BIOAUGMENTATION OF AN ACTIVATED SLUDGE PROCESS

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ABSTRACT

This study investigated the performance of a bacterial product Free Flow (NCH Suomi Oy) as a bioaugmentation tool at doses 0, 20 µl and 120 µl of an Activated Sludge Process on a laboratory scale. The flow rate of wastewater to the Activated Sludge Process plant was 18 l/d. Sludge settling, sludge activity, examination of composition of wastewater microbes present and biofloc formation under microscope, reductions in suspended solids, COD₇, BOD₇ₐₜᵤ and enteric microbes were studied. The percentage performances in the BOD₇ₐₜᵤ removal were 99% for both 120 µl and 20 µl doses and were higher than the 0 dose (97% p < 0.05). The percentage performances in the COD₇ removal were 97% for both 120 µl and 20 µl doses and these reductions were higher than the 0 dose (91% p < 0.05). The percentage performances in suspended solids reduction were 88% for the 120 µl dose, 87% for the 20 µl dose and 84% for the 0 dose. The log₁₀ reductions in microbial communities covered were; E. coli 120 µl dose 3.2, 20 µl dose 2.9, and 0 dose 2.6. With the Intestinal Enterococci population, the 120 µl dose performed (2.9, 20 µl dose 2.6, and 0 dose 2.4 log reductions. With the F-RNA coliphage population, the 120 µl dose performed 3.6, 20 µl dose 3.3, and 0 dose 2.8 log reductions. Mean MLSS level at the 120 µl dose phase was 6.5 g/l and was higher than the 20 µl (4.7 g/l and 0 dose 3.4 g/l p < 0.05). The 20 µl dose MLSS was also higher than the 0 dose (p < 0.05). MLVSS followed the same trend, 120 µl dose was 2.2 g/l, 20 µl dose 1.7 g/l and 0 dose 1.4 g/l. Mean sludge activity from dehydrogenase test of sludge recorded (2.9 µg formazan/MLSS), (3.5 µg formazan/MLSS) and (5.6 µg formazan/MLSS) at the 120 µl, 20 µl and 0 dose phases respectively. Mean sludge volume index recorded were 68 ml/g, 48 ml/g and 55 ml/g at the 120 µl, 20 µl and 0 dose phases, respectively. Microbial occurrence varied, however during 20 µl dose, the floc was very good and characterized by the prevalence of Rotifera, crawling ciliates and stalked ciliates. Sludge settling recorded higher values during the 120 µl dose phase and lower settling trend was recorded during the no dose phase. Overall, after the application of the bioaugmentation agent, a higher reduction performance was recorded with BOD₇ₐₜᵤ and COD₇, MLSS increased favourably and floc improved. The 120 µl dose performed the most reduction with all the microbes covered and the most successful reduction was achieved with the F-specific RNA coliphage microbial population.
ACKNOWLEDGEMENT

Dedicated to my family. Special thanks to Eila Torvinen, Helvi Heinonen-Tanski and Anna Sipilä for your guidance in making this thesis. I acknowledge the stipends and logistics provided by NCH Suomi during the laboratory phase of this work. Special thanks go to the late Matti Pessi for his expert advice, I still remember you Matti. I thank God almighty.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-CA</td>
<td>3-Chloroaniline</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Analytical Communities</td>
</tr>
<tr>
<td>ASP</td>
<td>Activated Sludge Process</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological Oxygen Demand</td>
</tr>
<tr>
<td>BOD\textsubscript{7ATU}</td>
<td>Biological Oxygen Demand; 7 Day Allythiourea method</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>COD\textsubscript{Cr}</td>
<td>Chemical Oxygen Demand; dichromate method</td>
</tr>
<tr>
<td>DCP</td>
<td>Dichlorophenol</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydrogenase Activity</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EEC</td>
<td>European Economic Community</td>
</tr>
<tr>
<td>EFF</td>
<td>Effluent</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>INF</td>
<td>Influent</td>
</tr>
<tr>
<td>MLSS</td>
<td>Mixed Liquor Suspended Solids</td>
</tr>
<tr>
<td>MLVSS</td>
<td>Mixed Liquor Volatile Suspended Solids</td>
</tr>
<tr>
<td>NCH</td>
<td>National Chemsearch Corporation</td>
</tr>
<tr>
<td>P-removal</td>
<td>Phosphorus removal</td>
</tr>
<tr>
<td>RAS</td>
<td>Return Activate Sludge</td>
</tr>
<tr>
<td>SA</td>
<td>Sludge Activity</td>
</tr>
<tr>
<td>SPP</td>
<td>Species</td>
</tr>
<tr>
<td>SRT</td>
<td>Sludge Retention Time</td>
</tr>
<tr>
<td>SS</td>
<td>Suspended Solids</td>
</tr>
<tr>
<td>TBGA</td>
<td>Tryptone Bile-Glucuronide Agar</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>TTC</td>
<td>Triphenyltetrazolium Chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>VSS</td>
<td>Volatile Suspended Solids</td>
</tr>
<tr>
<td>WAS</td>
<td>Wasted Activated Sludge</td>
</tr>
<tr>
<td>WW</td>
<td>Wastewater</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater Treatment Plant</td>
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1. INTRODUCTION

The goals of any form of wastewater treatment are to reduce the organic matter, pathogens and nutrients content to the standard requirement (Frigon et al. 2013; Riffat 2013). These ensure ecosystem health of receiving waters and reduce health risk to humans. Microorganisms, constituents and detrimental effects of wastewater (WW) vary widely depending on its source. According to Riffat (2013) the microorganisms play a significant role in the purification of wastewater by converting biodegradable organic matter to forms that are able to ensure the stability of wastewater while some are pathogenic. The activities of these microorganisms can occur naturally in systems such as rivers and streams which make them possess a kind of self-purification ability. These abilities have been overwhelmed by pollution hence the need for engineered treatment systems for the enhancement and efficient management of WW.

One of the current biologically engineered systems for the treatment of municipal WW is the Activated Sludge Process (ASP). The process involves the activation of a mass of microorganisms which have the ability to stabilize organic matter in wastewater (Metcalf and Eddy, 2003). The basic ASP, according to Riftat (2013) consists of three components:

(1) An aeration tank where microorganisms are kept in suspension aerated.
(2) A sedimentation tank or clarifier and
(3) A recycler system for returning settled solids to the system.

To improve removal of targeted components of wastewater in the ASP system, Leu and Stenstrom (2010) suggest bioaugmentation. Bioaugmentation is defined by Lindbergen et al. (1998) as the application of indigenous or non-indigenous natural or genetically modified organisms to polluted hazardous waste sites or bioreactors in order to accelerate the removal of undesired compounds. Bioaugmentation approach may depend on the goal(s) of the process such as using nitrifying bacteria to increase the rate of nitrification (Ni et al. 2008) or removal of heavy metals using bacteria metabolism (Mejare and Bulow, 2001). Bioaugmentation has also been used to improve floc formation in ASP (Lindbergen et al. 1998); as well as improved removal of suspended solids (Stephenson and Stephenson, 1992).

Bioaugmentation of wastewater treatment systems has become necessary due to challenges to meet standard requirements or contaminant removal by wastewater treatment plants (WWTPs). In bioaugmenting WW, it is essential that the parameters that affect the
microorganism’s performance are kept at right levels since these organisms operate within specific physico-chemical (temperature, pH, oxygen, shear stress, nutrients etc.) range. Microorganisms are particularly at risk during the start-up of the process since the stability of WWTP’s are vulnerable to rapid changes in physico-chemical parameters.

Some works such as those by Abeyasinghe et al. (2002) and Saler et al. (2003) report of the failure of bioaugmentation process because the bacteria used might have been grazed upon by predatory bacteria. This occurs when conditions are conducive for predatory microorganism(s) over the bioaugmented microorganisms. To overcome these initial bioaugmentation challenges, an engineered system with the ability to generate an overwhelming number of bacteria of importance to wastewater and perform array of wastewater treatment functions will be ideal.

BioAmp, a bio additive dispenser from NCH Corporation and administered by NCH Suomi Oy is a system programmed to dispense high amounts of bacteria. These bacteria are packaged in a form of a tablet (Free Flow) and belong to Biosafety Class 1 and therefore do not represent any threats to human or animal health or the environment.

The aim of this thesis work was to assess the performance of Free Flow bacterial product on wastewater treatment by monitoring physical, chemical and microbiological parameters and microscopic monitoring of the activated sludge before and during Free Flow addition.
2. LITERATURE REVIEW

2.1 WASTEWATER

Wastewater that is treated in a municipal WWTP can have various sources and forms. These include both solid and liquid wastes carried in water form from households, institutions such as hospitals, schools, restaurants etc. and industry. In addition to these sources are influences from rainwater or storm water flow as well as run-offs from storms to the WWTP.

2.1.1 Constituents of wastewater

Municipal WW is characterized by both organic and inorganic compositions. The organic components of WW include major part (75%) total suspended solids (TSS) Metcalf and Eddy, (2003), which consist of food waste, human waste, silt, paper etc. (Riffat 2013). Biodegradable organic matter which contains varied ports of proteins and carbohydrates (Peavy et al. 1985) and enteric microbes including pathogens (Metcalf and Eddy, 2003). Municipal WW composition as shown in Table 1 depends on the processes and products from its source.

**Table 1** General composition of municipal wastewater (Metcalf and Eddy, 1979) and in Finland (Karttunen et al. 2004).

<table>
<thead>
<tr>
<th>Contaminants</th>
<th>Unit</th>
<th>Weak</th>
<th>Medium</th>
<th>Strong</th>
<th>Finland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solids, total (TS)</td>
<td>mg L⁻¹</td>
<td>350</td>
<td>720</td>
<td>1200</td>
<td>350-600</td>
</tr>
<tr>
<td>Suspended solids (SS)</td>
<td>mg L⁻¹</td>
<td>100</td>
<td>220</td>
<td>350</td>
<td>150-200</td>
</tr>
<tr>
<td>Volatile</td>
<td>mg L⁻¹</td>
<td>80</td>
<td>165</td>
<td>275</td>
<td>120-150</td>
</tr>
<tr>
<td>Settleable solids</td>
<td>mg L⁻¹</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>BOD₅ at 20° C</td>
<td>mg L⁻¹</td>
<td>110</td>
<td>220</td>
<td>400</td>
<td>125-175⁷ATU*</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD)</td>
<td>mg L⁻¹</td>
<td>250</td>
<td>500</td>
<td>1000</td>
<td>300-450</td>
</tr>
<tr>
<td>Nitrogen (total as N)</td>
<td>mg L⁻¹</td>
<td>20</td>
<td>40</td>
<td>85</td>
<td>25-40</td>
</tr>
<tr>
<td>Phosphorus (total as P)</td>
<td>mg L⁻¹</td>
<td>4</td>
<td>8</td>
<td>15</td>
<td>6-8</td>
</tr>
<tr>
<td>Inorganic</td>
<td>mg L⁻¹</td>
<td>3</td>
<td>5</td>
<td>10</td>
<td>25-75</td>
</tr>
</tbody>
</table>

*BOD determination in Finland is done on 7 days incubation (BOD⁷ATU)
From the nutrients composition of WW, nitrogen and phosphorus (Table 1) are a major cause of aquatic pollution (Peavy et al. 1985). They trigger the growth of aquatic organisms leading to eutrophication and eventual death of the aquatic body if not treated to remove the nutrients. Various strategies to remove these nutrients exist

2.1.2 Biological Oxygen Demand and Chemical Oxygen Demand

Wastewater organic constituents include total suspended solids (TSS) carbohydrates, proteins, lipids, urea, organic synthetics such as pharmaceutical products, agricultural chemicals such as pesticides, fertilizers etc. (Metcalf and Eddy, 2003; Riffat, 2013). Other organic contents of municipal WW are synthetic detergents such as soap. To measure these organic contents, the most common methods are the determination of the biological oxygen demand (BOD) and chemical oxygen demand (COD) of the WW as shown in Table 1. The BOD test measures the amount of oxygen that will be required by organisms to breakdown the biodegradable organic content of WW and also quantifies the concentration biodegradable organic matter in the WW. It involves the incubation of the WW sample for 5 days (BOD$\delta$) (Metcalf and Eddy, 2003). The incubation duration in Finland is 7 days (BOD$\gamma$) (Karttunen et al. 2004). The procedure is described in Chapter 4 of this text. The COD test measures the amount of oxygen consumed in when the organic matter in the WW is degraded chemically. The procedure is described in Chapter 4 of this text. These two parameters are very critical to wastewater treatment because they affect aquatic life by depleting the oxygen content. They are thus included in the list of USEPA as conventional pollutant (BOD) and non-conventional pollutant (COD) (Riffat, 2013). The reduction of these parameters is therefore a requirement for all WWTPs.

2.1.3 Biological composition

Municipal WW have varied forms of organisms present including bacteria, protozoa, fungi, algae and viruses (Riffat, 2013). These organisms play different roles. Some are pathogenic and cause diseases (Metcalf and Eddy, 2003). The pathogenic microorganisms are generally the indicators of fecal contamination capacity of the WW and hence one of the most important goals in treating WW while others are involved in the decomposition of organic matter to stabilize WW. The stabilization processes include nitrification and denitrification (Alleman, 1984). Some of the microorganisms such as protozoa are usually aerobic or facultative and also consume other microorganisms such as bacteria, algae etc. and are
therefore essential in biological treatment to maintain an equilibrium between the various microorganisms (Johnstone and Horan, 1996).

2.2 TREATMENT METHODS OF WASTEWATER
Wastewater must be managed in a way that its hazardous effect is reduced since it can threaten human life if not properly disposed. Moreover Babcock Jr. (1991) has stated that thermal destruction techniques are currently not favourable because residuals and secondary products formed during high-temperature treatment of wastewater which could be released with stack gases are perceived as dangerous to public health. Levels of wastewater treatment according Riffat, (2013), are primary treatment, which involves physical removal of suspended solids through sedimentation and can also include using chemicals to enhance coagulation and flocculation; secondary treatment which involves biological treatment such as ASP for the degradation of organic matter and nutrients and solids reduction and tertiary treatment, which involves the removal of residual suspended solids and disinfection for pathogen removal. Treatment can combine the two or more of the three levels depending on the nature of pollutants and level of removal (Rittmann and McCarty, 2001; Metcalf and Eddy, 2003 and Riffat, 2013). According to Riffat (2013), wastewater engineering has progressed from the collection and open dumping to collection and disposal without treatment to collection and treatment before disposal all the way to collection and treatment to prior to reuse. Attention is now focused completely on the reuse and safe disposal.

Since disposal or receiving points include wetlands, streams, rivers, oceans etc. which are habitats, it has necessitated the enactment of regulations and laws to govern these wastewater management practices. In the European Union (EU) for example, three major directives govern the basis for all EU nations to form their own legislation;


(2) Drinking water directive (98/83/EC) of 1998 concerning potable water.

(3) Water framework directive (2000/60/ EC) of 2000 concerning management of surface and ground water resources.

Although natural waters which are mostly the receiving points of WW possess some form of self-purification ability, the ever increasing human and industrial activities has also increased pollutants received in natural reservoirs. The self-purification ability of such waters is easily
overwhelmed by the enormous WW generation therefore the need for treatment before dumping. According to Tchobanglous and Burton (1991), removal of unwanted substances from wastewater is done through physical unit operations, chemical unit process and biological unit processes. From these methods, there are process primary treatment, secondary treatment and tertiary treatment.

2.2.1 Activated sludge process

Activated sludge process (ASP) according to Riffat (2013) is an aerobic, most widely used suspended growth process used in the biological treatment of municipal and industrial WW. Tchobanglous, (1979) has reported that the ASP has been in use for nearly 80 years. It is a secondary step process for treating primary effluents which involves the production of activated mass of microorganisms capable of aerobic stabilization of organic matter in wastewater (Metcalf and Eddy, 2003). Suspended growth is a growth process where microorganisms are kept in suspension in a biological reactor and constantly mixed and where the suspension converts biodegradable organic matter by feeding on it as food. The basic process involves (Figure 1, Babock Jr. 1991, Ėkos et al. 2011):

1. An aeration tank: where primary effluent flows and microorganisms are kept in suspension, aerated and feed on the organic matter to degrade it and convert to a mixture of cell mass and other waste.

2. A sedimentation tank or clarifier: which separates the mixture from the aeration tank into effluent and settled solids.

3. The settled solids are removed as underflow sludge is recycled from the clarifier to the aeration tank or wasted to control the biomass concentration.

Fig.1 Basic schematic representation of the ASP (Adapted from Ėkos et al. 2011).
The ASP is governed by the microorganism characteristics and the physical configuration of the tank and these affect the biological kinetics and the process kinetics (Metcalf and Eddy, 1979). Typical instance is the process of N-removal; in this case, if it is designed for nitrification-denitrification process which requires an ASP built with both anoxic and aerobic parts as shown in Figure 1. The presence of bacteria such as *Nitrosomonas*, *Nitrococcus*, and *Nitrobacter* drives the nitrification and *Pseudomonas*, *Micrococcus*, *Bacillus* and *Alcaligenes* are involved in the denitrification process (Metcalf and Eddy, 2003). In order to maintain sufficient nitrification rate in the ASP, dissolved oxygen (DO) concentration has to be maintained at 1.5 to 2 mg/l and alkalinity at the level of at least 1 - 1.5 mmol/l in the ASP (Eckenfelder and Grau, 1992). The ASP can also be used to perform P-removal by chemical precipitation. The bio-P bacteria, such as *Acinetobacter* spp. also perform P-removal by storing P as an energy reserve (Rybicki, 1997).

Reported variations on ASP for different goals in treating wastewater include for example the process described by Lindberg and Carlsson (1996) who controlled NO$_3^-$ concentration using external carbon dosage for microorganisms based on a generalized minimum variance. Samuelson and Carlson (2001) used carbon as a controller in ASP and designed a feed forward strategy based on a simple mass balance. This mass balance determination ensures the amount of constituents coming in the effluent comparing to the wasted activated sludge (WAS). This process again is able to determine the level of degradation in the ASP. By extension, the components which were not treated and passed through the ASP untreated can be determined. Cho *et al.* (2002) proposes a cascade control strategy for controlling the NO$_3^-$ in both the effluent and the last anoxic compartment of the ASP.

The ASP has been described as efficient in metabolizing a vast number of organic compounds and to oxidize or reduce polymerized compounds containing nitrogen, phosphorus, sulfur etc. (Eckenfelder and Grau, 1992). However it is also widely subjected to fluctuating flows, temperatures and changes in the influent wastewater concentration and composition.

### 2.2.2 Mixed liquor suspended solids and Mixed liquor volatile suspended solids

An integral part of the ASP is mostly microorganisms and WW materials in suspension. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) are used as a measure of microorganism’s concentration in the aeration tank (Woodside and Kocurek, 1997). MLSS consists of both the volatile and inert solids, while MLVSS consists of only volatile solids. MLVSS is determined by igniting the MLSS and it describes the organic fraction of MLSS and is used to more closely approximate the biologically active
portion of the solids, MLSS is widely used to monitor the ASP because the analytical procedure is faster (Woodside and Kocurek, 1997). The MLSS available ensures a continuous breakdown of organic waste material in the WW which then lowers BOD and improves effluent quality which is the goal of the operation of ASP. The amount of MLSS in the process therefore has a significant effect on the operation. A higher MLSS is most likely to cause bulking which can result in some of the particles flowing to the effluent limiting the effluent quality if sludge recycle rate is not increased accordingly.

2.2.3 Floc formation

Efficient performance of ASP is based on floc formation. Floc is an aggregate of microorganisms, colloidal matter and macromolecules of wastewater bounded together by macro and micro nutrients in the WW (Stypka, 1998). The size and nature of the flocs influence the rate of settling in the clarifier hence final effluent quality. A well formed floc is able to settle out well and separate from the effluent.

Assessing the performance of ASP on the basis of settling trend is quite complex. The influence of microorganisms in relation to the biological activity of sludge and hydraulic disturbances within the system that affect the ability of the clarifier to separate and concentrate the activated sludge from the effluent and with the sludge itself are known confounding factors. From these confounding factors the quality of the effluent is affected by the following:

1. Sludge bulking due to excessive growth of filamentous organisms for example *Nocardia* spp. resulting in sludge settling poorly

2. Pinpoint floc consisting of small floc particles which are non-settleable suspended solids present in the supernatant after the sludge has settled.

3. Floating sludge due to the nitrogen gas produced during denitrification.

Stypka (1998) has reported that factors like; amount and type of filamentous microorganisms, floc size, specific surface area, surface charge, amount of extracellular polymers, the amount of divalent cations (e.g. Ca$^{2+}$, Mg$^{2+}$), floc strength, floc density and hydrophobicity are responsible for an efficient settling process. However, the clarification criterion is important only during drastic changes in hydraulic load for a short period of time, because if the hydraulic loading persists the thickening or solids handling criterion will in time become the governing one (Laquidara and Keinath, 1983).
2.2.4 Sludge activity

Due to inconsistencies in assessing the performance and sludge characteristics using MLSS or MLVSS, alternative methods have been developed based on chemical analysis. A sludge activity method measures the activity of sludge from adenosine triphosphate (ATP) of viable cells present in activated sludge based on triphenyltetrazolium chloride-dehydrogenase activity (TTC-DHA) or assimilation of glucose (Gray, 1990). According to Stypka (1998) by estimating the biological capacity of the activated sludge system, loading rates can be estimated based on the functional relationship between the concentration of wastewater and sludge activity.

2.2.5 Aeration and pH

Aeration during the operation of ASP is to supply oxygen to the microorganisms for their metabolism, which in turn will sustain the continuous degradation of organic matter (Metcalf and Eddy, 2003). A minimum of 1.5 to 2 mg/l of oxygen is required in the aeration tank, this ensures a proper operation of the system. Low oxygen to the ASP is implicated in the growth of filamentous bacteria which causes among others settling challenges (Ritmann and McCarty, 2001). An optimum pH between 6.5 and 7.5 according to Ritmann and McCarty, (2001) is required for the normal growth of microorganisms relevant in the ASP. In the operation of the ASP, it is important that both pH and aeration are kept constant.

2.2.6 Microscopic examination in ASP

The operation of ASP is based on the activities of microorganisms’ degradation of organic material to achieve the desired wastewater effluent results. The need in using the microscope analysis to observe microscopic organisms in the ASP provide a tool for process control and analysis of the progress of the ASP based on the presence of the organisms in the treatment process. According to Gerardi (2008), treatment controls of ASP has its own profile of organisms when operating steady-state condition and enables the operation to correlate the organism present with existing operational conditions whether acceptable or unacceptable.

2.3 BIOAUGMENTATION

Various definitions on bioaugmentation exist. Vogel, (1996) defined bioaugmentation as the addition of microorganisms to enhance a specific biological activity. According to Vogel, (1996), the usage of bioaugmentation is supported by studies showing the incompetence of indigenous microorganisms in some cases and the apparent enhanced bioremediation rate after the addition of competent microorganisms.
2.3.1 Goals in bioaugmenting ASP

2.3.1.1 Protective function

Young (1976) and Wojnowska-Baryla and Young (1983) have stated that the reason for bioaugmenting ASP is to improve degradation and decrease problems associated with it. To this end, researchers such as Jingbo et al. (2010) in a protective function used bioaugmentation to accelerate the start-up of ASP at low temperature and were successful in stabilizing the performances of three biological municipal wastewater treatment processes at low temperatures. Low temperatures have been reported to have a negative effect on the microbial community in the ASP (Martin et al., 2005). This phenomenon has the danger of being ignored especially if the ASP is on a laboratory scale where temperature is monitored. In real life ASP however, the process is subjected to ambient conditions. As a protective function, bioaugmentation was used to protect the microbial community in the ASP from harmful compounds. This function has been studied by Boon et al. (2000) by inoculating 3-chloroaniline (3-CA) degrading strain Comamonas testosteroni I2 gfp. The inoculum recorded improved nitrification by maintaining the desired microbial activity, sludge settling and removal of COD after the bioaugmentation of the ASP with Comamonas.

2.3.1.2 Degradation and improved removal function

Apart its protective function, Babcock Jr. et al., (1991) investigated the biodegradation of 1-Naphtylamine in ASP including its percentage removal by non-biological mechanical removal (through sorption and volatilization) using enrichment culture in Salicyclic acid medium consisting mineral base and vitamins in yeast extract. An important conclusion in that work was the relationship between the inoculum size of Naphthylamine degrading culture and increased average degradation rates; the larger inoculum provided a greater increase in the rate. Wang et al., (2013) employed Acinetobacter spp. as a bioaugmentation agent and assessed the effect on nicotine degradation in an ASP. This strategy was successful in 98% removal of nicotine followed by a stable 80-90% COD removal. Enzymes employed in bioaugmenting the ASP can be substrate specific meaning they act on platforms that are only aligned or oriented to act on. However Chapman, (1971) reported that enzymes in the bioaugmentation process are often non-substrate specific and can be induced by compounds of similar structure, degradation products or earlier precursors. These conflicting reports make it difficult in following the progress of the potency of the enrichment cultures involved in the bioaugmentation process in ASP. An example of substrate specific bioaugmentation of ASP is the inhibition of NH$_4$ concentration from methanogenic activity by anaerobic microorganisms.
with sodium acetate as the carbon source (Yue et al. 2011). Another bioaugmentation goal was the degradation of 2,4-dichlorophenol (DCP) using yeast. This degradation of DCP in wastewater had previously been studied by Beltrame et al., (1982) and Chudoba et al., (1989) using synthetic WW. Workers such as Manconi et al., (2007) used a suspended culture of sulfur utilizing denitrifying bacteria with *Thiobacillus denitrificans* as a bioaugmenting agent as a cost-effective alternative to the conventional heterotrophic denitrification process and simultaneous sulfide removal. Ditröi et al., (2008) used mixed microbial cultures that use starch as a substrate to improve aeration in ASP.

2.3.1.3 Improved nitrification

Workers such as Leu and Stenstrom, (2010) investigated some of the known approaches in bioaugmentation for improved nitrification in the ASP and compared three of the approaches.

1) The parallel plants approach which used acclimated biomass grown in long Sludge Retention Time (SRT) plant to augment a low (SRT) plant.

2) The Enricher-Reactor (ER) approach which uses an offline reactor (Enricher) to produce augmented cultures and later transferred to the main reactor.

3) The ER-RAS approach which grew enrichment culture in a re-aeration reactor which is then added to the main reactor as the augmentation product.

The study revealed that all three approaches increased the SRT of nitrifying biomass and provided improvements for nitrogen removal, but the parallel plants approach (1) created a much higher biomass concentration in the main reactor.

2.3.2 Community structure after bioaugmentation

The general microbial composition of an ASP comprises heterotrophic bacteria engaged in denitrification as well as protozoan communities. Some bioaugmentation strategies are known to maintain the microbial community structure in the ASP. Yu et al. (2011) investigated the effects of predation of protozoa on the nitrification performance and microbial community during bioaugmentation with *Nitrosospira europaea*, *Nitrosospira mobilis* and found enhanced activity and community of the nitrifiers and inhibiting the activities of rotifers simultaneously. The rotiferans are known predators in ASPs (Lee and Welander, 1994; Lee and Oleszkiewicz, 2003). Lee and Oleszkiewicz, (2003) discovered a general increase in protozoan population including zooflagellates and nematodes though they admitted to intensive aeration and this could have also favoured for protozoan growth. This phenomenon
of an overgrowth of protozoa after bioaugmentation has also been reported by Bouchez et al. (2000).

2.3.3 Common challenges and limitations of ASP with mixed microbial communities as the bioaugmentation tool

The biological treatment of wastewater by mixed microbial communities is known to be interrupted by different factors. This may include environmental conditions such as temperature, O₂, and inconsistencies in the ASP operation. Apart from these potential challenges, Britton, (1994); and Spain et al. (1991) have reported that treatment of wastewater by a mixed microbial community often face disruptions from organism and inorganic chemicals present in the wastewater. Henze (1991) has reiterated that the disruptions of the process have a negative effect on sludge settling in ASP. Ekama et al. (1997) has also identified pumped flows as a potential source of challenge for secondary clarifiers in that square wave discharges and also the size and the features of the clarifier have a major influence on the effect of hydraulic transients. ASPs are known to perform well for the case of the easily degraded components of the wastewater, but not for hazardous components which may be intermittently present and which may be toxic to bacteria, or slow to degrade.
3. AIM OF THE WORK

The objectives of this study were to;

Assess the performance of Free Flow bacterial product by using a BioAmp system for the growth of the bacteria and dosing into a pilot-scale activated sludge process over a three month period and monitoring physical, chemical and microbiological parameters before and during Free Flow addition (bioaugmentation).

Conduct microscopic monitoring to study the biofloc formation and microbial composition before and during Free Flow addition.
4. MATERIALS AND METHODS

4.1 ACTIVATED SLUDGE PROCESS PLANT

Laboratory scale Activated Sludge System was set up at the joint water laboratory of University of Eastern Finland and Savonia University of Applied Sciences in the Technopolis building. The plant consisted of a primary clarifier, an activated sludge pool which was divided into an anoxic zone and an aerobic zone, a final clarifier and systems for returning sludge. (Figures 2 and 3). The flow rate of WW to the Activated Sludge Process plant was 18 l/d. Removal of organic material and nitrification take place in the aerobic zone. Phosphorus is removed simultaneously with ferric sulphate. Denitrification takes place in the anoxic zone.

![Diagramatic representation of the simulation plant showing the various sections. From right to left: primary clarifier, anoxic zone, aerobic zone and final clarifier.](image)

The simulation plant was equipped with a balancing tank where the WW was held temporarily and thoroughly mixed before it was pumped to the primary clarifier, a ferric sulphate pump for P-removal, pH control which pumped Ca(OH)₂ to the system to neutralize increasing acidity caused by denitrification. Pumps for return and excess sludge as well as nitrate cycling (Figure 4) and an aeration pump which controlled the oxygen (O₂) level in the aeration section.
4.2 BIOAUGMENTATION WITH FREE FLOW

Each Free-Flow tablet contained twelve strains of bacteria; *Pseudomonas fluorescens*, *Pseudomonas putida*, four strains of *Bacillus subtilis*, *Bacillus licheniformis* *Bacillus thuringiensis*, two strains of *Bacillus amyloliquefaciens* and two strains of *Bacillus simplex*. A BioAmp GT machine (NCH Corp.) of dimensions 45cm (W) x 75 cm (H) x 23 cm (D) operating on 240V power was also set up at same location for the growth and dispensing process of bacteria to the ASP. The BioAmp was programmed to empty and cleanse the system every 24 hours after each day of growth. It was equipped with a back flow preventer...
to prevent the flow of water from the BioAmp machine into the drinking water system. Free-Flow was dosed manually to the active sludge process. Enough subsample (around 30 ml) was pipetted from the 3.5 liters volume of the grown bacterial suspension in a BioAmp machine into a glass beaker 15-30 minutes before the BioAmp dispensing time. From this subsample, both the 20 μl and 120 μl were pipetted into the primary clarifier of the plant. BioAmp then started the growing cycle. The pipette was totally emptied by sucking some water from the primary clarifier into the pipette and emptying it back there a few times. Each phase of the bioaugmentation lasted approximately one month.

4.3 SAMPLING

Wastewater samples for the ASP were obtained weekly from Lehtoniemi WWTP (Kuopio, Finland). The plant treats municipal wastewater from Kuopio city with a population of about 92,626 (Malo et al. 2011). Wastewater flow to the plant is 18,700 m³/day = 18700 m³/day x 365 = 6,657,200 m³/year and uses the ASP with chemical P-removal treatment processes (Kuopion vesi, 2015). The wastewater sample is brought from Lehtoniemi and was stored in a cold room at a temperature of approximately 7 °C. Wastewater was pumped from there four times a day to the balancing tank (Figure 4A), where it was constantly mixed to prevent settling and pumped to the system at a uniform rate of 18 l/day. Influent samples were taken from the balancing tank by grab sampling with a clamped beaker and transferred to a measuring cylinder. Effluent samples were taken from effluent collected overnight from the final clarifier into a can and was grabbed by clamped beaker and transferred into a measuring cylinder.

Activated sludge samples were taken from the aeration section by grab sampling with a clamped graduated beaker. Excess sludge was collected in a glass jar after being pumped out as programmed and transferred to the appropriate crucible for the procedure.

Apart from the sludge activity parameter which was done once a week, sampling frequency was twice per week and parallel samples were taken for each sample parameter. The different samples were subjected to analyses according to Table 2.
Table 2 Summary of parameters analysed from different samples.

<table>
<thead>
<tr>
<th></th>
<th>Activated Sludge</th>
<th>Excess sludge</th>
<th>Wastewater Influent</th>
<th>Wastewater Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Settling test</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLSS</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLVSS</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>Microscopic Analysis</td>
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<td>Sludge Activity</td>
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<tr>
<td>O₂</td>
<td>*</td>
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<tr>
<td>pH</td>
<td>*</td>
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<td></td>
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</tr>
<tr>
<td>TSS</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>VSS</td>
<td>*</td>
<td>*</td>
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<td></td>
</tr>
<tr>
<td>COD&lt;sub&gt;Cr&lt;/sub&gt;</td>
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<td>*</td>
<td></td>
<td></td>
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<td>*</td>
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<td></td>
</tr>
<tr>
<td>Microbial density</td>
<td>*</td>
<td>*</td>
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<td></td>
</tr>
</tbody>
</table>

### 4.4 SLUDGE ANALYSES

#### 4.4.1 Activated sludge settling test

100 ml of activated sludge from the aeration tank was transferred into a 100 ml measuring cylinder and allowed to settle for 30 minutes. The volume of the settled sludge was read from the interface of the sludge and water. Foaming and bubbles were avoided for accurate results by pouring gently. Sludge Volume Index (SVI) was determined mathematically from Equation 1.

\[
SVI = \frac{SV}{mlss}
\]  

Equation 1

\[
SV = \text{sludge volume after 30 minutes of settling}
\]

\[
mlss = \text{MLSS concentration}
\]
4.4.2 MLSS and MLVSS.

Previously washed and dried crucible was weighed and the weight recorded. 40 ml of sludge sample was transferred into the crucible and dried in a drying chamber at 105 °C for 2 hours. The dried sample was placed in a desiccator to cool and weighed. The MLSS of the sludge was determined mathematically from Equation 2.

\[
MLSS = \frac{1000(m_2-m_1)}{V}, \quad \text{Equation 2}
\]

\[m_1= \text{weight of crucible}\]
\[m_2= \text{weight of crucible + dried sample}\]
\[V= \text{volume of sludge taken}\]

The sample for determination of MLSS was ignited in a furnace at 550 °C for approximately 2 hours. The ignited sample was then placed in a desiccator to cool and weighed after cooling. The MLVSS of the sludge sample was determined mathematically from Equation 3.

\[
MLVSS = \frac{1000(m_2-m_3)}{V}, \quad \text{Equation 3}
\]

\[m_2= \text{weight of dried sample+ crucible}\]
\[m_3= \text{weight of ignited sample+ crucible}\]
\[V= \text{volume of sludge taken}\]

4.4.3 Sludge activity

The underlying assumption for this procedure is the influence that composition of wastewater has on the biochemical activity of sludge. The procedure measures the biochemical sludge activity by quantifying the dehydrogenase activity (DHA) of sludge in a relatively short time. Sludge activity in activated sludge was determined spectrophotometrically by using (bar coded) a LCK 318 cuvette test (concentration range 5 – 200 μg) according to the manufacturer’s instructions (Hach Company, Loveland, CO.). Dehydrogenases converts 2,3,5–triphenyltetrazolium chloride (TTC) Formazan hence the unit μg formazan. 4.3 ml of activated sludge was taken from the aeration tank and transferred into a graduated syringe. 0.5 ml of buffer solution of the LCK 318A was added. With the help of a syringe extension, the content of the syringe was transferred into another syringe slowly to avoid bubbles. The new syringe was stoppered and inverted slowly few times to mix and incubated at room
temperature for 1 hour. The syringe stopper was removed after 1 hour and replaced by a Screw-on membrane filter (LCW904; pore size 1.2 µm) and the sample filtered and the filtrate discarded. The membrane filter and the syringe was screwed loosely onto solution B (LCK 318B) bottle and solution B was slowly drawn into the syringe through the membrane filter to 4.6 ml mark on the syringe and was left to stand for 10 minutes. The content of the syringe was then slowly and carefully filtered into the barcode cuvette after 10 minutes, tightly closed and inverted a few times to mix and read on a Hach DR-3900 spectrophotometer (Hach Company, Loveland, CO).

The sludge activity was calculated per MLSS according to Equation 4:

\[
\text{Sludge activity (S.A.) in } \mu \text{g formazan/mlss} = \frac{\mu \text{g formazan}}{\text{MLSS}}
\]

Equation 4

4.4.4 Microscopic analysis

The principle was to observe the presence of microbes whose activities are relevant to the ASP process and also bio floc formation as also investigated by (Gerardi, 2008). An aliquot of the activated sludge from the aeration tank was taken using a dropper and one drop was placed on a microscope slide and covered with a cover glass (size 24x24 mm).

Care was taken to prevent the activated sludge from extending beyond the edges of the cover glass. The prepared slide was examined interchangeably under 200x, 500x and 1000x magnification using the Olympus BX51 microscope (Olympus Tokyo, Japan). The result was determined on the basis of percentage frequency of occurrence of the relevant microbe at each study phase.

4.5 WATER ANALYSES

4.5.1 pH and O₂

By means of a holder, fixed to the tank, an HQ 40d multimeter pH probe (Hach Company, Loveland, CO.) was placed in the aeration and the anoxic part of the ASP. Approximately 10 minutes was allowed for stabilization after which the pH result was recorded.

Oxygen and temperature of the activated sludge were similarly recorded daily using HQ 40d multimeter oxygen probe as described above. Probe was calibrated weekly.
4.5.2 TSS AND VSS

A Whatman GF/A 55 mm glass fiber filter (pore size 1.6 µm) was placed in a pre-washed filtration funnel and was filtered with deionised water using a vacuum pump. With cleaned forceps, the filter was removed from the funnel carefully to avoid breaking and placed in a petri dish. The petri dish was placed in a Memmert drying chamber (GmbH + Co. KG, Germany) and dried at 105 °C for approximately 1 hour. It was placed in a desiccator to cool and weighed. For the TSS, a pre-washed and dried funnel was placed on a vacuum pump and the pre-treated filter placed in it using clean forceps. 100 ml of gently mixed influent sample was poured gently on the filter paper and filtered. Care was taken to avoid spillage as much as possible. The filter was placed back in the petri dish and put in the Memmert drying chamber and dried out at 105 °C for approximately 1 hour. After drying, it was placed in the desiccator to cool and weighed after cooling. TSS was calculated with Equation 5 according to SFS-EN 872 protocol.

\[
\text{TSS} = \frac{1000(m_2 - m_1)}{V},
\]

Equation 5

\(m_1\) = weight of petri dish and empty filter paper

\(m_2\) = weight of petri dish and filtered sample

\(V\) = volume of filtered sample

For the VSS, a filtered sample used to determine TSS was placed in a Carbolite furnace (United Kingdom) and ignited in at 550 °C for approximately 2 hours. It was placed in the desiccator to cool and weighed after cooling. VSS was determined mathematically from Equation 6.

\[
\text{VSS} = \frac{1000(m_2 - m_3)}{V},
\]

Equation 6

\(m_2\) = weight of dried filtered sample + petri dish

\(m_3\) = weight of ignited filtered sample

\(V\) = volume of filtered sample

The effluent sample was filtered, dried, ignited and determined for the TSS and VSS similarly as described above using 250 ml of the sample.
4.5.3 (COD$_{Cr}$)
COD$_{Cr}$ in the influent and effluent samples were determined spectrophotometrically by using LCK 114 (concentration range 150 – 1000 mg/L) and LCK 314 (concentration range 15 – 150 mg/L) cuvette tests according to the manufacturer’s instructions (Hach Company, Loveland, CO.). The temperature of a Hach L5 200 COD thermostat was pre-raised to approximately 148 °C. 2 ml of both influent and effluent samples were transferred into their respective vials and tightly closed and inverted to evenly mix with default reagent. The vial was wiped with a dry wipe and placed in the reactor for 2 hours. After 2 hours, the vials were allowed to cool and inserted into the cell holder of a Hach DR. 3900 spectrophotometer and the COD levels read.

4.5.4 BOD$_7$ ATU
BOD$_7$ ATU:- in the influent and effluent samples were determined using Oxitop system (WTW, Weilheim, Germany). The analysis was done following the system Oxitop control operating manual. The Oxitop- C measuring heads were cleaned off from dirt and grease to avoid interference in communication between Oxitop- C measuring head and WTW Oxitop OC 110 meter. The BOD$_7$ bottle heads were constantly monitored to assess the progress of oxygen consumption.

4.5.5 Reductions
The percentage (%) reduction of COD$_{Cr}$, BOD$_7$ ATU, TSS and VSS from influent to effluent were determined mathematically from Equation 7.

\[
\% \text{ Reduction} = \left( \frac{\text{Parameter}_{\text{inf.}} - \text{Parameter}_{\text{eff.}}}{\text{Parameter}_{\text{inf.}}} \right) \cdot 100
\]

Equation 7

4.5.6 Microbial analysis
100 ml of both influent and effluent water samples were collected for the analyses.

4.5.6.1 E. coli
Spread plate technique was used to culture the samples and Harlequin TBGA agar (Tryptone Bile-Glucuronide Agar) (LabM, Lancashire, UK) was used as the growth medium. The TBGA was prepared following the manufacturer’s instruction. Dilution series of -1 to -6 for influent sample and 0 to -3 for effluent sample based on preliminary trial dilutions were used and incubated at 44 +/- 1 °C for 18-24 hours inverted. Two parallel plates were prepared.
4.5.6.2 Intestinal Enterococci

Enterococci were cultured using Slanetz- Bartley medium (ISO7899-2) (LabM, Lancashire, UK) for the detection and enumeration of Enterococci in water samples. The growth media so prepared was dispersed into plates and stored (approximately 8-15°C). For culturing intestinal Enterococci, appropriate volumes of the serial dilutions made (-1 to -6 for influent and 0 to -3 for effluent) were inoculated to the plates labeled accordingly using appropriate aseptic techniques in the hood. The plates were left in the hood for a while to dry. They were later transferred into the incubator and incubated upside down at 37 °C for 44 hours approximately. Two parallel plates were prepared for the analysis.

4.5.6.3 F-specific Coliphages

Host (E.coli strain 15597) (ISO 10705-1) was cultured prior to incubations by taking small amount from Tryptone Glucose Yeast Agar (TYGA) and inoculated into 100 ml of TYG broth. It was incubated in a shaker at 37°C overnight and stored at approximately 4-10 °C. For the inoculations, 5 ml of prepared E.coli culture was transferred into 100 ml of TYG broth and incubated in a shaker for 2 hours. Tubes containing 2 ml of previously prepared semisolid TYGA (ssTYGA) were placed in a water bath (50 °C) and melted to liquid and later transferred to another water bath to cool. Serial dilutions prepared (-1 to -6) for influent and effluent (0 to -3) were labeled. 1.0 ml of the serial dilutions was transferred into the ssTYGA tubes according to each corresponding label. This was followed by addition of 0.1 ml triphenyltetrazolium chloride (TTC) in ethanol (prepared from dissolving approximately 0.16 g TTC in 2 ml ethanol). 0.2 ml of host bacterium culture cooled after incubating in shaker for 2 hours was then transferred into ssTYGA tubes. The tubes were swirled gently to mix and the entire content poured onto the TYGA in plates labeled accordingly. The plates were immediately and gently moved in one directional circular motion to spread on the agar.

Care was taken as much as possible not to create bubbles on the plate. The plates were left in the hood for a while to solidify and then placed in the incubator upside down at 37 °C for approximately 18 hours.

4.5.7 Reporting microbial results

Colonies or plaques formed were counted from plates and results were calculated as weighted mean and reported as Colony forming units per ml (Cfu/ml) or Plaques forming units per ml (Pfu/ml) according to Equation 8.
Mathematically weighted mean (Cfu/ml) = $\frac{\sum C_{inf}}{\sum V_{inf}}$, \hspace{2cm} \text{Equation 8}

$\sum C_{inf} =$ total colonies counted in the influent

$\sum V_{inf} =$ total volume of sample inoculated

4.6 DATA ANALYSIS AND STATISTICAL PROCEDURES

Figures of all parameters, average values and reductions for chemical and physical parameters, standard deviations, geometric means and logarithm reductions for microbiological numbers between the different phases of bioaugmentation were drawn using Microsoft Excel. Analysis of data was done using Minitab 17 statistical software. Test of normality was at Anderson-Darling ($\alpha = 0.10$). Non-parametric Mann-Whitney ($\alpha = 0.05$) analysis was used to test data which was not normally distributed. One-way analysis of variance (anova) was used to test the differences between the three dose phases with normally distributed data. Pearson’s correlation analysis was used to draw a correlation matrix between SVI, SA, MLSS and $O_2$. 
5. RESULTS

5.1 SLUDGE ANALYSES

5.1.1 Sludge settling

The 30 minutes settling test showed that sludge from the 120 µl dose phase had higher settling values over the 0 and 20 µl dose phases sludge (p < 0.05) as shown in Figure 5. There was no difference in the sludge settling trend between 0 and 20µl dose phases.

![Fig. 5 Settling trends of sludge (ml/L) from aeration tank recorded during the study period](image)

5.1.2 SVI

During the study period, mean SVIs recorded were for 120 µl 68 ml/g ±32; for 20µl 48 ml/g ± 3.1 and for 0 µl 55 ml/g ± 12 and is represented in Figure 6. These different values recorded from the different dose phases did not differ significantly between each phase.

![Fig. 6 Mean Sludge Volume Index (SVI ml/g) (from aeration tank) recorded at each phase of the process. Bars represent standard deviation (SD).](image)
5.1.3 MLSS and MLVSS

Mean MLSS concentration recorded at the different dose phases were; 120 µl 6.6 g/l ±1.1; 20 µl 4.7 g/l ±0.7; and 0 µl 3.5 g/l ±0.7 as shown in Figure 7. The bioaugmented phases recorded higher MLSS concentrations over the non bioaugmented (p < 0.05). The 120 µl dose also generated a higher MLSS concentration than the 20 µl dose (p < 0.05). There was a corresponding trend with the MLVSS where the highest mean value of 2.3 g/l ±0.5 was recorded with the 120 µl dose (p < 0.05) and lowest mean value of 1.4 g/l ±0.3 was recorded during the 0 µl dose phase. The 20 µl dose phase recorded 1.8 g/l ±0.3 and was also higher than the 0 µl dose (p < 0.05). Excess sludge levels recorded during the study period followed similar trend as in the MLSS. The highest mean excess sludge level of 5.8 g/l ±1.0 was recorded at 120 µl dose phase and the lowest mean level of 3.2 g/l ±0.9 was recorded during the 0 dose phase.

![Mean MLSS and MLVSS from aeration tank concentration (g/l) recorded at each phase of the process. Bars represent standard deviation (SD).](image)

5.1.4 Sludge activity

During the study period, the bioaugmented phases generated the following mean sludge activity values as presented in Figure 8. 120 µl 2.9 µg/l ±0.3 and 20 µl 3.5 µg/l ±0.5. The 0 dose phase recorded 5.6 µg/l ±2.9. These values were not different across the different dose phases.
5.1.5 Microscope examination of biofloc and the presence of microorganisms

Varied presence of wastewater microorganisms and biofloc formation were observed across all three phases of the process. At the 0 dose phase of the process, the presence of formed flocs as well as the presence of microorganisms such as stalked ciliates *Vorticella* and *Epitylis* were observed as shown in Figure 9 A, B and D. However, scattered or unformed flocs were also observed as shown in Figure 9 C. *Amoeba* although not shown in Figure 9 were also present and were present at the point where less unformed flocs occurred. The presence of some fibre-like structures were also observed, these were however not filamentous organisms that is associated with settling challenges.

![Microorganisms and bioflocs formation observed under microscope during 0 dose phase.](image)
At the 20 µl dose phase, formation of flocs was quite pronounced and was marked by elevated presence of *Rotifera*, as shown in Figure 10 A. Stalked ciliates such as *Epistylis* were also present as shown in Figure 10 D. However, scattered and unformed flocs were also observed at some point during this phase and particularly during these periods were the presence of numerous crawling ciliate *Aspidisca* as presented in Figure 10 B. Fibre-like structures were observed at some point during the 20 µl dose phase as seen in Figure 10 C.

![A](image1.png), [B](image2.png), [C](image3.png), [D](image4.png)

**Fig. 10** Microorganisms and biofloc formation observed under microscope during 20µl dose phase.

Dense floc as seen in Figure 11 B, as well as scattered and unformed flocs as shown in Figure 11 A, C and D were observed at different periods during the 120 µl dose phase of the study. The microbial presence varied widely. The presence of testate *Amoeba* was also observed during the period of unformed floc as shown in Figure 11 C. However, the presence of stalked ciliates *Vorticella* as shown in Figure 11 D was also observed although their presence was not pronounced.
There were clear changes in the microbial composition of the sludge at the different dosing phases as shown in Figure 12. During 0 dose period, naked amoebas and free ciliates dominated the system. Also some flagellates and testate amoebas were observed, while the presence of crawling and stalked ciliates and multi-celled organisms was lower. At the 20 µl dose phase, *Rotifera*, crawling ciliates and stalked ciliates clearly dominated. During the 120 µl dose, the dominating organisms were nematodes, flagellates and testate amoeba. Also all other groups were seen at this dose.

![Figure 11](image1.png)  
*Fig. 11* Microorganisms and biofloc formation observed under microscope during 120µl dose phase.

![Figure 12](image2.png)  
*Fig. 12* Observed percentage of occurrence of microorganisms at each phase of the bioaugmentation process during the study period.
5.1.6 Pearson’s correlation analysis of sludge parameters

Correlation matrix at the non-dose phase of the study between SVI, SA, MLSS and O$_2$ revealed mixed correlations. There was a strong correlation between SVI and SA as well as a weak correlation between MLSS and O$_2$ as presented in Table 2. The correlations between the other parameters were insignificant.

**Table 3** Correlation matrix for SVI; (N=7), SA; (N=4), MLSS; (N=14) and O$_2$; (N=20) during the 0 dose phase of the study. Figures represent correlation coefficients and p-values.

<table>
<thead>
<tr>
<th></th>
<th>O$_2$ (mg/l)</th>
<th>MLSS</th>
<th>Sludge Activity</th>
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</thead>
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<tr>
<td>MLSS (g/l)</td>
<td>0.39; p &gt; 0.05</td>
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<tr>
<td>Sludge Activity (µg/l)</td>
<td>-0.31; p &gt; 0.05</td>
<td>0.06; p &gt; 0.05</td>
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</tr>
<tr>
<td>SVI (ml/g)</td>
<td>0.22; p &gt; 0.05</td>
<td>0.32; p &gt; 0.05</td>
<td>0.62; p &lt; 0.05</td>
</tr>
</tbody>
</table>

At the 20 µl dose phase, correlation between SVI, SA, MLSS and O$_2$ revealed a strong correlation between SVI and SA and weaker correlations between SVI and MLSS and a weak correction between MLSS and O$_2$ (Table 3).

**Table 4** Correlation matrix for SVI; (N=7), SA; (N=4), MLSS; (N=14) and O$_2$; (N=20) during the 0 dose phase of the study. Figures represent correlation coefficients and p-values.

<table>
<thead>
<tr>
<th></th>
<th>O$_2$ (mg/l)</th>
<th>MLSS</th>
<th>Sludge Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLSS (g/l)</td>
<td>0.42; p &gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sludge Activity (µg/l)</td>
<td>0.06; p &gt; 0.05</td>
<td>0.28; p &gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>SVI (ml/g)</td>
<td>0.26; p &gt; 0.05</td>
<td>0.45; p &lt; 0.05</td>
<td>0.68; p &lt; 0.05</td>
</tr>
</tbody>
</table>

At the 120 µl dose phase, a strong correlation was recorded between SVI and O$_2$ and. A weak positive correlation was recorded between SVI and SA as represented in Table 4.

**Table 5** Correlation matrix for SVI; (N=7), SA; (N=4), MLSS; (N=14) and O$_2$; (N=20) during the 0 dose phase of the study. Figures represent correlation coefficients and p-values.

<table>
<thead>
<tr>
<th></th>
<th>O$_2$ (mg/l)</th>
<th>MLSS</th>
<th>Sludge Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLSS (g/l)</td>
<td>0.15; p &gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sludge Activity (µg/l)</td>
<td>0.22; p &gt; 0.05</td>
<td>0.36; p &gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>SVI (ml/g)</td>
<td>0.77; p &lt; 0.05</td>
<td>0.37; p &gt; 0.05</td>
<td>0.44; p &lt; 0.05</td>
</tr>
</tbody>
</table>
### 5.2 WATER ANALYSES

#### 5.2.1 pH and oxygen

Oxygen content and pH recorded during the study period are represented in Figure 13. The pH during the 20 µl dose and the O₂ during the 0 dose showed a generally higher trend than during the other phases of the study. At the 20 µl dose phase, the O₂ trend recorded was generally low. The 13th pH value during the 0 µl dose phase appeared to be lower from the trend and this could be as a result of system challenges during the 0 µl dose phase.

![Graph showing pH and O₂ (mg/l) measured from aeration tank during the study period.](image)

**Fig. 13** pH and O₂ (mg/l) measured from aeration tank during the study period.

#### 5.2.2 Suspended solids

Figure 14 represents the amounts of suspended solids in the influent at each phase of the process. The highest mean suspended solids value of 200 mg/l ±45 was received during the 20 µl phase. The 120 µl phase had 185 mg/l ±33 and 0 dose phase 172 mg/l±26. The organic portion of the influent (VSS) was also highest 178 mg/l ±39 at the 20 µl dose phase and lowest 155 mg/l ±20 at the 0 dose phase. VSS recorded at the 120 µl phase was 160 mg/l ±54.

In the effluent, the highest suspended solids 28 mg/l ±19 was recorded during the 0 dose phase and the lowest 19 mg/l ±16 was recorded during the 20 µl dose phase as shown in Figure 15.
The mean percentage reduction in suspended solids during the bioaugmented phases were $\mu l$ 88% ± 7 and 87% ± 14 with the 120 $\mu l$ and 20 $\mu l$ doses, respectively. The 0 dose phase recorded 84% ± 9. The reductions in the suspended solids concentration were not statically different throughout all the phases of the study. However during the 20 $\mu l$ dose phase, the reduction values had a wider variation as represented in Figure 16.
5.2.3 COD\textsubscript{Cr} and BOD\textsubscript{7 ATU}

Figure 17 represents the mean COD\textsubscript{Cr} and BOD\textsubscript{7 ATU} in the influent samples during the study. The 20 µl phase of the study had the highest levels of both COD\textsubscript{Cr} = 501 mg/l ±100; BOD\textsubscript{7 ATU} = 257 mg/l ±51. The lowest values were at the 0 dose phase COD\textsubscript{Cr} = 390 mg/l ±55; and BOD\textsubscript{7 ATU} 170 mg/l ±25.

The highest mean COD\textsubscript{Cr} and BOD\textsubscript{7 ATU} in the effluent were recorded during the 0 dose phase (COD\textsubscript{Cr} 33 mg/l ±24; BOD\textsubscript{7 ATU} = 5.3 mg/l ±0.5) while the lowest were recorded during the 20 µl dose phase (COD\textsubscript{Cr} = 15 mg/l ±5.6; BOD\textsubscript{7 ATU} 1.6 mg/l ±0.9) as shown in Figure 18.
The percentage reductions in the COD$_{C_r}$ as shown in Figure 19 revealed that the 120 µl and 20 µl Free Flow applications recorded similar percentage reduction levels: 120 µl = 97% ±0.5 and 20 µl = 97 % ±1.1. These were higher than 91% ±7.0 recorded with the 0 dose phase (0 and 20 µl p < 0.05; 0 and 120 µl p < 0.05. Mann-Whitney test). The percentage BOD$_{7ATU}$ removals recorded 97 % ±0.4 at the 0 dose phase. The bioaugmented phases recorded 120 µl = 99% ±1.0 and 20 µl = 99 % ±0.4 as represented in Figure 19. The bioaugmented phases performed higher reductions compared to the non-bioaugmented phase; (0 and 20 µl p < 0.05; 0 and 120 µl p < 0.05. One way anova). However, there was no difference statistically between the reductions at the bioaugmented phases.
5.2.4 Microbial density

There was a large variation in the concentrations of the microbial groups studied in the influent samples. The lowest values for all groups were recorded in the first periods of the study (marked with 0 dose) as shown in Figure 20.

![Fig. 20](image)

**Fig. 20** Geometric means of microbial density in the Influent samples; *Escherichia coli* (E. coli), Intestinal *Enterococci* (Ent) and F- specific RNA coliphages (F-RNA) at each phase of the process. Bars represent Geometric standard deviation (GSD).

Microbial density in the effluent was several orders of magnitude lower than was in the influent, from (Figure 21).

![Fig. 21](image)

**Fig. 21** Geometric means of microbial density in the Effluent; *Escherichia coli* (E. coli), Intestinal *Enterococci* (Ent.) and F- specific RNA coliphages (F-RNA) recorded at each phase of the process. Bars represent Geometric standard deviation (GSD).

At the 0 dose phase, mean logarithmic reduction was 2.7 ±0.3 for *E. coli*, 2.4 ±0.4 for Intestinal *Enterococci* and 2.9 ±0.5 for F-coliphages. At the 20 µl dose phase, mean logarithmic reduction was 2.9 ±0.5 for *E. coli*, 2.6 ±0.6 for Intestinal *Enterococci* and 3.3 ±0.5 for F-specific coliphages and at the 120 µl dose phase, mean logarithmic reduction was 3.2 ±0.5 for *E. coli*, 2.9 ±0.6 for Intestinal *Enterococci* and 3.7 ±0.5 for F-specific coliphages.
**Fig. 22** Mean $\log_{10}$ reduction in microbial density recorded at each phase of the process during the study period. *Escherichia coli* (E. coli), Intestinal *Enterococci* (Ent.) and F-specific RNA coliphages (F-RNA). Bars represent standard deviation (SD).

The reductions during the bioaugmented phases (20 µl and 120 µl doses) compared with the 0 dose phase were not different across all the microbes. However, the mean reduction in the F-specific coliphages population at the 120 µl dose phase was higher ($p < 0.05$) compared to the 0 dose phase.
6. DISCUSSION

6.1 SLUDGE CHARACTERISTICS

A significantly higher mean MLSS was recorded during the bioaugmented phases of the study over the non-bioaugmented phase. The 120 µl dose phase recorded the highest MLSS concentration followed by the 20 µl dose phase. Since the bioaugmentation is addition of microorganisms to the ASP, it perhaps led to increase in MLSS. According to the operation of conventional ASP, the activities of the desired microorganisms in breaking down suitable degradable organic matter in wastewater leads to an increase in the original microorganism present in the ASP which is seen in the measurement of MLSS. It follows that the application of a dose of bacteria which will lead to an increase in microorganisms population will most likely lead to a further increase in MLSS. A higher application of bacteria will then increase the microorganism activities leading to a higher MLSS. This was the case in this study as the 120 µl dose produced a significantly higher (6.6 g/l ±1.1) MLSS compared to the 20 µl dose (4.7 g/l ±0.7). This increment in biomass has been found by Speece et al. (1973) who realized an increase in MLSS upon the increase of microorganism in contact with organic matter. They also realized an increase in biomass before an increase in microorganism, indicating storage of substrate in some form showing regular performance of the ASP. Norman and Tramble (2011) also recorded an increase in MLSS after bioaugmenting wastewater treatment plant and comparing with a baseline with no bioaugmentation. The work of Norman and Tramble (2011) revealed an increase in MLSS with different doses of bioaugmentation agent in line with this study. The trend in the MLSS results was also recorded with the MLVSS and this was in line with Gerardi, (2002) who stated that an increase in volatile content of MLSS represents an increase in the bacterial population, whereas a decrease represents a decrease in the bacterial population. It is worth noting that excess sludge removed was also consistent with this trend.

The sludge settling test conducted revealed that at the phase where a higher dose of mixed culture of microorganisms (120 µl Free Flow) was added to the ASP, settling recorded higher (Figure 5) values. A lower (20 µl Free Flow) and no dose followed in that order. Varied reasons could be attributed to this phenomenon, however the production of more MLSS could have been the most probable cause. This is because the higher MLSS concentration and hence more sludge production during the bioaugmentation phase was consistent throughout the study. Settling during the second (62ml/l) and the fifth (75ml/l) weeks of the 120 µl dose
phase (Figure 5) was higher as a result of poor settling event. One of the reasons for poor settling in the ASP is the presence of filamentous organisms such as *Sphaerothilus natans*, Type 1701, *Nocardia* spp. Type 021N, Type 0041, Type 0092 and *Microthrix parvicella* (Stypka, 1998). The presence of filamentous microorganisms however was never encountered in this study as shown in the microscope examination. Novak et al. (1993) worked on the phenomenon of non-filamentous bulking and have assigned the fact that the kind or source of wastewater used may support the formation of Zoogleal growth if the possibility of insufficient amount of certain nutrients in the sludge exists among other reasons. According to Stypka, (1998), significant production of exocellular polymers which is a characteristic of Zoogleal bulking could also be related to the change of wastewater composition. In this study, wastewater for the ASP was obtained weekly over a four-month period with contrasting whether conditions and therefore the differences in the composition of the wastewater used could have impacted on the settling of the process in line with (Stypka, 1998). Presumably, brief operational difficulties encountered at the 120 µl dose phase such as blockages in the tubes which resulted in the accumulation of sludge in the anoxic and aeration sections and accumulation of Ca(OH)\(_2\) in the aeration tank where sample for the settling test was grabbed. Although this anomaly was rectified immediately, it cannot be fully overlooked considering that sampling proceeded at those periods. These factors could contribute to the distorted settling recorded during those weeks. It is worth noting that most researchers have not defined a correlation between system challenges in ASP and settling. Stypka (1998) therefore proposes more work in determining how fundamentals of ASP such as design, operating conditions and wastewater characteristics affect sludge settling. Again the dynamics of settling will be a complex event. Generally settling depends on the extent of floc formation and floc formation according to Murthy and Novak (1998) is not well understood. From its composition of microbial aggregates, filamentous organisms, organic and inorganic particles and exocellular polymers (Bruus et al. 1992; Higgins and Novak, 1997 a,b), it will be difficult to make a definite conclusion on an aspect of the composition which is a problem in the formation and by extension settling in the final clarifier.

A higher SVI (68 ml/g ±32) was recorded at the 120 µl dose phase while the lowest (48 ml/g ± 3.1) was at the 20 µl phase. A deviation in the SVI trend with the settling test was that the 0 dose recorded a higher SVI than the 20 µl phase. This situation is an indication that settling challenges might have occurred at the 0 dose phase. The settling character of the 20 µl dose
phase was generally higher than the 0 dose phase. These values were however well under 100 ml/g which is most desired in the normal operation of ASP (Ritmann and McCarty, 2001).

Correlation matrix between SA, SVI, MLSS and O₂ at all dose phases revealed mixed statistical relevance. There was strong correlation between SVI and SA across all the study phases. There was also a weak positive correlation between MLSS and O₂. These mixed or irregular correlations could be as a result of the discrepancies in data numbers.

Sludge Activity was high at the 0 dose phase and low during the bioaugmentation phases. The differences were not significant. According to Gray (1990), assessing SA measures the viable cells. It will then follow that since more living cells has been introduced by way of the Free Flow doses, there will be a corresponding rise in the SA. This was not achieved. However many different wastewater characteristics including the presence of metals is an inhibitory factor to sludge biological activity (Stypka, 1998). This study did not cover heavy metals analysis in the sample therefore the distortions in the SA levels could not be accounted for.

During 0 dose period, naked Amoeba and free ciliates dominated the system. Also some flagellates and testate amoebas were observed, while the presence of crawling and stalked ciliates and multi-cellular organisms was lower. The dominating organisms indicate a young, not a very stable system (Gerardi, 2008). The presence of flagellates and amoeba, which consume organic matter like bacteria do, indicates a lower bacterial activity (Rittman and McCarty, 2001). At the 20 µl dose phase, Rotifera, crawling ciliates and stalked ciliates clearly dominated. All of these organisms need a stable floc to survive, so it seems that during 20 µl dose the floc was very good, as seen from (Fig. 20 A, C and D). The amount of flagellates and Amoeba was low, indicating a good bacterial activity in the sense that these flagellates and Amoeba were out competed for nutrition as they consume the same food as bacteria thereby reducing their numbers. During the 120 µl dose, the dominating organisms were nematodes, flagellates and testate Amoeba. Also all other groups were seen during this dose. It seems that the environment has been changing, as the dominating groups are indicators of both a young and a mature system. The indicators of strong floc (crawling and stalked ciliates) were observed only scarcely. The problems with Ca(OH)₂ has had its effect on microorganisms. Again the periodic low oxygen content of the system during the rectification of Ca(OH)₂ challenges might have reduced the Rotifera population at this phase as these microorganisms are known to be sensitive to this negative phenomenon(Gerardi, 2008).
On the overall, the influence of the components of the influent on the basis of spatial and temporal variability cannot be overlooked. This is the case for both industrial and municipal wastewater with industrial wastewater regarded as having more intense variation in components. These variations in components have an impact on degree of degradability and final effluent concentration (Orhon et al., 2009).

6.2 WATER ANALYSES
6.2.1 TSS
The influent TSS recorded during the study period revealed that the sample had a higher (200 mg/l ±45) concentration of TSS during the 20 µl dose phase while the 0 dose phase had the lowest concentration of TSS. The nature of wastewater varies between space and time. This is the case for both industrial and municipal wastewater with industrial wastewater regarded as having more intense variation in components (Riffat, 2013). From this context, the differences in the influent suspended solids recorded during the study period could have only been as a result of the different times when the wastewater sample was obtained from the WWTP since by inspection, the physical appearance of the sample was different for the different sampling times. Moullec et al. (2011) stated that real wastewater is difficult to handle in laboratory since its composition changes over days, whereas a steady composition is necessary to perform reproducible experiments. These variations in influent quality have an impact on degree of degradability and final effluent concentration (Orhon et al. 2009). The reduction in the TSS from the influent to the effluent showed that, although the influent fed to the ASP at the non-dose (0 dose) phase had the lowest load of suspended solids, the bioaugmented phases recorded slightly higher mean reductions even with high suspended solids load. The effluent TSS again shows that the addition of the Free Flow agent resulted in a clearer effluent than when no Free Flow was added. By extension, the increment in the MLSS during the introduction of Free Flow doses might have improved the settling of the sludge. This hypothetical situation is confirmed in Jin et al. (2003), when they quoted that a good effluent quality is achieved with proper sludge settling and MLSS concentration. It can then be assumed in principle that the higher sludge settling values at the 120 µl were from a higher MLSS production and not settling challenges since a clearer effluent was recorded from the TSS reduction results. These results came in the face of system challenges that at one point or the other could have possibly affected the microbial activity in the process. This notwithstanding, the mean TSS of secondary wastewater treatment facility and the reduction recorded in this study were within the Urban Wastewater Treatment Directive (91/271/EEC)
of the European Union and 1973 USEPA standard for secondary plants treating municipal wastewater (35 mg/l). It is also worth noting that the mean percentage reduction during the 0 dose phase of the process was however below the standard requirement by USEPA which sets 85% removal efficiency as the minimum. The highest mean reduction was recorded by a higher dose (120 µl) although the difference between the lesser dose (20 µl) was not statistically significant. This implies the TSS removal efficiency increased upon the introduction of Free Flow agent to the process aiding the process to surpass the minimum USEPA and EU requirements.

6.2.2 COD\textsubscript{Cr} and BOD\textsubscript{7ATU}

The reduction performance in COD\textsubscript{Cr} between the bioaugmented phases revealed no significant statistical difference compared with the non dose phase. Similarly, the reduction performance in BOD\textsubscript{7ATU} between the bioaugmented phases revealed no statistical significant difference, however the bioaugmented phases performed higher reductions over the non dose phase. This was a departure from the trend recorded with the MLSS where each dose resulted in a significantly different concentration. Nonetheless, a dose-response relationship appeared to exist between the bioaugmented and the non bioaugmented phases in COD\textsubscript{Cr} and BOD\textsubscript{7ATU}. The resulting reduction performance from these phenomena is better biodegradation of organic materials and a corresponding clearer effluent in BOD and COD. It is important to note that the phenomenon where minimal BOD reduction is achieved between different doses of mixed microbial culture could be due to potential inhibitory factors such as unfavorable temperature variations. Kikot et al. (2010) found pH instability as an inhibitor compared to metals in their bioremediation studies. This is in contrast to the findings of Stypka (1998) on metals as the inhibitor of SA. In line with Kikot et al. (2010) however, the challenges encountered with the Ca(OH)\textsubscript{2} deposition events in the aeration tank during the 120 µl dose phase might have impacted negatively on the reduction performance of the microbes. This notwithstanding, the mean reductions in both BOD\textsubscript{7ATU} and COD\textsubscript{Cr} met the US EPA revised for 2012 Riffat (2013) standard requirements of 45 mg/l (BOD\textsubscript{7}) and 40 mg/l (COD).

6.2.3 Microbial density

A reduction in E. coli, Enterococci and F-RNA coliphage populations covered in this study was achieved over the non-bioaugmented phase on the application of Free Flow. This is an indication that conditions were created for their being used as part of floc formation. The presence of Amoeba during the 0 dose phase and the subsequent reduction in their presence
during the bioaugmented phases clearly indicated that more nutrients and other microbes were being consumed from increasing Protozoan population resulting in high MLSS concentration as well as dense floc which settled out. From Rittmann and McCarty, (2001), most microbes in wastewater are attached to flocs outside or within, hence well formed floc will settle out resulting in a more hygienic effluent. This study recorded a reduction in the microbial population covered in the effluent and this improvement could possibly be due to the performance of Free Flow since the slight process disturbances encountered could have slowed their activities. The 120 µl dose performed the highest reduction with all the microbes covered and the most successful reduction was achieved with the F-specific RNA coliphage microbial population.
7. CONCLUSIONS

Compared to the non bioaugmented phase of this study, sludge parameters covered including MLSS, MLVSS and sludge settling, increased upon the addition of the different doses of the bioaugmentation agent with the highest increase occurring with the highest dose of 120 µl. Also floc formation improved and microbial occurrence varied in favour of improved performance characterized by the prevalence of Rotifera, crawling ciliates and stalked ciliates. The increasing trend recorded with the sludge parameters was also recorded with wastewater parameters which included increased reduction in TSS, VSS, COD\textsubscript{Cr}, BOD\textsubscript{7ATU} and microbial population (E. coli, Enterococci and F-specific RNA coliphage) resulting in a clearer effluent. The 120 µl dose performed a higher reduction with all the microbes covered and the most successful reduction was achieved with the F-specific RNA coliphage microbial population. However, the application of the doses could not influence TSS reduction levels significantly. A shift in the trend of the sludge parameters was recorded with SA and SVI, the SA recorded at 0 µl dose phase was higher than found during the bioaugmented phases and the probable cause could have been inhibitory events while higher SVI was recorded during the 120 µl dose phase and lowest was recorded at the 20 µl. However the SVI recorded during the 120 µl phase was within 100 ml/g which is an indication of good settling properties of the sludge formed in the ASP.
REFERENCES


Drinking water directive (98/83/EC) of 1998


Urban Wastewater Treatment Directive (91/271/EEC) of the European Union


Water framework directive (2000/60/EC) of 2000


