RESEARCH REPORT

# Acetylcholinesterase inhibition and micronucleus frequency in oysters (*Crassostrea corteziensis*) exposed to chlorpyrifos

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## Abstract

Chlorpyrifos (CPF) is an Organophosphorous pesticide (OP) that has been widely used for both agricultural and domestic pest control. To date, there is little information regarding the effects of this pesticide on aquatic organisms, particularly oysters. The aim of this study was to evaluate Acetylcholinesterase (AChE) activity and Micronucleus (MN) frequency in the oyster *Crassostrea corteziensis* in laboratory exposure with CPF (20, 40, 60, 80, and 160 µg/L) and in a field study. The results showed that AChE was reduced 60 - 82 % in oysters exposed to CPF, relative to the negative control. Similar AChE results were observed in oysters collected from the Boca de Camichín Estuary in Nayarit, Mexico; with respect to genetic damage, evaluated through MN, treatment with CPF did not induce the MN frequency, nor did the oyster from the field study exhibit an increase in this biomarker. These results suggest that *C. corteziensis* is a sensitive model for evaluating the acute toxicity of OP in laboratory studies as well in the field. In addition, it generates prospects on studying mechanisms through which the oyster could possess resistance to genotoxic agents, as well as its being a reliable model for evaluating the genotoxic effects of xenobiotics through the MN technique.

**Key Words**: acetylcholinesterase; micronucleus; *Crassostrea corteziensis*; chlorpyrifos

## Introduction

Marine pollutants have consequences at multiples biological levels (Mix, 1986; Malins *et al.*, 1988; Bolognesi, 1990; Gopal and Pathak, 1993) and have been traditionally documented in terms of chemical concentrations of contaminants; however, these measurements do not provide estimations of harmful effects on living organisms and are now complemented with the measurement of additional biomarkers that are indicative of such effects. Biomarkers represent integral and measurable biochemical and physiological changes in organisms exposed to contaminants, thus indicating initial responses to environmental perturbations and contamination (McCarthy and Shugart, 1990; Bengtson and Henshel, 1996; Roy *et al.*, 1996). For

example, the measurement of Acetylcholinesterase (AChE) activity is widely used to evaluate the exposure and effects of anti-AChE compounds (Galgani and Bocquené, 2000). AChE is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid in the nerve synapse. AChE inhibition is directly linked with the action mechanism of the Organophosphorus (OP) and carbamate pesticides that block the action of this enzyme (Reigart and Roberts, 1999). Measurement of AChE activity is widely employed in several species, including aquatic species (Cajaraville *et al.*, 2000; Singh and Sharma, 2005; Bernal-Hernández, *et al.*, 2010).

The rapid increase in the production and use of OP and carbamate pesticides has raised concerns regarding their potential negative effects on humans and non-target wildlife populations. Pesticides enter waterways from agricultural and urban run-off, groundwater discharge, and after direct application (Schulz and Leiss, 1999), and may be transported to estuaries and coastal waters (Magni *et al.*, 2006; Ismail *et al.*, 2014).

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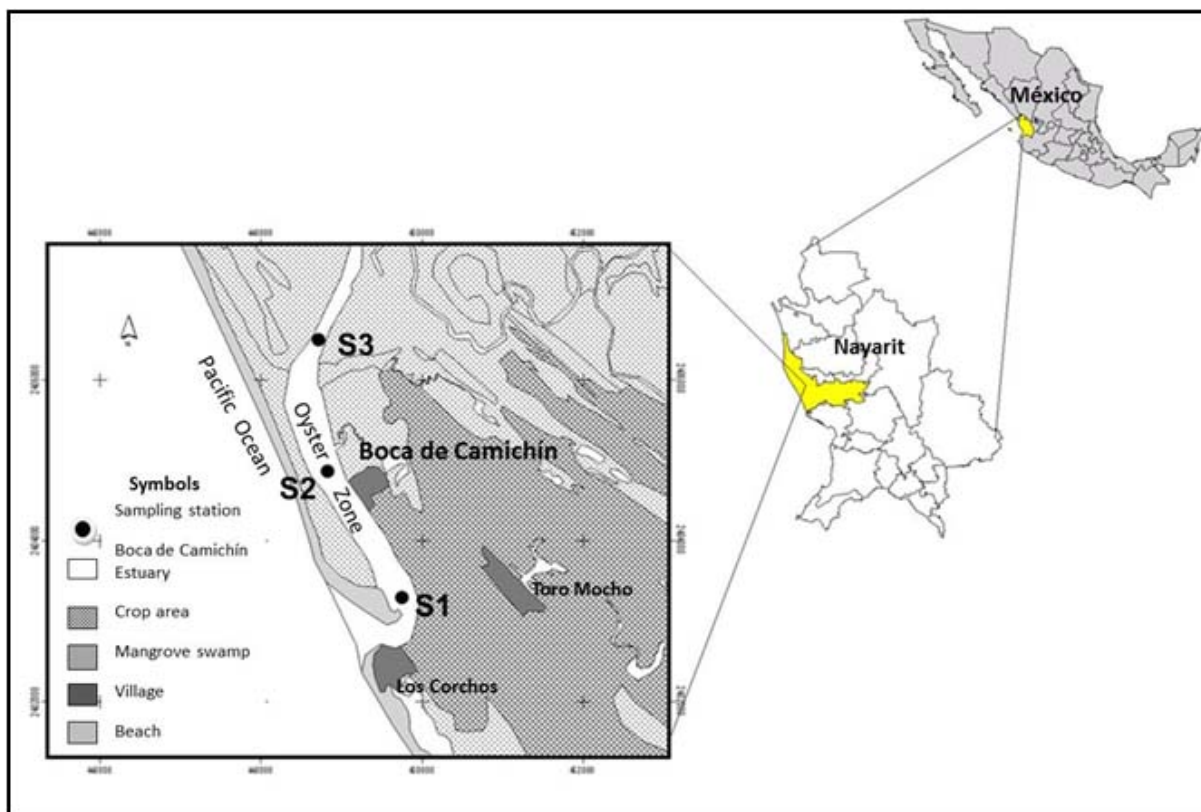CP 63155 Tepic, Nayarit, México
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**Fig. 1** Map of the Boca de Camichín Estuary showing the three sampling stations. S1: Station 1; S2: Station 2, and S3: Station 3.

Chlorpyrifos (CPF) is an OP utilized for domestic and agricultural pest control. Although CPF is a pesticide used worldwide, there is little information concerning its acute and chronic effects on aquatic organisms, particularly oysters. Some reports have suggested that CPF has the potential to cause neuro- and genotoxic effects in aquatic organisms, such as inhibition of AChE and the formation of micronuclei (MN), respectively (Shugart, 1995). In some cases, the formulated pesticides are found to be more toxic than the active ingredient, particularly to aquatic organisms (Ali *et al.*, 2009).

MN frequency is one of the most widely used genotoxicity biomarkers in aquatic organisms. MN are small intracytoplasmic masses of chromatin resulting from chromosomal breakages during cell division. An increase in the frequency of micronucleated cells is thought to result from chromosomal and genomic damage caused by clastogens or spindle poisons (Bolognesi and Fenech, 2012). At present, the MN assay is applied in laboratory and field studies using hemocytes and gill cells from bivalves, or simultaneously in both cell types (Barsiene *et al.*, 2006; Bolognesi and Fenech, 2012). However, few studies have reported MN frequency in oysters.

The oyster *Crassostrea corteziensis* is a species native to the eastern Tropical Pacific where it is naturally distributed in mangrove zones from Mexico to Peru (Stuardo and Martínez, 1975). This species

supports artisanal and commercial fisheries in northwestern Mexico. The Boca de Camichín Estuary situated in Nayarit state is one of the largest oyster producers in Mexico. This ecosystem is important not only for its oyster production, but also for its biologic diversity. High agricultural activity around the estuary is reported, with elevated use of pesticides. One of the most utilized insecticides in the ecosystem is CPF (González-Arias *et al.*, 2010; Rojas-García *et al.*, 2011). Therefore, the aim of this study was to determine AChE activity and MN frequency in order to evaluate the degree of exposure to CPF and the integrity of genetic material, respectively, *in vivo* and in field study.

**Materials and Methods**

*Sampling collection*

For *in vivo* and in-field study, *Crassostrea corteziensis* oysters approximately 4 - 8 cm in shell length were collected at the Boca de Camichín Estuary in Nayarit state, Mexico. This area is characterized by its biological diversity and in part to the high-priority National Wetlands (Robledo-Marenco *et al.*, 2006). Three sampling sites at the estuary were chosen (Fig. 1), taking into account the hydrographic and biological characteristics of the system. One site was localized toward the head of the estuary (S1: 21º44′06.8″N, 105º29′19.06″), another station was localized downstream and near

the mouth of the estuary (S2: 21°44′53.0″N, 105°29′39.0″W), and one was situated in the middle of the oyster-culture zone (S3: 21°45′41.1″N, 105°29′43.5″). The oysters were collected at each of the three sites at two levels: from the upper and the lower part of the oyster string. Gills were dissected immediately and frozen until use to screen for total protein, AChE activity, and MN frequency.

*Exposure to CPF under* in vivo *conditions*
The oysters were carried to the laboratory, placed in 40-L tanks filled with filtered seawater, and acclimated for 15 days, during which time they were fed 2 L/day of microalgae species: *Chaetoceros* spp. ($6 \times 10^5$ cells/mL). For exposure to CPF, oysters were placed in aquariums with 10 L of aerated water and were acclimated to estuary conditions (26 ‰; 32 ± 1 °C). Ten organisms were utilized for each treatment. The organisms were divided into seven lots as follows: one for negative control (maintained under the same condition, but without treatment), one for positive control (treatment with mitomycin C as MN control) and five for CPF treatments (20, 40, 60, 80, and 160 µg/L). The organisms were incubated in 12-h:12-h dark-light cycles for > 96 h. Each experiment was run in triplicate. At the end of exposure, the organisms were washed, opened, and the gills were excised to screen for total protein, AChE activity, and MN frequency. For the present study, CPF (44.5 % Emulsifiable concentrate [EC]) with the Magnum L-480 brand was employed.

*Total protein content and AChE activity*
Gills were homogenized (1:2 W/V) in an extraction buffer (20 mM Tris-Base, 1 mM EDTA, 1 mM DTT, 500 mM sucrose, 150 mM KCl, 0.1 mM PMSF, pH 7.6) (Monserrat *et al.*, 2002). The homogenate was centrifuged at 9,000 rpm for 30 min at 4 °C. The pellet was discarded and the supernatant (S9 fraction) was used to determine protein and AChE activity. Total protein was determined in fraction S9 according to the Lowry method (Lowry *et al.*, 1951) using Bovine serum albumin (BSA) as the standard. AChE activity was

performed according to Ellman *et al.* (1961), modified by Monserrat *et al.* (2002). Enzyme activity was determined in triplicate using 300 µL of supernatant, 2700 µL of phosphate buffer (pH 8, 0.1 M), 100 µL of 0.5 mM Ellman's reagent (DTNB), and 20 µL of 0.5 mM acetylthiocholine iodide as substrate. The rate of absorbance change at 412 nm was recorded over 120 sec at 25 °C using a Spectronic Genesys 10 Bio spectrophotometer (Genesys, WI, USA).

*Micronucleus (MN) determination*
*Cell preparation*
This technique was based on the methodology described by Bolognesi *et al.* (1999) with some modifications. To prepare the cell suspension, gills were excised and cut into fragments with dissection scissors in 5 mL of saline solution. The cell suspension was centrifuged at 1,500 rpm at 25 °C for 5 min and then fixed in 5 mL of Carnoy's fixative solution (CFS) (3:1 methanol:acetic acid) for 20 min. Afterward, the cell suspension was centrifuged and washed three times with CFS. The pellet was resuspended in 1 mL of CFS and treated with 1 mL of trypsin (0.00015 %). Trypsin activity was blocked by the addition of 5 mL of CFS. The cell suspension was centrifuged and washed twice with CFS. Finally the samples were resuspended in 1 mL of CFS and were maintained at 4 °C until slide preparation.

*Exposure to mitomycin C as positive control*
Mitomycin C (MMC) is a DNA cross-linking agent that has been extensively used as a reference mutagen in numerous MN studies (Das and Nanda, 1986; Majone *et al.*, 1987; Scarpato *et al.*, 1990, Williams and Metcalfe, 1992). Oysters were placed in aquariums with 10 L of water (26 ‰). Prior to the exposure, they were acclimatized for 24 h. Subsequently, a group of 10 organisms with one replicate were exposed to MMC (2.5 mg/L) for 48 h. At the end of treatment, the oysters were sacrificed and their gills were separated to determine the presence of MN. The negative control group was run with one replicate.
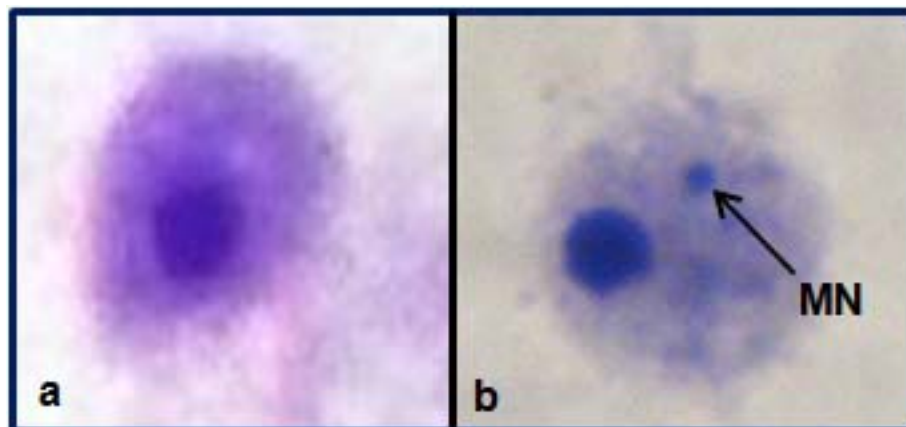


**Fig. 2** Representative gill cells from *Crassostrea corteziensis* (a) and a micronucleated gill cell (b). The micronucleus (MN) is indicated by an arrow beside the main nucleus. Staining with the Merck Hemacolor kit (100x).
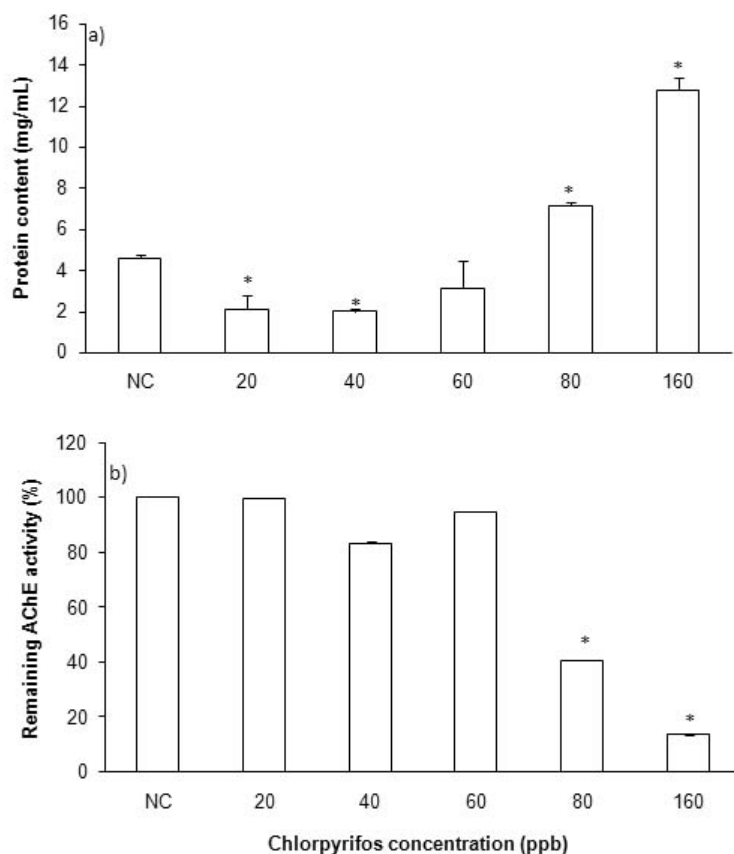
**Fig. 3** Total protein content (a) and remaining acetylcholinesterase activity in oyster (*Crassostrea corteziensis*) gills (b) exposed to different chlorpyrifos concentrations for 96 h. Data are expressed as means ± Standard deviations (SD) of three independent determinations. NC, Negative control. The asterisk (*) indicates a significant difference from control animals (NC) at $p < 0.05$.

*Criteria for scoring MN*

MN were identified according to following criteria described by Fenech *et al.* (2003): (1) round and ovoid-shaped nonrefractory particles in the cytoplasm; (2) chromatin-like color and structure; (3) diameter of 1/3 - 1/20 of the main nucleus, and (4) particles completely separated from the main nucleus. Figure 2 shows the cell morphology and micronucleated cell observed in the MN test. Slides were analyzed by light microscope (Carl Zeiss, Axiostar plus, Göttingen, Germany) at a magnification of 100x for nuclear abnormalities (MN) and 1,000 gill cells were scored. Only intact cells were scored. All MN were checked by a second operator to ensure that unambiguous micronucleated cells were exclusively scored.

*Statistical analysis*

The results were expressed as the average of three independent experiments. The results were analyzed by Mann-Whitney *U* test and Dunn multiple comparison tests. *p* values < 0.05 were considered statistically significant. Statistical analyses were conducted using the Stata ver. 8.0 program (Stata statistical software; Stata

Corporation, College Station, TX, USA) and GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

**Results and Discussion**

The treatments with CFP did not cause mortality in the oysters, but we observed that the organisms closed their valves for 12 h. There are reports that suggest that these oysters are able to isolate themselves from a contaminated environment by closing their valves (Kramer *et al.*, 1989; Mersch *et al.*, 1993). In addition, in bivalve molluscs, contact with toxic compounds causes a reduction in filtration activity; thus, this might reduce exposure time (Van der Gaag *et al.*, 1990; Wrisberg *et al.*, 1992).

*Total protein content and AChE activity*

The results showed that *C. corteziensis* oysters exposed to 80 and 160 µg/L had the highest protein concentrations (8.50 and 12.77 mg/mL, respectively) compared with those of the negative control; the opposite was observed at 20 and 40 µg/L (2.32 and 2.41 mg/mL) ($p < 0.05$) (Fig. 3a). In addition, significant variations in remaining AChE activity of gills exposed to different concentrations of CFP
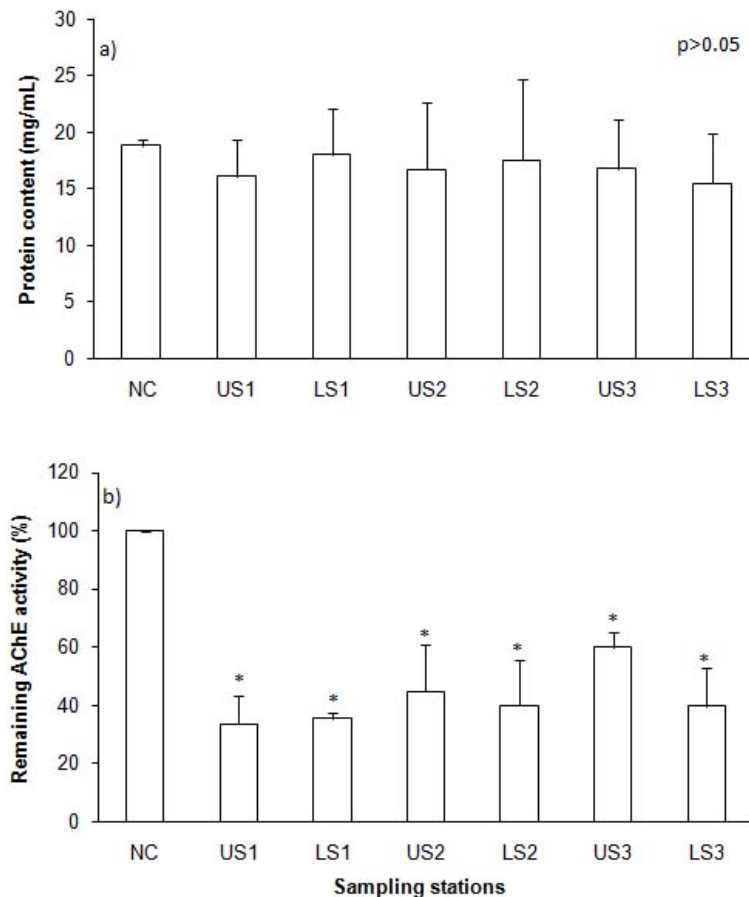
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**Fig. 4** Total protein content (a) and remaining acetylcholinesterase activity (b) in *Crassostrea corteziensis* oysters collected from the Boca de Camichín Estuary. Data are expressed as means ± Standard deviations (SD) of three independent determinations. NC, Negative control; S1, Sampling site 1; S2, Sampling site 2; S3, Sampling site 3. U: Upper part of string; L: Lower part of string. The asterisk (*) indicates a significant difference from control animals (NC) at $p < 0.05$.

during 96 h were recorded (Fig. 3b). At the highest CFP treatments (80 and 160 µg/L), the remaining AChE activity was 40 and 18 % with respect the control group, which confirms the inhibitory effects of CFP on oyster exposure.

Proteins and other oyster components vary considerably among species and among individuals of the same species, also depend on age, gender, size, environment, and season of the year, and are closely related with food organisms (Maeda-Martínez *et al.*, 2001). In this regard, average total protein content in the gills of oysters in this study was 5.93 mg/mL in CPF-treated organisms and 4.59 mg/mL in the negative control group. It has been reported that protein concentrations in the tissues of gills can be affected both positively and negatively by stress-associated physiological changes, as well as by the presence of chemicals (Rank *et al.*, 2007).

On the other hand, in the field study no changes in protein content in *C. corteziensis* oysters was observed (Fig. 4a), but all of the organisms collected at the estuary exhibited lower AChE activities with respect the negative control, as illustrated in Figure 4b; this indicates the presence

of anticholinesterase compounds in the estuary and, possibly, OP pesticides.

Oysters have been proposed as bioindicators of aquatic pollution, in addition to mussels (Bebianno *et al.*, 1993, 1994; Viarengo *et al.*, 1998; Blasco and Puppo, 1999; Rodríguez-Ortega *et al.*, 2001), and data regarding the measurement of AChE activity in oyster tissues are available in the literature (Mora *et al.*, 1999; Dellali *et al.*, 2001; Bernal-Hernández *et al.*, 2010). Previous studies suggest that AChE activity is found in the gills, mantle, and adductor muscles of bivalves (Bocquene *et al.* 1997; Escartín and Porte 1997; Radenac *et al.*, 1998; Mora *et al.*, 1999; Monserrat *et al.*, 2002; Damiens *et al.*, 2004). Gills were chosen for measurement of esterase activities because gill tissue exhibited high activity levels and higher sensitivity to contaminants with respect to those of other tissues (Mora *et al.*, 1999). Also, oyster gills are easy to dissect and have a relatively important and constant mass (apparently independent of field conditions) (Mora *et al.*, 1999).
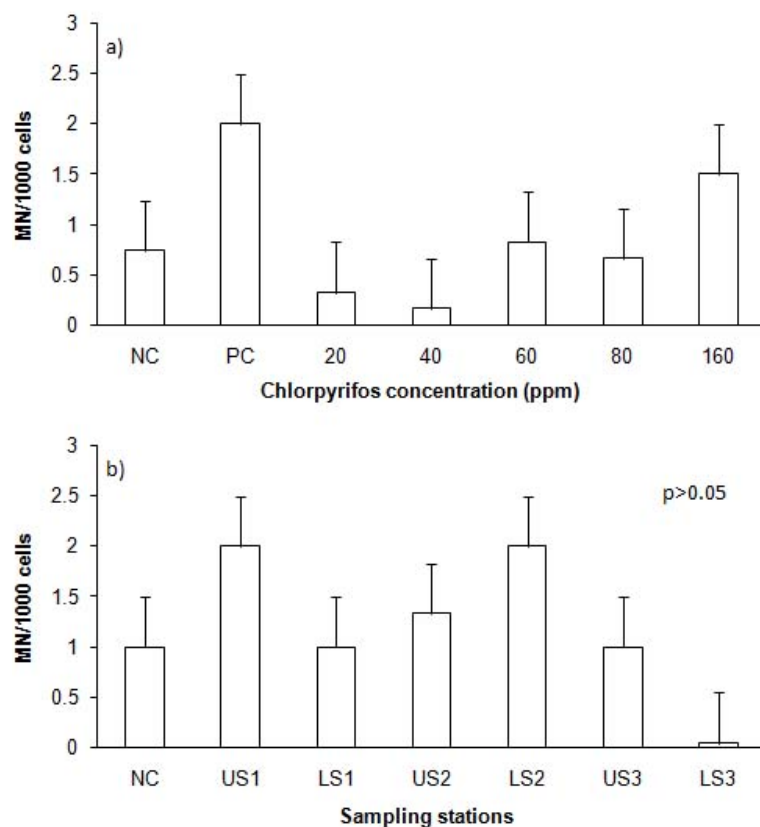
**Fig. 5** Micronucleus frequency (MN) in gill cells of the oyster *Crassostrea corteziensis* exposed to chlorpyrifos for 96 h (a) and oysters collected in the Boca de Camichín Estuary (b). Values represent the means ± Standard deviations (SD) of three independent experiments performed in triplicate. NC, Negative control; PC, Positive control: organisms exposed to 2.5 mg/mL of Mitomycin C (MMC). S1, Sampling site 1; S2, Sampling site 2; S3, Sampling site 3. U: Upper part of string; L: Lower part of string. The *p* value was determined by the Mann-Whitney *U* test and statistical significance was set at *p* < 0.05.

*Micronuclei frequency*

MN frequencies in the different CPF treatments, as well as the control organisms, are depicted in Figure 5a. In this study, we observed a low incidence of micronucleated gill cells in CPF-exposed *C. corteziensis*, and no statistically significant differences in MN frequencies were observed between control organisms and those exposed to CPF (*p* > 0.05). On the other hand, in oyster gill tissues taken from the different sample sites at the Boca de Camichín Estuary, none had significant differences in MN number with respect to that of the negative control (Fig. 5b). Our results agree with those reported in an oyster-farming area contaminated by cadmium and copper (Burgeot and Galgani, 1995). These authors found that MN frequency was not very sensitive to a pollution gradient and that it exhibited high interindividual variability. These results are similar to those reported in other bivalves, such as *Macoma balthica* and *Mytilus edulus* (Barsiené *et al.*, 1996). Regarding MN frequency in oyster species, few studies are available in the literature. In *Crassostrea gigas*, Burgeot *et al.* (1995) found a baseline MN frequency of 2.8, while in *Crassostrea virginica*, Weis *et al.* (1995) found a baseline MN frequency of

4.8. Few works are available on baseline MN frequency in bivalve molluscs. In this regard, Table 1 shows that baseline MN levels reported in the literature in this organism range from 0.54 - 5.7 MN/1,000 cells.

Surprisingly, MMC did not cause an increase in MN number in oysters. To confirm the quality of the MMC reagent, we conducted human lymphocyte cultures with significant MN induction results (data not shown). MMC is a known mutagenic and clastogenic agent. Sister chromatid exchanges, chromosome aberrations, and MN comprise genetic effects that can be observed during exposure *in vivo* to this compound (Rudd *et al.,* 1991; Salvadori *et al.,* 1994). Our findings are important because the genotoxic effect of this compound is widely demonstrated in other species, such as mussels and fish. In these species, a lower concentration of MMC than that employed in this work causes an increase of 30 - 50 % in the baseline MMC number (Majone *et al.,* 1989; Grisolia, 2002); this was also observed in mammal species such as rodents (Hayashi *et al.*, 1992; Holtz *et al.*, 2003).

We observed that *C. corteziensis* oysters collected from the Boca de Camichín Estuary had AChE-level activity similar than that of oysters

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**Table 1** Micronucleus (MN) frequency in bivalve organisms

| Organism | Tissue | Compound | MN/ 1,000 cells | SD | Reference |
|---|---|---|---|---|---|
| *Mytilus galloprovincialis* | Gill | Zinc chloride | 2.2 | ND | Majone *et al.,* 1987 |
| *Crassostrea gigas* | Heart cells | Benzo[a]-pyrene | 2.8 | ±2.3 | Burgeot *et al.,* 1995 |
| *Dreissena polymorpha* | Hemolymph | Clastogens: Mitomycin C (MMC) | 1.2 | ±1.0 | Mersch *et al.,* 1996 |
| | | Bleomycin | 4.4 | ± 1.8 | |
| | | Di-methylar-sinic acid | 6.9 | ± 2.4 | |
| | | Potassium chromate | 3.2 | ± 1.1 | |
| | Gill | Mitomycin C (MMC) | 6.1 | ± 1.4 | |
| | | Bleomycin | 6.7 | ± 3.1 | |
| | | Dimethyl-arsinic acid | 5.6 | ± 1.9 | |
| | | Potassium chromate | 5.4 | ± 2.6 | |
| *Mytilus trossulus* | Gill | PCB, DDT, HCB, HCH, and Polybrominated diethyl ethers (PBDE) | 6 | ND | Kopecka *et al.,* 2006 |
| *Mytilus edulis* | Gill | Crude oil | 2.4 | ND | Baršienė and Andreikė-Naitė, 2007 |
| *Macoma balthica* | Gill | Contaminated zone | 1.2-3.63 | ND | Baršienė *et al.,* 2008 |
| *Mytilus edulis* | Gill | Contaminated zone | 1.7-3.3 | ND | Baršienė *et al.,* 2008 |
| *Mytilus galloprovincialis* | Hemolymph and gills | Urban and industrial pollution | 5.5-5.1 | ND | Taleb *et al.*, 2009 |
| *Crassostrea corteziensis* | Gill | Chlorpyrifos | 1.2 | ±1.1 | This study |
| *Cerastoderma edule* | Hemolymph | Chemical contaminant | 0.4 | ±0.6 | Ruiz *et al.,* 2013 |
| *Mya arenaria* | Hemolymph | Organotin compounds, arsenic, Polyaromatic hydro-carbons (PAH) and PCB | 15.4 | ND | Debenest *et al.,* 2013 |
| *Mytilus galloprovincialis* | Digestive glands | $Hg^{2+}$ | 2.3 | ND | Pytharopoulou *et al.*, 2013 |
| *Ruditapes philippinarum* | Gill | PAH and trace metals such as Cr and Ni | 4–16 | ND | Sacchi *et al.,* 2013 |

ND: Not determined; SD: Standard deviation; PCB: Polychlorinated biphenyl; DDT: Dichlorodiphenyltrichloroethane; HCB: Hexachlorobenzene; HCH: Hexachlorocyclohexane; Cr: Chromium; Ni: Nickel.

exposed under *in vivo* conditions to CPF, which caused marked inhibition of AChE activity but did not induce the presence of MN. These results confirm that AChE activity in *C. corteziensis* oysters can serve as a sensitive biomarker to assess exposure to OP, such as CPF (Bernal-Hernández *et al.*, 2010). On the other hand, these results generate perspectives regarding the mechanisms through which oyster could possess resistance to genotoxic agents, as well as whether this is a reliable model for evaluating the genotoxic effects of xenobiotics.

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