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Potential oestrogenic effects (following the OECD test guideline 440) and thyroid dysfunction induced by pure cyanotoxins (microcystin-LR, cylindrospermopsin) in rats

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ABSTRACT

Potential endocrine-disrupting properties of cyanotoxins, such as microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are of concern due to their increasing occurrence, the scarcity of reports on the topic (particularly for CYN) and the impact of human's health at different levels. Thus, this work performed for the first time the uterotrophic bioassay in rats, following the Organization for Economic Cooperation and Development (OECD) Test Guideline 440, to explore the oestrogenic properties of CYN and MC-LR (75, 150, 300 μg/kg b.w./day) in ovariectomized (OVX) rats. Results revealed neither changes in the wet and blotted uterus weights nor in the morphometric study of uteri. Moreover, among the steroid hormones analysed in serum, the most remarkable effect was the dose-dependent increase in progesterone (P) levels in rats exposed to MC-LR. Additionally, a histopathology study of thyroids and serum levels of thyroids hormones were determined. Tissue affectation (follicular hypertrophy, exfoliated epithelium, hyperplasia) was observed, as well as increased T3 and T4 levels in rats exposed to both toxins. Taken together, these results point out that CYN and MC-LR are not oestrogenic compounds at the conditions tested in the uterotrophic assay in OVX rats, but, however, thyroid disruption effects cannot be discarded.

1. Introduction

In the last years, the augment of water eutrophication, anthropogenic activities and climate change have produced an increase of cyanobacteria, organisms that are capable to form cyanobacterial blooms (Metcalf and Codd, 2020). These blooms can produce a wide range of secondary metabolites and bioactive compounds, with many considered to be toxins (cyanotoxins) (Diez-Quijada et al., 2021a). Cyanotoxins reach humans mainly orally through the consumption of contaminated water and food, although inhalation and dermal exposure during recreational activities are also common (Buratti et al., 2017). The World Health Organization (WHO) and the European Food Safety Authority (EFSA) have catalogued cyanobacteria as an emerging health issue due to the high toxic risks that their exposure can produce in humans (Testai et al., 2016). Among cyanotoxins, cylindrospermopsin (CYN) and microcystins (MCs) are the most relevant worldwide due to their wide distribution, bioaccumulation capacity and toxic effects (Weralupitiya et al., 2022).

CYN is an alkaloid consisting of a tricyclic guanidine moiety combined with hidroxymethiluracil and due to its zwitterionic nature is a highly water-soluble compound (Pichardo et al., 2017). The toxin is produced by different cyanobacterial genera, being more frequently reported from the genera *Raphidiopsis, Aphanizomenon, Anabaena* and *Umezakia* (World Health Organization, 2020a; Díez-Quijada et al., 2018). The toxin primarily targets the liver, but it is also a general cytotoxin that injures the eye, spleen, kidney, lungs, thymus, heart, etc. (Gutiérrez-Praena et al., 2012; Pichardo et al., 2017). Concerning the mechanisms of action, CYN is well known by its inhibition of protein and glutathione synthesis (Runnegar et al., 1995; Froscio et al., 2003), induction of oxidative stress (Puerto et al., 2011; Guzmán-Guillén et al., 2013) and genotoxicity (Puerto et al., 2018; Díez-Quijada et al., 2019), and recently, its immunomodulatory effects have been also reported

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(Diez-Quijada et al., 2022).

MCs are produced by different species from the genera *Microcystis, Anabaena,* and *Planktothrix*, among others (Catherine et al., 2017; World Health Organization, 2020b). These toxins are cyclic heptapeptides composed of five common amino acids, plus a pair of variable L-amino acids, and there are a lot of MCs variants, but microcystin-LR (MC-LR) is the congener most widely distributed, and with more toxicological *in vitro* and *in vivo* studies performed (Testai et al., 2016; Pichardo et al., 2005, 2007; Puerto et al., 2009). MCs are hepatotoxins and tumour promoters due to their strong potent inhibition of protein phosphatases 1 and 2 A (PP1 and PP2A), induction of oxidative stress (Puerto et al., 2011) and effect on cell signalling pathways (Bouaïcha et al., 2019). Although the liver is the main target organ of MCs, other effects have been reported including reproductive toxicity (Zhang et al., 2021) via apoptosis, autophagy, cytoskeletal destruction, reproductive tumours and endocrine disruption (Liu et al., 2017; Chen et al., 2021).

Regarding to endocrine disruption, the endocrine system is a collection of glands that secrete different hormones directly into blood circulation and that includes different axes like hypothalamic-pituitary-gonad (HPG) and hypothalamic-pituitary-thyroid (HPT) (Brüggemann et al., 2018). Each axis regulates different functions of the body, including metabolism, growth, and development via axis-specific hormones. The HPG axis coordinates reproduction by steroid hormones (Miller and Auchus, 2011). Among them, estradiol (E_2) and testosterone (T) are the main hormones in females and males, respectively. E_2 regulates development, maturation and functioning of the female reproductive tract (Drummond, 2006; Knobil and Neill, 2006; Rosenfeld et al., 2001). E_2 also exerts negative and positive feedback on the pituitary and hypothalamus and thus regulates gonadotropins (luteinizing hormone (LH), follicle-stimulating hormone (FSH)) and gonadotropin-releasing hormone (GnRH) secretion, respectively (Clarke et al., 2012). HPT axis regulates energy metabolism and development by thyroid hormones (Chen et al., 2021). The endocrine system can be altered by different substances called endocrine disruptors (EDs) (Sabir et al., 2019). The WHO defined an ED as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population" (WHO/IPCS, 2002). Different number of substances including pesticides, environmental pollutants, plastics, metals, food contaminants, and personal care products, are noticed as EDs (Tijani et al., 2013; EFSA, 2018). Moreover, cyanobacterial blooms could be a source of diverse EDs (Jia et al., 2019). In that sense, cyanotoxins were proposed as potential EDs due to their estrogenic potency and capacity of interference in the signalling of intracellular receptors that are important for hormonal regulation, reproduction and development of vertebrates (Rogers et al., 2011). Several studies have also demonstrated that MCs can disrupt the synthesis of steroid hormones resulting in dysfunction of endocrine system and reproductive toxicity, especially MC-LR (Liu et al., 2016). Globally, MCs and CYN have shown endocrine disruption activity mediated by different mechanisms, as it has been recently reviewed (Casas-Rodriguez et al., 2022), although in the case of CYN the number of studies is very scarce and the information of the estrogenic potency of CYN is very limited (World Health Organization, 2020a).

It is important to highlight that only a few works determined the potential endocrine disruption activity of cyanobacterial cells or pure cyanotoxins through the interactions with signalling pathways of several molecular receptors (estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) and retinoid receptor (RAR) (Stěpánková et al., 2011; Sychrová et al., 2012; Jonas et al., 2015; Mallia et al., 2020), and none of them was performed following the requirements of international protocols, such as guidelines of the Organization for Economic and Cooperation Development (OECD). It has been demonstrated that extracts from complex cyanobacterial biomasses showed greater estrogenic potency than extracts of single cyanobacterial species (Stěpánková et al., 2011). Some EDs effects have been detected in extracts and exudates of several cyanobacteria (Sychrová et al., 2012; Jonas et al., 2015), although the estrogenic potency revealed the involvement of different compounds not identified, probably phytoestrogens.

Taking all this into account, the aim of this work was to assess the potential effects induced *in vivo* by pure CYN and MC-LR as potential estrogen and thyroid disrupters. An uterotrophic assay in ovariectomized (OVX) rats has been performed following the OECD 440 guideline (OECD 440, 2007), and the level of different steroids hormones (E_2, T, LH, FSH), progesterone (P) and thyroid hormone levels, such as triiodothyronine (T3), thyroxine (T4), thyrotropin (thyroid-stimulating hormone, TSH) were analysed to determine the potential effects of these pure toxins. In addition, histopathological changes in uterus and thyroid, and a morphometric measurement of endometrial epithelium have been also included for quantitative comparison.

2. Materials and methods

2.1. Chemical and reagents

CYN (95% purity) and MC-LR (99% purity) were obtained from Enzo Life Sciences (Lausanne, Switzerland). E_2 (CAS No. 57-63-6) was purchased from Dicsa (Zaragoza, Spain). Kits for the determination of E_2 (AR E−8800 R), T (AR E−8000 R) and P (AR E−8700 R) serum levels were purchased from LDN (Nordhorn, Germany). MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel (RPTMAG-86 K) was provided by Sigma-Aldrich (Madrid, Spain) to determine the LH, FSH, and TSH. T3 and T4 were quantified by electrochemiluminescence immunoassay (ECLIA) and measured in the Cobas 6000 equipment (Roche Diagnostics).

2.2. Animal housing

For both toxins, CYN and MC-LR, the studies were performed at the Central Service of Experimental Animals (SAEX) of the University of Córdoba (Spain), under the codes 21-CAM-06 (CYN) and 22-CAM-05 (MC-LR) in conformity with the OECD Guideline 440 (OECD 440, 2007). All animals received human care in agreement with the Directive for the protection of animals used for scientific purposes (Directive, 2010/63/UE, Decision, 2020/569/UE and RD 1386/2018). All the methods employed have been authorized by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project no. June 26, 2021/102 in both cases).

For the experimental set up, 60 female Sprague Dawley rats (strain Crl:SD), 30 for each individual study (CYN or MC-LR) of 5 weeks, were supplied by Charles River (Charles River Laboratoires, Les Oncins, Saint Germain Nuelles, France). After a 1-week acclimation period (room temperature 22 ± 3 °C, 50–60% of relative humidity, 12 h light/dark cycle) rats were ovariectomized following OECD 440 (2007) protocol and prior to cyanotoxin administration they were left for two weeks to stabilise the uterus. Rats were fed with standard laboratory diet (Altromin - LASQCdiet® Rod 14-R, Altromin Spezialfutter GmbH & COoK, Lage, Germany) and water *ad libitum*. The rats were assigned to the control and dose groups (6 rats/group), by randomized weight distribution so that mean body weight (b.w.) of each group is not statistically different from any other group. The sample size, six females for each dose level, was chosen according to the OECD 440 test guideline (OECD 440, 2007).

2.3. Rat model for uterotrophic assay in OVX rats

2.3.1. Preliminary studies with EE

Before the uterotrophic assay, the laboratory demonstrated its proficiency and the responsiveness of the animal model by establishing the dose response of the reference oestrogen EE. Thus, 36 Sprague-Dawley female rats were randomly distributed in 6 groups (6 rats/group): control (vegetal oil) and five experimental groups treated with different doses of EE (0.1, 0.3, 1.0, 3.0 and 10.0 μg/kg b.w./day) (Kanno et al.,

2001). The product was administered by gavage, previously dissolved in a minimal amount of ethanol 95% v/v and then diluted in vegetal oil as vehicle (sunflower oil, Sanjosol, Aceites San Jose Import-Export S.L, Sevilla, Spain). After oral administration for 3 days at 24 h intervals, the animals were sacrificed 24 h after the last administration. The protocol included daily measurement of animal body weights. The end points of interest were the mean wet and blotted uterine weight and their respective standard deviations (SD) (Table 1). Moreover, data of mean body weights and their SD, and the body weight gain (±SD) of treated groups relative to the vehicle control group were also included (Table 1).

2.3.2. Uterotrophic bioassay in OVX rats

The uterotrophic assay, one for each pure cyanotoxin, CYN or MC-LR, was performed according to the OECD 440 test guideline (OECD 440, 2007), and the protocol used in both studies was described previously by Kanno et al. (2001). The test followed the schedule shown in Fig. 1, as described by Lee et al. (2022).

Once the animals were acclimatized for OVX, rats were anesthetized (Isoflurane, Sigma Aldrich) and then the abdominal cavity was opened. The ovaries were removed from both ends of the fallopian tube. A minimum of 14 days between ovariectomy and the first day of administration of each toxin was followed to allow the uterus to regress to a minimum stable baseline.

To determine the uterine effects of CYN or MC-LR, two assays were performed, one for each pure toxin. In each assay rats were randomly divided in 5 groups (6 rats/group): (1) OVX control; (2) OVX + EE (10 μg/kg b.w./day), (3) OVX + toxin (75 μg/kg b.w./day CYN or MC-LR); (4) OVX + toxin (150 μg/kg b.w./day CYN or MC-LR); (5) OVX + toxin (300 μg/kg b.w./day CYN or MC-LR). In the case of OVX control, rats were administered with the vehicle used with the treated groups, Milli-Q water for both cyanotoxins.

The three exposure doses for each toxin were chosen following the criteria of the guideline 440, so that they ensure animal survival and do not induce significant toxicity or distress to the animals after three consecutive days. Considering previous toxicological studies with CYN in Sprague–Dawley rats (Diez-Quijada et al., 2021b), slight signs of damage were observed after 28 days oral exposure to 75 μg CYN/Kg b.w. such as changes in several biomarkers (triglycerides levels and aspartate aminotransferase activity (AST)). Previously, another work from our team found histopathological changes in stomach and liver of Wistar rats orally exposed to 7.5–75.0 μg/kg b.w.) in an *in vivo* combined assay of the micronucleus test (MN) and comet assay (Díez-Quijada et al., 2019). Consequently, 75 μg CYN/Kg b.w. was chosen as the lower dose to be tested in the present study and increasing dose levels were chosen using a factor of two (150 and 300 μg CYN/Kg b.w.). In the case of MC-LR, its LD50 by the i.p. route is approximately 25–150 μg/kg b.w. in mice (Fawell and James, 1994), and even higher values have been reported by Fawell et al. (1999). But for comparative purposes with CYN, the same doses of 75, 150 and 300 μg/kg b.w./day for MC-LR were selected.

CYN or MC-LR were administered orally in a single daily dose for three consecutive days at 09:00 a.m., and similarly a positive control of EE, and a negative control were included. Twenty-four hours after the last dose, rats were weighed and sacrificed. Body weight, feeding activity (food and water consumption) and general clinical symptoms such as changes in behavior, skin, fur, eyes, mucous membranes, etc. were checked to confirm the animal's condition as specified by OECD test guideline 440 (OECD 440, 2007).

2.4. Organ weights, histopathological and morphometric studies

At the end of the study, and after the sacrifice of the animals, which was performed as described in Diez-Quijada et al. (2021b) the following organs were extracted and weighted: uterus (wet and blotted weights), uterine tissues, thyroid, liver, brain, spleen, thymus and kidney. To compare the uterotrophic effect of both toxins with their controls, the relative uterine weight, wet and blotted, was calculated for each animal. The relative uterine weight was calculated as the ratio of uterine weight to final body weight (b.w.), according to Hu et al. (2013).

At postmortem examination, the uteri and thyroids were immersed in 10% neutral buffered formalin for 24 h, routinely processed using an automatic processor, and embedded in paraffin wax. Samples from the right and left uterine horns were taken from above the junction, processed routinely and embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin and eosin (H&E) for the histological study. The morphometric study of uteri was carried out measuring the uterine epithelial cell height from the apical cell surface to the basement membrane. All measurements were made in areas where luminal folds were not present taking care to avoid measuring sections that were cut obliquely (Varayoud et al., 2008, 2016). Thus, only simple cuboidal or columnar epithelium was measured. The right and left uterine horns from each rat were photographed with 400 magnification and up to 15 measurements of the epithelial cell height were done in each photography with the ruler tool included in the image analyzer software. All measurements were made by means of a photomicroscope (BX43F, Olympus®, Japan) coupled to a photographic camera (XC50, Olympus®, Japan) connected to a computer with an image analyzer software (CellSens, Olympus®, Japan). The average of the epithelial cell height obtained from the right and left horns of each rat were expressed as means of micrometers ± SD.

2.5. Serum hormones quantification

2.5.1. Multiplex assay

At the end of the exposure period, blood samples were obtained by intracardiac injection from the rats. Levels of LH, FSH, and TSH were measured in serum using a MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel. First, 200 μL of assay buffer (catalog number: LE-ABGLP) were added to each well of a 96 wells plate provided and incubated on a plate shaker during 10 min at room temperature. Then, the blank, serial dilutions of the 5 standard and the samples (25 μL) were added to the corresponding wells. Antibody-Immobilized Beads of each hormone were sonicated, vortexed and mixed in Bead Diluent (catalog number: LHE-BD), then 25 μL were added to each well, followed by incubation overnight at 4 °C on a plate shaker, and washing 3 times with 200 μL of wash buffer (catalog number: L-WB). Detection antibodies (50 μL) (LH, FSH and TSH) were also added to the wells and incubated for 30 min on a plate shaker at room temperature. Streptavidin-Phycoerythrin (50 μL) was added to each well and the plate was incubated with the same

Table 1
Parameters measured in Sprague-Dawley rats on the validation test. Data are presented as mean ± SD (standard deviation). The significance levels observed are *$p < 0.05$ and ***$p < 0.001$ in comparison to the control group.

	CONTROL	EE 0.1 μg/kg b.w./day	EE 0.3 μg/kg b.w./day	EE 1 μg/kg b.w/.day	EE 3 μg/kg b.w./day	EE 10 μg/kg b.w./day
Body weight (g)	305.70 ± 30.22	306.60 ± 43.11	302.70 ± 11.29	296.50 ± 29.45	295.70 ± 15.76	295.8 ± 16.36
Body weight gain (g)	77.50 ± 16.39	70.80 ± 10.64	74.17 ± 9.28	70.17 ± 8.28	73.17 ± 11.99	64.50 ± 6.28
Wet uterus (g)	0.1158 ± 0.0152	0.0948 ± 0.0088	0.1143 ± 0.0078	0.1463 ± 0.0280	0.2945 ± 0.0497***	0.3172 ± 0.1214***
Blotted uterus (g)	0.1072 ± 0.0141	0.0872 ± 0.0069	0.1072 ± 0.0087	0.1375 ± 0.0280	0.2508 ± 0.0257	0.2570 ± 0.0415*
Wet uterus weight/b.w. (%)	0.038 ± 0.002	0.031 ± 0.004	0.038 ± 0.003	0.049 ± 0.009	0.100 ± 0.021***	0.105 ± 0.034***
Blotted uterus weight/b.w. (%)	0.035 ± 0.002	0.029 ± 0.004	0.035 ± 0.003	0.046 ± 0.009	0.085 ± 0.011***	0.087 ± 0.014***

B.w.: body weight; EE: ethynyl estradiol.

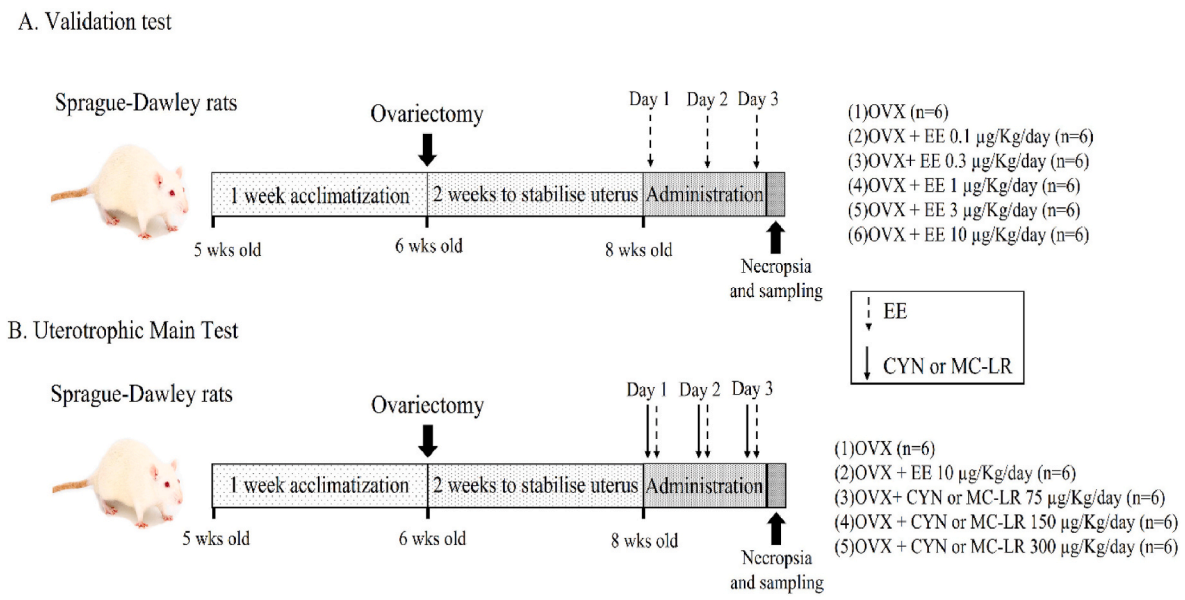

Fig. 1. Scheme of work for preliminary studies with EE and uterotrophic assays with cyanotoxins.

conditions. Finally, the plate was washed 3 times with 200 µL of wash buffer, beads were resuspended in assay buffer (100 µL) and the plate was read on a Bio-Plex® 200 Multiplex system (Bio-Rad Laboratories Inc., Hercules, CA, USA) available at the Biology Service of the Centro de Investigación, Tecnología e Innovación (CITIUS) of the University of Sevilla. For readings with the Bio-Plex 200 instrument, the following settings were set: Reporter Gain (PMT) Low; DD Gates: 5000 (low) and 25,000 (high); Bead Events: 50. The Minimum Detectable Concentrations (MinDC) of the hormones were 7.62, 3.28 and 0.87 pg/mL for FSH, LH, and TSH respectively. Data from the multiplex analysis was obtained using the Bio-Plex Manager™ version 4.1.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.5.2. ELISA and ECLIA assays

E_2, T, and P, were determined by competitive ELISA using the corresponding available kits and following the manufacturer's instructions. Absorbance was measured at 450 nm in a BioTek Synergy HTX plate reader (BioTek® Instruments Inc., Winooski, VT, USA) available at CITIUS. MinDC of the hormones were 0.002, 0.06 and 0.15 ng/mL for E_2, T, and P respectively.

The determination of thyroid hormones (T3 and T4) was carried out by ECLIA in Cobas 6000 (Roche's Diagnostics, Sant Cugat del Vallés, Spain) following the manufacturer's recommendations. The development of immunoassays is based on the use of a ruthenium-complex and tripropylamine (TPA). The chemiluminescence reaction for the detection of the reaction complex is initiated by applying a voltage to the sample solution resulting in a precisely controlled reaction (Serdarevic, 2018).

2.6. Statistical analysis

All data were analysed using GraphPad Prism 9.0.0 (Graph Pad Software, La Jolla, CA, USA). The data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed to evaluate potential differences in the different variables studied. The Kolmogorov–Smirnov test was applied to check for normality assumption. Comparisons were made with the Tukey–Kramer multiple comparisons test (when statistically significant) or with the Kruskal–Wallis test, followed by Dunn's multiple comparison tests (non-normality). Differences were considered significant from * $p < 0.05$.

3. Results

3.1. Uterotrophic assay: baseline positive control study

The main endpoints of this study, wet and blotted uterine weights were shown in Table 1, as well as the body weight (g) and body weight gain (g) for all the control and the five exposed groups (0.1–10.0 µg EE/Kg/day). At the end of the experiment, rats body weight (Table 1) showed no significant differences, with weights between 295.70 and 306.60 g. Body weight and uterine weight showed no correlations, between these two variables, in agreement with Kanno et al. (2001). In the same way, no significant differences in body weight gain (Table 1) were found, with gains between 64.50 and 77.50 g.

The uterine weight increase is the adequate response of the female to sufficient exposure to oestrogen agonist (EE), and significant increases were detected in both parameters, wet and blotted uterus weight at the higher doses assayed of 3 or 10 µg EE/Kg/day, in comparison to the control group. Similar significant differences were found when compared relative uterine wet or blotted weight/final b.w. for the higher doses of 3.0 and 10.0 µg EE/Kg/day with the control group. These results agree with Kanno et al. (2001) which carried out a validation study of the Rat Uterotrophic bioassay in rats between 19 laboratories, and all of them reported increases in uterine weights using EE. The authors indicated that the response begins with the essential interaction of the oestrogen with a high affinity receptor in uterine tissues that initiates a series of responses and at the end, induced uterine weight increase, being this weight increase due to a combination of water inhibition in the tissue and the uterine lumina and a hypertrophic response of the uterine tissues (Kanno et al., 2001).

3.2. Uterotrophic main test

3.2.1. Body weight, organ weight and food and water consumption. Uterus absolute and relative weights

During the 3 days of treatment, neither mortality nor physiological stress was observed in the rats treated with CYN or MC-LR. Following OECD 440 (2007) protocol, the body weight, and the food and water consumption of all rats were measured daily. The changes of body weight, and body weight gain (g), and food (g/day) and water consumption (mL/day) are shown for CYN and MC-LR in Tables 2 and 3, respectively. No differences were found in all these four parameters in

Table 2
Parameters measured in the uterotrophic assay after the exposure to different CYN (75, 150 and 300 μg/kg b.w./day) and EE (10 μg/kg b.w./day) doses in Sprague Dawley rats. Data are presented as mean ± SD (standard deviation). The significance levels observed are ***p < 0.001 in comparison to the control group and ###p < 0.001 in comparison to the EE-treated group.

	CONTROL	EE 10	CYN 75	CYN 150	CYN 300
Body weight (g)	310.30 ± 22.54	300.70 ± 15.42	302.70 ± 23.93	309.00 ± 24.58	291.30 ± 28.18
Body weight gain (g)	135.30 ± 21.63	120.70 ± 17.60	130.70 ± 21.32	133.30 ± 14.83	111.20 ± 18.17
Food consumption (g/day)	26.17 ± 2.74	24.28 ± 2.39	25.11 ± 2.13	25.33 ± 2.38	23.85 ± 1.86
Water consumption (mL/day)	28.19 ± 5.99	30.81 ± 2.82	36.00 ± 9.39	30.60 ± 4.02	31.08 ± 3.35
Wet uterus (g)	0.1203 ± 0.0210	0.3118 ± 0.0520***	0.1093 ± 0.0115###	0.1043 ± 0.0111###	0.1068 ± 0.0096###
Blotted uterus (g)	0.1045 ± 0.0084	0.2372 ± 0.0109***	0.0993 ± 0.0125###	0.1000 ± 0.0103###	0.1002 ± 0.0107###
Wet uterus weight/b.w. (%)	0.039 ± 0.008	0.104 ± 0.020***	0.035 ± 0.003###	0.034 ± 0.002###	0.037 ± 0.004###
Blotted uterus weight/b.w. (%)	0.034 ± 0.004	0.079 ± 0.005***	0.032 ± 0.003###	0.032 ± 0.002###	0.035 ± 0.004###
Thyroid (g)	0.037 ± 0.017	0.025 ± 0.005	0.028 ± 0.005	0.024 ± 0.003	0.023 ± 0.003
Liver (g)	14.84 ± 2.10	14.35 ± 0.57	14.63 ± 1.73	14.22 ± 1.84	12.64 ± 1.94
Brain (g)	2.05 ± 0.10	1.96 ± 0.08	2.05 ± 0.11	1.98 ± 0.04	1.83 ± 0.12
Spleen (g)	0.74 ± 0.10	0.71 ± 0.07	0.76 ± 0.06	0.73 ± 0.14	0.67 ± 0.07
Thymus (g)	0.99 ± 0.12	1.00 ± 0.02	1.29 ± 0.22	1.01 ± 0.23	1.03 ± 0.20
Kidney (g)	1.07 ± 0.11	1.06 ± 0.12	1.08 ± 0.08	1.04 ± 0.10	1.10 ± 0.12

B.w.: body weight; EE: ethynyl estradiol.

Table 3
Parameters measured in the uterotrophic assay after the exposure to different MC-LR (75, 150 and 300 μg/kg b.w./day) and EE (10 μg/kg b.w./day) doses in Sprague Dawley rats. Data are presented as mean ± SD (standard deviation). The significance levels observed are ***p < 0.001 in comparison to the control group and ###p < 0.001 in comparison to the EE-treated group.

	CONTROL	EE 10	MC-LR 75	MC-LR 150	MC-LR 300
Body weight (g)	334.80 ± 23.78	321.30 ± 18.70	328.50 ± 23.15	333.50 ± 22.55	331.70 ± 15.72
Body weight gain (g)	110.80 ± 18.41	95.17 ± 7.11	103.30 ± 17.05	110.00 ± 11.45	102.20 ± 7.96
Food consumption (g/day)	25.27 ± 2.74	24.81 ± 1.53	25.18 ± 1.76	26.03 ± 1.72	25.89 ± 2.14
Water consumption (mL/day)	35.01 ± 7.02	34.55 ± 4.24	35.40 ± 8.64	37.05 ± 3.90	37.60 ± 5.35
Wet uterus (g)	0.1263 ± 0.0165	0.4615 ± 0.0804***	0.1210 ± 0.0133###	0.1185 ± 0.0088###	0.1233 ± 0.0187###
Blotted uterus (g)	0.1183 ± 0.0173	0.3007 ± 0.0125***	0.1127 ± 0.0126###	0.1118 ± 0.0085###	0.1172 ± 0.0187###
Wet uterus weight/b.w. (%)	0.038 ± 0.004	0.144 ± 0.029***	0.037 ± 0.004###	0.036 ± 0.003###	0.037 ± 0.004###
Blotted uterus weight/b.w. (%)	0.035 ± 0.004	0.094 ± 0.007***	0.034 ± 0.004###	0.033 ± 0.003###	0.035 ± 0.005###
Thyroid (g)	0.034 ± 0.007	0.035 ± 0.005	0.036 ± 0.009	0.033 ± 0.006	0.033 ± 0.005
Liver (g)	13.10 ± 1.30	13.50 ± 1.41	12.34 ± 1.22	12.96 ± 1.68	13.11 ± 1.06
Brain (g)	2.05 ± 0.05	1.99 ± 0.13	2.05 ± 0.06	1.95 ± 0.05	2.01 ± 0.02
Spleen (g)	0.81 ± 0.15	0.67 ± 0.11	0.69 ± 0.09	0.77 ± 0.04	0.68 ± 0.06
Thymus (g)	1.07 ± 0.11	1.02 ± 0.04	1.08 ± 0.11	1.04 ± 0.10	1.03 ± 0.13
Kidney (g)	1.09 ± 0.07	1.07 ± 0.09	1.07 ± 0.09	1.07 ± 0.08	1.05 ± 0.06

B.w: body weight; EE: ethynyl estradiol.

the rats of the treated groups (CYN or MC-LR) in comparison to their respective control groups.

The wet and blotted uterus weights (g) and their relative uterine weight to final body weight (%) are shown in Tables 2 and 3, as well as the weight of the different organs extracted from the experimental groups (CYN or MC-LR), EE-treated groups and negative control group. In the assay of CYN (Table 2), the group treated with EE showed, as expected, significant differences with the control group on the absolute and the relative wet and blotted uterus weights. However, CYN-treated group did not show significant differences compared to the control group, and all CYN-treated groups showed significant differences with EE-treated group, with significant lower values in comparison to EE-group. All these results indicated that CYN has no significant effect on the uterus weight at any dose assayed.

Similar results were obtained for MC-LR (Table 3), with significant higher values of absolute and relative wet and blotted uterus weights in the EE-treated group when compared to the control group. In contrast, no significant differences were found between control group and MC-LR-treated groups. Only significant differences were found in MC-LR treated rats in comparison to EE-treated group.

In addition, no significant differences were found in the organs weight studied, (thyroid, liver, brain, spleen, thymus and kidney) for both toxins, CYN and MC-LR in comparison to their respective control and EE groups.

3.2.2. Morphometric study of uteri and thyroid histopathology

The uterus morphometric study (Fig. 2) revealed that uteri from the EE-treated rats (C+) of both CYN and MC-LR experiments showed a significant statistical increase in the uterine luminal epithelial cell height compared to the negative control group reaching values between

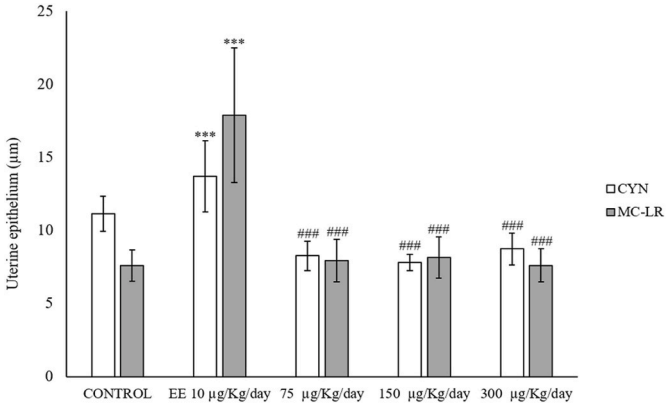

Fig. 2. Morphometry of the uterine epithelium (μm) obtained in the uterotrophic assay after the exposure for three days to different doses of CYN or MC-LR (μg/Kg b.w./day). Values are expressed as mean ± SD. The significant levels observed are ***p < 0.001 compared to the control group and ###p < 0.001 compared to the EE group.

14 and 18 μm with MC-LR showing the higher increase. However, the epithelial cell heights from rats administered with 75, 150 or 300 μg/kg b.w./day doses in both CYN and MC-LR groups presented values similar to the negative controls displaying differences statistically significant with C+ groups.

Thyroids (Fig. 3) of the negative controls from both CYN and MC-LR experiments presented the normal histological architecture showing round follicles, lined by a single layer of cuboidal epithelial cells, which are separated from each other by a loose fibroconnective tissue. The lumen of the follicle contains colloid which is an acidophilic material composed by proteins secreted by the follicular cells. Conversely, thyroids of the groups treated with EE, from both CYN and MC-LR experiments displayed areas of hypertrophy showing follicles lined by cuboidal to columnar epithelium with central follicles tightly packed and smaller than normal with little colloid or none, and sometimes disorganization of the follicles. Thyroids from rats administered with the higher doses of 150 and 300 μg/kg b.w./day of CYN or MC-LR presented features similar to the EE treated rats although slightly less severe displaying areas with hypertrophy and occasional disorganization of the follicles. Also, few follicles with lack of colloid and/or with exfoliated epithelium mixed with little colloid were sometimes found. Rats administered with the lowest dose of 75 μg/kg b.w./day of CYN or MC-LR showed mild focal hyperplasia and presence of exfoliated epithelium in few follicles with an architecture similar to the negative control.

3.3. Hormones measurement

3.3.1. Steroids hormones

As explained in section 2.5 we measured different hormones in both uterotrophic assays, for both toxins, CYN and MC-LR. Results for steroid hormones are showed in Fig. 4. In the case of serum levels of E_2 (Fig. 4A), negative control (C-) showed similar values for both assays, 1.99 ± 0.21 pg/mL (CYN) and 1.83 ± 0.29 pg/mL (MC-LR), and significant differences were found in the case of EE-group in comparison to its control group in the CYN assay. In CYN-treated rats, similar values of E_2 were found, ranging between 1.92 and 2.38 pg/mL, and no significant differences were detected in comparison to its C- group. For MC-LR assay, similar results were obtained at the different doses assayed, with values of E_2 ranging between 1.79 and 1.93 pg/mL, and no differences were found in comparison with the C- group. For both toxins, only significant differences were found at the intermediate dose of 150 μg CYN/Kg b.w./day or at the lower dose of 75 μg MC-LR/Kg b.w/day when compared with its respective EE groups.

Regarding to T (Fig. 4B), values for negative control groups were 101.43 ± 2.29 pg/mL, and 117.54 ± 18.83 pg/mL for CYN and MC-LR assays, respectively. Similar levels for toxin-treated groups were found with values of T ranging between 96.99 and 136.40 pg/mL, and 111.89–137.39 pg/mL for CYN and MC-LR, respectively. Only rats treated with 75 μg/kg b.w/day of CYN showed significant differences in comparison to its C- group.

For P (Fig. 4C), in the case of CYN assay, similar values were found in all groups (2507.95–3773.14 pg/mL) with no significant differences when compared with the C- group. For MC-LR a dose-dependent effect on P concentrations has been found, with values ranging between 5739.98, 9669.49 and 14,669.44 pg/mL for 75, 150 and 300 μg/kg b.w./day MC-LR groups, respectively. Moreover, significant differences were found at the higher doses assayed (150 and 300 μg MC-LR/Kg b.w/day) as well as in the case of EE group in comparison to the C- group.

Regarding to hormones measured with milliplex assay (LH and FSH), results showed similar values of LH (Fig. 4D) for the C- group for both assays (13.59 ± 2.34 and 14.11 ± 2.28 pg/mL for CYN and MC-LR assays, respectively). In the assay with CYN, a decrease in LH concentration as toxin concentration increased was observed, with values of 21.14 ± 1.97, 16.30 ± 2.12 and 14.81 ± 3.30 pg/mL for 75, 150 and 300 μg CYN/Kg b.w./day groups, respectively. Only in the lowest dose assayed of 75 μg CYN/Kg b.w./day group significant differences in comparison to the C- group were found. Similar LH levels were observed in all MC-LR-treated groups with a range of 13.46–15.28 pg/mL, and no significant differences were detected when compared with the controls.

In the case of FSH (Fig. 4E), values for negative controls were 775.63 ± 149.83 pg/mL, and 802.36 ± 283.94 pg/mL for CYN and MC-LR assays, respectively. However, a decreased in the FSH levels were found in EE-treated groups with values of 411.97 ± 67.40 pg/mL, and 510.15 ± 16.18 pg/mL for experiments with CYN and MC-LR, respectively. Similar and variable results were obtained in all the groups treated with CYN, showing a slight decrease in FSH levels as toxin concentration increased, with values of 1207.44 ± 574.51, 1176.10 ± 352.66 and 836.45 ± 308.31 pg/mL for 75, 150 and 300 μg CYN/Kg b.w./day groups, although no significant differences can be established with respect its C- group. Significant differences were found at doses of 75 and 150 μg CYN/Kg/day groups in comparison to EE-treated group. For MC-LR, very similar values were found in toxin-treated groups, with a range of 758.07–1005.17 pg/mL. Similar to CYN assay, EE-treated group showed the lowest concentration of FSH with 510.15 ± 16.18 pg/mL, and significant differences were found at the lowest dose of 75 μg MC-LR/Kg b.w/day group in comparison to its EE group.

3.3.2. Thyroid hormones

Different thyroid hormones, including T3, T4, and TSH, were measured in the blood serum and their results are showed in Table 4. Regarding T3 results, the C- groups showed similar values for both assays, with values of 76.17 ± 2.80 and 76.34 ± 2.36 ng/dL for CYN and MC-LR assays, respectively. The EE-treated group showed the highest T3 values in both experiments, 98.37 ± 5.02 and 94.37 ± 5.89 ng/dL for CYN and MC-LR assays, respectively. In both experiments, the EE-treated groups showed significant differences when compared with their respective C- groups. In the case of CYN-treated groups, T3 values were similar in all the exposed groups, and significant differences were found in comparison to the C- group. For MC-LR-experimental groups, the T3 values showed a slight increase as toxin concentration increased (79.82–86.58 ng/dL), and significant differences in comparison to C- group were found for the higher doses of 150 and 300 μg MC-LR μg/Kg b.w./day groups, and for EE group.

The T4 value for negative control in CYN assay was 4250 ± 397.40 ng/dL. CYN-treated groups showed an increment in T4 values at all doses assayed in comparison to negative control, with values ranging between of 5858.00 ± 650.89 and 5427.50 ± 436.69 ng/dL for 75 and 300 μg CYN/Kg b.w. groups, respectively. Significant differences were found in all groups in comparison to the C-. In the MC-LR assay, the higher T4 values were found at the higher doses of MC-LR assayed and significant differences were found in the EE, 150 and 300 μg MC-LR/Kg b.w./day groups, in comparison to C- group.

The T3/T4 ratio is an index which reflects the thyroid function and action of hormones on tissues (Couderc et al., 2016; Amereh et al., 2019). Molar ratios of T3/T4 remained unchanged with no significant differences in any assayed group.

The last thyroid hormone measured was TSH, and for CYN-treated rats a dose-dependent decrease was found, although only significant differences in comparison to C- group were found at the lowest dose group of 75 μg MC-LR/Kg b.w./day. For MC-LR assay, similar values of TSH were found in toxin-treated groups, and no significant differences were found in comparison to C- and EE groups.

4. Discussion

To evaluate *in vivo* the estrogenic potential of EDs, the uterotrophic bioassay in rodents has been applied to several compounds, using immature rats (de Lima Toccafondo Vieira et al., 2008; Hu et al., 2013) or ovariectomized female rats and mice (OVX) (Koda et al., 2007; Ohta et al., 2012; Okuda et al., 2010; Montagnini et al., 2018; Varayoud et al., 2016; Lee et al., 2022). The uterotrophic assay is a classical *in vivo* test to evaluate estrogenic activity at a level that includes the whole organ

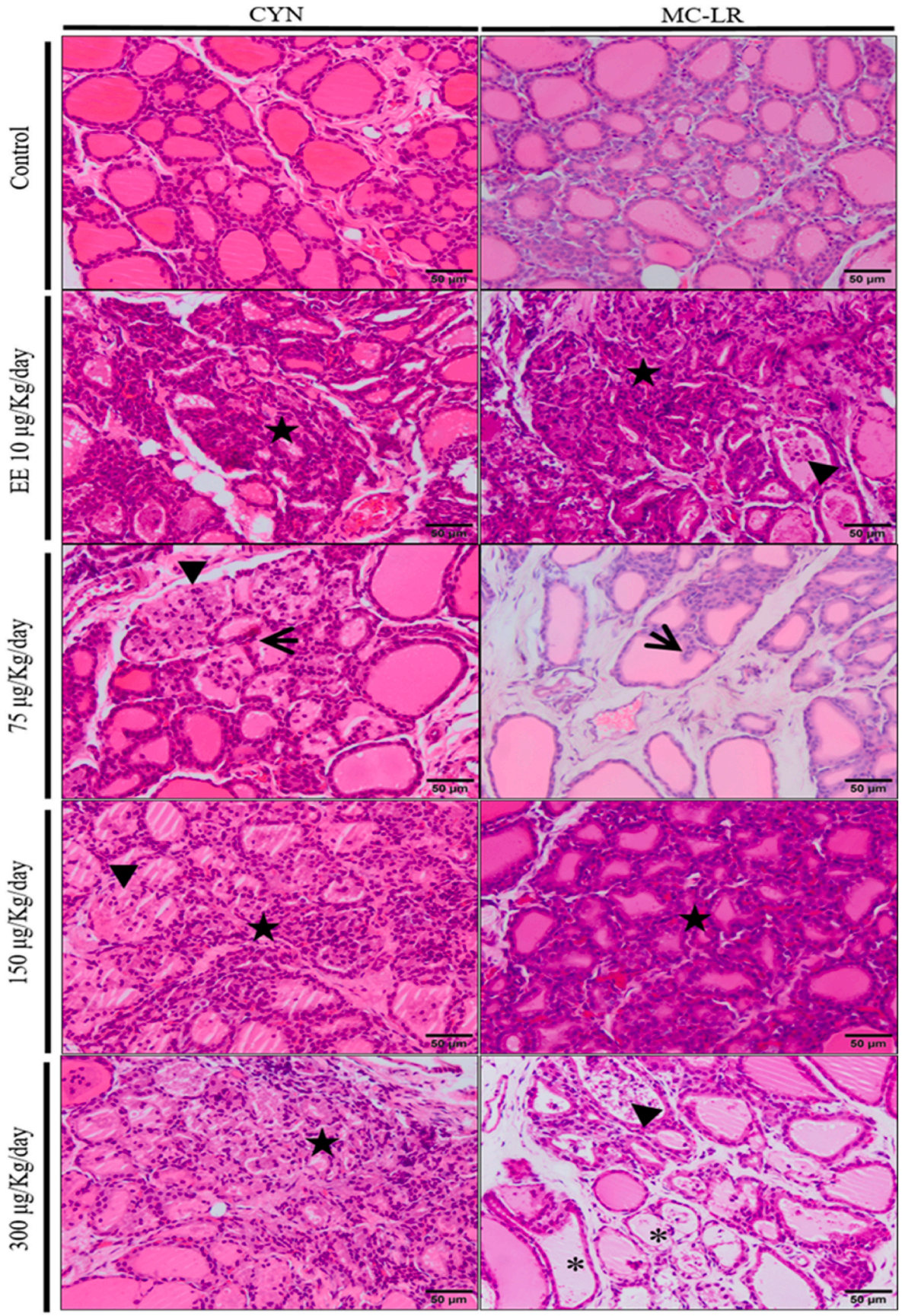

Fig. 3. Histopathological changes in thyroid from rats administered with cylindrospermopsin (CYN) or microcystin-LR (MC-LR) at different doses: 75, 150 or 300 µg/kg/day compared to the negative control and EE-treated group (EE 10 µg/kg/day). Star: Follicular hypertrophy; Arrowhead: exfoliated epithelium; Arrow: Hyperplasia; Asterisk: lack or very pale colloid.

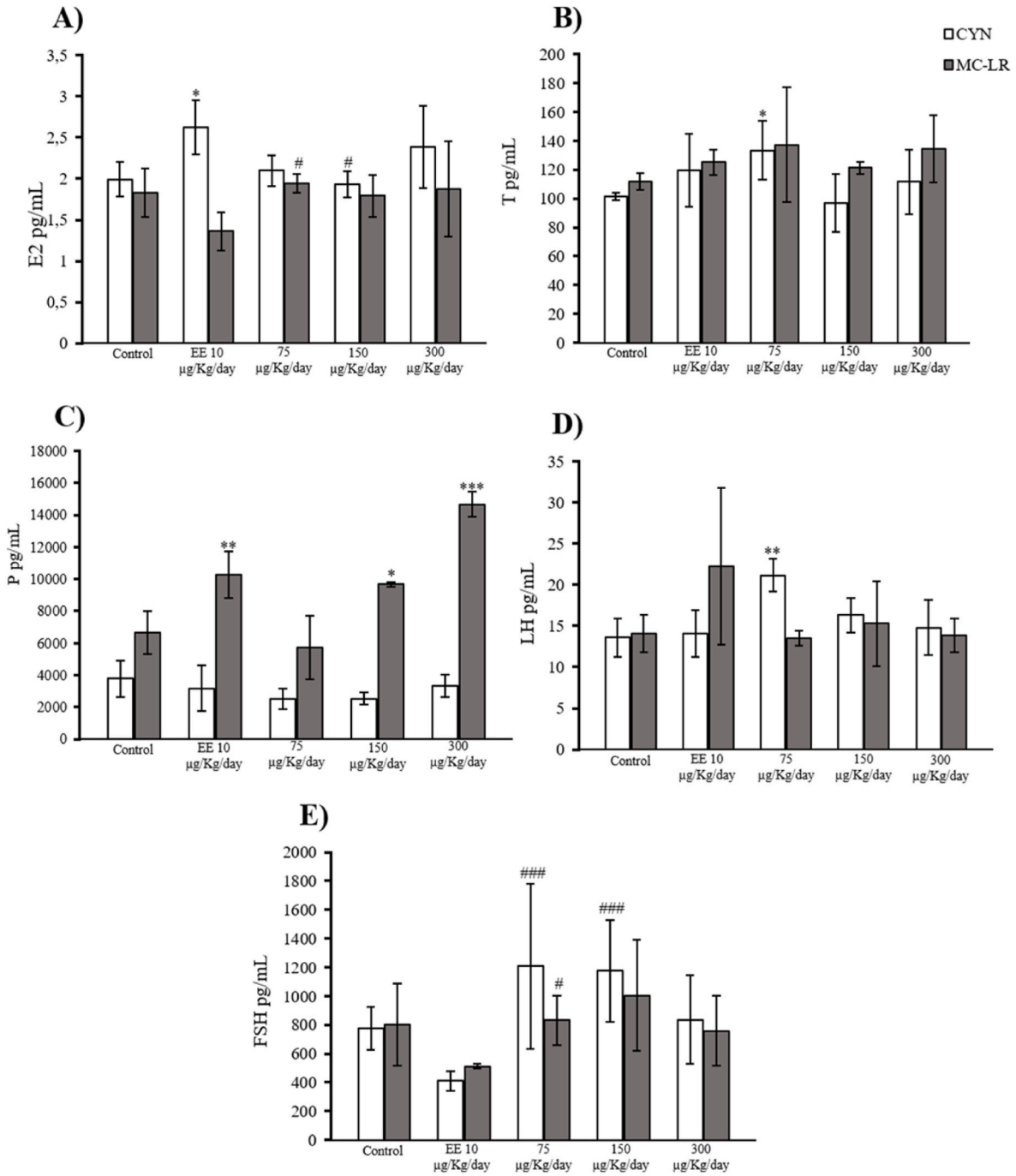

Fig. 4. Values obtained in serum of Sprague-Dawley rats for (a) estradiol; (b) testosterone; (c) progesterone; (d) luteinizing hormone and (e) follicle stimulating hormone after exposure for three days to different doses of CYN or MC-LR (µg/Kg b.w./day). Values are expressed as mean ± SD. The significant levels observed are *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the control group and #$p < 0.05$ and ###$p < 0.001$ compared to the EE group.

(Varayoud et al., 2016). In the case of cyanotoxins, the scarce works found in the scientific literature leading with their potential estrogenic activity were performed with extracts or exudates of cyanobacteria and green algae, using several *in vitro* assays (Stěpánková et al., 2011; Sychrová et al., 2012; Jonas et al., 2015; Mallia et al., 2020). In addition, only Oziol and Bouaïcha (2010) evidenced the estrogenic potential of pure MC-LR at low concentrations, *in vitro*, probably by indirect interaction with ERs. Liu et al. (2018) demonstrated the estrogenic activity of pure CYN by the yeast estrogen screen (YES) assay, and that the toxin modulated the E_2 estrogenic activity, resulting in non-monotonic responses. Furthermore, as it has been previously mentioned, none of these assays have been conducted under OECD guidelines. Based on the scientific literature reviewed (Casas-Rodriguez et al., 2022), the cyanotoxins CYN and MCs are potential endocrine disrupters, but research is required, particularly in the case of pure CYN, considering the use of OECD guidelines. In fact, as far as we know, no *in vivo* studies using the uterotrophic assay have been performed until now for either of the two pure toxins.

In this work we have investigated for the first time the potential estrogenic activities of CYN or MC-LR *in vivo,* in OVX rats, following the protocol described by OECD 440 (2007). In this OVX rat model, both toxins showed no significant effects on uterine status, because after treatment, no obvious differences were found between the body weights, body weight gains, and the absolute and relative wet and blotted uterine

Table 4
Thyroid hormones values obtained in serum of Sprague-Dawey rats after the exposure to different doses of CYN, MC-LR (75, 150 and 300 μg/kg b.w/day) or EE (10 μg/kg b.w./day)for three days. Values are expressed as mean ± SD. The significant levels observed are *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the control group.

	CONTROL	EE	75	150	300
CYN					
T3	76.17 ± 2.80	98.37 ± 5.02***	93.07 ± 4.72***	89.79 ± 2.82***	90.45 ± 7.15**
T4	4250.00 ± 397.40	5825.00 ± 769.77*	5858.00 ± 650.89***	5755.00 ± 986.99*	5427.0 ± 436.69***
T3/T4	0.0155 ± 0.0005	0.0160 ± 0.0004	0.0163 ± 0.0021	0.0154 ± 0.0023	0.0166 ± 0.0028
TSH	8.01 ± 2.05	9.51 ± 1.56	11.84 ± 1.14*	9.53 ± 3.54	8.06 ± 1.67
MC-LR					
T3	76.34 ± 2.36	94.37 ± 5.89***	79.82 ± 3.92$^{\#}$	85.70 ± 8.56*	86.56 ± 2.68**
T4	4824.00 ± 103.82	5752.00 ± 133.30**	5037.50 ± 221.57	5944.00 ± 413.92**	5800.00 ± 698.37**
T3/T4	0.0180 ± 0.0013	0.0170 ± 0.0017	0.0162 ± 0.0016	0.0149 ± 0.0005	0.0150 ± 0.0024
TSH	9.10 ± 0.42	12.38 ± 3.45	9.23 ± 0.47	9.47 ± 3.00	9.34 ± 1.47

EE: ethynyl estradiol.

weights of the rats in the negative control group (C-) and those of the rats in all the three treated groups for each toxin (CYN or MC-LR experimental groups). The assays were correctly performed because in rats that were given the dose of 10 μg/kg b.w./day EE (EE group) the absolute and relative uterine weights were significantly increased ($p < 0.05$) in comparison to control rats. This last fact agrees with the results reported by Lemini et al. (2003) in OVX mice, that showed that the uterine weight increased significantly to more than twice the control when EE was administered at the same dose assayed of 10 μg/kg b.w./day for 3 days.

The sensitivity of the uterotrophic assay has been questioned due to the negative results reported for several well-known estrogen-mimics, and according to some authors, to limit the assessment of a potential ED solely by the uterotrophic assay could result in a potential false negative result (Varayoud et al., 2016). Thus, we have complemented the assay with the histopathological evaluation of different organs, in order to identify potential estrogenic effects that could be missed. Following this suggestion, we have carried out a morphometric study of the uteri, and a histopathological evaluation of thyroids. In the present work, in uterus histology, from the apical cell surface to the basement membrane of rats treated with CYN or MC-LR, no significant differences were measured when compared to their respective C- group. Only the uterine epithelial cell heights were increased significantly in the case of the EE treated (C+) group in comparison to C- or rats exposed to CYN or MC-LR. In summary, and according to the results obtained in the current work, none of the toxins CYN or MC-LR, under the conditions tested (OCDE 440) showed a positive response in the uterotrophic bioassay.

No previous information has been found in the scientific literature related to the effects of CYN or MC-LR on uterus of OVX rats. In normal and mature rats, the studies available dealing with the effects of both cyanotoxins, especially CYN, on the ovary and uterus are very scarce, and mainly focused on ovaries changes. In the case of pure CYN, no changes in the weight of uterus and ovaries of rats after 28-days oral repeated exposure to 18.75, 37.5 or 75 μg CYN/Kg/day were observed; in addition, no histological changes were observed in both organs in treated rats when compared with control rats (Diez-Quijada et al., 2021b). In contrast, rats subchronically exposed to low doses of MC-LR (20 μg/kg/day group) showed a significant reduction of the relative ovary weight and pathomorphological changes in ovaries (Wu et al., 2014). In mice, chronic exposure to MC-LR at environmental levels leaded to decreased developmental follicles and a reduction of gonadosomatic index (GSI) (Wu et al., 2015).

In relation to the histopathological study of the thyroids, both toxins induced changes in the exposed rats, in a dose-dependent manner. Thus, at the higher doses assayed (150 or 300 μg/kg b.w./day) the rats exhibited areas with hypertrophy and disorganization of the follicles, and these lesions were similar to those observed in the group administered with EE, although with less intensity. The effects of chronic E_2 treatment on the thyroid gland structure of OVX rats were studied by Abdel-Dayem and Elgendy (2009) and smaller size of follicles, having small amount of colloid were reported, in agreement with the results obtained in the present work (central follicles were tighltly packed and smaller than normal with little colloid). Consequently, both toxins induced thyroid endocrine disruption, because the pathological changes observed in the thyroids of exposed rats (150–300 μg/kg b.w./day) were of the same order than those induced after administration of 10 μg EE/Kg b.w./day. Rats exposed to the lowest dose (75 μg/kg b.w./day of CYN or MC-LR) globally showed an architecture similar to the negative control. In the present work, despite adverse follicular cell hypertrophy has been found after exposure to each toxin, thyroid weights of both studies were all within normal limits. This fact agrees with the results analysed in a critical evaluation of 124 repro screening studies performed in mammals carried out by Beekhuijzen et al. (2019), in which there were 13 studies with histopathological findings in the thyroid, and only one presented increased thyroid organ weight.

The thyroid toxicity of MCs in mammals has been poorly studied (Casas-Rodriguez et al., 2022). In mice injected i.p. with MC-LR for 4 weeks, weight loss with thyroid disfunction was reported, although no histopathological study of thyroids was performed (Zhao et al., 2015). The chronic oral exposure of MC-LR (0–40 μg/L) in mice for 6 months, induced cell apoptosis without detectable structural changes in their thyroid tissues (Chen et al., 2019). In rats, thyroids of female rats exposed to a single i.p. injection at doses of 36.5, 54.75, or 73 μg MC-LR/Kg b.w. showed broken nuclei, necrosis of follicular epithelial cells and reduced intracellular colloid, and there were also exfoliated epithelial cells in the follicular cavity, with the most severe lesions appearing in the group of rats treated with the highest dose assayed (Chen et al., 2021). These last results agree with those obtained in the present work, although the experimental conditions, such as exposure route (i.p. injection versus oral gavage), the number and the range of doses administered of MC-LR were different. As far as we know, in the case of CYN no pathological studies have been found to date in mammals (Casas-Rodriguez et al., 2022).

The morphometric measurement of the endometrial epithelium could be directly correlated with changes in the sex steroids (E_2 and P) in the rodent uterus (Wood et al., 2007). In the present work, although no differences were found in the case of E_2 for CYN or MC-LR at any dose assayed, significant differences of P levels were found at the higher doses of MC-LR (150 and 300 μg MC-LR/Kg b.w/day) with respect its C- group. These changes could indicate effects of MC-LR in the rodent uterus, in a dose-dependent manner. In the scientific literature, P levels in OVX rats ranged between 5000 and 8500 pg/mL, in agreement with the values found in the present work for C- controls (Kuba et al., 2006; Tahara et al., 2021); consequently, the higher values found in MC-LR treated rats could be interpreted as effects due to the toxin. Contradictory effects of MC-LR on P concentrations in mammals have been previously reported (Casas-Rodriguez et al., 2022). Thus, increased P values were found in mice exposed to MC-LR in the high dose MC-LR treated group

(40 μg/L, oral route) after three and six months of exposure, and E_2 serum levels decreased (Wu et al., 2015). In contrast, the same authors found P decrease in mice subchronically exposed to MC-LR (28 days, i.p injection), although no alterations in serum E_2 were reported. These discrepancies could be due to differences in the experimental conditions of the studies, such as dose, periods of exposure, and route of administration of MC-LR. In comparison to the present work, the experimental model chosen, rats instead mouse, could also influence the results obtained.

In the present study, T levels in controls and exposed OVX rats were very similar, with the only exception of rats treated with the lower dose of CYN (75 μg CYN/kg b.w./day) (133.40 ± 20.46 pg/mL). This change has no apparent relation to CYN doses, and it was not considered toxicologically relevant, because levels of T in OVX rats (without toxin) were of the same order and oscillated between 80 and 170 pg/mL (Tivesten et al., 2006; Bonilla-Becerra et al., 2017). In addition, values of LH and FSH were also evaluated. The only statistically significant higher LH levels in the CYN-treated rats administered with the lowest dose in comparison to C-, could be considered as a sporadic change, not related to CYN dose. Although after MC-LR exposure our results did not show any changes in both hormone levels, Chen et al. (2021) reported higher concentrations of LH in female (not OVX) exposed to several concentrations of the toxin (36.5–73 μg MC-LR/kg b.w.). Further experiments are needed to clarify these contradictory results.

Thyroid disruption could be explained through several pathways, including alterations in the hypothalamus and/or pituitary status, hormone synthesis and secretion, regulation transport and metabolism, and/or interference with a receptor (Hoseini et al., 2016). To date a scarce number of assays (Chen et al., 2019, 2021) have been carried out in female rodents to study the potential thyroid disruption by MC-LR and no studies have been performed in the case of CYN (Casas-Rodriguez et al., 2022). Although thyroid histology did not necessarily reflect hormone levels (Schmutzler et al., 2007) in our case, OVX rats exposed to CYN or MC-LR, showed histopathological changes previously mentioned in thyroids, and in addition increments in T3 and T4 serum values, in comparison to their C- groups, while TSH values only increased in rats treated with 75 μg/kg b.w./day of CYN. These results are in contraposition with the results obtained by Chen et al. (2021) in rats injected with a single dose of MC-LR. These authors showed an increase in TSH concentrations in MC-LR treated rats (Chen et al., 2021).

Furthermore, in another study carried out by (Chen et al., 2019) in mice, the expression of thyroid hormones after oral administration of MC-LR for 6 months the expression of TSH was up-regulated compared with control at the doses of 20–40 μg MC-LR/L. Again, these contradictory results could be explained for the differences in dose administration (gavage oral vs injection) or the duration of the exposure (3 days vs 6 months). The T3/T4 ratios, unchanged after administration of every toxin at all doses assayed, were similar to other ED studies, like in the case of rats chronically exposed to pristine polystyrene nanoplastics (Amereh et al., 2019).

In general, the interferences by EDs on several levels of the HPT axis are diverse, and according to Schmutzler et al. (2007), they did not conform to classic mechanisms of endocrine regulation and feedback. Thus, hormone levels in 4-nonylphenol-treated (4-NP) rats did not shown concerted alterations, that is, higher T4 levels was not always accompanied by higher TSH. The 4-NP–treated animals showed significant decrease in the volume of epithelial compartments that resulted in a decrease of the epithelium versus colloid ratio by about 50%, and a significant increase in both T_3 and T_4serum levels, but, however, TSH was not decreased as it might have been expected from the morphologic data. All these data agree with the results obtained in the present work.

5. Conclusions

In summary, this work demonstrates that CYN and MC-LR after application of the uterotrophic assay in OVX rats, did not show estrogenic effects at the doses tested of each toxin (75–300 μg/kg b.w./day) because no significant differences in uterine weight were found. In addition, the morphometric study of the uteri did not reveal differences in the uterine luminal epithelial cells height after the administration of the toxins at the levels assayed. Despite the weights of thyroids were similar to control rats, both toxins induced thyroid histopathological changes in the experimental groups exposed to the higher doses (150–300 μg/kg b.w./day), similarly to the lesions induced after administration of 10 μg EE/Kg b.w./day. MC-LR showed a dose-dependent increase of P serum levels. Moreover, only specific increases in some thyroid hormones, T3 and T4 serum levels, were demonstrated for each toxin. Globally, the results obtained in the uterotrophic assay conducted under OECD guidelines in OVX rats (OECD 440) suggest that exposure to these pure toxins, CYN or MC-LR at the doses assayed did not act as estrogenic disruptors, although potential thyroid disruption has been demonstrated by the histopathological and hormone changes detected.

Credit author statement

Antonio Casas Rodríguez: Methodology, software, formal analysis, investigation, data curation, writing-original draft preparation, visualization. **Rosario Moyano:** Conceptualization, writing-original draft preparation. **Verónica Hernández-Molina:** Methodology, data curation, writing-original draft preparation. **Ana María Cameán:** Conceptualization, formal analysis, investigation, resources, writing-original draft preparation, writing-review and editing, supervision, project administration, funding acquisition. **Ángeles Jos:** Conceptualization, formal analysis, investigation, resources, writing-original draft preparation, writing-review and editing, supervision, project administration, funding acquisition.

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Institutional Review Board statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Ethical Animal Experimentation Committee of The University of Córdoba and by The Junta De Andalucía (Project No June 26, 2021/102 in both cases).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Camean AM reports financial support was provided by Spanish Ministerio de Ciencia e Innovación. Casas-Rodriguez A reports financial support was provided by Spanish Ministerio de Ciencia e Innovación. Molina-Hernandez V reports financial support was provided by Regional Government of Andalusia-FEDER.

Data availability

Data will be made available on request.

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References

Abdel-Dayem, M.M., Elgendy, M.S., 2009. Effects of chronic estradiol treatment on the thyroid gland structure and function of ovariectomized rats. BMC Res. 2, 173. https://doi.org/10.1186/1756-0500-2-173.

Amereh, F., Eslami, A., Fazelipour, S., Rafiee, M., Zibaii, M.I., Babaei, M., 2019. Thyroid endocrine status and biochemical stress responses in adult male Wistar rats chronically exposed to pristine polystyrene nanoplastics. Toxicol. Res. 8 (6), 953–963. https://doi.org/10.1039/c9tx00147f.

EFSA (European Food Safety Authority)with the technical support of the Joint Research Centre (JRC) Andersson, N., Arena, M., Auteri, D., Barmaz, S., Grignard, E., Kienzler, A., Lepper, P., Lostia, A.M., Munn, S., Parra Morte, J.M., Pellizzato, F., Tarazona, J., Terron, A., Van der Linden, S., 2018. 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA J. 16 (6). https://doi.org/10.2903/j.efsa.2018.5311, 5311, 135.

Beekhuijzen, M., Rijk, J.C.W., Meijer, M., de Raaf, M.A., Pelgrom, S., 2019. A critical evaluation of thyroid hormone measurements in OECD test guideline studies: is there any added value? Reprod. Toxicol. 88, 56–66. https://doi.org/10.1016/j.reprotox.2019.07.014.

Bonilla-Becerra, S.M., de Oliveira, M.G., Calmasini, F.B., Rojas-Moscoso, J.A., Zanesco, A., Antunes, E., 2017. Micturition dysfunction in four-month old ovariectomized rats: effects of testosterone replacement. Life Sci. 179, 120–129. https://doi.org/10.1016/j.lfs.2017.05.006.

Bouaïcha, N., Miles, C.O., Beach, D.G., Labidi, Z., Djabri, A., Benayache, N.Y., Nguyen-Quang, T., 2019. Structural diversity, characterization and toxicology of microcystins. Toxins 11 (12), 714. https://doi.org/10.3390/toxins11120714.

Brüggemann, M., Licht, O., Fetter, É., Teigeler, M., Schäfers, C., Eilebrecht, E., 2018. Knotting nets: molecular junctions of interconnecting endocrine axes identified by application of the adverse outcome pathway concept. Environ. Toxicol. Chem. 37 (2), 318–328. https://doi.org/10.1002/etc.3995.

Buratti, F.M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., Funari, E., 2017. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch. Toxicol. 91 (3), 1049–1130. https://doi.org/10.1007/s00204-016-1913-6.

Casas-Rodriguez, A., Cameán, A.M., Jos, A., 2022. Potential endocrine disruption of cyanobacterial toxins, microcystins and cylindrospermopsin: a review. Toxins 14 (12), 882. https://doi.org/10.3390/toxins14120882.

Catherine, A., Bernard, C., Spoof, L., Bruno, M., 2017. Microcystins and nodularins. In: Meriluoto, J., Spoof, L., Cood, G.A. (Eds.), Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis. John Wiley & Sons, Hoboken, NJ, pp. 109–126. https://doi.org/10.1002/9781119068761.ch11.

Chen, J., Bian, R., Li, J., Qiu, L., Lu, B., Ouyang, X., 2019. Chronic exposure to microcystin-LR reduces thyroid hormone levels by activating p38/MAPK and MEK/ERK signal pathway. Ecotoxicol. Environ. Saf. 173, 142–148. https://doi.org/10.1016/j.ecoenv.2019.02.024.

Chen, L., Shi, T., Wang, Y.T., He, J., Zhao, X., Wang, Y.K., Giesy, J.P., Chen, F., Chen, Y., Tuo, X., Chen, J., Xie, P., 2021. Effects of acute exposure to microcystins on hypothalamic-pituitary-adrenal (HPA), -gonad (HPG) and -thyroid (HPT) axes of female rats. Sci. Total Environ. 778, 145196 https://doi.org/10.1016/j.scitotenv.2021.145196.

Clarke, I.J., Campbell, R., Smith, J.T., Prevot, V., Wray, S., 2012. Neuroendocrine control of reproduction. In: Fink, G., Pfaff, D.W., Levine, J.E. (Eds.), Handbook of Neuroendocrinology. Elsevier, Amsterdam, pp. 197–236.

Couderc, M., Marchand, J., Zalouk-Vergnoux, A., Kamari, A., Moreau, B., Blanchet-Letrouvé, I., Le Bizec, B., Mouneyrac, C., Poirier, L., 2016. Thyroid endocrine status of wild European eels (*Anguilla anguilla*) in the Loire (France). Relationships with organic contaminant body burdens. Sci. Total Environ. 550, 391–405. https://doi.org/10.1016/j.scitotenv.2015.12.136.

de Lima Toccafondo Vieira, M., Duarte, R.F., Campos, L.M., Nunan, E.deA., 2008. Comparison of the estrogenic potencies of standardized soy extracts by immature rat uterotrophic bioassay. Phytomedicine 15 (1–2), 31–37. https://doi.org/10.1016/j.phymed.2007.06.006.

Decision 2020/569/EU. Commission Implementing Decision (EU) 2020/569 of 16 April 2020 Establishing a Common Format and Information Content for the Submission of the Information to be Reported by Member States Pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes and Repealing Commission Implementing Decision 2012/707/EU. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020D0569&from=EN.

Díez-Quijada, L., Guzmán-Guillén, R., Prieto Ortega, A.I., Llana-Ruíz-Cabello, M., Campos, A., Vasconcelos, V., Jos, Á., Cameán, A.M., 2018. New method for simultaneous determination of microcystins and cylindrospermopsin in vegetable matrices by SPE-UPLC-MS/MS. Toxins 10 (10), 406. https://doi.org/10.3390/toxins10100406.

Díez-Quijada, L., Llana-Ruiz-Cabello, M., Cătunescu, G.M., Puerto, M., Moyano, R., Jos, A., Cameán, A.M., 2019. *In vivo* genotoxicity evaluation of cylindrospermopsin in rats using a combined micronucleus and comet assay. Food Chem. Toxicol. 132, 110664 https://doi.org/10.1016/j.fct.2019.110664.

Diez-Quijada, L., Benítez-González, M.D.M., Puerto, M., Jos, A., Cameán, A.M., 2021a. Immunotoxic effects induced by microcystins and cylindrospermopsin: a review. Toxins 13 (10), 711. https://doi.org/10.3390/toxins13100711.

Diez-Quijada, L., Moyano, R., Molina-Hernández, V., Cameán, A.M., Jos, Á., 2021b. Evaluation of toxic effects induced by repeated exposure to Cylindrospermopsin in rats using a 28-day feeding study. Food Chem. Toxicol. 151, 112108 https://doi.org/10.1016/j.fct.2021.112108.

Diez-Quijada, L., Casas-Rodriguez, A., Guzmán-Guillén, R., Molina-Hernández, V., Albaladejo, R.G., Cameán, A.M., Jos, A., 2022. Immunomodulatory effects of pure cylindrospermopsin in rats orally exposed for 28 days. Toxins 14, 144. https://doi.org/10.3390/toxins14020144.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September, 2010. On the protection of animals used for scientific purposes. Available online:. https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF.

Drummond, A.E., 2006. The role of steroids in follicular growth. Reprod. Biol. Endocrinol. 4, 16. https://doi.org/10.1186/1477-7827-4-16.

Fawell, J.K., James, H.A., 1994. United Kingdom: Allen House. The Listons, Liston Road, Marlow, Bucks. SL7 IFD.

Fawell, J.K., Mitchell, R.E., Everett, D.J., Hill, R.E., 1999. The toxicity of cyanobacterial toxins in the mouse: I microcystin-LR. Hum. Exp. Toxicol. 18 (3), 162–167. https://doi.org/10.1177/096032719901800305.

Froscio, S.M., Humpage, A.R., Burcham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ. Toxicol. 18 (4), 243–251. https://doi.org/10.1002/tox.10121.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M., 2012. Time-dependent histopathological changes induced in Tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral and intraperitoneal route. Ecotoxicol. Environ. Saf. 76 (2), 102–113. https://doi.org/10.1016/j.ecoenv.2011.10.008.

Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán, A.M., 2013. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). Chemosphere 90 (3), 1184–1194. https://doi.org/10.1016/j.chemosphere.2012.09.027.

Hoseini, S.M., Hedayati, A., Taheri Mirghaed, A., Ghelichpour, M., 2016. Toxic effects of copper sulfate and copper nanoparticles on minerals, enzymes, thyroid hormones and protein fractions of plasma and histopathology in common carp *Cyprinus carpio*. Exp. Toxicol. Pathol. 68 (9), 493–503. https://doi.org/10.1016/j.etp.2016.08.002.

Hu, Y., Zhang, Z., Sun, L., Zhu, D., Liu, Q., Jiao, J., Li, J., Qi, M., 2013. The estrogenic effects of benzylparaben at low doses based on uterotrophic assay in immature SD rats. Food Chem. Toxicol. 53, 69–74. https://doi.org/10.1016/j.fct.2012.11.043.

Jia, Y., Chen, Q., Crawford, S.E., Song, L., Chen, W., Hammers-Wirtz, M., Strauss, T., Seiler, T.B., Schäffer, A., Hollert, H., 2019. Cyanobacterial blooms act as sink and source of endocrine disruptors in the third largest freshwater lake in China. Environ. Pollut. 245, 408–418. https://doi.org/10.1016/j.envpol.2018.11.021.

Jonas, A., Scholz, S., Fetter, E., Sychrova, E., Novakova, K., Ortmann, J., Benisek, M., Adamovsky, O., Giesy, J.P., Hilscherova, K., 2015. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular *in vitro* and zebrafish embryos assays. Chemosphere 120, 321–327. https://doi.org/10.1016/j.chemosphere.2014.07.074.

Kanno, J., Onyon, L., Haseman, J., Fenner-Crisp, P., Ashby, J., Owens, W., Organisation for Economic Co-operation and Development, 2001. The OECD program to validate the rat uterotrophic bioassay to screen compounds for *in vivo* estrogenic responses: phase 1. Environ. Health Perspect. 109 (8), 785–794. https://doi.org/10.1289/ehp.01109785.

Knobil, E., Neill, J.D., 2006. In: Neil, J.D. (Ed.), Knobil and Neill's Physiology of Reproduction, vol. 2. Gulf Professional Publishing, Houston, TX.

Koda, T., Morita, M., Imai, H., 2007. Retinoic acid inhibits uterotrophic activity of bisphenol A in adult ovariectomized rats. J. Nutr. Sci. Vitaminol. 53 (5), 432–436. https://doi.org/10.3177/jnsv.53.432.

Kuba, T., Wu, H.B., Nazarian, A., Festa, E.D., Barr, G.A., Jenab, S., Inturrisi, C.E., Quinones-Jenab, V., 2006. Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Horm. Behav. 49 (4), 441–449. https://doi.org/10.1016/j.yhbeh.2005.09.007.

Lee, N., Lee, J.H., Won, J.E., Lee, Y.J., Hyun, S.H., Yi, Y.-D., In, G., Han, H.D., Lee, Y.J., 2022. KRG and its major ginsenosides do not show distinct steroidogenic activities examined by the OECD test guideline 440 and 456 assays. J. Ginseng Res. https://doi.org/10.1016/j.jgr.2022.09.002.

Lemini, C., Jaimez, R., Avila, M.E., Franco, Y., Larrea, F., Lemus, A.E., 2003. *In vivo* and *in vitro* estrogen bioactivities of alkyl parabens. Toxicol. Ind. Health 19 (2–6), 69–79. https://doi.org/10.1191/0748233703th177oa.

Liu, W., Chen, C., Chen, L., Wang, L., Li, J., Chen, Y., Jin, J., Kawan, A., Zhang, X., 2016. Sex-dependent effects of microcystin-LR on hypothalamic-pituitary-gonad axis and gametogenesis of adult zebrafish. Sci. Rep. 6, 22819 https://doi.org/10.1038/srep22819.

Liu, H., Wang, W., Zhuang, D., Cheng, X., Zhang, H., 2017. The mechanisms of reproductive toxicity induced by MCs. Life Sci. 14 (7), 26–34. https://doi.org/10.7537/marslsj140717.05.

Liu, J., Hernández, S.E., Swift, S., Singhal, N., 2018. Estrogenic activity of cylindrospermopsin and anatoxin-a and their oxidative products by Fe^{III}-B*/H_2O_2. Water Res. 132, 309–319. https://doi.org/10.1016/j.watres.2018.01.018.

Mallia, V., Ivanova, L., Eriksen, G.S., Harper, E., Connolly, L., Uhlig, S., 2020. Investigation of *in vitro* endocrine activities of *microcystis* and *Planktothrix* cyanobacterial strains. Toxins 12 (4), 228. https://doi.org/10.3390/toxins12040228.

Metcalf, J.S., Codd, G.A., 2020. Co-occurrence of cyanobacteria and cyanotoxins with other environmental health hazards: impacts and implications. Toxins 12 (10), 629. https://doi.org/10.3390/toxins12100629.

Miller, W.L., Auchus, R.J., 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr. Rev. 32 (1), 81–151. https://doi.org/10.1210/er.2010-0013.

Montagnini, B.G., Pernoncine, K.V., Borges, L.I., Costa, N.O., Moreira, E.G., Anselmo-Franci, J.A., Kiss, A.C.I., Gerardin, D.C.C., 2018. Investigation of the potential effects of triclosan as an endocrine disruptor in female rats: uterotrophic assay and two-generation study. Toxicology 410, 152–165. https://doi.org/10.1016/j.tox.2018.10.005.

OECD, 2007. Test No. 440: Uterotrophic Bioassay in Rodents: A Short-Term Screening Test for Oestrogenic Properties, *OECD Guidelines For the Testing Of Chemicals*. OECD Publishing, Paris. https://doi.org/10.1787/9789264067417-en. Section 4.

Ohta, R., Takagi, A., Ohmukai, H., Marumo, H., Ono, A., Matsushima, Y., Inoue, T., Ono, H., Kanno, J., 2012. Ovariectomized mouse uterotrophic assay of 36 chemicals. J. Toxicol. Sci. 37 (5), 879–889. https://doi.org/10.2131/jts.37.879.

Okuda, K., Takiguchi, M., Yoshihara, S., 2010. *In vivo* estrogenic potential of 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, an active metabolite of bisphenol A, in uterus of ovariectomized rat. Toxicol. Lett. 197 (1), 7–11. https://doi.org/10.1016/j.toxlet.2010.04.017.

Oziol, L., Bouaïcha, N., 2010. First evidence of estrogenic potential of the cyanobacterial heptotoxins the nodularin-R and the microcystin-LR in cultured mammalian cells. J. Hazard Mater. 174 (1–3), 610–615. https://doi.org/10.1016/j.jhazmat.2009.09.095.

Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Camean, A.M., Repetto, G., 2005. The use of the fish cell lines RTG-2 and PLHC-1 to compare the toxic effects produced by microcystins LR and RR. Toxicol. Vitro 19 (7), 865–873. https://doi.org/10.1016/j.tiv.2005.06.016.

Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Cameán, A.M., Repetto, G., 2007. Acute and subacute toxic effects produced by microcystin-YR on the fish cell lines RTG-2 and PLHC-1. Toxicol. Vitro 21 (8), 1460–1467. https://doi.org/10.1016/j.tiv.2007.06.012.

Pichardo, S., Cameán, A.M., Jos, A., 2017. *In vitro* toxicological assessment of cylindrospermopsin: a review. Toxins 9 (12), 402. https://doi.org/10.3390/toxins9120402.

Puerto, M., Pichardo, S., Jos, A., Cameán, A.M., 2009. Comparison of the toxicity induced by microcystin-RR and microcystin-YR in differentiated and undifferentiated Caco-2 cells. Toxicon 54 (2), 161–169. https://doi.org/10.1016/j.toxicon.2009.03.030.

Puerto, M., Jos, A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20 (8), 1852–1860. https://doi.org/10.1007/s10646-011-0723-0.

Puerto, M., Prieto, A.I., Maisanaba, S., Gutiérrez-Praena, D., Mellado-García, P., Jos, Á., Cameán, A.M., 2018. Mutagenic and genotoxic potential of pure Cylindrospermopsin by a battery of *in vitro* tests. Food Chem. Toxicol. 121, 413–422. https://doi.org/10.1016/j.fct.2018.09.013.

Real Decreto 1386/2018, 2018. Real Decreto 1386/2018, de 19 de Noviembre, por el que se Modifica el Real Decreto 53/2013, de 1 de Febrero, por el que se Establecen las Normas Básicas Aplicables Para la Protección de los Animales Utilizados en Experimentación y Otros Fines Científicos, Incluyendo la Docencia. BOE Núm. 280. Available online: https://www.boe.es/boe/dias/2018/11/20/pdfs/BOE-A-2018-15797.pdf.

Rogers, E.D., Henry, T.B., Twiner, M.J., Gouffon, J.S., McPherson, J.T., Boyer, G.L., Sayler, G.S., Wilhelm, S.W., 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and microcystis reveals endocrine disrupting effects of Cyanobacteria. Environ. Sci. Technol. 45 (5), 1962–1969. https://doi.org/10.1021/es103538b.

Rosenfeld, C.S., Wagner, J.S., Roberts, R.M., Lubahn, D.B., 2001. Intraovarian actions of oestrogen. Reproduction 122 (2), 215–226. https://doi.org/10.1530/rep.0.1220215.

Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Lu, S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Pharmacol. 49 (2), 219–225. https://doi.org/10.1016/s0006-2952(94)00466-8.

Sabir, S., Akhtar, M.F., Saleem, A., 2019. Endocrine disruption as an adverse effect of non-endocrine targeting pharmaceuticals. Environ. Sci. Pollut. Res. Int. 26 (2), 1277–1286. https://doi.org/10.1007/s11356-018-3774-4.

Schmutzler, C., Gotthardt, I., Hofmann, P.J., Radovic, B., Kovacs, G., Stemmler, L., Nobis, I., Bacinski, A., Mentrup, B., Ambrugger, P., Grüters, A., Malendowicz, L.K., Christoffel, J., Jarry, H., Seidlovà-Wuttke, D., Wuttke, W., Köhrle, J., 2007. Endocrine disruptors and the thyroid gland–a combined *in vitro* and *in vivo* analysis of potential new biomarkers. Environ. Health Perspect. 115 (Suppl. 1), 77–83. https://doi.org/10.1289/ehp.9369. Suppl 1.

Serdarevic, N., 2018. The comparison between different immunoassays for serum carbohydrate Antigen (CA 19-9) concentration measurement. Acta Inf. Med. 26 (4), 235–239. https://doi.org/10.5455/aim.2018.26.235-239.

Stěpánková, T., Ambrožová, L., Bláha, L., Giesy, J.P., Hilscherová, K., 2011. *In vitro* modulation of intracellular receptor signaling and cytotoxicity induced by extracts of cyanobacteria, complex water blooms and their fractions. Aquat. Toxicol. 105 (3–4), 497–507. https://doi.org/10.1016/j.aquatox.2011.08.002.

Sychrová, E., Štěpánková, T., Nováková, K., Bláha, L., Giesy, J.P., Hilscherová, K., 2012. Estrogenic activity in extracts and exudates of cyanobacteria and green algae. Environ. Int. 39 (1), 134–140. https://doi.org/10.1016/j.envint.2011.10.004.

Tahara, A., Takamatsu, H., Ohtake, A., Tanaka-Amino, K., Kaku, S., 2021. Effects of neurokinin 3 receptor antagonist fezolinetant on hot flash-like symptoms in ovariectomized rats. Eur. J. Pharmacol. 905, 174207 https://doi.org/10.1016/j.ejphar.2021.174207.

Testai, E., Buratti, F.M., Funari, E., Manganelli, M., Vichi, S., Arnich, N., Biré, R., Fessard, V., Sialehaamoa, A., 2016. Review and analysis of occurrence, exposure and toxicity of cyanobacteria toxins in food. EFSA Support. Publ. 13, 1–309. https://doi.org/10.2903/sp.efsa.2016.EN-998.

Tijani, J.O., Fatoba, O.O., Petrik, L.F., 2013. A review of pharmaceuticals and endocrine-disrupting compounds: sources, effects, removal, and detections. Water, Air, Soil Pollut. 224, 1770. https://doi.org/10.1007/s11270-013-1770-3.

Tivesten, A., Bollano, E., Nyström, H.C., Alexanderson, C., Bergström, G., Holmäng, A., 2006. Cardiac concentric remodelling induced by non-aromatizable (dihydro-) testosterone is antagonized by oestradiol in ovariectomized rats. J. Endocrinol. 189 (3), 485–491. https://doi.org/10.1677/joe.1.06722.

Varayoud, J., Durando, M., Ramos, J.G., Milesi, M.M., Ingaramo, P.I., Muñoz-de-Toro, M., Luque, E.H., 2016. Effects of a glyphosate-based herbicide on the uterus of adult ovariectomized rats. Environ. Toxicol. 32 (4), 1191–1201. https://doi.org/10.1002/tox.22316.

Varayoud, J., Monje, L., Bernhardt, T., Muñoz-de-Toro, M., Luque, E.H., Ramos, J.G., 2008. Endosulfan modulates estrogen-dependent genes like a non-uterotrophic dose of 17 beta-estradiol. Reprod. Toxicol. 26 (2), 138–145. https://doi.org/10.1016/j.reprotox.2008.08.004.

Weralupitiya, C., Wanigatunge, R.P., Gunawardana, D., Vithanage, M., Magana-Arachchi, D., 2022. Cyanotoxins uptake and accumulation in crops: phytotoxicity and implications on human health. Toxicon 211, 21–35. https://doi.org/10.1016/j.toxicon.2022.03.003.

WHO/IPCS (World Health Organization/International Programme on Chemical Safety), 2002. Global Assessment of the State-Of-The-Science of Endocrine Disruptors. WHO/PCS/EDC/02.2, p. 180. https://apps.who.int/iris/handle/10665/67357.

Wood, G.A., Fata, J.E., Watson, K.L., Khokha, R., 2007. Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus. Reproduction 133 (5), 1035–1044. https://doi.org/10.1530/REP-06-0302.

World Health Organization, 2020a. Cyanobacterial Toxins: Cylindrospermopsins. Background Document for Development of WHO Guidelines for Drinking-Water Quality and Guidelines for Safe Recreational Water Environments. World Health Organization, Geneva. https://apps.who.int/iris/handle/10665/338063.

World Health Organization, 2020b. Cyanobacterial Toxins: Microcystins. Background Document for Development of WHO Guidelines for Drinking-Water Quality and Guidelines for Safe Recreational Water Environments. World Health Organization, Geneva. https://apps.who.int/iris/handle/10665/338066.

Wu, J., Shao, S., Zhou, F., Wen, S., Chen, F., Han, X., 2014. Reproductive toxicity on female mice induced by microcystin-LR. Environ. Toxicol. Pharmacol. 37 (1), 1–6. https://doi.org/10.1016/j.etap.2013.10.012.

Wu, J., Yuan, M., Song, Y., Sun, F., Han, X., 2015. MC-LR exposure leads to subfertility of female mice and induces oxidative stress in Granulosa cells. Toxins 7 (12), 5212–5223. https://doi.org/10.3390/toxins7124872.

Zhang, S., Du, X., Liu, H., Losiewic, M.D., Chen, X., Ma, Y., Wang, R., Tian, Z., Shi, L., Guo, H., Zhang, H., 2021. The latest advances in the reproductive toxicity of microcystin-LR. Environ. Res. 192, 110254 https://doi.org/10.1016/j.envres.2020.110254.

Zhao, Y., Xue, Q., Su, X., Xie, L., Yan, Y., Steinman, A.D., 2015. Microcystin-LR induced thyroid dysfunction and metabolic disorders in mice. Toxicology 328, 135–141. https://doi.org/10.1016/j.tox.2014.12.007.