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Sex hormonal disruption by cyanobacterial bioactive compounds

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Abstract

Aims: This study investigated the effect of some cyanobacterial exudates on the level of sex hormones in a mammalian model and to identify the molecules that could act as endocrine disruptor.
Methods and Results: In this study, the cytotoxicity of purified exudates of some axenic cyanobacterial cultures (*Nostoc ellipsosporum*, *Nostoc muscorum*, *Anabaena oryzae* & *Anabaena*. sp.) was evaluated against normal melanocyte cell line (HFB4). The intraperitoneal injection of the cyanobacterial exudates demonstrated a marked disturbance in the serum level of testosterone, progesterone, follicular stimulating hormone (FSH) and luteinizing hormone (LH) of male mice. GC-MS and LC–MS/MS analysis showed the presence of some sterol-like compounds in the cyanobacterial filtrates.
Conclusions: This work demonstrated that the nontoxic cyanobacterial species have the ability to produce some bioactive compounds into their surroundings that can disrupt the mammalian reproductive hormones.
Significance and Impact of Study: The cyanobacterial extracellular bioactive molecules can affect on the production of the sex hormones via positive and negative feedback and may be a risk to human beings.

Introduction

Cyanobacteria are widespread photosynthesizing Gram-negative bacteria. They are not only capable of modifying their habitat through fixation of atmospheric nitrogen but also capable of producing biologically active natural products. A diverse group of cyanobacterial secondary metabolites have been reported as anticancer, antifungal, antiviral, anti-inflammatory and antimalarial (Stewart *et al.* 1988; Stratmann *et al.* 1994; Reshef *et al.* 1997; Dey *et al.* 2000; Bernardo *et al.* 2004; Patel *et al.* 2006; Rastogi and Sinha 2009). In addition, many investigations have focused on a group of cyanobacterial metabolites belonging to the classes of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) (Hallam *et al.* 1988; Barrios-Llerena *et al.* 2007).

Despite of the beneficial impact of cyanobacteria such as nitrogen fixation, CO_2 fixation and hydrogen production (Yu and Takahashi 2007), certain species of cyanobacteria are known to produce a variety of potent toxins. These harmful compounds can cause a range of effects including skin irritation, gastrointestinal tract distress, neurotoxicity, hepatotoxicity effects following ingestion of water containing toxic blue-green algal cells or the toxins released by ageing cells (Cox *et al.* 2005; Banack *et al.* 2007; Jaiswal *et al.* 2008; Oziol and Bouaicha 2010).

Cyanobacteria have the potential to produce mass populations in natural and controlled water bodies. Large masses of these organisms can occur under conditions of eutrophication that causes number of problems such as shading of the macrophytes and phytoplanktons in the water column and the depletion of oxygen in water. Moreover, the cyanobacterial blooms release certain metabolites into their environments that act as antigrazing agents to discourage phytoplankton grazers in marine and freshwater environments (Cruz-Rivera and Paul 2006). Some of these metabolites could have a harmful effect on the populations of aquatic life (Robin *et al.* 2006; Paul 2008).

Oestrogens are natural compounds produced by some organisms, which are responsible for metabolic, behavioural and morphological changes that happen during various phases of reproduction. They affect cell proliferation and differentiation, development and activity of tissues participating in reproduction (Janosek *et al.* 2006; Speranza 2010).

Recently, a severe problem has emerged in the water environment, specifically, the contamination of food and water by endocrine disrupting compounds (Anway *et al.* 2005; Timms *et al.* 2005) that may adversely affect the reproduction function of human beings and wildlife. The oestrogenic compounds are characterized by their ability to bind with the oestrogen receptors and mimic the action of oestrogen hormones naturally produced by an organism (Fisher 2004).

Endocrine disrupting compounds are now widely distributed in the environment as a result of different human activities; pesticides, industrial products, drugs, fattening agents as well as natural products such as human and animal wastes. Moreover, some plant-derived compounds called phytoestrogens are naturally formed and were proven to possess oestrogen-like activity (Strauss *et al.* 1998; Henley and Korach 2006).

Considerable amounts of scientific resources have been employed to clarify the potential risk of the secondary metabolites of cyanobacteria. Some of these compounds might be toxic and linked with health problems of human and aquatic organisms. Nevertheless, nonlethal and chronic exposure to the cyanobacterial toxins may cause severe organ damage and impairment also resulting in an accumulation of these metabolites within exposed organisms (Sipia *et al.* 2002; Xie *et al.* 2005). Furthermore, the exposure of mice to some toxic cyanobacterial extracts has induced a harmful effect on the male reproductive system and some sex hormones (Ding *et al.* 2006).

Algal blooms are formed in the canals, banks and drains of Fayoum (Egypt) during spring and summer seasons. These blooms that were dominated with the cyanobacterial genera *Anabaena* and *Nostoc* showed negative impacts on fish during summer season (R.M. Ali, unpublished data). So, the main objective of this study was to investigate whether the local nontoxic cyanobacterial isolates can release any endocrine disruptors that may affect on the sex hormones in male mice and to identify these molecules.

Materials and methods

Isolation and growth of the cyanobacterial strains

Water samples were collected from the agricultural drains in Fayoum during summer season (2010). Samples were filtered through sterilized Whatman No. 41 filter paper and then suspended in 5-ml sterilized BG11 medium (Rippka *et al.* 1979). One to two drops of each suspension were inoculated on solid BG11 and incubated for about 2 weeks in culture room at 25 ± 1°C under controlled continuous illumination of 40 μEm^{-2} s^{-1}. The plates were examined and the best colonies were selected, picked up and restreaked to new agar plates. Restreaking and subculturing were repeated several times to obtain unialgal cultures. To get axenic cultures of the test organisms, the tested algae were grown in liquid cultures for 12 days to attain vigorous growth. About 20 ml of each culture were centrifuged at 3000 ***g*** for 10 min, and the algal pellets were then streaked on peptone or yeast extract solid medium. Those which proved not to be axenic, streaks were repeated till they became axenic. Inocula from axenic cultures were taken into sterilized liquid medium to be ready for the desired experiments. The purified algae were identified as *Nostoc muscorum*, *Nostoc ellipsosporum, Anabaena oryzae* and *Anabaena* sp. (Prescott, 1978).

Chlorophyll 'a' content was used to monitor the cyanobacterial growth. Cultures were centrifuged at 3000 ***g*** for 10 min to harvest the cells. In this case, supernatants were discarded and chlorophyll 'a' content in the biomass was extracted using the standard acetone extraction method described in APHA (1999). After extraction, chlorophyll 'a' was determined spectrophotometrically at 750 and 665 nm using a PYE Nnicom, SP8-100 UV spectrophotometer. To estimate the dry weight of the algal biomass, the harvested cells were washed out three times with sterile medium. The pellets formed after the last centrifugation were used for determination of pellet dry weight after drying at 105°C to constant weight.

Preparation and purification of the cyanobacterial exudates

Batch cultures of the cyanobacterial strains were grown on BG11 medium for 28 days under the previous conditions. Cells were separated from extracellular products of laboratory cultures by centrifugation at 3000 ***g*** for 10 min. The biomass was subjected to chlorophyll 'a' content and dry weight analysis (Table 1) while the supernatant was stored frozen at −20°C. The extracellular organic compounds of the cyanobacterial filtrates were purified using C18 solid-phase extraction discs (Empore, 3M, Minneapolis, MN, USA). Prior to extraction, the discs were conditioned with methanol and water. Two hundred millilitre of each strain filtrate was extracted with 47-mm discs by applying vacuum to maintain a flow rate of 0·5–5 ml min^{-1}. The sterol-like compounds were eluted from the C18 discs three times with 5 ml of

Table 1 Growth parameters of the cyanobacterial cultures (*Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena.* sp.) after 4 weeks

	Nostoc ellipsosporum	*Nostoc muscorum*	*Anabaena oryzae*	*Anabaena. sp.*
Chl 'a' (mg l^{-1})	6·7	7·9	9·5	10·4
D.wt (mg l^{-1})	791	684	672	579

methanol each time, which was blown down to dryness and stored frozen. The content was dissolved in physiological saline solution (1 mg min^{-1}) for intraperitoneal injection of the mice. Another set of the purified exudates were dissolved in methanol/water (65/35, v/v) and used for the GC-MS and LC–MS/MS analysis.

Cytotoxicity of the cyanobacterial exudates

According to Singab *et al.* (2012), one mg of each of the purified frozen cyanobacterial exudates was subjected to Sulforhodamine-B (SRB) assay of cytotoxic activity at concentration of 0–200 μg ml^{-1}using normal melanocyte cell line (HFB4). The HFB4 cells were obtained from the American Type Culture Collection at National Cancer Institute, Cairo, Egypt. Cells were seeded in 96-well microtiter plates at a concentration of 5×10^5 cell per well in a fresh medium and were left to attach to the plates for 24 h. Then, they were incubated with the different concentrations of the cyanobacterial exudates and incubation was continued for 24 h. After that the cells were fixed with 50 μl cold 50% trichloroacetic acid for 1 h at 4°C. Wells were washed five times with distilled water and stained for 30 min at room temperature with 50 μl of 0·4% SRB dissolved in 1% acetic acid. The wells were washed four times with 1% acetic acid. The plates were air-dried, and the dye was solubilized with 100 μl per well of 10 mmol l^{-1} tris-base (pH 10·5) for 5 min on a shaker at 1600 rpm. The optical density was measured spectrophotometrically at 564 nm with an ELISA microplate reader (Meter tech. Σ 960, Fisher Bioblock, Illkirch, France). The percentage of cell survival was calculated as the concentrations of the cyanobacterial exudates required to produce 50% inhibition of cell growth (IC50) according to the following equation::

$$\text{Survival fraction} = \text{OD (treated cells)}/\text{OD (control cells)}$$

Animals, treatment and hormonal assay

Twenty-five male albino mice weighing 20–25 g were obtained from the National Cancer Institute (NCI), Cairo University, Egypt. The subjected mice were housed in a temperature controlled room under 12:12 h light/dark cycle and had free access to food pellets and tap water *ad libitum*. The minimal number of animals needed to obtain statistical significance was used, and all efforts were made to minimize animal suffering. On the day of experiment, mice were divided into five groups, four of them received three intraperitoneal injections of 25 mg kg^{-1} of the purified cyanobacterial exudates in saline at 24-h intervals (total dose: 75 mg kg^{-1}) while the control mice group was given equal dose of physiological saline only. One day postinjection, mice were decapitated and blood samples were placed at room temperature for *c.* 30 min then, the tubes were centrifuged at 3000 ***g*** for 10 min, and the supernatants were collected. Serum concentrations of total testosterone, free testosterone and progesterone were measured by radioimmunoassay (RIA) using a commercial kit obtained from BioSource (Nivelles, Belgium). Meanwhile, LH and FSH were measured by direct chemiluminescence (ADVIA Centaur, Bayer Co., Bayer AG, Leverkusen, Germany).

Analysis of sterol-like compounds

Gas chromatography–mass spectrum analysis

Gas chromatography–mass spectrum analysis was carried out in Faculty of Science (Fayoum University) on an Agilent 5973 single quadrupole mass spectrometer (Palo Alto, CA, USA), coupled with an Agilent 6890 gas chromatograph used with an Agilent DB-5.625 (30 × 0·25 mm inner diameter × 0·25 μm) analytical column (inlet temperature, 275°C; injection volume, 2 μl; J&W Scientific, Folsom, CA, USA). The total GC run time was 53 min, and the carrier gas was helium. The initial oven temperature was held at 80°C for 2 min and then increased by 10°C min^{-1} till it reached 290°C, after which it was held at this temperature for 30 min. The injector temperature was 290°C, and the split ratio was 1 : 30. NIST Mass Spectral library was used to identify the compounds in the extracts using hexane as a control. The closest match with the highest probability in the library was recorded.

Liquid chromatographic tandem mass spectrometric analysis

The used MS system was a Quattro Micro mass spectrometer (Waters, Milford, MA, USA). High purity nitrogen was used as the drying and electrospray ionization (ESI) nebulizing gas and was set at 100 and 500 lh^{-1}, respectively. Argon was used as the collision gas with a gas cell pirani pressure of 3·87 e−3 mbar. The capillary voltage was set at 3·5 kV, and the source block and desolvation temperatures were 100 and 300°C, respectively. Chromatography was performed using a Waters Alliance 2695 (Waters, Milford). The column was a Water

Symmetry® C18, 2·1 × 50 mm, 3·5 μm particle size, with a guard column. The flow rate was 0·3 ml min^{-1}, and an injection volume of 10 μl was used. The run time of the electrospray positive ionization (ESI+) mode was 60 min and the mobile phase consisted of methanol/water (65/35, v/v) containing 0·3% formic acid.

Statistical methods

Results are presented as mean ± standard error of the mean. Data were compared for significant differences using Statistica Program (version 5, Analyse-it Software, Ltd., Leeds, UK) and Student's *t*-test. The levels of significance chosen were $P < 0·05$ and $P < 0·01$.

Results

Cytotoxicity activity of the cyanobacterial exudates

By comparing the cytotoxicity activity of the different cyanobacterial exudates on melanocyte cell line (HFB4), a low cytotoxic action was observed with *N. ellipsosporum* (IC50 83·4 μg ml^{-1}), *N. muscorum* (IC50 89·6 μg ml^{-1}), *A. oryzae* (IC50 76·3 μg ml^{-1}) and *A.* sp (IC50 86·1 μg ml^{-1}) as compared to standard doxorubicin, which showed cytotoxic activity at IC50 2·9 μg ml^{-1}.

Effect of the cyanobacterial exudates on the sex hormones level

Data showed in Fig. (1) clarified a sharp change in the serum level of sex hormones of the male mice that were intraperitoneal injected with the purified cyanobacterial filtrates of *N. ellipsosporum, N. muscorum, A. oryzae & A.* sp. A highly significant decrease in the total and free testosterone was recorded with the exudates of *N. ellipsosporum* (95·3 & 96·6%), *N. muscorum* (94·7 & 96·3%) and *Anabaena* sp. (93·9 & 96·1). Meanwhile, *A. oryzae* showed an enhancement in the total and free testosterone levels (21·5 & 4·3%). Interestingly, the progesterone level in the treated mice was massively reduced due to the injection of the cyanobacterial filtrates; 76·4% for *N. ellipsosporum*, 67·1% for *N. muscorum*, 71·7% for *A. oryzae* and 59·7% for *A.* sp. Similarly, the level of FSH hormone was significantly declined in case of *N. muscorum* (31·8%) *and A. oryzae* (40·9%), while with *N. ellipsosporum* a nonsignificant change was obtained (4·5%). On the other hand, the exudate of *Anabaena* sp. recorded a massive increase in FSH level (1509·1%). At the same time, the LH level was sharply increased with *N. ellipsosporum* (169·2%), *N. muscorum* (123·1%) and *Anabaena sp.* (976·9%) while an inhibition of the LH level was recorded with *A. oryzae* (15·4%).

Identification of sterol-like hormones

The GC-MS analysis of the purified filtrates of the tested cyanobacterial cultures showed the presence of some sterol-like compounds (Fig. 2). The chemical composition of the bioactive compounds of the cyanobacterial exudates was compared using GC-MS. The identity of most peaks was determined by direct comparison to NIST GC-MS chemical library. The metabolites indicated by peaks with retention times as shown in Table 2. In case of *N. ellipsosporum*, compounds with retention times (17·15, 16·32, 15·95 & 13·67 min) showed a similarity of 52·1% to corticosterone, 69·8% to 2-methoxyesterone, 74·6% to hydrocortisone and 55·4% to androsterone. Another set of peaks with retention times (20·99, 17·05, 18·56 & 19·36 min) have been recorded in case of *N. muscorum* exudates and showed resemblance to corticosterone (81·9%), hydroxyprogesterone (67·7%), methyltestosterone (53·2%) and hydrocortisone (54·6%). At the same time, some sterol-like compounds were identified in the exudates of *A. oryzae* with similarity percentage with similarity 76·8% to corticosterone, 88·7% to hydrocortisone and 57·3% to hydroxyprogesterone at retention times 17·89, 16·08 and 10·39 min, respectively. Also, the filtrate of the *Anabaena sp.* culture contained some sterol-like molecules with similarity percentage 76·2% with corticosterone (17·64 min), 51·4% with boldenone (18·32 min) as well as 49·3% with hydroxyprogesterone (17·21 min).

Due to the low similarity percentage between steroidal compounds of the cyanobacterial exudates and the available eukaryotic steroids, LC–MS/MS was used with cholesterol as reference that was broken down into product ions 88·30, 106·22 and 256·34 g mol^{-1}. The latter was considered as a nucleus for the sterol-like molecules. This nucleus is composed of four fused rings; three cyclohexane rings and one cyclopentane ring (Fig. 3). Consequently, some sterol-like molecules with molecular mass 328·1, 504·3, 354·7 and 859·8 g mol^{-1} were identified in the exudates of *N. ellipsosporum* at retention time 0·26, 0·52, 0·77 and 2·31 min, respectively, as shown in Fig. 4. Similarly, the filtrate of *N. muscorum* culture contained some sterol-like compounds with molecular mass 677·8, 801·0 and 936·8 g mol^{-1} at retention time 0·37, 0·94 and 2·30 min, in that order. In case of *A. oryzae*, another set of the sterol-like compounds with molecular mass 304·5, 471·1, 318·8 and 462·1 g mol^{-1} was identified in their exudates at retention time 0·47, 2·88, 4·10 and 4·68 min, respectively. At the same time, an additional group of these compounds was spotted in the filtrate of *A.* sp. with molecular mass 705·1, 614·3 and 698·6 g mol^{-1} at retention time 1·29, 1·60 and 2·53 min, correspondingly.

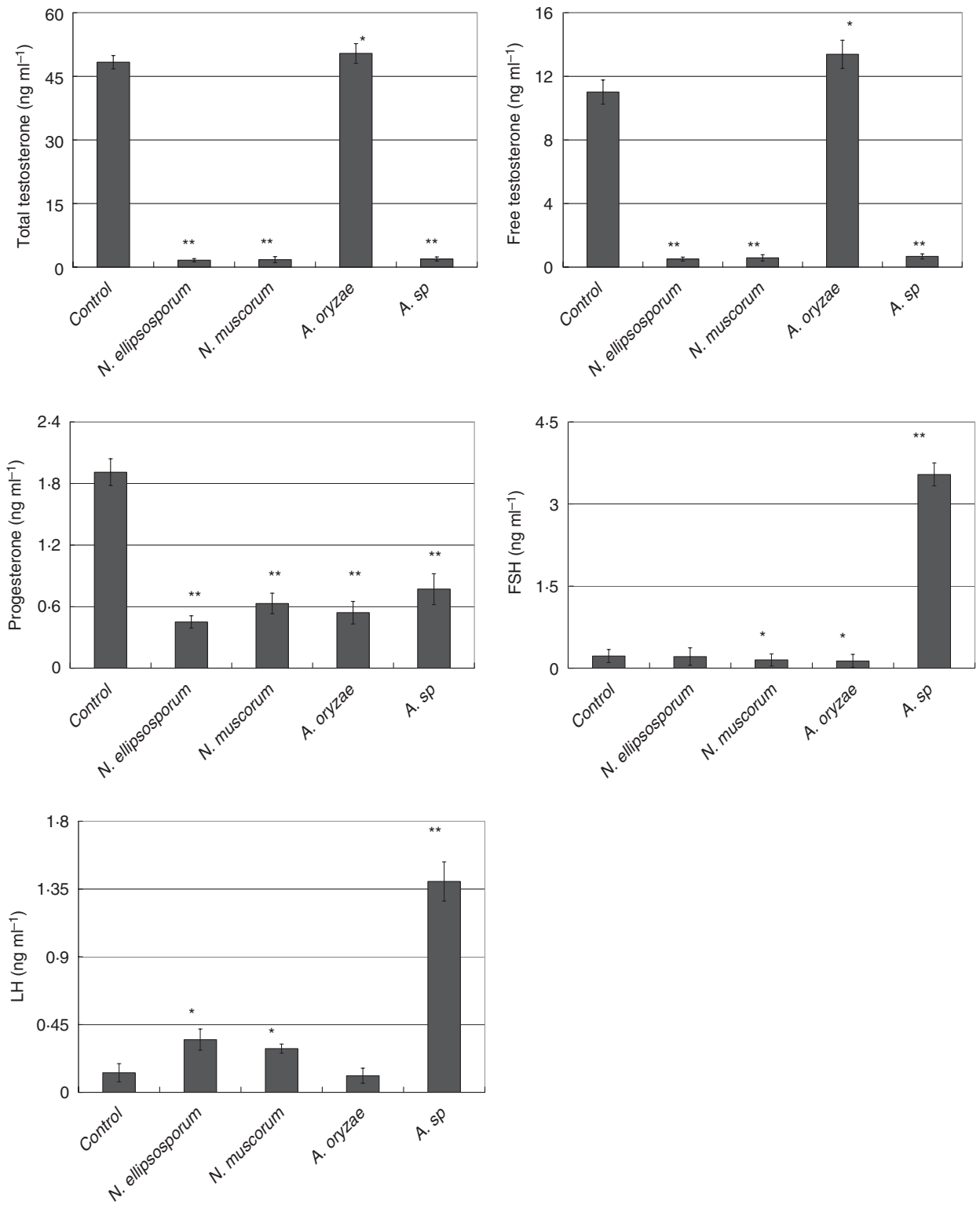


Figure 1 Levels of testosterone, progesterone, follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) in the serum of male mice intraperitoneal injected with cyanobacterial exudates of *Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena.* sp. Significant (*) and highly significant differences (**) are depicted.

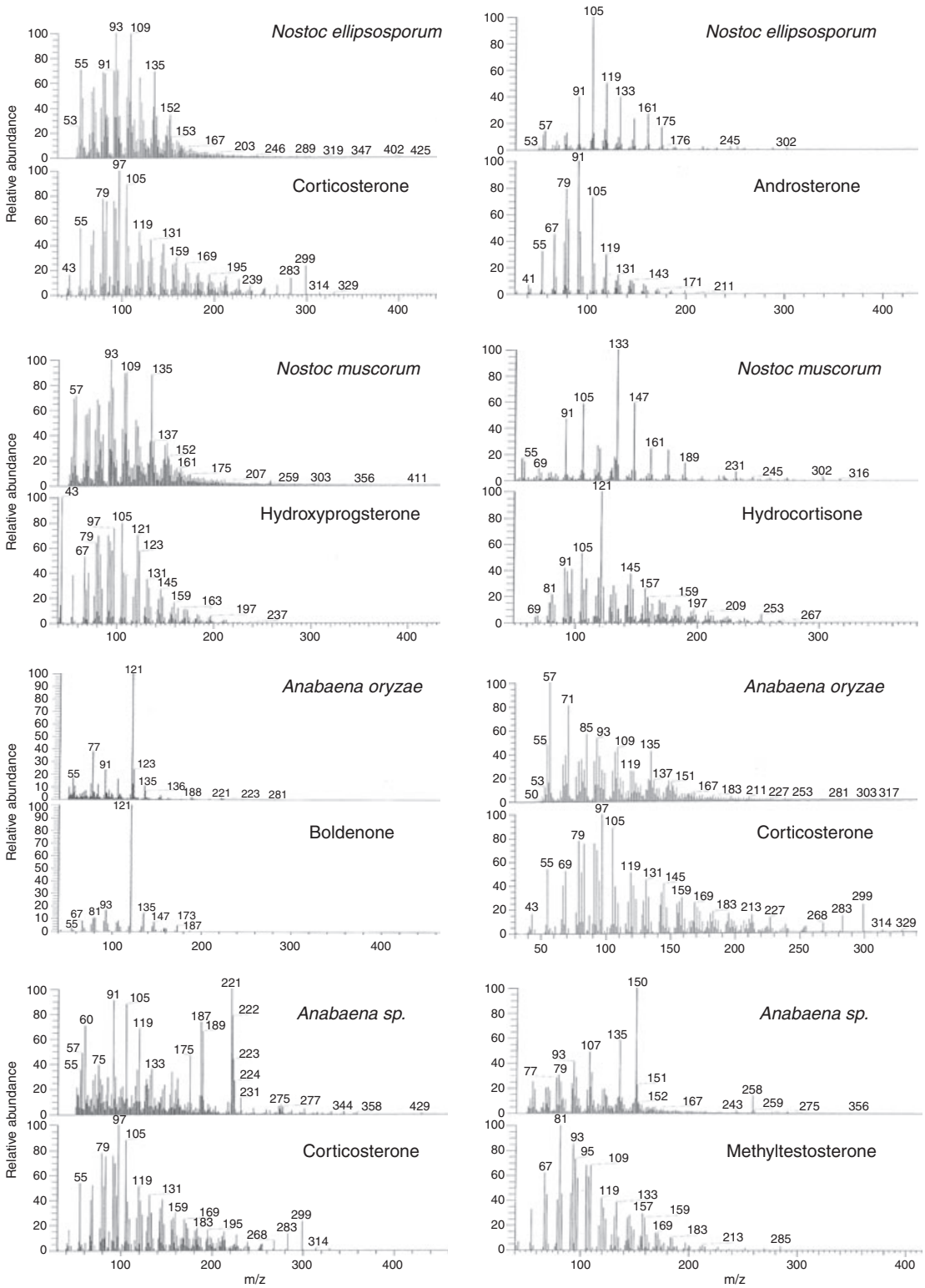


Figure 2 Gas chromatography–mass spectra of some sterol-like compounds from the cyanobacterial exudates of *Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena. sp.* Data are representative of at least two independent biological replicates for each culture condition.

Table 2 Composition of bioactive compounds from the cyanobacterial exudates of *Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena.* sp. revealed by gas chromatography–mass spectrometry (GC-MS). Data are representative of at least two independent biological replicates for each culture condition

Retention time (min.)	Molar mass ($gmol^{-1}$)	Close match	Similarity (%)
Nostoc ellipsosporum			
17·13	358	Corticosterone	52·1
16·32	358	2-methoxyesterone	69·8
15·95	316	Hydrocortisone	74·6
13·67	302	Androsterone	55·5
Nostoc muscorum			
20·99	429	Corticosterone	81·9
17·05	416	Hydroxyprogesterone	67·7
18·56	356	Methyltestosterone	53·2
19·36	361	Hydrocortisone	54·6
Anabaena oryzae			
1789	414	Corticosterone	76·8
16·08	429	Hydrocortisone	88·7
10.39	355	Hydroxyprogesterone	57·3
Anabaena sp.			
17·64	461	Corticosterone	76·2
18·32	281	Boldenone	51·4
17·21	339	Hydroxyprogesterone	49·3

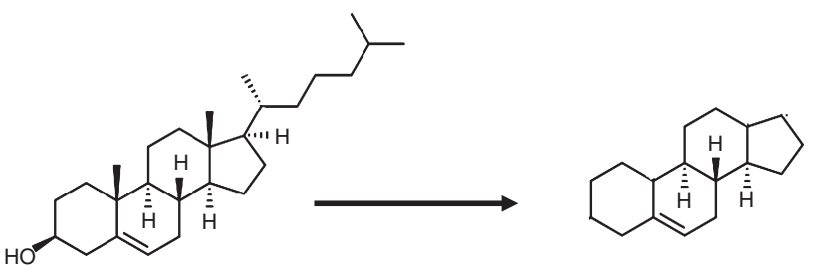
Discussion

In the environment, there are many natural or synthetic molecules that may disrupt the endocrine system in mammals. These compounds have been shown to significantly affect the reproductive system and in some cases result in abnormalities including reduced gonadal size, feminization of genetic males as well as low sperm count and quality (Carreau *et al.* 2007; Min and Lee 2010).

In fact, most of cyanobacteria are nontoxic and are common members of the marine, brackish and fresh waters as well as soils. The current study showed low cytotoxic effect of cyanobacterial exudates on the cell line HFB4 but intriguingly their injection demonstrated sharp disturbance in the level of sex hormones (testosterone, progesterone, FSH and LH). The fluctuation in the sex hormonal concentrations was attributed to the presence of some bioactive compounds in the cyanobacterial exudates. These compounds might have a disruptive effect on the mammalian endocrine system exclusively the reproductive hormones. This hypothesis is consistent with Korpinen *et al.* (2006) and Hisem *et al.* (2011) who clarified a dysregulation effect of cyanobacterial species on normal activity of reproductive hormones and the reproductive system of invertebrates. Moreover, the exposure of vertebral models to the cyanobacterial extract induced a negative consequence on the reproductive system such as reduced body weight, damaged testes, low quality of mature sperm in addition to an inhibition in the levels of some sex hormones in blood serum (Ding *et al.* 2006; Damkova *et al.* 2009).

Although sterols are characteristic of eukaryotic organisms and serve as precursors of steroid hormones (Lindsey *et al.* 2003; Summons *et al.* 2006), some studies reported the occurrence of sterols, diterpenoids and triterpenoids in a diverse range of cyanobacterial strains (Kohlhase and Pohle 1998; Hai *et al.* 1996; Jaki *et al.* 2000). Furthermore, many cyanobacterial species have been reported to produce another group of compounds called hopanoids that are closely related to sterols in their structure. These compounds play an important role in the improvement in plasma membrane strength (Simonin *et al.* 1996).

The presence of some sterol-like compounds in the cyanobacterial filtrates was confirmed by the GC-MS and LC-MS/MS analysis. These compounds could be correlated with the disruption of the sex hormonal levels through the interference with the hormone-dependent signalling pathways. Sterol-like compounds can increase or block the metabolism of naturally occurring steroid hormones by activating or antagonizing nuclear hormone receptors especially the oestrogen and androgen hormone receptors (Tabb and Blumberg 2006). Moreover, these substances caused perturbations in the



Cholesterol
(Molar mass 386·65 g mol^{-1})

Nucleus of cholesterol
(Molar mass 256·32 g mol^{-1})

Figure 3 The nucleus of the sterol-like molecules (256·3 g mol^{-1}) resulted from the breaking down of cholesterol. This nucleus is composed of four fused rings; three cyclohexane rings and one cyclopentane ring.

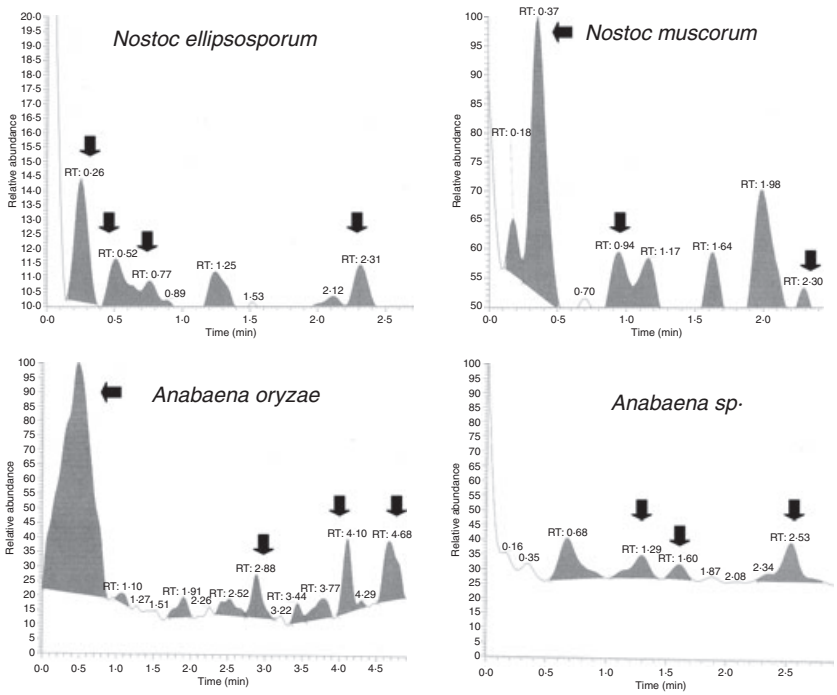


Figure 4 LCMS/MS chromatograms for the analysis of the cyanobacterial exudates *(Nostoc ellipsosporum, Nostoc muscorum, Anabaena oryzae and Anabaena*. sp.). Black arrows indicate the beaks that contain the sterol-like nucleus (256·32).

level of gonadotropin-releasing hormone, which in turn affect the synthesis and the release of LH and FSH (De Coster and van Larebeke 2012). This explanation is in agreement with Sychrova *et al.* (2012) who reported an oestrogenic potency of aqueous extracts and exudates of some cyanobacteria with oestrogen receptor signalling. Meanwhile, Rogers *et al.* (2011) confirmed the production of an oestrogen-like compound by the blue-green alga *Microcystis,* which tested positive for some oestrogenic biomarkers and also demonstrated a clear effect on larval zebra fish. Furthermore, some cyanotoxins such as nodularin-R, microcystin-LR and cylindrospermopsin were recorded to have an oestrogenic activity *in vitro* using different mammalian cell lines. These compounds act as endocrine disruptor at low concentrations and are toxic for the tissues of reproduction (Young *et al.* 2008; Oziol and Bouaicha 2010).

The current study demonstrated that the nontoxic cyanobacterial species have the capability to produce some extracellular bioactive compounds into their surroundings that can disrupt the mammalian reproductive hormones. This effect was attributed to the presence of sterol-like compounds in their filtrate that was confirmed with the GC-MS and LC–MS/MS analysis. Consequently, cyanobacteria could potentially contribute to a hazardous effect on mammalian health via producing some endocrine disruptors with oestrogenic activity. Further studies are required to investigate the oncogenic effect of these compounds on the mammalian reproductive system.

Conflict of interest

No conflict of interest declared.

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