

Bioaccumulation level of cadmium concentration in wild population of black-headed oriole *oriolus brachyrhynchus* (swainson, 1837) from some selected locality in benue state, Nigeria

Abstract

An investigation to determine cadmium concentration in Black-headed oriole *Oriolus brachyrhynchus* was carried out using Atomic Absorption Spectrometry (AAS). The organs are feather, liver heart, skin, nestling feather, egg and the carcass. A total of 30 birds were caught using mist net and 150 of these organs were obtained, whereas 9 eggs and 9 nestling were collected from different nest in the study locations. The concentration measured in mg/kg in the organs from the various locations Buruku, Daudu and Adegga were compared using descriptive statistics. Cadmium concentration in the feather has the highest mean value of 4.26 ± 0.59 mg/kg in Daudu in the liver; 4.58 ± 0.49 mg/kg in Buruku, heart; 3.65 ± 1.13 mg/kg in Buruku and in skin; 0.41 ± 0.35 mg/kg in Buruku whereas carcass has 6.68 ± 0.63 mg/kg in Buruku. Equally, cadmium concentration in egg; 0.10 ± 0.01 mg/kg was highest in Daudu and nestling feather; 4.19 ± 0.33 mg/kg in Buruku. A very significant correlation ($r^2=0.778$) existed between feathers and carcass ($P<0.01$), $r^2=0.662$ between weight of feather and heart ($P<0.05$). Furthermore, a negative correlation ($r^2=-0.702$) existed between weight of skin and heart ($P<0.05$), and ($r^2=-0.723$) between liver and skin ($P<0.05$). Cadmium concentration in maize was highest 3.72 ± 0.49 mg/kg in Daudu and in rice; 1.53 ± 0.04 mg/kg in Buruku while 3.76 ± 1.46 mg/kg in Daudu. In soil and water cadmium concentration was highest 6.04 ± 0.19 mg/kg and 0.07 ± 0.1 mg/l in Buruku respectively. The reason for high cadmium concentrations in the species from this study is due to agricultural activities such as application of fertilizer, production of bricks, bush burning and emission from exhaust of vehicles. However this call for concern since the permissible limit for wild birds has been exceeded.

Keywords: cadmium, bioaccumulation, black headed, concentration, locality

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Abbreviations: AAS, atomic absorption spectrometry; RCBD, randomized complete block design; PPM, parts per million; WF, weight of feather; CdCF, cadmium concentration in feather; WL, weight of liver; CdCC, cadmium concentration in carcass; WC, weight of carcass; CdCL, cadmium concentration in liver; WS, weight of skin; CdCS, cadmium concentration in skin; WH, weight of heart; CdCH, cadmium concentration in heart

Introduction

Black-headed oriole basically measures 21 centimeters 8.3 in length and weighs 42 to 57g.¹ The adult upperparts are yellow-olive in colour; the head to upper breast is black and the periphery of the wings has a small white patch. It has a brownish pink beak. The under parts are yellow, the tail feathers are black and have broad yellow tips.² The juvenile has olive upperparts. The head is olive, and the throat is streaked with yellow. It has a dusky beak and black streaked breast.² The Black-headed oriole (*Oriolus brachyrhynchus*) is a species of bird in the family Oriolidae that is native to Africa. The adult upperparts are yellow-olive, and the under parts are yellow in colour. It is rated as a species of least concern on the International Union for Conservation of Nature List of Endangered Species.³ Cadmium is present in volcanic activities and forest fires which have been reported as natural sources of cadmium air emissions.⁴ Cadmium releases to the environment may result either from these natural sources, or

from anthropogenic sources⁵ such as production of cement clinker, manufacturing of bricks, production of phosphorus, nitrogen or potassium based fertilizers, waste and waste water treatment, paper and wood production processing, animal and vegetable products from the food and beverage sector. In addition, car exhaust may contribute to cadmium concentration in soils near roads. Low levels of dissolved cadmium may also be present in surface waters. Cadmium is of great concern to health of bird species and the environment due to their ability to travel long distances in the atmosphere before deposition and it is a common component of commercial fertilizers used in agriculture⁶ in Buruku, Daudu and Adegga. Cadmium waste streams from industries mainly end up in soils.⁷ Bird species are usually exposed to cadmium through ingestion of food⁸ from farmland where crops are cultivated in contaminated soils, atmospheric deposition, and water and from inhalation. When cadmium is present in soils it can be extremely dangerous, as the uptake through food will increase rapidly over time.⁹ Birds are predominantly susceptible to the effects of anthropogenic activities in the environment and they have been recognized as good indicators of environmental contamination because of their abundance, wide distribution, feeding at different tropic levels, and their long life span.¹⁰⁻¹² With global change and increasing levels of industrial, commercial and agricultural contaminants it is important to study particular species living in a sensitive habitat¹³ such as agricultural ecosystem for example Daudu, Buruku and Adegga in Benue State, Nigeria because cadmium is capable of causing kidney

damage¹⁴ in population of wild birds. Cadmium may also enter the environment mainly through the ground, because it is found in manures and pesticides. In Nigeria, studies of cadmium concentration in wild birds are limited due to poor funding of research. The main objective of the study is to determine, weather the cadmium concentration in the various parts of the birds differs with location.

Materials and methods

Study Area

The study sites were located at Buruku on Latitude 7°27'35.6"N and Longitude 9°12'20.5"E in Buruku LGA, Daudu on Latitude 7°55'53.0"N and Longitude 8°34'53.9" E in Guma LGA and Adegan on Latitude 7°01'47.4" N and Longitude 8°15'28.0"E in Obi LGA of Benue State in Figure 1. Sampling technique: Using selective sampling technique which is a non- probability sampling technique which is a non- probability sampling techniques based on personal choice without statistical bias. The study sites are not a conservation area and the local people are predominantly peasant farmers. A total of 28 hectares of farmland area was selected in each of these study sites. The 28 ha sampling plot is further divided into four sampling units of 7 ha, where bird capturing activities took place and ethical permit was obtained from Department of Forest Protection and Management, Ministry of Water Resources and Environment Benue State, Nigeria. Mist nets were set in each sampling unit and the nets were checked after every 2-3 hours to search for a catch. Three replicates of nestling feathers and eggs collected were stored in plastic sampling bottles from the tree species used as breeding ground by birds. Ten birds were trapped at the various locations, slaughtered and weighed before they were defeathered, the feathers were dried in an oven and kept in polyethene bags and sealed tightly. The defeathered birds are dissected to remove the skin, heart and liver which were kept in a polyethene bags and stored in a dessicator for cadmium analysis. Three replicates of Rice, maize and guinea corn were also collected directly from different farmland as component of biological pathway for cadmium contamination in Black-headed oriole. Three replicates of water and soil samples were equally collected from the study areas for cadmium concentration investigation as component of biological pathway.

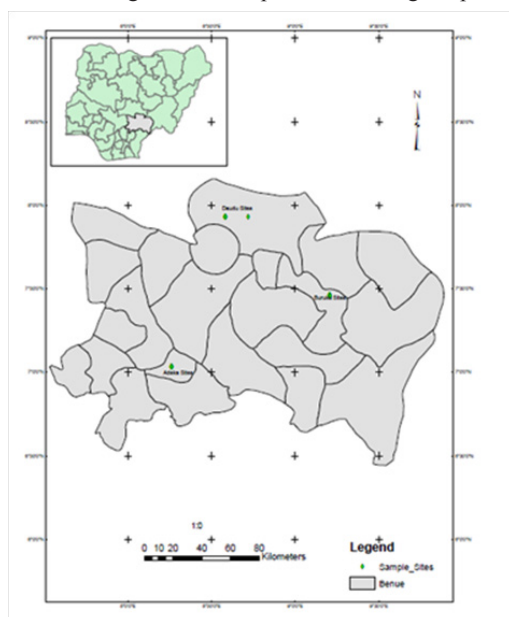


Figure 1 Map of benue state showing samples sites.

Sample cleaning

The feather samples collected were cut into about 0.3cm using a stainless steel scissors and first rinsed in ethanol, then washed three times in distilled water and then finally washed again in ethanol in accordance to the recommendation of International Atomic Energy Agency.¹⁵ These was placed in crucibles and dried in an oven at 75°C±5°C for 25 minutes. About 0.2mg of treated feather sample was weighed and stored in an inert plastic container of 10cm³ capacity, corked tightly and kept for cadmium analysis using a Flame Atomic Absorption Spectrometry. The skin, heart, liver and the carcass after the vital parts are removed were dried in an oven equipped with circulation system at 60°C for 48h, and homogenized using a porcelain mortar. Approximately 0.2mg of dry sample was treated with 7ml of concentrated nitric acid and heated for 20min in the microwave oven, as described by Edison B.¹⁶ The resultant solution was transferred into a 100ml volumetric flask and made up to volume with distil water. The solution was stored at 4°C in polyethylene bottles until cadmium was analyzed, using a Flame Atomic Absorption Spectrometry. The same procedure was followed for rice, maize, guinea corn, water and soil collected and digested for this study.

Quality control

To ensure accuracy of results, two blanks were prepared for the avian parts, cereal and water namely; A and B. Blank A comprises of 7ml of concentrated nitric acid and 2ml of hydrogen peroxide which were heated for 30 minutes and the content is allowed to cool down to a room temperature then transferred into a volumetric flask and diluted to a final volume of 100ml with distil water whereas, blank B was 100ml of distil water only. The contaminants present in blank A and B were subtracted from the contaminants present in parts of the birds, crops and water collected and digested for this study to obtained the actual concentration of contaminants present. Similarly, two blanks were also prepared for soil samples namely C and D. Blank C comprises of 7ml of freshly prepared aqua-regia (3ml HNO₃+9ml HCL) in the ratio 1:3 which was heated for 45 minutes and the content is allowed to cool down to a room temperature then transferred into a volumetric flask and diluted to a final volume of 100ml with distil water whereas, blank D was 100ml of distil water only. The contaminants present in blank C and D were subtracted from the contaminants present in all the soil samples collected and digested for this study to get the actual concentration of contaminants in soil. In addition to further ensure accuracy, the AAS machine was calibrated using a known concentration of metals. These metals were run in the AAS machine to determine the absorbance and concentration. Calibration curves were obtained by plotting concentration against absorbance.

Experimental design and data analysis

Using Randomized Complete Block Design (RCBD) the data was analyzed, where locations represented the blocks and the bird species the treatment. Pearson correlation was use to test the correlation between weight of parts and cadmium concentrations in parts (P<0.05). The Summary statistics of cadmium concentration (mg/kg) in selected parts of Black-headed orioles was express in mean values with their respective standard deviation, minimum and maximum values and results were presented in tables and bar chart.

Results

For cadmium concentration analysis, a total of 168 organs were obtained from Black headed oriole trapped from different location

in Benue State for the investigation. Table 1 shows the summary statistics of cadmium concentration in selected organs of Black-headed orioles expressed in mean values with their respective standard deviation, minimum and maximum values. Cadmium concentration in feathers of Black headed oriole ranged from 1.69-3.24mg/kg in birds trapped from Buruku with a mean of 2.43±0.55mg/kg whereas the concentration in birds trapped from Daudu ranged from 3.50-5.18mg/kg with a mean of 4.26±0.59mg/kg and birds from Adega ranged between 0.54-1.56mg/kg with a mean of 1.13±0.32mg/kg. However Daudu has the highest cadmium concentration of 4.26±0.59 mg/kg in the feathers of the bird species. The concentration in liver of the birds trapped from Buruku ranged from 3.89-5.31mg/kg with a mean 4.58±0.49mg/kg whereas cadmium concentration in liver of birds trapped from Daudu ranged from 2.73-5.00mg/kg with a mean of 3.74±0.71mg/kg but the concentration in liver of birds trapped from Adega ranged from 1.69-4.41mg/kg with a mean of 3.12±1.30mg/kg. However, birds trapped from Buruku have the highest cadmium concentration in the liver of the birds. The concentration in hearts of the birds trapped from Buruku ranged from 2.02-4.83mg/kg with a mean of 3.65±1.13mg/kg whereas cadmium concentration in heart of birds trapped from Daudu ranged from 1.89-4.61mg/kg with a mean of 3.31±1.27mg/kg but the concentration in heart of birds trapped from Adega ranged from 1.43-2.98mg/kg with a mean of 2.02±0.69mg/kg. Therefore, Buruku has the highest cadmium concentration in the heart of the birds. For the skin, the concentration in the birds from Buruku ranged from 0.13-1.17mg/kg with a mean of 0.41±0.35mg/kg whereas the ones trapped from Daudu ranged from 0.11-1.00mg/

kg with a mean of 0.30±0.31mg/kg but cadmium concentration in skin of birds from Adega range from 0.11-0.20mg/kg. However, Buruku has the highest cadmium concentration of 0.41±0.35mg/kg in the skin of the birds (Table 1). Cadmium concentration in carcass of Black headed oriole from Buruku after the vital organs were removed ranged from 5.83-7.51mg/kg with a mean of 6.68±0.63mg/kg whereas cadmium concentration in the carcass of birds trapped from Daudu ranged from 4.78-6.48mg/kg with a mean of 5.61±0.63mg/kg but the concentration in carcass of birds from Adega ranged from 1.76-4.15mg/kg with a mean of 2.63±0.72mg/kg. Buruku has the highest cadmium concentration in the carcass of the studied species. Cadmium concentration in eggs of Black headed oriole collected from Buruku ranged from 0.05-0.11mg/kg with a mean 0.07±0.32mg/kg whereas the concentration in eggs of birds collected from Daudu ranged between 0.09-0.11mg/kg with a mean of 0.10±0.01mg/kg but the concentration in eggs of birds collected from Adega ranged from 0.01-0.09mg/kg. Daudu has the highest cadmium concentration in the eggs of the birds (Table 1). Cadmium concentration in nestling feathers of Black headed oriole from Buruku ranged from 3.89-4.54mg/kg with a mean 4.19±0.33mg/kg whereas cadmium concentration in nestling feather of birds trapped from Daudu ranged from 3.50-4.79mg/kg with a mean of 3.97±0.71mg/kg but cadmium concentration in the nestling feather of birds trapped from Adega ranged from 0.54-1.17mg/kg with a mean of 0.85±0.32mg/kg. However, Buruku has the highest cadmium concentration in the nestling feathers of bird species (Table 1).

Table 1 Summary statistics of cadmium concentration (mg/kg) in selected organs of Black-headed orioles trapped from Benue State, Nigeria

| Location and Tissues of Black-Headed Orioles | N | Mean±SD | Minimum | Maximum |
|--|----|-----------|---------|---------|
| Buruku | | | | |
| Feathers | 10 | 2.43±0.55 | 1.69 | 3.24 |
| Liver | 10 | 4.58±0.49 | 3.89 | 5.31 |
| Heart | 10 | 3.65±1.13 | 2.02 | 4.83 |
| Skin | 10 | 0.41±0.35 | 0.13 | 1.17 |
| Carcass | 10 | 6.68±0.63 | 5.83 | 7.51 |
| Eggs | 3 | 0.07±0.32 | 0.05 | 0.11 |
| Nestling Feathers | 3 | 4.19±0.33 | 3.89 | 4.54 |
| Daudu | | | | |
| Feathers | 10 | 4.26±0.59 | 3.5 | 5.18 |
| Liver | 10 | 3.74±0.71 | 2.73 | 5 |
| Heart | 10 | 3.31±1.27 | 1.89 | 4.61 |
| Skin | 10 | 0.30±0.31 | 0.11 | 1 |
| Carcass | 10 | 5.61±0.63 | 4.78 | 6.48 |
| Eggs | 3 | 0.10±0.01 | 0.09 | 0.11 |
| Nestling Feathers | 3 | 3.97±0.71 | 3.5 | 4.79 |
| Adega | | | | |
| Feathers | 10 | 1.13±0.32 | 0.54 | 1.56 |
| Liver | 10 | 3.12±1.30 | 1.69 | 4.41 |
| Heart | 10 | 2.02±0.69 | 1.43 | 2.98 |
| Skin | 10 | 0.15±0.05 | 0.11 | 0.2 |
| Carcass | 10 | 2.63±0.72 | 1.76 | 4.15 |
| Eggs | 3 | 0.04±0.04 | 0.01 | 0.09 |
| Nestling Feathers | 3 | 0.85±0.32 | 0.54 | 1.17 |

Metal levels are in parts per million (PPM) and are presented as wet weight for eggs and dry weight for other organs. However, Table 2 shows very high positive significant correlation ($r^2=0.778$) between cadmium concentration in the feathers and in the carcass ($P<0.01$) whereas a significant positive correlation ($r^2=0.662$) also existed between weight of feather and weight of heart ($P<0.05$). There was

also a similar correlation although negative ($r^2=-0.702$) between weight of skin and weight of heart ($P<0.05$). Another negative correlation ($r^2=-0.723$) also existed between cadmium concentration in the liver and cadmium concentration in the skin ($P<0.05$) (Table 2).

** = ($P<0.01$), * = ($P<0.05$)

Table 2 Correlation between wet weight of organs and cadmium concentration in organs

| | WF | WC | WL | WS | WH | CdCF | CdCC | CdCL | CdCS | CdCH |
|------|--------|--------|--------|---------|--------|---------|--------|---------|--------|------|
| WF | 1 | | | | | | | | | |
| WC | 0.54 | 1 | | | | | | | | |
| WL | -0.272 | -0.104 | 1 | | | | | | | |
| WS | -0.151 | -0.348 | -0.593 | 1 | | | | | | |
| WH | 0.662* | 0.438 | 0.201 | -0.702* | 1 | | | | | |
| CdCF | -0.196 | -0.154 | -0.17 | -0.159 | 0.188 | 1 | | | | |
| CdCC | -0.503 | -0.456 | -0.111 | 0.055 | -0.281 | 0.778** | 1 | | | |
| CdCL | 0.148 | 0.321 | 0.176 | -0.001 | 0.056 | 0.605 | -0.420 | 1 | | |
| CdCS | -0.224 | -0.566 | 0.066 | -0.15 | 0.087 | 0.673 | 0.526 | -0.723* | 1 | |
| CdCH | -0.147 | 0.258 | 0.195 | -0.21 | 0.531 | -0.046 | -0.32 | 0.649 | -0.164 | 1 |

From the analysis, a total of 27 crop samples were collected from Buruku, Daudu and Adegá for cadmium concentration investigation (Table 3). The concentration in maize harvested from farmland in Buruku ranged from 1.69-2.33mg/kg with a mean 1.90 ± 0.37 mg/kg whereas cadmium concentration in maize harvested from Daudu ranged from 3.37-4.28mg/kg with a mean of 3.72 ± 0.49 mg/kg, but cadmium concentration in maize harvested from Adegá ranged from 0.38-0.82 with a mean of 0.55 ± 0.24 mg/kg. However, maize collected from Daudu had the highest cadmium concentration in the maize harvested from the studied areas. The concentration in rice harvested from farmland in Buruku ranged from 1.49-1.56mg/kg with a mean 1.53 ± 0.04 mg/kg whereas cadmium concentration in

rice harvested from Daudu ranged from 0.65-1.82mg/kg with a mean of 1.34 ± 0.61 mg/kg, but the concentration in rice harvested from Adegá ranged from 0.53- 0.96mg/kg with a mean of 0.70 ± 0.23 mg/kg. Therefore, rice harvested from Buruku had highest cadmium concentration from the studied areas. The concentration in guinea corn harvested from farmland in Buruku ranged from 1.55-1.98mg/kg with a mean 1.77 ± 0.22 mg/kg whereas cadmium concentration in guinea corn harvested from Daudu ranged from 2.07-4.66mg/kg with a mean of 3.76 ± 1.46 mg/kg, making it the highest cadmium concentration in the guinea corn harvested from the study areas but cadmium concentration in guinea corn harvested from Adegá ranged from 1.07-1.59mg/kg with a mean of 1.31 ± 0.26 mg/kg (Table 3).

Table 3 Summary statistics of cadmium concentration (mg/kg) in crops foraged on frequently by black-headed orioles from the study locations

| Locations | N | Mean±SD | Minimum | Maximum |
|------------------------------------|---|---------------|---------|---------|
| Buruku | | | | |
| Maize <i>Zea mays</i> | 3 | 1.90 ± 0.37 | 1.69 | 2.33 |
| Rice <i>Oryza sativa</i> | 3 | 1.53 ± 0.04 | 1.49 | 1.56 |
| Guinea Corn <i>Sorghum bicolor</i> | 3 | 1.77 ± 0.22 | 1.55 | 1.98 |
| Daudu | | | | |
| Maize <i>Zea mays</i> | 3 | 3.72 ± 0.49 | 3.37 | 4.28 |
| Rice <i>Oryza sativa</i> | 3 | 1.34 ± 0.61 | 0.65 | 1.82 |
| Guinea Corn <i>Sorghum Bicolor</i> | 3 | 3.76 ± 1.46 | 2.07 | 4.66 |
| Adegá | | | | |
| Maize <i>Zea mays</i> | 3 | 0.55 ± 0.24 | 0.38 | |
| Rice <i>Oryza sativa</i> | 3 | 0.70 ± 0.23 | 0.53 | 0.96 |
| Guinea Corn <i>Sorghum bicolor</i> | 3 | 1.31 ± 0.26 | 1.07 | 1.59 |

A total of nine samples of soil and water were collected from different location for cadmium concentration investigation (Figure 2 & 3). Soil samples from Buruku ranged from 5.83-6.22mg/kg whereas Daudu has cadmium concentration that ranged from 5.04-6.08mg/kg and samples from Adegá ranged from 1.83-3.42mg/kg (Figure 2). However, soil sample from Buruku had the highest cadmium

concentration of 28.32mg/kg in soil. Water samples from Buruku range from 0.06-0.08mg/l, whereas cadmium concentration in water samples collected from Daudu ranged from 0.06-0.07mg/l. Water samples from Adegá ranged from 0.04-0.04mg/l (Figure 3). Therefore water samples from Buruku had the highest cadmium concentration of 0.08mg/l.

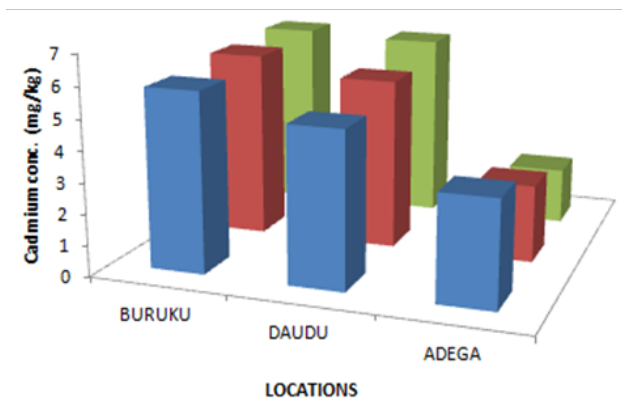


Figure 2 Cadmium concentration in soil samples.

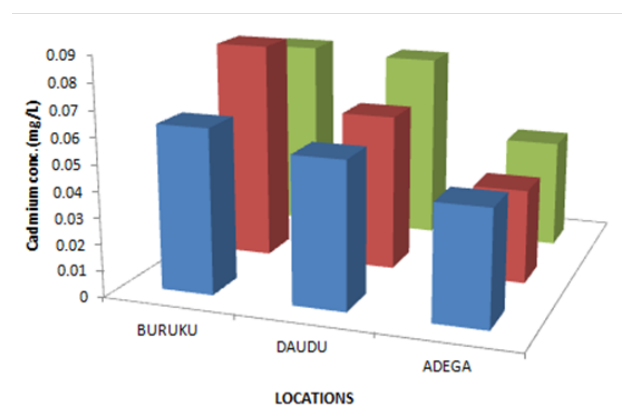


Figure 3 Cadmium concentration in water sample.

Discussion

Concentration of contaminants in the said species varies and this variation can be attributed to environmental factors, bioaccumulation and biomagnifications of contaminants in different organs. This present research work is at variance with report of¹⁷ who recorded a lower cadmium concentration that ranged from 0.81-2.96mg/kg in feathers of white stock in Poland. Similarly, this work is also at variance with the report of¹⁸ who reported a lower cadmium concentration that ranged from 0.02-1.5mg/kg in droppings of Cattle Egret in India. Cadmium concentration recorded in the present study was much lower than those found in droppings of Vulture which ranged from 13.93±1.18mg/kg as reported Bravo¹⁹ in Venezuela. This may be due to the differences in the feeding behaviour and diet feed on by both birds. While Black-headed feeds mainly on grains, vultures are scavengers, therefore gradual accumulation of this metal from scavenging may be responsible. Our research work is also at variance with the work of²⁰ who reported a higher cadmium concentration of 19±4mg/kg, 14±3mg/kg and 48±9mg/kg in feathers of shorebirds (Red knot, Sanderling and Semipalmated sandpiper respectively) migrating through Delaware Bay New Jersey in USA. Equally, cadmium concentration in eggs of Black headed orioles in this present research work is at variance with the research work of²¹ who also reported a higher cadmium concentration of 0.82±0.25mg/kg and 5.21±0.17mg/kg in yolk and shell of Black headed gull in Poland. Lower values of cadmium concentration of 0.694mg/kg and 0.915mg/kg were also reported in juvenile and adult birds respectively²² in Lesser Scaups wintering in Indiana Harbour canals in USA and also the work of²³ who reported cadmium concentration of

1.274mg/kg and 0.469mg/kg in liver and breast muscles of mallards and pheasants respectively from Slask in Poland. Metal accumulation in birds is a function of environmental pollution, so that a careful managed environment with strict adhesion to environmental laws will produce low metal concentration. This current work recorded a higher cadmium concentration compared to the report of²⁴ who recorded cadmium concentration of 1.09mg/kg in food crops in some selected farmland in Benue State. From our research findings, cadmium concentration of 2.0mg/kg²⁵ as recommended by World Health Organization in crop plants has been exceeded. Therefore, cadmium concentrations from the study areas do not conform to the acceptable limits of 2.0mg/kg basically recommended for food crops²⁴ as established by regulatory bodies such as WHO and FAO. This study is in agreement with the report of²⁶ who emphasized on the implication of excessive application of fertilizers, pesticides and herbicides on crops which may equally lead to buildup of left over chemical in crop plants. However, phosphate fertilizers contain cadmium and this can easily accumulate in crops in form of residue especially in this 21st century in West Africa where farmers are over depending on chemicals both in urban and rural areas. In addition, sewage sludge and contaminated fertilizers are usually considered as important sources of cadmium contamination.²⁷ Cadmium is considered to be a pollutant in phosphate fertilizers^{4,28} which is added to soil and food crops through normal farming practice.²⁹ Therefore, birds that basically forage on these crops are bound to accumulate the residue in their organs. However, this present work recoded lower cadmium concentration in water compared to the report of²⁵ who recorded a cadmium concentration that ranged from 0.004-0.129mg/l in water samples collected from Pakistan. The maximum permissible limit for Cadmium in water is 0.01mg/l as recommended by World Health Organization²⁹ and the permissible limit for soil is 0.7mg/kg.³⁰ These permissible limits have been exceeded from all indication. Therefore, water and soil can easily be considered as a potential threat to birds of these localities if the biological pathway is put into consideration.

Conclusion

The reason for high cadmium concentrations in the species from this study is due to agricultural activities such as application of fertilizer, production of bricks, bush burning and emission from exhaust of vehicles. However this call for concern since the permissible limit for wild birds is exceeded. The possible causes of cadmium concentrations in water and crops may be attributed to increase in the use of chemical fertilizers on farmland.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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