption of the thyroid gland can lead to alteration in embryo development like resorption of tail, limb development and gonadal differentiation in frogs (Carr and Patino, 2010). Many water pollutants are known to disrupt thyroid hormone functions. Heavy metals, POPs such as DDT and its metabolites, Bisphenol-A (BPA) and related compounds, dioxins, phthalates, plasticizers, PCBs and PAHs can alter thyroid functions in fish and amphibians (Kashiwagi et al. 2009, Carr and Patino, 2010). Furthermore, it is generally accepted that all endocrine organs can be disrupted by EDCs. There is increasing evidence that the endocrine and immune systems are interacting with each other. Many EDCs can alter immune functions and result in immunotoxicity (Kuo, et al. 2012).

2.6.4 Immunotoxicity

Immunotoxicity is an adverse effect caused by some toxic substances. The immune system is made up of various organs, tissues and cells. The immune system functions through two highly regulated pathways, namely innate and adaptive responses. While the innate response does not have a memory of previous exposures to antigen, the adaptive response is highly specific to an antigen and retains a memory of previous exposure to antigens. The innate response is the first line defense mechanism of the body, that uses physical barriers, chemical defense mechanisms such substances found in secretory, cellular and inflammation responses (Bols et al. 2001).

One of the important immune cells involved in immune responses are macrophages. Macrophages are phagocytic cells. Macrophges are produced by precursor bone marrow cells, and are released into the circulatory system as monocytes. The monocytes differentiate to form mature macrophages. Macrophages function as the control switch of several immune responses including recognition and clearing of any foreign materials, pathogens and tumor cells by phagocytosis and induction of inflammatory reactions (Omer et al. 2012). During inflammatory reactions, macrophages are involved in balancing between pro-inflammatory and anti-inflammatory responses. The balancing is achieved by two distinct macrophage subpopulations, classically activated M1 macrophages and alternatively activated M2 macrophages (Laskin, 2009).

The classically activated M1 macrophages are characterized by pro-inflammatory phenotype and respond to inflammatory cytokines, toxic compounds, pathogens and microbial products by secreting cytotoxic reactive oxygen, nitrogen intermediates and pro-inflammatory mediators (Laskin, 2009; Laskin et al. 2011). Secretion of reactive

oxygen, nitrogen intermediates and pro-inflammatory mediators is important for induction of bactericidal activity and inflammatory reactions. An important reactive nitrogen intermediate secreted by macrophages is nitric oxide (NO). In addition to NO secretion, among the pro-inflammatory mediators secreted by activated M1 macrophages are IL-6 and several other cytokines and chemokines. IL-6 has many functions including inflammatory reactions and bactericidal activity (Barnes et al. 2011; Mihara, et al. 2012). Apart from bactericidal activity and inflammatory action of IL-6, high level of IL-6 promotes polarization of macrophages to alternatively activated M2 macrophages (Fernando et al. 2014).

The alternatively activated M2 macrophages are induced by many factors including, IL-6, IL-4, toll-like receptors (TLRs) agonists and glucocorticoid hormones (Montovani et al. 2004; Laskin, 2009). Stimulation of alternatively activated M2 macrophages induces suppression of the immune response by producing low levels of pro-inflammatory cytokines and initiation of tissue repair processes (Laskin, 2009).

Many pollutants present in wastewater can disrupt the immune response by either suppression of immunity or stimulation of immune response (Bahadar, et al. 2014). The suppression of immune function is characterized by enhanced disease susceptibility and inability to eliminate cancer cells. There are many pollutants in wastewater that can induce immune suppression effects (Milla et al. 2011; Nygaard et al. 2013; Leme et al. 2014). Examples of pollutants found in wastewater that can induce immunosuppression includes corticosteroids, PAHs, PCBs, pesticides and some heavy metals (Bahadar, et al., 2014).

Stimulation of immune response on the other hand, is characterized by exaggerated immune response presented as hypersensitivity/allergy or increased autoimmunity. While very few pollutants can induce autoimmunity, there are many wastewater pollutants that are known to induce immune stimulation resulting in hypersensitivity/allergy (Milla et al., 2011; Nygaard et al. 2013; Leme et al. 2014). Examples of common pollutants in wastewater that can induce inflammatory responses characterized by hypersensitivity reactions include textiles dyes, pathogens and their breakdown products (Nygaard et al. 2013; Leme et al. 2014).

Immune stimulation leading to hypersensitivity involves many cell types, such as macrophages and activated T cells, which release cytokines and chemokines that attract cytotoxic T cells. Excessive activation of macrophages is associated with increased secretion of high levels of inflammatory mediators and cytotoxic effects. Consequently, excessive inflammatory mediators, which are associated with tissue repair processes, lead to immunotoxic effects as a result of cytotoxicity, genotoxicity and inflammatory reactions.

2.6.4.1 Cytotoxicity

Cytotoxicity is an absolute cell death caused by various toxic compounds, as a result of either cell necrosis or apoptosis. Whereas necrosis is cell death following cell lysis, apoptosis is a programmed cell death. Necrosis is characterized by swelling of cells and loss of membrane integrity that lead to cell lysis and release of cytoplasm contents. In contrast, apoptosis is a complex and highly regulated mechanism of cell death characterized by several morphological and biochemical changes. Morphological changes of apoptosis are characterized by DNA fragmentation, condensation of nuclear

chromatin, cell shrinkage, a disintegration of mitochondria, membrane blebbing, and formation of apoptotic bodies (Orrenius, et al., 2010). On the other hand, biochemical changes of apoptosis are characterized by many complex signaling networks that regulate intracellular pathways via gene expression and/or protein activity. Common apoptosis signaling pathways include dysregulation of Ca^{2+} ions, stimulation of G protein-dependent signaling pathways, induction of oxidative stress, endoplasmic reticulum stress and ATP depletion. Other signaling pathways are disruption of outer mitochondria membrane, alterations of anti-apoptotic-apoptotic Bcl-2 proteins ratio. There are also up-regulation of p53 and apoptosis-related gene expression; activation of caspases and cell surface death receptors and their ligands like TNFα and Fas ligand; and overall disturbances in protein synthesis (Agalakova and Gusev, 2012).

Many pollutants can induce recruitment or repression of the apoptosis signaling pathways through different or shared molecular mechanisms (Orrenius et al., 2010; Agalakova and Gusev, 2012; Mrema et al., 2013). For instance, heavy metals and organochloride compounds can induce oxidative stress by generating reactive oxidative species (ROS). High levels of ROS induce modification of cellular macromolecules, alter protein functions and initiate cell death (Circu and Aw, 2010). Increased levels of ROS can also lead to activation of c-Jun N-Terminal Kinases (JNKs) and NF-kB, which up-regulate pro-apoptotic genes that modulate mitochondrial apoptotic proteins by phosphorylation (Dhanasekaran and Reddy, 2008).

2.6.4.2 Inflammation
Inflammation is an immune response induced by many factors including trauma, pathogens, toxic chemicals or pollutants among many others. The primary role of an

inflammatory response is protection of the host against inflammatory inducers. Thus, when immune cells like macrophages are exposed to pollutants such as pathogens, they respond by releasing many soluble inflammatory mediators. The release of inflammatory mediators induced by pathogens or LPS is triggered through germline-encoded pattern recognition receptors (PRRs). The receptors expressed in macrophages include Toll-like receptors (TLRs) and Retinoic acid-inducible gene-I-like receptors (RLRs) (Kawai and Akira, 2009).

The TLRs like TLR4 localized on cell membrane can sense pathogens outside the cell membrane and upon activation induce production of pro-inflammatory mediators. Similar to pathogen-induced inflammatory response, chemical induced inflammatory responses activate similar receptors to induce production of pro-inflammatory mediators. Some important pro-inflammatory mediators produced by macrophages are IL-6 and TNF (Laskin, 2009). Consequently, the produced TNF induces production of cytotoxic reactive oxygen species (ROS) and reactive nitrogen species (RNS). The secreted ROS and RNS like NO are released so as to kill the inflammation inducers such as invading pathogens. The excessive secretion of ROS, RNS and TNF can also induce tissue damage as a result of cytotoxicity (Chen, et al. 2008).

In situations of persistent inflammatory induction, such as exposure to pollutants, efforts of inflammation resolution arise. Resolution of inflammation is induced by anti-inflammatory mediators secreted by alternatively activated M2 macrophages to restore the homeostasis. The alternatively activated M2 macrophages secrete anti-inflammatory cytokines and growth promoters required for tissue repair (Laskin, 2009). Failure to

restore the normal homeostasis, lead to pathological lesions due to altered inflammatory mediators and cytotoxicity.

2.7 Biomarkers of immunotoxicity in macrophages

Biomarkers are measurable changes in organisms, cells and tissue secretions that are caused by exposure to pollutants. Therefore, alterations of structure and functions of macrophages exposed to pollutants are important biomarkers of immunotoxicity. The changes in macrophages may include absolute cell death due to cytotoxicity and changes in secretions as a result of inflammatory reactions. The inflammatory reactions in macrophage are normally characterized by secretion of many inflammatory mediators like reactive oxygen species (ROS), reactive nitrogen species (RNS) and pro-inflammatory cytokines (Laskin, 2009; Laskin et al. 2011). The following is an overview of macrophage biomarkers of immunotoxicity which were evaluated in the present study.

2.7.1 Cytotoxicity

Cytotoxicity is a fate of cells exposed to toxic agents. Cells exposed to toxic compounds like pollutants, may lead to cell death due to necrosis or apoptosis. Necrosis is cell death following swelling of cells and loss of membrane integrity that lead to cell lysis and release of cytoplasm contents. On the other hand, apoptosis is a programmed cell death, characterized by biochemical changes, DNA fragmentation and condensation of nuclear chromatin. Other features are cell shrinkage, disintegration of mitochondria, membrane blebbing, and formation of apoptotic bodies (Orrenius, et al. 2010). Generally, cytotoxic cells are characterized by suppressed metabolism, membrane intergrity and cell lysis.

Therefore, cytotoxicity is measured by cell viability assays. The assays are based on assessment of cell functioning parameters that include cell membrane permeability,

enzyme activity, co-enzyme and ATP production (Riss et al. 2011). Examples of enzyme based methods are tetrazoliun dye reduction assays. The tetrazoliun dye reduction assays is based on the principle of the breakdown of tetrazolium to water soluble formazan dye by the action of dehydrogenase enzyme. The most common tetrazoliun dye reduction assays are MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT and WST assays. The latter two, XTT and WST assays are used with an intermediate electron receptor that can penetrate the cells to facilitate reduction of tetrazolium into soluble formazan coloured product. The soluble formazan product is then easily recorded on the assay plate.

The tetrazoliun dye reduction assays have been extensively applied in many toxicity studies. For example, WST-1 assay was used to investigate the cytotoxicity of water and sediment samples from mining area using a catfish ovary cell line (Ternjej et al. 2013). In another study, Albrecht et al. (2009) used the WST-1 assay to evaluate cytotoxic effects of nanoparticles of different sizes in primary alveolar macrophages. In a similar study, Scheel et al. (2009) used XTT test to determine the cytotoxic effects of different nanoparticles in RAW264.7 cell line.

2.7.2 Interleukin-6

Interleukin 6 (IL-6) is a cytokine secreted by many cells such as T-lymphocytes, monocytes and macrophages during inflammatory responses. It is a pro-inflammatory and anti-inflammatory cytokine with many functions including inflammatory reactions and acute phase responses (Barnes et al. 2011; Mihara, et al. 2012). Furthermore, IL-6 enhances lymphocytes functions by activating T-cytotoxic reactions and promoting of differentiation B-lymphocytes into plasma cells. IL-6 is produced during both acute and

chronic inflammation. Expression and secretion of IL-6 in macrophages are regulated by activation of NF-kappa B (NFκB) (Xing et al. 2010). Secretion of IL-6 is induced by many other inflammatory cytokines like TNF and various inflammatory inducers like organic pollutants, pathogens or their breakdown products (Scheller et al. 2011; Kolasa et al. 2013). Therefore, IL-6 is used extensively as a biomarker inflammation during infection (Paquette et al. 2012). Similarly IL-6 is used as a biomarker of exposure to inflammatory environmental pollutants. Exposure of mice to air pollutants can induce inflammatory responses characterized by increased secretion of many pro-inflammatory cytokines including IL-6 (Xu et al. 2013). Similarly, the inflammatory responses in situations of air pollution have been associated with involvement of macrophages (Hiraiwa and van Eeden, 2013).

Secreted IL-6 is normally determined quantitatively by enzyme linked immunosorbent assay (ELISA). The assay is based on the principals of a double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA). Briefly the assay uses anti-IL-6 antibody adsorbed onto microplate. IL-6 present in test sample binds to antibodies adsorbed to microplate. Then a biotinylated anti-IL-6 antibody binds to the IL-6 captured by the first antibodies in the microplate. Following incubation, unbound biotin-conjugated anti-IL-6 antibody is removed by washing. Then Avidin-horse radish peroxidise (HRP) conjugate is added to the biotin- conjugated IL-6 antibodies. After incubation, the unbound Avidin-HRP is removed again by washing. Finally, substrate solution that reacts with HRP is added. This results in the breakdown of the substrate to coloured product. The amount of coloured product formed is proportional to the amount of IL-6 present in samples.

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The IL-6 DAS ELISA has been applied extensively for determination of IL-6 in clinical samples (Fan et al. 2012) and in whole blood culture model for assessment of inflammatory activity in environmental water (Pool et al. 2003; Pool and Magcwebeba, 2009). In fact, the use of IL-6 inflammatory markers determined by DAS ELISA in whole blood culture, as well as in isolated white blood cell culture, has been evaluated for monitoring water quality (Wichmamn et al. 2004;) and for routine assessment of efficiency of treatment facilities performance (Adebayo et al. 2014).

2.7.3 Nitric Oxide

Nitric oxide (NO) is a small molecule that belongs to reactive nitrogen species (RNS) that has many biological activities. It is a very highly reactive molecule that is involved in many cells signalling pathways and also killing of pathogens (Omer et al. 2012). NO can further modulate many important inflammatory responses including vascular blood flow, release of antioxidants and more inflammatory mediators (Wallece, 2005).

NO is synthesized in many cells from L-arginine induced by nitric oxide synthase (NOS) enzyme. The enzyme (NOS) exists in three different forms, namely neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). The latter two, eNOS and iNOS are constitutive forms that are expressed in many cells including macrophages (Connelly et al. 2003). While iNOS expression in macrophages is induced by many inflammatory cytokines and pathogen or LPS, the eNOS expression is induced by many physiological factors including steroid hormones (Mihara et al. 2012). For example, Nevzati et al. (2015) measured the effect of estrogen on eNOS expression *in vitro*. The results showed that estrogen induced increaed level of eNOS expression through estrogenic receptor mediated mechanism. Similarly,

androgenic compounds can induce NO synthesis. The induction of NO synthesis by testosterone is through androgenic receptor dependent activation of eNOS (Yu et al. 2010).

The NO produced has a very short half-life, whereby excess amount is instantly converted and stored in the form of nitrite (Shiva, 2013). The level of nitrite converted is usually used to estimate the amount of NO secretion (Liu et al. 2012; Kim et al. 2014). The NO produced is commonly determined using Griess reaction assay. The assay has been extensively used to evaluate inflammatory reactions (Szliszka et al. 2011) and anti-inflammatory responses in RAW264.7 macrophages (Lee and Park, 2011; Lee and Park, 2015).

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Chapter 3: The Effects of Endocrine Disrupting Chemicals on Biomarkers of Inflammation Produced by Lipopolysaccharide Stimulated RAW264.7 Macrophages *

3.1 Abstract

Endocrine disrupting chemicals (EDCs) are common pollutants in the environment and can induce disruption of the endocrine and immune systems. The present study evaluated the effects of selected common environmental EDCs on secretion of inflammatory biomarkers by RAW264.7 cells. The EDCs investigated were Estradiol (E2), 5α-dihydrotestosterone (DHT), and Bisphenol A (BPA). To evaluate if the effects caused by EDCs were modulated by steroid hormone receptors, antagonists of estrogen and androgen receptors were used. The steroid receptor antagonists used were Tamoxifen, an estrogen receptor antagonist, and Flutamide, an androgen receptor antagonist. Secretion of biomarkers of inflammation, namely nitric oxide (NO) and interleukin 6 (IL-6), were monitored. The NO was determined using Griess reaction and IL-6 was measured by enzyme linked immunosorbent assay (ELISA). Although 5μg/mLE2, DHT, and BPA were not toxic to RAW264.7 cell cultures, the same treatments significantly ($p<0.001$) reduced both NO and IL-6 secretion by lipopolysaccharide (LPS)-stimulated RAW264.7 cell cultures. The suppression of NO and IL-6 secretion indicate inhibition of inflammation by DHT, E2, and BPA. The inhibitory effects of DHT, E2 and BPA are partially mediated via their cellular receptors, because the effects were reversed by their respective receptor antagonists.

Flutamide reversed the effects of DHT, while Tamoxifen reversed the effects of E2 and BPA. In conclusion, E2, BPA, and DHT inhibit the synthesis of inflammation biomarkers by LPS-stimulated RAW264.7 cells. The inhibitory effects of EDCs can be partially reversed by the addition of an estrogen receptor antagonist for E2 and BPA, and an androgenic receptor antagonist for DHT. The inhibition of inflammatory response in stimulated RAW264.7 cells may be a useful bioassay model for monitoring estrogenic and androgenic pollutants.

Keywords: Estradiol; 5α-dihydrotestosterone; Bisphenol A; anti-inflammatory; nitric oxide; interleukin 6

3.2. Introduction

Endocrine disrupting chemicals (EDCs) are environmental compounds that can interfere with biosynthesis, secretion, action, or metabolism of endogenous hormones, resulting in altered normal hormone actions (Kloas and Lutz 2006; Zoeller, et al. 2014). They include compounds such as industrial chemicals, agricultural chemicals like pesticides, natural and synthetic hormones, and pharmaceuticals. These chemicals are widely distributed in the environment and especially in wastewater. Common examples reported in the environment are estrogenic steroids like Estradiol (E2) and Bisphenol A (BPA) (Sun et al. 2013; Sun et al. 2014) and androgenic steroids like testosterone and 5α-dihydrotestosterone (DHT) (Bellet et al. 2012; Liu et al. 2011). While BPA is an industrial chemical with many applications, testosterone and E2 are natural hormones of male and female animals, respectively.

Both estrogenic and androgenic chemicals are common pollutants in municipal wastewater. They are persistent in the environment and, in most cases, are resistant to removal by common conversional wastewater treatment techniques. Therefore, some of these pollutants can pass through wastewater treatment plants (WWTPs) and contaminate the environment. For example, Liu et al. (2011) reported the presence of estrogenic E2 among several steroids in the environment after analysis of sludge and surface water samples. In another study, Sun et al. (2013) reported some E2 and BPA activities in both sewage and reclaimed water.

Similar to estrogenic EDCs, androgenic steroids are also common in sewage and surface water bodies. Bell et al. (2012) reported high androgenic activities in sewage samples. Along with other androgenic hormones, 5α-dihydrotestosterone (DHT) has also been reported in sewage (Liu et al. 2011). DHT is a more biologically active form of testosterone. It is a derivative of testosterone synthesized from testosterone by enzymatic action of 5-reductase (Pham 2002; Wu 2013).

Exposure to environmental estrogenic and androgenic compounds causes many adverse health effects on the endocrine system, leading to several disorders in humans and animals (Kloas and Lutz 2006; Zoeller, et al. 2014; Kuo et al. 2012; Bergman et al. 2013). The most common reported effects are reproductive disorders, including reproductive anomalies, sexual dysfunctions, and cancers of reproductive origin (Rogers et al. 2013). Apart from disruption of reproductive structure and functions, estrogenic and androgenic compounds are also known to induce immunotoxicity (Nakamura et al. 2010).

They induce immunotoxicity by interfering with activation and survival of immune cells; and alteration of cytokines and synthesis of inflammatory mediators (Kuo et al. 2012). The altered immune response is normally characterized by either suppression of immunity or stimulation of immune response. The immune suppression is normally characterized by enhanced disease susceptibility and inability to eliminate cancer cells (Schwartz, 2000; Marsland et al. 2002). On the other hand, the overstimulation of the immune system is characterized by immune hypersensitivity (Warrington et al. 2011).

The inflammatory reaction is an important immunological response, which is induced by many inflammatory factors. These inflammatory factors include pathogens, injury, organic compounds, toxins, and endotoxins like lipopolysaccharide (LPS) (Williams 2011). The resulting inflammatory responses involve many immune cells. One of these types of cells is macrophages, which are phagocytic cells derived from circulating monocytes. They form the first line of the innate part of the immune system. When macrophages are stimulated, they ingest and digest invading pathogens, dead cells, and any other foreign substances (Carrillo et al. 2017). Therefore, macrophage stimulation is one of the early immune responses to infection. For example, exposure of macrophages to LPS, a gram negative bacteria membrane component, induces an inflammatory response. The induction of an inflammatory response by LPS is through activation of a Toll-like receptor (TLR-4), which is expressed on the cell surface of macrophages (Carrillo et al. 2017; Billack 2006).

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Estrogenic and androgenic compounds can also induce an immune response in macrophages through their specific receptors, namely, estrogen receptors (ERs) and androgen receptors (ARs), respectively (Murphy et al. 2009; Lai et al. 2012). Both ERs and ARs are members of the nuclear receptor family of ligand-dependent transcription factors (Contrò et al. 2015). Therefore, exposure of macrophages to estrogenic and androgenic compounds may activate or inhibit expression of nuclear factor kappa B (NFκB), which leads to alteration of inflammatory mediators and cytokine secretion (Billack 2006). Inhibition of an inflammatory response induced by estrogen has been reported in wound healing (Campbell et al. 2010). On the other hand, suppression effects of testosterone on inflammation have been associated with many metabolic diseases (Muraleedharan and Jones 2010). In fact, recently, there is evidence in literature showing that a low testosterone level correlates with occurrence of metabolic diseases, characterized by increased expression of biomarkers of inflammation (Bianchi, 2018).

Activated macrophages initiate inflammatory responses by inducing secretion of many inflammatory mediators and pro-inflammatory cytokines (Newton and Dixit, 2012). Examples of common inflammatory mediators and pro-inflammatory cytokines secreted by macrophages are nitric oxide (NO) and interleukin 6 (IL-6), respectively (Barnes et al. 2011). IL-6 is a pro-inflammatory cytokine with many functions, including supporting chronic inflammatory reaction, induction of acute phase reactions, and inducible nitric oxide synthase (iNOS) (Barnes et al. 2011; Mihara et al. 2012). Inducible nitric oxide synthase (iNOS) is a key enzyme in NO production. NO is a small molecule secreted by many tissue cells including macrophages. Therefore, secretion of both NO and IL-6 in

macrophages is extensively used as a biomarker of inflammation studies (Pool and Magcwebeba, 2009; Avdagić et al. 2013; Piva et al. 2013).

Inflammatory responses have been studied in vitro using established cell lines like mouse RAW264.7 macrophages. This cell line was developed from mouse ascites leukemia cells. When RAW264.7 cells are stimulated, they secrete inflammatory mediators like NO and IL-6. Stimulation of RAW264.7 cells with LPS has been used extensively to study the immunomodulating effects of natural products and herbs (Kim 2012; Chen and Zhang 2014; Choi et al. 2014; Srisook et al. 2015). However, there are relatively very few reports on the use of RAW264.7 cells to study anti-inflammatory effects of common pollutants like DHT, E2, and BPA. The present study was done to evaluate the in vitro effects of common EDCs, such as DHT, E2, and BPA, on cytotoxicity and inflammatory biomarker secretion by LPS-stimulated RAW264.7 macrophages. This study also evaluated whether effects of the EDCs on inflammatory biomarkers are mediated via their respective steroid receptors.

3.3. Material and Methods

3.3.1. Reagents
Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4, Dimethyl sulfoxide (DMSO), 5α-dihydrotestosterone (DHT), Estradiol (E2), Bisphenol A (BPA), Flutamide, and Tamoxifen were purchased from Sigma-Aldrich Chemie Gmbh, Munich, Germany. The LPS, DHT, E2, BPA, flutamide, and tamoxifen were dissolved in DMSO (Sigma-Aldrich, Germany).

3.3.2. Cells Culture

A mouse macrophage RAW264.7 cell line, purchased from American Type Culture Collection (ATCC TIB-71, Manassas, VA, USA), was used in this study. The RAW264.7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Lonza, Cape Town, South Africa), supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% *v/v* antibiotic/antimycotic mixture (Sigma-Aldrich), 0.5% v/v gentamycin (Sigma-Aldrich), and 1% v/v glutamax (Gibco, Life Technology, Carlsbad, CA USA). The cells were cultured in 96-well plates at a density of 5×10^5 cells/mL in a humidified incubator at 37 °C and 5% CO_2 until confluent. At confluence, cells were treated as follows: Normal medium for negative control and medium supplemented with 1μg/mL lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Germany) as a positive control. Some cell cultures stimulated with LPS (1μg/mL) were treated with 5μg/mL of each of DHT, E2, and BPA alone or in combination with 2μg/mL of flutamide or tamoxifen. The optimal concentration of compounds was determined by serial dilution (data not shown). The dilution of DHT, E2, and BPA started with 1 in 100 of 10μg/mL of compound. The concentrations of EDCs starting from 1 in 200 (5μg/mL) and further dilutions did not affect cell viability and were not different from the control. Optimal concentrations of flutamide and tamoxifen treatment were also determined by serial dilution, starting from 1 in 500 dilutions of 5μg/mL of each. The concentrations of flutamide and tamoxifen, starting from 1 in 1000 (2.5μg/mL), and further dilutions alone had no suppression effects on cell viability, NO, and IL-6 secretion in RAW264.7 cells. The cells were cultured in medium supplemented with LPS (1μg/mL) overnight. Then, cell cultures stimulated with LPS were treated with 5μg/mL of each of DHT, E2, and

BPA alone or in combination with 2μg/mL of flutamide or tamoxifen and incubated at 37°C and 5% CO_2 overnight. After overnight incubation at 37 °C and 5% CO_2, culture supernatants were collected for NO and IL-6 assays. The cells remaining on the plate were used for cell viability assays. Each assay was carried in four replicates.

3.3.3. Cell Viability

The cell viability was determined using the chromogenic WST-1 assay. The assay is based on the breakdown of the water-soluble (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium by the dehydrogenase enzyme to produce water-soluble formazan dye that can be monitored spectrophotometrically. In brief, the assay procedure was as follows: After removal of cell supernatant from culture, each plate well received 100μL of medium supplemented with 10% WST-1 reagent (Roche, Basel, Switzerland). The absorbance was read immediately after addition of WST-1 medium and a second reading was done after incubation for 30 min at 37 °C and 5% CO_2. The change in absorbance at 450 nm over 30 min was used as a measure of cell viability.

3.3.4. Nitric Oxide Determination

Nitric oxide (NO) secreted by cells into the cell culture was determined using the Griess reaction. Cell culture supernatant in 96-well plates (Nunc, Roskilde, Denmark) was mixed with an equal volume of Griess reagent made up of 1% m/v sulphanilamide (Sigma-Aldrich, Germany), 0.01% m/v naphthyl ethylenediamine dihydrochloride (Sigma-Aldrich), and 2.5% phosphoric acid. The mixture was allowed to react for 15 min at room temperature. The colour developed was measured at 540 nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation). The concentration of NO was

determined from a standard curve generated using 1.56–100μM sodium nitrite (Sigma-Aldrich, Germany).

3.3.5. Interleukin-6 Determination

Interleukin 6 (IL-6) in culture supernatant was determined using a double antibody sandwich enzyme linked assay (DAS ELISA), with a commercial kit (e-Bioscience, Ready-Set-Go, Waltham, MA, USA). The assays were done on Nunc Maxisorp 96-well plates (Nunc, Denmark). The ELISA kit contains all reagents required for the assay and the manufacturers' assay protocol was followed. In brief, the protocol involved coating the ELISA plate with capture antibody, anti-mouse IL-6 diluted in coating buffer (PBS), and incubated overnight at 37 °C. Then, the plate was washed five times in wash buffer made of PBS with 0.1% v/v Tween. After washing, the plate was blocked with assay diluent for 1 h at room temperature. After another five washes, IL-6 standard and cell culture supernatant were added to each well accordingly and incubated for two hours at room temperature. The plate was washed again five times, then detection antibody, biotinylated anti-mouse IL-6, was added to each well and incubated for 1 h at room temperature. After another wash, Avidin—horse radish peroxidise (HRP) conjugate—was added and incubated for 30 min at room temperature. The plate was washed seven times, then TMB substrate was added and incubated in the dark for 15 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 stop solution, and the absorbance was read at 450 nm with a multiskan microplate reader (Multiskan Ex, Thermo Electron Corporation).

3.3.6. Statistical Analysis

The data are presented as mean ±SD (n=4), which were statistically analyzed with one way variance analysis (ANOVA) using sigmastat (SigmaStat software, Inc., CA, USA). The results of cell viability are presented as a percentage of the negative control. The mean values of NO and IL-6 concentration for each treatment were compared with the positive control. P-value <0.001 was considered statistically significant.

3. 4. Results

3.4.1. Effects of Selected EDCs on Cell Viability

The effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on viability of stimulated RAW264.7 cells are shown in Figure 3.1. The cell viability is presented as a percentage of the negative control. None of the treatments reduce RAW264.7 cell viability as compared with the positive control treated with LPS (1µg/mL).
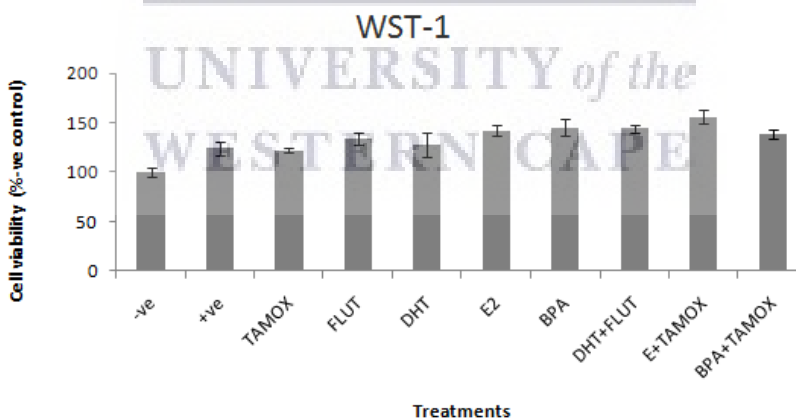

Figure 3.1 Effects of 5α-dihydrotestosterone (DHT), Estradiol (E2), and Bisphenol A (BPA) alone and in combination with flutamide or tamoxifen on viability of RAW264.7 cells, stimulated with 1µg/mL lipopolysaccharide (LPS). The stimulated cells were treated with 5µg/mL of DHT, E2, or BPA; and flutamide (FLUT) or

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tamoxifen (TAMOX) at 2µg/mL; negative control was treated with normal medium and positive control was treated with LPS (1µg/mL). The results of cell viability are presented as percentage of negative control.

3.4.2. Effects of Selected EDCs on NO Production

The effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on NO production in stimulated RAW264.7 cells were determined by NO assay in cell culture supernatant. The results of the effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on NO secretion in RAW264.7 cells stimulated with 1µg/mL LPS are shown in Figure 3.2.
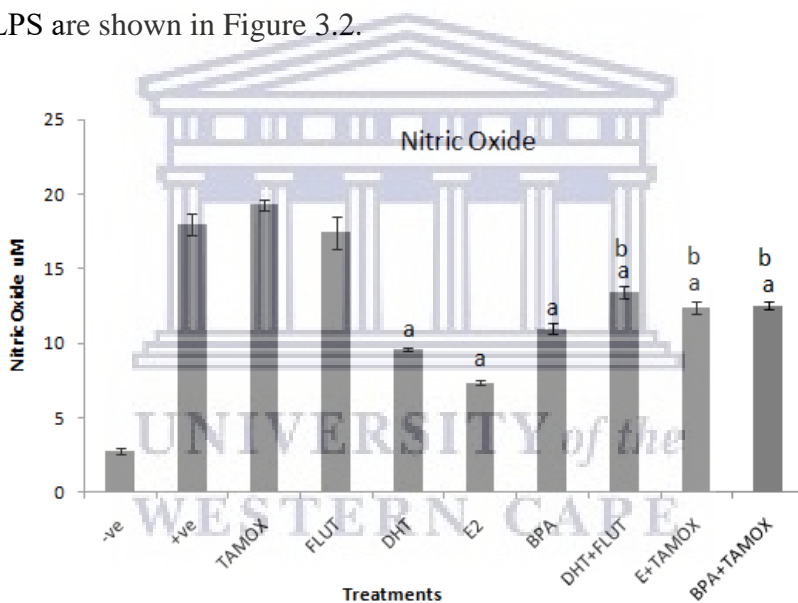

Figure 3.2 Effects of DHT, E2, and BPA alone and in combination with tamoxifen (TAMOX) or flutamide (FLUT) on secretion of nitric oxide (NO) in supernatant of RAW264.7 cells stimulated with 1µg/mL LPS. The stimulated cells were treated with 5µg/mL of DHT, E2, and BPA alone and 2µg/mL of flutamide or tamoxifen. Negative control was treated with normal medium and positive control treated with LPS (1µg/mL). The results are presented as mean ±SD; **a-**indicates that NO level is

significantly ($p<0.001$) lower than the positive control; **b-**indicates that NO level is significantly ($p<0.001$) higher than levels of NO secreted by cells treated with DHT, E2, and BPA alone.

The results reveal that exposure of the stimulated cells to tamoxifen or flutamide alone had no significant effect on NO secretion. Exposure of the stimulated culture cells to 5μg/mL of DHT, E2, and BPA significantly ($p<0.001$) decreased NO secretion when compared to the positive control. The strongest suppression effect was induced by E2 (7.4 ± 0.13 μM). Next to E2 was DHT (9.6 ± 0.13 μM) and relatively weak suppression of NO production was induced by BPA (10.9 ± 0.4 μM). The inhibitory effects of EDCs were reversed by the addition of their respective antagonist compounds. Addition of flutamide, a chemical with anti-androgenic effects, significantly ($p<0.001$) reversed the anti-inflammatory effects of DHT. The NO production induced by DHT alone was 9.6 ± 0.13 μM, whereas DHT in combination with flutamide induced 13.4 ± 0.13 μM of NO. Similarly, addition of tamoxifen, a chemical with anti-estrogenic effects, significantly ($p<0.001$) reversed the effects of both estradiol and BPA. E2 alone induced 7.4 ± 0.13 μM, while E2 in combination with tamoxifen increased NO production to 12.4 ± 0.42 μM. BPA alone induced 10.9 ± 0.4 μM of NO. BPA in combination with tamoxifen induced 12.5 ± 0.28 μM of NO.

3.4.3. Effects of Selected EDCs on IL-6 Secretion

The effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on secretion of IL-6 in stimulated RAW264.7 cells was determined in cell culture supernatant, using a double antibody sandwich enzyme linked assay (DAS ELISA). The effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on

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secretion of IL-6 in stimulated RAW264.7 cells are shown in Figure 3.3. The results show that exposure of cells to DHT, E2, and BPA alone significantly ($p<0.001$) reduced the IL-6 response in stimulated RAW264.7 cells.
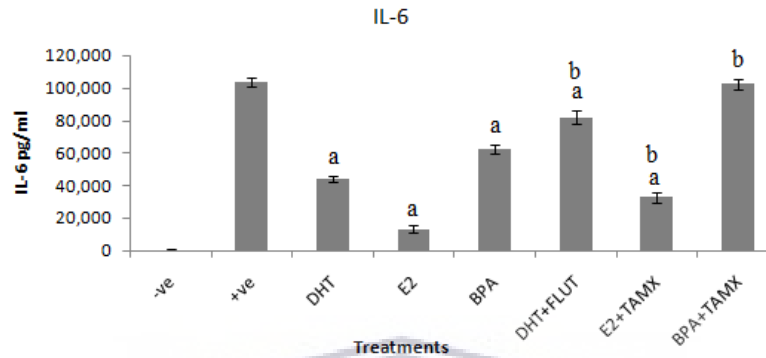

Figure 3.3 Effects of DHT, E2, and BPA alone and in combination with flutamide or tamoxifen on IL-6 secretion in RAW264.7 cells stimulated with 1μg/mLLPS. The stimulated cells were treated with 5μg/mLof DHT, E2, and BPA and 2μg/mLof flutamide (FLUT) or tamoxifen (TAMOX). Negative control was treated with normal medium and positive was control-treated with LPS (1μg/mL). The results are presented as mean ±SD; **a-**indicates that IL-6 level is significantly ($p<0.001$) lower than the positive control; **b-**indicates that IL-6 level is significantly ($p<0.001$) higher than levels of IL-6 secreted by cells treated with DHT, E2, and BPA alone.

The results show that the positive control treated with LPS (1μg/mL) secreted IL-6 as high as 103,914±2620 pg/mL. Addition of DHT alone to stimulated cultures suppressed IL-6 secretion to 43,943±1750 pg/mL. DHT in combination with flutamide secreted 81,871±4098 pg/mL of IL-6. The highest IL-6 suppression effect was evident in cells treated with E2. Treatment of stimulated RAW264.7 cell culture with E2 suppressed IL-6 to 13,329±1971 pg/mL. Tamoxifen or flutamide alone had no suppression effect on IL-6

secretion in stimulated RAW264.7 cell culture (data not shown). Addition of tamoxifen with an antagonistic effect significantly reversed the anti-inflammatory effects of E2. The combination of E2 and tamoxifen secreted 32,871±3093 pg/mL of IL-6. A relatively low suppression effect on IL-6 secretion was induced by BPA treatment. BPA alone induced 62,671±2903 pg/mL of IL-6, which was reversed when combined with tamoxifen to 102,529±3235 pg/mL of IL-6.

3.5. Discussion

Pollutants with estrogenic and androgenic effects are common in wastewater treatment facilities and receiving water bodies (Sun et al. 2014; Faul et al. 2014). As a result, there have been increasing concerns over their association with several adverse effects to the environment, animals, and humans (Sun et al. 2013). The most common adverse health effect reported is endocrine disruption, especially reproductive system modulation (Knez, 2013). Apart from the disruption of the reproductive system, an increasing number of reports suggest that androgenic and estrogenic compounds can also cause immune disruption. The disruptions of immune functions occur in many different cell types of the immune system, including macrophages (Kuo et al. 2012; Bergman et al. 2013; Rogers et al. 2013; Nakamura et al. 2010; Murphy et al. 2009). Based on the possible effects of estrogenic and androgenic compounds on the immune system, the present study evaluated whether exposure of macrophages to common EDCs such as DHT, E2, and BPA modulate inflammatory biomarker production. These EDCs were selected for investigation due to literature available showing that they can be found in most water bodies impacted by human activity (Liu et al. 2011; Manickum and John, 2014). The study used a murine macrophage RAW264.7 cell line as a model for inflammatory

responses. The inhibition of inflammation was assessed using NO and IL-6 as biomarkers of an inflammatory response. The results show that 5μg/mL of DHT, E2, and BPA had no negative effect on RAW264.7 cell viability and were therefore not toxic.

The results further show that exposure of LPS-stimulated RAW264.7 cells to 5μg/mL of DHT, E2, or BPA suppressed inflammatory biomarker secretion. The inhibition of inflammatory effects ischaracterized by significant decreases of NO and IL-6 secretions, compared with the positive control stimulated with 1μg/mL LPS. These results indicate that DHT, E2, and BPA suppressed both NO and IL-6 secretion in stimulated RAW264.7 cells.

The actions of DHT through androgen receptors (ARs) are similar to those of testosterone, because DHT is a more biologically active form of testosterone. DHT is a derivative of testosterone synthesized from testosterone by enzymatic action of 5-reductase (Pham 2002; Wu 2013). Its actions are also through ARs, whose expression has been detected in various immune cell types, such as macrophages (Murphy et al. 2009; Lai et al. 2012). The involvement of AR in the anti-inflammatory effects of DHT was confirmed by the results of combined DHT and flutamide treatment. The combined treatment of flutamide with DHT reversed the inhibition of inflammatory biomarker secretion by RAW264.7 cells. This is probably because flutamide is a selective antagonist of the AR (Brooke et al. 2015). A similar inhibition response in macrophage RAW264.7 cells culture might be induced by environmental androgenic pollutants. This is because androgens are common in sewage effluents and surface water bodies (Liu et al. 2011;

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Manickum and John, 2014). Therefore, these results suggest that anti-inflammatory activities of androgens, which are common in wastewater, can be assessed by the use of stimulated RAW264.7 cells culture. However, molecular mechanisms of androgen action on AR in macrophages require further studies.

Similarly, inhibitions of inflammatory biomarkers were evident upon treatment of stimulated RAW264.7 cells with E2 and BPA. The treatment of stimulated RAW264.7 cells with E2 and BPA suppressed inflammatory biomarkers by decreasing secretion of both NO and IL-6. The effects were more evident with E2 than BPA treatment. Both E2 and BPA can bind to estrogenic receptors (ERs), which are expressed in many immune cells including macrophages. The difference of E2 and BPA effects could be associated with differences in their affinity to ERs (Shanle and Xu, 2010; Kovats, 2012). E2 is normally characterized by stronger estrogenic activities compared to BPA, which is characterized by weaker estrogenic activities (Sun et al. 2013). Involvement of ERs in E2 and BPA inhibition of inflammatory biomarkers was confirmed by the results where LPS-activated RAW264.7 cells were exposed to combinations of E2 and BPA with tamoxifen. Tamoxifen is an ER antagonist (An, 2016). Likewise, anti-inflammatory activities of estrogen might be induced by estrogenic pollutants. Estrogenic hormones have been frequently detected in sewage effluents and surface water bodies (Faul et al. 2014; Manickum and John, 2014). Hence, the findings of the present study suggest that anti-inflammatory activities of estrogenic pollutants can be evaluated by using stimulated RWA264.7 cell culture. Nevertheless, molecular mechanisms involving ER in macrophages may require further studies.

3.6. Conclusions

The results of the present study show that DHT, E2, and BPA, which are common EDCs in wastewater, can disrupt inflammatory response and hence immune function. The results show further that DHT, E2, and BPA can inhibit inflammatory mediator production in macrophages, partly through their respective steroid hormone receptors. These findings show that stimulated RAW264.7 macrophages may be a useful model for evaluation of inflammatory mediator inhibition activities of estrogenic and androgenic contaminants that are common in wastewater.

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Chapter 4: The assessment of inflammatory activity and toxicity of treated sewage using Raw264.7 cells *

4.1 Abstract

Toxicity and inflammatory activity of wastewater samples were evaluated using RAW264.7 cells as a bioassay model. The RAW264.7 cell cultures were exposed to sterile filtered wastewater samples collected from a sewage treatment plant. Cell viability was evaluated using WST-1 and XTT assays. Inflammatory effects of samples were assessed by determination of nitric oxide (NO) and interleukin 6 (IL-6). The NO was estimated using the Griess reaction and IL-6 was measured by enzyme linked immunoassay (ELISA). All samples had no toxicity effects to RAW264.7 cells, however they significantly ($P<0.001$) induced NO and IL-6 production. The highest NO (12.5 ±0.38μM) and IL-6 (25383.84 ±2327 pg/ml) production was induced by post bio-filtration sample. Final effluent induced the lowest inflammatory response, which indicates effective sewage treatment. In conclusion wastewater samples can induce inflammatory activities in RAW264.7 cells. The RAW264.7 cells therefore, can be used as a model for monitoring the quality of treated sewage.

Keywords: Effluent, Environmental assessment, Pollution, Wastewater, Sewage.

4.2 Introduction

Sewage is normally composed of many types of pollutants. Raw sewage may contain high levels of pollutants like nutrients, inorganic chemicals, organic micropollutants,

microorganisms and microbial products like endotoxins (da Silva, et al. 2010; Carlson et al. 2013). When inadequately treated, it can pollute water bodies. In municipal effluents common pollutants are microbes, heavy metals, steroids, pharmaceuticals, personal care products, industrial and domestic chemicals (Naidoo and Olanaran 2013; Faul et al. 2014; Du et al. 2014). Discharged municipal effluents can therefore be a source of contamination to receiving surface water and drinking water (Stackelberg et al. 2004; Naidoo and Olanaran 2013). The contaminated water has been associated with many health problems including immunotoxicity (Gust et al. 2013). Many of the pollutants in wastewater can be immunotoxic due to induction of inflammatory reactions (Wichmann, et al. 2004; Kim et al. 2014; Xu et al. 2013).

The inflammatory reaction is one of the defence mechanisms of the immune system, which is induced by the presence of pathogens. The first line of defence system is innate immune system, which is mediated by phagocytes such as neutrophils and macrophages (Newton and Dixit 2012). When macrophages are activated, they initiate an inflammatory response by producing a wide range of inflammatory mediators and pro-inflammatory cytokines (Newton and Dixit 2012). Common inflammatory mediators and pro-inflammatory cytokines secreted by macrophages includes nitric oxide (NO), interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα) (Barnes et al. 2011). IL-6 is a pro-inflammatory cytokine with many functions (Mihara, et al. 2012). In inflammatory reactions, IL-6 induces acute phase responses and also supports chronic inflammation processes (Barnes et al. 2011; Mihara et al. 2012).

The pro-inflammatory cytokines are also known for induction of inducible nitric oxide synthase (iNOS), a key enzyme in NO production. The iNOS enzyme catalyzes production of NO from L-arginine. The NO produced is a highly reactive molecule important for cell signalling and killing of pathogens (Omer et al. 2012). Therefore NO is normally used as a biomarker of inflammation. Unfortunately, NO has a very short half-life hence excess is instantly converted and stored in the form of nitrite (Shiva 2013). The level of nitrite produced is therefore used in many studies to estimate NO production (Liu et al. 2012; Kim et al. 2014).

The production of NO and IL-6 has been used as biomarkers of inflammatory responses in many studies. There are *in vivo* studies which used NO and IL-6 as biomarkers of inflammatory reaction (Avdagic et al. 2013; Piva et al. 2013). Increased production of NO is a known biomarker of inflammatory response in the respiratory system (McCluskie et al. 2004). Therefore exhaled NO has been used to monitor respiratory inflammation associated with air pollutants (Berhane et al. 2014). Similarly, increased secretion of IL-6 has been also reported to be a sensitive biomarker of inflammatory activity (Pool et al. 2000). In fact there is an increase of studies using *in vitro* assays for IL-6 as a biomarker of inflammatory. For instance, there has been extensive use of whole human blood culture to detect IL-6 as a biomarker for inflammatory responses (Pool et al. 2003; Pool and Magcwebeba 2009; Faul et al. 2014). Others are using isolated peripheral human mononuclear cell culture (Wichmann et al., 2004; Abedayo, et al., 2014).

The use human blood culture for routine monitoring of inflammatory activies in water may be hindered by ethical issues. Alternative to using human blood culture, the

inflammatory response can be evaluated using established cell lines. One of the most widely used cell lines in inflammatory studies is mouse macrophages like RAW264.7 cells. The RAW264.7 cells are from mouse ascites leukaemia induced cells. Once activated, RAW264.7 cells express inflammatory mediators and pro-inflammatory cytokines such as NO and IL-6 respectively (Kim et al. 2014; Xu et al. 2014). As a result the cells have been used widely in many inflammatory response studies. For example macrophage RAW264.7 cells have been used in studying the inflammatory effects of environmental *Mycobacterium* spp (Huttumen et al. 2000). The RAW264.7 cells have been also used in inflammatory effects of air pollutants (Chauhan et al. 2004). Furthermore, the cell line has been used extensively for testing of inflammatory and anti-inflammatory effects of natural products (Sziliszka et al. 2013; Yang et al. 2014; Xu et al. 2014).

Despite extensive literature searches, no research report on the use of RAW264.7 cells for testing inflammatory activities of water or wastewater was found. In the present study, we have evaluated the effects of wastewater samples on RAW264.7 cells. The current study focused on the cytotoxicity and inflammatory activity of these samples to determine if the RAW264.7 cell line is a sensitive model for sewage quality monitoring.

4.3 Material and methods

4.3.1 Sewage water samples
Water samples were collected from Stellenbosch wastewater treatment works (SWTW) in Cape Town, South Africa. The processes involved at the plant are shown in Figure 4.1. Water samples were collected at four different points, namely influent (1), post biofilter (2), post sludge (3) and final effluent (4). Water samples were collected in clean glass

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bottles. An aliquot of each sample was immediately checked for total coliforms and *E. coli*. Another aliquot was   sterile filtered using 0.45μM filters and stored in sterile tubes at -20ºC till used in cell culture. The rest of the water sample was used immediately for C-18 extraction using DS-18 solid phase extraction (SPE) column (Supelco, Sigma). The extract was analysed for steroid concentrations. The steroid concentration and bacteriology measurement were done as a routine checks for plant efficiency. Total coliforms and *E.coli* were determined using pre-made ReadyCult coliforms medium (Merck, Germany). Steroids concentrations were determined using commercial available ELISA kits (DRG, Germany). The uses of ELISA kits for determination of levels of steroids in water have been previously validated (Swart and Pool, 2007). The minimum detection limits of the assay are: estrone 2.21 pg/ml, estradiol 9.7 pg/ml, ethinyloestradiol 0.1 ng/l, progesterone 0.45 ng/ml, and testosterone 83 pg/ml. Samples were diluted accordingly with diluted wash buffer; and assay procedure provided in the kits were followed. The performance efficiency of the plant based on bacteriological and steroids concentrations are summerized in table 4.1 and table 4.2, respectively.
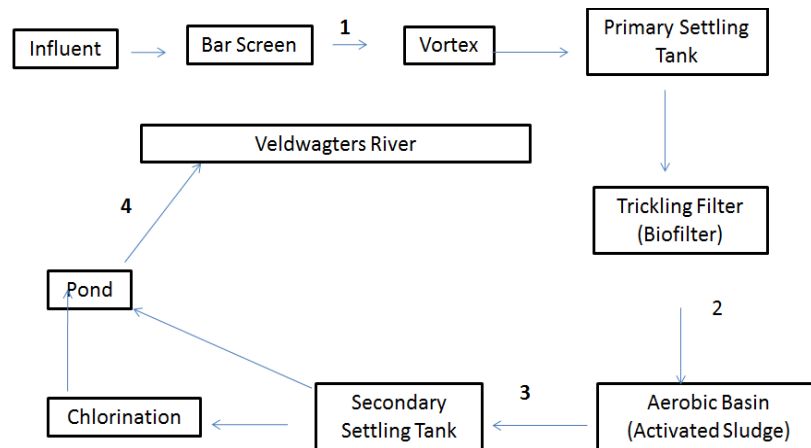
Figure 4.1 Stellenbosch wastewater treatment works (SWTW) layout: showing wastewater treatment stages and water sample collection points. **1**-Influent sample, **2**-Post-biofilter sample, **3**-Post activated sludge sample and **4**- Final effluent sample.

Table 4.1 Bacteriological quality of water samples as determined by concentration of total coliforms and *Escherichia coli* at different stages treatment in Stellenbosch STW

Sample	Total coliforms	*E.coli*
Influent	>1000 cfu/mL	>1000 cfu/mL
Post-Biofilter	>1000 cfu/mL	>1000 cfu/mL
Post-Sludge	>1000 cfu/mL	>1000 cfu/mL
Effluent	<1 cfu/mL	<1 cfu/mL

cfu: colony forming units; ml: millilitre

Table 4.2 Concentration of steroid hormones in water samples (N=4) from SWTW (Booysen, 2014).

Sample ID	Estrone		Estradiol		Ethinylestradiol		Progesterone		Testosterone	
	Conc (pg/mL)	SD	Conc (pg/mL)	SD	Conc (pg/mL)	SD	Conc (pg/mL)	SD	Conc (pg/mL)	SD
Influent	371.26	4.85	192.08	13.69	49.65	15.66	209.82	13.0	181.19	2.57
Post-biofilter	369.54	0.80	145.55	14.04	55.61	12.12	779.64	9.33	153.05	3.90
Post-sludge	117.54	4.25	12.23	1.28	5.63	1.17	10.25	1.41	7.23	0.09
Final Effluent	203.80	18.81	29.62	1.55	10.78	1.11	12.58	0.00	6.87	1.19

4.3.2 Cell culture

Mouse macrophage RAW264.7 cell line ATCC-TB-71 was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax, at 37ºC and 5% CO_2. The cells were cultured in 96 well plates at density of 5×10^5 cells/ml till were almost confluent. At confluent cell culture were treated as follows: normal medium for negative control, medium supplemented with 1μg/ml lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma, Germany) as positive control, medium containing sewage samples at 1 in 10 and 1 in 100 dilutions in respective wells. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for NO and IL-6 assays. The cells on a plate were used for cell viability assays.

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4.3.3 Cell viability

Cell viability assays was determined using chromogenic based water-soluble tetrazolium salts WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays. The assays were done using XTT reagent mixture (Roche, Germany) and WST-1 reagent (Roche, Germany). The assays procedures were followed according to manufacturer's protocol provided.

4.3.4 Nitric oxide

Secretion of NO was determined in culture supernatants using the Griess reaction in 96 well plates (Nunc, Denmark). The supernatant was mixed with equal volume of Griess reagent made up of 1% sulphanilamide (Sigma, Germany), 0.01% naphthy ethylenediamine dihydrochloride (Sigma, Germany) and 2.5% phosphoric acid. The colour developed after 15 minutes incubation was measured at 540 nm using a microplate reader (Thermo electron). The concentration of NO was determined from a standard curve generated using $100\mu M – 1.56\mu M$ sodium nitrite (Sigma, Germany).

4.3.5 Interleukin 6

Interleukin 6 was determined in cell culture supernatant using a commercially available mouse IL-6 ELISA kit (e-Bioscience, Germany). The assay is a double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA). The assays were done on Nunc Maxisorp 96 well plates (Nunc, Denmark). All reagents and assay diluent were provided in the kit; and the assay kit's assay procedure was followed accordingly.

4.3.6 Statistical analysis

The data are presented as mean ±standard deviation (SD), which were statistically analyzed with one way variance analysis (ANOVA) using sigmastat (SigmaStat software,

Inc., CA). The mean values of each treatment were compared with control. Sample size for all assays was four. The P-value <0.001 was considered as statistically significant.

4.4 Results

4.4.1 Cell viability

Water samples at 1/10 and 1/100 dilutions in the culture medium were tested for effects on cell viability. All data were compared to the negative and LPS stimulated culture controls. The XTT and WST1 results for all the samples were similar indicating that the samples had no toxic effects to RAW264.7 cell viability (Figure 4.2 and 4.3).
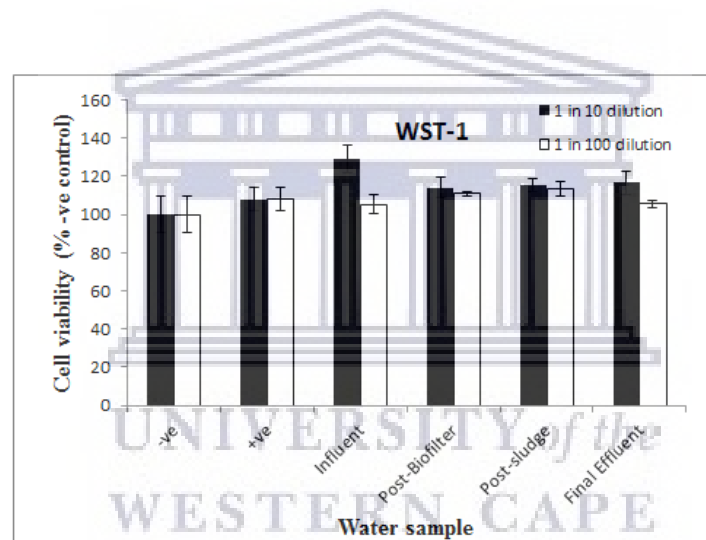
Figure 4.2 Effects of water samples on RAW264.7 cells viability as determined by WST-1 assay. Negative control was treated with normal medium, positive control treated with LPS (1µg/ml) and sample treatment at 1 in 10 and 1 in 100 dilutions.
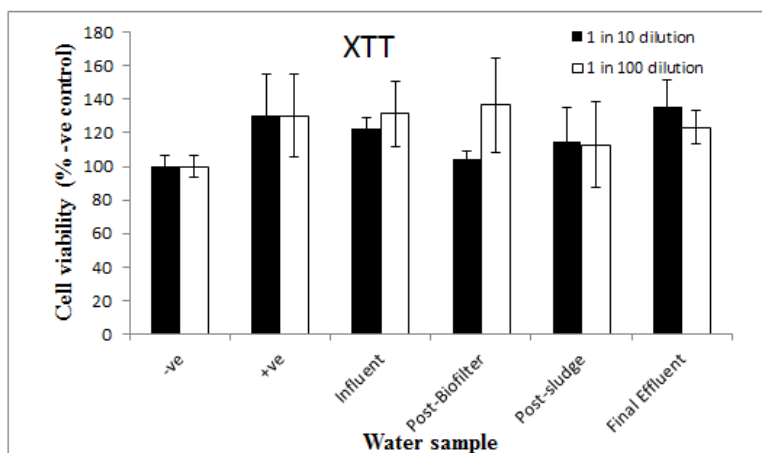
Figure 4.3 Effects of water samples on RAW264.7 cells viability as determined by XTT assay. Negative control was treated with normal medium, positive control treated with LPS (1µg/ml) and water samples treatment at 1 in 10 and 1 in 100 dilutions.

4.4.2 Effects of water samples on NO production

The effects of water samples on NO production in RAW264.7 cell culture were determined in cell culture supernatant. All water samples at 1 in 10 dilution increased production of NO significantly ($P<0.001$) higher than the negative control (Figure 4.4). The highest effect on NO production was induced by post-biofilter sample (12.5±0.38 µM). The post sludge sample induced relatively decreased effect, while the lowest induction was evident with influent (5.2±0.47 µM) and final effluent samples (5.7±0.21 µM). Among the water samples at 1 in 100 dilutions, post biofilter sample induced significantly ($P<0.001$) higher NO production compared to the negative control.
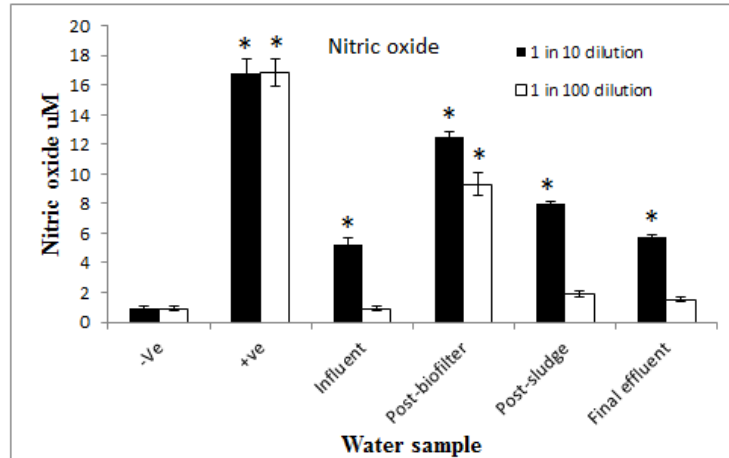
Figure 4.4 Effects of water samples on NO production in RAW264.7 cells. Negative control received normal medium alone, positive control was treated with LPS (1µg/ml), water samples tested at 1 in 10 and 1 in 100 dilutions. * indicates that NO level is significantly (P<0.001) higher than the negative control.

4.4.3 Effects of water samples on IL-6 secretion

The effects of water samples on IL-6 secretion in RAW264.7 cell culture were determined in cell culture supernatant. Water samples from influent, post-biofilter, and post-sludge at 1 in 10 dilution significantly (P<0.001) induced production of IL-6 in RAW264.7 cells compared to the negative control (Figure 4.5). The post-biofilter water sample induced the highest secretion of IL-6 (25 383.84 ±2327 pg/ml). Induction of IL-6 by effluent sample was similar to the negative control in both dilutions. Among all water samples tested at 1 in 100 dilutions post biofilter sample induced significantly (P<0.001) higher production of IL-6 than the negative control.
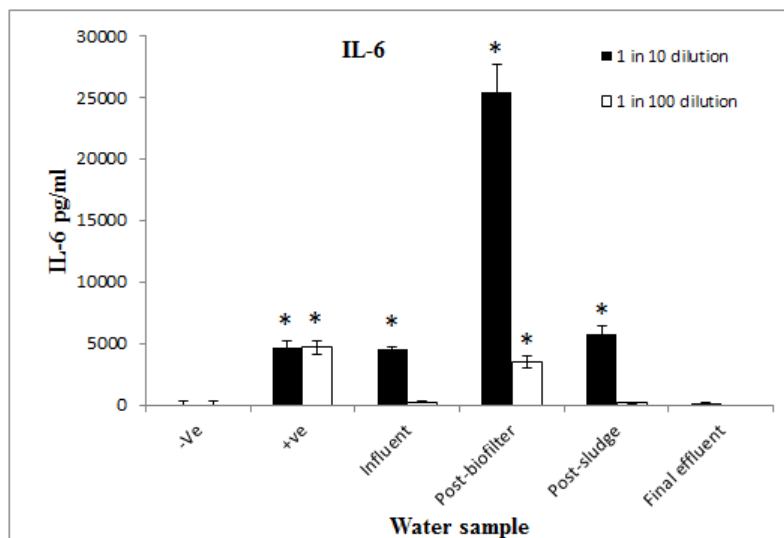
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Figure 4.5 Effects of water samples on IL-6 secretion in RAW264.7 cells. Negative control received normal medium alone, positive control was treated with LPS (1μg/ml), water samples treatment at1 in 10 and 1 in 100 dilutions. * indicates that the IL-6 induced by the treatments is significantly (P<0.001) higher than the negative control.

4.5 Discussion

Detection of pollutants in wastewater using chemical methods is a common technique of assessment of pollutants. However, because of the complex mixture of pollutants in wastewater, analysis of each pollutant is not easy. Alternatively, biological assays are usually developed for assessment of biological effects of wastewater samples. In the present study wastewater samples from SWST were assessed for toxicity and inflammatory activities using RAW264.7 cells. None of the water samples tested resulted in significantly different WST-1 nor XTT based metabolic assay compared to controls, which implies that samples had no toxic effects to RAW264.7 cells. Lack of toxicity of wastewater or contaminated water has been reported in previous studies (Pool et al.,

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2000; Hendricks and Pool 2012). This observation may be attributed to lower concentration of pollutants required to induce immunotoxicity as compared to that required for induction of cell death (Heymery et al. 2014).

Stimulation of RAW264.7 cells induces production of NO and other pro-inflammatory cytokines including IL-6 (Liu et al. 2012; Kim et al. 2014; Xu et al. 2014). In the present study, all samples at concentrations of 1 in 10 dilution induced NO production in RAW264.7 cells significantly ($P<0.001$) higher than negative control. Similar inflammatory response was observed in the induction of IL-6 secretion due to influent, post-biofilter and post sludge samples. The increase of NO and IL-6 production in this study show that the water samples induced inflammatory responses in RAW264.7 cells. The induction of IL-6 secretion has been reported as a sensitive biomarker for monitoring inflammatory activities of water (Pool et al. 2000). Due to this, it has been used as a biomarker assay for water quality. Indeed, IL-6 has now been used in many water assessment studies using human blood culture (Pool et al. 2003; Pool and Magcwebeba, 2009; Faul et al. 2014; Abedayo, et al. 2014).

The results also show that the highest inflammatory activity in RAW264.7 cells was induced by post bio-filter sample. The sample induced significantly ($P<0.001$) higher production of both NO and IL-6 than the rest of water samples. The high levels of NO and IL-6 is an indication of increased inflammatory activities due to post bio-filter water sample. The inflammatory effects of this sample could be associated with the presence of high content of inflammatory pollutants. A number of organic pollutants common in

wastewater can induce inflammatory activities (Kim et al. 2014; Xu et al. 2013). The increase in inflammatory responses in post bio-filter sample can also be associated with high levels of steroids in this sample (Table 2). Some studies showed that estradiol can induce proinflammatory cytokines IL-6 and iNOS through oestrogen receptor (ERα) and ERβ in macrophages (Calippe et al. 2010). Another possible source of high inflammatory activities in the post-biofilter sample could be associated with presence of microbial products like endotoxins due to high microbial activities in the bio-filter process. The process in trickling filters is dominated by high microbial activities.

The results further show that influent water sample induced relatively low inflammatory responses as determined by NO level. The influent sample is normally a rich mixture of pollutants. Some of the pollutants in influent may have anti-inflammatory effects. This sample has a high concentration of steroid hormones. Some steroids present in the water sample like progesterone and testosterone can suppress inflammatory activities (Rettew et al. 2008; Corcoran et al. 2010). Progesterone can suppress production of IL-6 and NO as well as expression of iNOS and NF-kB in macrophages (Sue et al. 2009). Therefore the low inflammatory response induced by the influent might be due to contaminants with anti-inflammatory activities in the sample.

The final effluent sample induced relatively low inflammatory response, characterized by low level of NO and undetectable IL-6 secretion. This water sample was collected at the post chlorination point just before discharged into a receiving river. The low inflammatory activities of the sample from this point could be due to effects of

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disinfection done by chlorination at the preceding process. The results show that the treatment processes at SWTW remove most of the inflammatory pollutants. The findings in this study correlate well with previous studies using whole human blood culture in the assessment of water quality (Pool et al. 2003; Pool and Magcwebeba 2009; Faul et al. 2014). Similar trends of results have also been reported in studies using isolated human peripheral mononuclear cell culture (Wichmann et al. 2004; Abedayo et al. 2014). In order to avoid ethical issues surrounding uses of human blood, the induction of inflammatory responses in RAW264.7 cells observed in this study therefore, provides an alternative bioassay model to using of human blood culture.

In conclusion, water samples collected from SWTW did not induce cytotoxic effects to macrophage RAW264.7 cells. However, the same samples induced inflammatory responses by increasing NO production and IL-6 secretion in RAW264.7 cells. The increase of NO production and IL-6 secretion in RAW264.7 cells can be used as biomarkers for inflammatory activity and monitoring water quality. The results also give evidence that RAW264.7 cells can be used as a model for monitoring inflammatory pollutants in water and wastewater in particular.

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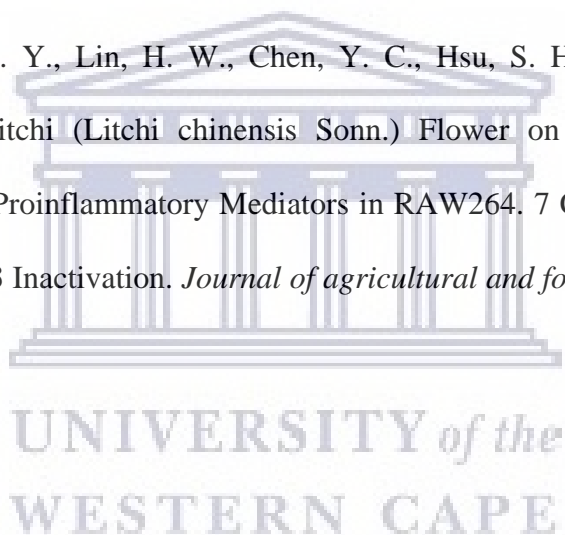
Chapter 5: Evaluation of cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by coagulation-flocculation methods *

5.1 Abstract

Effective treatment of textile effluent prior to discharge is necessary in order to avert the associated adverse health impacts on human and aquatic life. In the present investigation, coagulation/flocculation processes were evaluated for the effectiveness of the individual treatment. Effectiveness of the treatment was evaluated based on the physicochemical characteristics. The quality of the pre-treated and post-flocculation treated effluent was further evaluated by determination of cytotoxicity and inflammatory activity using RAW264.7 cell cultures. Cytotoxicity was determined using WST-1 assay. Nitric oxide (NO) and interleukin 6 (IL-6) were used as biomarkers of inflammation. NO was determined in cell culture supernatant using the Griess reaction assay. The IL-6 secretion was determined using double antibody sandwich enzyme linked immunoassay (DAS ELISA).

Cytotoxicity results show that raw effluent reduced the cell viability significantly (P<0.001) compared to the negative control. All effluent samples treated by coagulation/flocculation processes at 1 in 100 dilutions had no cytotoxic effects on RAW264.7 cells. The results on inflammatory activities show that the raw effluent and effluent treated with 1.6g/L of Fe-Mn oxide induced significantly (P<0.001) higher NO

production than the negative control. The inflammatory results further show that the raw effluent induced significantly (P<0.001) higher production of IL-6 than the negative control. Among the coagulants/flocculants evaluated $Al_2(SO_4)_3.14H_2O$ at a dosage of 1.6 g/L was the most effective to remove both toxic and inflammatory pollutants. In conclusion the inflammatory responses in RAW264.7 cells can be used as sensitive biomarkers for monitoring the effectiveness of coagulation/flocculation processes used for textile effluent treatment.

Keywords: Textile effluent, Toxicity, Inflammation, Coagulation/Flocculation, Interleukin 6, Nitric oxide.

5.2 Introduction

Environmental pollution due to textile industry activities has become a global concern that has attracted considerable attention among researchers. The textile industry is one of those industries that utilize high volumes of clean water and complex chemicals during processing operations (Oller et al. 2011). As a result there is a huge amount of wastewater generated that is composed of different complex chemical substances. The chemicals in the wastewater are mostly high molecular weight, non-biodegradable and highly recalcitrant in the environment (Zapata et al. 2009a; Zapata et al. 2009b). Examples of common pollutants in textile wastewater include heavy metals, detergents, reactive dyes, dye fixing agents and hydrocarbon compounds used as softeners (Ghaly et al. 2014). In addition, textile effluents usually have extremely high pH values, strong colour, high chemical oxygen demand (COD), biochemical oxygen demand (BOD), turbidity, total organic carbon (TOC), suspended and dissolved solids and soluble organic compounds among others (Mazumber, 2011; Martín et al. 2011and Hussaini et al. 2013).

The potential adverse effects on humans and the entire ecosystem due to the direct disposal of textile effluent into the aquatic environment without proper treatment have been widely reported (Gatidou et al. 2007; El-Gohary and Tawfik, 2009; Pothitou and Voutsa, 2008). According to Martín et al., (2011) direct discharge of coloured wastewater can cause eutrophication and reduce the available dissolved oxygen level. Such adverse conditions make aquatic life difficult and tend to accelerate genotoxicity and microtoxicity in aquatic organisms (Foo and Hameed, 2010; Verma et al. 2012). Similarly, continuous exposure to untreated coloured wastewater may lead to effects such as suppression of the immune system, the respiratory system, cause neurobehavioral disorders, leukemia, hyperventilation, eye infections and lung edema among others (Verma et al. 2012). Furthermore, reactive dyes, which form the main component in textile effluents, can also cause harmful effects like acute toxicity (Klemola et al. 2007; Verma, 2008; Verma, 2010), carcinogenic effects (Marthur et al. 2005) and contact allergies (Ryberg et al. 2006; Malinauskiene at al. 2011).

Thus, considering the negative impacts of direct discharge of untreated textile wastewater on humans and the ecosystem, textile effluents need to be treated before being discharged into the environment. The review of literature revealed that several physico-chemical and biological methods such as microfiltration, activated carbon adsorption, coagulation, flocculation, activated sludge treatment either in the form of a pre or post-treatment step have been exploited to treat these effluents. However, one of the major environmental concerns is the removal of colour, suspended solids and perhaps reduction of COD in the effluent before discharge into the environment (Zayas et al. 2007; Oller et al. 2011). The presence of these complex mixtures make the treatment quite extensive, time demanding

and as such single step clean-up systems are normally not effective for complete colour or COD reduction, instead a combinatory approach is required (Oller et al. 2011).

The use of coagulation-flocculation is one of the most widely utilised and practiced techniques in developed and developing countries due to its simplicity as well as effectiveness in the removal of colour, suspended matter and COD reduction (Ciabatti et al. 2010; Oller et al., 2011; Verma et al. 2012). Different coagulant-flocculant mixtures such as ferric salts, aluminium salts, synthetic organic polymers, polyelectrolytes and lime have been applied to treat conventional wastewater (Bolto and Gregory, 2007; Verma et al. 2012). These coagulants/flocculants accelerate the rate of aggregation of colloidal particles by reducing the electrostatic surface charges between the coagulants/flocculants and particles in the acidic pH region (Sher et al. 2013). The overall efficiency of the process depends on factors such as coagulant-flocculant dosage and type, stirring speed, reaction time, effluent pH and others (Bolto and Gregory, 2007; Verma et al. 2012).

In most cases during treatment of wastewater from textile industries, great attention has been placed on colour removal. As a result the assessment of effectiveness of the techniques employed is normally based on the physico-chemical properties of the treated effluent such as removal of colour, COD and TDS. For instance the reductions of COD and TDS by coagulation and precipitation methods have been reported (Parmer et al. 2011; Sabur et al. 2012). Sabur et al. (2012) found that both processes were pH dependent and at pH 6, 90.17 and 74.09 % COD and TDS reduction were observed. Mukhlish et al. (2013) have applied the coagulation-flocculation process to treat textile

effluent and achieved a 61.3 % COD reduction at the optimum dosage of 2.5 kg/m^3 of CaO and 2.0 kg/m^3 of FeSO$_4$ respectively. Very recently, Di Bella et al. (2014) applied coagulation-flocculation as a pre-treatment step for saline wastewater and found that 50 mg/L of aluminium sulphate was effective for the removal of 70-80 % TOC.

Although reactive dyes are dominant components in textile effluents and are well known to cause numerous adverse biological effects, assessment of such biological effects are relatively rare. Several studies have demonstrated that reactive dyes can cause allergic reactions (Ryberg et al. 2006; Malinauskiene at al. 2012; Nygaard et al. 2013). Allergic reaction is a chronic inflammatory response involving many immunological factors and cells like basophils, eosinophils, T-cells and macrophages (Kang and Biswas, 2013). The role of macrophages in allergic reactions has been detailed in allergic asthma (Moreira and Hogaboam, 2011) and in atopic dermatitis (Kasraie and Werfel, 2013). In early inflammatory phase of allergic reaction, macrophages play a key role in initiating inflammatory processes. After activation by foreign bodies such as an allergen, macrophages release many inflammatory factors like NO and pro-inflammatory cytokines such as IL-6 (Kasraie and Werfel, 2013). The IL-6 is a pro-inflammatory cytokine involved in many reactions like acute phase responses and chronic inflammation processes (Mihara, et al. 2012; Barnes et al. 2011). Secretion of IL-6 is induced by inflammatory agents such as pollutants, pathogens or their product through activation of receptors. Expression and secretion of IL-6 in macrophages are regulated by activation of NF-kB (Scheller, et al. 2011). NO is synthesized by enzymatic oxidation of L-arginine by nitric oxide synthase (NOS) (Koyabashi 2010). In macrophages, the main NOS is inducible NOS (iNOS), however endothelial NOS (eNOS) isomer is also expressed

(Connelly et al. 2003). The synthesised NO is very reactive with a short half-life (Koyabashi 2010). The excess of NO produced is readily converted to nitrite, which is used to estimate the NO produced.

Inflammatory responses have been studied *in vitro* using established macrophage cell lines. One of the cell lines used widely in inflammatory studies is the mouse macrophage RAW264.7 cell line (Roponen, et al. 2001; Jeon et al. 2014). When RAW264.7 cells are stimulated they can secrete NO and IL-6 (Kim et al. 2014). Therefore, both NO and IL-6 levels in RAW264.7 cells can be useful biomarkers of inflammatory activities (Xu et al. 2014; He et al. 2014). Despite an extensive literature search, there are few or no studies that report on the evaluation of inflammatory activities of textile effluent and on efficient removal of the inflammatory pollutants. The current study focused on the evaluation of coagulant/flocculant treatment processes to effectively remove of toxic and inflammatory substances from textile wastewater. The study also investigated the effects of treatment procedures on pH, COD and TOC of the pre and post-treatment wastewater.

5.3 Material and methods

5.3.1. Wastewater samples

Wastewater samples that are odoriferous and dark in colour were supplied by a textile industry in Cape Town. This industry has different production lines where wastewaters are being generated and so the most concentrated effluents stored at the central compartment was collected and used for the treatment. The samples were stored in 200 L litre acid washed plastic drums. Prior to the sampling, the drums were washed with diluted HNO_3 and millipore water and thereafter rinsed with the effluents. For the laboratory investigations, the samples were stored in plastic vials at 4ºC.

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5.3.2. Wastewater treatment

In order to identify the best suitable coagulant or flocculant for the treatment of effluent, the coagulant-flocculant dosages were optimised. The wastewater sample was subjected to gravitational settling for 30 minutes due to the presence of a substantial quantity of suspended solids. But, it was found that the suspended solids were poorly settled within 30 minutes. The following analytical grade chemicals $Al_2(SO_4)_3.14H_2O$, $Ca(OH)_2$, $FeCl_3$, $FeSO_4.7H_2O$ were procured from Sigma Aldrich and were used without to any further treatment. Raw ferromanganese wad (Fe-Mn) oxide was obtained from Vaal Triangle, about sixty kilometres South of Johannesburg and was also used without further purification. The study was performed by varying the mass of Fe-Mn oxide, $Al_2(SO_4)_3.14H_2O$, $FeSO_4.7H_2O$, $FeCl_3$, and $Ca(OH)_2$ from 0.2–1.0 g in a batch mode (Table 5.1).

Table 5.1 Experimental protocol for wastewater treatment

Sample treatment	Treatment protocol
Raw effluent	Before treatment
Fe-Mn oxide	Addition of 0.2 – 1.0 g of Fe-Mn oxide to 0.5 L effluent, stirred rapidly for 120 seconds and then slowly for 60 minutes
$Al_2(SO_4)_3.14H_2O$	Addition of 0.2- 1.0 g $Al_2(SO4)_3.14H_2O$ to 0.5 L effluent, stirred rapidly for 120 seconds and then slowly for 60 minutes
$FeSO_4.7H_2O$	Addition of 0.2- 1.0 g $FeSO_4.7H_2O$ to 0.5 L effluent, stirred rapidly for 120 seconds and then slowly for 60 minutes
$FeCl_3$	Addition of 0.2- 1.0g $FeCl_3$ to 0.5 L effluent, stirred rapidly for 120 seconds and then slowly for 60 minutes
$Ca(OH)_2$	Addition of 0.2 – 1.0 g of $Ca(OH)_2$ to 0.5 L effluent, stirred rapidly for 120 seconds and then slowly for 60 minutes

Firstly, 500 ml each of wastewater sample was added to five beakers to which were added specified amounts of the coagulant and flocculants without adjusting the pH of the effluent with either acid or base. Thorough mixing was performed for 1 min at 200 rpm, and then the coagulation-flocculation was carried out at a speed of 20 rpm for 20 min. Finally, the wastewater mixture content in the beaker was allowed to settle for 60 minutes and the supernatant was collected for analysis. The experiments were performed at room temperature ($25^{o}C \pm 2$) and constant pH of 6.02. The parameters measured for the supernatant before and after treatment were COD, TOC and pH. The experiments were repeated three times and the average result was computed including the standard deviation of the mean. The best treated samples obtained at the optimal dosage were further evaluated for toxicity and inflammatory activities.

5.3.3. Chemical analysis

The collected sample was characterized for pH, electrical conductivity (EC), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Biochemical oxygen demand (BOD), Total organic carbon (TOC), and Chemical Oxygen Demand (COD) as shown in Table 5.2. All the parameters were measured in accordance with "Standard methods for treatment of water and wastewater" (American Public Health Association, 1999).

5.3.4. Cell culture

Mouse macrophage RAW264.7 cells, American Type Culture Collection (ATCC-TB-71) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Gibco, Life Technology). The cells were cultured in 96 well plates at density of $5x10^5$ cells/ml in a humidified incubator

at 37ºC and 5% CO_2 until confluence. Then the medium was replaced with fresh culture medium containing sterile filtered textile effluent water samples treated with adsorption and coagulation techniques. To the cell cultures samples were added as follows: normal medium for negative control, medium supplemented with 1μg/ml lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma, Germany) as positive control, medium containing effluent samples at 1 in 100 dilutions in respective wells. The effluent samples included raw effluent, effluent treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$. Effluent samples were sterilized using 0.45uM filters and applied to culture medium at 1 in 100 dilutions. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays.

5.3.5. Cell viability

Cell viability was determined using chromogenic based water-soluble tetrazolium salts (WST-1). The assay is based on the principle of the breakdown of tetrazolium to water soluble formazan dye by the action of dehydrogenase enzyme. Briefly, the assay procedure was as follows; after removal of cell supernatant from culture, each well received 100 μl of medium supplemented with 10% WST-1 reagent (Roche, Germany). The absorbance was read immediately after addition of WST-1 medium and a second reading was done after incubation for 30 minutes at 37ºC and 5% CO_2. The change in absorbance at 450 nm over 30 minutes was used as a measure of cell viability.

5.3.6. Nitric oxide determination

Nitric oxide (NO) production was determined in culture supernatants using the Griess reaction in 96 well plates (Nunc, Denmark). The supernatant was mixed with equal

volume of Griess reagent made up of 1% sulphanilamide (Sigma, Germany), 0.01%

naphthyl ethylenediamine dihydrochloride (Sigma, Germany) and 2.5% phosphoric acid.

The colour developed after 15 minutes incubation was measured at 540 nm using a

microplate reader (Thermo electron). The concentration of NO was determined from a

standard curve generated using 100 μM – 1.56 μM sodium nitrite (Sigma, Germany). NO

value of each water sample was subtracted from respective treatment as reading of

background blank.

5.3.7. Interleukin-6 determination

Interleukin 6 in cell culture supernatant was determined using a commercially available

mouse ELISA kit (e-Bioscience, Germany). The assay system is a double antibody

sandwich enzyme linked immunosorbent assay (DAS ELISA). The assays were done on

NUNC Maxisorp 96 well plates (Nunc, Denmark). The assay kit's procedure was

followed as provided in the kit. Briefly the protocol involved coating of a plate overnight

at 4 ℃ with capture antibody (anti-mouse IL-6 diluted in coating buffer, PBS). The plate

was washed five times in wash buffer consisting of PBS with 0.1% Tween. The plate was

then blocked with assay diluent for 1 hour at room temperature. After another five times

washing, IL-6 standard or cell culture supernatants were added to each well accordingly.

The plate was then incubated for two hours at room temperature. The plate was washed

again five times, after which the detection antibody (biotinylated anti-mouse IL-6 in

block diluent) was added to each well and incubated for 1 hour at room temperature.

After another five times wash, Avidin - horse radish peroxidise (HRP) conjugate was

added and incubated for 30 minutes at room temperature. The plate was washed seven

times, then substrate TMB solution was added and incubated in the dark for 15 minutes at

room temperature. The reaction was stopped with 0.5M H_2SO_4 stop solution, and the absorbance read at 450 nm with a microplate reader (Thermo electron).

5.3.8. Statistical analysis

The data are presented as mean ±standard deviation (SD), which were statistically analyzed with one way variance analysis (ANOVA) using sigmastat (SigmaStat software, Inc., CA). The mean values of each treatment were compared with control. P-value <0.001 was considered as statistically significant.

5.4. Results

5.4.1. Effluent sample chemical characteristics

The results of chemical oxygen demand (COD), total organic content (TOC) and pH values of effluent before treatment and after treatment with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ are shown in Table 5.3. Treatment with 1.6 g/L of Fe-Mn oxide produced no appreciable reduction in COD and TOC value compared to the raw effluent, although a significant reduction in odour was noticed. With the addition of 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ to the raw effluent, 63.13 % and 75.5% reduction in COD and TOC value respectively was observed. Similarly, addition of 1.6 g/L of $FeSO_4.7H_2O$ to the raw effluent decreased the COD and TOC value by 45.6 % and 61.1 % respectively. The maximum COD and TOC removal at optimal dosage of 1.6g/L of $FeCl_3$ was 59.65 and 72.26 % respectively. The optimal concentration of $Ca(OH)_2$ for treating effluent was 1.6 g/L with 38.1 and 56.8 % reduction in COD and TOC respectively.

Table 5.2 Physicochemical characteristic of the raw textile wastewater

Parameters	Symbols	Value
pH		5.99 ± 0.03
Electrical conductivity		1238.6 ± 1.53
Alkalinity as carbonate	CO_3^{2-}	212.3 ± 12.5
Ortho phosphate	PO_4^{3-}	203 ± 2.62
Sulphate	SO_4^{2-} dissolved	190.3 ± 0.61
Total phosphorus	P	199 ± 3.62
Turbidity		8145.3 ± 5.74
Dissolved organic carbon	DOC	2438.7 ± 1.52
Total organic carbon	TOC	5952 ± 2.64
Chemical oxygen demand	COD	20457 ± 1.52
Biochemical oxygen demand	BOD	807 ± 1.00
Total suspended solids	TSS	2758.7 ± 8.08
Total dissolved solids	TDS	744 ± 6.55
Chlorides	Cl-	55.7 ± 0.09
Flourides	F	16.3 ± 0.14
Colour	Dark	
Odour	Highly unpleasant (odiferous)	

All values are in mg/L except for pH, electrical conductivity (mS/cm), turbidity (NTU)

Table 5.3 Chemical oxygen demand (COD), total organic content (TOC) and pH values of effluent before and after treatment at optimal conditions

Sample treatment	pH	COD (mg/L)	TOC (mg/L)
Before treatment (raw effluent)	5.99 ± 0.03	20457 ± 1.52	5952 ± 2.64
After treatment with 1.6g/L of Fe-Mn oxide	5.74 ± 0.06	$19\,987 \pm 3.42$	$5\,893 \pm 2.12$
After treatment with 1.6g/L of $Al_2(SO_4)_3.14H_2O$	2.44 ± 0.15	$7\,543 \pm 0.45$	$1\,456 \pm 0.31$
After treatment with 1.6g/L of $FeSO_4.7H_2O$	5.03 ± 0.26	$11\,134 \pm 16.57$	$2\,310 \pm 17.81$
After treatment with 1.6g/L of $FeCl_3$	1.88 ± 0.04	$8\,369 \pm 11.45$	$1\,650 \pm 12.53$
After treatment with 1.6g/L of $Ca(OH)_2$	11.32 ± 1.00	$12\,653 \pm 4.18$	$2\,568 \pm 5.34$

5.4.2. Effects of effluent samples on cell viability

The effects of effluent samples on RAW264.7 cell viability are shown in Figure 5.1. Raw effluent reduced cell viability significantly ($P<0.001$) as compared to negative control. None of the effluent samples effluent treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ induced toxicity in RAW264.7 cell culture.

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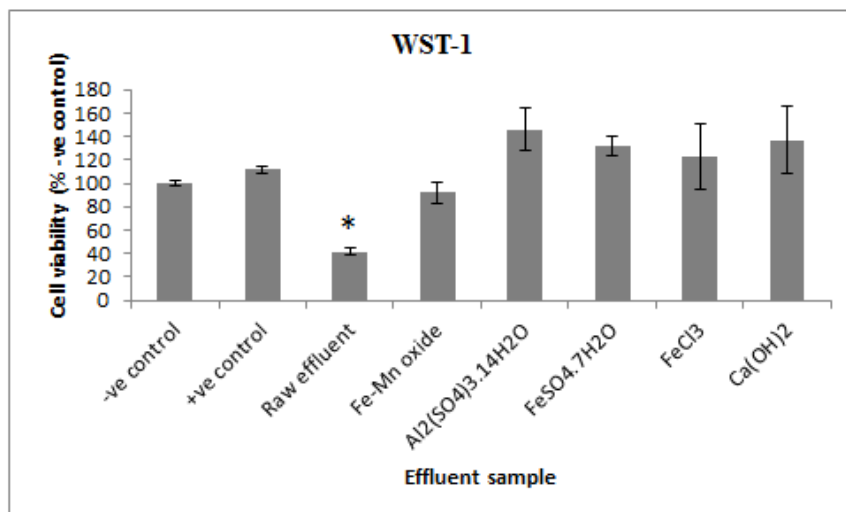
Figure 5.1 Effects of effluent samples treated with coagulation/flocculation techniques on RAW264.7 cells viability as determined by WST-1 assay. Negative (-ve) control was treated with normal medium, positive (+ve) control was treated with LPS (1 μg/ml), and effluent samples 1 in 100 dilutions as follows: raw effluent; effluent treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$. *indicates that cell viability is significantly (P<0.001) lower than the negative control.

5.4.3. Effects of effluent samples on nitric oxide production

The effects of effluent samples on NO production in RAW264.7 cell culture were determined in cell culture supernatant. The effects of effluent samples on secretion of NO by RAW264.7 cells are shown in Figure 5.2. Raw effluent and effluent treated with Fe-Mn oxide induced significantly (P<0.001) higher NO production than the negative control. The effluent samples treated with 1.6g/L of $Al_2(SO4)_3$, 1.6g/L of $FeSO_4$, 1.6g/L of $FeCl_3$ and 1.6g/L of $Ca(OH)_2$ did not induce significant amounts of NO.
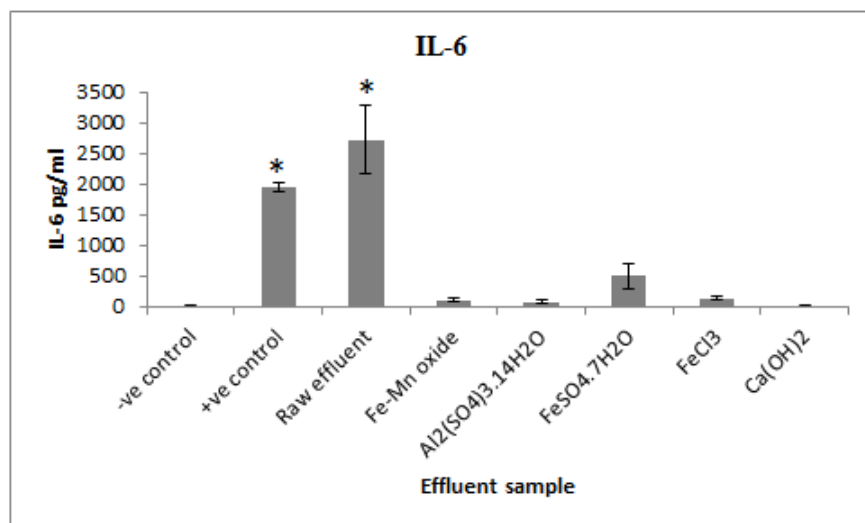
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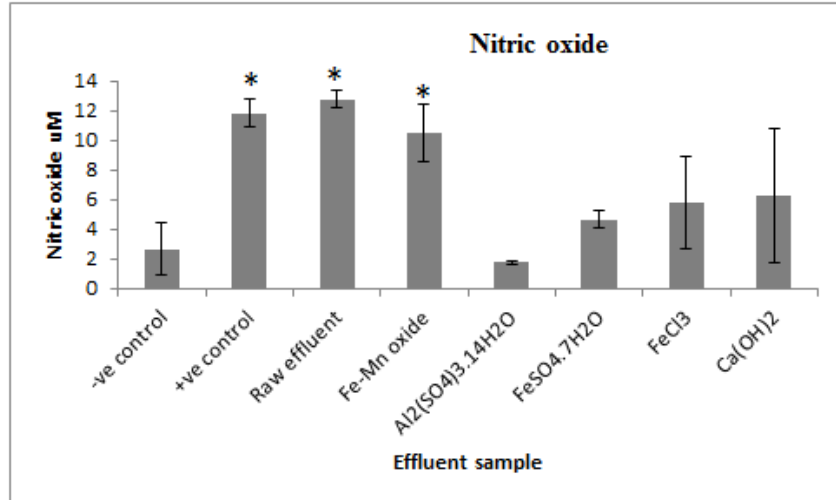
Figure 5.2 Effects of effluent samples treated with adsorption and coagulation methods on induction of NO production in RAW264.7 cells culture. Negative (-ve) control was treated with normal medium, positive (+ve) control was treated with LPS (1μg/ml), and effluent samples 1 in 100 dilutions as follows: raw effluent; effluent treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$. *indicates that NO level is significantly (P<0.001) higher than the negative control.

5.4.4. Effects of effluent samples on IL-6 secretion

The effects of effluent samples on IL-6 secretion in RAW264.7 cell culture were determined in cell culture supernatant. The effects of effluent samples on IL 6 secretion by RAW264.7 cells culture are shown in Figure 5.3. Raw effluent induced significantly (P<0.001) higher IL-6 secretion than the negative control. Effluent treated with 1.6g/L of $FeSO_4$ induced production of IL-6, with non-significant different from the control. Effluent samples treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO4)_3$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ had no significant effects on production of IL-6.

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Figure 5.3 Effects of effluent samples treated with adsorption and coagulation methods on IL-6 secretion in RAW264.7 cells culture. Negative (-ve) control was treated with normal medium, positive (+ve) control was treated with LPS (1µg/ml), and effluent samples 1 in 100 dilutions as follows: raw effluent; effluent treated with 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$. *indicates that IL-6 level is significantly (P<0.001) higher than the negative control.

5.5 Discussion

Different physicochemical methods have been developed for treatment of wastewater. In the present study the raw textile effluents produced by the factory had an average COD and TOC value of 20457 ± 1.52 and 5952 ± 2.64 mg/L respectively. The COD value of the raw wastewater supplied was greater than 20,000 mg/L. It is evident that the wastewater does not meet the applicable 5000 mg/L COD maximum effluent discharge limits for discharge into water sources as per City of Cape Town wastewater and industrial effluent bylaw schedule 2 page 10

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(www.capetown.gov.za/en/Water/Documents/wwater_bylaw_eng.pdf). The high COD, TOC, pH and intense colour can be attributed to the use of complex organic substances that are highly non-biodegradable dyes (Anouzla et al., 2009). High values of these indicator parameters revealed the pollution status of the effluent and thus direct discharge into the environment may cause adverse environmental and health effects, especially on aquatic species. In order to improve the effluent characteristics and comply with South African National Water Act waste discharge standards (DWA 2010 guidelines and City of Cape Town wastewater and industrial effluent bylaws), different dosages of coagulant/flocculants were added to the effluents and after the treatment the COD and TOC were evaluated.

In the present study textile effluent samples were treated separately with specified amount of coagulant/flocculant. Thereafter the optimal dose for each coagulant/flocculant was determined. Based on the effective reduction of the chemical oxygen demand (COD) and total organic carbon (TOC) value, $Al_2(SO_4)_3.14H_2O$ at a dose of 1.6 g/L seemed to be the most effective treatment option. The optimum dose of 1.6g/L for $Al_2(SO_4)_3.14H_2O$ reduced the COD and TOC value by 63.13 % and 75.5% respectively. The treatment with this coagulant/flocculant removed the complex organic substance. Similar observations have been reported by others (Kumar et al. 2008; Dulov et al. 2011). In order to further evaluate the efficiency of coagulant/flocculants to remove organic substances, treated samples were assessed for induction of toxicity and inflammatory activities in macrophage RAW264.7 cells.

The cell viability results show that raw effluent induced toxicity to RAW264.7 cells after 24 hours exposure. The results imply that raw effluent contains toxic pollutants, which can induce cytotoxicity effects to RAW264.7 cells. On the other hand, all effluents treated with optimized coagulation/flocculation processes did not induce toxic effects. Lack of toxicity partly indicates the efficiency of the treatment techniques. The lack of toxicity also could partly be due to low concentration of effluent samples used at 1 in 100 dilutions. Similar observations are commonly reported in previous toxicity studies (Pool et al., 2000; Hendricks and Pool, 2012). The observation has been associated with high concentrations of pollutants required to induce toxicity as compared to that required for induction of other response like immunotoxicity (Heymery et al. 2014).

The results of inflammatory response in RAW264.7 cells after 24 hours incubation with effluent samples show that raw effluent and effluent treated with 1.6g/L of Fe-Mn oxide induced inflammatory response by inducing significantly ($P<0.001$) higher NO production than the negative control. Wastewater sample treated with 1.6g/L of $Al_2(SO_4)_3.14H_2O$ induced the lowest level of NO. Wastewater samples treated with 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ induced NO production with no statistically difference to the negative control. Although the levels of NO induced by 1.6g/L of $FeSO_4.7H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ are not significant, the use of these coagulant/flocculants or the residual toxins in the water might have effects on NO production. The effect of ferric iron (Fe^{3+}) has been reported to regulate NO through transcription of iNOS (Weiss et al. 1994). Thus the presence of iron ions can induce NO production through increased expression of iNOS (Galleano et al. 2004). Similarly, wastewater treated with 1.6g/L of $Ca(OH)_2$ also induced a non-significant NO

production. The production of NO in macrophage is normally induced by inflammatory agents via an iNOS pathway, which is a calcium independent pathway (Mattila and Thomas, 2014). However, macrophages such as RAW2646.7 cells can also express eNOS, which is a calcium dependant isomer (Schmidt, et al. 1992; Connelly et al. 2003). Therefore the presence of calcium (Ca^{2+}) in treated sample from the coagulant/flocculants can influence NO production. In the same vein, the COD was still high after treatment, so the effect could be due to the residual toxins in the treated water.

The results of inflammatory responses further show that raw effluent induced significantly ($P<0.001$) higher level of IL-6 secretion than the negative control. Effluent treated with 1.6g/L of FeSO4.7H2O increased IL-6 secretion with no statistically different from the control. Treatment of effluents using 1.6g/L of Fe-Mn oxide, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $FeCl_3$, and 1.6g/L of $Ca(OH)_2$ did not induce IL-6 secretion. The increase of IL-6 secretion is an indication of the inflammatory response. Increased IL-6 secretion has been used as a sensitive biomarker for monitoring inflammatory activities in water (Pool et al. 2000; Abedayo, et al. 2014). Therefore the high level of IL-6 induced by raw effluent is an indication of the presence of inflammatory pollutants in textile effluent. The increased IL-6 secretion in the effluent sample treated with 1.6g/L of $FeSO_4.7H_2O$ can indicate inadequate removal of pollutants as effluent is characterized by high COD value or is due to effects of Fe^{2+} on the function of macrophages (Ward et al. 2011).

5.6 Conclusion

The results of this study show that raw effluent from a textile industry can induce cytotoxicity in RAW264.7 cells and inflammatory activities by increasing both NO

production and IL-6 secretion. Treatment of textile industrial effluent using 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ is the most effective coagulation/flocculation processes for removing both toxic and inflammatory pollutants. The induction of IL-6 secretion in RAW264.7 cells is a more sensitive biomarker than NO production for evaluation of efficiency of coagulation/flocculation treatment of textile effluent. The induction of the inflammatory response in macrophage RAW264.7 cells can be used as a model bioassay system for monitoring the effectiveness of coagulation/flocculation processes in the treatment of textile industry wastewater.

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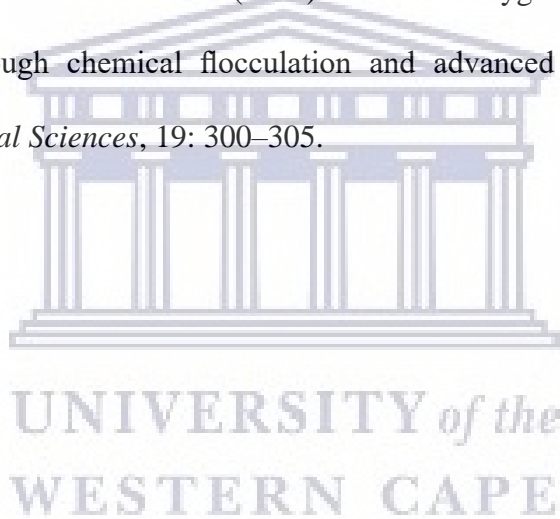
Chapter 6: Toxicity Study of a Textile Effluent Treated with Electrohydraulic Discharge and Coagulant/Flocculants *

6.1 Abstract

Exposure to complex organic substances present in textile wastewater has been considered a threat to human health and aquatic organisms. Development of appropriate treatment mechanisms, as well as sensitive monitoring assays, is considered important in order to safeguard and protect the delicate natural equilibrium in the environment. In this study, combined coagulation/flocculation and electrohydraulic discharge (EHD) system were explored for treatment of textile wastewater. Pre- and post-treatment samples were used to evaluate process efficiencies. Process efficiencies were evaluated using physicochemical characteristics, and cytotoxicity and inflammatory activities induced in macrophage RAW264.7 cell line. The RAW264.7 cell line was evaluated as an alternative to animals and human blood culture models, whose routine applications are hindered by stern ethical requirements. The toxicity of effluent was evaluated using WST-1 assay. The inflammatory activities were evaluated in RAW264.7 cell culture supernatant using nitric oxide (NO) and interleukin 6 (IL-6) as biomarkers of inflammation. The levels of NO and IL-6 were determined using the Griess reaction assay and double-antibody sandwich enzyme-linked immunoassay (DAS ELISA), respectively. Overall, the results of this study show that combined approaches and not the single EHD system are sufficient for complete removal of chemical oxygen demand

(COD) and total organic carbon (TOC), toxicity and inflammatory activities in textile wastewater. The study shows that induction of NO and IL-6 secretions in macrophage RAW264.7 cells is a very sensitive model system to monitor the efficiency of textile effluent treatment processes.

Keywords: Coagulation/flocculation, Textile effluent, Toxicity, Inflammation, Electrohydraulic discharge, Nitric oxide, Interleukin 6.

6.2 Introduction

The increase in industrial activities has been recognised as a fundamental ingredient of economic growth and improvement of standard of living. On the other hand, industrial expansion and textile activities in particular remain environmentally unfriendly. This is because textile industry uses a large amount of water and generates a large amount of wastewater. Textile wastewater is normally characterised by strong colour, chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total organic carbon (TOC) (Chequer et al. 2013). Direct discharge of raw textile wastewater effluent into the environment has been widely acknowledged to cause water pollution with various adverse environmental effects on human and aquatic species (Lacasse and Baumann 2004; Savin and Butnaru 2008). To avoid toxic effects of effluent, various conventional wastewater treatment processes have been implemented (Oller et al. 2011; Maletz et al. 2013). The conversional methods include coagulation, flocculation, biological treatment, precipitation, adsorption and chlorination, among others (Kasprzyk-Hordern et al. 2009; Maletz et al. 2013). However, the efficiency of these methods is low in terms of decomposition of non-biodegradable dyes. To achieve complete removal of dye molecules, a combination of different conversional and advanced oxidation treatment

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technologies is recommended (Oller et al. 2011; Enjarlis 2013; Anvari, et. 2014). Advanced oxidation technologies involving electrohydraulic discharge (EHD) systems have been widely regarded as promising and effective treatment techniques particularly in the decomposition of persistent organic pollutants present in textile wastewater (Sharma et al. 2011; Oller et al. 2011; Olivier et al. 2013).

Discharge of inadequately treated textile effluent has been associated with many biological effects. For example textile dyes are known to cause toxicity, carcinogecity, mutagenicity and allergic reactions (Puvaneswari et al. 2006; Malinauskiene et al. 2012). Allergic reactions are forms of chronic inflammatory reaction involving many inflammatory factors including cells such as basophils, eosinophils, T-lymphocytes and macrophages (Kang and Biswas 2013). Inflammatory activities of textile effluent treated with EHD may be due to inflammatory pollutants such as dyes which are the main components of textile effluents (Nygaard et al. 2013; Leme et al. 2014). Another possible cause of toxicity and inflammatory effects in effluent treated by the EHD method is the presence of intermediate products such as reactive radicals. The intended effect of generated reactive radicals is to react with pollutants in water leading to the reduction of organic pollutants. However, excess reactive radicals such as the hydroxyl radical ($\bullet$OH) form more stable radicals such as hydrogen peroxide (H_2O_2) which can be detected in environmental water (Li et al. 2012).

To ascertain efficiency removal of pollutants in wastewater, evaluation of quality of effluent is very important. In most cases, the quality of effluent has been evaluated based on physicochemical parameters such as pH, COD and TOC (Sonune et al. 2015; Osuolale

and Okoh, 2015). However, these physicochemical parameters lack information on the efficiency removal of biological effects (Sacan and Balcioglu 2006). Therefore, in order to assess the efficiency removal of pollutants with biological effects, appropriate bioassays must be used. Several studies have reported on evaluation of performance of wastewater treatment methods by bioassays using organisms like invertebrates and fish (Meriç et al. 2005; Soni et al. 2006; Rizzo 2011; Nagel-Hassemer et al. 2011). Other studies evaluated inflammatory effects of wastewater and environmental water using whole human blood culture (Pool and Magcwebeba 2009; Faul et al. 2014) or isolated peripheral human mononuclear cell culture (Wichmann et al., 2004; Adebayo, et al., 2014). The induction of inflammatory reaction by oxidative stress is similar to stimulation with bacterial lipopolysaccharides (LPS) and dyes, which is via NF-kB leading to the synthesis of inflammatory modulators such as nitric oxide (NO) and interleukin 6 (IL-6) (Hoesel and Schmid 2013). NO and IL-6 are secreted by many cells including macrophages. These inflammatory mediators can be induced in animals and blood culture; however, their routine uses for assessment of effluent quality may be hindered by ethical requirements. In order to avoid stern ethical requirements, similar inflammatory responses can be induced in established cell lines. One of the cell lines commonly used in inflammatory response studies is a mouse macrophage RAW264.7 cell line. The cell line has been successfully used in many inflammatory studies as well as in anti-inflammatory studies (Kim et al. 2014; Xu et al. 2014). Nevertheless, so far there is no study reported on bioassay using RAW264.7 cells for evaluating the performance of the electrohydraulic discharge system combined with conversional methods for treatment of textile effluent. Therefore, the present study aimed at evaluating the efficiency of the

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electrohydraulic discharge system combined with coagulant/flocculants treatment in terms of reduction of the COD, TOC, toxicity and inflammatory activities in textile effluent.

6.3 Material and Methods

6.3.1 Wastewater Cample Collection

The wastewater samples were collected from a nonwoven textile industry in Cape Town South Africa and stored in 200L acid-washed plastic drums. The raw effluent was taken from the central storing tank that received effluent from all the processing units in the factory. The effluent is completely dark in colour and highly odoriferous. Prior to the sampling, the drums were washed with diluted HNO_3 and millipore water and thereafter rinsed with the effluents. The samples were transported to the laboratory and refrigerated at 4 °C prior to laboratory investigations.

6.3.2 Coagulation and Flocculation Processes

The collected textile effluent was subjected to gravitational settling for 30 minutes due to the presence of a substantial quantity of suspended solids although the suspended particulate matter was poorly settled within 30 minutes. Thus, a coagulation-flocculation study was undertaken to remove the colour and other suspended particulate matters. The study was performed by varying the mass of either $Al_2(SO_4)_3$, or $Ca(OH)_2$ or Fe- Mn oxide from 0.2–1.0g. Analytical-grade $Al_2(SO_4)_3.14H_2O$ and $Ca(OH)_2$ were procured from Sigma-Aldrich and were used without any further treatment. Raw ferromanganese wad (Fe- Mn) oxide was obtained from Vaal Triangle, Gauteng and was also used without further purification. Firstly, the specified dose of the coagulant/flocculant was added to a 0.5L wastewater sample. Thorough mixing on magnetic stirrers was performed

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for 1min at 100rpm, and then, flocculation was carried out at a speed of 20rpm for 20min. Finally, the wastewater content in the beaker was allowed to settle for 40min, and the supernatant was collected for analysis. The experiments were performed at room temperature (25 ºC±2) and constant pH of 6.02. The experiment was repeated twice and the average values obtained from the duplicate of experiments were recorded.

6.3.3 Electrohydraulic Discharge Treatment
The raw textile effluent and the best treated sample with optimised dose of coagulant/flocculant were further subjected to EHD treatment. A power supply set at 25V, delivering a current of 3A and a power of 75W, was directly connected in series to a transformer that steps the AC voltage up to a DC peak voltage of ~ 8kV directly delivered into the EHD reactor. A 0.5mm diameter silver electrode directly connected to the high voltage (output of the transformer) was immersed in a 50g/L of sodium chloride electrolyte placed in the inner tube of a single-cell reactor. This reactor tube containing the electrode was placed in a 1.8L beaker filled with 1.5L of raw effluent. An air pump with a high and low flow speed switch was connected to both air flow meter and the single-cell reactor tube for air (mostly oxygen) production. A flow rate of 3.0L/min was used. The reactor consisted of an inner and outer tube. The diameter of the inner tube was approximately equal to 1mm and that of the outer tube was 7mm. The reactor was 23cm long with an inlet and outlet for air circulation. The ground electrode was submerged into the raw effluent as the case may be in the beaker to complete the circuit and also grounded to the earth to avoid any electrocution. The EHD experiment was conducted for 60 min and sampling was done every 10 min (Table 6.1).

Table 6.1 Experimental protocol for wastewater treatment

Sample treatment	Treatment protocol
Raw effluent	Before treatment
Electrohydraulic discharge system alone	Treated with electrohydraulic discharge alone for 60 min
$Al_2(SO_4)_3.14H_2O$ and electrohydraulic discharge system	1.6g/L of $Al_2(SO_4)_3.14H_2O$ and electrohydraulic discharge treatment for 60 min
$Al_2(SO_4)_3.14H_2O$, $Ca(OH)_2$ and electrohydraulic discharge system	1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $Ca(OH)_2$ and electrohydraulic discharge treatment for 60 min
Fe-MnO$_2$, $Al_2(SO_4)_3.14H_2O$, $Ca(OH)_2$ and electrohydraulic discharge system	1.6 g/L of Fe-MnO$_2$, 1.6g/L of $Al_2(SO_4)_3.14H_2O$, 1.6g/L of $Ca(OH)_2$ and electrohydraulic discharge treatment for 60 min

6.3.4 Chemical Characterization

The raw and treated effluent were analysed for physicochemical characteristics such as the COD, TOC and pH value. All the physicochemical characteristics were measured in accordance with "Standard methods for treatment of water and wastewater" (American Public Health Association, 1999). Aliquot of each sample was sterile filtered using a 0.45μM filter paper and stored at -20ºC.

6.3.5 Cell Culture

Mouse macrophage RAW264.7 cell line from American type collection ATCC-TB-71 was maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany) and 1% Glutamax (Gibco, Life Technologies), in a humidified incubator at 37 ºC and 5% CO_2. The cells were cultured at

5×10^5 cells/mL in 96-well plates till confluence. After confluence, medium was replaced with fresh culture medium containing the effluent samples at 1 in 100 dilutions in respective wells. Control treatments included normal medium for negative control and medium supplemented with 1μg/mL lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma, Germany) as positive control. The cell culture was incubated overnight at 37 ℃ and 5% CO_2. After the overnight incubation, culture supernatants were collected for NO and IL-6 assays. The cells were used for cell viability assays.

6.3.6 Cell Viability

Toxicity of effluent samples was evaluated by cell viability assays using the chromogenic-based water-soluble tetrazolium WST-1 assay. The WST-1 assay is based on the principle of breakdown of tetrazolium to water-soluble formazan dye by the action of dehydrogenase enzyme. The assay was done using WST-1 reagent (Roche, Germany). Briefly, after removal of culture medium, each cell culture well received 100μl of medium supplemented with 10% WST-1 reagent. The absorbance was read immediately after addition of WST-1 medium, and a second reading was done after incubation for 30 min at 37 ℃ and 5% CO_2. The change in absorbance at 450nm over 30 min was used as a measure of cell viability.

6.3.7 Nitric Oxide Assay

Nitric oxide (NO) production was determined in culture supernatants using the Griess reaction in 96-well plates (Nunc, Denmark). The supernatant was mixed with equal volume of Griess reagent made up of 1% sulphanilamide (Sigma, Germany), 0.01% naphthyl ethylenediamine dihydrochloride (Sigma, Germany) and 2.5% phosphoric acid. The colour developed after 15 min incubation was measured at 540nm using a microplate

reader (Thermo Electron). The concentration of NO was determined from a standard curve generated using 100 –1.56μM sodium nitrite (Sigma, Germany).

6.3.8 Interleukin 6 Assay

Interleukin 6 in cell culture supernatant was determined using a commercially available mouse ELISA kit (e-Bioscience, Germany). The assay system is a double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA). The assays were done on NUNC Maxisorp 96-well plates (Nunc, Denmark). The assay kit's procedure was followed as provided in the kit. Briefly the protocol involved coating of a plate overnight at 4 ºC with capture antibody (anti-mouse IL-6 diluted in coating buffer, PBS). The plate was washed five times in wash buffer consisting of PBS with 0.1% Tween. The plate was then blocked with assay diluent for 1 h at room temperature. After another five times of washing, IL-6 standard or cell culture supernatants were added to each well accordingly. The plate was then incubated for 2 h at room temperature. The plate was washed again five times, after which the detection antibody (biotinylated anti-mouse IL-6 in block diluent) was added to each well and incubated for 1 h at room temperature. After another five-time wash, Avidin - horse radish peroxidase (HRP) conjugate was added and incubated for 30 min at room temperature. The plate was washed seven times; then, substrate TMB solution was added and incubated in the dark for 15 min at room temperature. The reaction was stopped with 0.5 M H_2SO_4 stop solution, and the absorbance read at 450nm with a microplate reader (Thermo electron).

6.3.9 Statistical Analysis

The data are presented as mean ±Standard deviation (SD) and were statistically analysed with one-way variance analysis (ANOVA) using Sigmastat (SigmaStat software, Inc.,

CA). The mean values of each treatment were compared with those of the control. The *P* value <0.001 was considered statistically significant.

6.4. Results and Discussion

6.4.1 Physicochemical Characteristics of Effluent Samples

The results of the physicochemical characterisation of raw textile effluents supplied by the factory are shown in Table 6.2. The high value of electrical conductivity indicates that a significant amount of ionized species is present in the raw effluent, and thus, direct discharge might have an adverse impact upon aquatic and other species. The total suspended solids (TSS) and total dissolved solids (TDS) reveals suspended organic and inorganic matter in the raw effluent, and the individual values are well above the recommended limit. The COD value of the raw wastewater supplied is greater than 20,000mg/L, and water with high value of COD is toxic to aquatic life and the entire ecosystem. It is evident from Table 2 that the wastewater does not meet the applicable 5000mg/L COD minimum effluent discharge limit values as contained in City of Cape Town wastewater and industrial effluent bylaw schedule 2 page 10 (www.capetown.gov.za/en/Water/Documents/wwater_bylaw_eng.pdf). The high COD, TOC, BOD, TDS, TSS, pH and intense colour can be attributed to the use of complex organic substances that are highly non-biodegradable. It is imperative, based on the obtained value that the effluents need to be treated prior to discharge into the environment as this will definitely have adverse effects on the environment, especially on the aquatic species.

Table 6.2 Physicochemical characteristic of the raw wastewater

Parameters	Symbols	Value
pH		5.99 ± 0.03
Electrical conductivity		1238.6 ± 1.53
Alkalinity as carbonate	CO_3^{2-}	212.3 ± 12.5
Nitrate + nitrite	NO_3-	17.5 ± 0.70
Nitrite	NO_2-	11.7 ± 1.15
Ortho phosphate	PO_4^{3-}	203 ± 2.62
Sulphate	SO_4^{2-} dissolved	190.3 ± 0.61
Total phosphorus	P	199 ± 3.62
Turbidity		8145.3 ± 5.74
Dissolved organic carbon	DOC	2438.7 ± 1.52
Total organic carbon	TOC	5952 ± 2.64
Chemical oxygen demand	COD	20457 ± 1.52
Biochemical oxygen demand	BOD	807 ± 1.00
Total suspended solids	TSS	2758.7 ± 8.08
Total dissolved solids	TDS	744 ± 6.55
Chlorides	Cl-	55.7 ± 0.09
Flourides	F	16.3 ± 0.14
Colour	Dark	
Odour	Highly unpleasant (odiferous)	

All values are in milligrams per litre except for pH, electrical conductivity (mS/cm) and turbidity in Nephelometric Turbidity Units (NTU).

6.4.2 Effects of Coagulation/Flocculation Processes and the EHD System on Physicochemical Properties

It had earlier been observed that application of individual coagulant/flocculants mentioned above was not effective to reduce the COD and TOC values to the limit set by City of Cape Town, South Africa (www.capetown.gov.za/en/Water/Documents/wwater_bylaw_eng.pdf). Thus, after pre-treatment with coagulation/flocculation processes, the optimal conditions were determined, and the treated effluents were further subjected to electrohydraulic discharge treatment. The results of combined treatment processes are presented in Table 3. Coagulation/flocculation process was applied as a form of pre-treatment to remove

colour, suspended particulate matters and other interfering matrix and thereafter subjected to advanced oxidation process such as electrohydraulic discharge treatment. The results presented in Table 6.2 represent an optimum dosage of each coagulant and flocculant in combination with the electrohydraulic discharge system at a specific pH. With EHD alone, 11.7 and 16.7 % COD and TOC removal was observed. The maximum COD and TOC removal for aluminium sulphate/EHD treatment was 77.8 and 83.4%, respectively. Combined treatment with 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$, and electrohydraulic discharge system successfully reduced the COD and TOC value by 95.3 and 96.23%, respectively. The combination of 1.6 g/L Fe-Mn oxide, 1.6 g/L $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L $Ca(OH)_2$, followed by electrohydraulic discharge system, further enhanced the COD and TOC reduction by 96.79 and 96.6%, respectively. Compared with the raw effluent, it can be seen that the most feasible and effective combined treatment is 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$ and electrohydraulic discharge system as well as 1.6 g/L of Fe-Mn, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$ and electrohydraulic discharge system with significant reduction in COD and TOC values.

Table 6.3 COD, TOC and pH values before and after flocculation/EHD treatment for 60 minutes

Sample	pH	COD (mg/L)	TOC (mg/L)
Before treatment	5.99 ± 0.03	20457 ± 1.52	5952 ± 2.64
After treatment with electrohydraulic discharge system alone	5.83 ± 0.16	$18,057 \pm 2.36$	4953 ± 5.46
After treatment with 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ + electrohydraulic discharge system	4.56 ± 0.24	$4,532 \pm 0.43$	986 ± 0.71

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After treatment with 1.6 g/L of Al$_2$(SO$_4$)$_3$.14H$_2$O 1.6 g/L of Ca(OH)$_2$ + electrohydraulic discharge system	9.63 ± 1.23	958 ± 0.06	224 ± 0.05
After treatment with 1.6 g/L of Fe-Mn oxide, 1.6 g/L of Al$_2$(SO$_4$)$_3$.14H$_2$O, 1.6 g/L of Ca(OH)$_2$ + electrohydraulic discharge system	8.51 ± 0.09	656 ± 0.89	200 ± 1.23

The raw textile effluent was treated using the EHD system; the EHD produces reactive radicals such as O$_3$, OH, H$_2$O$_2$ and UV, which degrade organic compounds. From Table 6.3 there was only a small reduction of about 10% in the COD, TOC, and pH values of the raw and treated effluent by the EHD even after 60 min. This might be due to the competition between the radical scavengers such as nitrates, sulphates, carbonates and chlorides and the organic pollutants present in the effluents for free reactive species produced by the EHD system. Depending on the solution pH, the scavenging effect lowers and suppresses the oxidation potential of the free reactive species and that possibly affects the process efficiency leading to lower COD and TOC values (Bacardit et al. 2007; Oller et al. 2011). Equally, the formation of a series of intermediate products and the possible occurrence of secondary reactions among the by-products might also be responsible for lower degradation efficiency by the EHD (Kiwi et al. 2000). According to Enjarlis (2013) incomplete degradation of organic pollutants gives rise to formation of different intermediate products that can affect the overall process efficiency. It is also noteworthy based on the results presented in Table 3 that the raw effluent contained an appreciable amount of carbonate, nitrate, sulphate, phosphates and chlorides. These major species control the solution pH and quench the free reactive species. Wu et al. (2008) had

earlier reported that the quenching effect of hydroxyl radical by carbonates appreciably contributed to impeding the COD and TOC reduction levels. Treatment of the raw effluent with EHD is potentially less effective due to the presence of radical scavengers (Kiwi et al. 2000). Furthermore, interesting behaviour was noticed with the treatment of the raw effluent by combination of aluminium sulphate/EHD. The combined approach lowers the COD and TOC values to 4532 and 986 mg/L with a removal rate of 77.8 and 83.4%, respectively. This observed trend indicates that a significant portion of organic pollutants and interfering species have been removed from the raw effluent as sludge by the aluminium sulphate whereas a portion transformed into intermediate compounds due to the influence of the free radicals produced by the EHD. Thus, both the aluminium sulphate and EHD played a crucial role in lowering the COD and TOC values. Not only that, it has been previously demonstrated that continuous exposure of the treated effluent to ozone, UV, OH and H_2O_2 at a minimum dose is effective for complete degradation of organic pollutants in wastewater (Zayas et al. 2007). Furthermore, after 60 min of treatment with combination of 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L of $Ca(OH)_2$ followed by electrohydraulic discharge system, the COD and TOC values were further decreased to 95.3 and 96.23%, respectively. This was due to the destabilization of organic flocs which settled very fast and resulting in almost stable COD values. Correspondingly, with the addition of 1.6 g/L $Ca(OH)_2$, the pH value increased to 9.36, which is slightly higher than the favourable pH range (6-7). At high pH value, decomposition of ozone takes place prior to eventual reaction with pollutants in the presence of hydroxyl ions (Muruganandham et al. 2014). As the solution pH increased, the concentration of hydroxyl radical increased and later combined to produce hydrogen

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peroxide. Thus, the combined effect of hydrogen peroxide/ozone at high pH value eventually increased the decomposition of organic compound bonding framework. This view has been widely expressed in a number of studies (Jung et al., 2012). The obtained COD and TOC values are lower than the City of Cape Town permissible limit. At this level, treated wastewater is safe to be discharged into the city of Cape Town wastewater system. Table 3 shows that with the incorporation 1.6 g/L of Fe-Mn oxide and 1.6 g/L of aluminium sulphate/calcium hydroxide/EHD system, the COD and TOC values further reduced by 96.79 and 96.6%, respectively. This is due to the improved generation of free reactive species caused by absence of interfering anions. Integration of coagulation-flocculation and electrohydraulic discharge system is considered to be advantageous for lowering the COD and TOC levels in the effluent. On the other hand, COD and TOC values cannot be completely removed from raw effluent. The residual COD and TOC in the treated effluent may plausibly be due to the formation of intermediate macromolecules or secondary reactions. Overall, there was a substantial decrease in the investigated parameters of the treated effluent compared with the raw effluent with the exception of EHD itself alone and 1.6 g/L of $Al_2(SO4)_3$/EHD treatment. The decrease was due to the synergistic effect of the individual coagulant/flocculant agents as well as the EHD system. The treated effluents were further subjected to toxicity and inflammatory test.

6.4.3 Effects of Coagulation and Flocculation Processes/EHD System on Cell Viability

The toxicity of raw effluent and effluent treated with electrohydraulic discharge alone or combined coagulation/flocculation processes and electrohydraulic discharge system were

tested for their effects on RAW264.7 cell viability. Raw effluent and effluent treated with electrohydraulic discharge alone reduced cell viability significantly ($P < 0.001$) compared with negative control (Figure 6.1). Samples treated with 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ combined with electrohydraulic discharge system, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$ and electrohydraulic discharge system or 1.6 g/L of Fe-Mn oxide, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$ and electrohydraulic discharge system had no significant effect on the viability of RAW264.7 cells.
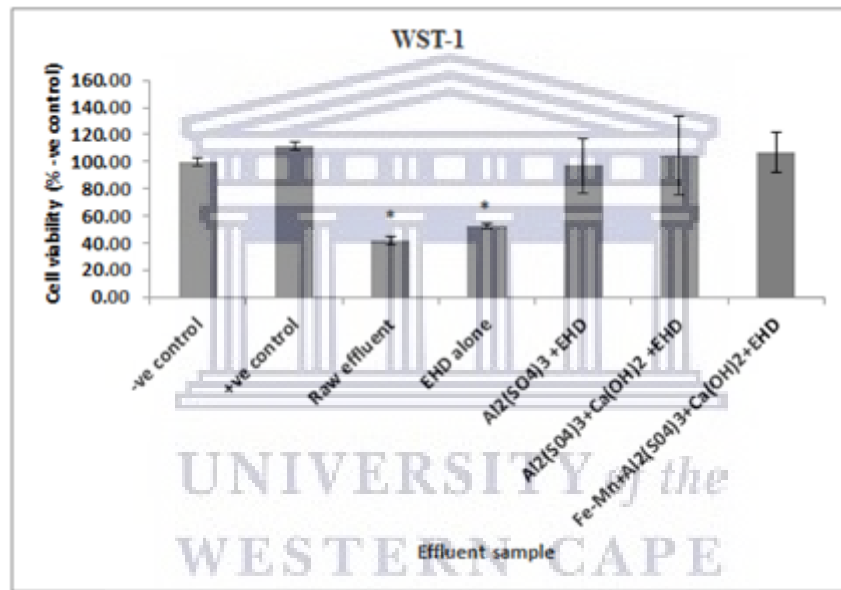
Figure 6.1 Effects of effluent samples treated with different methods on RAW264.7 cells viability as determined by WST-1 assay. Negative (-ve) control was treated with normal medium; positive (+ve) control was treated with medium containing LPS (1 µg/ml). Wastewater samples were diluted to 1 in 100 in medium as described in methods. The results are presented as mean ±SD and * indicate that cell viability is significantly ($P < 0.001$) lower than negative control.

The decrease of cell viability induced by raw effluent and effluent treated by EHD alone may be due to toxicity effects of pollutants in the samples. The raw sample was characterised by high values of COD and TOC, which may be due to high content of organic pollutants. Toxicity of textile effluent has been previously reported using *in vivo* bioassays (Soni 2006; Verma 2008). Toxicity due to specific reactive dyes in raw effluent has been reported by several studies (Klemola et al. 2007; Verma 2008). Similarly, toxicity of effluent treated with EHD alone could be partly attributed to inadequate removal of toxic pollutants by the EHD system. Inefficient degradation using EHD could be due to the presence of scavenging molecules in the effluent sample that react with produced intermediate by-products (Kiwi et al. 2000). This is because the performance of the EHD system depends on generation of free reactive radicals species such as hydroxyl radical ($\bullet$OH), perhydroxyl radical ($\bullet$OOH), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), ultraviolet light and shock waves (Bruggeman and Locke 2013). The free radicals are very reactive and non-selective, especially hydroxyl radicals, which cause degradation of complex organic substances (Malik 2010; Jiang et al. 2014). The degradation of dyes is achieved by cleavage of the double bond of dye molecules (Jiang et al. 2014). However, it has been observed that most of the free radicals have a very short life, hence may disappear before decolouration is complete (Tahara and Okubo 2014). Furthermore, the hydroxyl radical ($\bullet$OH) is quickly converted to H_2O and H_2O_2, which is more stable and can be detected in water (Li et al. 2012). Therefore, accumulation of H_2O_2 can also be a source of toxicity in effluent treated with EHD alone.

6.4.4 Effects of Coagulation and Flocculation Processes /EHD System on Inflammatory Activities

Inflammatory effects of effluent samples were determined in RAW264.7 cell supernatant by NO and IL-6 as biomarkers of inflammation. The effects of effluent samples on NO production were determined in cell culture supernatant. Samples from raw effluent, effluent treated with electrohydraulic discharge alone and combined $Al_2(SO4)_3$/EHD induced significantly (P <0.001) higher NO production in RAW264.7 cell culture than the negative control (Figure 6.2). Effluent samples treated with $Al_2(SO_4)_3.14H_2O$/Ca(OH)$_2$/EHD system as well as Fe-Mn oxide/$Al_2(SO_4)_3.14H_2O$/Ca(OH)$_2$/EHD system did not induce significant different amounts of NO compared with negative control. Similar trend of inflammatory activities of effluent samples was also observed in the induction of IL-6 secretion as shown in Figure 6.3. Raw effluent, effluent treated with electrohydraulic discharge alone and combined $Al_2(SO4)_3$/EHD induced significantly (P <0.001) higher IL-6 secretion in RAW264.7 cell culture than negative control. Effluent samples treated with $Al_2(SO_4)_3.14H_2O$/Ca(OH)$_2$/EHD system as well as Fe-Mn oxide/$Al_2(SO_4)_3.14H_2O$/Ca(OH)$_2$/EHD system had no significant effects on production of IL-6 in RAW264.7 cell culture compared with negative control.
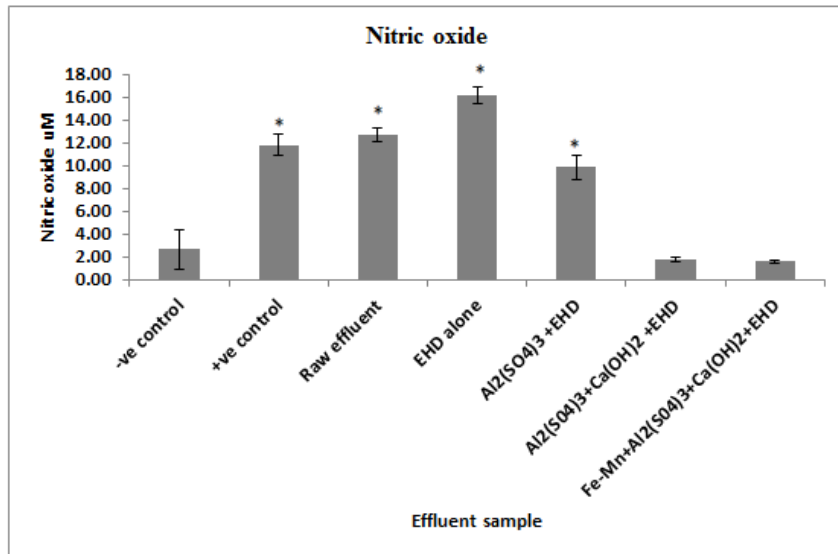
Figure 6.2 Effects of raw and effluent samples treated with different methods on induction of NO production in RAW264.7 cells culture. Negative (-ve) control was treated with normal medium, positive (+ve) control was treated with medium containing LPS (1 μg/ml), and raw and treated effluent samples were diluted at 1 in 100 dilutions in medium. The results are presented as mean ±SD and * indicate that NO production is significantly ($P < 0.001$) higher than negative control.

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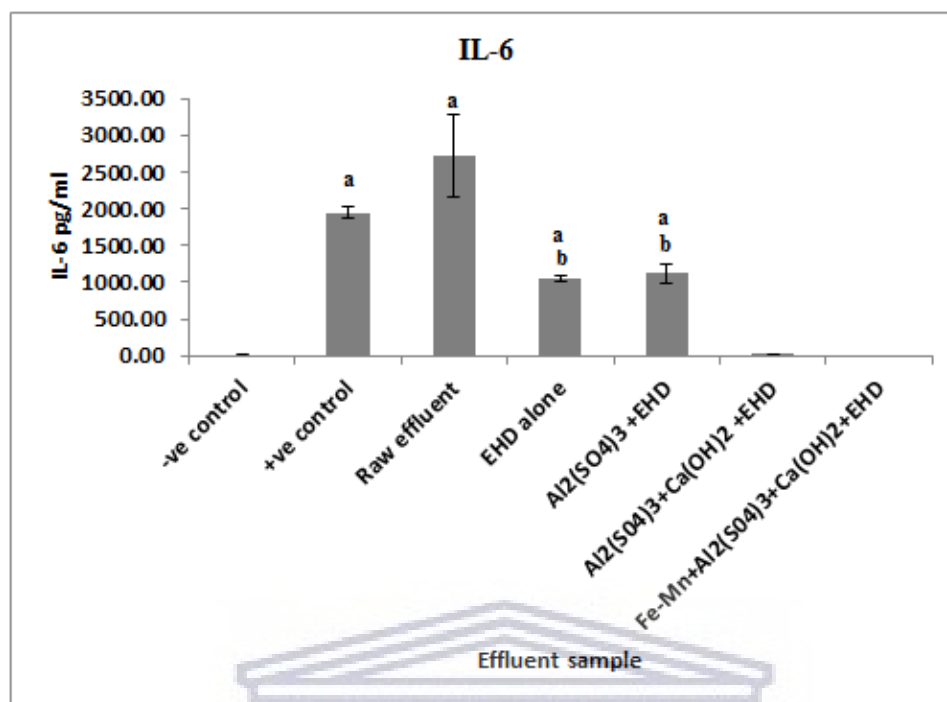
Figure 6.3 Effects of raw and effluent samples treated with different methods on induction of IL-6 secretion in RAW264.7 cells culture. Negative (-ve) control was treated with normal medium, positive (+ve) control was treated with LPS (1μg/ml), wastewater sample 1 in 100 dilutions in medium. The results are presented as mean ±SD and (a) indicate that IL-6 secretion is significantly (P <0.001) higher than the negative control. (b) indicate that IL-6 secretion is significantly (P <0.001) lower than raw effluent

Both NO and IL-6 results showed a similar trend of inflammatory activities. A significant increase in NO and IL-6 induced by raw effluent may be associated with high content of inflammatory pollutants in the sample. Presence of dyes may be the main causes of inflammatory activities in raw effluent samples. Several studies have also associated textile dyes with allergic reactions (Malinauskiene at al. 2012; Nygaard et al. 2013).

Allergic reactions are due to chronic inflammation, in which macrophages secrete inflammatory mediators. Treatment of raw effluent with EHD alone significantly (P <0.001) reduced levels of NO and IL-6 as compared with raw effluent. However, the levels of both NO and IL-6 were still significantly (P <0.001) higher than the negative control. In addition to presence of inflammatory pollutant as result of incomplete removal, inflammatory activities in effluent treated with EHD alone may also be due to the presence of excessive intermediate free radicals. Excessive free radicals may lead to oxidative stress, which induces inflammatory mediators and pro-inflammatory cytokine through NF-kB pathways (Hoesel and Schmid 2013). Similar effects of oxidative stress have been studied in macrophages of the respiratory system (Hnizdo and Vallyathan 2003; Kirham 2007).

Addition of 1.6 g/L $Al_2(SO_4)_3.14H_2O$ alone followed by EHD system did not reduce inflammatory pollutants. As a result, effluent samples induced secretion of inflammatory cytokine IL-6 and NO production in RAW264.7 cells. The inflammatory activities could be due to two possible sources. One is incomplete removal of inflammatory pollutants, which correlates well with high values of physicochemical properties of the sample. The sample is characterised by high value of COD. High COD is an indication of inefficiency of $Al_2(SO_4)_3.14H_2O$ to remove organic pollutants. At low pH (4.56), aluminium precipitates rapidly. Due to the rapid precipitation of aluminium, it led to short contact time that could be responsible for the incomplete removal of pollutants that are responsible for inflammatory activities.

Samples treated with EHD after addition of 1.6g/L of $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L of $Ca(OH)_2$ or 1.6 g/L of Fe-Mn oxide, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L of $Ca(OH)_2$ did not induce significant amounts of NO and IL-6. This is an indication of efficient removal of inflammatory pollutants which correlates well with low values of COD and TOC. The presence of $Ca(OH)_2$ in the pre-treatment process might have improved faster settling of pollutants hence increasing efficiency removal of COD (Yang et al. 2012). Similar observations have been reported on efficiency of $Ca(OH)_2$ to decolourise wastewater containing dyes (Al-Hemiri et al. 2007). Furthermore, calcium hydroxide at a pH value of 9.63 dissociated in water and released highly reactive free radicals (hydroxyl ions) which are very reactive and very effective in removal of dyes, bacteria and other organic pollutants. Therefore, based on the results of this study, the best combined techniques are 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L of $Ca(OH)_2$ or 1.6 g/L of Fe-Mn oxide, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$ and 1.6 g/L of $Ca(OH)_2$ followed by electrohydraulic discharge system. Wastewater samples treated with these combined techniques achieved above 95% effective for COD and TOC with neither toxicity nor inflammatory activities.

6.5 Conclusions

In this study, the physicochemical characteristics of the textile wastewater samples revealed high value of parameters such as COD, BOD, TOC, TSS and TDS among others. In addition to high values of physicochemical properties, the effluent also was characterised by high toxicity and inflammatory activities. Treatment of the raw effluent by combination of coagulation/flocculation process with electrohydraulic discharge system produced satisfactory results. The maximum and adequate COD and TOC removal of 96.79 and 96.6 %, respectively, was realised with a combination of 1.6 g/L of

Fe-Mn oxide, 1.6 g/L of $Al_2(SO_4)_3.14H_2O$, 1.6 g/L of $Ca(OH)_2$ and EHD system. Similarly, the combined system removed toxic and inflammatory pollutants based on the results of NO and IL-6 in RAW264.7 cell cultures. This study demonstrated that a single treatment approach is not effective in removing all pollutants. However, a combined treatment approach effectively removed complex organic pollutants. The study has also shown that performance of the combined methods can be evaluated using biomarkers of inflammation such as NO and IL-6 in macrophage RAW264.7 cells as a model system.

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Chapter 7: Summary and future perspectives

Global population growth and increased economic activities have been associated with increased discharge of wastewater to the environment. As a result there have been continued efforts of developing effective wastewater treatment technologies. In order to verify performance efficiency of the developed technologies, various assessment assays have been implemented. The most commonly methods used to evaluate quality of effluent and water resources are physicochemical parameters. The commonest parameters used for assessment of effluent and water quality include pH, BOD, COD, TOC, TSS and colour. Other common methods are analysis of specific pollutant and microbial characteristics.

Unfortunately, determination of physicochemical parameters and analysis of specific pollutants in wastewater lack information on the efficiency removal of biological effects such as cytotoxicity, endocrine disruption and immunotoxicity. Therefore, in order to ascertain the efficient removal of pollutants in a mixture of wastewater with such biological effects, the use of biomarkers of effects is considered ideal. To achieve this, biological effects of wastewater such as immunotoxicity have been monitored using animal studies, human whole blood culture and isolated peripheral human mononuclear cell culture. Although these techniques are recommended due to their precision, simplicity and low cost, their routine applications are hindered by ethical issues. In order to avoid stern ethical requirements, the use of established cell lines characterized by inflammatory responses can be developed as strategies towards to replacement of *in-vivo*

studies and blood cultures. Therefore, the aim of study was to use mouse macrophage RAW264.7 cell line as a cell culture model for monitoring of environmental pollutants in wastewater. This aim was achieved through four objectives.

The first objective was to evaluate effects of endocrine disrupting chemicals on biomarkers of inflammation produced by lipopolysaccharide stimulated RAW264.7 macrophages (**paper I in Chapter 3**). Endocrine disrupting chemicals (EDCs) are common pollutants in municipal wastewater and environment. In the environment, they are persistent in nature and in most cases are resistant to removal by most of treatment techniques. In the present study, stimulated RAW264.7 cells were exposed to selected common EDCs namely; Estradiol (E2), 5α-dihydrotestosterone (DHT) and Bisphenol A (BPA) alone or in combination with flutamide and tamoxifen. This study demonstrated that DHT, E2 and BPA at concentration of 5μg/ml had no cytotoxic effects in stimulated RAW264.7 cells. However, the same treatments induced suppression of NO and IL-6 secretion in stimulated RAW264.7 cells. The suppression of NO and IL-6 indicates that DHT, E2 and BPA can induce anti-inflammatory activities. The anti-inflammatory effects were reversed by their respective antagonist compounds. The combined treatment of flutamide with DHT resulted in reverse effects of anti-inflammatory of DHT alone. Similarly, combination of estrogenic E2 or BPA with tamoxifen reversed the effects of each compound. The reverse effects of flutamide and tamoxifen are due to their selective antagonism with androgenic (AR) and estrogenic receptors (ERs) respectively. Both androgenic and estrogenic receptors are expressed in macrophages. Therefore, this study has shown that stimulated RAW264.7 cells culture can be a useful model for evaluation

of anti-inflammatory activities of common androgenic and estrogenic pollutants in wastewater.

The second objective was to assess toxicity and inflammatory activity of municipal wastewater samples using RAW264.7 cell culture model (**paper II in Chapter 4**). Raw municipal sewage is normally composed of many types of pollutants, including various organic compounds, microorganisms and microbial products. In the present study sewage samples were collected from Stellenbosch wastewater treatment works (SWTW) in Cape Town, South Africa. The samples collected and analysed were influent, post bio-filtration, post activated sludge treatment and final effluent. The final effluent was characterized by the lowest (<1 cfu/ml) for both total coliforms and *E.coli* bacterial count. Then RAW264.7 cell cultures were exposed to dilute and sterile filtered influent, post bio-filtration, post activated sludge treatment and final effluent. The results of this study show that all diluted wastewater samples did not induce cell toxicity. The results show further that all wastewater samples induced NO and IL-6 production in RAW264.7 cells. The highest inflammatory activities were induced by post bio-filtration wastewater sample. Final effluent sample collected at the post chlorination point just before discharged into a receiving river induced the lowest inflammatory response. The lower inflammatory activity in final effluent indicates effective removal of pollutants upon sewage treatment. The findings of this study show that sewage samples can induce inflammatory responses in RAW264.7 cells. The results of this study also give evidence that macrophages RAW264.7 cells can be used as a model for monitoring the quality of treated municipal sewage.

The third objective was to evaluate cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by coagulation-flocculation methods, using RAW264.7 cell culture model (**paper III in Chapter 5**). The RAW264.7 cell cultures were exposed to sterile filtered wastewater samples from raw effluent and effluent treated with various coagulation-flocculation methods. The coagulation-flocculation methods were performed by varying the mass of Fe-Mn oxide, $Al_2(SO_4)_3.14H_2O$, $FeSO_4.7H_2O$, $FeCl_3$, and $Ca(OH)_2$ from 0.2–1.0 g in a batch mode. The raw effluent was characterized by high physicochemical parameters namely; COD, TOC, BOD, TDS, TSS, pH and intense colour. Treatment of raw effluent with 1.6 g/L of Fe-Mn oxide produced no appreciable reduction in COD and TOC value compared to the raw effluent. Physicochemical parameters also show that the most effective treatment was $Al_2(SO_4)_3.14H_2O$ at a dose of 1.6 g/L. The treatment reduced the COD and TOC value by 63.13 % and 75.5% respectively. The results of this study also show that raw effluent induced cytotoxicity by reducing cell viability. The results on inflammatory activities show that the raw effluent and effluent treated with 1.6g/L of Fe-Mn oxide induced NO production. The inflammatory results further showed that the raw effluent induced also production of IL-6 in RAW264.7 cells culture. Based on the results of this study, it is obvious that among the coagulants/flocculants evaluated, $Al_2(SO_4)_3.14H_2O$ at a dosage of 1.6 g/L was the most effective to remove both toxic and inflammatory pollutants. Therefore, the inflammatory responses in RAW264.7 cells can be used as sensitive biomarkers for monitoring the effectiveness of coagulation/flocculation processes used for textile effluent treatment.

The last objective of this study was to evaluate toxicity and inflammatory activities of a textile effluent treated with electrohydraulic discharge and coagulant/flocculants (**paper IV in Chapter 6**). Electrohydraulic discharge (EHD) system is one of the advanced wastewater treatment technique that is praised for effective treatment of biological active and persistent organic pollutants. However, the presence of radical scavengers in textile effluent and high cost of energy consumption in the course of wastewater treatment limits the industrial application of this technique. Thus, in order to achieve complete removal of pollutants with minimum cost, a combination of coagulant/flocculants and EHD system was tested for treatment of a textile wastewater. Pre- and post-treatment samples were used to evaluate process efficiencies. Process efficiencies were evaluated using physicochemical characteristics, and cytotoxicity and inflammatory activities induced in macrophage RAW264.7 cell line. The RAW264.7 cell cultures were exposed to sterile filtered raw effluent sample and effluent after treatment with EHD in absence and presence of coagulant/flocculants. Raw effluent was characterized by high parameters of COD, TOC, BOD, TDS, TSS, pH and intense colour. Treatment of textile with EHD alone successfully reduced the COD and TOC value by 95.3 and 96.23 % respectively. However, the results of this study also show that a single treatment approach is not effective in removing all toxic and inflammatory pollutants. On the other hand a combined treatment approach effectively removed toxic and inflammatory pollutants based on the results of NO and IL-6 in RAW264.7 cell cultures. In fact, the best combined techniques achieved above 95% effective for COD and TOC with neither toxicity nor inflammatory activities. Therefore, this study confirms that induction of NO and IL-6 secretions in macrophage RAW264.7 cells are very sensitive model systems to

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monitor the efficiency of textile effluent treated with electrohydraulic discharge and /or combined with other processes.

Based on the results of this study, precision of data achieved by established macrophage RAW264.7 cells and avoidance of stern ethical restriction of using other biological assays, it is evident that macrophage RAW264.7 cell culture can be used to monitor pollutants that disrupt immune function. Similarly, macrophage RAW264.7 cell culture model can be used to assess efficiency removal of inflammatory pollutants in sewage and wastewater effluent. However, due the fact that biological effects should be evaluated on more than one level that is battery-type programme, future research should consider developing and implementation of more biomarkers of immunotoxicity in macrophage RAW264.7 cells.
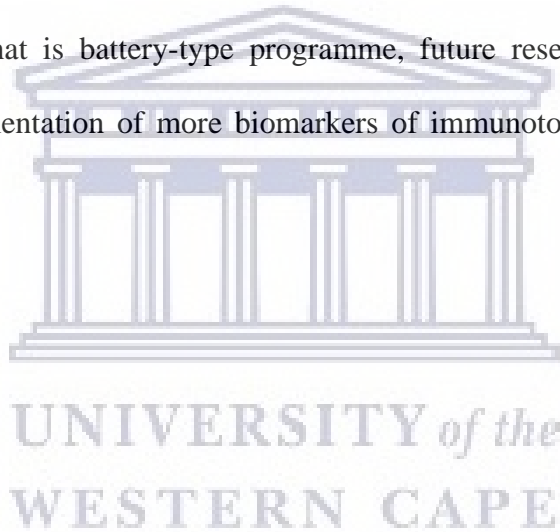
Addendum: Optimization of reagents concentration and standard curves of assays

Optimization of reagents concentration and standard curves of assays

Introduction

Optimal concentrations of reagents used in these studies were determined to avoid adverse effects of reagents to cell culture. The reagents optimized are Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4, Dimethyl sulfoxide (DMSO), 5α-dihydrotestosterone (DHT), Estradiol (E2), Bisphenol A (BPA), Flutamide, and Tamoxifen. The LPS, DHT, E2, BPA, flutamide, and tamoxifen were dissolved in DMSO (Sigma-Aldrich, Germany).

Materials and Methods

Cells Culture

A mouse macrophage RAW264.7 cell line, purchased from American Type Culture Collection (ATCC TIB-71, Manassas, VA, USA), was used in studies. The RAW264.7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Lonza, Cape Town, South Africa), supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% *v/v* antibiotic/antimycotic mixture (Sigma-Aldrich), 0.5% v/v gentamycin (Sigma-Aldrich), and 1% v/v glutamax (Gibco, Life Technology, Carlsbad, CA USA). The cells were cultured in 96-well plates at a density of 5×10^5 cells/mL in a humidified incubator at 37 °C and 5% CO_2 until confluent. At confluence, cells were treated as follows: Normal medium for negative control and medium supplemented with 1μg/mL lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Germany) as a positive control. The optimal concentration of each compound was determined by serial

dilution. After overnight incubation at 37 °C and 5% CO_2, culture supernatants were collected for NO and IL-6 assays. The cells remaining on the plate were used for cell viability assays. Each assay was carried in four replicates.

Cell Viability

The cell viability was determined using the chromogenic WST-1 assay. The assay is based on the breakdown of the water-soluble (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium by the dehydrogenase enzyme to produce water-soluble formazan dye that can be monitored spectrophotometrically. In brief, the assay procedure was as follows: After removal of cell supernatant from culture, each plate well received 100μL of medium supplemented with 10% WST-1 reagent (Roche, Basel, Switzerland). The absorbance was read immediately after addition of WST-1 medium and a second reading was done after incubation for 30 min at 37 °C and 5% CO_2. The change in absorbance at 450 nm over 30 min was used as a measure of cell viability.

Optimization of DMSO dilution

Macrophage RAW264.7 cell cultures at confluent were treated with serial dilution of DMSO to determine effect on cell viability. The cells were treated with DMSO starting from 1 in 100 dilutions. Then the cells were cultured in a humidified incubator at 37ºC and 5% CO_2 overnight. After overnight incubation, the cells on a plate were used for cell viability assays.

Optimization of reagents dilution

The optimal concentration of reagents were determined in RAW264.7 cell cultures at confluent treated with serial dilution of LPS, DHT, E2, BPA, Flutamide and Tamoxifen.

The reagents were dissolved in DMSO to determine their effect on cell viability, NO production and IL-6 secretion. The cells were treated with LPS at different dilution starting from 1μg/ml. The other set of cells were treated with DHT, E2, and BPA starting with 1 in 100 dilutions of 10μg/ml of compound. Flutamide and tamoxifen treatment were diluted serially starting from 1 in 500 dilutions of 5μg/ml of each. Then the cells were cultured in a humidified incubator at 37ºC and 5% CO_2 overnight. After overnight incubation, culture supernatants were collected for NO and IL-6 assays. The cells on a plate were used for cell viability assays.

Nitric oxide determination

Nitric oxide (NO) secreted by cells into the cell culture was determined using the Griess reaction. Cell culture supernatant in 96-well plates (Nunc, Roskilde, Denmark) was mixed with an equal volume of Griess reagent made up of 1% m/v sulphanilamide (Sigma-Aldrich, Germany), 0.01% m/v naphthyl ethylenediamine dihydrochloride (Sigma-Aldrich), and 2.5% phosphoric acid. The mixture was allowed to react for 15 min at room temperature. The colour developed was measured at 540 nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation). The concentration of NO was determined from a standard curve generated using 1.56–100μM sodium nitrite (Sigma-Aldrich, Germany).

Interleukin 6 determination

Interleukin 6 (IL-6) concentration in culture supernatant was determined using a double antibody sandwich enzyme linked assay (DAS ELISA), with a commercial kit (e-Bioscience, Ready-Set-Go, Waltham, MA, USA). The assays were done on Nunc

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Maxisorp 96-well plates (Nunc, Denmark). The ELISA kit contains all reagents required for the assay and the manufacturers' assay protocol was followed. In brief, the protocol involved coating the ELISA plate with capture antibody, anti-mouse IL-6 diluted in coating buffer (PBS), and incubated overnight at 37 °C. Then, the plate was washed five times in wash buffer made of PBS with 0.1% v/v Tween. After washing, the plate was blocked with assay diluent for 1 h at room temperature. After another five washes, IL-6 standard and cell culture supernatant were added to each well accordingly and incubated for two hours at room temperature. The plate was washed again five times, then detection antibody, biotinylated anti-mouse IL-6, was added to each well and incubated for 1 h at room temperature. After another wash, Avidin—horse radish peroxidise (HRP) conjugate—was added and incubated for 30 min at room temperature. The plate was washed seven times, then TMB substrate was added and incubated in the dark for 15 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 stop solution, and the absorbance was read at 450 nm with a multiskan microplate reader (Multiskan Ex, Thermo Electron Corporation).

Results

Optimization of DMSO dilution

The optimal concentration of DMSO was determined after exposure of RAW264.7 cells to a serial dilution of DMSO startining from 1 in 100 dilutions. Figure 1 shows the cell viability of RAW264.7 cells stimulated with LPS (1µg/ml) in varying DSMO dilution starting from 1 in 100 dilutions.
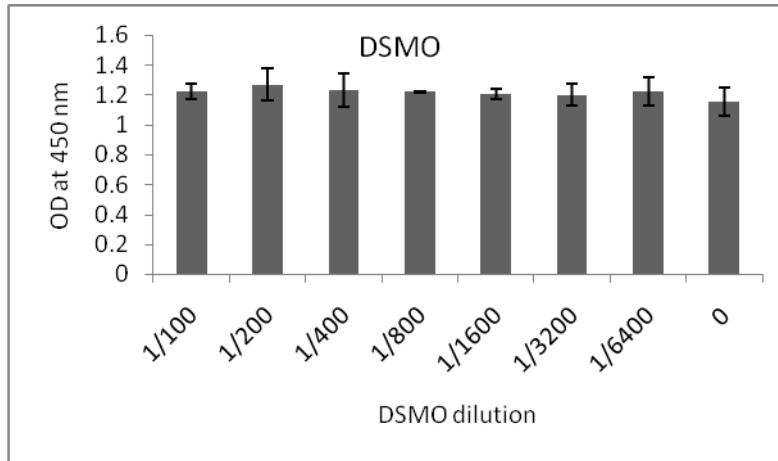
Figure 1. Optimization of DMSO dilution on viability of stimulated (LPS 1μg/ml) RAW264.7 cells in different concentration starting from 1 in 100 dilutions. The control was treated with LPS 1μg/ml alone.

Then optimal concentration of DSMO was determined on secretion of NO in stimulated RAW264.7 cell culture. Figure 2 shows the effect of DSMO dilution on secretion of NO in stimulated cells.
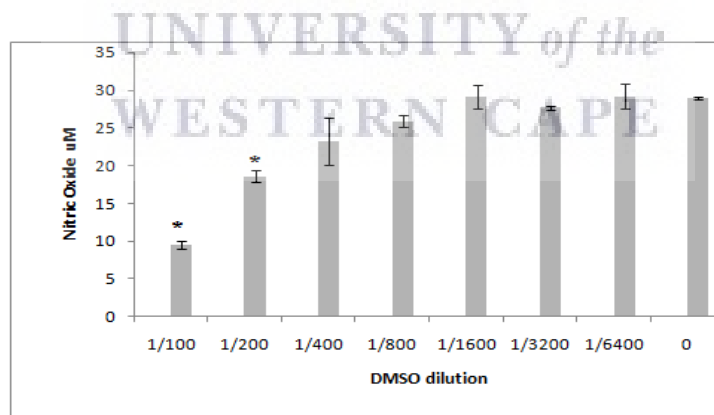
Figure 2. Optimization of DMSO dilution on secretion of NO in stimulated RAW264.7 cells starting from 1 in 100 dilutions. The control was treated with LPS 1μg/ml alone.

The results of effects of DMSO dilution on secretion of NO in stimulated RAW264.7 show that stimulated RAW264.7 cell treated with the highest concentration of DMSO reduced NO secretion. Therefore, all reagents were reconstituted in DMSO concentration such that DMSO concentration is not higher than 1 in 800 dilutions.

Optimization of LPS concentration

In order to determine the optimal concentration of LPS to use with no toxic effect to cells, RAW264.7 cell culture was exposed to a serial dilution of LPS starting from 1μg/ml. The optimal concentration of LPS was determined on RAW264.7 cell viability and secretion of NO and IL-6. The effect of LPS dilution on cell viability is shown in Figure 3. The results of cell viability show that all exposures had no effect on cell viability as compared with control which receive plain medium.
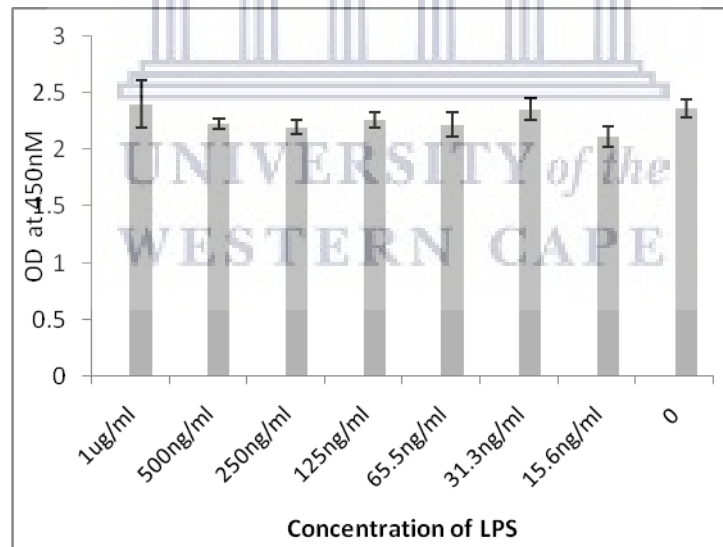
Figure 3. Optimization of concentration of LPS on RAW264.7 cells viability. RAW264.7 cells culture was treated with serial dilution of LPS starting from 1μg/ml, and control was treated with normal plain medium.

The effect of LPS dilution on NO production is shown in Figure 4. The results show that the control which received plain medium produced lowest level of NO. The RAW264.7 cell cultures exposed to 1μg/ml and 500ng/ml of LPS produced the highest amount of NO.
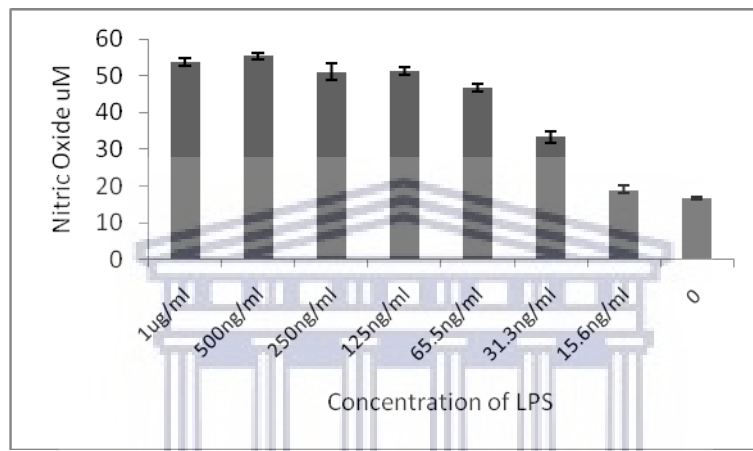
Figure 4. Optimization of LPS concentration on secretion of NO in RAW264.7 cells culture. RAW264.7 cells culture was treated with serial dilution of LPS starting from 1μg/ml, and control was treated with normal medium.

The effect of LPS dilution on IL-6 secretion in RAW264.7 cells culture is shown in Figure 5. The figure shows that the control treatment, which received plain medium and RAW264.7 cell culture that received lowest (15.6ng/ml) concentration of LPS did not secrete IL-6. The highest level of IL-6 was secreted by RWA264.7 cells treated with 1μg/ml and 500ng/ml.

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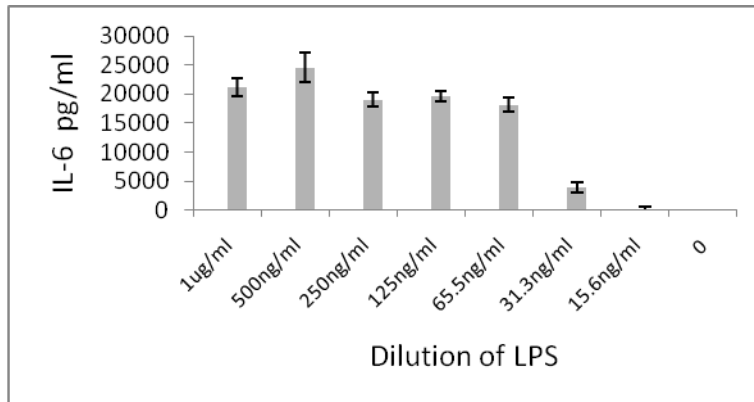
Figure 5. Optimization of LPS dilution on secretion of IL-6 in RAW264.7 cells starting from concentration of 1μg/ml. The RAW264.7 cells culture was treated with serial dilution of LPS starting from 1μg/ml, and control was treated with normal medium.

Optimization of EDCs dilution

The optimal concetration of reagents namely, DHT, E2, BPA, flutamide and tamoxifen were determined on RAW264.7 cell viability and inflammatory responses. The effects of DHT dilution starting from 1 in 100 (10μg/ml) on cell viability, and NO secretion are as shown in Figure 6 and Figure 7 respectively.
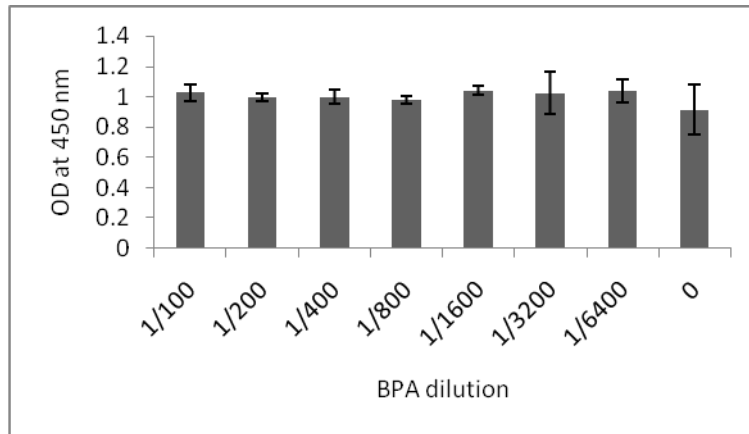
8
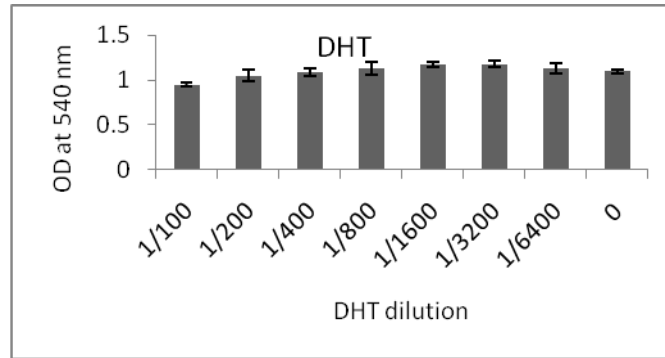
Figure 6. Optimization of DHT dilution starting from 1 in 100 (10µg/ml) dilutions on viability of RAW264.7 cells stimulated with 1µg/ml LPS. Control culture was treated with LPS (1µg/ml) alone.
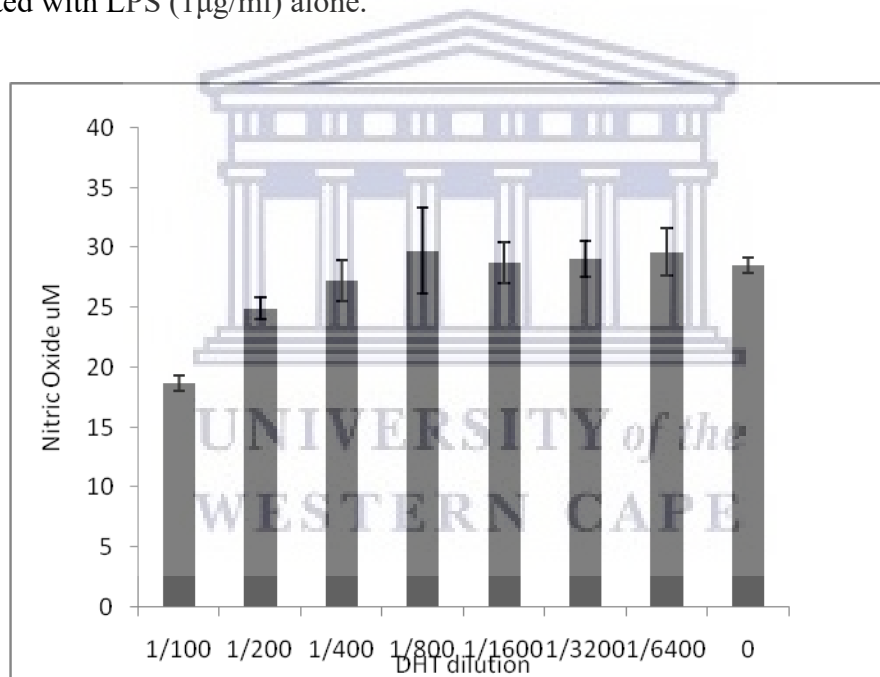
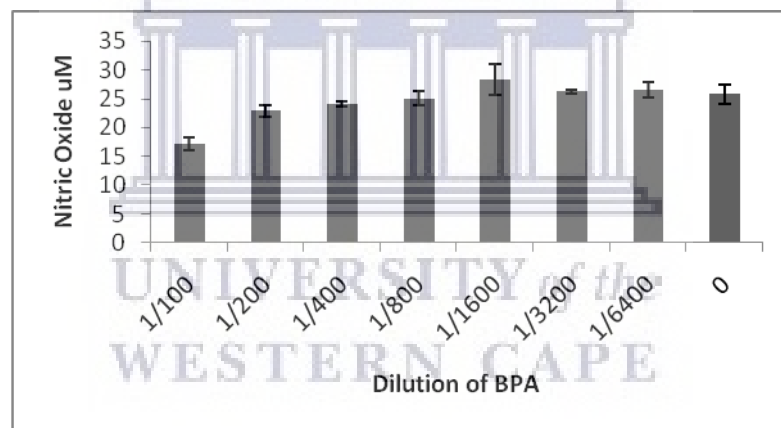Figure 7. Optimization of DHT dilution starting from 1 in 100 (10µg/ml) dilutions on secretion of NO in RAW264.7 cells stimulated with 1µg/ml LPS. Control culture was treated with LPS (1µg/ml) alone.

The results of effect of DHT dilution on cell viability show that DHT at concentrations starting from 1 in 200 (5µg/ml) and further dilutions did not affect cell viability and were

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not different from the control. The results of the effects of DHT concentration on NO production, show that treatment with DHT at concentration of 1 in 100 (10µg/ml) reduced NO production in stimulated RAW264. 7 cell culture. The DHT dilution starting from 1 in 400 (2.5µg/ml) and control culture secreted same levels of NO as control.

The effects of estradiol concentration on cell viability and NO production in RWA264.7 cells stimulated with 1µg/ml LPS are as shown in Figure 8 and Figure 9 respectively.
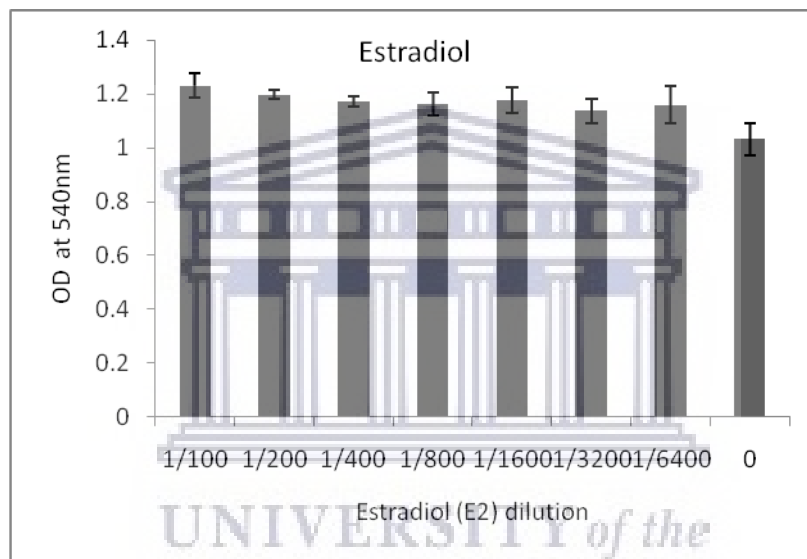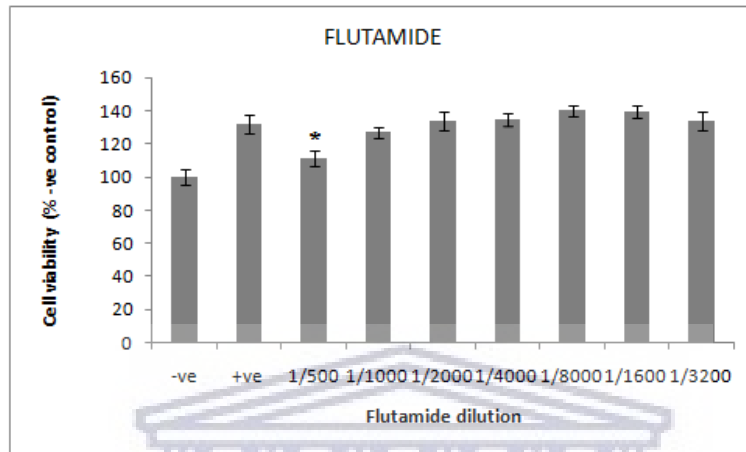

Figure 8. Optimization of estradiol (E2) dilution starting from 1 in 100 (10µg/ml) dilutions on viability of RAW264.7 cells stimulated with 1µg/ml LPS. Control culture was treated with LPS (1µg/ml) alone.
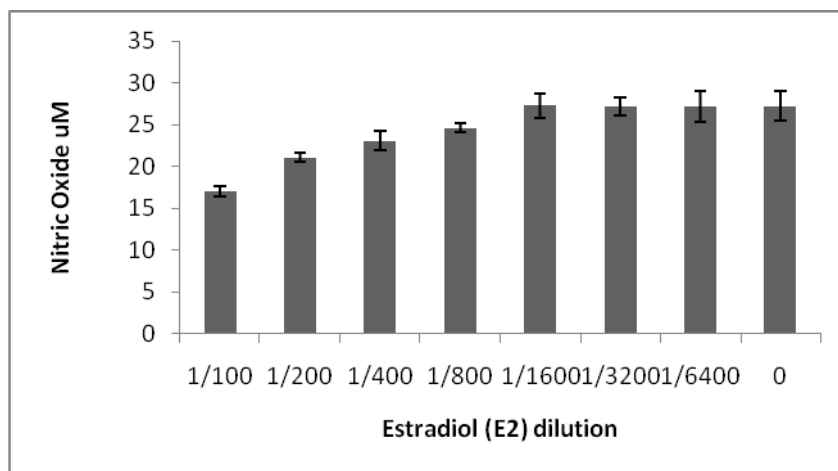
Figure 9. Optimization of estradiol (E2) dilution starting from 1 in 100 (10μg/ml) dilutions on NO secretion in RAW264.7 cells stimulated with 1μg/ml LPS. Control culture was treated with LPS (1μg/ml) alone.

The results of the effects of estradiol dilution on viability of stimulated RWA264.7 cells show that E2 at all concentrations had no effects on cell viability. The results imply that E2 at all concentration had no cytotoxic effects to RAW264.7 cells. The results of E2 dilution on secretion of NO in stimulated RAW264.7 cells show that high concentrations of E2 suppressed NO secretion as compared to the control.

The effects of BPA dilution on cell viability and secretion of NO in stimulated RWA264.7 cells are presented in Figure 10 and Figure 11 respectively. The results of the effects of BPA concentration on cell viability show that all BPA concentrations starting from 1 in 100 (10μg/ml) had no significant effects on cell viability of stimulated RAW264.7 cells. The results of dilution effects of BPA on secretion of NO in stimulated RAW264.7 cells show that the highest concetration of 1 in 100 (10μg/ml) had stronger suppression effects compared to other concentrations.
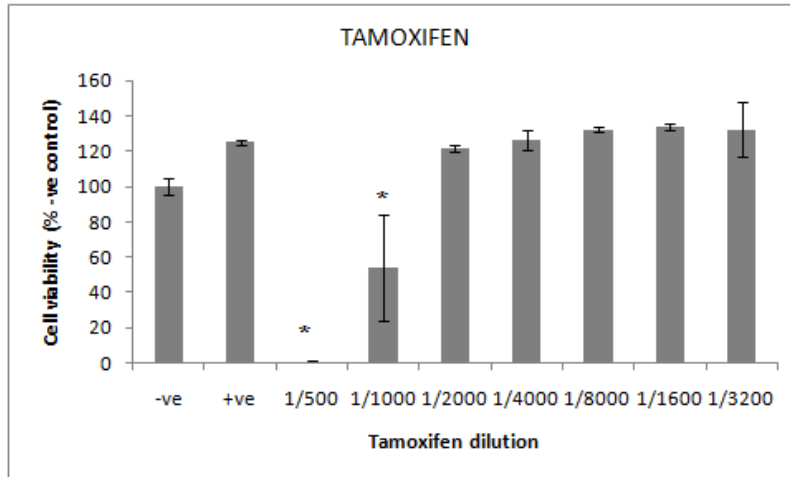
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Figure 10: Optimization of BPA dilution starting from 1 in 100 (10μg/ml) dilutions on viability of RAW264.7 cells stimulated with 1μg/ml LPS. Control culture was treated with LPS (1μg/ml) alone.

Figure 11: Optimization of BPA dilution starting from 1 in 100 (10μg/ml) dilutions on secretion of NO in RAW264.7 cells stimulated with 1μg/ml LPS. Control culture was treated with LPS (1μg/ml) alone.

The effects of flutamide concentration on cell viability and secretion of NO in RAW264.7 cells stimulated with 1μg/ml LPS are presented in Figure 12 and figure 13 respectively. The results of cell viability show that flutamide alone at all concentrations

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did not suppress cell viability. The results of effects of dilutions on secretion of NO reveal that flutamide alone at the highest concentration of 1 in 500 (5μg/ml) suppressed NO secretion in RAW264.7 cells stimulation with 1 μg/ml LPS.
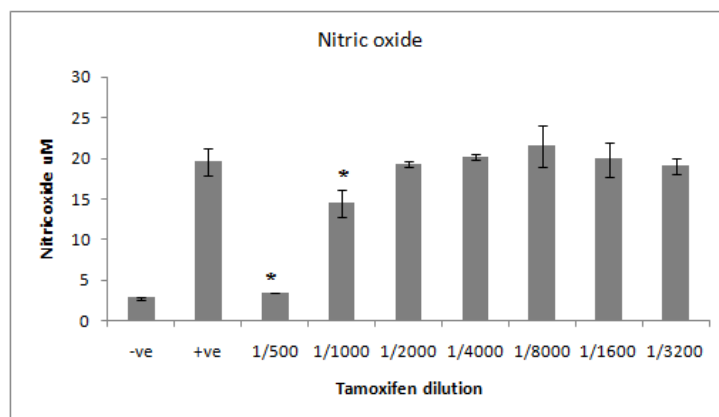

Figure 12: Optimization of flutamide dilution starting from 1 in 500 (5μg/ml) dilutions on viability of RAW264.7 cells stimulated with 1μg/ml LPS. Negative control was treated with normal medium and positive control was treated with LPS (1μg/ml).
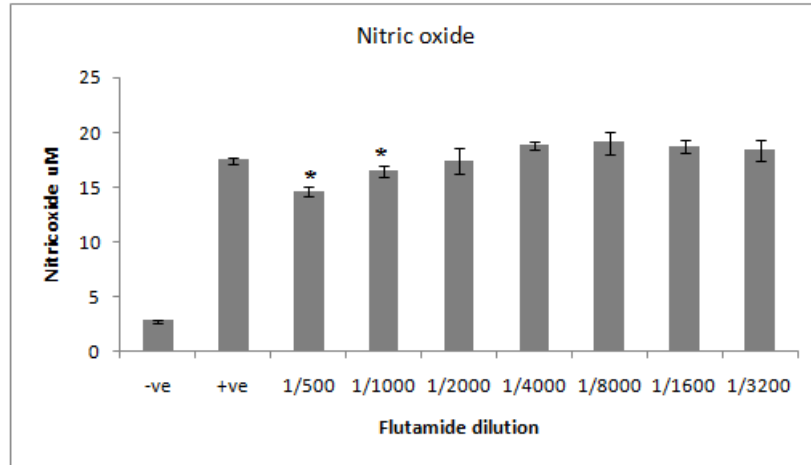
Figure 12: Optimization of flutamide dilution on secretion on NO starting from 1 in 500 (5μg/ml) dilutions on RAW264.7 cells stimulated with 1μg/ml LPS. Negative control was treated with normal medium and positive control was treated with LPS (1μg/ml).

The effects of tamoxifen concentration on cell viability and secretion of NO in RAW264.7 cells stimulated with 1μg/ml LPS are presented in figure 13 and figure 14 respectively.

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Figure 13: Optimization of tamoxifen dilution starting from 1 in 500 (5μg/ml) dilutions on viability of RAW264.7 cells stimulated with 1μg/ml LPS. Negative control was treated with normal medium and positive control was treated with LPS (1μg/ml).

The results of the effects of tamoxifen on viability of stimulated RAW264.7 cells show that tamoxifen alone at the highest concetration of 1 in 500 (5μg/ml) reduced cell vialiabilty and hence it is cytotoxic. The results show further that tamoxifen at the highest contration of 1 in 500 (5μg/ml) suppressed NO secretion. The concentrations of tamoxifen below 1 in 1000 (2.5μg/ml) did not suppress secretion of NO in stimulated RAW264.7 cells.

Figure 14: Optimization of tamoxifen dilution starting from 1 in 500 (5μg/ml) dilutions on NO secretion in RAW264.7 cells stimulated with 1μg/ml LPS. Negative control was treated with normal medium, positive control treated with LPS (1μg/ml).

Therefore, 2μg/ml of tamoxifen and flutamide was considered optimal concentration for the subsequent experiments. The effects of optimal concentration of flutamide (2μg/ml) and tamoxifen (2μg/ml) on IL-6 secretion in RAW264.7 cells stimulated with 1μg/ml LPS are as presented in figure 15.

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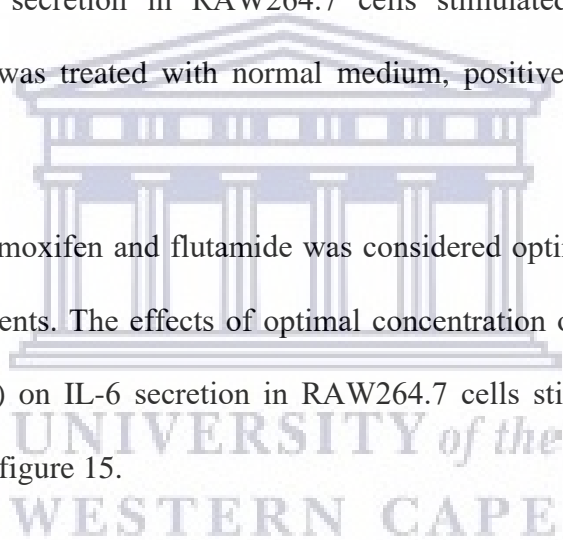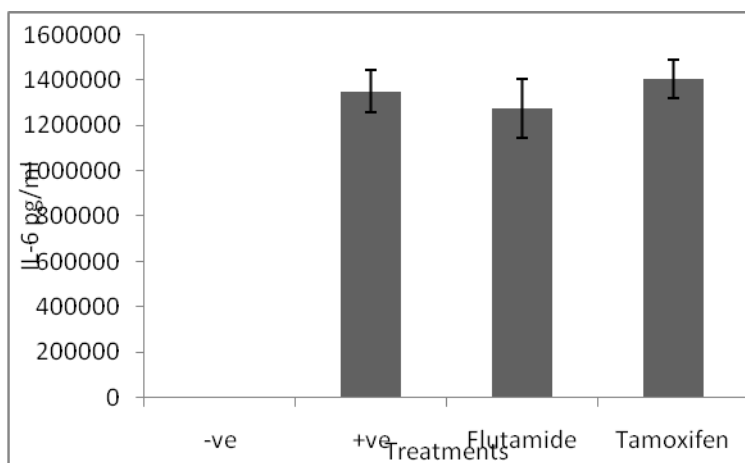
Figure 15: Effect of optimal concentration of flutamide (2μg/ml) and tamoxifen (2μg/ml) on IL-6 secretion in RAW264.7 cells stimulated with 1μg/ml LPS. Negative control was treated with normal medium, positive control treated with LPS (1μg/ml).

The results of the effects of flutamide (2μg/ml) or tamoxifen (2μg/ml) alone on IL-6 secretion in stimulated RAW264.7 cells revealed that both flutamide (2μg/ml) and tamoxifen (2μg/ml) alone had no suppression effects on IL-6 secretion in RAW264.7 cells stimulated with LPS (1μg/ml).

Standard curves of assays:

Standard curves of assays in RAW264.7 cells culture stimulated with 1μg/ml LPS

A standard curve of NO assay in supernatant of RAW264.7 cells culture stimulated with 1μg/ml LPS is presented in figure 16. The standard curve showed a linear relationship between OD (450 nM) and NO (μM) concentration with good correlation ($R^2=0.999$) observed.

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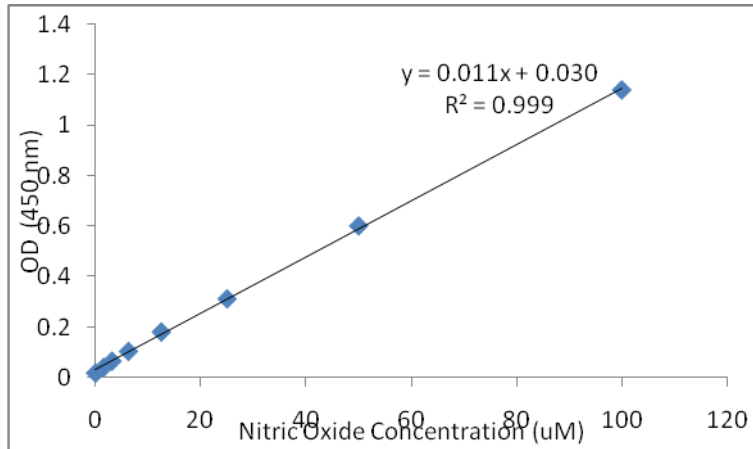
Figure 16. A typical standard curve for NO assay showing a linear relationship of OD (450 nM) and NO (μM) concentration in supernatant of RAW264.7 cells culture stimulated with 1μg/ml LPS.

Figure 17 shows a standard curve obtained for the IL-6 assay in supernatant of RAW264.7 cells culture stimulated with 1μg/ml LPS. The standard curve shows a linear relationship between OD (450nm) and IL-6 (pg/ml) concentration in supernatant of RAW264.7 cells culture with a good correlation ($R^2 = 0.995$) observed.
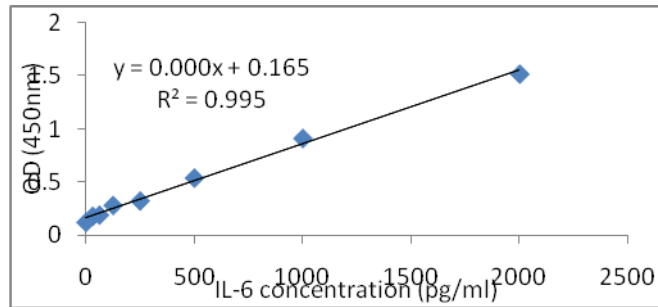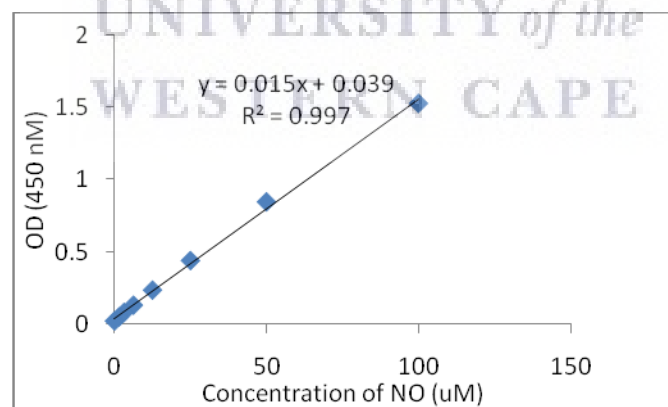
Figure 17. A typical standard curve for IL-6 ELISA showing a linear relationship of OD (450nM) and IL-6 (pg/ml) concentration in supernatant of RAW264.7 cells stimulated with 1µg/ml LPS.

Standard curves of assays in RAW264 cell culture treated with sewage samples

A standard curve of NO assay in supernatant of RAW264.7 cells culture treated with sewage samples is presented in figure 18. The standard curve shows a linear relationship between OD (450nM) and concentration of NO produced with a good correlation of $R^2=0.997$.

Figure 18. A typical standard curve for NO assay showing a linear relationship of OD (450nM) and NO (uM) concentration in supernatant of RAW264.7 cells treated with sewage samples.

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Figure 19 shows a standard curve of IL-6 ELISA in supernatant of RAW264.7 cells treated with sewage samples. The standard curve shows a polynomial relationship between OD (450nM) and concentration of IL-6 (pg/ml) secreted with a good correlation of R^2=0.997 observed.
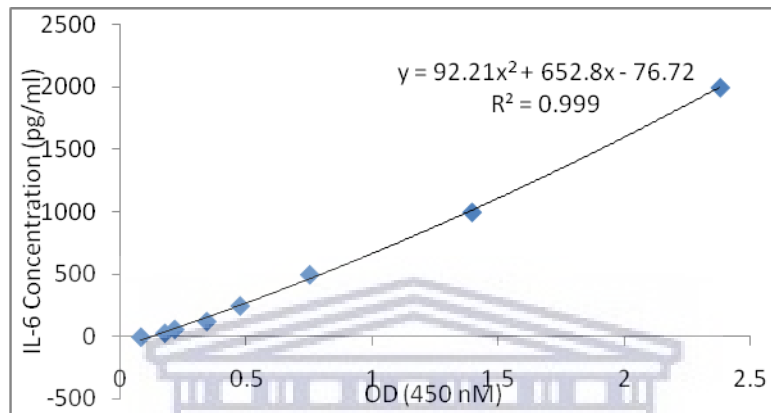

Figure 19. A typical standard curve for IL-6 ELISA showing a polynomial relationship of OD (450nM) and IL-6 (pg/ml) concentration in supernatant of RAW264.7 cells treated with sewage samples.

Standard curves of assays in RAW264.7 cells treated with effluent samples treated with adsorption and coagulation techniques.

A standard curve obtained for the NO assay in supernatant of RAW264.7 cells treated with effluent samples treated with adsorption and coagulation techniques is presented in figure 20. The standard curve shows a linear relationship between OD (450nM) and concentration of NO produced with a good correlation of R^2=0.999.
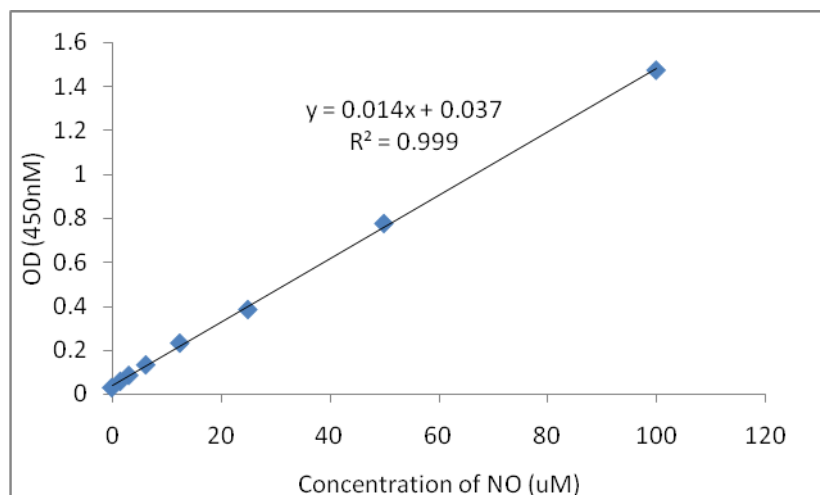
Figure 20. A typical standard curve for NO assay showing a linear relationship of OD (450nM) and NO (uM) concentration in supernatant of RAW264.7 cells treated with effluent samples treated with adsorption and coagulation techniques.

Figure 21 shows a standard curve obtained for the IL-6 ELISA in supernatant of RAW264.7 cells treated with textile effluent samples treated with adsorption and coagulation techniques. The standard curve shows a linear relationship between OD (450nM) and concentration of IL-6 secreted with a good correlation of $R^2=0.999$.
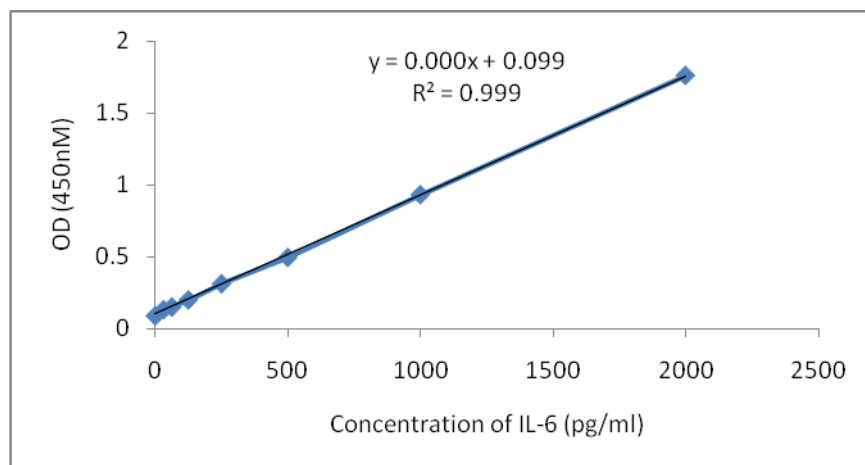
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Figure 21. A typical standard curve for IL-6 ELISA showing a linear relationship of OD (450nM) and IL-6 (pg/ml) concentration in supernatant of RAW264.7 cells treated with effluent samples treated with adsorption and coagulation techniques.

Standard curves of assays in supernatant of RAW264 cell culture treated with electrohydraulic discharge alone or combined with coagulation/flocculation processes

A standard curve obtained for the NO assay in RAW264.7 cell culture supernatant treated with electrohydraulic discharge alone or combined with coagulation/flocculation processes is presented in figure 22. The standard curve shows a linear relationship between OD (450nM) and concentration of NO produced with a good correlation of R^2=0.999.
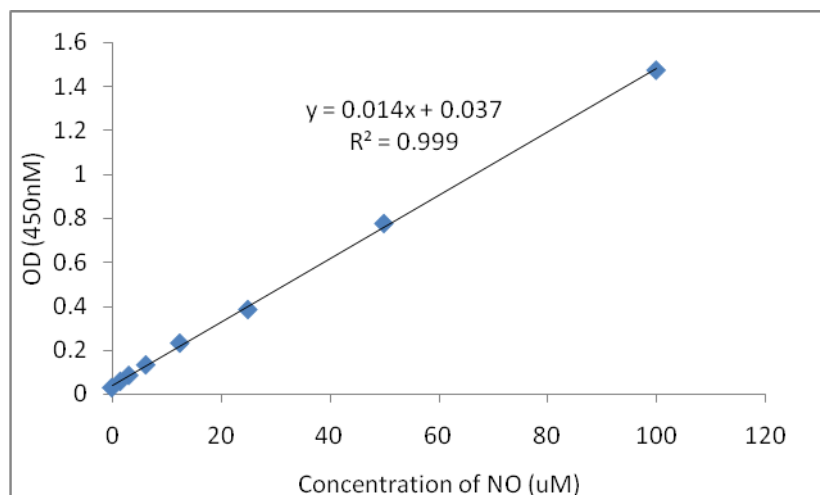
Figure 22. A typical standard curve for NO assay showing a linear relationship of OD (450nM) and NO (uM) concentration in RAW264.7 cell culture supernatant treated with electrohydraulic discharge alone or combined coagulation/flocculation processes.

Figure 23 shows a standard curve obtained for the IL-6 ELISA in RAW264.7 cell culture supernatant treated with electrohydraulic discharge alone or combined coagulation/flocculation processes. The standard curve shows a linear relationship between OD (450nM) and concentration of IL-6 secreted with a good correlation of R^2=0.999.
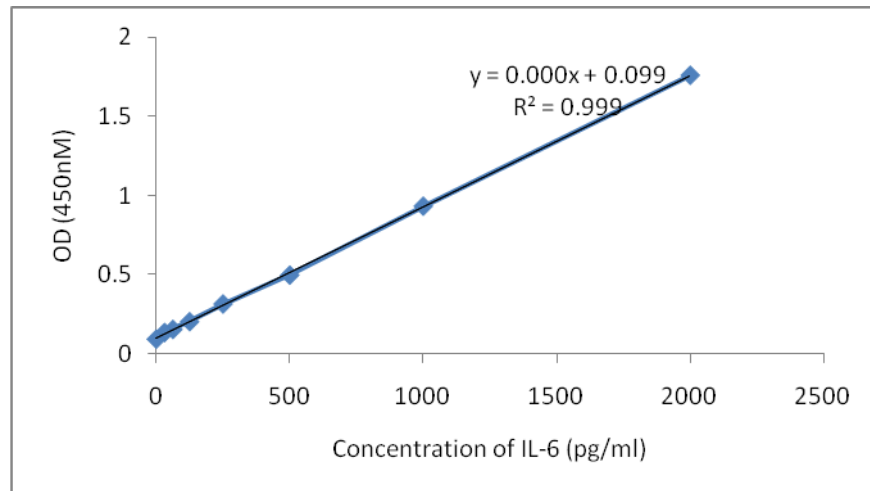
Figure 23. A typical standard curve for IL-6 ELISA showing a linear relationship of OD (450nM) and IL-6 (pg/ml) concentration in supernatant of RAW264.7 cells treated with electrohydraulic discharge alone or combined with coagulation/flocculation processes.

Conclusion

The optimal concentrations of reagents determined and used in this study had no adverse effects to RAW264.7 cell cultures. Generally, standard curves of both NO and IL-6 assays always showed a relationship between OD (450nM) and concentration of NO and IL-6 secreted respectively with a good correlation.

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