Genotoxic and Mutagenic Effects of the Antifouling Biocide Chlorothalonil on the Estuarine Fish Micropogonias Furnieri, Desmarest, 1823

Muryllo Santos Castro
Universidade Federal do Maranhão: Universidade Federal do Maranhao
Larissa Cristine Carvalho Penha
Universidade Federal do Maranhão: Universidade Federal do Maranhao
Thamires Alexsandra Torres
Universidade Federal do Maranhão: Universidade Federal do Maranhao
Marianna Basso Jorge
Universidade Federal do Maranhão: Universidade Federal do Maranhao
Luis Fernando Carvalho-Costa
Universidade Federal do Maranhão: Universidade Federal do Maranhao
Gilberto Fillmann
Universidade Federal do Rio Grande
Ricardo Luvizotto-Santos (luvizottosantos@hotmail.com)
Universidade Federal do Maranhão: Universidade Federal do Maranhao https://orcid.org/0000-0002-3828-8930

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Abstract

Chlorothalonil is a widely used fungicide in agriculture, and as biocide in antifouling paints. Although it causes toxic effects on non-target organisms and can bioaccumulate in fish tissues, little is known about its sublethal effects. Thus, we evaluated the genotoxic and mutagenic effects of chlorothalonil in *Micropogonias furnieri*, an estuarine and commercially important fish with potential as a test organism for ecotoxicology assays. We showed that chlorothalonil exerts genotoxic (DNA damage) and mutagenic (micronuclei and nuclear abnormalities) in a dose-dependent manner (0.35 and 3.5 μg g^{-1}). As the genomic instability may lead to carcinogenesis, our data can assist decision-makers with evidence for banning this compound since any benefit to portuary activities and maritime navigation is outweighed by the cost to aquatic ecosystems and to human health.

Introduction

Chlorothalonil (2,4,6,6-tetrachloroisophthalonitrile) (CHLT) is a broad-spectrum non-systemic fungicide from the isophthalonitrile group widely used in agriculture (Wu et al. 2012). Moreover, it has been used as biocide in the manufacture of antifouling paints, increasing its presence in the aquatic environment (Gallo and Tosti 2015). As a consequence, this biocide is found in high concentrations in port regions such as the Mediterranean, United Kingdom, Greece, and Korea (Voulvoulis et al. 2000; Sakkas et al. 2002; Lee et al. 2011).

The antifouling boosters prevent the establishment and growth of aquatic organisms - bacteria, microalgae, and invertebrates - on submerged or semi-submerged surfaces (Yebra et al. 2004). Its usage is increasing since fouling organisms cause damage to the vessel's structure, loss of speed due to hull irregularities, increase in fuel consumption, and longer mooring period (Brito et al. 2014). However, these biocides can affect non-target species from marine and estuarine environments in addition to fouling organisms (Sakkas et al. 2002), such as fish (Caux et al. 2015).

CHLT has a moderated Log K_{ow} of 2.64, low solubility in water (0.6 mg L^{-1}) and is extremely susceptible to photodegradation due to a half-life up to four weeks in seawater (Davies 1987) and eight to nine days in estuarine water (Caux et al. 2015). The high toxicity of chlorothalonil is related to the multiple reactive electrophilic centers in the molecule which depletes the levels of glutathione (GSH), a major player in organisms defenses against xenobiotics (Long and Siegel 1975).

Due to its toxic potential, this biocide has been banned from the United Kingdom (Thomas and Brooks 2010), and recently from the European Union and Switzerland due to its carcinogenic potential (Kiefer et al. 2020). In Brazil, ANVISA (Brazilian Health Regulatory Agency) created a model for reevaluating pesticides based on risk criteria for human health, and ranked Chlorothalonil as a potential carcinogen (ANVISA, 2019). However, little is known about the genotoxic and mutagenic potential (*i.e.*, sublethal or chronic effects) of CHLT to non-target aquatic organisms. Such studies can be used for Health

Surveillance given that their predictive character as indicator of carcinogenic effects might be useful to define which substances and concentrations are allowed to be used for each purpose.

Biomarkers are valuable tools to study the effects of toxicants on organisms. The comet assay, micronucleus test, and nuclear abnormalities are the most used ones to assess the potential risk of chemicals to cause DNA damage and mutation. Fishes are among the most widely used organisms for detecting harmful effects of xenobiotics, even when exposed to low concentrations, due to their high sensitivity and capacity to bioaccumulate (Yancheva et al., 2015). *Micropogonias furnieri* (whitemouth croaker) is a native estuarine fish that has been used for biomonitoring, since it is abundant and commercially relevant along the Brazilian coast (Marcovecchio, 2004; Amado et al., 2006). Thus, we used *M. furnieri* as ecotoxicological test organism to assess the potential of genotoxic and/or mutagenic effects associated with exposure to sublethal concentrations of CHLT.

Methodology

Test organism

M. furnieri was collected in estuarine streams in the town of Raposa (Maranhão State, Northeast Brazil: 2º25'19.8"S 44º05'29.4"W), using a 30 mm mesh cast-net (SisBio-IBAMA License N. 55187-1). Juvenile specimens (total length < 250 mm; Juras, 1984) were pre-selected *in situ* (n= 80) and transported in a recipient with local water and constant aeration to the laboratory. At the laboratory, they were measured, weighed (length < 250 mm; weight = 18.3 g ± 4 g), and forwarded to a pre-treatment following Blaylock et al (2005). Briefly, they first received a freshwater bath for 3-5 minutes and then were acclimatized for 11 days (25 ^{o}C ± 1 ^{o}C, 12C/12D photoperiod, salinity = 20 g/kg and constant aeration) in 310 L PVC tanks with canisters filters at a density of 1 g L^{-1}. Fishes were fed *ad libitum* with shrimp twice a day.

Exposure treatments

After acclimatized, fishes were randomly distributed into four experimental groups (20 individuals per group): (1) a negative control that received an intraperitoneal dosage of the "vehicle" (physiological solution for fish and dimethyl sulfoxide-DMSO in the proportion 99:1 (v:v)); (2) a positive control that received the "vehicle" and cyclophosphamide (50 µg g^{-1}), a well known genotoxic and mutagenic agent (Anderson et al. 1995); (3) received the "vehicle" and CHLT at 0.35 µg g^{-1}; and (4) received the "vehicle" and CHLT at 3.5 µg g^{-1}.

Intraperitoneal injections were used to avoid the generation of toxic biocide residues and to achieve biocide accumulation in tissues. According to Kinkel et al. (2010), intraperitoneal injections are more effective for delivering a defined quantity of a chemical to each fish based on weight, particularly in experiments related to metabolic studies. The CHLT lowest dose was based on the bioconcentration factor of 18 of the marine fish *Galaxias sauratus* after 14 days of exposure to 20 µg L^{-1} of CHLT

dissolved in water (Davies 1988). The lowest concentration was considered as the non-lethal one that accumulates in wet tissues; the highest one — one order of magnitude higher — was considered as the sub-lethal. The specimens were weighed and received an intraperitoneal dosage of 1 μL g^{-1} according to each treatment. After injections, fishes were kept for 96 h (without feeding) in the same conditions of acclimatization and then anesthetized with eugenol (100 mg L^{-1}). A sample of peripheral blood was taken from the brachial artery (Research ethics committee authorization: CEUA-UFMA N.23115.008075/2016-12) to assess the potential of genotoxic and/or mutagenic effects.

Genotoxic and mutagenic assays

The comet assay was performed according to Cestari et al. (2004) modifications on Singh et al. (1988) and Tice et al. (2000) protocols. After the blood sample (10 μL) be diluted in 1 mL of fetal bovine serum (GoldLab, Brazil), 10 μL of this solution were mixed with 120 μL of low melting fusion point agarose (0.5%) (Agargen, Brazil). This mixture was poured on glass slides covered with regular agarose (1.5%) (Agargen, Brazil). Slides were covered with coverslips and remained at 4°C for 10 to 20 minutes. After removing the coverslip, slides were dipped in ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris pH 10, 10% DMSO, and 1% Triton X-100) and left for 1h at 4°C. Slides were disposed in a horizontal electrophoresis vat placed in an ice-filled plastic box to keep the material refrigerated. The gel electrophoresis (25 V and 300 mA) took place for 25 minutes in alkaline buffer (10 M NaOH, 0.2 M EDTA) and distilled water (pH > 13) and transferred to a neutralization solution (0.4 M Tris/HCl, pH 7.5) for 15 minutes, and allowed to dry at room temperature. Slides were fixed with absolute ethanol for 5 minutes, stained with ethidium bromide (30 μg ml^{-1}), and analyzed under a fluorescence microscope (BX51/BX52-Olympus; filter 516-560ηm; 40x objective).

The tail sizes of the erythrocyte nucleoids (100 nucleoids/slide/individual) were classified according to the damage in the DNA (= nucleoid tail size) into five classes: 0 - no damage (<5%); 1 - low level of damage (5-20%); 2 - medium level of damage (21-40%); 3 - high levels of damage (41-94%); and 4 - total damage (> 95%) (Speit and Hartmann 1995). The damage score was obtained by multiplying the number of nucleoids in each class by the value of the respective class, and divided by the number of nuclei (= cells) analyzed, according to the formula by Speit and Hartmann (1995):

Score = [(0 x n 0) + (1 x n 1) + (2 x n 2) + (3 x n 3) + (4 x n 4)] / total number of cells

The micronucleus test followed the technique described by Hooftman and Raat (1982). A blood sample was smeared on a slide, fixed using methanol for 10 minutes, and left drying at room temperature. Slides were stained with the Panoptic hematological kit (Laborclin, Brazil) for 20 minutes, washed in running water, and dried at room temperature. The frequency of micronuclei in erythrocytes was determined by analyzing 1,000 cells per sample, considering only red blood cells with intact nuclear and cytoplasmic membranes. Micronuclei were classified according to Hooftman and Raat (1982) and the nuclear

abnormalities (nuclear bud, apoptotic fragments, bilobed cells, and binucleated cells) according to Barsiene et al. (2006)

Data analysis

The normal distribution and homogeneity of the data sets were evaluated by the Shapiro Wilk and Levene tests, respectively. Since the data sets did not fit a normal distribution, we used the Kruskal-Wallis test, followed by the Dunnet test ($p<0.05$), to evaluate the statistical significance of the differences for each biomarker among treatments.

Results

M. furnieri showed to be resistant to manipulation (capture and transportation) and acclimation to laboratory conditions as no mortality or incidence of disease during these periods were observed. The fish was also sensitive to CHLT and cyclophosphamide (positive control).

Both concentrations of CHLT caused DNA damage ($p <0.05$) in *M. furnieri* with a 2.8-fold increase in the damage scores (genotoxic effects) compared to the negative control and at a similar magnitude as the positive control (Fig. 1A). The micronucleus frequency (Fig. 1B) also revealed mutagenic effects ($p <0.05$) for both dosages of CHLT (0.35 and 3.5 μg g^{-1}), with an increase of 3.1 and 7.0-fold, respectively, compared to the negative control ($p <0.05$). The higher dosage caused micronuclei similarly as the positive control. The effect was dose-dependent only for micronucleus ($p <0.05$).

We also observed statistically significant effects in the formation of nuclear buds compared to the negative control, with a 2.8-fold increase for the positive control in the lowest concentration (0.35 μg g^{-1}) and a 1.9-fold increase in the highest one (3.5 μg g^{-1}), but no statistical differences between dosages and the positive control (Fig. 2A). The positive control and CHLT (both dosages) also induced the formation of apoptotic fragments, causing an increase of 14.5, 6.0 and 13.8-fold higher than the negative control (Fig. 2B). The number of bilobed cells was also significantly higher for the positive control and CHLT exposures than the negative control, with an increase of 2.9, 3.0 and 6.9-fold, respectively (Fig. 2C). On the other hand, despite increased frequency of binucleated cells, no statistical ($p > 0.05$) effects were observed for both the positive control and CHLT treatments (Fig. 2D). For nuclear abnormalities, only the frequencies of apoptotic fragments and bilobed cells were dose-dependent ($p < 0.05$).

Discussion

M. furnieri is suitable as a test organism species for ecotoxicological assays. Although it has been used as a biomonitor for aquatic pollution (*e.g.*, Kehrig et al. 2002; Marcovecchio 2004; Amado et al. 2006; Tortelli et al. 2006; Seriani et al. 2011), there are no cases in the literature using the species for ecotoxicological assays. The species follows the criteria as an appropriate test organism species, such as: highly available and abundance, trophic level, environmental significance, wide distribution,

commercial importance, easy manipulation, and sensibility to chemical exposure (Segner and Baumann 2015). Plus, as a native and commercial species, the results can be translated to their wild counterparts.

The biomarkers show that CHLT cause DNA damage (comet assay) and mutation (micronucleus test and nuclear abnormalities, except for binucleated cells) in a dose-dependent manner, sometimes similar to the positive control. These effects may be associated to an increase in oxidative stress — increasing the production of reactive oxygen species (ROS) — and/or to a decrease in the antioxidant defenses, such as glutathione (Pompella et al. 2003; Gagné 2014; Bacchetta et al. 2017; García-Medina et al. 2017). A mutagenic effect of CHLT was also shown for the guppy *Poecilia vivipara* (Lopes et al. 2019) when exposed to 1 and 10 μg L^{-1} of CHTL concentration. Micronuclei, as well as all other anomalies, have a similar origin, such as failure in cell repair, DNA replication damaged, improperly condensed chromatin, chromosome fragments without telomeres, and nucleus centromeres (Lindberg et al. 2007).

The mechanism of chlorothalonil action resembles reactions involving both low and high molecular-weight thiols, and its toxicity resides in inhibiting thiol-dependent enzymes (Tillman et al. 1973). Arvanites and Boerth (2001), working with fungi confirmed that CHTL toxicity was associated with the rapid conjugation of cellular thiols derivatives with CHTL, specifically with thiol-rich enzymes, such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and GSH, depleting cellular GSH reserves. It is known that GSH is an important protein in the cellular defense, being able to act also against toxic xenobiotics such as drugs, pollutants, and carcinogens compounds. It is important to note that exposure to CHLT also causes an increase in enzyme gluthatione *s*-transferase (GST) activity in fish (Davies 1985; Lopes et al. 2019) reducing GSH availability, and decreasing its protective role in the cell's antioxidant defense, especially, in ROS neutralization.

Recent studies with different kinds of biomarkers have been used to identify CHLT potential for toxicity on different kinds of organisms (Table 1), revealing three groups of responses: genetics, biochemicals, and physiological. Genetic responses include: (1) induced transcription of genes involved in oxidative stress, lipid peroxidation, reactive oxygen species, and DNA damage in fish (Garayzar et al. 2016); (2) decreased expression of genes related to immunity, reproduction, and xenobiotic clearance in fish (Garayzar et al. 2016); (3) increased micronucleus in fish (Lopes et al. 2019); and (4) teratogenic effects in Ascidians (Gallo and Tosti 2015).

Table 1
Effects of chlorothalonil in non-target organisms.

Organism	Effects	Reference
Zebrafish	Increase: GST, CAT, SOD, LPO, and ROS.	Da Silva Barreto et al., (2020)
Mussel	Increase: SOD, and GST. Decrease: GCL, LPO, and protein carbonylation.	Guerreiro et al., (2020)
Guppy	Increase: GCL (glutamate-cysteine-ligase), GSH, LPO, ROS, and micronucleus. Decrease: GST, and sperm quality.	Lopes et al., (2019)
Mussel	Increase: cellular adhesion, phagocytic activity. Decrease: hemocyte viability, and air survival capacity.	Guerreiro et al., (2017)
Polychaeta	Increase: GST, and LPO. Decrease: ACAP, GSH, and AChE.	Da Silva Barreto et al., (2017)
Zebrafish	Increase: transcription of genes related to division and DNA damage. Decrease: expression of genes related to immunity, reproduction, and xenobiotic clearance.	Garayzar et al., (2016)
Zebrafish	Exerts: estrogen receptor α (ERα) agonist activity, thyroid receptor b (TRb) agonistic, and antagonistic activities.	Zhang et al., (2016)
Ascidians	Deleterious effects on gametes and fertilization; Interference in embryonic development as induction in larval malformation; Teratogenic effects.	Gallo and Tosti (2015)
Amphibians	Increase: numbers of liver granulocytes and melanomacrophages, corticosterone, immune cell levels, and liver damage.	Mcmahon et al., (2011)
Rainbow trout	Increase: phagocytic leukocytes, respiratory burst, and phagocytic cells.	Shelley et al., (2009)
Rat	Increase: PLOOH (phospholipid hydroperoxides), PCOOH (phosphatidylcholine hydroperoxide), PEOOH (phosphatidylethanolamine hydroperoxide). Decrease: GSH.	Suzuki et al., (2004)

CAT: Catalase, SOD: Superoxide dismutase, LPO: lipid peroxidation, GCL: glutamate cysteine ligase, ACAP: Antioxidant capacity against peroxyl radicals, Erα: estrogen receptor α, TRb: thyroid receptor b, PLOOH: phospholipid hydroperoxides, PCOOH: phosphatidylcholine hydroperoxide, PEOOH: phosphatidylethanolamine hydroperoxide.

Organism	Effects	Reference
Oyster	Decrease: phagocytic capacity, ROS; and pyridine nucleotide.	Baier-Anderson and Anderson (2000a)
Striped bass	Decrease: cell viability, GSH synthesis. Suppress: baseline and stimulated O_2 production by macrophage NADPH oxidase.	Baier-Anderson and Anderson (2000b)

CAT: Catalase, SOD: Superoxide dismutase, LPO: lipid peroxidation, GCL: glutamate cysteine ligase, ACAP: Antioxidant capacity against peroxyl radicals, Erα: estrogen receptor α, TRb: thyroid receptor b, PLOOH: phospholipid hydroperoxides, PCOOH: phosphatidylcholine hydroperoxide, PEOOH: phosphatidylethanolamine hydroperoxide.

Biochemical responses include alterations in biomarkers of oxidative stress and increase in lipoperoxidation in estuarine polychaete (*Laeonereis acuta)* (Da Silva Barreto et al. 2017), mussels (*Perna perna*) (Guerreiro et al. 2020), fish (*Poecilia vivipara*) (Lopes et al. 2019; Da Silva Barreto et al. 2020) and in rat hepatocytes (Suzuki et al. 2004). Physiological effects include: (1) decreasing total motility, progressive motility and mitochondrial functionality of sperm quality in fish (Lopes et al. 2019); (2) alterations of the immune system in fish (Baier-anderson and Anderson 2000b; Shelley et al. 2009), mollusks (Baier-anderson and Anderson 2000a; Guerreiro et al. 2017), and amphibians (Mcmahon et al. 2011); (3) disruptive effects in fish hormones (Zhang et al. 2016); and (4) disruptive effects in reproduction of Ascidians (Gallo and Tosti 2015).

In addition to be potentially genotoxic and mutagenic, CHLT is also a potential carcinogen. The United States Environmental Protection Agency (US EPA) ranks CHLT as a suspected carcinogen to humans based on studies with mice (US EPA, 1999). In Brazil, the National Health Surveillance Agency created a model for reevaluating pesticides based on risk criteria for human health. In this assessment, Chlorothalonil is ranked as a potential carcinogen for humans (ANVISA, 2019). Other studies have shown clearly the carcinogenic effects of chlorothalonil in fish (Lopes et al., 2019, Garayzar et al., 2016 and Gallo and Tosti, 2015). Our results also suggest that CHLT may be involved with carcinogenesis because it causes genomic instability and mutation, which might overwhelm the DNA repair system.

Banning CHLT as occurred in the United Kingdon, European Union and Switzerland (Kiefer et al 2020) should be in sight of the Brazilian health authorities. Any benefits to portuary activities and maritime navigation provided by CHLT-based antifouling paints is outweighed by the risks of its biomagnification through the food chain — eventually reaching humans — the carcinogenic potential and the damage to aquatic ecosystems.

Conclusion

The estuarine fish *M. furnieri* has the potential for use as a test organism in ecotoxicological assays. We also show that chlorothalonil causes DNA damage and mutation, probably, by depleting GSH availability, a major player in the cell's antioxidant defense. As the genomic instability and mutation may lead to carcinogenesis, our data can assist decision-makers with evidence for banning this compound from

antifouling paints since any benefit to portuary activities and maritime navigation is outweighed by the cost to aquatic ecosystems and human health.

Declarations

1. Ethics approval

Research ethics committee authorization: CEUA-UFMA N.23115.008075/2016-12 (attached).

2. Consent to participate.

Not applicable in this section.

3. Consent for publication.

Not applicable in this section.

4. Availability of data and materials.

Not applicable in this section.

5. Competing interests

The authors declare that they have no competing interests

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7. Authors' contributions

MSC and RLS conceived and designed the study. MSC, LCCP, TAT, LFCC, MBJ conducted the literature search. MSC, LCCP, TAT was involved in the analysis and interpretation of data. MSC, LCCP, TAT, LFCC, MBJ and RLS drafted the manuscript. The study was supervised by LFCC, MBJ, GF and RLS. All authors read and approved the manuscript.

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Figures

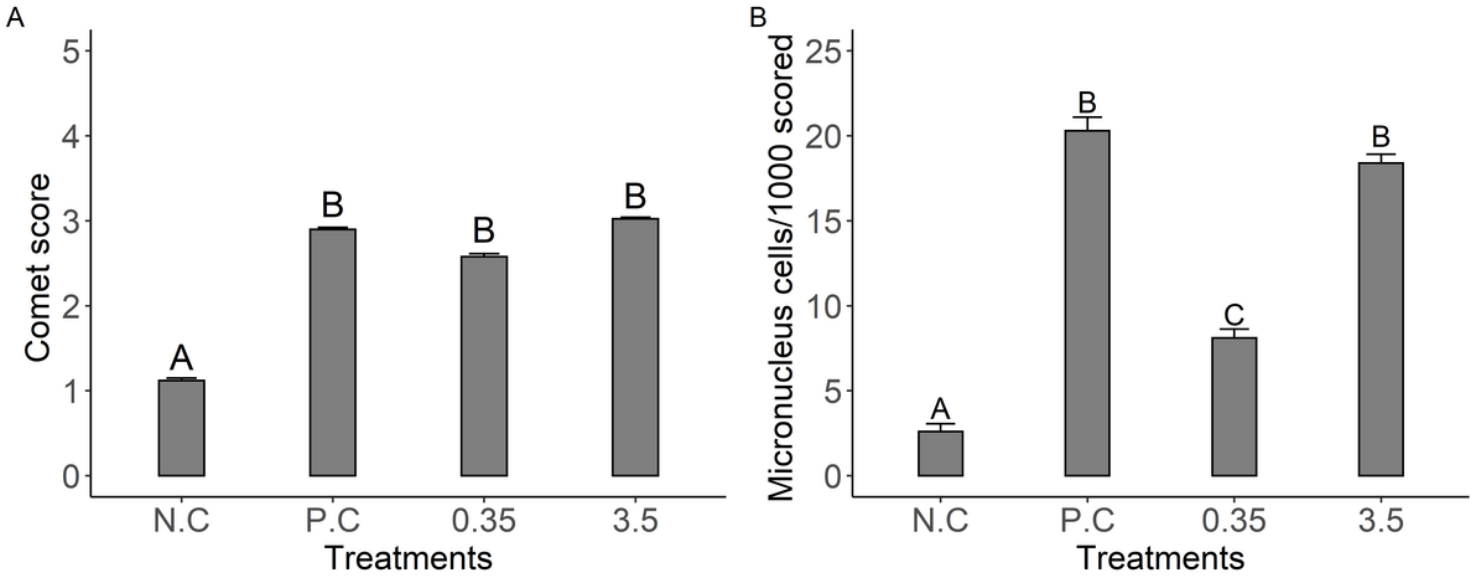

Figure 1

Mean (and standard error of the mean) damage scores (comet assay) (A) and frequency of micronuclei (B) for each treatment of M. furnieri exposed to chlorothalonil (0.35 µg g-1 and 3.5 µg g-1) compared to the negative (NC: saline solution and DMSO) and positive controls (PC: cyclophosphamide 50 µg g-1). Different letters above bars indicate statistically significant differences among treatments (Kruskal Walis - Dunnet, $p < 0.05$).

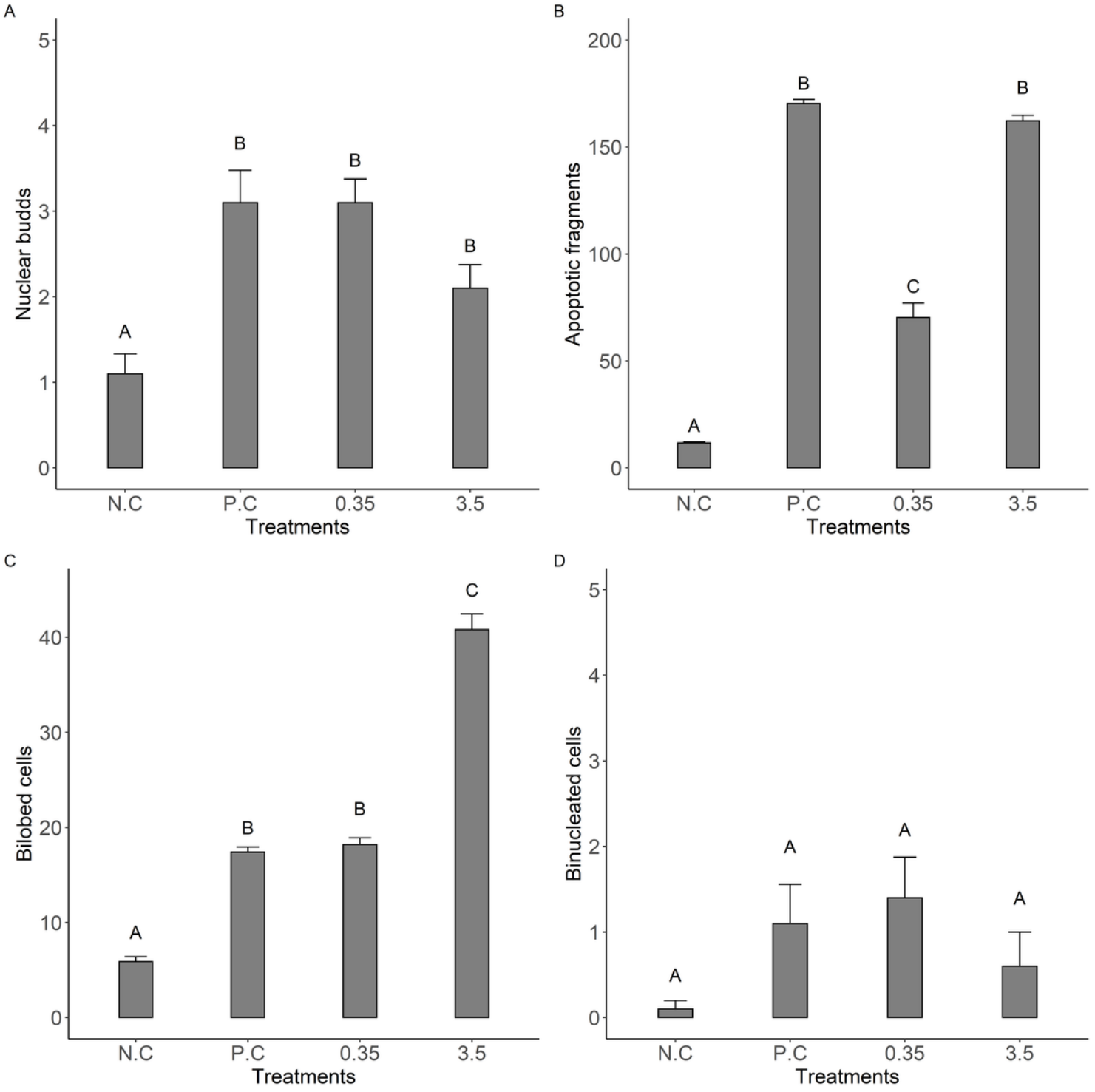

Figure 2

Mean (and standard error of the mean) frequencies of nuclear abnormalities (A: nuclear buds; B: apoptotic fragments; C: bilobed cells; D: binucleated cells) for the different treatments of M. furnieri exposed to chlorothalonil (0.35 µg g-1 and 3.5 µg g-1) in comparison to the negative (NC: saline solution and DMSO) and positive controls (PC: cyclophosphamide 50 µg g-1). Different letters above bars indicate statistically significant differences among treatments (Kruskal Walis - Dunnet, $p < 0.05$).