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Receptor-mediated estrogenicity of native and chemically dispersed crude oil determined using adapted microscale reporter gene assays

Sarah Johann[a,*], Milena Esser[a], Leonie Nüßer[a], Dag Altin[b], Henner Hollert[c], Thomas-Benjamin Seiler[a,*]

[a] Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
[b] BioTrix, NO-7022 Trondheim, Norway
[c] Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

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ABSTRACT

Endocrine disrupting compounds (EDCs) emerged as a major concern for water quality in the last decade and have been studied extensively since. Besides typical natural and synthetic estrogens also petroleum product compounds such as some PAHs have been identified as potential EDCs, revealing endocrine disruption to be a relevant mode of action for crude oil toxicity. Hence, in the context of a comprehensive retro- or prospective risk assessment of oil spills the implementation of mechanism-specific toxicity such as endocrine disruption is of high importance. To evaluate the exposure risk for the aquatic biota, research focuses on water-soluble fractions underlying an oil slick that could be simulated via water-accommodated fractions (WAF). Against this background human (ERα-CALUX®) and yeast based (A-YES®) reporter gene bioassays were successfully optimized for the application in estrogenicity evaluation of the water-accommodated fraction (WAF) from a crude oil. Combining different approaches, the estrogenicity of the WAFs from a naphthenic North Sea crude oil was tested with and without the addition of a chemical dispersant addressing specific aspects of estrogenicity including the influence of biotransformation capacities and different salinity conditions. Both the WAF free from droplets (LEWAF) as well as the chemically dispersed WAF (CEWAF) gave indications of an ER-mediated estrogenicity with much stronger ERα agonists in the CEWAF treatment. Resulting estradiol equivalents of the WAFs were above the established effect-based trigger values for both bioassays. Results indicate that the dispersant rather increased the fraction of ER-activating crude oil compounds instead of interacting with the receptor itself. Only slight changes in estrogenic responses were observed when cells capable of active metabolism (T47D) were used instead of cells without endogenous metabolism (U2-OS) in the recombinant ER transactivation CALUX assay. With the yeast cells a higher estrogenic activity was observed in the experiments under elevated salinity conditions (6‰), which was in contrast to previous expectations due to typical decrease in dissolved PAH fraction with increasing salinity (salting-out effect) but might be related to increased cell sensitivity.

1. Introduction

The endocrine system plays a crucial role in the regulation of several physiological processes including reproduction, growth, development and behavior. The Endocrine Society defines endocrine disrupting compounds (EDCs) as “exogenous chemicals, or mixture of chemicals, that can interfere with any aspect of hormone action” (Zoeller et al., 2012). EDCs emerged as a major concern for water quality and have been studied extensively in the last decades (Bergman et al., 2013; Campbell et al., 2006; Leusch et al., 2010). EDCs are nowadays being retroperspectively linked to negative impacts on wildlife proven on different biological organization levels (Wagner et al., 2017). A set of studies has further concluded that endocrine disruption plays a crucial role in effect-based risk management monitoring programs (Brack et al., 2019; Hecker and Hollert, 2011; Koenemann et al., 2018). Examples of adverse effects related to endocrine disruption in aquatic biota are the

Abbreviations: EDC, endocrine disrupting compounds; ERα, estrogen receptor α; ERα-CALUX®, ERα-chemical-activated luciferase gene expression; AYES®, *Arxula*-Yeast Estrogen Screen; (LE/CE/HE)-WAF, low energy/chemically enhanced/high energy water-accommodated fractions; NNS crude oil, naphthenic North Sea crude oil; PAH, polycyclic aromatic compounds; SOP, standard operation procedure; GC, gas chromatography; BDS, BioDetection Systems; POP, persistent organic pollutants

* Corresponding authors at: Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, 52074 Aachen, Germany.
E-mail addresses: sarah.johann@rwth-aachen.de (S. Johann), seiler@bio5.rwth-aachen.de (T.-B. Seiler).

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imposex phenomenon in mollusks (e.g. reviewed in Oehlmann et al., 2007), intersex occurrence in fish (e.g. Jobling et al., 2005; Kidd et al., 2007), or dysregulation of thyroid function in marine mammals (Villanger et al., 2011). In this context, several chemicals or groups of chemicals are identified to act as xeno-estrogens, which the present study focuses on.

Fresh- and drinking water research considers several natural and synthetic estrogens as well as industrial compounds as the main drivers for estrogenic activity (Jarošová et al., 2014). However, also persistent organic pollutants (POPs) including PCBs or petroleum product compounds such as (hetero-) PAHs have been identified as potential EDCs (Brinkmann et al., 2014). This emphasizes endocrine disruption as a relevant mode of action in the assessment of adverse effects from crude oil, following the increase in oil consumption and transport with an accompanying risk of acute and diffuse oil spills around the world. Against this background, the EU-funded project GRACE focusing on a holistic approach towards the hazardous impact of oil spills in cold climate conditions implements a comprehensive toxicity assessment including the herein presented estrogenicity (Jørgensen et al., 2019).

Estrogenicity can be investigated via cell-based reporter gene assays in which the EDCs interact with nuclear receptors like the estrogen receptor (ER), leading to an interference with natural regulations in the hormone system (Wagner et al., 2017). For assessing the hazard potential of a complex environmental sample *in vitro* bioassays have many advantages that complement chemical analysis data. They do not require *a priori* information on the identity of bioactive substances and can provide mechanistic insight into their mode of actions. Additionally, they are cost and time efficient and, with respect to the 3R principle of reducing and replacing animal testing, are ethically more accepted (Brack et al., 2019; May et al., 2009). I*n vitro* bioassays are suitable screening tools to identify the estrogenic potential of complex mixtures like crude oil water-accommodated fractions (WAF) that cover additive, synergistic or antagonistic interactions with molecular receptors. However, studies on receptor-mediated estrogenicity of crude oil are limited and especially studies focusing on the dissolved and particulate fractions that are directly bioavailable for the aquatic biota (WAF approach) are particularly scarce.

The present study addressed the ER-mediated endocrine disruptive potential of WAFs from native as well as chemically dispersed crude oil in the ERα-CALUX® and the A-YES® bioassay. Both assays, which are currently validated in ISO guidelines (ISO, 2018a,b), are commonly used in surface water quality assessment and have been demonstrated to be highly sensitive and robust screening tools (Escher et al., 2015; Hettwer et al., 2018; Leusch et al., 2010; Sonneveldet al., 2005).

In general, several observed adverse effects in organisms exposed to crude oil have successfully been linked to PAH-induced AhR-dependent and -independent mechanisms (e.g. reviewed in Incardona, 2017). It has been demonstrated that the metabolization via the vertebrate biotransformation system of crude oil constituents can markedly increase the mechanism-specific toxicity including endocrine disruption (Brinkmann et al., 2014; Mollergues et al., 2017; van den Berg et al., 2003). As both human U2-OS (ERα-CALUX®) and yeast *A. adeninivorans* (A-YES®) cells have very limited capability for metabolization (Pieterse et al., 2015), the human T47D cell line that endogenously expresses the ER and biotransformation associated enzymes (Legler et al., 1999; Spink et al., 1998) was included in the present study to overcome this shortcoming.

The yeast *A. adeninivorans* is characterized by a high salinity tolerance (Hahn et al., 2006), and hence the A-YES® assay can detect the estrogenic potential of water samples in a broad range of salinity conditions (Hettwer et al., 2018) making this assay especially applicable for petroleum product toxicity testing in marine and brackish water conditions.

As crude oil WAFs are unique and complex matrices containing hundreds of compounds with varying volatility or hydrophobicity depending on the chemistry of the oil, specific and crucial method adaptions were integrated.

Against this background, the current study addressed the following questions regarding the estrogenic potential of crude oil WAFs: (a) Do dissolved and particulate fractions act as ER agonists?, (b) How does the dispersant influence the estrogenicity?, and (c) Do intrinsic (metabolic capability) and extrinsic (salinity) factors influence the estrogenic potential?

2. Material and methods

2.1. Oil and dispersant samples

A naphthenic North Sea (NNS) crude oil, chemically characterized by high amounts of low molecular weight hydrocarbons such as decalins and naphthalenes, was selected for the present study. To investigate the potential influence of a chemical dispersant on crude oil toxicity, the third generation dispersant Finasol OSR 51® (Total Special Fluids, France) was used. The commercially available Miglyol 812® (Caesar and Loetz GmbH, Germany) consisting of medium-chain triglycerides was selected as an inert model oil with characteristics (e.g. viscosity) comparable to the NNS crude oil.

In order to differentiate between specific aspects of the petroleum-induced estrogenicity, oils and dispersant were investigated individually and combined in different exposure scenarios of WAFs as summarized in Fig. 1. Cells were exposed to the WAFs from the crude oil alone (low energy water-accommodated fractions, LEWAF) and the WAFs from dispersed crude oil (chemically-enhanced water-accommodated fractions, CEWAF). Additionally, the dispersant alone was investigated by preparing high energy water-accommodated fractions (HEWAF) containing corresponding dispersant amounts to the CEWAF in order to ensure comparability of the treatments. To evaluate further the potential changes in toxicity of the dispersant in the presence of an oil, the combination of Finasol OSR 51® and the inert oil Miglyol 812® was realized in separate experiments with a HEWAF. To proof the inertness of the Miglyol 812® a HEWAF with inert oil only was prepared as well.

2.2. Preparation of water-accommodated fractions

In general, the preparation of LEWAFs, CEWAFs and HEWAFs was performed according to Singer et al. (2000). Briefly, WAFs were prepared in aspirator glass flasks (500 mL) by application of oil or a dispersant-oil mixture (1:10) on the surface of ultrapure water (300 mL) at 10 °C at an oil-to-water (w:v) ratio of 1:50 (LEWAF) or 1:200 (CEWAF), respectively. HEWAFs of dispersant alone were prepared with dispersant loadings corresponding to the amounts added for the CEWAF (1:10 part of 1:200 stock).

In case of elevated salinity testing (A-YES® assay) the ultrapure water was artificially re-mineralized up to a salinity of 6‰ (Tropic Marin® sea salt, Dr. Biener GmbH, Germany) before it was acclimatized at 10 °C and loaded with the oil, dispersant or dispersant-oil mixtures,

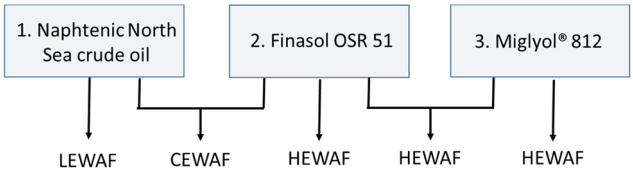

Fig. 1. Overview of different water-accommodated fractions (WAFs) prepared according to Singer et al. (2000) and used for the investigation of ER-mediated estrogenicity. Low-energy- (LEWAF), chemically enhanced- (CEWAF) and high energy (HEWAF) water-accommodated fractions of a crude oil, a third generation dispersant and an inert oil were investigated in the mechanism-specific *in vitro* bioassays ERα-CALUX® (human cell lines) and A-YES® (yeast). Approaches of individual samples and combinations are indicated by arrows.

respectively. The LEWAF setup was carefully stirred with low energy avoiding a vortex in the water phase while CEWAFs and HEWAFs were stirred at higher stirring speeds (25% vortex). WAFs were incubated stirring at 10 °C for 40 h, followed by 1 h settling time. Afterwards, water fractions were carefully drained off (via valve at flask bottom) and immediately used for the exposure.

2.3. Cell culture

2.3.1. Human osteosarcoma U2-OS cells

Human osteoblastic osteosarcoma U2-OS cells have been stably transfected with the human estrogen receptor α (ERα) and a reporter gene construct expressing a luciferase gene. The reporter gene is expressed under the control of responsive elements for the activated ER (Sonneveld et al., 2005). U2-OS cells were purchased from BioDetection Systems BV (BDS), Amsterdam, The Netherlands. Cells were cultured as described in Legler et al. (1999).

2.3.2. Human T47D breast adenocarcinoma cells

The T47D human breast adenocarcinoma cell line endogenously expressing the ER was stably transfected with pEREtata-Luc as described by Legler et al. (1999). Cultivation conditions were similar to those for the U2-OS cells.

2.3.3. Arxula adeninivorans cells

The yeast *A. adeninivorans* has also been stably transfected with the human ERα. Deviating from the aforementioned U2-OS cells the receptor activates the reporter gene phyK, which encodes the enzyme phytase (Hettwer et al., 2018). The *Arxula*-Yeast Estrogen Screen (A-YES®) test kit was purchased from New Diagnostics GmbH, Dresden, Germany, supplying the yeast cells as freeze-dried aliquots.

2.4. ERα-CALUX® assay

The ERα chemical-activated luciferase reporter gene expression (ERα-CALUX®) assay was performed according to the ISO/DIS 1904-3 (ISO, 2014) for water quality assessment and the standard operation procedures (SOP) of BioDetection Systems (BDS) as described in Kuckelkorn et al. (2018). The bioassay was conducted separately with U2-OS and T47Dluc cells with consistent assay procedures expect for the reference compound calibration series (see below).

Methodical and material adaptions concerning the crude oil testing were elaborated in the framework of the present study and are presented and discussed in more detail in section 3.1. In particular, glass-coated 96-well plates (WebSeal Plate$^+$, VWR, Germany) and plate-covering glass plates were used to reduce loss of hydrophobic substances in the WAFs during exposure due to absorption. For the ERα-CALUX® assay with native water samples a 3x-concentrated assay medium was prepared from cell culture medium powder (Sigma, D2902), which was finally supplemented with FCS (charcoal stripped, Biowest, France), non-essential amino acids and penicillin-streptomycin.1x-concentrated assay medium was prepared by diluting the 3×-concentrated assay medium with sterile ultrapure water.

Briefly, cells were seeded in a 96-well plate at a density of $1 \times 10^5 mL^{-1}$ in 1x-concentrated assay medium. 24 h after seeding, cells were exposed to dilution series of the standard reference compound 17-ß estradiol (E2, 0.1 pM–330 pM for U2-US, 0.1 pM–33 pM forT47D) and LEWAF, CEWAF or HEWAF samples. For all WAF samples a serial dilution (1:2) of the 100% stock solution was prepared in sterile ultrapure water shortly prior to the application. Each dilution was complemented with 3× concentrated assay medium (1:3) to guarantee equally nutrient supply comparable to normal culturing conditions in all dilutions steps. WAF dilutions and the corresponding E2 calibration series were tested in triplicates.

To avoid non-specific cytotoxic effects, the highest test concentrations for each WAF was determined in a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay first described by Mosmann (1983) (data can be found in SI, Fig. 1). Exposure concentrations in the ERα-CALUX® were based on a 1:2 dilution series with the undiluted stock (1:50; LEWAF) or 50% of stock (1:200, CEWAF) used as the highest test concentrations identical for tests with T47D and U2-OS cells. To ensure a direct comparability of dispersant HEWAF with the CEWAF treatment, all HEWAFs were prepared in a dilution series from 50% of stock (1:200).

After 24 h of exposure the medium was removed, cells were lysed (25 mM Tris, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% TritonX®-100) for 15 min and luciferase activity was measured with the application of 100 µL luciferin substrate mixture (20 mM Tricine,1.07 mM $(MgCO_3)_4Mg(OH)_25$, 2.67 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM EDTA, 1.5 mM DTT, 539 µM D-Luciferin, 5.49 mM ATP) and 100 µL reaction stop reagent (0.2 M NaOH) using a luminescence reader (Glomax 96-microplate reader, Promega, Madison, USA). The intensity of the luminescence signal correlates with the activation of the ERα.

2.5. A-YES® assay

The A-YES® was performed according to the ISO/DIS 19040-2 (ISO, 2018a) for water quality assessment and the SOP by New Diagnostics (New Diagnostics GmbH, Freising, Germany) as detailed in Hettwer et al. (2018) with minor modifications necessary for application to crude oil testing. Methodical and material adaptions concerning the crude oil testing were elaborated in a series of pretests. As described for the ERα-CALUX® assay, polystyrene material was avoided by using glass coated 96-deep well plates (WebSeal Plate$^+$, VWR, Germany) in particular.

Briefly, after washing and re-dissolving the freeze dried yeast cells were reactivated by incubating at 31 °C and 450 rpm for 1 h. Exposure concentrations of the standard reference compound 17ß-estradiol (1–80 ng L^{-1}) and the different WAF samples were prepared in ultrapure water (low salinity conditions) or artificially re-mineralized dilution water (elevated salinity conditions at 6‰), respectively. A 1:2 dilution series from the 100% stock solution was prepared with the undiluted stock solution as the highest test concentration as no cytotoxic effects were observed in the pretests. Sterile medium mixes for inoculation (low and high salinity) of the test kit were prepared to obtain the same salinity conditions for the E2 and the WAF treatment. Sample dilutions and corresponding E2 calibration series were tested in triplicates (WAF dilutions) and duplicates (E2), respectively.

Exposure solutions and yeast inoculation media were combined in the deep-well plate and incubated at 31 °C and 900 rpm for 22 h.

Afterwards, the cells were separated from the exposure medium by centrifugation (10 min, 700*g*) and the supernatant (50 µL) was transferred to a new 96-well plate (CytoOne®, StarLab International GmbH, Hamburg, Germany). The substrate p-nitrophenyl phosphate (50 µL) was added to the supernatant and the phytase activity was photometrically detected at 405 nm using a microplate reader (Infinite® M200, Tecan Group, Switzerland). Additionally, the growth rate of the yeast was photometrically detected at 630 nm in the re-dissolved yeast pellet.

2.6. Data analysis and EEQ calculations

2.6.1. EEQ calculation

The estrogenic activity in both assays was quantified by means of the calibration reference compound E2. In the present study at least 3 independent experiments per sample fulfilling predefined validity criteria were generated. The validity criteria were based on data evaluation elaborated by BDS (ERα-CALUX®) and New Diagnostics (A-YES®), respectively, and are described in detail in the ISO guidelines (Hettwer et al., 2018; ISO, 2018a,b). Briefly, validity criteria of the ERα-CALUX® assay included curve fit quality ($R^2 > 0.98$), EC_{50} range of reference compound (1.9–19 pM), minimum induction factor (> 5) and quality of

technical replicates (Z-factor > 0.6). Quality criteria of the A-YES® included calibration series EC_{50} range (7–35 ng L^{-1}), curve slope, cell growth (optical density of blank, maximum optical density) and variability in technical replicates.

17ß-estradiol equivalents (EEQ) were extrapolated from the E2 calibration curve using non-linear regression models (Wagner et al., 2017). EEQ values were calculated for each test concentration corrected for the volume of the original sample (per L water) according to the methods given by BDS and New Diagnostics using templates in Microsoft Excel (ER-CALUX® e-bds-019 version q, solver function) and the software BioVal® (A-YES®). This approach using a single-point estimation, provides more detailed information on the sample's concentration-response relationship and with this also insight into curve shape and comparability to the ideal E2 curve shape. As a consequence, in case that more than one concentration resulted in an EEQ value above quantification or detection limits (LOD, LOQ), the EEQ of the treatment was presented in a range EEQ, taking into account differences between responsive dilutions. For treatments resulting in EEQ values likewise above and below the quantification limits across the different replicates, a maximum EEQ instead of a mean EEQ was included.

2.6.2. Concentration-response curves of sample interaction with the ERα

Graphs of the WAF treatment-induced estrogenicity were prepared using Prism 6.0 (GraphPad, San Diego, USA). Luminescence values of 3 technical replicates were corrected for the corresponding blank value. The corrected signals were then normalized to the maximum induction of the E2 reference by dividing the luminescence values by the maximum value of E2. A logistic 4-parameter non-linear regression with variable slope and fixed top and bottom (1, 0) was used to fit concentration-response curves to the data of 3 or 4 experimental replicates (Eq. (1)).

$$y = bottom + \frac{(top - bottom)}{1 + 10^{(\log EC50 - x) * hillslope}} \quad (1)$$

Logistic 4-parameter non-linear regression with variable slope to fit concentration-response curves to normalized relative luminescence data. Top and bottom were set to 1 and 0, respectively. The model was applied in Prism 6 (GraphPad, San Diego, USA).

2.7. Chemical analysis of WAFs and particle size distribution of CEWAF droplets

LEWAF stock solutions in both low and elevated salinity media were analyzed for a set of target PAHs characteristic for the investigated oil sample using solid phase micro-extraction (SPME) according to the methods described in Potter and Pawliszyn (1994). Details are shown in the SI, Section 2. Oil droplet size distribution formed in the CEWAF was analyzed using a Zetasizer Nano-ZS (Malvern Instruments Ltd. Worcestershire, United Kingdom). Details can be found in the SI, Section 3.

3. Results

3.1. ERα-CALUX® procedure optimization to crude oil toxicity testing

As crude oil WAFs are complex sample types containing hydrophobic, absorptive compounds, methodical and material adaptions had to be established in a first step to guarantee the reliable implementation in validated bioassays. When comparing the WAF-induced estrogenic potential in the ERα-CALUX® assay performed in polystyrene plates to the WAF response in glass-coated well plates (Fig. 2, panel A) a clear difference in the WAF compound's interaction with the ER α can be observed. While WAF exposed cells in glass-coated well plates showed a maximum response of 40% of the maximum E2 induction, the exposure in polystyrene well plates reached relative induction values > 100% of the maximum E2 response. Hence, the application of a polystyrene well plate would dramatically overestimate the endocrine disruptive potential of the dispersed crude oil probably due to an elution of xenoestrogenic compounds from the plate material by CEWAF components.

In general, U2-OS cells grew normal in the glass-coated well plates and showed expectedly sensitive responses in the ERα-CALUX® as indicated by the almost identical concentration-response curves of the reference compound E2 in glass-coated and polystyrene plates (Fig. 2, panel B). Hence, the application of glass-coated well plates in crude oil WAF toxicity testing is suitable for reliable responses.

Among other specific material adaptions including well plate covering material of glass instead of polystyrene to avoid cell contamination during the incubation time, the application equipment used for cell exposure towards freshly prepared WAF dilutions was evaluated. No clear differences in resulting biological activity of WAF samples were observed between volumetric pipettes with polypropylene tips and a Hamilton glass syringe (Fig. 2, panel C) used for WAF application, probably due to a sufficiently short contact time of the solutions in the pipetting equipment. Hence, in the interest of a higher throughput polypropylene tips were selected to load WAF dilutions on the well plates containing the cell layer.

3.2. ER-mediated estrogenicity in the ERα-CALUX® bioassay with U2-OS cell

Results obtained from the ERα-CALUX® assay indicate that sample compounds of both LEWAF and CEWAF do interact with the ERα in the highest test concentrations, as the luciferase induction increased with increasing sample concentrations (Fig. 3, panels A and B). The dispersed crude oil exposure resulted in a stronger receptor-mediated estrogenicity with relative luminescence values reaching almost double of the maximum induction by the crude oil alone. Mean estradiol equivalents (EEQ) for both treatments were calculated as 1.34 ng E2 L^{-1} and 3.06 ng E2 L^{-1}, respectively (Table 1).

All HEWAFs of dispersant alone, dispersed inert oil and inert oil alone did not activate the ERα above the quantification- or even detection limit (Fig. 3 panels C-D, Table 1), with the exception of one replicate of the dispersant HEWAF. In this experiment a maximum EEQ of 0.69 ng E2 L^{-1} was calculated, which is still an EEQ close to the quantification limit determined for most samples measured using this bioassay.

3.3. ER-mediated estrogenicity in the ERα-CALUX® bioassay with T47D cells

In compliance with the ER activation in U2-OS cells also in T47D cells WAF from both native and chemically dispersed crude oil samples did interact with the ERα, resulting in a concentration-related increase of luminescence (Fig. 4). Again, as shown in Table 2, the dispersed crude oil (mean EEQ: 2.69 ng E2 L^{-1}) induced a stronger receptor-mediated estrogenicity compared to WAF from the crude oil alone (mean EEQ: 0.74 ng E2 L^{-1}).

In comparison, the concentration-response relationship of ER activation differed between both cell lines with respect to curve shape and maximum induction. Even though the maximum ERα activation was higher for U2-OS cells (EEQ LEWAF: 2 ng E2 L^{-1}) than for T47D cells (EEQ LEWAF: 0.87 ng E2 L^{-1}), the general response over all exposure concentration was higher in T47D cells (Fig. 4, panels C and D), which was furthermore consistent for both treatments. However, taking into account the range of EEQ values due to the single-point estimation in EEQ calculations, a high variance especially in the CEWAF treatment becomes obvious and differences in EEQ values for both cell types were not statistically significant (see SI Fig. 3).

3.4. ER-mediated estrogenicity in the A-YES® bioassay with Arxula yeast

To investigate the influence of medium salinity changes on crude oil estrogenicity the A-YES® assay was performed with all WAF

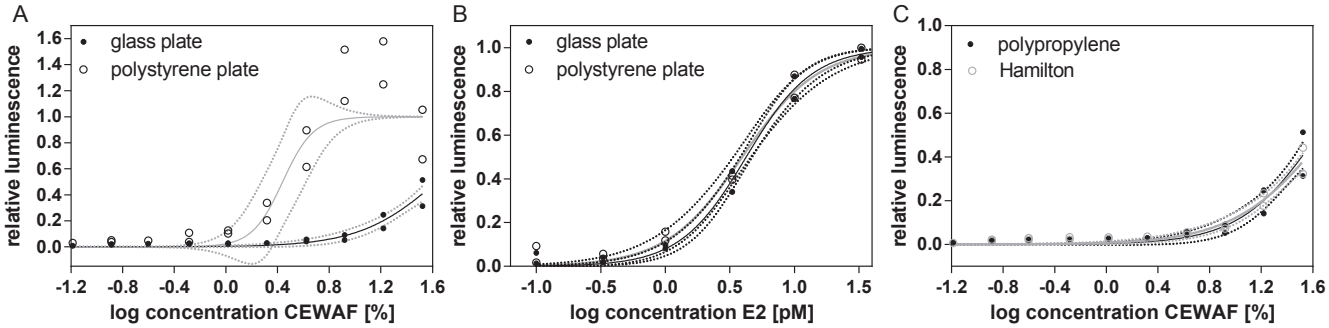

Fig. 2. Material adaption steps for crude oil toxicity testing in *in vitro* based small-scale assays. Direct comparison of different approaches including glass coated well plates (WebSeal Plate+, VWR, Germany) versus polystyrene well plates (CytoOne®, StarLab International GmbH, Hamburg, Germany) for crude oil CEWAF (A) and the reference compound E2 (B) as well as WAF application in glass-coated well plates using a Hamilton syringe versus volumetric pipette with polypropylene tips (C) in the ERα-CALUX® procedure. Dots represent the relative luminescence of 2–3 independent experiments normalized to the maximum E2 luminescence. Concentration-response curves and corresponding 95% confidence bands were fitted using a 4 parameter, non-linear regression model with variable slope in Prism 6, where bottom and top were set to 0 and 1, respectively. Equation: Y = 1/(1 + 10^((LogEC50-X)*HillSlope)).

combinations prepared at low and elevated salinity conditions.

No activation of the ERα above the quantification- or even detection limit was observed for LEWAF from the crude oil as well as for HEWAF from the inert model oil exposure independent of the salinity regime used for WAF preparation (Table 3). Again, out of all WAF treatments the CEWAF from the crude oil showed the highest endocrine disruptive potential with resulting EEQs of maximum 6.8 ng E2 L^{-1} (low salinity) and 9.9 ng E2 L^{-1} (elevated salinity), respectively. The HEWAF of the dispersant alone as well as the HEWAF of the dispersed inert oil did activate the ERα in 1 out of 3 or 4 independent replicates in a quantifiable manner. As no estrogenicity of the inert oil was observed it can be assumed that a response in both HEWAFs was caused by the dispersant itself. Hence, a slight estrogenic potential of the dispersant cannot be excluded.

In direct comparison, a clear trend for a stronger receptor-mediated estrogenicity of WAFs prepared in elevated salinity conditions than for WAFs prepared in low salinity conditions was observed with dispersed crude oil and dispersant alone showing a 1.5–2.4 times higher maximum response. However, a high variability of calculated EEQ values and replicates with non-quantifiable estrogenic response have to be considered.

4. Discussion

4.1. ER-mediated estrogenicity of naphthenic North Sea crude oil WAFs

Both native and dispersed crude oil indicate an ER-mediated estrogenicity with stronger ERα agonism (> 2-fold) in the CEWAF treatment. Though based on human ERα the result of this reporter gene assay on receptor-mediated estrogenicity are likewise relevant for the

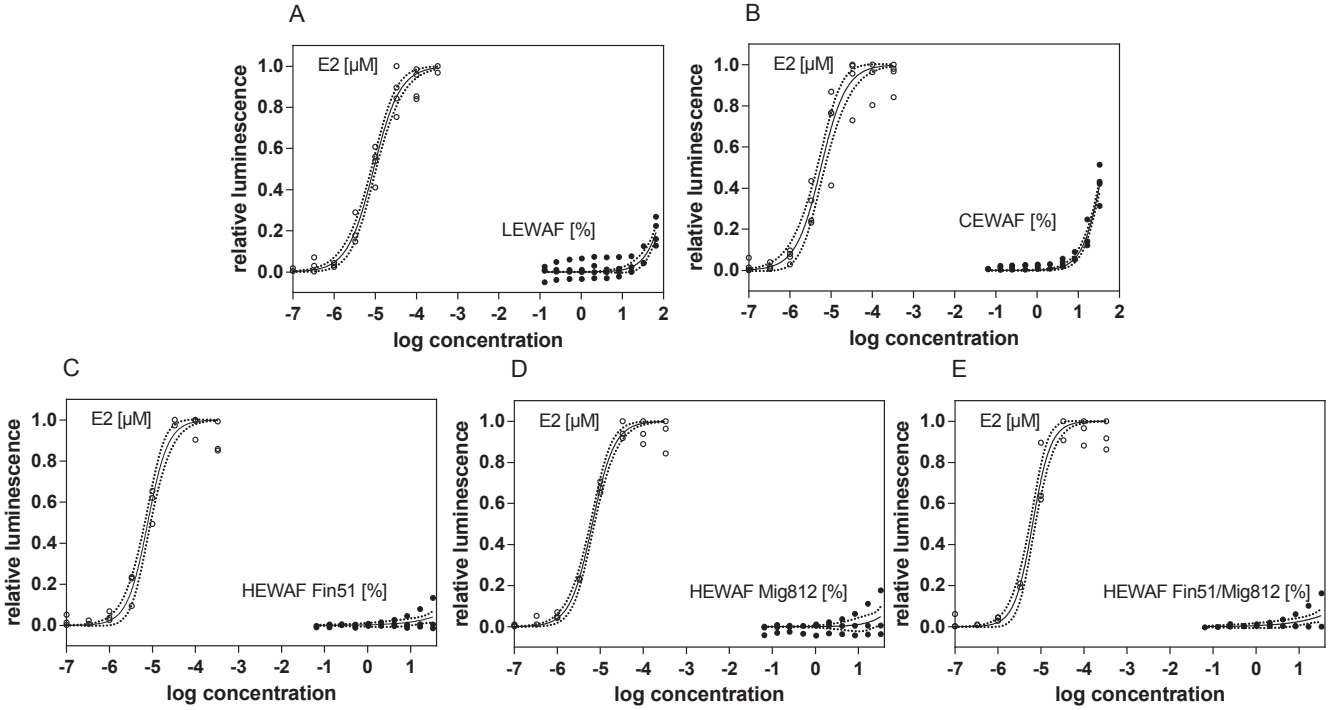

Fig. 3. Relative activity of the human ERα in U2-OS cells exposed to WAF dilutions in the ERα-CALUX® assay. Relative luminescence is shown for the naphthenic North Sea crude oil (LEWAF), dispersed crude oil (CEWAF, with Finasol 51), dispersant alone (HEWAF Finasol 51), inert oil (HEWAF Mig812) and dispersed inert oil (HEWAF Finasol 51/Mig812) exposure, respectively. Dots represent the relative luminescence of sample dilutions (filled circles) normalized to the highest luminescence measured for the E2 standard (n = 3–4). Concentration-response curves and corresponding 95% confidence bands were fitted using a 4 parameter, non-linear regression model with variable slope in Prism 6, where bottom and top were set to 0 and 1, respectively. Equation is presented before.

Table 1
Calculated 17ß-estradiol equivalents (EEQ) of WAF dilutions in the ERα-CALUX® bioassay with U2-OS cells. EEQ values of 3–4 independent experiments as well as the corresponding limits of detection (LOD) and limits of quantification (LOQ) after the exposure to naphthenic North Sea crude oil (LEWAF), dispersed crude oil (CEWAF, with Finasol 51), dispersant (HEWAF Finasol 51) and corresponding combinations with inert oil (HEWAF Mig812, HEWAF Mig812/Fin51) are shown. EEQs were extrapolated from a non-linear model of the E2 calibration curve. EEQs are presented in a range for samples with more than the highest exposure concentration inducing EEQ values (single-point estimation). Treatments indicated by the same letter (a,b) did not differ significantly from each other (normal distribution, equal variance fulfilled; One Way ANOVA with Tukey post hoc test, $p < 0.05$).

Treatment	EEQ [ngE2 L^{-1}]	LOD [ngE2 L^{-1}]	LOQ [ngE2 L^{-1}]	EEQ_{mean} [ngE2 L^{-1}]	SD [ngE2 L^{-1}]
LEWAF	< LOQ	0.45	1.34		
	0.86	0.09	0.28	**max 2.0***	
	2.00	0.36	1.07	(1.18)** a	(0.72)**
	1.15	0.18	0.53		
CEWAF	2.57–7.93	0.03	0.08		
	3.73	0.23	0.68	**3.06 b**	**0.54**
	2.47–2.65	0.12	0.35		
	3.13–3.37	0.11	0.34		
HEWAF Finasol 51	0.69	0.06	0.19		
	< LOQ	0.31	0.93	**max 0.69***	
	< LOD	0.27	0.81	(0.43)** a	(0.28)**
HEWAF Mig812	< LOD	0.12	0.37		
	< LOD	0.30	0.90	**< LOQ**	
	< LOQ	0.11	0.33	(0.12)** a	(0.06)**
HEWAF Mig812/Finasol 51	< LOQ	0.14	0.41		
	< LOQ	0.11	0.34	**< LOQ**	
	< LOD	0.27	0.82	(0.17)** a	(0.04)**

* For samples with replicates < LOD/LOQ no mean EEQ was calculated, instead maximum response was given.
** Mean EEQ value with SD including LOQ/2 or LOD/2 for replicates with < LOD/LOQ.

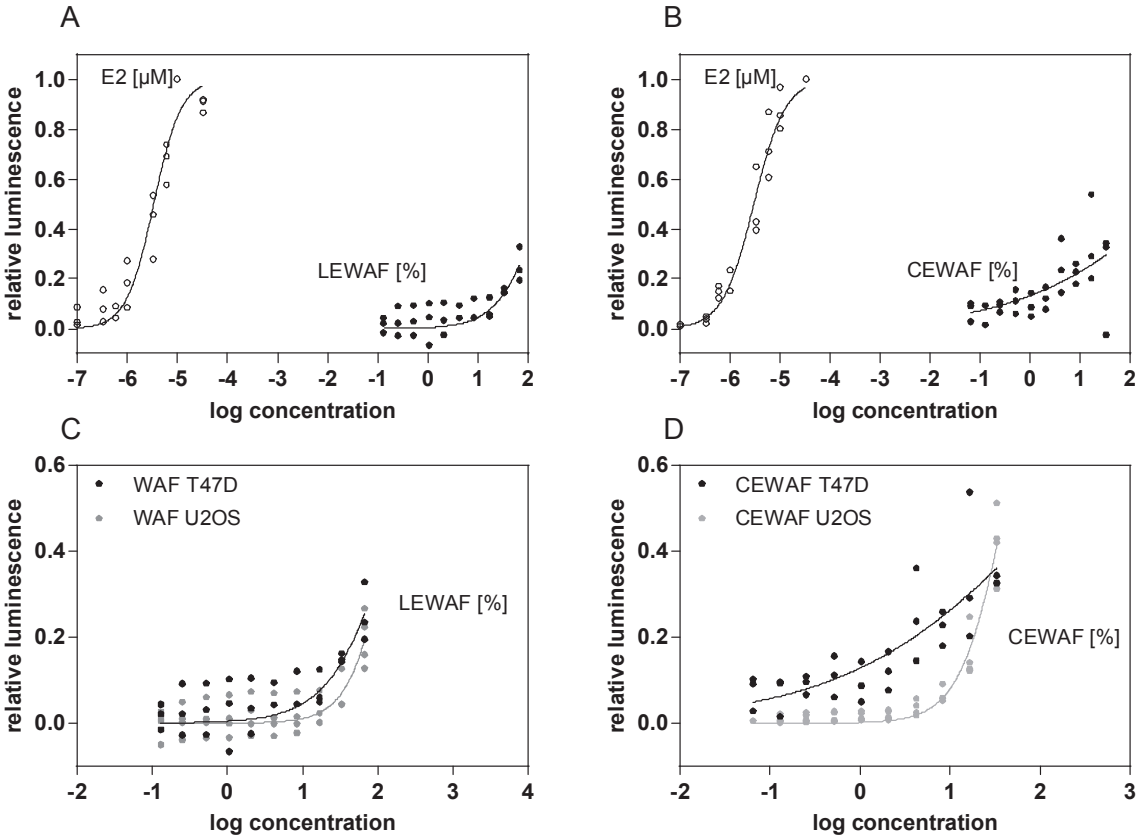

Fig. 4. Relative activity of the human ERα in T47D cells exposed to WAF dilutions in the ERα-CALUX® assay. Relative luminescence is shown for the naphthenic North Sea crude oil (LEWAF, panel A) and the dispersed crude oil (CEWAF, with Finasol 51, panel B). Panels C and D show a direct comparison of relative luminescence in both cell lines. Dots represent the relative luminescence of sample dilutions (filled circles) normalized to the highest luminescence measured for the E2 standard (n = 3–4). Concentration-response curves and corresponding 95% confidence bands were fitted using a 4 parameter, non-linear regression model with variable slope in GraphPad Prism 6, where bottom and top were set to 0 and 1, respectively. Equation is presented before.

Table 2
Calculated 17ß-estradiol equivalents (EEQ) of WAF dilutions in the ERα-CALUX® bioassay with T47D cells. EEQ values of 3 independent experiments as well as the corresponding limits of detection (LOD) and limits of quantification (LOQ) after the exposure to naphthenic North Sea crude oil (LEWAF) and dispersed crude oil (CEWAF, with Finasol 51) are shown. EEQs were extrapolated from non-linear model of the E2 calibration curve. EEQs are presented in a range for samples with more than the highest exposure concentration inducing EEQ values > LOQ (single-point estimation). Treatments were normal distributed and of equal variance and varied significantly from each other according to One Way ANOVA with Tukey post hoc test for pairwise comparison ($p < 0.05$).

Treatment	EEQ [ngE2 L^{-1}]	LOD [ngE2 L^{-1}]	LOQ [ngE2 L^{-1}]	EEQ_{mean} [ngE2 L^{-1}]	SD [ngE2 L^{-1}]
LEWAF	0.86–0.87	0.08	0.26		
	0.46–0.76	0.05	0.16	**max 0.87**	
	< LOQ	0.57	1.71	(0.78)**	(0.14)**
CEWAF	0.89–2.68	0.06	0.20		
	0.63–5.92	0.04	0.11	**2.69**	**0.79**
	0.4 – 5.6	0.04	0.12		

*For samples with replicates < LOD/LOQ no mean EEQ was calculated, instead maximum response was given.
** Mean EEQ value with SD including LOQ/2 or LOD/2 for replicates with < LOD/LOQ.

aquatic vertebrates, as the ER is conserved across the vertebrates. In more detail, significant structural homologies between human and, e.g., fish ER (Judson et al., 2010; Nelson and Habibi, 2013) as well as a similar ligand binding specificity have been reported (Yost et al., 2014). However, deviating from mammals, teleost fish have at least 3 ER nuclear subtypes (Hawkins et al., 2000; Nelson and Habibi, 2013) with the ERß suggested to be critically involved in the teleost estrogenic response (Yost et al., 2014). Nonetheless, the expression of these nuclear receptors is dependent on the tissue and the developmental stage, and the activities of the different subtypes are still not fully understood.

Table 4 provides an overview of comparable studies using *in vitro*-based methods to evaluate crude and refined petroleum product estrogenicity. Reported estrogenic response varied among the confined set of available studies from no to high potencies. In general, most studies investigating the estrogenic potential of petroleum products used sample extracts for cell exposure instead of water soluble fractions. Studies focusing on the estrogenicity via MCF-7 proliferation assay did not determine estrogenic responses for a broad variety of oil extracts (Arcaro et al., 2001; Ssempebwa et al., 2004; Zhu et al., 2012). While none of the tested crude and refined oils were found to contain ERα-activating compounds in yeast estrogen screen assays (Vrabie et al., 2010), which is in contrast to the present results obtained using the A-YES® assay, mammalian cell based systems were able to determine ERα-induced estrogenicity (Vrabie, 2011). Interestingly, some oil extracts that were reported to not interact with the ERα in previous studies did show anti-estrogenic responses or high activities for other ER subtypes (Arcaro et al., 2001; Vrabie et al., 2010), highlighting the importance of considering other modes of actions behind the endocrine disruption.

Besides crude oils also produced water discharge extracts from offshore oil and gas production installations in the North Sea, including samples of the platform producing the present NNS crude oil, have been investigated for their estrogenic potential (Thomas et al., 2009, 2004; Tollefsen et al., 2007). All water extracts were found positive for the presence of ER agonists (Thomas et al., 2009, 2004; Tollefsen et al., 2007). Compared to the NNS crude oil WAF from the present study the estrogenic potency of the corresponding water extract had been lower, which was rather based on dilution effects and water sampling subsequent to a treatment process. Nonetheless, studies showing estrogenicity of production water demonstrate that oil-originating estrogen active compounds do enter the marine environment, and thus emphasize the importance of endocrine disruption to be included in oil risk assessment.

The high variance across several *in vitro* based studies on estrogenicity might be related to a very limited comparability due to unique characteristics of each petroleum product and to experimental setup conditions including sample preparation and selected *in vitro* assay. In the context of yeast estrogen screen, for example, discrepancies are most likely related to assay-specific detection limits with the A-YES® being 2–4-times more sensitive (LOQ around 2.5 ng E2 L^{-1}) compared to other yeast-based estrogen screens (LOQ around 5–10 ng E2 L^{-1})

Table 3
Calculated 17ß-estradiol equivalents (EEQ) of WAF dilutions in the A-YES® bioassay. EEQ values of 3–4 independent experiments as well as the corresponding limits of detection (LOD) and limits of quantification (LOQ) after the exposure to naphthenic North Sea crude oil (LEWAF), dispersed crude oil (CEWAF, with Finasol OSR 51), dispersant (HEWAF Fin51) and corresponding combinations with inert oil (HEWAF Mig812, HEWAF Mig812/Finasol 51) are shown. EEQs were extrapolated from non-linear model of the E2 calibration curve. EEQs are presented in a range for samples with more than the highest exposure concentration inducing EEQ values > LOQ (single-point estimation). Data were not normal distributed and Kruskal-Wallis One Way ANOVA on ranks did not detect significant differences between the treatments.

Treatment	Low salinity					Elevated salinity (6‰)				
	EEQ [ngE2 L^{-1}]	LOD [ngE2 L^{-1}]	LOQ [ngE2 L^{-1}]	EEQ_{mean} [ngE2 L^{-1}]	SD	EEQ [ngE2 L^{-1}]	LOD [ngE2 L^{-1}]	LOQ [ngE2 L^{-1}]	EEQ_{mean} [ngE2 L^{-1}]	SD
LEWAF	< LOD	2.94	6.11			< LOQ	3.52	4.82		
	< LOQ	2.38	3.06	**< LOQ**		< LOD	3.17	4.69	**< LOQ**	
	< LOD	4.35	6.67	(1.7)**	(0.4)**	< LOQ	1.51	2.17		
CEWAF	< LOQ	2.94	6.11			5.8–9.9	3.52	4.82		
	3.9–6.80	2.38	3.06	**max 6.8***		5.7–9.6	3.17	4.69	**6.8**	**1.6**
	< LOD	4.35	6.67	(3.5)**	(1.7)**	4.1–5.7	1.51	2.17		
HEWAF	< LOQ	2.94	6.11			4.9–9.9	3.52	4.82		
Finasol 51	4.0–6.20	2.38	3.06	**max 6.2***		11.9–14.6	3.17	4.69	**9.0**	**3.7**
	< LOD	4.35	6.67	(3.4)**	(1.5)**	4.0–8.7	1.51	2.17		
HEWAF	< LOD	3.48	3.97			< LOD	4.19	5.65		
Mig812	< LOD	3.22	5.50	**< LOQ**		< LOD	4.77	8.08	**< LOQ**	
	< LOQ	2.27	3.03	(1.6)**	(0.1)**	< LOQ	1.53	2.35	(1.9)**	(0.6)**
	< LOD	3.57	5.78							
HEWAF	< LOD	3.48	3.97			< LOQ	4.19	5.65		
Mig812/Finasol51	< LOD	3.22	5.50	**max 13.1***		< LOQ	4.77	8.08	**max 7.5***	
	13.1	2.27	3.03	(5.5)**	(6.6)**	4.6–7.5	1.53	2.35	(4.3)**	(1.6)**
	< LOQ	3.57	5.78							

* For samples with replicates < LOD/LOQ no mean EEQ was calculated, instead maximum response was given.
** Mean EEQ value with SD including LOQ/2 or LOD/2 for replicates with < LOD/LOQ.

Table 4
Overview of studies investigating the crude and refined petroleum product induced estrogenicity. A variety of oil types were tested for general estrogenic or ER-mediated response using different *in vitro* based bioassays. Columns characterizing details on the study setup show limitations of results comparability to the present study.

Sample type	Sample preparation	Bioassay	Endpoint	Response	Reference
Clarrified slurry oil, light and heavy crude oil	DMSO extract (0.1%)	MCF-7 focus assay	Postconfluent growth (multicellular nodules/foci):estrogenicity	Not active	Arcaro et al. (2001)
Clarrified slurry oil, light and heavy crude oil	DMSO extract (0.1%)	MCF-7 focus assay	Postconfluent growth (multicellular nodules/foci):anti-estrogenicity	Active	Arcaro et al. (2001)
Fresh and waste crankcase oil	DMSO extract (0.1%)	MCF-7 focus assay	Postconfluent growth (multicellular nodules/foci):estrogenicity	Not active	Ssempebwa et al. (2004)
Fresh and waste crankcase oil	DMSO extract (0.1%)	MCF-7 focus assay	Postconfluent growth (multicellular nodules/foci):anti-estrogenicity	Active	Ssempebwa et al. (2004)
Fresh C-heavy oil	Artificial medium with oil	MCF-7 MTS/PES assay	MCF-7 cell growth	Not active	Zhu et al. (2012)
Biologically degraded C-heavy oil	Artificial medium with oil	MCF-7 MTS/PES assay	MCF-7 cell growth	Slightly active (73 d degradation)	Zhu et al. (2012)
Crude and refined petroleum products	Toluene extracts	Recombinant yeast estrogen screen	ERα activity	Not active	Vrabie et al. (2010)
Crude and refined petroleum products	Toluene extracts	Recombinant yeast estrogen screen	ERβ activity	Active	Vrabie et al. (2010)
Crude and refined petroleum products	Toluene extracts	Recombinant mammalian estrogen screen (U2-OS)	ERα activity	Active	Vrabie (2011)
Crude and refined petroleum products	Toluene extracts	Recombinant mammalian estrogen screen (U2-OS)	ERβ activity	Active	Vrabie (2011)
North Sea produced water discharges (5 platforms)	SPE extracts (MeOH/DCM)	Recombinant yeast estrogen screen	ERα activity	Active	Thomas et al. (2004)
North Sea produced water discharges (5 platforms)	HPLC fractionated SPE extracts (MeOH/DCM)	Recombinant yeast estrogen screen	ERα activity	Active (petrogenic naphthenic acid)	Thomas et al. (2009)
North Sea produced water discharges (10 platforms)	SPE extracts (MeOH/Hexane)	Recombinant yeast estrogen screen	ERα activity	Active	Tollefsen et al. (2007)
North Sea produced water discharges (10 platforms)	Oil and particulate fraction	Recombinant yeast estrogen screen	ERα activity	Not active	Tollefsen et al. (2007)

(Hettwer et al., 2018).

In order to evaluate the estrogenic potential with respect to water quality assessment, trigger values have been suggested for defining thresholds below which no environmental risk is expected. A set of concepts based on different perspectives and protection goals including human health (Brand et al., 2013) or environmental risk assessment (e.g. Escher et al., 2018) established trigger values for bio-equivalents (e.g., EEQ) obtained from specific *in vitro* bioassays. With focus on surface waters and municipal effluents ERα-CALUX® specific effect-based trigger (EBT) values in the range of 0.5 ng E2 L^{-1} (Jarošová et al., 2014; Kunz et al., 2015; van der Oost et al., 2017) down to 0.1 ng E2 L^{-1} (Escher et al., 2018) were suggested. Also for the A-YES® an EBT-EEQ of 0.56 ng E2 L^{-1} has been calculated (Escher et al., 2018). WAFs of the NNS crude oil used in the present study exceeded those values, indicating a potential risk of endocrine disruptive activity for the aquatic environment.

A maximum EEQ instead of a mean EEQ was included for treatments with E2 equivalents above and below the quantification limit across the replicates representing a worst case scenario. In literature, other approaches using LOD, LOD(LOQ)/2 or 0 instead of a maximum value can be found and were used here as additional information for statistical analysis. However, it has to be considered that replacing non-quantifiable estrogenicity by one methods of choice can considerably over- or underestimate the risk.

Though observed at WAF concentrations that are unlikely to be found in the environment after an oil spill, a chronic exposure with low doses, e.g., from several diffuse spills or production waters might lead to endocrine disruption in biota. Furthermore, though good correlations between *in vitro* and *in vivo* results have been reported for selected estradiol derivates (Schenk et al., 2010; Sonneveld et al., 2005; Wehmas et al., 2011) *in vitro*-based methods do have limitations when it comes to risk extrapolation to the aquatic environment due to lacking toxicokinetics.

4.2. The influence of metabolization in ER-mediated estrogenicity of crude oil samples

As reviewed earlier, several studies focusing on single PAHs concluded an estrogen-disruptive potential with differences in strength of ER agonistic response depending on the individual compound (Zhanget al., 2016). For the most prominent PAHs in the NNS crude oil WAFs from the present study, namely naphthalene, fluoranthene and phenanthrene (see SI, Table 1), especially the hydroxylated metabolites are known for a stronger ER agonism compared to their parent compounds. 2-OH derivates of phenanthrene and fluorene have at least displayed weak agonistic interactions with the ERα (Fertuck et al., 2001; Hayakawa et al., 2007; Wenger et al., 2009), while the parent compounds were found to be completely inactive (Hayakawa et al., 2007). Furthermore, a stronger binding affinity of hydroxylated PAHs compared to their parent compounds has been confirmed previously (Fertuck et al., 2001; Hayakawa et al., 2007; van Lipzig et al., 2005). However, results from a receptor binding affinity assay cannot directly be extrapolated to estrogenic responses in reporter gene assays (Fertuck et al., 2001; Wagner et al., 2017). In contrast, also no estrogenic potential or stronger response of parent compounds compared to metabolites have been reported for the WAF relevant PAHs (Lee et al., 2017; Villeneuve et al., 2002), which is rather in compliance with the current study as overall no higher ER agonistic potential of crude oil WAFs in metabolically active T47D cells was observed. Nonetheless, the ER activation in T47D cells for most CEWAF concentrations was above the response in U2-OS cells, indicating a trend of hydrocarbon bio-activation in this approach. However, also the metabolization of dispersant components cannot be excluded as the HEWAF was not investigated in T47D cells.

The application of a phase I metabolizing system (S9), typically originating from rat liver homogenates, is another approach to simulate vertebrate biotransformation in metabolically inactive cell lines (Kuckelkorn et al., 2018; Mollergues et al., 2017). However, as the exact mechanisms underlying the S9-induced alteration are still not fully understood and also high variabilities in quality and composition of S9 mixtures (Mollergues et al., 2017) are known, this approach was not chosen for the current study.

4.3. The influence of salinity in ER-mediated estrogenicity of crude oil samples

It is well known that besides physical-chemical characteristics of a unique crude oil also external parameters affect the partitioning of petrogenic oil components in the oil:water system. For most PAHs, including the relevant naphthalene and phenanthrene a decreased solubility caused by the “salting out effect” is observed when salinity is increased (Eganhouse and Calder, 1976; Saranjampour et al., 2017; Whitehouse, 1984; Xie et al., 1997). However, a clear salting out effect cannot be generalized for all complex PAH mixtures (Eganhouse and Calder, 1976) and hence should analytically be addressed for individual scenarios. Furthermore, it has to be kept in mind that the PAHs partitioning is far more sensitive to small changes in temperature compared to changes in salinity (Whitehouse, 1984).

For the low and elevated salinity scenarios used in the current study only marginally reduced PAH concentrations were observed when salinity was increased (see SI Section 2, Table 1) leading to the conclusion that the stronger ER-activation under elevated salinity conditions is not linked to partitioning changes of oil components but rather related to the changes in the test system itself. When incubated in higher salinity media *A. adeninivorans* cells grow in a smaller morphological habitus. Hence, the surface-volume relationship is increased leading to the assumption that the sensitivity of the test system is increased in brackish and marine conditions. Against this background, a direct comparability of resulting EEQs from low and elevated salinity conditions is limited. Nonetheless, the A-YES® assay has been proven to be a sensitive screening tool detecting estrogenicity in a range comparable to human-receptor mediated assays and can be recommended for the application in brackish and marine water research.

4.4. The role of dispersant in ER-mediated toxicity

In compliance with the present study a higher toxicity of crude oil CEWAFs compared to LEWAFs has been reported in several studies (e.g.Dussauze et al., 2015; Ramachandran et al., 2004; Rial et al., 2014). A dispersant serves as an additional source of dissolved as well as particulate hydrocarbons (Redman and Parkerton, 2015). Even though the influence of the dispersant is discussed controversially (e.g. reviewed in Bejarano et al., 2014), Prince (2015), only a limited number of studies addressed the role of a dispersant in experimentally relevant conditions, thus allowing a direct comparison to CEWAFs.

The results of the present study allowing a direct comparison indicate a very limited contribution of the dispersant itself to the high estrogenic potential of the CEWAF. This leads to the assumption that the amount of dissolved hydrocarbons is increased or other, more potent oil components become bioavailable in this approach. An increased fraction of dissolved hydrocarbons in the CEWAFS is likely related to the droplet induced accelerated equilibrium partitioning. However, a slight ER-agonistic potential of the dispersant cannot be excluded based on occasionally occurring quantifiable EEQs. In accordance with the current findings, also other dispersants have been identified to only marginally mediate ER activity when tested in a comparable concentration range in a reporter gene *in vitro* assay (Judson et al., 2010). However, like crude oils also dispersants are complex mixtures, some containing the surfactants nonylphenol ethoxylates, which can be degraded to the endocrine disruptive nonylphenol (Judson et al., 2010), and hence would cause a stronger estrogenic activity of the respective dispersants.

Nonetheless, the increased availability of particulate hydrocarbons in form of oil droplets (micelles), the extent of which is strongly dependent on oil characteristics (e.g. viscosity) or mixing energy (Redman and Parkerton, 2015), is of high importance for a CEWAF toxicity assessment. In the static exposure system of the present study dispersed droplets are expected to migrate to the surface over time and not being bioavailable for cell exposure. However, droplets in the nanometer or low micrometer range would stay in the water column with a very limited surfacing tendency. We were able to show droplets between 100 and 700 nm stably across different CEWAF dilutions (see SI Section 3, Fig. 2). According to Stoke law those NNS crude oil droplets would surface with a maximum velocity of 0.003–0.15 mm h^{-1} under ideal freshwater conditions, and hence are expected to be directly available during the exposure period (0.48 mm/24 h) for, e.g., direct physical interactions such as phagocytosis, which changes the toxic load to the cells. Hence, among other aspects including high oxygen consumption in CEWAF approaches due to dispersant dependent chemical reduction, droplets have to be considered as an additional route of exposure in CEWAF toxicity interpretation.

Another question with respect to dispersant toxicity was whether an oil could also change the toxicity of a dispersant. Even though no clear conclusion due to very limited responses of the dispersant itself was possible, a slight trend towards a reduced estrogenicity was observed when the dispersant was incubated together with the inert oil. The inertness of Miglyol 812 was proven in both assays independent of salinity conditions. Hence, an oil might also influence the toxicity of a dispersant.

5. Conclusion

Method optimization of established bioassays with respect to testing of complex petrogenic oil samples was successfully demonstrated in the present study. Besides applying the methods to a light fresh crude oil, sensitivity has to be verified in additional experiments using other refined petroleum products like heavy fuel oils as well as artificially weathered crude oils simulating different drifting time at sea. Additionally, method optimization should be further expanded by including, e.g., passive dosing approaches, which are rapidly emerging as stable exposure scenarios for hydrophobic compounds (Bera et al., 2018). As the present study indicated stable particulate oil droplets in the nanometer range, further research should focus especially on this fraction of particulate oil, since it is directly bioavailable for the exposed biota.

The importance of using these sensitive small-scale assays to identify contaminated water bodies is further highlighted considering the very low environmental quality standards for (xeno-)estrogens (low ng L^{-1} range) that are close to the actual detection or quantification limits in chemical analysis.

Furthermore, as indicated in the current discussion, other modes of endocrine disruption like androgen receptor interactions or ER inhibitions should be included in future research. In general, also the potential detoxification via active metabolism should be investigated in more detail with respect to *in vitro* based methods application in risk assessment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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