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Long-term toxicity of lindane through oxidative stress and cell apoptosis in *Caenorhabditis elegans*☆

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ABSTRACT

Lindane persists in the environment and bioaccumulates as an organochlorine pesticide and can pose risks to ecological environments and human health. To explore the long-term toxicity and underlying mechanisms of lindane, *Caenorhabditis elegans* was chosen as an animal model for toxicological study. The indicators of physiological, oxidative stress and cell apoptosis were examined in nematodes chronically exposed to environmentally relevant concentrations of lindane (0.01−100 ng/L). The data suggested that exposure to lindane at doses above 0.01 ng/L induced adverse physiological effects in *C. elegans*. Significant increases of ROS production and lipofuscin accumulation were observed in 100 ng/L of lindane-exposed nematodes, suggesting that lindane exposure induced oxidative stress in nematodes. Exposure to 10−100 ng/L of lindane also significantly increased the average number of germ cell corpses, which indicated cell apoptosis induced by lindane in *C. elegans*. Moreover, chronic exposure to 100 ng/L lindane significantly influenced the expression of genes related to oxidative stress and cell apoptosis (e.g., *isp-1, sod-3, ced-3, and cep-1* genes). These results indicated that oxidative stress and cell apoptosis could play an important role in toxicity induced by lindane in nematodes.

1. Introduction

Organochlorine pesticides (OCPs) have been extensively sprayed to abate the risk of pests and diseases (Saravanan et al., 2011). Lindane (γ-HCH), one of the highly effective OCPs, was widely used between 1950 and 1980 in medical and agricultural products (Li, 1999; Vijgen et al., 2011). After excessive use, lindane is regarded as a persistent organic pollutants (POPs) owing to slow hydrolyzation and degradation (Fang et al., 2017; Kathleen and Robert, 1999; Stockholm, 2010; Walker et al., 1999). Lindane can persist and cause pollution in a variety of environmental matrices, such as in water (20.9 μg/L), soil (28.76 ng/g) and sediments (67.1 ng/g) (Kovacik et al., 2018b; Mishra and RCS, 2013; Wei He, 2012; Yang et al., 2012). Moreover, the high bioaccumulation of lindane has an adverse influence for non-target organisms, such as fish, bird, and human beings, and draws serious attention to its toxic effects (Nandan and Nimila, 2012; Sudaryanto et al., 2005; Trukhin and Boyarova, 2019). Until now, lindane has been known to induce cytotoxicity, reproductive toxicity, neurodevelopmental toxicity, endocrine disruption, and even death (Agrahari et al., 2019; Croom et al., 2015; Khezri et al., 2017; Singh and Canario, 2004). Nonetheless, studies of the long-term toxicity and underlying mechanisms of lindane have been very scarce, especially in nonmammals exposed to environmental concentrations.

Caenorhabditis elegans (*C. elegans*), a non-parasitic animal, was an excellent model organism to evaluate the potential toxicity of xenobiotic pollutants (Liu et al., 2019). It has the unique characteristics of a short growth cycle, easy culturing, stimulus sensitivity, a well-described molecular background, and is highly homologous with human genes (Leung et al., 2008). It has been proven that the toxicity from exposure to toxicants is quite similar to that conducted in mammals, such as rats and rabbits (Ju et al., 2013; Li et al., 2013; Lumsden et al., 2016). In *C. elegans*, the toxic effects are evaluated by growth and locomotion behaviors, and the mechanisms of toxicity are assessed by ROS production, cell apoptosis and

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genes expression (Ding et al., 2018; Roh and Choi, 2008; Yang et al., 2016; Zhang et al., 2011; Zhou et al., 2016).

Although banned in recent years, the concentration of lindane ranging from 100 pg/L to 20.9 μg/L has still been detected in a variety of environmental water bodies (Abraham et al., 2017; He et al., 2012; Kovacik et al., 2018b; Lakaschus et al., 2002). In this paper, *C. elegans* were exposed to lindane at environmentally relevant concentrations (0, 0.01, 0.1, 1, 10 and 100 ng/L) for 10 d. The growth and locomotive behaviors were examined as physiological endpoints. Furthermore, the indicators of oxidative stress and cell apoptosis were investigated to explore the mechanisms in nematodes. This study highlights the potential risks of lindane in the environment and provides new insight into its underlying mechanisms in nematodes.

2. Materials and methods

2.1. Target strain for exposure and chemicals

Bristol N2 were cultured on nematode growth medium (NGM) plates with *E. coli* OP50 as a food source (Brenner, 1974). Synchronized nematodes were obtained using a bleaching mixture, and eggs were transferred to NGM plate with food (Donkin and Dusenbery, 1993). All nematodes were cultured at 20 °C in a sterile incubator.

Lindane was dissolved with dimethyl sulfoxide (DMSO) and diluted in K medium at the final DMSO concentration of 0.01% for all doses (Williams and Dusenbery, 1990). The nematodes of L4 larvae were exposed to lindane at nominal concentrations of 0.01, 0.1, 1, 10, and 100 ng/L for 10 days in 12-well sterile tissue culture plates. The nematodes were also treated with 0.01% DMSO as a vehicle control. All the exposures were performed in an incubator at 20 °C in the presence of food. Nematodes were fed UVC-killed *E.coli* to eliminate the potential confounding effects of bacterial metabolism. Moreover, a mitotic inhibitor was added to the culture to suppress offspring production during the exposure period. Subsequently, nematodes in every group were washed 3 times. Independent experiments were carried out in quadruplicate.

2.2. Evaluation of physiological indicators

Lethality was evaluated by the percentage of survival animals. After chronic exposure, inactive ones were scored under a dissecting microscopy. The nematodes will be judged to be dead if they cannot respond to the stimulus using a small metal wire. One hundred animals were examined per treatment. Body length is directly related to development in *C. elegans*, and locomotion behaviors are major indicators reflecting the effects on the nervous system. According to the previous description, these indicators were determined (Chen et al., 2019). After rinsing to remove impurities, fifty nematodes killed by heat were picked out and placed on a microscope (Olympus BX51, USA) for measurement of body length. Locomotion behaviors were externalized by frequency of head thrash and body bend. To assay body bends, fifty nematodes for each treatment were transferred into a second plate without food, and body bends were counted for 20 s by eyes under a dissecting microscope. A body bend was a change in the direction of the nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis. To examine the head thrash, fifty nematodes for each treatment were picked onto 60 μL of K medium on top of agar without food. A thrash was defined as a change in the direction of bending at the middle body.

2.3. Evaluation of oxidative stress and cell apoptosis indicator

After chronic exposure, nematodes were pre-incubated in different reagents. (1) To assess ROS production, nematodes were pre-incubated in 10 μM of CM-H_2DCFDA for 2 h at 20 °C. (2) To determine cell apoptosis, nematodes were treated with 25 μg/mL of acridine orange (AO) for 60 min at 20 °C without light and allowed to recover for 60 min on the bacterial lawns in order to repel the excessive dye in intestinal lumen.(3) To analyze lipofuscin accumulation, nematodes were put in 1 mM of levamisole. After pre-incubation, fifty nematodes for each treatment were put on 2% agar pads. The fluorescent images were obtained with an Olympus BX51 fluorescence microscope and analyzed by Image J to measure relative fluorescence intensity.

2.4. qRT-PCR

The purified total RNA was extracted by Trizol reagent, and the cDNA was synthesized by reverse-transcription with gDNA Dispelling SuperMix (Tiangen, China). The qRT-PCR was performed to test the expression of related genes by using Applied Biosystems StepOnePlus System. The results were analyzed by using the $2^{-\triangle\triangle Ct}$ method and expressed as the relative gene expression. All gene analysis for each concentration were carried out in triplicate. The primer sequences are shown in Tables S1 and S2.

2.5. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) when compared to the control. The statistical analyses were conducted using SPSS 18 software (SPSS Inc., USA). Significant differences were performed by analysis of variance (ANOVA) and the Tukey post-hoc test. Probability levels of 0.05 and 0.01 were considered statistically significant.

3. Results and discussion

3.1. Physiological effects of lindane exposure

After exposure to lindane, the physiological impacts were investigated in nematodes. As shown in Fig. 1A, chronic exposure to lindane at doses of 0–100 ng/L did not affect survival of *C. elegans*. In comparison with the control group, lindane at doses of 0.01–10 ng/L did not significantly affect body length in *C. elegans* after chronic exposure (Fig. 1B). On the contrary, a significant decrease of body length was observed when nematodes were exposed to lindane at a dose of 100 ng/L. In addition, 0.01–100 ng/L of lindane exposure significantly decreased head thrashes in nematodes (Fig. 1C). Similarly, exposure to 0.01–100 ng/L lindane obviously reduced body bends in *C. elegans* (Fig. 1D).

The above results show that both growth and locomotion behavior were suppressed after chronic exposure to lindane, which suggested developmental toxicity and neurotoxicity in nematodes. Previous studies have indicated that lindane exposure is harmful for the nervous system and triggers frequent changes of normal behaviors and the presence of abnormal behaviors in organisms (Mack et al., 2014; Saravanan et al., 2011). Similarly, lindane induced toxicity of the nervous system, such as rat and euryhaline fish (Croom et al., 2015; Nandan and Nimila, 2012). Recently, it has been consistently reported that the significant decrease of swimming behavior was detected in 0.5 mg/L of lindane-exposed zebrafish (Zhang et al., 2020). Therefore, the lindane-induced neurotoxicity could be one of the main toxicities in organisms. In addition, this study also shows that chronic exposure to lindane (0.01 ng/L) resulted in physiological effects in *C. elegans*. However,

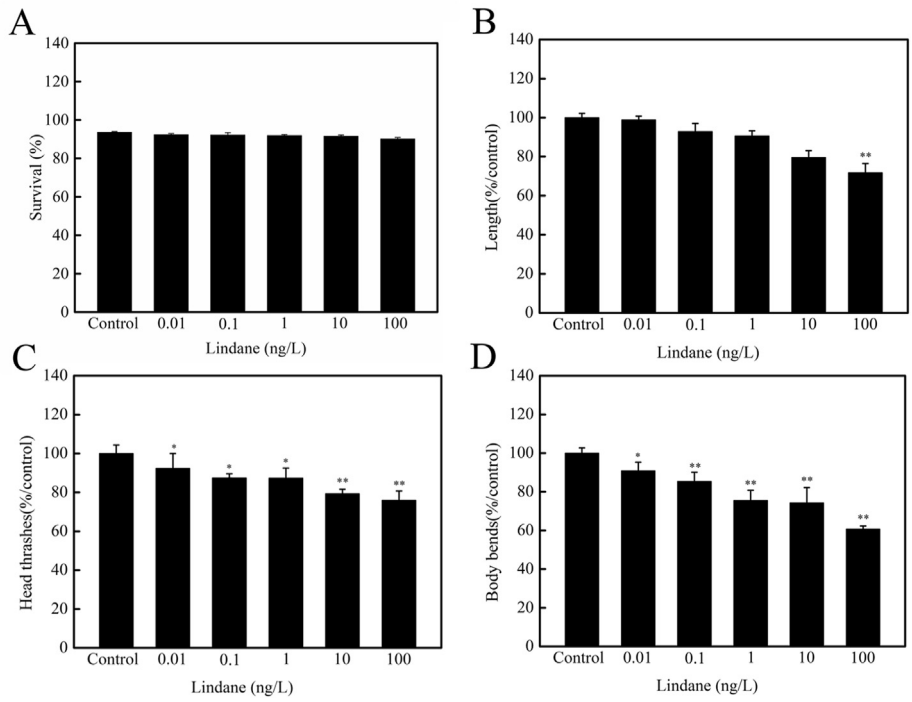

Fig. 1. Physiological effects of lindane treatments in *C. elegans*. (A) Survival. (B) Body length. (C) Head thrashes. (D) Body bends. *$p < 0.05$ or **$p < 0.01$ indicate significant differences.

the concentrations of lindane are much higher in the environment. For example, the concentrations of lindane were in the range of 0.6 ng/L to 20.9 μg/L in the environment (He et al., 2012; Kovacik et al., 2018a; Lakaschus et al., 2002). Therefore, special attention has been drawn to potential risks of lindane at environmental concentrations after chronic exposure.

3.2. Effects of lindane treatments on oxidative stress

Considering that oxidative stress is a major mechanism for the toxic effect of xenobiotic pollutants, ROS production and lipofuscin accumulation were assayed in nematodes. As shown in Fig. 2B and 10-100 ng/L of lindane exposure significantly enhanced the ROS production ($p < 0.05$) in nematodes. In addition, chronic exposure to lindane at a dose of 100 ng/L obviously elevated lipofuscin accumulation in nematodes (Fig. 2D).

Oxidative stress can be induced by an excessive increase of ROS in *C. elegans* (Mates et al., 2008), and lipofuscin accumulation is promoted by oxidative stress (Kim et al., 2013). Thus, ROS production and lipofuscin accumulation are frequently used as indicators of oxidative stress in nematodes. In this study, lindane exposure induced oxidative stress in nematodes. Lindane exposure (100 mg/kg body weight) caused oxidative stress by ROS generation in rats (Vijaya Padma et al., 2011). Similarly, exposure to lindane resulted in oxidative stress through increasing the production of ROS (Banerjee et al., 1999). Moreover, lindane exposure has been shown to trigger oxidative stress and result in toxic effects in various organisms (Guo et al., 2016; Hong et al., 2014; Shi et al., 2017). Therefore, oxidative stress could play an important role in the toxicity induced by lindane.

3.3. Effects of lindane treatments on cell apoptosis

A series of studies has indicated that oxidative stress induced by toxicants causes apoptotic cell death (Collins et al., 2008; Hunt et al., 2019; Krishnaswamy et al., 2010). Therefore, germ cell corpses were scored in lindane-exposed nematodes. As shown in Fig. 3B, chronic exposure to 0.01–1 ng/L of lindane did not obviously affect the formation of germ cell corpses compared with control; however, 10–100 ng/L of lindane exposure obviously increased number of germ cell corpses ($p < 0.01$) in *C. elegans*.

Environmental stress, such as through exposure to pesticides, is the main way to trigger cell apoptosis in *C. elegans* (Carlson et al., 2000; Kannan et al., 2000). Cell apoptosis, associated with oxidative stress, is an active gene-regulated process (Saradha et al., 2009; Vaux and Korsmeyer, 1999). In this paper, chronic exposure to lindane induced cell apoptosis in nematodes. A previous study indicated that treatment with lindane at higher concentrations (>75 μM) significantly increased apoptosis in the mouse thymocytes (Olgun et al., 2004). It has been reported that cell apoptosis could attribute to the toxicity of lindane in organisms (Olgun et al., 2004; Palmerini et al., 2017). Similarly, exposure to lindane changed the level of anti-apoptotic protein (Bcl-xL) in a dose-dependent manner, which ultimately caused cytotoxicity in rats (Zucchini-Pascal et al., 2009). Therefore, cell apoptosis might be involved in the toxic effects induced by lindane.

3.4. Effects of lindane exposure on genes expression

To further examine the molecular basis of oxidative stress and cell apoptosis in the regulation of toxicity, expression of related gene was investigated in *C. elegans* (Table S3). Chronic exposure to lindane (100 ng/L) obviously enhanced the expression of *skn-1, mev-1, isp-1, sod-1, sod-2, sod-3, sod-4,* and *sod-5,* but significantly reduced expression of *ctl-1* in *C. elegans* (Fig. 4 and Fig. S1). These genes play a key role in mediating oxidative stress in *C. elegans* (Yu et al., 2020). Regarding the genes required for cell apoptosis, chronic exposure to lindane significantly increased expression of *dpl-1, efl-1, efl-2, egl-38, ced-3, ced-4, ced-9,* and *pax-2*, but caused a significant reduction in the expression of *ced-13, cep-1, egl-1,* and

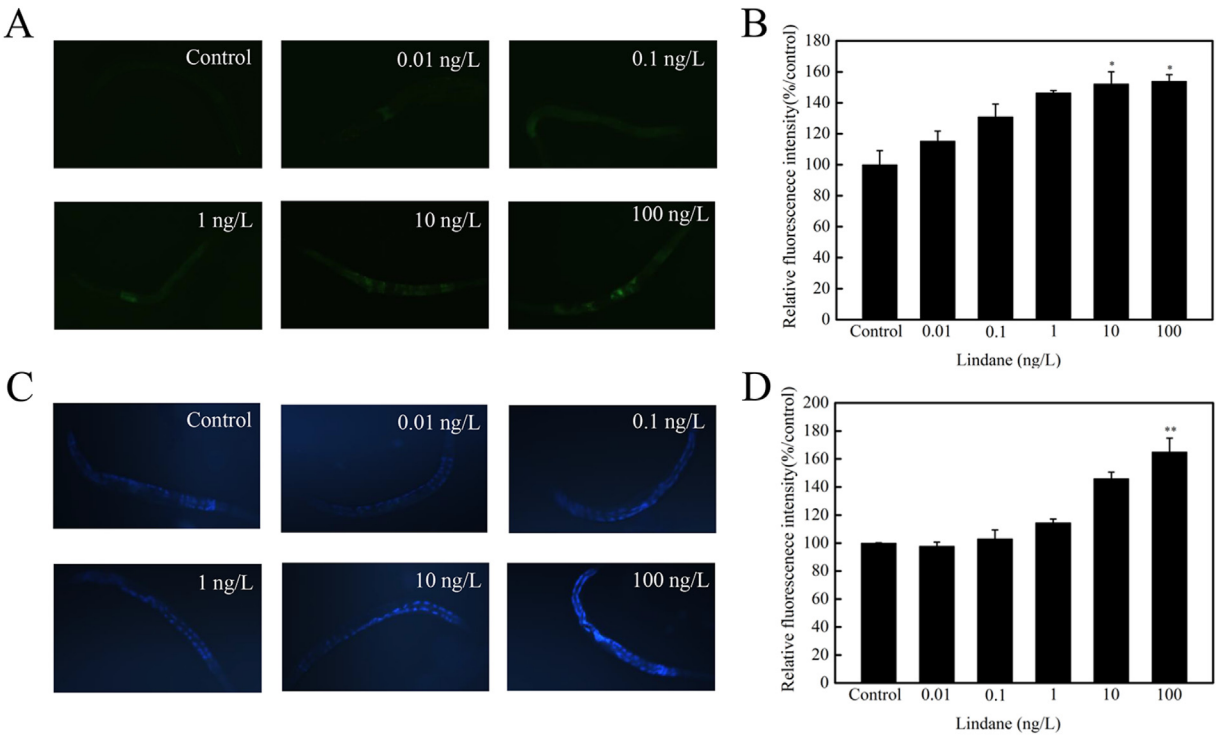

Fig. 2. Effects of lindane treatments on oxidative stress in *C. elegans*. (A) Representative images showing ROS production. (B) Comparison of ROS production. (C) Representative images showing lipofuscin accumulation. (D) Comparison of lipofuscin accumulation. $*p < 0.05$ or $**p < 0.01$ indicate significant differences.

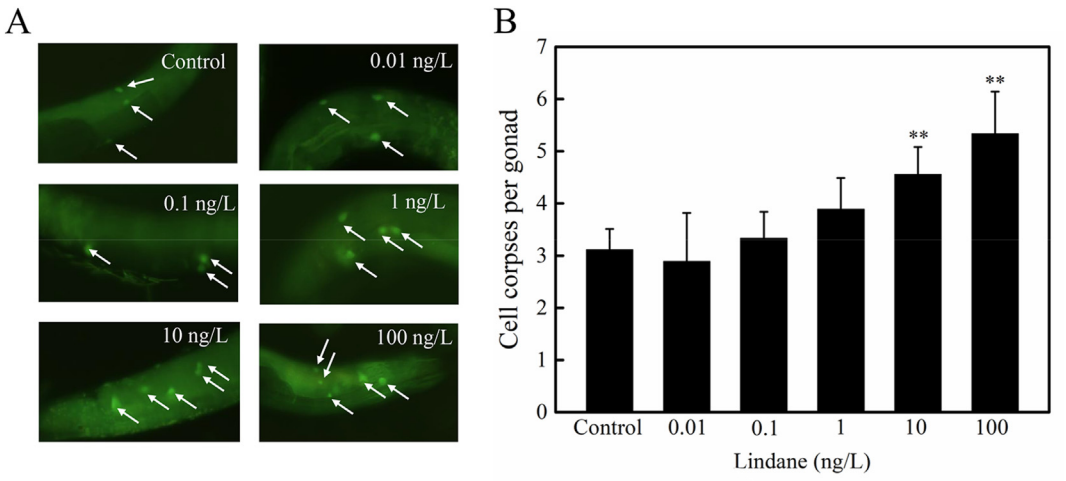

Fig. 3. Effects of lindane treatments on cell apoptosis in nematodes. (A) Representative pictures showing germline apoptosis. Arrowheads indicate the apoptotic cells in gonad. (B) Comparison of the number of apoptotic cells in unilateral gonad arm of nematodes. $**p < 0.01$ indicate significant differences.

sir-2.1 in nematodes compared to the control. It has been proven that these genes were involved in the apoptosis pathway in *C. elegans* (Polli et al., 2014).

The data showed that the expression of genes related to oxidative stress and cell apoptosis was significantly influenced in nematodes, which further suggests lindane exposure induces oxidative stress and cell apoptosis. Previous reports have shown that oxidative stress induced by lindane can alter the levels of related genes and enzyme activity (Parmar et al., 2003). Owing to the increase of ROS generation, activities of several antioxidant enzymes were decreased in organisms after lindane exposure (Hfaiedh et al., 2011; Vijaya Padma et al., 2011). Similarly, paraquat exposure significantly influenced the expression of *sod-3* in nematodes compared with control (Kronberg et al., 2018). Moreover, the significant increase in expression of the p53 gene were detected in mouse parietal granulosa cells exposed to 1–10 μM of lindane, suggesting cell apoptosis induced by lindane (Palmerini et al., 2017; You et al., 2018). To summarize, lindane exposure influences the expression of related genes, which illustrate that oxidative stress and cell apoptosis could play a critical role in lindane-induced toxicity in *C. elegans*.

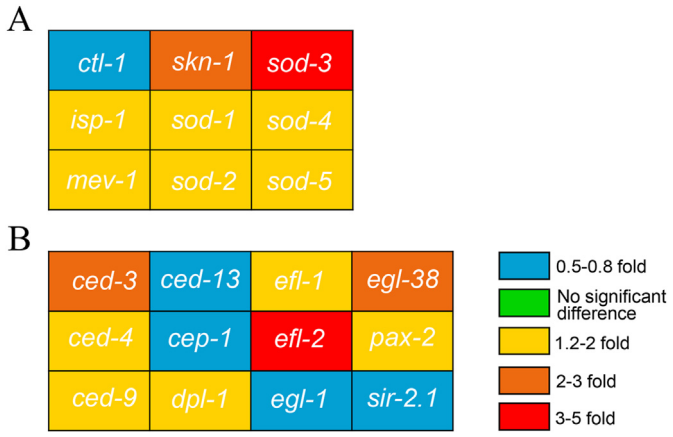

Fig. 4. Effects of lindane treatments on genes expression in 100 ng/L of lindane-exposed nematodes. (A) Expression of genes related to oxidative stress. (B) Expression of genes related to cell apoptosis. Gene expressions were normalized against *tba-1* gene and expressed as mean compared to the control (control = 1).

4. Conclusion

Chronic exposure to lindane at low dose caused adverse physiological effects concomitant with increase of the ROS production, lipofuscin accumulation, and germ cell corpses in *C. elegans*. The expression of genes related to oxidative stress and cell apoptosis was also significantly influenced in nematodes. Thus, the oxidative stress and cell apoptosis could play a critical role in toxicity induced by lindane in *C. elegans*. This study highlighted potential risks of lindane in the environment and provided an important insight into the mechanisms of lindane exposure in nematodes.

Author statement

Yunjiang Yu: Conceptualization, Resources, Writing- Reviewing and Editing, Haibo Chen: Writing - original draft, Investigation, Data curation, Xin Hua: Formal analysis, Investigation, Zhengdong Wang: Visualization, Liangzhong Li: Validation, Zongrui Li: Validation, Mingdeng Xiang: Validation, Ping Ding: Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116036.

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