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Microbial and Heavy Metal Analysis of Irrigation Water and Vegetables Grown and Consumed in Abakaliki Metropolis

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In this research, the Heavy metal and Microbial content of vegetables and its irrigation water in Abakaliki were studied. The samples were collected from Onuebonyi Inyimagu village in Izzi Local Government Area (LGA) and Iyiudele farm located at Abakaliki LGA. The samples were analyzed for heavy metals (lead, cadmium chromium arsenic and mercury,) using Atomic absorption spectrophotometer (AAS). The microbial count was done using digital colony counting machine while the microbial cells were further identified to species level using 16S rDNA and ITS rDNA sequencing analysis for bacterial and fungal cells respectively at CABI identification services UK. The results of the heavy metal analysis showed that cadmium was within tolerable limit for food while lead, arsenic, chromium and mercury where above maximum acceptable limit according to relevant national and international food and water regulatory agencies. The high levels of these heavy metals on the vegetables portents significant health risk for the consumers of these vegetables as high levels of these heavy metals in human body have been linked to so many cardiovascular and other organ dysfunctions. The result of the microbial analysis also showed that the microbial load of both the irrigation water and the vegetables where above acceptable limit according to international Commission for microbiological specification of food (ICMSF) and other necessary food and water quality regulatory agencies. The contaminating organisms were identified as *Escherichia coli* and *Escherichia fergusonii*) (504743) and *Aspergillus tamari* (504744a) according to the result of 16S rDNA and ITS rDNA sequencing analysis. Thus, the findings of this study showed that there is an urgent need for government to take steps to provide clean water for irrigation purposes in Abakaliki metropolis. This will go a long way in ensuring that vegetables produced in Abakaliki are free from potentially dangerous microbes and heavy metals and are generally of better quality.

Keywords: Heavy metals, microbial load, AAS, Sequencing, ITS rDNA, 16S rDNA, *Escherichia coli, Aspergillus tamari.*

Vegetables are rich sources of vitamins, minerals, fibre and also have beneficial anti-oxidative effects. Farmers use shallow well water for irrigation as is often the cheapest and most accessible water from farm[1, 2]. Irrigation practices require a large volume of water as 80-95% of the mass of plants is composed of moisture[3]. Most stream/well waters Contain essential plant nutrients such as N, P, K, Ca, Mg, and Fe as well as varieties of other inorganic substances from domestic and industrial sources, including potential toxic elements and heavy metals such as Pb, Cd, Ni, Cr, and Hg. These metals play important roles

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in the metabolic pathways during the growth and development of plant when available in required concentrations[4].

Vegetables absorb various kinds of heavy metals when available in irrigation water; however intake of heavy metal contaminated vegetables may pose risks to human health. This is because heavy metals have the ability to accumulate in the leaving organisms (bioaccumulation) and at elevated level can be toxic[1]. For instance when lead exceeds its safe value concentration, it causes hepatic and kidney damages, haemolytic Anaemia and methanoglobinemia. The acceptable limit for human consumption of lead for all food in solid form is 6ppm while for foods in liquid form is 1ppm[5, 6]. Cadmium exacts its effects on human health when present at higher concentration and causes severe diseases such as tubular growth, excessive salivation, gastrointestinal irritation, cancer, kidney damage, diarrhea and vomiting[7, 8].

Vegetables accumulate heavy metals in their edible and non- edible parts, leafy part of vegetables accumulate higher amounts of heavy metals than their fruits[4, 9]. Knowledge of metal-plant interactions is important for the safety of the environment and for reducing the risk associated with the introduction of trace metals into the food chain. Consequently, the metal can inactivate many important enzymes resulting in inhabitation of photosynthesis respiratory rate and other metabolic process in plant[10]. Heavy metal contamination of fresh product is a problem as cases of food borne illness are increasing daily[11]. Vegetables have great nutritional values but they also harbor microbes due to untreated irrigation or sewage fertilizer and poor handling after harvest.

Food borne pathogens such as *Listeria monocytogenus*, *Salamonella spp, shigella spp*, *Campylobalter Spp, Campylobalter spp*, *staphylococcus auerus. Clotirdium butilinum, E. coli* and *Aspergillus* spp have been the main causes of illnesses during the past decade and have all been isolated from vegetables irrigated with water contaminated with domestic and industrial wastes[12, 13]. Consumption of vegetables contaminated with Heavy metals and pathogenic microbes portent a great health risk to both human and animals and calls for urgent attention. Thus, the purpose of this study is to determine the levels of microbial and heavy metals contaminations of vegetables cultivated in Abakaliki metropolis Ebonyi State, Nigeria.

MATERIALS AND METHODS

Samples

The samples used for this study were Irrigation water (shallow well water and pond water), pumpkin (vegetable 1 and vegetable 2 Irrigated with shallow well water and pond water respectively).

Source of Samples

Shallow well water and Vegetable I were collected from Iyi Udele farm at Abakaliki Ebonyi State. Pond water and vegetable 2 were collected at Onuebonyi Inyinmagu village, Abakaliki, Ebonyi State. Samples were collected for two months (December, 2016 and January 2017)

Heavy Metal Analysis

Methods for the Heavy Metal Analysis

Heavy metal analysis was conducted using Varian AA240 Atomic Absorption spectrophotometer according to the method of APHA 1995 (American Public Health Association). The analysis was done at Spring Laboratory Services, Awka, Anambra State, Nigeria.

Preparation of Reference Solution and the Heavy Metal Determination

A series of standard metal solutions in the optimum concentration ranges were prepared, the reference solutions were prepared by diluting the single stock element solutions with distilled water containing 1.5 ml concentrated nitric acid/ litre. A calibration blank was prepared using all the reagents except for the metal stock solutions. The samples were introduced into the AAS machine and the quantity of each heavy metal was determined based on the working principle of Atomic absorption spectrophotometer. Atomic absorption spectrophotometer's working principle is based on the sample being aspirated into the flame and atomized when the AAS's light beam is directed through the flame into the monochromator; and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Each metal has its own characteristic absorption wavelength and a source lamp composed of that element was used, making the method relatively free from spectral or radiation interferences. The amount of energy of the characteristic

wavelength absorbed in the flame is proportional to the concentration of the element in the sample. Calibration curve for each metal was prepared by plotting the absorbance of standards versus their concentrations. The Curves were printed from the AAS Machine.

Total Viable Bacterial and Fungal Counts

Total viable bacterial and fungal count Ten- fold serial dilution was used for both fungal and bacterial count. All media used (Nutrient agar and Saboraud Dextrose Agar) were prepared according to manufacturer's instruction (BIOTECH India) and autoclave for 15 minutes at 121°C and 15 psi. The prepared media were allowed to cool to about 40°C in a water bath and were then poured into sterile petri- dishes containing 1 ml aliquot of the appropriate dilutions (peptone water as diluents) prepared from the samples. The sample solutions were prepared by adding 1 g and 1ml of the samples into 10 ml of peptone water for the vegetable and irrigation water respectively. The plates were incubated for 3 days at room temperature and colonies formed were counted and expressed in colony forming unit per gram CFU/g/ml.

Bacterial and Fungal Identification

The Bacterial and fungal colonies were subcultured on Nutrient and Sabouraud Dextrose Agar (SDA) and sent to CABI for molecular identification.

Methods for Molecular Identification of Isolates

Fungal samples were processed using ITS rDNA sequencing analysis using the FASTA algorithm with the Fungus database from EBI. Bacteria samples were processed using partial 16S rDNA sequencing analysis. All procedures were validated and processing undertaken in accordance with CABI's in-house methods as documented in TPs 61-68 and TP70 for bacteria and TPs 72-80 for filamentous fungi. All original samples were subjected to a purity check. Molecular assays were carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA in vitro. The quality of the PCR product was assessed by undertaking gel electrophoresis. Polymerase Chain Reaction PCR purification step was carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1). DyeEx™ 2.0 (Qiagen, UK) modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionised formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available in European Molecular Biology Laboratory (EMBL) via the European Bioinformatics Institute (EBI).

RESULTS AND DISCUSSION

LEAD

Lead is a widely distributed heavy metal. Its significant source of exposure is often from diet and water consumption[14, 15]. The findings of this study showed that both the irrigation water and the vegetables irrigated with the water were contaminated by lead (Tables 1 & 2).The contamination of the irrigation water by lead could be as a result of industrial and domestic sewage that are channeled from most household and industrial site within the metropolis to these streams where the shallow wells are dogged and used for irrigation of this vegetables in Abakaliki metropolis. The vegetables might have absorbed this metal from the soil as well as the irrigation water. The levels

of lead contamination in the shallow well water and its vegetables are beyond international acceptable limits. The standard for irrigation water approved by National Environmental quality standards (NEQs) for lead is 0.5 mgL^{-1} , while maximum acceptable limit for lead in food according to FSANZ, is 0.1mg/kg[16, 17]. According to European Food Safety Authority, the maximum acceptable limit of lead in vegetables is 0.1mg/kg[14]. Lead exposure through contaminated food and water has been established to directly link with the risk of developmental neurotoxicity in young children, cardiovascular effects and nephrotoxicity in adults. According to Food Safety Authority of Ireland, Short-term exposure to high levels of lead can cause brain damage, paralysis (lead palsy), anaemia and gastrointestinal symptoms. Longer-term exposure can cause damage to the kidneys, reproductive and immune systems in addition to effects on the nervous system[17]. The levels of the lead contamination were significantly higher in the second month when compared with the first month of this study. The increase in the lead concentration in the second month could be as a result of increased evaporation which often characterized the month of January in Nigeria thus resulting in further concentrating the heavy metal content of the irrigation water and consequently the vegetables. This high level of lead in these vegetables represent a significant health risk as some of these are often consume raw.

Arsensic

The result of this study showed that the irrigation water and the vegetables contain intolerable levels of arsenic (Table 1 & 2). Arsenic can exist in both organic and inorganic form and have been established to enter the food chain through contaminated water and soil. This implies that both irrigation water and the soil in which vegetables are cultivated in Abakaliki metropolis must have been contaminated with the arsenic through domestic and industrial wastes that are usually dump at the irrigation site within the metropolis. The maximum tolerable limit of arsenic in food is 0.002mg/kg[9, 7]. Arsenic especially in its inorganic form has been classified as human carcinogen according to International Agency for Research into Cancer (IARC)[15, 17, 18]. Its exposure has also been associated with skin, vascular and nervous system disorders. The significant increase

Table 1. Heavy Metal Analysis for Month 1 (December)

Heavy metals (ppm)	SW	V1	PW	V2
Lead	7.135± 0.002^a	9.231± 0.002^b	6.469± 0.011^c	6.613± 0.001^d
Arsenic	2.437± 0.001^a	3.651± 0.001^b	2.132± 0.002^c	2.801± 0.003^d
Chromium	0.061± 0.01^a	0.088± 0.01^b	0.009± 0.03^c	0.012± 0.02^d
Cadmium	0.152± 0.005^a	0.161± 0.004^b	0.138± 0.005^c	0.162± 0.006^d
Mercury	9.402± 0.001^a	12.314± 0.001^b	0.000± 0.002^c	0.000± 0.002^d

Values are means of triplicate determinations and standard deviation (±SD). Means with different superscript along the row are significantly different ($p<0.05$)
SW = Shallow well water, V1 = Vegetable 1, PW = Pond Water, V2 = Vegetable 2

Table 2. Heavy Metal Analysis for Month 2 (January)

Heavy metals (ppm)	SW	V1	PW	V 2
Lead	6.781± 0.001^a	10.124± 0.005^a	6.724± 0.007^a	7.027± 0.005^a
Arsenic	4.174± 0.002^a	4.921± 0.001^a	2.271± 0.01^a	2.213± 0.03^a
Chromium	0.087± 0.05^a	0.098± 0.01^a	0.013± 0.01^a	0.026± 0.05^a
Cadmium	0.168± 0.03^a	0.187± 0.02^a	0.153± 0.01^a	0.192± 0.01^a
Mercury	11.507± 0.001^a	13.478± 0.005^a	0.000± 0.005^a	0.000± 0.01^a

Values are means of triplicate determinations and standard deviation (±SD). Means with different superscript along the row are significantly different ($p<0.05$)
SW = Shallow well water, V1 = Vegetable 1, PW = Pond Water, V2 = Vegetable 2

Table 3. Microbial Analysis for month 1(December)

Microbial count	SW	V1	PW	V2
Bacterial count	2.5 x± 0.05 x 10^{7a}	1.8 x± 0.01 x 10^{6b}	3.5 x± 0.04 x 10^{4c}	1.7 x± 0.05 x 10^{6d}
Fungi count	7.0 x± 0.02 x 10^{3a}	3.6 x± 0.02 x 10^{4b}	1.2 x± 0.05 x 10^{4c}	3.2 x± 0.01 x 10^{4d}

Values are means of triplicate determinations and standard deviation (±SD). Means with different superscript along the row are significantly different ($p<0.05$)
SW = Shallow well water, V1 = Vegetable 1, PW = Pond Water, V2 = Vegetable 2

Table 4. Microbial Analysis for month 2 (January)

Microbial count	SW	V1	PW	V2
Bacterial count	8.8 x± 0.06 x 10^{8a}	6.2 x± 0.06 x 10^{8b}	6.9 x± 0.05x 10^{7c}	6.0 x± 0.01 x 10^{7d}
Fungi count	4.2 x± 0.10 x 10^{5a}	1.8 x± 0.07 x 10^{4b}	2.1 x± 0.05 x 10^{5c}	1.3 x± 0.04 x 10^{5d}

Values are mean of triplicate determinations and standard deviation (±SD). Means with different superscript along the row are significantly different ($p<0.05$)
SW = Shallow well water, V1 = Vegetable 1, PW = Pond Water, V2 = Vegetable 2

in the arsenic concentrations in the second month showed other Environmental Factors could trigger even further increase in the future and thus need to be urgently addressed and continuously monitored.

Chromium

The samples were also found to be contaminated with chromium (Tables 1 & 2). However the concentrations of the Cr metal on the irrigation water and the vegetables were within tolerable limit. The maximum acceptable limit of chromium in vegetables is 1ppm[5, 19], while the result of this study showed that all the samples analyzed were below 1ppm. The standard for irrigation water and approved by NEQS (National Environmental Quality standards) for chromium is 1.0Ng/mil^{-1}. The maximum permissible limit of chromium for plant is 1.30mg/kg recommended by WHO (World Health Organization)[20, 21]. This finding is similar the finding of[15,22]) in which the disclosed concentration of chromium in some of the water samples studied were within tolerable limit but need to be continuously monitored.

Cadmium

The finding of this research revealed that the concentrations of Cadmium were slightly higher than the maximum acceptable limits of Cadmium in foods (0.1mg/kg) ([18, 23]. The standard for irrigation water approved by NEQS (National environmental quality standards for cadmium is 0.10 $Ngml^{-1}$. While according to European Union regulation, the maximum acceptable limit of Cadmium in leafy vegetables is 0.02mg/kg[9]. This result is similar to the work of other researchers in which they revealed that high concentrations (above acceptable international standards) of Cd content in untreated waste water increased their concentration in the applied soils[4, 8]. The natural occurrence of Cd has been established to be from industrial and agricultural sources of contamination and the absorption of Cd in human being is not important according to European Parliament and Council Regulation[24]. Many organ dysfunctions have been linked to Cd in humans such as renal dysfunctions and bone demineralization. The International Agency for Research on Cancer has also classified Cd as a group 1-human carcinogen[6, 14]. This goes to show that consumers of these vegetables are expose to high health risk and are bound to have many organ failures in the future if something is not done urgently to address this potentially health/environmental disaster.

Mercury

The level of mercury contamination of the irrigation water from the shallow well and its vegetables were above tolerable limits. While the irrigation water from pond water and its vegetable were found to contain no mercury (Tables 1 & 2). It has been established that environmental

contaminations of mercury can both be from natural sources and from anthropogenic emissions such as industrial activities and mining. According to European Food Safety Association (EFSA) and US food and Drug Administration (FDA) the minimum acceptable limit of mercury in food is 0.5mg/kg and 1µg/l for water[5, 17]. The findings of this study showed that the shallow well water and the vegetable irrigated with the water have high mercury contaminations and is of serious concern considering that this vegetable are consumed in high volume within the metropolis. Excessive exposure to mercury through contaminated foods and water has been associated with a wide spectrum of adverse health effects including damage to the central nervous system (neurotoxicity) and the kidney[2, 16]. There was also significant increase in the concentrations of the mercury in the vegetable in the second month when compared with the first month. This further showed that there is an urgent need not only to continuously monitor the extent of heavy metal contaminations of the irrigation water but also for Government to take urgent steps to stop the use of such water for Irrigation purposes in Abakaliki metropolis.

Result of the Total Viable Count/Molecular Identification for the Fungi and Bacteria Isolates

The result of this research showed that both the irrigation water and the vegetables were heavily contaminated by microorganisms (Tables 3 & 4). The levels of the microbial contaminations of the vegetables were beyond maximum acceptable limits according to international commission on microbiological specification of food[25]. According to this Agency the maximum acceptable limit of bacteria count in food products is 10^3 cfu/ml. In this study, the numbers of the microbial count were above this limit and thus represent potential hazard as these vegetables are often consumed raw. Microorganisms present in fruits and vegetables are directly related to the water used in irrigating it and the hygienic practices during their cultivation, harvesting, post harvest handling, processing and distribution of the product[26]. This agrees with findings of this study as the high microbial load on the vegetables could be from both the irrigation waters and the soil.

The result of the molecular identification of the most dominant bacteria contaminant in the samples further confirm the potential danger associated with these vegetables as the bacteria isolate shows top matches (97 -98%) to the species *Escherichia coli* and *Escherichia fergusonii.* Thus, the isolates were identified as *Escherichia coli* and *Escherichia fergusonii* (CABI Identification services UK). *Escherichia coli* and *Escherichia fergusonii* belong to the family *Enterobacteriaceae* and have been established to be distributed worldwide and can be found in soil, water, plants, food products, animals and man. Some members of this species (including *E. coli*) are pathogenic to man and have been categorized as Hazard group 2 or 3 organisms by Advisory Committee on Dangerous Pathogens (ACDP) (UK). This correlates already established scientific facts that a wide range of microbial pathogens have been found in water and can be transferred to crops during irrigation. *E. coli, Salmonella spp. and Vibrio spp*. were recovered from irrigation stream water in Nigeria and these organisms were also present on the irrigated plants[25, 27]. The sources of this microbial contamination could be from domestic and industrial sewage that are often channeled to the irrigation water. Dumping/channeling of domestic and industrial wastes to irrigation site has been observed to be common practices among residents in Abakaliki metropolis. Lack of fresh water for irrigation has forced growers to utilize any type of available water, including wastewater, from domestic and industrial sewage[1, 13]. The sequence obtained from the fungi isolated from the vegetable samples showed top matches at 100 % identity to multiple sequences from strains of *Aspergillus tamari* including sequence HQ340111 from reference culture collection strain NRRL 427. This is a common species especially in the tropics, with brown to olive colonies and large thick-welled roughened conidia. It is frequently isolated from soil, seeds and other plant-based substrata and is known to produce the mycotoxin cyclopiazonic acid. It is assigned by ACDP (UK) to hazard group 2 thus substrate containing this organism is designated as potentially dangerous and should be discarded (ACDP, UK).

CONCLUSION

The findings of this research revealed that irrigated vegetables grown in Abakaliki Metropolis contain high concentrations of potentially toxic

heavy metals. The microbial content of the vegetables were also above safe limits and the nature of the isolated microorganisms are potentially dangerous. Therefore, there is an urgent need for an action plan that will ensure these waters are not used for irrigation purposes while better quality water are provided to farmers so that vegetables grown in Abakaliki are irrigated with water of better quality.

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