Elsevier Editorial System(tm) for Chemosphere
Manuscript Draft

Manuscript Number:

Title: DECONTAMINATION TRENDS IN THE AQUACULTURED FISH GILTHEAD SEABREAM (Sparus aurata) AFTER FEEDING LONG-TERM A PCDD/F SPIKED FEED

Article Type: Research Paper

Section/Category: Persistent Organic Pollutants and Dioxins

Keywords: Dioxin exposure, bioaccumulation, chemical markers, food

Corresponding Author: Dr. Esteban Abad Holgado,

Corresponding Author's Institution: Inst. d'Investigacions Quimiques, i Ambientals, Dept. d'Ecotecn.

First Author: Esteban Abad Holgado

Order of Authors: Esteban Abad Holgado; Manuela Ábalos; Jordi Parera; Alicia Estevez; Montserrat Solé; Mari Carmen Fabregat; Benjamin Piña; Anna Navarro

DECONTAMINATION TRENDS IN THE AQUACULTURED FISH GILTHEAD SEABREAM (*Sparus aurata*) AFTER FEEDING LONG-TERM A PCDD/F SPIKED FEED

Manuela Ábalos[1], Jordi Parera[1], Alicia Estévez[2], Montserrat Solé[3], Mari Carmen Fabregat[3,4], Benjamin Piña[4], Anna Navarro[4], Esteban Abad1*

[1]Laboratory of Dioxins, Environmental Chemistry Department, IDÆA-CSIC. Jordi Girona 18, 08034 Barcelona, Spain

E-mail: esteban.abad@idaea.csic.es

[2]IRTA Centre de Sant Carles. Ctra. Poble Nou Km 6, Sant Carles de la Ràpita, 43540 Tarragona, Spain

[3]Institut de Ciències del Mar (CMIMA-CSIC). Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

[4] Environmental Chemistry Department, IDÆA-CSIC. Jordi Girona 18, 08034 Barcelona, Spain

DECONTAMINATION TRENDS IN THE AQUACULTURED FISH GILTHEAD SEABREAM (*Sparus aurata*) AFTER FEEDING LONG-TERM A PCDD/F SPIKED FEED

Manuela Ábalos[1], Jordi Parera[1], Alicia Estévez[2], Montserrat Solé[3], Mari Carmen Fabregat[3,4], Benjamin Piña[4], Anna Navarro[4], Esteban Abad1*

[1]Laboratory of Dioxins, Environmental Chemistry Department, IDÆA-CSIC. Jordi Girona 18, 08034 Barcelona, Spain

E-mail: esteban.abad@idaea.csic.es

[2]IRTA Centre de Sant Carles. Ctra. Poble Nou Km 6, Sant Carles de la Ràpita, 43540 Tarragona, Spain

[3]Institut de Ciències del Mar (CMIMA-CSIC). Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

[4] Environmental Chemistry Department, IDÆA-CSIC. Jordi Girona 18, 08034 Barcelona, Spain

Abstract

Aquacultured fish gilthead seabream (*Sparus aurata*), previously exposed to low levels of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) trough the diet for a 13 months period, were fed on a clean feed for another 22 months. Gilthead seabream is a protandrous hermaphrodite species and this "decontamination" period coincided with the stages of sex differentiation, maturity and reproduction of the fish. PCDD/F levels in the fish tissues (i.e. muscle, liver, perivisceral fat and gonads), expressed in pg WHO-TEQ/g fresh weight, showed a general decreasing trend during the "decontamination" period. However, this general trend varied among tissues and was also dependent on sex and lipid contents. Toxicological effects affecting fish behaviour and hepatic marker responses were also evaluated. The results pointed out that exposure to PCDD/Fs did not have an impact on fish development and reproduction, since the proportion of sexes found after the sex reversal process was within the normal range described for this species. In addition, long-term exposure to low PCDD/F levels did not significantly affect the response of most of the biochemical markers considered. On the contrary, some of them (e.g. EROD activity) showed variations in their responses during the sex differentiation process and onwards. Finally, the hepatic AhR mRNA levels increased during dioxin exposure but they returned to values typical for non-exposed fish after the "decontamination" period.

Keywords: Dioxin exposure, bioaccumulation, chemical markers, food

1. Introduction

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/Fs), also referred as "dioxins", are among the most known families of compounds belonging to the category of persistent organic pollutants (POPs). Due to their stable structure and lipophilic character, dioxins tend to bioaccumulate and biomagnify through the trophic chain. The toxicity of these contaminants has been largely studied (Safe, 1986; van den Berg et al., 1994) . In particular, the International Agency for Research on Cancer (IARC) declared the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as carcinogenic for humans (McGregor et al., 1998) . Dioxins have also been recognized as endocrine disruptors for living organisms (Safe, 1999). In 1997, the World Health Organization (WHO) revaluated the toxic equivalency factors (TEFs) assigned to PCDD/Fs for the calculation of the toxic equivalents (TEQs) (van den Berg et al., 1998).

The main source of human exposure to PCDD/Fs is diet, accounting for more than 90% of the total daily intake (Liem et al., 2000). Maximum levels of dioxins have been set by the European Union (EU) for a large number of foodstuffs, including the muscle meat of fish (Commission Regulation (EC) No. 1881/2006). In this sense, a project was designed with the aim to follow the bioaccumulation of PCDD/Fs in a highly appreciated aquacultured fish species in the Mediterranean (i.e. gilthead seabream, *Sparus aurata*) being the animals exposed to these pollutants trough the diet. For that purpose, two groups of fish were considered, one was fed a commercial feed and the second one was fed the same feed but spiked with low levels of dioxins. The experiment started with juveniles (~15 g) gilthead seabream and lasted 13 months, a period similar to that needed to achieve commercial size (Ábalos et al., 2008). Dioxins were determined in muscle (flesh) and liver tissues. After feeding the spiked feed for 3 months PCDD/F concentrations in muscle were 5.50 pg WHO-TEQ/g fresh weight (f.w.), being slightly above the maximum established at the EU Regulation (4 pg WHO-TEQ/g f.w.). The levels remained similar during the rest of the experimental period; although after 13 months of exposure they decreased and were below the maximum set. After finishing the evaluation of PCDD/F concentrations during this exposure period, the next aim of the project paid attention to the levels of these contaminants when the previously exposed animals were fed a "clean" feed for a given period of time ("decontamination" period). Thus, fish were kept in the tanks for an additional 22 months and fed a non-spiked commercial feed. A similar experiment (Brambilla et al., 2007) showed a depletion of the levels of selected PCDD/Fs and polychlorinated biphenyls (PCBs) in muscle of farmed rainbow trout (*Oncorhynchus mykiss*) attributed to both, clearance and growth dilution.

In addition to the bioaccumulation study, another objective included in the first part of our project was to evaluate the toxicological effects for the fish derived from PCDD/F exposure. Toxicity mediated

action of dioxins occurs trough the aryl hydrocarbon receptor (AhR) (Hahn, 2001). Exposure to PCDD/Fs in fish, and in particular to the most toxic congener (TCDD), has been reported to negatively affect their cardiovascular function, their immune system and growth. Moreover, exposure has been associated with fish mass mortality events (Giesy and Snyder, 1998). PCDD/Fs have also been proved to be antiestrogenic compounds. In our study, during the exposure period to dioxins several hepatic markers were evaluated. In general, it was observed a significant increase in the 7-ethoxyresorufin O-deethylase (EROD) activity and in AhR gene expresión after ~300 days of exposure. However, no effect on the antioxidant enzymes catalase (CAT) and total glutathione peroxidase (t-GPX) was found. On the other hand, differences in fish growth were not observed between dioxin-exposed and non-exposed animals and no mortalities were recorded due to dioxin intake (Ábalos et al., 2008).

Besides the interest on following the trend of the levels of PCDD/Fs, the response of several hepatic markers as well as some aspects of fish behaviour during the "decontamination" period were also considered. It has to be taken into account that gilthead seabream is a protandrous hermaphrodite species (Zohar et al., 1978). In captivity, all fish function as males during the first year of their life. Afterwards, from the first or second year of life onwards a certain percentage of the males undergo sex reversal, so that over succeeding years the ratio of females in the population increases (Zohar et al., 1978 and 1984). All males in the population start to develop ovaries at the end of the spawning season (around May), with the final commitment to changing sex into mature females made by September (Zohar et al., 1978). Males that enter into the ambisexual state but ultimate do not become females, develop testes once more and become functional males again. The studies of Happe and Zohar (Happe and Zohar, 1988) showed that the proportion of males reversing sex is socially controlled, and careful attention should be paid to establishing groups of broodstock, or modifying their composition, otherwise it might lead to a sex ratio that is detrimental to spawning. In addition, it is well established that estrogens and the enzyme needed for their synthesis, the cytochrome P450 aromatase (CYP19) play an important role in the development of sex determination and sex differentiation processes (Guiguen et al., 2010; Cheshenko et al., 2008; Wong et al., 2006). Dioxins may disrupt this mechanism, as AhR is known to affect the reproductive pathway both by interacting with the estrogen receptor (ER) mediated response and by affecting expression of key enzymes on steroid metabolism, such as CYP19 (Cheshenko et al., 2008). Therefore, it was interesting to check whether an early exposure to PCDD/Fs had effects on fish development and reproduction during the "decontamination" period.

The aim of the present study was to determine the levels of PCDD/Fs in several fish tissues and the toxicological effects affecting fish behavior (i.e. growth, sex distribution and sex reversal, spawning and egg quality) and hepatic marker responses during the "decontamination" period, similarly to what was performed during the first 13 months of the project when the animals were exposed to PCDD/Fs.

Three sampling campaigns were carried out corresponding to the periods of gilthead seabream sex differentiation, maturity and reproduction. In contrast to the former study, PCDD/F concentrations were also determined in perivisceral fat and gonads (formerly only muscle (flesh) and liver). Due to the lipophilic character of dioxins they tend to accumulate in fat. In this sense, it must be noticed that the percentage of fat in the muscle of gilthead seabream is very low and most of the fat is located in the liver and perivisceral fat. The fat present in these two tissues is mobilized during the spawning season in order to obtain energy necessary to carry out this process and a part is incorporated in the egg in the form of oil droplets. Therefore, it is likely that the PCDD/Fs associated to the fat will be also mobilized during this process. In order to evaluate the toxicological effects, in addition to markers already included in our previous work (i.e. induction of cytochrome P450 1A (CYP1A) dependent EROD activity, the conjugating enzyme glutathione S-transferase (GST), the antioxidant enzymes (CAT, t-GPX) and AhR gene expression), other components of the mixed function oxygenase (MFO) system (i.e. (NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide reductases), uridinediphosphate glucuronyltransferase (UDPGT), superoxide dismutase (SOD) and glutathione reductase (GR) activities were also included in the present study.

2. Materials and Methods

2.1. Fish rearing

Gilthead seabream juveniles (~15 g) were stocked during 13 months (exposure period) in four tanks. The experiment was designed to be carried out in duplicate, thus in two of the tanks, namely D-1 and D-2, fish were fed a spiked feed, while fish in the other two tanks were considered as control and fed with non-spiked feed (Ábalos et al., 2008). Once the fish reached a commercial size (400 g, June 2005), only the two groups fed with dioxin spiked feed were kept in their corresponding tanks for a further 22 months ("decontamination" period), being fed a commercial fish diet without dioxin addition until April 2007.

2.2. Sampling procedure

During the "decontamination" period fish were sampled to check growth in weight in June and December 2005; February, September and December 2006 and April 2007. In addition, fish were sacrificed in three sampling campaigns (December 2005, December 2006 and April 2007) for muscle, liver, perivisceral fat and gonads dioxin analysis. Similar to what was done during the exposure period, samples of the tissues, once dissected in ice and frozen at -20ºC, were immediately sent to the laboratory and stored at -20 ºC until analysis. Liver samples were also taken to perform the analysis of chemical markers in this tissue. The protocols to collect and preserve these samples are described

elsewhere (Ábalos et al., 2008). The number of animals sacrificed in each campaign together with the tissues considered for analysis is reported in Table 1.

In December 2006, once the sex reversal process took place, all the remaining fish (N=25) were collected and checked for sex distribution. Fish were sexed by abdominal stripping and/or cannulation to collect sperm (if males) and/or oocytes (if females), respectively. Two broodstocks, one per tank, were formed with 4 males and 4 females each, being the remaining fish (N=9, 6 males and 3 females) sacrificed for analysis. From January to April 2007 normal spawning activity of the fish ocurred. In April 2007, all the animals from the two tanks were finally sacrificed for analysis.

2.3. PCDD/F analysis

For PCDD/F analysis, composite samples of the different tissues were prepared from a determined number of fish in each case. Separate analyses were carried out for males and females from December 2006. In this sense, a female fish from each tank in April 2007 was not included in the composites since they did not show any spawning activity. Gonads were only analyzed in December 2006 and April 2007, when male and female fish could be distinguished. Finally, perivisceral fat was not considered for analysis in April 2007 since only small amounts of this tissue were found in two fish at the end of the "decontamination" period.

The methodology applied for the determination of PCDD/Fs in the tissue samples was previously described (Ábalos et al., 2008). After extraction, clean-up and purification the final extracts obtained were analyzed by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) on a 6890N Network GC System Agilent gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) coupled to a Autospec Ultima NT high resolution mass spectrometer (EBE geometry) (Micromass, Manchester, UK), using a positive electron ionization (EI+) source operating in the SIM mode. Quantification was carried out by the isotopic dilution method.

2.4. Biochemical determinations

Biochemical markers. Biochemical markers were determined in the microsomal and cytosolic fractions obtained from individual livers (aprox. 1.5 g) as described in Raldúa et al. (2008). In this case, when male and female fish were present no distinction was made among them, thus liver samples from a certain campaign were considered altogether as a group for analysis. Briefly, homogenates followed 3 step centrifugations (500g x 10 min, 10,000g x 20 min and 100,000g for 60 min) obtaining the cytosolic (supernatant) and microsomal (pellet). Total protein content was measured in both fractions by the method of Bradford et al. (1976), using bovine serum albumin (BSA) as standard. All

enzymatic activities were carried out in duplicate at 25º C, and in a final reaction volume of 1 ml or 3 ml (CAT). EROD and UDPGT activities were performed after incubation at 30ºC.

MFO parameters such as EROD activity and NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide reductases were measured in the microsomal fraction. Conjugating enzymes such as total GST and UDPGT were measured in the cytosolic and microsomal fraction, respectively. The antioxidant enzymes CAT, t-GPX, GR and SOD were determined in the cytosol as described in detail elsewhere (Raldúa et al., 2008; Fernández-Díaz et al., 2006).

All biomarkers data are calculated as mean values ± standard error mean (SEM). Significant differences among groups were tested by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test. Homogenous groups are indicated by the same upper letter. Level of significance was set up to $p<0.05$.

AhR gene expression. RNA isolation and quantification was performed as described elsewhere (Ábalos et al., 2008). Total RNA was reverse transcribed to cDNA using Omniscript RT-PCR kit (Qiagen, Valencia, CA, USA) and stored at −20 ºC. Specific transcripts were quantified by real time PCR in a ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primers used were the following Ábalos et al., 2008:

AhR: 5' TCTGAAGGGGACCTACTGCTCC 3' (sense)/5'AAGCCCAGGTAGTCCTTGATTGTAG 3' (antisense).

Actin: 5'CCTGGACTTCGAGCAGGAGA 3' (sense)/5' TCTCATTGCCGATGGTGATG 3' (antisense).

CYP4501A1: 5' TTCCYGAATACGGACGGCA 3' (sense)/ 5' CGTGCAATGACCTCTCCGAT 3' (antisense).

Relative mRNA abundance values were calculated according to Eq. (1) using threshold cycle (Ct) values from triplicate assays as previously described (Pfaffl, 2001).

$$\frac{mRNA_{Tg}}{mRNA_{Act}} = \frac{E_{Act}^{Ct,Act}}{E_{Tg}^{Ct,Tg}} \times 1000 \qquad (1)$$

3. Results

3.1. PCDD/F levels

The concentrations of PCDD/Fs, expressed in pg WHO-TEQ/g of fresh weight (f.w.) or lipid weight (l.w.), in different fish tissues from the animals sampled in June and December 2005, December 2006 and April 2007 are shown in Table 2. Mean values are reported together with the standard deviation (SD) of the results obtained from the two experimental tanks. In general, PCDD/F levels progressively decreased in the tissues during the "decontamination" period although some increases were observed in particular cases that are discussed in the next Section. Differences were found among tissues, sexes and also depending on the way the values were reported (i.e. fresh or lipid weight basis). The relative standard deviation (RSD%) between the animals from the two tanks was below 25% in most of the cases; higher values, up to a 75%, were occasionally obtained and are discussed further on.

The percentage of fat determined in the different samples is also presented in Table 3. These results are relevant in order to understand the PCDD/F trends observed in some particular cases.

3.2. Fish behavior

Fish growth. The growth (in weight) of the fish is shown in Figure 1 and Table 4. Growth was calculated and expressed three ways: (1) as weight gained per fish (g/fish), (2) as percentage of weight gain (%) and (3) as specific growth rate (SGR, % bw day-1). Similar to what was observed during the exposure period the fish continued growing normally during the "decontamination" period. SGR values were around 0.2 % day-1 reaching the animals a final average weight of 1265 g in December 2006. At final sampling date, in April 2007, the fish had lost weight (Final average weight: 1134 g) as a consequence of the spawning effort.

Sex distribution and sex reversal. In December 2006 all the fish were checked for sexual distribution. Among the 25 remaining fish, 14 were males (56%) and 11 were females (44%). After sampling fish for analysis, two broodstocks were formed with 4 males and 4 females each. However, when the fish were finally sacrificed in April 2007, once the spawning season was coming to an end, the population in each broodstock consisted of 5 females and 3 males. That is one of the males at each broodstock had changed to female.

Spawning and egg quality. Normal spawning season took place from December 2006 until April 2007. Eggs were collected initially everyday for quality check, but once the spawnings started to be produced daily, eggs were sample only once per week to check egg and larval quality.

3.3. Biochemical markers and AhR gene expression

The response of several biochemical markers is shown in Table 5. Long-term (13 months) dioxin exposure had little effect ($p>0.05$) on EROD activity and MFO reductases, while during the "decontamination" period a significant depletion on these activities was evidenced. All MFO components (i.e. EROD, NAD(P)H cytochrome c reductases and NADH ferrycianide reductase) evolved similarly and showed a good correlation among them ($r=0.506$-0.820; $p<0.05$). Conjugating enzymes (GST and UDPGT) also evolved similarly ($r=0.388$; $p<0.01$) although UDPGT correlated better with MFO reductases ($r=0.652$-0.672; $p<0.01$). Contrasting these phase II activities in control group vs. exposed group, despite both were elevated in the dioxin fed fish, only UDPGT activity reached significance. After 6 months decontamination (December 2005), both transferases were enhanced but decreased thereafter to values close to those of controls, although UDPGT activity remained significantly lower to controls. As far as the antioxidant enzymes concerns, only SOD activity was elevated in exposed fish vs. controls, while the other antioxidant enzymes were similar in both groups. During the "decontamination" period CAT activity was clearly reduced ($p<0.05$), whereas t-GPX and GR although initially decreased, this was not significant ($p>0.05$) and after 22 months decontamination were enhanced. In December 2006, SOD activity almost doubled the values at the end of the exposure period (June 2005) and it was 10-fold higher than the controls. This coincides with a strong depletion on EROD activity, the value being 33 and 27 times lower to those from the exposed fish and the controls (June 2005), respectively. On the other hand, while CAT was positively related to MFO parameters and UDPGT, the antioxidants SOD, t-GPX and GR showed no relationship or this was negative in relation to the other hepatic markers.

Relative abundances of mRNA corresponding to CYP1A and AhR were analyzed by qRT-PCR in control, exposed and decontaminated fish. No significant differences (Kruskal-Wallis, $p>0.05$) were observed in CYP1A expression among the three groups. In contrast, dioxin exposure increased levels of hepatic AhR mRNA by approximately four-fold, whereas decontaminated animals showed levels indistinguishable from those of the control fish included in the first part of the study (Figure 2).

4. Discussion

4.1. PCDD/F levels

During the exposure period to PCDD/Fs the levels of these contaminants were determined only in muscle and liver tissue, while during the "decontamination" period other tissues (i.e. perivisceral fat and gonads) were also included for analysis. The reason is that both, the size of the gonad and the amount of perivisceral fat are very small until the fish reach a certain weight. Therefore, during the exposure period the contribution of these tissues to PCDD/F bioaccumulation was considered negligible. On the other hand, separate analyses were carried out in males and females from December 2006 onwards in order to check possible differences in the bioaccumulation process between sexes.

Several studies have dealt with the trends in PCDD/F levels in different types of animals (i.e. pigs, broilers and hens) and tissues (i.e. fat, liver) when a withdrawal period is carried out following a previous exposure to the contaminants (Hoogenboom et al., 2004; Traag et al., 2006) . In general, PCDD/F concentrations decreased rapidly after a few weeks on clean feed. For the case of fish, Brambilla et al. (2007) carried out a study in which they followed the levels of several PCDD/Fs and PCBs in the muscle of farmed rainbow trout (Oncorhynchus mykiss) for a 3 months period after the fish had previously been exposed to the pollutants for 1 month. A significant decrease on the concentrations was also observed during the withdrawal period.

Therefore, according to the results of the abovementioned studies a gradual decrease in the levels of PCDD/Fs was expected during the "decontamination" period when compared with the concentration found at the end of the exposure period (June 2005) (Ábalos et al., 2008). The variation in the concentration of PCDD/Fs, expressed in fresh weight, in muscle and liver tissue of females sampled from June 2005 to April 2007 followed this decreasing trend. However, when these same results were expressed in lipid basis the levels of the contaminants showed an increase from December 2006 to April 2007 due to the significant loss of fat in both females' tissues during the last 4 months of the "decontamination period". Moreover, this decrease in the percentage of fat in female tissues varied depending on the tank where the fish were collected from. The fat content in the muscle of females decreased from 6.6% to 1.4% in D-1 and from 7.4% to 3.1% in D-2. Similarly, in the case of the liver tissue the percentages of fat diminished from 18.2% to 5.8% and from 25.4% to 18.3%, for D-1 group and D-2 group females, respectively. Therefore, a higher decrease in the fat content of the female tissues was observed in the D-1 group from December 2006 to April 2007. These differences between the two tanks gave rise to a high RSD% for the PCDD/F concentrations determined in the muscle and liver tissue of females in April 2007 when the results were expressed in fresh weight (i.e. 41% (muscle) and 75% (liver)); while the RSD% was considerably lower (i.e. 14% (muscle) and 2% (liver)) when the results were expressed in lipid basis.

In the case of male fish, the levels of PCDD/Fs gradually decreased in muscle and liver tissue from June 2005 to December 2006. Afterwards, the concentrations showed an increase again in both tissues in April 2007. Contrary to what was observed in females, the increase observed in these two tissues during the last months of the study was reflected both, in the results expressed either in fresh weight or in lipid basis. This increase seemed to indicate that part of the contaminants that were transferred from muscle, liver tissue and perivisceral fat to the gonads during the reproductive period were reallocated once the spawning period finished.

PCDD/F concentrations in perivisceral fat were first determined in June 2005, when this tissue represented about 1.5% of the total fish weight. At that moment only perivisceral fat from the D-1

group was analyzed. Nevertheless, this data made possible to see a decrease in the PCDD/F levels in this tissue from June 2005 to December 2006 and, particularly in the last sampling campaign both in males and females. The decrease is consistent with the fact that perivisceral fat is mobilized during the spawning season, being the levels of pollutants reduced at the same time.

PCDD/F levels were also determined in the gonads in December 2006 and April 2007, once the sex reversal took place and it was possible to distinguish between males and females. In the case of females, animals from the D-2 group showed similar PCDD/F concentrations, expressed in fresh weight, in the two sampling campaigns (approx. 0.60 pg WHO-TEQ/g f.w). On the contrary, the levels for D-1 group females increased from 0.31 to 0.65 pg WHO-TEQ/g f.w. Thus, the RSD% between the two tanks in December 2006 was 51% when the results were expressed in fresh weight. This increase might be related to the delay in egg release of D-1 group that started spawning on January 22nd, compared to D-2 group that started on January 15th. Therefore, in December 2006 the amount of fat and consequently the amount of contaminants mobilized to the gonads in the fish of D-1 group were less than the fat and contaminants mobilized in the case of the D-2 group. In addition, this would also explain that in December 2006 the RSD% expressed in lipid basis was only 13%. In the case of males, the fat content in the gonads was also mobilized during the spawning season being the pollutants removed at the same time. Therefore, the PCDD/F levels in the gonads, expressed in fresh weight, diminished from December 2006 to April 2007. However, when the results were expressed in lipid basis the concentrations increased due to the lower fat percentage that remained in male gonads at the end of the spawning period.

4.2. Fish behaviour

No effects of dioxin feeding were observed in the general behaviour, health and growth of the fish neither along the exposure period neither during the "decontamination" period. In addition, the proportions of sexes in December 2006 might be considered between the normal findings for this species. Furthermore, at the end of the spawning season a further sex change had occurred in each broodstock with one of the previously considered males changing to female.

As it has been already mentioned, dioxins are known to act as antiestrogens normally acting through the AhR inducing transcription of the phase I biotransformation CYP1A, among other target genes and its associated proteins, reducing the synthesis of vitellogenin or impairing gonadal development (Cheshenko et al., 2008; Denslow and Sepúlveda, 2007). However, the nature of the effect is determined by dose-dependent routing and cross-talk (concentration- and time-dependent interactions) between different classes of nuclear receptors (Goksoyr, 2006). This, or an adaptation of the fish to a chronic exposure to the endocrine disruptor, may have occurred in the present experiment since no changes in the normal proportion of sexes or in the frequency of sex reversal were observed in the fish

fed during 13 months with a low-dose dioxin-enriched diet followed by a 22 months "decontamination" period.

No effect was either observed in the spawning frequency, the quality of the spawned eggs and/or the quality of the larvae. Although a lower fertility rate than the normal findings (about 1 million eggs per female) was noted, these results must be carefully evaluated because (1) the frequency of spawning and quantity of eggs released were only checked in a weekly basis and the total amount of eggs produced during the spawning season was not recorded, and (2) not all the females contributed to the spawnings. From our own experience in previous studies involving a reduced number of broodfish, one female usually dominates and spawns whereas the other females contribute rarely and in a very low number of eggs per spawn (personal observations).

4.3. Biochemical markers

EROD activity is considered a very robust biomarker of AhR agonists exposure in several species, including gilthead seabream (Pretti et al., 2001; Ortíz-Delgado et al., 2002). Although formerly EROD activity was measured in the S9 fraction, comparisons are feasible as a correlation between EROD activity in S9 and microsomes was seen ($r = 0.871$; $n = 31$). During the first part of the project, after the fish were fed the dioxin-enriched diet for 10 months, EROD activity was doubled in the exposed group in relation to controls whereas GST and the antioxidants CAT and t-GPX were not significantly affected (Ábalos et al., 2008). However, at the end of the exposure period (June 2005) differences between the control and the exposed groups were shortened and lost significance. The reason for this lack of response after a longer exposure can be explained by similar findings in field observations. That is, in a field study with Atlantic killfish, Fundulus heteroclitus, an adaptation in fish from a heavily polluted site, that conferred them resistance to induction by AhR agonists, was seen (Bello, et al., 2001). The authors explained that the resistance acquired may be due to a genetic adaptation, although non genetic mechanisms, such as induction of proteins that repress AhR signalling, more likely to play a role in our case study, could also be involved.

During the "decontamination" period a great response in EROD (and SOD) activity was seen in the fish in December 2006. This coincides with either male specimens undergoing sex change into females or specimens already recently turned into females. Although sex hormones were not measured in these specimens, steroids such as testosterone but more significantly estradiol are reported to be strong EROD inhibitors in several fish species, including gilthead seabream (Teles et al., 2005; Pérez-Carrera et al., 2007). Other MFO components showed a similar behaviour to EROD. That is, dioxin exposure did not significantly affect any of the reductases considered but some were also significantly affected during the sex reversal and onwards. Similarly, a relative lower sensitivity of reductases towards AhR ligand compounds has been reported for Sparus aurata injected with □-naphtoflavone, where a 58-fold

increase in EROD activity was not reflected in an elevation on NADPH cytochrome c reductase activity or phase II GST and UDPGT responses (Pretti et al., 2001).

In fact, conjugating enzymes GST and UDPGT responses in relation to dioxin exposure are more controversial probably due to the unspecific nature of the substrates used: CDNB (1-chloro-2,4-dinitrobenzene) and pNP (p-nitrophenol), respectively. In our study, UDPGT activity differed significantly between groups (exposed vs. control in June 2005 and fish after 6 and 18 months decontamination), while no significant differences were seen in GST, similarly to what was already observed in the first part of the study (Ábalos et al., 2008). A lower response to TCDF, in relation to EROD activity, was recorded in Atlantic killfish, Fundulus heteroclitus, UDPGT activity. Although fish from a polluted site expressed 60% of the activity of those from the reference site, TCDF injection of those fish did not affect their UDPGT activity neither in those from the reference nor polluted sites. On the contrary, GST response varied depending on the sex and origin of the fish (Bello et al., 2001). Similarly to our findings, carp UDPGT was down-regulated during sexual maturation (Sikoki et al., 1989) while in eelpout no significant variations were recorded in phase II enzymes during the spawning season (Ronisz et al., 1999).

At the end of the dioxin-fed period an oxidative stress situation was evidenced in the exposed group by enhanced SOD activity (produces H2O2 from O2-) although this was not reflected in other antioxidant activities either in here or in the former study (Ábalos et al., 2008). In fact, not many fish studies have explored the effect of dioxin exposure over the antioxidant responses and those that did, they failed to see an effect (Pretti et al., 2001; Palace et al., 1996) despite evidences of an effect in other vertebrates (Senft et al., 2002) and in killfish embryonic stages exposed to a dioxin-like chemical such as PCB126 (Arzuaga et al., 2006). On the contrary, in the case of gilthead seabream sex change had a more profound effect over the antioxidant response since CAT and SOD were both affected, while no significant variations were recorded in CAT, Se-GPX and GR during the spawning season in male eelpout (Ronisz et al., 1999).

Dioxin exposure did not induce changes in CYP1A gene expression levels in the first part of the study, whereas levels of hepatic AhR mRNA were significantly higher in exposed fish than in controls (Ábalos et al., 2008). Our results indicate that CYP1A mRNA levels remained stable during all the process of exposure and decontamination, while AhR mRNA levels in exposed fish returned to values typical for non-exposed fish after a decontamination period of almost two years. These results confirm our previous observations that hepatic AhR mRNA levels constitute a sensitive marker for dioxin exposure.

5. Conclusions

Concentrations of PCDD/F in muscle and liver tissue of aquacultured gilthead seabream exposed to a low dioxin level trough the diet showed a marked increase after 3 months of exposure. Later, these levels remained similar during a long-term exposure period (13 months). When dioxin exposure was stopped, and the fish were fed on a clean feed for another 22 months, PCDD/F levels in the different tissues analyzed showed a decreasing trend. However, this general trend varied among tissues and was also dependent on sex and lipid contents. No effects of the low dioxin level exposure during the ongrowing period were observed on the fish in terms of growth, survival, behaviour, sex determination and reproduction, being the proportion of sexes of the exposed group similar to the ratios obtained in fish farms for this species. Egg spawning time and duration were also similar to what is commonly observed in farmed fish as well as the quality of the eggs and larvae obtained. Several hepatic responses were evaluated in order to relate toxicological effects to dioxin exposure. EROD activity in exposed fish showed a significant increase compared to non-exposed fish after 10 months of feeding the dioxin spiked feed. However, at the end of the exposure period (13 months) these differences were reduced. Afterwards, during the "decontamination" period, some biochemical markers showed significant variations in their responses that could be more linked to the sex differentiation process than to a reduced dioxin-supply. The levels of hepatic AhR mRNA, which increased during dioxin exposure, also returned to values similar to that of non-exposed fish after the "decontamination" period.

6. Acknowledgments

This project was supported by the Spanish National Plan for Research PN2003 ref. AGL2003 02283-ALI and "Proyectos Intramurales de 30 de Marzo de 2004. Consejo Superior de Investigaciones Científicas (CSIC), Ministerio de Educación (200680I106)". Additional funding from Ministerio Ciencia e Innovación (CGL2008-01898) is also acknowledged. M. Ábalos acknowledges an I3PDR contract from CSIC in the framework of the I3P project. Thanks are due to M. Monllaó and O. Bellot (IRTA) for their help during fish rearing and tissue sampling.

References

Ábalos, M., Abad, E., Estévez, A., Solé, M., Buet, A., Quirós, L., Piña, B., Rivera, J., 2008. Effects on growth and biochemical responses in juvenile gilthead seabream '*Sparus aurata*' after long-term dietary exposure to low levels of dioxins. Chemosphere 73, S303–S310.

Arzuaga, X., Wassenberg, D., Di Giulio, R., Elskus, A., 2006. The chlorinated AHR ligand 3,3',4,4',5-pentachlorobiphenyl (PCB126) promotes reactive oxygen species (ROS) production during embryonic development in the killifish (*Fundulus heteroclitus*). Aquat. Toxicol. 76, 13–23.

435 Bello, S.M., Franks, D.G., Stegeman, J.J., Hahn, M.E., 2001. Acquired Resistance to Ah Receptor
436 Agonists in a Population of Atlantic Killifish (*Fundulus heteroclitus*) Inhabiting a Marine Superfund
437 Site: In Vivo and in Vitro Studies on the Inducibility of Xenobiotic Metabolizing Enzymes. Toxicol.
438 Sci. 60, 77–91.

439 Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of
440 protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

441 Brambilla, G., Dellatte, E., Fochi, I., Iacovella, N., Miniero, R., Di Domenico, A., 2007. Depletion of
442 selected polychlorinated biphenyl, dibenzodioxin, and dibenzofuran congeners in farmed rainbow trout
443 (*Oncorhynchus mykiss*): A hint for safer fish farming. Chemosphere 66, 1019–1030.

444 Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008. Interference of endocrine
445 disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction
446 of teleost fish. Gen. Comp. Endocr. 155, 31–62.

447 Commission Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in
448 foodstuffs. Off. J. Eur. Union L364, 5–24.

449 Denslow, N., Sepúlveda, M., 2007. Ecotoxicological effects of endocrine disrupting compounds on
450 fish reproduction. In: The Fish Oocyte: From Basic Studies to Biotechnological Applications. Babin
451 PJ, Cerdà J, Lubzens E (eds.), Springer, Netherlands, pp. 255–322.

452 Fernández-Díaz, C., Kopecka, J., Cañavate, J.P., Sarasquete, C., Solé, M., 2006. Variations on
453 development and stress defences in *Solea senegalensis* larvae fed on live and microencapsulated diets.
454 Aquaculture 251, 573–584.

455 Giesy, J.P., Snyder, E.M., 1998. Xenobiotic Modulation of Endocrine Function in Fishes. In:
456 Principles and Processes for Evaluating Endocrine Disruptors in Wildlife. Kendall RJ, Dickerson RL,
457 Giesy JP, Suk WA (eds.), SETAC Press, Pensacola, FL, pp. 155–237.

458 Goksoyr, A., 2006. Endocrine Disruptors in the Marine Environment: Mechanisms of Toxicity and
459 their Influence on Reproductive Processes in Fish. J. Toxicol. Env. Heal. A 69, 175–184.

460 Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2010. Ovarian aromatase and estrogens: A pivotal
461 role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocr. 165, 352–366.

462 Hahn, M.E., 2001. Dioxin toxicology and the aryl hydrocarbon receptor: Insights from fish and other
463 non-traditional models. Mar. Biotechnol. 3, S224–S238.

Happe, A. and Zohar, Y., 1988. Self fertilization in the protandrous hermaphrodite Sparus aurata: development of the technology. In: Reproduction in fish – Basic and applied aspects in endocrinology and genetics. Zohar Y, Breton B (eds.), INRA, Paris, France, pp. 177–180.

Hoogenboom, L.A.P., Kan, C.A., Bovee, T.F.H., Van der Weg, G., Onstenk, C., Traag, W.A., 2004. Residues of dioxins and PCBs in fat of growing pigs and broilers fed contaminated feed. Chemosphere 57, 35–42.

Liem, A.K.D., Fürst, P., Rappe, C., 2000. Exposure of populations to dioxins and related compounds. Food. Addit. Contam 17, 241–259.

McGregor, D.B., Partensky, C., Wilbourn, J., Rice JM, 1998. An IARC evaluation of polychlorinateddibenzo-p-dioxins and polychlorinated dibenzofurans as riskfactors in human carcinogenesis. Environ. Health Perspect. 106, 755–760.

Ortíz-Delgado, J.B., Sarasquete, C., Behrens, A., Gonzalez de Canales, M.L., Segner, H., 2002. Expression, cellular distribution and induction of CYP1A in gilthead seabream, Sparus aurata, brain. Aquat. Toxicol. 60, 269–283.

Pérez-Carrera, E., García-López, A., Martín del Río, M.P., Martínez-Rodríguez, G., Solé, M., Mancera, J.M., 2007. Effects of 17beta-estradiol and 4-nonylphenol on osmoregulation and hepatic enzymes in gilthead sea bream (*Sparus auratus*). Comp. Biochem. Physiol. C 145, 210–217.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29:e45.

Pretti, C., Salvetti, A., Longo, V., Giorgi, M., Gervasi, P.G., 2001. Effects of beta-naphthoflavone on the cytochrome P450 system, and phase II enzymes in gilthead seabream (Sparus aurata). Comp. Biochem. Physiol. C 130, 133–144.

Palace, V.P., Klaverkamp, J.F., Lockhart, W.L., Metner, D.A., Muir, D.C.G., Brown S.B., 1996. Mixed Function Oxidase Enzyme Activity and Oxidative Stress in Lake Trout (Salvelinus namaycush) Exposed to 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). Environ. Toxicol. Chem. 15, 955–960.

Raldúa, D., Padrós, F., Solé, M., Eljarrat, E., Barceló, D., Riva, M.C., Barata, C., 2008. First evidence of polybrominated diphenyl ether (flame retardants) effects in feral barbel from the Ebro River basin (NE, Spain). Chemosphere 73, 56–64.

Safe, S. H., 1986. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Annu. Rev. Pharmacol. Toxicol. 26, 371–399.

494 Safe, S. H., 1999. Effects on Male and Female Reproductive Systems, in: Endocrine Disruptors. Naz
495 RK (ed.), CRC Press, Boca Raton, FL, pp. 187–221.

496 Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Hutchinson, R.J., Shertzer, H.G., 2002. Dioxin
497 increases reactive oxygen production in mouse liver mitochondria. Toxicol. Appl. Pharm. 178, 15–21.

498 Sikoki, F., Tubb, R., and Curtis, L., 1989. Elevation of sex-steroids and inhibition of UDP-
499 glucuronyltransferase are out of phase during gonadal maturation in the common carp. Comp.
500 Biochem. Physiol. 92 C, 267–272.

501 Ronisz, D., Larsson, D.G.J., Förlin, L., 1999. Seasonal variations in the activities of selected hepatic
502 biotransformation and antioxidant enzymes in eelpout (Zoarces viviparus). Comp. Biochem. Physiol.
503 C 124, 271–279.

504 Teles, M., Pacheco, M., Santos, M.A., 2005. *Sparus aurata* L. liver EROD and GST activities, plasma
505 cortisol, lactate, glucose and erythrocytic nuclear anomalies following short-term exposure either to
506 17ß-estradiol (E2) or E2 combined with 4-nonylphenol. Sci. Total Environ. 336, 57–69.

507 Traag, W.A., Kan, C.A., Van der Weg, G., Onstenk, C., Hoogenboom, L.A.P., 2006. Residues of
508 dioxins (PCDD/Fs) and PCBs in eggs, fat and livers of laying hens following consumption of
509 contaminated feed. Chemosphere 65, 1518–1525.

510 Van den Berg, M., De Jongh, J., Poiger, H., Olson, J.R., 1994. The Toxicokinetics and Metabolism of
511 Polychlorinated Dibenzo-p-Dioxins (PCDDs) and Dibenzofurans (PCDFs) and Their Relevance for
512 Toxicity. Crit. Rev. Toxicol., 24, 1–74.

513 Van den Berg, M,, Birnbaum, L., Bosveld, A.T.C., Brunström, B., Cook, P., Feeley, M., Giesy, J.P.,
514 Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Liem,
515 A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M.,
516 Younes, M., Waern, F., Zacharewski T., 1998. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs,
517 PCDFs for Humans and Wildlife. Environ. Health Persp. 106, 775–792.

518 Wong, T.T., Ijiri, S., Zohar, Y., 2006. Molecular Biology of Ovarian Aromatase in Sex Reversal:
519 Complementary DNA and 50-Flanking Region Isolation and Differential Expression of Ovarian
520 Aromatase in the Gilthead Seabream (*Sparus aurata*). Biol. Reprod. 74, 857–864.

521 Zohar, Y., Abraham, M., Gordin, H., 1978. The gonadal cycle of the captivity-reared hermaphroditic
522 teleost *Sparus aurata* (L.) during the first two years of life. Ann. Biol. Anim. Bioch. Biophys. 18, 877–
523 882.

524 Zohar, Y., Billard, R., Weil, C., 1984. La reproduction de la daurade (*Sparus aurata*) et du bar
525 (Dicentrarchus labrax): connaissance du cycle sexuel et contrôle de la gamétogenèse et de la ponte. In:
526 "L'Aquaculture du Bar et des Sparides". Barnabé G, Billard R (eds.), INRA, Paris, France, pp. 3–24.

527

528 Table 1.- Number of fish sacrificed from the two experimental groups (D-1 and D-2) in each sample
529 campaign.

Campaign	Number of fish sacrificed		Tissues analyzed
	Males	Females	
December 2005	3 (D-1); 3 (D-2)		Muscle, liver, perivisceral fat
December 2006	4 (D-1); 2 (D-2)	1 (D-1); 2 (D-2)	Muscle, liver, perivisceral fat, gonad
April 2007	3 (D-1); 3 (D-2)	5 (D-1); 5 (D-2)	Muscle, liver, gonad

530

531

532

533 Table 2.- PCDD/F concentrations in the different fish tissues. Results are expressed in pg WHO-TEQ/g in upperbound levels. Mean values ± SD of n=2
534 tanks are reported in each case.

535

Campaign	Muscle				Liver				Perivisceral fat		Gonad			
	Males		Females		Males		Females		Males	Females	Males		Females	
	Fresh weight	Lipid weight	Fresh weight	Lipid weight	Fresh weight	Lipid weight	Fresh weight	Lipid weight	Lipid weight		Fresh weight	Lipid weight	Fresh weight	Lipid weight
June 2005	2.21 ± 0.53	49.0 ± 0.8			12.92 ± 0.70	56.1 ± 1.4			55.3 *					
December 2005	1.64 ± 0.46	37.2 ± 4.4			9.10 ± 2.02	37.2 ± 4.7			44.9 ± 2.2					
December 2006	0.53 ± 0.04	10.1 ± 1.1	0.86 ± 0.14	12.3 ± 1.1	3.94 ± 0.89	13.2 ± 1.0	3.14 ± 0.65	14.5 ± 0.6	14.1 ± 2.5	19.5 ± 1.1	0.27 ± 0.01	8.6 ± 0.3	0.49 ± 0.25	11.3 ± 1.5
April 2007	1.20 ± 0.20	26.4 ± 2.0	0.52 ± 0.21	22.8 ± 3.1	4.31 ± 0.34	28.8 ± 6.2	2.76 ± 2.06	22.8 ± 0.4			0.14 ± 0.02	13.3 ± 0.9	0.62 ± 0.04	17.1 ± 2.3

* Single measure

536 Table 3.- Percentages of fat (%) in the different fish tissues from the two experimental groups (D-1 and
537 D-2).

538

539

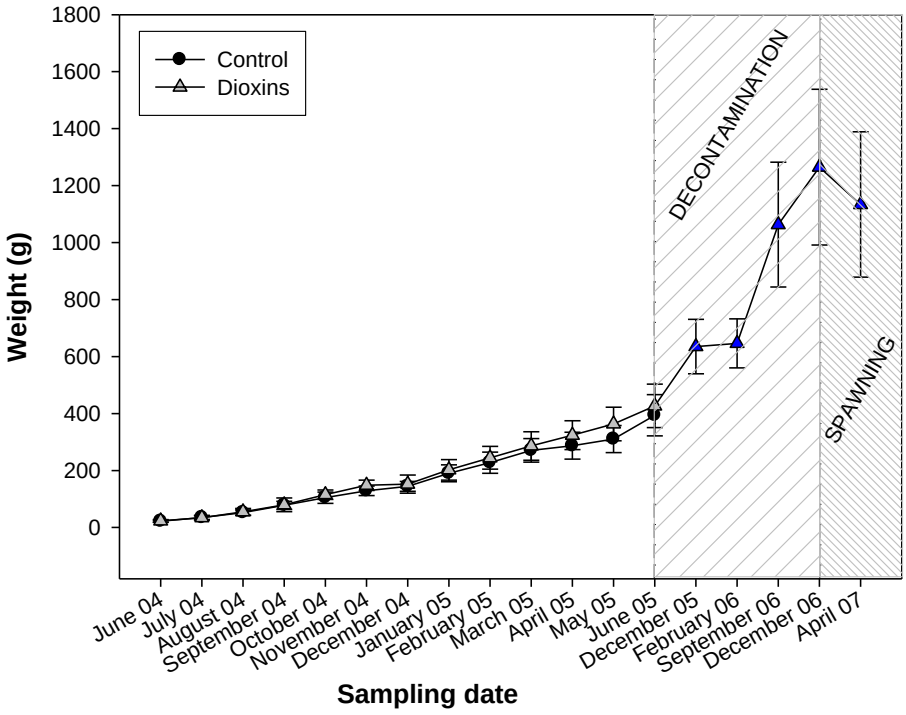
Campaign	Muscle				Liver				Gonad			
	Males		Females		Males		Females		Males		Females	
	D-1	D-2	D-1	D-2	D-1	D-2	D-1	D-2	D-1	D-2	D-1	D-2
June 2005	5.3	3.7			23.9	21.9						
December 2005	4.9	3.8			26.2	21.8						
December 2006	5.5	5.1	6.6	7.4	26.5	32.8	18.2	25.4	3.5	3.1	2.5	6.5
April 2007	3.8	5.4	1.4	3.1	13.7	17.5	5.8	18.3	1.0	1.1	3.4	3.9

540

541

542 Figure 1.- Increase in body weight of exposed/decontaminated fish and control fish. Bars indicate the
543 SD from *n*=2 tanks (*n*=5 fish per tank) in each case.

544



545

546 Table 4.- Monthly growth of the fish. Mean values of *n*=2 tanks (*n*=5 fish per tank) are presented.

547

Month	**Average weight (g)**	**Weight gain (g/fish)**	**Weight gain (%)**	**SGR (%bw day^{-1})**
June 05	426.9	63.5	17.5	0.53
December 05	635.1	208.2	48.8	0.22
February 06	646.1	11.0	1.7	0.03
September 06	1063.1	417.0	64.5	0.24
December 06	1265.0	201.9	19.0	0.19
April 07	1133.8	-131.2	-10.4	-0.09

Weight gain (%) = (w_f-w_i/w_i * 100)
Specific growth rate (SGR, %bw day^{-1}) = 100 * (e^G-1); being G = Ln (w_f/w_i)/ t_f-t_i
w_f, w_i, t_f, t_i are the final and initial weight (w) and the final and initial time (t), respectively

548

549

550 Table 5. MFO parameters, conjugating and antioxidant enzyme activities in microsomes and cytosol of
551 gilthead seabream, *Sparus aurata*.

	June 2005		December 2005	December 2006	April 2007
	Controls (n=10)	Exposed (n=10)	Decontaminated for 6 months (n=6)	Decontaminated for18 months (n=9)	Decontaminated for 22 months (n=16)
MFO parameters					
EROD[1]	533 ± 48 *ab*	653 ± 84 *b*	362 ± 72 *b*	19.7 ± 3.8 *c*	163.4 ± 54.5 *c*
NADPH cyt c red[2]	20.3 ± 0.8 *c*	21.5 ± 0.6 *cd*	24.4 ± 1.0 *d*	16.7 ± 0.9 *b*	12.9 ± 1.2 *a*
NADH cyt c red[2]	47.9 ± 3.2 *c*	45.4 ± 2.2 *bc*	38.0 ± 2.1 *ab*	40.9 ± 2.8 *bc*	31.3 ± 1.8 *a*
NADH ferricyanide red[2]	1490 ± 68 *a*	1604 ± 60 *a*	1621 ± 69 *a*	1103 ± 133 *b*	982.8 ± 62.6 *b*
Conjugating enzymes					
GST[2]	437 ± 31 *ab*	462 ± 28 *bc*	608 ± 46 *b*	513.6 ± 40.7 *bc*	361.7 ± 39.9 *a*
UDPGT[2]	386 ± 37 *b*	493 ± 32 *c*	639 ± 29 *d*	272.5 ± 37.4 *a*	235.2 ± 18.1 *a*
Antioxidant enzymes					
CAT[3]	356 ± 14 *a*	360 ± 19 *a*	245 ± 10 *b*	134.1 ± 12.4 *c*	161.5 ± 25.3 *c*
SOD[4]	1.0 ± 0.1 *a*	5.3 ± 1.5 *b*	4.9 ± 2.3 *b*	9.4 ± 0.8 *c*	6.4 ± 0.8 *bc*
t-GPX[2]	88.4 ± 2.3 *a*	80.8 ± 2.9 *ab*	61.5 ± 5.4 *b*	69.8 ± 5.5 *ab*	87.3 ± 8.0 *a*
GR[2]	4.9 ± 0.2 *a*	4.3 ± 0.4 *a*	3.7 ± 0.6 *a*	3.6 ± 0.4 *a*	7.3 ± 0.7 *b*

552 Mean values ± SEM from *n*=number of fish measured, are reported in each case. Different letters
553 indicate statistical significance ($p<0.05$).
554 [1] pmol/min/mg prot
555 [2] nmol/min/mg prot
556 [3] μmol/min/mg prot
557 [4] a.u/mg prot
558

559 Figure 2.- AhR mRNA abundance in control, exposed and decontaminated fish. Boxplot show medians
560 (horizontal black bars), 2nd and 3rd quartiles (boxes) and total ranges (vertical bars) of values for each
561 population, expressed as ‰ of reference gene (ß-Actin), are shown. Letters in brackets indicate
562 statiscally homogeneous sets of samples (ANOVA plus Tukey's).

563
564
565