Chapter 20
Genotoxic Effect of Amoxicillin on Peripheral Blood of Common Carp (*Cyprinus carpio*)

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20.1 Introduction

Along the history, mankind has created many different products to prolong and improve their quality of life, and most of them have resulted in efficiently accomplishing the task; however, a few of them have resulted in catastrophic results like thalidomide, methyl parathion, DDT, etc., which have triggered an international concern about the products we have created and the products we actually use daily that could represent a risk for the environment. In the light of the potential impact of these substances, a new definition has raised, emerging pollutants. The emerging pollutants are defined as synthetic or naturally occurring chemicals that are not commonly monitored in the environment but which have the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. In some cases, release of emerging pollutants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods were developed. In other cases, synthesis of new chemicals or changes in

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L. M. Gómez-Oliván (ed.), *Pollution of Water Bodies in Latin America*,
https://doi.org/10.1007/978-3-030-27296-8_20

use or disposal of existing chemicals can create new sources of emerging pollutants (Geissen et al. 2015).

Emerging pollutants are categorized in more than 20 classes related to their origin; one of the most prominent classes is the pharmaceutical. Within the pharmaceutical class, we found a humongous group, the antibiotics, composed of a diverse amount of structures, functional groups, uses, and spectra. The occurrence and fate of antibiotics in the environment has become the subject of recent investigations due to their potential health effects on nontarget species as well as the increased emergence of resistant pathogenic bacteria (Mojica and Aga 2019). Therefore, it is not a surprise that they have been detected in the μg/L range in different water bodies around the world (Johnson et al. 2015; Kummerer 2003; Zheng et al. 2012; Watkinson et al. 2009; Gibs et al. 2013).

This work particularly focuses in a semi-synthetic broad-spectrum antibiotic used worldwide in high amounts. Amoxicillin, an antibiotic, can enter into the aquatic environment through diverse pathways; it could be from point sources as effluents of manufacturing plants and effluents from hospitals but also from nonpoint sources as municipal effluents from households, effluents from wastewater treatment plants, disposal of unused or expired compounds in the drainage, runoff from fields where animals have been treated, direct dosage of antibiotics in the water for aquaculture, and so on (Elizalde-Velázquez et al. 2017; Sarmah et al. 2006).

Besides its global high consumption (Elizalde-Velázquez et al. 2016), amoxicillin is excreted unchanged in urine ($\leq$ 85%) and feces ($\leq$ 10%) of humans and animals treated with the bactericide compound, which means a huge and continual disposal of this emerging pollutant in environmental waters. In a past work, we described the oxidative damage produced by amoxicillin in different organs of *Cyprinus carpio* once it was transformed by biotic factors into amoxicilloic acid (Elizalde-Velázquez et al. 2017); our work sums to other past studies that also described the toxic effects of amoxicillin in diverse aquatic organisms. Andreozzi et al. (2004) described that amoxicillin induced high toxicity in the blue algae *S. leopoliensis*; Oliveria et al. (2013) report that the antibiotic induces premature hatching, oedemas, and malformations in embryos of *Danio rerio*, as well as alterations in its normal enzymatic activity; and Liu et al. (2015) found that amoxicillin induced toxicity in the cyanobacteria *Microcystis aeruginosa* after a short-term exposure.

Due to its oxidative damage, it may be possible that this bactericide compound could also be potentially genotoxic. In fact some previous studies that test the genotoxic activity of this compound have reported positive results in mammalian cells. Arabski et al. (2005) reported that amoxicillin induces strand breaks and base modification in DNA of human peripheral blood lymphocytes and in *Helicobacter pylori* using the conventional comet assay. Li et al. (2007) described the damage produced by this beta lactam antibiotic in the DNA of human AGS and NB4 cell lines and in Chinese hamster cell lines, possibly by intracellular induction of reactive oxygen species, and more recently, it was reported the genotoxic activity of this compound in an aquatic species. Anlas and Ustuner (2019) reported that amoxicillin induces

DNA damage in *Oncorhynchus mykiss* erythrocytes due to an induction of reactive oxygen species and a deficient DNA repair activity of rainbow trout; however, some other studies differ with this results. Cahill et al. (2004) reported negative result measuring the genotoxic effect of amoxicillin using the green screen assay, and Istifli and Topaktas (2009) reported that amoxicillin does not exert genotoxic effects in human peripheral blood lymphocytes in vitro.

The aim of this study was to evaluate the in vivo genotoxic effect induced by three different concentrations of amoxicillin (10 ng/L, 10 μg/L, 10 mg/L) on the peripheral blood of the freshwater teleost fish *C. carpio* using the comet assay, so the genotoxic information of this emerging pollutant in aquatic species could be extended.

20.2 Materials and Methods

20.2.1 Test Substance

Amoxicillin trihydrate (CAS number 61336-70-7, >98.0% purity) ($C_{16}H_{19}N_3O_5S{\cdot}3H_2O$), 365.40 Da, was purchased from Tokyo Chemical Industry Co., LTD.

20.2.2 Specimen Collection and Maintenance

The test was carried out on 330 healthy adult common carps (*Cyprinus carpio*) with an average length of 17.93 ± 0.46 cm and an average weight of 48.72 ± 6.4 g, obtained from the aquaculture facility in Tiacaque, State of Mexico. Carps were acclimated to test conditions for 30 days prior to the experiment, temperature between 20 ± 2 °C, oxygen concentrations between 85 and 90%, water pH between 7.5 and 7.8, and natural light/dark photoperiods. During acclimation, carp were fed with Pedregal Silver™ fish food, and three-fourths of the tank water was replaced every 24 hours in order to maintain a healthy environment.

20.2.3 Experimental Design

For the test, we used static systems maintained at room temperature with natural light/dark photoperiods, provided with constant aeration and no food to specimens during the exposure period of time. For the genotoxic evaluation, amoxicillin was tested in three different concentrations (10 ng/L, 10 μ/L, 10 mg/L). The ng/L and μg/L concentrations are based on environmental reported data, while the mg/L concentration was

used to monitor the amoxicillin behaviour by analytical techniques (Elizalde-Velázquez et al. 2017). For each concentration, a kinetic of 12, 24, 48, 72, and 96 h was run, each system with six carps, and the assays were performed in triplicate. A free amoxicillin system with six carps was set up for each exposure time as control group, and a cyclophosphamide system was used only at 10 mg/L as a positive control. At the end of each exposure period of time, peripheral blood was obtained from anesthetized specimens with clove oil (Yamanaka et al. 2011) by puncture of the caudal vessel.

20.2.4 Analytical Measurements

The analytical analysis of amoxicillin and its degradation products in the blood of *Cyprinus carpio* was performed using a LC-10 AD system coupled to a L-ECD-6A electrochemical detector and a SPD-M10A diode array detector (Shimadzu, Kyoto, Japan) fitted to a Rheodyne injection valve (20 µL sample loop), using a Phenomenex Synergi Hydro-RP HPLC column (150 × 4.60 mm, 4 lm). The procedure was adapted from Gozlan et al. (2013a, b), with the following settings: injection volume 20 µL, flow rate 1 mL/min, column temperature 28 °C, and mobile phase isocratic 95/5 (v/v) water [pH adjusted to 2.5 with 99% trifluoroacetic acid (TFA, spectrophotometric grade)] and methanol (HPLC grade). Data was recorded at 230 nm UV absorption. The settings for the EC detection were adapted from Brooks et al. (1981): EC detector was operated in direct mode at +1.17 V vs. an Ag/AgCl reference electrode.

20.2.4.1 Water

Samples of 10 mL were collected from each test systems and frozen at −20 °C until its analysis by the analytical techniques. Samples were brought to room temperature 1 hour before its analysis and mixed in a vortex mixer. For the water analysis, we do not perform any treatment prior its injection in the HPLC. Amoxicillin was used as the standard for the determination of the retardation factors (RFs). Gozlan et al. (2013a) use this technique since the RFs of amoxicillin and amoxicilloic acid are very similar. Based on this assumption, two calibration curves (0.01–10 mg/L) were constructed for amoxicillin and its analysis in the UV and EC detectors.

20.2.4.2 Blood

For the analytical analysis of the blood of *Cyprinus carpio*, we used the homogenization-deproteinization method of Brooks et al. (1981); fresh blood samples were homogenized with buffer phosphate solution and stored protected from the light at −20 °C. One hour before the analysis, frozen samples were brought to room temperature; each sample of 0.2 µL was mixed with 150 µL of Milli-Q water and 50 µL of perchloric acid at 70% (Merck, Germany). Each mixture was centrifuged after a thorough mixing on a vortex mixer; the centrifugation setting was

2000 × g and 4 °C for 5 min on a Hermle Z233 MK2 centrifuge. The supernatant was removed and then analysed by HLPC-EC-UV.

20.2.5 Comet Assay (Tice method et al. 2000)

The comet assay was performed according to Tice et al.'s (2000) methodology with some modifications. Peripheral blood was diluted with cold phosphate buffer solution (1:15); then 25 μL of the past solution was mixed with 75 μL of normal melting point agarose (0.7%); this mixture (100 μL) was then spread into the slides. To solidify the agarose, the slides were immediately protected from light and kept at 4 °C for 15 min; once the agarose was solidified, the slides were immersed in cold lysing solution (2.5 M NaOH, 10 M ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 10% dimethyl sulphoxide (DMSO), and 1% Triton, at pH 10) for 1 hour at 4 °C and protected from light.

Then slides were placed in an electrophoresis chamber for 20 minutes with a cold alkaline solution (300 mM NaOH and 1 mM EDTA) at pH 13, protected from light, to allow the unwinding. Electrophoresis was performed at 300μAmp, 25 V, and pH > 13 for 20 min. Slides were then stopped and washed three times with a neutralization buffer (0.4 M trizma base) at pH 7.4. Finally, the slides were stained with 20 μL of ethidium bromide and were examined with an epifluorescence microscope attached to an image analyser equipped with a program for measurement of the cell nucleus. A total of 100 measurements per triplicate per sample were made, and the %DNA damage in the tail (T/N) was obtained measuring the length of the tail (T) and the width of the nucleus (N). Measuring was done with a Zeiss Axiophot KS400 microscope equipped with epifluorescence and a 510–560 nm filter.

20.2.6 Statistical Analysis

Results of the comet assays were statistically evaluated by one-way analysis of variance (ANOVA), and differences between means were compared using the nonparametric tests Kruskal-Wallis and Dunn, with P set at <0.05. The differences with respect to time were tested. Statistical determinations were performed with SPSS v10 software (SPSS, Chicago IL, USA).

20.3 Results

Figure 20.1. It shows the results of the comet assay in lymphocytes of *Cyprinus carpio* exposed to 10 ng/L, 10 μ/L, and 10 mg/L of amoxicillin. The figure shows clearly a concentration-dependent increase in the groups exposed at 12 and 48 hours. For the lowest concentration (10 ng/L), an increase compared to the control group

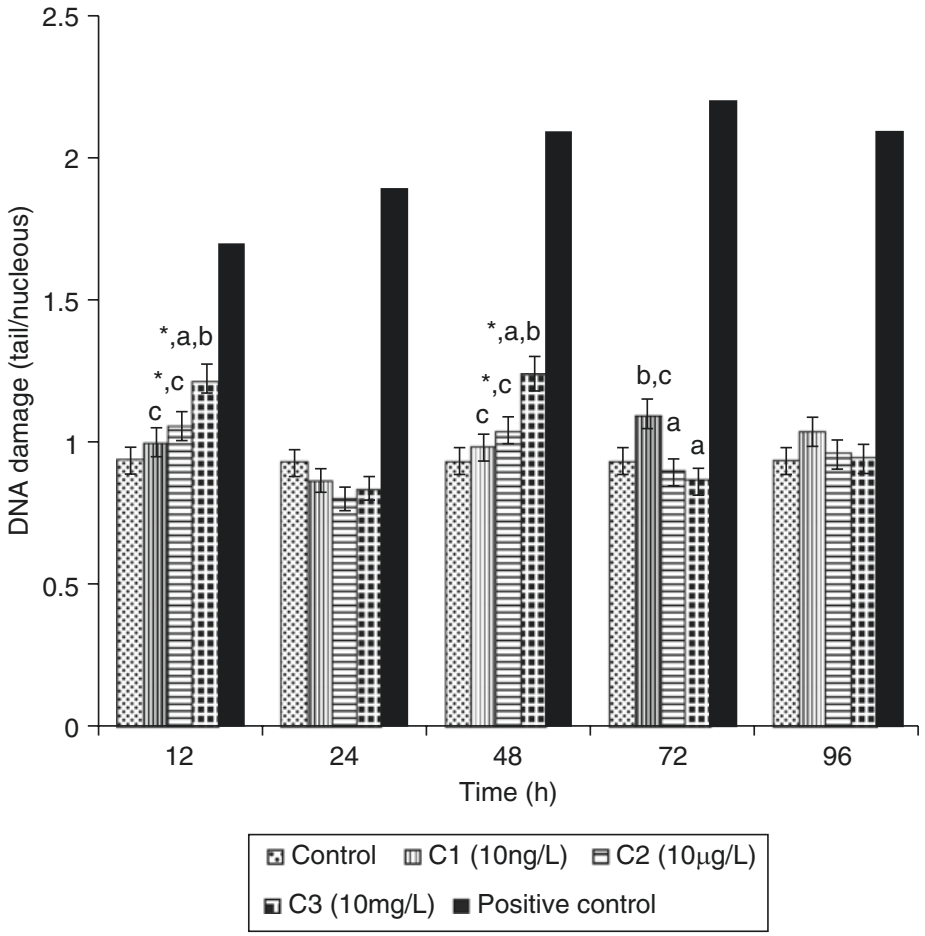

Fig. 20.1 Determination of the DNA damage via the comet assay in blood cells of *Cyprinus carpio* exposed to AMX. The bars represent the mean ± SEM of the index values of damage of five specimens by concentration and by exposure time. The assay was carried out in triplicate. Significantly different from ∗control group (*Kruskal-Wallis and Dunn*, $p < 0.05$)

was recorded at 12, 48, 72, and 96 hours of 7, 6, 18, and 11%, respectively (without significant differences), and at 24 hours, a decrement was obtained compared to the control group of 6% (without significant differences). The middle concentration (10 μ/L) shows a significant increase compared to the control group of 8 and 7% at 12 and 48 hours, but a decrement was obtained compared to the control group at 24 hours of 7% (without significant differences). Finally, for the higher concentration (10 mg/L), an increase compared to the control group was recorded at 12 and 48 hours of 31 and 32%, respectively, but again at 24 hours, a decrement was recorded compared to the control group of 11% (without significant differences)

Table 20.1. It shows the results of the analytical analysis of the water looking for amoxicillin and its main degradation product amoxicilloic acid. It shows clearly that amoxicillin was not detected in any test system at any concentration (10 ng/L, 10 μ/L, 10 mg/L) and at any time (12, 24, 48, 72, 96 h). Amoxicillin completely disappears from the water since the beginning of the experiment, even at 12 hours, which was our shorter time of analysis. On the other hand, amoxicilloic acid, present

Table 20.1 Amoxicilloic acid quantification/detection in water systems

E. time (h)	10mg/L		10 µg/L		10ng/L	
12	AMA1:**3.9 ± 0.88**	AMA2:**6.0 ± 0.95**	AMA1:**D**	AMA2:**D**	AMA1:**D**	AMA2:**D**
24	AMA1:**4.8 ± 0.9**	AMA2:**4.2 ± 0.98**	AMA1:**D**	AMA2:**D**	AMA1:**D**	AMA2:**ND**
48	AMA1:**5.1 ± 0.66**	AMA2:**2.6 ± 0.63**	AMA1:**D**	AMA2:**ND**	AMA1:**D**	AMA2:**ND**
72	AMA1:**4.8 ± 1.02**	AMA2:**0.9 ± 0.99**	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**
96	AMA1:**4.7 ± 0.75**	AMA2:**0.4 ± 0.74**	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**

Values are the mean of five replicates ± SE
ND no detected

Table 20.2 Amoxicilloic acid quantification/detection in blood

E. time (h)	10 mg/L		10 µg/L		10 ng/L	
12	AMA1:**D**	AMA2:**ND**	AMA1:**D**	AMA2:**ND**	AMA1:**D**	AMA2:**ND**
24	AMA1:**D**	AMA2:**ND**	AMA1:**D**	AMA2:**ND**	AMA1:**D**	AMA2:**ND**
48	AMA1:**D**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**
72	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**
96	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**	AMA1:**ND**	AMA2:**ND**

D detected, *ND* no detected

as two isomers 5R6R and 5S6R, was detected and quantified in all the concentrations and at all the times for the higher concentration (10 mg/L) and only detected until the 48 hours for the two lower concentrations (10 ng/L, 10 µ/L).

Table 20.2. It shows the results of the analytical analysis of the blood of *Cyprinus carpio* looking for amoxicillin and its main degradation product amoxicilloic acid. Considering the results in the water systems previously analysed, amoxicillin was neither detected nor quantified in the blood at any exposure time and at any concentration. However, one of the stereoisomers (5S, 6R) of amoxicilloic acid was detected but not quantified in the blood at the highest concentration tested (10 mg/L) but only until 48 hours after exposure and also detected but not quantified for the two lower concentrations (10 ng/L, 10 µ/L) but only until 24 hours after exposure.

20.4 Discussion

In a previous paper, we assess the oxidative stress that amoxicillin produces in *Cyprinus carpio* as well as its transformation in water and within some tissues as the brain, gills, the liver and the kidney, concluding that amoxicillin was transformed

into amoxicilloic acid by abiotic factors due to the presence of bacteria capable to produce beta-lactamase enzymes which lead to the opening of the beta-lactam ring, producing the transformation of amoxicillin into amoxicilloic acid. Furthermore, as this metabolite was detected and even quantified in the water and in all of the tissues analysed, at all concentrations and at all the times tested, it was concluded that the amoxicilloic acid was responsible for the oxidative stress produced in the different tissues of *Cyprinus carpio* (Elizalde-Velázquez et al. 2017).

Now in this research, we are investigating if the amoxicillin represents a genotoxic compound for the environment, assessing its potential to produce damage in the DNA of *Cyprinus carpio* using the comet assay. In the past, some studies have been performed to assess the genotoxic effects of amoxicillin in different species; however, the scientific literature has diverse results: some of them state that amoxicillin is not a genotoxic compound, while some others state the contrary. Istifli and Topaktas (2009) tested the genotoxic effect of amoxicillin in vitro using human peripheral blood lymphocytes, and they concluded that amoxicillin did not induce any sister chromatid exchange (SCE), did not increase chromosomal aberration (CA), and did not induce micronucleus (MN), and Cahill et al. (2004) reported also a negative result measuring the genotoxic effect of amoxicillin in the yeast *Saccharomyces cerevisiae* using the green screen assay.

On the other hand, some other research studies have reported genotoxic effects of amoxicillin in different species. For example, Arabski et al. (2005) tested the genotoxic potential of amoxicillin in human peripheral blood lymphocytes and gastric mucosa cells using the alkaline comet assay, and they conclude that amoxicillin can induce DNA damage in both cell lines causing strand breaks and base modifications, as a result of the production of reactive oxygen species; however, they suggest that amoxicillin needs a cellular activation before it can induce any DNA damage. Interestingly, Li et al. (2007) reported similar results as Arabski et al. (2005). They tested the genotoxic potential of amoxicillin in human and hamster culture cells using a modified comet-assay technique (comet nuclear extract or NE), concluding that amoxicillin induces DNA damages by action of reactive oxygen species, causing oxidation and opening rings of purine and pyrimidine bases. Finally, Anlas and Ustuner (2019) reported a study of the genotoxic activity of amoxicillin in *Oncorhynchus mykiss*; they tested the genotoxic potential of amoxicillin in rainbow trout erythrocytes using the comet assay and the micronucleus test, concluding that amoxicillin has genotoxic effects on fish, increasing the micronuclei frequency and the percent of tail DNA in cells, as well as suggesting that amoxicillin does not directly affect the DNA. Rather, it induces the DNA damage indirectly by increasing the production of reactive oxygen species, which leads to apoptosis and oxidative stress in fish species.

From the above, it is important to remark that three different scientific researches conclude that amoxicillin induce DNA damage indirectly by increasing the production of reactive oxygen species (Arabski et al. 2005; Anlas and Ustuner 2019; Li et al. 2007). As stated at the beginning of the discussion, in a previous work we suggest that amoxicillin induce oxidative stress in the brain, gill, liver and kidney of *Cyprinus carpio* by action of a hypersensitivity reaction, which induces an increase in the reactive oxygen species (Elizalde-Velázquez et al. 2017). Blood is another

susceptible tissue of oxidative damage since, in addition to fulfilling diverse functions such as the transport of xenobiotics throughout the body, it also transports proteins like albumin, lymphocytes, and haemoglobin, as well as other biomolecules to all body tissues, which are target of the free radical attack (Sanjuan-Reyes et al. 2013). Figure 20.1, as we described in the results section, shows that amoxicillin indeed has genotoxic effects in the lymphocytes of peripheral blood of *Cyprinus carpio*, increasing the tail of DNA cells particularly at 12 and 48 hours after its exposure to this bactericide chemical. Gathering the information of the works described above, with our previous work results and the current results of this research paper, it can be said that amoxicillin induces DNA damage in the lymphocytes of the peripheral blood of *Cyprinus carpio*, due to an increase in the reactive oxygen species.

Arabski et al. (2005) suggest that amoxicillin needs a cellular activation to induce the DNA damage, since amoxicillin did not induce DNA strand breaks in isolated plasmid DNA; therefore, a cellular activation of the drug might be associated with the free radical generation. About this point in our past work, we also demonstrate that amoxicillin was transformed into amoxicilloic acid by the cleavage of the beta-lactam ring due to the presence of beta-lactamase enzymes. Amoxicilloic acid in contrary to amoxicillin has reports of toxicity, specifically by inducing the activation of the immune response, since its structure is capable to create adducts with proteins, which after being recognized as strange for the body can trigger a hypersensitivity reaction that could explain the generation of reactive oxygen species, and as a result, it may induce oxidative stress or a genotoxic effect in cells (Elizalde-Velázquez et al. 2017). Table 20.1 and Table 20.2 show the results of the analytical analysis of all the water systems and the blood of *Cyprinus carpio*, and it is evident that amoxicillin was completely transformed into amoxicilloic acid since amoxicillin was not even detected at the shorter time of analysis (12 h) for both water and blood. Therefore, we suggest that the activation process that Arabski et al. (2005) refer could be the transformation of amoxicillin into amoxicilloic acid; furthermore, as described above amoxicilloic acid is responsible for the elevation of the intracellular reactive oxygen species, which also could explain what Anlas and Ustuner (2019) suggest that amoxicillin does not directly affect the DNA. Rather, it induces the DNA damage indirectly by increasing the production of reactive oxygen species, which leads to apoptosis and oxidative stress in fish species.

Finally, Arabski et al. (2005) and Li et al. (2007) reported that the DNA lesions of amoxicillin could be repaired within 60 min and 6 hours after the exposure to the antibiotic; however, both experiments were performed in human cell lines. Particularly Li et al. (2007) report that mammalian glycosylated enzymes OOG1 and OOG2 are enzymes capable to repair the damage caused by amoxicillin in the DNA by excision of the damaged bases. However, amoxicillin DNA damage may pose potential genotoxic problems to those that are genetically or physiologically deficient in the capacity to remove the oxidative DNA damage (Arabski et al. 2005). In fact, Anlas and Ustuner (2019) remark that fish cells have a low DNA repair activity compared to mammalian cells and therefore may be more susceptible to genotoxic agents.

20.5 Conclusion

Amoxicillin transformed by biotic factors into amoxicilloic acid may be capable to induce oxidative DNA damage to blood lymphocytes of *Cyprinus carpio* by generation of reactive oxygen species. Available data is not enough to conclude whether amoxicillin has genotoxic activity or not as well as if it represents a risk for the environment; therefore, scientific guild must strive to generate more genotoxic studies in different species to assess its genotoxic effects and to assess the DNA repair capacity in different bioindicators.

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