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# Influence of $Zn^{2+}$ and $Mg^{2+}$ on Some Kinetic Parameters of Alkaline Phosphatase in Agba River Water

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**ABSTRACT**
The level of alkaline phosphatase (ALP) activity in fresh water body was investigated using filtered and unfiltered Agba River water to assess the level of contamination and the role of cofactors-containing effluents in the degree of acceptable quality of Agba River water. Alkaline phosphatase catalyses phosphorous recycling, which is important for zooplankton and phytoplankton growth. The activity of alkaline phosphatase in water can be used to determine if there is pollution in the water or not. Higher activity of alkaline phosphatase was observed in unfiltered Agba River when compared to the filtered sample and this may be as a result of pollution by phosphoesters or due to the presence of both bound and free forms of the enzyme. The influence of increase in concentration of zinc and magnesium ions on the kinetic parameters ($K_m$ and $V_{max}$) of unfiltered and filtered Agba River alkaline phosphatase was also determined. $Zn^{2+}$ had a higher stimulatory effect (with $V_{max}$ of 0.50-5.00 nmol/min for unfiltered and 0.43-2.00 nmol/min for filtered sample) on the enzyme rate of reaction when compared to that of $Mg^{2+}$ (with $V_{max}$ of 0.63-2.50 nmol/min for unfiltered and 0.22-0.71nmol/min for filtered sample) as depicted by the increase in $V_{max}$ as the concentration of $Mg^{2+}$ and $Zn^{2+}$ increased. The $K_m$ values decreased as the concentration of $Zn^{2+}$ and $Mg^{2+}$ increased. The presence of ALP in Agba River may serve as a means by which water pollution, through phosphorus contaminants like detergents, fertilizers and animal wastes from homes and industries, can be reduced. Also, the presence of $Zn^{2+}$ and $Mg^{2+}$ in the discharged pollutants may further strengthen the reduction of fresh water contamination by ALP.

**Keywords:** Zinc ion, Magnesium ion, Alkaline phosphatase, Agba River water, $K_m$, $V_{max}$

## INTRODUCTION

Phosphatases are a group of orthophosphorus mono- and diester phosphohydrolases capable of cleaving orthophosphate groups from organic phosphate compounds. These enzymes have been characterized as alkaline, neutral, or acid phosphatases, based on the optimum pH for the observed hydrolytic cleavage of the esterified phosphate group. In most cases these enzymes are rather unspecific with respect to the organic phosphate substrate as well as to the pH at which the hydrolysis occurs (Flint and Hopton, 1977a; Torriani, 1960).

Although these enzymes act as extracellular enzymes, they are associated with specific membrane-cell wall components and are localized, in gram-negative bacterial cells, in the periplasmic space (Bhatti, 1978) or on the cell wall (Cheng *et al*., 1971). Phosphate enzymes are located at the periphery of algal and bacterial cells (Kuenzler and Perras, 1965). Free phosphatase results from the natural loss of phosphatase from the periplasmic region and environmental stress such as cation or osmotic shock.

The presence and activity of phosphatases in natural systems relates directly to phosphorus cycling and its availability to other organisms such as algae and macrophytes. Phosphatase activity in natural systems is modulated by mineral forms of phosphorus as well, as specific inhibitors or repressors of phosphatase production and activity (Flint and Hopton, 1977, Tyler, 1974). The occurrence of phosphatases in aquatic, terrestrial and sewage systems has been documented (Berman, 1969; Berman, 1970; Flint and Hopton, 1977b).

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Phosphate for primary production can be supplied by remineralization of organic phosphorous compounds, by release of phosphate by zooplankton (Moegenburg and Vanni 1991, Gulati *et al*., 1995, Vadstein *et al*., 1995, Hantke *et al*., 1996) and phagotrophic protozoa (Nagata and Kirchman, 1992), and by external input from land. Alkaline phosphatases (ALP) can be found on the outer surface of the cell where they cleave a variety of phosphate monoesters in the surrounding water (Kuenzler, 1965), thereby allowing phytoplankton to utilize organic phosphates when in-organic phosphate is no longer available (Kuenzler and Perras, 1965).

Phosphorus is a major limiting nutrient in many aquatic ecosystems, especially in temperate freshwater lakes (Hecky and Kilham, 1988). Phytoplankton primary productivity, biomass, and community composition strongly depend on phosphorus availability. The intensity of ALP expression is commonly used as an indicator of phosphorus deficiency in algae (Cotner and Wetzel 1992). The major activity performed by ALP in water is the recycling of phosphorous. This is required for the growth of phytoplankton in water. The importance of dissolved organic phosphorous components as a phosphate source for phytoplankton growth was raised by Curies and Kalff (1984). However, most algal species can use organic phosphate for growth only if hydrolyzed by phosphatase enzymes (Cembella *et al*., 1984).

Surface water is a term used to describe water sources occurring naturally on the ground (Oloyede *et al.,* 2003; Amadi *et al.,* 2006). Accessibility and availability of fresh and clean water is the key to sustainable development and an essential element in health (Adekunle *et al.,* 2004; Odeyemi *et al.,* 2010). Surface water bodies in developing countries are predisposed to pollution. Water is generally said to be polluted when its acceptable quality has been altered by man's activities through anthropogenic inputs such that its intended usage for commercial or domestic purpose is hampered (Amadi *et al.,* 2006; Ibeh and Mbah, 2007).

There are many published reports on Nigerian rivers (Umeham, 2000; Amadi *et al.,* 2003; Oloyede *et al*., 2003; Ekaise and Anyasi, 2005; Akaninwor and Egwim, 2006; Ibeh and Mbah, 2007; Victor *et al.,* 2007) but there is limited data on Agba River in Ilorin. Ilorin is located on latitude 8°30'N and Longitude 4°35'E with an area of about 100 km (Kwara State Diary 1997). The city is situated in the transitional zone between the forest and savanna region of Nigeria. The geology of Ilorin consists of Pre-Cambrian basement complex with an elevation that varies from 273m to 333m in the West having an isolated hill (Sobi Hill) of about 394m above sea level and 200m to 364m in the East. Oyegun, 1983 further asserted that a large part of Ilorin town is laid by sedimentary rock, which contains both primary and secondary laterites and alluvial deposits. The most important river is Asa River which flows in south-northern direction. The other rivers that drain into Asa River are River Agba, River Alalubosa, River Okun, River Osere and River Aluko. Agba River is located in the Agba dam area of Ilorin.

It has been shown that alkaline phosphatase have three classes of metal binding sites, which have been designated to perform catalytic, regulatory and structural roles. The alkaline phosphatase of *E. coli* is a dimer composed of two identical subunits with four atoms of zinc and two atoms of magnesium (Kim and Wyckof, 1991). Removal of the zinc ions leads to loss of catalytic activity while replacement of the zinc ions by other divalent cations resulted in lower maximal activity. Three metal ions (two $Zn^{2+}$ and one $Mg^{2+}$) in ALP active sites are essential for enzymatic activity. The inhibition of alkaline phosphatase by excess $Zn^{2+}$ has been proposed to be due to the replacement of $Mg^{2+}$ by $Zn^{2+}$ at one site of the enzyme (PetitClerc and Fecteau, 1977). The objective of this study is to ascertain the presence and level of alkaline phosphatase in Agba River water and also to determine the effect of certain reaction conditions on the kinetic parameters of ALP in the fresh water of Agba River. This may serve as necessary indicator to monitor levels and extent of pollution.

## MATERIALS AND METHODS

*Chemicals*

The substrate, paranitrophenyl phosphate (pNPP), $ZnSO_4$ and $MgSO_4$ are products of Sigma Chemical Chemical, England. Bicarbonate buffers and NaOH are prepared in the laboratory using the standard procedures.

*Collection and preparation of sample*

The fresh water used for the experiment was from Agba River (Figure 1), Agba dam area, Ilorin, Kwara State, Nigeria. The water was collected at the bank of the river, where there is phytoplankton growth. The depth of the collection was noted which is about 10cm deep. The temperature of the water was maintained at $0^0$C on collection with ice packs to avoid the denaturization of the enzyme. The water sample was divided into two parts: one part was filtered with a filter paper and the other was not filtered. The filtered sample was labeled A and the unfiltered labeled B.

*Assay of ALP activity*

The activity of alkaline phosphatase in both the filtered and unfiltered water samples was assayed by monitoring the rate of hydrolysis of p-NPP at 25$^o$C in 0.1 M $Na_2CO_3$-$NaHCO_3$ (sodium carbonate – bicarbonate) buffer (pH 10.1) as described by Ahlers (1975). Alkaline phosphatase catalyzes the hydrolysis of p-NPP to yield para–nitrophenol, which is bright yellow (other reactants and products are colourless in aqueous solution), the intensity of which is proportional to the enzyme activity. Reactions were initiated by the addition of the appropriate concentration of the substrate, pNPP. Incubation was allowed for 10 minutes before stopping the reaction by the addition of 0.1 M KOH. The absorbance was read at 400 nm against a blank of the buffered substrate on a Specronic-21 UV-Vis spectrophotometer and the corresponding activities recorded. The activity of the enzyme was determined in the absence and presence of $Mg^{2+}$ and $Zn^{2+}$ respectively and ALP activity expressed in nmol/ min. The concentrations of $Zn^{2+}$ and $Mg^{2+}$ investigated were 0.2, 0.4, 0.6, 0.8 and 1.0mM at pNPP concentrations ranging from 0.63-5.1mM.

In order to determine the concentration dependent effect of $Mg^{2+}$ within optimal levels, the reaction medium was set up essentially as described by Wright and Plummer (1972) but $Mg^{2+}$ concentration was varied in the range of 0.2-1.0mM. The concentration range of pNPP was kept constant for all $Mg^{2+}$ concentrations studied and incubation was allowed for 10 minutes before stopping the reaction by the addition of 0.1M NaOH. The absorbance was monitored at 400nm against a blank of the buffered substrate and the corresponding activities were recorded.

## RESULT

The hydrolysis of pNPP over a range of concentrations (3.33 – 25.67 mM) by filtered and unfiltered Agba river alkaline phosphatase obeyed Michaelis-Menten kinetics as shown in Figure 1 and the double reciprocal plot is shown in Figure 2. This allows for the estimations of Michaelis constant and maximum rates. The good fit of the data to Michaelis–Menten equation affords a simple way of analyzing the data.

An analysis of the roles of $Mg^{2+}$ and $Zn^{2+}$ in the activation of unfiltered and filtered Agba River ALP was carried out by investigating the kinetics of pNPP hydrolysis in the presence of 0.2, 0.4, 0.6, 0.8 and 1.0mM of the two metal ions separately. The Michaelis-Menten plots for ALP activity from filtered and unfiltered Agba River in the presence of $Mg^{2+}$ are shown in Figures 3 and 5 respectively and their double reciprocal plots are shown in Figures 4 and 6 respectively.

The effects of $Mg^{2+}$ on the enzyme $K_m$ for pNPP and $V_{max}$ values of ALP from both unfiltered and filtered Agba River were obtained from Lineweaver-Burk plots (Table 1). Kinetic analysis showed that a more progressive increase in activation occurred in unfiltered Agba River ALP when compared with filtered Agba River ALP when $Mg^{2+}$ concentration was raised from 0.2mM to 1.0mM as observed by the difference in $V_{max}$ values in the two samples. Increasing $Mg^{2+}$ concentration in both unfiltered and filtered Agba River from 0.2 mM to 1.0 mM resulted in a decrease in the $K_m$ for pNPP. The activation of both unfiltered and filtered Agba River ALP by $Zn^{2+}$ followed a pattern similar to

that of $Mg^{2+}$ in affecting both the $V_{max}$ and the $K_m$ (Figures 7-10).

The effects of $Zn^{2+}$ on the enzyme $K_m$ for pNPP and $V_{max}$ values of ALP from both unfiltered and filtered Agba River were obtained from Lineweaver-Burk plots (Table 2). Similar to that in the presence of $Mg^{2+}$, a progressive increase in activation was observed in the activity of ALP both from unfiltered and filtered samples as the concentration of $Zn^{2+}$ was increased from 0.2mM to 1.0mM. This is indicated by the rise in $V_{max}$ values (Table 2). A corresponding decrease in $K_m$ values for pNPP was also obtained. Comparing activation of ALP by the two ions ($Mg^{2+}$ and $Zn^{2+}$), a higher degree of activation was observed in the presence of $Zn^{2+}$.

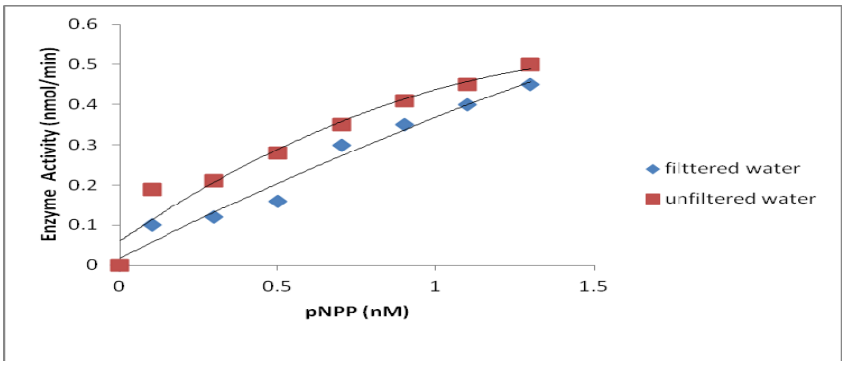


Figure 1: ALP activity in Agba River

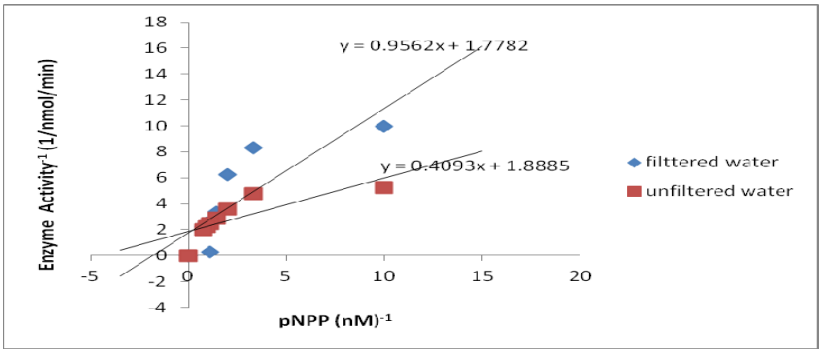


Figure 2: Double-reciprocal plot of ALP-catalyzed hydrolysis of p-NPP

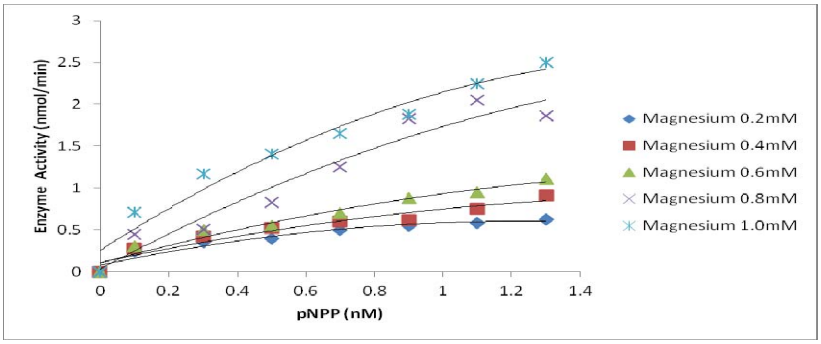


Figure 3: Michaelis-Menten curve of the influence of various concentrations of $Mg^{2+}$ on ALP activity in unfiltered Agba River

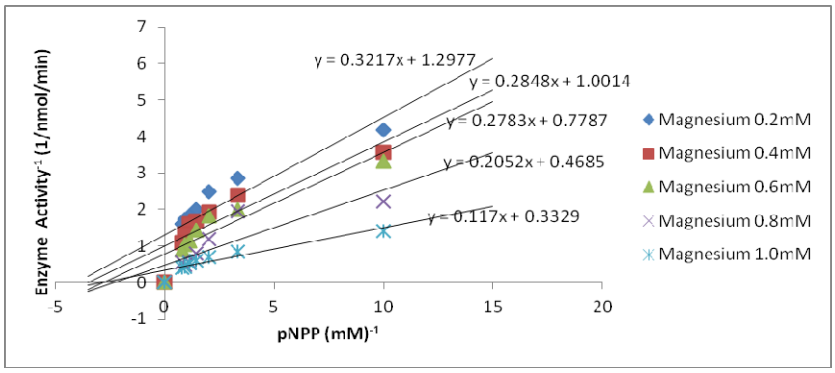


Figure 4: Double-reciprocal plot of the effect of various concentrations of $Mg^{2+}$ on the rate of ALP (from unfiltered Agba River)-catalyzed hydrolysis of p-NPP

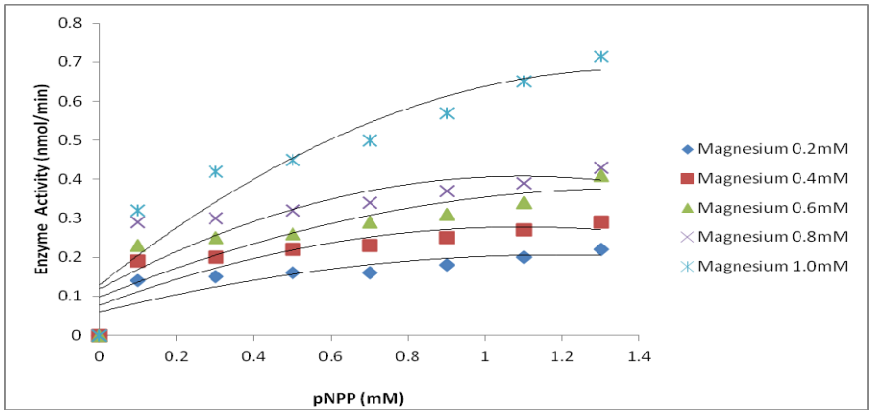


Figure 5: Michaelis-Menten curve of the influence of various concentrations of $Mg^{2+}$ on ALP activity in filtered Agba River

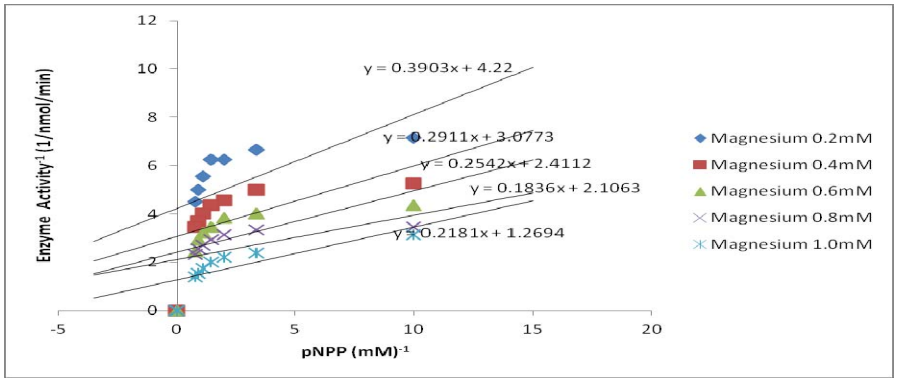


Figure 6: Double-reciprocal plot of the effect of various concentrations of $Mg^{2+}$ on the rate of ALP (from filtered Agba River water)-catalyzed hydrolysis of p-NPP

Table 1: Influence of $Mg^{2+}$ concentrations on the kinetic parameters of alkaline phosphatase in Agba river water

| $Mg^{2+}$(mM) | $V_{max}$ (nmol/ml) | | $K_m$ | |
|---|---|---|---|---|
| | Filtered | Unfiltered | Filtered | Unfiltered |
| 0.2 | 0.221 | 0.625 | 0.475 | 2.411 |
| 0.4 | 0.29 | 0.909 | 0.385 | 1.850 |
| 0.59 | 0.41 | 1.111 | 0.379 | 1.800 |
| 0.80 | 0.434 | 1.86 | 0.333 | 1.981 |
| 1.00 | 0.714 | 2.500 | 0.147 | 1.083 |

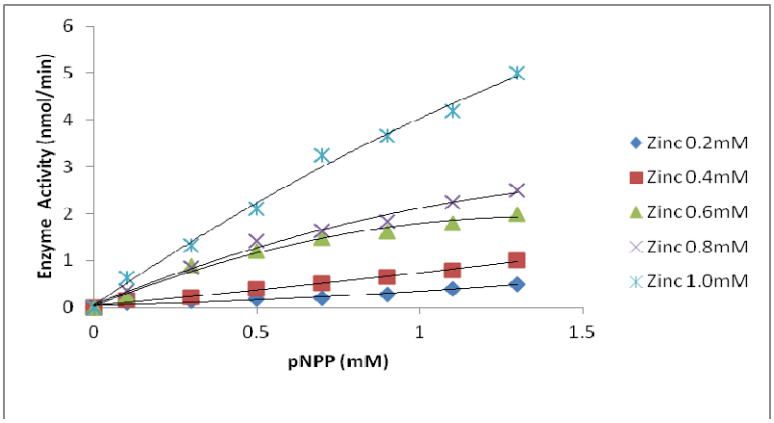


Figure 7: Michaelis-Menten curve of the influence of various concentrations of $Zn^{2+}$ on ALP activity in unfiltered Agba River water

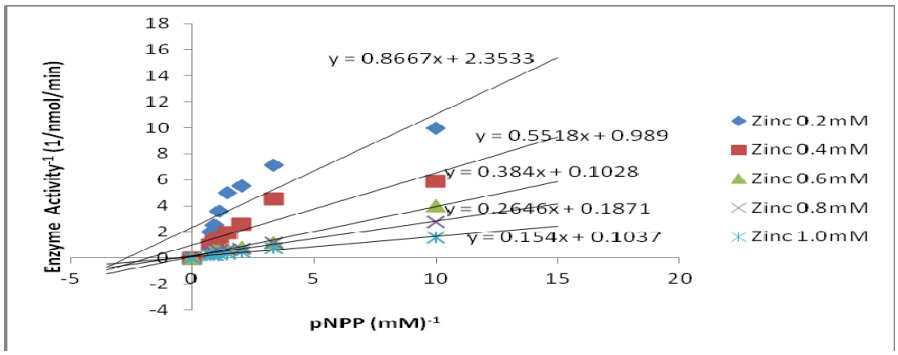


Figure 8: Double-reciprocal plot of the effect of various concentrations of $Zn^{2+}$ on the rate of ALP (from unfiltered Agba River)-catalyzed hydrolysis of p-NPP

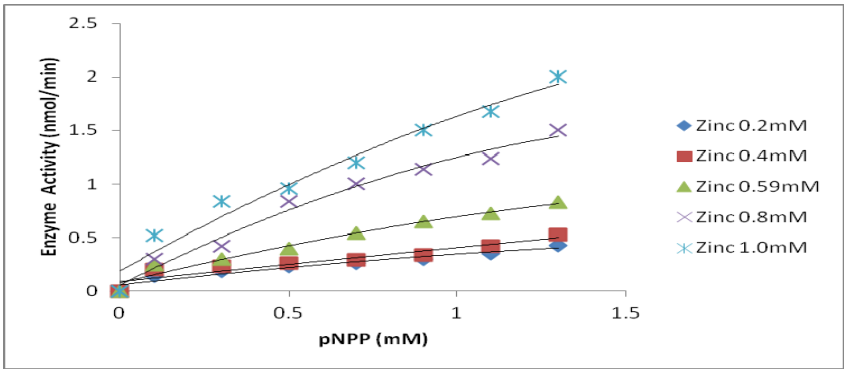


Figure 9: Michaelis-Menten curve of the effect of various concentrations of $Zn^{2+}$ on ALP activity in filtered Agba River water

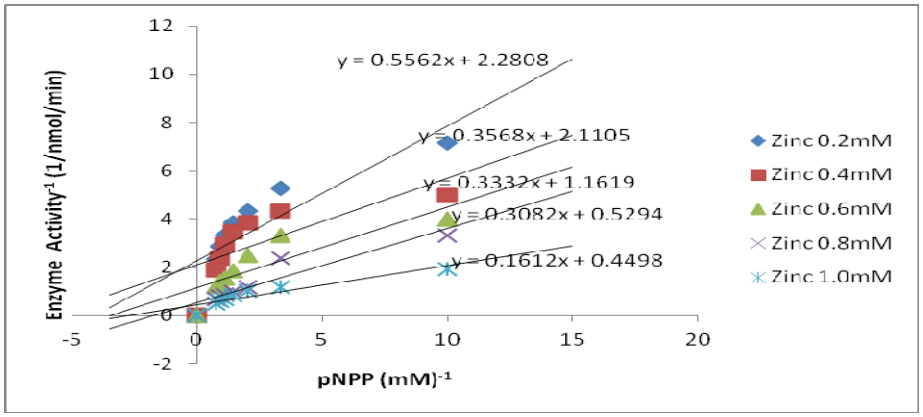


Figure 10: Double-reciprocal plot of the effect of various concentrations of $Zn^{2+}$ on the rate of ALP (from filtered Agba River)-catalyzed hydrolysis of p-NPP

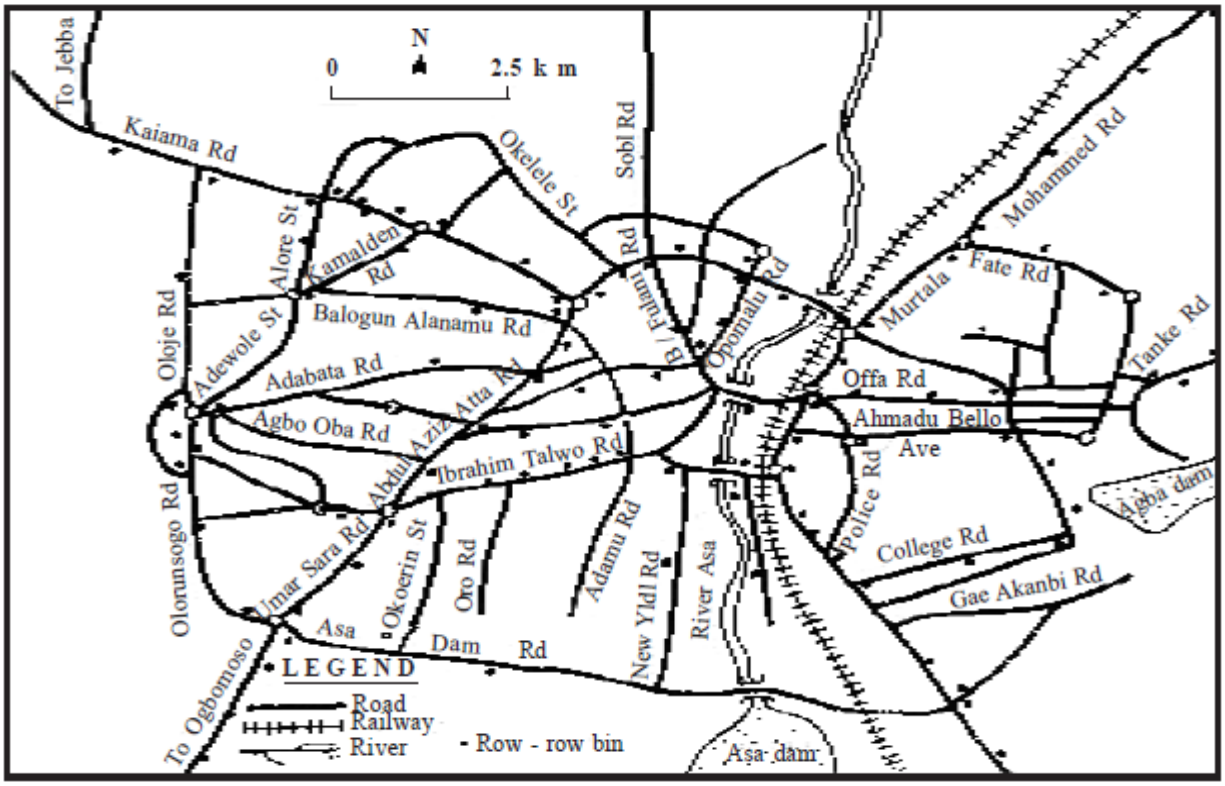


Figure 1: Map of Ilorin Metropolis showing locations of Dumpsters (Roro bins)
*Source:* Ministry of Lands and Surveys, Ilorin, Nigeria.

Table 2: Influence of $Zn^{2+}$ concentrations on the kinetic parameters of alkaline phosphatase in Agba River water

| $Zn^{2+}$(mM) | $V_{max}$ (nmol/min) | | $K_m$ | |
|---|---|---|---|---|
| | Filtered | Unfiltered | Filtered | Unfiltered |
| 0.2 | 0.43 | 0.50 | 1.866 | 2.466 |
| 0.4 | 0.53 | 1.00 | 1.212 | 1.823 |
| 0.59 | 0.83 | 2.00 | 0.888 | 1.823 |
| 0.80 | 1.5 | 2.5 | 0.569 | 1.692 |
| 1.00 | 2.0 | 5.0 | 0.614 | 2.377 |

## DISCUSSION

The occurrence of phosphatases in aquatic, terrestrial and sewage systems has been documented (Berman, 1969; Browman, and Tabatabai. 1978). The presence of alkaline phosphatase (ALP) in natural water bodies is further confirmed in this study by the dephosphorylation of pNPP by both filtered and unfiltered Agba River water**.** Higher activity of ALP observed in the unfiltered sample may be as a result of planktons and zooplanktons which are present in both bound and free forms. The bound form is not diffusible and therefore not present in the filtered sample**.** Filtration removes two types of material: firstly, bacteria and phytoplankton, which may alter dissolved phosphorus concentrations by uptake, breakdown of organic/polymeric fractions and release phosphorus on death by lysis, secondly, particulate materials which may adsorb or release phosphorus on standing (Helen *et al*., 2002). It is only the free form that is present in the filtered water, but both free and bound form are present in the unfiltered sample and they release more alkaline phosphatase, than in filtered sample.

The use of phosphatase enzymes to determine bioavailable organic phosphorus was first reported by Strickland and Solarenzo (1966). Thereafter, this method has been used to investigate the cycling of organic phosphorus in aquatic environments and its contribution to biological phosphorus supply (Francko and Heath, 1979; Hino, 1989; Cooper *et al*., 1991). The higher activity of ALP observed in the unfiltered sample of Agba river therefore shows its participation in phosphorous recycling. The phosphorus may also be as a result of phosphoester from pollution of water with fertilizers or other chemicals (Matavulj *et al*., 1978, Tadano *et al*., 1993). Phosphorus is a necessary nutrient for plants to live, and is the limiting factor for plant growth in many freshwater ecosystems. The addition of phosphorus increases algal growth.

The activation of ALP-catalyzed hydrolysis of pNPP observed in this study by the two metal ions is exerted via both $V_{max}$ and $K_m$ effects. Metalloenzymes may contain one or more sets of metal binding sites whose occupancy by the same or different metal atoms critically affects catalysis, structural stability, or both. McCracken and Meighen (1981) established the existence of three classes of metal binding sites in ALP. These are designated "catalytic" "structural" and "regulatory". $Zn^{2+}$ occupies the catalytic and structural sites, while $Mg^{2+}$ ions are bound at the regulatory site (Bosron *et al*., 1975; Anderson *et al.*, 1978**)**. Brunel and Cathala (1972), in explaining the role of metal ions in metal-activated enzymes systems proposed that the metal ion can act as a bridge between the enzyme and its substrate or it can induce conformational changes and thereby convert an inactive or partially activated form of an enzyme into a catalytically active or more active form. Arise *et al*. (2005) has shown that $Mg^{2+}$ performs more of a structural role by inducing a conformational change in ALP that brings about activation of the enzyme molecule, thus confirming the increasing activation of ALP by the increasing concentration of $Mg^{2+}$ in this study. The effect of $Mg^{2+}$ concentrations on the Michaelis constant ($K_m$) suggests that $Mg^{2+}$ activates ALP by increasing the affinity of the enzyme for pNPP. This study also established the activation of ALP by $Zn^{2+}$ as earlier shown by Olorunniji *et al.* (2007). The observation that $Zn^{2+}$ affects $K_m$ may suggest that $Zn^{2+}$ induces its activation effect on ALP through its interaction with the free enzyme. The observed increase in $V_{max}$ due to increasing $Zn^{2+}$ concentration most likely reflects the catalytic requirement for the metal ion in phosphate ester hydrolysis (Holtz *et al*., 1999).

In summary, the study revealed that unfiltered Agba River displayed a higher level of alkaline phosphatase activity which implies that it may contain a higher concentration of dissolved phosphorous available for use by planktons and zooplanktons and thus increase their growth rate in the river. Pollution of fresh water by phosphorus-containing pollutants from factories may be significantly reduced if these effluents contain compounds such as $MgSO_4$ and $ZnSO_4$ which are ALP activators.

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