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Wastewater Protozoan-Driven Environmental Processes for the Protection of Water Sources

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1. Introduction

Since the middle of the last century, while the population doubled, water use has tripled. The world's freshwater reserves are dropping year after year and becoming a scarce commodity. On the one hand, the utilization of synthetic detergents, the increased use of agricultural inorganic fertilizers and the population explosion have led to the acceleration of the process of pollution of lakes and other surface water. Reports have shown an increasing percentage of rivers and streams that are contaminated with chemical and microbial pollutants (Momba et al., 2004; 2005; Bahlaoui et al., 1997; Clarke et al., 2008; Pennil et al., 2008). This is compounded by the alterations in the hydrological cycle, associated with the global climate change that increases the magnitude and frequency of runoff events (Rose et al., 2001). On the other hand, the world is faced with problems related to the management of wastewater due to the extensive industrialization, increasing population density and a highly urbanized society (McCasland et al., 2008). Recycling municipal and industrial wastewater is therefore essential for reducing the negative impact of pollution on the freshwater reserves and also for protecting public health by safeguarding water supplies against the spread of waterborne diseases (Bitton, 1999).

The first part of this chapter discusses the importance of wastewater treatment for the protection of water resources. The second part sheds light on the role protozoa play in the excess removal of phosphate and nitrate in wastewater treatment plants, with emphasis on the removal efficiency of two ciliates (*Aspidisca, Trachelophylum*) and one flagellate (*Peranema*). The third part reveals the predation potential of these protozoan species on pollution indicators and pathogenic bacteria (*Escherichia coli* O157:H7, *Salmonella typhimurium, & Shigella flexneri* spp.).

2. Wastewater treatment for the protection of water resources

Before wastewater treatment was required, raw wastewater was discharged directly into streams and lakes. Despite significant and prolonged environment damage, major environment legislation did not take effect until the 1970s (Gerardi & Zimmerman, 2005). To maximize the health and environmental benefits associated with the use and discharge of wastewater, great deals of legislation and several guidelines have been developed. The World Health Organization (WHO) Guidelines for the use of effluents were developed in

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1973, with revised editions in 1989 and 2006. The World Health Organisation (WHO) establishes the limit for nitrates in drinking water at 50 mg NO3/L (or 11.3 mg NO3-N/L). However, the US Environmental Protection Agency (EPA) has set this limit at 10 mg NO3/L (2.3 mg NO3-N/L) (WHO, 2006).
Because guidelines must be considered in the context of national environmental, social, economic and cultural conditions, several countries have formulated their own regulations. In the United State of America, the Clean Water Act was amended in 2002. The objective of the Act is to restore and maintain the chemical, physical and biological integrity of the United States water and to prevent, reduce and eliminate pollution (Clean Water Act, 2002). The European Community, on the other hand, has established the maximum admissible levels of nitrates plus nitrites in drinking water at 50 mg NO3/L (Directive 98/83/EC). With regard to discharges, the admissible concentration depends on the receiving environment, and usually ranges between 10 and 30 mg NO3-N/L for discharges into fresh water and 50 mg NO3-N/L for discharges into seawater. These limits are lower if the discharges occur in "sensitive areas", ranging between 10 and 15 mg Total-N/L (Directive 91/271/EEC). In South Africa, the National Water Act of 1998 was enacted. The Act makes provision for the legal requirements, registration and authorization for the discharge of wastewater into a water sources (National Water Act, 1998). Also in the Act are the general wastewater discharge limits. According to the Act, there must be compliance with the set limits before wastewater can be discharged into receiving water bodies or used for irrigation. The Act of 1998 established the limits ranging between 2 and 6 mg/L for ammonia nitrogen, between 1.5 mg/L and 15 mg/L for nitrate/nitrite and between 1 and 10 mg/L for orthophosphate as phosphorus in the effluent discharge. These guidelines are an integrated and preventive management framework for maximizing the public health benefits of wastewater use and discharge.
Wastewater collection and treatment systems have been designed and built for the following purposes: (i) to provide clean water for cities undergoing rapid industrialization, (ii) to protect the quality of the waters receiving the effluent from wastewater treatment plants, and (iii) to control the outbreaks of communicable diseases. Outbreaks of communicable diseases are often related to poor sanitary conditions (Gerardi and Zimmerman, 2005). This section focuses on the characteristics of wastewater and the most common biological processes used for wastewater treatment, with the emphasis on nitrogen and phosphorus removal.

2.1 Characteristics of wastewater

The chemical characteristics of wastewater that are of special concern include pH, dissolved oxygen (DO), oxygen demand (chemical and biological), nitrogen (nitrite and ammonia), phosphate and metals (heavy and trace) (Larsdotter, 2006). The pH values of lower than 5 and greater than 10 indicate the presence of industrial waste and non-compatibility with biological operations (Gray, 2002). The DO is required for the respiration of aerobic microorganisms as well as other aerobic life forms. Eutrophication due to excessive amounts of nutrients contributes to the depletion of dissolved oxygen (Momba et al., 1997). The high concentration of inorganic phosphate and nitrogen in lakes and other aquatic environments is often the major cause of algal and bacterial blooms. These degrade the quality of water sources by giving the water an offensive appearance, odour and taste, and they consume oxygen. The discharge of nitrogen into receiving waters exerts a high oxygen demand at night, which adversely affects fish and other aquatic life, and has a negative

impact on the beneficial use of water resources for drinking or recreation. Nitrogen compounds discharged into the environment can also cause hazards to human health. Furthermore, nitrates can form nitrosamines and nitrosamides, potentially carcinogenic compounds (Ono et al., 2000). The prevention of eutrophication can be achieved by removing phosphate and nitrogen from wastewater by chemical and biological methods or a combination of the two. The chemical removal used in most countries is expensive and results in the accumulation of large quantities of chemical waste sludge. This disadvantage has led to intensified investigations into biological phosphate and nitrogen removals, which provide a more cost-effective alternative to chemical treatment methods.

Wastewater not only constitutes an important source of chemical pollutants, but also a reservoir of pathogenic microorganisms. The major microorganisms found in wastewater influents are viruses, bacteria, fungi, protozoa and helminthes. On a daily basis, a large number and diversity of pathogenic disease-causing organisms enter sanitary sewer systems and wastewater treatment plants. These pathogens enter sewer systems from domestic wastewater and industrial wastewaters through inflow and infiltration. Pathogenic bacteria, viruses and protozoan parasites can be found in the sewer system in the wastewater, sediment, and biofilm and at wastewater treatment plants in wastewater, sludges, bioaerosols, contaminated surfaces, foam, recycle streams and scum (Gerardi, 2006, Momba & Sibewu, 2009). The biological removal mechanisms of pathogenic organisms include antibiosis, exposure to biocides, predations, and attack by litic bacteria, natural die-off and competition for limiting nutrients or trace elements (Green et al., 1997). Predation of bacteria by protozoa has been reported to be the main mechanism that contribute to the removal of bacteria in wastewater treatment plants, and, more importantly, ciliates have been actually found to play a dominant role (Curds, 1992; Pauli et al., 2001).

2.2 Biological phosphorus and nitrogen treatment processes

The biological phosphorus and nitrogen removal systems have been extensively investigated for municipal wastewater treatment and significant advances have been made in the area of engineering (design) and technology (implementation and operation) of the single activated sludge system. A large number of biological processes have been developed for the combined removal of nitrogen and phosphorus. Many of these processes use a form of activated sludge system process, but employ a combination of anaerobic, anoxic and aerobic zones to accomplish nutrient removals. Activated full-scale sludge systems have been successfully designed and implemented to progressively include aerobic COD removal and nitrification, anoxic denitrification and anaerobic/anoxic/aerobic excess phosphorus removal. This implementation has been aided by the development of a suite of steady state design and kinetic simulation models (Ekama et al., 84; Wentzel et al., 1992).

The activated sludge process was originally developed for carbon, nitrogen and phosphorus removals. The basic process for the simultaneous biological removal of phosphorus and nitrogen was proposed by Barnard in 1976 and is known as the Phoredox process in South Africa and Bardenpho or Modified Bardenpho in the United States. The Bardenpho activated sludge process is a four-stage process designed mainly for removing nitrogen, whereas the Phoredox activated sludge process is a five-stage process designed to remove nitrogen and phosphate. Nevertheless, the two systems consist of a sequence of primary anoxic, primary aerated, secondary anoxic, and a secondary aerated basin, followed by a clarifier. In the Phoredox activated sludge process, the incorporation of an anaerobic zone at

some point in the process allows the release of phosphate; and phosphate, after being released from the biomass in the anaerobic zone, is reincorporated in the biomass during aerobiosis, together part or all the influent phosphate (Gerber et al., 1986; Momba and Cloete, 1996a,b).

In addition to the Phoredox and Bardenpho processes, other biological nutrient-removal (BNR) systems have been developed and implemented. These new BNR systems include the A^2 /O process, the University of Cape Town or UCT process, the modified UCT process and the Virginia Initiative Process or VIP. The A^2 /O™ process was developed in the United States of America. It is one of the simplest biological phosphorus removal systems. The returned activated sludge is mixed with the incoming wastewater and this mixed liquor passes through an anaerobic zone and then through an aerobic zone (Muyima et al., 1995). This process provides relatively low phosphorus removal due to high nitrate recycle to the anaerobic zone. Its layout is similar to the modified Phoredox in general, but the reactor has all its stages divided into complete mixed cells. The process is used for high-loaded activated sludge systems (Rybicki, 1997). The UCT process is derived from the Phoredox process. In this process, both the return activated sludge and the aeration tank contents are recycled to the anoxic zone, and the contents of the anoxic zone are then recycled to the anaerobic zone. This recycle sequence decreases the chance of introducing residual nitrate into the anaerobic zone. The internal recycle can be controlled to maintain zero nitrates in the effluent from the anoxic reactor, thereby ensuring that no nitrates will be returned to the anaerobic reactor. For weak wastewater, the UCT process can achieve both phosphorus and partial nitrogen removal to 6.8 mg/L (Water Environment Federation, 1996; Gray, 2002). In the modified UCT process, the anoxic zone is subdivided into parts: the first anoxic zone receives sludge recycle while an anoxic-anaerobic mixed liquor recycle is taken from it. The second anoxic zone part receives aerobic-anoxic mixed liquor recycle. The advantage of this process is that the first anoxic part is designed to reduce only the nitrate nitrogen in the return sludge, which prevents nitrogen intrusion in the anaerobic zone (Muyima et al., 1995; Rybicki, 1997; Tchobanoglous, 2003). The VIP was developed by Hampton Roads Sanitation District and the engineering firm CH2M Hill. In this process, which is similar to the UCT process, all the zones are staged to consist of at least two completely mixed cells in series (Metcalf and Eddy, 2003). The process includes the anaerobic, anoxic and aerobic zones.

2.3 Microorganisms involved in the biological nutrient removal

The concentration of total phosphate should not exceed 2 mg/L for installations of 10,000 - 100,000 i.e., and 1 mg/L for installations designed for more than 100,000 i.e. The respective criteria for nitrogen are 15 and 10 mg/l. Member states are expected to comply for discharges above 15,000 i.e. (Bowker & Stensel, 1990). Biological wastewater treatment systems rely on the interaction and metabolisms of microorganisms. These systems essentially depend on the capacity of the microbial community to recycle elements by way of biogeochemical cycles.

The composition of the microbial community is determined by the type of nutrient content of the wastewater and a number of other selective pressures such as mean cell retention time, aerobiosis, anaerobiosis, temperature and other extrinsic factors (Cloete & Muyima, 1997). In biological wastewater treatment systems, bacteria and other microorganisms breakdown and metabolize the COD to 10-100mg/L (Eckenfelder et al., 1992). Water is aerated and microorganisms convert the organic carbon to carbon dioxide and into cell

biomass. Biomass is separated from the treated wastewater in the clarifier for recycling or wasting to solids-handling process.

Over the past three decades, most investigations have been concentrated on the important role of bacteria in the biological treatment of wastewater influents. The bacterial population has become important in the operation and control of the biological phosphorus and nitrogen removal processes. Parallel to the development in engineering and technology of the activated sludge system, significant advancements have been made in the bacteriological and biochemical analytical methods. In these areas, researchers have increasingly moved away from pure culture work to a number of new analytical techniques for the study of in situ bacterial population, for example, ATP analysis (Nelson & Lawrence, 1980), DNA analysis (Liebeskind & Dohmann, 1994), quinine profiling (Hu et al., 1999) and the use of fluorescent probes for ribosomal RNA (Wagner et al., 1994). The 16S RNA-based clones libraries or denaturing gradient gel electrophoresis have resulted in a number of high-diversity groups of bacteria involved in enhanced biological nutrient removal (Zeng et al., 2003).

Phosphate release in the anaerobic zone followed by excess phosphate uptake in the aerobic zone constitutes the main characteristics of an activated sludge system. The active biomass is returned to the reactors after settling out in a clarifier. Polyphosphate and insolubilised mineral phosphate are important fractions of activated sludge, because phosphate removal efficiency in the activated sludge process depends mainly on the sludge phosphate content.

The polyphosphate is accumulated in the sludge, accompanied by potassium and magnesium accumulation. Magnesium and potassium are therefore known to be essential to enhanced phosphate removal (Toerien et al., 1990; Momba % Cloete, 1996). Sludge age is also an important factor in the removal of phosphate, because the time that the microorganisms are given to break down the waste products has a significant effect on effluent quality. Sufficient time must be permitted for microorganisms to be in contact with the waste to accomplish the treatment. A sludge age ranging from 15 to 20 days is therefore required for the removal of phosphate in activated sludge systems (Toerien, 1990). Previous investigators have also shown the role of carbon sources in nutrient removal from wastewater (Kargi et al., 2005; Akpor et al., 2008).

Studies have shown that *Acinetobacter* sp. is of little significance in phosphate removal when compared to members of other phylogenic groups of bacteria (Momba & Cloete, 1996a,b; Band et al,. 1999; Jeon et al., 2003). Other bacteria such as *Aeromonas, Vibrio, Pseudomonas,* and coliforms have been implicated in dominant polyphosphate-accumulating organisms (PAO) (Momba and Cloete, 1996a,b; Snaidr et al., 1997; Seviour et al; 2003). A series of studies by Momba and Cloete (1996a,b) on the relationship between biomass concentrations and phosphate uptake have demonstrated the role of initial biomass concentration of PAO bacteria such *Acinetobacter junii, A. radioresistens, Pseudomonas fluorescens* and *Escherichia coli* to remove phosphate from a mixed liquor medium in a laboratory-activated sludge scale system using different initial biomass cell concentrations (from 10^4 to 10^8 cells/mL). In this study, phosphate removal was biomass and growth-stage related. The results showed a relationship between a high initial cell density and phosphate uptake. *Acinetobacter junii* and *P. fluorescens* at a high initial biomass concentration of 10^8cell/mL removed all the 28.25 mg/L phosphate during the entire duration of the 24 h growth study. Low initial biomass concentrations triggered the release of phosphate once transferred into the mixed liquor. The release of phosphate increased during active growth and uptake occurred when cells

reached the stationary growth phase. The rate of the phosphate removal increased during the stationary growth phase for the *A. junii* and during the logarithmic growth phase for *P. fluorescens*. Enhanced phosphate uptake in both cases was related to the final cell yield in the culture media. *Acinetobacter radioresistens*, and *E. coli* at 10^6 cells/mL and/or 10^7cells/mL (initial cell density) removed phosphate during the first hour of the lag growth phase (17.14mg/L and 15.14mg/L for *A. radioresistens*, 6.64mg/L for E. coli). Some accumulated phosphate was released back into the medium during active growth. Both these species removed some phosphate during the stationary growth phase. *Pseudomonas fluorescens* removed more phosphate compared to *A. radioresistens* and *E. coli* with a specific rate of 3.00 – 28.50 for *P. fluorescens* , 4.92 – 17.14 mg/L for *A. radioresistens* and 0.50 -8.50 mg/L for E. coli. Finally, the most favorable net phosphate removal from mixed liquor was obtained in all cases by using a high initial biomass concentration (Momba & Cloete, 1996a, b).

The biological nitrogen removal process generally results from the combined nitrification and denitrification processes (Wentzel, 1991; Carrera et al., 2003; Oguz, 2005). In the nitrification process, the first step is the conversion of ammonia to nitrite by Nitrosomonas, while the second step is the further oxidation of nitrite to nitrate, which is commonly accepted to be carried out by Nitrobacter (Antoniou et al., 1990; Sedlak, 1991). It has been estimated that 80 % of the energy generated by nitrifier autotrophs is used to fix carbon (iv) oxide (Painter, 1970; Prosser, 1989; Sabalowsky, 1999). Unlike autotrophic nitrification where nitrification is required in order to generate energy necessary for growth, it is generally accepted that heterotrophic nitrification is not linked to cellular growth (Prosser, 1989; Pennington & Ellis, 1993).

The biological denitrification process enables the transformation of oxidized compounds by a wide spectrum of heterotrophic bacteria that convert nitrate to harmless nitrogen gas (Foglar et al., 2005). The necessary condition for denitrification to take place in activated sludge systems is the presence of a facultative microbial mass. Many common denitrifiers found in activated sludge systems appear to be capable of heterotrophic nitrification, which appears to occur simultaneously with denitrification (Prosser, 1989; Pel et al., 1997). Common denitrifiers reported in activated sludge systems include *Achromobacter, Aerobacter, Alcaligenes*, *Bacillus*, *Brevibacterium, Denitrobacillus, Flavobacterium, Lactobacillus, Micrococcus, Brevibacterium, Pseudomonas, Spirillum, Proteus, Xanthomonas, Staphylococcus* and *Paracoccus* (Gray, 1990; Metcalf & Eddy, 1999; Sabalowsky, 1999).

The efficiency of wastewater treatment plants by activated sludge system is not only linked to the bacterial population but also to the protozoa (Nicolau et al., 1997). According to their locomotive, protozoa are commonly placed in five groups, which include amoebae, flagellates, free-swimming ciliates, crawling ciliates and stalked ciliates. Most of the protozoa found in the sludge are ciliates and they can be classified in four groups: free-swimming, crawling, attached and carnivorous. Ciliated protozoa are currently used as biotic indicators due to the fact they reduce the concentration of bacteria and suspended particles in the treatment process, resulting in the production of effluents of good qualities (Curds & Cockburn, 1970a; b; Madoni et al., 1993, Salvadö et al., 1995).

The role of protozoa in the decomposition of sewage has been reported since 1964. Johannes (1964, 1965) was the first investigator to emphasize the role of protozoa in the regeneration of phosphorus, a role traditionally assigned mainly to bacteria (Fenchel, 1986, 1988). High concentrations of ciliates and other protozoa have been found to be characteristics of

decomposing sewage (Johannes, 1964; 1965; Caron et al., 1985; Andersen et al., 1986). In aquatic ecosystems, ciliates and phagotrophic microflagellates have been reported to play an important role in the regeneration and mineralization of nitrogen in large quantities while grazing (Gast & Horstmann, 1983; Andersen et al., 1986). Studies have also revealed that flagellated and ciliated protozoa account for a major portion of nitrogen recycling (uptake and excretion) in both marine and freshwater habitats (Caron & Goldman, 1988; Pace & Funke, 1991).

In normal conditions, the concentrations of protozoa are larger than 10^6protozoa/L, and the concentration of 10^7protozoa/L corresponds to very good pollution abatement. On the contrary, concentrations lower than 10^5 protozoa /L are indicative of a poor efficiency of the plant (Drakides, 1978). In terms of biomass, protozoa represent between 0.17% and 0.44% of the sludge during the colonization phase but can represent up to 9% at steady-state (Madoni, 1994a). Curds and Cockburn (1970b) have established relationships between the abundance of some species and the sludge loading: they have associated them with the quality of the effluent, depending on the biological oxygen demand (BOD). A recent study by Sibewu and coworkers (2008) indicated a high diversity of protozoan population in four South African activated sludge systems, which included 68 protozoan genera made up of 44 ciliates, 16 flagellates and eight others. Although the average density of ciliates was 10^4 cells/mL in all aerobic zones, the plants that had total protozoan genera of 27 or 26 and a BOD_5 < 25 mg/mL produced highly-purified effluents.

Despite the evidence that the protozoan community plays a role in the recycling of wastewater and other aquatic ecosystems, increasing knowledge about the relationship between the protozoan biomass and enhanced phosphorus and nitrogen uptake is highly important for the production of effluent of a high standard and for the protection of water sources.

3. Role of protozoa in the excess removal of phosphate and nitrogen in wastewater systems

The average concentration of total phosphorus (inorganic and organic forms) in wastewater is in the range of 10–20 mg/L, much of which comes from phosphate builders in detergents. Common forms of phosphorus in wastewater are orthophosphate (PO4) (50–70 percent of phosphorus), polyphosphates, and phosphorus tied to organic compounds. Orthophosphate comprises approximately 90% of phosphorus in biologically treated effluents (Meganck & Faup, 1988). The total nitrogen is comprised of organic nitrogen, ammonia nitrogen, nitrite and nitrate. The concentrations of each of the types of nitrogen in the influent wastewater treatment of wastewater treatment depend on the composition of the discharges to the collection system and the conditions in the collection system prior to entering the wastewater treatment plant. Typical range values for typical strength domestic wastewater as determined by Metcalf and Eddy (1991) are as follows: 12–50 mg/L for ammonia nitrogen, 8–35 mg/L for organic nitrogen and 20–85 mg/L for total nitrogen. Since phosphorus and nitrogen are limiting nutrients and are mainly responsible for eutrophication of surface waters, they must be removed by wastewater treatment processes before discharge of the effluents into surface waters.

Case study- Protozoan biomass relationship to enhanced phosphate and nitrate removal

In a recent study conducted by Akpor and Momba-workers (2010), the relationship between protozoan biomass concentrations and phosphate and nitrate removal was investigated in

sterile mixed liquors obtained from the anaerobic zone of an activated sludge system. The study was carried out in a shaking flask environment using three initial biomass concentrations (10, 100 and 1000 cells/mL) of two ciliates (*Aspidisca, Trachelophyllum*) and one flagellate (*Peranema*). The three organisms were isolated from the aerobic zone of the same activated sludge system. To enhance nutrient removal from mixed liquor in the presence of the protozoan isolates, sodium acetate (5 g/L) as a carbon source and potassium (0.18 g/L KNO3.) were added to the medium. Samples were taken every 24 h to determine phosphate and nitrate for a period of 96 h. Figure 1 shows an example of the pattern of the relationship between protozoan biomass and enhanced nutrient removal.

The results of the study revealed no remarkable removal of phosphate or nitrate during the first 24 h, as there was no specific increase in protozoan densities. A drastic decrease in phosphate and nitrate contents occurred with a progressive increase in protozoan biomasses (Fig. 1). This trend was observed at all initial biomass concentrations for all protozoan species (results not shown). Between 24 and 96 h, the increases in the protozoan densities corresponded to a phosphate decrease from initial ranges of 55.42–57.36 mg/L, 50.27–51.17 mg/L and 50.01–50.83 mg/L to final ranges of 2.46–11.90 mg/L, 0.61–11.80 mg/L and 1.29–13.89 mg/L, in the presence of *Aspidisca, Trachelophyllum* and *Peranema*, respectively. Nitrate concentrations were observed to decrease from initial ranges of 23.84 to 25.90 mg/L, 23.94 to 25.84 mg/L and 26.12 to 26.54 mg/L to final ranges of 0.11 to 6.32 mg/L, 0.16 to 5.60 mg/L and 0.24 to 9.04 mg/L, in the presence of *Aspidisca, Trachelophyllum* and *Peranema*, respectively.

The COD and the DO concentrations in the mixed liquor were also taken into consideration. The study revealed no COD removal in the presence of acetate as a carbon source. At initial biomass concentration of 10^3 cells/mL, COD increases of 50.58 % for *Aspidisca*, 53.79 % for *Trachelophyllum* and 60.18% for *Peranema* were recorded (Fig 1). However, a DO decrease of over 93 % occurred in mixed liquor from the first 24 h of the experimental study and this was irrespective of the protozoan isolates and the initial biomass concentrations (Akpor & Momba, 2010).This study by Akpor & Momba (2010) revealed a direct relationship between decreases in phosphate and nitrate concentrations and the protozoan biomass. An increase in protozoan growth automatically triggered a corresponding nutrient removal and a decrease in DO concentration. It is also important to note that the growth rates of the three protozoa in mixed liquor were dependent on the initial biomass concentration of the inoculums. This study shows the need to create an environment for the proliferation of the test protozoa in activated sludge systems (Akpor & Momba, 2010).

A progressive increase in the population of protozoa with time has been reported by Petropoulos and coworkers (2005). As stated above, Momba & Cloete (1996a; b) have also reported the relationship between bacterial biomass and enhanced phosphorus removal. These studies and the current study by Akpor & Momba (2010) demonstrate the important of high concentrations of biomass for the most favourable net nutrient removal from wastewater. Hence, the introduction of high initial biomass of bacteria and protozoan species into the activated sludge system may be one of the possible means of enhancing the phosphate and nitrate uptake rate. Therefore, it would be necessary to determine in the activated sludge system which strains of these organisms are capable of nutrient removal and which initial biomass concentrations are able to result in excess uptake of phosphate and nitrate.

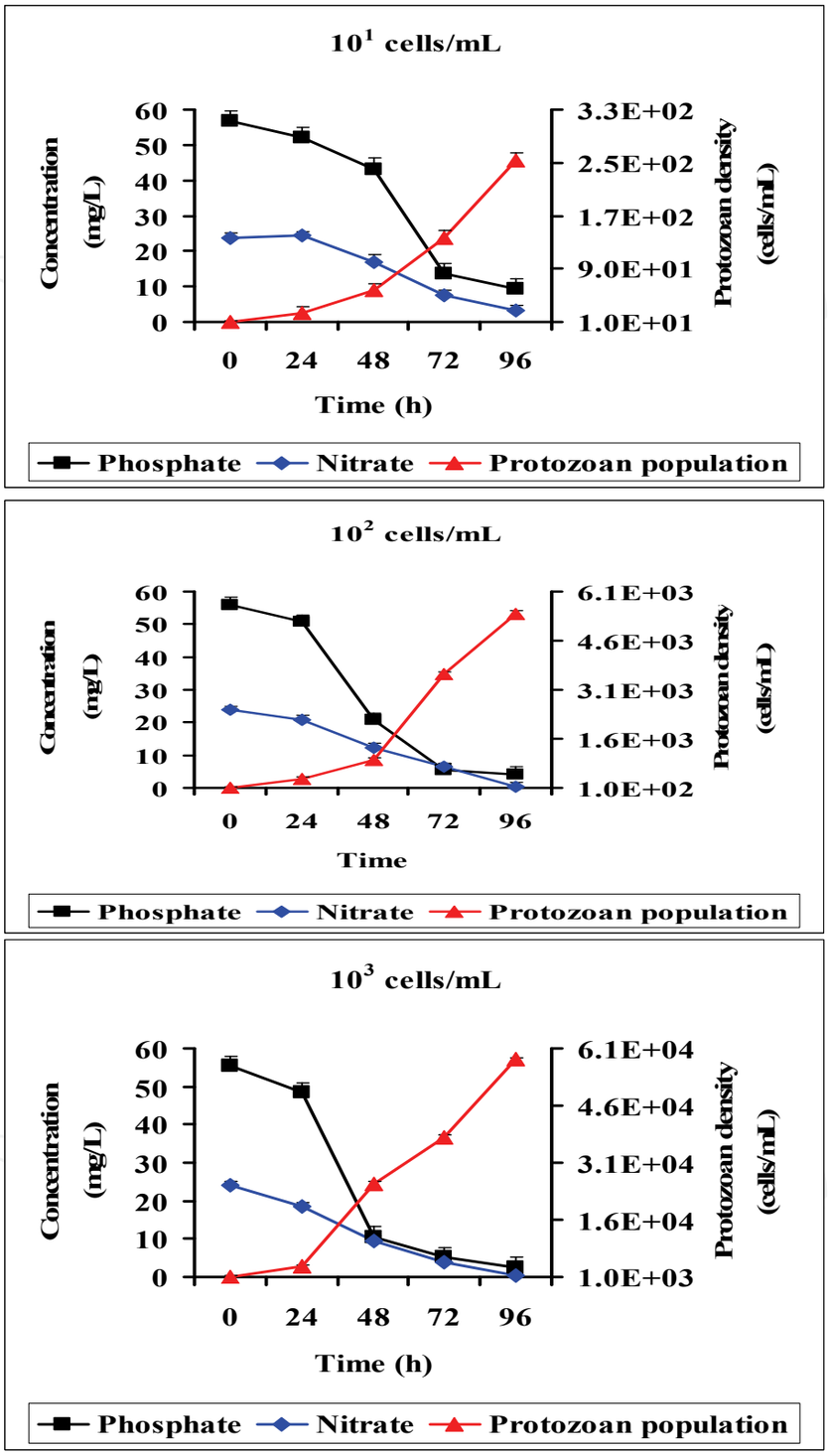

Fig. 1. An example of the relationship between nutrient (phosphate and nitrate) removal and the growth of *Aspidisca* sp in the presence of acetate as carbon source at the different initial biomass concentrations. Similar patterns were also noted for *Trachelophyllum* and*Peranema.* (Full results for these two protozoan species can be found in Akpor & Momba, 2010)

4. Predation potential of wastewater protozoa on human pathogenic bacteria

Previous studies have reported that the presence of protozoa in aeration tanks of wastewater treatment plants reflects an improvement in the effluent quality (Curds & Cockburn, 1970b; Sibewu et al., 2008). Researchers have focused on the significance of protozoa as reducers of bacterial concentrations and suspended particles in biological wastewater treatment plants (Curds & Cockburn, 1970a, b; Al-Shahwani & Horan, 1991; Madoni et al., 1993; Salvado et al., 1995). Two mechanisms have been proposed by which protozoan predation participates in the removal of indicator and pathogenic bacteria in biological wastewater treatment plants. The most commonly proposed mechanism is that predators graze on free bacteria and on bacteria attached to the flocks (Heinbokel, J.F., (1978). The second proposed mechanism is that suspension-feeding predators remove suspended particles as the particles flow through the wastewater treatment plant. Predators that graze on attached bacteria potentially free up the sites for future bacterial attachment, while the suspension-feeding predators directly remove particles from the mobile phase (Weber-Shirk & Dick, 1999). The feeding of flagellates and ciliates on bacteria represents the oldest predator–prey interaction that is known in nature. Flagellates and ciliates can control bacterial density in many ecosystems (Berninger et al., 1991). The majorities of investigations on the predation of bacteria by protozoa have focused either on laboratory strains of protozoa or on marine isolates (Omori and Ikeda, 1984; Laybourn-Parry et al., 2000; Scott et al., 2001; Pedros-Alio et al., 2000).

During the past decade, studies have demonstrated that different bacterial strains are not equally vulnerable to grazers and have evolved different mechanisms to resist capture, ingestion or digestion by bacterivories (Hahn & Höfle**,** 2001; Jürgens & Matz. 2002). This has first been documented for Legionella pneumophila, which can multiply inside amoeba such as Acanthamoeba (Omar et al., 2000). Phenotypic bacterial properties that have been identified to influence grazing mortality are size and morphology (Gonzalez et al., 1990; Šimek & Chrzanowski, 1992, Šimek et al., 2001),, swimming speed (Matz & Kjelleberg 2005), toxic pigments (Matz et al., 2004), the physico-chemical surface structure (Monger et al., 1990; Matz & Jürgens 2001) and cell-to-cell communication (Okada et al., 2005). These findings have indicated that predation by protozoa is an important factor that contributes to the diversification of bacterial traits and might select specific antipredator adaptations in bacteria. It is therefore interesting to describe this phenomenon during the biological treatment of wastewater by activated sludge. Hence the influence of protozoan grazing (grazing rate, clearance rate and ingestion rate) on pollution indicator and enteric bacteria pathogens from mixed liquor wastewater was investigated and the optimum density of protozoa required for the removal of pathogenic bacteria was identified in our laboratory. The results of this study are discussed below.

Case study- Protozoan biomass relationship to pathogenic bacteria removal from wastewater

To investigate the protozoan biomass relationship to pathogenic bacteria removal, three strains of human enteric pathogenic bacteria frequently found in wastewater treatment plants [(*pathogenic Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (CCRC 1077) and *E. coli* O157:H7 (ATCC 43895)] and three protozoan isolates (*Aspidisca sp., Trachelophylum sp.* and *Peranema* sp.), which were previously screened for phosphate and nitrate removal efficiency (Akpor et al., 2007) were used. For each selected protozoa, three different initial

biomass concentrations (10^2cell/ml, 10^3cell/ml and 10^4cells/ml) were considered. The grazing experiments were carried out using a modification of the protocol outlined by Sherr and Sherr (1993). Each experiment consisted of duplicate batches containing one protozoa species and one of live pathogenic bacterial strains. Experiments were conducted in Erlenmeyer flasks containing 250 mL of modified wastewater mixed liquor, which is a simulated environment for the bacteria and protozoa isolated from the wastewater system (Momba & Cloete, 1996). For each protozoan species, flasks were separately inoculated with initial protozoa biomass (10^2,10^3 and 10^4 cells/mL and then placed in the dark in a water bath at 25°C for 30 min to allow the protozoa to recover from handling shock and to acclimatise. For each pathogenic bacterium, an initial concentration of 10^6cfu/mℓ was separately added to each flask, followed by incubation in the dark with shaking (100 ± 10 rpm) at room temperature. Five milliliter aliquots were collected from each flask on an hourly basis for the first 12 h and thereafter every 18 h and 24 h. Protozoan densities were estimated by dispensing approximately 1mL of the sample on improved Neubauer hemocytometer and viewing directly under the microscope at 400 X magnifications (Axioplan Carl Zeiss GmbH equipped with phase contrast, bright field and epifluorescence, HBO 50 illuminator and a digital imaging system). Spread plate procedure and selective media (XLD agar for *Salmonella typhimurium* and *Shigella flexneri* and Fluorocult *E coli* O157:H7 agar for E coli O157:H7) were used to determine the concentration of live bacteria. The plates were incubated at 37°C for 24h. The protozoan concentrations were calculated using the following formula:

$$\text{Number of cells/mL} = (C \times V)/(A \times D \times F) \quad (1)$$

where C is the number of organisms counted, F the number of fields counted, D (mm) the depth of the counting chamber, A (mm^2) the area of a field and V (mm^3) the volume of the counting chamber (APHA 2001). Growth rates (μ) (d^{-1}) were calculated using the following equation:

$$\mu = (\ln F_t - \ln F_0)/\Delta t \quad (2)$$

where F_0 and F_t denote the concentrations of the protozoa at the beginning and at the end of the time interval (Δt) of exponential increase during the study period (APHA 2001).
The grazing rates (G) (per hour) were calculated by the equation:

$$G = (N_0 - N_t)/[(N_0 - N_t)/2]/\Delta t, \quad (3)$$

where N_0 and N_t refer to the concentrations of bacteria at the beginning and the end of the time interval (Δt) of decline. The clearance rates (C) (nl /flagellate/h) were calculated by the equation:

$$C = (G \times 1{,}000{,}000)/F_m, \quad (4)$$

where F_m (Protozoa/mL) is the mean concentration of the flagellate. F_m was calculated for the time of an exponential increase, according to the method of Heinbokel (1978):

$$F_m = (F_t - F_0)/(\ln F_t - \ln F_0) \quad (5)$$

where F_0 and F_t refer to the concentrations of the protozoa at the beginning and at the end of the time interval, respectively.

The ingestion rates (I) (bacterial cells/protozoa/hour) were estimated by the equation (Wu et al., 2004):

$$I = (N_0 - N_t)/F_m/\Delta. \quad (6)$$

The results indicated a relationship between the protozoan biomass and their grazing, clearance and ingestion rates on pathogenic bacteria. During the 24 h of the predation experiments, there were no significant changes in protozoan densities, which ranged from 2 × 10^4 to 9.8 × 10^4 cells/mL, from 2 × 10^4 to 9.1 × 10^4 cells/mL and from 2 × 10^4 to 8.7 × 10^4 cells/mL when the three protozoa had *E. coli, Salmonella typhimurium* and *Shigella flexneri* as their sole sources of food, respectively. No significant differences in protozoan growth rates were noted between the initial protozoan biomass of 10^2cells/mL and that of 10^3cells/mL in the presence of the three enteropathogenic bacterial strains. However, significant differences in growth rates were recorded between the initial protozoan biomass of 10^4cells/mL and those of the two former initial biomasses [P = 0.054 9 one-way ANOVA)]. For all initial protozoan densities, the maximum protozoan growth rates were observed when grazing on *E. coli*, with *Peranema* having the highest rates, ranging between 0.14 and 0.17h^{-1} during the study period (Tables 1–3). The growth experiment therefore indicated a strong relationship between a higher protozoan biomass and a higher growth rate, although in various densities all the three protozoa species were able to predate successfully on the enteropathogenic bacteria, and that bacterial food concentrations of 10^6bacteria/mL allowed a positive growth (Tables 1-3).

For all protozoan species, lower protozoan biomass concentrations of 10^2cells/mL and 10^3cells/mL significantly resulted in lower grazing rates (P = 0.002 [t test]), clearance rates (P = 0.002 [t test]) and ingestion rates (P = 0.007 [t test] compared to the higher protozoan biomass of 10^4cells/mL (Tables 1–3). This clearly showed that the three mechanisms of protozoan predation on pathogenic bacteria increased with a higher protozoan biomass

Strain	**Mechanisms**	***A***	***B***	***C***
E. coli O157:H7	Grazing rate (cell^{-1} h^{-1})	32	42	28
	Clearance rate (nl cell^{-1} h^{-1})	20	60.36	13.27
	Ingestion rate (bacteria cell^{-1} h^{-1})	240	715	181
	Growth rate (d^{-1})	0.13± 0.01	0.12± 0.03	0.14± 0.01
Salmonella typhimurium	Grazing rate (cell^{-1} h^{-1})	4.7	21	4
	Clearance rate (nl cell^{-1} h^{-1})	12.89	52	12.04
	Ingestion rate (bacteria cell^{-1} h^{-1})	153	394	133
	Growth rate (d^{-1})	0.09± 0.02	0.09± 0.02	0.06± 0.2
Shigella flexneri	Grazing rate (cell^{-1} h^{-1})	38	38	23
	Clearance rate (nl cell^{-1} h^{-1})	38	68.2	9.4
	Ingestion rate (bacteria cell^{-1} h^{-1})	366	502	148
	Growth rate (d^{-1})	0.10± 0.01	0.10± 0.01	0.08± 0.02

A- Aspidisca sp, B - Trachelophyllum sp and C - Peranema sp.

Table 1. Predation of initial protozoan density of 10^2cells/ml on enteric pathogenic bacteria

Strain	Mechanisms	*A*	*B*	*C*
E. coli O157:H7	Grazing rate (cell^{-1} h^{-1})	36	51	33
	Clearance rate (nl cell^{-1} h^{-1})	21.34	59.4	15.43
	Ingestion rate (bacteria cell^{-1} h^{-1})	232	728	181
	Growth rate (d^{-1})	0.14± 0.01	0.13± 0.03	0.15± 0.01
Salmonella typhimurium	Grazing rate (cell^{-1} h^{-1})	5	30	8
	Clearance rate (nl cell^{-1} h^{-1})	15	36	10
	Ingestion rate (bacteria cell^{-1} h^{-1})	160	403	123
	Growth rate (d^{-1})	0.10± 0.02	0.10± 0.02	0.06± 0.2
Shigella flexneri	Grazing rate (cell^{-1} h^{-1})	33	44	30
	Clearance rate (nl cell^{-1} h^{-1})	35	40	8.9
	Ingestion rate (bacteria cell^{-1} h^{-1})	358	489	111
	Growth rate (d^{-1})	0.11 ± 0.01	0.11± 0.01	0.09± 0.02

A- Aspidisca sp, B - Trachelophyllum sp and C - Peranema sp.

Table 2. Predation of initial protozoan density of 10^3cells/ml on enteric pathogenic bacteria

Strain	Mechanism	*A*	*B*	*C*
E. coli O157:H7	Grazing rate (cell^{-1} h^{-1})	78	83	60
	Clearance rate (nl cell^{-1} h^{-1})	44	94.1	60
	Ingestion rate (bacteria cell^{-1} h^{-1})	930	1035	440
	Growth rate (d^{-1})	0.16± 0.01	0.16± 0.03	0.17± 0.01
Salmonella typhimurium	Grazing rate (cell^{-1} h^{-1})	15	52	14
	Clearance rate (nl cell^{-1} h^{-1})	30	58.6	77
	Ingestion rate (bacteria cell^{-1} h^{-1})	920	730	320
	Growth rate (d^{-1})	0.11± 0.02	0.16± 0.02	0.09± 0.2
Shigella flexneri	Grazing rate (cell^{-1} h^{-1})	77	77	62
	Clearance rate (nl cell^{-1} h^{-1})	29	98.21	55
	Ingestion rate (bacteria cell^{-1} h^{-1})	540	1078	430
	Growth rate (d^{-1})	0.11± 0.01	0.11± 0.01	0.15± 0.02

A- Aspidisca sp, B - Trachelophyllum sp and C - Peranema sp.

Table 3. Predation of initial protozoan density of 10^4cells/ml on enteric pathogenic bacteria

concentration. The results of this study showed that the ingestion rate was significantly higher ($P < 0.001$) compared to the grazing rate and to the clearance rate. This was irrespective of the protozoan initial biomass concentrations. In general, the highest grazing, clearance and ingestion rates were significantly noted with *Trachelophylum* when predating on *E. coli* and *Shigella flexneri* compared to *Aspidisca* and *Peranema* (test, $P \leq 0.002$ in all cases) (Tables 1–3).

In terms of food preference, *Escherichia coli* O157:H7 remained the most preferred prey for both ciliates and the flagellate, followed by *Shigella flexneri*, and *Salmonella typhimurium* was found to be the least preferred prey among protozoa in most cases. This study clearly demonstrated that protozoa generally play a big role in the removal of indicators and pathogenic bacteria in activated sludge systems. To reveal whether this holds true for the majority of bacteria, more investigations are needed.

5. Conclusion

The various perspectives on the role of microorganisms in water and water resources have been highlighted in order to gain a deeper understanding of the intricate web of relationships between man, microbes and the environment. Microorganisms do not exist in a vacuum. They interact with the environment, causing harmful and beneficial effects which are in a delicate balance.

It is well known that wastewater treatment consists of physical, chemical and biological treatment processes. All these processes may be required to produce effluent of a high quality that can result in the protection of water sources. Individual wastewater treatment processes cannot be applied in a universal fashion. Each process has limitations and should be used in combination with several other processes to deliver effluent of an appropriate quality.

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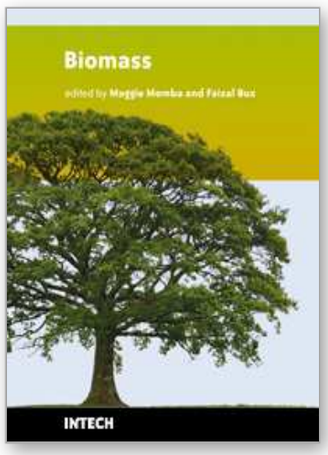

Biomass

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Due to demands placed on natural resources globally and subsequent deterioration of the environment, there is a need to source and develop appropriate technology to satisfy this requirement. For decades mankind has largely depended on natural resources such as fossil fuels to meet the ever increasing energy demands. Realizing the finite nature of these resources, emphasis is now shifting to investigating alternate energy source governed by environmentally friendly principles. The abundance of biomass and associated favorable techno-economics has recently changed global perceptions of harnessing biomass as a valuable resource rather than a waste. To this end this book aims to make a contribution to exploring further this area of biomass research and development in the form of a compilation of chapters and covering areas of ecological status of different types of biomass and the roles they play in ecosystems, current status of biomass utilization and deriving energy and other value added products from biomass. In this context biomass can be defined as large plants and trees and different groups of microorganisms. This book will serve as an invaluable resource for scientists and environmental managers in planning solutions for sustainable development.

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