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EFFECT OF FERTILIZERS ON HAEMOCYANIN (THE OXYGEN BINDING PROTEIN IN SNAIL)

Agbo, A.C.[1*]; Eneh, C.A.[2]; Onwubiko, H.A.[1]

[1] Department of Biochemistry, Faculty of Biological sciences, University of Nigeria, Nsukka
[2] Department of Agricultural economics, University of Nigeria, Nsukka
* Corresponding Author, aloysiusagbo30@gmail.com

Abstract

Giant African snails (Achatina achatina) uses haemocyanin as their oxygen binding protein. Haemocyanin is a copper containing protein present in the animal's haemolymph. It binds and transports oxygen and can exist in two conformations viz oxy-haemocyanin and deoxy-haemocyanin depending on the availability of oxygen. This work examines the effects of two fertilizers (NPK 20:10:10 and Urea) on haemocyanin. In the preliminary experiment results in which the environmental effect of these fertilizers on Achatina achatina were carried out revealed a negative change in function consequent upon weight loss and death. Haemocyanin on reacting with ions such as those contained in the fertilizers for example nitrogen ion looses its function due to conversion to met-haemocyanin which has a reduced oxygen affinity. In determining the molecular cause of death in Achatina achatina, various concentrations of both fertilizers were reacted with purified haemocyanin and the spectra of the mixture read. Result showed significant decrease in the magnitude of absorbance maxima as compared to normal control. The decrease suggests that haemocyanin's oxygen binding affinity decreased. Varying the amount of fertilizers was to determine if concentration has any effect. In comparing the results of the different concentrations, observation was made that as the concentration of fertilizer increased, the absorbance decreased. Fertilizers therefore affects the pathophysiology of Achatina achatina by disrupting the activity of its oxygen carrier leading to a diseased condition and eventual death.

Keywords: Effect of NPK fertilizer; African Giant snail; Haemocyanin

Introduction

Fertilizers – particularly inorganic fertilizers consists mainly of salts of ammonium nitrate, phosphate and potassium (Aoun *et al.,* 2010). The combination of these fertilizer salts and/or primary nutrients, including secondary and macronutrients are essential for plant growth thus, fertilizer is used for intensive and extensive agriculture, ranching, and horticultural crop production to, increase soil fertility and ensure sustainable crop production (Otero, 2005; Gong *et al*., 2009). Whilst production productive, extensive use of Nitrogen fertilizer causes high nitrogen uptake in plants, resulting in high content of nitrate in food and drinking water (Bryan *et al.,* 2010; Lundberg *et al.,* 2009). In spite of the foregoing there is, in this modern era of agricultural practice interest on, high use of chemical fertilizers, synthetic pesticides, and high yield crop varieties with resultant positive effects especially on crop yield but however, effects of, soil degradation, decreased soil organic matter, decreased water quality and, increased greenhouse gas emissions amongst others. All these negative impacts is without doubt having harmful and deadly effects on different forms of life particularly that of animals especially (Harter, 2009).

The manufacture of fertilizers is generally at a high temperature and high pressure, in the presence of several highly hazardous chemicals, dust and gases. These products are absorbed high in the airway

(Geetha *et al.,* 2001) while, a main source of nitrates-contaminating-ground water and surface water. Besides, when fertilizers are discriminately applied, it causes air pollution of nitrogen compounds (NO, N_2O, NO_2) emissions (Serpil, 2012). Exposure to fertilizer is most commonly associated with contact dermatitis (Vacher and Vallet, 1968).

On the other hand scientifically, several epidemiological studies have reported that occupational exposure to very high levels of nitrate in employees resulted in a, significant increase in body burdens of nitrate and nitrite in saliva (Moller, 1995). When there is a primary concentration of nitrate in drinking water of 50 mg/L which, exceeds the value of the bowel in adult's digestive and urinary systems, toxic effect of inflammation is seen. Fertilizer exposure has also hampered iron-contained-in-haemoglobin and as a result, blood oxygen transport function was lost, thereafter too, in addition, infants were found strangled to death (Serpil, 2012).

In studies with rats, daily doses of 200 mg, 400 mg and 600 mg of NH_4NO_3 per kg received *ad libitum* for a period of three weeks induced a proportional increase in serum glucose; an increased enzymatic activity of transaminases (GOT, GPT) and increase in the Urea and creatinine serum (Boukerche *et al*., 2007).

In studying the toxicological effects of Urea fertilizer on fish, Soma & Susanta (2014) submitted that, when Urea is mixed with water bodies, it was found to diminish fish production and also caused mortality. In some cases urea, was reported to cause pernicious physiological changes in fishes (Soma & Susanta, 2014). Since the molluscan haemocyanin is analogous to the mammalian haemoglobin, one will not be wrong to say that the effects of the components of chemical fertilizers especially nitrate on the molluscan oxygen carrier (Haemocyanin) will, be the same as is submitted by Serpil (2012) above.

Commercial molluscicides are widely used by rice farmers to control the Golden Apple Snail (GAS) – *Pomacea canaliculata* in the Philippines. In addition, misuse of these synthetic pesticides not only pollutes the environment but also poses hazards to applicators, farm workers, work animals, and other non-target organisms such as fish, frogs and surrounding arthropods. When applied before planting, inorganic fertilizers reduced (GAS) population altering, the physiological and biochemical functions of these organisms and even the end consumer – man – in some cases (DeLaCruz *et al.,* 2001). Not much in literature has been done on the effect of fertilizer on snail's haemocyanin. Thus, the need for this study.

The genetics and biotech industries have assured us they can deliver increased agricultural yields through, promising leaps in genetic agricultural potential of 3% to 4% per year (Fixen, 2007; Jepson, 2008). However, history suggests that genetic advances may not be able to solve the world's food shortage (Roberts, 2009). The discourse so far on improving food availability without having adverse effects on the environment is, seemingly focused on the US where technological advancement is fully enforced and appreciated. In Africa and in Nigeria, in particular, where better ways of ensuring food security such as production of genetically modified foods is not fully functional, it is logical to say that the use of other means such as fertilizer use will be even higher (Fixen, 2007; Jepson, 2008; Roberts, 2009). Consequently, this higher use of fertilizer however, logically relate to physiological and biochemical function effect on man and to some other lower animals like reduced Golden Apple Snail population (DeLaCruz *et al.,* 2001) and invariably the African giant snail (*Achatina achatina*) which belongs to a group of animals that makes use of haemocyanin as their oxygen binding protein. Thus, the need for this study.

Fertilizer

Food is a basic need for animal existence. Thus, in the food attempt to

ensure adequate food availability man have, devised various means of improving crop yield. The State of the future (2008) report identified; better rain-fed agriculture and irrigation management, genetic engineering for high yielding crops, precision agriculture, drought tolerant crops, and several other elements as critical long-term strategies to feed the world and, required for new agricultural approaches (Roberts, 2009). The said report, State of the future (2008) however, said little of fertilizer especially as a critical long-term sustainable element for environmental-toxically-free-agricultural sustainability (Roberts, 2009). That notwithstanding, fertilizer use is, still a very important aspect of the agricultural and food industry.

Going forward through 2020, Fixen (2007) estimated the extra production from a 3% annual increase in maize yield in the USA would require an additional 18% Nitrogen, 21% Phosphorus and, 13% Potassium compared to average fertilizer use from 2004 to 2006 (Roberts, 2009). This discourse and rise in fertilizer use in agriculture is without doubt having certain positive effects besides adverse effect on the environment and it dwellers. The use of phosphate fertilizer for instance is essential in agriculture because they supply farmlands with nutrients for growing plants. However, heavy metals might be included as impurities in natural materials and minerals so, heavy metals can also be present in phosphate fertilizers or other chemical fertilizers (Cheraghi *et al*., 2012).

The Food and Agricultural Organisation (FAO) (2008) classify fertilizers as:

Straight fertilizers**:** These contain one of the three major nutrients N, P or K and, Compound/complex fertilizers: Theses contain at least two out of the three major nutrients.

NPK Fertilizer

NPK fertilizer is a widely used fertilizer in Nigeria. This fertilizer has the chemical elements Nitrogen, Potassium and Phosphorus as its main constituents. These are mineral elements needed in large quantities for normal plant growth. NPK fertilizer is formulated in different Nitrogen, Phosphorus and Potassium composing ratios say, NPK 15:15:15 and NPK 20:10:10 (FAO, 2008).

Urea Fertilizer

According to the Food and Agricultural Organisation (FAO) (2008), Urea fertilizer can be free flowing or granulated. Urea is one of the most widely used nitrogen fertilizer in the world. It is also commonly found in nature since it is expelled in the urine of animals. According to the International Plant Nutrition Institute (2000), the production of Urea fertilizer involves controlled reaction of Ammonia gas (NH_3) and carbon dioxide (CO_2) with elevated temperature and pressure. The molten Urea is formed into spheres with specialised granulation equipment or hardened into a solid pill while falling from a tower. During the production of Urea, two urea molecules may inadvertently combine to form a molecule called biuret, which can be, damaging when sprayed onto plant foliage. Most commercial urea fertilizer contains only low amounts of biuret due to carefully controlled conditions during manufacturing. However, special low-biuret urea is available for unique applications (International Plant Nutrition Institute, IPNI, 2000).

Fertilizer Manufacture Process/Active Components in Fertilizer

Straight fertilizers such as Calcium ammonium nitrate (CAN), Ammonium nitrate, Ammonium sulphate, Urea, Single superphosphate, Triple superphosphate, Potash (Potassium chloride) and combined types such as, Mono-ammonium phosphate, Di-ammonium phosphate are well defined products made using well defined processes. Compound or complex fertilizers such as NPK are, more difficult to define as there is an infinite number of N/P/K-ratios and, the processes applied in their production are numerous (European Fertilizer Manufacturers Association (EFMA), 2000).

The product name "NPK" is normally followed by three numbers to indicate the percent of N, P_2O_5 and, K_2O which the product fertilizer contains. According to European Fertilizer Manufacturers Association (EFMA) (2000), NPK fertilizer production makes use of chemicals classified as:

Acids: Nitric acid, phosphoric acid, sulphuric acid

Gases**:** Ammonia, nitrogen oxides and compounds of fluorine, chlorine and sulphur

Dust: NPK dust which is regarded as inert

Snails

Snails are invertebrate animals belonging to the class gastropoda and phylum molusca. There are over 1000 species of snails in the world distributed in various habitats viz land, fresh water etc. The Giant African snail, *Achatina achatina* is one of the numerous species of snails.

Fig. 1: African Giant snail (*Achatina achatina*) (adapted from: Falkner *et al.*, 2010)

Habitat

Raut & Barker (2002) suggested that *Achatina achatina* is tolerant of a wide variety of environmental conditions. The US fish and wild life service's analysis of the worldwide geographic distribution of *A. achatina* suggests that this snail is most closely associated with tropical and subtropical moist broadleaf forest and, tropical and subtropical dry broadleaf forest (US Fish and Wildlife service, 2015). These snails are active over a temperature range of about 48-90 degrees Fahrenheit (9-32 degrees Celsius) but can survive both in lower and higher temperatures by, burrowing into the soil (Capinera, 2011).

Achatina achatina is able to survive in many and different varied environments. These many and different varied environments are usually areas rich in calcium. Thus, *Achatina achatina* thrive in locations with milestone, marl and places with concrete and cement (USDA-APHIS, 2011)

Taxonomic hierarchy and taxonomic standing of snail

According to Tiller (1989), the taxonomic hierarchy and taxonomic standing of snail is thus:

Kingdom: Animal

Phylum: Mollusca

Class: Gasstropoda

Order Stylommatophora

Family: Achatinidae
Genus: Achatina
Specie: *Achatina*

Morphology

The morphology of snail (*Achatina achatina*) shows an invertebrate with an un-segmented body covered by an exoskeleton or shell made of calcium carbonate. *Achatina achatina* according to GSID (2010) average about 5 to 10 cm with adults potentially exceeding 20 cm in shell length (GSID, 2010). *Achatina achatina* has a narrow conical shell, which is twice as long as it's width and contains 7 to 9 whorls when fully grown. The shell is generally reddish brown in colour with weak yellowish vertical markings. Colouration however of Snail (*Achatina achatina*) varies with environmental conditions and diet but a light coffee colour is common generally (GSID, 2010).

Achatinids are generally nocturnal forest dwellers but have the potential to adapt to disturbed habitats. Concealed habitats are generally preferred however, individuals may colonize more open habitats in the event of overcrowding. Achatinids often become more active during periods of high humidity (e.g., after rainfall) however, the occurrence of large numbers of individuals especially during daylight may indicate high population density (White-McLean, 2011).

Impacts on the Environment

Direct costs of snail (*Achatina achatina*) presence or rearing to the natural environment may include herbivory of; altering nutrient cycles associated with large volumes of plant material that is feed on through the achatinid gut; adverse effects on indigenous gastropods particularly when there may arise competition (Raut & Baker 2002).

In tropical agriculture, the cost of snail (*Achatina achatina*) presence or rearing to the natural environment and the cost of the natural environment on snail (*Achatina achatina*) is fourfold. First, there is, the loss of crop and crop volumes cum yield caused by herbivory of snail (*Achatina achatina*). Secondly, damage may be caused to the snail (*Achatina achatina*) by, the spread of disease through the transmission of plant pathogens. Thirdly, there is the cost associated with the control of the pest and finally, there are the opportunities lost with enforced changes in agricultural practice such as limiting certain crops to be grown in a region especially those resistant to snail invasion (Raut & Baker, 2002). Irrespective of crop and crop specie resident in the snail (*Achatina achatina*) environment, the seedling or nursery stage in the snail (*Achatina achatina*) development stages is the most vulnerable stage. In more mature plants, the nature of the damage varies with the specie, sometimes involving defoliation and in others involving damage to the stems, flowers or fruits (Raut & Baker 2002).

Aestivation

The giant African snail is usually active (eating, reproducing) during the dry season (Hodasi, 1979). As the dry season approaches, the animal retreats into a dormant state called aestivation, where it endures many months or years of prolonged dryness.

During the dry season, ambient temperatures in the tropics vary between, 28 degree Celsius to 33 degree Celsius with a drop in relative environmental humidity. As a result, the rate at which snail's loose water by desiccation increases. This problem can only be solved by aestivating. During aestivation (Hodasi, 1979) *Achatina achatina* and other pulmonate land snails bury themselves deep in the ground, under rocks or leaves. Here snails withdraw into their shell and secrete layers of a thin film and calcified mucous memebrane (epiphragm) across their apertures (Rokitka & Herreid, 1975).

Haemocyanin

Oxygen is the driving force of living things. Its importance in life can never be overemphasised. Animal's need for oxygen has driven them into evolving and developing various means to gain this vital resource necessary for existence. These

include; simple diffusion for the lower animals; organs and systems for the more advanced ones. These operates in processes that may involve various biochemical reactions. However, these processes may be adversely affected by physiological or environmental changes (Mariano *et al*, 1995).

There are various proteins used by animals in moving oxygen in their body; haemoglobin in vertebrates, haemocyanin in molluscs, crustacean and arthropods and, haemerythrin in annelids. Hemocyanins are copper proteins found in the hemolymph of several invertebrate species belonging to the phyla of Mollusca and Arthropoda where, there is a need for the oxygen transport and/or storage function. The biological role of this family of proteins is based on the reversible binding of dioxygen to a dinuclear copper active site. The protein exists in two functional forms, deoxy-hemocyanin and oxy-hemocyanin. The, relative abundance of these proteins (deoxy-hemocyanin and oxy-hemocyanin) depend on oxygen concentration (Mariano *et al*, 1995).

The mollusc's haemocyanin is a metalloproteien having two copper (Cu) atoms to bind a single oxygen (O_2) atom. Haemocyanins are among the most important copper containing proteins, representing up to 95% of the hemolymph of crustaceans (Sellos *et al*., 1997; Jayasree *et al*., 2001; Zheng *et al*., 2016; Fredrick *et al*., 2012). Hemocyanins are extracellular negatively charged proteins involved in numerous physiological functions, like protein storage, osmoregulation, oxygen transport and enzyme activities (Paul *et al*., 1997; Decker *et al*., 2007; Markl, 2013). They can perform as antiviral agents against a variety of viruses. It has been pointed out that they hold asleep the infection of white spot syndrome virus (WSSV) *in vivo* (Lei *et al*., 2007). Also, haemocyanin can respond with anti-human proteins known as antigen (Zheng *et al*., 2016).

The antiviral properties of haemocyanin are not limited to invertebrate systems but predominantly those systems associated to molluscs. Indeed, they can induce potent immuno-stimulatory responses in mammalian hosts and, show notable anti-viral (Nesterova *et al.*, 2011), anti-cancer (Dolashka *et al*., 2011; Lammers *et al*., 2012), anti-parasitic (Guo *et al.*, 2009 and 2011) and therapeutic potential (Dolashka- Angelova *et al*., 2008; Becker *et al*., 2009; Zanjani *et al.*, 2014). Previous studies reported that hemocyanins can act as antimicrobial agents in the form of, activators and as source of antimicrobial proteins (Kawabata *et al*., 1995; Velayutham *et al*., 2016).

Haemocyanins appear to have evolved from a molecule similar to the enzyme tyrosinase (Schneider *et al*., 1984). Though however, they – haemocyanins have diverged sufficiently such that the proteins are structurally similar only at the oxygen binding sites (Ling *et al*., 1994).

Occurrence of copper in proteins

The occurrence of copper in proteins is not widespread. Only about eight classes of proteins have copper bound at their "active sites". These proteins with copper at their active sites include the ceruloplasmins, cytochrome oxidases, galactose oxidases, haemocyanins, laccases, plastocyanins, superoxide dismutases and tyrosinases. Two of these eight classes are directly involved in respiration. The first – cytochrome oxidase proteins are, directly involved in the final stages of electron transport in the electron transport chain. These oxidases are very complex multi subunit-proteins that have some sub-units which possess copper (Bonaventura & Bonaventura, 1977; Herskovits, 1988).

The second, haemocyanin is functionally analogous to vertebrate haemoglobins (Bonaventura & Bonaventura, 1977). The haemocyanins are oligomeric copper containing proteins which are involved in oxygen transport in molluscs and confer the blue colour in the

blood of a snail as described in the literature by the Dutch naturalist Swammerdam. Because haemocyanin uses copper atoms in binding oxygen, they are metalloproteins and, the nature of the copper oxygen bond is reversible (Bonaventura & Bonaventura, 1977).

Characteristics of the haemocyanins are, their high molecular weights and their dissociation into subunits. This dissociation-association reactions of haemocyanins depend on, the protein concentration, ionic environment, pH and other factors (Herskovits, 1988).

Structure of Haemocyanin

Haemocyanins are respiratory proteins occurring freely dissolved in the hemolymph of many arthropods and molluscs. Arthropod hemocyanins are found as single hexamers (1-6mers) or multiples of hexamers (2-6mers, 4-6mers, 6-6mers, 8-6mers). Each arthropod hemocyanin subunit (ca. 72 kDa) folds into three domains characterized by different folding motifs according to Volbeda & Hol (1989), Hazes *et al*. (1993), Magnus *et al*. (1994) of, domain I with five or six a-helices; domain II with a four alpha-helix bundle and the active site containing two copper ions; and domain III with a seven-stranded antiparallel b-barrel (Volbeda & Hol, 1989; Hazes *et al*., 1993; Magnus *et al*., 1994).

In contrast, molluscan haemocyanins are cylindrical decamers, didecamers or multidecamers of a ca. 350 or 400 kDa polypeptide subunit. This folds into a chain of seven or eight functional units (FUs). Each functional unit (FU) is composed of two different structural domains termed, alpha (from alpha-helix domain) and beta (from beta-sandwich domain) domain. Domain alpha folds into a four alpha-helix bundle carrying the copper active site, and domain beta into a six-stranded anti-parallel b-barrel (Cuff *et al*., 1998). The alpha-domain of a molluscan hemocyanin functional unit (FU) corresponds functionally to domain II of an arthropod hemocyanin and, the b-domain a molluscan hemocyanin functional unit (FU) corresponds functionally to domain III respectively (Loewe, 1978; Decker & Sterner, 1990; Van-Holde & Miller, 1995).

The oxygen-binding behaviour of hemocyanins is usually characterized by a low to moderate oxygen affinity that can be modulated by a variety of factors such as an adaptation to the species-specific ecophysiology. Cooperativity of oxygen binding is comparatively low in molluscan hemocyanins (with Hill coefficients around 2), but can be exceptionally high in arthropod hemocyanins (with Hill coefficients up to 9) (Loewe, 1978; Decker & Sterner, 1990; Van-Holde & Miller, 1995).

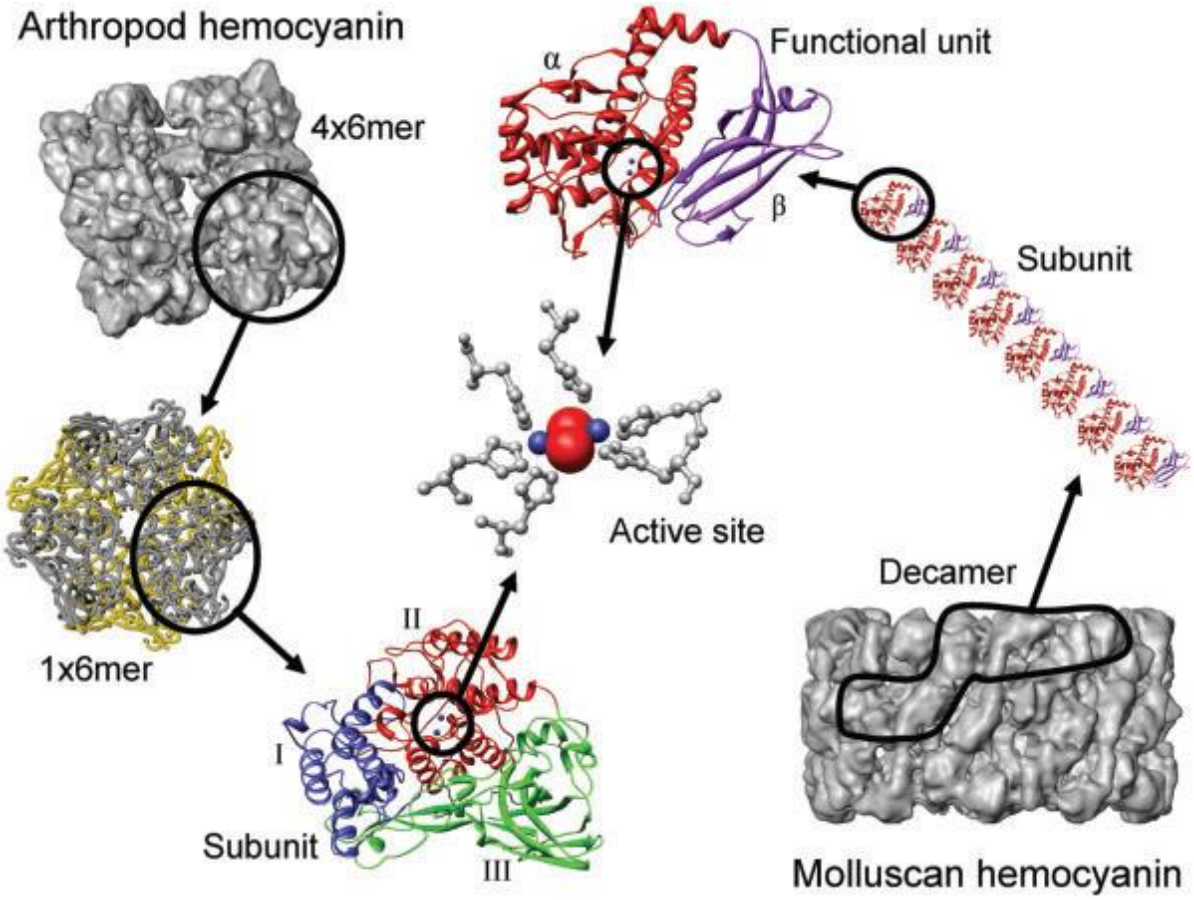

Fig. 2. Structural levels of arthropod and molluscan hemocyanins. (Adapted from Decker *et al*, 2007).

Note that in the case of arthropod hemocyanin, the subunit polypeptide carries a single active site, whereas in molluscan hemocyanin, the subunit polypeptide contains seven or eight functional units (FUs), each with an active site (Decker *et al*, 2007).

Oxygenation and Active Site Composition of Haemocyanin

In contrast to mammalian haemoglobin, the haemocyanin is directly suspended in the haemolymph. Oxygenation leads to a colour change from colourless to blue. In the free-state, two copper atoms has an oxidation number of one (1). Binding of one oxygen molecule causes an increase in copper's oxidation number to two (2). The copper atoms of haemocyanin are bound as prosthetic groups coordinated by histidine residues. Haemocyanins active site is composed of two (2) copper cations which are coordinated to the protein through the driving force of imidazole rings of six (6) histidine residues.

Oxygenated haemocyanins display two (2) main absorption bands (with the exception of the band at 280nm) at around 340nm and 570nm (Nickerson & Van-Holde, 1971). These copper bands disappear upon deoxygenation (Nickerson & Van-Holde, 1971). Studies by Ling *et al.* (1994) led to the assignment of these bands as follows: the transition around 340nm corresponds to charge transfer from the imidazole groups of histidine residues to Cu^{2+} (Im------>Cu^{2+}) and that around 570nm to (O_2^{2-}------->Cu^{2+}) charge transfer (Nickerson & Van-Holde, 1971).

During oxygenation, the copper-copper distance within an active site changes from 3.67 to 3.68 Armstrong. Whereas, the copper-copper distance between adjacent active site remains

unchanged (1.95 Armstrong) in both oxy and deoxygenated haemocyanins (Nickerson & Van-Holde, 1971).

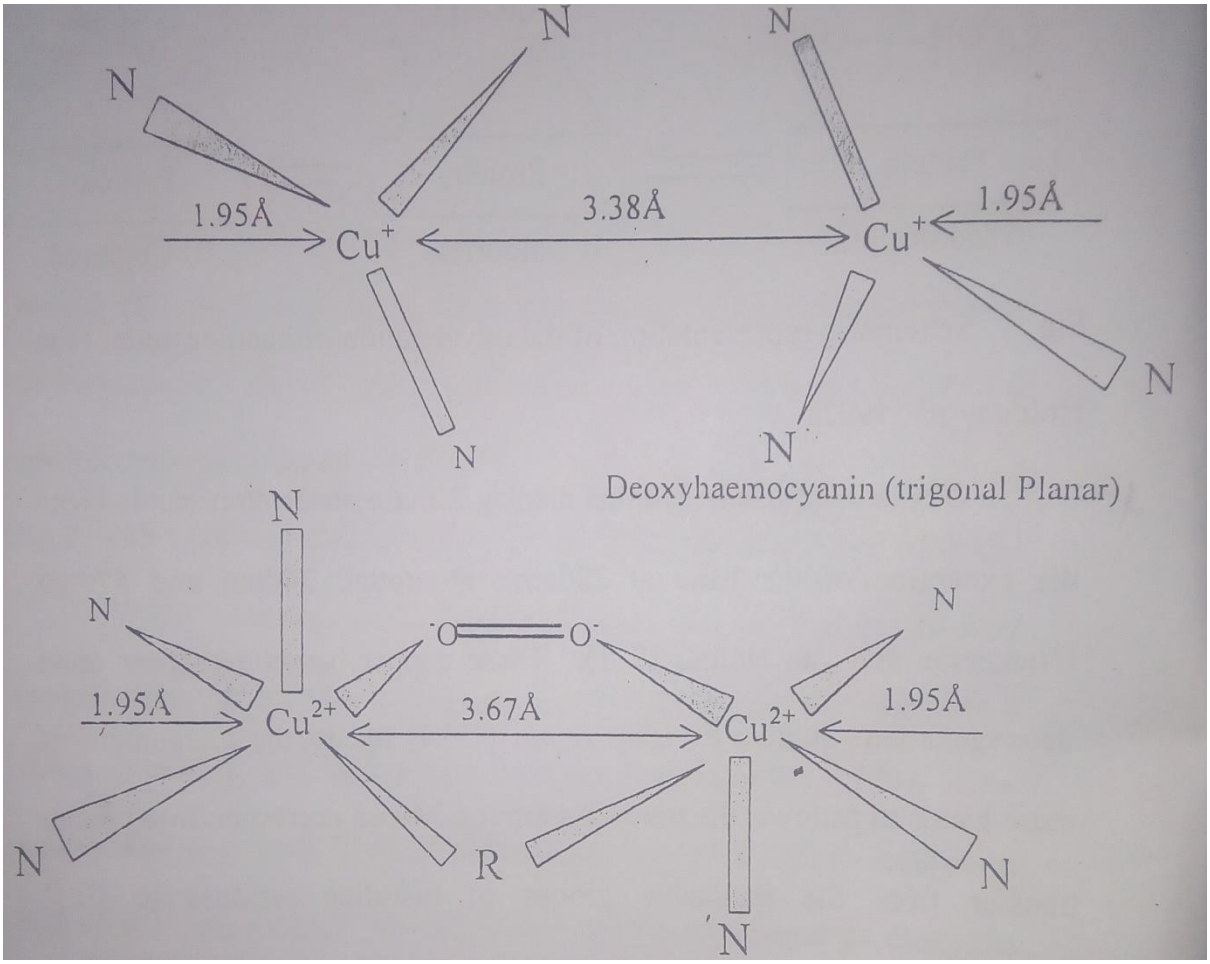

Fig.3: Spectroscopically effective active site picture of oxy and deoxyhaemocyanin (adapted from: Edward, 1983). R= Internal ligand, N=Nitrogen

Modulators

In a study titled, Are there unidentified co-factors in haemocyanin-containing bloods that alter their respiratory properties in response to some transient environmental stimulus? Mangum (1980) reported that with respect to gastropod blood, the answer seems to be convincingly no. In their exhaustive analysis of the effect of inorganic ions on *Busycon haemocyanin*, the study attempted to restore the respiratory properties of native blood by adding salts, one by one to purified haemocyanin (Mangum, 1980).

In the presence of physiological cations of only Na^+, Ca^{2+}, Mg^{2+}, and Cl^-, oxygen equilibrium properties differ a little or not at all from those of native blood. The hypothesis of an unknown co-factor is unwarranted by the results (Mangum, 1980). Mangum (1980) further reported that like most haemocyanins, the oxygen carrier in Busycon blood is strongly pH dependent, and that their success in restoring its oxygenation properties was probably facilitated by their ability to measure pH at P_{50} such that they were able to distinguish H^+ effects from salt effects in their poorly buffered preparations of whole blood.

Effect of fertilizer on haemocyanin

Fertilizers have proven to be very potent tools used in ensuring food availability and security. All plants require

a good amount of minerals to grow and provide food for man. Nature has designed it that these minerals can only be obtained naturally by plants from the soil and subsequently obtained by animals from plant diet. Most farm area have enough of these mineral however, a vast percentage of these all important minerals have been lost through various processes of leaching, erosion and harmful farm practices. Some of these nutrients may also not be originally available. To ensure plant growth and yield, and by extension animal survival and proper functioning, these nutrients must be made available for plants (Truchot, 1992; Morris, 1990).

However, the consistent use of fertilizer over the years does not just have gains only. Its improper or indiscriminate use have been proven by research to pose a lot of harmful and deleterious effects on the environment destroying and reducing animal population, posing health challenges to farmers, contributing to climate change and eutrophication of water bodies causing, aquatic mortality (Truchot, 1992; Morris, 1990).

Inorganic modulators of haemocyanin oxygen affinity

Truchot (1992) pointed out that oxygen affinity might be changed by modifying the intrinsic structure of the haemocyanin or by adding effectors or modulator substances. Morris (1990) defined effectors as having a constant influence over oxygen affinity under normal conditions. Whereas modulators is defined as substances that respond to environmental conditions or stress by a change in concentration thereby, influencing haemocyanin oxygen affinity (Morris, 1990).

Inorganic ions

It has long been known that the ionic composition of the haemolymph affects haemocyanin oxygen affinity. In his thorough study, Truchot (1992) was able to show that both Mg^{2+} and Ca^{2+} increased oxygen affinity in *Carcinus maenas*, and that Mg^{2+} increased the Bohr effect at physiological levels at the same time (Truchot, 1975). Other ions such as Cl^- have a variable effect, increasing oxygen affinity in some species such as *Penaeus setiferus* (Brouwer *et al.*, 1977) and *C. Maenas (*Truchot, 1975) and decreasing it in others such as, *Limulus polyphemus* (Brouwer *et al.*, 1977). In *Carcinus maenas* however, the chloride effect only became significant at high extra physiological values (Truchot, 1992).

pH

Bridges (2001) reported that when organisms are subjected to either to activity or to environmental hypoxia then, tissue anoxia occurs at some stage either, through an increase in the oxygen consumption or through a decrease in oxygen in oxygen transport. This in turn the study (Bridges, 2001) further reported leads to tissue anaerobiosis with the consequent formation of metabolites which in turn may provide H^+ that affects the acid-base status of the haemocyanin. The number of proton formed depends upon the length and severity of the hypoxia (Bridges, 2001).

Soma & Susanta (2014) in studying the toxicological effects of Urea fertilizer on fish submitted that, when Urea is mixed with water bodies, it was found to diminish fish production and also cause mortality. In some cases, urea was reported to cause pernicious physiological changes in fishes. The study (Soma & Susanta, 2014) submitted that, due to indiscriminate use of Urea fertilizer in agricultural fields which are, ultimately washed out and continuously added to the water bodies, the aquatic flora and fauna life is affected.

According to Nasiruddin *et al.* (2012), *Channa punctatus* when exposed to sub-lethal concentration of malathion revealed a declining trend of red blood cells (RBCs), haemoglobin and increasing trend of white blood cells (WBCs). Experiment was also done with plant seed extract on air breathing fish. In the said study and experiment, Ansari & Waleema (2009) reported that nutrient and chemical

fertilizers were the main cause of pollution of water bodies.

Whilst an issue of concern, the above study corroborated the study conducted by Soma & Susanta (2014) on the effect of Urea fertilizer on *Heterpnuestes fossilis*. In the study, fishes were exposed to Urea for seven and fourteen days. The study revealed a decreasing trend in red blood cells (RBCs) after treatment with urea. The study also observed the same trend in the haemoglobin and lymphocytes of the fish species *Heterpnuestes fossilis* (Soma & Susanta, 2014).

In studies with rats, daily doses of 200 mg, 400 mg and 600 mg NH_4NO_3 per kg received *ad libitum* for a period of three weeks induced a proportional increase in serum glucose, an increased enzymatic activity of transaminases and increase in the Urea and creatinine serum (Boukerche *et al.,* 2007).

Whilst literature on the effect of fertilizer on snail's haemocyanin is rare however, available data shows that high phosphorus and high nitrogen fertilizers have a negative impact on the growth rate of juvenile snails. Such observations were supported by determining antioxidant parameters in the digestive gland of snails.

Results indicated a significant increase in the catalase activity of snails that were exposed to ½ LC_{50} concentrations of balanced and high phosphorus fertilizers (Fatima *et al*., 2017). Furthermore, studies on the effect of three herbicides on snail biochemistry by Amina *et al.* (2019) indicated that the herbicides increased alanine aminotransferase ALT and aspartate aminotransferase in the haemolymph of *B. alexandrina* and significantly decreased total protein and albumin content. Light microscopical examinations of haemocytes monolayers of *B. alexandria* snails showed three different cell types (small cells, granulocytes and halinocytes). All three herbicides caused abnormalities in cell shapes (Amina *et al.*, 2019).

In addition, flow cytometric analysis showed that the total number of dead haemocytes in the haemolymph significantly increased in groups treated with herbicides compared to control group. Phagocytosis in groups treated with herbicides highly significantly increased compared to the control group indicating a very strong response of the snails in the treated group to herbicides. This foregoing finding also was, consistent with the result of the alkaline comet assay of DNA damage of snails. The study (Amina *et al.*, 2019) demonstrated that, herbicides have a genotoxic effect on snails (Amina *et al.*, 2019).

While phenomenal, the studies above allure to fertilizers and/or herbicides as snail toxicants. Thus, when snails were treated with fertilizers and/or herbicides like as revealed above, a change in enzymes activity and a distortion in cell shapes were observed.

Materials and methods

Reagents/Chemicals

Chemicals used in carrying out this research work were of the purest grade obtained from the Department of Biochemistry, University of Nigeria, Nsukka. In any case also, some were not available in the department. They were sourced commercially within the locality. The chemicals/reagents used in the research work include:

a. NPK 20:10:10 fertilizer
b. Urea [$CO(NH_2)_2$] fertilizer
c. Ammonium sulphate $NH_4(SO_4)_2$
d. 0.05M Tris-HCl buffer (pH 7.5) (Nippon Soda Co. Ltd, Japan),
e. Calcium chloride ($CaCl_2$)
f. Standard buffer tablets (pH 4.0 and 9.0) (Alpha Chemika, India).

g. Distilled water (Pure and Industrial Chemistry department, University of Nigeria, Nsukka)

Equipment/instrument

All equipment/instruments were obtained and used at the Department of biochemistry laboratories, Faculty of Veterinary medicine laboratory and National Centre for Energy Research and Development, University of Nigeria Nsukka. The equipment's/instruments used include: Digital electronic weighing balance (Jenway, UK), UV-Visible Spectrophotometer (Jenway, UK; Model 6405, UK), Centrifuge machine Max-4000rpm (Labsco, Germany), pH metre (Jenway, UK; Model 295, UK), Micropipette (Pipetman, France), EDTA bottles (Meus, Piove di Sacco, Itally), Refrigerator (Thermocool premier, Japan).

Animal Procurement

Thirty mature African giant snails *(Achatina achatina)* were bought from the Nsukka Local market in Enugu state. The snails were divided into two groups of fifteen snails each.

The first group were kept in a raffia basket half filled with collected wet soil. The basket had openings for air passage. The snails in this first group were fed with water melon pills and sprinkled with water daily to maintain a humid environment. They were kept in the laboratory with temperature range of 25-27 degrees Celsius until they were de-shelled for haemocyanin extraction.

The other group of fifteen snails were further divided into three groups of five individual snails. The snail in each group were weighed and their weights recorded. Each of the snail were labelled using a permanent marker and a masking tape such that the weight of each snail was indicated also on the label tag.

The snails were labelled A, B and C. Group A members were labelled thus: A1, A2, A3, A4 and A5. The same format of labelling was also used for snails in groups B and C. Three raffia baskets with the same specifications as described above – in the control – were half filled with wet sand and labelled A, B and C. Snails of each group were put in the basket with the corresponding label. The top of each basket was covered with a flat thick wood to prevent the snails from absconding. They were kept in the laboratory for 24 hours.

Fertilizer Procurement.

Two fertilizers were purchased commercially from the local market in Nsukka Enugu State. The fertilizers are NPK 20:10:10 and Urea fertilizer. Both fertilizers were in the granulated forms.

Method

About 96.22g of both fertilizers were taken. The NPK 20:10:10 fertilizer was evenly applied to basket A housing snails of group A. Urea fertilizer was applied to basket B housing snails of group B. Snails in basket C was used as the control group. The three groups of snails were fed daily with pilled water melon and sprinkled with clean water to provide a humid environment. They were monitored for 14 days after which the snails were again weighed to determine weight gain or loss before and after the experiment.

Haemolymph collection

The snails were washed with clean water to remove dirt and soil debris on their shells. They were put in a plastic bowl containing clean water and left in it for four hours. This was done to provide a hypoxic environment for the snails which will facilitate the collection of the haemolymph.

A hammer was used to break the snail shells at the tail end. The haemolymph were collected through the opened posterior region of each snail. Twisting leads to the puncture of the pervisceral haemocal which is, posterior to the buccal mass (Lukong et al., 2012; Lukong & Onwubiko, 2004a, b). The haemolymph (blue in colour) was collected in a conical flask placed in an ice bath. The snails were placed in funnels containing filter papers. The funnels enhanced haemolymph collection while the filter papers held back mucous materials to prevent contamination of the haemolymph.

Purification of haemocyanin

Haemocyanin makes up 90-95% of haemolymph protein in snails (Ghiretti, 1966). Haemocyanin was purified thus; to a 100ml haemolymph contained in a conical flask, a 30% ammonium sulphate precipitation was achieved by dissolving 16.4g of ammonium sulphate into the haemolymph over a period of two minutes while shaking vigorously. The mouth of the conical flask was tied with a thin nylon and rubber band. The mixture was then kept in the fridge at a temperature of 4 degrees Celsius for 12 hours for effective precipitation to occur.

The precipitated mixture was poured into centrifuge tubes and centrifuged with an 800D centrifuge for 40 minutes at a speed of 4000 rpm. After centrifugation, the supernatant was collected and the pellet discarded. A 55% precipitation was achieved by adding 14.8g ammonium sulphate per 100ml haemolymph solution. The sample was then kept to stand in a fridge for 12 hours. The mixture was centrifuged again for 90 minutes at a speed of 5000 rpm. The supernatant was removed using a Pasteur pipette and the pellet was dissolved in 0.5M Tris-HCl buffer of pH 7.5.

Preparation of reagents

0.1M Tris-HCl Buffer, pH 7.5 stock solution

Exactly 6.0g Tris HCl was dissolved in 700ml distilled water in a beaker. The pH of the solution was adjusted to pH 7.5 with the use of a pH metre and concentrated HCl. The solution was then transferred into a measuring cylinder and made up to the 1000ml mark with distilled water. 800ml of the solution was measured into a beaker. Exactly 1.6g $CaCl_2$ was dissolved into the 800ml solution.

Preparation of sample for dialysis

Exactly 1ml of the buffer was pipetted into haemolymph pellets contained in centrifuge tubes. The solution was shaken vigorously before transferring into dialysis bags. After dialysis, the haemocyanin was transferred back into the tubes and stored at 4 degree Celsius until when used.

Preparation of urea fertilizer stock solution

Exactly 1.0g of urea fertilizer was dissolved in a 100ml distilled water contained in a beaker. The mixture was shaken thoroughly for effective dissolution of the urea.

Preparation of NPK fertilizer stock solution

Exactly 1.0g of NPK 20:10:10 fertilizer was dissolved in a 100ml distilled water contained in a beaker. The mixture was shaken thoroughly for effective dissolution of NPK.

Test

Haemocyanin best dilution was obtained by running different volumes of haemocyanin with different volumes of buffer. The preferred dilution was obtained as 2000 micro ml of buffer to 400 micro ml of haemocyanin. The buffer was run as blank. Raw haemocyanin (i.e without dilution) was run on the UV Visible spectrophotometer. A control experiment was run at 2000 micro ml of buffer to 400 micro ml of haemocyanin. 8 test tubes were set up each, containing 2000 micro ml of buffer and 400 micro ml of haemocyanin. To four of the test tubes, varying amount of the urea fertilizer solution were added. The final mixture of haemocyanin, buffer and urea solution were transferred into a curvette and their spectra obtained.

To another four of the test tubes, varying amounts of the NPK 20:10:10 fertilizer solution were added. The final mixture of haemocyanin, buffer and NPK 20:10:10 fertilizer solution were transferred into a curvette and their spectra obtained.

Table 1: Dilutions of solutions used in the preparation of Haemocyanin (Hc) for urea treatment

Tubes	Volume of Hc added (micro litre)	Volume of urea added (micro litre)	Volume of buffer added (micro litre)
1	400	10	2000
2	400	20	2000
3	400	30	2000
4	400	40	2000

Table 2: Dilutions of solutions used in the preparation of haemocyanin (Hc) for NPK treatment

Tubes	Volume of Hc added (micro litre)	Volume of NPK added (micro litre)	Volume of buffer added (micro litre)
1	400	10	2000
2	400	20	2000
3	400	30	2000
4	400	40	2000

Results and discussion

Preliminary study (environmental effect of fertilizers on *Achatina achatina)* and tabular presentation of measured weights of *Achatina achatina* and fertilizer

The weight of the snails used for the first part of the experiment i.e to determine the environmental effects of fertilizers on snail is as presented below. The table shows the weights of the snails before and after treatment with fertilizers.

Table 3: NPK 20:10:10 fertilizer treated snails

Snail label	Weight in gram before treatment with fertilizer	Weight in gram after treatment with fertilizer/when dead	Weight loss in gram
A1	80.32	51.63	28.69
A2	68.39	59.27	9.12
A3	77.87	46.69	31.18
A4	56.55	39.33	17.22
A5	87.50	59.71	27.79

Table 4: Urea fertilizer treated snails

Snail label	Weight in gram before treatment with fertilizer	Weight in gram after treatment with fertilizer/when dead	Weight loss in gram
B1	70.64	51.20	19.44
B2	101.89	71.15	30.74
B3	63.31	41.75	21.56
B4	71.86	46.50	25.36
B5	74.97	59.47	15.50

Table 5: Experimental control snails

Snail label	Weight in gram before experiment	Weight in gram after experiment	Weight loss/gain in gram after experiment (+ or -)
C1	62.01	61.61	0.4
C2	63.14	67.06	3.92
C3	75.67	86.45	10.78
C4	104.46	108.34	3.88
C5	70.90	72.90	2.00

Table 6: Weight of fertilizer used in treatment

Fertilizer	Weight in gram
NPK 20:10:10	96.22
Urea	96.22

Absorption spectrum of oxyhemocyanin in the absence of any reactant

Result from figure 4 shows the absorption spectrum of unreacted oxyhemocyanin in the spectral range of 200-800nm. The result reveals an absorbance characterized by a peak at 348nm.

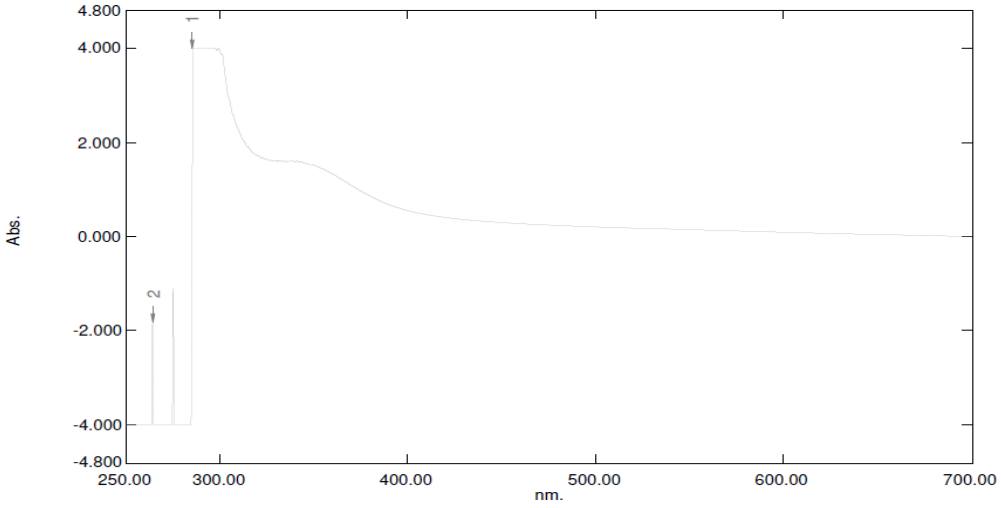

Figure 4: Absorption spectral of 400µl Haemocyaninin 50mM Tris-HCl buffer, pH 7.5 at 25˚C.

Spectrum report of 10µl of NPK 20:10:10+ 400µl oxy-haemocyanin.

Result from figure 5 shows the spectrum report of 10µl of NPK 20:10:10+ 400µl 0xy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

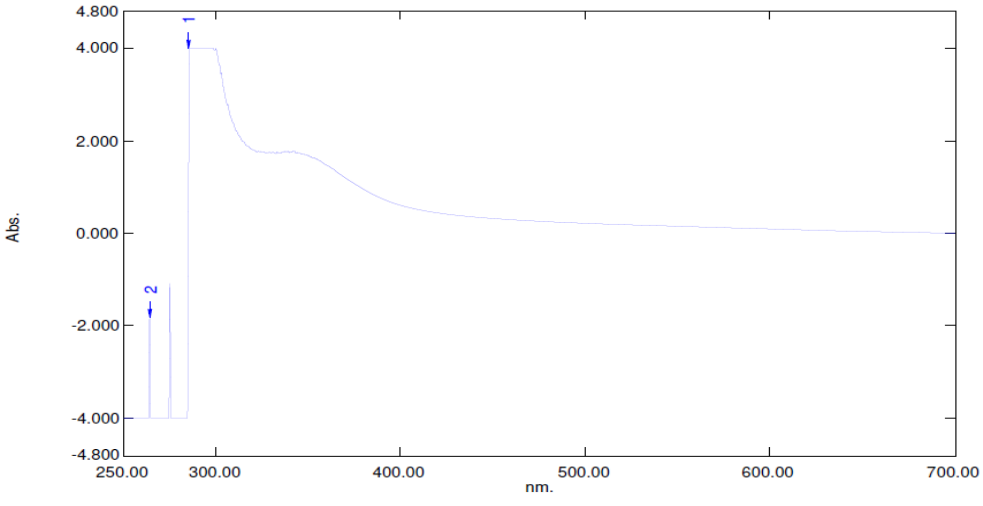

Figure 5: Absorption spectral of 10µl Haemocyaninin 50mM Tris-HCl buffer, pH 7.5 at 25˚C

Spectrum report of 20µl of NPK 20:10:10+ 400µl oxy-haemocyanin.

Result from figure 6 shows the spectrum report of 20µl of NPK 20:10:10+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

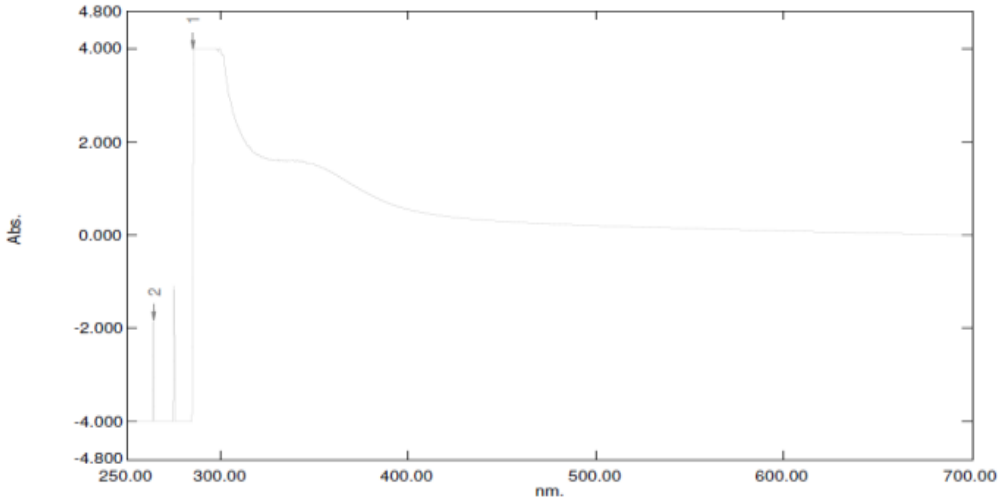

Figure 6: Spectrum report of 20µl of NPK 20:10:10+ 400µl oxy-haemocyaninin 50mM Tris-HCL buffer, pH 7.5 at 25˚C.

Spectrum report of 30µl of NPK 20:10:10+ 400µl oxy-haemocyanin.

Result from figure 7 shows the spectrum report of 30µl of NPK 20:10:10+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

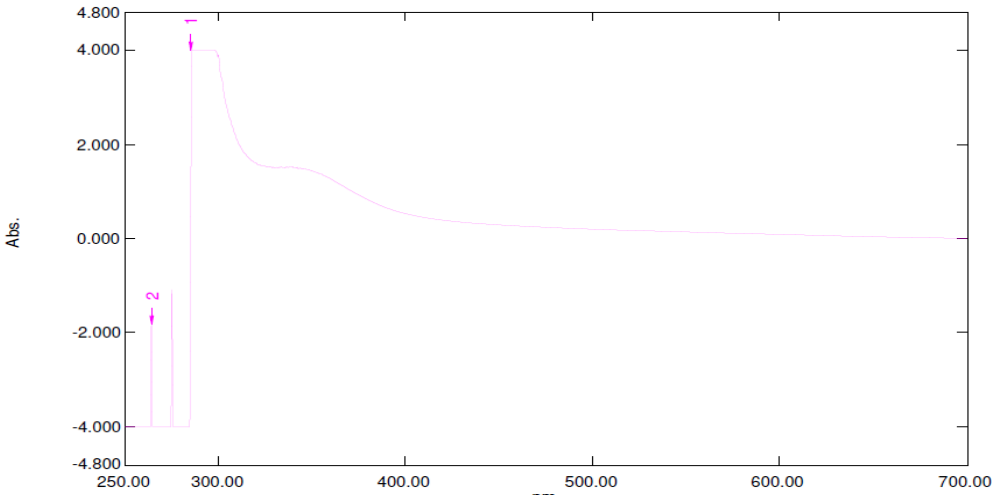

Figure 7: Spectrum report of 30µl of NPK 20:10:10+ 400µl oxy-haemocyaninin 50mM Tris-HCL buffer, pH 7.5 at 25˚C.

Spectrum report of 40µl of NPK 20:10:10400µl oxy-haemocyanin.

Result from figure 8 shows the spectrum report of 40µl of NPK 20:10:10+

400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

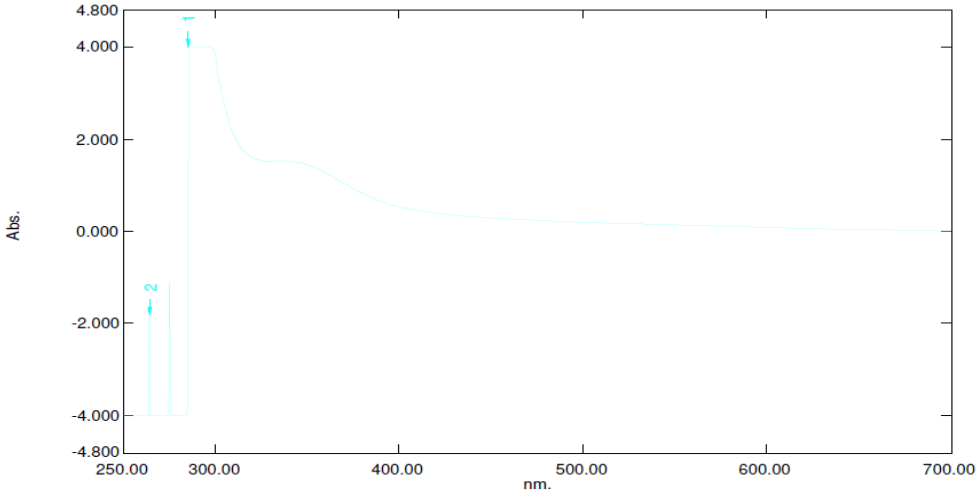

Figure 8: Spectrum report of 40µl of NPK 20:10:10+ 400µl oxy-haemocyaninin 50mM Tris-HCL buffer, pH 7.5 at 25˚C.

Spectrum report of 10µl of Urea+ 400µl oxy-haemocyanin

Result from figure 9 shows the spectrum report of 10µl of Urea+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

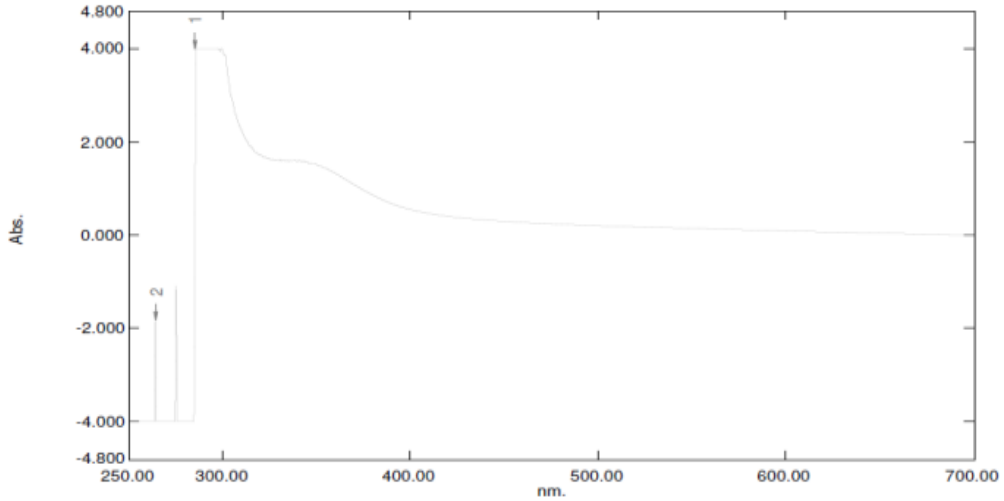

Figure 9: Spectrum report of 10µl of Urea + 400µl oxy-haemocyanin in 50mM Tris-HCL buffer, pH 7.5 at 25

Spectrum report of 20µl of Urea+ 400µl oxy-haemocyanin.

Result from figure 10 shows the spectrum report of 20µl of Urea+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

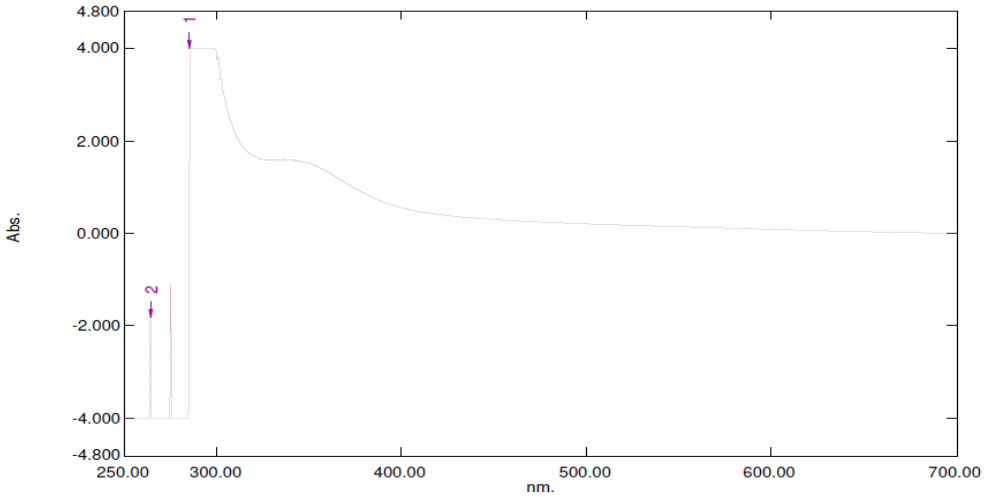

Figure 10: Spectrum report of 20µl of Urea+ 400µl oxy-haemocyaninin 50mM Tris-HCL buffer, pH 7.5 at 25˚C.

Spectrum report of 30µl of Urea+ 400µl oxy-haemocyanin.

Result from figure 11 shows the spectrum report of 30µl of Urea+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

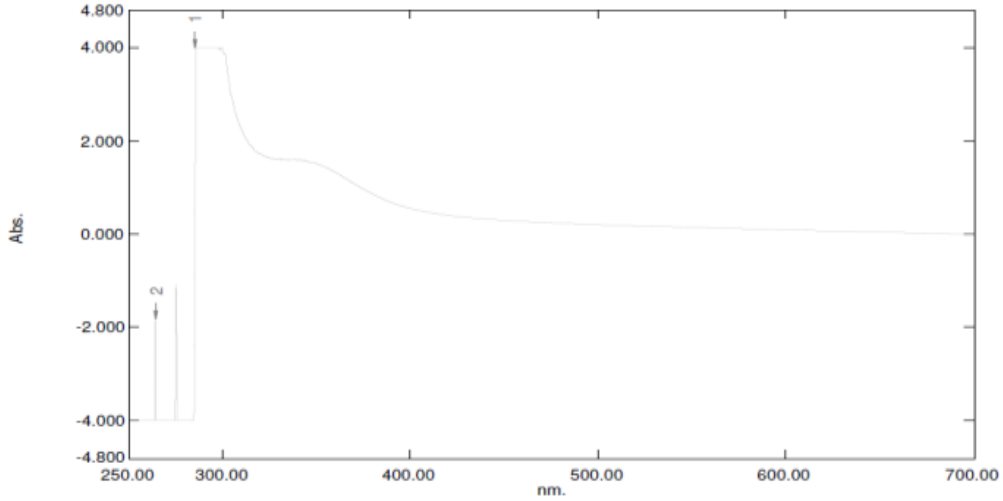

Figure 11: Spectrum report of 30µl of Urea + 400µl oxy-haemocyanin in 50mM Tris-HCl buffer, pH 7.5 at 25˚C.

Spectrum report of 40µl of Urea+ 400µl oxy-haemocyanin.

Result from figure 12 shows the spectrum report of 40µl of Urea+ 400µl oxy-haemocyanin in the spectral range of 200-800nm. The result reveals a significant decrease in the magnitude of the absorbance maxima at 348nm compared to the normal control.

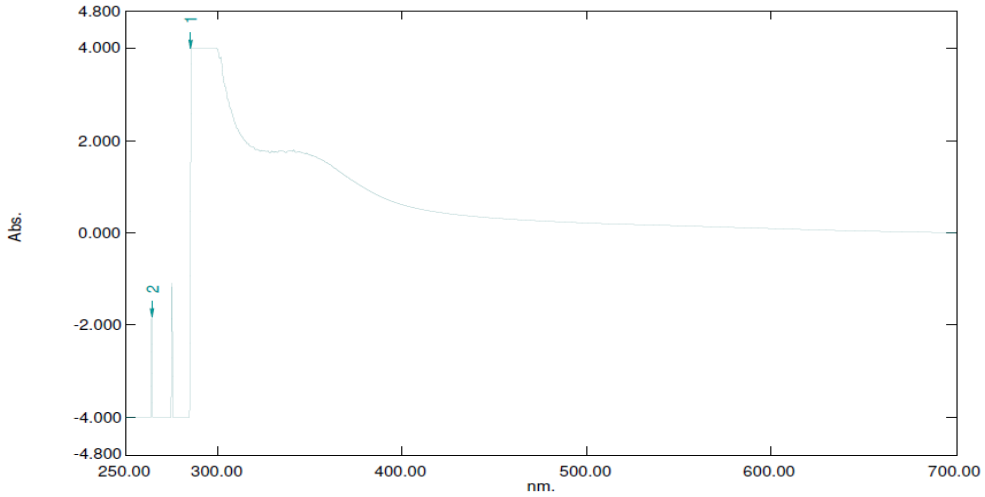

Figure 12: Spectrum report of 40µl of Urea+ 400µl oxy-haemocyanin in 50mM Tris-HCl buffer, pH 7.5 at 25˚C.

Effect of different volumes of NPK 20:10:10 and Urea on absorbance values of oxy-hemocyanin.

The result from table 7 shows the effect of different volumes of NPK 20:10:10 and Urea on absorbance values of oxyhemocyanin compared to the unreacted hemocyanin. The different volumes of the Urea had more reduction in the absorbance values compared to the different volumes of NPK 20:10:10. Within the different volumes of NPK 20:10:10, 40µl of NPK 20:10:10 had the highest reduction in absorbance values compared to 10µl, 20µl and 30µl of Urea. Finally, within the different volumes of Urea, 40µl had the highest reduction in absorbance values compared to 10µl, 20µl, and 30µl of Urea.

Table 7: Oxy-Hemocyanin absorbance values at 348nm absorbance band

	Band
Groups	**348nm**
Hemocyanin	
10µl of NPK 20:10:10	3.863nm
20µl of NPK 20:10:10	3.649nm
30µl of NPK 20:10:10	3.296nm
40µl of NPK 20:10:10	1.767nm
10µl of Urea	1.320nm
20µl of Urea	0.951nm
30µl of Urea	2.874nm
40µl of Urea	0.251nm

Effect of different volumes of NPK 20:10:10 and Urea on concentration of oxy-hemocyanin.

The result from table 8 shows the effect of different volumes of NPK 20:10:10 and Urea on concentration of oxy-hemocyanin compared to the unreacted hemocyanin. The different volumes of the NPK 20:10:10 had more reduction in the oxy-hemocyanin concentration compared to the different volumes of Urea. Within the different volumes of NPK 20:10:10, 40µl of NPK 20:10:10 had the highest reduction in oxy-hemocyanin concentration compared

to 10µl, 20µl and 30µl of NPK 20:10:10. Finally, within the different volumes of Urea, 40µl had the highest reduction in oxy-hemocyanin concentration compared to 10µl, 20µl, and 30µl of Urea.

Table 8: Oxy-Hemocyanin concentration at different volumes of NPK 20:10:10 and Urea.

	Band
Groups	**348 (ml mg^{-1} cm^{-1})**
Hemocyanin	
10µl of NPK 20:10:10	2.740
20µl of NPK 20:10:10	2.588
30µl of NPK 20:10:10	2.338
40µl of NPK 20:10:10	1.253
10µl of Urea	0.936
20µl of Urea	0.674
30µl of Urea	2.038
40µl of Urea	0.178

This study evaluated the effect of two fertilizers (NPK 20:10:10 and Urea) on snail's haeemocyanin. The preliminary study which involved the environmental effects of the two fertilizers on the snail showed that on treatment with the fertilizers, the snails manifested changes in behaviour and functions.

In both groups of the snails treated, it was observed on the first day that the snails recoiled far into their shells with just a part of their body visible. This was observed in all individual snail treated. Their sensitivity was reduced as their tentacles rarely protruded when monitored. Few from both groups made slow movements occasionally. Feeding also reduced minimally in both groups. The control group however maintained normal movement and sensitivity and fed well on their food. More negative observations were made on the second day from both groups.

However, the physical changes observed from both groups differed slightly. One snail in group A, that is, snail A2 had, the margin of its shell cracked or broken. Four of the snails in group B, that is, those treated with Urea fertilizer and in particular snails B1, B3, B4 and B5 had produced watery greyish substances which was less viscous than slime. This was not observed in those in group A, that is, those treated with NPK 20:10:10. However, they recoiled farther into their shells than observed on the first day.

Movement and sensitivity and feeding in both groups dropped more drastically than observed on the first day. Those in the control maintained normal functioning. On the third day, motility was totally lost in both groups. They all appeared to be lifeless, however death could not be ascertained as there was no parameter or equipment to measure death and it could not also be concluded that they were dead as there was the high possibility of them aestivating due to the unfavourable condition.

The condition of each snail from both groups remained the same on the fourth and fifth day as was observed on the third day suggesting the snails might have tried to aestivate. However, on the sixth day of the experiment, potent foul smell was perceived in the lab. This suggested the death of the snails. On individual observation, all the treated snails were confirmed dead by the decaying smell oozing out from their shells. Only the snails in the two baskets infested with fertilizers were confirmed dead. Those in the control group still sensed, moved and fed normally. On the seventh day, maggots were seen

crawling inside and outside of the snail's shells. A loss in weight as shown in tables 3 and 4 and, eventual death suggests that the fertilizers had altered the physiology of the snails as compared to the control group where its snails gained weight and did not die as at the end of the experiment.

Haemocyanin, the oxygen carrying protein in snails oxidises normally within the mollusc. However, when oxidation is induced by an environmental factor such as fertilizer, the homeostatic condition of the snail is adversely altered as more haemocyanin are oxidised than can be balanced by the animal. When this happens, several free radicals and superoxides are formed, which eventually disrupts the physiology of the snail causing several diseased condition of which haemolytic anaemia is the most common.

In a study by Hepp *et al.* (1979) it, was shown that the reaction that gives met-haemocyanin from *Octopus vulgaris* haemocyanin was promoted by various anions including azide, fluoride and acetate. Kinetic data indicated that the reaction mechanism is different from that currently accepted involving a peroxide displacement of bound dioxygen through an associative chemistry in on an open axial position of the copper ions.

In a similar study by Mariano *et al.* (1995), it was suggested that the protonated form of the anion is likely to be due to the species reacting with the oxygenated form of the protein. Furthermore it is also proposed that protonation of bound dioxygen generates an intermediate hydroperoxodicopper (II) complex to which the exogenous anion is also bound. This intermediate is accumulated and precedes the release of hydrogen peroxide by reaction with water. Upon dialysis, it leads to the met-haemocyanin form.

In studying the toxic impact of urea on haematological parameter of air breathing fish *Heteropnuestes fossilis* by Soma & Susanta (2014), comparison of control and treated fish showed changes in blood parameters due to toxic effects of fertilizer. Initial increase in both studied total count (TC) of red blood cells (RBCs) and haemoglobin concentration and then gradual decrease of these two parameters with the increase of doses, indicating slow recovery from adverse effects of urea in the fishes. Differential leukocyte count indicated significant response due to toxic effect of this fertilizer. Though the count of lymphocytes, heterophils and eosinophils decreased, the gradual increase of basophils, neutrophils and thrombocytes took place with increasing dose of urea. Elevation in the number of these cells revealed recovery and exhibited resistance of this fish to environmental xenobiotic compound like urea.

Incubation of *Octopus* oxy-hemocyanin in 100 mM Fluorine- or Nitrogen solutions resulted in a time-dependent reaction leading to the disappearance of oxy-hemocyanin. This process was detected by the decrease of the 348-nm absorption band assigned to a *Oz*--Cu(I1) ligand to metal charge transfer (LMCT) transition. Furthermore, this reaction produced a permanent modification of the active site structure that was not reversed upon removal of the anions by exhaustive dialysis. (Mariano *et al.*, 1995).

From these similar studies to this study, it is established that oxidation of haemocyanin leads the deoxy form of the protein. Oxidation by anions leads to the formation of met-haemocyanin. Spectrophotometric studies are used to observe the changes in absorption. Oxidation clearly leads to shift in the absorption band and reduces oxygen affinity.

Also, from the result in table 7, the significant decrease in the magnitude of the absorbance means that haemocyanin's oxygen binding affinity is decreasing, as the concentration of the fertilizer increases. The magnitude of this decrease implies that the more the concentration of fertilizer, the more toxic its effects on the protein and by extension on the snail. Similarly, Urea

fertilizer with corresponding concentration as NPK fertilizer caused more significant decrease effect in absorbance implying that urea has more toxic effect than NPK.

Conclusion

Haemocyanin's function as an oxygen carrying protein is greatly affected by toxicants like fertilizers (NPK 20:10:10 and Urea) specifically. This toxicity effect amounts to a change in the pathophysiology of the snail and is manifested as diseased conditions. This eventually causes death in the animal.

Recommendations

Fertilizers are toxic to the protein (haemocyanin) that carries the all essential oxygen in snails. This no doubt contributes to, a decline in the population as snails habitat in farm lands where these fertilizers are applied. Recommendations are that fertilizers should not be applied in excess or indiscriminately. It is shown by this study that the more the fertilizer, the more harmful the impact especially on snails. Secondly, better and improved fertilizers with less toxic effects should be used by farmers.

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