



Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Chronic effects of a binary insecticide Acer 35 EC on Nile tilapia *Oreochromis niloticus* through a multi-biomarker approach

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HIGHLIGHTS

- Even at doses below the environmental concentrations, Acer 35 EC impairs the physiology and health status of Nile tilapia.
- Acer 35 EC affects many systems including immune, reproductive and nervous systems.
- The adverse effects of Acer 35 EC are dependent on time and sex.
- Insecticide Acer 35 EC affects females more than males.

ARTICLE INFO

Article history:

Received 14 May 2020
 Received in revised form
 1 October 2020
 Accepted 2 October 2020
 Available online xxx

Handling Editor: Jim Lazorchak

Keywords:

Acetamiprid
 Lambda-cyhalothrin
 Biomarkers
 Nile tilapia
 Chronic effects
 Integrated biomarker response

ABSTRACT

Acer 35 EC is a widely used insecticide (a binary mixture of lambda-cyhalothrin and acetamiprid) in pest control in many West African countries, particularly in the cotton culture in north Benin. The aim of this study was to investigate the chronic effects of Acer 35 EC on Nile tilapia *Oreochromis niloticus* juveniles using a multi-biomarker approach under laboratory conditions. For this purpose, fish were exposed to sublethal concentrations of Acer 35 EC (0, 1 and 10% of LC₅₀- 96 h value). After 28 and 56 days of exposure, several biomarkers were measured in males and females including enzymatic activities related to detoxification and oxidative stress, neurotoxicity and immune responses, sex steroid hormones (testosterone, 17β-estradiol and 11-keto-testosterone) and histological alterations of liver, kidney and gonads. An Integrated Biomarker Response (IBR) was then calculated. The results showed a reduction of cholinesterase activity in muscles, and intercellular superoxide anion production in both sexes. Female steroidogenesis and gametogenesis were affected, especially testosterone levels and oocyte growth. More alterations were observed in liver after exposure to Acer 35 EC. In both sexes, IBR values were higher after 56 days than after 28 days of exposure. In conclusion, based on a large set of biomarkers and IBR values, the chronic exposure to low doses of insecticide Acer 35 EC seems to impair different physiological functions in Nile tilapia juveniles on a time-dependent manner, with a stronger impact on females than on males.

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1. Introduction

Cotton cultivation is a strategic sector for many countries around the world because of its economic importance. In Benin, the cotton sector, like that of other countries in the sub-region (Burkina Faso, Mali, Ivory Coast), forms the basis of the rural and agro-industrial economy. In fact, every year it provides nearly 70 billion CFA francs to about 2 million producers, and contributes 80% to export revenues and 3.8% to the gross domestic product (Togbé et al., 2014). While cotton production represents an essential income, particularly in developing countries where the sector employs 7% of the labour force, it causes serious environmental and social damage (CTB, 2011). Indeed, cotton production consumes the world's largest amount of pesticides with 16% of all pesticides used on the planet. This is due to the fact that the cotton plant is very sensitive to pest and disease attacks, leading to the use of large quantities of inputs such as mineral fertilizers and synthetic pesticides for its production. In Benin, more than 2000 tons of pesticides are used each year for cotton production (Agbohessi et al., 2012).

Among the synthetic pesticides used in Benin for the phytosanitary treatment of cotton, insecticides and herbicides are the two main classes usually used (Gouda et al., 2018; Adechian et al., 2015). Among them, insecticides play a major role. Indeed, in the Beninese context, cotton consumes nearly 90% of the insecticide market (Ton, 2001), with the remainder being used in vegetable and cowpea crops and corn cultivation, respectively (Pazou, 2005). All families of insecticides (organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids) have been used in Benin for cotton cultivation. Studies by Gouda et al. (2018) show that the main insecticides used today are binary acaricides or aphicides (Cotonix 328 EC, Lambda super, Acer 35 EC, Thalix 112 EC etc.) belonging to families such as neonicotinoids (acetamiprid), avermectins (emamectin), organophosphates (chlorpyrifos-ethyl) and pyrethroids (lambda-cyhalothrin). Acetamiprid and lambda-cyhalothrin are two of the mainly used active ingredients (Gouda et al., 2018). Dognon et al. (2018) showed that Acer 35 EC, which contains lambda-cyhalothrin (20 g/L) and acetamiprid (15 g/L), was the binary insecticide most commonly applied by cotton farmers. In Benin, to our knowledge, there are no data available in the scientific literature regarding the concentrations of acetamiprid and lambda-cyhalothrin found in rivers located in cotton areas. The only available data were reported from water reservoirs located in cotton-growing areas. The concentrations of acetamiprid residues found in the reservoirs of northern Benin ranged between 0.20 and 7.7 µg/L (Zoumenou et al., 2019). For lambda-cyhalothrin, the concentrations varied between 0.18 and 1.17 µg/L in the same area (Zoumenou, 2019). In the world, the reported concentrations of acetamiprid residues detected in water ecosystems are respectively 6.31 µg/L in Brazil, 34.4 ng/L in China and 40 ng/L in the United States (Yi et al., 2019; Hladik and Kolpin, 2016; Carbo et al., 2008). Those concentrations are lower than the LC50-96 h determined in Nile tilapia *Oreochromis niloticus* (Guedegba et al., 2019) but may constitute a risk for a good ecological status of water bodies because of its high solubility in water (Pietrzak et al., 2019).

Studies on both, acute and subchronic toxicity of lambda-cyhalothrin show that it is extremely toxic to aquatic species (Bibi et al., 2014; Muthukumaravel et al., 2013; Saravanan et al., 2009; Kumar et al., 2011). These studies also reveal that the main toxicological effect for lambda-cyhalothrin is neurotoxicity attributable to synthetic pyrethroids. Indeed, studies on juveniles of the spotted snake head *Channa punctatus* (Kumar et al., 2009) and on fry of the common carp *Cyprinus carpio* (Bibi et al., 2014) showed that lambda-cyhalothrin causes a significant decrease in acetylcholinesterase (AChE) activity. Alterations such as darkening of skin

colour, hyperactivity, increased loss of balance, rapid swimming, convulsions, increased surface activity, and accelerated opercula movements, characteristic for signs of nervous system damage, have also been observed (De Moraes et al., 2013; Kumar et al., 2011). Further effects on functions of the fish organism have been reported. For example, lambda-cyhalothrin affects biochemical parameters such as the lipid, glucose, and protein metabolism in the liver and the serum of fish exposed to it (Muthukumaravel et al., 2013; Kumar et al., 2012; Parthasarathy and Joseph, 2011; Saravanan et al., 2009). Numerous histopathological changes in the kidney and liver have been observed in different fish species (Li et al., 2014; Muthukumaravel et al., 2013; Velmurugan et al., 2007) as well as immunosuppressive effects on contaminated organisms, decreasing both, the innate and adaptive immune responses (Yekeen et al., 2013; Ogueji and Ibrahim, 2012). Finally, effects on endocrine functions have been reported, such as decreased plasma levels of sex steroids and thyroid hormones, and increased levels of corticosteroids (Saravanan et al., 2009).

In contrast, acute toxicity studies of acetamiprid have shown that it is very low toxic for aquatic vertebrates (Raj and Joseph, 2015; Alam et al., 2014; Siddiqui and Wanule, 2010). However, only few studies exist on the impact of acetamiprid on aquatic species. Behavioural changes, disturbances in calcium, phosphate and urea homeostasis, and a decrease in plasma albumin concentration have been reported in rohu *Labeo rohita* exposed to acetamiprid (Alam et al., 2014). Moreover, Zhang and Zhao (2017) showed that acetamiprid disturbed amino acid metabolism, the TCA cycle, and the balance of neurotransmitters in the head, blood serum, and liver of zebrafish (*Danio rerio*) after exposure. Exposure to acetamiprid also showed significant developmental toxicity in zebrafish embryos with various effects on behaviour, growth, morphology, hatchability, and death at high concentrations (Ma et al., 2019). Similarly, histopathological alterations of gills have been observed in goldfish *Carassius auratus*, affecting respiratory rate and oxygen consumption (Păunescu et al., 2011). On the other hand, the increase in the number of leukocytes found in the spotted snake head indicates a stimulation of the immune system following exposure to acetamiprid (Siddiqui and Wanule, 2010). The effects of acetamiprid on other aspects of fish physiology have not been addressed.

Testing the acute toxicity of Acer 35 EC showed that it was highly toxic to Nile tilapia following the international classification of toxicity of substances based on their median lethal concentration LC₅₀ (very highly toxic LC₅₀ < 0.1 mg/L; highly toxic 0.1–1 mg/L; moderately toxic 1–10 mg/L; slightly toxic 10–100 mg/L and practically non-toxic LC₅₀ > 100 mg/L) (Guedegba et al., 2019). The same study revealed that Acer 35 EC was more toxic to Nile tilapia juveniles than acetamiprid but less toxic than lambda-cyhalothrin, the two active ingredients of Acer 35 EC. So far, no studies have been conducted on the chronic toxicity of Acer 35 EC in aquatic species.

In order to assess the biological impact of contamination in aquatic ecosystems, diagnostic tools, including biomarkers, have been developed and are widely used. These biomarkers (biological responses) are ecotoxicological tools to assess the actual impact of different chemicals on different functions of exposed organisms (Sanchez et al., 2012; Beliaeff and Burgeot, 2002). Currently, there is a growing interest in the use of biomarkers to monitor environmental quality. A more interesting approach is an integrative one based on the responses provided by a set of complementary biomarkers targeting different physiological functions that provide a comprehensive diagnosis of the state of disruption of organisms in their environment (Sanchez et al., 2012; Flammarion et al., 2002). Such an *in-situ* approach is complex and would require the

monitoring of animals and water contamination in the field. Preliminary laboratory approaches are needed to assess the effects of these pesticides on these organisms using a set of complementary biomarkers.

The objective of this study was to evaluate the effects of the binary insecticide Acer 35 EC (a mixture of acetamiprid and lambda-cyhalothrin) on the biochemical, physiological and histological responses of juveniles of Nile Tilapia exposed to chronic doses under laboratory conditions with a multi-biomarker approach.

2. Materials and methods

2.1. Fish

Nile tilapia juveniles (mean weight 14.77 ± 3.72 g and mean length of 9.72 ± 0.74 cm) were bought from the Centre de Recherche et d'Incubation Aquacole du Benin (CRIAB, Calavi, Republic of Benin). They were transported in oxygenated plastic bags and acclimated to laboratory conditions during 12 days in plastic tanks (1000 L) at the Research Laboratory Aquaculture and Aquatic Ecotoxicology (LaRAEAq). During acclimation, fish were fed twice a day to satiation with a commercial dry feed (Coppens pellets 2 mm, 45% crude proteins, 12% crude fat, The Netherlands). All experiments were carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals (EU Directive 2010/63/EU for animal experiments).

2.2. Experimental design and pollutant concentrations

Acer 35 EC is an emulsifiable insecticide containing 20 g/L of lambda-cyhalothrin and 15 g/L of acetamiprid, according to the manufacturer (Sinochem Agro Co., LTD. It has been introduced into the cotton technical route in Benin since the 2014–2015 agricultural campaign and is used in the third window in the phytosanitary treatment calendar for cotton. Acer 35 EC was obtained from the national ministry of agriculture official services. Before using the insecticide in the contamination experiment, the actual concentration of the two main compounds lambda-cyhalothrin and acetamiprid was assayed. Standards of lambda-cyhalothrin, acetamiprid and trifluralin D14 were purchased from Dr Ehrenstorfer (Augsburg, Germany) with a purity of 99%. The presence of the active substances in the Acer 35 EC was checked by GC-MS and their concentrations were estimated. Acer 35 EC was diluted 500 000 times and 50 000 times in acetone, for lambda-cyhalothrin and acetamiprid analysis, respectively. The GC-MS analysis was performed in presence of a constant amount of 120 pg of trifluralin D14, used as internal standard, which was added to both samples and calibration solutions of lambda-cyhalothrin (50–450 pg/ μ L) and acetamiprid (10–90 pg/ μ L). For GC-MS analysis, pesticides were separated on a Focus GC gas chromatographer (Thermo Fisher Scientific) using an Equity 5 column (30 m \times 0.25 mm \times 0.25 μ m) (Sulpeco, Bellefonte, PA, USA) and analyzed with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific). Results were calculated using Xcalibur Software (ThermoFinnigan). Helium gas was applied as carrier gas. The temperature program was 50 °C for 1 min, followed by an increase in 20 °C per min to 100 °C and hold for 1 min, then 10 °C per min to 250 °C and hold for 1 min, then an increase in 20 °C per min to 300 °C and hold for 2 min; total run time was 42 min. The pesticides were detected using selected ion monitoring (SIM) mode in four segment windows. The analysis indicated a concentration of 39 g/L of lambda-cyhalothrin (instead of 20 g/L as indicated in the container) and 22 g/L of acetamiprid (instead of 15 g/L).

The experiment was conducted at two concentrations:

0.0017 μ L/L (corresponding to 0.066 and 0.0374 μ g/L of lambda-cyhalothrin and acetamiprid respectively) and 0.017 μ L/L (corresponding to 0.66 and 0.374 μ g/L of lambda-cyhalothrin and acetamiprid respectively) of the commercial insecticide Acer 35 EC with one control group (no pesticide). These concentrations correspond to 1% and 10% of the 96-h LC₅₀ value of Acer 35 EC for *O. niloticus* respectively, as determined by Guedegba et al. (2019). The higher concentration (0.017 μ L/L) was in the range of environmental concentrations detected in water reservoirs in the cotton area of Northern-Benin (Zoumenou et al., 2019). Each treatment was conducted in three replicates. Healthy fish with similar size were randomly distributed in 9 glass aquaria (volume = 100 L, 20 females and 20 males per aquarium). Each aquarium was equipped with a continuous air flow via a sponge biological filter (professional 3, 350 T EHEIM, Germany) for aeration and waste elimination (remaining food, faeces). Fish were maintained under semi-static renewal conditions with 75% (corresponding to 75 L) of the experimental solution (water and insecticide) replaced every 72 h exposing fish to a freshly prepared solution. The water was changed early in the morning, in one go, before feeding the fish, without any effect on fish behaviour and feeding activity. The fish were fed twice a day. The experiment was carried out for 56 days which approximately corresponds to the duration of the phytosanitary treatment of cotton in Benin.

During the exposure experiment, water samples were collected twice a week: at 0 h (right after) and 72 h after renewal in each tank and stored at –20 °C until their extraction to evaluate the insecticide concentration. The samples were collected 18 times along the experiment. For each sampling time, the water sample at 0 h (right after the renewal) was pooled with the one taken after 72 h in order to reduce the number of samples and the cost of the analysis. Table 1 presents the concentrations of active ingredients (lambda-cyhalothrin and acetamiprid) of Acer 35 EC measured in the experimental solution. Acetamiprid and lambda-cyhalothrin contained in Acer 35 EC were analyzed at the CER-group in Marloie (Belgium). Lambda-cyhalothrin was extracted and analyzed by a Gas Chromatography-Electron Capture Detector (GC-ECD) and acetamiprid was extracted and analyzed by an Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). For lambda-cyhalothrin extraction, 20 mL of experimental water sample were put in a polypropylene centrifuge tube and were spiked with 50 μ L of internal standard solution (IS). Then, 10 mL of hexane were added to the mix. After agitation for 15 min and centrifugation 5 min at 4500 g, the hexane-layer was transferred to a new polypropylene centrifuge tube. This extraction was repeat once. The 2 combined hexane extracts were evaporated under a gentle stream of nitrogen at 30 °C. Finally, the residual was dissolved in 120 μ L of hexane prior to analysis. Briefly, the GC system used for lambda-cyhalothrin analysis, consisted of an Agilent 6890 N (Agilent, Palo Alto, CA, USA) equipped with an auto sampler (Agilent 7683b) and coupled to the Micromass Quattro micro GC quadrupole mass spectrometer (Waters Corporation, Manchester, UK). The GC separation was achieved using a silica DB-5MS capillary column (30 m \times 0.25 mm id. \times film thickness 0.25 μ m) (J&W Scientific, Folsom, CA, USA). Splitless injections of 3 μ L of the final extract in hexane were carried out at 280 °C. Helium was used as carrier gas at a constant flow rate of 2.0 mL/min. The GC interface temperature was set to 300 °C. For acetamiprid extraction, once conditioned with methanol (MeOH) (10 mL) and ultra-pure water (10 mL), 20 mL of experimental water samples were loaded onto an Oasis HLB cartridge. Then, the target fraction was eluted from the cartridge by 6 mL of MeOH. Extraction solution was evaporated under a gentle stream of nitrogen at 30 °C. And, the remaining residue was dissolved in 500 μ L of MeOH/water (20:80, v/v) prior to analysis. For LC-MS/MS analysis, acetamiprid was

Table Table 1

Measured concentration of active ingredients (lambda-cyhalothrin and acetamiprid) of Acer 35 EC contained in exposed solution. Pool of water from 18 samples collected 0 and 72 h after renewal.

Time of sampling	Exposed concentrations of Acer 35 EC	Measured concentration of active ingredients ($\mu\text{g/L}$)	
		Lambda-cyhalothrin	Acetamiprid
0 h after renewal	Control	< LOQ	< LOQ
	A-0.0017 $\mu\text{L/L}$	0.6926	0.309
	A-0.017 $\mu\text{L/L}$	2.7781	3.0173
72 h after renewal	Control	< LOQ	< LOQ
	A-0.0017 $\mu\text{L/L}$	0.0173	0.257
	A-0.017 $\mu\text{L/L}$	0.1439	2.3090

analyzed using a Waters Xevo TQ-S Micro mass spectrometer (Waters, Manchester) coupled to a Waters Acquity UPLC i-Class Plus with flow through needle sample manager. The chromatographic separation was performed in gradient mode during 30 s with 20% B (methanol + 0.05% formic acid + 10 mM of ammonium formate), followed by a linear increase to 100% B during 4.5 min and held for 1 min at 100% B. The column and autosampler were maintained at 50 °C and 15 °C, respectively. The injection volume was 10 μL . Mass spectrometry analysis was operated with an electrospray (ESI) source in positive mode.

2.3. Organ and blood sampling

Fish were sampled two times: at 28 days and 56 days of exposure to the pollutant. At each sampling, five females and five males were randomly caught in each aquarium. Fish were anesthetized by immersion into a solution of Ethyl 3-aminobenzoate methanesulfonate salt (MS-222, 400 mg/L, Sigma, Belgium). Blood was removed from the caudal vein using heparinised syringes, centrifuged at 7500 g for 10 min (VWR 2416 Centrifuge) and aliquots of plasma were stored at -80 °C until steroid hormones and complement analyses. After blood sampling, fish were euthanized by cervical dislocation. Fish were measured (cm), weighed (g) and dissected for kidney, liver, spleen, muscle, gonads and brain. Brain, muscle and a part of liver were immediately frozen in liquid nitrogen and stored at -80 °C for antioxidant and cholinesterase activity analysis. Gonads, kidney and a part of liver were stored in formalin (10% VWR, Belgium) for histological analyses. Spleen tissues were placed in Eppendorf tubes containing 500 μL of Leibovitz medium (L-15 medium, Sigma, Belgium) for superoxide anion production assay. Entire fish bodies were also collected in each aquarium, stored at -20 °C until analysis. Lambda-cyhalothrin and acetamiprid residues were analyzed in fish muscles applying the same method used to determine their concentration in water (see 2.2.). Before analysis, homogenized fish ($5 \text{ g} \pm 0.05$) were placed in polypropylene centrifuge tube and spiked with 50 μL of internal standard solution (IS). The mixture allowed to stand for 15 min at room temperature and 5 mL of acetonitrile were added to the mix. Then, magnesium sulfate (2.0 g) was added and shaken vigorously for 1 min. The sample was centrifuged at 4650 g for 5 min. For lambda-cyhalothrin extraction in muscle 2 mL of the upper acetonitrile layer was transferred into a new polypropylene tube which contains the clean-up sorbents: PSA (300 mg) and C18 (300 mg) and shaken vigorously for an additional minute. After centrifugation ($4500 \text{ g} \times 5 \text{ min}$), 1 mL was taken and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was reconstituted in 120 μL of hexane before analysis. For acetamiprid extraction, 1.2 mL of the upper acetonitrile layer was transferred into a micro centrifuge tube which contains the clean-up sorbents: MgSO₄ (50 mg), PSA (50 mg) and C18 (50 mg) and shaken vigorously for an additional minute. After centrifugation at 10000

$\text{g} \times 5 \text{ min}$, the supernatant was taken and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was reconstituted in 1 mL of a mixture of methanol and water (20:80, v/v) before analysis.

2.4. Cholinesterase and antioxidant enzyme activities

2.4.1. Preparation of liver, brain and muscle extracts

Cholinesterase enzyme activity and oxidative stress biomarkers were measured in post-mitochondrial fraction S9. Frozen brain, muscle and liver samples were homogenized in ice-cold phosphate buffer (100 mM, pH 7.8, 9 vol for one volume of tissue) supplemented with 20% of glycerol and 0.2 mM phenylmethylsulfonyl fluoride (Sigma, France) as a serine protease inhibitor. Tissues were crushed twice using tissue homogenizer Precellys 24 (Bertin) at 6000 rpm during 20 s with 10 s of interval. The post-mitochondrial fraction S9 was obtained after centrifugation of the homogenate at 10000 g, during 15 min at 4 °C. Total protein content was measured in the homogenate using the method developed by Bradford (1976) before performing biochemical assays for cholinesterase and antioxidant biomarkers.

2.4.2. Neurotoxicity biomarkers

Cholinesterase (ChE) activity was determined in post-mitochondrial fraction from brain and muscle tissues using the method of Ellman et al. (1961) with acetylcholinesterase (Sigma, France) as a standard. Homogenate was normalized to 0.5 g/L of total protein concentration and 4.5 μL of diluted homogenate was mixed with 90 μL of phosphate buffer (100 mM, pH 7.4) containing 210 mM acetylthiocholine iodide (Sigma) and 21 mM dithiobis-2-dinitrobenzoic acid (DTNB) (Sigma, France). The absorbance was recorded at 405 nm for 10 min and the results were expressed as units per gram of protein.

2.4.3. Oxidative stress biomarkers

All the following measures were carried out on liver homogenate at room temperature in microtiter plates, using a microplate multimode reader (Synergy H4–Bio-Tek instruments).

GSH content was measured according to Vandeputte et al. (1994) method using the reaction between glutathione –SH group and DTNB. A protein precipitation was carried out on 100 μL of diluted sample (normalized to 2.5 g/L of total protein) with 25 μL of trichloroacetic acid (25%, Sigma, France) followed by a centrifugation at 2000 g and 4 °C during 15 min. The obtained supernatant (10 μL) was added to 60 μL of a mixture containing phosphate buffer (100 mM), EDTA (10 mM), NADPH (2 mM), DTNB (10 mM) and to 60 μL of glutathione reductase standard (2.83 U/mL in phosphate buffer 100 mM). The absorbance was recorded at 405 nm for 10 min and the results were expressed as μmol per g of protein.

Glutathione-S Transferase (GST) activity was determined

following the method of Habig et al. (1974) using chlorodinitrobenzene (CDNB) (Sigma, France) as substrate and purified GST from equine liver (Sigma, France) as a standard. The reaction mixture (45 μ L) contained 1 mM CDNB. One mM GSH (total reduced glutathione) in 50 mM phosphate buffer (pH 6.5) was added to 55 μ L of diluted homogenate (0.25–0.3 g/L of total protein) and the absorbance was recorded at 340 nm for 10 min. Results were expressed as units per gram of protein.

Catalase (CAT) activity was determined according to Beers and Sizer (1952) and Babo and Vasseur (1992) using the spectrophotometric measure of hydrogen peroxide (H_2O_2) breakdown at 240 nm. The assay mixture contained 100 μ L of phosphate buffer (100 mM), H_2O_2 (28 mM) and 100 μ L of diluted homogenate (0.04 and 0.4 g/L of total protein). Results were expressed as unit per g of protein.

Glutathione reductase (GR) activity was measured as described by Carlberg and Mannervik (1985). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm. To 45 μ L of the assay mixture containing phosphate buffer (100 mM, pH 7.6), NADPH (2 mM), oxidized glutathione (GSSG) (20 mM), 55 μ L of the diluted sample (normalized to 2.5 g/L of total protein) was added following a spectrophotometric measure for 30 min. Results were expressed as unit per g of protein.

Glutathione peroxidase (GPx) was determined according to Paglia and Valentine (1967). A volume of 10 μ L of diluted sample (normalized to 2 g/L of total protein) was added to 56.6 μ L of the reaction mixture containing phosphate buffer (100 mM, pH 7.6), GSH (30 mM), NADPH (3.6 mM) and GR (30 U/mL). The addition of 33 μ L of cumene hydroperoxide (0.552 mM) initiated the reaction and the activity was recorded right after at 340 nm for 2 min. Results were expressed as unit per g of protein.

2.5. Immune biomarkers

2.5.1. Intracellular superoxide anion production

The assay of spleen intracellular superoxide production was adapted from Fatima et al. (2007) modified by Milla et al. (2010). After dissection, spleens were weighted and gently mashed through a 100 μ m nylon mesh grid into a Petri dish containing 0.5 mL of L-15 medium (Sigma, Belgium) in presence of 0.5 mL L-15. The cell suspension obtained was washed twice as following: after centrifugation at 500 g for 5 min, the supernatant was discarded, 0.5 mL of fresh culture medium was added to the cell suspension and mixed gently. Aliquots (100 μ L) of the cell suspension were placed into 1.5 mL polypropylene tubes. After 30 min of incubation at room temperature, 100 μ L of nitroblue tetrazolium (NBT, 1 mg/mL dissolved in PBS, pH 7.4) was added to the samples. The relative standard curve was made with 100 mL of pooled cell suspension and 0–150 μ L of NBT. After 1 h of incubation at room temperature, centrifugation (500 g, 5 min) and supernatant removal, cells were fixed with 280 μ L of methanol. The NBT deposited inside the cells was then dissolved, first by adding 240 mL of KOH (2 M) to solubilize cell membranes and then by adding 280 mL of N-dimethylformamide to dissolve blue formazan with gentle shaking. After 3500 rpm centrifugation, the supernatant was transferred in cuvettes for spectrophotometer and the absorbance was read at 550 nm.

2.5.2. Alternative complement pathway

The procedure of the plasma alternative complement pathway using rabbit red blood cells (RRBC) as targets was adapted from Sunyer and Tort (1995) and Milla et al. (2010). A serial dilution from 1/4 to 1/2048 into veronal buffer (IDVert, France) was performed for each plasma sample (60 μ L of total volume) in a round-bottomed 96-well plate. Then, 10 μ L of RRBC were added in each

well and the plate incubated at 37 °C during 120 min with gentle mixing every 20 min. A positive control (100% haemolysis) consisting of 10 μ L of RRBC lysed in 60 μ L of distilled water and a negative control (spontaneous haemolysis) consisted of 10 μ L of RRBC incubated with veronal buffer was also performed in the same plate. The plate was centrifuged at 2000 g and 4 °C during 5 min and 35 μ L of the supernatant was transferred to flat bottom 96 well plates to measure the absorbance at 405 nm.

2.6. Sex steroid biomarkers

Plasma levels of testosterone (T), 17 β -estradiol (E2) and 11-ketotestosterone (11 KT) were determined by radioimmunoassay (RIA) according to Fostier and Jalabert (1986) adapted by Mandiki et al. (2004). For each steroid, 25 μ L of plasma were extracted twice with 1 mL of cyclohexane-ethyl acetate (v/v) (VWR, Belgium). All samples and standards were assayed in triplicate. All the radioactive hormones were purchased from Amersham Pharmacia (Buckinghamshire, England), T and E2 antibodies were provided by Laboratoire d'Hormonologie de Marloire (Belgium), and anti-11KT was kindly given by Prof A. Fostier (INRA, Rennes, France). The intra-assay coefficients of variation were 5.56, 5.22 and 6.48% for T, 11 KT and E2 respectively and the detection limit ranged from 5 to 10 pg/mL.

2.7. Histological analysis

Liver, kidney and gonad samples were fixed in 10% neutrally buffered formalin (NV MLS, Belgium) and then transferred into a solution of acetic formaldehyde 4% for 24 h. Fixed tissues were dehydrated by increasing concentrations of ethanol, infiltrated with xylene and then embedded in paraffin. Sections of 5 μ m and 6 μ m were cut from the paraffin block for ovary, kidney and liver respectively. Ovary sections were stained with a trichrome (Trigreen): hematoxylin Gill III (Merck), phloxine B (0.5%, Merck) and light green (0.5%, FLUKA) for oocyte maturation stage determination and with hematoxylin, eosin, safran (HES) for histological alterations. Testis, liver and kidney sections were stained with HES trichrome. All sections were examined using light microscopy with a 10–40x range of magnification. As *O. niloticus* is a "plurimodal spawner", different stages of vitellogenesis were found in females. The oocyte maturation stages were assessed according to Rinchard and Kestemont (1996), El-Saba et al. (2013) and Agbohessi et al. (2015a, 2015b):

- **Stage 1:** Protoplasmic oocyte or oocyte with vacuole free cytoplasm.
- **Stage 2:** Cortical alveoli (early or late vacuolation, in the form of vesicles polysaccharide).
- **Stage 3:** Early Vitellogenesis oocyte. Vitellogenic oocytes accumulate yolk globules, and cortical alveoli are pushed at the periphery of the cytoplasm.
- **Stage 4:** Late Vitellogenesis oocyte. Vitellogenic oocyte accumulate yolk globules in all cytoplasm.
- **Stage 5:** Final maturation stage. Appearance of micropyle and migration of the germinal vesicle to the micropyle.
- **Stage 6:** Stage post-ovulatory. Folded follicular cells, after expulsion of the oocyte.

The percentage of each stage was determined on one hundred cells counted per ovary. The diameter of twenty vitellogenic oocytes (stage 4) from each ovary was measured with the computer program Olympus cellSens Dimension after scanning the slide using an Olympus optical microscope with multiple heads.

The presence of histological alterations for each organ was

evaluated semi-quantitatively using a protocol developed by [Bernet et al. \(1999\)](#) and adapted by [Van Dyk et al. \(2012\)](#) for liver alterations and by [Silva and Martinez \(2007\)](#) for kidney alterations. Pathological changes were classified into four reaction patterns including: (1) circulatory disturbances, (2) regressive changes, (3) progressive changes, (4) inflammatory responses. For each organ, an index was calculated for each reaction pattern, e.g. for the liver: Circulatory liver index (IL-C), regressive liver index (IL-R), inflammatory liver index (IL-I) and progressive liver index (IL-P). The sum of these indexes yields the total organ index: IL for the liver, IK for the kidney, IT for the testis and IO for ovaries, calculated according to [Bernet et al. \(1999\)](#) using the following formula:

$$I_{org} = \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

where: org = organ; rp = reaction pattern; alt = alteration; a = score value; w = importance factor.

The indexes were classified depending on the severity of the histological response according to [van Dyk et al. \(2009a, 2009b\)](#) classification which is based on the scoring system developed by [Zimmerli et al. \(2007\)](#).

Class I ($I_{org} < 10$): Normal tissue structure with slight histological alterations.

Class II ($10 \leq I_{org} \leq 25$): Normal tissue structure with moderate histological alterations.

Class III ($26 \leq I_{org} \leq 35$): Pronounced alteration of organ tissues.

Class IV ($I_{org} > 35$): Severe alteration of organ tissues.

In addition to these indexes, we determined the prevalence of histopathological features. The prevalence of an alteration was calculated at each site as following:

Prevalence of a histological alteration = (number of fish with the alteration/total number of fish) \times 100.

2.8. Integrated biomarker response index (IBR)

The biomarker results were calculated with the "Integrated Biological Responses index version 2" (IBRv2) as described by [Beliaeff and Burgeot \(2002\)](#) and modified by [Sanchez et al. \(2013\)](#). This index is based on the reference deviation between disturbed and undisturbed states ([Sanchez et al., 2013](#)). In this study, the deviation between biomarkers measured at 28 and 56 days in fish exposed to two different concentrations of Acer 35 EC were compared to those measured in control fish. At each exposure time and for each biomarker (X_i), the ratio between the mean value obtained for each exposed group and the control value (X_0) was log-transformed ($Y_i = \log(X_i/X_0)$). Then, the general mean (m) and standard deviation (s) of Y_i were calculated for all groups at each time and Y_i values were standardized by the formula: $Z_i = (Y_i - m)/s$. The mean of standardized biomarker response Z_i for each group and the mean of the reference biomarker data Z_0 (control group) were used to define the biomarker deviation index A following $A = Z_i - Z_0$. This allowed us to create the basal line centred at 0 and represent biomarker variation. The sum of the absolute value of A parameters represent the integrated multi-biomarker value IBRv2 ($IBRv2 = \sum |A|$). At each exposure time and for each group ($A = 0.0017$ and $A = 0.017 \mu\text{L/L}$), the calculated A values were reported in a star plot representing the reference deviation of each biomarker ([Sanchez et al., 2013](#)). The area above 0 reflects biomarker induction while the area below 0 indicates biomarker inhibition.

2.9. Statistical analysis

All parameters were expressed as mean \pm standard deviation. All statistical analyses were performed using Statistica for

Windows, version 5.0 Software. For all dependant variables, the normality and the homoscedasticity of data were previously tested using Kolmogorov-Smirnov and Bartlett tests, respectively. If necessary, data were log-transformed. For each sex, data were analyzed by two-way analysis of variance (ANOVA II) with pesticide concentration Acer 35 EC and exposure time as fixed factors. A Turkey post-hoc was performed to test the position of significant differences between the different conditions. The level of significance used in all tests was $p < 0.05$. Data are represented as the mean \pm standard deviation and tanks (glass aquaria) were used as the statistical unit ($n = 3, 5$ fish). In addition, data are presented according to the factor for which a significant effect is observed.

3. Results

3.1. Residues of pesticide in muscle of Nile tilapia exposed to Acer 35 EC

As shown in [Table 2](#), residues of lambda-cyhalothrin and acetamiprid were highly concentrated in fish muscles of Nile Tilapia exposed to the higher Acer 35 EC concentration whatever the duration of exposure.

On the one hand, the fish sampled at 56 days of exposure to the higher concentration, accumulated approximately twice lambda-cyhalothrin residues compared to fish sampled at 28 days of exposure to the same concentration. Fish exposed to the lower concentration and sampled at 56 days accumulated slightly less lambda-cyhalothrin compared to those sampled at 28 days.

On the other hand, the fish sampled at 56 days of exposure showed less acetamiprid residues in their muscles compared to those sampled at 28 days of exposure.

3.2. Neurotoxicity biomarkers

In the brain, no significant difference was observed between Acer 35 EC concentrations in both, females and males ([Fig. 1a](#)). However, ChE activity was significantly higher at 28 days of exposure in females of all groups ($p \leq 0.001$) than after 56 days of exposure. In muscles, ChE activity varied significantly depending on Acer 35 EC concentrations only in females ([Fig. 1b](#)). Females exposed to the low concentration of Acer 35 EC showed the lowest ChE activity compared to the control group ($p < 0.05$) while those exposed to the higher concentration showed an intermediate ChE activity compared to the two other groups.

3.3. Oxidative stress biomarkers

Changes in antioxidant enzymes and reduced glutathione content measured in the liver were summarized in [Table 3](#). Only exposure time induced significant changes in the activities of glutathione S-transferase (GST, $p < 0.001$), glutathione reductase (GR, $p < 0.05$) and catalase (CAT, $p < 0.01$) for all males with higher values after 56 days of exposure. No significant changes were observed in glutathione peroxidase (GPx) while both Acer 35 EC concentrations and exposure time affected significantly the levels of reduced glutathione content (GSH, $p < 0.05$ and $p < 0.01$ respectively). On the one hand, males exposed 56 days to the different Acer 35 EC concentrations showed higher values than those exposed 28 days. On the other hand, males exposed to the higher Acer 35 EC concentration showed GSH higher levels than those exposed to the lower concentration ($0.0017 \mu\text{L/L}$). In females, only GSH and GST varied significantly depending on the exposure time ($p < 0.05$ and $p < 0.001$ respectively) with higher values after 56 days of exposure.

Table Table 2
Lambda-cyhalothrin and acetamiprid residues in muscle of Nile tilapia exposed to Acer 35 EC.

Pesticides ($\mu\text{g}/\text{Kg}$ of muscle)		Lambda-cyhalothrin	Acetamiprid
28 days of exposure	Control	< LOQ	< LOQ
	A-0.0017 $\mu\text{L}/\text{L}$	20.2	9.1
	A-0.017 $\mu\text{L}/\text{L}$	49.1	27.1
56 days of exposure	Control	< LOQ	< LOQ
	A-0.0017 $\mu\text{L}/\text{L}$	13.8	2.4
	A-0.017 $\mu\text{L}/\text{L}$	94.3	9.7

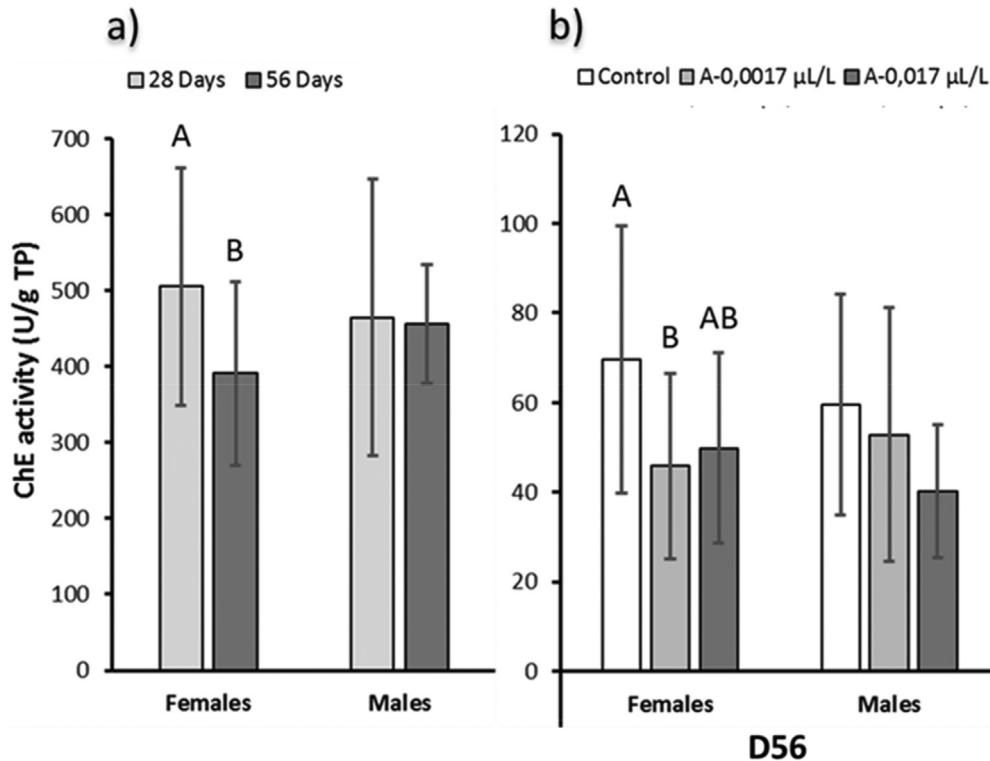


Fig. A.1. Changes in ChE activity (U/g total protein) in the brain (a) depending on the exposure time (28 days, grey box and 56 days, dark box) and in muscles (b) depending on Acer 35 EC concentrations (A-0.0017 $\mu\text{L}/\text{L}$, grey box and A-0.017 $\mu\text{L}/\text{L}$, dark box) compared to the control group (white box) of *O. niloticus* juveniles (females and males). Data are expressed as mean \pm standard deviation. Different letters (A, B) indicate significant differences ($p < 0.05$).

Table Table 3

Changes in antioxidant enzyme activities (U/g total protein) and reduced glutathione content ($\mu\text{mol}/\text{g}$ total protein) in liver homogenate of *O. niloticus* juveniles (females ♀ and males ♂) exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 $\mu\text{L}/\text{L}$ and A-0.017 $\mu\text{L}/\text{L}$) compared to the control group. Different capital letters (A, B) indicate significant differences between Acer 35 EC concentrations and lower-case letters (a, b) indicate significant differences among the exposure time ($p < 0.05$).

Exposure time	Treatments	GST	GSH	GPx	GR	CAT	
♀	28 days	Control	870.63 \pm 437.82 ^b	4.1 \pm 1.06 ^b	44.81 \pm 6.53	7.85 \pm 2.28	400240 \pm 131714
		A-0.0017 $\mu\text{L}/\text{L}$	541.91 \pm 199.38 ^b	4.17 \pm 1.29 ^b	46.4 \pm 6.69	6.65 \pm 0.56	558785 \pm 242609
		A-0.017 $\mu\text{L}/\text{L}$	606.81 \pm 129.46 ^b	3.87 \pm 1.4 ^b	43.7 \pm 5.98	6.25 \pm 0.61	519149 \pm 236946
	56 days	Control	3446.96 \pm 901.2 ^a	5.64 \pm 1.49 ^a	44.11 \pm 2.05	7.14 \pm 2.31	367947 \pm 9938
		A-0.0017 $\mu\text{L}/\text{L}$	4099.86 \pm 149.95 ^a	3.69 \pm 0.38 ^a	38.36 \pm 1.52	8.86 \pm 0.96	324974 \pm 123428
		A-0.017 $\mu\text{L}/\text{L}$	3828.41 \pm 368.96 ^a	6.66 \pm 0.76 ^a	38.82 \pm 1.37	6.68 \pm 1.56	428987 \pm 109019
♂	28 days	Control	856.64 \pm 307.71 ^b	5.12 \pm 0.65 ^{Ab}	51.61 \pm 18.24	5.66 \pm 0.86 ^b	346382 \pm 216530 ^b
		A-0.0017 $\mu\text{L}/\text{L}$	782.14 \pm 333.74 ^b	5.23 \pm 1.18 ^{Ab}	39.57 \pm 5.07	8.5 \pm 1.97 ^b	327433 \pm 206221 ^b
		A-0.017 $\mu\text{L}/\text{L}$	909.41 \pm 166.5 ^b	5.87 \pm 2.8 ^{Ab}	42.76 \pm 13.2	9.29 \pm 3.33 ^b	203764 \pm 115008 ^b
	56 days	Control	5589.33 \pm 1127.73 ^a	8.16 \pm 2.36 ^{ABb}	59.48 \pm 8.73	10.31 \pm 2.32 ^a	407946 \pm 94474 ^a
		A-0.0017 $\mu\text{L}/\text{L}$	4560.7 \pm 267.64 ^a	7.25 \pm 1.7 ^{Bb}	51.06 \pm 5.73	10.64 \pm 2.14 ^a	518592 \pm 135107 ^a
		A-0.017 $\mu\text{L}/\text{L}$	6079.99 \pm 730.66 ^a	13.07 \pm 3.01 ^{Aa}	54.4 \pm 4.75	11.33 \pm 0.55 ^a	740993 \pm 88476 ^a

3.4. Immune biomarkers

Acer 35 EC concentrations significantly affected the spleen superoxide production (NBT) in females (Fig. 2a, $p < 0.05$). Females

showed lower levels in NBT after exposure to Acer 35 EC compared to the control group regardless of exposure time. In males, a significant difference related to the interaction between Acer 35 EC concentrations and exposure time was observed in superoxide

production ($p < 0.05$). While there was no significant difference between the different Acer 35 EC concentrations after 28 days of exposure, males exposed to the low Acer 35 EC concentration (0.0017 $\mu\text{L/L}$) showed significant lower NBT levels compared to the control group after 56 days of exposure ($p < 0.05$). Moreover, NBT levels increased significantly throughout the exposure time only in the control group ($p < 0.05$).

In males, no significant changes were observed between the different groups in complement activity (Fig. 2b), while in females, complement activity levels changed significantly depending on the interaction between Acer 35 EC concentration and exposure time ($p < 0.01$). Females exposed 28 days to the low concentration showed significantly higher levels than those exposed 28 days to the high concentration. Moreover, a significant decrease in complement activity was observed only in females exposed to the low Acer 35 EC concentration.

3.5. Sex steroid biomarkers

Plasma testosterone (T) levels varied significantly depending on both, Acer 35 EC concentrations ($p < 0.05$) and exposure time ($p < 0.01$) only in females (Fig. 3a). On the one hand, T levels were significantly lower after 28 days of exposure in females regardless of Acer 35 EC concentration ($p < 0.05$). On the other hand, females exposed to the high Acer 35 EC concentration showed higher T levels compared to the control group regardless of exposure time ($p < 0.05$), while females exposed to the low Acer 35 EC concentration showed intermediate T levels. In males, no significant differences were observed in testosterone levels.

Plasma 17 β -estradiol (E2) levels varied depending on the exposure time only in females (Fig. 3b). Females showed significantly higher E2 levels after 56 days of exposure ($p < 0.05$) regardless of Acer 35 EC concentration. No significant differences were observed in E2 levels in males.

Plasma 11-ketotestosterone (11 KT) levels varied significantly depending on exposure time only in males ($p < 0.01$, Fig. 3c). After 56 days of exposure, males showed significantly higher levels of

11 KT compared to after 28 days of exposure. No significant differences were observed in 11 KT levels in females.

3.6. Histology and histopathology biomarkers

3.6.1. Ovary histology

Only the four first stages were observed in ovary sections (Fig. 4). After 28 days of treatment, there was no significant difference between the groups. However, after 56 days of treatment, all females exposed to Acer 35 EC showed a lower proportion of late vitellogenic oocytes compared to the control group ($p < 0.05$). Only exposure time induced significant changes in the proportion of protoplasmic oocyte and cortical alveoli stages ($p < 0.05$ and $p < 0.01$ respectively) with a high proportion after 28 and 56 days respectively.

Oocyte diameter (diameter of vitellogenic oocyte) varied significantly depending on the interaction between Acer 35 EC concentrations and exposure time ($p < 0.001$, Fig. 5). While there was no significant difference after 56 days of exposure, females exposed to the high Acer 35 EC concentration (0.017 $\mu\text{L/L}$) showed significantly higher oocyte diameters compared to the two other groups after 28 days of exposure ($p < 0.001$). Moreover, oocyte diameter increased significantly throughout the exposure time in the control group ($p < 0.05$) and decreased significantly throughout the exposure time in females exposed to the high concentration ($p < 0.001$).

3.6.2. Liver histopathology

In liver, 11 different alterations were identified and were classified into progressive changes (cellular hypertrophy), regressive changes (glycogen depletion, hyaline inclusions, hepatocyte deformation, fat vacuolisation of hepatocytes and nuclear pleomorphism mainly nuclear hypertrophy), circulatory disturbances (haemorrhage and sinusoid congestion) and inflammatory responses (presence of melano macrophages centres and granuloma and infiltration of immune cells in hepatic parenchyma). Neoplastic changes were not observed in liver tissues.

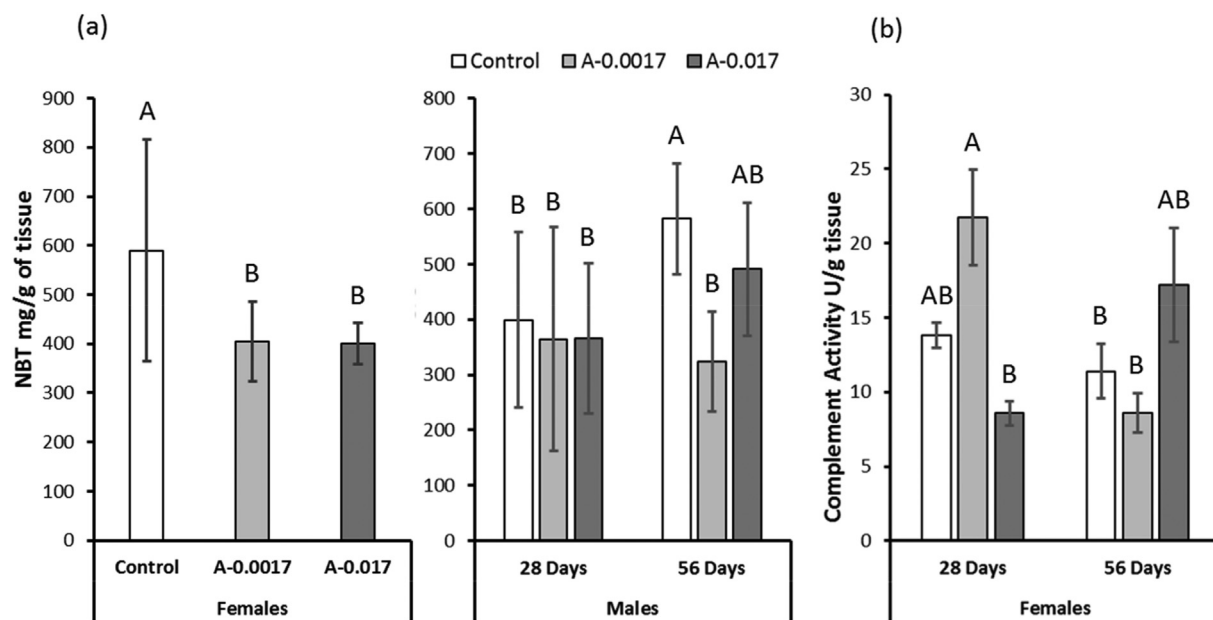


Fig. A.2. Changes in superoxide production (a) and complement activity (a) in spleen homogenate and plasma of *O. niloticus* juveniles (females and males) exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 $\mu\text{L/L}$, grey box and A-0.017 $\mu\text{L/L}$, dark box) compared to the control group (white box). Data are expressed as mean \pm standard deviation. Different letters (A, B) indicate significant differences ($p < 0.05$).

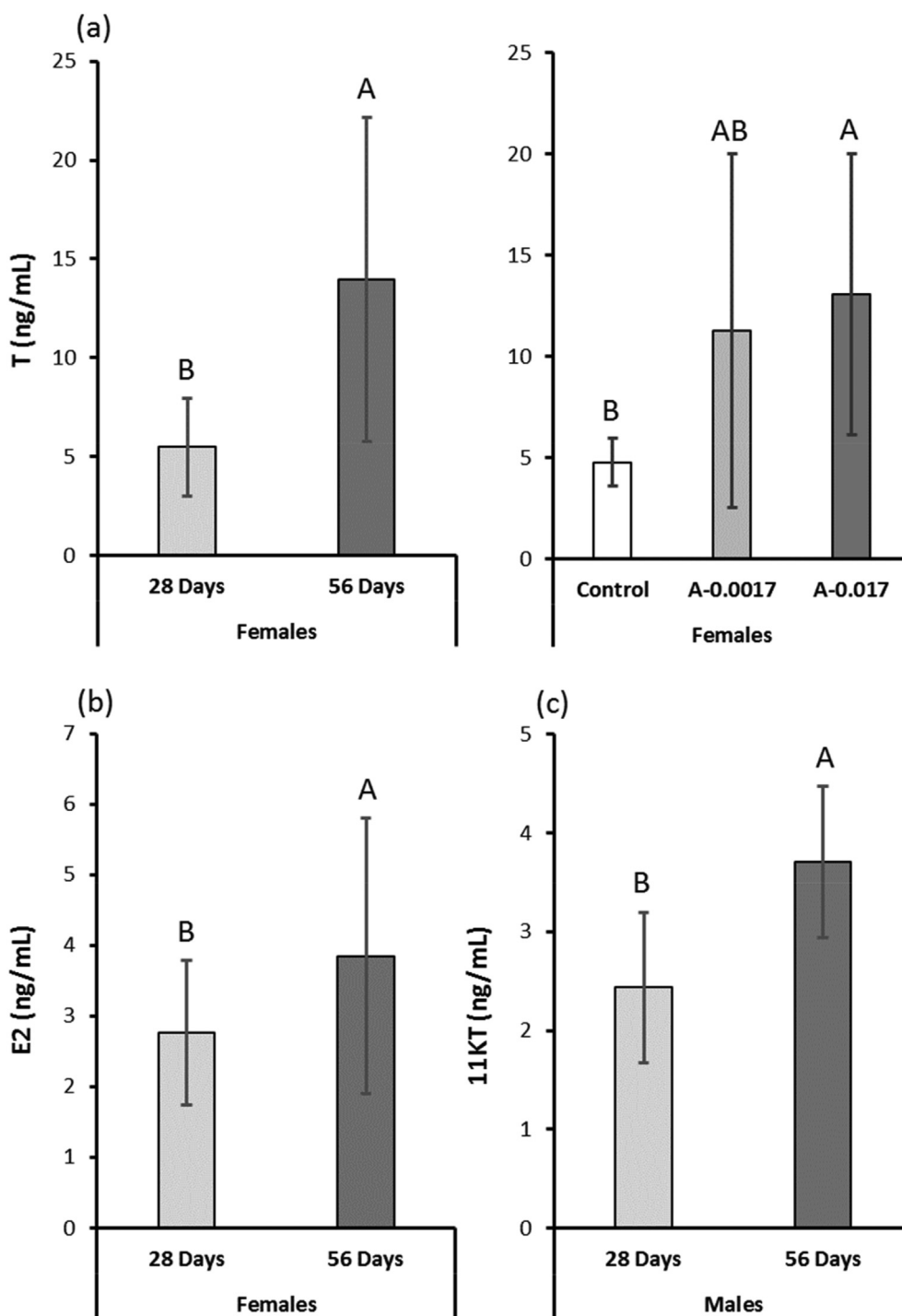


Fig. A.3. Changes in plasma sex steroid levels (ng/mL): testosterone (a), 17β-estradiol (b) and 11-ketotestosterone (c) of *O. niloticus* juveniles exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 μL/L, grey box and A-0.017 μL/L, dark box) compared to control group (white box). Data are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

The liver indexes for circulatory (IL-C), inflammatory (IL-I), progressive (IL-P) and regressive (IL-R), changes were calculated for each sex (Table 4). In males, none of the indexes showed significant differences between the groups except IL-I, which decreased significantly after 56 days for the males exposed to 0.0017 μL/L of Acer 35 EC. In females, while IL-C and IL-P did not vary significantly among treatments and exposure time, IL-I and IL-R showed significant differences between groups. The inflammatory index

showed a significant difference between females exposed to Acer 35 EC after 56 days of exposure compared with the control ones ($p < 0.05$). After 28 days of exposure, females exposed to 0.0017 μL/mL of Acer 35 EC showed a higher IL-R ($p < 0.01$) than the other groups. The total liver index (IL) was calculated according to Bernet et al. (1999) and showed significant differences depending on the Acer 35 EC exposure ($p < 0.001$) only in females (Fig. 6). Females exposed to Acer 35 EC showed a higher total liver index than the

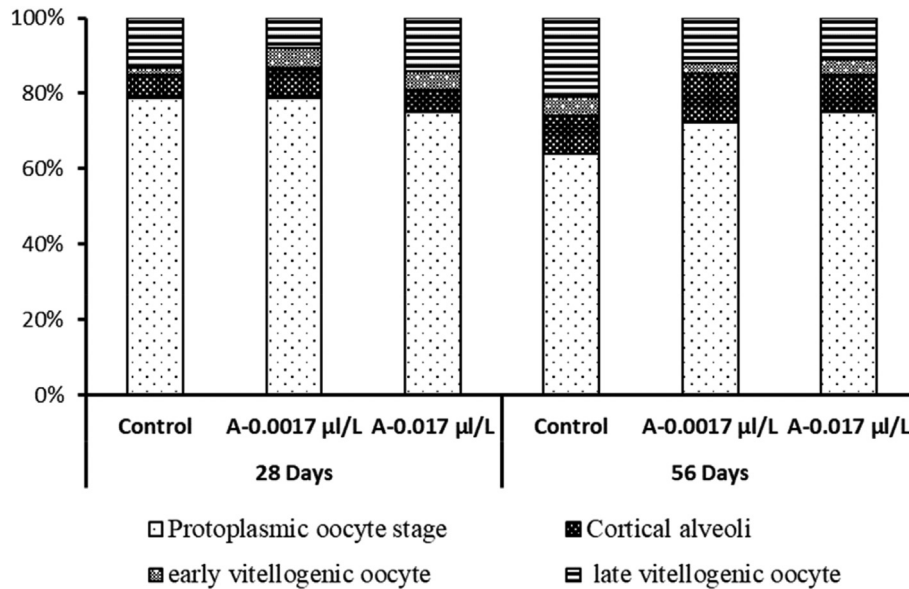


Fig. A.4. Changes in percentages (%) of oocyte stages of *O. niloticus* females exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 µL/L and A-0.017 µL/L) compared to the control group.

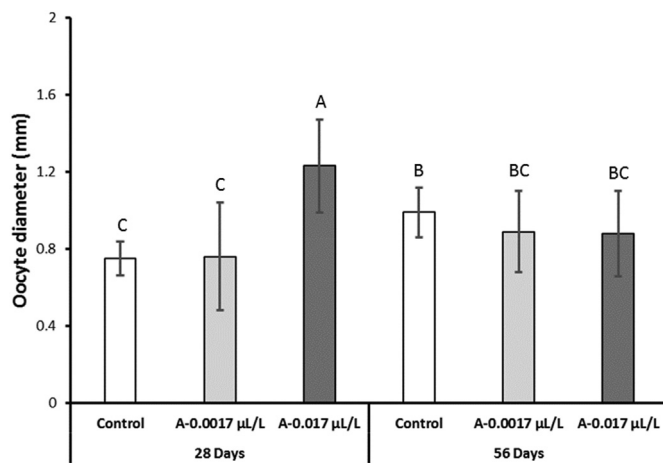


Fig. A.5. Changes in oocytes diameter (mm) of *O. niloticus* females exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 µL/L, grey box and A-0.017 µL/L, dark box) compared to the control group (white box). Data are expressed as mean \pm standard deviation. Different letters indicate significant differences ($p < 0.05$).

control ones. Exposure to 1% LC50 - 96 h dose (0.0017 µL/L) had a more deleterious effect on liver tissues than the exposure to 10% LC50 - 96 h dose (0.017 µL/L of Acer 35 EC).

Concerning the class of histological alterations, all males showed a normal tissue structure with moderate histological alterations (class II: $10 \leq IL \leq 25$). Females exposed to 0.0017 µL/L of Acer 35 EC showed pronounced alteration of the liver tissue (class III: $26 \leq IL \leq 35$) while the other groups showed a normal tissue structure with moderate histological alterations.

3.6.3. Kidney histopathology

Circulatory (sinusoid congestion) and inflammatory (presence of melano macrophages centres) changes did not vary significantly except for females after 56 days of exposure for the latter one (Table 5). Females exposed to the higher dose of Acer 35 EC (0.017 µL/L) showed a higher inflammatory changes index than the control ones ($p < 0.01$). The progressive changes (tubular regeneration) index varied significantly depending on the exposure time in males and females ($p < 0.05$) with higher values after 28 days of exposure than after 56 days. Regressive changes (occlusion of tubule lumen, tubular degeneration, vacuolisation of tubule cells and

Table Table 4

Changes in circulatory (IL-C), inflammatory (IL-I), progressive (IL-P) and regressive (IL-R) alterations in liver tissues of *O. niloticus* juveniles (females ♀ and males ♂) exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 µL/L and A-0.017 µL/L) compared to the control group. Different capital letters (A, B) indicate significant differences between treatments and lower-case letters (a, b) indicate significant differences among the exposure time ($p < 0.05$).

Exposure time	Treatments	IL-C	IL-I	IL-P	IL-R	
♀	28 days	Control	3.47 \pm 0.39	4.80 \pm 0.81	0.13 \pm 0.13	9.00 \pm 1.26 ^B
		A-0.0017 µL/L	3.64 \pm 0.32	7.79 \pm 1.01 ^a	0.14 \pm 0.14	14.79 \pm 1.55 ^A
		A-0.017 µL/L	3.20 \pm 0.22	6.27 \pm 0.64	0.00	9.60 \pm 1.15 ^B
	56 days	Control	3.86 \pm 0.51	2.43 \pm 0.36 ^B	0.00	8.36 \pm 1.03
		A-0.0017 µL/L	4.27 \pm 0.57	4.64 \pm 0.88 ^{Ab}	0.55 \pm 0.31	13.36 \pm 1.55
		A-0.017 µL/L	3.80 \pm 0.33	5.60 \pm 0.78 ^A	0.00	12.07 \pm 1.39
♂	28 days	Control	3.73 \pm 0.21	5.33 \pm 0.94	0.00	13.00 \pm 1.69
		A-0.0017 µL/L	4.07 \pm 0.28	5.00 \pm 0.87 ^a	0.80 \pm 0.43	14.00 \pm 1.29
		A-0.017 µL/L	3.73 \pm 0.38	5.13 \pm 0.97	0.00	14.33 \pm 1.19
	56 days	Control	3.86 \pm 0.33	5.00 \pm 1.13	0.07 \pm 0.07	11.29 \pm 1.23
		A-0.0017 µL/L	4.07 \pm 0.49	3.00 \pm 0.71 ^b	0.78 \pm 0.43	15.64 \pm 1.82
		A-0.017 µL/L	3.53 \pm 0.43	4.33 \pm 0.67	0.13 \pm 0.13	14.40 \pm 1.51

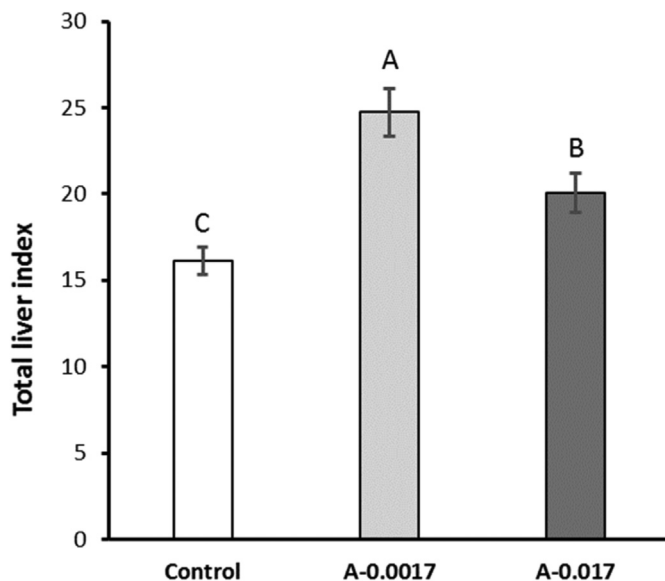


Fig. A.6. Changes in the total liver index of *O. niloticus* females exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 $\mu\text{L/L}$ and A-0.017 $\mu\text{L/L}$) compared to control group. Data are expressed as mean \pm standard deviation. Different letters (A, B, C) indicate significant differences ($p < 0.05$).

presence of hyaline droplets) were less important in all males and females after 56 days of experiment ($p < 0.01$). After 28 days, *O. niloticus* juveniles exposed to the highest concentration of Acer 35 EC showed lower values of IK-R compared to control ones regardless of the sex. Like in liver, neoplastic changes were not observed in the kidney. No significant difference was observed in the general kidney index between the different groups. All males showed normal tissue structure with moderate histological alterations (class II: $10 \leq \text{IK} \leq 25$). Females showed normal tissue structure with moderate histological alterations except the ones exposed to 0.0017 $\mu\text{L/L}$ of Acer 35 EC that showed pronounced alteration of liver tissue (class III: $26 \leq \text{IK} \leq 35$).

3.6.4. Gonad histopathology

Four and five types of alterations, in ovaries and in testis respectively, that had been classified as regressive changes (RC), were identified. Oocyte vacuolisation, detachment of basal membrane and connective tissue accumulation were highly present in 86.2, 30.2 and 43.4% of ovaries after 28 and 56 days. Atretic follicles were found in higher proportions (60–75%) in exposed fish compared to control ones (32.5%) after 56 days. In males,

vacuolisation of the testicular parenchyma, presence of immature cells in lobular lumen and fibrosis were present in all treatments regardless the exposure period. The prevalence of lobular disorganization was always higher in exposed fish (60%) compared to control ones (20%). The different alterations observed were found to be more prevalent regardless of the sex in fish exposed during 28 days (IT = 17.2, IO = 16.7) than in fish exposed during 56 days (IT = 14, IO = 10.38). No significant changes were observed in total ovaries and testis index among the treatments and/or the exposure time. Regarding ovarian and testis alterations, all the fish showed normal tissue structure with moderate histological alterations (class II: $10 \leq \text{IO/IT} \leq 25$).

3.7. IBR

In females, ovary (IO) and liver (IL) indexes, catalase (CAT) and complement (ACH50) activities are the biomarkers that showed a different response between the exposed females and the control group after 28 days of exposure (Fig. 7). After 56 days, liver index, testosterone (T), 11-ketotestosterone (11 KT) spleen superoxide anion production (NBT), Cholinesterase (ChE) activity in muscles and Glutathione peroxidase activity (GPx) are the biomarkers that presented an important variability between the exposed females and the control ones. In males, very few biomarkers (ChE in the brain and glutathione reductase GR) showed a difference between exposed and control fish after 28 days of exposure. After 56 days, glutathione reduced content (GSH) and CAT showed a response under exposure to high concentrations of Acer 35 EC. The IBR values were not different between the fish exposed to low and high concentration of Acer 35 EC regardless the sex and the exposure duration (Fig. 7). However, IBR values in both sexes were higher after 56 days (from 11.46 to 18.13) than after 28 days (range: 8.43–10.14) of exposure. They also were higher in females (IBR > 17) than in males (IBR < 13) after 56 days of exposure.

4. Discussion

In this study, the toxic effects of the binary insecticide Acer 35 EC at chronic concentrations for 28 and 56 days were assessed using a wide range of biochemical and histological responses in Nile tilapia juveniles. The results showed that Acer 35 EC affected the physiology of Nile tilapia juveniles by many ways. It disturbed the nervous system by inhibiting the activity of ChE in females' muscle exposed during 56 days to a dose equivalent to 1% LC50 of Acer 35 EC, representing only 10% of the concentrations found in water reservoirs in the cotton area of Northern Benin. It caused oxidative stress by increasing the GSH content in males exposed during 56

Table Table 5

Changes in circulatory (IK-C), inflammatory (IK-I), progressive (IK-P) and regressive (IK-R) alterations in kidney tissues of *O. niloticus* juveniles (females ♀ and males ♂) exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 $\mu\text{L/L}$ and A-0.017 $\mu\text{L/L}$) compared to a control group. Different capital letters (A, B) indicate significant differences between treatments and lower-case letters (a, b) indicate significant differences among the exposure time ($p < 0.05$).

	Exposure time	Treatments	IK-C	IK-I	IK-P	IK-R
♀	28 days	Control	3.92 \pm 0.47	2.15 \pm 0.52	2.77 \pm 0.43 ^a	17.00 \pm 1.65 ^{Aa}
		A-0.0017 $\mu\text{L/L}$	3.00 \pm 0.67	3.78 \pm 1.21	3.33 \pm 0.53 ^a	15.89 \pm 1.94 ^{ABa}
		A-0.017 $\mu\text{L/L}$	4.21 \pm 0.33	3.07 \pm 0.37	3.07 \pm 0.37 ^a	11.71 \pm 1.34 ^B
	56 days	Control	4.08 \pm 0.43	1.86 \pm 0.44 ^B	0.36 \pm 0.17 ^b	10.57 \pm 0.91 ^b
		A-0.0017 $\mu\text{L/L}$	4.25 \pm 0.57	3.83 \pm 0.76 ^{AB}	1.42 \pm 0.43 ^b	11.41 \pm 1.25 ^b
		A-0.017 $\mu\text{L/L}$	3.80 \pm 0.55	5.00 \pm 0.99 ^A	1.36 \pm 0.45 ^b	11.36 \pm 0.85
♂	28 days	Control	2.80 \pm 0.29	1.00 \pm 0.27	2.55 \pm 0.47 ^a	16.55 \pm 1.89 ^{Aa}
		A-0.0017 $\mu\text{L/L}$	3.46 \pm 0.48	1.62 \pm 0.64	3.31 \pm 0.36 ^a	12.77 \pm 1.39 ^{ABa}
		A-0.017 $\mu\text{L/L}$	3.42 \pm 0.40	1.54 \pm 0.33	3.23 \pm 0.46 ^a	11.38 \pm 0.98 ^B
	56 days	Control	3.38 \pm 0.49	2.21 \pm 0.54	0.64 \pm 0.31 ^b	9.14 \pm 1.16 ^b
		A-0.0017 $\mu\text{L/L}$	4.62 \pm 0.43	2.21 \pm 0.57	1.07 \pm 0.35 ^b	9.50 \pm 0.97 ^b
		A-0.017 $\mu\text{L/L}$	4.08 \pm 0.29	1.62 \pm 0.4	1.77 \pm 0.41 ^b	11.46 \pm 1.27

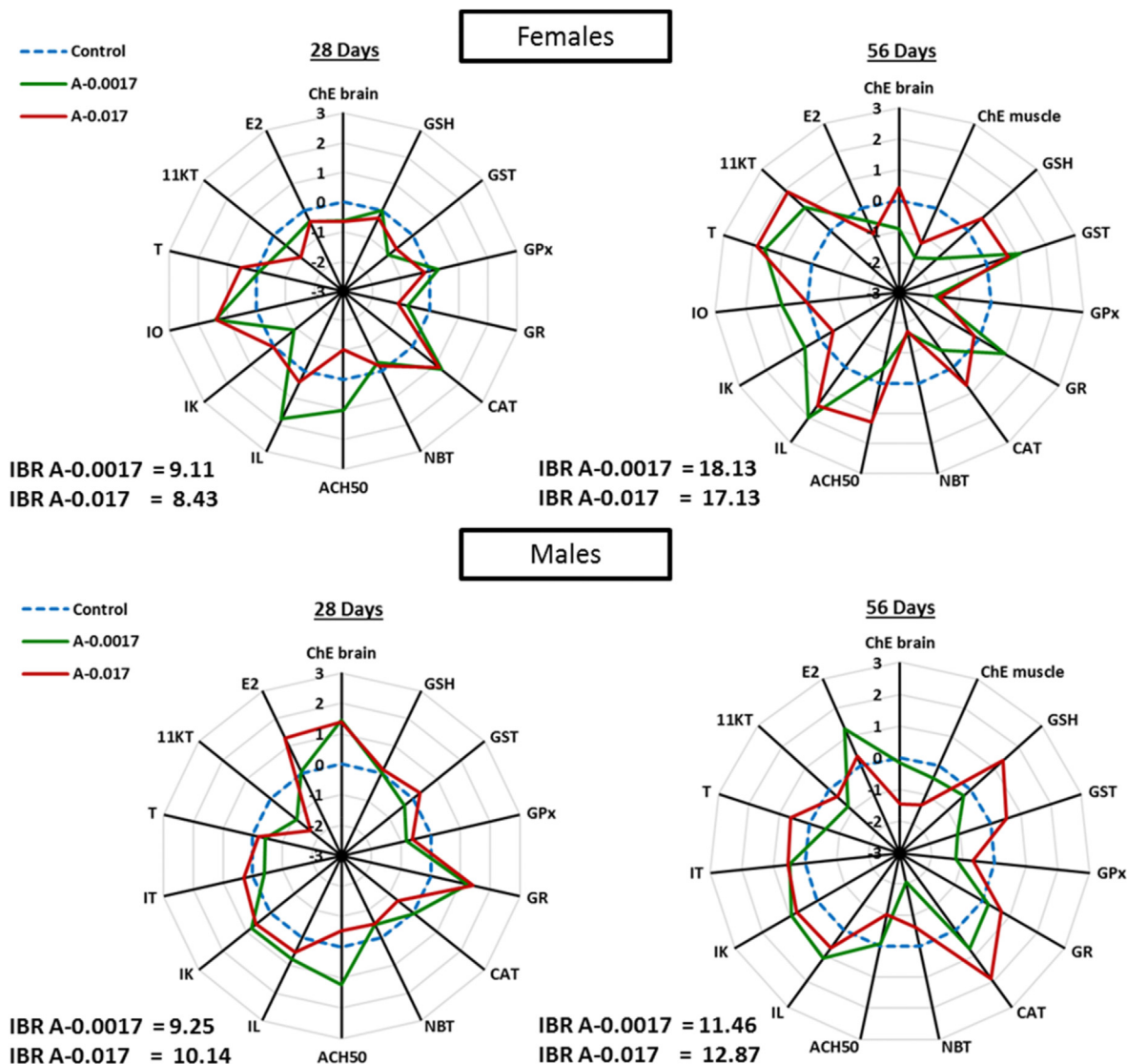


Fig. A.7. Integrated biomarker response index (IBR) of *O. niloticus* juveniles (females and males) exposed during 28 or 56 days to two sublethal concentrations of Acer 35 EC (A-0.0017 $\mu\text{L/L}$ and A-0.017 $\mu\text{L/L}$) compared to a control group.

days to 10% LC50 of Acer 35 EC the dose equivalent to the environmental concentration. It weakened the immune system by inhibiting the production of superoxide anions (in both sexes after 56 days of exposure at the higher concentration but only in males exposed to the lower one) and complement activity in females exposed to the lower concentration at D28. It disrupted the reproductive system by stimulating the testosterone production in females exposed to the higher concentration, decreasing the number of advanced vitellogenic oocytes and reducing the oocyte diameter after 56 days of exposure. Histopathology of the liver and kidney of exposed fish also showed alterations.

4.1. Pesticide residues in muscles of Nile tilapia exposed to Acer 35 EC

The analyses of acetamiprid residues in Nile tilapia showed that fish sampled after 56 days of exposure contained less residues in their muscles. A bioconcentration factor (BCF) was calculated on the basis of the two concentrations measured in water at 72 h intervals (concentration measured in water just after water renewal

and those measured in water 72 h later). The range of BCF calculated in fish sampled at 28 days of exposure was between 29.45 and 35.41 and between 8.98 and 11 in fish exposed to 1% and 10% Acer 35 EC LC50 of respectively. In fish sampled at 56 days the BCF range was between 7.77 and 9.34 and between 3.21 and 4.20 in fish exposed to 1% and 10% of LC50 of Acer 35 EC respectively. This indicates that acetamiprid was eliminated by fish. Indeed, acetamiprid is highly soluble in water and not very lipophilic ($\log K_{ow} = 0.8$), thus having a low bioconcentration potential (PHSS, 2014; Nakayama et al., 1997). In addition, studies showed that rats exposed to acetamiprid eliminated 90% of the residues accumulated in their body through the gastro-intestinal tract and excreted 53–65% with urine, already 1 h after administration (WHO/FAO, 2011). Accordingly, acetamiprid appears to be rapidly excreted, which limited its concentration in tissues and reduces its toxicity (PHSS, 2014).

For lambda-cyhalothrin, our results showed that the residues found in the muscle of Nile tilapia were higher in fish exposed to the higher concentration (10% of Acer 35 EC LC50) especially in fish sampled after 56 days of exposure. Results also showed that the fish

exposed to the lower concentration (1% of Acer 35 EC LC50) and sampled after 56 days of exposure had slightly less residues than those sampled at 28 days of exposure. This result shows that lambda-cyhalothrin is not as easily eliminated as acetamiprid. Indeed, lambda-cyhalothrin is a very lipophilic compound which can readily accumulate in tissues rich in lipids (Soderlund et al., 2002), thus having a high bioconcentration potential (Maund et al., 2012). This is in agreement with our results which showed that the range of BCF calculated (on the basis of the two concentrations measured in water at 72 h intervals) for lambda-cyhalothrin in fish sampled at 28 days of exposure was between 29.16 and 1167.63 and between 17.67 and 341.209 in fish exposed to 1% and 10% of Acer 35 EC LC50 respectively. In fish sampled at 56 days the BCF range was between 19.92 and 797.68 and between 33.94 and 655 in fish exposed to 1% and 10% of Acer 35 EC LC50 respectively. These results confirmed the bioconcentration nature of lambda-cyhalothrin and showed that this compound is eliminated very little during an exposure. This is in agreement with studies carried out by ARLA (2017) in carp which showed that lambda-cyhalothrin was eliminated after exposure (78% of the accumulated residues were eliminated after 28 days of depuration).

4.2. Biomarkers of neurotoxicity

Cholinesterases (ChE), carboxylic ester hydrolases, are a family of enzymes that play an essential role in neural and motor functions (Alves et al., 2015; Wilson, 2010). These enzymes are responsible for the removal of the neurotransmitter acetylcholine (ACh) from the synaptic cleft by hydrolysis (Habig and DiGiulio, 1991). In this study, our results showed that Acer 35 EC reduced ChE activity in the muscle of females exposed for 56 days to the lower concentration (1% of LC50). Previous studies have reported that lambda-cyhalothrin as well as neonicotinoid insecticides (in which acetamiprid belongs) can reduce AChE activity (Bibi et al., 2014; Kumar et al., 2009; Azevedo-Pereira et al., 2011). The inhibition of cholinesterase activity observed in our study could be attributed to both compounds lambda-cyhalothrin and acetamiprid. This inhibition could lead to an accumulation of ACh, inducing a continuous and excessive stimulation of nerve/muscle fibres, which results in tetany, paralysis and eventually death. Disruptions in cholinesterase activity in general and AChE activity in particular can also reduce cell metabolism, induce cell membrane deformations, disrupt metabolic and nerve activity (Suresh et al., 1992), cause ionic reflux and differential membrane permeability (Tolosa et al., 1996) and affect feeding, escape and reproductive behaviour (Bretaud et al., 2000). The difference of cholinesterase activity in brain was also observed in the two exposure periods in females. This could be due to differences in the size of Nile tilapia juveniles (Flammarion et al., 2002). Indeed, previous studies indicate that baseline ChE activities in the brain of several species of fish differ with body size and age. For example, Chandrasekara and Pathiratne (2007) found that small Nile tilapia (fry, 3–4 cm) had significantly higher brain AChE activity than larger juvenile fish (fingerlings, 6–8 cm; subadults, 10–12 cm).

4.3. Biomarkers of oxidative stress

According to Livingstone (2001), oxidative stress caused by pesticides in aquatic organisms leads to the production of ROS and the modification of antioxidant enzymes.

Excessive ROS production and its harmful effects are controlled by cellular antioxidant systems (Dorts et al., 2012). In general, organisms under stress conditions use antioxidant enzymes to adapt to environmental stress. In our study, among the antioxidant enzymes analyzed, only GSH showed a difference due to the exposure

of fish to the insecticide Acer 35 EC. As the most abundant low molecular weight thiol in the cell, GSH is considered to play a central role in protecting cells from damage caused by oxidants (Woods et al., 1999). The increase in GSH content measured in male tilapia exposed to the higher concentration of Acer 35 EC after 56 days of exposure compared to males exposed to the lower concentration was also observed by Piner and Üner (2012) in Nile tilapia liver exposed to lambda-cyhalothrin. Shukla et al. (2017) and Vieira et al. (2018) also observed an increase in GSH activity in the brain of zebrafish and Sábalo *Prochilodus lineatus* exposed to the neonicotinoid imidacloprid. The apparent increase in GSH levels with a concomitant increase in GST activity (although not significant) suggests an adaptive and protective role for this biomolecule against oxidative stress induced by Acer 35 EC. Although trends were observed between treatments, the high individual differences in measured antioxidant enzymes probably explain the insignificant effects recorded in this study.

4.4. Biomarkers of immunotoxicity

The immune system is a physiological function essential to maintain the health of the individuals. The sensitivity of the immune system to environmental disturbances has been extensively documented, making these components potential biomarkers for xenobiotic contamination (Diaz-Resendiz et al., 2015; Zelikoff et al., 2000). Phagocytosis, mainly involving macrophages and neutrophils, is one of the most important immune processes during the activation of the immune system in response to the detection of pathogens (Magnadóttir, 2006). The inhibition of macrophage and neutrophil activities measured in the spleen of fish exposed to Acer 35 EC even at low doses in this study is similar to the inhibition of macrophage activity found in rohu exposed to alpha-permethrin (Nayak et al., 2004). Similarly, inhibition of phagocytic activity has been demonstrated in Nile tilapia following exposure to sub-lethal concentrations of diazinon (Giron-Pérez et al., 2007) and chlorpyrifos (Diaz-Resendiz and Giron-Pérez, 2014; Giron-Pérez et al., 2006). Inhibition of phagocytic activity may result from a reduction in phagocytic cell count, as suggested by the decrease in neutrophils observed in African catfish *Clarias gariepinus* after exposure to lambda-cyhalothrin (Ogueji and Ibrahim, 2012). Thus, the decrease in phagocytic activity caused by Acer 35 EC may lead to a reduction in individual resistance to infection, as shown in rohu following exposure to alpha-permethrin (Nayak et al., 2004). Among the components of the immune system, the complement system plays a central role in the innate and adaptive response by intervening in the inflammatory process, cell lysis, leukocyte chemoattraction, phagocytosis and modulation of the immune response (Boshra et al., 2006). In this study, a decrease in the complement activity was observed in female individuals exposed to the higher concentration of Acer 35 EC insecticide compared to individuals exposed to the lower one after 28 days of exposure. Similarly, in females exposed to the lower concentration, there was a decrease in complement activity measured at 28 and 56 days of exposure. This could be explained by the immunosuppressive power of lambda-cyhalothrin observed by Yekeen et al. (2013) and Fawole and Yekeen (2014). Mondal et al. (2009) also demonstrated the immunosuppressive power of acetamiprid in female rats, which would induce a decrease in the level of immunoglobulin and lymphoid cell number at the germinal centres of the spleen, significantly reducing the cellular and humoral immune response (Mondal et al., 2014). These results once again indicate the sensitivity of Nile tilapia females to Acer 35 EC insecticide.

4.5. Reprotoxicity biomarkers

The results of our study show that Acer 35 EC significantly stimulates testosterone production in females exposed for 56 days, beginning already after 28 days of exposure. These results are consistent with those of Lewis et al. (2012) in juveniles and adult males of the freshwater amphipod *Gammarus pseudolimnaeus* exposed to bisphenol. On the contrary, a decrease in testosterone concentration was reported by Agbohessi et al. (2015a) in African catfish exposed to endosulfan suspected endocrine disrupters, by Oruç (2010) in Nile tilapia exposed to chlorpyrifos and by Saravanan et al. (2009) in frogs exposed to lambda-cyhalothrin. The increase in testosterone concentration observed in this study may be due to a disruption in the conversion of testosterone by granulosa cells to 17 β -estradiol (E2) via the action of the aromatase enzyme (P450arom) or by inhibiting the activity of the aromatase enzyme (Ankley et al., 2005; Monod et al., 1993). This is supported by the non-significant decrease in E2 concentration observed in the same exposed females. These results may reflect a trend towards masculinization of females following exposure to Acer 35 EC insecticide, especially since a trend of increasing 11 KT concentration has been observed in exposed females. The determination of aromatase activity in the gonads would have been useful for confirming these results.

The lower number of advanced vitellogenic oocytes and an increasing trend of the young stages (protoplasmic oocyte and cortical alveoli stages) suggest a delayed maturation in females exposed to Acer 35 EC insecticide. Agbohessi et al. (2015a) also observed this effect in African catfish exposed to Tihan and Chatterjee et al. (1997) in fossil catfish exposed to carbofuran, both suspected endocrine disrupters. The oocyte growth retardation mentioned above is confirmed by the significant decrease in oocyte diameters measured in females exposed to Acer 35 EC.

4.6. Histopathology of the liver, kidney and gonads

In liver, significant differences were found for histopathological effects related to inflammatory responses and regressive changes. Our results showed that the value of the inflammatory response index (IL-I), found in males exposed to the lower concentration of Acer 35 EC is lower than those found in the control and the group exposed to the higher concentration. However, inflammatory responses include alterations such as presence of melano macrophages centres, granuloma and infiltration of immune cells in hepatic parenchyma. This low value observed is due to the low occurrence and low prevalence observed for these alterations in these males (20% prevalence for immune cell infiltration and 0% for granuloma). In contrast, females exposed to Acer 35 EC and sampled at D56 presented a high value of the IL-I index compared to control group. This is partially due to a higher prevalence of the infiltration of immune cells in hepatic parenchyma observed in exposed fish (45.45 and 71.43% respectively for females exposed to 1 and 10% of the LC50) compared to controls (7.14%). The highest values of the inflammatory index observed in exposed females could reflect a more intense immune activity. Indeed, the presence of a significant number of immune cells would indicate that fish immune defense mechanisms are triggered to counteract the deleterious effects of Acer 35 EC (Coimbra et al., 2007). However, our results on the activity of complement and macrophages and neutrophils showed a decrease in the activity of the immune system. Nevertheless, during the study of the immune response to a stressor, it is not uncommon to see opposite effects between the organs studied.

In addition, females exposed to the lower concentration showed a high value of their indices of regressive changes. This could be explained by the high prevalence observed at the level of tissue structure (64.29%) in these females compared to the other groups (33.33 for the control group and 46.67% for the females exposed to the higher concentration). A difference in prevalence was also observed at the level of nuclear pleomorphism between exposed and control females. These different alterations can lead to a dysfunction of hepatic activities such as nutrient absorption, metabolism of carbohydrates, vitamins, lipids or detoxification of xenobiotics. Our results also showed that the total histopathological index (IT) was higher in exposed females compared to the control. More specifically, the index calculated for females exposed to the lower concentration was between 26 and 35 corresponding to class 3, i.e. pronounced alterations in the liver.

In the kidney, Acer 35 EC caused in females exposed to the higher concentration an increased inflammatory index value compared to that of the control group at D56. In addition, in both sexes, Acer 35 EC led to a decrease of the regressive index in individuals exposed to the higher concentration compared to control groups at D28. However, for the total histopathological index (IT), no differences were observed between the control groups and the exposed ones. The main histological alterations, namely renal tubular lumen occlusion, the presence of melanoma macrophages centres, and the observation of regenerating and degenerating renal tubules corresponding to inflammatory responses and regressive changes, have often been observed in individuals exposed to Acer 35 EC. Similar observations have been made in mrigal carp *Cirrhinus mrigala* exposed to lambda-cyhalothrin (Velmurugan et al., 2007) or in Nile tilapia exposed to cypermethrin (Korkmaz et al., 2009). Following a damage to the kidney, usually a regeneration process starts with an increase in the apparition of new nephrons and, consequently, new tubules (Cormier et al., 1995). Consequently, the presence of regenerating tubules has been suggested as a biomarker of exposure to xenobiotics (Cormier et al., 1995). However, the development of new nephrons also occurs throughout the life of the fish and is particularly frequent in juveniles (Reimschuessel, 2001). Such alteration can disrupt the flow of filtrate and delay the process of reabsorption and secretion into the tubule (Silva and Martinez, 2007). Thus, it appears that Acer 35 EC has little effect on kidney histology.

Vacuolation, connective tissue accumulation, atresia and basement membrane detachment of oocytes, all classified as regressive changes, were the main alterations observed in females exposed to Acer 35 EC. In this experiment, among the high significance alterations of factor (3), the atresic pre-ovulatory follicles identified at 56 days after exposure, are the most frequently observed alterations with higher values occurring in females exposed to Acer 35 EC. An increase in atresia was also observed in African catfish exposed to endosulfan (Agbohessi et al., 2015a), in Nile tilapia exposed to deltamethrin (Bayar et al., 2014), in Mozambique tilapia exposed to DDT (Mlambo et al., 2009), and in goldfish exposed to atrazine (Spanò et al., 2004). Several authors have shown ovarian regression in fish treated with estrogenic pesticides (Pieterse et al., 2010; Mlambo et al., 2009; Spanò et al., 2004). This suggests a greater impact of Acer 35 EC at low doses. The average histopathological index is between 10 and 25 (class 2) for all treatments, regardless of the period, which corresponds to normal tissue with moderate histological changes.

Among the alterations observed in male gonads in our study, lobule disorganization was the one with the more frequent occurrence in individuals exposed to Acer 35 EC insecticide (60% in exposed fish compared to 20% in control ones). Agbohessi et al.

(2015b) also reported lobule disorganization in Guinea tilapia *Tilapia guineensis* and African catfish living in a cotton basin heavily impacted by pesticides. Similar effects were described for *Cichlasoma dimerus* exposed to endosulfan (Da Cuna et al., 2013) and in Mozambique tilapia exposed to DDT (Mlambo et al., 2009).

4.7. Integrated biomarker response (IBR)

In this study, the calculated IBR values showed that Acer 35 EC insecticide affects females (9.11, 8.43 and 18.13, 17.13 for females exposed to 1 and 10% LC50 of Acer 35 EC respectively at 28 and 56 days of exposure) more than males (9.25, 10.14 and 11.46, 12.87 for males exposed to 1 and 10% LC50 of Acer 35 EC respectively at 28 and 56 days of exposure). In addition, the IBR reveals that, even at the lower concentrations, Acer 35 EC impacted the exposed fish (values close to the IBR for the two concentrations tested at D28 and D56 in males and females). Also, a longer exposure time increases the effects of Acer 35 EC. The high number of biomarkers used in this study follows the recommendations of Beliaeff and Burgeot (2002), who pointed out that the selection of a suitable biomarker battery can avoid false negative responses obtained with a single biomarker and allow information to be summarized as a multivariate data set. According to Broeg and Lehtonen (2006), because of its mathematical basis, the IBR becomes more robust as the number of biomarkers increases. The number of biomarkers included in the IBR calculation plays an important role in the "relative weight" of each biomarker in the final value of the index. When the set is relatively large, for example 6–8, the weight of a factor is significantly reduced compared to the case where 3–4 biomarkers only are used (Damiens et al., 2007).

5. Conclusion

This study is the first to report the effects of the binary insecticide Acer 35 EC, composed of the active ingredients lambda-cyhalothrin and acetamiprid, on different vital functions of Nile tilapia. The results obtained in this study revealed that, even at low concentrations (*i.e.* 1% of the LC50-96 h and about 10% of the environmental concentrations of the compounds of Acer 35 EC, found in the water reservoirs of the cotton area of Northern Benin, according to Zoumenou (2019) and Zoumenou et al. (2019)), the components of Acer 35 EC affect fish exposed to it. Similarly, all functions of exposed fish were affected in one way or another by Acer 35 EC, particularly at the concentration of the compounds Acetamiprid and Lambda-Cyhalothrin (10% of the LC50-96 h) in the range of those found in the Northern Benin water reservoirs. The results of the calculated biomarker indices also revealed that the longer the exposure period, the greater the effects. Individual differences in fish, particularly in Nile tilapia, were at the root of the insignificant differences observed in some endpoints despite the strong downward or upward trends observed. This study ultimately showed that Acer 35 EC insecticide has a negative impact like most insecticides on the survival of aquatic organisms in general and fish in particular. This experimentation has also highlighted the interest of IBR to depict the overall effect of contaminants on organisms. This indicator could be of particular interest for field studies, provided that pesticide contamination of water is measured in parallel (by regular sampling and/or the use of passive integrative samplers).

Credit authors statement

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Conceptualization, Supervision, Writing - review & editing, Imen Ben Ammar: Formal analysis, Writing - original draft, Writing - review & editing, Loïc François: Investigation, Noëlle Oreins: Investigation, Olivier Palluel: Investigation, Syaghalirwa N. M. Mandiki: Supervision, Writing - review & editing, Thierry Jauniaux: Validation, Resources, Jean-Marc Porcher: Investigation, Marie-Louise Scippo: Supervision, Investigation, Patrick Kestemont: Conceptualization, Funding acquisition, Supervision, Writing - review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The present study was supported by ARES-CCD (Académie de Recherche et d'Enseignement Supérieur-Commission de la Coopérative au Développement of Belgium) as part of the AQUATOX Bénin project. The authors are grateful to the Cell and Tissue Laboratory of the University of Namur (Belgium) and particularly to Daniel VAN VLAENDER and Benoît BALAU for their invaluable help in histological analyses. The authors express their thanks to Christelle Robert, Nathalie Gillard and Mathieu Dubois from BU laboratory, CER Marloie (Belgium) for the determination of the acetamiprid and lambda-cyhalothrin content in water and tissues. They also are thankful to the master degree students from the Department of Science and Technique of Animal Production of the University of Parakou (Benin) who participated in sampling and some analysis. Thanks to Carolin Mayer for the English editing of the manuscript.

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