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The *Comamonas testosteroni* steroid biosensor system (COSS)—Reflection on other methods☆

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ABSTRACT

Natural and synthetic steroid hormones are released uncontrolled into the environment and are considered as pollutants with regard to their endocrine activity and negative influence on all kind of organisms. Due to their widespread presence, endocrine activity even at low concentrations, and their potential adverse effects in both the environment and human health, there is an increasing need for the development of rapid, sensitive and quantitative techniques for measuring trace levels of these steroids. In addition to classical analytical methods like GC–MS, LC–MS and others, several techniques have been established that are based on human nuclear steroid receptors as reporter systems. However, many of these systems require human or yeast cell culture and are therefore time consuming and expensive, while others suffer from too low sensitivity or cover only one specific steroid compound. These are some of the main reasons that limit current techniques for environmental application. The remarkable ability of certain microorganisms to transform and degrade the steroid nucleus and to respond with the induced expression of steroid regulated genes lead us to explore, whether the steroid signalling machinery of *Comamonas testosteroni* could be used to construct a steroid sensoring system that is sensitive, rapid, easy to perform, and which could also be applied to detect environmental steroid mixtures at low concentrations. Both whole *C. testosteroni* mutant cells as well as the cytosol thereof were used as new and sensitive fluorescence based biosensor systems for the successful determination and quantification of a variety of different steroids. We could show that our COSS (*Comamonas testosteroni* Steroid Sensor) is able to detect testosterone, estradiol and cholesterol in concentrations of 29 pg/mL, 0.027 pg/mL, and 9.7 pg/mL, respectively. The sensitivity of the COSS together with the fact that it is very fast, reproducible and can be used for high-throughput screening in a microplate format makes it suitable for the detection of single steroid hormones or steroid hormone mixtures in environmental samples at low costs. In summary, the COSS is able to detect steroid hormone effects at the molecular level through activation of bacterial steroid-sensing systems. In the future, it may be further developed as a useful tool for the integrative assessment of ecotoxicological potentials caused by hormonally active agents and endocrine-disrupting compounds.

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1. Introduction: steroid hormones are of ecotoxicological concern

Natural sex hormones and a rising number of man-made synthetic steroids are becoming increasingly important as potential environmental contaminants because they can interfere with the hormone system of any organism. Importantly, steroid hormones have biological activities already at low concentrations, because they mediate their effect by transactivation of their cognate receptors that bind to hormone responsive elements (HRE) at their natural target genes [1]. By mimicking physiological hormones or disrupting signal pathways as endocrine disruptors, these environmental steroids can misregulate or interfere with normal biological responses [2]. Such effects were shown in aquatic organisms, in animals in field studies [3], as well as in laboratory studies with molluscs [4], fish [5], amphibians [6,7], birds [8], and mammals [9]. Especially, the exposure of aquatic organisms to environmental steroid hormones and their metabolites may be significant in view of their occurrence in treated effluents of sewage treatment plants, landfill leaches and contaminated waters [10].

Even in concentrations near the detection limit natural and synthetic sex hormones can cause adverse effects. For example, at environmentally relevant concentrations 17β-estradiol and the synthetic 17α-ethinylestradiol lead in fish to induction of the yolk precursor vitellogenin, effects on gonadal histology, reduction of fecundity and reproductive success [11,12]. Already at

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around 0.1 ng/L 17α-ethinylestradiol induces the expression of vitellogenin in fish [13] and affects sex differentiation [14].

Recently, synthetic gestagens were shown to affect fish reproduction: levonorgestrel at around 1 ng/L, drospirenone at around 6 μg/L [15]. Interestingly, levonorgestrel at somewhat higher concentrations (3.3 ng/L) even lead to a clear masculinization of female fish, which was most probably due to the androgenic partial effect of this steroid hormone [15]. It should be noted, here, that on the basis of a theoretical estimation (according to the European Medicines Agency [16]), the environmental concentration of levonorgestrel can reach a level of 2 ng/L, which underlines that the effects on fish caused, at least by levonorgestrel, may be of environmental relevance.

Due to their incomplete removal during wastewater treatment processes, their widespread presence in the environment, and their endocrine activity even at low concentrations [17,18], these endocrine disruptors have therefore received increased attention in water quality management and health care [19]. However, the monitoring of hormonal active steroids in the environment affecting human health is a major challenge not only for governmental institutions.

The increasing environmental and public risk requires, in a first step, the development of novel approaches to detect these compounds in the environment. In a later phase it is envisaged to prevent disturbances in the development and functionality of the human and animal reproductive systems by bioremediation of environmental steroid contaminations.

2. Specific detection and quantification of steroid hormones

2.1. Classical methods

A variety of classical analytical techniques for specifically estimating steroids have already been reported such as high pressure liquid chromatography (HPLC), liquid chromatography coupled with fluorescence measurement or mass spectrometry (MS), or gas chromatography coupled with mass spectrometry (GC/MS) [20,21]. However, not of all these techniques are suitable for environmental probes because the sample preparation is either time consuming or the limit of detection is insufficient to detect environmental concentrations. Analytical methods have been developed to successfully determine ultra-traces of target endocrine disruptors in the aquatic environment by gas chromatography coupled to tandem mass spectrometry (GC–MS–MS) as well as detecting estrogens in different matrices by liquid chromatography tandem mass spectrometry (LC–MS–MS) [22,23].

On the other hand, due to the high specificity of these methods for definite steroid molecules, other steroids with a slightly altered configuration or when their precise chemical structure is not known are sometimes not identified by these methods.

2.2. Surface plasmon resonance sensing (SPR)

Novel developments for the quantitative and specific evaluation of low levels of estrogens are highly sensitive immunoassays using a surface plasmon resonance (SPR) system [24] or electrochemical immunosensors [25].

The surface plasmon resonance (SPR) immunoassay was used for the quantitative evaluation of low levels of the estriol metabolite estriol-16-glucuronide (E3-16G) in liquid media [24]. As the authors state, this method is simple to apply and does not require any sample pre-treatment, such as hydrolysis, extraction, or derivatization. Quantification of E3-16G is performed in aqueous samples by means of competition between injected free E3-16G (pre-mixed with the anti-E3-16G-antibody) and E3-16G conjugated to ovalbumin through oligoethylene glycol (E3-16G-OEG-OVA) immobilised on a CM5 BIAcore biosensor chip via amine coupling to develop inhibition immunoassays. The biosensor response is obtained by binding of just the antibody to the conjugate which minimizes the distance to the biosensor surface, thereby increasing the corresponding SPR signal. A further advantage of this format is that the result could be obtained in less than 10 min.

With this system, a detection limit for E3-16G of 76 pg/mL was achieved using a rabbit anti-sheep primary antibody as a binding agent. Interestingly, the detection limit was further improved by using synthesized gold colloids (15 nm) as high mass labels conjugated to the primary antibody. In this Au nanoparticle-enhanced assay, the concentration of E3-16G in aqueous samples could be determined within 7.5 min at a level as low as 14 pg/mL [24].

According to the authors, the sensitivity of the assay indicates its potential applicability in monitoring E3-16G in applications including the menstrual cycle, foetal well-being, detecting high-risk pregnancy, and to monitor environmental liquid, waste waters, and drinking waters.

2.3. Electrochemical immunosensor

Another technique which can offer a specific, sensitive and fast means of detection of estradiol is the electrochemical immunosensor [26]. This method is based on the extent of competitive binding between sample estradiol and labelled estradiol to its specific capture antibody that has been immobilised on the immunosensor surface [27].

In a recent approach, this immunosensor featured a gold particle/Protein G-scaffold, to which a monoclonal anti-estradiol capture antibody was immobilised to facilitate the competitive immunoassay between sample estradiol and a horseradish peroxidase-labelled 17β-estradiol conjugate. Upon constructing this molecular architecture on a disposable gold electrode in a flow cell, amperometry was conducted to monitor the reduction current of benzoquinone produced from a catalytic reaction of horseradish peroxidase. This current was then quantitatively related to 17β-estradiol present in a sample [25].

Regarding sensitivity, a detection limit of 6 pg/mL was yielded with blood serum samples. This low detection limit was attributed to the favourable characteristics of the immunosensor to the gold nanoparticle/Protein G-scaffold, where the gold nanoparticles provided a large electrochemically active surface area that permits immobilisation of an enhanced quantity of all components of the molecular architecture [25].

2.4. Are these techniques suitable for environmental probes?

The structural diversity of the multitude of steroid hormones suspected in the environment demands the need to develop detection methods that would identify these substances by their hormonal activity rather than by their chemical structure. In addition, a screening for every single steroid hormone in the environment would be very costly and impracticable for environmental monitoring purposes. Therefore, the assessment of potential endocrine risks requires test systems that do not need structural knowledge of the agents but are based on an effect-oriented approach.

3. Steroid determination via their biological activity

3.1. Effect-oriented assays

To monitor hormonally active steroids in the environment and to help understand the mechanisms by which they interact with

their biological targets, the development of novel investigation methods has become an important task. Biosensors have shown the potential to monitor steroids using nuclear hormone receptors as recognition elements.

Steroids bind to and activate nuclear hormone receptors with subsequent activation or repression of transcription of respective genes harbouring hormone responsive sequences as *cis*-regulatory elements. This reflects that such nuclear receptors act as ligand-dependent transcription factors, which mediate the effects of hormones to regulate the expression of specific genes, which in turn affect reproduction and development.

Since the activity of steroid hormones can be measured by binding to and activation of their cognate receptors, *in vitro* assays for assessment of endocrine activities may provide quantitative estimates of the total activity of particular receptor-mediated responses. Several reporter gene constructs have been established in the past for analyses of biological effects at the molecular and biochemical level. These nuclear hormone receptor-based assays provide an effect-oriented identification of hormone-active substances by a well defined molecular mechanism.

3.2. The estrogen screen (E-Screen)

An important biosystem for detecting estrogenic activities is the estrogen-screen (E-screen) which has been developed to asses the estrogenicity of environmental chemicals using the proliferative effect of estrogens on their target cells as an end point [28]. This assay is performed with the human breast cancer cell line MCF-7 expressing the estrogen receptor α (ERα) constitutively and measures the estrogen-induced increase of the number of cells. Cell proliferation depends on binding of estrogens or xeno-estrogens with the receptor. The basic end point of the E-screen assay is the cell number relative to the hormone-free control. The proliferative effect of a hormone is defined as the ratio between the highest cell number achieved with the hormone and the cell number of the negative control.

The E-screen requires full culturing of MCF-7 cells. Subconfluent cells are harvested and seeded e.g. into in 24-well plates and exposed to the sample. Six days later (day 6) the assay is terminated during the late exponential phase of proliferation. Hormone-dependent cell growth is measured e.g. by sulforhodamin B staining at 492 nm. The absorbance measured correlates with estrogen or xeno-estrogen-induced growth.

In addition to estrogens, the E-screen bioassay is a reliable tool to assess estrogenicity on a large number of compounds. These xeno-estrogens are found among antioxidants, plasticizers, polychlorinated biphenyl (PCB) congeners, and pesticides.

The E-screen is an *in vitro* cell-based assay found to be appropriate to determine estrogenic activity in environmental extracts from river grab samples and wastewater treatment plant effluents samples [29]. Although the system can detect estrogenic compounds with high sensitivity (the limit of detection for 17β-estradiol is e.g. 0.32 ng/L), the method is time consuming and laborious, since beside full cell culturing it needs long exposure periods of the cells to the samples (ca. 6 days) [30].

3.3. Yeast estrogen screen (YES) and yeast androgen screen (YAS)

Another two widely used receptor/reporter assays for detecting estrogenic and androgenic compounds are the yeast estrogen screen (YES) [31] and the yeast androgen screen (YAS) [32]. Recombinant yeast cells contain either a stably transfected human estrogen receptor α (YES) or androgen receptor (YAS) within their main chromosome together with an expression plasmid carrying receptor specific-responsive sequences that control the reporter gene *lacZ*. Binding of an estrogen or androgen to the hormone receptors in the yeast cell leads to the activation of the receptor thereby inducing the expression of the *lacZ* gene. The enzyme β-galactosidase, which is then produced, is secreted into the medium and catalyzes the metabolism of the yellow chlorophenol red β-D-galactopyranoside into a red product that can be measured spectrophotometrically at 540 nm.

The YES and YAS have been applied for the detection of estrogens/androgens in environmental waterways, aquifers, wastewater treatment systems and dairy manure [33–35]. In addition, the YES has been used extensively to measure endocrine responses to a variety of non-steroidal hormonal active substances such as polychlorinated biphenyls, polynuclear aromatic hydrocarbons (PAH) and other compounds [36–39].

The YAS was originally developed for the identification of androgenic as well as anti-androgenic contaminants of environmental samples [40], and may also be suitable to detect activities of anabolic androgens in human urine samples. The YAS assay was able to detect stanazolol and norandrosterone metabolites in concentrations of 80 ng/mL and 36 ng/mL, respectively [41].

The yeast-based assays may be carried out in 96-well microtiter plates. After 72 h incubation the microtiter plates can be read in microplate readers at absorbance of 540 nm to detect colour change of the assay medium, and at 620 nm to measure turbidity as an indicator of yeast growth [42].

To enhance the sensitivity, the colorimetric (*LacZ*) yeast assays described above have been further developed to yeast-based bioreporters using the green fluorescent protein [43], the firefly luciferase [44] or the *Photorhabdus luminescens lux* operon. For example, by substitution of the *lacZ* gene in the YES and the YAS assay the *Saccharomyces cereviseae* bioluminescent bioreporters BLYES (Bioluminescent Yeast Estrogen Screen) [45] and BLYAS (Bioluminescent Yeast Androgen Screen) [46] have been generated. As the authors state, one significant advantage of bioluminescence assays compared with colorimetric assays is speed. Quantifiable bioluminescence using BLYES and BLYAS was observed in 60 min with maximum bioluminescence observed in 3–4 h [45,46]. In contrast, the colorimetric assays required 3 days before a response was measured and for target compounds or environmental samples with low estrogenicity, 5 days of incubation were required for detection of the estrogenic response [35,36].

With regard to sensitivity, the EC_{50} values were 6.3×10^{-10} M of the BLYES for 17β-estradiol and 1.1×10^{-8} M of the BLYAS for 5α-dihydrotestosterone [47]. However, regarding speed, still it is necessary to perform time-consuming yeast cell cultivation to obtain recombinant yeast cells containing the human estrogen or androgen receptors stably integrated into their main chromosome together with expression plasmids carrying the reporter genes.

3.4. Enzyme-linked receptor assay (ELRA)

As valid for the systems described so far, the development of the enzyme-linked receptor assay (ELRA) was motivated by the intension to provide a fast and sensitive instrument for detecting estrogenic and xeno-estrogenic agents in environmental samples [48]. The receptor assay is based on the principle of a competitive enzyme-linked immunoassay (ELISA) that analyses a ligand protein interaction. The essential difference is the use of the human estrogen receptor α (ERα) instead of an antibody as a linking protein. Namely, the ELRA measures the competitive binding of sample estrogens (or xeno-estrogens) against estradiol supplied as a BSA-coating conjugate to the dissolved ERα.

The ELRA uses the recombinant human ERα, which has been produced in transformed yeast (*S. cereviseae*), and can be carried out in a 96-well microplate format using the experimental setup known from ELISA. In detail, the microplates are coated with a 17β-estradiol-BSA conjugate and a dilution series of sample estrogens or

xeno-estrogens and incubated with the ERα. After a washing step, a biotinylated mouse anti-ER antibody is added to each well. Sample estradiol or xeno-estrogens binding to the ERα are determined by a streptavidin–peroxidase–biotin complex with subsequent measurement of the peroxidase activity at the wavelength of 450 nm using a commercial ELISA multiplate reader [48].

It has been stated that the ELRA is a subcellular test procedure that can be applied to detect the estrogenicity of single substances, chemical mixtures, or even more complex matrices such as environmental samples by use of the human ERα under laboratory conditions. It may therefore ensure an efficient environmental monitoring with low hardware requirements and high sample throughput [48].

Regarding time, after overnight coating of the microtiter plates the test can be performed within more or less 4 h. The detection limit of the ELRA for 17β-estradiol was reported to be 0.1–0.2 μg/L [48]. Hence, in comparison with the E-screen and the YES, the ELRA excels in shorter assay duration, but has lower sensitivities for reference substances like 17β-estradiol (YES = 3 ng/L, MCF-7 test = 0.27 ng/L) [49].

3.5. Bipartite recombinant yeast system (BRYS)

In interesting approach is the bipartite recombinant yeast system (BRYS) which was developed to discriminate and characterize subtype-selective estrogen receptor (ER) ligands [50]. The idea is based on the fact that there exist two different subtypes of the estrogen receptor, ERα and ERβ, which are thought to mediate different biological effects. For this purpose, either the ERα or ERβ was introduced into the yeast system combined with a reporter plasmid.

Although being products from two separate genes, both ERα and ERβ are nuclear receptors that share a similar architecture [51]. A common amino-terminal region of each receptor plays an important role in target-gene transcription. The central region of both receptors is involved in DNA binding and receptor dimerization. The carboxyl-terminal domain of the receptors is crucial for ligand binding, nuclear translocation, receptor dimerization, and modulation of target-gene expression associated with coregulators [52]. The variability in the carboxyl-terminal domain (only 58% similarity) of both ER subtypes is the bases for the respective binding affinities of estrogenic ligands and their variable agonistic transcriptional activities [53].

The expression of ERα and ERβ depends strongly on the tissue or even on the different cell types of the same tissue. While in some tissues both isoforms are expressed on a similar level, in other tissues either ERα or ERβ seems to predominate [54]. The different expression pattern of the two isoforms might play an important role for the function of physiological estrogens. On the other hand, ERα and ERβ may have different affinities to synthetic ER modulators, agonists and antagonists which is of great interest with regard to medical application [51]. For example, selective estrogen receptor modulators (SERMs) like tamoxifen are used in cancer treatment and exert an antagonistic effect in the breast while acting as an agonist in the uterus and bone [55]. Raloxifene has even a higher tissue selectivity and is used in the treatment of postmenopausal osteoporosis.

To establish the system [50], yeast cells were subsequently transformed with the reporter gene plasmids and the ER expression plasmids, the latter being under the control of the inducible metallothionine promoter (CUP1). Under the induction of copper ion, either ERα or ERβ are expressed which form estrogen-ER complexes that are recruited to estrogen responsive elements (ERE) of the reporter plasmids. Subsequently, the transcription of the *lacZ* reporter gene which encodes β-galactosidase is initiated. By measuring the β-galactosidase activity in lysates of the transformed yeast cells, the estrogenic effects of different ligands can be analyzed.

In the study by Liang et al. [50], a variety of specific estrogen receptor agonists were tested to validate this system. There was obvious an anticipative discrimination between the agonistic activities when these chemicals were identified and characterized. Regarding sensitivity, 4.7 pM (1.3 ng/L) estradiol was the lowest concentration that could be detected with the ERα system and 0.12 nM (33.5 ng/L) with the ERβ system. The results indicate that this yeast system could be used to *in vitro* screen for subtype-selective ER ligands.

However, despite the possibility to be performed in 96-well plates, the yeast cells for the BRYS have first to be cultured and then be incubated with the samples in the microplates for 24 h at 30 °C. After lysis for another 2 h, β-galactosidase activity can be measured. Hence, with the BRYS it takes just 2 days to complete an entire assay with a larger number of samples [50].

3.6. Reflectometric interference spectroscopy (RIfS)

In nature, ERα does not only bind to its ligand(s), rather it also interacts with coactivators or corepressors through a specific binding site. For example, estradiol binding causes the ERα to adopt a conformation typical for a bound agonist, whereas raloxifene or tamoxifen cause the ERα to adopt a conformation typical for an antagonist [56]. An agonist-type conformational change of ERα (estradiol) leads to formation of a binding site for coactivators, an antagonist-type conformational change (tamoxifen, raloxifene) prevents coactivator interaction [57].

The reflectometric interference spectroscopy (RIfS) biosensor system utilizes the specific interaction between the ERα and short peptides as coactivators. This system does not only allow to discriminate between ligands and nonligands of the ERα, but also between agonists and antagonists of the ERα.

The RIfS monitors binding of molecules to sensitive bionic interfaces through the change in the apparent optical thickness of the sensing layer [58]. A RIfS biosensor chip (glass) surface with immobilised DAPEG (diaminopoly(ethylene glycol)) is covalently modified with biotin–streptavidin and biotinylated peptide α/β 1 [59], the latter consisting of 15 specific amino acids. The interaction of the ligand binding domain of the ERα with the coactivator peptide and the ligand itself is monitored with RIfS. In principle, the change in apparent optical thickness of the chip is recorded by interference of white light reflected at the interface of a multilayer system using a diode array spectrometer.

The major advantage of RIfS, apart form the short time necessary (about 1 h) and the avoidance of labels which might interfere in the biological process, is the ability to determine kinetic and thermodynamic data of the monitored interactions [60]. In addition, the biosensor is applicable to complex biological matrices, even crude cell extracts, and might therefore be used for environmental samples. However, the major drawback of this technology is the low sensitivity of detection which is in the range of 10 μg/mL 17β-estradiol (measured after 30 min incubation with a lysate containing 0.01 mg/mL of the ERα ligand binding domain). This is especially true when compared with fluorescence-based methods. Another problem is non-specific binding to the sensor surface which makes it difficult to distinguishing between specific and non-specific effects [61].

3.7. Summary of human steroid receptor-based biosensors

Overall, major disadvantages of steroid biosensors that are based on human nuclear receptors are their time consuming procedures and their high costs. In addition, with most of these techniques only one specific steroid compound or a relatively small

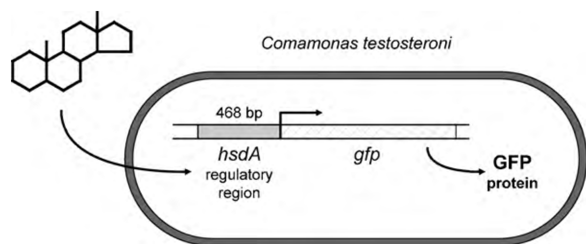

Fig. 1. The Comamonas testosteroni Steroid Sensor (COSS). The regulatory region of the *hsdA* gene (468 bp) was cloned upstream of the *gfp* gene coding for the green fluorescent protein (GFP). Upon steroid exposure, the expression of the *gfp* gene is induced via the *hsdA* regulatory region. The enhanced expression of the GFP protein was recorded in a fluorescence microplate reader (Tecan, Austria GmbH). For further details cf. text.

group of compounds can be detected, because the systems do only use a specific type of human nuclear hormone receptor. Therefore, new methods for steroid detection and quantification are urgently needed that are cheap, easy to perform, are sensitive enough to detect even lowest concentrations in the environment, and cover a broader spectrum of different substances in one assay.

4. A bacterial steroid-inducible gene and its regulatory region as novel biosensor system for steroid determination

4.1. The Comamonas testosteroni Steroid Sensor (COSS)

The remarkable ability of the bacterium *C. testosteroni* to use steroids both as carbon source and also as signal molecules for the induction of appropriate steroid degrading enzymes led us to explore if the regulatory region of steroid sensitive genes in *C. testosteroni* could be used as a sensitive biosensor system for steroid determination in the environment.

C. testosteroni is a Gram-negative bacterium found in soil and water [62], but has also been found in humans [63]. This bacterium is able to grow on steroids or aromatic hydrocarbons as sole carbon and energy source and may therefore play an important role in the biodegradation of these compounds in the environment [64]. We have shown in earlier investigations that 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is a key enzyme in the degradation of these complex ring structures by *C. testosteroni* [65–67]. The enzyme catalyzes the interconversion of hydroxy- and oxo-groups at position 3 of the steroid ring structure. Interestingly, 3α-HSD/CR is expressed only at very low and basal levels in the absence of steroids, but can undergo a several fold induced expression in the presence of steroids like testosterone [65,67,68].

Previously, we have elucidated the complex regulation of the 3α-HSD/CR gene (*hsdA*) from *C. testosteroni* and identified a variety of *cis*- and *trans*-regulating elements for *hsdA* expression [69–71]. It turned out that 3α-HSD/CR expression is controlled by two repressors (RepA and RepB), an activator (ActA) and a testosterone-inducible regulator (TeiR) that drives steroid-dependent gene regulation via its kinase activity. In a recent work, we replaced *hsdA* within the *C. testosteroni* chromosomal DNA by the gene coding for the green fluorescent protein (GFP). In the resulting *C. testosteroni* mutant (CT-GFP5-1), GFP was under transcriptional control by the *hsdA* upstream regulatory region, including the *cis*-acting promoter and operator sequences, and the corresponding *trans*-acting factors [72]. The bacterial system obtained was named COSS (*C. testosteroni* steroid sensor) and was tested for its capability to detect a variety of different steroids (Fig. 1).

In a first set of experiments, whole CT-GFP5-1 bacterial cells were exposed to different concentrations of testosterone, estradiol and cholesterol in 96-well black plates at room temperature. The fluorescence was determined in a GENios Pro Fluorescence Microplate Reader from Tecan, Austria GmbH. Within this cell-based assay, the fluorescence signals indeed increased dependent on steroid concentration and time. The determination range obtained was 57–450 pg/mL (0.195–1.56 nM) for testosterone, 1.6–14.4 pg/mL (0.006–0.054 nM) for estradiol, and 19.3–154.4 pg/mL (0.050–0.390 nM) for cholesterol. Compared to testosterone, the sensitivities for estradiol were much higher, which is probably due to testosterone binding (scavenging effect) to Rep B (discussed in Ref. [72]). Importantly, the maximum fluorescence could already be detected as early as after 30 min.

To further optimize the COSS assay, a cell-free bacterial bioassay for steroid determination was established. The idea was to simplify the system, since cell-free assays do not require culturing of fresh cells before each detection. A cell-free protein synthesis method was reported that was used to screen for soluble protein expression constructs [73]. For our system, the cytoplasm of *C. testosteroni* CT-GFP5-1 mutant cells was used in 96-well black plates. After isolation of the cytoplasm, the plasmid pTOPO-3αGFP5 was added, which harbours the *hsdA* regulatory region upstream of the *gfp* gene, together with different concentrations of testosterone, estradiol and cholesterol [72]. The results of testosterone, estradiol and cholesterol determination in this cell-free bioassay yielded an up to 2 orders of magnitude higher sensitivity than that of the cell-based bioassay. The determination range obtained was 29–576 pg/mL (0.1–2.0 nM) for testosterone, 0.027–0.532 pg/mL (0.0001–0.002 nM) for estradiol, and 9.7–194 pg/mL (0.0245–0.512 nM) for cholesterol (Fig. 2). As observed for the cell-based assay, the sensitivities for estradiol were much higher compared to testosterone. Disadvantages of the COSS assay are the limited detection range for the steroids and the high background of fluorescence observed in both the cell-based and cell-free systems. These applications are the focus of on-going investigations.

4.2. Concluding remarks

In vitro tests like our COSS, using the regulatory region of steroid sensitive genes in bacteria, are obviously effective in detecting steroids and may be commonly used in the first stage screening of hormonal compounds in the environment. Advantages of our COSS are a fast, cost-effective, and less complex tool that can be used for high-throughput screening in a microplate format (Table 1). Moreover, the COSS is robust and not susceptible towards cytotoxic interferences like the common cell-culture methods. In addition, the cytoplasm could be stored at −20 °C at least for 1 month.

Current methods of risk assessment usually focus on the assessment of single substances. This is in contrast to the exposure situation in the environment, where organisms are typically exposed to a variety of steroid hormones as mixtures and at very low concentrations. With the reporter systems developed so far, using human steroid receptors expressed in yeast cells or human cell culture, it is difficult to assess the effects of a complex mixture of steroids hormones (Table 1).

It is an important improvement that a microbial steroid-dependent gene regulatory system (which is rather non-specific for one steroid) is now used for steroid hormone detection instead of a eukaryotic (yeast, human cells) system harbouring a specific human steroid receptor (ERα, ERβ, AR). The new microbial fluorescence based COSS may serve as a first stage screening tool for identification of compounds that require further characterization. Furthermore, the COSS was motivated by the intention to provide a fast and sensitive instrument for detecting hormonal active agents in environmental samples and to ensure an efficient environmental monitoring with low hardware requirements and high sample throughput. Since steroid compounds in the environment have such diverse concentration-effect relationships, and in view of its

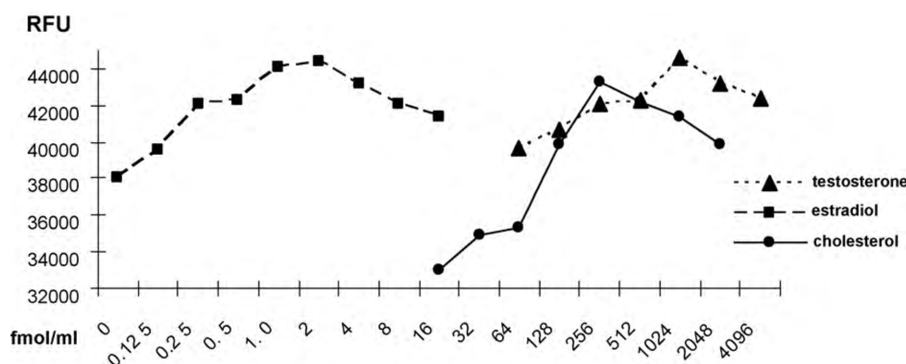

Fig. 2. Determination of testosterone, estradiol and cholesterol with the COSS bioassay. Different concentrations of testosterone, estradiol and cholesterol were assayed in the cell-free *Comamonas testosteroni* Steroid Sensor (COSS) system as described in [72]. RFU, relative fluorescence units. For further details cf. text.

Table 1
Comparison of a variety of steroid detection methods.

Method	System	Time	Limit of detection/range	Advantage	Disadvantage	Applicable as biosensor to environmental probes	Literature
Classical (e.g. GC/MS; LC/MS)	Analytical chromatography	(Time consuming sample preparation)	~1 pg/mL	High sensitivity	Specific for only *one* compound; time consuming sample preparation	–	[20]
Surface plasmon resonance	Immunoassay	10 min	14–76 pg/mL	High sensitivity	Specific for only *one* compound	–	[24]
Electrochemical detection	Immunoassay	<1 h	6 pg/mL	High sensitivity	Specific for only *one* compound	–	[25]
Estrogen (E)-screen	MCF-7 human breast cancer cell line	1 week	0.32 pg/mL	High sensitivity	Specific for only estrogenic compounds; time consuming cell culture	(+)	[28]
Yeast estrogen screen (YES)	Yeast transformed with human ERα; colorimetry	3–5 days	YES: 109 pg/mL (4.0×10^{-10} M)	High sensitivity; 96 well-plate	Specific for only estrogenic or androgenic compounds; time consuming procedure	(+)	[45] [32]
Yeast androgen screen (YAS)	Yeast transformed with human AR; colorimetry	3–5 days	YAS: 36-80 ng/mL	High sensitivity; 96 well-plate			
Bioluminescent yeast estrogen/androgen screen (BLYES/BLYAS)	Yeast transformed with human ERα/AR; bioluminescence	3–4 h	BLYES: 171 pg/mL (6.3×10^{-10} M) for Estradiol BLYAS: 3.8 ng/mL (1.1×10^{-8} M) for 5α-DHT	High sensitivity; 96 well-plate	Specific for only estrogenic or androgenic compounds, respectively	+	[45] [46]
Enzyme-linked receptor assay (ELRA)	Competetive binding to recombinant ERα; POD colorimetry	4 h	100 pg/mL	High sensitivity 96 well-plate	Specific for only estrogenic compounds	+	[48]
Bipartite recombinant yeast system (BRYS)	Yeast transformed with either human ERα or ERβ; colorimetry (β-galactosidase)	2 days	Erα: 1.3 pg/mL (1.3 ng/L) ERβ: 33.5 pg/mL (33.5 ng/L)	High sensitivity; 96 well-plate; differentiates between ERα and ERβ-ligands	Specific for only estrogenic compounds; time consuming	(+)	[50]
Reflectometric interference spectroscopy (RIfS)	Binding of ERα LBD to glass chip; DAD	1 h	10 μg/mL	Discrimination between ERα-agonists and ERα-antagonists	Low sensitivity; specific for only ERα-ligands; non-specific binding to the sensor surface	(+)	[61]
Comamonas testosteroni steroid biosensor system (COSS)	Steroid-sensitive bacterial reporter genes	30 min	test: 29.0 pg/mL estra: 0.027 pg/mL chol: 9.7 pg/mL	Rapid, easy to perform, cost-effective; simple; 96 well-plate, high-throughput	High background	+	[72]

AR, androgen receptor; DAD, diode array detector; ER, human estrogen receptor; estra, estradiol; LBD, ligand binding domain; POD, peroxidase; test, testosterone; chol, cholesterol; applicability as biosensor for environmental probes: + (yes); (+) (with limitations); – no.

proven sensitivity, functionality and fastness, it is recommendable to standardize the COSS as an environmental test system.

In conclusion, our COSS is a microbial cell-free method that is very sensitive, can be performed faster and easier than eukaryotic steroid reporter systems, is more robust against interferences and shows less variation in the results. The system is easy to handle, cost efficient and needs no high-tech equipment. But most importantly, this biological test system does not require detailed knowledge of the chemical structure of a steroid hormone and might therefore be suitable to detect previously unidentified hormonally active substances, especially those with endocrine-disrupting potential.

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