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Comparative teratogenicity of Chlorpyrifos and Malathion on *Xenopus laevis* development

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Abstract

The embryotoxic potential of chlorpyrifos (CPF) and malathion (MTN), two organophosphorus insecticides (OPs), was evaluated by modified Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). CPF and MTN were not embryolethal even at the highest concentration tested (6000 μg/l), but both exhibited a powerful teratogenicity. The probit analysis of malformed larva percentages showed a TC_{50} of 161.54 μg/l for CPF, and a TC_{50} of 2394.01 μg/l for MTN. Therefore, CPF teratogenicity was about 15 times higher than MTN. Larvae of both exposed groups were mainly affected by ventral and/or lateral tail flexure coupled with abnormal gut coiling. Histopathological diagnosis displayed abnormal myotomes and myocytes with marked hypertrophies localized at the cell extremity, probably due to a break away of myofibril extremities at the intersomitic junction level. We speculate that this muscular damage was related to inhibition of acetylcholinesterase that showed a clear concentration-response in CPF and MTN exposed larvae. The teratogenic effects of these anti-cholinesterase compounds on *Xenopus laevis* myogenesis suggest a possible role played by OPs on induction of congenital muscular dystrophy.

Keywords: Chlorpyrifos; FETAX; Malathion; Muscular damage

1. Introduction

In recent decades, many pesticides have been subjected to careful investigations to assess their harmful effects on the environment. While highly stable chlorinate hydrocarbons are undoubtedly ecotoxic

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compounds, the hazard effects of rapidly degraded organophosphates insecticides (OPs), widely used in agriculture and in households, are less evident. OPs ability to poison insects is the result of their anticholinesterase activity. The usual symptoms in insects roughly follow the general pattern of nerve poisoning like hyper excitability, tremors, convulsions and paralysis that lead to death. Many other supporting or contradicting data for the theory of OP anticholinesterase activity could be described, but most evidence, at present, strongly support the view that cholinesterase inhibition in vertebrates is analogous to what happens in insects. In both insects and vertebrates it is common practice to separate OP poisoning symptoms into muscarinic, nicotinic and central nervous. Of these, the nicotinic effect is the result of action on somatic nerve elements which cause an over stimulation followed by paralysis of voluntary muscles (Matsumura, 1975). Indeed, OP neurotoxicity mediated by the phosphorylation and subsequent inhibition of acetylcholinesterase (AChE) is most noted in mammals, humans included (Oehmichen and Besserer, 1982; Finkelstein et al., 1988). Even if OPs have not been classified as teratogenic compounds in mice and rats (Bleyl, 1980; Robinson et al., 1986; Ruckman et al., 1999), there are growing data showing that they are teratogenic on the grounds of experimental concentrations in non-mammal developing embryos, such as amphibians (Richards and Kendall, 2002) and birds (Meiniel, 1981).

In this paper, with the aid of the modified Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), a powerful and flexible bioassay for developmental toxicants (Dumont et al., 1983; Bantle et al., 1990; Vismara et al., 1993; Bernardini et al., 1994), we studied the teratogenic potential of chlorpyrifos (CPF) and malathion (MTN), comparing the rate of AChE inhibition with the degree of the teratogenic effects. Considering that AChE is required for embryo muscular development (Behra et al., 2002) and OPs are able to induce muscular damages in mammals (Gupta et al., 1987; Karalliedde and Henry, 1993; De Bleecker et al., 1994; John et al., 2003), we studied at histological level the malformations induced by CPF and MTN on the tail musculature, since previous works had shown that *Xenopus laevis* larvae exposed to these compounds displayed an abnormal tail flexure with impairment of motility (Vismara et al., 1996; Richards and Kendall, 2002).

2. Materials and methods

2.1. Chemicals and solutions for the bioassay

The organophosphorus insecticides chlorpyrifos (*O*,*O*-diethyl *O*-[3,5,6-trichloro-2-pyridyl] phosphorothioate; CPF) and malathion (S-[1,2-dicarbethoxyethyl] *O*,*O*-dimethyldithiophosphate; MTN) with over 99% purity were supplied by Labservice Analytica S.r.l, Italy. All analytical grade reagents, human chorionic gonadotropin (HCG), 3-amino-benzoic acid ethyl ester (MS 222), Triton X-100, 4′,6-diamidino-2-phenylindole (DAPI), phalloidin-TRITC were obtained from Sigma-Aldrich S.r.l., Italy. The control FETAX solution composition in mg/l was NaCl 625, $NaHCO_3$ 96, KCl 30, $CaCl_2$ 15, $CaSO_4$-$2H_2O$ 60, and $MgSO_4$ 70, pH 7.5–8.5 (Dawson and Bantle, 1987). The De Boer-Tris (DBT) solution in mg/l was NaCl 6,900, KCl 186, $CaCl_2$ 200 buffered to pH 7.5 with 10 mM Tris–HCl.

2.2. Animals

Adult males and females *X. laevis* were from the Centre d'élevage de Xénopes du CNRS, Rennes, France. The animals were acclimated for at least 6 weeks in aquaria with dechlorinated tap water at $22 \pm 2\,^{\circ}C$, alternating 12 h light–dark cycles and fed a semi synthetic diet from Mucedola S.r.l., Settimo Milanese, Italy, three times a week.

2.3. Frog Embryo Teratogenesis Assay-Xenopus (FETAX)

We used a modified version of FETAX (Bernardini et al., 1994) as follows.

2.3.1. In vitro fertilisation

For a single bioassay, six females were injected with 900 IU while two males with 500 IU of HCG. About 16 h later, eggs obtained by massaging the females' abdomens were put in a 140-mm plastic Petri dishes and then artificially inseminated with a sperm suspension previously obtained by mincing adult male testes in 1–2 ml of cold DBT solution (Vismara et al., 1993). One minute later, 30 ml of FETAX solution were added to each Petri dish. Successful insemination was

detected when the eggs were oriented with the dark animal pole side up. A first screening performed 3 h post fertilisation, p.f., enabled us to remove the unfertilised and necrotic eggs; this was followed by a second screening, 5 h p.f., stage 8, blastula, in which normal cleavage was ascertained (Niewkoop and Faber, 1956).

2.3.2. CPF and MTN experimental groups

Ten undejellied normal blastulae from the same female were put in a 40 mm Petri dishes to make control and exposed groups. Each Petri dish contained 20 ml of control or test solution. This procedure was followed for the six females of the bioassay. Blastulae (stage 9, 8 h p.f.) were exposed to eight CPF concentrations ranging from 50 to 6000 μg/l or to five MTN concentrations ranging from 375 to 6000 μg/l. The exposure was suspended at stage 47 of free swimming larva (120 h p.f.). The single bioassay was repeated four times for CPF and three times for MTN under the same experimental conditions. During the bioassay the dishes were kept in a thermostatic chamber at $23 \pm 0.5\,^{\circ}C$ and each day the test solutions were renewed and the dead embryos removed. The number of dead embryos was recorded.

2.3.3. Data collection

At the end of exposure time (stage 47), the number of dead embryos was calculated, while the surviving larvae were anaesthetised with MS 222 (100 mg/l) for morphological examination. The number of malformed larvae as well as the frequency of single malformations was recorded. When in the control group of one female the mortality and malformation rates were greater than 20%, all groups of that female were discarded (Bernardini et al., 1994).

2.3.4. Statistical analysis

The relationship between the CPF and MTN concentrations and outcomes, percentage of dead embryos and malformed larvae, was investigated by resorting to chi-square and probit analysis (Finney, 1971). The effective concentrations at 120 h p.f. are called, depending on the case, lethal (LC_{50}) or teratogenic (TC_{50}). The Teratogenic Index, T.I., useful in estimating the teratogenic risk associated with the tested compounds, was the ratio LC_{50}/TC_{50} (Dawson and Bantle, 1987).

2.4. Histological studies

Stage 47 control larvae and those exposed to CPF or MTN at 3000 μg/l were fixed in 10% formaldehyde in PBS, repeatedly rinsed in PBS, dehydrated in a graded alcohol series and embedded in paraffin. Transversal and para-sagittal 5 μm sections were dewaxed, hydrated in a graded ethanol series and stained with Mayer's haemalaun and eosin. Further control and exposed larvae were fixed for 2 h in modified Karnowsky's liquid (2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M PBS, pH 7.4), dehydrated in graded alcohol series and embedded in epoxy resin Epon 812. Transversal and para-sagittal 0.5 μm semithin sections were stained with crystal violet and basic fuchsin. Slides were examined under a light Zeiss Axioplan MC 100 microscope. Images were taken with a colour digital Image Pro Plus version 4.5.1, Media Cybernetics.

2.5. Confocal microscopic analysis

Stage 47 control larvae and those exposed to CPF or MTN at 3000 μg/l were anaesthetised with MS 222 (100 mg/l). Whole-mount tails were fixed in 4% paraformaldehyde in PBS buffer 120 mM for 1 h and washed in Triton X-100 (0.4% in PBS buffer 120 mM) for 20 min and then with PBS 120 mM for 15 min. Actin was stained with 0.5 μg/ml phalloidin-TRITC and nuclei with 20 μg/ml DAPI for 30 min. The tails were washed in PBS 120 mM, mounted in a glycerol-based medium and stored at $4\,^{\circ}C$ in the dark. Myocyte actin filaments and nuclei were visualised with a Leica TCS-NT confocal microscope, Leica Microsystem, Heidelberg, Germany. Focal series of horizontal planes of section were monitored for TRITC using a Ar/Kr laser with a long pass filter LP 590 and for UV using a Ar ion laser with a long pass filter LP 450.

2.6. AChE activity analysis

Stage 47 control larvae and those exposed to CPF at 100, 250 and 3000 μg/l and to MTN at 1500, 3000 and 6000 μg/l were used for AChE activity evaluation, determined in three independent experiments from five pools of five whole body larvae. Each assay was performed in triplicate using a modified Ellman procedure (Ellman et al., 1961) with acetylthiocholine io-

dide (0.075 M) as a substrate. The Ellman protocol was adapted to quantify AChE activity in *X. laevis* larvae (Gindi and Knowland, 1979). Briefly, whole body larvae were homogenised in Tris buffer (1% Triton X-100 in 0.05 M Tris–HCl, pH 7.4) at 1:10 (w/v) and centrifuged for 5 min at 15,000 g. The presence of thiol groups masks the low levels of AChE causing an incorrect evaluation of enzyme activity; for this reason, before adding the substrate, a 10 min pre-incubation with 0.33 mM 5,5′-dithio-bis (2-nitrobenzoic acid) of extracts was necessary. Changes in optical density were noted every 15 s for 15 min at 412 nm. Preliminary experiments were conducted to define conditions for sample concentration required for linear rates of substrates hydrolysis. AChE activity was calculated as nmol of acetylthiocholine iodide hydrolysed per min per gr larvae and the results were expressed as percentages of AChE inhibition versus control. At the testing of the differences of the arithmetical means between specific group of samples (CPF and MTN 3000 μg/l) the *t*-test was applied. Conclusion about the rejection of the null hypothesis of the equality and the differences, was done at $P < 0.001$.

3. Results

Stage 35–36 embryos exposed to CPF and MTN moved normally but immediately after they showed defects of neuromuscular activity such as spasms, tremors and, at the highest concentrations, paralysis that later seriously compromised swimming of stage 47 larvae.

3.1. CPF and MTN embryotoxicity

The CPF embryotoxic responses are given in Table 1. The compound was not embryolethal even at the highest 6000 μg/l concentration. On the other hand, a good concentration-response was observed in malformed larva percentages. There was a significant difference between control malformed larva percentage and that at 100 μg/l, while 100% of the larvae were malformed at 750 μg/l (Table 1). The percentages of malformed larvae investigated by probit analysis allowed to calculate a TC_{50} of 161.54 μg/l (Fig. 1). Because there was no mortality even at 6000 μg/l, the LC_{50} and thus the T.I. could not be calculated. Since T.I. would be many times greater than 3, according to Dawson and Bantle (1987), CPF must be considered a powerful teratogenic compound. Unlike the control (Fig. 2A), CPF larvae were mainly affected by ventral and/or lateral tail flexure coupled with abnormal gut coiling (Fig. 2B, C).

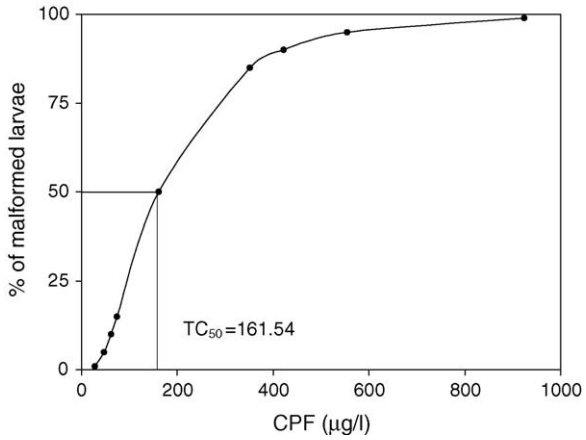

Fig. 1. TC_{50} predicted value of *X. laevis* larvae exposed to CPF, calculated by probit analysis.

MTN also showed no lethality at any concentrations used (Table 2), but like CPFs, it was able to induce malformations with a good concentration-response. There

Table 1
CPF embryotoxicity on *X. laevis* (stage 47)

	Concentrations	CPF (μg/l)							
		50	100	250	500	750	1000	3000	6000
Utilized embryos (*n*)	790	140	137	149	150	90	90	180	90
Dead embryos (*n*)	5	3	5	5	1	7	5	10	6
Mortality (%)	0.6	2.1	3.6	3.4	0.7	7.8	5.5	5.6	6.7
Living larvae (*n*)	785	137	132	144	149	83	85	170	84
Malformed larvae (*n*)	36	10	52	90	142	83	85	170	84
Malformed larvae (%)	4.6	7.3	39.4*	62.5**	95.3**	100**	100**	100**	100**

* $P < 0.05$.
** $P < 0.01$.

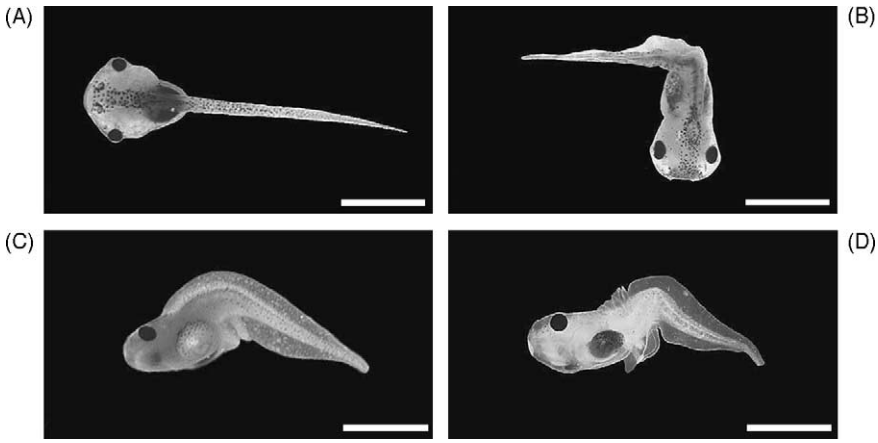

Fig. 2. Stage 47 *X. laevis* larvae. (A) dorsal view of a control larva; (B), (C) and (D) abnormal tail flexure coupled with abnormal gut coiling; (B) dorsal view; (C) lateral view of larva exposed to CPF; and (D) lateral view of larva exposed to MTN. Bars = 3 mm.

was a significant difference between control and exposed groups starting from 1500 μg/l, but 100% of malformed larvae was not reached even at 6000 μg/l (Table 2). The percentages of malformed larvae investigated by probit analysis allowed to calculate a TC_{50} of 2394.01 μg/l (Fig. 3) and, here too, MTN must be considered a powerful teratogenic compound. Moreover, the MTNs were mainly affected by abnormal tail flexure, malformation easily comparable to that observed in CPFs (Fig. 2D).

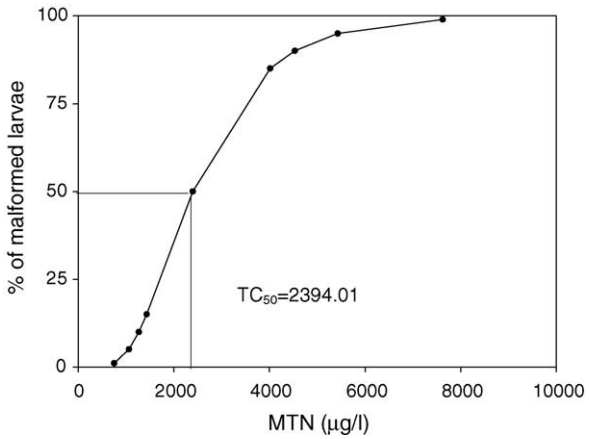

Fig. 3. TC_{50} predicted value of *X. laevis* larvae exposed to MTN, calculated by probit analysis.

3.2. Histological studies

The histological screening was done to identify the main histopathological features of myotomes, myocytes and notochord. In the controls the myocytes were normally orientated in parallel to the notochord, occupying the whole length of the myotomes, and were attached at regular intersomitic boundaries

Table 2
MTN embryotoxicity on *X. laevis* (stage 47)

	Concentrations	MTN (μg/l)				
		375	750	1500	3000	6000
Utilized embryos (n)	152	93	113	113	169	117
Dead embryos (n)	13	8	8	12	17	14
Mortality (%)	8.5	8.6	7.1	10.6	10.1	12
Living larvae (n)	139	85	105	101	152	103
Malformed larvae (n)	14	11	9	23	113	98
Malformed larvae (%)	10.1	12.9	8.6	22.8**	74.3**	95.1**

** $P < 0.01$.

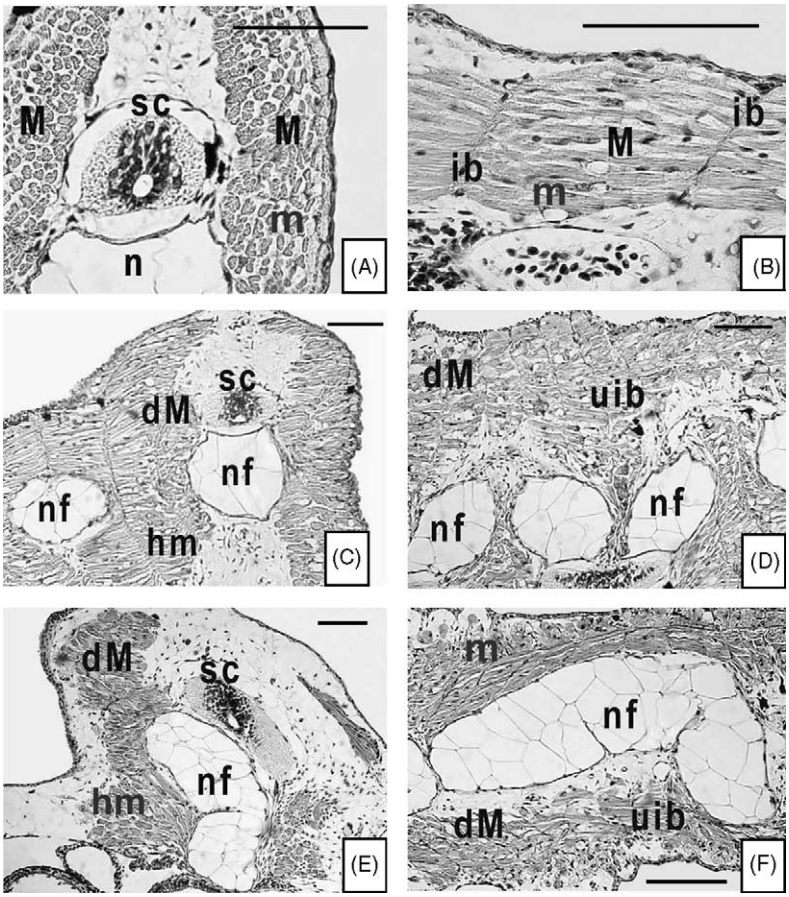

Fig. 4. Paraffin sections of stage 47 *X. laevis* larvae. (A) transversal; (B) para-sagittal sections of control larvae; (C) transversal; (D) para-sagittal sections of CPF exposed larvae (3000 μg/l); (E) transversal; and (F) para-sagittal sections of MTN exposed larvae (3000 μg/l). sc: Spinal cord; M: myotomes; n: notochord; m: myocytes; ib: intersomitic boundaries; nf: notochord flexure; dM: distorted myotomes; hm: horizontal myocytes; uib: undeveloped intersomitic boundaries. Bars = 100 μm.

(Fig. 4A, B). In CPFs notochord flexure was evident and myocytes had not a correct orientation, a few being oriented even perpendicular to the notochord. Myotomes were smaller and seriously distorted than in the controls, while the intersomitic boundaries were only partially developed (Fig. 4C, D). The pathologies found in MTNs were similar and easy comparable to those observed in CPFs. The notochord, myotomes and myocytes showed a phenotype entirely altered and sometimes the myocytes were oriented perpendicularly to the notochord (Fig. 4E, F). Semi-thin sections of control larvae showed regular myocytes with normal contractile apparatus, lipid droplets and yolk granules (Fig. 5A, B). On the contrary in CPFs and MTNs, myocytes were completely distorted, rich in lipid droplets, pointed in different direction and with no clear cellular boundaries. The most outstanding pathologies were the heavy disarrangement of contractile structures and the marked hypertrophies at the cell extremities (Fig. 5C, D).

3.3. Confocal microscopic analysis

Confocal microscopic analysis of control myotomes confirmed the normal morphology observed in paraffin and resin sections. The multinucleate myocytes occupied the whole length of the myotomes and attached at regular intersomitic boundaries (Fig. 6A). The images showed characteristic myocytes packed with cross-striated myofibrils (Fig. 6B). In CPFs many

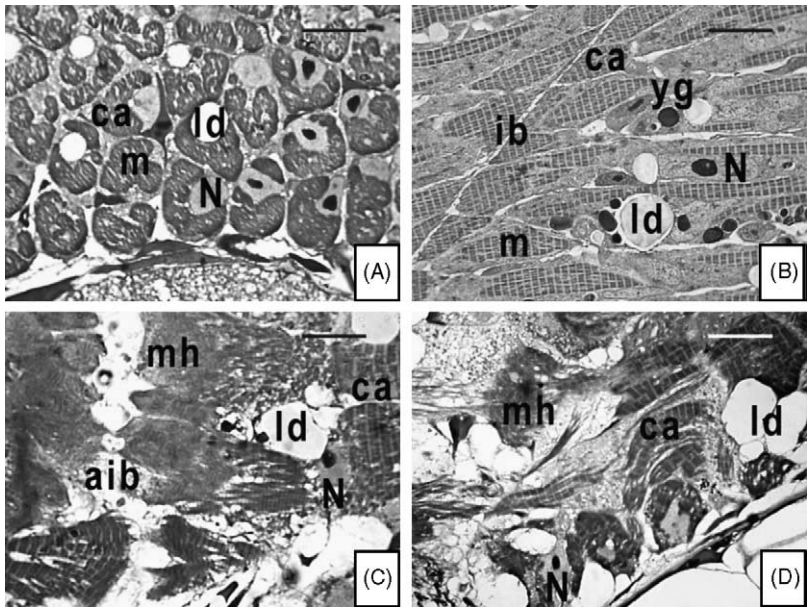

Fig. 5. Semi-thin sections of stage 47 *X. laevis* myotomes. (A) transversal; (B) para-sagittal sections of control larvae; (C) para-sagittal section of CPF exposed larva (3000 μg/l); and (D) para-sagittal section of MTN exposed larva (3000 μg/l). m: Myocytes; N: nucleus; ca: contractile apparatus; ld: lipid droplets; ib: intersomitic boundary; yg: yolk granules; aib: alterated intersomitic boundaries; mh: myocyte hypertrophies. Bars = 20 μm.

dramatic alterations were present. The myocytes were smaller, completely distorted and with contractile apparatus, when present, made of myofibrils that seldom showed cross striations (Fig. 6C). This confocal analysis showed that the hypertrophies observed in paraffin and resin sections were due to a break away of myofibrils extremities (Fig. 6E). This last alteration of the contractile apparatus was most evident in MTNs (Fig. 6D, F).

3.4. AChE activity analysis

AChE activity inhibition was investigated in *X. laevis* larvae exposed to concentrations lower and higher than TC_{50} for both CPF (TC_{50} = 161.54 μg/l) and MTN (TC_{50} = 2394.01 μg/l). As showed in Fig. 7a good concentration-response was observed between CPF and MTN concentrations and percentages of AChE inhibition. In CPFs, the inhibition was 75.71% at 100 μg/l, 79.73% at 250 μg/l whereas it reached 94.12% at 3000 μg/l. Instead, in MTNs, 58.45% of AChE inhibition was observed at 1500 μg/l, 74.26% at 3000 μg/l and 95.02% at 6000 μg/l. At the same concentration of 3000 μg/l, the AChE inhibition was significantly higher ($P < 0.001$) in CPF than in MTN exposed larvae.

4. Discussion

The present paper showed no embryolethal effects of CPF and MTN in *X. laevis* embryos exposed from stage 9 (8 h p.f.) to 47 (120 h p.f.), even at the highest concentrations tested. Previous studies on this species found a LC_{50} value of 14,600 μg/l for CPF in embryos exposed for 96 h starting from stage 14 (16 h p.f.) (Richards and Kendall, 2002) and of 10,900 μg/l for MTN in embryos exposed for 96 h starting from stage 2 (1.5 h cleavage initiated) (Snawder and Chambers, 1989). These data evidenced a relative tolerance in terms of mortality of *X. laevis* embryos to OP pesticides. The tolerance to OPs changes from species to species and inside the same species, and is influenced by several factors such as sensitivity of the target, disposition and metabolism (Chambers and Carr, 1995). In *X. laevis* tadpoles, the relative insensitivity

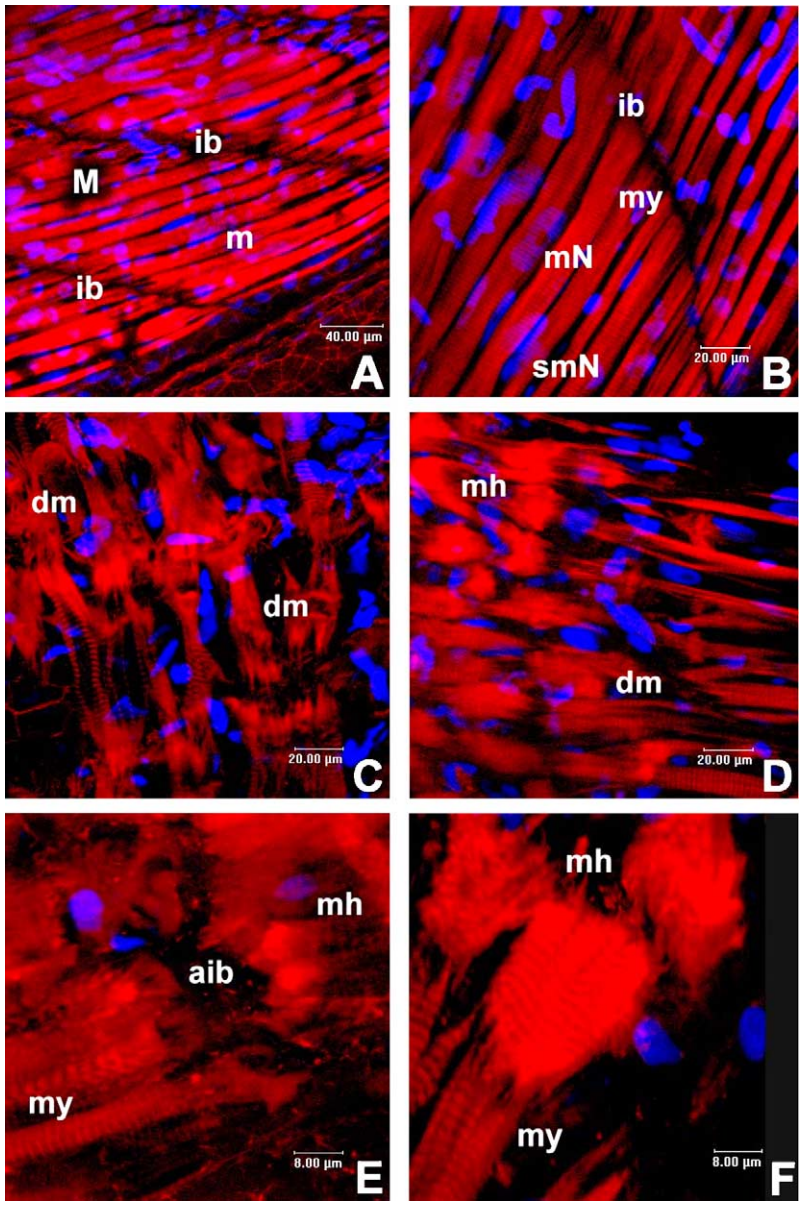

Fig. 6. Confocal microscopic analysis of stage 47 *X. laevis* whole-mount tails. (A) myotomes; (B) myocytes of control larva; (C) myotomes; (E) myocytes of CPF exposed larvae (3000 μg/l); (D) myotomes; and (F) myocytes of MTN exposed larvae (3000 μg/l). Red, actin stained with phalloidin-TRITC; blue, nuclei stained with DAPI. M: myotomes; m: myocytes; ib: intersomitic boundaries; mN: myoblast nucleus; smN: secondary myoblast nucleus; my: myofibrils; dm: distorted myocytes; mh: myocyte hypertrophies.

to OPs was ascribed to AChE resilience toward these inhibitors (Shapira et al., 1998), while in *Bufo arenarum* the tolerance to OPs was related to a reduction in glutathione content and increase in glutathione S-transferase activity (Anguiano et al., 2001). Moreover, OP acute toxicity depends on the activation to oxon metabolites mediated by cytochrome P450 and *X. laevis* developmental stages considered in this study

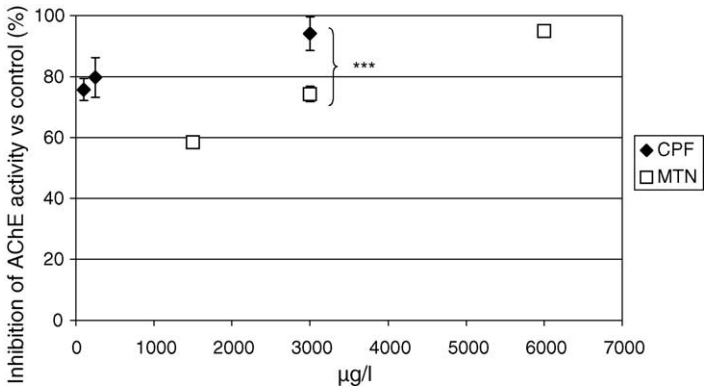

Fig. 7. Inhibition of AChE activity in stage 47 *X. laevis* larvae after exposure to CPF (♦) and MTN (□). Values were based on the assumption that control larvae had 0% of inhibition of AChE activity. Each value represents the mean ± S.D. obtained from three independent assays carried out in triplicate on five pools of larvae. When not given, S.D. bars were smaller than the used scale. Key: ***$P < 0.001$ between the marked points.

lack a competent metabolic activation system (Fort et al., 1988; Bantle et al., 1999), which starts to be sufficiently expressed only after 120 h p.f. (Colombo et al., 1996).

Beside a high tolerance in terms of mortality, we found that both compounds caused 100% of malformed larvae at relatively low concentrations among those tested. *X. laevis* embryos showed different ranges of sensitivity towards the pesticides examined and CPF resulted nearly 15 times more teratogenic than MTN. In addition to these results, a substantial difference in AChE inhibition between the two compounds was found in this study. The degree of AChE inhibition in CPFs and MTNs showed a good concentration-response and at 3000 μg/l, a statistically significant difference was found between CPFs and MTNs, with an almost complete inhibition in CPFs. These differences in CPF and MTN toxicity could be explained by several contributing factors. Lund et al. (2000) reported a higher half-life and octanol/water partition coefficient for CPF than MTN and, according to Serrano et al. (1997), this can influence a different persistence of the two compounds in the test solutions, as well as their levels of accumulation in tissues. Boone and Chambers (1997) reported that AChE target enzyme sensitivity is the main factor of high CPF toxicity. Moreover, the sterical hindrance, smaller in CPF than in MTN, would make CPF more accessible to the AChE active centre justifying its higher inhibitory potency.

A strong correlation between AChE inhibition and malformation percentages was observed both for CPF ($r^2 = 0.97$, $P < 0.01$) and MTN ($r^2 = 0.90$, $P < 0.01$) and, although the CPF and MTN embryotoxicity in *X. laevis* was at different concentrations, similar terata occurred.

Larvae scored as malformed, showed often gross alterations in the tail flexure coupled with a great decrease of neuromuscular activity, and these results are well comparable to those of other authors (Snawder and Chambers, 1989; Vismara et al., 1996; Richards and Kendall, 2002). It is well known that in non-target organisms, such as amphibians, fishes, birds and mammals, AChE inhibition is the primary manifestation of toxicity during OP exposure (Thomson et al., 1991; Sultatos, 1994; Calumpang et al., 1997; Fulton and Key, 2001) and that OP compounds produce typical signs of anti-cholinesterase toxicity such as complex posturing movements, body shaking or skeletal muscle fasciculation associated with muscle fibre damages in mammals (Gupta et al., 1987; Karalliedde and Henry, 1993; De Bleecker et al., 1994; John et al., 2003). The abnormal tail flexure observed in CPFs and MTNs could be the consequence of the cholinergic phase, where the AChE inhibition causes repetitive firing of muscle fibres leading to the axis tail folding and, at high concentrations, to paralysis. Previous data on lower vertebrates showed that newly hatched catfish larvae exposed to MTN developed deformed tail, as a consequence of uncontrolled and continuous contraction of the tail musculature (Lien et al., 1997). This hypothe-

sis was confirmed by Behra et al. (2002) who noticed that the first movements of zebrafish embryos homozygous for AChE gene mutation were characterised by tail twitching. During the further development, mutants showed severe myopathy characterised by disruption of myocyte myofibrils due to the lack of AChE activity. On avian embryos OPs induced gross malformations, such as variable degrees of vertebral fusion, probably due to the muscular cell sarcolemma depolarisation, followed by uninterrupted muscle contractions. Therefore, the concurrent vertebral organogenesis was seriously distorted (Meiniel, 1981). Also in *X. laevis*, Snawder and Chambers (1993) found abnormal notochord morphogenesis after MTN exposure.

Tail flexures that we observed in exposed embryos were histologically characterised by abnormal notochord curvature and disorganised myotomes. Since early in the normal amphibian myogenesis, myotomal cells change in position from perpendicular to parallel relative to axial notochord (Kielbówna, 1981; Daczewska, 2001), a possible mechanism for histopatological myotome damages in CPFs and MTNs may be related to an early alteration of the normal myotomal cell orientation. Therefore, myocytes were perpendicular rather than parallel to the axial notochord (Fig. 4C, E). Moreover, like in zebrafish mutants lacking of AChE activity (Behra et al., 2002), the extensive and marked hypertrophies localised at myocyte extremity level, as well as myofibril disorders (Fig. 5C, D), could be due to a myofibril break away and a perturbation of myocyte contractile apparatus (Fig. 6E, F). In detail, our hypothesis is that the terminal sarcomeric actin filaments may lose their connection with the trans–membrane protein complex at the intersomitic junction (ISJ), a highly specialised sarcolemma domain that ensure the integrity of the muscle fibres during contraction (Peng and Chen, 1992).

The perturbation of myocyte contractile apparatus could be ascribed to an overstimulation of the postsynaptic membrane, caused by AChE inhibition, leading to excessive Ca^{2+} influx as reported in OP treated mammals (Wecker et al., 1986; Karalliedde and Henry, 1993; De Bleecker et al., 1994). Alteration of intracellular Ca^{2+} homeostasis has been associated with cell death in a variety of system (Orrenius et al., 1989). The involvement of calcium as the mediator of muscle necrosis has been described by Leonard and Salpeter (1979) who referred Ca^{2+}-activated proteases which specifically remove Z-disks in mammalian skeletal muscles. A similar mechanism was also observed in venom-induced muscle degeneration (Harris et al., 2003). The snake venom determined a depolarisation of the muscle fibres followed by a hyperconcentration of Ca^{2+} into the cytosol. Since the rapid elevation of Ca^{2+} was responsible for the activation of intermediate filament specific proteases (Nelson and Traub, 1983), a hydrolysation of desmin and titin was consequent, and this represents an early feature of stress- and disease-related muscle degeneration (Harris et al., 2003). In particular desmin, which is the major intermediate filament in skeletal muscle, is crucial to interlink adjacent sarcomeres, forming a collar around the Z-disks, and to strengthen the tethering of myofibrils to the plasma membrane. The loss of desmin would lead to the loss of register between adjacent sarcomeres and would expose titin to hydrolytic attack leading to the disaggregation of the sarcomeres (Harris et al., 2003). In *X. laevis* myotomal muscle with truncated forms of desmin, Cary and Klymkowsky (1995) reported structural defects at the ISJ, confirming the important role of intermediate filaments in the organisation and stabilisation of myofibril-membrane attachment sites. The abnormal development of the ISJ would also determine an incorrect differentiation of the dystrophin–glycoprotein complex exhibiting a phenotype very similar to that observed in myocytes of mdx mouse, which lacks of dystrophin (Cary and Klymkowsky, 1995).

In conclusion the CPF and MTN teratogenic effects observed during *X. laevis* myogenesis suggest a possible role played by anti-cholinesterase compounds on induction of congenital muscular dystrophy not genetically inherited.

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