ANTIMICROBIAL RESIDUES AND FISH - BORNE ZOONOTIC TREMATODES (FZT) IN RAINBOW TROUT FARMING IN LAO CAI PROVINCE, VIETNAM

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ABSTRACT

The rainbow trout farming industry began to develop in Vietnam in 2004. Since then, this industry has grown rapidly and made a positive contribution to local economic development. The presence of parasitic flukes and residues of drugs and chemicals is quite common in aquaculture. In this study, we determined the residues of Chloramphenicol, furazolidone (AOZ), furaltadone (AMOZ), Ciprofloxacin, Oxytetracycline, Malachite green (MG), and Leucomalachite green (LMG), as well as fish-borne zoonotic trematodes (FZT) in farmed rainbow trout at several farms and restaurants in the three largest rainbow trout farming areas in Lao Cai province, including Ngu Chi Son, Lao Chai, and San Sa Ho communes, from 2019 to 2022. The results from 173 collected samples showed no detection of FZT metacercariae, AOZ, AMOZ, and Oxytetracycline residues in the fish meat. Chloramphenicol and Ciprofloxacin were detected only once in 2019, with levels of 0.33 and 1.84 µg/kg, respectively. The residues of LMG and MG significantly decreased from 2019 to 2022. The levels of MG and MLG in these four years were 304.6, 343.9, 0.49, and 3.94 µg/kg, respectively. These positive results were obtained through the dissemination and training activities in Lao Cai province under the frame of the ICI Project Phase III, funded by Finland. However, it is necessary to continue implementing appropriate measures to minimize the residues of prohibited substances in farmed rainbow trout meat.

Keywords: *Antimicrobial, residue, zoonotic trematodes, rainbow trout.*

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1. INTRODUCTION

Global aquaculture production has grown significantly in recent years. The share of rainbow trout (*Oncorhynchus mykiss*) trade, a freshwater salmonid, has also intensely increased. Despite the availability of several vaccines, bacterial disease's rates in fish farms have increased due to intensive aquaculture. It led to widespread use of antibiotics. Since trout farming is mainly in open environments, antibiotic use may have a strong impact on the emergence, selection and/or dissemination of resistant microorganisms.

Since 2004, the Research Institute for Aquaculture No.1 (RIA1) has cooperated with Finland for the first time to successfully bring rainbow trout eyed eggs to trial farming in Sapa town, Lao Cai province. Up to now, cold-water fish farming has developed strongly in 25 provinces/cities across the country, typically in Lam Dong, Lao Cai, Son La, and Lai Chau provinces [1]. Lao Cai province currently has more than 300 cold water fish farming establishments, mainly in Sapa town, Van Ban, and Bat Xat districts, mainly rainbow trout, and sturgeon. By the end of 2020, the total production of cold water fish in the province reached over 670 tons, an increase of nearly 300 tons compared to 2015. Farming facilities create jobs for thousands of local workers. However, extreme weather events with temperature increases, prolonged drought, frequent rains, and floods have, and will, cause unpredictable material losses to cold-water fish farmers in mountainous areas. The problem of

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disease and quality management of aquatic products is a dilemma and uses drugs to prevent and treat diseases outside the permitted list, which will affect the quality of fish [2]. Twenty different antimicrobial products were used for disease prevention and treatment in shrimp and marine fish cultures in three provinces in Northern Vietnam, including banned products such as Chloramphenicol, Enrofloxacin, and malachite green [3]. A survey using questionnaires in 7 provinces of Vietnam on the use of antibiotics in fish and shrimp farming reported that antibiotics were widely used in fish farms (64%) and shrimp farming (24%) [4].

Malachite green (MG) is a dye that has been used to make colors such as silk, wool, leather, cotton, and paper products [5, 6]. MG is also used to treat fungal and protozoal infections in fish and fish eggs farming industries around the world [7, 8, 9, 10, 11]. MG causes damage to liver, spleen, and kidney, and produces teratogenic, genotoxic, and carcinogenic effects. In waterborne exposure, MG is absorbed into fish tissues and transformed into the metabolite, colourless compound, leucomalachitegreen (LMG). This metabolite remains in fatty tissues for extended periods [7, 9, 12, 13]. The high temperature does not guarantee a full breakdown of the residue of MG and LMG which may occur in fish muscles [14]. Therefore, the use of MG in food-producing animals is not authorized in many countries including Vietnam [15, 16]. However, MG is still illegal to use in fish farms due to its low cost, easy availability, and effectiveness. The usage of MG in aquaculture in Vietnam for parasite and fungal disease treatments in brackish and marine aquaculture was reported [3]. However, the use and residue of MG and LMG for rainbow trout farming in Vietnam have not been reported yet.

Raw fish (e.g. sashimi) is a favorite dish widely served in restaurants around the world. In Vietnam, rainbow trout is also favored by fresh raw fish dishes. Eating raw or otherwise inadequately cooked fish can be associated with risks of acquiring fishborne zoonotic trematode (FZT) infection. An investigation of 483 specimens of nine freshwater fish species for FZT metacercariae in Ninh Binh province showed that all 9 fish species were infected with FZT, with a mean prevalence of 56.1% and a mean density of 33,245 [17]. A study on the prevalence of FZT infection in 180 fish farmers in Nam Dinh province reported that 32.2% of fish farms were infected with FZT [18]. A high prevalence of zoonotic trematodes was identified in more than half of the fish species investigated in Yen Bai province [19]. Similar to residues of antibacterial and banned substances e.g. MG, until now, there are no reports on the existence of FZT metacercaria in rainbow trout cultured in Vietnam.

2. MATERIAL AND METHODS

2.1. Study areas and sample collection

Rainbow trout samples were collected in three villages including Ngu Chi Son, Lao Chai, and San Sa Ho communes in Sapa town, Lao Cai province. These areas are the most concentrated cold water farming households of Sapa town in particular and Lao Cai province in general. In addition, rainbow trout samples were also collected at several restaurants in Sapa town for analysis of antimicrobial residue and FZT metacercaria presence. A total of 173 samples were collected from April 2019 to October 2022, divided into two periods:

- 1st period from 2019 - 2020: 79 fish samples from 15 farms and 6 restaurants were collected in seven investigations.

- 2nd period from 2021 - 2022: 116 fish samples from 21 farms and 4 restaurants were collected in 10 investigations.

The collected samples were stored in an icebox and transported to the National Agro-Forestry-Fisheries Quality Assurance Department (NAFIQAD) – Branch 1 in Hai Phong province for chemical residue analysis. FZT metacercaria samples were transferred to Research Institute for Aquaculture No. 1 (RIA1) for analysis.

2.2. Sample analysis

Antimicrobial residues including Chloramphenicol, Furazolidone (AOZ), Furaltadone (AMOZ), Ciprofloxacin, Oxytetracycline, Malachite green (MG), and Leucomalachite green (LMG) in fish were analyzed by a quantitative liquid chromatography–tandem mass spectrometric (LC–MS/MS) followed the NAFIQAD internal ISO-accredited methods: 05.2/CL1/ST 03.69 (AOZ, AMOZ), 05.2/CL1/ST 03.71 (Ciprofloxacin, Oxytetracycline) 05.2/CL1/ST 03.73 (MG, LMG).

FZT metacercaria was analyzed following the instruction of Phan T. V. and T. N. Bui (2013) [20]. Briefly:

Sample preparation: Fish was weighed before sample processing. Each fish was processed separately. The market size of fish ≥ 300 g was sliced vertically from head to tail, taking representative slices from different parts to make up a total of 100 g. Samples were ground in Pepsin solution (MERCK) in a preheated incubator at a temperature of 37^{0}C for 2 to 3 hours. The ratio of sample to pepsin solution was 2/3 (dissolve 6 g of pepsin in 1000 ml of distilled water, then add 8 ml of HCl). Samples were stirred with a glass rod for 15 - 20 minutes to ensure a uniform and complete digestion process. After complete digestion, samples were filtered by a metal sieve with a mesh size of 1 x 1 mm and physiological saline solution (0.85% saline). Allow the sample to settle after filtration and discard the supernatant from the glass beaker (approximately 1/2 of the volume). Continue adding physiological saline solution, allow it to settle, and discard the supernatant until the liquid in the glass beaker becomes clear.

Classification: Each portion of the sample liquid from the glass beaker was poured onto a Petri dish (12 x 12 mm) for examination under a dissecting microscope (Olympus CZ21). The FZT metacercaria species were classified directly based on their morphological characteristics using the classification key described by Phan T. V. and T. N. Bui (2013) [20].

2.3. Data analysis

Differences in antimicrobial residue contents between years were analyzed using SPSS statistical software (version 23, IBM Corp). First, the data were tested for normality using the Shapiro – Wilk test. If the normality condition was satisfied, ANOVA was used to determine whether there were any significant differences between the groups. A nonparametric Kruskal – Wallis was used if the normality condition was not satisfied. A significant difference was recognized when $p < 0.05$. Principal Component Analysis (PCA) was performed by Statistica 12 (StatSoft). The mean values of each chemical residue content in each investigated event were used for PCA analysis.

3. RESULTS AND DISCUSSION

3.1. Occurrence frequency of FZT metacercaria and antimicrobial residue in fish

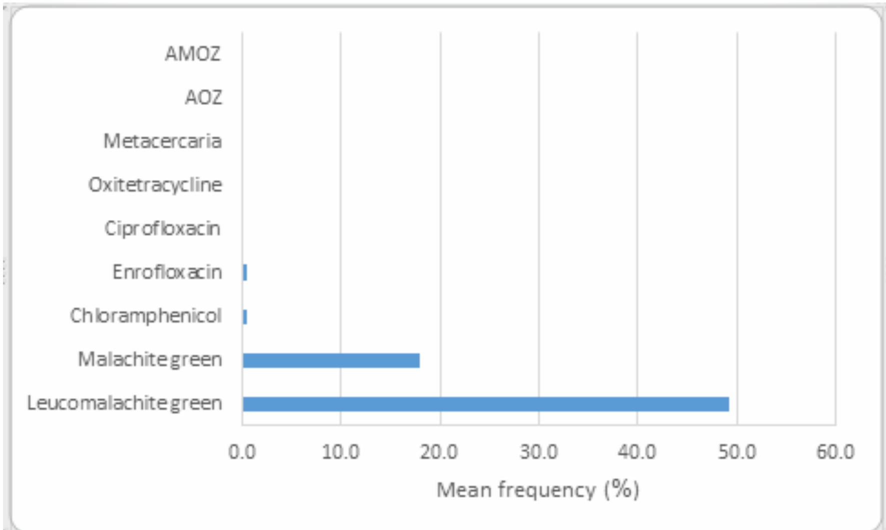

Figure 1a. Mean frequency of metacercaria and antimicrobial residues detection in fish in the period 2019 - 2022

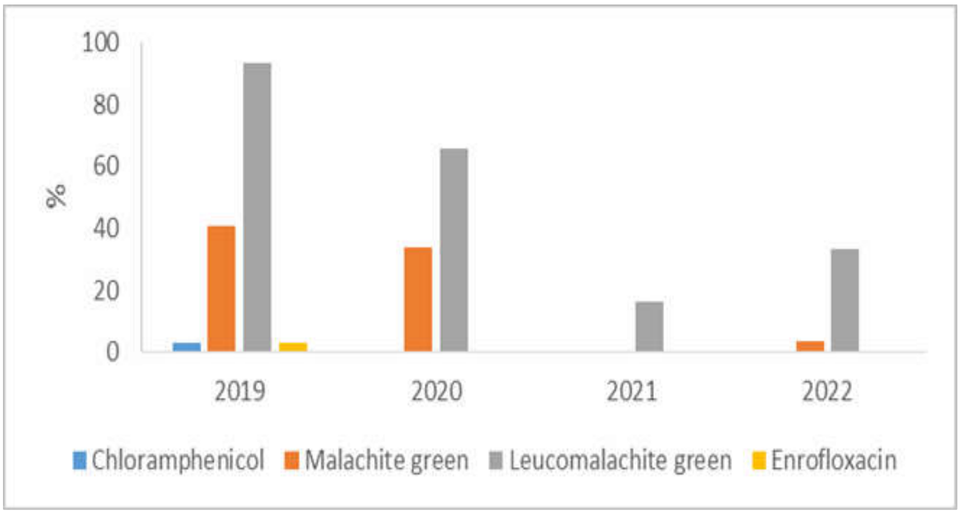

Figure 1b. Occurrence frequency of antimicrobial residues detection in fish from 2019 to 2022

Figure 1. Frequency of chemical residues detection in fish

From April 2019 to October 2022, we conducted 20 investigations, collecting a total of 173 fish samples. No AMOZ, ciprofloxacin, and FZT metacercaria were detected in fish tissue during the whole investigation. The analysis results showed that the residue of LMG was the highest, reaching 49.13% in fish meat, followed by MG at 17.92% (Figure 1a). Enrofloxacin and Chloramphelicon were only detected once in August 2019. Figure 1b demonstrates a obvlously decreasing trend in the chemical residue in fish meat. In 2019, the detected ratio of LMG was 93.75%, which decreased to 65.96%, 16.1%, and 33.33% in 2020, 2021, and 2022, respectively. Similarly, the detection rate of MG decreased from 40.63% in 2019 to 34.04%, 0%, and 3.51% in 2020, 2021, and 2022, respectively (Figure 1b). Previous works on FZT metacercarial infections have shown that many species of freshwater and brackish water fish play the roles as the source of FZT infections in humans [17 - 22]. The intermediate host, the snail, can play an important role in the transmission of these zoonotic diseases [23]. Farmed fish exposed to FZT cercariae released by snails are common in fish ponds [24]. The route of FZT transmission is more complex than in the presence of intermediate hosts, snails, in aquaculture ponds because the exchange of pond water with surrounding habitats such as rice fields and irrigation canals and these surrounding habitats can be a source of snails and free-swimming FZT cercariae, and contributes to FZT infection in farmed fish. However, FZT Cercariae emergence from snails was significantly reduced or stopped during the winter months [25]. One possible explanation for the absence of FZT in this study is that the rainbow trout culture environment is cold water, so there is little existence of snail species as an intermediate host. Moreover, rainbow trout are mainly raised in cement tanks or composite tanks, water is taken from the stream into the culture tank and flowed out, so the existence of snails in the tank is absent.

The presence of MG/LMG residues in fish samples was reported in several other studies. In Croatia from 2009 to 2011, MG residues were found in muscle tissues of 42 carp (*Cyprinus carpio*) and 30 rainbow trout (*Oncorhynchus mykiss*) samples collected from fish farms with a detected frequency of 68.1% [26]. In Malaysia MG and LMG were detected in muscle tissue of tilapia (*Oreochromis* hybrid), African catfish (*Clarias gariepinus*), barramundi (*Lates calcarifer*), hybrid grouper (*Epinephelus fuscoguttatus*% × %*Epinephelus lanceolatus*) and striped catfish (*Pangasius hypophthalmus*) with detected frequencies of 20 - 60% [27]. The Czech Republic reported 1.3% and 16.3% non-compliant results for MG and LMG, respectively, out of 80 aquaculture product samples. According to EFSA

2.5% and 8.4% MG/LMG samples were over the thresholds in Austria and Poland, respectively. Germany, Lithuania, and Spain reported 12.6% (out of 269 samples), 80% (out of 5 samples), and 100% (out of 2 samples) of non-compliant results for LMG in suspect samples, respectively. Lithuania and Spain reported 40% (out of 5 samples) and 100% (out of 2 samples) of non-compliant results for MG, respectively [28]. From 2017–2019, the Rapid Alert System for Food and Feed in the European Union (RASFF) recorded 11 cases of elevated concentrations of MG and LMG in fish and fish products. Among them, in 6 cases the products originating from Vietnam had exceeded the allowable amount, with the range of MG and LMG from 2 - 40.44 µg/kg in catfish and barramundi.

3.2. Chemical residues variation in rainbow trout tissue in Lao Cai province

10 investigations were conducted in 4 years from 2019 to 2022, with a total of 195 collected fish samples. Similarly to the trend in frequency of occurrence, the content of chemical residues also shows a decreasing trend during the period of 2019 to 2022 (Figure 2). The level of LMG tended to increase from April 2019 (169.0 µg/kg) to June 2020 (630 µg/kg) (Table 1). However, the level of LMG decreased significantly in 2021, with the highest detected level being only 0.7 µg/kg. In 2022, LMG slightly increased, ranging from 0.18 to 13.69 µg/kg. Malachite green fluctuated between 1.8 and 58.2 µg/kg from 2019 to 2020. In 2021, no LMG residue was detected in fish meat. In 2022, it was detected only twice, with the highest concentration being 0.45 µg/kg (Figure 2a). Table 1 shows that chloramphenicol and enrofloxacin were detected only in August 2019, with levels of 0.33 and 1.84 µg/kg, respectively. There were no detections of these two chemicals in the following years.

Table 1. The chemical residues in rainbow trout flesh investigated from 2019 - 2022

Sampling date	Malachite green (µg/kg)	Leucomalachite green (µg/kg)	Chloramphenicol (µg/kg)	Enrofloxacin (µg/kg)
April 10th 2019	4.62±9.71	169.04±287.89	ND	ND
May 30th 2019	11.20±29.07	357.23±677.63	ND	ND
August 12th 2019	6.42±11.0	394.07±723.65	0.33±087	1.84±4.88
March 18th 2020	58.16±101.29	580.87±1001.50	ND	ND
May 4th 2020	0.18±0.72	16.08±56.51	ND	ND
June 1st 2020	2.97±4.62	630.47±982	ND	ND
December 25th 2020	1.83±4.87	155.87±421.59	ND	ND
July 20th 2021	ND	1.11±1.88	ND	ND
September 24th 2021	ND	0.73±10.8	ND	ND

October 22nd 2021	ND	0.68±1.81	ND	ND
November 23rd 2021	ND	ND	ND	ND
December 20th 2021	ND	ND	ND	ND
January 19th 2022	ND	ND	ND	ND
February 15th 2022	ND	ND	ND	ND
March 15th 2022	ND	ND	ND	ND
April 18th 2022	0.45±1.18	3.74±1.87	ND	ND
May 17th 2022	ND	0.18±0.47	ND	ND
June 29th 2022	ND	0.33±0.76	ND	ND
September 7th 2022	ND	13.69±26.97	ND	ND
October 27th 2022	0.09±0.32	6.38±14.61	ND	ND

Note: ND: Not detected

According to EFSA (2020), exposure to food contaminated with MG/LMG at or below the Minimum Required Performance Limit (MRPL) of 2 µg/kg does not represent a health concern [29]. In our study, the mean content of MG and LMG was below the MRPL in 2021, with a content of 0.49 µg/kg. However, the mean contents of MG/LMG were higher than MRPL in 2019, 2020, and 2022, with levels of 304.6, 343.9, and 3.94 µg/kg, respectively. Although the malachite green and its metabolites significantly reduced in 2021 and 2022 compared to 2019 and 2020 ($p<0.05$). However, the contents of MG and LMG were still above the threshold in 2022. The results indicated that the content of MG and LMG has been well controlled recently. However, the situation is still a potential hazard to public health. The excessed MG residue in fish meat is widely observed in the world. Pipoyan *et al.* (2020) reported that 34.5% of investigated samples exceeded the MRPL in Armenia [30]. The investigation in Malaysia illustrated that residues of MG and LMG were highest in domestic striped catfish and lowest in red tilapia, with 8.1% of 149 samples above 2 µg/kg. The Czech Republic reported the LMG exceeded resides at levels of 7.73 - 13.4 µg/kg in rainbow trout originating from this country and Italy. Barani and Tajik. (2017) reported that the usage of MG in Iranian fish farms is frequent. MG was detected in 108 fish samples, corresponding to 61.0% of the total examined samples, ranging between 0.35 and 7.12 µg/kg. 18 out of 177 samples contained residue of MG higher than 2 µg/kg [31].

Chloramphenicol is a broad-spectrum antibiotic with well-known bacteriostatic properties effective against both Gram-positive and Gram-negative bacteria, and other groups of microorganisms [32]. Chloramphenicol can be quickly eliminated from fish tissue. Bilandžić *et al.* (2011) suggested that trout muscle tissue could be compliant with health requirements for consumption 10 days after withdrawal from chloramphenicol treatment [33]. In this study, the content of chloramphenicol was detected only one time at a concentration of 0.33 µg/kg, which may not harm consumers. Enrofloxacin is a fluoroquinolone antimicrobial agent. The antibiotic was used for rainbow trout against the main pathogenic bacteria *Aeromonas salmonicida*, *Yersinia ruckeri,* and *Flavobacterium psychrophilum* [34]. The content of enrofloxacin in our study was much lower than the level of MRL 100 µg/kg, which was set by the Commission of the European Union for the sum of the levels of enrofloxacin and ciprofloxacin in tissues from fishes in general [28]. With only one time detected during four years and low content, enrofloxacin is supposed not to be the chemical residue problem in rainbow trout culture in Lao Cai province.

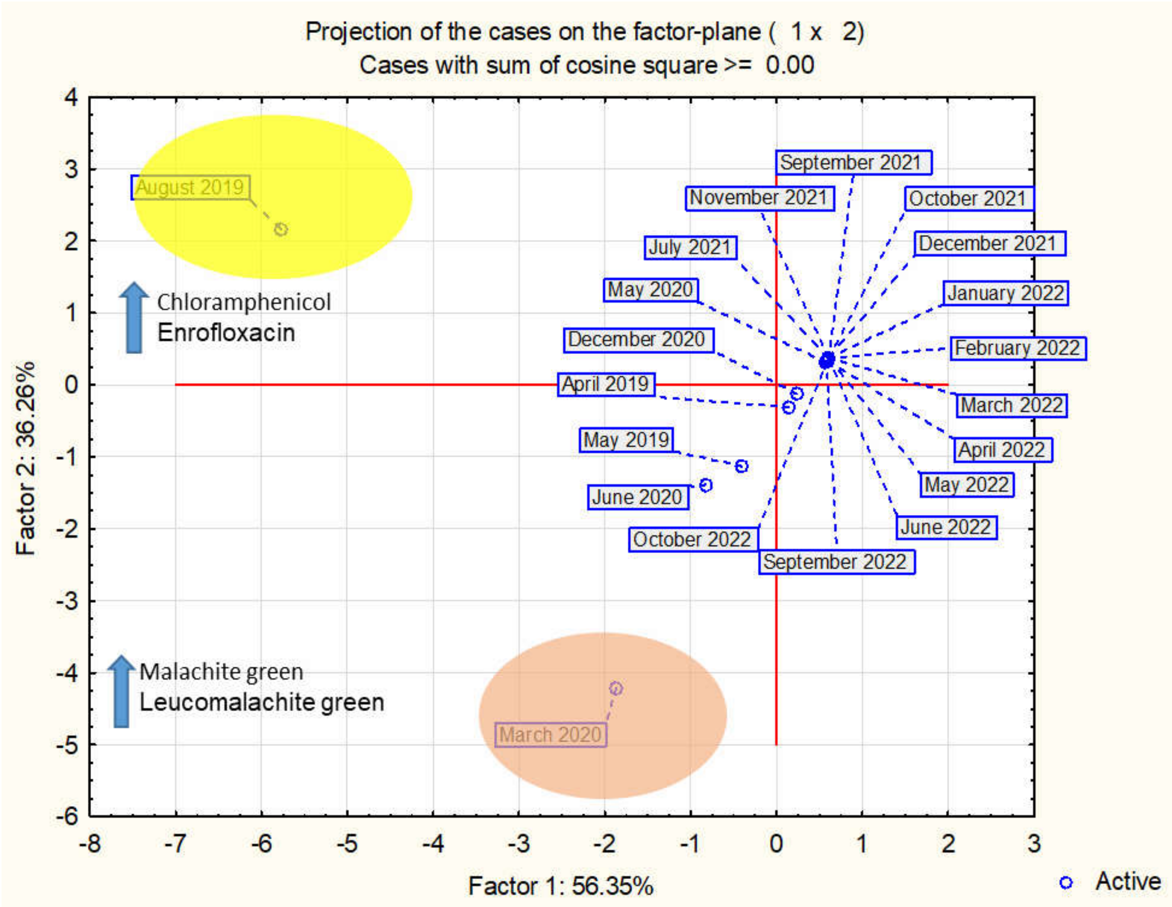

Figure 2. Principal component analysis of chemical residues in rainbow trout from 2019 - 2022.

Note: Principal component analysis (PCA) projection of cases (A) and variables (B) on factor 1 vs. 2. Variables are average concentrations of chemical residue in each investigation event.

We applied principal component analysis (PCA) to explore potential relationships between chemical residues and sampling time (Figure 2). The value of antimicrobial residue in each sampling event was labeled and presented in the form of month and year, for example, sampling on September 7th, 2022 was presented as September 2022. The projection of cases and variables on the principal component (PC) 1 and PC 2 plane in figure 2 shows a clear separation by time characteristics. Two PCs explain 92.6% of the total variance. Factor one was the negatively correlated content of LMG, MG, chloramphenicol, and enrofloxacin residues. Which is representative of a better chemical residue control situation in 2021 and 2022. While high content of enrofloxacin and chloramphenicol was seen in August 2019. High levels of LMG and MG were seen in March 2020. These results demonstrate significant achievements in our efforts to minimize chemical residues in rainbow trout. These accomplishments have been made possible with the support of ICI Project Phase III (2019-2022), funded by Finland, which has implemented a range of awareness-raising and training solutions. However, it is important to continue implementing measures to ensure that the levels of these chemicals remain within safe limits.

4. CONCLUSION

Leuko malachite green and malachite green are the most common chemical residues present in rainbow trout.

The leukomalachite green and malachite green content in rainbow trout decreased significantly in 2021 and 2022.

It is necessary to continue implementing measurements to effectively control the levels of chemical residues in rainbow trout.

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