

## CHARACTERIZATION ANALYSIS OF WILD BIRD POISONING CASES THROUGH SPRAYING SEEDS ARTIFICIALLY CONTAMINATED WITH PESTICIDES

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**Abstract.** The purpose of this study is to analyze the characteristics of wild birds fell victim to pesticides poisoning in South Korea. The main outbreak of pesticide poisoning intensively occurred in winter, when food is rapidly reduced, and hungry birds easily fell victim to food contaminated with pesticides, resulting in ecologically fatal mass mortality. From 2017 to 2018 in South Korea, 1379 wild birds fell victim to pesticide poisoning. In detail, 832 (60.33%) waterfowls such as Baikal teals, and 547 (39.67%) forest birds such as brown-eared bulbuls died. The major pesticides identified in wild birds died of acute pesticide poisoning were carbofuran, monocrotophos and phosphamidon. Taken together, it is pesticide poisoning caused by artificially spraying rice seeds contaminated with pesticides that gives rise to mass death of wild birds in winter in South Korea, and continuous public education and dedicated institutions are needed to prevent this accident.

**Keywords:** *carbamate, organophosphate, stomach contents, carcasses, avin*

### Introduction

Pesticides, which refer to all drugs used in agricultural work. The classification of pesticides is flexible, and it may change according to functions and forms, but it is generally divided into purpose of use, chemical composition, formulation, and toxicity. In particular, it is classified into inorganic pesticides, natural organic pesticides, and organic synthetic pesticides according to chemical components, and the most used organic synthetic pesticides are further classified into organic phosphorus (OP), organic chlorine (OC), and carbamate (CB) (Riah et al., 2014; Karami-Mohajeri et al., 2011). Pesticides minimize the loss of crops from pests, thereby increasing the yield and reducing the number of labor and working hours required for agricultural work, by improving work efficiency (Peshin et al., 2014). Despite this usefulness of pesticides, their extensive use in the environment is one of the major threats to wild animals and

poses serious problems, especially for wild birds (Brown et al., 1996). The exposure of wild animals to pesticides is largely divided into a long-term exposure to low concentrations (chronic exposure) and acute exposure due to artificial spraying (Mineau, 2005). In particular, acute exposure attributed to spraying pesticides on food to kill wild animals deliberately can cause mass mortality in certain areas within a short time (Grilo et al., 2021; Fernández-Vizcaíno et al., 2022).

From the perspective of managing chronic exposure of pesticides in ecosystems, interest in the toxic effects of pesticides on the ecosystem, began with the book of Rachel Carson (*Silent Spring*), led to establishing regulations, such as the Stockholm Convention, on the use of highly toxic pesticides (Gay, 2012). In accordance with the Stockholm Convention, several pesticide components have been designated as a prohibited and restricted substance such as dichlorodiphenyl trichloroethane (DDT) and toxaphene among persistent organic pollutants (POPs) (El-Shahawi et al., 2010; Xu et al., 2013). However, in terms of acute exposure, mass mortality of wild animals caused by pesticide spraying continues to occur worldwide (Goldstein et al., 1999). Although many bird conservation organizations are making various efforts to combat wild bird pesticide poisoning, the misconception about wild birds as a food source in specific areas and deliberate spraying of pesticides for disease control in the poultry industry remains unchanged (Mendelssohn and Paz, 1977; Mineau, 2002).

Wild birds in Korea have been killed off by various factors such as pesticide poisoning (Kwon et al., 2004), botulism bacteria (Son et al., 2018) and transparent window collision (Park et al., 2012). Particularly, mass mortality caused by pesticide poisoning occurs very frequently in winter, and even raptors including vultures are suffering from secondary poisoning (Kwon et al., 2004). Such phenomenon is attributed to disturbed habitats of migratory birds by ongoing and rapid industrialization, such as reclamation of tidal flats over the past few decades, as well as decreasing food resources directly threatening bird ecosystems (Zhai et al., 2020). For this reason, the Ministry of Environment of Korea enacted the Act on the Protection and Management of Wildlife in 2011, and in 2014, the field of wild animal disease was strengthened. However, pesticide poisoning continues to kill wild birds in South Korea. Therefore, the purpose of this study is to analyze the characteristics of wild bird population death due to pesticide poisoning and to propose environmental health risk management measures against deliberate application of pesticides.

## Materials and methods

### *Carcasses collection and preparation*

From January 2017 to December 2018, 1519 carcasses of wild birds were found in South Korea. Immediately after spotting the deaths in the field, the carcasses were examined and investigated for highly pathogenic avian influenza (HPAI) infection using the avian influenza virus antibody rapid test kit and transferred to biosafety Level3 (BL3) laboratory of National Institute of Environmental Research (NIER). The samples were observed for specific lesions through autopsy. The contents of the stomach were separated and stored at -20 until pesticide analysis. In addition, a detailed examination of HPAI infection was performed with sampling of swabs a cloacal/throat and spleen dissection for polymerase chain reaction (PCR) analysis. We classified the entire group as dead by pesticide poisoning when the average value of pesticide components in the stomach contents of the found wild bird carcass group exceeded the LD<sub>50</sub> value.

### ***Chemicals and reagents***

Standard materials and reagents were selected according to the chemical characteristics, such as organophosphate, organochlorine and carbamate, by referring to the USA PAN (Pesticide Action Network), and were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany) and AccuStandard, Inc (New Haven, CT, USA). Individual stock solutions were prepared by weighing out the powder or liquid, and dissolving them in 50 mL of HPLC-grade acetone (Sigma-Aldrich) for analyzing the pesticides. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method materials were obtained from commercial suppliers. For extraction contents of the stomach, Association of Official Analytical Chemists (AOAC International, USA) containing Na-acetate 1.5 g and MgSO<sub>4</sub> 6 g was used. For sample purification, EMR-Lipid dSPE (Enhanced Matrix Removal-Lipid dispersive Solid-phase Extraction, Agilent, USA) and EMR-Lipid Final Polish (2 mL centrifuge tubes containing MgSO<sub>4</sub> 1.6 g and NaCl 0.4 g Agilent, USA) were used.

### ***QuEChERS pretreatment method***

The pretreatment of samples was performed using the AOAC 2007.01 method of quick, easy, cheap, effective, rugged, and safe (QuEChERS, Association of Analytical Communities Official Method) (Lehotay, 2007) and the purification was performed using the EMR method (Han et al., 2016). For sample extraction, 5 g of the homogenized sample and 15 mL of acetonitrile containing 1% acetic acid were added to a 50 mL tube and soaked for 1 min. After that, extraction tube consisting of 1.5 g of Na-acetate and 6 g of MgSO<sub>4</sub> were added and soaked for 10 min at 250 rpm, and then centrifuged at 5000 rpm for 10 min. In the case of sample purification, after adding 5 mL of distilled water to 1 g of EMR-Lipid dSPE tube, EMR dSPE-lipid kit (NaCl:MgSO<sub>4</sub> = 2:8) was added, soaked for 1 min, and centrifuged for 5 min at 3500 rpm. After 5 mL of the supernatant was added to the EMR-lipid polish kit, vortexing and centrifugation were performed, and then the solvent layer was separated and analyzed according to the method.

### ***Pesticide analysis***

The pretreatment of the samples was performed using the AOAC 2007.01 method. The determination of pesticide was done by Thermo scientific TSQ 8300 EVO with Trace 1310 (GC-MS/MS) equipped with DB-5MS (30 m × 250 μm × 0.25 μm, J&W Scientific, USA). Helium (purity ≥ 99.999%) was used as a carrier gas with a flow rate of 1.0 mL/min. The injector temperature was 280°C and the oven temperature was programmed as follows: 60°C (hold 10 min) increased to 160°C at 20°C/min, raised to 300°C at 5°C/min (hold 4 min). The injection volume was 1 μL in splitless mode. The samples were ionized using positive electron ionization (EI) mode. Argon (purity ≥ 99.999%) was used as Q2 collision gas with a flow rate of 50 mL/min. Pesticide was identified and quantified in selected reaction monitoring mode at 45~650 m/z under the following condition: interface temperatures at 280°C, ion source at 280°C, and multiplier voltage of 1000 V, respectively. LC-Orbitrap analysis was performed using LCMS-8030 (Shimadzu, Kyoto, Japan) coupled to Nexera UHPLC (Shimadzu, Kyoto, Japan) with electrospray (ESI, positive mode). The analytical column was a Kinetex C18(100 X 2.1 mm i.d., 2.6 μm, Phenomenex, USA) and the column oven temperature was 40°C. The injection volume was 4 μL and the mobile phase was eluted at 0.2 mL min<sup>-1</sup>. Mobile phases were 0.1%

formic acid and 5 mM ammonium formate in water (A), and 0.1% formic acid and 5 mM ammonium formate in methanol (B). It was used by changing the ratio over time at a flow rate of 200 µl/min. The ratio of methanol was 0% until 2 min, and it was raised to 100% by 25 min, maintained for 5 min, and lowered to 0% by 35 min and maintained until the final 40 min.

### Data analysis

For analysis of regional characterization, the QGIS program (version 3.10.10-A Coruña) was used to analyze the found location of the wild bird carcasses and the wintering sites of wild birds. The information of winter migratory birds during November 16~17, 2018 on the Korean peninsula was analyzed based on winter waterbird census of Korea provided by the National Institute of Biological Resources. The measured results of the LC-Orbitrap were analyzed using instrument program and library. The accurate mass program used in LC-Orbitrap was performed using TraceFinder 3.3 (Thermo Scientific, MA, USA), and MS library patterns were compared using the NIST library owned in the equipment and the library created by the MZ Cloud website, and the isotope pattern and ddMS2 cleavage pattern were compared to confirm the target pesticides.

## Results and discussion

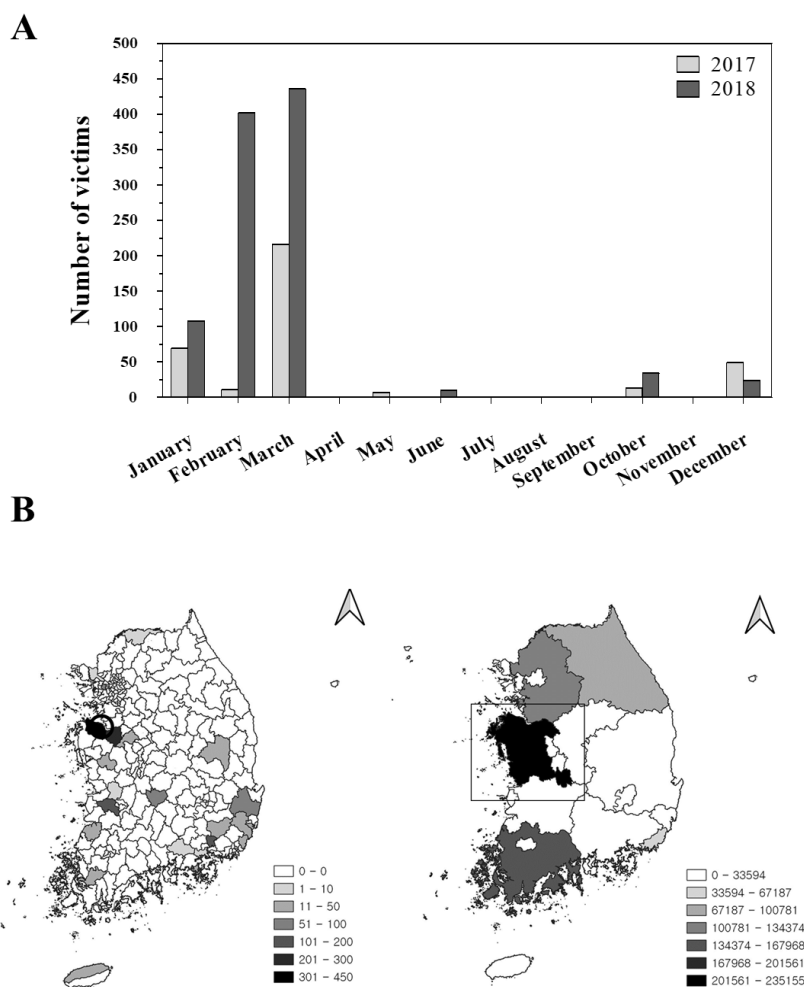
### Characteristics of dead bird clusters and avian species

From January 2017 to December 2018 in South Korea, a total of 1379 wild birds, including migratory birds and resident birds, have been killed with pesticides (Table 1).

**Table 1.** Summary of species of wild bird victims of pesticide poisoning from 2017 to 2018 in South Korea

Avian species	2017	2018	Total (%)
<i>Waterfowl</i>	55	777	832 (60.33)
Baikal teal ( <i>Anas formosa</i> )	8	560	568 (41.19)
Spotbill duck ( <i>Anas poecilorhy-ncha</i> )	40	82	122 (8.85)
Mallard ( <i>Anas platyrhynchos</i> )	-	102	102 (7.40)
Eurasian coot ( <i>Fulica atra</i> )	-	17	17 (1.23)
White-fronted goose ( <i>Anser albifrons</i> )	7	4	11 (0.80)
Whooper swan ( <i>Cygnus cygnus</i> )	-	7	7 (0.51)
Tundra bean Goose ( <i>Anser serrirostris</i> )	-	4	4 (0.29)
Common teal ( <i>Anas crecca</i> )	-	1	1 (0.07)
<i>Forest birds</i>	310	237	547 (39.67)
Brown-eared bulbul ( <i>Hypsipetes amaurotis</i> ),	174	69	243 (17.62)
Rook ( <i>Corvus frugilegus</i> )	69	86	155 (11.24)
Pigeon ( <i>Columba rupestris</i> )	52	27	79 (5.73)
Crow ( <i>Corvus corone</i> )	3	34	37 (2.68)
Vulture ( <i>Aegypius monachus</i> )	3	17	20 (1.45)
Sparrow ( <i>Passer montanus</i> )	9	-	9 (0.65)
Bohemian waxwing ( <i>Bombycilla garrulous</i> )	-	4	4 (0.29)
<b>Total</b>	<b>365</b>	<b>1014</b>	<b>1379 (100.00)</b>

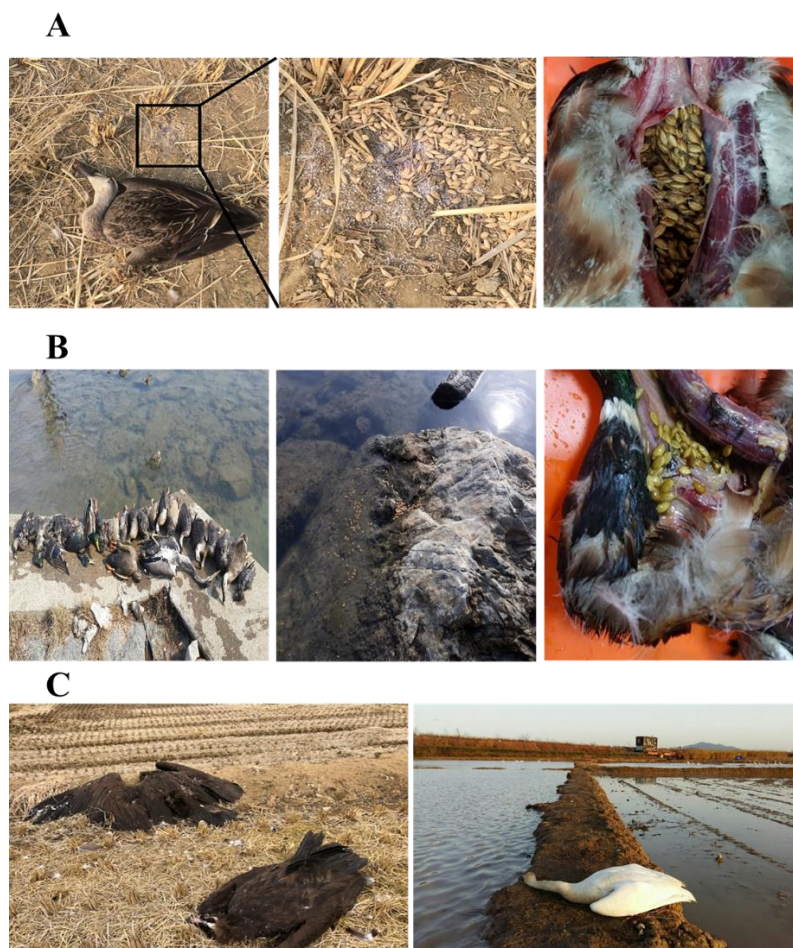
Among wild birds that died collectively, there were 994 (78%) migratory birds such as mallard and spot-billed duck, and 385 (28%) resident birds such as brown-eared bulbul. The main discovery period of dead birds is from January to March, during which more than 90% of them died of pesticide poisoning. Out of the total mass mortality, the number of occurrences from late spring to autumn was less than 5% (*Fig. 1a*).



**Figure 1.** Seasonal and regional analysis of wild bird victims due to pesticide poisoning on the South Korea from 2017 to 2018. (a) Seasonal distribution of wildlife victims. (b) Area analysis of mass death of wild birds (left) and winter waterbird census (right). The circle marks Sapgyo Lake and the rectangular box indicates the Chungcheongnam-do province

More than 1 million winter birds periodically visit and use the Korean Peninsula as over-wintering sites migrating from their breeding areas in Mongolia and Siberia approximately from October to March (Cha et al., 2015). This study found that it was mostly in the vicinity of wintering sites where dead migratory bird clusters occurred due to pesticide poisoning, and the forest birds were identified as park areas or agricultural areas near the city. The largest number of wild birds (620, 45%) died of pesticide poisoning in the Chungcheongnam-do where about 30% of winter migratory birds stay during a visit to South Korea in winter (*Fig. 1b*).

Table 1 shows the characteristics of wild bird species sacrificed with pesticides. The number of waterfowls sacrificed was 832 (60.33%), most of which were ducks, such as Baikal teals, spot-billed duck and mallards. In particular, 560 of Baikal teals were sacrificed intensively around Sapgyoho Lake for about 3 weeks from February to March 2018. Also, from February 12 to 18, 2018, 65 spot-billed duck were found dead in the urban farmland. As for forest birds, 547 were died due to pesticide poisoning, accounting for about 40% of the total victims. The main species were brown-eared bulbul, rook and pigeon. In some cases, where mass mortality was found, traces of artificially spraying pesticides on rice seeds were observed (Fig. 2a and b). Also, whooper swan and vultures designated as Near Threatened (NT) group by International Union for Conservation of Nature (IUCN), were also the victim of pesticide poisoning (Fig. 2c). In particular, vulture died from secondary pesticide poisoning.



**Figure 2.** Photo of the wild bird carcasses that died of pesticide poisoning. (a) Pesticide and rice seeds found around the carcass of a spot-billed duck in the field (left and center). Rice seeds found in the crop (right). (b) The carcass of a mallard found in a river and rice seeds contaminated with pesticides (left and center). Rice seeds found in the esophagus during autopsy (right). (c) Wild bird carcasses on the IUCN red list. Vulture (left), whooper swan (right)

Since HPAI occurs mainly in winter and is separated from migratory birds, wild birds are suspected as the main source of HPAI in Korea (Lee et al., 2008). HPAI is an

acute infectious disease of birds and causes great damage when infected in poultry (Swayne and Suarez, 2000). In particular, in 2014, when HPAI began to spread in large quantities in Korea, the government ordered all poultry farms across the country to temporarily suspend movement to prevent the spread of infection. Due to these measures, the poultry related industry suffered a lot of economic loss.

In a short period of time since the investigation of Sapgyo Lake where Korea's major HPAI virus was found (Swayne and Suarez, 2000), we also detected dead wild bird clusters affected by pesticides poisoning in the surrounding area of the Sapgyo Lake. Also, the area near Lake Sapgyo is one of the major wintering areas for migratory birds in Korea (Fig. 1b). Among the sacrificed wild birds, high levels of carbofuran (38~821 mg/kg) were found in the carcasses of vultures. Among the sacrificed wild birds, high levels of carbofuran were found in the stomach contents (MEAN: 38.67 mg/kg) and liver (MEAN: 0.24 mg/kg) of vulture carcasses, as well as in their vomit (MEAN: 8.33 mg/kg). Carbofuran has LD<sub>50</sub> values of 0.238 mg/kg to 12 mg/kg in various species of birds (Smith, 1993). Thus, these results suggest secondary poisoning by ingesting various ducks and geese and their carcasses, including Bikal teals (in stomach content: MEAN 485.33 mg/kg) and mallards (in stomach content: MEAN 204.34 mg/kg). In the case of forest birds that became victims of pesticide poisoning such as brown-eared bulbul and rooks, they were found to live in groups with very loud cries. Especially, crows have been conventionally considered an ominous sign and people tend to avoid them in Korea. Therefore, it is regarded that major deaths of the wild birds are largely attributed to spraying artificial pesticides in limited areas motivated from the above reasons.

#### *Analysis of detecting pesticides in carcasses of wild birds*

In wild migratory birds sacrificed from pesticide poisoning, 64.25% of the deaths were affected by carbamate pesticides such as carbofuran and 35.75% affected by organic phosphorus pesticides such as monocrotophos, respectively (Table 2). However, all carcasses of wild birds were confirmed negative as a PCR result of the avian influenza virus.

*Table 2. Analysis of pesticide found in sacrificed wild birds*

Group	2017		2018		Total	
	Victims	Range (mg/kg)	Victims	Range (mg/kg)	Victims	%
<i>Carbamate</i>	145		741		886	64.25
<i>Carbofuran</i>	127	4.4~696.3	733	2.2~6041.6	860	62.36
<i>Methomyl</i>	18	77.9~230.7	8	15.0~28.2	26	1.89
<i>Organophosphate</i>	220		273		493	35.75
<i>Monocrotophos</i>	101	2.1~104.3	75	12.7~389.4	176	12.76
<i>Phosphamidon</i>	119	27.9~106.0	54	1.7~767.2	173	12.55
<i>Fenthion</i>	-		120	14.5~650.1	120	8.70
<i>Terbufos</i>	-		24	0.5~479.8	24	1.74
<b>Total</b>	<b>365</b>		<b>1014</b>		<b>1379</b>	<b>100.00</b>

In 2017, approximate 50% of the migratory birds died from pesticide poisoning occurred in a colony of Baikal teals living in large herds, and the analysis of gastric contents confirmed that it was carbofuran poisoning that was a cause of death.

Carbofuran found in the stomach contents was detected at a very high concentration (max 6041.6 mg/kg, LD<sub>50</sub> 0.2~7 mg/kg) compared to other pesticides, and the rice seeds found together with the carcass were contaminated with high concentrations of carbofuran up to 5552 mg/kg. It is thought that this was deliberately sprayed to sacrifice the starving wild birds in winter. The detection ranges of the organophosphorus pesticide, monocrotophos, was 2.1 to 389.4 mg, while that of phosphamidon was analyzed to be 1.7 to 767.2 mg/kg. These concentrations mostly exceeded the LD<sub>50</sub>(oral) concentrations in birds for monocrotophos and phosphamidon are 0.94~6.5 and 3.6~7.5 mg/kg, respectively (Kim et al., 2016).

Before the Wildlife Protection and Management Act began to be strengthened in 2012 in Korea, the Animal and Plant Quarantine Agency (QIA) conducted a pesticide poisoning test on wild birds found across the country and announced the results of pesticides poisoning cases (Kwon et al., 2004; Kim et al., 2016). In previous studies conducted from 1998 to 2002 and from 2003 to 2013, 759 birds (Kwon et al., 2004) and 304 wild birds (Kim et al., 2016, 2008; Jang et al., 2010). for each respective year were found to be killed by pesticide poisoning. Organophosphorous pesticides such as phosphamidone and monocrotopus were mostly detected in carcasses sacrificed from 1998 to 2013, and the wild birds sacrificed were migratory birds of the waterfowl such as mallards and Baikal teals.

In this study, the major pesticides that caused acute poisoning were analyzed as carbamates. These results are different from the research results reported for about 20 years since 1998 in which organophosphorus pesticides such as monocrotophos and phosphamidon have been known as major poisoning-inducing insecticides. The reason for this difference is that in the 2018, 560 Baikal teals were sacrificed due to ingestion of rice seeds contaminated with a high-concentration of carbofuran intentionally sprayed near the wintering site. With the exception of the Baikal teal case, the damage from organophosphorus pesticide poisoning accounts for 35.75% of the total death, which is similar to previous studies.

The purpose of the introduction of pesticide positive list system (PLS) is to strengthen food safety management by controlling non-standard pesticides with a uniform standard of non-detection level and to manage each pesticide within the allowable daily intake (ADI) range. Carbofuran is an insecticide that requires careful handling due to its high toxicity, and has permeability to crops, thus being used for various pests that are difficult to control with soil mixing treatment (Gupta, 1994). In PLS, the use of carbofuran as an insecticide in leafy vegetables is prohibited, and its use in agriculture is restricted. Carbofuran granule has a similar size and shape of the seeds that birds eat (*Fig. 2a*). In addition, it has a high solubility (in water, 351 mg/L at 25°C) (Fenoll et al., 2013) and can be easily permeated into seeds in water. Therefore, given these physical chemical properties of carbofuran, the chemical may easily sacrifice wildlife in a way to spray with seeds, or directly contaminate seeds.

In other words, it is regarded that the high concentration of carbofuran, which was identically detected in the rice seeds around the carcasses and in the stomach contents of wild birds, clearly caused acute pesticide poisoning resulting in a mass death. After the mass death of wild birds due to pesticide poisoning, the Ministry of Environment has installed a notice board regarding the enhanced wildlife protection and management in areas where pesticides poisoning takes place and major migratory bird habitats, and actively informed not to spray poisons. However, despite such efforts, the arrest rate of pesticide sprayers is very low.



In order to solve the root cause, it is necessary to immediately obtain various information immediately from the field through the development of a systematic manual for responding to mass mortality of wild birds so that it can be utilized in the detailed epidemiological investigation in the future. In addition, it is necessary to continuously conduct pesticide poisoning monitoring tests, which are useful as a diagnostic method to identify the cause of wild bird death. Also, the diagnosis of heavy metals and botulinum other than pesticides should be included by securing precise analysis capability and expanding national facilities. In particular, if pesticide poisoning or avian botulism is confirmed in the case of group death of birds suspected of being infected with HPAI, it can be indirectly supposed that the cause of death is not related to infectious diseases, which can be a means to prevent wastage of national quarantine capabilities.

In summary, most of the wild birds that died from pesticide poisoning in Korea between 2017 and 2018 occurred in large quantities in a short period of time due to acute poisoning because of intentionally spraying pesticides over food. Also, until recently, many wild birds have been victimized by pesticide poisoning in Korea. Consequently, in 2020, a National Institute of Wildlife Disease Control and Prevention (NIWDC) was established with the aim to manage the outbreak of wildlife diseases, including HPAI and African swine fever. Therefore, in order to prevent accidents such as pesticide poisoning of wild birds, it is necessary to further develop One-Health system that manages people, animals, and the environment in an integrated manner and continuous education for those who have a wrong perception of wildlife. In addition, sample diagnosis and analysis for various wild animal diseases is required to continuously maintain a professional response in surveillance, epidemiological investigation, and quarantine along with systematic research such as technology development through NIWDC.

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