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LONG TERM EXPOSURE TO LOW DOSE NEUROTOXIC PESTICIDES AFFECTS HATCHING, VIABILITY AND CHOLINESTERASE ACTIVITY OF *ARTEMIA* sp.

Chiara Gambardella¹*, Daniela Nichino², Camillo Iacometti², Sara Ferrando², Carla Falugi², Marco Faimali¹
¹ISMAR - CNR, Via de Marini 6, 16149 Genova Italy
²DISTAV, University of Genoa, Viale Benedetto XV, 16132 Italy

*corresponding author: Chiara Gambardella
ISMAR-CN- Via de Marini 6
16149 Genova, Italy

E-mail: chiara.gambardella@ge.ismar.cnr.it, chiagamba@gmail.com

**ABSTRACT**

The brine shrimp *Artemia* was used as a model organism to test toxicity of several neuroactive pesticides (chlorpyrifos (CLP), chlorpyrifos oxon (CLP ox), diazinon (DZN), carbaryl (CBR)) following exposure to far below than lethal doses. Cysts were exposed to the pesticides in order to test a scenario similar to actual coastal environment contamination, by analyzing different responses. Cysts were rehydrated in water containing the pesticides at concentrations ranging from 10⁻¹¹ to 10⁻⁵ M, for 72, 96 and 192 h, respectively. For these exposure times, morpho-functional and biochemical parameters, such as hatching speed and viability were investigated in the larvae together with cholinesterase (ChE) activity quantification and histochemical localization. Finally, ChE inhibition was also compared with conventional selective ChE inhibitors.

Results showed that CLP ox and CBR caused a significant dose-dependent decrease in hatching speed, followed by high percentages of larval death, while CLP and DZN were responsible for irregular hatching patterns. In addition, the pesticides mostly caused larval death some days post-hatching, whereas this effect was negligible for the specific ChE inhibitors, suggesting that part of pesticide toxicity may be due to molecules other than the primary target.

ChE activity was observed in the protocerebrum lobes, linked to the development of pair eyes. Such activity was inhibited in larvae exposed to all pesticides. When compared to conventional selective inhibitors of ChE activities, this inhibition demonstrated that the selected pesticides mainly affect acetylcholinesterase and, to a lesser extent, pseudocholinesterases. In conclusion, the brine shrimp is a good model to test the environmental toxicity of
long term exposure to cholinergic pesticides, since changes in hatching speed, viability and ChE activity were observed.

**Keywords**: carbaryl; chlorpyrifos; crustaceans; diazinon; organophosphates; oxonization.

1. **Introduction**

Organophosphate (OP) and carbamate (CB) insecticides are the most toxic pesticides in the world (Barahona and Sanchez Fortun, 2007) used since the 1980s, replacing chlorinated biphenyl compounds in agricultural areas before being banned because of their long persistence in the environment. Both insecticides are released several times every year at high concentration, therefore their presence in the environment may be considered to be persistent. These pesticides end up in the waterways, thus, the aquatic environment is of a major concern (Moses et al., 1993).

OP and CB insecticides exert a neurotoxic action on several aquatic and coastal species (Sanchez Fortun et al., 1997; Pesando et al., 2003; Varò et al., 2006), by acting on the cholinergic system and irreversibly binding to the active site of the acetylcholinesterase enzyme (AChE, E.C: 3.1.1.7, Aluigi et al., 2005). This is a key neurotransmission enzyme, since its main task is to cleave acetylcholine, detaching it from the neuromuscular receptors, thus enabling intercellular signaling. In addition, detoxifying enzymes are well known to act on OP and CB pesticides, by fully blocking AChE activity, producing oxon derivatives (Sultatos, 1994).

OP pesticides belong to a wide class of organophosphorus molecules, generally formed by organic pyrophosphoric acid salts. First used as a chemical weapon during the Second World War, they were later employed as pesticides (Khurana and Prabhakar, 2000). The main OP compounds are chlorpyrifos (CLP) and diazinon (DZN). CLP has been recently included in the European priority list of pollutants present in seawater and freshwater, since, at environmentally relevant concentrations (EQS—Environmental Quality Standard CPF 0.1 µg/L; 2013/39 EU), it may potentially cause deleterious effects. Therefore, it has been extensively studied and its action mechanism and toxicity are well known (John and Shaike, 2015). DZN is used worldwide as a broad-spectrum contact insecticide, introduced in 1965 (pesticide information profile, PIP, 1993; National pesticide Information Center, NPIC, US) and its use in raw water is still ongoing (Aggarwal et al., 2013).

CB pesticides derive from carbamic acid and their action mechanism is very similar to OPs – mainly through inhibition of cholinesterase enzymes – which are their primary targets (Quistad et al., 2005). Carbaryl (CBR), known to be a reversible AChE inhibitor (Casida, 1963), first introduced in 1956, has been the mostly used carbamate pesticide worldwide, more than all the others combined.
OPs and CBs toxic effects in coastal waters have been studied on several species, including echinoderms (Pesando et al., 2003) and crustaceans (Barahona and Sanchez-Fortun, 1999; Varò et al., 2006). The dose responsible for causing an effect on 50% of aquatic species population (EC50) could be calculated, namely $10^{-5}$ and $10^{-6}$ M (Falugi et al., 2011). Such concentrations are far higher than the environmental doses used in agriculture and ranging between about $3*10^{-10}$ and $3*10^{-8}$ M, depending on different conditions of use throughout the year (Banks et al., 2005). Also, the effects on coastal organisms of both insecticides at low concentrations but for long term exposure in water are still unknown. Therefore, the aim of this study was to carry out long term exposure experiments, in order to assess the risk to coastal aquatic organisms, in terms of growth and reproductive success, related to these neurotoxic pesticides at concentrations likely to be found in the environment, throughout the year and during seasonal crop treatments. CLP, CLP derivate (CLP oxon), DZN and CBR were selected as pesticides to expose cysts of the brine shrimp Artemia, a small crustacean resistant to chemicals, pesticides and to environmental stresses (Clegg, 1974; Browne, 1980; Varò et al., 1998 Sarabia et al., 2002; Nunes et al., 2006).

This invertebrate thrives in coasts and hypersaline ecosystems, usually close to agricultural areas (Bustos-Obregon and Vargas, 2010). The genus Artemia consists of several sibling species, including recognized sexual species and several parthenogenetic lineages with different ploidy levels (Gajardo et al., 2002; Baxevanis et al., 2006; Amat et al., 2007; Muñoz et al., 2008), and probably super species defined by the criterion of reproductive isolation (Browne and Bowen, 1991; Browne et al., 1991). Several taxa may occur in sympatry, developing concurrently or seasonally overlapping populations along the year (Amat, 1980; Amat et al., 1995). Because of their dominance in hypersaline systems, there are several ecological analyses of environmental factors that control the population dynamics of Artemia (Lenz, 1987). A characteristic of the life cycle of Artemia is that, under adverse environmental conditions, the reproduction mode switches from ovoviviparity to oviparity, producing resistant cysts which are embryos in diapause covered by a shell or chorion that confers protection (Varò et al., 2006). These dormant cysts, containing crypto biotic embryos at gastrula stage (G II), can be routinely purchased from commercial sources.

Artemia is a cosmopolitan organism, characterized by a wide array of intrinsic characteristics (i.e. easy rearing, short life-cycle, large offspring production, Varò et al., 2015), that make it a good model for testing toxicity (Migliore et al., 1997; Nunes et al., 2006; Gambardella et al., 2014, 2017; Mesarić et al., 2015) in estuarine and hypersaline environments. Since its habitat is characterized by high salinity water, it can be easily polluted by all selected pesticides, that are widely used in farming. In this regard, the bioaccumulation and ecotoxicological effects of OPs and CBs have been demonstrated in Artemia populations (Varò et al., 1998, 2002, 2006; Falugi and Raineri, 1983). For example, short-exposure to
OPs (dichlorvos and CLP) does not affect survival but induces more than 80% cholinesterase inhibition of two species of the genus Artemia (Artemia salina and Artemia parthenogenetica, Varò et al., 2002). CLP pesticide also affects free swimming Artemia nauplii survival (Varò et al., 2006) and the cholinergic system at high concentration levels (Falugi and Raineri, 1983).

Literature reports short and long term toxicity on this model by some of the selected pesticides, that have established the LC50 values and the most sensitive stages for the same pesticides used here (Table I). However, no studies are available on the effects of these pesticides at environmentally relevant concentrations (0.1-2.55 µg/L, Phillips et al. 2007, Directive 2013/39/EU) in Artemia. Therefore, the aim of this study was to evaluate the effects of long term exposure of brine shrimp’ cysts to concentrations likely to be present in coastal waters. Such effects have been evaluated based on morpho-functional and biochemical parameters, such as hatching and offspring viability, and on ChE activities. This study is an attempt to understand the mechanisms through which neurotoxicity is exerted. In this regard, a comparison was carried out with the effects caused by specific inhibitors of different ChE molecules.

2 Materials and methods

2.1 Selected pesticides and anti-cholinesterase agents

For this study, we investigated CLP (CAS 2921-88-2, MW 350.59); its oxonized derivate, CLP oxon (CLPox, MW: 334.52), DZN (CAS 333-41-5. MW: 304.35), and the carbamate CBR (CAS 63-25-2, MW 201.23). In addition, the following anti-cholinesterase agents specific for AChE and pseudocholinesterases were used for toxic effect comparison: BW 284c51 (decamethonium bromide crystalline. MW 418.29 E.C. 208-772-2), iso-OMPA (tetraisopropyl pyrophosphoramide CAS: 513-00-8 MW: 342.36), DFP (di-isopropyl fluorophosphate, CAS 55-91-4, MW 184.15), and eserine sulfate (Physostigmine).

BW 284c51 and eserine sulfate are reversible and specific inhibitors of the ‘true’ AChE (E.C.:3.1.17). Eserine is used as a potent prophylactic antidote against organophosphate intoxication (Somani and Dube, 1989); iso-OMPA is the irreversible inhibitor of butyrylcholinesterase (BChE: E.C. 3.1.1.8), while DFP is the irreversible and unique inhibitor of propionylcholinesterase (PChE, E.C. 3.1.1.8) activity.

All these products were purchased from Sigma Chem. Co, and were produced by Pestanal and supplied with a certificate attesting purity (98%for DZN and 99.5% for the other products). Information about these pesticides was obtained from the technical sheets supplied by the producers and by Muccinelli (2006).

2.2 Experimental setup

Commercially available dehydrated cysts (stage GII, Blue Line, Macerata, Italy) of Artemia parthenogenetic population (diploid) were used for the experiments. Rehydration was performed in
artificial sea water (ASW, Instant Ocean) at 37 % salinity, according to Vanhaecke and Persoone (1984). During the experiments, cysts were continuously exposed to pesticides and observed after 72, 96 and 192 h. These sampling times correspond to Nauplius I and II (N I, II), metanauplius I-II (MN I, II) and metanauplius III (MN III), according to Benesch (1989) and Falugi and Raineri (1985), respectively (Scheme 1). In detail, Nauplius I was characterized by scarcely pigmented eye, presence of antennulae, primordia of the appendages and lip, not functional digestive system; Nauplius II was constituted by fully pigmented eye and the gut starting to open distally; metanauplius I showed primordia of the abdominal appendages and fully opened-gut lacking of the setae; metanauplius II was characterized by an elongated abdomen, setae on the appendages, primordia of the nervous ganglias from the protocerebral to the mandibular ones and protruding towards the first abdominal segments; metanauplius III was considered the larval stage with a complete development of the neuromuscular system. These sampling times were chosen in order to evaluate the long-term effects of low pesticide concentrations and their likely reversibility.

2.3. Pesticide concentrations
In order to compare pesticide effects, we used molarity to define the concentrations, because solutions with the same molarity contain the same number of solute molecules (Avogadro’s number).

A stock 10⁻⁴M solution was obtained by dissolving the pesticides in distilled water at room temperature. Each pesticide has a different solubility (DZN: 40 mg/L; CLP and CLP oxon: 2 mg/L; CBR: 40 mg/L). Thus, concentrations 10⁻⁵, 10⁻⁷, 10⁻⁹ and 10⁻¹¹M were obtained for successive dilutions in ASW from stock solutions and used to carry out the experiments as described in the supplementary table S1.

2.4. Cyst Exposure
Cysts were exposed to concentrations between 10⁻⁵ and 10⁻¹¹M of each pesticide. The concentrations between 10⁻⁷ and 10⁻¹¹M are in the range of estimated environmental concentrations (Banks et al., 2005). The 10⁻⁵M concentration – equal to the EC50 dose of most OPs for invertebrates (Falugi et al., 2011), including crustaceans (Baek et al., 2015) was used for comparison.

Tests were carried out in multi-well capsules and performed in ultra-filtered ASW 0.22 μm, without a renewal of the medium. In each well, about 50 cysts in 2.5 ml ASW were covered with the lid and maintained at T= 25°C. Hatching percentage was monitored at different times (72 h, 96 h, 192 h) under a stereomicroscope (Zeiss, Germany). After 48 h and 120 h, lyophilized Chlorella (Sun Chlorella Corp, London Branch, UK) was provided at 1 mg/L concentration. The last observation (192h) aimed to check any expected decay in
pesticide activity and recovery of damaged larvae. The test was performed in triplicates for each assessed biomarker, i.e. hatching percentage, ChE analysis, ChE histochemical localization, and mortality. After 96 h of exposure to pesticides, samples were collected and frozen at -20°C for quantifying ChE activities. Other samples were fixed in 70% ethanol for 5 min and immediately rinsed and used for histochemical localization of ChE activities. After 192 h, dead larvae were counted under the microscope in order to measure any LC50 (lethal concentration for 50% of the population).

2.5. Specificity of ChE activity
ChE activity of controls (unexposed cysts) was monitored during brine shrimp development, by following the following exposure times: 0-18-24-36-48-60-72-96-120 hours. In addition, at the same exposure times, ChE activity of cysts exposed to the selective inhibitors (BW284C51, iso-OMPA, Eserine, DFP) was measured, as a specificity control.

2.6. Histochemical staining of ChEs
Histochemical AChE reaction was carried out in toto, according to the thiocholine method (Karnovsky and Roots, 1964). The basis of the method is that the thiocholine ester is used as substrate to hydrolyze cholinesterase: the liberated thiocholine is captured by Cu^{++} ions, precipitating as colorless copper thiocholine that is converted to brownish CuS by treatment with yellow ammonium sulfide. Therefore, the colour is produced directly at the site of enzymatic activity. Specific controls were performed by pre-exposing the larvae to 10^{-4} M of the above listed conventional inhibitors for 30 minutes. A negative control was also performed by omitting the substrates. Histochemical AChE reaction was carried out for enzyme activity localization, using a mixture of 0.1 M Na citrate, 0.03 M copper sulphate, and 0.005M potassium ferricyanide, buffered at pH 6.0.

The substrate was acetylthiocholine iodide (ATChI), which is hydrolysed by AChE, and, at a slower rate, by pseudocholinesterases (butyrylcholinesterase, BChE). Reaction specificity was controlled either by omitting the substrate, or by pre-incubation for 30 min with 10^{-4} M BW284 c51 and 10^{-4} M iso-OMPA, a specific BChE activity inhibitor, routinely used in the laboratory for this purpose.

2.7. Quantitative analysis of ChE activities
AChE activity was measured by applying the quantitative method of Ellman et al. (1961), which was modified ad hoc for the Jenway (6405 Ultra Violet/Visible, Barloworld Scientific Ltd T/As Jenway, Gransmore Green, UK) spectrophotometer. The larvae were collected and maintained for 2 weeks at 20°C and then homogenated and sonicated for 25 min in a bath sonicator (FALC, mod. LBS1, Italy).
The larvae were passed through a very thin syringe needle (Ultrafin 29G, 12.7 mm length) in the presence of 1% triton X100, and centrifuge for 30 seconds at 8000 rpm. The colorimetric reaction was recorded for 10 min at 412 nm in the spectrophotometer. Each measurement was performed in triplicate.

2.8. Statistical analysis

All data are expressed as means ± standard deviation of the 3 replicates. Comparison of means was carried out using Student t-test and analysis of variance (ANOVA). When data failed to meet the assumption of normality and homoscedasticity tests even after arcsine transformation, data were compared using the non parametric Kruskal Wallis test. Data were considered significantly different when P<0.05.

3 Results

3.1 Hatching and survival

3.1.1 Unexposed cysts

In normal conditions, unexposed cysts hatched gradually in a time-dependent way, following a linear trend (R² = 0.9171, Fig.1A). The difference in the number of larvae between sampling times was significant (P<0.05, Fig. 1 A). After 72 h, about 20% of the cysts released swimming larvae, at different development stages (nauplius I and II; N I, II). After 96 h, 70% of the larvae were released, about half of them had reached metanauplius II stage (MN II, while the remaining ones were still at previous stages (metanauplius I, MN I). At 192 h, almost all larvae (~98%) had been released, reaching the metanauplius II and III stage (MN II, III). No dead larvae were observed at any sampling time (Fig. 1B).

3.1.2 Exposed cysts

In figure 2, all results are reported on hatching success of A. salina cysts exposed to the pesticides and on lethality observed after 192 h.

Chlorpyrifos (CLP)

The percentage of hatched larvae after 72 and 96 h exposure to CLP (Fig. 2 A) was not significantly different between controls and treated samples (P>0.05). During the following sampling time (192 h), hatching percentage was similar for all concentrations including controls (P>0.05), except for 10⁻⁵M CLP, which significantly reduced the number of hatched cysts (P<0.05), inducing about 40% mortality (Fig. 2B). In addition, larval swimming was slow and scarcely directed to the light source (not shown), while mortality and larval behavior in the other sampling times and concentrations did not differ from controls.
Clorpyrifos oxon (CLPox)

Exposure to CLPox significantly reduced the number of hatched cysts compared to controls (P<0.05), depending on sampling time increase (Fig. 2C). All data were different from controls, except for 10^{-11}M after 96 h exposure (P>0.05). After this sampling time, hatching failed to progress, and larval development was delayed, below the MN I stage. Phototropism was not impaired at the first samplings, but was subsequently lost (96 and 192 h). Observed mortality was not different from controls at 72 and 96 h sampling times, while at 192 h dose-dependent mortality of hatched larvae was observed (Fig. 2C). The percentage of dead larvae caused by 10^{-5} M CLP ox (Fig. 2D) is very low in the graphic, because most cysts had failed to hatch at the previous sampling times.

Diazinon (DZN)

Exposure to DZN for 72 h significantly inhibited hatching percentage only at the highest DZN tested concentration (10^{-5}M, P<0.05, Fig. 2E). Prolonged exposure (96 h) caused no significant effects in hatched larvae; conversely, after 192 h exposure hatching stopped, showing delayed, suffering or dead larvae (Fig. 2F). Mortality was significantly dose-dependent (P<0.05), with a linear trend increasing with drug concentration (R^2= 0.9777).

Carbaryl (CBR)

The effect on hatching after CBR exposure was significantly dose-dependent, compared to controls (P<0.05) at all sampling times (Fig. 2 G), with the only exception of cysts exposed to 10^{-11}M: their hatched percentage and swimming characteristics very similar to controls (P > 0.05). However, the percentage of delayed and dead larvae was quite high (about 40%, Fig. 2H). Lethality was observed after 192 h at all concentrations, reaching about 40% at 10^{-11}M. Also, most surviving larvae were severely delayed.

3.1.3 Exposure to selective inhibitors of ChEs

Results on samples exposed to selective inhibitors are shown in Fig. 3. Noteworthy, samples developed faster than controls, reaching maximum hatching (about 100% larvae) at 96 h. Moreover, at 192 h the larvae showed complete recovery: no dead or anomalous larvae were found after exposure to BW284C51, iso-OMPA and eserine.
The samples obtained from cysts exposed to BW284C51 showed accelerated dose-dependent hatching compared to controls at both exposure times (72 h, 96 h; Fig. 3A). This difference was significant at 10^{-7} and 10^{-5} M treatments compared to control only after 72 h exposure (P<0.05). Behavioral abnormalities as previously described were also observed in samples exposed to 10^{-5}M BW284C51.

**Iso-OMPA**

After 72 h and 96h, exposure to iso-OMPA did not cause a significant decrease in hatched larvae, compared to controls (P>0.05, Fig. 3B). Larval behavior looked normal, with phototropic movement, preeminently in the water column/surface. No mortality was observed at any concentration.

**Eserine**

The percentage of hatched larvae obtained from cysts exposed to eserine showed a dose-dependent increase if compared to controls, depending on exposure times (Fig. 3C). This difference was significant in all treatments compared to controls at all exposure times (P<0.05). Behavior was not affected.

**DFP**

DFP exposure accelerated hatching after 72 and 96 h, with a swimming larvae percentage higher than controls (Fig. 3D). At 72 h, this acceleration was significantly different from controls (P<0.05) from 10^{-7} M and resulted to be dose-dependent, while no differences were observed in swimming behavior compared to controls. At 96 h, all the cysts had hatched, with a higher percentage than controls. After 192 h, the number of dead larvae increased (Fig. 3E). Surviving larvae showed features of advanced metanauplius stage, with irregularly shaped naupliar eye, and developed 2 ocelli of the pair eyes (Fig. 6 L, M). The amount of dead larvae was not dose-dependent.

### 3.2. Cholinesterase activity

#### 3.2.1. Unexposed cysts and ChE activity specificity

ChE activity of unexposed cysts was time-dependent. The activity of dehydrated cysts was 19 U/mg protein. Activity decreased during morphogenetic events (18 h), and increased along with larval development. At 96 h, it was more than 90 U/mg protein (Fig. 4 A).

ChE activity specificity in unexposed cysts and larvae was assessed by pretreating the homogenates of the different developmental stages, from cysts to MN III, with specific inhibitors. Results show that iso-OMPA
inhibition was less than half, compared to BW284c51 (Fig. 4B); eserine and DFP inhibition was very similar to BW284c51, except for the early stage (18-24 h, Fig. 4 C, D).

3.2.2. Exposed cysts - ChE activity at 96 h after exposure
Exposure to CLP at all concentrations significantly inhibited enzyme activity, compared to control samples (P<0.05, Fig. 5A). CLPox oxonized molecule (Fig. 5B) showed a similar trend, with a significant dose-dependent effect (P<0.05). ChE inhibition by DZN exposure (Fig. 5C) was significantly different from controls (P<0.05) but not dose-dependent. Exposure to 10^{-5} M DZN was shown to be similar to 10^{-11} M, suggesting that at 96 h inhibition decay occurs, while homeostatic response is greater at high concentrations. After 96 h exposure, CBR (Fig. 5D) showed an enzyme activity recovery. In this case, dose-dependence followed an opposite trend, since recovery increased with concentration (P<0.05).

3.2.3 Comparison between effects of pesticides and conventional ChE inhibitors
ChE activity Specificity
Table 2 shows the inhibition power of analyzed pesticides, compared to controls and to the specific ChE inhibitors. DZN seems to be the most effective pesticide of those selected in this model, with an inhibition percentage higher than the other pesticides, including CLP. CLPox is very active also after a 96 h exposure, since it almost totally stops the activity of control larvae, in a manner proportional to its concentration. Moreover, its main target seems to be AChE activity, as shown by comparing its inhibition percentage with the one by iso-OMPA. CBR seems to be less effective on AChE. Conversely, CBR seems more active in inhibiting BChE activity, if compared with BW and iso-OMPA exposure. If compared to eserine, conversely, AChE in *Artemia* exposed for 96 h was shown to be scarcely sensitive to this pesticide, while sensitivity to DFP varies depending on pesticide concentration.

3.2.4 Histochemical localization of ChE activity
Histochemical results (Fig. 6) match quantitative ChE activity results, because at 96 h development, ChE activity disappeared from cephalic sense organs in a dose-dependent way. Control larvae (Fig. 6A) presented ChE activity localized in the naupliar eye, in the protocerebrum and in the connective nerves protruding towards the presumptive area of the pair eyes, in the suboesophageal ganglion and in the fibers connecting contralateral abdominal ganglia pair. Exposure to BW caused a progressive dose-dependent loss in enzyme activity from cephalic to distal sites (Fig. 6B-D). Exposure to CLP (Fig. 6E, F), CLPox, CBR, DFP and DZN (Fig. 6 G-N) caused complete activity loss at higher concentrations.
4. Discussion

According to the outcome of this work, long-term exposure at doses lower than the LC50 for the different pesticides affects hatching, survival, and ChE activity of the brine shrimp *Artemia*.

4.1 Effects on hatching and survival

Long term exposure to pesticides at coast environmental-like concentrations significantly affects hatching and larval survival, already affected by insecticides, according to previous results on the same model exposed to OPs (Varò et al., 2006; Venkateswara Rao et al., 2007). As suggested by these authors, OP uptake in brine shrimp may be inhibited by the thick envelope of resistant cysts likely to decrease penetration into brine shrimp embryos. Conversely, hatchability results were shown to be related to pesticide toxicity. This observation may be applicable also to CBs, as shown by our findings.

Both CB and OP exposure on *Artemia* hatching at low concentrations either enhanced or delayed brine shrimp development, but caused not developmental abnormalities, unlike other marine invertebrates exposed to the same pesticides (Buznikov et al., 2001; Falugi and Aluigi, 2012). These findings can be explained by the fact that *Artemia*—a proterostome organism—has a non-regulatory development. Thus, neurotoxic poisoning does not interfere with positional information, as is the case in other invertebrates, such as sea urchins, where dramatic morphological larval abnormalities caused by pesticides have been reported in the larvae (Aluigi et al., 2008).

None of the pesticides used in this study affected brine shrimp larvae survival at the first sampling times (72 and 96 h), while mortality was observed after 192 h. These results confirm the data available in the literature on CLP, DZN and CBR, known to exert a lethal activity at $10^{-5}$M concentration (Baek et al., 2015; Barahona and Sanchez-Fortun, 1999; Bustos Obregon and Vargas, 2010; Varo’ et al., 2006). They also provide new insight into the toxicity of oxonized derivates. In addition, it can be noted that exposure to DZN is responsible for a dose-response effect in *Artemia* larvae, in line with previous studies on this OP compound (Bustos-Obregon and Vargas, 2010).

Regarding OP insecticides, the results on hatching success after 192h suggest that DZN could be considered as the most aggressive compound, followed by CLPox and CLP. In the case of carbamate CBR, which is very effective on hatching inhibition (similar to CLPox effect observed in this study), a mortality trend decreasing with concentration was only apparent, since it depended on the number of hatched cysts: only few of them were able to hatch, thus, the percentage of dead larvae versus the initial number of cysts was very low. It is worth noting that exposure to low pesticide concentrations in this model has caused quite a different effect from the one found in other vertebrate and invertebrate models, where CBR was the most active toxicant,
followed by CLPox and CLP, while DZN was the least effective one (Falugi et al., 2011). Conversely, the specific ChE inhibitors (DFP) used for comparison caused no mortality at any concentrations. Generally, all the cysts had hatched at 192 h. However, in the samples exposed to DFP, some larvae percentage was dead at the bottom of the tanks at 192 h, independently from pesticide concentration. DFP effect may be due to the fact that DFP is a very aggressive OP compound (Kumar et al., 2010), having been synthesized during the II World War as a nervine weapon. Moreover, DFP is an efficient inhibitor of both BChE and AChE activities (Gupta, 2006). In some models, it is 10-100 times more active on BChE.

On the whole, the difference in mortality effects between OP pesticides and specific ChE inhibitors suggests that mortality may be due to pesticide interference with molecules other than ChEs. Actually, several authors have demonstrated other target proteins besides ChEs, and identified other molecular-level protein biomarkers as targets of reactive OP compounds (Murray et al., 2005; Thompson et al., 2010). These authors have found a number of responsive proteins in rat brain homogenates exposed to OPs, such as DZN and CLP. Although these authors failed to identify the protein targets, each of the tested OPs bound to a different collection of proteins, suggesting that different OPs may produce their own specific form of toxicity (Murray et al., 2005). These effects would also be due to species selectivity and acquired resistance, attributable in part to structural differences in binding subsites, receptor subunit interfaces, or transmembrane regions (Casida and Durkin, 2013), including a direct effect of pesticides on ACh receptors, such as muscarinic ones (Casida and Quistad, 2004). A similar mechanism could also be suggested for brine shrimps: further investigations on target proteins of OP pesticides should be conducted to confirm this hypothesis.

4.2 Effects on ChE activity

ChE activity was assessed at 96 h, due to low doses requiring a long time to exert any effects. Not all exposures allowed larvae to survive up to 192 h. Generally, cholinergic pesticides cause an irreversible blockade of ChE catalytic site (Sultatos, 1994; Falugi et al., 2011). In the analyzed stages of Artemia, ChE activity was always inhibited by the pesticides compared to controls, with a non dose-dependent pattern in all cases, not always in line with the effects on hatching. This may be due to different causes, such as different pesticide selectivity on AChE and BChE molecules. Increase in AChE activity after exposure to higher DZN and CBR concentrations shows some sort of paradox effect, as already reported in a number of different models (Aluigi et al., 2005; Aluigi et al., 2010). Enhanced ChE activity is possibly due to homeostatic effects, as well as to general toxicity causing inflammatory responses. In different models, including humans, increased non-neuromuscular acetylcholine was related to inflammation and/or wound healing (Wessler and Kirkpatrick, 2008), which may be followed by a rapid AChE increase as homeostatic mechanism. Actually, ChE molecules, besides their well-known role at neuromuscular synapses and nerve endings, are likely to play a number of roles in modulating
cell-to-cell communication mediated by ion fluxes, from fertilization to embryonic development. This process has been known for several years (Buznikov et al., 1996), while new roles are being assumed, such as apoptosis regulation (Zhang et al., 2002; Aluigi et al., 2010) and inflammation dynamics (Wessler and Kirkpatrick, 2008; de Oliveira et al., 2012; Gambardella et al., 2016).

The irregular responses shown by different CLP and DZN concentrations may also be due to different causes, such as: 1- different permeability of the cysts to different molecules; 2- likely bioaccumulation/decay during exposure time; 3- likely hormetic effect of used doses, 4- binding mode (reversible or irreversible) to ChE molecule active site; 5- larvae homeostatic responses and differentiation stage. As a matter of fact, studies on toxicity of neuroactive pesticides and ChE inhibitors in *Artemia* have shown that cholinesterases sensitivity to ChE inhibitors varies with age (Sanchez-Fortun et al., 1995-1997; Barahona and Sanchez Fortun, 1999, 2007). These authors reported that larvae exposed at 72 h are the most sensitive stage. This report is in agreement with AChE activity increase in control samples, at 60-72 and 96-120 h of their development, corresponding to the beginning of ganglion chain elongation. At this time, in non-exposed subjects, AChE activity was double than in exposed cysts and continued to increase, along with differentiation and growth of the cholinergic neuromuscular system and sense organs. With CBR, which reversibly binds to the active site, after 96 h exposure, a recovery in enzyme activity was observed. In this case, dose-dependence showed an opposite trend, since recovery increased with concentration (P<0.05). This may be tentatively explained by arguing that the stronger the first CBR effect (demonstrated by the dose-dependent decrease in hatching) the greater the following homeostatic recovery.

4.3. Comparison with specific inhibitors of ChE and AChE activity

By comparing CLP and CLPox with iso-OMPA inhibition percentage (specific inhibitor of pseudocholinesterase BChE: E.C. 3.1.1.8) most of the target activity is represented by AChE. Conversely, when compared with eserine, AChE form present in *Artemia* exposed for 96 h is scarcely sensitive to eserine. This suggests the presence of an ancestral AChE form, as demonstrated by Massoulié et al. (1993), who reported that a ‘true’ AChE of vertebrate nerve endings appeared only after the evolution of gnatoctomes. In other models, eserine, being a carbamate, strongly inhibits AChE activity with a slightly different mechanism than OPs. Also being a reversible inhibitor, it has a strong short-term action. However, in living organisms, the effect is not durable; at high drug concentrations, the homeostatic effect prevails, as demonstrated by the effects from cyst exposures.

CBR, when compared to BW and iso-OMPA exposure, seems more active in inhibiting BChE activity. Therefore, our results obtained with long exposure times to low doses, seem to demonstrate that pesticides in coastal waters are likely to impinge on the developmental success and viability of the species inhabiting...
shallow waters near agricultural sites or estuarine areas, through both physiological and biochemical mechanisms.

5. Conclusions
Our findings show that, despite low drug concentrations and a model which is very resistant to extreme environmental conditions, cholinergic pesticides may affect relevant endpoints in *Artemia* through long exposure times. Actually, exposure to pesticides at concentrations far lower than EC50, similar to concentrations in environmental waters, has been found to affect hatching rate, viability, and enzyme functions. Exposure to neurotoxic pesticides such as CLP, DZN and CBR at doses higher than those used in this work were reported to affect ChE, and in particular AChE activity in a dose dependent way (Varò et al., 2015) with a decreasing trend proportional to inhibitor concentration. Conversely, long-term exposure to very low doses showed an irregular or opposite trend in both hatching and AChE activity, possibly due to homeostatic response to low doses. Dose-dependent inhibition of AChE activity was only exerted by CLPox (Amitai et al., 1998; Cobana et al., 2016) and CBR (Falugi et al., 2011), which has been demonstrated to have a strong inhibition potential, much higher than the other tested compounds. Some damage was always done by the pesticides, as shown by the mortality rate observed after the MN II-III stage. This damage may be due to abnormal AChE activity distribution, as demonstrated by the histochemical reaction. Actually, the lack of AChE activity in the protocerebrum – associated with the main cephalic sense organs – may affect food recognition /intake.
In this light, *Artemia* cysts may be a good model to test environmental toxicity. While this model is quite resistant to chemicals, hatching speed, mortality, and ChE activity values of exposed samples are generally significantly different from controls. Thus, it should be taken into account to determine the presence of minimal amounts of neurotoxic pesticides in environmental waters, provided that the exposure is long enough to allow for bioaccumulation, oxonization, and chemical interaction with different cellular mechanisms.

6. References


PIP: Pesticide Information Profile (PIPs) http://extoxnet.orst.edu/pips/searchindex.html


**LEGENDS to figures:**

**Scheme 1.** *A. salina* nauplius (N) and metanauplius (MN) stages. In the present study were used nauplius I and II (NI, NII), metanauplius I-II (MN I, II) and III (MN III) resulting from 72, 96 and 192 h post-hatching respectively.

**Fig. 1:** Unexposed samples. **A:** percentage of *A. salina* hatched cysts at the different sampling times (0, 72, 96, 192 h). **Differences among the data were always significant (**P**<0.05). **B:** Corresponding stages of larvae at 192 h: percentage of larvae belonging to different stages at 192 h. MN= metanauplius. **P**<0.05

**Fig. 2:** Percentage of hatched *Artemia* larvae (A, C, E, G) after 72h, 96h and 192 h and percentage of mortality after 192 h (B, D, F, H) after exposure to different concentrations of chlorpyrifos (A, B), chloropyrifos oxon
(C, D), diazinon (E, F) and carbaryl (G, H). Data are the mean ± standard deviations of the 3 replicates of exposed cysts. **Significant differences (*)P<0.05 among the control and exposed groups (A, C, E, G) are indicated starting from the first significant concentration.** Percentage of dead larvae was calculated against the initial number of exposed cysts.

**Fig. 3:** Percentage of hatched *Artemia* larvae (A-D) after 72h, 96h and 192 h and percentage of mortality after 192 h (E) after exposure to different concentrations of selective inhibitors of cholinesterases (ChEs): BW284C51 (A), iso-OMPA (B), eserine (C) and DFP (D, E). Data are the mean ± S.D of the 3 replicates of exposed cysts. **Significant differences (*)P<0.05 among the control and exposed groups (A-D) are indicated starting from the first significant concentration.** The percentage of dead larvae was calculated against the initial number of exposed cysts.

**Fig. 4:** *A. salina* unexposed samples: A) cholinesterase (ChE) activity along normal development increase in a time-dependent manner. B-D) Percentage of inhibition of ChE activity, by the different inhibitors (BW284C51 and iso-OMPA (B), eserine (C), DFP (D) evaluated by pretreating for 30 min the homogenates of normally developing *A. salina* stages (0, 18, 24, 36, 48, 60, 72, 96, 120 h). *P<0.05

**Fig. 5:** Percentage of ChE activity (Y) of *Artemia* larvae after 96h exposure to different concentrations of chlorpyrifos (A), chlorpyrifos oxon (B), diazinon (C) and carbaryl (D). Data are the mean ± standard deviations of the 3 replicates of exposed cysts. *P<0.05

**Fig. 6:** Histochemical localization of ChE activity in 96h old larvae after exposure to inhibitors and pesticides. A= control; B- D= 10⁻⁹, 10⁻⁷, 10⁻⁵M BW; E, F= 10⁻⁷, 10⁻⁵M CLP; G= 10⁻⁷ CLPox; H= 10⁻⁵ CBR; I-M=10⁻⁵M, 10⁻⁷, 10⁻⁵M DFP; N= 10⁻⁷ DZN. br= protocerebrum; c= connective nerves; sg= sub esophageal ganglion, pg= pair ganglia throwing nerves to the mandibula. Bar represents 100 µm.
water (ASW, Instant Ocean) at 37 ‰ salinity, according to Persoone (1983). During the experiments, cysts were continuously exposed to pesticides and observed after 72, 96 and 192 h. These sampling times correspond to Nauplius I and II (N I, II), metanauplius I – II (MN I, II) and metanauplius III (MN III), according to Benesch (1989) and Falugi and Raineri (1985), respectively (Scheme 1). In detail, Nauplius I was characterized by scarcely pigmented eye, presence of antennulae, primordia of the appendages and lip, not functional digestive system; Nauplius II was constituted by fully pigmented eye and the gut starting to open distally; metanauplius I showed primordia of the abdominal appendages and fully opened-gut lacking of the setae; metanauplius II was characterized by an elongated abdomen, setae on the appendages, primordia of the nervous ganglia from the protocerebral to the mandibular ones and protruding towards the first abdominal segments; metanauplius III was considered the larval stage with a complete development of the neuromuscular system. These sampling times were chosen in order to evaluate the long-term effects of low pesticide concentrations and their likely reversibility.

2.3. Pesticide concentrations

In order to compare pesticide effects, we used molarity to define the concentrations, because solutions with the same molarity contain the same number of solute molecules (Avogadro’s number).

A stock 10$^{-4}$ M solution was obtained by dissolving the pesticides in distilled water at room temperature. Each pesticide has a different solubility (DZN: 40 mg/L; CLP and CLP oxon: 2 mg/L; CBR: 40 mg/L). Thus, concentrations 10$^{-5}$, 10$^{-7}$, 10$^{-9}$ and 10$^{-11}$ M were obtained for successive dilutions in ASW from stock solutions and used to carry out the experiments as described in the Supplementary Table S1.

2.4. Cyst exposure

Cysts were exposed to concentrations between 10$^{-5}$ and 10$^{-11}$ M of each pesticide. The concentrations between 10$^{-7}$ and 10$^{-11}$ M are in the range of estimated environmental concentrations (Banks et al., 2005). The 10$^{-5}$ M concentration – equal to the EC50 dose of most OPs for invertebrates (Falugi et al., 2011), including crustaceans (Baek et al., 2015) – was used for comparison.

Tests were carried out in multi-well capsules and performed in ultra-filtered ASW 0.22 μm, without a renewal of the medium. In each well, about 50 cysts in 2.5 ml ASW were covered with the lid and maintained at T = 25 °C. Hatching percentage was monitored at different times (72 h, 96 h, 192 h) under a stereomicroscope (Zeiss, Germany). After 48 h and 120 h, lyophilized Chlorella (Sun Chlorella Corp, London Branch, UK) was provided at 1 mg/L concentration. The last observation (192 h) aimed to check any expected decay in pesticide activity and recovery of damaged larvae. The test was performed in triplicates for Scheme 1.

Scheme 1. A. salina nauplius (N) and metanauplius (MN) stages. In the present study were used nauplius I and II (NI, NII), metanauplius I–II (MN I, II) and III (MN III) resulting from 72, 96 and 192 h post-hatching respectively.
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Fig. 2. Percentage of hatched Artemia larvae (A, C, E, G) after 72 h, 96 h and 192 h and percentage of mortality after 192 h (B, D, F, H) after exposure to different concentrations of chlorpyrifos (A, B), chlorpyrifos oxon (C, D), diazinon (E, F) and carbaryl (G, H). Data are the mean ± standard deviations of the 3 replicates of exposed cysts. Significant differences (*P < 0.05) among the control and exposed groups (A, C, E, G) are indicated starting from the first significant concentration. Percentage of dead larvae was calculated against the initial number of exposed cysts.
4. Discussion

According to the outcome of this work, long-term exposure at doses lower than the LC50 for the different pesticides affects hatching, survival, and ChE activity of the brine shrimp Artemia.

4.1. Effects on hatching and survival

Long-term exposure to pesticides at coastal environmental-like concentrations significantly affects hatching and larval survival, already affected by insecticides, according to previous results on the same model exposed to OPs (Varò et al., 2006; Venkateswara Rao et al., 2007). As suggested by these authors, OP uptake in brine shrimp may be inhibited by the thick envelope of resistant cysts likely to decrease penetration into brine shrimp embryos. Conversely, hatchability results were shown to be related to pesticide toxicity. This observation may be applicable also to CBs, as shown by our findings.

Both CB and OP exposure on Artemia hatching at low concentrations either enhanced or delayed brine shrimp development, but caused no developmental abnormalities, unlike other marine invertebrates exposed to the same pesticides (Buznikov et al., 2001; Falugi and Aluigi, 2012). These findings can be explained by the fact that Artemia—a protostome organism—has a non-regulatory development. Thus, neurotoxic poisoning does not interfere with positional information, as is the case in other invertebrates, such as sea urchins, where dramatic morphological larval abnormalities caused by pesticides have been reported in the larvae (Aluigi et al., 2008).

None of the pesticides used in this study affected brine shrimp larvae survival at the first sampling times (72 and 96 h), while mortality was observed after 192 h. These results confirm the data available in the literature on CLP, DZN and CBR, known to exert a lethal activity at $10^{-5}$ M concentration (Baek et al., 2015; Barahona and Sànchez Fortùn, 1999; Bustos-Obregon and Vargas, 2010; Varò et al., 2006). They also provide new insight into the toxicity of oxonized derivates. In addition, it can be noted that exposure to DZN is responsible for a dose-response effect in Artemia larvae, in line with previous studies on this OP compound (Bustos-Obregon and Vargas, 2010).

Regarding OP insecticides, the results on hatching success after

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Fig. 3. Percentage of hatched Artemia larvae (A–D) after 72 h, 96 h and 192 h and percentage of mortality after 192 h (E) after exposure to different concentrations of selective inhibitors of cholinesterases (ChEs): BW284C51 (A), iso-OMPA (B), eserine (C) and DFP (D, E). Data are the mean ± S.D of the 3 replicates of exposed cysts. Significant differences (*P < 0.05) among the control and exposed groups (A–D) are indicated starting from the first significant concentration. The percentage of dead larvae was calculated against the initial number of exposed cysts.
Fig. 4. *A. salina* unexposed samples: A) cholinesterase (ChE) activity along normal development increase in a time-dependent manner. B–D) Percentage of inhibition of ChE activity, by the different inhibitors (BW284C51 and iso-OMPA (B), eserine (C), DFP (D) evaluated by pretreating for 30 min the homogenates of normally developing *A. salina* stages (0, 18, 24, 36, 48, 60, 72, 96, 120 h). *P < 0.05.

Fig. 5. Percentage of ChE activity (Y) of *Artemia* larvae after 96 h exposure to different concentrations of chlorpyrifos (A), chlorpyrifos oxon (B), diazinon (C) and carbaryl (D). Data are the mean ± standard deviations of the 3 replicates of exposed cysts. *P < 0.05.
Fig. 4. A. salina unexposed samples: A) cholinesterase (ChE) activity along normal development increase in a time-dependent manner. B–D) Percentage of inhibition of ChE activity, by the different inhibitors (BW284C51 and iso-OMPA (B), eserine (C), DFP (D) evaluated by pretreating for 30 min the homogenates of normally developing A. salina stages (0, 18, 24, 36, 48, 60, 72, 96, 120 h). *P < 0.05.

Fig. 5. Percentage of ChE activity (Y) of Artemia larvae after 96 h exposure to different concentrations of chlorpyrifos (A), chlorpyrifos oxon (B), diazinon (C) and carbaryl (D). Data are the mean ± standard deviations of the 3 replicates of exposed cysts. *P < 0.05.
suggest that DZN could be considered as the most aggressive compound, followed by CLPox and CLP. In the case of carbamate CBR, which is very effective on hatching inhibition (similar to CLPox effect observed in this study), a mortality trend decreasing with concentration was only apparent, since it depended on the number of hatched cysts: only few of them were able to hatch, thus, the percentage of dead larvae versus the initial number of cysts was very low. It is worth noting that exposure to low pesticide concentrations in this model has caused quite a different effect from the one found in other vertebrate and invertebrate models, where CBR was the most active toxicant, followed by CLPox and CLP, while DZN was the least effective one (Falugi et al., 2011). Conversely, the specific ChE inhibitors (DFP) used for comparison caused no mortality at any concentrations. Generally, all the cysts had hatched at 192 h. However, in the samples exposed to DFP, some larvae percentage was dead at the bottom of the tanks at 192 h, independently from pesticide concentration. DFP effect may be due to the fact that DFP is a very aggressive OP compound (Kumar et al., 2010), having been synthesized during the II World War as a nervine weapon. Moreover, DFP is an efficient inhibitor of both BChE and AChE activities (Gupta, 2006). In some models, it is 10–100 times more active on BChE.

On the whole, the difference in mortality effects between OP pesticides and specific ChE inhibitors suggests that mortality may be due to pesticide interference with molecules other than ChEs. Actually, several authors have demonstrated other target proteins besides ChEs, and identified other molecular-level protein biomarkers as targets of reactive OP compounds (Murray et al., 2005; Thompson et al., 2010). These authors have found a number of responsive proteins in rat brain homogenates exposed to OPs, such as DZN and CLP. Although these authors failed to identify the protein targets, each of the tested OPs bound to a different collection of proteins, suggesting that different OPs may produce their own specific form of toxicity (Murray et al., 2005). These effects would also be due to species selectivity and acquired resistance, attributable in part to structural differences in binding sub-sites, receptor subunit interfaces, or transmembrane regions (Casida and Durkin, 2013), including a direct effect of pesticides on ACh receptors, such as muscarinic ones (Casida and Quistad, 2004). A similar mechanism could also be suggested for brine shrimps: further investigations on target proteins of OP pesticides should be conducted to confirm this hypothesis.

Table 2

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>CLP</th>
<th>CLPox</th>
<th>DZN</th>
<th>CBR</th>
<th>BW284C51</th>
<th>ISO-OMPA</th>
<th>Eserine</th>
<th>DFP</th>
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<tbody>
<tr>
<td>10^{-11} M</td>
<td>36.06±9.08</td>
<td>73.89±4.10</td>
<td>84.75±1.71</td>
<td>35.28±3.96</td>
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<td>79.08±4.14</td>
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<td>24.80±1.71</td>
<td>88.86±1.96</td>
<td>25.92±8.29</td>
<td>10.85±1.82</td>
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<td>10^{-7} M</td>
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<td>81.58±4.09</td>
<td>89.44±1.02</td>
<td>23.96±4.73</td>
<td>44.13±6.48</td>
<td>37.22±7.75</td>
<td>38.82±1.01</td>
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<td>10^{-5} M</td>
<td>47.32±6.06</td>
<td>86.71±2.25</td>
<td>84.19±1.80</td>
<td>34.19±4.82</td>
<td>48.96±16.80</td>
<td>26.00±8.22</td>
<td>55.36±0.59</td>
<td>24.18±11.13</td>
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</tbody>
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Fig. 6. Histochemical localization of ChE activity in 96 h old larvae after exposure to inhibitors and pesticides. A = control; B-D = 10^{-9}, 10^{-7}, 10^{-5} M BW; E, F = 10^{-7}, 10^{-5} M CLP; G = 10^{-7} CLPox; H = 10^{-5} CBR; I-M = 10^{-5} M, 10^{-7}, 10^{-9} M DFP; N = 10^{-7} DZN. br = protocerebrum; c = connective nerves; sg = sub esophageal ganglion, pg = pair ganglia throwing nerves to the mandibula. Bar represents 100 μm.
The dose responsible for causing an ecotoxicological effect on crustaceans (several species, including echinoderms) is very similar to OPs worldwide, more than all the others combined. Falugi et al., 2011

As far as aquatic species are concerned, the most active toxicant at concentrations likely to affect survival but not reproduction in coastal waters has been studied on *Artemia salina* (98% for DZN and 99.5% for the other products). Information about the effects of propionylcholinesterase (PChE, E.C. 3.1.1.8) activity.

Table 1

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Exposure Time</th>
<th>Endpoint</th>
<th>LC50</th>
<th>Max. Sensitivity</th>
<th>Reference</th>
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<tr>
<td>CLP</td>
<td>cysts</td>
<td>hatching</td>
<td>0.259 mg/L = -10^{-6} M</td>
<td>72h</td>
<td>Bae et al. (2015), Varò et al. (2006)</td>
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<td>CLP ox</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>DZN</td>
<td>at hatching</td>
<td>larvae</td>
<td>10^{-7} M</td>
<td>72h</td>
<td>Bustos-Obregon and Vargas (2010)</td>
</tr>
<tr>
<td>CBR</td>
<td>72 h</td>
<td>viability</td>
<td>1.74*10^{-3} M</td>
<td>72h</td>
<td>Barahona and Sánchez Fortia (1999)</td>
</tr>
</tbody>
</table>

Table 2

Inhibition percentage of metanauplii II stage after exposure to the pesticides and selective ChE inhibitors at each concentration, expressed as average ± standard deviations after 96 h of rehydration.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>10^{-11} M</th>
<th>10^{-9} M</th>
<th>10^{-7} M</th>
<th>10^{-5} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLP</td>
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<td>86.71 ± 2.25</td>
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<tr>
<td>DZN</td>
<td>84.75 ± 1.71</td>
<td>90.93 ± 1.13</td>
<td>89.44 ± 1.02</td>
<td>84.19 ± 1.80</td>
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<td>CBR</td>
<td>35.28 ± 3.96</td>
<td>24.80 ± 1.71</td>
<td>23.96 ± 4.73</td>
<td>34.19 ± 4.82</td>
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<tr>
<td>BW284C51</td>
<td>59.25 ± 5.03</td>
<td>88.86 ± 1.96</td>
<td>44.13 ± 6.48</td>
<td>48.96 ± 16.80</td>
</tr>
<tr>
<td>ISO-OMPA</td>
<td>2.04 ± 8.23</td>
<td>25.92 ± 8.29</td>
<td>37.22 ± 7.75</td>
<td>26.00 ± 8.22</td>
</tr>
<tr>
<td>Eserine</td>
<td>0.03 ± 2.04</td>
<td>10.85 ± 1.82</td>
<td>38.82 ± 1.01</td>
<td>55.36 ± 0.59</td>
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<tr>
<td>DFP</td>
<td>41.23 ± 8.46</td>
<td>31.73 ± 10.39</td>
<td>11.73 ± 9.98</td>
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