

Cytogenotoxicity assessment of monocrotophos and butachlor at single and combined chronic exposures in the fish *Catla catla* (Hamilton)

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Abstract Cytogenotoxic effects in the form of micronuclei and deformed nucleus, nuclear buds, binucleated cells, vacuolated nucleus, vacuolated cytoplasm, echinocytes, and enucleus induced by two compounds belonging to two different chemical classes of agrochemicals (monocrotophos and butachlor) at sublethal concentrations (0.625, 1.3, and 2.3 ppm and 0.016, 0.032, and 0.064 ppm) in single and combined chronic exposures were studied under laboratory conditions for a period of 35 days in the economically important Indian fish *Catla catla*. Statistically significant duration-dependent increases in the frequencies of micronucleus (MN) and other cytological anomalies were observed. Compared to single exposures, a twofold increase in micronuclei frequency was noted at combined exposures indicating the synergistic phenomenon. Binucleated and enucleated cells appeared only in fishes exposed to sublethal concentrations of butachlor. The present study is the first of its kind in exploring a significant positive correlation between micronuclei and other nuclear anomalies suggesting them as new possible biomarkers of genotoxicity after agrochemical exposures. The study

highlights the sensitivity of the assay in exploring various predictive biomarkers of genotoxic and cytotoxic events and also elicits the synergistic effects of agrochemicals in apparently healthy fishes. *C. catla* can be considered as a suitable aquatic biomonitoring sentinel species of contaminated water bodies.

Keywords *Catla catla* · Cytome assay · Micronucleus · DNA damage · Synergism · Agrochemicals

Introduction

The impact of the green revolution provokes the application of pesticides that becomes indispensable in agriculture. Agrochemical residues eventually end up in the aquatic ecosystems due to rain and surface runoff and, owing to their stability, may be deposited even at sublethal concentrations in the tissues of aquatic biota like fishes and pose serious threat to the consumers at different trophic levels (Aktar et al. 2009).

Genetic biomarkers are widely measured in ecotoxicology as molecular toxic end points of major environmental pollutants (Wessel et al. 2007; Mussali-Galante et al. 2013). Cytogenetic biomarkers play a key role in assessing the impact of pollutants in apparently healthy sentinel aquatic organisms such as fishes. Among the cytogenetic end points, the erythrocyte micronucleus assay has gained popularity over other basic cytogenetic assays to assess mutagenic and genotoxic effects of chemicals due to its sensitivity, simplicity, and reliability for detecting cytogenetic DNA damage (Heddle et al. 1983; Al-Sabti and Metcalfe 1995; Cavas and Konen 2007; Bopp et al. 2008; Anbumani and Mohankumar 2012).

Pesticides occurring in nature are normally not present individually, but in complex mixtures (Gilliom et al. 2006). Complex mixtures of pesticides dissolved or adsorbed to soil particles have the potential to exacerbate the living conditions

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of aquatic organisms causing them to exhibit stress at relatively low concentrations leading to sublethal effects such as decline in fecundity, immune suppression, endocrine dysfunction, carcinogenicity, and genotoxicity at individual and population hierarchies (Frankart et al. 2002; Misumi et al. 2005; Tarrant et al. 2005; Glaser 2006).

Monocrotophos (MCP), an organophosphate (OP) ester, is a widely used insecticide across the world and is known to cause genotoxicity in various organisms including fishes, mussels, mice (Chauhan et al. 2005; Ali and Kumar 2008; Hoda and Sinha 1993; Pavlica et al. 2000; Sharma et al. 2007), and man (Grover et al. 2003). MCP is the largest selling agrochemical in India (Banu et al. 2001; Bhushan et al. 2013) with a usage level of 1815 metric tonnes. The recommended level of MCP in the agricultural field is about 10.97 ppm (Senapati et al. 1988). Butachlor, on the other hand, is an organochlorine (OC) compound of the chloroacetanilide family widely used to control weeds in rice, barley, cotton, and peanut crops in India with 3000 metric tonnes as the consumption rate (Gobi and Gunasekaran 2010; Yadav et al. 2013). The application rate of butachlor in the paddy waters is around 4.8 ppm (USGS 2011). It is reported to induce a wide variety of toxicological effects in fish, frog, human peripheral blood, and mammalian cells (Panneerselvam et al. 1999; Dwivedi et al. 2012; Liu et al. 2011; Yadav et al. 2013). Both agrochemicals are extensively used in Asia and were found in contaminated water bodies (Farombi et al. 2008; Ismail et al. 2014).

Reports are available on acute toxicity studies with pesticides singly and in binary mixtures in fishes (Abdelghani et al. 1997; Belden and Lydy 2006; Verbruggen and van den Brink 2010). DNA damage at single exposures has been explored in nontarget organisms; however, studies are sparse on combined sublethal exposures under controlled laboratory conditions. Sublethal exposures to agrochemicals disrupt the organism's metabolic activity thereby reducing the "fitness" of natural population where DNA damage is one of the fitness parameter. Hence, an attempt was made to study the cytogenetic effects induced by two compounds belonging to two different chemical classes of agrochemicals at lower sublethal concentrations in single and combined chronic exposures under controlled laboratory conditions in economically important sentinel fish *Catla catla*.

Materials and methods

Experimental fish specimens and chemicals

The freshwater fish *C. catla* (Hamilton, Family: Cyprinidae) was chosen for the study, as it is commonly available in India throughout the year and is an edible species of increased market value. It has also been proved to be a sensitive

indicator of environmental stress (Tilak et al. 1981). Fingerlings weighing between 10 and 12 g and of length 11 ± 2.0 cm were procured from a commercial fish farm and transported to the laboratory in oxygenated bags and released into 50-L glass aquaria filled with dechlorinated tap water. They were then acclimatized for 21 days under laboratory conditions with natural photoperiod and fed with oil cake. The fecal matter and other waste materials were siphoned off daily to reduce ammonia content in water that was renewed once in 2 days with dechlorinated tap water. The water quality parameters were analyzed and maintained within the normal range (pH 7.1 ± 0.2 to 7.6 ± 0.3 , dissolved oxygen 8.26 ± 0.09 to 8.84 ± 0.05 mg/L, temp. 22 ± 2 °C, and hardness in terms of CaCO₃ was 200 ± 0.0 ppm, respectively).

For the present study, technical-grade monocrotophos (36 % SL) with trade name Parryfos (MCP; CAS no. 6923-22-4) (3-hydroxyl-*N*-methyl-*cis*-crotonamide dimethyl phosphate) manufactured by Coramandel Fertilizers Ltd., India, and butachlor (50 % emulsifiable concentrate), a widely used herbicide with trade name Weedar (CAS no. 23184-66-9) [*N*-(butoxymethyl)-2-chloro-2', 6'-diethylacetanilide)] from Sree Ramcides Chemicals Ltd. India, were used.

Determination of acute toxicity

A total of 360 fish from the acclimatized batch were used to determine the acute toxicity bioassays for monocrotophos technical and butachlor 50 % emulsifiable concentrate (EC) in a semi-static system (i.e., exchange of test solution every 24 h) for the determination of 96-h median lethal concentration (LC₅₀) as per OECD acute fish toxicity test no. 203 (OECD 1992). Stock solutions of MCP and butachlor were prepared by dissolving in acetone and water, respectively. Acclimatized *C. catla* fish specimens were randomly exposed to five different concentrations of MCP (4.5, 6.8, 10, 15, and 23 ppm) and butachlor 50 % EC (0.05, 0.1, 0.2, 0.4, and 0.8 ppm) having ten fish per concentration based on the range finding study results (0.1, 1, 10, 50, and 100 ppm). Experiments were conducted in triplicates, and fish maintained in tap water and 0.1 % acetone in tap water was considered as control and solvent control, respectively. Approximately seven to ten fishes were introduced in the 50-L capacity glass aquaria. The fish were under close observation for a period of 96 h. The LC₅₀ determination was done by inspecting the fish twice daily, taking note of the number of mortalities observed on specific intervals (i.e., 3, 6, 24, 48, 72, and 96 h). The concentration that caused 50 % mortality was calculated using TOXSTAT 3.5 version software.

In vivo exposure to MCP, butachlor, and its combination

The fish specimens were exposed to three different concentrations (sublethal I 2.5 ppm; sublethal II 1.3 ppm, sublethal III 0.625 ppm) of MCP and sublethal I 0.064 ppm; sublethal II

0.032 ppm, and sublethal III 0.016 ppm of butachlor. Sublethal concentrations I, II, and III were chosen based on the 1/5th, 1/10th, and 1/20th of the LC_{50} values of MCP and butachlor, respectively. For combined exposures, sublethal concentrations 2.5+0.064, 1.3+0.032, and 0.625+0.016 ppm of MCP and butachlor were used. Experiments were carried out in duplicate and exposures continued up to 35 days. Samplings were done at intervals of day 3, 7, 14, 21, 28, and 35 at the rate of ten fishes per duration. The concentration of acetone was maintained 0.1 % in test solution and solvent control. Mortality was not observed throughout the study during chronic exposures, and fish were found normal with unperturbed behavioral responses. On each sampling day, blood was collected from fish anesthetized with MS222 by caudal vein puncture technique using a heparinized syringe (2.7 % EDTA as anticoagulant). Since the specimens used in the present study were fingerlings, repeated blood sampling was difficult. Hence, for the sake of practical convenience, specimens were randomly selected, sampled, and discarded.

Erythrocyte micronucleus cytome assay

A modified micronucleus assay named erythrocyte micronucleus cytome assay (EMNCA) was standardized and has been described in detail elsewhere (Anbumani and Mohankumar 2012). From each replicate, two slides per fish were made and a total of 1000 cells were scored blindly for each slide using a light microscope (Nikon PFX Japan, Optiphot-2, oil immersion lens, 100/1.25). The established criteria for identifying micronuclei and other anomalies were strictly followed to ensure authentic scoring (Fenech 2007).

Classification of anomalies

Cytological abnormalities observed in the present study include micronucleus (MN), deformed nucleus (DN), nuclear bud (NBu), vacuolated nucleus (VN), binucleated cells (BNC), vacuolated cytoplasm (VC), echinocytes (EC), and enucleus (EN), and the description of this anomalies has been described in detail elsewhere (Anbumani and Mohankumar 2012).

Statistical analysis

Statistical analyses were performed using Prism 3 (GraphPad Software, Inc). All data from the assays were tested for normality using Shapiro–Wilk’s test. Since the data showed normal distribution, one-way ANOVA followed by a post hoc test (Tukey) was performed. The level of statistical significance was set at $p < 0.01$ and $p < 0.05$. Correlation was calculated according to Spearman, and the significance level was set at 95 % ($\alpha = 0.05$).

Results

For the determination of median lethal concentration (LC_{50}), the number of fish that died at 3, 6, 12, 24, 48, and 96 h post exposure was considered. Aside from mortality, fish behavior and toxic symptoms induced by monocrotophos and butachlor were also observed and recorded. Some of the toxicity symptoms observed were pigmentation, surface air gulping, excessive mucous secretion, rapid opercular movement, loss of equilibrium, and lateral lying at the bottom of the aquaria. Interestingly, dead fish exposed to all lethal concentrations of monocrotophos exhibited “curved spine” symptom along with aforementioned manifestation, a unique symptom of OP toxicity. The 96-h LC_{50} values of MCP and butachlor 50 % EC were determined as 12.6 ppm (95 % confidence interval 10.5–13.9 ppm) and 0.315 ppm (95 % confidence interval 0.2–0.4 ppm), respectively.

Micronuclei induced by MCP, butachlor, and their combination

A statistically significant increase in the frequency of MN was noted in fishes exposed to sublethal concentrations I, II, and III of MCP, butachlor, and their combination with respect to corresponding control and solvent control. The frequency of MN is shown in Table 1. Concentration- and duration-dependent increase in the frequency of micronuclei was observed in all three sublethal concentrations. Peaks in the induction of MN appeared on day 28, 21, and 7 in fishes exposed to MCP, butachlor, and its combination, respectively. On par with single treatments, a twofold increase in MN frequency was noted at combined exposures indicating the synergistic action of pesticides. MCP- and butachlor-induced MN and other aberrations are shown in Fig. 1.

Nuclear abnormalities induced by MCP, butachlor, and its combination

The sum of total frequencies of DN+NBu+VN termed as erythrocyte nuclear abnormalities as a function of sampling time are presented in Fig. 2a–c. Apart from the formation of MN, fish exposed to concentration range 0.625 to 2.5 ppm of MCP, 0.016 to 0.064 ppm of butachlor, and its combination induced a significant increase in the frequencies of DN, NBu, and VN ($p < 0.0001$). Frequency of NBu was increased two- to threefold above the MN values peaking on day 28.

Interestingly, binucleated cells were observed only in fish exposed to all three sublethal concentrations (0.016, 0.032, and 0.064 ppm) of butachlor (Table 2). An enhanced frequency of BNCs was observed after combined exposures.

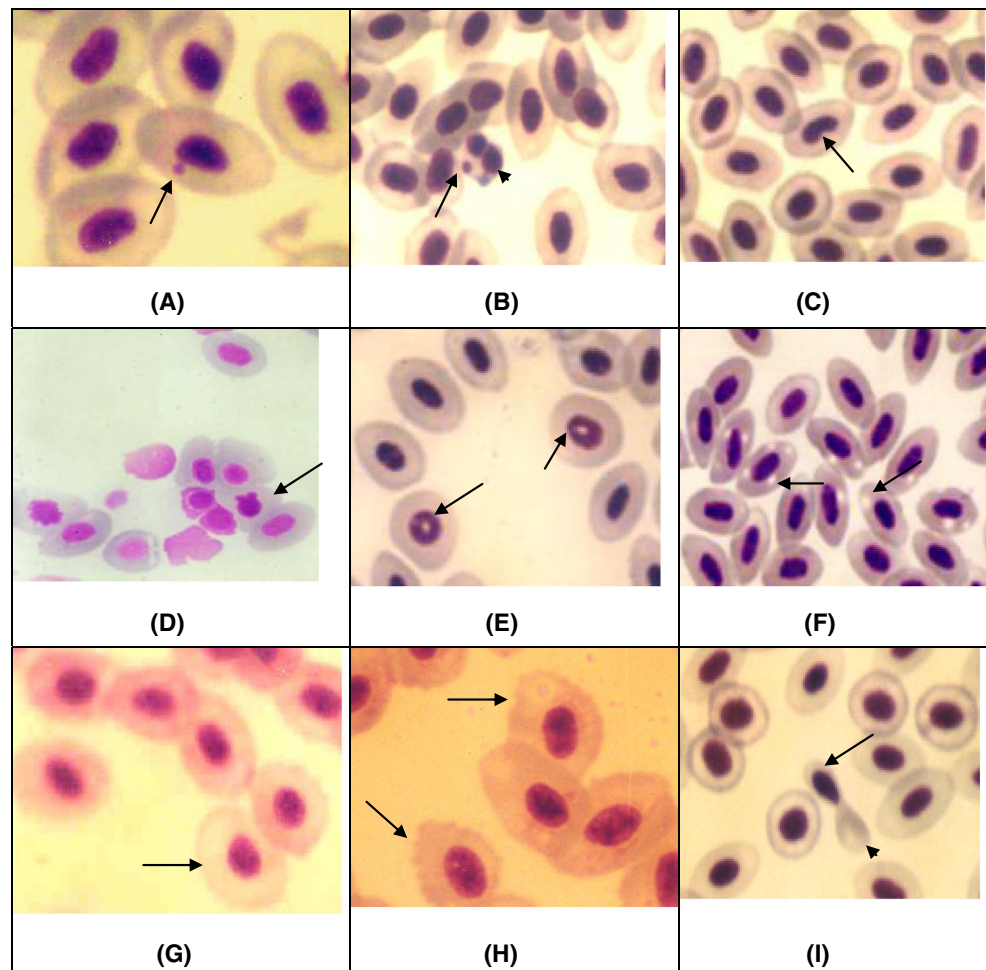
Table 1 Frequency of MN at chronic combined exposures in the fish *Catla catla* exposed to MCP and butachlor

Agrochemical used	Exposure type	Frequency of MN (mean±SE)					
		Day 3	Day 7	Day 14	Day 21	Day 28	Day 35
MCP (monocrotophos)	Control	0.60±0.22	0.80±0.29	0.50±0.22	1.0±0.33	0.70±0.26	0.70±0.26
	Solvent control	0.80±0.29	1.10±0.27	0.60±0.30	1.20±0.32	0.90±0.27	0.50±0.22
	0.625 ppm	0.70±0.26	1.80±0.41	2.10±0.34*	5.60±0.67 ^{aa}	12.40±0.98 ^{aa}	0.71±0.22
	1.3 ppm	1.10±0.31	2.60±0.37*	3.50±0.52*	7.90±0.23 ^{aa}	17.40±1.18 ^{aa}	1.0±0.25
	2.5 ppm	1.80±0.24*	3.40±0.40*	6.0±0.36*	12.70±0.50 ^{aa}	21.70±0.47 ^{aa}	1.30±0.21
Butachlor 50 % EC	0.016 ppm	0.50±0.22	0.50±0.16	2.20±0.38	6.70±0.74 ^{aa}	1.20±0.32	0.40±0.22
	0.032 ppm	0.80±0.29	1.50±0.30*	3.0±0.51*	14.80±0.84 ^{aa}	2.40±0.47	0.60±0.26
	0.064 ppm	1.10±0.27	2.70±0.30*	5.10±0.48*	20.20±0.62 ^{aa}	2.80±0.24	0.80±0.24
MCP+butachlor	0.625+0.016 ppm	5.40±0.40 ^{aa}	9.90±0.62 ^{aa}	3.20±0.51 ^{aa}	1.40±0.34	0.80±0.24	0.30±0.15
	1.3+0.032 ppm	12.70±1.03 ^{aa}	20.0±1.68 ^{aa}	8.30±0.83 ^{aa}	2.30±0.39*	1.90±0.54	0.60±0.26
	2.5+0.064 ppm	17.10±0.76 ^{aa}	56.60±1.16 ^{aa}	12.0±1.03 ^{aa}	5.0±0.57*	3.60±0.52*	1.0±0.25

*Statistically significant at 0.05 % level with respect to control and solvent control; ^{aa} statistically significant at 0.0001 % level with respect to control and solvent control

Compared to individual treatments, frequencies of nuclear abnormalities were found to be synergistically elevated in a concentration- and duration-dependent manner at all three sublethal concentrations of combined exposures.

Fig. 1 Micronuclei and other cellular anomalies in the fish *Catla catla* (Ham.) to sublethal concentrations of MCP and butachlor at single and combined exposures. **a** Micronuclei (MN), **b** binucleated cell (BNC) (arrow head) with micronuclei, **c** deformed nucleus (DN), **d** nuclear bud (NBu), **e** vacuolated nucleus (VN), **f** vacuolated cytoplasm (VC), **g** anisochromasia (AN), **h** echinocyte (EC), **i** cytodieretic pattern showing invagination of cytoplasm responsible for microcyte (MC) and enucleus (EN) (arrow head) formation



Cytoplasmic abnormalities induced by MCP, butachlor, and its combination

Cytoplasmic abnormalities, the sum of frequencies of VC, AN, and EC observed to a considerable extent in fish exposed to 0.625 to 2.5 ppm of MCP, 0.016 to 0.064 ppm of butachlor, and their combinations were shown in Fig. 3a–c. Enucleated cells (EN) appeared in fish exposed to all sublethal concentrations of butachlor (Table 2). Similar to nuclear abnormalities, statistically significant increases in cytoplasmic abnormalities were observed after MCP, butachlor, and combination exposures, exhibiting peaks on day 7, 14, and 21, respectively.

Correlation between MN and other anomalies

Significant positive correlations between micronucleus, individual nuclear, and total erythrocyte nuclear abnormalities were noted in fish exposed to MCP, butachlor, and their combinations even at lower concentrations (Table 3; Fig. 4a–d) demonstrating them as prospective markers of genotoxic exposure.

Discussion

The erythrocyte micronucleus assay (EMNA) is a simple, reliable, yet sensitive cytotoxic assay and hence a preferred bioindicator of environmental genotoxicity over other cytogenetic techniques. A recent study from this laboratory has shown that apart from MN, various types of cytological anomalies could also be considered as biomarkers of exposure, thus justifying renaming this assay as the erythrocyte micronucleus cytome assay (EMNCA) (Anbumani and Mohankumar 2012).

MN formation after MCP, butachlor, and its combined exposures

Micronuclei are envisaged to be formed as a result of symmetrical and asymmetrical chromatid and chromosome exchanges or whole chromosome fragments that fail to be included in the daughter nuclei at the completion of telophase during mitosis due to lack of spindle attachment during the segregation process in anaphase (Fenech 2011). The frequency of MN and other nuclear aberrations in fish erythrocytes can serve as a relevant parameter in the assessment of genotoxic pollutants and may represent a valuable tool as a biological dosimeter in polluted environments providing an indirect evidence of the frequency of chromosomal and DNA damage and an insight into the risk of human health arising from presence of genotoxic environmental contaminants in aquatic ecosystems (Al-Sabti 1991).

In the present study, a statistically significant increase in MN was noticed in fish exposed chronically to sublethal concentrations of monocrotophos, butachlor singly, and in combination in a time- and concentration-dependent manner. These observations are in good agreement with the results of earlier studies with pesticides and heavy metals, in natural and laboratory conditions in fishes (Das and Nanda 1986; de Flora et al. 1993; Nepomuceno and Spano 1995; Nepomuceno et al. 1997; Campana et al. 1999; Ateeq et al. 2002; Cavas and Ergene-Gozukara 2003; Summak et al. 2010; Polard et al. 2011). Ali and Kumar (2008) observed a significant increase in DNA strand breaks in the fish *Channa punctatus* exposed to MCP concentrations ranging between 1.59 and 4.78 mg/L. The present study shows that cytogenetic biomarkers using the sensitive EMNCA could detect DNA damage at much lower pesticide concentrations (0.625–2.5 mg/L) in the fish *C. catla*.

The peak in the appearance of MN in peripheral erythrocytes differs between two different classes of compounds used in the present study. MCP exposure showed a peak on day 28, whereas butachlor and combined exposures exhibited peaks on day 21 and 7, respectively. A two- to threefold increase in MN frequency was noted in fish exposed to a mixture of MCP and butachlor, indicating the synergistic action at lower subchronic concentrations. Similar to the present study, Bony et al. (2008) observed statistically significant DNA damage seen as strand breaks using the comet assay in the fish *Salmo trutta fario* contaminated by a mixture of vineyard pesticides in the Morcille River. Devaux et al. (1998) and Flammarion et al. (2002) reported a threefold induction in DNA strand breaks in erythrocytes of chub (*Leucis cephalus*) polluted by complex mixture of organic compounds and metals. Winter et al. (2004) also observed a twofold increase in the DNA damage in both feral and caged fish in UK rivers exhibiting different levels of contaminants.

The difference in the induction of MN at different sampling times could be related to the blood cell kinetics and erythrocyte replacement rate as proposed by Campana et al. (1999). An early manifestation of MN peaking on day 7 indicates a synergistic action of MCP and butachlor probably due to the combined action of OP and OC functional groups. Quantitative structure activity relationship (QSAR) studies will shed more information towards the synergistic mechanisms.

Another plausible reason for the peak in MN formation might be due to the time taken for the production of new cells that experience DNA damage to reach peripheral circulation from the cephalic kidney or the spleen under stressful conditions of pesticide exposure. It could be due to the inhibition of apoptosis as demonstrated by Decordier et al. (2002) and further confirmed by Polard et al. (2011). In the present study, elevated levels of MN were observed at different time points of exposure to MCP or butachlor and this might be due to the release of cells experiencing DNA damage in the

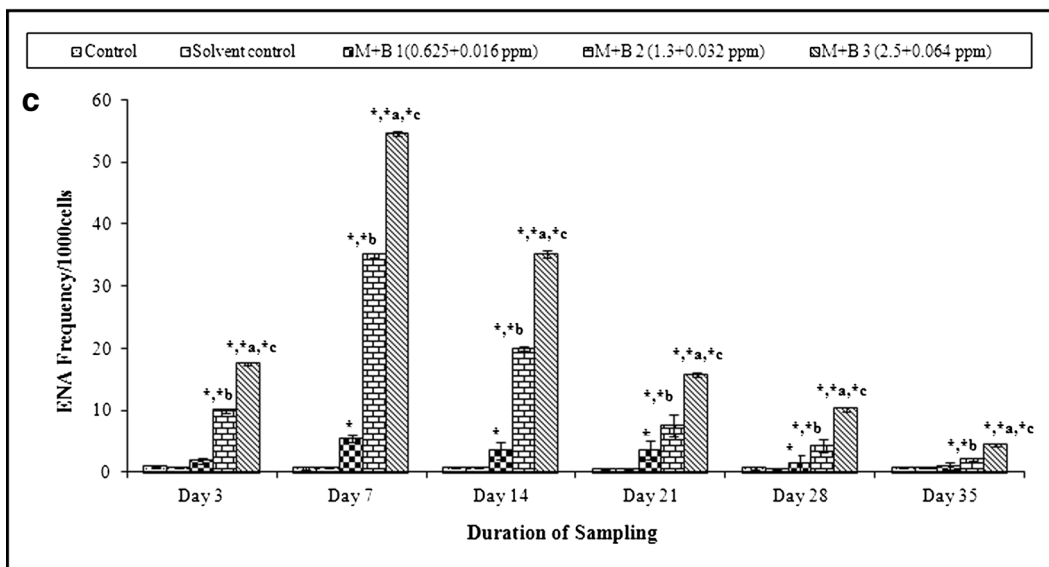
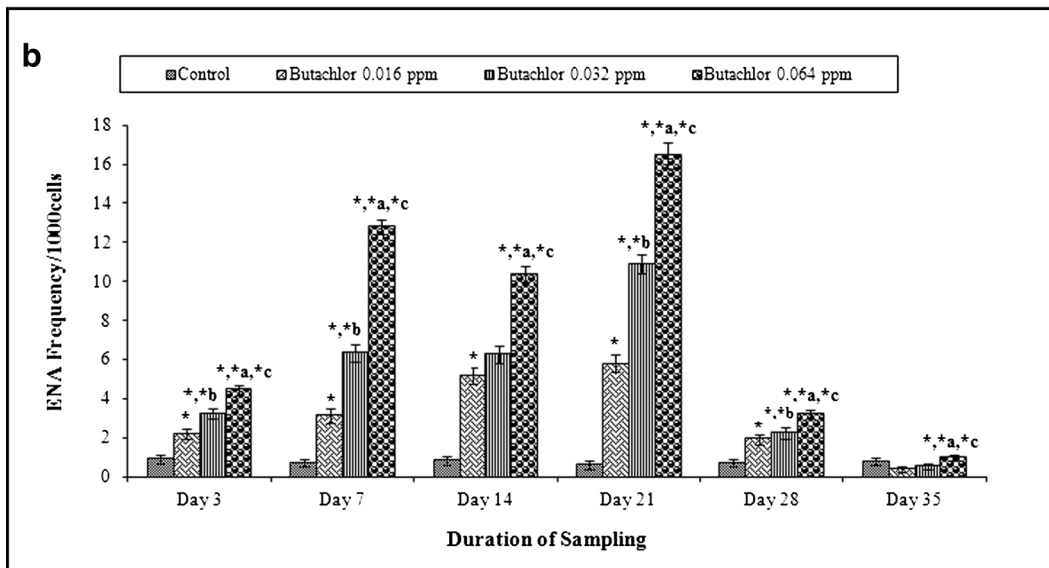
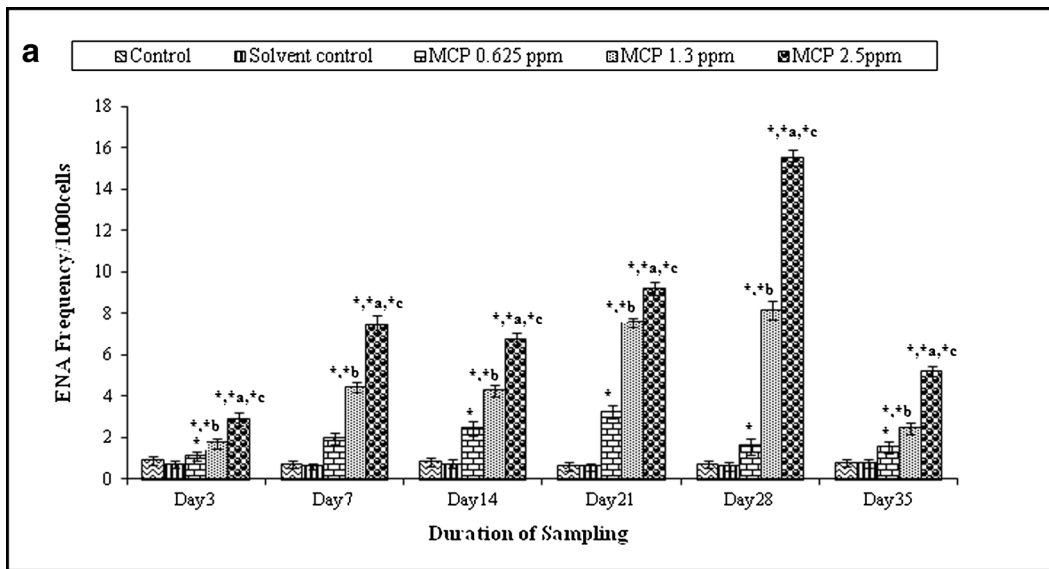


Fig. 2 a Frequencies (mean±SEM) of erythrocyte nuclear abnormalities (ENA)/1000 cells in fish exposed to sublethal concentrations of monocrotophos. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control and solvent control; ^asignificant at $p<0.001$ level between 2.5 and 1.3 ppm; ^bsignificant at $p<0.001$ level between 1.3 and 0.625 ppm; ^csignificant at $p<0.001$ level between 2.5 and 0.625 ppm. **b** Frequencies (mean±SEM) of erythrocyte nuclear abnormalities (ENA)/1000 cells in fish exposed to sublethal concentrations of butachlor. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control; ^asignificant at $p<0.001$ level between 0.064 and 0.032 ppm; ^bsignificant at $p<0.001$ level between 0.032 and 0.016 ppm; ^csignificant at $p<0.001$ level between 0.064 and 0.016 ppm. **c** Frequencies (mean±SEM) of erythrocyte nuclear abnormalities (ENA)/1000 cells in fish at combined exposures to sublethal concentrations of monocrotophos and butachlor. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control and solvent control; ^asignificant at $p<0.001$ level between M+B1 and M+B2; ^bsignificant at $p<0.001$ level between M+B2 and M+B3; ^csignificant at $p<0.001$ level between M+B1 and M+B3

hematopoietic stem cells and subsequent maturation taking place over these days in peripheral blood cells.

Mode of action of monocrotophos and butachlor has been well established by numerous researchers (Wauchope et al. 1992; Beal 1995; Yamano and Morita 1995; Ashby et al. 1996; Perry et al. 1998; Lushchak 2011). The decrease in the MN formation and other cytological abnormalities after various intervals may indicate repair of damaged DNA, loss of heavily damaged cells, or both as proposed by Banu et al. (2001). This inverse relationship between time of exposure and DNA damage may be due to toxicity of xenobiotics that could perturb enzymatic processes in the formation of DNA damage (Rank and Jensen 2003). Another putative mechanism could be gene activation of metabolizing enzymes such as cytochrome P450 in various tissues that provides a defensive mechanism against the persistent organic pollutants (Wong et al. 2001). Similar repair mechanism was observed with pesticides like malathion and monocrotophos in isolated human lymphocytes and fish (Blasiak et al. 1999; Banu et al. 2001). It is well known that, chemical toxicants like pesticides induced DNA damage through oxidative stress via free-radical generation. Thus, in the present study, MCP and butachlor synergistically induced elevated oxidative stress and its subsequent DNA damage in the form of micronuclei and other anomalies in the sentinel fish, *C. catla*. Oxidative enzyme profile studies in this fish at chronic exposures of these two different classes of agrochemicals are required to address these possibilities.

MCP-, butachlor-induced nuclear anomalies at single and combined exposures

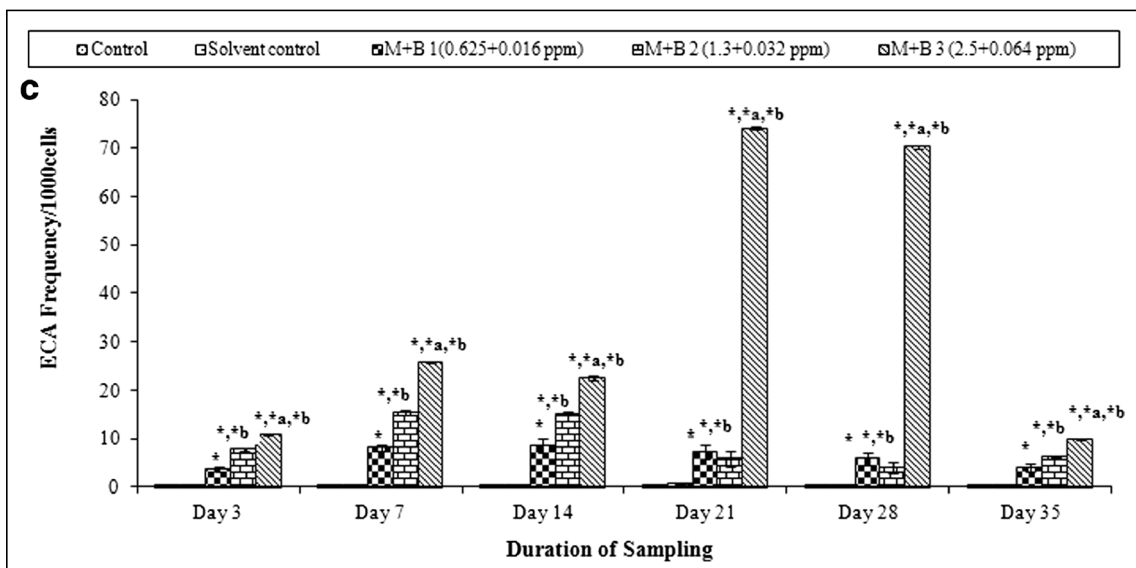
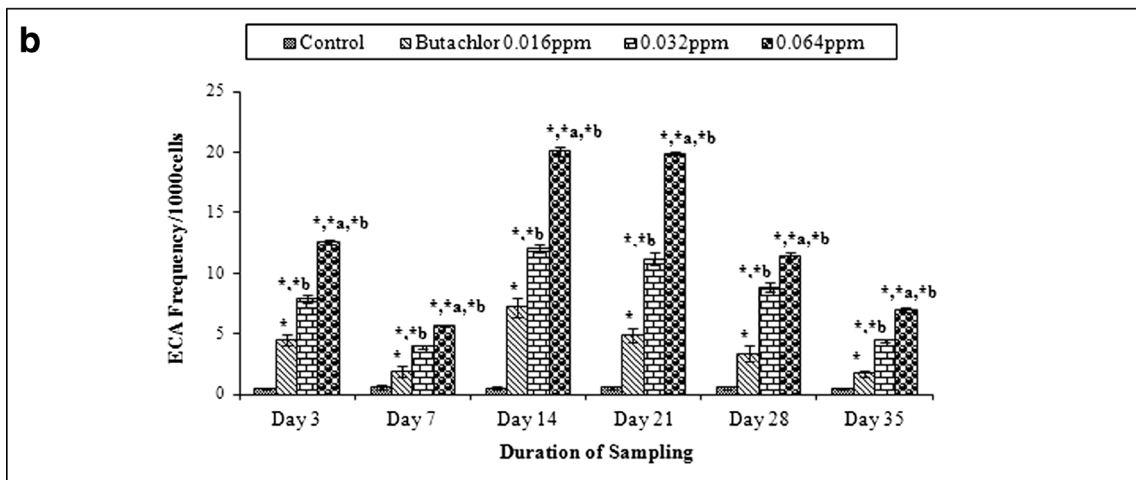
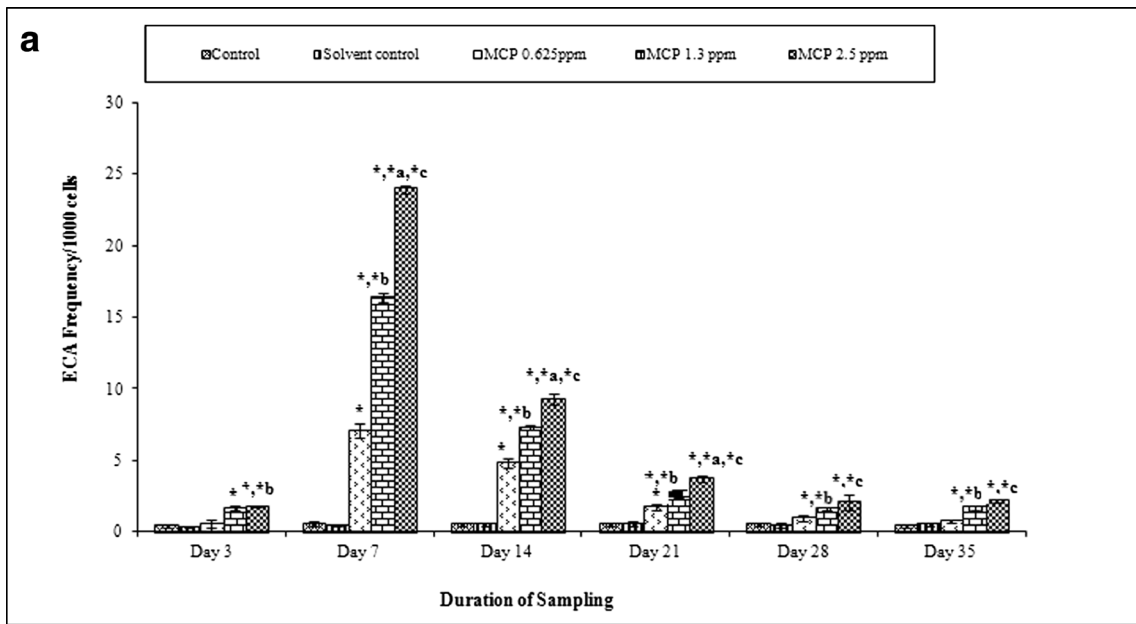
Apart from the appearance of micronuclei, simultaneous expression of morphological nuclear abnormalities (NAs) received considerable attention in the field of aquatic

Table 2 Frequency of binucleated and enucleated cells at chronic exposures in the fish *Catla catla* exposed to butachlor

Aberration	Concentration (ppm)	Duration of sampling and frequency (mean±SE)						
		Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	
BNC	Control	0±0	0±0	0±0	0±0	0±0	0±0	0±0
	0.016	0.30±0.21	2.20±0.51	4.40±0.60	13.80±0.71	3.60±0.81	2.0±0.63	
	0.032	1.40±0.34	5.60±0.60 ^{a,b1}	6.90±0.40 ^{a,b1}	36.70±2.20 ^{a,b}	6.10±0.70	2.60±0.47	
EN	0.064	1.0±0.36	6.20±0.57 ^{a,c}	11.70±0.79 ^{a,a,c}	59.80±2.43 ^{a,b,c}	9.50±0.60 ^{a1,c}	2.10±0.70	
	Control	0.10±0.10	0.40±0.22	0.20±0.13	0.20±0.20	0.50±0.16	0.20±0.20	
	0.016	0±0	0.50±0.22	0.80±0.35	0±0	0.30±0.15	0.40±0.22	
	0.032	0.10±0.10	3.0±0.39 ^{a,b}	1.80±0.38 ^{a,b}	0.20±0.13	0.50±0.30	0.70±0.26	
	0.064	0.20±0.13	2.40±0.49 ^{a,c1}	0.90±0.31	0.40±0.22	0.30±0.21	0.60±0.26	

BNC binucleated cell, EN enucleated cells

*Significant at $p<0.001$ w.r. to control; **significant at $p<0.05$ w.r. to control; ^a significant at $p<0.001$ between 0.064 and 0.032 ppm; ^{a1} significant at $p<0.05$ between 0.064 and 0.032 ppm; ^b significant at $p<0.001$ between 0.032 and 0.016 ppm; ^{b1} significant at $p<0.001$ between 0.064 and 0.016 ppm; ^c significant at $p<0.05$ between 0.064 and 0.016 ppm; ^{c1} significant at $p<0.05$ between 0.064 and 0.016 ppm



◀ **Fig. 3 a** Frequencies (mean±SEM) of erythrocyte cytoplasmic abnormalities (ECA)/1000 cells in fish exposed to sublethal concentrations of monocrotophos. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control and solvent control; *^asignificant at $p<0.001$ level between 2.5 and 1.3 ppm; *^bsignificant at $p<0.001$ level between 1.3 and 0.625 ppm; *^csignificant at $p<0.001$ level between 2.5 and 0.625 ppm. **b** Frequencies (mean±SEM) of erythrocyte cytoplasmic abnormalities (ECA)/1000 cells in fish exposed to sublethal concentrations of butachlor. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control; *^asignificant at $p<0.001$ level between 0.064 and 0.032 ppm; *^bsignificant at $p<0.001$ level between 0.032 and 0.016 ppm; *^csignificant at $p<0.001$ level between 0.064 & 0.016 ppm. **c** Frequencies (mean±SEM) of erythrocyte cytoplasmic abnormalities (ECA)/1000 cells in fish at combined exposures to sublethal concentrations of monocrotophos and butachlor. ANOVA followed by Tukey post hoc test. *Significant at $p<0.001$ with respect to corresponding control; *^asignificant at $p<0.001$ level between 0.064 and 0.032 ppm; *^bsignificant at $p<0.001$ level between 0.032 and 0.016 ppm; *^csignificant at $p<0.001$ level between 0.064 and 0.016 ppm

genotoxicology (Ayllon and Garcia-Vazquez 2000; Pacheco and Santos 2002; Cavas and Ergene-Gozukara 2005;

Bolognesi et al. 2006; Guilherme et al. 2008; Osman et al. 2011). The correlation between the frequencies of MN and nuclear abnormalities emphasizes the importance of other nuclear abnormalities as prospective biomarkers (Osman and Harabawy 2010).

In the present study, along with MN, various nuclear abnormalities like deformed nucleus, nuclear bud, vacuolated nucleus, and binucleated cells were observed in fish exposed to sublethal concentrations of MCP and butachlor at single and combined treatment regimes. Although there are several studies on the induction of NAs in aquatic organisms exposed to chemicals under laboratory conditions (Venier et al. 1997; da Silva and Fontanetti 2006; Cavas and Ergene-Gozukara 2003; Cavas and Ergene-Gozukara 2005), reports on agrochemical-induced nuclear abnormalities were not well investigated and present investigation is the first of kind in reporting the agrochemical-induced nuclear anomalies at combined chronic exposures.

Ateeq et al. (2002) observed several nuclear and cytoplasmic anomalies in the fish *Clarias batrachus* exposed to

Table 3 Correlation data of agrochemical-induced nuclear anomalies in the fish *Catla catla*

Data	Pesticide concentration (ppm)	r value	p value
Frequency of MN w.r.t NBu	MCP		
	2.5	0.92	0.0076
	1.3	0.99	0.0001
	0.625	0.98	0.0002 (Fig. 4a)
	M+B combination		
	M+B1	0.93	0.0054
	M+B2	0.83	0.0380
Frequency of MN w.r.t DN	M+B3	0.88	0.0192 (Fig. 4b)
	Butachlor		
	0.032	0.82	0.0442
	M+B combination		
	M+B1	0.91	0.0118
Frequency of MN w.r.t BNCMN w.r.t. VN	M+B2	0.89	0.0151
	Butachlor		
	0.064	0.99	<0.0001
	0.032	0.99	<0.0001
	0.016	0.89	0.0333 (Fig. 4c)
Frequency of MN w.r.t ENA	0.064	0.82	0.0454
	MCP		
	2.5	0.94	0.0037
	1.3	0.87	0.0230
	0.625	0.98	0.0005
	Butachlor		
	0.032	0.82	0.0439
	0.016	0.85	0.0288
	M+B combination		
M+B1	0.89	0.0147	
M+B2	0.88	0.0190 (Fig. 4d)	

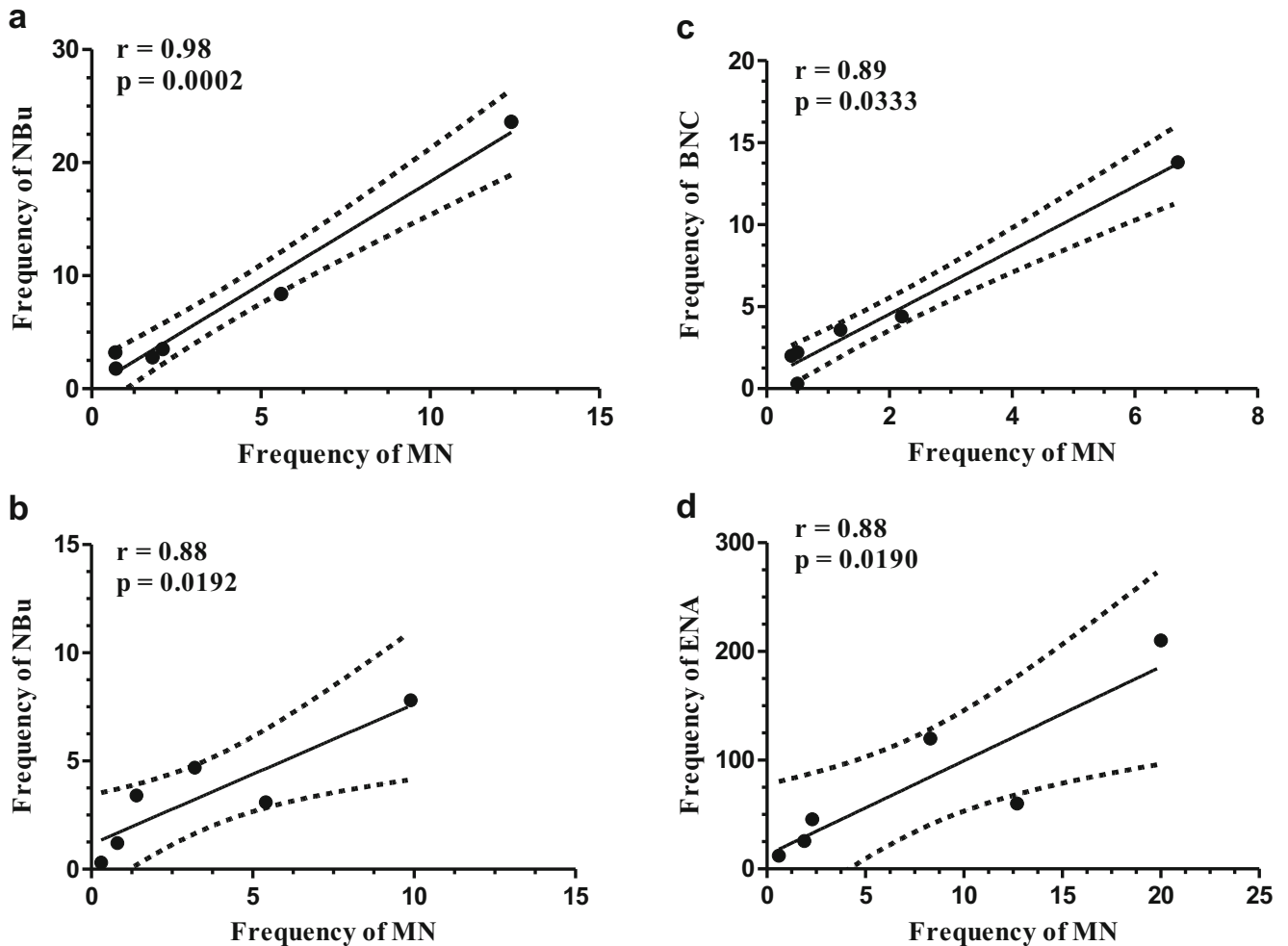


Fig. 4 **a** Correlation between MN and NBu in the fish *Catla catla* exposed to sublethal concentration (0.625 ppm) of monocrotophos technical. Spearman correlation was used and the level of significance set at 95 % ($\alpha=0.05$). **b** Correlation between MN and NBu in the fish *C. catla* at combined exposures to sublethal concentration of monocrotophos and butachlor (0.625+0.016 ppm). Spearman correlation was used and the level of significance set at 95 % ($\alpha=0.05$). **c** Correlation between MN and

BNC in the fish *C. catla* exposed to sublethal concentration (0.016 ppm) of butachlor. Spearman correlation was used and the level of significance set at 95 % ($\alpha=0.05$). **d** Correlation between MN and ENA in the fish *C. catla* at combined exposures to sublethal concentration of monocrotophos and butachlor (1.3+0.032 ppm). Spearman correlation was used and the level of significance set at 95 % ($\alpha=0.05$)

sublethal concentrations (1–2.5 mg/L) of butachlor and 2, 4-D and thereby proposed nucleus as a specific biomarker to butachlor exposure. In the present study also, binucleated and enucleated cells were observed in fishes exposed to butachlor even at concentrations much lower than those used by Ateeq et al. In the present study, the frequencies of MN and NBu increased in a time-dependent manner showing a linear correlation. Similar observations were made by others (Bolognesi et al. 2006; Ergene et al. 2007; Cavas 2008). A threefold increase in the formation of nuclear buds was observed in fish exposed to MCP. Butachlor-exposed fish showed a fourfold increase in deformed nucleus than MCP, whereas in combined exposures, it was significantly increased than MCP or butachlor.

Statistically significant increase in binucleated cells was observed in butachlor-exposed individuals and to a greater

extent in combined exposures. This may be attributed to blocking of protein synthesis and thereby inhibition of cell division as proposed by Panneerselvam et al. (1999). This observation suggests a synergistic action of butachlor with other chemicals to inhibit cytokinesis.

MCP-, butachlor-induced cytoplasmic abnormalities at single and combined exposures

Cytoplasmic abnormalities like vacuolated cytoplasm, anisochromasia, echinocytes, and enucleus were also noted in the fish *C. catla* exposed to sublethal concentrations of MCP or butachlor at single and combined exposures. Several ambiguities exist in elucidating the mechanism of formation of these cytoplasmic anomalies. Several red cells were bilobed with a marked constriction of the cytoplasm and dislocation of

the nucleus to the periphery; a possible cytodieretic pattern connected with the process of cell denucleation and erythroplastid formation in pesticide exposed fishes. The erythroplastids found in the peripheral blood revealed the presence of a small nucleus termed as microcyte. This is probably derived from the cytoplasm splitting of erythrocytes, in the plane of the short axis at the periphery of the nucleus in organisms exposed to pollutants (Barni et al. 2007). The eccentric or peripheral splitting plane of bilobed cells thus leads to the formation of enucleus (Blum et al. 1988; Fijan 2002). Ellis (1984) suggested the bilobed cell with cytoplasmic constriction to be a consequence of pathological fragmentation or a form of senescence. Vacuoles observed in the present study could be due to cell injury induced by MCP and butachlor in erythrocytes leading to intracellular reduction in oxidative phosphorylation with a resultant drop in the ATP level (Ateeq et al. 2002).

Conclusion

Based on our experimental findings, chronic exposures of MCP and butachlor at sublethal concentrations resulted in increased genotoxic events in the form of MN and other cytological anomalies synergistically in the fish *C. catla*. This concentration-dependent synergistic increase in MN and other cellular abnormalities during the 35-day study period following pesticide exposures clearly reveals clastogenicity and genotoxicity in peripheral erythrocytes. This study illustrates the cytogenotoxic effects of agrochemicals at combined exposures under laboratory conditions at very low sublethal concentrations. The present study explored a significant positive correlation between MN and nuclear anomalies after agrochemical exposures, thereby suggesting them as alternate biomarkers of exposure. The study highlights the importance and sensitivity of erythrocyte micronucleus cytochrome assay (EMNCA) in determining the synergistic effects of sublethal concentrations of agrochemicals and also suggests *C. catla* as a suitable aquatic biomonitoring sentinel species.

Conflict of interest None declared.

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