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Abstract: Normal functioning of the endocrine system is essential for the proper development and reproduction of animals. Substances interfering with its homeostasis are called endocrine disruptors (EDs) and may represent a risk for the health of the organism. One of the mechanisms of endocrine disruption that has attracted great attention in recent years concerns alterations in the normal functioning of the estrogen receptor (ER), but far less attention has been paid to those substances interfering with the thyroid axis, which, in fish, plays several critical roles in a variety of biological functions. In aquaculture, feedstuffs can be a source of hormones or persistent pollutants which act as potential EDs. In this study, the main purpose was to assess the possible estrogenic and thyrogenic activities of 32 commercial fish feeds. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay using sea bass ERα (sbER α) was developed and validated. Potential thyroidal disruption was screened with a cell line permanently transfected with luciferase as reporter gene under the control of avian (av) thyroid receptor α (THRα). The results obtained showed that 11 and 18 out of 32 assayed feeds were able to activate the sbERα or the avTHRα1, respectively. The present study is pioneer in demonstrating thyrogenic activity in fish diets commercially available and widely used in aquaculture. Given that maintaining the homeostasis in the endocrine system is critical for the proper development and reproduction of fish, any estrogenic or thyrogenic activity caused by the feedstuffs should be taken into account with regards to its potential impact on farmed fish.

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Dr. Mosconi has always worked on fish physiology and in the last years has moved to the study of the interaction of pollutants with the reproductive physiology of fish.

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Dr. Ortiz-Zarragoitia has a wide expertise in the field of ecotoxicology. Since the article we are sending deals with estrogenic and thyrogenic activities of fish feed, Dr Ortiz-Zarragoitia can act as reviewer due to his knowledge about the effect of pollutants on the endocrine system of fish.

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Dr. Hoogenboom has a broad expertise in the field of residue contaminants in feed and food. He has performed a variety of studies related with the detection of very different contaminants in feed, including estrogenic substances, growth-promoting agents, veterinary drugs and environmental dioxin-like substances.

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Madrid, 11th October 2011

Dear Dr. Gatlin,

Please, find together with this letter all the uploaded documents corresponding to the manuscript entitled "Assessment of estrogenic and thyrogenic activities in fish feeds", by Alba Quesada, Ana Valdehita, Mª Luisa Fernández-Cruz, Esther Leal, Elisa Sánchez, Mónica Martín-Belinchón, José M. Cerdá Reverter y José M. Navas.

The estrogenicity and thyrogenic activity of fish feed extracts was assessed by means of cell lines containing the estrogen receptor or the thyroid receptor and reporter genes under the control of the corresponding receptor. For the assessment of estrogenic activities a new cell line containing the sea bass estrogen receptor has been developed. Although some estrogenic activity could be detected, strikingly a very strong thyroidal activity caused by fish feed extracts was observed.

English language has been checked by professional translators.

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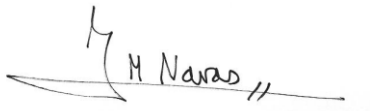
Thank you very much for your time and effort.

Sincerely yours,



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Highlights

Estrogenic and thyrogenic activities in fish feed extracts were assessed

A new stably transfected cell line with the sea bass estrogen receptor was used

Estrogenic activity was detected in 8 out of 32 tested pellets

A strong activation of the thyroid receptor was caused by 21 of the 32 tested feeds

Assessment of estrogenic and thyrogenic activities in fish feeds

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Abstract

Normal functioning of the endocrine system is essential for the proper development and reproduction of animals. Substances interfering with its homeostasis are called endocrine disruptors (EDs) and may represent a risk for the health of the organism. One of the mechanisms of endocrine disruption that has attracted great attention in recent years concerns alterations in the normal functioning of the estrogen receptor (ER), but far less attention has been paid to those substances interfering with the thyroid axis, which, in fish, plays several critical roles in a variety of biological functions. In aquaculture, feedstuffs can be a source of hormones or persistent pollutants which act as potential EDs. In this study, the main purpose was to assess the possible estrogenic and thyrogenic activities of 32 commercial fish feeds. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay using sea bass ERα (sbER α) was developed and validated. Potential thyroidal disruption was screened with a cell line permanently transfected with luciferase as reporter gene under the control of avian (av) thyroid receptor α (THRα). The results obtained showed that 11 and 18 out of 32 assayed feeds were able to activate the sbERα or the avTHRα1, respectively. The present study is pioneer in demonstrating thyrogenic activity in fish diets commercially available and widely used in aquaculture. Given that maintaining the homeostasis in the endocrine system is critical for the proper development and reproduction of fish, any estrogenic or thyrogenic activity caused by the feedstuffs should be taken into account with regards to its potential impact on farmed fish.

Keywords: fish feed, endocrine disruptors, thyroid hormones, estrogen, TR and ER.

1. Introduction

Endocrine disruptors (EDs), as defined by the World Health Organization (WHO), are "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effect in an intact organism, its progeny, or (sub) populations" (WHO/IPCS 2002). The impact of EDs is of particular concern in teleost fish since these animals are exposed to waterborne contaminants during their whole life span. Intensive fish culture makes feedstuffs an alternative vehicle for the incorporation of persistent EDs. The principal potential sources of feed contamination are ingredients of animal origin, and fish aquaculture diets are not an exception (Pelissero and Sumpter, 1992; Mantovani et al., 2009). Long-term sustainability of intensive aquaculture requires the replacement of fish meal and fish oils in aquafeeds by vegetable equivalents (Drew et al., 2007; Gatlin et al., 2007; Glencross et al., 2007). However, plant meals used as substitutes also contain substantial quantities of EDs that negatively affect fish physiology (Pelissero and Sumpter, 1992; Matsumoto et al., 2004; Beresford et al., 2011). Soybean meal is the main source of vegetable protein present in animal diets, although a large number of studies have shown that a high dietary percentage of soybean meal may result in decreased growth and reproductive changes in fish (Pelissero and Sumpter, 1992; Drew et al., 2007). The poor growth rates exhibited by fish fed diets rich in soy flour have been attributed to the presence of estrogenic isoflavones, e.g. daidzein and genistein, in the bile of these fish (Kausik et al., 1995). In fact, estrogenicity of commercial fish feeds has already been assessed using yeast estrogen-screen assays (Matsumoto et al., 2004) or *in vivo* experiments (Beresford et al., 2011).

Estrogenic substances can emulate the action of the endogenous estrogen via activation of the estrogen receptors ERs which work as ligand-activated transcription factors. Following agonist binding, the receptor undergoes a conformational change which enhances its affinity for DNA, where it interacts with specific sequences called estrogen responsive elements (ERE), inducing the

74 expression of estrogen-dependent genes (Beato and Klug, 2000). These genes are mainly related to
75 reproduction, differentiation and growth. However in teleosts, estrogens are involved in immune
76 system regulation and several studies have related estrogen-like disruptors with immunosuppression
77 (Milla et al., 2011). For all the above, anti/estrogenic compounds have received substantial attention
78 in recent years (Hotchkiss et al., 2008).
79 Unfortunately, far less attention has been paid to the detection of substances that may disrupt the
80 hypothalamus-pituitary-thyroid axis (HPT). The thyroid of fish secretes L-thyroxine (T4) into the
81 circulation. T4 enters target cells, where it undergoes monodeiodination to 3,3',5-triiodo-L-
82 thyronine (T3). Thyroid hormones (THs) play critical roles in growth, metabolism and development
83 in all vertebrates (Yen, 2001). But, in fish and amphibians, the thyroid axis also plays a key function
84 in normal development and metamorphosis, larval stages being particularly sensitive to the
85 disruption of the HPT axis (Blanton and Specker, 2007; Carr and Patiño, 2011). There is also
86 evidence that TH may be involved in gonadal sex differentiation, probably via its action on
87 aromatase activity (Mukhi et al., 2007), but also in the proliferation of Sertoli and Leidyg cells and,
88 by extension, in the testis development and function (Matta et al., 2002).
89 In recent years, many chemicals have been suspected of acting as thyroid disruptors including some
90 polychlorinated biphenyls (PCBs), tetrabromobisphenol A (TBBPA) and polybrominated diphenyl
91 ethers (PBDEs) (Boas et al., 2006). These substances may compete with the endogenous hormones
92 for binding to transport proteins (transthyretin) and/or to TH receptors (TR), acting as either
93 agonists or antagonists and disrupting TH homeostasis (Kashiwagi et al., 2009; Boas et al., 2006).
94 THRs, together with the steroid receptors, belong to the nuclear receptor family and act as ligand-
95 dependent transcription factors which bind to a specific region of the DNA named TRE (thyroid
96 hormone responsive element). Previous studies have found considerable levels of PCBs and dioxin-
97 like substances in fish feeds (Berntssen et al., 2010), where they could mimic endogenous TH and
98 potentially lead to thyroid disruption. To the best of our knowledge, the presence of thyroidal

disruptors in fish diets has never been tested. The aim of this study was to simultaneously assess the potential estrogenic and thyrogenic activity of 32 commercial fish diets using hormone receptor-mediated reporter gene activation. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay, using sea bass ERα (sbERα) was developed (Muriach et al., 2008). The assay was validated using 17ß-estradiol (E2) analogues and ER antagonists and through the screening of sewage effluent samples, previously reported as containing considerable estrogen loads (Carbonell et al., 2010). Potential thyroid disruption was screened with a reporter gene under the control of avian (av) THRα1 (Jugan et al., 2007). We demonstrate that extracts from 11 of the 32 assayed fish diets activated sbERα, while, 18 diets activated avTHRα1.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (≥98% purity), tamoxifen (≥99% purity), 17-α-estradiol (≥ 98% purity), 17-α-methyltestosterone (≥ 97% purity), 3′, 5-Triiodo-l-thyronine (T3, ≥98% purity), etnylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), methanol (≥99.9% purity), tricineKOH, bovine serum albumina (BSA), $MgCl_2$, isobutylmethylxanthine (IBMX), dithiothreitol (DTT), ATP, Coenzyme A hydrate (CoA) and luciferin were purchased from Sigma-Aldrich (Madrid, Spain).. Fetal bovine and horse serum (FBS and FHS), ultraglutamine, penicillin-streptomycin (10.000 U/ml), hygromycin, tripsin, geneticin, ultraglutamine 1, and cell culture Dulbecco's Minimal Essential Medium (DMEM) were obtained from Lonza (Barcelona, Spain). Phenol red-free DMEM was from PanBiotech (Zaragoza, Spain). The stock solutions of E2, 17-α-estradiol, tamoxifen and T3 were prepared in DMSO; 17α-methyltestosterone was dissolved in ethanol.

2.2. Extraction of EDs present in fish food

Thirty two commercially available fish feeds were tested for estrogenicity and thyroidal activity. The extraction of estrogenic and thyrogenic substances was carried out with methanol as previously described (Cerdá-Reverter et al., 1996; Rodríguez et al., 2000; Matsumoto et al., 2004) with minor modifications: 0.5 g of each diet were sonicated in 2.5 ml of methanol using Vibra Cell ™ ultrasonic probe (Sonic & Materials Inc., Newtown, CT, USA) at 18 KHz in three pulses of 15 seconds (70% amplitude). Homogenates were then centrifuged at 1700 xg for 10 minutes. Supernatants were vacuum–dried and resuspended in 300 µl of methanol. The extracts were maintained at -20 ºC until their assessment in the cellular assays.

Overloading tests were designed to evaluate the efficiency of the extraction method. Briefly, 300 µl of a solution of, either 100 µM E2 or 100 nM T3, were added to 0.5 g of a diet showing no estrogenic or thyrogenic activity and extracted as above. The resulting extracts were named as E2–feed and T3-feed. In order to ensure the proper recovery of the hormones in the methanol and their stability during the extraction process, hormone solutions of E2 and T3 were submitted to the same extraction process. These extracts were named E2-MeOH, and T3-MeOH and contained a concentration of either 100 µM E2 or 100 nM T3, respectively.

Additionally, in a set of fish feeds, this process was performed using hexane as described by Ramos et al. (2004) in order to extract non-polar substances.

2.3. Generation of HER-LUC cell line

The HEK-293 cell line, stably expressing sbERα (Muriach et al., 2008) was cotransfected, in proportion 50:1, with a construct (ERE-TK-LUC) containing the luciferase gene under the control of tandem repetitions of the estrogen responsive element (ERE; Muriach et al., 2008) and a construct carrying resistance to puromycin (Muñoz et al., 2005). Cells were grown in 96-well plates and selected with DMEM (Invitrogen) containing 10% foetal bovine serum (Invitrogen),

149 penicillin (100 units/ml), streptomycin (100 μg/ml) and puromycin (8μg/ml; Invitrogen) in a
150 humidified atmosphere of 5% CO_2 at 37ºC. Luciferase activity was tested after incubating
151 resistant clones in 96-well plates (15,000 cells/well) with assay medium (DMEM medium +
152 0.1mg/ml bovine serum albumin, BSA + 0.1 mM, IBMX) containing 10^{-6}- 10^{-12} M estradiol.
153 Forty eight hours post-treatment cells were, washed twice with saline phosphate buffer,
154 resuspended into 100 μl of reporter lysis buffer (Promega) and stored at -80 ºC until luciferase
155 activity determinations. Lysed cells were pelleted by centrifugation for 30s at 15000 xg, and 20
156 μl of the supernatant were mixed with 200 μl of luciferin reagent (20 mM TricineKOH, pH 7.8,
157 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 270 μM CoA, 530 μM ATP, 400 μM luciferin).
158 The light emitted was measured in a luminometer (Junior, EG&G, Berthold). The most sensitive
159 cell clone was named as HER-LUC and was selected for subsequent studies. To evaluate
160 unspecific responses due to any basal luciferase transcription activity, HEK 293 cells were
161 transiently transfected only with ERETKLUC construct and exposed to equivalent E2
162 concentrations.
163
164 *2.4. Cell culture*
165 HER-LUC cells were grown in 75 cm^2 flasks under 5 % CO_2 humidified atmosphere at 37ºC in
166 DMEM supplemented with 10% FBS, 1% antibiotic mixture (Penicillin/Streptomycine) and 2%
167 Ultraglutamine.
168 The PC-DR-LUC cell line derived from PC-12 cells stably expressing the avTRα1 (Muñoz et al.,
169 1993) and the luciferase reporter gene (Jugan et al., 2007) was used to assess the thyroidal activity
170 of the fish feeds. Cells were grown in 75 cm^2 flasks under 5% CO_2 humidified atmosphere at 37 °C,
171 in DMEM containing 4.5 g/L glucose, 15% serum (10% horse serum + 5% foetal calf serum), 1%
172 antibiotic mixture (penicillin/streptomycin), 1 mM ultraglutamine, 0.8 mg/mL geneticin and 0.8
173 mg/mL hygromycin B. The cells were split weekly with 0.5% trypsin/0.02% EDTA.

2.5. Luciferase reporter gene assay optimization using HEK-sbERα-ERETKLUC

Cells were seeded into 96-well, white, opaque cell culture plates (Perkin Elmer, Groningen, The Netherlands) at a density of 25 x 10^4 cells per well in DMEM medium. The response of HER-LUC to E2 was evaluated in different culture medium conditions. First, experiments were focused on the study of the effects of FBS or phenol red on sbERα-mediated luciferase activation. Cells were grown in DMEM containing FBS or charcoal-treated FBS and, lastly, serum-free medium. The effect of phenol red was evaluated by using phenol red-free medium.

The response to different agonists and antagonists was evaluated by incubating HER-LUC cells with different concentrations of test compounds (17β-estradiol, 17α-estradiol and 17α-methyltestosterone) -ranging from 10^{-9} to 10^{-3} M.

Similarly, to evaluate the possibility that the induction of luciferase could be mediated by a factor other than ER activation, HER-LUC cells were pretreated with the ER antagonist tamoxifen at concentrations ranging from 0.039 to 12.5 µM for two hours. Then, cells were treated with E2 0.25 µM. After 24 h, the medium was discarded, and cell viability and luciferase activity were measured as described in the following section.

To assure the ability of the cell line to detect estrogenicity in field samples, water from sewage treatment work effluents was extracted as described by Fernández et al., 2010. Extracts were reconstituted in 300 µl of DMSO. Serial dilutions in the culture medium were prepared for the cell exposure (ranging from 0.12 to 8.3 ml effluent/ml medium).

2.6. Screening of possible EDs in fish feed

Cell lines (HER-LUC and PC-DR-LUC) were exposed to different concentrations of fish feed extracts equivalent to a range from 0.008 to 16.7 mg fish feed/ml for 24 h. Positive (1µM of E2 or 1nM of T3) and negative (methanol 1%) controls were always added.

After incubation, cell viability was determined by measuring the cellular metabolic activity with the

resazurin method (O' Brien et al., 2000). Given that resazurin does not interfere with the luminiscence assay (data not shown), the cell viability assays were performed prior to luciferase assays in the same plate. 5 µl of the resazurin dye solution (ToxKit8, Sigma-Aldrich, Madrid, Spain) was added to each well and plates were incubated at 37 ºC and 5% CO_2 for 90 min. Fluorescence was then read with a Microplate-Reader (Tecan Genios Pro, Maenndorf, Switzerland) using 530 nm and 590 nm as excitation and emission wavelengths, respectively. As a positive control of cytotoxicity, 1mM SDS was also included in each assay. Luciferase activity was then measured using a luciferase reporter gene assay kit (Biodetection Systems, Amsterdam, the Netherlands) according to the manufacturer's instructions with small modifications. Briefly, 90 µl or 120 µl of phosphate buffered saline (PBS, pH 7.5) for the HER-LUC or the PC-DR-LUC cell lines, respectively and 30 µl of the lysis buffer were added. After 15 minutes, 80 µl (HER-LUC) or 50 µl (PC-DR-LUC) of the luciferase reagent was added and bioluminescence was measured in the culture plates using a luminometer (MicroBeta Trilux, Perkin Elmer, USA).

2.7. Data and statistical analysis

The results are presented as mean ± standard error of the mean (SEM) of at least two independent experiments (between two and six independent experiments were carried out, depending on the test). All the statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The luciferase fold induction was expressed as the ratio between mean value of light units from exposed and non-exposed control wells. The normal distribution of data was verified with the Shapiro-Wilk test. Statistical significance of luciferase induction compared with its control was tested by one-way repeated measures analysis of variance (ANOVA) followed by Dunnet´s test.

The estimation of the concentration-response function and the calculation of the EC_{50} (effective concentration 50, defined as the concentration causing a response 50% of the maximal response)

were made by fitting the luminescence results to a regression equation for a sigmoid curve: $y=max/[1+(x/EC_{50})b]+min$, where max is the maximal response observed, b is the slope of the curve and min is the minimal response. Relative transactivation activity (RTA) of each tested compound was calculated by normalizing its maximal luciferase fold induction with respect to that produced by 0.25μM of E2 (set at 100%). Relative agonistic activities (RAA) allow chemicals to be ranked according to their potency for estrogen receptor activation and were calculated by dividing the EC_{50} value of the E2 by the EC_{50} value of the compound of interest. The RTA of each fish feed was calculated by normalizing its maximal luciferase fold induction with respect to that produced by the corresponding positive control (PC) (fish feed spiked with either 0.5 nM T3 or 0.25 μM E2). The lack of a complete dose-response curves in some of the fish feeds for either of the assays meant that the EC_{50} values could not be calculated in those cases.

3. Results

3.1. Characterization of HER-LUC cell assay

In order to establish the best conditions to perform the luciferase reporter gene assays with the HER-LUC cell line, the interferences caused by phenol red, FBS or hormones present in FBS were evaluated. Fig.1 shows typical dose-response curves obtained by treating cells with increasing concentrations of E2 for 24h under different culture conditions. In complete media (containing phenol red and 10% FBS), the maximal response was reached at a concentration of 0.25 μM E2 with EC_{50} = 0.029 μM. The results obtained with phenol red-free medium showed that E2 efficacy (maximal response) was approximately half that obtained with complete medium, indicating the activating effect of phenol red on ER. In this case, the EC_{50} value was 0.025 μM, pointing to a similar potency of stimulation as obtained with the complete medium. Serum deprivation during the treatment resulted in an E2 efficacy similar to that obtained with phenol red-free medium and an

249 EC_{50} of 0.0069 μM. Although these conditions led to an increase in the potency of the assay, cells
250 cultured without serum appeared extremely weak, and were easily detached and lost during
251 exposure to feed extracts. Data from cells grown in phenol red-free media and charcoal-treated
252 serum (to avoid the presence of hormones) resulted in the typical sigmoidal response curve. In these
253 conditions, the EC_{50} was 0.032 μM and the maximum response was obtained at concentration of
254 0.25 μM. Thus, these were the conditions selected to carry out the assays.
255 Prior to the assessment of the fish feeds, the responsiveness of the new HER-LUC cell line to 17α-
256 estradiol, 17β-estradiol, 17α-methyltestosterone and tamoxifen were evaluated. All the selected
257 chemicals have been proposed by the OECD as reference chemicals in *in vitro* estrogenicity studies
258 (OECD Guideline 455, 2009). Figure 2 shows dose-dependent response curves corresponding to the
259 four chemicals. 17β-estradiol was the most potent and efficient estrogenic compound with
260 EC_{50}=0.032 μM and a maximal response caused by 0.25 μM, followed by 17α-estradiol, which was
261 approximately 100 times less potent than 17β-estradiol (EC_{50}=2.854 μM), and achieved its
262 maximum induction (RTA 84.6 %) at 80 μM. As expected, 17α-methyltestosterone was seen to be a
263 much weaker agonist, with an EC_{50} of 380 μM. The maximum response of 17α-methyltestosterone
264 was obtained at 950 μM (RTA of 15% compared with that of E2). Relative agonistic activities
265 (RAA) varied within five orders of magnitude; 17α-estradiol had an RAA of 0.01, while in the case
266 of 17α-methyltestosterone it was $8.53x10^{-5}$ (RAA E2=1). The antagonist tamoxifen inhibited the
267 maximal response induced by E2 in a dose-dependent manner. The IC_{50} was 3.56 μM and
268 concentrations of tamoxifen above 12.5 μM completely antagonized the E2-induced response. The
269 exposure of cells to the same range of tamoxifen doses did not induce luciferase activity. The
270 possible cytotoxicity of all compounds was evaluated by means of the resazurin method (O' Brien
271 et al., 2000). None of the tested agonists or the antagonist tamoxifen was toxic.
272 When the cells were transfected only with ERETKLUC construct in order to evaluate unspecific
273 responses, the results showed a lack of any response to the E2 treatment.

3.2. Estrogenic activity of sewage water

In order to evaluate the screening capacity of the newly developed system with complex environmental samples, cells were also exposed to extracts of waste water effluents previously reported as containing considerable estrogen loads (Carbonell et al., 2011). Concentrations ranging from 0.12 to 8.3 ml effluent/ml medium were able to induce full dose-response curves (Fig. 3), indicating the suitability of the new cell line for the detection of estrogenicity. Interestingly, relative transcription activities (RTA) reached 100% of that produced by 0.25 µM of E2, thus allowing the equivalents of estradiol present in these samples to be calculated.

3.3. Evaluation of the efficiency of the extraction

Figure 4 shows the typical dose-dependent response curves obtained after stimulation of HER-LUC (4A) or PC-DR-LUC (4B) cells with increasing doses of E2 and T3, respectively.

In HER-LUC cells, both E2 and E2 submitted to the extraction procedure (E2-MeOH) showed similar efficacy and potency (EC_{50} values of 0.032 and 0.036 µM, respectively), meaning that the extraction process itself does not affect the stability of the hormone and that the recovery was maximal with MeOH. When the fish feed was overloaded with E2 and extracted, the potency of stimulation was similar (EC_{50}=0.051µM). However, the extraction efficiency was reduced to 66%. These results indicate that the added hormone might be strongly complexed with other substances present in the fish feed so that the extraction is not complete.

In PC-DR-LUC cells the EC_{50} values for T3 and T3-MeOH were 0.068 and 0.127 nM, respectively, these results show that T3-MeOH presented lower stimulation potency than intact T3, although the amplitude of the response was similar in both cases. In the dose-response curve belonging to T3-overloaded feed, a lower efficacy was observed than in the T3 or in the T3- MeOH curves, 62% of the induction obtained with T3 being reached, with an EC_{50} = 0.168 nM.

To improve hormone recovery, a double extraction with methanol was performed, but this did not

lead to an increase of the maximal signal (efficacy) in the curves obtained with the extracts of the spiked feed (data not shown). Taking into account that the complex matrix prevents higher recoveries, the dose-response curves obtained with the hormone-overloaded feed extracts (E2-feed, T3-feed) were used as reference when assessing the estrogenicity or thyroid activity in the fish feeds.

3.4. *Estrogenic activity of fish feed extracts*.

The potential estrogenicity of commercial fish feeds was tested in HER-LUC cells. Two types of extraction, using methanol and hexane, were assayed to detect polar and non-polar compounds, respectively. The equivalent concentrations of fish feed to which cells were exposed ranged from 0.008 to 16.7 mg fish feed/ml. Extracts obtained with hexane did not stimulate sbERα-induced luciferase activity (data not shown). Previously, cell viability was evaluated through resazurin transformation into the fluorescent resorufin, expressed as relative fluorescence units (RFU). Fish feed concentrations inducing a decrease in RFU $\geq$ 80% were considered cytotoxic and excluded from the analysis. In the three diets (F3, F9 and F31) extracted with methanol, the RFU was enhanced significantly compared with the control levels (Fig. 5).

As regards estrogenicity assessment, 11 of the 32 fish feeds significantly induced luciferase activity. The induction of luciferase activity in 6 representative samples at two consecutive concentrations (the maximum concentration inducing an effect and the preceding concentration), is shown in Figure 5. Three feeds (F2, F7 and F9) were able to induce a two-fold increase in luciferase activity when tested at 1, 0.13 and 0.26 mg/ml respectively. In addition, F27 and F31 induced a weak significant increase in the luciferase activity of about 50% at a concentration of 0.015 and 0.06 mg/ml, respectively. F3 showed the highest estrogenic activity, inducing a three-fold increase when tested at a concentration of 16 mg/ml (EC_{50} = 9.57 mg/ml). Table 1 shows the maximum RTA of the 11 fish feeds with estrogenic activity. In this case, the RTA value is relative to that of the positive

control (E2-feed). In ten of the tested diets, the RTA represented between 1.05 and 3.05 % but the estrogenicity of the F3 feed was 6.05% of that obtained in the E2-feed.

3.5. Thyroidal activity of fish feed extracts.

The potential disrupting effect at the TR level was also assessed in the same commercial fish diets (concentrations ranging from 0.008 to 16.7 mg fish feed/ml). Again, extracts done with hexane had no effect on the reporter activity (data not shown). For the methanol extracts, the cytotoxic criteria considered, measured with the resazurin method, were the same described above. In this case, none of the diets provoked an increase in the fluorescence at any concentration. The thyroidal activity assessment shows that 18 out of 32 assayed fish feeds induced a significant luciferase activation when compared to the control levels. Increasing concentrations of the extracts resulted in increased induction of luminescence, with maximum responses observed at concentrations ranging from 4.1 to 16.7 mg fish feed/ml. 14 out of 18 positive diets were able to induce a full dose-response curve and thus, EC_{50} values could be derived (Table 1). All EC_{50} values were in the same order of magnitude ranging from 1.09 to 5.23 mg of fish feed/ml. The induction of luciferase activity in 12 representative feeds at two consecutive concentrations (the maximum concentration provoking the effect and the preceding concentration) is shown in figure 6. The representative dose-response curves for F4 (6A) and F32 diets (6B) are shown as insets.

The RTAs are shown in Table 1. In 9 diets, RTAs were between 10-20% whereas 7 diets exhibited RTAs between 20-30%. Finally, diet F19 and F20 showed RTAs above 35%.

4. Discussion

In the present study, potential endocrine disruption by fish diets was assessed by means of *in vitro* bioassays. Estrogenic activity was evaluated by using the newly developed HER-LUC reporter gene

assay, whose development, optimization and validation is described in the present work. This reporter assay allowed the quantification of sbERα-mediated luciferase activity by hormones (E2, 17-α-estradiol), analogues or environmental samples. The bioassay is based on the HEK-293 cell line, stably expressing the sbERα and the luciferase under the control of EREs. The use of sbERα is of particular interest and was chosen taking into account that sea bass is a carnivorous species of great importance for Mediterranean aquaculture. Disruption at thyroidal level was screened with an already established reporter gene assay which expresses luciferase gene under the control of TRα1 of avian origin (Jugan et al., 2007). Our study is in the first to demonstrate thyrogenic activity in fish diets commercially available and widely used in aquaculture.

Previous reports about endocrine disruption of feed or environmental samples use the responses obtained with different concentrations of the hormone standard as a reference to determine the endocrine activity of the studied sample (Matsumoto et al., 2004). In this study, dose-response curves obtained with the extracts of T3 or E2-overloaded feed were used as reference. This approach allows a more accurate estimation of the activity observed in the feed samples, avoiding the under and over-estimation of activities, since extraction and treatment of the original hormone and of the sample are influenced by the same factors. Validation of the HER-LUC system demonstrated the previously described estrogenic effect of phenol red (Berthois et al., 1986). In addition, serum deprivation during treatment resulted in weaker reporter gene induction when cells were exposed to E2, again as previously reported (Ackermann et al., 2002). Moreover, the lack of serum appears to affect cell fitness since they are not able to overcome the high concentrations of fish feed extracts, resulting in cytotoxicity. Although the amplitude of the response to E2 diminished, the potency (EC_{50}) was in the same order of magnitude when using complete, phenol red-free, and charcoal treated serum/phenol red-free media. In light of these results and to reduce the phenol red and serum effects, all the assays were performed in phenol red-free medium supplemented with charcoal-treated serum. In these conditions, the EC_{50} value for E2 was 32 nM.

Similar EC_{50} values were reported in previous reporter gene assays using fish ERs (Matthews et al., 2000; Ackermann et al., 2002; Cosnefroy et al., 2009). In the present work, the maximal induction factor obtained with HER-LUC cells was 54 fold. Previous studies have demonstrated that divergence in the amino acid sequences of the ligand binding domain of ER (Pakdel et al., 2000) resulted in lower affinity of rainbow trout ER (rtER) compared with human ER (hER) (Le Dréan et al., 1995). Indeed, the clearly discernible differences in sensitivity of the HER-LUC cells to estrogenic compounds could also be related with a lower binding affinity of sbERα for estrogens and estrogen-like substances than observed in rtER or hER. More experiments focusing on sbER binding affinity are required to corroborate this hypothesis.

To verify the correct functioning of the bioassay, the response to three agonists exhibiting different binding affinities for ER was assessed. Moreover, the effects of tamoxifen on E2-induced luciferase activity were also evaluated (Brzozowski et al., 1997; Navas and Segner, 2001). The detection of estrogenic activity in E2 and 17-α-estradiol demonstrated the specificity of the cell line to detect estrogenic substances with diverse efficacy. The RAA of 17α-estradiol was 100 times lower compared with E2 induction, which is in good agreement with the results obtained by Sonneveld et al. (2006). The representative hormone for the androgen receptor (17-α-methyltestosterone) showed a low agonistic response ($RAA=8.53E^{-5}$). In addition, cells transfected only with ERETKLUC construct were not responsive to E2 treatment, confirming that the luminescence response is only due to the activation of the ER. The inhibition of the estrogenic response due to pre-treatment with tamoxifen in a dose-dependent manner demonstrates that the induction of luciferase activity by E2 is specifically mediated by sbERα. However, we did not observe the partial agonistic action of tamoxifen at the low concentrations previously reported by other authors using mammalian cells or yeast expressing hER (Legler et al., 1999; Andersen et al., 1999). Similar results were obtained in previous experiments using rtER, suggesting species-specific differences in the ER response to tamoxifen. To further characterize the bioassay, the ability to detect estrogenicity in environmental

399 samples was tested. Cells were exposed to extracts of effluents from wastewater treatment plants.
400 The response obtained was similar in amplitude to that of E2, allowing calculation of the estrogenic
401 potential and demonstrating the potential use of this cellular system for the study of environmental
402 estrogenicity.
403 The estrogenicity caused by commercial fish feeds has been reported in previous studies (Miyahara
404 et al., 2003; Matsumoto et al., 2004; Beresford et al., 2011). In the present work, 11 out of 32 diets
405 induced sbERα-mediated transcriptional activity. The viability of the cells after exposure to feed
406 extraction was assessed simultaneously to the reporter assays by means of the resazurin method.
407 The decrease in fluorescence can be attributed to cell death and was useful for discarding cytotoxic
408 concentrations in both cell lines. Interestingly, three feeds (F3, F9 and F31) in HER-LUC cells
409 resulted in an increased fluorescence at specific concentrations. The increase in fluorescence has
410 been previously used to estimate cell proliferation (Freitas et al., 2010). However, in our case, this
411 increase was not due to cell proliferation, which was assessed by means trypan blue stain (data not
412 shown). These results were probably related with an increase in the general activity of the cells in
413 an attempt to metabolize the feed extract. The estrogenic capacity of these diets in the HER-LUC
414 cells was weak, as shown by the RTA values. However, estrogenic diets have been shown to
415 severely impact fish reproductive physiology (Pelissero and Sumpter, 1992). Our previous studies
416 demonstrated that estradiol-supplemented diets have profound negative effects on food intake levels
417 and growth performance in sea bass, suggesting that estrogenic diets could induce similar effects
418 (Leal et al., 2009). Although RTA levels in the diets seemed low, it should be taken into account that
419 absolute doses delivered daily to animals via food intake could reach 10^3-10^5 times those reported
420 in the assays depending on the fish size and food intake level.
421 To our knowledge, this work is the first to report the detection of thyroidal activity of fish feed
422 extracts. The present study shows that 56% (18 out of the 32) of the methanol extracts from fish
423 diets tested have thyromimetic effects. Thyroid activity of fish diets was screened with the PC-DR-

LUC reporter gene assay expressing luciferase under the control of TRα1 of avian origin (Jugan et al., 2007). The differences in receptor binding affinities or receptor-DNA interactions should be considered. For instance, rainbow trout nuclear TR showed a lower affinity for T3 than other animal species receptors (Ichikawa et al., 1989). When receptor-DNA interactions were compared among species, the receptors from rainbow trout and dog liver were similar. In spite of these differences, the use of an already established assay, as a first approach to assessing potential thyroid disruption in fish feeds, was considered the correct option. Future approximations should include the development of a cell line transfected with a TR from a fish species (e.g. rainbow trout or sea bass), not described to date.

A number of studies have reported the disruption of thyroid homeostasis by numerous industrial chemicals, such as polychlorinated biphenyls (PCBs), dioxins, flame retardants, including polybrominated diphenyl ether (PBDEs), phenolic compound and their halogenated derivatives, phthalates, and pesticides (Boas et al., 2006). The presence of thyroid endocrine disruptors in wastewater treatment plants, river and drinking water (Jugan et al., 2009) has also been evaluated, most activity being related with hydrophobic compounds. However, in our case the extracts performed with hexane did not provoke any induction of the TR-mediated luciferase activity, or the inhibition of the activation caused by treatment with T3. Thus, we might speculate that the thyroidal activity found in the fish feeds is mainly due to polar compounds, including hormones and some pollutants, but not to industrial chemicals exhibiting low polarity. However, this assumption must be confirmed through more specific analyses.

Thyroid hormones are essential in metamorphic transformation processes and play a crucial role in the postnatal maturation of different organs during early development (Flamant and Samarut, 2003; Blanton and Specker, 2007; Carr and Patiño, 2011). Experiments have demonstrated that deiodinase activity regulates pigmentation in zebrafish (Walpita et al., 2009) and treating flatfish with T4 increases the rate of albinism (Yoo et al., 2000). Positive correlation has been found between

449 thyroid status and reproductive status (Cyr and Eales, 1996). In zebrafish the exogenous
450 administration of TH produced strongly male-biased cohorts, whereas the testis weight and
451 gonadosomatic index was 100% higher in tilapia treated early with goitrogens than in control fish.
452 Treated tilapia also exhibited retarded growth (Matta et al., 2002). The presence of thyromimetic
453 compounds in fish diets could severely compromise early developmental processes, resulting in
454 unwanted characteristics in reared fish.
455 In conclusion, this report describes for the first time the simultaneously assessment of estrogenic
456 and thyroidal activities in commercial fish feed. Although the estrogenic activity was weak, in more
457 than half of the diets a very high thyroidal activity was detected. In addition, five of the fish feeds,
458 provoked disruption at both levels simultaneously. Moreover, given that fish receive food
459 continuously, the observed effects could be multiplied with time and with the quantity of ingested
460 food, so that the estrogenic or thyroidal activity observed should be taken into account with regards
461 to its potential impact on fish population in aquaculture.
462
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469

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625

626

Table 1: Maximal estrogenic/ thyroidal activity of fish feeds determined by HE-LUC/ PC-DR-LUC assay. Significance: *: $p<0.05$; **: $p<0.01$.

Fish feed sample	ER induction			TR induction		
	C Max	RTA	EC_{50} (mg feed/ml)	C Max	RTA	EC_{50} (mg feed/ml)
F1	-	-	NDR	4.1**	9.96±2.60	1.31
F2	1.04**	3.05 ± 1.45	NDR	4.1*	11.82±1.45	1.61
F3	16.4**	6.05 ± 2.30	9.57	4.1**	17.49±2.08	2. 03
F4	-	-		8.2**	23.78±2.18	3.00
F5	-	-		8.2**	14.55±1.14	1.71
F6	-	-		8.2**	16.30±2.37	1.40
F7	0.13**	2.78 ± 0.81	NDR	4.1**	24.24±1.61	2.97
F8	-	-		4.1*	13.68±1.50	1.76
F9	0.26**	2.04 ± 0.28	NDR		-	
F10	0.065**	1.38 ± 0.25	NDR		-	
F11	0.065**	2.25± 0.27-	NDR	8.2**	29.19 ± 5.71	1.30
F12	0.065**	1. 41 ± 0.14	NDR	-	-	
F14	0.065*	1.19 ± 0.09	NDR	-	-	
F16	-	-		4.1**	23.06±1.96	1.77
F17	-	-		4.1*	20.78±1.01	1.70
F18	-	-		8.2**	22.69±4.52	3.21
F19	0.065**	1.39 ± 0.39	NDR	8.2**	47.64±5.68	1.09
F20	-	-		16.4**	35.46±0,008	NDR
F21	-	-		16.4**	18.10±5.95	NDR
F23	-	-		16.4*	13.48±5.10	NDR
F27	0.0325*	1.05 ± 0.09	NDR	-	-	
F30	-			8.2*	18.34±7.04	2.32
F31	0.065*	1.20 ± 0.39	NDR	-	-	
F32	-		-	16.4**	27.33±10.93	5.23

CMax: Concentration inducing maximal effect (mg fish feed/ml). RTA: Relative transactivation activity (percentage of induction with respect to maximal E2-feed or T3-feed response (0.25µM E2 or 0.5nM T3, respectively). NDR: No dose response was observed impeding the calculation of EC_{50} value.

Figure 1

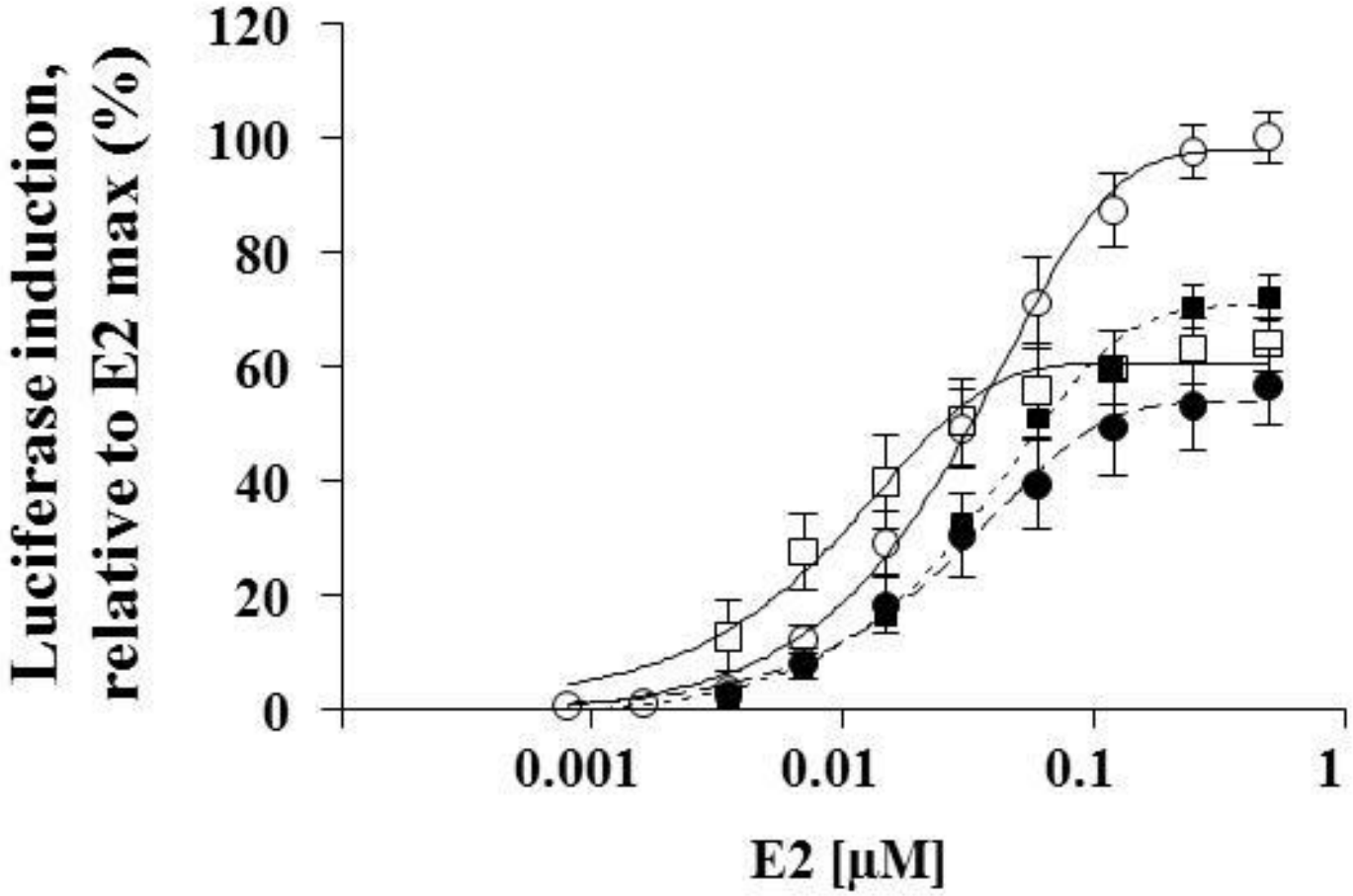

Luciferase induction, relative to E2 max (%)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
E2 [µM]

Figure 2

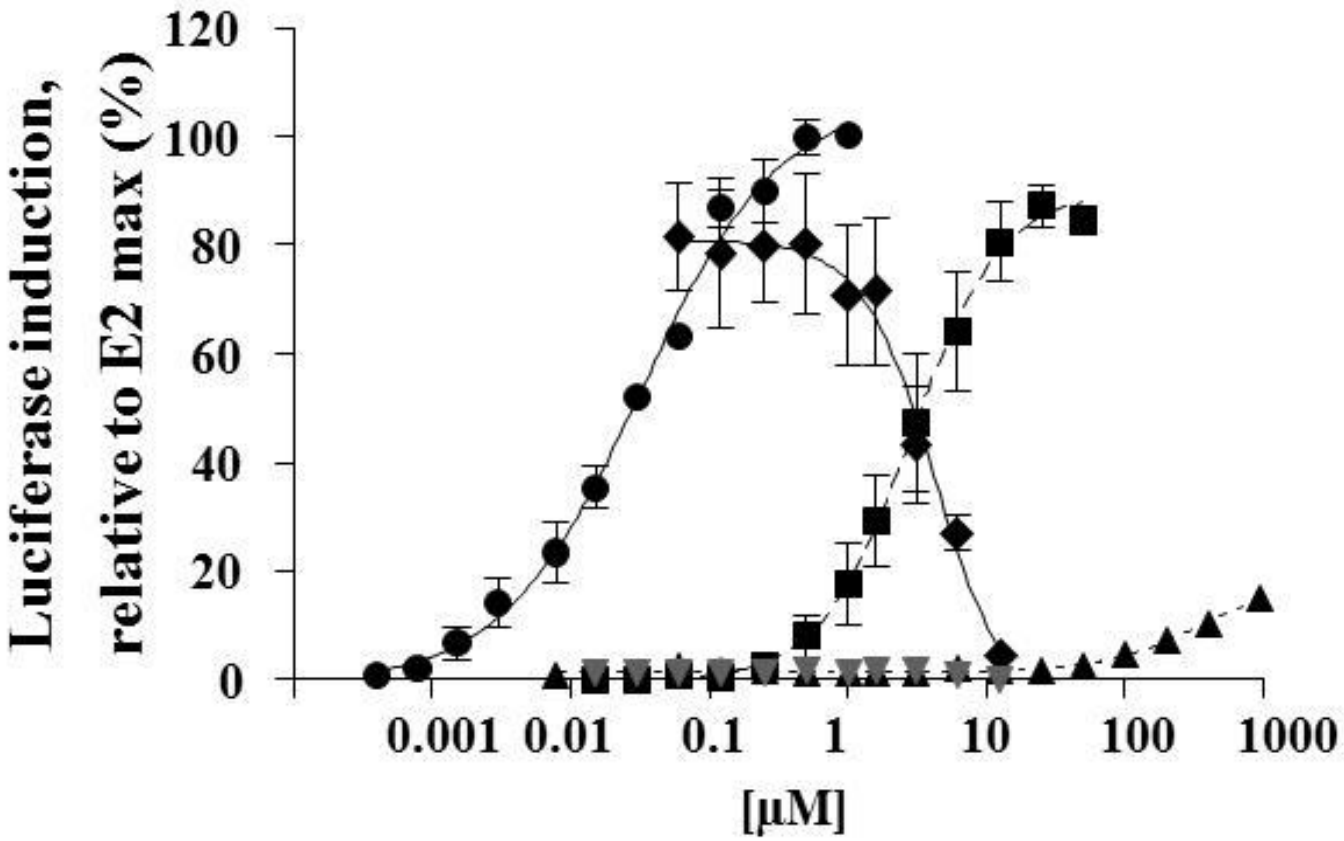

Luciferase induction, relative to E2 max (%)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
[µM]

Figure 3

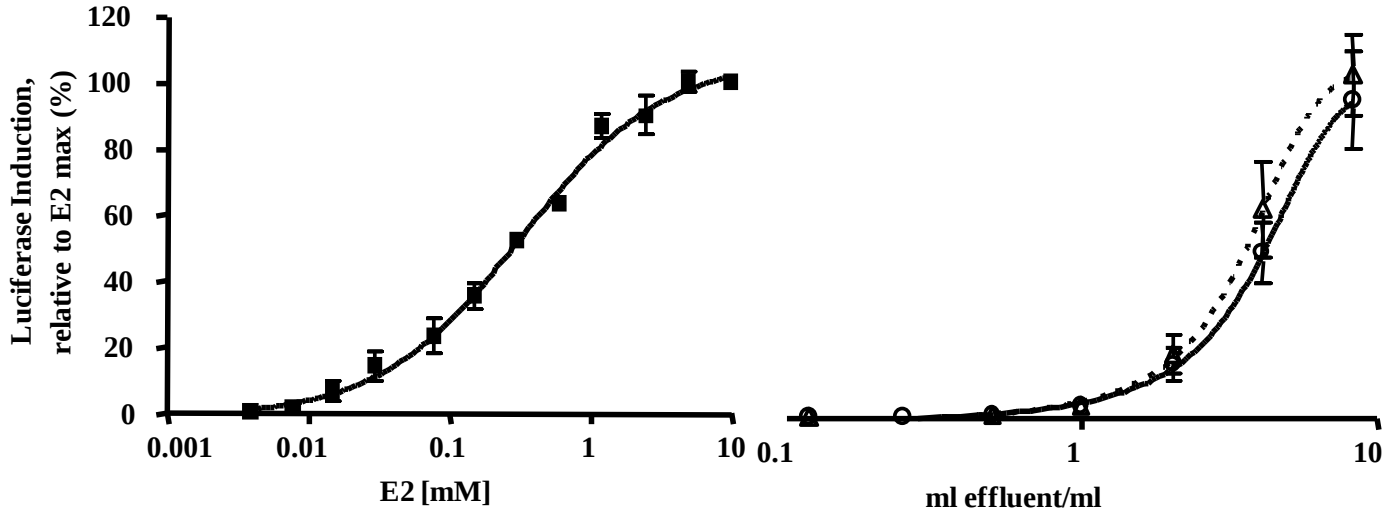

Luciferase Induction, relative to E2 max (%)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
E2 [mM]
0.1
1
10
ml effluent/ml

Figure 4

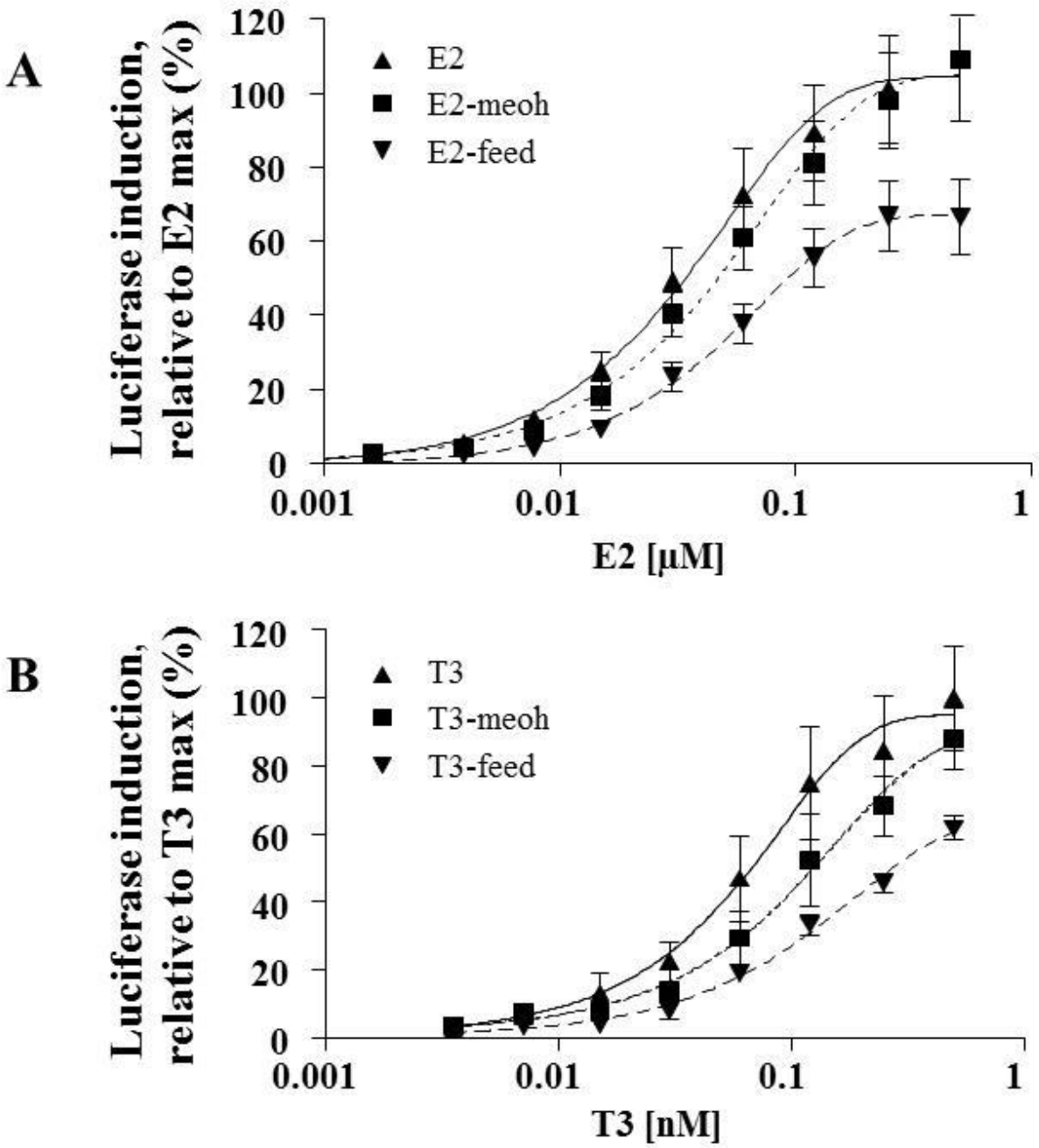

A
Luciferase induction, relative to E2 max (%)
120
100
80
60
40
20
0
E2
E2-meoh
E2-feed
0.001
0.01
0.1
1
E2 [µM]
B
Luciferase induction, relative to T3 max (%)
120
100
80
60
40
20
0
T3
T3-meoh
T3-feed
0.001
0.01
0.1
1
T3 [nM]

Figure 5

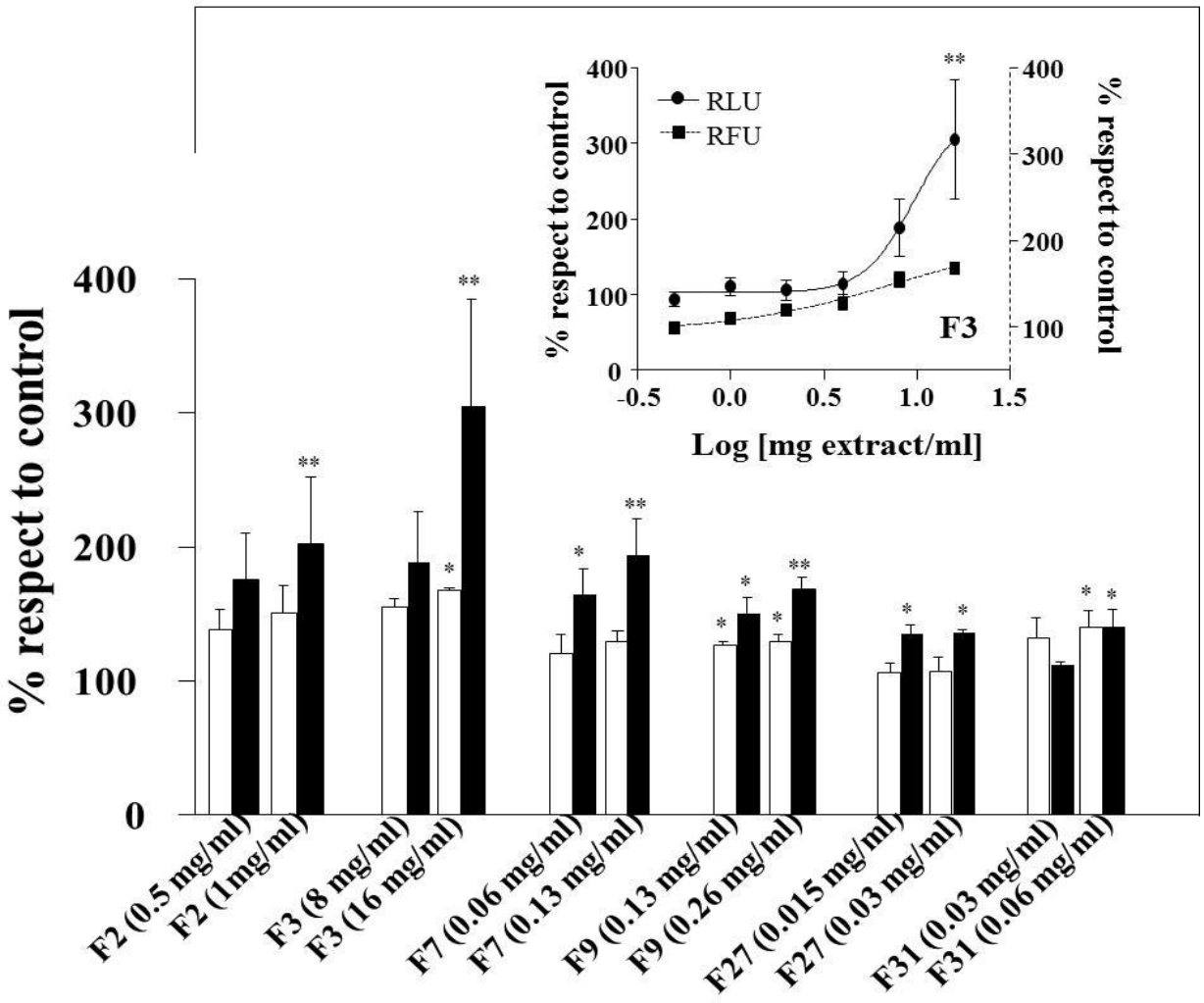
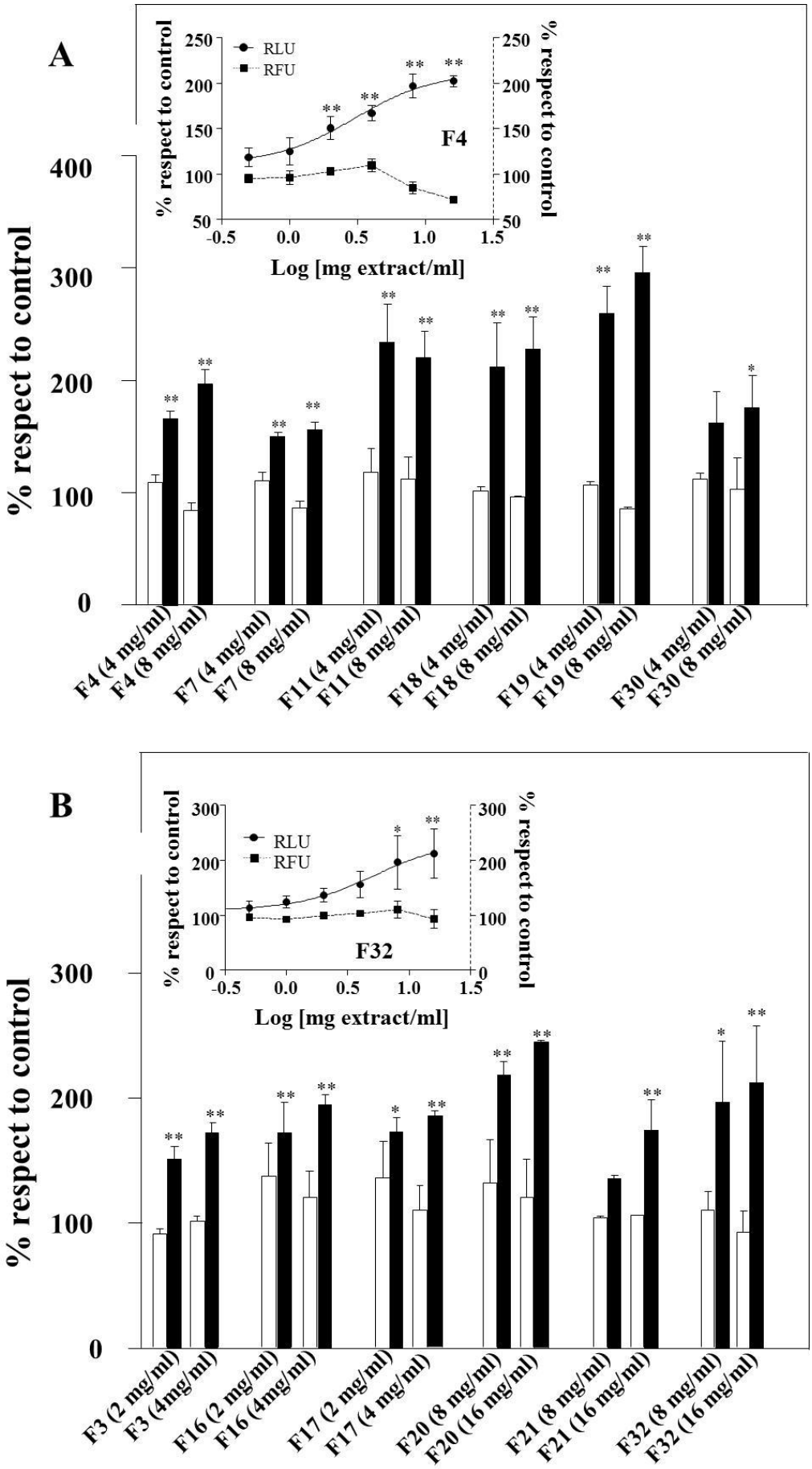

% respect to control
400
300
200
100
0
**
*
F2 (0.5 mg/ml)
F2 (1mg/ml)
F3 (8 mg/ml)
F3 (16 mg/ml)
F7 (0.06 mg/ml)
F7 (0.13 mg/ml)
F9 (0.13 mg/ml)
F9 (0.26 mg/ml)
F27 (0.015 mg/ml)
F27 (0.03 mg/ml)
F31 (0.03 mg/ml)
F31 (0.06 mg/ml)
RLU
RFU
F3
Log [mg extract/ml]
-0.5
0.0
0.5
1.0
1.5

Figure 6


A
% respect to control
400
300
200
100
0
F4 (4 mg/ml)
F4 (8 mg/ml)
F7 (4 mg/ml)
F7 (8 mg/ml)
F11 (4 mg/ml)
F11 (8 mg/ml)
F18 (4 mg/ml)
F18 (8 mg/ml)
F19 (4 mg/ml)
F19 (8 mg/ml)
F30 (4 mg/ml)
F30 (8 mg/ml)
RLU
RFU
F4
% respect to control
Log [mg extract/ml]
B
F32
F3 (2 mg/ml)
F3 (4mg/ml)
F16 (2 mg/ml)
F16 (4mg/ml)
F17 (2 mg/ml)
F17 (4 mg/ml)
F20 (8 mg/ml)
F20 (16 mg/ml)
F21 (8 mg/ml)
F21 (16 mg/ml)
F32 (8 mg/ml)
F32 (16 mg/ml)

List of figures:

Figure 1: Luciferase induction of HER-LUC cells after exposure to E2 in media containing phenol red and serum (○), media without phenol red (●), serum-free media (□) and phenol red-free and charcoal-treated serum media (■). The results are shown as % with respect to the maximal induction caused by E2 in complete media (considered 100%). Data points represent mean ± SEM of six independent experiments performed in duplicate.

Figure 2: Dose-response curves for the tested agonists, E2 (●); 17α-estradiol (■) and 17α-methyltestosterone (▲). The antagonist tamoxifen was tested both alone (▼) and coincubated with 0.25μM E2 (◆). Data points represent mean ± SEM of at least three independent experiments performed in duplicate and are shown as % with respect to the maximal induction caused by E2

Figure 3: Dose response curves of E2 (A) and two waste water effluents (B): effluent 1 (○) and effluent 2 (△**).** Data points represent mean ± SEM of three independent experiments performed in duplicate and are shown as % with respect to the maximal induction caused by E2.

Figure 4: Dose response curves in HER-LUC (A) and PC-DR-LUC cells (B), after exposure to their corresponding pure hormone, hormone extracted with methanol and spiked-fish feed. Data points represent mean ± SEM of five-nine independent experiments performed in duplicate. The results are shown as % relative to maximal induction of pure hormones, E2 (A) and T3 (B) (considered 100%).

Figure 5: Estrogenic activity represented as relative luminescence units (RLU, black) and cytotoxicity as relative fluorescence units (RFU, white) in HER-LUC cells. The data represented show the effect of the maximal concentration inducing the estrogenic effect and its immediately previous concentration. Columns represent the mean of at least two independent experiments ±

SEM. Significance: *: $p<0.05$; **: $p<0.01$. The inset shows the full dose response curve belonging to one of the samples (F3).

Figure 6: Thyroidal activity represented as relative luminescence units (RLU, black) and cytotoxicity as relative fluorescence units (RFU, white) in PC-DR-LUC cells. The figure shows the induction of luciferase activity in 12 representative feeds at the two consecutive concentrations provoking the maximum effect. Columns represent the mean of at least two independent experiments ± SEM. Significance: *: $p<0.05$; **: $p<0.01$. The insets show the full dose response curves belonging to two of the samples F4 (6A) and F32 (6B). In the case of F4, the highest concentration (16 mg/ml) resulted cytotoxic to the cells, and although shown in the graph, was excluded from the RTA and EC_{50} calculations.