773272 2-5

Taylor & Francis
Taylor & Francis Group

AGREEMENT IN RELATION TO COPYRIGHT IN AN ARTICLE FOR A TAYLOR & FRANCIS/ROUTLEDGE JOURNAL

In order to ensure both the widest dissemination and protection of material published in our Journal, we ask Authors to transfer to the Publisher, Taylor & Francis, the rights of copyright in the Articles they contribute. This enables Taylor & Francis to ensure protection against infringement. The transfer of copyright must be clearly stated in writing.

PLEASE PROVIDE US WITH THE FOLLOWING INFORMATION, REVIEW OUR POLICIES, AND CONFIRM YOUR ACCEPTANCE OF THE TERMS OF THE ATTACHED ARTICLE PUBLISHING AGREEMENT BY SIGNING THIS FORM AS INDICATED BELOW.

Article (the "Article") entitled:
Studies on Phytoremediation of Copper with Pteridium aquilinum (Bracken fern) in the presence of biostimulants and Bioassay ulsing Clarias gariepinus juveniles
Author(s):
Flora Eyibio Olaifa, Anamese Jennifer Omekam
To be published in the journal (the "Journal"):
International Journal of Phytoremediation (1522-6514)

YOUR STATUS

☐ **I am the sole author of the Article**
Please indicate if any of the statements below also apply to you:
- ☐ I am a UK, Canadian or Australian Government employee and claim Crown Copyright
- ☐ I am a US Government employee and there is no copyright to transfer
- ☐ I am an NIH employee and there is no copyright to transfer. I submit this form together with an NIH addendum.
- ☐ I am a contractor of the US Government (includes NIH contractors) under contract number: .... I am required to sign this form.

✓☐ **I am one of multiple co-authors of the Article and confirm I have the consent of my co-authors to sign this agreement on their behalf**
Please indicate if any of the below also apply to you and your co-authors:
- ☐ All of my co-authors are UK, Canadian or Australian Government employees and Crown Copyright is claimed/not claimed (circle one)
- ☐ One or more of my co-authors, but not all of them, are UK, Canadian or Australian Government employees and Crown Copyright is claimed/not claimed (circle one)
- ☐ All of my co-authors are US Governmental employees and there is no copyright to transfer
- ☐ The work was performed by contractors of the US Government under contract number: ....

☐ **The copyright in the Article belongs to my employer (is a "work made for hire") and I am granting licence to publish as an authorized representative of my employer. My Title and Company are stated in the section below.**

ASSIGNMENT OF PUBLISHING RIGHTS

I hereby assign to Taylor & Francis the copyright in the above specified manuscript (government authors not transferring copyright hereby assign a non-exclusive licence to publish) and any accompanying tables, illustrations, data and any other supplementary information intended for publication in all forms and all media (whether known at this time or developed at any time in the future) throughout the world, in all languages, for the full term of copyright, to take effect if and when the article is accepted for publication. If I am one of several co-authors, I hereby confirm that I am authorized by my co-authors to grant this Licence as their agent on their behalf. For the avoidance of doubt, this assignment includes the rights to supply the article in electronic and online forms and systems.

I confirm that I have read and accept the full terms of the Journal's article publishing agreement attached to this form including my author warranties, and have reviewed the Journal's policies on Author Rights.

Signed: [signature] Name Printed: DR FLORA EYIBIO OLAIFA

Title and Company (if employer representative): ______________________ Date: 30TH JUNE, 2013

Please return only this page completed and physically signed. You may submit by fax, postal mail, email, or upload to CATS.

THIS FORM WILL BE RETAINED BY THE PUBLISHER FOR ADMINISTRATIVE PURPOSES.

Taylor & Francis
Taylor & Francis Group

ARTICLE PUBLISHING AGREEMENT-COPYRIGHT ASSIGNMENT

ASSIGNMENT OF COPYRIGHT

1. In consideration of the publication of your Article and subject to the provisions of the accompanying publishing agreement information form, you assign to us with full title guarantee all rights of copyright and related rights in your Article. So that there is no doubt, this assignment includes the assignment of the rights (i) to publish, reproduce, distribute, display and store the Article worldwide in all forms, formats and media now known or as developed in the future, including print, electronic and digital forms, (ii) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other derivative works based on the Article and all provisions elaborated in 1(i) above shall apply in these respects, and (iii) to sub-license all such rights to others. In the event the Article is not accepted and published by us or is withdrawn by you before acceptance by us, the assignment of copyright set out in this agreement shall cease to be effective and all rights assigned by you to us in relation to the Article shall revert to you.

PUBLISHER RESPONSIBILITIES

2. We shall prepare and publish your Article in the Journal. We reserve the right to make such editorial changes as may be necessary to make the Article suitable for publication, or as we reasonably consider necessary to avoid infringing third party rights or law; and we reserve the right not to proceed with publication for whatever reason.

AUTHOR RIGHTS

3. You hereby assert your moral rights to be identified as the author of the Article according to US copyright law.

4. You are permitted to use the material in the ways described in the Schedule of Author"s Rights providing that you meet all the conditions set out in the Schedule. These are rights which are personal to you and cannot be transferred by you to anyone else.

AUTHOR WARRANTIES

5. You hereby warrant that you have secured the necessary written permission from the appropriate copyright owner or authorities for the reproduction in the Article and in the Journal of any text, illustration, or other material. You warrant that, apart from any such third party copyright material included in the Article, the Article is your original work, and does not infringe the intellectual property rights of any other person or entity and cannot be construed as plagiarising any other published work. You further warrant that the Article is not currently under submission to, nor is under consideration by or has been accepted by any other journal or publication, nor has been previously assigned or licensed by you to any third party. Without prejudice to the provisions of Clause 3 above you undertake that the fully reference-linked version of scholarly record will not be published elsewhere without our prior written consent.

6. In addition you warrant that the Article contains no statement that is abusive, defamatory, libelous, obscene, fraudulent, nor in any way infringes the rights of others, nor is in any other way unlawful or in violation of applicable laws.

7. You warrant that wherever possible and appropriate, any patient, client or participant in any research or clinical experiment or study who is mentioned in the Article has given consent to the inclusion of material pertaining to themselves, and that they acknowledge that they cannot be identified via the Article and that you will not identify them in any way.

8. You warrant that you shall include in the text of the Article appropriate warnings concerning any particular hazards that may be involved in carrying out experiments or procedures described in the Article or involved in instructions, materials, or formulae in the Article, and shall mention explicitly relevant safety precautions, and give, if an accepted code of practice is relevant, a reference to the relevant standard or code.

9. You undertake that you will keep us and our affiliates indemnified in full against all loss, damages, injury, costs and expenses (including legal and other professional fees and expenses) awarded against or incurred or paid by us as a result of your breach of the warranties given in this agreement.

10. If the Article was prepared jointly with other authors, you warrant that you have been authorized by all co-authors to sign this Agreement as agent on their behalf, and to agree on their behalf the order of names in the publication of the Article. You shall notify us in writing of the names of any such co-owners.

GOVERNING LAW

11. This agreement (and any dispute, proceeding, claim or controversy in relation to it) is subject to US law and the jurisdiction of the United States. It may only be amended by a document signed by both of us.

Subject: International Journal of Phytoremediation - Decision on Manuscript ID BIJP-2011-0199.R1

From: jingsong@issas.ac.cn (jingsong@issas.ac.cn)

To: floraolaifa@yahoo.com; jomckam@yahoo.com;

Date: Friday, January 25, 2013 9:00 AM

25

25-Jan-2013

Dear Dr Olaifa:

Ref: Studies on Phytoremediation of Copper with Pteridium aquilinum (Bracken fern) in the presence of biostimulants and Bioassay uising Clarias gariepinus juveniles

Our referees have now considered your paper and have recommended publication in International Journal of Phytoremediation. We are pleased to accept your paper in its current form which will now be forwarded to the publisher for copy editing and typesetting. The reviewer comments are included at the bottom of this letter.

You will receive proofs for checking, and instructions for transfer of copyright in due course.

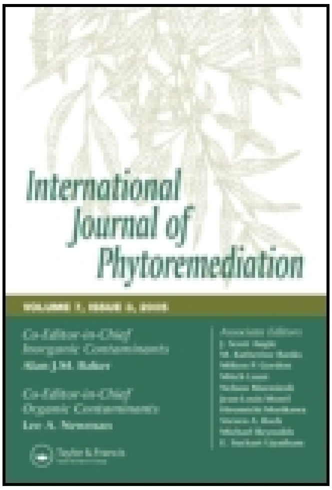
The publisher also requests that proofs are checked and returned within 48 hours of receipt.

Thank you for your contribution to International Journal of Phytoremediation and we look forward to receiving further submissions from you.

Sincerely,
Dr Song
Editor in Chief, International Journal of Phytoremediation
jingsong@issas.ac.cn

Reviewer(s)' Comments to Author:

There are now over 1050 Taylor & Francis titles available on our free table of contents alerting service! To register for this free service visit: www.informaworld.com/alerting.



International Journal of Phytoremediation
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bijp20

Studies on Phytoremediation of Copper Using Pteridium Aquilinum (Bracken Fern) In the Presence of Biostimulants and Bioassay Using Clarias Gariepinus Juveniles

Flora Eyibio Olaifa [a] & Anamese Jennifer Omekam [a]
[a] Department of Aquaculture and Fisheries Management , University of Ibadan , Ibadan , Nigeria
Accepted author version posted online: 08 Jul 2013.Published online: 27 Sep 2013.

To cite this article: Flora Eyibio Olaifa & Anamese Jennifer Omekam (2014) Studies on Phytoremediation of Copper Using Pteridium Aquilinum (Bracken Fern) In the Presence of Biostimulants and Bioassay Using Clarias Gariepinus Juveniles, International Journal of Phytoremediation, 16:3, 219-234, DOI: 10.1080/15226514.2013.773272

To link to this article: http://dx.doi.org/10.1080/15226514.2013.773272

International Journal of Phytoremediation, 16:219–234, 2014

ISSN: 1522-6514 print / 1549-7879 online
DOI: 10.1080/15226514.2013.773272

STUDIES ON PHYTOREMEDIATION OF COPPER USING *PTERIDIUM AQUILINUM* (BRACKEN FERN) IN THE PRESENCE OF BIOSTIMULANTS AND BIOASSAY USING *CLARIAS GARIEPINUS* JUVENILES

Flora Eyibio Olaifa and Anamese Jennifer Omekam
Department of Aquaculture and Fisheries Management, University of Ibadan, Ibadan, Nigeria

A study was carried out to evaluate the uptake of copper from water containing 10 mg/L copper by* Pteridium aquilinum *(bracken fern) and* Clarias gariepinus *in the presence of five plant growth stimulants: Nitrogen: phosphorus: potassium (15-15-15: an inorganic fertilizer), pig, cattle, poultry, and a mixture of pig/cattle manures. A plant growth stimulant differentiated each treatment. A 96-hour bioassay using* C. gariepinus *was carried out at the end of the experiment to test the efficacy of the clean up by* P. aquilinum*. The control experiment contained no copper or plant growth stimulant. Fish survival, uptake of copper by* P. aquilinum, C. gariepinus, *concentration of copper in water, hematology and histopathology of the fish were assessed. Higher concentrations of copper were reported in* P. aquilinum *than in water or* C. gariepinus*. Low fish mortality was reported with the highest being 20% in the cattle manure–containing treatment.

KEY WORDS: *P. aquilinum*, copper, bioassay, biostimulants, *Clarias gariepinus*, hematology, histology

INTRODUCTION

Copper is an essential element to living organisms, a component of enzymes and oxygen carrier in the blood of crustaceans like shrimps and lobsters (COPPERINFO 2001; The Analyst 2002). It participates in several processes in plants like photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production and disease resistance (Rana 2008, Kanoun-Boule′ *et al.* 2009). At high concentrations, it may be poisonous though the effects of metals on ecosystems are related to bioavailability and not to the total metal concentration (ICME 1998; Kabata-Pendias and Pendias 2001).

Copper enters the drinking water from copper pipes and additives for controlling algal growth in water such as copper sulphate and other copper-based algaecides. Copper compounds are also used to prevent fish diseases and parasites in fish culture (Palacios and Risbourg 2006). Copper is present in the natural state in water as a trace element less than 5 μ/l (Alabaster and Llyod 1982). Its toxicity is attributed to the cupric (Cu^{2+}) form, the

Address correspondence to Flora Eyibio Olaifa, Department of Aquaculture and Fisheries Management, University of Ibadan, Ibadan, Nigeria. E-mail: floraolaifa@yahoo.com; fe.olaifa@mail.ui.edu.ng

species commonly found in environment. It readily complexes with inorganic and organic substances and adsorbs onto particulate matter. Acute toxic effects of copper include death of animal, death or slow growth in plants while chronic toxic effects may be shortened lifespan, reproductive problems including lowered fertility and changes in appearance or behaviour. Its toxicity to aquatic life varies with physical and chemical conditions of the water such as hardness, alkalinity, pH, dissolved oxygen and temperature.

Various methods exist for cleaning up heavy metals but many of them are expensive (Jadia and Fulekar 2009). Phytoremediation is the use of plants to remediate soils, sludge, sediments and water contaminated with organic and inorganic chemicals (Khellaf and Zerdaoui 2010). Plants are grown in a contaminated matrix, for a required growth period, to remove contaminants from the matrix, or facilitate immobilization (binding/containment) or degradation (detoxification) of the pollutants (Jadia and Fulekar 2009; Huang *et al*. 2004; Kakitani *et al*. 2006). It is a technique that deals with pollution cheaply and sustainably (Rai 2009; Rajkumar *et al*. 2009; Khellaf and Zerdaoui 2010). Plants that have been used for phytoremediation include *Glycine max, Eichornia crassipes*, *Lemna spp* and *Pteris vittata* (Ma *et al*. 2001; Khellaf and Zerdaoui 2010). Other aquatic plants like *Hydrilla vercillata* Casp., Elodea (*Elodea canadensis* Rich) and *Salvinia* sp. have been tested for removal of heavy metals like iron, copper and nickel from metal solution (Begum and Hare Krishna 2010).

Many plants accumulate metals in their tissues without showing symptoms of toxicity (Sabreen and Sugiyama 2008). Metal resistance depends on tolerance or avoidance mechanisms (Papazoglou *et al*. 2005). There is a dearth of information on remediation studies in which bioassays using fish are included to test the effectiveness of the clean up process. Sequel to an earlier study assessing the toxicity of copper on *C. gariepinus* juveniles (Olaifa *et al*. 2004) which reported high mortalities, this work was undertaken to assess the uptake of copper by *C. gariepinus*, and *P. aquilinum* in the presence of plant growth stimulants with emphasis on fish survival, haematology and histopathology of the fish and bioconcentration of copper by *P. aquilinum* and *C. gariepinus* juveniles.

MATERIALS AND METHODS

Ferns are classified as *Ophioglossaceae*, *Marattiaceae* and *Leptosporangiate*. The last group includes aquatic ferns *Marsileaceae* and *Salviniaceae*, land ferns including *Pteridaceae* (Hasebe *et al*. 1994) one of which is *Pteridium aquilinum*.

P. aquilinum is a large perennial fern occurring in several habitats- dry to wet forests, meadows, clearings, sandy soils, road sides, lake-shores, bogs, burned areas, coast, tablelands, wetlands and to a lesser extent, the slopes. It has deep underground stems, stiff fronds, can survive intensive fire and has hardy persistent and extensive root system called rhizome. *P. aquilinum* produces no seeds or flowers but spores in bodies called sori which occur in continuous lines under the fronds. The spores are minute and can travel long distances by wind.

Determination of Physico-Chemical Characteristics of the Water

Water quality characteristics monitored included dissolved oxygen, temperature, pH, alkalinity and nitrate. A mercury-in-glass thermometer (Paragon Scientific Ltd, Birkenhead, Wirral, UK) was dipped into each tank for two minutes with the mercury bulb fully immersed

Table 1 Activities and time taken for each activity during this Study

Activities	Number of Days
Acclimatization of *P. aquilinum*	21
Exposure of *P. aquilinum* to copper in water	14
Acclimatization of *C. gariepinus*	14
Bioassay using *C. gariepinus*	96 hours (4 days)
Total number of days	53

and temperature readings taken. Other parameters were nitrate and phosphate (Murphy and Riley 1962), alkalinity (Eaton *et al*. 2005); hydrogen ion concentration (pH) was measured using Jenway 3015 pH metre, 0.0 accuracy (Genway, Staffordshire, UK) and dissolved oxygen using Winkler's method was calculated as D.O (in mg/l) = (ml of titrant) (N) (8) (1000) / Sample volume in ml where N = 1 and represents the normality of the solution used to titrate the sample.

Collection, Acclimatization and Introduction of *P. Aquilinum* into Experimental Tanks

P. aquilinum was obtained from the fish farm of the Department of Aquaculture and Fisheries Management, University of Ibadan and acclimatized for three weeks. The ferns were placed together with attached soils in plastic bowls, lightly wetting for three days to detach soil, drain out and grown in well water throughout study. The activities carried out and experimental design used are shown on Tables 1 and 2.

Preparation of toxicant. Hydrated copper chloride was used as toxicant at the rate of 10 mg/L for all treatment groups and required amounts were obtained by calculation (Reish and Oshida 1987; Odiete 1999).

Table 2 The Design of the Experiment

Treatments	Contents
Control 1	Copper (0 mg/l), manure (0 mg/l), *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* juveniles (10)
2	NPK(15:15:15) (10 mg/l), copper (10 mg/l), *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* (10)
3	Pig manure (10 mg/l), copper (10 mg/l), *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* juveniles (10)
4	Cattle manure (10 mg/l) copper (10 mg/l), *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* juveniles (10)
5	Poultry manure (10 mg/l), copper (10 mg/l), *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* juveniles (10).
6	10 mg/l pig + cattle manure, 10 mg/l copper, *P. aquilinum* (4 fronds on rhizome), *C. gariepinus* juveniles (10)

Collection and Acclimatization of Fish

Clarias gariepinus juveniles (numbering 200 with mean weight 40 g and length 22 cm)
were purchased from a fish farm in Ibadan, Nigeria. The fish were kept in bowls with 20 L of water and acclimatized for two weeks before introduction into the experimental tanks. During acclimatization of the fish, water was replaced every other day and fish fed twice daily with multi-purpose compounded feed at 3% of their body weight. The fish were randomly allocated to six groups (five treatments and one control). Each group had two replicates and 10 fish per replicate. Feeding of fish was stopped 24 h before the experiment and no aeration was used.

Introduction of plants into experimental tanks. *P. aquilinum* stands were transferred into each experimental tank and held in place using stakes and masking tapes (Plate1). Water in each tank was made up to 20 litres. Nitrogen- phosphorus- potassium (N:P:K 15:15:15) fertilizer, pig, cattle, poultry and a combination of pig and cattle manures were added to tanks as growth stimulators for *P. aquilinum* at the rate of 10 mg/l of water (Ndimele 2009). A 96-h bioassay (Reish and Oshida 1987) using *Clarias gariepinus* juveniles was carried out from day 49–53 (Tables 1 and 2). Table 5 shows the metal content in the manures used.

Plate 1 Experimental Set up showing ferns in glass aquaria (color figure available online).

Table 3 Water Quality during the acclimatization of *P. aquilinum*

Temp (°C)	Dissolved oxygen	pH	Alkalinity	Nitrate	Phosphate	Potassium
27	6.5	7.5	20	1.424	0.2315	1.07

Digestion of Samples and Determination of Metal Contents of Water, *C. Gariepinus* and *P. Aquilinum*

Whole *P. aquilinum* samples (fronds and rhizomes) from each treatment and manure were digested using perchloric and hydrochloric acid (Pratt 1965; Isaac and Korbor 1971). 0.5 g of the dried, milled whole *P. aquilinum* and manure samples were weighed out into a 25-ml volumetric flask. 5 ml of the mixture of perchloric and hydrochloric acid solution was added and heated on a hot plate for an hour at 200°C until a clear solution was obtained. The flasks were removed from heat and allowed to cool. Deionised water was added and made up to the 25 ml mark. Copper was analyzed using a Buck Scientific VGP 210 atomic absorption spectrophotometer. Metal concentration was calculated from AAS readings as described by Odiete (1999).

Wet fish sample was weighed out (2 g) into an open beaker and 10 ml of freshly prepared 1:1 nitric acid– hydrogen peroxide added. The beaker was covered with a watch glass till initial reaction subsided in about 1 h, placed in a water bath on a hot plate. The temperature was gradually raised to 160°C, boiled gently for 2 h to reduce the volume to

Table 4 Water Quality Parameters of Water at the beginning and end of the Experiment

Parameters	Control	NPK	Pig	Cattle	Poultry	Pig & Cattle
Temp. (°C)						
Onset	27	26.5	27	27	26.5	27
After	27	27	26.5	26	26.5	26.5
D.O (mg/l)						
Onset	6	5.4	5.7	5.2	5.1	4.9
After	5.2	4.8	4.6	4.7	4.4	3.8
pH						
Onset	6.4	8.9	7.2	7.6	8.6	8
After	8.2	7.6	6.9	7.1	7.4	7.1
Alkanility (mg/l)						
Onset	24	26	28	26	30	28
After	54	84	222	252	128	130
NO_3 (mg/l)						
Onset	2.21	1.92	1.56	1.60	1.22	2.13
After	1.60	2.42	0.71	1.03	2.49	4.62
Phosphate						
Onset	0.08	18.1	0.38	0.15	0.92	1.54
After	18.52	139.0	48.30	82.40	8.79	70.3
K (mg/l)						
Onset	0.68	2.11	0.72	0.87	0.35	0.59
After	1.05	2.51	2.00	2.87	1.09	2.03
Cu (mg/l)						
Onset	0	0.01	0.02	0.03	0.05	0.10
After	0.39	0.46	0.69	0.56	0.11	0.70

Table 5 Concentrations of metals in the manure in mg/kg)

Manure	Calcium	Magnesium	Potassium	Sodium	Manganese	Iron	Copper	Zinc
Pig	3.53	0.49	1.28	0.06	0.004	0.54	0.01	0.02
Cattle	2.32	0.64	3.55	0.09	0.005	0.33	0.003	0.01
poultry	10.39	1.46	0.45	0.45	0.32	0.58	0.04	0.22
Pig + cattle	1.04	0.53	2.08	0.25	0.05	0.53	0.07	0.02

2–5 ml. The digests were cooled, transferred to 25 ml volumetric flasks and made up to mark with de-ionized water (FAO/SIDA 1983). The digests were kept in plastic bottles and copper concentrations determined using an atomic absorption spectrophotometer. The actual concentration of each metal was calculated as PPMR multiplied by dilution factor where PPMR is the AAS reading. The dilution factor is volume of digest divided by volume of sample digested. Bioconcentration factor was calculated as the concentration of copper in fish or plant tissue (mg/g) divided by the initial concentration of copper in the water (mg/l) (Khellaf and Zerdaoui 2009).

Water samples (from the well, acclimatization and experimental tanks) were filtered before analysis for copper using atomic absorption spectrophotometer. All data obtained were subjected to a one-way analysis of variance while the means were compared for significant differences using Duncan's multiple range test (Duncan 1955).

Collection of Blood and Histopathology Samples

Blood sampling and analyses were carried out (Blaxhall and Daisley 1973; Dacie and Lewis 1975; Jain 1986; Schmitt *et al.* 1999). For histopathology, the internal organs were exposed by dissection, and the gills, kidneys, livers, and intestines observed for gross lesions and stored in formalin. Small portions of each organ were fixed and put through series of dehydration in graded concentrations of xylene. They were embedded in wax, sectioned at 5 μ and transferred onto glass slides. The thin sections were stained with heamotoxylin and eosin (H and E) dyes for examination under the light microscope for histological changes (MAFF 1984).

Table 6 Number and Time of Mortality of Fish During the 96- hour Bioassay of fish exposed to 10 mg/l copper in water at the end of the Experiment

Biostimulant	Number of test fish	Mortality (Replicate 1)	Time of death (hours-Replicate 1)	Mortality (Replicate 2)	Time of death (hours-Replicate 2)
Control	10	—	—	—	—
NPK	10	1	74	1	96
Pig manure	10	—	—	—	—
Cattle manure	10	1	74	1	74
Poultry manure	10	—	—	—	—
Pig & Cattle manure	10	1	74	2	76

Table 7 Percentage Mortality of *C. gariepinus* during the 96- hour Bioassay

Biostimulant	Number of test fish	Mortality (Replicate 1)	Mortality (Replicate 2)
Control	10	—	—
NPK	10	10	10
Pig manure	10	—	—
Cattle manure	10	10	10
Poultry manure	10	—	—
Pig & Cattle manure	10	10	20

RESULTS AND DISCUSSION

A study was undertaken to assess the uptake of copper by *P. aquilinum* and *C. gariepinus* from water in the presence of different plant growth stimulants. A bioassay was also carried out to test the efficacy of the clean up process using *C. gariepinus* juveniles as test organisms. Before the experiment, chromium, cadmium, lead and nickel were undetected but cobalt was 0.95–3.80 mg/l and copper 0–0.1 mg/l in the well water used. The concentration of copper in the well water was within the range obtained by other workers (Ajani and Akpoilih 2010). All other results obtained during this study are presented in Tables 3–10 and Figures 2–10.

The water quality characteristics during this study are presented (Tables 3 and 4). There was little variation in water temperature during the study. Lower D.O. concentrations were observed at the end of the study though they were still within the recommended concentrations for warm water fish (Boyd 1982). The decrease in D.O. could have been due to biological activities occurring in the water such as decomposing organic matter present in manure and uptake for respiration by fish and *P. aquilinum*. The low D.O. in the water was also indicated by the appearance of foam on water surface in all treatments except the control. It has also been reported that the presence of metals reduce the concentrations of oxygen in water (Tawari-Fufeyin *et al*. 2008). All other water quality characteristics were adequate for fish survival. Significant increases ($p < 0.05$) in alkalinity and phosphate were also observed at the end of the study above the observations made during acclimatization and at the onset of the study with the highest reported for cattle manure. Potassium (K) increased marginally in all treatments at the end of the study.

Table 8 Concentrations of copper in *C. gariepinus*, Water and *P. aquilinium* Exposed to water containing 10 mg/L copper and different plant growth stimulants

Biostimulant	Initial concentration of copper in water (10 mg/l)	Actual concentration of copper in fish and plant tissues			
		Cu concentration in fish (mg/g)	Cu conc. in plant (rep 1) (mg/g)	Cu conc. in plant (rep 2) (mg/g)	Final conc. of Cu in water (mg/L)
Control	0	0.06	31.20	13.85	0.09
NPK	10	0.08	286.00	167.50	0.46
Pig manure	10	0.03	225.50	317.50	0.69
Cattle manure	10	0.06	1058.00	2442.00	0.56
Poultry manure	10	0.12	942.50	1774.00	0.11
Pig & Cattle manure	10	0.05	1007.00	425.50	0.70

Table 9 Biocncentration of copper in *C. gariepinus* and *P. aquilinum* exposed to 10 mg/l copper and different plant growth stimulants

Biostimulant	Initial copper concentration in water	Bioconcentration factor in fish	Bioconcetration factor in *P. aquilinum* (Replicate 1)	Bioconcentration factor in *P. aquilinum* (Replicate 2)
Control	10	—	—	—
NPK	10	0.008	28.6	16.75
Pig manure	10	0.003	22.5	31.75
Cattle manure	10	0.006	105.8	244.2
Poultry manure	10	0.012	94.25	177.4
Pig & cattle manure	10	0.005	100.70	42.55

After introduction of 10 mg/l of copper in all treatments yellowing of leaves and suppressed shoot development were observed over the study period except in the treatment 5 with poultry manure which produced the highest number of growing shoots of *P. aquilinum* (13 extra shoots) during the period. High concentration of copper may account for the suppressed root growth and leaf yellowing of leaves observed among plants (Baker and Walker 1990; Omwoma *et al*. 2010). This study showed that *P. aquilinum* has the potential to accumulate copper with the highest uptake being 2442 mg/kg copper in treatment 4 containing cattle manure.

On introduction of fish into the system, they swam excitedly in between the roots of the plants. Weakness, slow response to touch and suspension at the water surface were observed 25 hours after introduction of fish and mortality was first recorded at 74 hours (Tables 6 and 7) and ranged between 10 and 20% during the study and differed from previous findings (Olaifa *et al*. 2004). The lower mortality could be due to the uptake of the copper by *P. aquilinum* during the two weeks between introduction of copper and introduction of *C. gariepinus* into the system. During this period, the water in the tanks turned green due to algal growth (Plate 1). Copper compounds are used for algal control in water bodies but the presence of algae signified a decrease in copper concentrations during the two-week period before introduction of the fish. Also the plant growth stimulants might have helped to stimulate algal growth in water. It has been observed that the toxicity of copper reduces in the presence of organic or inorganic substances because copper forms complexes with

Table 10(a) Mean haematological indices of *C. gariepinus* juveniles exposed to water with *P.aquilinium*, 10 mg/l copper and different plant biostimulants

Biostimulants	Packed Cell Volume (%)	White Blood Cells (10^{12}/L)	Plasma Protein (g/dl)	Haemoglobin (g/L)	Platelet (m/μl)	Red blood cells (10^{12}/L)
Initial	36.00a	24550.00a	6.000a	12.050a	254000.00a	3.2950
Control	21.00	13700.00b	2.550b	6.750	100500.00b	1.3250
NPK	19.00	11300.00b	3.700b	5.900	173000.00ab	1.3700
Pig	24.50	15625.00ab	3.650b	7.950	142000.00ab	1.9150
Cattle	25.50	12800.00b	3.600b	7.800	124000.00ab	2.1200
Poultry	20.50	10475.00b	3.850b	6.750	101000.00b	1.2500
Pig & Cattle	21.50	19200.00ab	5.400ab	6.750	155000.00ab	1.1300
ns				ns		ns

Note: Numbers on the same row with same letters are not significantly different.

Table 10(b) Mean hematological indices of *C. graiepinus* juveniles exposed to water with *P. aquilinum,* 10 mg/l copper and different plant biostimulants

Biostimulants	Lymphocyte ($\times 10^9$/l)	Neutrophil (%)	Eosinophil ($\times 10^9$/l)	Monocyte ($\times 10^9$/l)	Albumin (g/dl)
Initial	48.00c	46.50a	2.50ab	3.00	1.250b
Control	58.00bc	39.50ab	1.00b	1.50	1.250b
NPK	59.50bc	38.00ab	1.00b	1.50	2.200ab
Pig	76.00a	19.50c	2.00ab	2.50	1.800ab
Cattle	66.00ab	29.00bc	3.00a	2.00	1.950ab
Poultry	72.50ab	24.00c	1.50ab	2.00	2.000ab
Pig & Cattle	68.00ab	28.50bc	1.50ab	2.00	2.300a
				ns	

Note: ns = not significant.
Numbers on the same rows with different letters are significantly different ($p < 0.05$).

them and toxic effects of copper are reduced (Oronsaye and Ogunbor 1998). Significantly higher ($p < 0.05$) concentrations of copper were recorded in *P. aquilinum* than either in water or fish at the end of the experiment (Tables 8 and 9 and Figure 1) with the highest uptake and bioconcentration factor of copper (2442 mg/g; 244) respectively by *P. aquilinum* observed in the treatment with cattle manure.

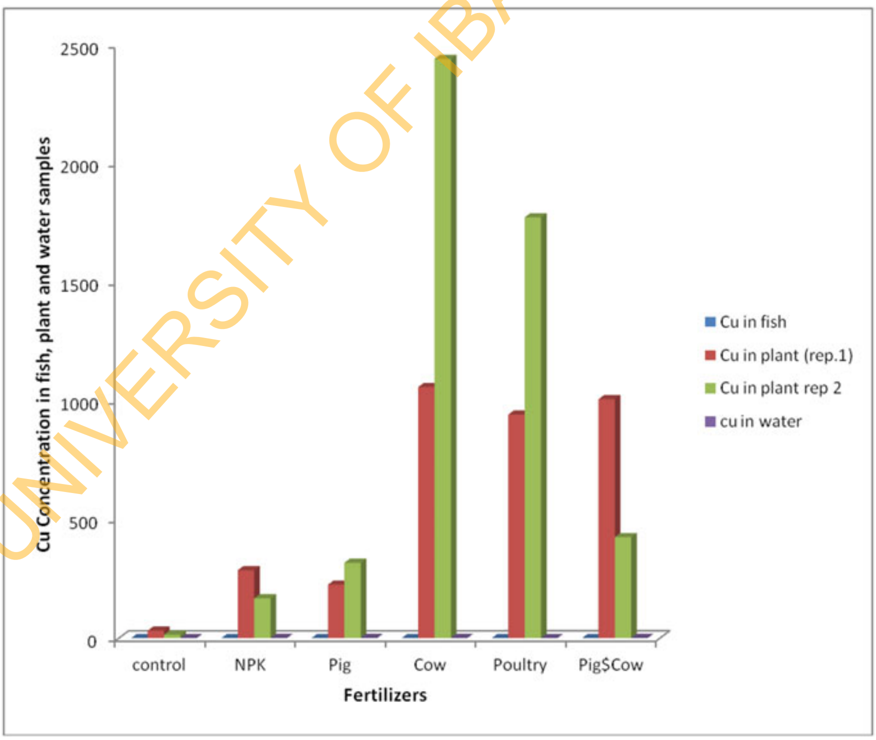


Figure 1 Chart showing copper concentrations in fish, plant and water samples after digestion in all treatments (color figure available online).

Histopathology of *C. gariepinus* Exposed to 10 mg/L Copper in water containing various plant growth stimulants and *P. aquilinium*:

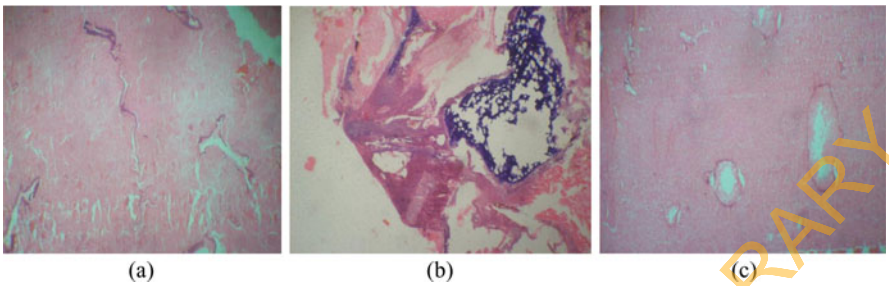


Figure 2 Organs of control fish with no visible lesions, (a) kidney (×250), (b) gill (×250), and (c) liver (×250). (color figure available online).

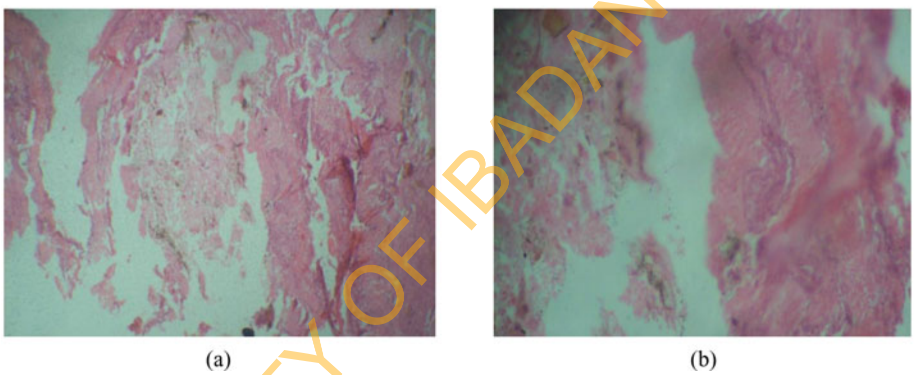


Figure 3 Intestines of *C. gariepinus* exposed to 10 mg/l cattle manure and copper showing mucosal erosion (a) intestine (replicate 1, ×250), and (b) intestine (replicate 2, ×250). (color figure available online).

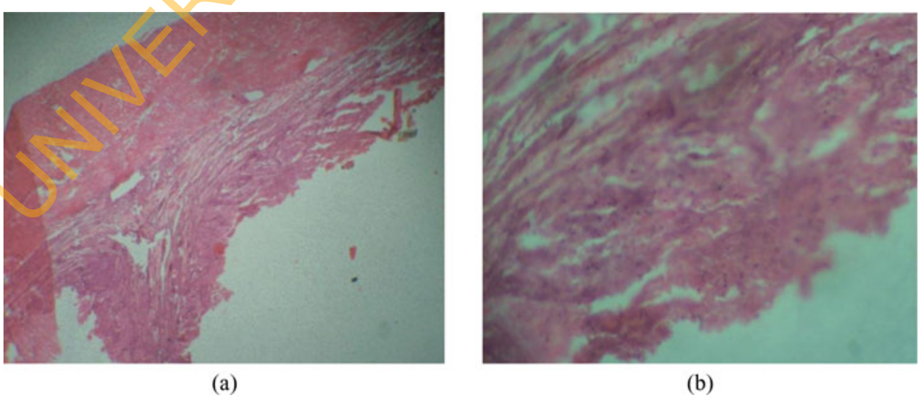


Figure 4 Intestines of *C. gariepinus* exposed to 10 mg/l of NPK showing mild epithelial sloughing (a) intestine (replicate 1, ×250), and (b) intestine (replicate 2, ×250). (color figure available online).

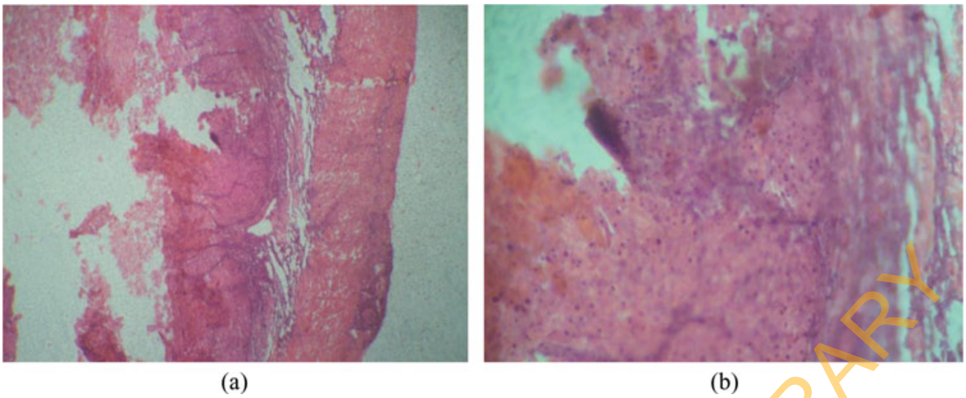


Figure 5 Intestines of *C. gariepinus* exposed to copper and poultry manure with intestinal villi absent and showing mucosal erosion (×250), (a) Intestine (replicate 1, ×250), and (b) intestine replicate 2 (×250). (color figure available online).

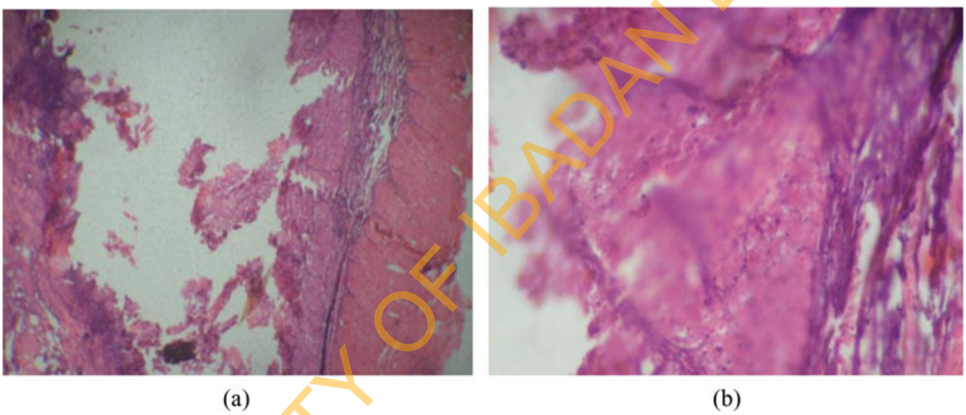


Figure 6 Intestines of fish exposed to copper and pig manure with severe epithelial sloughing, (a) intestine (replicate 1 × 250), and (b) intestine (replicate 2, ×250). (color figure available online).

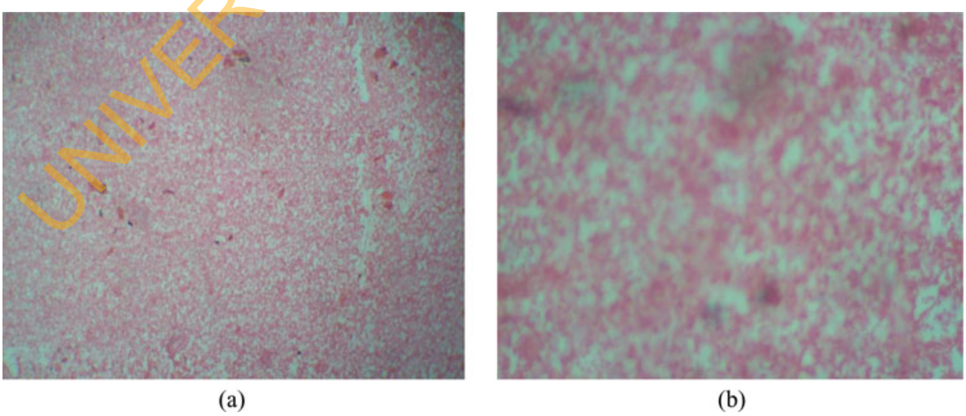
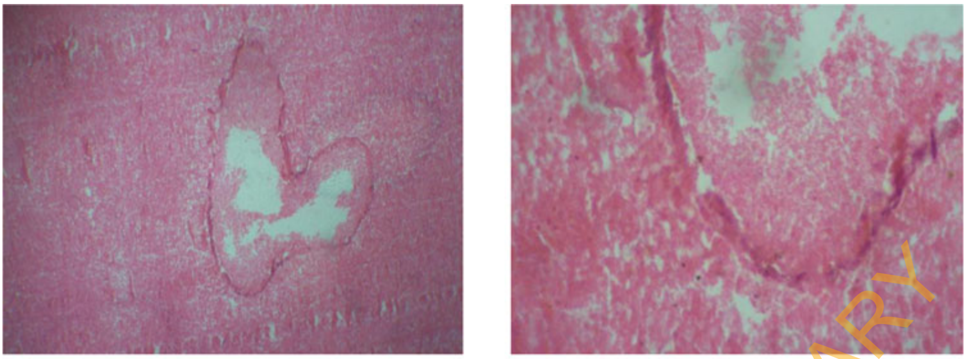


Figure 7 Livers of *C. gariepinus* exposed to copper and cattle manure (10 mg/l) showing mild diffuse vacuolation of the hepatocytes, (a) liver (replicate 1, ×250), and (b) liver (replicate 2 ×250). (color figure available online).

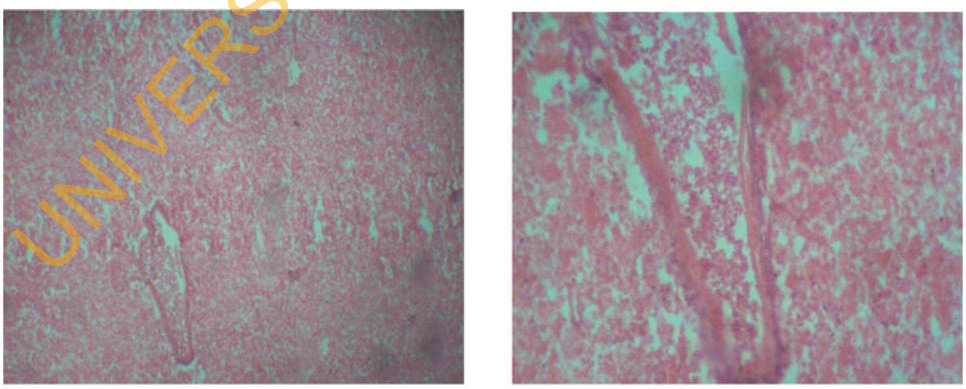


(a) (b)

Figure 8 Livers of *C. gariepinus* of exposed to 10 mg/l copper and NPK with moderate central venous congestion, and mild diffuse vacuolation, (a) liver (replicate 1, ×250), and (b) liver (replicate 2, ×250). (color figure available online).

The hematology of fish (Table 10 a, b) showed no significant differences ($p > 0.05$) in packed cell volume, red blood cells, hemoglobin and monocytes between the control and treatments. However, all the initial blood parameters were greater ($p < 0.05$) than those observed during the study except for lymphocytes and albumin. These observations were similar to those of other workers (Singh *et al*. 2008) after exposure of *Oreochromis mossambicus* to copper and zinc.

Haemoglobin concentration indicates the supply of an organism with oxygen. Exposure of fish to copper during the experiment could have caused stress- a response to any factor which disturbs homeostasis. Stress reactions involve various physiological changes including alteration in blood composition and immune mechanisms (Svoboda 2001). The red blood cell count is a good index to determine fish health (Adeyemo *et al*. 2008). Studies have shown that when the water quality is affected by toxicants, any physiological changes will be reflected in the values of one or more of the haematological parameters (Van Vuren



(a) (b)

Figure 9 Livers of fish exposed to 10 mg/l poultry manure and copper showing mild central venous congestion and diffuse vacuolation (×250), (a) liver (replicate 1, ×250), and (b) liver (replicate 2, ×250). (color figure available online).

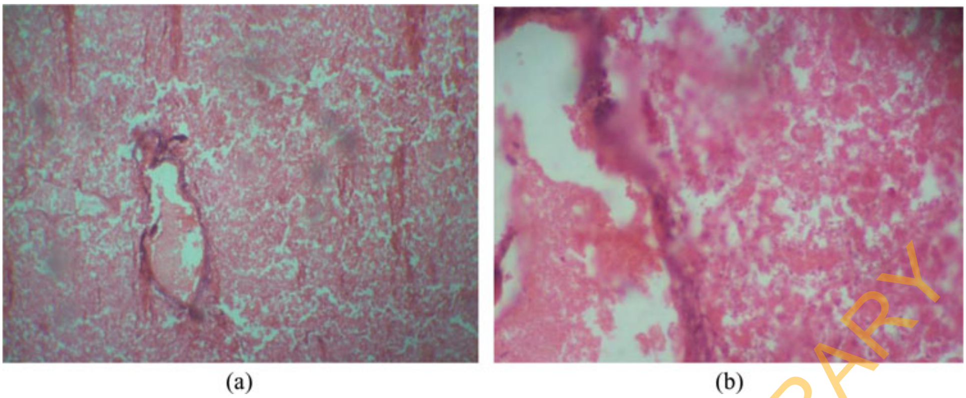


Figure 10 Livers of fish exposed to 10 mg/l copper and pig-cattle manure mixture showing moderate central venous congestion, with mild diffuse vacuolation, (a) liver (replicate 1, ×250), and (b) liver (replicate 2 × 250). (color figure available online).

1986; Adeyemo 2008). A decrease in both haemoglobin and haematocrit are possibly the consequences of anaemia and haemodilutuion (Vosyliene 1999). Lymphocytes increased in all treatments during the study which could have been due to compromised immune system caused by stress of exposure to copper and the different manures. Non specific stresses also cause white blood cells to increase. Monocytes are precursors of macrophages and play an important role in non specific immunity and inflammatory response (Vosyliene 1999).

HISTOPATHOLOGY

The histopathology of the fish is presented in Figures 2–10. Lesions were not observed in the gills and kidneys of all treatments and the control. The liver and intestines of all treatments except the control showed morphological changes such as vacuolation, venous congestion of the liver and epithelial erosion of the intestines. The liver is the main organ of biotransformation and excretion of xenobiotics and their presence rapidly causes structural, biochemical and molecular changes (Bernet *et al*. 1999; Fernandes *et al*. 2008; Kaoud and El-Dashan 2010; Rodrigues *et al*. 2010). The kidney regulates electrolytes, water balance and maintains a stable internal environment. Both the kidney and liver can serve as indicators of pollution (Pathan *et al*. 2010).The changes observed during this work were less severe than other reports (Saxena and Saxena 2008; Kaoud and El-Dashan 2010; Pathan *et al* 2010).

CONCLUSION AND RECOMMENDATION

The locally available bracken fern showed good quality as an accumulator of copper in the presence of biostimulants. Further studies need to be conducted to establish these qualities with and without plant growth stimulants. Field studies will also need to be carried out to establish this finding outside laboratory conditions.

REFERENCES

Adeyemo OK, Ajani F, Adedeji OB, Ajiboye OO. 2008. Acute Toxicity and Blood Profile Adult *Clarias gariepinus* Exposed to Lead Nitrate. Inter J Hematol 2.

Ajani EK, Akpoilih BU. 2010. Effect of chronic dietary copper exposure on haematology and histology of common carp (*Cyprinus carpio L.*). J Appl Sci Environ Manage 14:39–45

Alabaster JC, Lloyd R. 1982. Copper in water quality, criteria for freshwater fish. London (UK): Butterworths. p. 188–216.

Baker AJM, Walker PL. 1990. Ecophysiology of metal uptake by tolerant plants. In: Shaw A, editor. Heavy metal tolerance in plants – Evolutionary Aspects. Boca Raton (FL): CRC Press, p. 155–177.

Begum A, Hare Krishna S. 2010. Bioaccumulation of Trace metals by aquatic plants. Int J Chem Tech Res 2:250–254.

Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T. 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J Fish Diss 22:25–34.

Blaxhall PC, Daisley KW. 1973. Routine haematological methods for use with fish blood. J Fish Biol 5:771.

Boyd CE. 1982. Water quality management for pond fish culture. Amsterdam (The Netherlands): Elsevier Scientific Publishing Company. p. 318.

COPPERINFO. 2001. Copper and aquatic life. International Copper Association. http: //www. copperinfo.com/health/aquatic.shtml

Dacie JV, Lewis SM. 1975. Practical haematology, 5th ed. Longman Edinburgh (NY): Churchill Livingstone. p. 285.

Duncan RM. 1955. Multiple Range and Multiple F-tests. Biometrics 11:1–42.

Eaton AD, Clesceri, LS, Rice EW, Greenberg AE, Franson MAH. 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. Washington D.C. American Health Association. *Source:* USEPA 200.9 Revision 2.2.

FAO/SIDA. 1983. Manual of methods in aquatic environmental research, part 9. Analyses of metals and organochlorines in fish. FAO Fisheries Technical Paper. p. 212.

Fernandes C, Fernandes AF, Ferreira M, Salgado M. 2008. Oxidative stress response in gill and liver of *Liza saliens*, from the Esmoriz-Paramos coastal lagoon, Portugal. Arch Environ Contam Toxicol 55:691–700.

Hasebe MT, Omori M, Nakazawa, Sano T, Kato, Iwatsuki K. 1994. *rbcL* gene sequences provide evidence for the evolutionary lineages of *Leptosporangiate* ferns. Proc Natl Acad Sci USA 91:5730–5734.

Huang WH, Poynton CY, Kochian LV, Elless MP. 2004. Phytofiltration of arsenic from drinking water using hyper-accumulating ferns. Environ Sci Technol 38:3412–3417.

ICME. 1998. Expert Workshop on the Atmospheric Transport and Fate of Metals in the Environment. Antwerp, Belgium, p. 14, Report available from ICME, Ottawa.

Isaac AR, Korbor JD. 1971. Atomic absorption and flame photometry. Technique and uses soil plant and water analysis. In: Walsh LM, editor. Instrumental Methods for Analysis of Soils and plant tissues. Madison (WI): American Soil Science Society.

Jadia CD, Fulekar MH. 2009. Review-Phytoremediation of heavy metals: Recent Techniques. Afr J Biotechnol 8:921–928.

Jain NC. 1986. Schalm's Veterinary Haematology. 4th ed.: Lea and Febiger.

Kabata-Pendias A, Pendias H. 2001. Trace elements in soils and plants. London (UK): CRC Press.

Kakitani T, Hata T, Kajimota T, Imamura Y. 2006. Designing a purification process for chromium, copper, and arsenic contaminated wood. Waste Management 26:453–458.

Kaoud HA, El-Dahshan AR. 2010. Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish. Nature and Science 8:147–156.

Kanoun-Boule′M, Vicentea JAF, Nabaisa C, Prasad MNV, Freitas F. 2009. Ecophysiological tolerance of duckweeds exposed to copper. Aqua Toxicol 91:1–9.

Khellaf N, Zerdaoui M. 2009. Phytoaccumulation of zinc by the aquatic plant, *Lemna gibba* L. Bioresour Technol 100:1637–1640.

Khellaf N, Mostefa Zerdaoui M. 2010. Growth response of the duckweed *Lemna gibba* L. to copper and nickel phytoaccumulation. Ecotoxicology 19:1363–1368.

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic. Nature. 409:579–579.

MAFF. 1984. Manual of veterinary investigation. Laboratory techniques. Volume 2, Reference Book 390. Third Edition Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationery Office, London, UK.

Murphy J, Riley JP. 1962. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 2:31–36.

Ndimele PE. 2009. Evaluation of Phyto-remediative properties of Water Hyacinthb *(Eichhornia crassipes* (Mart.) Solms) and Biostimulants in Restoration of Oil-Polluted wetlands the Niger Delta. A PhD thesis submitted to the Faculty of Agriculture and Forestry, University of Ibadan.

Odiete WO. 1999. Environmental Physiology of Animals and Pollution.1st ed. Lagos (Nigeria): Diversified Resources Ltd. p. 261.

Olaifa FE, Olaifa AK, Onwude TE. 2004. Lethal and sub-lethal Effects of Copper to the African Catfish (*Clarias gariepinus*) Juveniles. Afr J Biomed Res 7:65–70.

Omwoma S, Lalah JO, Ongeri DMK, Wanyonyi MB. 2010. Impact of fertilizers on heavy Loads in Surface Soils in Nzoia Nucleus Estate Sugarcane Farms in Western Kenya. Bull Environ Contam Toxicol 85:602–608.

Oronsaye JAO, Ogunbor EO. 1998. Toxicity of copper to *Oreochromis niloticus* Fingerlings in Ikpoba River. Indian J Animal Sci 68:1001–1003.

Palacios SP, Risbourg SB. 2006. Hepatocyte nuclear Structure and sub cellular distribution of copper in zebra fish *Brachydano rerio* and roach *Rutilus rutilus* (Teleostei, Cyprinidae) exposed to copper sulphate. Aquat Toxicol 77:306–313.

Pathan TS, Shinde SE, Thete PB, Sonawane DL. 2010. Histopathology of Liver and Kidney of Rasbora daniconius exposed to paper mill effluent. Res J Biol Sci 5:389–394.

Papazoglou EG, Karantounias GA, Vemmos SN, Bouranis DL. 2005. Photosynthesis and growth responses of Giant reed (Arundodonax L.) to heavy metals Cd and Ni. Environ Int 31:243–249.

Pratt PF. 1965. Digestion with Hydrofluoric and perchloric acids for total potassium and sodium.In: Black CA, editor. Methods of soil and plant analysis. Agronomy 9:1010–1021. Madison (WI): American Society of Agronomy Inc.

Rai PK. 2009. Heavy metal phytoremediation from aquatic ecosystems with special reference to macrophytes. Crit Rev Environ Sci Technol 39:697–753.

Rajkumar K, Sivakumar S, Senthilkumar P, Prabha D, Subbhuraam CV, Song YC. 2009. Effects of selected heavy metals (Pb, Cu, Ni, and Cd) in the aquatic medium on the restoration potential and accumulation in the stem cuttings of the terrestrial plant, *Talinum triangulare*. Linn Ecotoxicology 18:952–960.

Rana SVS. 2008. Metals and apoptosis: recent developments. J Trace Elem Med Biol 22:262–284.

Reish DL, Oshida OS. 1987. Manual of Methods in Aquatic Environment Research. Part 10: Short-term static bioassays. FAO Fisheries Technical Paper. 247:62 pages.

Rodrigues VR, Miranda-Filho KC, Gusmão EP, Moreira CB, Romano LA, Sampaio LA. 2010. Deleterious effects of water-soluble fraction of petroleum, diesel and gasoline on marine pejerrey *Odontesthes argentinensis* larvae. Sci Total Environ 408:2054–2059.

Sabreen S, Sugiyama S. 2008. Trade-off between cadmium tolerance and relative growth rate in 10 grass species. Environ Exp Bot 63:327–332.

Saxena MP, Saxena HM. 2008. Histopathological changes in the lymphoid organs of fish after exposure to water polluted with heavy metals .The Internet Journal of Veterinary Medicine. 5 (1). http:/www.ispub.com:80/journal/theinternet-journal-of-veterinary-medicine/volume5-number1/histopathological-changes-in-lymphoid-organs-of-fish-after-exposure-to-water-polluted-with-heavy-metals.html, accessed 20th November, 2012.

Schmitt CJ, Blazer VS, Dethloff GM, Tillitt DE, Gross TS, Bryant Jr. WL, DeWeese LR, Smith SB, Goede RW, Bartish TM, Kubiak TJ. 1999. Biomonitoring of Environmental Status and Trends (BEST) Program: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants. Information and Technology Report USGS/BRD-1999-0007.U.S.Geological Survey, Biological Resources Division, Columbia, p. 68.

Singh D, Nath K, Trivedi SP, Sharma YK. 2008. Impact of copper on haematological profile of freshwater fish, *Channa punctatus*. J Environ Biol 29:253–257.

Svoboda M. 2001. Stress in fishes—a review. Bull. VURH Vodnany 4:69–191.

Tawari-Fufeyin P, Igetei J, Okoidigun ME. 2008. Changes in the catfish exposed to acute cadmium and lead poisoning. Bioscience Research Communication 20:271–276. Klobex Academic Publishers.

The Analyst. 2002. Copper toxicity, online health analysis. ⟨0:emphasis ⟩http:www.digitalnaturopath.com/cono/c5/404/html⟨/0:emphasis⟩ accessed on 24th Nov, 2012

Van Vuren JHJ. 1986. The Effects of Toxicants on the Haematology of *Labeo* (Teleostei; Cyprinidae). Comp Biochem Physiol 83C:155–159.

Vosyliene MZ.1999. The effect of Heavy Metals on Haematological Indices of Fish (Survey). *Acta Zoologica Lithuanica*. Hydrobiologia 9:76–82.