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Gross anatomical based sub-chronic toxicity testing of target heavy metals using growth morphometrics of *Coptodon zillii*

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Abstract

This gross anatomical based toxicological study is aimed to assess the survival and growth rate juvenile Coptodon zillii exposed to potential target heavy metal from crude oil spill. Based on literature review on the environmental crude oil spill burdens of Niger Delta region of Nigeria , the following target chemicals (TC) were selected for the study: Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni) and Lead (Pb). The experimental type is a sub-chronicity fish exposure testing for twenty eight (28) days duration. The experimental set up involves a twenty two pieces (22) of twenty liter (20l) tanks of four (4) replicate tanks per target chemical (TC) containing ten(10) juvenile study fish per tank in a semi-static tank testing system. TC exposure concentrations above maximum allowable toxicant concentration (MATC) for fresh water were used for the study. At the end of the experiment survival and growth rate per tank of TC concentration is assessed. Results showed that survival rates were zero percent for TC concentration of Cu1, Cu2, Ni1, Ni2 and Pb2. There reduction in the relative growth rate (RGR) of fishes from exposed to the TC, except for fish exposed to TC concentrations of Ni4 and Pb3, where increase RGR was noticed. This study has shown that gross anatomical features of environmental bio-indicators can be used to validate MATC guideline concentration for fresh water ecological status.

Keywords: Gross Anatomy; Sub-chronic; Toxicity; Heavy Metals and Fish Survival; Growth

1. Introduction

Environmental contaminants are known to induce measurable biochemical changes in exposed aquatic organisms. Likewise, stressors can load or limit physiological systems, reduce growth, impair reproduction and predispose aquatic organisms to disease and reduce their capacity to tolerate additional stressors. Thus, the response of aquatic organisms to the effects of contaminants may manifest at all levels of biological organization, in a hierarchical scale that can be at cellular, organismal, populations, communities, and ecosystems. In this way, the measuring of a suite of indicators across such levels of biological organization is often necessary to assess ecological integrity. These indicators usually include biochemical, physiological, morphological or behavioural alterations. Morphological changes involve the assessment of gross anatomical or histological parameters. Gross anatomical toxicity testing parameters can be alterations in weight, length or gross morphological distortion of the test organism. These ecological indicators can therefore be defined as measurable alterations in sentinel organism.

Traditionally, toxicity tests focus on whole organism endpoints, with survival, growth and reproduction being the most measured parameters (McCarty 1986; Meador 2006, Klüver et al., 2016). Most whole organism toxicity tests performed are short-term high-dose experiments, acute tests in which mortality is often the only endpoint. Mortality, however, is a crude parameter in response to relatively high and therefore often environmentally irrelevant toxicant concentrations. At much lower and therefore environmentally more relevant toxicant concentrations, organisms may

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suffer from a wide variety of sublethal effects. Hence, toxicity tests gain ecological realism if sublethal endpoints are addressed in addition to mortality (Hellou et al., 2008)

Sublethal endpoints need much longer exposure times to become expressed. It is usually done in chronic toxicity testing. This method of toxicity testing is used to assess the effects of toxicants on sublethal endpoints like survival rate, growth and reproduction (Van der Geest et al., 1999; Barata et al., 2008).

Growth can be measured in two ways, as an increase in length and as an increase in weight. Often only the length or weight at the end of the exposure period is determined. This, however, includes both the growth before and during exposure. It is therefore more distinctive to measure length or weight at the beginning as well as at the end of the exposure, and then subtract the individual or average initial length or weight from the final individual length or weight. Growth during the exposure period may subsequently be expressed as percentage of the initial lengths or weight. Ideally the initial length or weight is measured from the same individuals that will be exposed. When organisms are sacrificed to measure the initial length or weight, which is especially the case for dry weight, this is not feasible. In that case a subsample from the individuals is taken apart at the beginning of the test (Van der Geest et al., 1999; Barata et al., 2008).

Heavy metal contamination of aquatic ecosystem has attracted great attention of researchers over the last few decades (Farombi et al., 2007). Metal in the aquatic environment are bioaccumulated by organisms either passively from water or by facilitated uptake. Excess metal concentration in an organism must be actively excreted, compartmentalized in cells or tissues, or metabolically immobilized. Some metal escape all these actions causing toxic and other adverse effects (Chapman et al., 1996; Rand et al., 1985). Some heavy metals, Chromium (Cr), cadmium (Cd), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn), are found in the list of organic and inorganic hazardous pollutants which is prepared by the United States Environmental Protection Agency (USEPA) (Akar and Tunali, 2005). However, it is important to note that most essential metals may be toxic when present in concentrations that are in excess of the guideline levels (Agbozu et al., 2007). According to Roberts (1989 and 2001) Cu, Pb, mercury (Hg), Zn, Cr, manganese (Mn) and iron (Fe) are the most common cause of metal poisoning. Jarup (2003) documented that the main threat to human health from heavy metals are associated with exposure to Pb, Cd, Hg, and arsenic (As) (arsenic is a metalloid but is usually classified as a heavy metal).

Methods and guidelines abound for characterizing fish toxicity for risk assessment and environmental protection. Full and partial life-cycle toxicity tests are important for assessing growth and reproduction over long-term exposure, but are labour intensive and costly. Tests with early-life stage (ELS) fish have become popular for their ease of use, low cost, and ability to generate a large amount of data, especially for an ever-increasing number of compounds without basic toxicity information (Mwador, J.P., 2021).

2. Material and methods

2.1. Study Location

The fish toxicity testing was done in Wet laboratory of the Department of Aquaculture and Fisheries Management, Faculty of Agriculture, University of Benin, Nigeria. The department undertakes high level human resource development through research, training and technology transfer to ensure sustainable development of forest resources as well as the environmental and social impact on these resources.

2.2. Study Specie

- **Fish selection:** EROCIPS (Emergency Response to coastal Oil, Chemical and Inert Pollution from Shipping), (2006), "Protocol for Selection of Sentinel Species" was basically used to select the appropriate sentinel specie for this study. The resident fish species, *Coptodon zillii* because it is a widely studied fish species (Harvey et al., 1999; Pietrapiana et al., 2002; Budzinski et al., 2004; EROCIPS, 2006; Marigo´mez et al., 2006; Martı´nez-Go´mez et al., 2006; Joly-Turquin et al., 2009), and it is a resident of the Niger Delta region of Nigeria. This region is crude oil production hub of Nigeria, with the highest level of crude oil spills and other crude oil related environmental crimes.
- **Fish Biodata:** The chosen bio-indicator fish specie for this study is *Tilapia zillii*, now known as *Coptodon zillii.* The fish specie is a resident of the Niger Delta region of Nigeria. *Coptodon zillii* has a maximum length of 40cm (SL) and a maximum published weight of 300 grams with a total of 13 to 16 Dorsal spines (GISD, 2019). The non-breeding colouration of *C. zillii* is dark olive on top and light olive to yellow-brown on the sides, often with an iridescent blue sheen. Lips are bright green and the chest is pinkish. Six to seven dark vertical bars cross two horizontal stripes on the body and caudal peduncle. Fins are olivaceous, covered in yellow spots with the dorsal

and anal fins displaying an outline of a thin orange band. Caudal fin often grey with pale interstices with dots covering the entire fin. Adults display a black spot outlined in yellow. C. zillii from 2 to 14cm (SL) have an entirely yellow to grey caudal fin with no dots, developing a greyish caudal fin with dots with increasing size. Spawning coloration is shiny dark green on top and sides, red and black on the throat and belly, and obvious vertical bands on the sides. Heads turn dark blue to black with blue-green spots. Eggs are green to olive green, sticky, 1-2 mm in diameter; relatively smaller than eggs of other cichlids (FishBase, 2008; GISD, 2019).

Figure 1 Picture of *C. zillii*

- **Fish Source: T**he fish for toxicity testing was acquired from Nigeria Institution for Oceanography and Maritime Research (NIORMR) in Sapele Delta State, Nigeria. The Institute is involved in farmers' field test and incubation validated research results and technologies. The test fish was positively identified by a taxonomist from NIOMR, to be the right species for the study. The test fish used was disease-free and appear healthy, behave normally, feed well, and had low mortality in cultures, during holding, and in test control. (USEPA, 2002).
- **Test Fish Guidelines:** Juveniles of *Coptodon zillii* were chosen for the study in tandem with ISO,1994 recommendation for chronic toxicity study (ISO, 1994; CEPA, 1999). Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as juvenile is required for all tests (ISO, 1994; CEPA, 1999). Fish were approximately the same age and were gotten from the same source. Since age may affect the results of the tests, this would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility (ISO, 1994; CEPA, 1999).

2.3. Study Design

- **Study Guideline:** International Standardization Organization (ISO) test guideline standard, ISO 10229:1994 – "Chronic Toxicity Test of Fish Water quality", was used for the laboratory study (ISO, 1994). The standard specified the method for the determination of the long-term toxicity of substances (pure chemicals, mixtures, wastewater etc.). This Standard allows for the use of a semi-static method. The endpoint response was a measure of the morphological changes of test fish exposed to a test substance for a period of 14 to 28 days. The standard permits to adapt this method for use with a wide variety of freshwater, marine and brackish water fish with appropriate modifications in test conditions (temperature, food, fish marking technique).
- **Experimental Set-up:** Sub-Chronic Toxicity studies were done in Department of Aquaculture and Fisheries Management, Faculty of Agriculture, University of Benin. The experimental duration was 28 days. Twenty-two pieces (22) of twenty-liter (20L) tanks were acquired for the experiment. Four replicate tanks per Target Chemical (TC) were labeled and used for the group treated. Hence, five TC of Copper, Cadmium, Chromium, Lead and Nickel, a total of Twenty (20) tanks were set up for the experiment.

Figure 2 Experimental set-up in the Wet-laboratory of the Department of Agriculture University of Benin

- **Test Chemical/Concentration:** Target Chemicals (TC), Cadmium (Cd0, Chromium (Cr), Copper (Cu), Nickel (Ni) and Lead (Pb) were chosen because of their cardiogenic quotient and presence in crude oil. A standard stock solution of 100mg/L of the TC (Cd, Cr, Cu, Ni and Pb) was prepared from analytical grade metallic salts of Cadmium Chloride (CdCl2), Lead II Nitrate (Pb(NO3)2), Nickel II Sulphate (NiSO4.(H2O)6), Copper Sulphate (CuSO4) and Potassium Dichromate (K2Cr2O7). Stock solution was prepared with de-ionized water in I-L volumetric. The maximum acceptable toxicant concentration (MATC) for estuarine fresh water was set as the concentration of stock solutions for the laboratory testing (Gheorghe et al., 2017). Two concentrations below and above the estimated MATC were used for the testing (Gheorghe et al., 2017). Therefore, TC concentrations 1 and 2 (e.g. Cui and Cu2) are the concentrations above MATC, while TC concentrations 3 and 4 (e.g. Cu3 and Cu4).

Table 1 The results of TC doses applied for the fish toxicity study

Target Chemicals (TC)	**MATC (mg/L)**	**FED: Dose above MATC (mg/L)**		**FED: Dose below MATC (mg/L)**	
		C1	**C2**	**C3**	**C4**
Cadmium (Cd)	0.00001	0.1	0.01	0.0001	0.0001
Chromium (Cr)	0.001	10.0	1.0	0.01	0.001
Copper (Cu)	0.002	20.0	2.0	0.02	0.002
Nickel (Ni)	0.025	25.00	2.5	0.05	0.025
Lead (Pb)	0.001	10.0	1.0	0.01	0.001

Key: TC = Target Chemicals; FED = Fish Exposure Dose; C1, C2, C3 and, C4 = Dose Concentrations of Heavy metal applied per Fish Tank; MATA = CCME, 2001

2.4. Test Chamber

Twenty (20) liter chemically inert vessels (plastic Tanks) were used for the study. Each tanked was stocked with 25 fish, a stock capacity that was enough to allow for proper growth and maintenance of dissolved oxygen concentration. This is in compliance with the guideline loading rate criteria (OECD, 2013). The test chambers was randomly positioned in the test area and shielded from unwanted disturbance. The test was carried out without adjustment of pH. Nevertheless, where there is evidence of marked changed in the pH of the Tank water after addition of the test substance, the test would be repeated, adjusting the pH of the stock solution to the tank water before addition of the test substance. The PH adjustment will be made (preferably with HC1 or Na0H) in such a way that the stock solution concentration is not

changed to any significant extent and that no chemical reaction or physical precipitation of the test substance is caused (ISO, 1994; CEPA, 2004; Gheorghe et al., 2017).

2.5. Test Solution Conditions

The test solution is fresh water, which was used to simulated fresh surface water. Water temperature was maintained between 20^0C to 25^0C (ISO, 1994; CEPA, 2004). The temperature of test solutions was measured by placing a thermometer directly into the test solutions. Temperature was recorded continuously in at least one vessel during the duration of each. DO concentration was maintained at not less than 60% of the maximum air saturation value throughout the test. DO and pH was checked at the beginning of the test and daily throughout the test period. Light quality was set at ambient laboratory illumination. Photoperiod was set at a minimum of ration of 12 hours light to 12hours dark, with a light intensity maintained at 10 to 20 μE/m2/s. Feeding was at least once daily, the quantity of food being kept constant and related to the initial fish weight, at least 2% body weight (ISO, 1994; CEPA, 2004).

2.6. Validity of Test

- For the conditions of validity, ISO (1994) and OECD (2013) conditions for the validity of test were adopted for this study:
- The mortality in the controls should not exceed 10% at the end of test.
- The dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test
- In semi-static procedures, aeration can be used, provided it does not lead to a significant loss of test substance
- There should be evidence that the concentration of the substance being tested has been satisfactorily maintained (it should be at least 80% of the nominal concentration) over the test period. The results should be based on measured concentration if the deviation from the nominal concentration is greater than 20%

2.7. Gross Anatomical Evaluation: Survival Rate and Growth Rate

Deaths were recorded on a daily throughout the experiment, which was used to evaluate the Survival Rate (SR) per tank. After acclimation, the fish were batched weighed in each treatment tank to estimate their initial mean batch weight and length. At the end of the experiment, fishes from each tank were once again weighed and lengths measures as the final batch weight and length. These data was used to evaluate the Relative Growth Rates (RGR) per tank (batch). The following equation was used to estimate RGR:

WG = Weight gain = 100 x (final body weight – initial body weight) / initial body weight

SGR = Specific growth rates (% day -1) = (lnWi – lnWo/T) x 100

Where; Wi = final weight, Wo = initial weight, and T = time in days

Relative growth rate (RGR) = (W2 – W1/W1) x 100;

Where; W2 and W1 are the final weight and initial weight, respectively.

3. Results

3.1. Growth and Survival Rate

Table 2 The fish growth rates (RGR & SGR) and survival rate (FSR)

TC	FED	FS	W0 (g)	W1 (g)	RGR	SGR/DAY	FISH SURV. RATE (FSR)	
							NO. OF DEATHS	% SURV.
Control		24	4.40	6.04	27.2	1.94	0	100
Cd	Cd1	21	3.91	4.64	15.7	1.12	2	90.5
	Cd2	21	4.45	5.50	23.6	1.69	0	100
	Cd3	20	4.20	5.58	24.7	1.76	2	90
	Cd4	21	3.92	4.55	13.9	0.99	0	100
Cr	Cr1	20	4.11	6.96	41.0	2.93	4	80
	Cr2	19	3.01	3.96	24.0	1.71	3	84.2
	Cr3	24	3.91	4.72	17.2	1.23	2	91.7
	Cr4	24	4.41	6.04	27.0	1.93	2	91.7
Cu	Cu1	20	3.86	0	NA	NA	20	0
	Cu2	20	3.91	0	NA	NA	20	0
	Cu3	19	3.96	6.48	39.7	2.84	3	84.2
	Cu4	22	4.02	5.02	19.9	1.42	9	59.1
NI	Ni1	24	4.04	0	NA	NA	24	0
	Ni2	19	3.76	0	NA	NA	19	0
	Ni3	22	4.20	5.36	21.6	1.5	3	86.4
	Ni4	22	4.90	6.84	28.4	2.0	2	90.9
Pb	Pb1	21	4.41	0	NA	NA	21	0
	Pb2	21	4.50	4.96	9.3	0.6	5	76.2
	Pb3	22	4.30	6.12	29.7	2.1	2	90.9
	Pb4	19	4.91	5.58	12.0	0.9	2	89.5

Key: TC = Target Chemicals: Cd1, Cd2…Cr1, Cr2… etc = Concentrations of Heavy metal applied per Fish Tank; Conc = Concentration; Con = Control; FED – Fish Exposure Dose; FS = Fish Stock Concentration per Tank; W0 = Initial Batch Weight after acclimation, before commencement of experiment; W1 = Final batch weight after at the end of the experiment; RGR = Relative Growth Rate; SGR = Specific Growth Rate; Surv. = Survival; FSR = Fish Survival Rate

Table 3 Pared Sample T Test results of comparing Relative Growth Rate of fish from the different FED tanks of Target Chemicals

Paired Samples Test										
FED		Paired Differences					T	Df	Sig. (2-tailed)	
		Mean RGR	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference					
					Lower	Upper				
Pair 1	Con - Cd1	4.1	5.0	2.5	-3.9	12.1	1.6	3	0.203	Not Significant
Pair 2	Con - Cd2	1.8	1.8	0.9	-1.1	4.6	2.0	3	0.143	Not Significant
Pair 3	Con - Cd3	1.8	1.8	0.9	-1.1	4.6	2.0	3	0.140	Not Significant
Pair 4	Con - Cd4	4.6	5.9	3.0	-4.8	14.0	1.5	3	0.220	Not Significant
Pair 5	Con - Cr1	-2.6	7.7	3.9	-14.9	9.7	-0.7	3	0.549	Not Significant
Pair 6	Con - Cr2	2.9	1.6	0.8	0.4	5.4	3.7	3	0.034	Significant
Pair 7	Con - Cr3	3.0	4.7	2.4	-4.6	10.5	1.2	3	0.300	Not Significant
Pair 8	Con - Cr4	.05	0.1	0.1	-0.1	0.2	.9	3	0.419	Not Significant
Pair 9	Con - Cu1	9.4	12.1	6.0	-9.7	28.6	1.6	3	0.215	Not Significant
Pair 10	Con - Cu2	9.4	12.1	6.0	-9.8	28.6	1.6	3	0.216	Not Significant
Pair 11	Con - Cu3	-1.9	7.5	3.7	-13.8	10.0	-0.5	3	0.650	Not Significant
Pair 12	Con - Cu4	2.7	3.2	1.6	-2.3	7.7	1.7	3	0.188	Not Significant
Pair 13	Con - Ni1	8.4	12.8	6.4	-12.0	28.8	1.3	3	0.282	Not Significant

Pair 14	Con - Ni2	9.7	11.9	5.9	-9.2	28.6	1.6	3	0.200	Not Significant
Pair 15	Con - Ni3	2.1	2.4	1.2	-1.8	6.0	1.7	3	0.181	Not Significant
Pair 16	Con - Ni4	-0.1	1.4	0.7	-2.4	2.2	-0.2	3	0.874	Not Significant
Pair 17	Con - Pb1	9.1	12.3	6.1	-10.6	28.7	1.5	3	0.239	Not Significant
Pair 18	Con - Pb2	5.5	8.4	4.2	-7.9	18.8	1.3	3	0.283	Not Significant
Pair 19	Con - Pb3	-0.1	1.8	0.9	-3.0	2.8	-0.1	3	0.905	Not Significant
Pair 20	Con - Pb4	5.0	7.2	3.6	-6.4	16.5	1.4	3	0.256	Not Significant

Remarks: The tabled showed that there was a significant difference in the RGR between the control fish and those from Cr2 FED tanks

4. Discussion

The fish survival rate (FSR) for TC concentrations above the MATC for fresh water medium showed that: Cu (Cu1, Cu2), N (Ni1 and Ni2) and Pb (Pb1) had no fish survival (FSR = O%). The FSR for TC concentrations below MATC showed that variable survival rate Cu3 (, Cu4), N (Ni1 and Ni2) and Pb (Pb1) had no fish survival (FSR = O%)

The result showed that target heavy metals have effect on the survival rate and growth of *Coptodon zillii*. The fish survival rate (FSR) showed that the target chemicals (TCs) concentrations of Cu (Cu1 and Cu2), Ni (Ni1 and Ni2) and Pb (Pb1) had no fish survival (FSR = 0%), while the rest had had survival rates ranging from 76.2 – 100 %. It was also observed that the TC concentrations with zero survival rate were above the MATC levels for fresh water ecology, except for Pb2, which, even with the high level of concentration still had FSR of 76.2%. The zero FSR recorded were due TCs dose (mg/l) related acute toxicity response of the test fish (ATSDR, 2001). Exposure to toxicant without causing death to organisms can still cause harm (Stephan, 1977) and survival of estuarine and marine organisms in relatively low concentrations of toxicant on the first day, does not necessarily indicate that they are resistant to the toxicant pollution (Mironov,1972).

Decrease relative growth rate (RGR) were recorded for all TC concentrations except for Cu3 and Ni4 where there was recorded growth increase. Nevertheless, there was only significant difference in the RGR between the experimental and control fish in Cr2 exposed. Decreased growth was reported on *Mesidotea etemon* by Percy (1978). A similar reduction in growth was also observed by Toussain et al. (2001) and Onusiriuka (2002) when they exposed Japanese Medaka fish and *Clarias gariepinus* to sub-lethal concentrations of chloroform and formalin respectively, better growths were reported in control groups of certain fish than those exposed to toxicants as observed in this study. This might be due to the fact that they were able to utilize the feeds or that the feeds were palatable. This observation was in agreement with the reports of Omoregie and Okpanach (1995) in *Tilapia zilli*: Omoregie et al. (1998) in *Oreochromis niloticus*; Omoregie and Onuogu (2000) in *Aphyosemion gardneri*. Most of the authorities often attributed the decline in growth rates to the impairment of feeding by fish in the toxicant polluted area as observed in this study. Several workers have reported similar findings (Shanmugavel et al., 1988; Toussain, et al., 2001). This might also be due to the presence of a dominant aggressive fish that caused an increased activity for others and consequentily a reduction in their growth rates as well as an increase in their sensitivity of the pollutant. Petroleum effluents have also been reported to decrease fish growth and survival (Omoregie et al., 1997; Paraquat by Babatunde (1997). Decrease in FSR and RGR of the treated fish species may be attributed to the stress they experience while adjusting to attain a tolerance level with the toxicant.

5. Conclusion

This study was toxicologically relevant. The study was able to demonstrate that heavy metal toxicity can be simulated in a laboratory setting. It has further proven that the target chemicals concentration above the guideline levels or regulatory standards of MATC were hazardous to the bio-indicator fish. Though there is need to further investigate the pharmacokinetics behind the hazardous exposure effects caused by the concentrations of Cu and Pb that were below MATC guideline, which was inconsistent with MATC regulatory provision.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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