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Haematological, Histological and Growth Characteristics of *Oreochromis mossambicus* Exposed to Effective Microorganisms in Organically Manured Aquadams

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ABSTRACT

The aim of this study is to investigate the effect of Effective Microorganisms (EM) on the water quality, growth performance, haematological and histological changes in *Oreochromis mossambicus*. Five different EM dosages 7000 L of water (1, 3, 7, 10 and 15 L) and the control were assigned to aquadams fertilized with chicken manure in a completely randomized design replicated twice. Bicarbonate alkalinity and potassium increased with higher dosage of EM. This could have probably created favourable conditions for the proliferation of phytoplankton. Ammonia and phosphate were not significantly affected by the different EM dosages. There was a significant difference ($p<0.05$) in the RBC, WBC, HGB, HCT, MCHC, MCH and MCV in the different EM treatments. However, there was a weak dose-response relationship between the haematological parameters and EM dosage. Vacuolation, cellular swelling, nuclei pleomorphism, increase in kupffer cells and dilated sinusoids were the histological alterations observed as the EM dosage increased. Fish yield was best described by a parabolic function. The haematological and histological parameters showed that the fish were under stress at the highest EM dosage and this led to poor growth performance.

Key words: Haematological, histological, growth, microorganisms, manure

INTRODUCTION

The use of EM is widespread in various parts of the world because it is a low cost technology. Globally there is a brisk trade in EM for aquaculture. It was originally developed to improve the quality of soils, but its use has been extended to human health, livestock, industrial waste water treatment and aquaculture. There are claims that it enhances soil fertility, increases crop yield and crop quality, helps to correct nutritional and physiological crop disorders, reduces the infestation of pests and diseases, accelerates the decomposition of organic waste, increases beneficial micro organisms in the soil and helps control pathogens through competitive exclusion (APNAN, 1999). However, the concept is controversial as most of the claims by EM proponents still have to be put to test. There are very few peer reviewed scientific papers on EM technology. Most of the references on EM technology are from conference papers and technical reports that have not been peer-reviewed.

There have been claims of EM success reported in aquaculture, mostly from China (Fanping *et al*., 1997; Huang *et al*., 1999; Zhang *et al*., 1999; He *et al*., 2005) and some limited

success in South Africa (Prinslo and Schoonbee, 1986; Hanekom *et al*., 1999). The mode of action of EM remains largely speculative. It is claimed that application of EM in fish ponds enhances nitrification, ammonia removal and sulphide oxidation and this will lead to improvement in the water quality (Qi *et al*., 2009). Furthermore, it is claimed that application of EM in fish ponds will reduce the virulence of disease since it will enhance the immune response of the fish against pathogenic bacteria and also outcompete pathogenic bacteria (Ali *et al*., 2011; Shalaby, 2011).

In South Africa there are various types of brands of EM produced namely EM-1, EM bokashi, EM FPE and EM 5 (APNAN, 1999). However, the most commonly used brand is EM-1 which is dominated by photosynthetic bacteria species *Rhodopseudomonas palustris*. The Department of Agriculture in South Africa distributed aquadams (7000 L plastic fish tanks) to selected villagers for them to culture *Oreochromis mossambicus*. The villagers have been encouraged to use chicken manure and EM-1 ostensibly to improve fish production. The effect of EM-1 on the fish and water quality parameters has not been investigated before. The main objective of this study was to determine the effect of EM-1 on the health status of fish and growth performance in different EM dosages after application of chicken manure.

MATERIALS AND METHODS

The experiments on EM technology were conducted at the Aquaculture Research Unit at the University of Limpopo, South Africa, between September, 2012 and March, 2013.

Preparation of EMs: The EM-1 was purchased at ZZ2, Mooketsi. The basic constituents included were: 40 L EM stock solution, 50 kg brown sugar, 25 L of molasses with water to make up a total of 1000 L. The tank was sealed airtight and had a pipe with a water bottle at the rear to release pressure. The solution was incubated at 28-30°C for 7 to 10 days.

Experimental design: Six treatments of EM-1 at 0, 1, 3, 7, 10 and 15 L dosages were assigned to aquadams in a complete randomized design field experiment and replicated twice. Aquadams are plastic tanks with a water carrying capacity of 7000 L. The aquadams were fertilized with chicken manure at an application rate of 0.26 kg m^{-2} $week^{-1}$. The fish weight (±0.6 g) was determined before the fish were stocked in the aquadams. *Oreochromis mossambicus* fingerlings were stocked in each aquadam at the rate of 0.35 fish per litre, two weeks after application of manure. The different dosages of EM-1 were applied a month after the stocking of fish and were reapplied monthly. The following parameters were determined.

Temperature (°C), dissolved oxygen (mg L^{-1}), pH were measured using a hand held multi-probe water meter (YSI m, 556 MPS). Water samples for ammonia (mg L^{-1}), nitrite (mg L^{-1}), total alkalinity as $CaCO_3$ (mg L^{-1}) and phosphorus (mg L^{-1}) were collected monthly and measured using standard methods (APHA, 1985).

Blood from fish was drawn through caudal venous puncture by means of an injector and 2 mL was decanted in tubes with EDTA. Red Blood Cells count (RBC), Haematocrit (HCT), Haemoglobin (HGB), White Blood Cells Count (WBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were analysed. The counts were done using a blood analyser, Systemex, XT-1800i.

Twenty fish samples per treatment were collected at the end of the experiment. The fish were weighed, dissected and liver was removed and weighed. The liver samples were stored in 10%

formalin solution for 24 h. The tissues were sliced and placed in cassettes. The tissues were then washed in tap water and dehydrated in a series of increasing ethanol concentrations (30, 50, 70%) and then through xylene. Finally the tissues were embedded in melted paraffin and allowed to harden. The tissue samples were sectioned using a wax cutter. The sections were put on slides and were stained with haematoxylin and eosin using the H and E rapid staining protocol and covered with a coverslip. Samples were analysed with the aid of light microscopy at 400×magnification. A semi-qualitative histological assessment protocol was used to quantify histological alterations observed in the liver. The liver histology of the twenty fish from each treatment were assessed individually by using Pierce *et al.* (1978)'s grading system (0-4). Each group was then assigned an average grade value according to the dominant histological characteristics identified.

The fish were weighed at the end of the experiment and the Specific Growth Rate (SGR), fish yield and fish production indices were calculated as follows.

Specific growth rate (SGR; g day^{-1}) was calculated according to Winberg (1965):

$$SGR\ (\%) = \left[\frac{\ln W_t - \ln W_0}{t}\right] \times 100$$

Where:

W_t = Final body weight (g)
W_0 = Initial body weight (g)
t = Time (days):

$$\text{Fish yield} = \frac{\text{Mass (g)}}{\text{Area (m}^2\text{)}}$$

$$\text{Fish production} = \frac{\text{Yield per volume (gm}^{-3}\text{)}}{\text{Time (days)}}$$

Statistical analysis: Data was tested for normality using the Shapiro-Wilk normality test. One-way Analysis of Variance (ANOVA) was used to determine the significant differences of the water quality parameters, haematological parameters, histological parameters and growth parameters. Tukey's *post hoc* analysis was used to determine which means are significantly different from each other. The statistical analysis was carried out using the statistical package and service solution (IBM version 21).

RESULTS

Phosphate, pH, temperature and ammonia did not differ significantly ($p>0.05$) between treatments. However, there were significant differences ($p<0.05$) in dissolved oxygen, potassium and bicarbonate alkahinity. Bicarbonate alkalinity and potassium showed an increasing trend with the increasing levels of EM dosage (Table 1). The phosphate levels were 0.17 mg L^{-1} in the control and marginally dropped to 0.16 mg L^{-1} in the 15 L EM dosage (Table 1). There was no particular trend discernible for ammonia and phosphate. Oxygen showed a decreasing trend with the increasing EM dosage (Table 1). Dissolved oxygen was 6.87 mg L^{-1} in the control and marginally decreased to 6.33 mg L^{-1} in the 15 L EM dosage. The ammonia level was 0.03 mg L^{-1} in the control and rose to 0.32 mg L^{-1} in the 7 L EM dosage before decreasing to 0.18 mg L^{-1} in the highest EM dosage (15 L EM) (Table 1).

Table 1: Mean±SE of major physico-chemical parameters analyzed for water quality of the different EM treatments

	EM-1 treatments					
Parameters	0 L EM	1 L EM	3 L EM	7 L EM	10 L EM	15 L EM
Bicarbonate alkalinity (mg L^{-1})	22.81±0.001^{a}	27.41±0.001^{b}	29.61±0.001^{c}	33.21±0.001^{d}	33.61±0.001^{e}	35.21±0.001^{f}
Potassium (mg L^{-1})	1.45±0.000^{a}	1.63±0.003^{b}	2.11±0.003^{c}	3.71±0.003^{d}	4.62±0.003^{e}	3.24±0.003^{f}
DO (mg L^{-1})	6.87±0.001^{a}	9.65±0.001^{b}	8.50±0.001^{c}	8.40±0.001^{d}	7.73±0.001^{e}	6.33±0.001^{f}
pH	9.42±0.011^{a}	9.31±0.008^{a}	8.73±0.002^{b}	7.81±0.001^{c}	7.83±0.001^{c}	7.85±0.001^{c}
Temperature (°C)	23.97±0.003^{a}	23.27±0.021^{a}	23.32±0.032^{a}	23.66±0.033^{a}	24.10±0.010^{a}	23.52±0.502^{a}
Phosphate (mg L^{-1})	0.17±0.001^{ab}	0.24±0.001^{b}	0.21±0.001^{cd}	0.18±0.001^{bc}	0.14±0.001^{a}	0.16±0.001^{ab}
Ammonia (mg L^{-1})	0.03±0.003^{a}	0.04±0.003^{a}	0.17±0.003^{b}	0.32±0.003^{b}	0.26±0.003^{c}	0.18±0.003^{d}

NB: Figures in the same row with different superscript are significantly different ($p<0.05$, ANOVA)

Table 2: WBC, RBC, HCT, MCV, MCH and MHCV of *O. mossambicus* exposed to different EM dosages

	Treatments					
Blood parameters	0 L EM	1 L EM	3 L EM	7 L EM	10 L EM	15 L EM
WBC (^3 µL)	483.03±2.89^{f}	548.57±2.89^{a}	425.43±2.89^{b}	375.97±2.89^{c}	298.80±2.89^{d}	644.49±2.89^{e}
RBC (^6 µL)	1.19±0.01^{d}	1.23±0.01^{a}	1.33±0.02^{b}	1.25±0.01^{a}	1.05±0.01^{c}	1.24±0.01^{a}
HGB (g dL^{-1})	5.33±0.06^{e}	4.93±0.06^{a}	4.83±0.06^{a}	5.13±0.06^{b}	4.43±0.06^{c}	6.03±0.06^{d}
HCT (%)	22.43±0.06^{a}	22.53±0.06^{a}	23.83±0.06^{b}	22.43±0.06^{a}	16.93±0.06^{c}	22.53±0.06^{a}
MCV (f L^{-1})	189.83±0.06^{e}	184.43±0.06^{a}	180.33±0.06^{b}	180.63±0.06^{b}	162.53±0.06^{c}	181.53±0.06^{d}
MCH (pg)	44.93±0.57^{c}	40.23±0.57^{a}	36.43±0.57^{b}	41.13±0.57^{a}	44.33±4.10^{c}	48.43±0.87^{d}
MCHC (g dL^{-1})	23.73±0.29^{e}	21.83±0.29^{a}	20.23±0.29^{b}	22.83±0.29^{e}	26.03±0.29^{d}	26.73±0.29^{d}

NB: Figures in the same row with different superscript are significantly different ($p<0.05$, ANOVA)

WBC count increased in the 1L EM dosage but it subsequently decreased with higher EM dosage before rising significantly ($p<0.05$) to the highest level in the 15 L EM dosage (Table 2). WBC was 483×10^{3} in the control and rose to 584×10^{3} in the 1 L EM dosage declined to 298 in the 10 L EM dosage before rising to 644×10^{3} in the 15 L EM dosage (Table 2). RBC, HCT and HGB did not show any particular trend with increasing EM dosage (Table 2). RBC was 1.19×10^{6} in the control rose to 1.33×10^{6} in the 3 L EM dosage and decreased to 1.24×10^{6} in the 15 L EM dosage. HGB and MCHC followed a similar trend (Table 2). It is however significant to note that the RBC count in the highest EM dosage (15 L EM) was significantly higher ($p<0.05$) than the control. HGB in the 15 L EM dosage was significantly higher ($p<0.05$) than the control. However, the HCT count was not significantly different ($p>0.05$) between the control and the 15 L EM dosage. Generally, MCV decreased as the dosage of EM increased. There was however, an increase of MCV at the 15 L EM dosage but the MCV count was still significantly ($p<0.05$) lower than the control. MCH and MCHC showed the same trend, an initial decrease with higher EM dosage followed by an increase. For both MCH and MCHC the highest counts were recorded in the 15 L EM dosage (Table 2) and they were significantly higher ($p<0.05$) than the control.

Liver histology in the 0, 1, 3 and 7 L treatments appeared relatively normal and were scored an overall grade of 2 (Table 3, Fig. 1a-e). There was vacuolation observed in all the fish livers. Liver histology of the fish in the 10 L EM showed vacuolation as well as hepatic cord disarray and scored an overall grade of 4 (Fig. 1f). Cellular swelling was observed in most of the fish livers in the 15 L EM treatment as well as vacuolation and nuclei polymorphism and were given a overall grade value of 3 (Table 3, Fig. 1g).

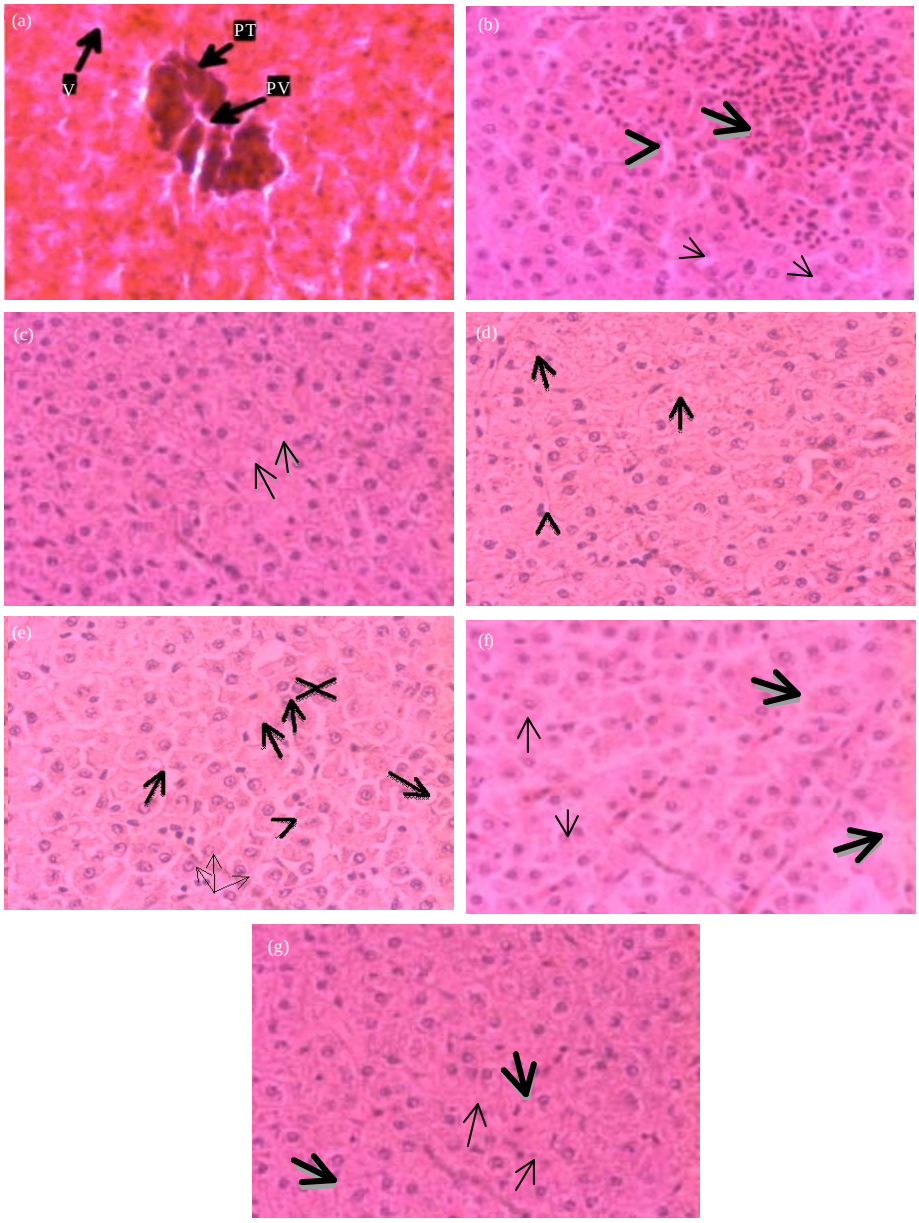

Fig. 1(a-g): Light micrographs of liver sections (H and E, 400x) of *O. mossambicus*, (a) Normal liver; Portal vein (PV); Pancreatic tissue (PT); Vacuole (V) adapted from Sadiq Bukhari *et al*. (2012), (b) 0 L EM treatment (Control); thick arrow showing kuppfer cells; thin arrow showing vacuoles and arrow head showing sinusoid, (c) 1 L EM treatments; arrows showing vacuoles, (d) 3 L EM treatment; thick arrows showing vacuoles and arrow head showing sinusoid, (e) 7 L EM treatment; thick arrows showing vacuolation, arrow heads showing dilated sinusoids and thin arrows showing nuclei pleomorphism, (f) 10 L EM treatment; thick arrows showing hepatic cord disarray; thin arrows showing nuclei pleomorphism and (g) 15 L EM treatment; cellular swelling with thick arrows showing vacuoles and thin arrows showing nuclei pleomorphism

Table 3: Number of *O. mossambicus* liver, given specific score according to their histological characteristics

	Treatment					
Score	0 L EM	1 L EM	3 L EM	7 L EM	10 L EM	15 L EM
0	-	-	-	-	-	-
1	-	1	9	5	-	-
2	18	19	11	15	-	2
3	2	-	-	-	3	18
4	-	-	-	-	17	-
Overall	2	2	2	2	4	3

Table 4: Mean±SD for growth parameters of *Oreochromis mossambicus* in the different EM treatment

Treatments (L EM)	SGR (g day^{-1})	Yield (kg ha^{-1})	Production (kg h^{-1} year^{-1})
0	1.50±0.003^{a}	424.64±0.02^{f}	1286.66±0.06^{f}
1	0.90±0.001^{b}	240.63±0.02^{a}	729.18±0.06^{a}
3	1.80±0.001^{a}	665.27±0.03^{b}	2015.94±0.05^{b}
7	2.20±0.001^{a}	962.50±0.02^{c}	2916.67±0.06^{c}
10	2.00±0.000^{a}	527.88±0.03^{d}	1629.91±0.06^{d}
15	2.00±0.000^{a}	410.49±0.03^{e}	1243.88±0.06^{e}

NB: Figures in the same column with different superscript are significantly different ($p<0.05$, ANOVA)

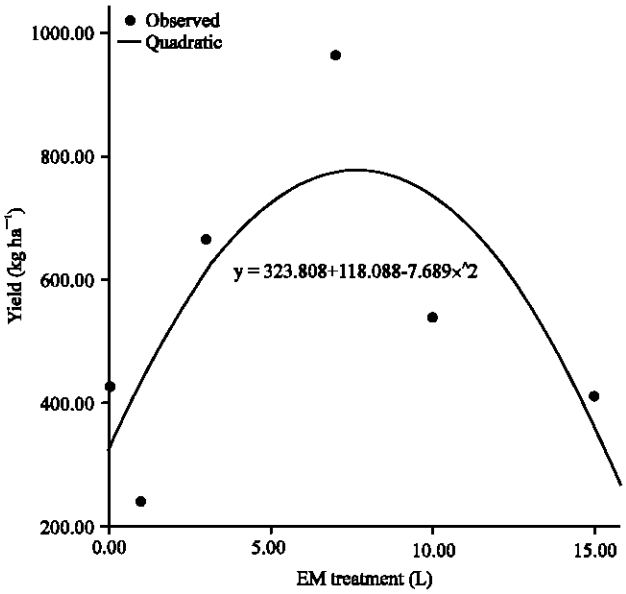

Fig. 2: A quadratic equation of fish yield and different EM treatments

There was a significant difference ($p<0.05$) in fish yield and production between the different EM treatments. The 7 L EM treatment gave the highest yield (Table 4). The specific growth rate showed no significant difference ($p>0.05$) in the different treatments (Table 4). The highest yield of 962.50 kg ha^{-1} along with a production value of 2916.67 kg ha^{-1} year^{-1} were realised in the 7 L EM dosage. The highest EM dosage produced the lowest yield (410.49 kg ha^{-1}) and production (1243.88 kg ha^{-1} year^{-1}) (Table 4). The relationship between fish yield and EM dosage (Fig. 2) was adequately described by the quadratic equation:

$$y = 323.808+118.088-7.689\ x^2$$

DISCUSSION

It has been claimed by proponents of EM (Shalaby, 2011; Zakaria *et al.*, 2010; El-Dakar *et al.*, 2004) that the application of EM will improve water quality by controlling the ammonia levels and increasing oxygen levels. This study, however, failed to reveal a positive influence of EM on key water quality parameters (phosphate, ammonia and dissolved oxygen). The mode of action of EM is claimed to be enhancement of organic matter degradation, nitrification and ammonia removal (Ali *et al.*, 2011). Nitrifying bacteria in a pond will always respond to the availability of substrate. Thus, if a pond has high ammonia levels, nitrifying bacteria will respond by multiplying rapidly and their abundance will decline when ammonia concentration decrease (Denev *et al.*, 2009; Ali *et al.*, 2011). A shortage of nitrifying bacteria does not arise if other environmental variables such as temperature, dissolved oxygen and pH are suitable. This probably explains why EM failed to show a positive influence on ammonia. It therefore, appears that the application of EM did not influence the rate of bacterially mediated processes such as nitrification.

Phosphate did not increase with increase in EM dosage. It is claimed by EM proponents that application of EM in ponds leads to a reduction in phosphate (Ali *et al.*, 2011; Parmar and Sindhu, 2013). The mode of action of EM that will lead to the reduction of phosphorus is supposedly the enhancement of phosphorus uptake in the fish ponds. However, no concrete evidence is available.

The increase of potassium with increasing EM dosage is probably because EM contains potassium solubilizing bacteria, *Bacillus* sp. (Kumar *et al.*, 2006). *Bacillus* sp. releases potassium from the organic manure, thus increasing the bioavailability of potassium (Han and Lee, 2005; Parmar and Sindhu, 2013). Dissolved oxygen generally decreased at high EM dosages. This is contradicts other studies (Ali *et al.*, 2011; Shalaby, 2011; Zakaria *et al.*, 2010) where the application of EM in fish ponds increased dissolved oxygen concentration. This is probably because in this study, at higher EM dosage (15 L) the Biological Oxygen Demand (BOD) increased as the EMs acted on organic matter associated with chicken manure.

Haematology is important in monitoring physiological and pathological changes in fish (Kori-Siapere *et al.*, 2005). Blood parameters vary in different fish species and this reflects adaptations to the varied environment conditions (Ramawamy and Reddy, 1978; Moyle and Cech, 1982; Arnold, 2005; Adeyemo, 2007). The RBC, HGB and MCHC count had no particular trend in the different EM dosages; however, HGB and MCHC were highest in fish exposed to 15 L EM dosage. This is possibly because of the low dissolved oxygen at high EM dosage which may necessitate production of more RBC to compensate for the low oxygen transportation and high energy demand (Orun *et al.*, 2003). Furthermore, increase in RBC has been reported by Adeyemo (2007) to indicate secondary responses to irritants. The high total dissolved solids associated with chicken manure may have acted as irritants to the fish.

The highest WBC count was observed in 15 L EM treatment. This was seen as a response of cellular immune system to infection. Sahan and Cengizler (2002) and Sahan *et al.* (2007) reported similar responses of fish to unfavourable conditions. Fifteen litres EM treatment had the highest HGB, MCH and MHCH levels and this may be as a result of the stress the fish were subjected to. MCV generally declined with increasing EM dosage. The HCT also had no distinctive trend; however, the lowest was in the 10 L EM treatment. The 10 L EM treatment also had the lowest RBC, HGB and MCV. A decrease in the mentioned haematological parameters indicates worsening of the organism's health status (Adeyemo, 2007). It has been claimed by the proponents of EM that

it enhances the immune system of fish (He *et al*., 2005; Condor-Golec *et al*., 2007). Fish in organically manured ponds are prone to infections by several pathogens (Osman *et al*., 2009). Application of EM-1 was thus supposed to boost the fish immunity. However, the blood parameters did not show that the immune system was boosted.

The major histological changes in the EM exposed fish were vacuolation, cellular swelling, nuclei pleomorphism, increase in kupffer cells and dilated sinusoids. The most severe histological alterations were observed in the 15 L EM and 10 L EM treatments and these were scored 4 and 3, respectively. The 7 L EM treatment had minor histological alterations while 0, 1 and 3L EM treatments appeared more normal and were all scored an overall grade of 2.

Vacuolation is characteristic of most fish livers but may differ in intensity depending on environmental stress, feeding habits and contaminant exposure. Large vacuoles force the nuclei to the periphery causing nuclear atrophy (Hibiya, 1982). This explains the presence of vacuoles in all the liver cells in all the treatments. However, a drastic increase in the vacuolated cells was observed in the 10 L EM treatment through to the 15 L EM. The degree of the vacuolation increased with increasing EM dosage. Vacuolation is associated with protein synthesis inhibition, energy deletion, disaggregation of microtubules or shift in substrate utilization (Van Dyk *et al*., 2007).

Cellular swelling was observed only in the livers of 15 L EM exposed fish. Hinton and Lauren (1990) reported that cellular swelling occurs because of the denaturation of volume-regulating ATPases or indirectly by disruption of cellular energy transfer processes required for ionic regulation. Both vacuolation and swelling of cells resulted in nuclei pleomorphism in the 7 L EM treatment through to 15 L EM treatment. Dilated sinusoids and increased kupffer cells were observed in the 0 L EM treatment. This indicated that there was a compromise to the immune system of the fish, thus the increase in kupffer cells was to destroy the pathogens that were present in the liver. This was expected because of the organic enrichment caused by the application of chicken manure, as it introduces bacteria in the water column. The livers of the fish that were exposed to 10 L EM treatment showed the denaturation of the hepatic cord structure. This indicates severe compromise of the health of the fish. This is further confirmed by the decrease in the RBC, HGB, WBC, HCT and MCV in the fish exposed to 10 L EM treatment, which indicates worsening of the health status of fish. It appears that the low dissolved oxygen levels at high EM dosages stressed the fish, thus opening them up to opportunistic infections.

There was a significant difference ($p<0.05$) of the fish yield and production in the different EM treatments. EM is claimed to increase phytoplankton and consequently the yield of fish (Tripathy and Ayyappan, 2005; Garg and Bahatnagar, 1996; Salton and El-Laithy, 2008). The fish yield showed a parabolic function. The role of EM in improving fish production in aquaculture systems remains dubious. The plot of EM dosage against fish yield showed a wide scatter and this again indicates the poor relationship between fish yield and EM dosage. Maeda *et al*. (1997) reported an increase in fish growth after application of EM. They suggested that this was achieved because EM suppressed the pathogenic bacteria and viruses. However, in this study as already indicated by the haematological and histological parameters high dosages of EM compromised the health of the fish.

CONCLUSION

Haematological and histological analysis showed that at the highest EM dosage (15 L) the health status of *Oreochromis mossambicus* was compromised. This was further confirmed by the low fish production and yield at the highest EM dosage. It is highly unlikely that EM-1 directly

caused harmful effects to the fish. The high EM dosage may have resulted in high BOD levels and this consequently led to low levels of dissolved oxygen saturation which was stressful to the fish.

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