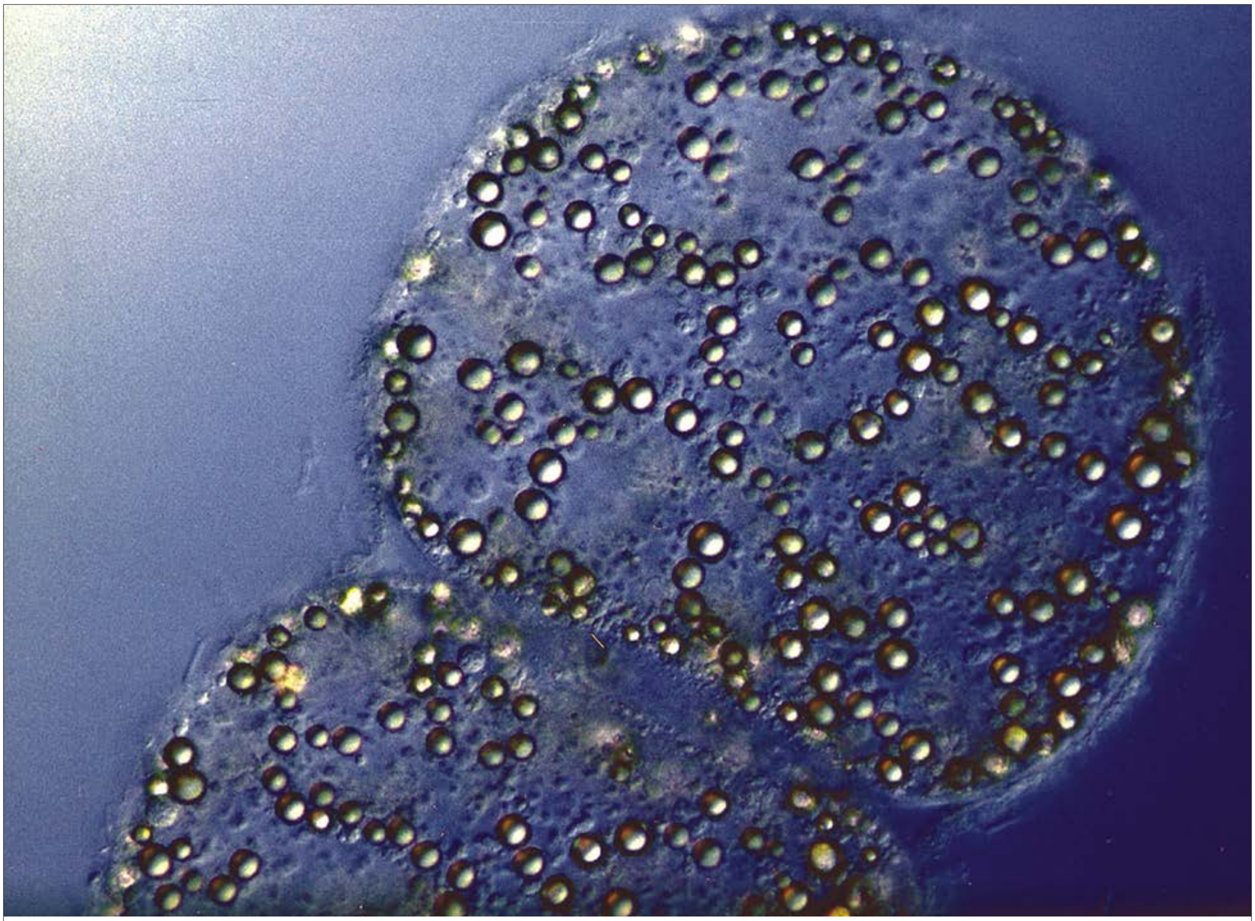

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Role of Protozoa on Faecal Bacteria Removal in Macrophyte and Algal Waste Stabilization Ponds

[1]Esi Awuah and [2]Samuel Fosu Gyasi
[1]Office of the Vice Chancellor, University of Energy and Natural Resources, Sunayi, Ghana
[2]Department of Energy and Environmental Engineering, University of Energy and Natural Resources, Sunayi, Ghana

Corresponding Author: Samuel Fosu Gyasi, Department of Energy and Environmental Engineering, University of Energy and Natural Resources, Sunayi, Ghana Tel: +233 268189473

ABSTRACT
Protozoa populations and the types of microbiota were determined at various depths (0.1, 0.35 and 0.63 m) in water lettuce, duckweed and algal ponds receiving medium strength sewage. The role of protozoa on the removal of faecal bacteria was also determined. In the water lettuce and duckweed ponds, protozoa were concentrated in the sediments while the algal ponds protozoa were mostly found in the sediments as well as on the surface. *Bodo* and *Vahlkampfia* protozoa were both common in all the 3 pond systems. The protozoan *Petalomonas* and *Chironomus* insect larvae were found only in the water lettuce and duckweed ponds. *Vorticella* and other unidentified ciliates were however unique to the algal ponds. The algal ponds had the highest number of species diversity and the highest number of protozoans, followed by water lettuce and duckweed. Other algae such as *Chlorella, Chlorococum, Phacus, Ulothrix* and some diatoms were also found but in small quantities. The presence of protozoa was found to have a significant effect in the removal of *E. coli* and *Salmonella* ($p<0.05$) in water lettuce pond system. In the duckweed and algal pond systems however, there was no difference between faecal bacteria removal with respect to protozoa. Results from this study has shown that the protozoa could play a major role in the removal of *E. coli* and *Salmonella* in water lettuce treatment ponds.

Key words: Stabilization ponds, macrophyte, algae, protozoa, faecal bacteria

INTRODUCTION
Waste Stabilization Ponds (WSP), often referred to as oxidation ponds or lagoons, are holding basins used for wastewater treatment where decomposition of organic matter is processed naturally. The activity in WSP is a complex process involving bacteria, protozoa, algae, other microbes and metazoans, to stabilize the wastewater and to reduce pathogen populations (Reinoso *et al*., 2008). In the tropics where enteric diseases are common, the removal of pathogens is of much importance. The natural processes involved in pathogen removal in algal ponds, such as UV light, sunlight induced factors such as high pH and DO, have been studied in detail by several authors (Curtis *et al*., 1992; Davies-Colley *et al*., 1999) and have been found to be effective in pathogen removal.

In macrophyte ponds, these mechanisms are largely absent due to the shading provided by the macrophyte cover on the surface (Gupta and Mithra, 2013). Attachment to plant surfaces and predation of pathogens by protozoa are potential natural mechanisms which could prevail in macrophyte ponds (Borowitzka and Moheimani, 2013). Theoretically high pH and DO could play a role because photosynthesis is still taking place but previous studies have shown that these factors do no play an important role in macrophyte-based ponds (Awuah *et al*., 2004). Predation of bacteria by protozoans has not been studied in detail in waste stabilization ponds but this could provide an important mechanism for pathogen removal.

Protozoa have been found to be effective in the removal of *Escherichia coli* (Espinosa-Garcia *et al*., 2014). Other protozoa have been observed to feed on faecal coliforms, diphtherial, choleral, typhal and streptococcal bacteria (McCambridge and McMeekin, 1979, 1980). It has been reported that protozoa improve the effluent quality of activated sludge plants, trickling filters and rotating biological contactors). Sinclair and Alexander (1989) also reported that slow growing bacteria are eliminated due to intense protozoa predation. Quantitative studies have also suggested that, one *Tetrahymena pyriformis* cell is able to digest 500 bacteria per hour which means that in 24 h one individual protozoan could remove about 1.2×10^4 of bacteria. This could therefore imply that, predation alone could remove significant amounts of bacteria pathogens in a given system (Gonzalez *et al*., 1990). In constructed wetlands however, predation of *Cryptosporidium* oocysts by ciliates such as *Euplotes patella* and *Paramecium caudatum* is known to act as a possible mechanism for pathogen removal (Stott *et al*., 2001).

In spite of the plethora of research that has gone into the investigations of WSP in recent times, the contribution of protozoa grazing involved in the removal of faecal bacterial in waste stabilization ponds is yet to be fully explored. Macrophyte ponds are now being utilized in developing countries in recent times for resource recovery but then their ability to remove pathogens and the mechanisms involved in this removal has not been fully investigated. An understanding therefore of the role of bacteria predation by protozoa in this removal mechanism of faecal bacteria in macrophyte and algal pond systems could contribute to improved design. This may also include the operation and maintenance practices of waste stabilization ponds for enhanced pathogen removal. In this present study, the role of protozoa in the removal of faecal bacteria in macrophyte and algal ponds would be investigated.

MATERIALS AND METHODS

This study was conducted in Kwame Nkrumah University of Science and Technology (KNUST), between June 2011 and August, 2012 as shown in Fig. 1.

Experimental design: The experimental set-up was carried out in a pilot-scale continuous flow system comprising of water lettuce (*Pistia stratiotes*), duckweed (*Spirodela polyrhiza*) and algal (natural colonization) pond systems which are connected separately as shown in Fig. 2. The set up displays an anaerobic pond with 2 days retention period which preceded the main set up. Each pond system consisted of 4 ponds operating in series and a retention period of 7 days in each pond with a total retention period of 28 days. The flow rate in each treatment system was 0.01 m^3 day^{-1}. The pond systems were operated in parallel according to the arrangement shown in Fig. 2.

Water lettuce ponds were maintained by harvesting once every week and twice a week in the case of duckweed ponds to ensure the systems run efficiently.

The types of microbiota populations present and the protozoan population profiles recorded were studied in the pond systems. To determine the effect of protozoa on faecal bacteria in the pond systems, protozoa were removed through filtration of wastewater.

Fig. 1: A map showing KNUST within Kumasi in the Ashanti Region of Ghana (Top) and that of Africa with Europe, Middle East and part of Asia (Down)

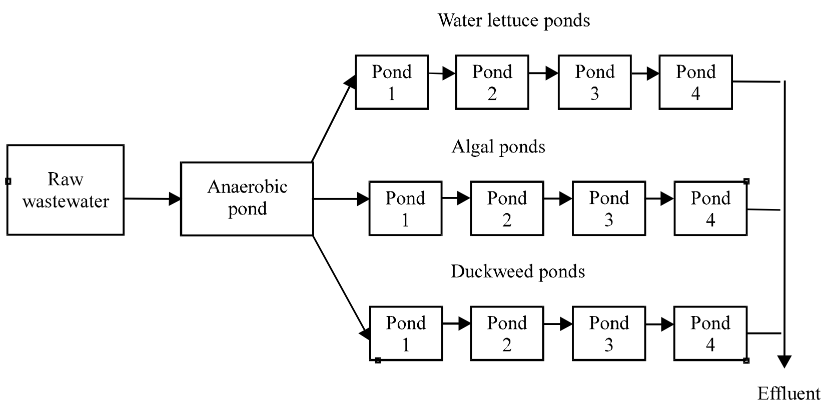

Fig. 2: Schematic diagram of bench-scale macrophyte and algal waste stabilization ponds

Procedure: One litre of wastewater was collected from the surface of the 2nd ponds (Pond 2) of each pond system. Ponds 2 were selected because this was the most productive ponds in terms of both the macrophyte and algal growth. For each of the 3 pond systems (i.e., Water lettuce, Algal and Duckweed ponds system) 500 mL of the 1 L wastewater in each case were filtered through a millipore nylon filter (Whatsman No. 1 filter paper, with a pore size of 7 μm) to remove protozoa and algae. The filtered samples were examined to ensure that there were no protozoa and algae. The non-filtered samples which were known to contain protozoa and all other microbiota that were present were also kept in a separate sterile labelled container.

The non-filtered and filtered samples were then divided into 3 equal volumes of 150 mL into 3 separate plastic cups of 200 mL capacity for each treatment. The set up was covered with aluminium foil to prevent the interference of light but loose enough to allow oxygen diffusion into the wastewater. The faecal bacteria populations were enumerated daily for 5 days using chromocult agar and incubated at 37°C (Byamukama *et al.*, 2000). *Escherichia coli* was confirmed in EC medium with acid and gas production as positive results whiles coliforms were cultured on Endo agar as metallic sheen colonies also indicated a positive results. In the same manner, *Salmonella* was confirmed as growth in tetrathionate base broth (Greenberg *et al.*, 1992). In all the 3 systems, the removal rates in the filtered and non-filtered treatments were calculated using Chick's law.

In determining the protozoa populations and profiles, the number per milliliter of each sample were assessed at the surface with an approximate depth of (0.10 m), middle portions with an approximate depth of (0.35 m) and at the bottom also with an approximate depth of (0.63 m). This was carried out each month beginning in August 2011 to January 2012. A total of 50 mL from 10 different sampling points at each 4 different depth from the surface to the bottom of each pond system were collected followed by manual shaking to mix the samples thoroughly.

Samples were collected once in every month in the mornings between 6-8 GMT, counted and identified using a Sedgewick rafter counting chamber employing the study protocol described by Greenberg *et al.* (1992). Identification of protozoa were based on shape, size, morphology, type of motility as well as the presence of cilia and flagella in accordance with the guidelines provided by Finlay *et al.* (1988).

RESULTS

Analysis of the results from the study with respect to the effect of protozoa on faecal bacteria removal showed an increase in bacteria numbers and this were recorded in all the treatments as shown in Table 1. The study showed that, for the Water lettuce treatment pond system, there was an increase in all the faecal coliform bacteria (*E. coli* 0.5±0.0, *Coliforms* 1.1±0.9, *Salmonella* 1.2±0.3 and *Enterobacteria* 0.3±1.1) k days^{-1} when the samples were filtered as shown in Table 1. However, the unfiltered counterpart (Indicating the presence of protozoans) showed a decline in faecal coliform numbers (Table 1). Differences were significant ($p<0.05$) for *E. coli* and *Salmonella*. This was also seen with the Duckweed treatment pond system with respect to coliform,

Table 1: Effect of the presence of protozoa on the removal of faecal bacteria

Treatment pond system	*E. coli*	Coliforms	*Salmonella*	Enterobacteria
	(k day^{-1})			
Water lettuce non-filtered	0.2±0.0	1.1±0.1	0. 6±0.3	0.7±0.4
Water lettuce filtered	0.5±0.0*	1.1±0.9*	1.2±0.3*	0.3±1.1*
Duckweed non-filtered	2.8±2.4ns	1.2±1.1	0.1±0.0	0.9±0.3
Duckweed filtered	2.3±2.1	2.2±1.2*	0.6±0.0*	1.1±0.8*
Algae non-filtered	0.3±0.0ns	1.3±0.9ns	0.3±1.2ns	1.3±0.0
Algae filtered	0.5±0.2	1.7±1.4	2.9±2.4	0.8±0.4*

Figures preceded by ± refers to the standard deviation, whiles the all other figures refers to the means, Ns: Not significant ($p>0.05$), *Significant ($p<0.05$) at 95% confidence interval salmonella and enterobacteria (2.2±1.2, 0.6±0.0 and 1.1±0.8 kday^{-1}, respectively) when the samples were filtered as shown in Table 1. However, with the exception of Enterobacteria (0.8±0.4 k days^{-1}) there were no significant difference (p>0.05) in die-off rates between filtered and non-filtered treatments for all algal treatment systems (Table 1).

Description of protozoa and other microscopic biota in the ponds: Microscopic investigation of the protozoan analysis showed the presence of several organisms in the wastewater treatment systems during the study as shown in Table 2. Protozoa of the genera *Bodo* and *Vahlkampfia* and algae of the genus *Euglena* were common to all the 3 pond systems (Table 2). Other microbiota like mites, copepods and nematodes were also found in the 3 pond systems. The protozoa of the genus *Petalomonas* and *Chironomus* insect larvae were seen only in the water lettuce and duckweed ponds while *Vorticella* and other unidentified ciliates species were unique to the algal ponds as shown in Table 2. The algal ponds had the highest number of species diversity and the highest number of protozoa, followed by water lettuce and duckweed. *Euglena* dominated in all the algal ponds at most times. The following algae were also

Table 2: Protozoa and other microscopic biota found in macrophyte and algal ponds

(a)

Treatment system	Amoebae	Ciliates	Flagellates	Algae	Metazoans
Raw sewage	-	2, 3, 4	1, 3	-	-
Anaerobic pond	2, 6, 8	2, 3, 4, 7, 10	3, 4	-	4
Water lettuce ponds					
Pond 1	3	2	1, 4	1, 6	1, 4
Pond 2	3, 4, 5	2	2	6	
Pond 3	3, 4, 5	1, 2, 6	2	6	
Pond 4	-	2, 9, 10	2	6	
Duckweed ponds					
Pond 1	-	2	1, 3	4, 6, 8	3
Pond 2	2, 7, 8	-	1	6	1
Pond 3	3, 8	-	1	6	2, 3
Pond 4	-	2	1, 4	6	1, 2, 3, 4
Algal ponds					
Pond 1	1, 2, 3,	3, 6, 7, 10	1	1, 2, 4, 6, 7, 8, 9	1, 3
Pond 2	2, 7, 8	2, 5, 8, 9	1	1, 2, 4, 5, 6, 7,8	1, 3, 4
Pond 3	2, 5	2, 5, 9	1	2, 3, 4, 5, 7, 8	1
Pond 4	2, 3, 4	2, 4, 5	1	1, 2, 4, 6, 7, 8	1, 3, 4

(b)

Cd	Amoebae	Cd	Ciliates	Cd	Flagellates	Cd	Algae
1	Spike-like heliozoan	1	Striped ciliate	1	*Bodo*	1	*Chlorococcum*
2	*Vahlkampfia*	2	Hopping ciliate	2	*Petalomona*	2	*Chlorella*
3	Oval amoeba, large	3	*Paramecium*	3	Spiral-shaped	3	*Chlamydomonas*
4	*Platymoeba*	4	Double fan-shaped ciliate	4	Larvae-like protozoa	4	Strands without cell walls
5	Small amoeba	5	*Vorticella*			5	Diatoms
6	Amoeba	6	Pear-shaped ciliate		*Metazoans*	6	*Euglena*
7	*Vanella*	7	*Tetrahymena*	1	Copepods	7	*Phacus*
8	Large spherical amoeba	8	Unidentified	2	*Chironomus* larvae	8	*Spirogyra*
		9	*Stentor* type 1	3	Mites	9	*Ulothrix*
		10	*Stentor* type 2	4	Nematodes		

Cd = Different codes for the different microbes seen in the algal ponds, although in small quantities: *Chlorella*, *Chlorococcum*, *Phacus*, *Ulothrix* and some diatoms. When floating algae covered the ponds, *Spirogyra* and some coenocyticalgae were dominant among the algal community (Table 2).

Protozoa population and profiles: The study also showed that, the protozoa populations in the raw sewage were always small in quantity except in October 2011 and December 2011 when spiral shaped protozoa appeared in large quantities (Table 2). The numbers however declined drastically in January (2012) as shown in Table 3.

A similar situation occurred in the 3 pond systems as most of the protozoa were small in size (<20 μm) with most of the small protozoa being flagellates Table 3. The medium sized protozoa (20-50 μm) were mostly ciliates whiles the large size protozoa (>50 μm) were mostly amoebae. The protozoa were however not evenly distributed within the ponds systems as shown in Table 3. Most

Table 3: Size distribution of protozoa from raw wastewater

	Size of organism in 1 mL of sample		
Month and Year	<20 μm	20-50 μm	>50 μm
August 2011	1	12	4
September 2011	40	0	21
October 2011	3620	2	1
November 2011	1	3	7
December 2011	920	0	0
January 2012	1	2	0

Table 4: Size distribution of protozoa in duckweed ponds at various depths

		Size of organism in 1 mL of sample		
Pond designation	Depth of pond	<20 μm	20-50 μm	>50 μm
1	0.10 m	141±156	0±1	36±50
	0.35 m	78±129	17±41	7±7
	0.63 m	2143± 4166	33±45	20±16
2	0.10 m	239±448	52±122	2±4
	0.35 m	84±176	2±4	1±1
	0.63 m	1783±1548	80±100	30±17
3	0.10 m	7±16	31±74	nil
	0.35 m	12±18	118±268	1±1
	0.63 m	590±395	845±1356	77±159
4	0.10 m	3±8	Nil	Nil
	0.35 m	518±1207	217±501	12±28
	0.63 m	585±1012	228±478	Nil

Table 5: Size distribution of protozoa in the anaerobic pond at various depths

	Size of organism in 1 mL of sample		
Depth of system	<20 μm	20-50 μm	>50 μm
0.10 m	4227±2962	77±188	15±20
0.35 m	1277±1267	115±128	16±13
0.63 m	54±131	68±163	51±63

Table 6: Size distribution of protozoa in water lettuce ponds at various depths

		Size of organism in 1ml of sample		
Pond designation	Depth of pond	<20 μm	20-50 μm	>50 μm
1	0.10 m	24±48	17±41	17±27
	0.35 m	28±35	9±22	5±8
	0.63 m	454±584	102±128	25±23
2	0.10 m	12±26	2±2	7±12
	0.35 m	61±150	54±102	9±11
	0.63 m	1615±1151	405±465	52±98
3	0.10 m	41±44	55±77	26±35
	0.35 m	34±64	29±56	3±4
	0.63 m	12488±20198	142±150	125±244
4	0.10 m	28±40	29±53	79±179
	0.35 m	176±167	39±22	44±44
	0.63 m	5057±8330	254±279	35±38

Table 7: Size distribution of protozoa in algal ponds at various depths

		Size of organism in 1 mL of sample		
Pond designation	Depth of pond	<20 μm	20-50 μm	>50 μm
1	0.10 m	1497±2277	209±448	68±80
	0.35 m	874±1526	46±73	14±17
	0.63 m	4218±9540	41±32	137±276
2	0.10 m	2129±2563	73±152	51±79
	0.35 m	373±818	14±24	6±7
	0.63 m	6137±8459	340±448	185±256
3	0.10 m	638±537	27±26	30±32
	0.35 m	784±456	118±864	30±41
	0.63 m	18083±23414	332±301	178±133
4	0.10 m	6157±10302	165±318	100±159
	0.35 m	951±921	106±149	40±48
	0.63 m	9973±11241	371±316	108±168

of the protozoa seen in this study were found in the sediments collected from the macrophyte ponds. The lowest protozoa populations were found at the surface of the macrophytes ponds (Table 3). The study further showed that protozoa populations in the anaerobic pond were higher than in the raw wastewater. The algal ponds however had the highest number of protozoa amongst the 3 pond systems as shown in Table 3.

The study showed that, the protozoa populations in the water lettuce ponds were higher than that of the duckweed ponds (Table 3). In the algal ponds high protozoa numbers were also observed on the surface as well as in the sediments. The duckweed ponds, which had the lowest protozoa population, showed complete absence at some depths (Table 4). Agglomeration of protozoa was also seen during the counting (Table 5-7).

DISCUSSION

Effect of protozoa on faecal bacteria removal: This is the first time a pilot study is being carried to investigate the influence of protozaons on faecal bacteria removal in Macrophyte and

Algal Waste Stabilization Ponds, Kumasi, Ghana. Results from the water lettuce based-treatment systems showed that the absence of protozoa could lead to higher faecal bacteria populations in effluents from waste stabilization ponds. This assertion is in line with a study conducted by Tharavathy and a group of researchers in India (Tharavathy *et al*., 2014).

The differences in the contribution of protozoa towards faecal bacteria removal in the three pond systems could be due to selective feeding by protozoa (Harvey *et al*., 2002; Finlay *et al*., 1988). Selective feeding by protozoa is based on size, motility and the growth condition of the protozoa themselves. This could vary between pond systems and even from pond to pond in the same pond system and this assertion was in line with the present study.

Differences in the number of protozoa and type of species in the 3 pond systems have been observed in this study (Tables 2-7). *Petalomonas* was present mostly in the water lettuce ponds (Table 2) and this flagellate might contribute to the removal of *E. coli* and *Salmonella* in this pond as reported by Reinoso *et al*. (2008). This was in agreement with this present study.

The effect of protozoa grazing on the elimination of bacteria was well recorded as part of this study. Predation by ciliates is considered to be a very important mechanism for bacteria removal in wastewater treatment plants (Curds and Vandyke, 1966; Curds, 1992; Decamp *et al*., 1999). In agreement with this study, an investigation reported that nano-flagellates could consume 12-74% of attached bacteria in a day and concluded that protozoa predation could be a major removal mechanism of pathogens in wastewater treatment systems.

The lack of significant differences in the presence and absence of protozoa in the duckweed and algal treatments meant that high protozoa population did not always correlate with effective removal of faecal bacteria by predation. In the duckweed pond the limited effect of protozoa on bacterial removal could be due to both selective feeding and the low numbers of protozoa (Table 4). A research indicated that for protozoa to be effective in the removal of bacteria through predation, their number should be more than 1000 mL^{-1}. Petropoulos *et al*. (2003) reported that low protozoa numbers resulted in increase in bacteria growth rate. This phenomenon is also very well known in rumen of cattle, where bacterial numbers may be doubled after removal of protozoa (Gijzen *et al*., 1988). This was consistent with the present study.

Increase in numbers of faecal bacteria numbers were observed in some of the treatments especially in the absence of protozoa (Table 1). These results showed that the ability of protozoa to effectively remove faecal bacteria was not always clear in macrophytes and algal ponds. In the laboratory, some positive results have been obtained by several authors (Curds, 1992; Decamp *et al*., 1999) but in the field the results could be different.

Protozoa profiles and populations: In the 3 pond systems, most of the protozoa were found in the sediments. The high numbers of protozoa (>1000 mL^{-1}) seen in all 3 pond systems suggested that predation of protozoa on faecal bacteria could be important in the sediments. Gijzen *et al*. (1988) and Awuah *et al*. (2004), have shown that number of bacteria in the sedimentswere always more than at the other depths. Experiments carried out in this study shows otherwise. This suggests that high numbers of protozoa could not be correlated with effective removal faecal bacteria as claimed by Decamp *et al*. (1999) at all times.

The profile of the protozoa shows that protozoa populations were not evenly distributed in the pond systems (Table 4, 6 and 7). Hence, in areas where there were no protozoa, effective removal of faecal bacteria through protozoa predation of faecal bacteria could not occur, indicating the presence of pockets inactivity. The low protozoa population especially at the surface of the macrophyte ponds showed that complete reliance on predation for faecal bacteria removal may not be adequate.

CONCLUSION

Results from this study has shown that, that protozoa could play a major role in the removal of *E. coli* and *Salmonella* in water lettuce treatment ponds. However, the removal of faecal bacteria may not be significant in duckweed and algal ponds especially when the protozoa numbers are low. Long retention periods in the presence of harsh environmental conditions could also be the main contributing factors in the removal of faecal bacteria in macrophyte and algal-based waste stabilization ponds. Design of macrophyte ponds therefore should consider promoting harsh environmental conditions and increase in depth for longer retention periods in the ponds.

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