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Western mosquitofish as a bioindicator of exposure to organochlorine compounds

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Abstract

The evaluation of the alochthonous and cosmopolitan mosquitofish species *Gambusia affinis* suitability as a bioindicator species and the induction of its liver cytochrome P450-dependent mixed function oxygenase (MFO), measured as the 7-ethoxyresorfin-*O*-deethylase (EROD) activity, as well as changes in DNA integrity, measured by the Fast Micromethod®, for the monitoring of organochlorine fresh water pesticide contamination, were the main aims of the study. The test mosquitofish were exposed under laboratory conditions to several doses (0.1, 10 and 100 $\mu g l^{-1}$) of lindane in experimental basins for up to 7 days, and a subsequent field study was carried out at five natural ponds in the south-western Istrian peninsula, Croatia, where up to 10 fish were collected from each pond. Results obtained during the studies showed positive correlations between the measured biomarkers in *G. affinis* liver (EROD activity and DNA integrity status) and lindane (laboratory experiment) or persistent organochlorine pollutant amounts in natural pond sediments (field study). The clear dose–responses of EROD activity and DNA integrity deterioration in *G. affinis* were recorded after exposure to 0.1–10 μg/l lindane and 96 h exposure to lindane, respectively. The results indicate that the mosquitofish *G. affinis*, due to its biological–ecological characteristics and the biomarker dose–response, is suitable for the monitoring of fresh water organochlorine pesticide contamination in general and lindane in particular.

Keywords: Biomarker; DNA integrity; EROD activity; *Gambusia affinis*; Lindane

1. Introduction

Several decades ago, the use of organochlorine chemicals markedly decreased due to their persistence in the environment and associated human health and environmental negative effects including the poisoning of non-targeted species (Bounias, 2003; Lopez et al., 2005). While tremendous efforts have been made in many countries to move towards the use of less toxic, less persistent, and less bioacumulative pesticides, organochlorine pesticides (OCPs) are still widely used in public health and veterinary science worldwide, particularly in countries in tropical regions. However, some of the OCPs are still used in agriculture to protect crops and are recommended by national health organizations to help avoid the spreading of harmful pests that cause human diseases such as malaria. According to their toxicity, persistence in environment, tendency for bioaccumulation, characteristic long-range atmospheric transport and adverse environmental and human health effects, several OCPs, including lindane, have been identified as persistent organic pollutants (UN-ECE, 1998). The identification of persistent organic pollutants, and in particular hazardous priority substances, such us OCPs, and their effects on biota, has been the main goal in numerous international environmental monitoring campaigns (Vallack et al., 1998).

Biomarkers are defined as physiological, biochemical, and histological changes that serve as indicators of exposure and/or the effects of xenobiotics at the sub-organism and organism level (Rand, 1995), or as a change in a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals (Peakall and Shugart, 1993). The changes at

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the molecular/biochemical level show short-term responses to xenobiotics, and although they are typically of low ecological relevance, they may allow the estimation of changes direction and the prediction of harmful deleterious effects in the whole ecosystem. While biochemical and molecular markers may serve as early indicators of pollution, they clearly provide direct evidence of exposure to toxic agents of same (Adams et al., 1989). Although they are typically of low ecological relevance the induction of EROD activity and decline of DNA integrity, may be used for the determination of specific xenobiotics and genotoxic exposure and thereby allowing the estimation of the direction of potentially harmful changes in the entire ecosystems.

The cytochrome P-450 (CYP) enzyme system is the major route in which a living organism can convert lipophilic xenobiotics into a water-soluble product (Phase I), thus making them suitable for elimination from the body. The Phase I reactions do not always produce less toxic and hydrophilic compounds and the Phase II enzyme-derived methylations, acylations, and conjugation reactions of those metabolites makes them water soluble and suitable for elimination from the body. Enzymes belonging to the cytochrome P-450 (CYP) 1A subfamily are induced by polycyclic aromatic hydrocarbons (such as benzo[*a*]pyrene B[*a*]P), polyhalogenated biphenyls, polychlorinated dioxins, and dibenzofurans and have been investigated extensively and validated as biomarkers of exposure for aquatic pollution monitoring in several fish species and their tissues (Williams et al., 1998).

DNA integrity, measured as strand brakes and alkali-labile sites may be used as an early signal of genotoxic contamination of the environment, although they do not specifically identify the agents of concerns (Mitchelmore and Chipman, 1998; Shugart, 2000). Over the past decade, several DNA damage/integrity techniques such as alkaline unwinding, alkaline elution, Comet assay and Fast Micromethod® have been used in environmental monitoring studies (Shugart, 2000, Jakšić et al., 2005). The Fast Micromethod® has been routinely employed and implemented in our laboratory, and due to its simplicity, accuracy and low time consumption represents the method of choice for DNA damage estimation (Batel et al., 1999; Bihari et al., 2002; Jakšić and Batel, 2003).

Mosquitofish, *Gambusia affinis* (Actinopterygii: Cyprinodontiformes: Poeciliidae) (Baird and Girardi, 1853), have been introduced all over the world from North and Central America at the beginning of 19th century in order to control the populations of mosquito larvae and prevent malaria. Their aggressive nature and high fecundity have had a major biological impact on many of the ecosystems into which this species was introduced. This species has a high tolerance to changes in temperature and salinity, which enables it to adapt to different environmental conditions and ecosystems. Due to these characteristics, its widespread occurrence in different regions, easy collection and laboratory cultivation, *Gambusia* sp. are widely used as bioindicator species (Tolar et al., 2001; Kavitha and Rao, 2007). The major drawbacks of *G. affinis* are their highly predaceous habits, they shredding other fish fins, alter zooplankton, insect and crustacean communities, and the relatively low sensitivity to the exposure with contaminants.

One of the most prominent OCPs, lindane (γ-HCH, gamma-1,2,3,4,5,6-hexachlorocyclohexane), although is forbidden in Croatia from July 2001, is still in use on Croatian islands and littoral areas for the prevention of ectoparasites such as fleas and lice on goats and sheep, and may introduced to ponds upon watering of the animals. Lindane slowly biodegrades in aerobic media and rapidly degrades under anaerobic conditions. It is very stable in both, fresh and salt-water environments and is resistant to photodegradation (Kidd and James, 1991). It usually disappears from the water by secondary mechanisms such as adsorption on sediment, biological breakdown by microflora and fauna, and adsorption by fish through gills, skin and food (Ulman, 1972), where it is stored in the body fat and is directly proportional to its concentration in the feedstock. Ferrando et al. (1992) reported a 15% lindane dissipation rate, after 24 h, in the natural waters of Albufera Lake, Spain, and in experimental tap water. Their results confirmed the first order of the lindane degradation reaction in both media and they calculated the half lives of lindane in natural and experimental water as 64.98 and 69.41 h, respectively. In a 96-h LC_{50} *and* time-to-death test, the effect of lindane on *G. affinis,* given by measured LC_{50} and LC_{70} values, were 263 and 290 μg lindane l^{-1}, respectively (Walton et al., 1997; Allenbach et al., 1999). The mortality was significantly greater during light than dark intervals (Walton et al., 1997). Furthermore, Sullivan and Lydy (1999) described the significant differences among survival curves of different *G. affinis* genotyping classes (phosphoglucose and malic enzyme) in a 96-h acute exposure to lindane. That research enabled us to create the time-dose pattern of *G. affinis* exposure to lindane basin water, to measure the response of a short-term biomarker, liver CYP1A1 modulation, and of a biomarker of exposure, DNA integrity changes, in order to evaluate the bioindicator species and the selected biomarker as a potentially useful tool in fresh water organochlorine contamination monitoring.

The several previous studies showed that biomarkers such as acetylcholinesterase, catalase and glutathione-*S*-transferase activity in the digestive gland of marine molluscs are sensitive to lindane exposure (Khessiba et al., 2005; Roméo et al., 2006). The significant increase of rat hepatic lipid peroxidation and DNA damage and time dependent effects of lindane and other OCPs were confirmed by Hassoun et al. (1993). However, the relatively low levels of DNA damage and no statistical differences between control and lindane exposed *Mytilus galloprovincialis*, as well as Zebra and Blue mussels collected along the pollution (polyaromatic hydrocarbons—PAHs, polychlorinated biphenyls—PCBs, lindane, heavy metals) gradient in

the Seine estuary (Raftopoulou et al., 2006; Rocher et al., 2006). Nevertheless, we prefer to investigate biomarkers of exposure to genotoxic contaminants and CYP1A-dependent enzymes in parallel in *G. affinis*, and the similar approach was provided by measurement of three different biomarkers (Ache, EROD activity and DNA strand breaks) in Zebra mussel from Lake Maggiore, Italy (Binelli et al., 2007).

The presented study reports on results of modulated CYP 1A activity (cytochrome P450 oxidase 1A subfamily member), measured as a 7-ethoxyresorufin-*O*-deethylase (EROD) activity and changes of DNA integrity in *G. affinis* liver, presented as a strand scission factors (SSFs) values, after laboratory exposure of fish to lindane in experimental basins. The same endpoints were measured in a subsequent study with fish collected at natural ponds in the nearby areas of different human activity. The aims of this study were to investigate the suitability of mosquitofish *G. affinis*, as a bioindicator species, and their liver EROD activity and DNA integrity, as molecular biomarkers, for the monitoring of organochlorine fresh water pesticide contamination due to their biological–ecological characteristics and the biomarker dose–response.

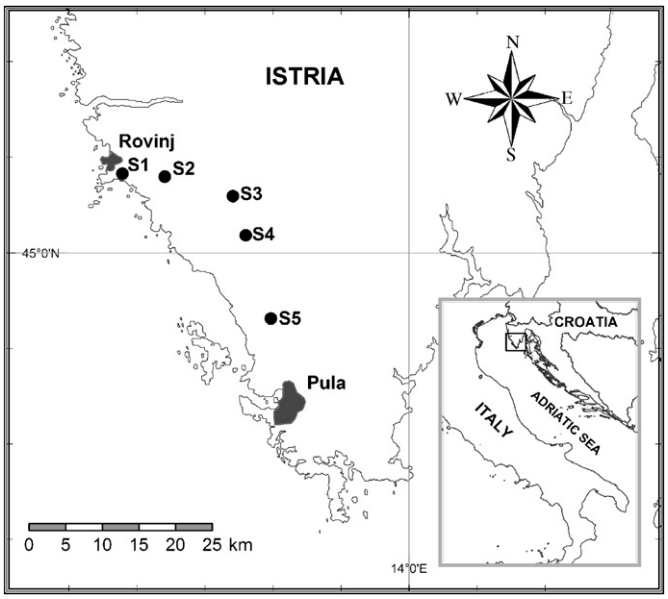

Fig. 1. *Gambusia affinis* and sediment sampling ponds in south-western Istrian peninsula, Croatia: S1—Španidiga, S2—Kukuletovica, S3—Bale, S4—Krnjaloža, and S5—Galižana.

2. Materials and methods

2.1. Test organism and study area

The study area extends over the south-western part of the Istrian peninsula, Croatia, between the two largest Istrian towns of Pula and Rovinj, with 60 000 and 20 000 inhabitants, respectively. All of the sampling sites are located near settlements, roads or close to agricultural areas. In this approximately 30 km long sampling area, where lindane is still widely used in veterinary medicine as an ectoparasitcide and in public health as an insecticide and rodenocide, we chose those sampling locations (ponds) which, except for the Bale pond, periodically serve as sheep and goat watering places Western mosquitofish, *G. affinis* were collected at five natural ponds: S1—Španidiga, S2—Kukuletovica, S3—Bale, S4—Krnjaloža and S5—Galižana (Fig. 1). The ponds geographic positions and brief descriptions s are given in Table 1. The sampled fish total body lengths were approximately 20–25 and 35–45 mm for male and female fish, respectively. All the sampled fish appeared normal and in good condition, and actively swimming in the ponds. The pH and temperature of each ponds water, as well as the amount of polychlorinated biphenyls and OCPs in their sediments are presented in Table 2. Those data were provided by the Public Health Institute of the Istrian Region, Pula, Croatia and were measured by gas liquid chromatography (UNEP/IOC/IAEA, 1993, 1995). This official IAEA method deals with the extraction of PCBs and OCPs by *n*-hexane with a two-step purging of the extracted compounds from the Fluorisil column by *n*-hexane and benzene, followed by gas chromatography separation on a SPB-5 column and detection by an electron capture detector. In general, the microbiological, physical and chemical indices of the health status of springs in the Istrian peninsula are quite good. The periodic laboratory tests of Istrian pond water and sediment have not confirmed the occurrence of xenobiotics that could affect CYP-1A dependent enzyme activities and DNA integrity, in the Bale pond (S3) (personal communication). The levels of those xenobiotics are very low and usually below the detection limit. The Bale pond (S3) is at a higher elevation above sea level than the other investigated ponds and has much smaller catchments area from which water may flow into the pond. Moreover, due to a consistent water level, elevated and spring-fed nature and stony bottom character the Bale pond (S3) was choose as a source of the water and test fish in our laboratory exposure test study as well as the reference sampling site in our field research. The fish from Bale pond (S3) were transferred to the laboratory, separated into two different adaptation basins, each filed with 50 l Bale pond water (25 °C, pH 6.5), for an adaptation period of 3 weeks. The fish were fed by commercial aquaria fish dry food once per day, which was discontinued during the test period.

2.2. Experimental design

2.2.1. Laboratory experiment—Lindane exposure

In this study, we used the commercial grade lindane, Gamacid® T-50 (Pliva d.d., Croatia), containing 500 g l^{-1} of active ingredient. The stock solutions, 5 mg ml^{-1} and 50 μg ml^{-1}, were prepared by dilution of commercial grade lindane in distilled water. To avoid direct contact of any fish to high doses of lindane the aliquots of stock solution were diluted in about 500 ml of Bale pond (S3) water, uniformly disposed, and gently mixed with the water in experimental basins. Twenty adult male and female fish, collected at the natural Bale pond (S3), were exposed in experimental basins with Bale pond water and different concentrations of lindane (0.1, 10, 100 μg l^{-1}) for a period of up to 7 days. Control fish were kept at the same Bale pond (S3) with no addition of lindane. The five male and female fish from each of eight experimental basins were harvested after 24, 96, and 168 h, and the livers were stored at cryotubes at −80 °C.

2.2.2. Field study

The livers from 10 adult male and female western mosquitofish, collected at five natural ponds (S1—Španidiga, S2—Kukuletovica, S3—Bale, S4—Krnjaloža, and S5—Galižana) in the south-western Istrian peninsula, Croatia, (Fig. 1) were taken immediately after collection and stored at cryotubes in liquid nitrogen.

2.3. Sample preparation

2.3.1. Microsomes preparation

Dissected fish livers, avoiding rupturing the gall bladder which may contain mixed function oxygenase (MFO) inhibitors, were weighed, washed with 150 mM KCl, and homogenized in a solution of 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 250 mM sucrose (pH 7.4) in a ratio of 1:5 (w:v) in Potter–Elvejham homogenizer.

Table 1
Sampling sites, codes, geographic positions and brief descriptions of the pond sampling sites

Sampling site	Code	Latitude λ	Longitude φ	Description
Kukuletovica	S1	45°04′N	13°41′E	Seasonally variable water level, 50 m^2, 1.5 m depth, mud bottom, adjacent to a main road and settlement
Španidiga	S2	45°03′N	13°43′E	Seasonally variable water level, 70 m^2, 0.8 m depth, mud bottom, adjacent to a road and quarry
Bale	S3	45°02′N	13°48′E	Annually consistent water level, 100 m^2, 3.5 m depth, stony bottom, elevated pond, spring-fed
Krnjaloža	S4	45°01′N	13°48′E	Annually consistent water level, 25 m^2, 1 m depth, mud bottom, agricultural area
Galižana	S5	44°56′N	13°52′E	Annually consistent water level, 75 m^2, 1 m depth, mud bottom, adjacent to settlement

Crude homogenates were centrifuged at 9000*g* for 15 min at 4 °C, and resultant supernatants (S9 fraction), without lipid phase, were transferred to ultracentrifuge tubes and recentrifuged at 100 000*g* for 90 min at 4 °C in a Sorvall OTD-COMBI ultracentrifuge. The precipitated microsomes were resuspended in 1 ml of 20% glycerol in homogenization buffer and separated into aliquots prior storage at −80 °C. The protein amounts were determined by Folin & Ciocaltheu's phenol reagent (Lowry et al., 1951).

2.3.2. Tissue homogenization for DNA integrity measurement

About 100 mg of fish livers were homogenized with 2 ml of 10% DMSO (dimethyl sulfoxide) solution in TE buffer (1 mM EDTA, ethylenediaminetetraacetic acid; 10 mM Tris–HCl, pH 7.4) under liquid nitrogen in a mortar with a pestle pre-cooled with liquid nitrogen. The pellets were collected in test tubes and stored at −80 °C. The amounts of DNA in dissolved sample aliquots were determined using YOYO®-1-iodide (Molecular Probes Inc., Eugene, OR, USA). Briefly, the 50 µl 4×10^{-7} M YOYO®-1-iodide in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) was added to 50 µl of diluted samples or DNA standard solutions in microplate wells, and after 10 min the fluorescence (excitatation 485 nm/emission 520 nm) was observed (Rye et al., 1993).

2.4. EROD activity

Seven-ethoxyresorfin-*O*-deethylase (EROD) activities in fish liver microsomes were determined according to the method described by Burke and Mayer (1974), with adaptations for microplate use. Briefly, 12.5 µM etoxyresorufin substrate and 200 µM NADPH cofactor (nicotinamide adenine dinucleotide phosphate reduced form) in 0.1 M phosphate buffer, pH 7.7 were incubated for 5 min in a microplate wells at 30 °C. The recorded fluorescence should remain constant over a period of 1 min and, after the addition of prepared microsomes, the resorufin production is followed by measuring the fluorescence rise (excitatation 537 nm/emission 583 nm) for a 5 min. At the end, a known amount of resorufin (0, 585 pM) is added to the each well and the fluorescence increases were recorded. The results were calculated by comparison of the average fluorescence increase in 1 min of enzyme reaction and the fluorescence increase after the resorufin addition. The results were expressed as pmol resorufin mg^{-1} protein min^{-1}.

2.5. Fast micromethod®

DNA integrity determination was performed by the Fast Micromethod® according to Jakšić and Batel (2003) with some minor modifications. The method is based on the selective character of the specific fluorochrome dye PicoGreen® (Molecular Probes Inc., Eugene, OR, USA) to make a very stabile complex only with double stranded (dsDNA), and that is used to detect DNA denaturation (reduction of dsDNA-PicoGreen® complex) under alkaline conditions. Sample homogenates, 100 ng DNA ml^{-1}, were lysed in microplates with lysing solution supplemented with PicoGreen® at 30 °C for about 30 min. After the addition of alkaline NaOH-EDTA solution time-dependent DNA denaturation was determined in the microplate by measuring the fluorescence (excitatation 485 nm/emission 520 nm) of the dsDNA-PicoGreen® complex (Fluoroscan II reader or Fluoroscan Ascent, Labsystems, Finland). After 5–10 min of denaturation, the SSFs were then calculated after correction for blanks as a measure of dsDNA amounts in examined and reference samples:

$$\mathrm{SSF} = \log\ 10 \times \frac{\text{Fluorescence units examinated sample}}{\text{Fluorescence units reference sample}}. \quad (1)$$

SSF < 0 indicates increasing frequencies of strand breaks and alkali labile sites (loss of DNA integrity) in samples. For practical reasons the SSFs in graphical presentations were multiplied by (−1).

2.6. Statistical analysis

The results of EROD activity and DNA integrity values are given as mean values of five replicates, with corresponding standard deviations. The data showed normal distributions and remain untransformed. The statistical analyses were carried out using Systat 10.2 software (SYSTAT Software Inc. Richmond, CA, USA). The statistically significant differences between biomarker (EROD activities and SSF × (−1)) values in control and lindane-exposed samples, and between the reference site sample (S3—Bale pond) and other sampling sites, were estimated by analysis of variance (ANOVA) with Tukey HSD post-hoc adjustment. The correlations between measured biomarker values and lindane (laboratory experiment) or OCP compounds (*in field*) levels were calculated by linear regression analysis. The positive correlation coefficient value indicates a positive association between the variables, while the value close to zero indicates no association between the variables, and the linkage between variations of both variables is given by the square of the correlation coefficient.

3. Results

3.1. Laboratory experiment—lindane exposure

The suitability of mosquitofish *G. affinis* as a bioindicator species, and the possible sensitivity of liver EROD activity and DNA integrity to OCPs were investigated by the exposure of fish to different concentrations of lindane (0.1, 10, 100 µg l^{-1}) in experimental laboratory basins. The results are shown in Figs. 2 and 3.

Exposure to lindane resulted in significant inductions of EROD activity in male and female mosquitofish. The male mosquitofish showed a significant induction of EROD activity of 96.0 ± 9.8 after 24 h, and 206.9 ± 3.8 pmol min^{-1} mg^{-1} after

Table 2
Sampling sites, codes, temperature and pH of pond water, δ-BHC, Lindane (γ-HCH), Aldrin, Dieldrin Endosulfan, pp-DDE, pp-DDD, and pp-DDT, total organochlorine pesticides and actual polychlorinated biphenyls (PCB) contamination levels-mass fractions (ω) in puddles dry sediment and brief descriptions of the pond sampling sites

Sampling site	Code	pH	Temperature (°C)	δ-BHC ($ng\,kg^{-1}$)	Lindane ($ng\,kg^{-1}$)	Aldrin ($ng\,kg^{-1}$)	Dieldrin ($ng\,kg^{-1}$)	Endosulfan ($ng\,kg^{-1}$)	pp DDE ($ng\,kg^{-1}$)	pp DDD ($ng\,kg^{-1}$)	pp DDT ($ng\,kg^{-1}$)	Organochlorine pesticides ($ng\,kg^{-1}$)	Polychlorinated biphenyls ($ng\,kg^{-1}$)
Kukuletovica	S1	6.5	28.5	0.10	0.34	0.34	0.42	0.35	0.84	–	–	2.39	0.93
Španidiga	S2	6.7	30.4	–	0.26	0.37	0.16	0.24	–	–	0.40	1.43	0.94
Bale	S3	6.5	29.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Krnjaloža	S4	6.5	29.0	–	0.40	0.16	–	–	–	0.26	0.68	1.50	4.29
Galižana	S5	6.4	28.6	–	0.11	0.24	0.31	0.31	2.10	–	0.15	3.22	2.00

NA, sediment not available.

96 h exposure in basins with lindane concentration of 100 and $10\,\mu g\,l^{-1}$, respectively (Fig. 2A).

Furthermore, significant effects were observed by exposure of female mosquitofish to $0.1\,\mu g\,l^{-1}$ of lindane. The induced EROD activities were 1.9 ($135.1 \pm 7.9\,pmol\,min^{-1}\,mg^{-1}$) and 3.4-fold ($127.8 \pm 7.5\,pmol\,min^{-1}\,mg^{-1}$) higher than in the controls, after 96 and 168 h, respectively. The EROD activities were 2.0-, 2.5-, and 3.4-fold higher after 24, 96, and 168 h exposure to $10\,\mu g\,l^{-1}$ of lindane and reached values of 107.0 ± 10.3, 174.7 ± 25.6, and $134.0 \pm 6.2\,pmol\,min^{-1}\,mg^{-1}$, respectively. The highest applied lindane concentration of $100\,\mu g\,l^{-1}$ showed moderate (1.7- and 2.1-fold) EROD activity induction of 88.1 ± 2.5 and $79.9 \pm 2.5\,pmol\,min^{-1}\,mg^{-1}$ after 24 and 168 h, respectively, although after 96 h the exposed samples showing only 60% ($44.5 \pm 1.6\,pmol\,min^{-1}\,mg^{-1}$) of the control EROD activity were recorded (Fig. 2B).

The 96 and 168 h exposure of fish to lindane concentrations ranging from 0.1 to $100\,\mu g\,l^{-1}$ resulted in a significantly lower DNA integrity status to male liver homogenates than in the control samples. The 96 h exposure to 10 and $100\,\mu g\,l^{-1}$ lindane caused significantly higher values than in the controls, and they reached SSF $\times(-1)$ values of 0.261 ± 0.053 and 0.294 ± 0.036, respectively. The 168 h exposure to the lowest lindane dose of $0.1\,\mu g\,l^{-1}$ showed SSF $\times(-1)$ values of 0.225 ± 0.008 compared to 0.002 ± 0.055 in the controls ($p < 0.001$), although remained at similar values to the controls for both applied higher doses (Fig. 3A).

In the case of female mosquitofish, the 24- and 168-h exposure to $10\,\mu g\,l^{-1}$, and 168 h to $0.1\,\mu g\,l^{-1}$ lindane showed significantly lower DNA integrity in liver homogenates than the control samples, respectively. However, after the 96-h exposure the results were quite the opposite. The highest, and most significantly ($p < 0.001$) different SSF $\times(-1)$ values from the control were measured after 24 h exposure to $10\,\mu g\,l^{-1}$ lindane (0.261 ± 0.053), and 168 h exposure to $0.1\,\mu g\,l^{-1}$ lindane (0.365 ± 0.020). After 168 h exposure to $10\,\mu g\,l^{-1}$ of lindane a less significant ($p < 0.05$) DNA integrity and SSF $\times(-1)$ value of 0.130 ± 0.018 was measured (Fig. 3B).

3.2. Chemical analysis

The gas liquid chromatography analysis of OCPs and PCBs in natural pond sediment samples did not shown a high level of contamination in all examined sediment samples. The mass fractions (ω) of OCPs and PCBs in pond sediments lay between 1.43 and 3.22 and 0.93 and $4.29\,ng\,kg^{-1}$, respectively. The highest observed grade of PCBs contamination, $4.29\,kg^{-1}$, was at Krnjaloža pond (S4) and the ratio of lindane in the total OCP content was 26.8%. The Galižana sediment pond (S5) showed a contamination level as high as the of Krnjaloža (S4) and the recorded OCPs levels ($3.22\,ng\,kg^{-1}$), was the highest among all four sediment samples. The Kukuletovica pond (S2) showed moderate levels of PCBs and OCPs

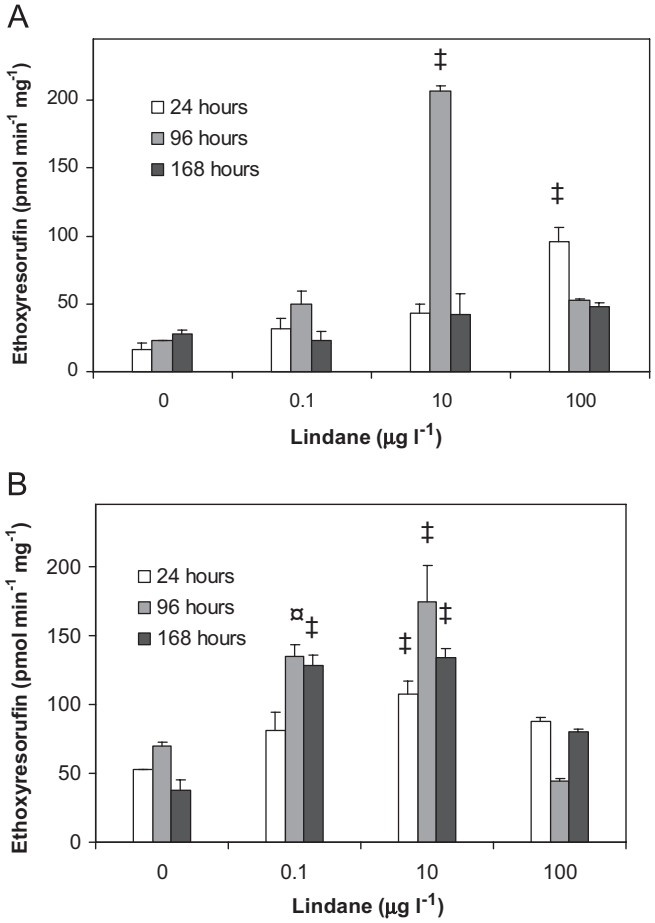

Fig. 2. EROD activity in livers of (A) male and (B) female *Gambusia affinis* exposed to 0.1, 10 and 100 µg l^{-1} lindane for 24, 96 and 168 h. The results are given as mean values with corresponding standard deviations. Statistically significant differences ($^{‡}P<$ 0.01 and $^{¤}P<0.05$) between control and exposed fish are indicated.

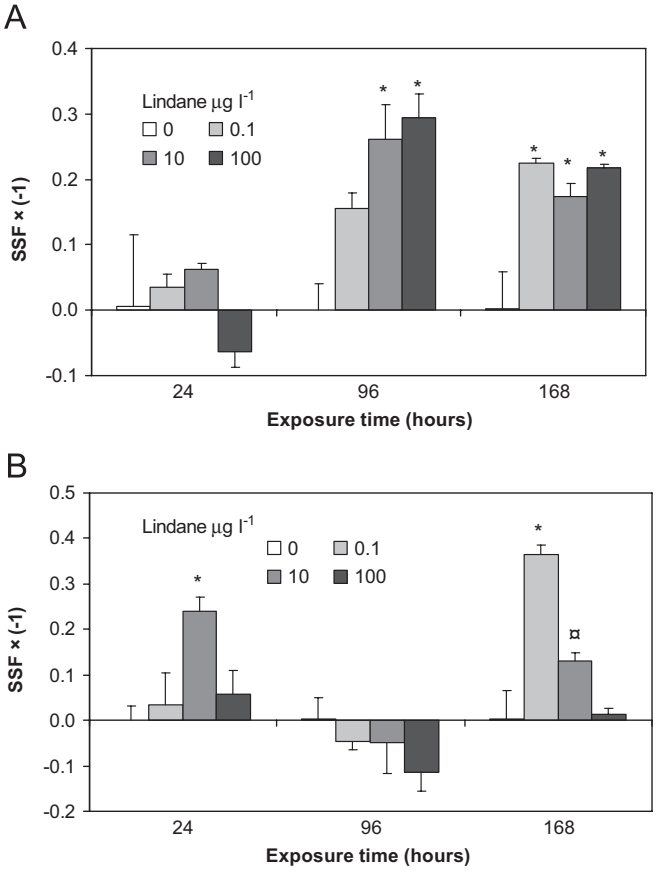

Fig. 3. Livers DNA integrity in (A) male and (B) female *Gambusia affinis* samples exposed to 0.1, 10 and 100 µg l^{-1} lindane for 24, 96, and 168 h. The results are given as mean values with corresponding standard deviations. Statistically significant differences ($^{*}P<0.001$ and $^{¤}P<0.05$) between control and exposed fish are indicated.

contaminations while Španidiga pond (S1) showed the lowest (Table 2).

3.3. Field study

The lowest liver EROD activities of 7.5±1.6 and 9.7±2.1 pmol min^{-1} mg^{-1} were recorded in male samples from Bale (S3) and female samples from Krnjaloža pond (S4), respectively. The only sampling site with, both male and female, specimens with liver EROD activity significantly higher ($p<0.01$ and $p<0.001$ respectively) than in the control Bale pond (S3) site was Galižana pond (S5). However, this male populations showed 1.7-, 3.4-, 9.7-, and 4.4-fold higher resorufin production rates (872.4±34.8 pmol min^{-1} mg^{-1}) than Kukuletovica (S1), Španidiga (S2), Bale (S3) and Krnjaloža (S4) male samples, respectively.

The female fish from Španidiga pond (S2) showed two-fold higher EROD activity (68.4±2.3 pmol min^{-1} mg^{-1}) than females from the reference Bale pond (S3) site (34.6±2.9 pmol min^{-1} mg^{-1}). The Galižana (S5) and Kukuletovica pond (S1) female mosquitofish shoved the highest measured values of resorufin production rates, 151.1±63.1 and 157.2±85.0 pmol min^{-1} mg^{-1}, respectively (Fig. 4). Those rates are 2.3, 4.5 and about 16-fold higher than resorufin production in the Španidiga (S2), Bale (3) and Krnjaloža (S4) samples, respectively.

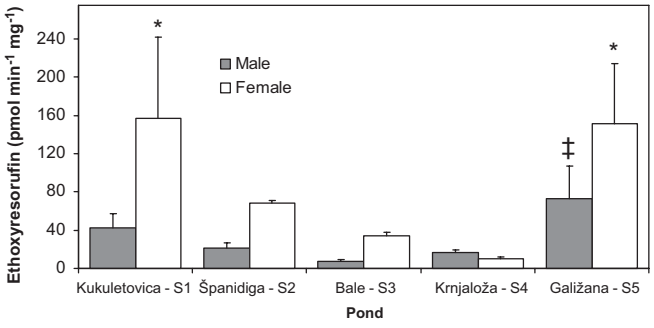

Fig. 4. EROD activity in (A) male and (B) female *Gambusia affinis* liver homogenates from ponds in south-western Istrian peninsula, Croatia. The results are given as mean values with corresponding standard deviations. The levels of statistical significance ($^{*}P<0.001$ and $^{‡}P<0.01$) with a reference site sample (S3—Bale pond) are indicated.

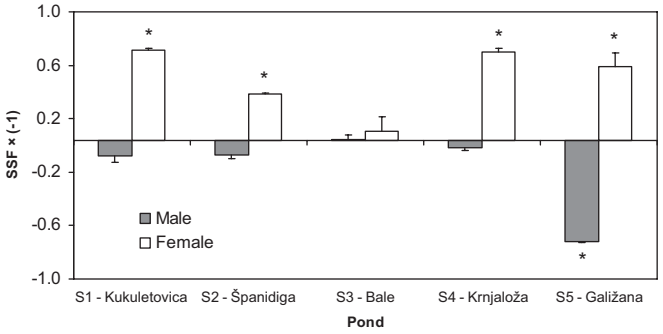

Fig. 5. DNA integrity in (A) male and (B) female *Gambusia affinis* liver homogenates from ponds in south-western Istrian peninsula, Croatia. The results are given as mean values with corresponding standard deviations. The levels of statistical significance ($^{*}P<0.001$ and $^{¤}P<0.05$) with a reference site sample (S3—Bale pond) are indicated.

Recorded DNA integrities in male *G. affinis* liver homogenates from all sampling sites were similar to the Bale control pond (S3). The exception is the Galižana pond (S5) where the measured $SSF\times(-1)$ value of -0.757 ± 0.010 is significantly ($p<0.001$) lower, not only than the control but also any of the other sample sites, which indicates a better DNA integrity status. However, in liver homogenates of female fish, the recorded $SSF\times(-1)$ vales ranged from 0.346 ± 0.003 for fish from Španidiga (S2) to 0.671 ± 0 020 for those from Kukuletovica pond (S1) and implies a significantly lower ($p<0.001$) DNA integrity status at all of those sampling sites in comparison to the Bale pond (S3) control (Fig. 5).

3.4. Correlation (linear regression) analysis

The correlation coefficients (r), achieved by linear regression analysis, between the *G. affinis* liver detoxification enzymes (EROD) activity or DNA SSF values and lindane (laboratory experiment) or persistent OCP amounts in natural pond sediments (field study), are presented in Table 3.

In our laboratory experiments, the male fish exposed to lindane showed highest correlations between EROD activity and lindane load (0–10 $\mu g\,l^{-1}$), then female fish, in entire exposure period. Although, the female fish showed the highest positive correlations between SSF values and lindane load than male fish, with exceptions for 96 h exposure where the achieved SSF values caused the negative correlation with lindane amounts (Fig. 3B). However, the positive correlations between SSF values and lindane 168 h exposition of male fish has not been unambiguously confirmed ($r=0.43$).

The correlation of the *G. affinis* liver detoxification enzymes (EROD) activity values with persistent organochlorine pollutant amounts in natural pond sediments (field study) were higher in male than female fish. As a possible consequence of unknown presented genotoxic agent the correlation between founded DNA SSF values were negative in male ($r=-0.95$) or even does not exist in female fishes ($r=0.18$).

Table 3
The correlation coefficients (r), achieved by liner regression analysis, between lindane contamination levels (0–10 $\mu g\,l^{-1}$) in laboratory basins water or organochlorine pesticides in ponds sediments (*in field*) and measured biomarkers (EROD activity and SSF values) in female and male *Gambusia affinis*

	Male		Female	
	EROD activity	DNA SSF	EROD activity	DNA SSF
Organochlorine pesticides (in pond sediments) (h)	0.99	−0.95	0.78	0.18
Lindane exposure (in laboratory basins)				
24	0.97[a]	0.84	0.85	0.99
96	0.99	0.81	0.79	−0.89
168	0.97	0.43[a]	0.56	1.00[b]

[a]Linear regression analysis of entire lindane dose range (0–100 $\mu g\,l^{-1}$).
[b]Linear regression analysis of two lindane levels (0 and 0.1 $\mu g\,l^{-1}$).

4. Discussion

The *Gambusia* species were well-known and utilized as a bioindicator organism in many studies and the toxicity of lindane has already been studied in that species (Walton et al., 1997; Allenbach et al., 1999; Sullivan and Lydy, 1999). The suitability of mosquitofish, as an indicator species for the exposure to OCPs has been validated by means of exposure under laboratory conditions to lindane for up to 7 days.

In our laboratory experiments, the female fish showed significant EROD activity induction with respect to a control at lower lindane doses and shorter exposure times than male specimens (Fig. 2B). The significant differences between the control and exposed male fish were found just after 24 h exposure to 100 $\mu g\,l^{-1}$ and after 96 h exposure to 10 $\mu g\,l^{-1}$ lindane in experimental basins (Fig. 2A). Differential sex specific physiological pathways may explain the discrepancy in *G. affinis* liver EROD activity between male and female fish, where male species showed significant induction only with the highest applied lindane doses. However, the basal EROD activities are approximately two-fold greater in female mosquitofish when compared to males. Observed EROD activities may be a consequence of faster recovery to normal levels in males than females, or may be a consequence of the collapse of the phase I metabolizing system at the high concentration of lindane tested in our experiment (100 $\mu g\,l^{-1}$), which is close to the LD50 of lindane in this species (Walton et al., 1997). That presumption would favour female *Gambusia* fish as a choice organism for CYP induction assessment in natural samples from areas under significant impact from OCPs, although its validity will depend on future insights into the dose–response range of the biomarkers. Moreover, the inducibility of the liver EROD enzyme system in male *Gambusia holbrooki* was confirmed by the exposure to synthetic estrogen 17α-ethynylestradiol and relatively high

doses of β-naphtoflavone (1.0–4.0 μg l^{-1}) (Aubry et al., 2005).

The measured DNA integrity status in male *G. affinis* liver homogenates after 96 and after the longer 168 h of exposure were significantly lower than in control samples. The 0.1 μg l^{-1} of lindane, after 168 h of exposure, showed higher SSF × (−1) values than those after 96 h, and the higher doses of 10 and 100 μg l^{-1} showed lower SSF × (−1) values after 96 h exposure than after 168 h (Fig. 3A). In female fish the highest SSF × (−1) values were measured in samples exposed for 168 h to 0.1 μg l^{-1} lindane. The samples exposed to a moderately high dose of lindane, 10 μg l^{-1}, showed significantly higher SSF × (−1) values than the control, even just after 24 h of exposure, although decreased to lower values after 168 h of exposure. The highest applied exposure dose of lindane, 100 μg l^{-1}, did not show any significant changes in measured DNA integrity status. However, all the female samples taken after 96 h of exposure to lindane in the experimental basins showed unexpected results, with the highest DNA integrity status in samples exposed to the highest doses of lindane. This DNA integrity status was also higher than in any other sample in the entire experiment. In the exposure test of *G. affinis* to 100 ppb benzo[*a*]pyrene for 48 h Batel et al. (1985) measured the high BPMO activity, and the disappearance of longer and increase of shorter DNA molecules, in order to nuclease S1 sensitive sites.

The study provided 15 years ago (Fingler et al., 1992) showed the presence of lindane in drinking water samples from Labin, a small town located in the karst area on the Istrian peninsula, in a lower than admissible concentrations of 0.1 μg l^{-1}. However, the same study perceived the presence of increased levels of chlorphenols, particular 2,3,4,6-tetrachlorphenol, and confirmed the prediction of possible transport of lipophilic compounds from distant industrialized and agricultural regions to the water sources in Istrian peninsula.

The field study, at five ponds in southwestern Istrian peninsula, Croatia, was performed afterwards. The measured EROD activities in female fish were higher than in male samples at all sampling locations except in those from Krnjaloža pond (S4) where the male and female samples showed very low EROD activity. The lowest EROD activity was recorded in male *Gambusia* samples from Bale pond (S3). Although the highest PCB amounts were found in Krnjaloža (S4) pond sediment the recorded EROD activity values were very low in both male and female samples. The male samples from Krnjaloža (S3), Španidiga (S2), Kukuletovica (S1), and Galižana (S5) showed EROD activities 2.2-, 2.8-, 5.6-, and 9.7-fold higher than males from Bale pond (S3). Furthermore, the observed EROD activity in females from Bale (S3), Španidiga (S2), Galižana pond (S5), and Kukuletovica (S1) were 3.6-, 7-, 15.6-, and 16.2-fold higher than in Krnjaloža pond (S4), respectively. The higher the absolute EROD values, the higher the standard deviations that were recorded, particularly in female samples. The variation coefficients were up to 36.6% and 48.0% in female samples from Kukuletovica (S1) and Galižana (S5), respectively (Fig. 4). Those results show a positive correlations between EROD activity in male ($r = 0.99$) and female ($r = 0.78$) fish and OCP mass fractions in sediments from related ponds. The moderate OCP level in Kukuletovica pond sediment (S1) induced the highest recorded EROD activity in female fish. That EROD activity was as high as in female fish from Galižana pond (S5) where the highest OCP level and moderate level of PCB were recorded. The lowest mass fractions of PCBs and OCPs was measured in Španidiga pond sediment (S2) where the induction of EROD activity in female fish were just seven-fold higher than in Krnjaloža (S4) and almost two-fold higher than in fish from Bale pond (S3). The measured results of EROD activity in female fish from Krnjaloža (S4), where the highest mass fractions of PCBs were found, and from Galižana, (S5) lead us to conclude about the possible inhibitory effect of high concentrations of PCBs and other non-investigated xenobiotic substances actions and mechanisms in pond sediments (Gravato and Santos, 2002; Oliveira et al., 2005; Edwards et al., 2007). Nevertheless, the results obtained herein suggest the applicability and inducibility of female *G. affinis* liver EROD activity by organic chlorine xenobiotics. Pacheco and co-workers (2002) investigated genotoxicity and the induction of MFO activity on female *Gambusia holbrooki* by bleached kraft pulp mill effluent in the Vouga River, Cacia, Portugal. In spite of documented toxicity near the effluent outlet, they found the significantly highest EROD activity induction at the most distant sampling site and may be consequence of the dilution of highly concentrated toxic compounds following previous inhibition.

Due to its characteristics the Bale pond was chosen as a control location for DNA integrity determination, which means that the DNA integrity of *G. affinis* samples from the Bale pond (S3) were used as a reference value for SSFs calculations (see Section 2.1). The male samples showed no significant differences among calculated SSF × (−1) values, except for the samples from Galižana pond (S5) whose SSF × (−1) values suggest an even better DNA integrity status than the control samples or possible additional DNA damage such as DNA–DNA and DNA–protein cross-links. However, significantly higher SSF × (−1) values than in the control samples were measured in female fish from all the other four sampling ponds. Moderate DNA integrity deterioration was recorded in samples from Španidiga pond (S2) where the lowest mass fractions of PCBs and OCPs were found. The female samples from Kukuletovica (S1) and Krnjaloža (S4) ponds, where moderate OCPs levels were found, showed the lowest DNA integrity among all sampling sites. The DNA integrity in *G. affinis* female liver homogenate from Galižana (S5), where the mass fraction of OCPs were the highest yet, had the lowest lindane fractions of all investigated ponds, as given by SSF × (−1) values were slightly lower than in samples from Kukuletovica (S1) and Krnjaloža (S4) (Fig. 5). Measured sediment mass fractions

of OCPs, and particularly lindane, and calculated SSF × (−1) values points towards a clear conclusion regarding induced DNA-damaging processes in female Gambusia samples from related locations, yet the impact of PCBs remains unclear. In fact, the highest PCB amounts found at Galižana (S5) and particularly Krnjaloža (S4) did not show any additional effect on female DNA integrity. Although EROD activity was the lowest in Krnjaloža (S4) quite high values were recorded in Galižana (S5) female samples. The measured EROD activity and SSF × (−1) values in female *G. affinis* liver homogenates showed significant differences among differently polluted sediment ponds in south-west Istria, Croatia. However, the positive linear correlation between DNA integrity deterioration in male and female fish liver and OCPs contamination of pond sediments remains unconfirmed (Table 3) as a possible consequence of uninvestigated genotoxic xenobiotics presences and actions. Recently, mutagenic effects on *Gambusia* species by agents present in the polluted waters of the Sarno River, Italy, were confirmed by Comet assay and micronucleus test (Russo et al., 2004), and the biomonitoring of environmental carcinogens by *G. affinis* as a bioindicator may be carried out by using the proliferating cell nuclear antigen antibody, PC10 (Lentz et al., 2003). The EROD activity and DNA strand breaks measured in Zebra mussel from the Lake Magiore, Italy, and the positive relation between EROD and organochlorine substances as well as between DNA strand brakes and PAHs were confirmed (Binelli et al., 2007).

The approach presented here to investigate biomarkers of exposure to genotoxic contaminants and CYP1A-dependent enzymes in parallel in *G. affinis* provide, for the first time, comprehensive data of lindane effects on EROD activity and DNA integrity in mosquitofish, helping to bridge a gap in ecotoxicological studies in this important bioindicator species.

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