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Cytotoxic and genotoxic effects of perfluorododecanoic acid (PFDoA) in Japanese medaka

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Abstract – This study investigated the cytotoxic and genotoxic potential of perfluorododecanoic acid (PFDoA), a perfluorinated carboxylic chemical (PFC) that has broad applications and distribution in the environment in Japanese medaka, *Oryzias latipes*. Micronucleus (MN) test and Comet assay were used for the toxicity study. Three groups of fish were exposed to 0.1 mg/L, 0.5 mg/L and 2.5 mg/L concentration of the chemical for 28 days. Another group served as control. Sampling of the fish blood and liver were done after days 1, 4, 7, 14, 21 and 28 for analysis of different erythrocyte abnormalities and damage to DNA using the MN test and Comet assay respectively. Results showed that there was a significant time and concentration dependent increase ($p < 0.05$) in percent tail length of DNA and frequency of erythrocyte abnormalities. Nuclear abnormalities observed include micronucleus, fragmented apoptotic cells, lobed nuclei, and bean-shaped cells. Increase in induction of erythrocyte abnormalities and percent tail length of DNA peaked at days 14 and 7, respectively, after which there was a gradual decline. The results indicate that sub-chronic exposure of PFDoA to Japanese medaka caused DNA damage with a simultaneous induction of different erythrocyte abnormalities.

Keywords: Comet assay / micronucleus test / DNA damage / PFDoA / Japanese medaka

Résumé – Effets cytotoxiques et genotoxiques de L'acide perfluorododecanoique (Pfddoa) sur Le medaka. Cette étude a étudié le potentiel cytotoxique et génotoxique de l'acide perfluorodécanoïque (PFDoA), un produit chimique carboxylique perfluoré (PFC) qui a de vastes applications et une large distribution dans l'environnement sur le medaka, *Oryzias latipes*. Le test du micronoyau (MN) et le test Comet ont été utilisés pour l'étude de toxicité. Trois groupes de poissons ont été exposés à des concentrations de 0,1 mg/L, 0,5 mg/L et 2,5 mg/L du produit chimique pendant 28 jours. Un autre groupe servait de contrôle. Le sang et le foie du poisson ont été prélevés après 1, 4, 7, 14, 21 et 28 jours pour l'analyse de différentes anomalies érythrocytaires et de dommages à l'ADN au moyen du test MN et du test Comet, respectivement. Les résultats ont montré qu'il y avait une augmentation significative ($p < 0,05$) de la longueur de la queue de l'ADN et de la fréquence des anomalies érythrocytaires en fonction du temps et de la concentration ($p < 0,05$). Les anomalies nucléaires observées comprennent des micronoyaux, des cellules apoptotiques fragmentées, des noyaux lobés et des cellules en forme de haricot. L'augmentation de l'induction d'anomalies érythrocytaires et le pourcentage de la longueur de la queue de l'ADN ont atteint un maximum aux jours 14 et 7 respectivement, après quoi il y a eu une baisse graduelle. Les résultats indiquent que l'exposition subchronique au PFDoA du medaka a causé des dommages à l'ADN avec une induction simultanée de différentes anomalies érythrocytaires.

Mots-clés : Test Comet / test du micronoyau / dommages à l'ADN / PFDoA / medaka

1 Introduction

The aquatic ecosystem is the final recipient of pollutants produced both naturally and from anthropogenic activities; and if these substances are allowed to accumulate and persist in the environment, life may become threatened (Fleeger *et al.*, 2003). The aquatic environment of countries that are highly populated receive a large quantity of waste either directly from agricultural activities, industry and urban settlements or indirectly as a result of airborne emissions being deposited in the atmosphere and this

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can cause contamination of the water bodies with complex chemicals (Frenzilli *et al.*, 2009).

One of the environmental pollutants of global concern is the perfluoroalkyl acids (PFAAs). The reason being that they are widely applied in fire retardants, lubricants, cosmetics and insecticides (Kennedy *et al.*, 2004). The constituent compounds found in these chemicals have differing lengths of chain including perfluorooctanoic acid (PFOA, C8), perfluorodecanoic acid and perfluorododecanoic acid (PFDoA, C12). PFAAs are not easily degraded due to their high energy C-F bond. As a result, they are persistent in soil, water, humans and wildlife (Kennedy *et al.*, 2004; Van de Vijver *et al.*, 2007; Tao *et al.*, 2008). According to Kennedy *et al.* (2004), experiments with animals have implicated PFDoA as the most toxic of all the 8–12 carbon chain length PFAAs, as such, environmental toxicologists and agencies have informed of the risk posed by this chemical to human populations and the environment at large.

Of the different carbon atom lengths of the perfluorinated organic chemicals, the PFOA and Perfluorooctane Sulfonate (PFOS) are the most widely studied. This is probably because of their surfactant and anti-wetting properties which makes them widely used in industrial products (Butenhoff *et al.*, 2006; USEPA, 2006). However, opinion varies on the genotoxic status of these perfluorinated carboxylic chemicals (PFCs). It was reported by Yao and Zhong (2005) that PFOA induced micronuclei in HepG2 cells and caused DNA strand breaks. It also increased 8-hydroxydeoxyguanosine (8-OH-dG) and intracellular reactive oxygen species (ROS). Earlier, Takagi et al., 1991 reported that PFOA induced 8-OH-dG in the liver of rat while Abdellatif (2003–2004) reported that PFOA does not significantly induce 8-OH-dG. PFOS was not reported to be genotoxic even though Kawamoto *et al.* (2008) reported that it induces a change in the potential of the membrane of paramecium, leading to an abnormal behavior in swimming. Due to these inconsistencies, Kawamoto *et al.* (2010) suggested the need to study PFOS and PFOA on different biological systems. Studying PFOA and PFOS alone in other living systems may not suffice as there are many other different carbon atoms of perfluorinated compounds. Hence, this study was designed to determine the genotoxic potential of PFDoA in the liver of Japanese medaka, *Oryzias latipes*.

PFDoA is used in the textile industry as a component of dye; as such easily find its way into the aquatic environment. Dyes are very visible when present in effluents and they can impact water aesthetics, turbidity and even the solubility of gas in the aquatic ecosystem receiving the effluents. Furthermore, certain water parameters are negatively impacted (Lanciotte *et al.*, 2004). The hazardous potential of textile effluents to the health of man and ecosystems has raised serious concerns. This is because there are some toxic substances in textile effluent such as surfactants, additives, detergents and dyes which can be terarogenic, mutagenic or carcinogenic to a wide range of organisms (Vanhulle *et al.*, 2008).

DNA damage is a key occurrence in carcinogenesis. Lord and Ashworth (2012) reported that DNA lesions occurring at specific genomic sites can cause changes in the sequence of nucleotide, resulting in mutagenesis and some other cellular responses. A sensitive, simple and well established test for identifying a wide spectrum of DNA lesions such as single and double strand breaks and alkali-labile sites in single cells is the (Singh *et al.*, 1988). Evaluation of the genotoxic potential of pollutants in the environment by analysing the DNA alterations in aquatic organisms has enjoyed wide acceptability, and it is a suitable method for detecting exposure in a broad range of species (Kolarević *et al.*, 2011; Rocco *et al.*, 2012; Sunjog *et al.*, 2012; Vuković-Gačić *et al.*, 2013). Because of its relevance as a very valuable fish biomarker, genotoxicity testing has been suggested to be a fundamental component of environmental risk assessment programmes (Van der Oost *et al.*, 2003). Furthermore, the micronucleus (MN) assay has been widely used as an all-inclusive method for evaluating damage in chromosome, which is scored specifically in once-divided binucleated cells containing micronuclei and other cell abnormalities. The frequency of micronuclei is a popular early cytotoxicity biomarker specifying chromosome breakage and/or total loss of chromosome (Xin *et al.*, 2014).

Many important reasons have contributed to the use of fish as indicator organisms in genotoxicity studies (Szefer *et al.*, 1990; Visn-Jeftic *et al.*, 2010). These include their position in the food webs, nutritive value to humans, ability to bioaccumulate toxic chemicals, sensitivity to low concentrations of mutagenic agents and even their aesthetic value. The kidney and liver are the major organs in animals for PFCs bioaccumulation (Hundley *et al.*, 2006). Additionally, the liver is the primary target organ for PFCs toxicity (Seacat *et al.*, 2003).

2 Methodology

2.1 Fish specimen and chemical

Matured female Japanese Madaka (*O. latipes*) embryos were hatched and raised for two months; first in large glass beakers, and thereafter, transferred into treatment tanks for acclimatization. The specimens had an average weight of 0.72 ± 0.04 g and average length of 2.7 ± 0.03 cm (±SD). The fishes were acclimatized in laboratory conditions using a continuous flow through system, where the water continually renews itself, waste and unused food flows out of the system. They were fed with commercial feed twice daily during this period. For this study, technical grade PFDoA was purchased. A stock solution of the chemical (50 mg in 1 L of distilled water), was prepared by dissolving in DMSO and mixing manually for 20 min and thereafter transferred into a sonicator for one hour to allow better dissolution of the chemical.

2.2 In vivo exposure

After a two week acclimatization period, they were exposed to three concentrations of PFDoA – 0.1, 0.5 and 2.5 mg/L prepared by taking the appropriate volumes from the stock solution of the chemical and making it up to 1 L using distilled water. Exposure was done as static renewal, with renewal done every 24 hr under the conditions of 16:h light: darkness. The toxicant was administered once every 24 hr to ensure its freshness. The fishes were divided into three groups with three replicates, fifteen fish in each tank, and forty five in each group. Some specimens were maintained in dechlorinated tap water and these served as the negative control. For the positive control, liver cells from *O. latipes* were treated with

2500 μM hydrogen peroxide for 30 min. The values of both negative and positive control used were based on the average of DNA damage from six samples on the first day. The experiment ran for a period of 28 days harvesting at intervals of 1, 4, 7, 14, 21 and 28 days. At each harvest, six fishes were sacrificed from each treatment group and from the positive and negative control for comparison, and their livers excised. Physicochemical parameters of the diluting water were monitored in this period.

2.3 Micronucleus (MN) assay

Blood samples from each group were collected by cutting the tails of the fish and taking blood in a heparinized microcapillary tube. The MN assay in erythrocytes was conducted following a modified version of the previously mentioned protocol (Udroiu, 2006). The peripheral blood erythrocytes from each fish were dropped onto three clean slides that were flattened by other slides to produce an evenly distributed blood smear, treated with a fixative (methanol) for 15 min at room temperature, air-dried, stained with 10% Giemsa in a phosphate buffer (PBS), washed twice with PBS and mounted. MN scoring was conducted on the cells that had been spread onto clean slides and air-dried. For micronuclei analysis, approximately 5000–8000 erythrocytes per concentration were observed at a 1000X magnification using an Olympus B50 fluorescence microscope. The frequencies of clearly outlined and typically shaped micronuclei in the peripheral blood erythrocytes were observed. The criteria, which were introduced by Fenech (2000), specified that scorable cells should be separate, easily distinguished and of approximately equal size. From this, the frequency of micronuclei was calculated and expressed as a percentage.

2.4 Comet assay

Alkaline Comet Assay was used to evaluate the genotoxicity of PFDoA in this experiment using a Trevigen Comet Assay Reagent Kit, USA for Single Cell Gel Electrophoresis Assay. Fish liver was chopped into pieces (1–2 mm^3), allowed to settle for 5 min and aspirated to get rid of medium. 1–2 mL of ice cold 20 mM ethylenediaminetetraacetic acid was added in 1X phosphate buffered saline PBS (Ca^{++} and Mg^{++} free), tissue was minced into very minute pieces and left to stand for a period of 5 min. The cell suspension was recovered, while preventing transfer of debris. Cells were counted, pelleted and suspended at 1×105 cells/mL in ice cold 1X PBS (Ca^{++} and Mg^{++} free). Cells were combined at 1×10^5/mL with molten Low Melting Agarose, at 37 °C at a ratio of 1:10 (v/v) i.e. 50 μL of cells in suspension at 1×10^5 /mL and 500 μL of molten agarose. From this, 50 μl was immediately pipetted onto CometSlide. Where necessary, side of pipette tip was used to spread agarose/cells over the sample area,ensuring total coverage of the sample area. Slides were placed flat at 4 °C in the refrigerator for 10 min. Thereafter, slides were immersed in 4 °C Lysis Solution overnight at 4 °C. Excess buffer was drained from slides and immersed in freshly prepared Alkaline Unwinding Solution at $pH>13$. The slides were allowed to stand in the Alkaline Unwinding Solution for 1 hr at 4 °C. The slides were placed in electrophoresis solution and made to pass through electrophoresis at 21 V for 30 min. Again, excess electrophoresis solution

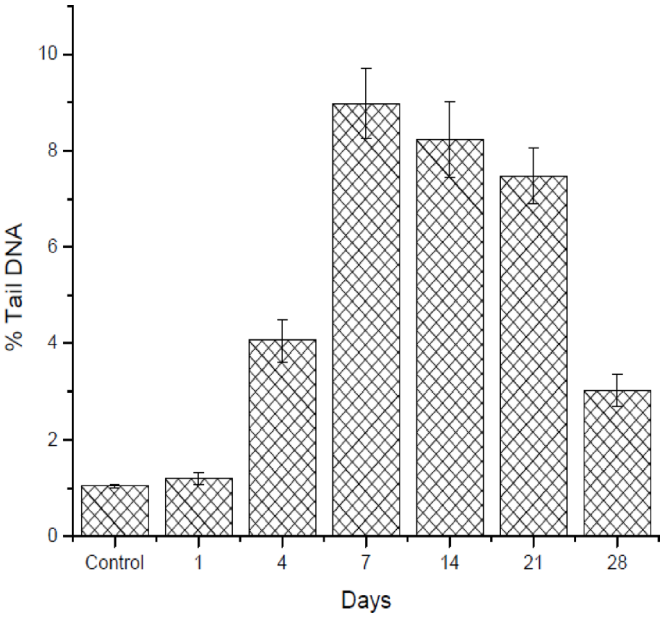

Fig. 1. Comparison of % Tail DNA and time at concentration 0.1 mg/L PFDoA.

was gently drained from slides, immersed two times in dH_2O for 5 min each, and then in 70% ethanol for 5 min. Samples were dried at 37 °C for 10–15 min to bring all the cells in a single plane for easier observation. 100 μL of diluted SYBR Green was placed onto each circle of dried agarose and stained for 30 min at room temperature in the dark. Slides were tapped softly to remove excess SYBR solution and briefly rinsed in water. They were then allowed to dry completely at 37 °C. Six specimens per concentration were observed. For each specimen, two slides preparation was done, 20 cells per slide, totaling 240 cells per concentration, were randomly scored. DNA damage was analysed using Trevigen comet assay kit. The percentage tail of DNA was adopted as the parameter for quantifying DNA damage.

2.5 Statistical analysis

One-way analysis of variance was employed using SPSS software (Standard Version 10.0) to compare the differences between means in % tail DNA in the different concentrations. Values were considered significant at 95% confidence level.

3 Results

Results of the physicochemical analysis of the test water used in the period of the experiment in the laboratory are as follows: Temperature – between 23.2 and 26.8 °C, pH – ranges from 6.04 to 7.14, Dissolved Oxygen – between 6.7 and 7.7 mg/L, Conductivity – 238–290 μs cm^{-1}.

DNA damage, expressed as % tail DNA was observed in the liver cells of fish in the period under observation. Each of the Figures 1–3 shows a time-dependent increasing damage in the DNA observed in fish liver cells after exposure to varying concentrations of PFDoA in the experiment. Nevertheless, damage to fish DNA was observed to be at the highest on day 7

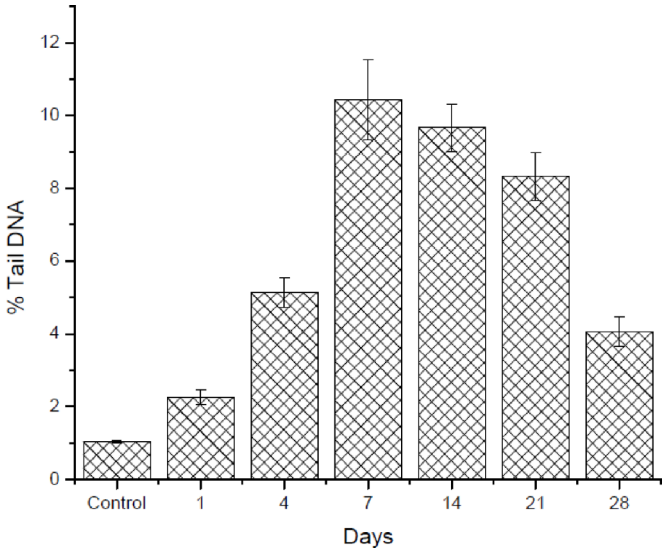

Fig. 2. Comparison of % Tail DNA and time at concentration 0.5 mg/L PFDoA.

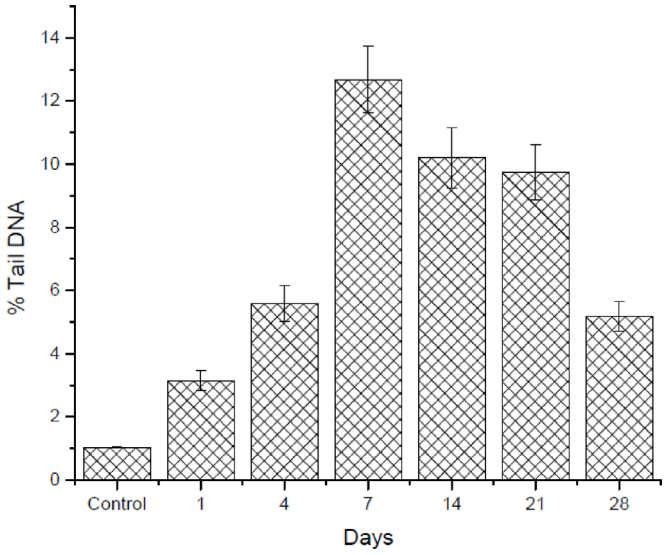

Fig. 3. Comparison of % Tail DNA and time at concentration 2.5 mg/L PFDoA.

in all the concentrations. After this period, DNA damaged was observed to reduce gradually. Also from the result, a concentration-dependent DNA damage was observed; the most pronounced damage noticed in the highest concentration. The tail length of DNA in fish exposed to the different concentrations of the chemical was higher in comparison with the values in the negative control. The positive control value also indicated a high degree of damage to fish liver cells. The respective values for the negative and positive control as calculated are 1.04 ± 0.03 and 6.15 ± 0.12. Some of the different comets observed are shown in Figure 4.

Similar to the results observed from the comet assay, PFDoA induced MN and some other cell abnormalities in fish blood. The frequency of MN and other forms of altered erythrocytes are presented in the table. Apart from MN, other nuclear alterations include lobed nuclei, fragmented apoptotic cells and bean-shaped nuclei. The different cell abnormalities did not show a consistent pattern of increase with time, however, total number of altered cells increased with increasing concentration till day 14 after which a decline was noticed. Furthermore, the frequency of altered cells increased with time.

Values with the same capital letter superscript, within the same week/fortnight are not significant while values with the same small letter superscript, against the same concentration are not significant ($p \geq 0.05$). (Mean values $\pm$ SE are for $n = 12$) during the observation period. These increases were significant ($p < 0.05$) in comparison with the negative control. The total number of the different cell abnormalities counted in the erythrocytes of both the control and exposed fishes is also presented in the table. This chemical shows potential to be an environmental toxicant.

4 Discussion

Assessing toxicity is important in determining how sensitive animals are to toxic agents, and can be used to measure the extent of damage to target organs and the resultant behavioral, biochemical and physiological alterations (Nwani *et al.*, 2010). That DNA damage is triggered off in the liver cells of *O. latipes* due to PFDoA exposure at different concentrations suggests its potential genotoxic and mutagenic properties. The negative control fishes had their DNAs intact, thus DNA damage can be said to be a result of the clastogenic action of the chemical. Environmental mutagen has been reported to increase both micronuclei and DNA migration in fish (Russo *et al.*, 2004).

Damage to the DNA of fish liver in the negative control group, as compared with those treated with different concentrations of PFDoA is low; hence the greater damage observed in exposed fish could only have been as a result of the toxic action of the chemical suggesting it to be genotoxic. Promoting DNA damage has been reported to be the first mechanism of action of genotoxic agents, which can possibly result in three outcomes: the damage can be repaired, the damage can become irreversible, or the damage may lead to cell death (Vicari *et al.*, 2012). According to Akcha *et al.* (2003), the absorption and biotransformation of genotoxic environmental pollutants could lead to the formation of DNA strand breaks in erythrocytes. Furthermore, the damage to fish DNA caused by PFDoA used in the present study might also have occurred due to the production of ROS. ROS such as hydroxyl radical (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), have been shown to produce damage such as strand breakage in DNA, enzyme inactivation and sometimes apoptosis (Peña-Llopis *et al.*, 2003; Banudevi *et al.*, 2006). Thus, it is possible that PFDoA could cause alterations in DNA of *O. latipes* resulting in formation of comets.

Reports from this study adds to public knowledge on the genotoxicity of the perfluorinated chemicals. As discussed above, the 8-carbon atoms of this group of compounds have been reported to both be genotoxic and non-genotoxic. There is the likelihood that PFDoA will also be toxic to previously studied living cells – human HepG2 and paramecium (and

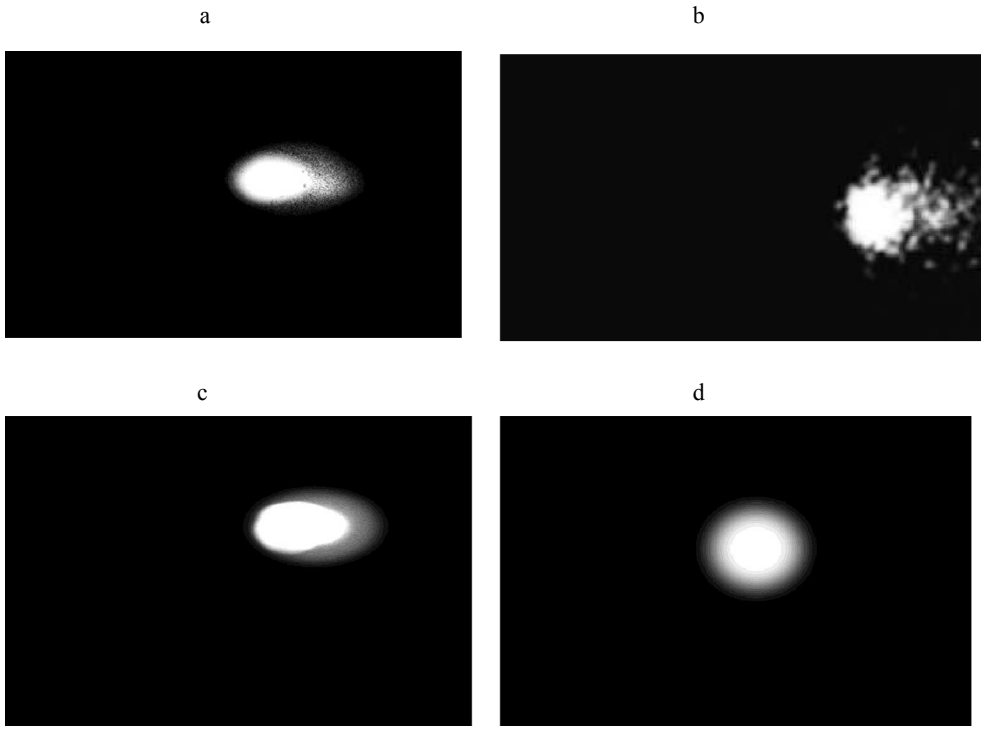

Fig. 4. Comets in fish DNA as a result of effects of PFDoA at concentrations (a) 0.1 mg/L (b) 0.5 mg/L (c) 2.5 mg/L and (d) 0.00 mg/L.

even other aquatic organisms) as it has been reported to be the most toxic of all PFCs. However, it will be interesting to see if with time, future genotoxic studies on PFDoA will report otherwise.

The reduction of the comets in DNA as observed in the tissues of fishes after day 7 may indicate repair of damaged DNA, loss of heavily damaged cells, or both (Banu *et al.*, 2001). The DNA repair systems may have led to this, and it could be explained using the threshold dependent repair theory. It proposes that the DNA repair enzymes get activated and that the rate of enzyme activity increases when a tissue accumulates toxicants above a threshold level, below which DNA repair operates only at a basal level (Ching *et al.*, 2001). Furthermore, knowledge of this will be a good starting point for environmental impact assessors even though further studies may still be essential.

An essential strategy for realizing better insight into the ability of organisms to repair damaged DNA, and other protective mechanisms for excreting the toxic chemicals may be a long-term genotoxicity study. Moreso, the absence of tail DNA at longer exposure as observed in this study could also be due to other mechanisms like toxicity of the contaminant preventing the enzymatic process of DNA damage (Rank and Jensen, 2003).

The result of the present study has shown that PFDoA has ability to cause different cell alterations in Japanese medaka (Tab. 1). It also showed that these alterations could be dependent on time and concentration. This result is similar to those reported by different researchers on the effects of different environmental pollutants in fish. The occurrence of a greater number of MN and other nuclear abnormalities in the treated fishes compared with the control in this study provides evidence of the cytotoxic potential of PFDoA. This probably means the fishes were under toxic stress. Because MN is usually given off along with the main nucleus, their presence would suggest their origin at a cell cycle that is more recent (Chandra and Khuda-Bukhsh, 2004). According to Jerbi *et al.*, (2011), the formation of micronucleated cells may be an indication of aneugenic and/or clastogenic actions, because the presence of MN can be related to entire chromosomes, caused by a malfunctioning of the spindle, or with chromosome fragments, derived from chromosome breakage.

This study showed progressive increase in the number of micronucleated erythrocytes and other abnormalities till day 14. This is in agreement with some (De Lemos *et al.*, 2001; Cavas *et al.*, 2005) studies that have reported decrease in the number of MN in fish erythrocytes after 14–21 of exposure. This could mean that DNA repair occurred after the day 14 onwards.

In conclusion, the present study has established the cytotoxic and genotoxic capability of PFDoA in fish. It also further proves the suitability of the MN and Comet assay as tools for evaluating potential environmental toxins. Because this chemical is a component of dye used in the textile industry, its hazardous potential to ecosystem and human health should be of great concern especially in countries with huge, active textile industry. The toxicity of the chemical to Japanese medaka provides a basis to project the potential harm that may be caused to other inhabitants of the ecosystem and those who depend on them. Therefore, it may be imperative to ensure careful, efficient use of this chemical so as to prevent adverse effects in the genetic components of aquatic ecosystems and man.

Conflict of interest

Authors declare that there is no conflict of interest in this research article.

Table 1. Time course numbers and frequencies of different cell alterations caused by concentrations of perfluorododecanoic acid in Japanese medaka.

Time (days)	Conc (mg/L)	Total number of cells scored	Alterations				Total number of Altered Cells	Frequency of Altered Cells (%) ±S.E
			MN	LN	FAC	B-SC		
1	0.00	6513	10	14	16	20	60	0.92 ± 0.026^{Aa}
	0.1	6798	15	23	23	21	82	1.21 ± 0.019^{Ba}
	0.5	7120	14	27	30	41	112	1.57 ± 0.036^{Ca}
	2.5	6907	20	37	31	26	114	1.65 ± 0.025^{Da}
4	0.00	6645	11	12	15	23	61	0.91 ± 0.017^{Aa}
	0.1	7343	16	24	30	48	118	1.61 ± 0.033^{Bb}
	0.5	6578	28	28	29	27	112	1.70 ± 0.028^{Cb}
	2.5	6998	20	32	46	32	130	1.86 ± 0.018^{Db}
7	0.00	6809	12	13	18	19	62	0.91 ± 0.031^{Aa}
	0.1	6787	20	30	37	34	121	1.78 ± 0.040^{Bc}
	0.5	7155	20	43	42	35	140	1.96 ± 0.060^{Cc}
	2.5	7483	24	46	37	44	151	2.02 ± 0.023^{Cc}
14	0.00	6850	12	12	16	20	60	0.87 ± 0.022^{Aa}
	0.1	7140	34	38	31	31	134	1.88 ± 0.013^{Bd}
	0.5	7151	32	54	36	42	166	2.32 ± 0.018^{Cd}
	2.5	6583	30	54	51	42	167	2.54 ± 0.030^{Dd}
21	0.00	5779	12	11	24	13	60	1.04 ± 0.013^{Aa}
	0.1	6782	20	40	27	25	112	1.65 ± 0.028^{Bb}
	0.5	7540	15	33	27	48	123	1.63 ± 0.028^{Cb}
	2.5	6987	21	35	58	28	142	2.03 ± 0.022^{Db}
28	0.00	6556	13	16	23	12	64	1.02 ± 0.025^{Aa}
	0.1	6494	13	28	14	26	81	1.25 ± 0.035^{Ba}
	0.5	7633	16	23	33	42	114	1.49 ± 0.028^{Ca}
	2.5	6735	25	32	37	21	115	1.71 ± 0.022^{Da}

MN: Micronucleus; FAC: Fragmented Apoptotic Cell
B-SC: Bean-shaped cell; LN: Lobed nucleus

Authors contribution

Ayanda Opeyemi Isaac carried out the experiment, analyzed the data, and prepared the draft manuscript

Min Yang monitored and supervised the progress of the experiment, analyzed the data and reviewed the draft manuscript

Zhang Yu monitored the progress of the experiment and reviewed the draft manuscript

Jinmiao Zha designed and supervised the experiment

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