



UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM

FACULTAD DE CIENCIAS DE LA VIDA

CARRERA DE INGENIERÍA EN BIOTECNOLOGÍA

**IMPACT OF MINING ACTIVITIES ON FOOD SAFETY:
BIOACCUMULATION OF METALS AND GENOTOXICITY
ASSESSMENT IN *Oreochromis niloticus* (TILAPIAS) FROM
NAPO, AMAZON-ECUADOR.**

Proyecto de Investigación previo a la obtención del Título de:
INGENIERA EN BIOTECNOLOGÍA

AUTORA

SAMANTHA DAYANARA VASCO VITERI

Napo – Ecuador

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Napo – Ecuador

2022

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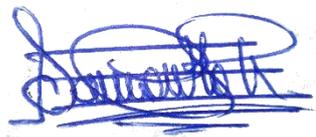
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AGRADECIMIENTO

A la Universidad Regional Amazónica Ikiam que me ha abierto las puertas del conocimiento y ha sido mi segundo hogar. A todos los docentes que han dejado una huella imborrable en mi vida; especialmente a Mariana por creer en mí y darme la oportunidad de aprender a su lado, y a Sol, mi hada madrina, por compartir su sabiduría e impulsarme a lograrlo. Al personal administrativo y de servicio que me ha ayudado y acompañado durante este proceso. A mis buenos amigos, por todos los momentos vividos juntos. A mi familia, por su apoyo y buenos deseos. A Jisa, por su paciencia y compañía, y a las cuatro patitas que me acompañaron todos estos años.

DEDICATORIA

Con todo mi corazón para mi abuelito Juanito, con quien me hubiera encantado compartir estos momentos. A mi mami, mi ejemplo de trabajo, dedicación, perseverancia y el motivo de este logro. A mi hermanita, la alegría y luz de mi vida. Y a ti, que si lees esto y sientes que no puedes más, para que sepas que si no te das por vencido, lo lograrás.

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Abstract:

In the province of Napo, former mining waste pits are being used to culture *Oreochromis niloticus* (tilapia). Given its popular consumption in the province, this study sought to determine the impact of mining activities on metal bioaccumulation, fish health and safe consumption of *O. niloticus* in Napo - Ecuador. The bioaccumulation of metals was analyzed by Atomic Absorption Spectroscopy with livers, gills and muscles of specimens from three commercial fish tanks (SP1, SP2 and SP3), where SP3 is a former mining pond. Food safety in the Kichwa of Napo was evaluated by calculating the hazard quotient (THQ) and the estimated weekly intake (EWI). Carcinogenic and non-carcinogenic risks were determined. Genotoxicity analysis was performed using the micronucleus assay. The metal concentration of Cd, Cu, Cr, Pb and Zn was in ascending order SP2<SP3<SP1. Intake of these fish exceeded permissible metal limits at all sites; however, there is no evidence of risk from consumption currently. The probability of carcinogenic risk from consumption of Pb and Cd is one in a million at all three sites. Genotoxicity analysis revealed that DNA damage is significantly higher in SP3 fish. It is concluded that, to varying extents, all three sites present a potential risk to human consumption and biota and that food produced in these areas needs continuous monitoring to ensure food safety.

Keywords: Mining, Amazon, Bioaccumulation, Food Safety, Genotoxicity.

Resumen:

En la provincia de Napo, antiguos pozos de residuos mineros se están utilizando para cultivo de *Oreochromis niloticus* (tilapia). Dado su popular consumo en la provincia, este estudio buscó determinar el impacto de las actividades mineras en la bioacumulación de metales, la salud de los peces y el consumo seguro de *O. niloticus* en Napo - Ecuador. Se analizó la bioacumulación de metales mediante Espectroscopía de Absorción Atómica con hígados, branquias y músculos de ejemplares de tres peceras comerciales (SP1, SP2 y SP3), donde SP3 es un antiguo depósito de relaves mineros. Se evaluó la seguridad alimentaria en los Kichwa del Napo mediante el cálculo del cociente de peligrosidad (THQ) y la ingesta semanal estimada (EWI). Se determinaron riesgos cancerígeno y no cancerígeno. El análisis de genotoxicidad se realizó mediante el ensayo de micronúcleos. La concentración de metales de Cd, Cu, Cr, Pb y Zn fue en orden ascendente SP2<SP3<SP1. La ingesta de estos peces sobrepasó los límites de metales permisibles en todos los sitios; sin embargo, no hay evidencia de riesgo de consumo actualmente. La probabilidad de riesgo cancerígeno por consumo de Pb y Cd es de una en un millón en los tres sitios. El análisis de genotoxicidad reveló que el daño en el ADN es significativamente mayor en los peces del SP3. Se concluye que, en distinta medida, los tres lugares presentan un riesgo potencial para el consumo humano y la biota y que los alimentos producidos en estas zonas necesitan monitoreo continuo para garantizar la seguridad alimentaria.

Palabras clave: Minería, Amazonía, Bioacumulación, Seguridad Alimentaria, Genotoxicidad.

1. Introduction

Metal pollution has become a major challenge for the 21st century because most metals are indestructible and hazardous to aquatic ecosystems [1]. This type of contamination is of great interest due to its effects on human health, since metals can form soluble complexes with water and be transported and distributed to other strata of the food chain, allowing bioaccumulation in other organisms [2]. Diet is an important route of exposure to these pollutants, even to populations that do not live near the mining area but are exposed to their contamination indirectly [3]. This is counterproductive to the goal of the governments and the United Nations (UN) to establish food safety-based programs since nutrition is one of the main factors in fertility and mortality rates [4]. Nevertheless, most of governmental plans on this matter are focused on the amount and availability of food, but not on its harmlessness [5].

This research focuses on farm raised *Oreochromis niloticus* (tilapia) because it constitutes an important economic activity and source of nutrients in the Province. *O. niloticus* is an introduced species to the upper Ecuadorian Amazon several decades ago [6]. It is among the most consumed foods in urban and rural areas of Napo because it is the main ingredient of the typical dish known as *Maito* [6]. In rural areas, *O. niloticus* farming dependence is on the rise due to population growth and the change in lifestyle from semi-nomadic to sedentary. [7,8]. Per capita fish consumption among Kichwa population of Napo is 134 g per day [7]. No information has been found on the sale and consumption of this fish in the Province. Fish consumption has been associated with the presence of trace metals in the human body [9]. This has also been reported in indigenous communities from Colombia, Peru, and Brazil [10,11]. However, the few studies that have been conducted on bioaccumulation of metals in the Ecuadorian Amazon, focus on Hg and not on other elements that pose a risk to food safety [12].

In Napo province, abandoned mining ponds are being used for *O. niloticus* farming. It is an alternative used by mining companies to compensate the landowners after the mining activities have ended [13]. These tilapias are marketed throughout the province with no health regulations and constitute a means of subsistence for local people. The most significant concern is the metal contamination to which these fish are exposed [13]. Several studies have been carried out on the consumption of fish affected by the

bioaccumulation of metals [3,14,15], concluding that when the concentration of metals exceed the permissible limits, the fish-consuming population is exposed to a potential health risk [3,16,17]. However, the metal concentrations in fishponds cannot only be attributed to mining. Trace metals can also be released from the Earth's Crust as a result of fish farming, they are also present in fish feed and some chemical products used for the maintenance of fishponds [18].

Some organs such as gills, muscle and liver are investigated to evaluate the concentration of metals, which varies according to the tissue [15,19]. Gills are the first route of entry of metals into the fish's organism as they are absorbed here and can bioaccumulate [20,21]. Liver is the main organ of detoxification and storage, so metals can also accumulate here [22]. Muscle is not an organ characterized by bioaccumulation, which only occurs when detoxification pathways have failed; nevertheless, the assessment of metal concentration in fish muscle is important in determining the consumption risk [3] In addition, blood samples allow assessment of the genetic, structural, and functional health of fish, and are key to the study because blood cells are sensitive to stress caused by contamination [15]. Micronucleus and nuclear abnormalities assay allow measuring the genetic instability of an individual because when there are alterations induced by genotoxic compounds or the presence of mutations in target genes, the genetic material is detached from the nucleus during cell division [23].

The objective of this study was to determine the impact of mining activities on metal bioaccumulation, fish health and safe consumption of *Oreochromis niloticus* in Napo Ecuador, by comparing farmed fish in former mining and non-mining areas. Fulton condition factor was calculated, to determine the physiological status of the fish. An analysis of the bioaccumulation of Cr, Zn, Pb, Cu and Cd in muscle, liver, and gill tissues of *O. niloticus* from mining and non-mining areas was carried out. Food safety was assessed through the calculation of the Estimated Weekly Intake of metals in food, Target Hazard Quotient, and the Cancer Risk of metal consumption indexes. Genotoxicity was studied by analyzing the nuclear abnormalities. Results from mining and non-mining areas were compared. The hypothesis was that the condition factor was going to be higher in fish from non-mining areas than in the mining area. It was also expected the bioaccumulation of metals in the three tissues to be higher in fish from the mining area than those from the non-mining areas. Genotoxicity was expected to be

higher in fish from mining ponds than in non-mining ponds. Consequently, the consumption of fish from the non-mining area was expected to be safer than the consumption of fish from the mining area.

2. Materials and Methods

2.1. Sampling

Three sampling points were selected considering popular commercial *O. niloticus*-farming ponds, located in Tena and Arosemena Tola cantons - Napo Province, Ecuador. Sampling Point 1 (SP1), known as Sapo Rumi; Sampling Point 2 (SP2), known as Atacapi; and Sampling Point 3 (SP3), known as Arosemena Tola. Of these, only SP3 is considered a mining area, while SP1 and SP2 are assumed to be free of mining activity (see Fig. 1). Nevertheless, they were not classified as control groups because the anthropogenic pressure to which these sites are exposed and the presence of metals due to natural origin are unknown. No water samples were taken, and no water quality parameters were found for SP1. SP2 presents total dissolved solids (TDS) 16.5 mg*L⁻¹, medium water quality index with presence of phytotoxicity and sediment toxicity [24]. SP3 water presents TDS: 16.25 mg*L⁻¹, turbidity of 10.2 NTU, dissolved Oxygen 81.5 mg*L⁻¹ with presence of phytotoxicity and sediment toxicity as well [25].

This method was based on Gusso et al (2016) [26]. Five specimens (average weight: 519.86 g) of both sexes of *O. niloticus* were collected per SP and transported alive to the laboratory in coolers with water from the same pool in which they lived to minimize tissue decay. The samples for all the analysis were taken the same day and euthanized by spinal cord severing. Each fish was measured and weighed to obtain the Condition Factor according to Equation 1.

$$KF = \frac{W}{L^3 \times 100} \quad (\text{Equation 1})$$

Where W is the body weight in grams and L is the body length in centimeters [26]. This factor gives a general idea of the condition and health of the fish [27]. Then, three

pseudo-replicates of each tissue (gills, liver, and muscle) were taken, they were stored in microtubes and Falcon tubes (50 ml for muscle), and frozen at -80 °C until analysis.

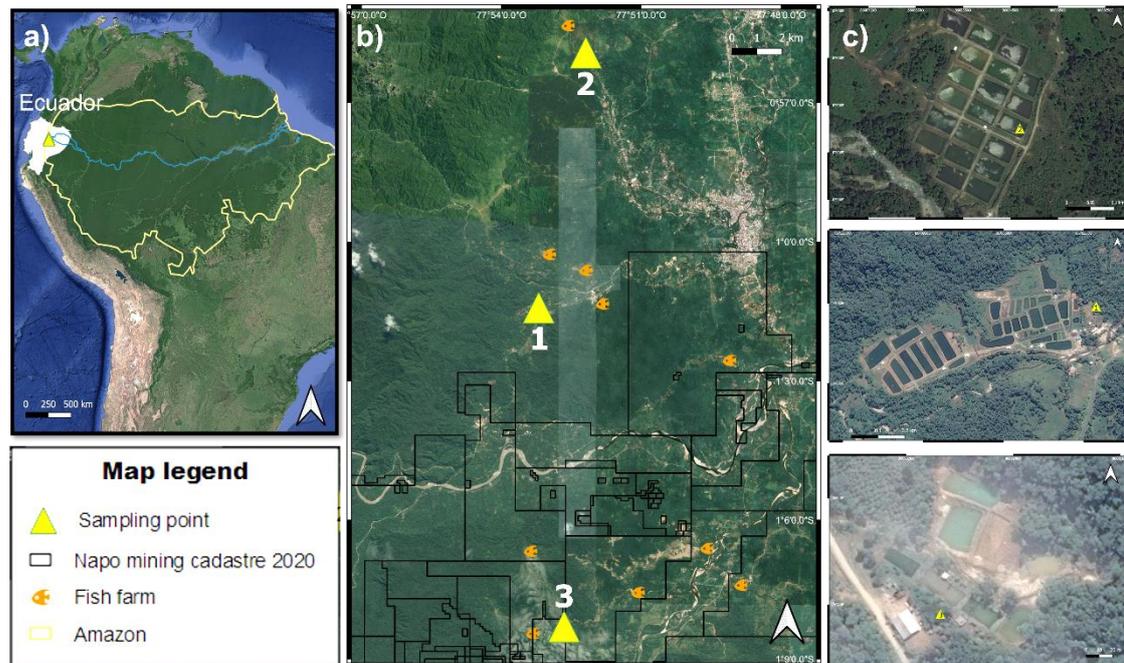


Figure 1. a) Location of the study area. b) Sampling points and gold mining concessions [24]. c) Approach to sampling points.

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2.2. Metal bioaccumulation analysis

A total of 135 samples (15 fish total, three tissues of each fish, three pseudo-replicates of each tissue) were let to thaw at room temperature, to determine the concentration of metals in tissues, the procedure was adapted from those described by AOAC (2005) [28]. The samples were homogenized and weighed in a crucible. The samples were dried in a drying oven at 105°C for 8 hours. The crucible was weighed again. Samples were calcinated in a furnace at 550 °C for 2h and let cool at room temperature obtaining black ashes, these ashes were hydrated with distilled water for 15 minutes, then they were solubilized with 100 µL 10% HNO₃ for 15 minutes. Finally, the samples were burned in the furnace again (550° C; 2h) obtaining white ashes that were hydrated and solubilized until they were clear. The calibration curve was prepared by making a multi-element mix with Cr, Zn, Pb, Cu and Cd standard solutions of 1000 [mg L⁻¹] from Merck. The samples were gauged with distilled water in volumetric balloons (25mL) then analyzed in an atomic absorption spectrophotometer with an air-acetylene burner for flame and graphite furnace (see Table 1) from Thermo Scientific and Thermo SOLAAR software determining

concentrations of Cr, Zn, Pb, Cu and Cd. The instrument limits of detection and quantification (LOD/LOQ) were estimated as 6 SD and 10 SD of 3 blank measurements, respectively ($p < 0.05$) (see Table 1). This analysis was performed at the Universidad del Azuay, city of Cuenca-Ecuador.

Table 1. Instrumental limits of detection and quantification (LOD/LOQ) ($\mu\text{g.g}^{-1}$). Atomic Absorption Spectroscopy. $p < 0.05$.

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Procedure with graphite furnace						
	MUSCLE		LIVER		GILLS	
	LOQ	LOD	LOQ	LOD	LOQ	LOD
Cd	4.50E-06	2.14E-06	4.50E-06	2.14E-06	4.50E-06	2.14E-06
Pb	1.02E-04	4.84E-05	1.02E-04	4.84E-05		
Cr	5.00E-06	2.37E-06	5.00E-06	2.37E-06	5.00E-06	2.37E-06
Procedure with flame						
Zn	0.00300	0.00145	0.00300	0.00145	0.00300	0.00145
Pb					2.31E-02	1.09E-02
Cu	0.00780	0.00371	0.00780	0.00371	0.00780	0.00371

2.3. Genotoxicity analysis

Blood samples were taken from the caudal vein of the fish. A drop of blood was placed on a slide plate and a smear was made; 3 repetitions were made for each fish. The slides were let dry at room temperature and fixed with 90% ethanol; they were allowed to dry again, fixed with Carnoy's solution (methanol/acetic acid 3:1) for 15 minutes and stained with 2% Giemsa in phosphate buffer at pH 6.8 for 15 minutes. The slides were observed under the microscope at 100X using immersion oil. The number of micronuclei (MN) and nuclear abnormalities (NA) was counted on a 1000-cell basis. This procedure was adapted from Nudi et al., (2010) and Prieto et al., (2008) [29,30].

2.4. Food Safety assessment

Food safety assessment was calculated with the muscle metals concentration alone. The population body weight used was 67.9 [kg] which was the mean weight of Ecuadorian population for 2015 [31]. Weekly per capita fish consumption in the Amazon was used to calculate weekly fish consumption. According to FAO this value varies according to the habitat of the person, so the value used was 134 [g/day], which is the global average for the Kichwa of Napo, since most of the population in Napo is considered indigenous

and the largest ethnic nationality is the Kichwa [7,32]. It is worth mentioning that this value does not include the consumption of fish consumed by the non-indigenous population, because the data found on fish consumption in Ecuador does not include the Amazon region. Since fish is not consumed daily, this value was multiplied by 4, considering that in the same study participants reported eating fish 57% of the days [7].

Estimated Weekly Intake (EWI) was calculated for each metal to determine the human consumption risk, using Equation 2 (adapted from WHO, 2008) [33]:

$$EWI = \frac{\text{Metal concentration } \left[\frac{\mu\text{g}}{\text{g}}\right] \times \text{Weekly Fish Consumption } [\text{g}]}{\text{Population Body Weight } [\text{kg}]} \quad (\text{Equation 2})$$

These results were compared with the current regulations of national and international organizations: USEPA (2000), EC (2006), Mercosur (2011), FAO/WHO (2014) and ANVISA (2013) [34–38]. Also, they were compared with the Provisional Tolerable Weekly Intake (PTWI), a reference value set by the joint FAO/WHO Expert Committee on Food Additive (JECFA) that represents the maximum weekly human exposure to pollutants with cumulative properties such as metals [20,33,39,40], which would make it possible to determine whether the consumption of these fish represents health risk.

Target Hazard Quotient (THQ) set by the U.S. Environmental Protection Agency was calculated to determine the risk of metal uptake from fish with non-carcinogenic effects according to Equation 3 [41]:

$$THQ = \frac{EDI}{RfD} \quad (\text{Equation 3})$$

Where EDI is the Estimated Daily Intake [mg/kg bw/day], which is calculated the same as *Equation 2* using Daily (instead of weekly) Fish Consumption; RfD is the oral reference dose that represents the continuous risk to which the population is exposed to toxins without presenting noticeable deleterious effects, being Cd= 0.001, Cr = 1.5, Cu= 0.04, Pb= 0.004, Zn= 0.3 [mg/kg] [41]. If the THQ is less than 1, there are no health risks for the population, the greater the THQ value, the greater the level of concern [41].

Cancer Risk assessment was obtained through the formula showed in *Equation 4*, which is used to determine the incremental probability of cancer development in a lifetime

exposure [41]. CSF_{ing} is the Carcinogenic Slope Factor by ingestion which is set by FAO/WHO, USEPA and USDOE [42]. The used values of CSF_{ing} for each metal were $Cd=0.38$, $Pb=0.0085$ [mg/kg bw/day] [43]. Only results between 10^{-6} and 10^{-4} were considered as predicted Cancer Risk [42].

$$CR_{ing} = EDI \times CSF_{ing} \quad (\text{Equation 4})$$

2.5. Statistical analysis

Statistical analyses were performed in SPSS Statistics version 21 [44]. The averages of pseudo-replicates and replicates were obtained for each SP. Normality tests were performed using the Shapiro-Wilks test, $n=15$. Kruskal Wallis for SP – KF, ANOVA for SP-NA and Two-factor ANOVA was performed for and SP*Tissue-Metal concentration and SP*PTWI-EWI.

3. Results and Discussion

3.1. Body Condition factor (KF)

All the fish caught for this study presented a quite homogeneous length; however, the KF of fish from SP3 was significantly higher than those from SP2 (Kruskal Wallis; $df = 2$; $p < 0.05$). KF value allows to determine the physiological condition of fish, through an association between length and weight [45]. It considers that individuals of similar length that show a greater mass have a better physiological condition [45]. One of the main factors that influences *O. niloticus* growth is the amount and quality of the food [46]. It has been reported that Cu and Zn are added to some fish feeds to accelerate the growth and development of *O. niloticus* [47]. Some aquaculture practices also recommend a maximum feed ration to increase the growth rate of *O. niloticus* and reduce operating costs [46]. Such practices do not consider the bioaccumulation of these metals in tissues which, according to Wang et al (2020), represents a potential threat to both fish and food safety [47]. Therefore, a higher KF would not necessarily represent a better physiological condition of the fish. Nevertheless, KF may be influenced by other factors such as water temperature and quality, fish tank population density, stress, trophic status of the habitat,

oxygen availability, and others [46,48]. Further studies are needed to determine the factors that are influencing KF value.

Table 2. Condition factor of *O. niloticus*.
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Sampling Points	n	Body weight [g]			Total length [cm]			Futon's condition factor (KF)		
		mean	±	SD	mean	±	SD	mean	±	SD
SP1	5	533.80	±	74.08	24.80	±	2.59	0.22	±	0.02
SP2*	5	461.60	±	36.68	23.80	±	0.84	0.19	±	0.01
SP3*	5	564.20	±	87.06	24.60	±	0.55	0.23	±	0.03

Results are shown as values +- S.D. *Shows significant difference between sampling points (Kruskal Wallis; df = 2; p < 0.05).

3.2. Metal bioaccumulation analysis

The accumulation analysis between sampling points showed that Pb ($221.06 \mu\text{g}\cdot\text{g}^{-1}$) and Cd ($0.11 \mu\text{g}\cdot\text{g}^{-1}$) present levels of concern in SP1. SP1 is 2.7 km away from the closer mining concession (see Fig. 1), although SP1 is not included in the mining cadaster, illegal mining and other anthropogenic pressures have been affecting the area for a longer period. Municipal Government of Pano confirms the presence of oil and gold in the zone and the artisanal gold mining in Achiyacu (next to SP1) and Pano rivers, soil degradation due to agricultural activities, and other anthropogenic pressures have also been reported [49]. Metal concentration found in the fish from SP2, located within the buffer zone of the Colonso-Chalupas Biological Reserve, may be influenced by indiscriminate deforestation, fish farming, sewage discharges into the river, garbage dumps, stone material extraction and, especially, livestock [50]. Metal presence also can have a natural origin as it can be released from the soil during the building of the ponds, or they may be present in the water [51]. However, determining whether the origin of these metals is natural or anthropogenic is not within the scope of this study.

The affinity of metals for tissues could depend on the route of entrance to the body; fish can uptake metals through water, food or epidermis permeation [47,52]. If the intake is from water, gills are the access route for metals [53]. Gills are the first target organ for heavy due to their role in respiration, osmoregulation, and excretion [17,21,54]. They also have been reported to work as a storehouse for some metals [53]. If the accumulation in gills is high, metals can reach other internal tissues by moving through the blood flow [53]. When compared, greater accumulation in gills than in other organs

may indicate recent contamination [45], or that the contamination is being produced by the respiration pathway rather than the food uptake [55,56].

Once metal ions are in the bloodstream, they are transported to detoxification or storage sites such as the liver or kidney [22]. When contamination enters through diet, the main organ exposed is the intestine, where metals are absorbed by endocytosis to the bloodstream [53]. Regardless of the route of intake, the liver accumulates high concentrations of metals [53] because it is in charge of the biotransformation, detoxification and derivation of xenobiotics [17,57]. Its response depends on the degree and kind of contaminants [17]. Greater accumulation in the liver than in other organs would indicate long exposure periods and difficulty of depuration [53].

It has been reported that *O. niloticus* muscle does not actively accumulate trace metals and has a high percentage of decontamination [53]. The reasons for this may be low quantity of metal-binding proteins, the presence of mucous that covers the skin and avoids permeation, and that other tissues do not transport metals to muscle because it is not in charge of detoxification processes [58]. Bioaccumulation in muscle occurs only when other detoxification routes have failed [3]. Nevertheless, muscle is the main edible tissue for humans, its analysis is important to study food safety.

Metal distribution in tissues is both species- and metal-specific [58]. According to Hamid Dar et al (2021), in fish, Cu mainly affects the liver, gills, and kidney of fish because it shows bioaccumulation affinity towards them [59]. Cr has higher affinity for the gills, Cd and Pb accumulate more in the kidney, liver and gills and the accumulation of Zn shows different patterns of bioaccumulation as it decreases when the body length increases [59]. An analysis for the accumulation of each metal in each SP and tissue is shown below.

In muscle, Cr was below the LOD (see Table 1). This result agrees with the findings of other authors, who indicate that despite the exposure of fish to chromium, little or no bioaccumulation has been observed [60,61] since it occurs only when the bioaccumulation in the liver and the kidney are excessive [61,62]. There is no significant main effect of sampling points $F(2,126)= 0.865$, $p>0.05$, $\eta^2=0.014$ on Cr concentration. A significant main effect of tissue on Cr concentration was found $F(2,126)=12.52$, $p<0.05$, $\eta^2= 0.16$, being concentration in gills ($M= 0.276 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.511$), significantly higher

than in liver $p < 0.05$ ($M = 0.012 \mu\text{g}\cdot\text{g}^{-1}$; $S.D. = 0.008$) (see Fig. 2a). As mentioned before, greater accumulation in gills may indicate recent exposure, and the contamination on other tissues may depend on the liver's ability to depurate. Some studies show similar results, since the greatest accumulation of chromium occurs in the gills of *O. niloticus* [63,64] as it is the most exposed organ [62]. Cr presence in gills can become lethal as the fish's respiration is disturbed and oxygen concentration decreases [21]. Decreased oxygen levels change the behavior of the fish and cause them to gather in a corner of the tank [21].

There is no significant effect of SP $F(2,126) = 0.480$, $p > 0.05$, $\eta^2 = 0.008$ on Pb concentration, which means that the three SPs are equally polluted. There is a significant effect of tissue $F(2,126) = 41.67$, $p < 0.05$, $\eta^2 = 0.398$ on Pb concentration. Pb bioaccumulation is significantly higher in gills $p < 0.05$ ($M = 181.834 \mu\text{g}\cdot\text{g}^{-1}$; $S.D. = 186.283$) than in liver ($M = 0.660 \mu\text{g}\cdot\text{g}^{-1}$; $S.D. = 0.681$) and muscle ($M = 0.090 \mu\text{g}\cdot\text{g}^{-1}$; $S.D. = 0.067$) (see Fig. 2c). High concentrations of Pb can be found in the gills due to metal ions exchange during respiration and osmoregulation [65]. Accumulation of Pb in the gills is higher when there is exposure to water contamination, compared to when the exposure is through feeding [65]. Considering concentrations in the three tissues, Pb is the one that accumulated the most. These results are consistent with other studies [65,66]. Among some toxic metals, lead is one of those that accumulate most in the organism because it easily binds oxygen and sulfur atoms with proteins and forms stable complexes [65]. Metals such as Pb, Hg and Cd are considered to cause public health hazards [64].

The mean effective concentration (ED50) in fish, above which strange swimming and accelerated opercular movement occurs, is about $0.273 \text{ mg}\cdot\text{L}^{-1}$ [67]. Pb toxic effects also include physiological, behavioral, and biochemical damage and increased susceptibility to oxidative stress [52,65]. The toxicity can depend on oxidative state of the metal, speciation, bioavailability, toxicokinetic and toxicodynamic [68].

There is a significant effect of SP $F(2,126) = 6.731$, $p < 0.05$, $\eta^2 = 0.097$ on Cu concentration, where SP1 ($M = 19.66 \mu\text{g}\cdot\text{g}^{-1}$; $S.D. = 1.36$) is significantly higher than SP2 ($M = 12.685$; $S.D. = 1.37$) $p < 0.05$. Mention was made earlier of the previous mining activity to which SP1 was exposed, which could justify its similarity to SP3 and its difference from SP2. The main sources of contamination by Cu in water and soil are mine tailings

and wastewater sludges [51]. Also, Cu is usually used to disinfect fish tanks from various pathogenic microorganisms and ectoparasites [54]. Use of Bordeaux broth (a mixture of copper sulfate, copper oxide and calcium hydroxide) has been noted in the area as well; this compound is used in agriculture and has been recommended by National Agricultural Research Institute of Ecuador (INIAP) [69,70].

A significant effect of the tissues $F(2,126)= 225.746$, $p<0.05$, $\eta^2= 0.782$ on Cu concentration was found (see Fig. 2b). Concentration of Cu in liver ($M=39.185 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 17.005$) is significantly higher than in gills ($M=7.43 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 1.43$) and muscle ($M=0.833 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.316$) $p<0.05$; being gills and muscle significantly different among them as well. This agrees with other authors, who mention that although Cu is first deposited in the gills, the highest concentrations are in the liver being the main organ where it accumulates and is the most affected [54,71]. High concentrations of Cu in the fish body can cause oxidative stress and hypoxia; in addition, it can affect processes such as gill ion transport, hematopoiesis, glucose metabolism, enzyme activities and the immune system [71].

There is no significant main effect of SP $F(2,126)= 0.265$, $p>0.05$, $\eta^2= 0.004$ on Cu concentration. A significant main effect of tissues $F(2,126)= 33.493$, $p<0.05$, $\eta^2= 0.347$ on Cu concentration was found. Zn concentration in muscle ($M=3.812 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.=0.666$) is significantly lower than Zn concentration in liver ($M= 7.259 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 3.106$) and gills ($M=7.202 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 2.662$) $p<0.05$. Low concentrations of Zn in muscle have been reported in other studies, although concentrations in gills and liver vary [61,72]. Zn is the second more abundant trace element in the organism and the higher concentrations are in the liver [73]. The presence of this element in water seems to be associated with active or inactive mine tailing ponds that also put at risk human water supplies [51]. Although there is evidence that Zn does not have carcinogenic properties in animals and humans, it is not known to be carcinogenic, when Zn is found in high concentrations, problems with homeostasis, apoptosis, cytotoxicity and even cell necrosis occur [74].

Some Cd concentrations in muscle were below the LOD (Table 1). This agrees with other studies that also report low concentrations of Cadmium in muscle [75,76]. There is a significant main effect of SP's $F(2,126)= 4.207$, $p<0.05$, $\eta^2= 0.063$ on Cd concentration, where Cd concentration is significantly higher in SP1 ($M= 0.38 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.006$) than

it is in SP3 ($M= 0.016 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.006$) $p<0.05$. It has been reported that one of the main sources of cadmium contamination in fish tanks is drainage from agriculture [77]. This would be justified by the fact that agriculture is the main economic activity in the sector; production is focused on products like cacao, corn, banana, cassava, among others [49]. In addition, poor agricultural management has been reported [49].

There is a significant main effect of tissues $F(2,126)= 22.264$, $p<0.05$, $\eta^2= 0.261$ on Cd concentration (see Fig. 2e). Concentration of Cd is significantly higher in liver ($M= 0.056 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.001$) than it is in gills ($M= 0.015 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.001$) and muscle ($M= 0.001 \mu\text{g}\cdot\text{g}^{-1}$; $S.D.= 0.001$) $p<0.05$. It has been reported that the liver is the main site of detoxification and bioaccumulation of Cd, the concentration in this organ is usually proportional to that present in the environment [53]. In fish, Cd accumulation can cause oxidative stress, skeletal deformities, and damage to organs such as gills, liver, kidneys and intestines [52,77]. It has been reported that increased temperature in the environment would enhance metal bioaccumulation and oxidative stress in *O. niloticus* [52]. This would indicate that in future years, with global warming, the impact of metal pollution will increase.

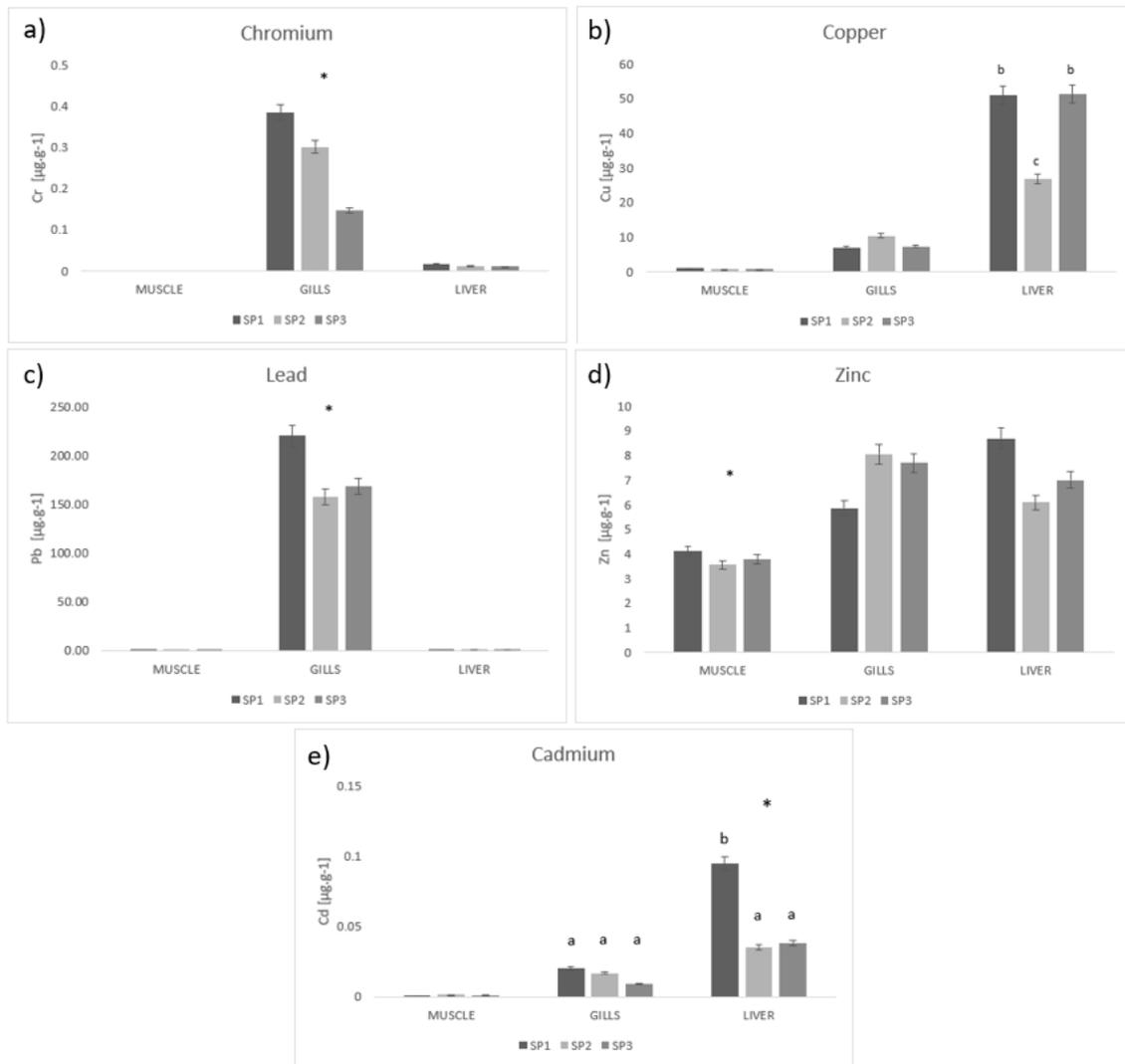


Figure 2. Bioaccumulation of metals.

Results are shown as means \pm E.R. *Shows significant difference in tissues. Different letters show significant differences between SPs.

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3.3. Metal genotoxicity analysis

MN and NA are evidence of damaged or broken chromosomes that have had an incorrect repairing process during cell division due to the presence of pollutants in the mitotic apparatus [14,26]. The frequency of MN found in this study is 4.83, 3.33, and 14.19 per 1000 cells, while the frequency of nuclear abnormalities (NA's) is 81.66, 45.27, and 183.71 per 1000 cells, for SP1, SP2, and SP3, respectively. Statistically significant effect of SP on the number of MNs $F(2,14)=1.291$; $p<0.05$; $\eta^2=0.177$ was found. SP3 (M= 13.40) presents higher amount of MN than SP2 (M= 7.80) and SP1 (M= 5.40).

Significant effect of SP on the amount of NAs $F(2,14)=2.495$; $p<0.05$; $\eta^2=0.065$ was found as well. SP3 (M= 36.60) presents higher numbers of NAs than SP2 (M= 12.60) and SP1 (M= 17.36). This shows that higher genotoxicity is caused in the former mining sampling point; this matches the KF results (see Table 2), where SP3 is higher too. Similar results were obtained in other study where they associate higher weight of the fish with higher genotoxicity [78]. This could be related to the time of exposure to trace metals in the medium. D'Costa et al (2017) state that longer exposure time to contaminants implies higher levels of genotoxicity in fish [14]. Genotoxicity could also be associated with the amount and type of food consumed by fish in this SP. As mentioned above, some of the fish feeds contain Cu and Zn [47], the concentrations of which are high in SP3 and could be influencing genotoxicity levels. Considering that *O. niloticus* growth depends on factors such as development time, amount of feed, temperature, among others [46], further study is needed.

These results also may suggest that currently no adaptive response is found in *O. niloticus* from all the SPs. The standard number of Nuclear Abnormalities in control groups for *O. niloticus* is around 0.16NA/1000 cells which is a mean value from other studies [30,79]. This shows that the three SP's display genotoxic effects. In lab conditions, the amount of NA increases progressively after 7 days of exposure, while the amount of MN tend to decrease [30]; so, this frequency of nuclear deformities may suggest that fishes from the three SPs have been exposed to pollutants for a long period. The results of genotoxicity analysis done with peripheral blood are associated with the body burden of metals in the organism and the destruction of the genetic material at a chromosomal level [26,30]. Further than assessing genotoxicity [17], this technique can also allow to detect cancer risk and diagnose other diseases [80]. In addition to the damages that all these metals accumulation can cause to fish, the consequences of these results in human health are of concern.

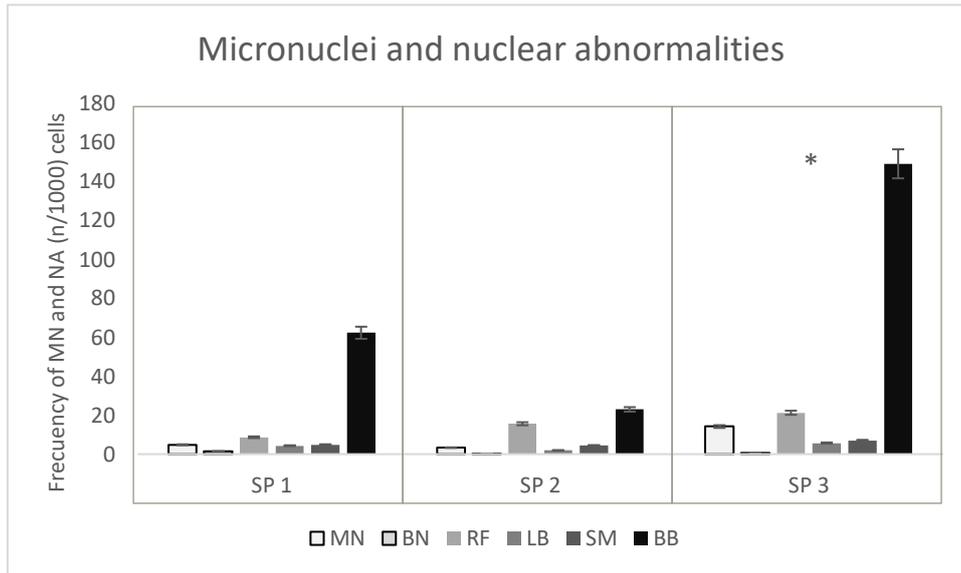


Figure 3. Frequency of nuclear abnormalities in erythrocytes of *Oreochromis niloticus*.

MN: Micronuclei, BN: Binucleated, RF: Reniform, LB: Lobed, SM: Segmented, BB: Blebbed. Frequency was calculated in 1000 cells. Results are shown as means \pm E.R. *Shows significant difference between SPs.

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3.4. Health risk assessment

National, and international regulations on contaminants and toxins in food INEN (2013), USEPA (2000), EC (2006), Mercosur (2011), FAO/WHO (2014) and ANVISA (2013) are based on marine fish only [34,35], so the results could not be compared. The Estimated Weekly Intake (EWI) was calculated and compared with the Provisional Tolerable Weekly Intake (PTWI) [33,39,40]. There is a significant effect of PTWI on EWI (two-way ANOVA; $F(3,48)= 778.719$; $p<0.05$; $\eta^2=0.980$), so Amazonian people are consuming more than 200% of the amount of metals that they should be consuming, from the three SPs, since the PTWI is the maximum allowed weekly human intake to these metals (see Fig. 4a and 4b).

There is a significant effect of SP on EWI (Two-way ANOVA; $F(2,74)=4.591$; $p<0.05$; $\eta^2=0.161$) with SP1 ($M= 8.278$) showing higher EWI than SP2 ($M= 6.885$) and SP3 ($M= 7.096$). This matches previous results that were already discussed. Since Cr concentrations in muscle were below LOD they were dismissed. The concentration of metals in muscle were $Zn>Cu>Pb>Cd$. Zn and Cu are essential metals so it may be reasonable that they present higher accumulation [51]. However, an excess of Zn

consumption could lead to reproductive system damage and growth affections [47]. High concentrations of Cu may produce harmful physiological and histological alterations and cause hepatic effects [47].

Pb [64], Cu and Cd have been reported to cause damage to renal system or nephrotoxicity [51]. 18% of annual deaths in the U.S. are attributed to the concentration of Pb in blood [81]. Pb is dangerous even in low concentrations since it can replace calcium in the body and lodge in the bones and blood system for years [82]. Children with history of Pb exposure have been reported to exhibit inattention, psychological and school deficits, poor cognitive development, behavioral problems, physical violence, and crime rates as they grew up [83,84]. A study conducted in the Amazon near a gold mine has shown that children who are more exposed to Pb, Hg and Mn contamination show decreases in their intellectual quotient (IQ), which may explain the poor school performance of Amazonian children.

The Target Hazard Quotient (THQ) for all sites is below the maximum level, which means that they do not pose risk of consumption currently (see Fig. 4c). The THQ represents the risk of non-carcinogenic effects of metals in human health, where values above one show an increasing probability of non-carcinogenic health problems [41]. Nevertheless, considering that the ED₀₁ is above the permissible limits, that Cr and Pb are more concentrated in the gills, which shows recent exposure, and the genotoxicity levels, it is necessary to keep permanent monitoring on the THQ of fishes from these areas. The more pollutants there are, the major additive or interactive effects they can cause on health [41].

Figure 4d shows that there is one in a million probability of developing cancer over 70 years [42] caused by consumption of *O. niloticus* in the three sites associated with Pb and Cd. Values for Zn and Cu were discarded since these metals are not carcinogenic and their CSF_{ing} is zero [43]. The possibility increases to one in 10000 as the CR value gets closer to 1×10^{-4} [43] which is unacceptable by most international regulatory agencies [85]. The results regarding health risk assessment depend on per capita fish consumption, body human weight, consumption frequency and exposure time [85]. These toxicological effects may vary from person to person.

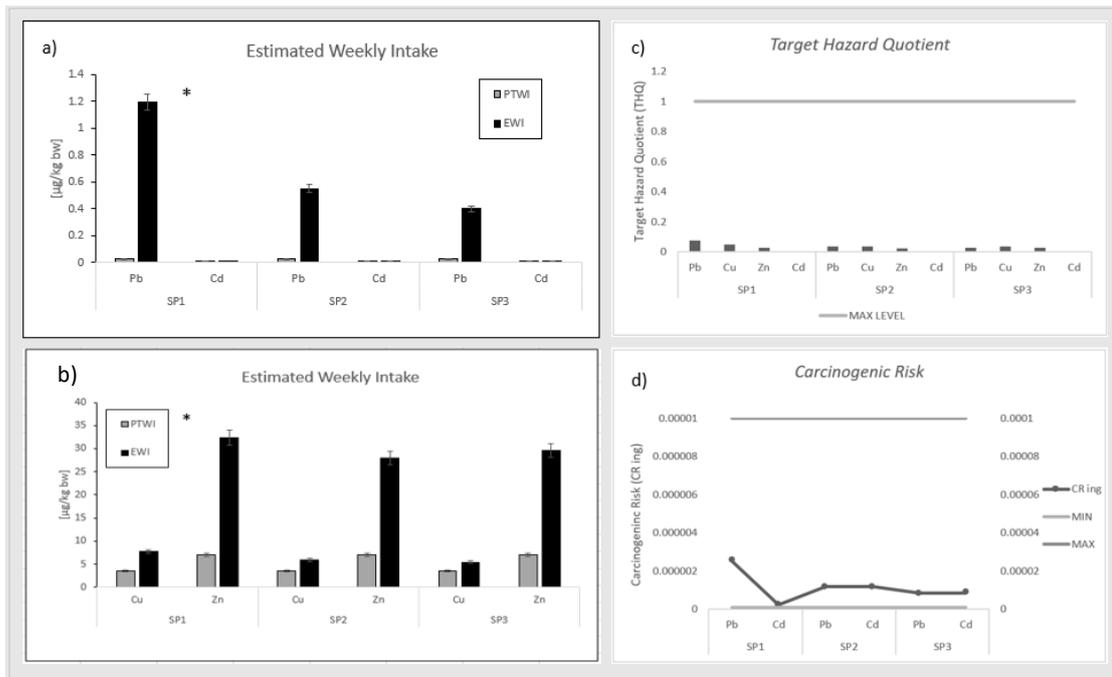


Figure 4. a and b) Comparison between Provisional Weekly Intake and Estimated Weekly Intake of metals. 4c) Target Hazard Quotient 4d) Carcinogenic Risk by metal ingestion.

Results are shown as values \pm E.R. * Shows significant difference between sampling points. Values within minimum and maximum limits are considered as predicted carcinogenic risk.

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The present investigation was carried out during the time of SARS-CoV-2 pandemic, so the limitations were basically the difficult mobility and access to laboratories. Also, the scarce information that could be obtained in the fish tanks, especially in the mining area; there was little local information on the consumption and sale of *O. niloticus*, as well as scarce data on the Amazon and regulations regarding the consumption of freshwater fish.

4. Conclusions

Contrary to what was expected in the hypothesis, fish from the mining area presented higher KF than the rest. The reason for this needs further investigation. Bioaccumulation presented concerning concentrations of Pb and Cd in SP1, which, despite not being within the mining cadaster, was found to be affected by illegal mining, poor agricultural activities, stone material extraction, and others. The results of the genotoxic analysis are consistent with the hypothesis since it was found that genotoxicity is higher in fish

cultured in the mining area. These results show irreversible damage in *O. niloticus* at a chromosomal level and a lack of adaptive response. In the food safety analysis, it was shown that, although there is currently no risk of consumption, the maximum tolerable consumption limits for metals are being exceeded in the three sampling points, which means a potential risk in the future. In addition, the presence and consumption of highly toxic metals such as Pb and Cd is of concern. Currently carcinogenic probability is one in a million, but it is noteworthy that this index considers one metal at a time and not their interaction together. These results depend on the amount of fish consumed and the weight of the population; since Kichwa people are high fish consumers, risks for the rest of the population would be lower. Future investigations may lead to analyze the metal concentration in blood or hair samples from people who live in the surroundings of mining areas.

5. Compliance with ethical standards

Conflict of interest The author declares that she has no financial interests or personal relationships that could influence this research.

Acknowledgements This research was possible thanks to the unconditional support of Dr. Mariana Capparelli and her team and Dr. Andrés Pérez, the help of the technicians from Universidad Regional Amazónica Ikiam and members of the Instrumental Chemical Analysis Laboratory in Universidad del Azuay.

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