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Vitamin D receptor antagonist activity in wastewater effluents—potential for endocrine disruption

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Abstract

Background Endocrine disrupting chemicals have been identified for a number of human endocrine systems, but there are no reports on vitamin D-antagonistic activities in environmental samples.

Objectives We have investigated if there are compounds present in the environment that can act as Vitamin D receptor (VDR) antagonists.

Methods Water samples were collected of the influent and effluent water from five Swedish wastewater treatment facilities and concentrated with solid phase extraction. VDR antagonistic properties of the samples were tested with a cell-based in vitro assay responsive to vitamin D signaling. Cytotoxicity was monitored by three different assays.

Results We observed a dose-dependent decrease in the VDR signaling in most studied samples, although the effect was overlapping with cytotoxicity for the influent samples. For effluent samples, we observed clear VDR antagonistic effects also in non-cytotoxic concentrations. The observed effects could not be explained by presence of natural organic matter or cadmium in the water.

Discussion The vitamin D endocrine system regulates a broad range of physiological processes, and disruption of this system could be associated with serious health consequences. In this study, we report environmental presence of compounds with VDR antagonistic properties, compounds which constitute a new group of potential endocrine disruptors. The VDR antagonism was observed in wastewater treatment facility effluent waters, which are discharged into water systems used as raw water for drinking water production. The findings reported in this study may indicate a potential hazard to human health and aquatic life. Future research is needed to investigate the presence of VDR antagonists in the environment, identification of the causative compounds, and studies of exposure of humans and aquatic organisms to these compounds.

Keywords Vitamin D receptor antagonists, Environmental pollutants, Vitamin D

Introduction

The biologically active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃, can be de novo synthesized in humans, is transported in the blood to the target site and act via a specific receptor. Therefore, 1 α , 25-dihydroxyvitamin D₃ is defined as a hormone rather than as a vitamin. 1 α , 25-Dihydroxyvitamin D₃ binds to the vitamin D receptor (VDR) and can thereby regulate a broad range of physiological processes. It is not only involved in the regulation of bone metabolism and calcium homeostasis,

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but it also affects the immune system, brain and fetal development, insulin secretion, and the cardiovascular system [2, 4, 10, 11, 21, 31]. Furthermore, vitamin D deficiency has been suggested to be involved in cancer development and vitamin D has been reported to, e.g., regulate cell proliferation and differentiation and apoptosis [12, 16, 33].

It is well-known that environmental pollutants can interfere with nuclear receptors in the endocrine system (e.g., estrogen receptors, thyroid receptors, and androgen receptor). Environmental pollutants with these properties are denoted as endocrine disrupting compounds (EDCs).

Presence of bioactive pollutants in the aquatic environment is a cause of concern both from an ecological perspective as well as from a human health perspective. Surface and ground water sources are often used for drinking water production, and chemical pollutants present in these sources may contaminate the drinking water. It has been concluded in an OECD report [26] as well as in a review [20], that disruption of or interference with the vitamin D signaling system by environmental pollutants could be associated with the risk of serious health consequences. Liu et al. [18] recently reported an association between per and polyfluoroalkyl ether sulfonic acids and vitamin D biomarkers in newborns, and inhibition of vitamin D-induced expression of genes involved in calcium transfer, cells was observed by Di Nisio et al. [6] in cells treated with perfluoro-octanoic acid (PFOA). However, until today, there has been no reports of vitamin D receptor agonistic nor antagonistic activities in environmental matrices.

The aim of this study has been to study whether there are compounds present in the environment that can disrupt the vitamin D signaling system by acting as VDR antagonists. This would constitute a new class of potential endocrine disrupters and a new target system for endocrine disrupters. We have tested this hypothesis

with a bioanalytical approach, adopting cell based in vitro assays to investigate the presence of VDR disrupting compounds in wastewater.

Methods

Water sample collection and preparation

Water samples were collected at five Swedish waste water treatment plants (WWTPs) in March and April 2018. Samples covered both the influent (untreated) water and the corresponding effluent (treated) water at each WWTP. A flow-proportional sampling mode was used for sample collection over a time of 24–144 h to integrate short-term fluctuations in the water composition. The treatment steps were the same for all studied WWTPs. These involved pretreatment (grit removal, settlement of sand or grit), primary treatment (sedimentation), secondary treatment (biological processes), post-sedimentation, and sludge handling (sludge thickener, digestion chamber). Details on the WWTPs, and sample labelling, are presented in Table 1. Water samples were collected in 12 L stainless steel containers, transported to the laboratory and stored at +4 °C awaiting sample preparation.

The extraction of the water samples (5 L) was conducted using an automatic solid phase extraction system (SPE–DEX, Horizon Technology, Salem, NH, USA) containing hydrophilic–lipophilic balance (HLB) extraction disks (Atlantic HLB-H Disks, diameter 47 mm; Horizon Technology, Salem, NH, USA). The disks were conditioned with 280 mL methanol and 420 mL Millipore water, and then the wastewater samples were loaded to the disks at a flow rate of 50 mL min⁻¹. After washing the disks twice with 24 mL 5% methanol in Millipore water, the disks were dried under vacuum for 30 min, and eluted 3 times with 25 mL methanol. The samples were then evaporated under a gentle nitrogen stream at 35 °C and reconstituted in 1 mL ethanol.

The enrichment and dilution of the samples were used to calculate the relative enrichment factor (REF),

Table 1 Information on the WWTPs included in this study

Location	City	Samples	Population equivalents	Average flow (m ³ /24 h)	Sampling time
WWTP1	Västerås	WW1 (influent) WW2 (effluent)	121 000	43 824	24 h
WWTP2	Örebro	WW3 (influent) WW4 (effluent)	140 000	40 200	24 h
WWTP3	Eskilstuna	WW5 (influent) WW6 (effluent)	95 000	43 655	120 h
WWTP4	Linköping	WW7 (influent) WW8 (effluent)	235 000	40 000	24 h
WWTP5	Uppsala	WW9 (influent) WW10 (effluent)	169 000	46 000	144 h

as previously described by Escher et al. [9]. The initial water volume of 5 L per sample was extracted to a volume of 1 mL, leading to an enrichment factor_{SPE} of 5000. When incubated with the cells, the concentrated water samples were diluted by 100 fold with the cell medium to get a final concentration of 1% ethanol and a REF of maximum 50. The concentrated water samples were then analyzed in a dilution series with a dilution factor of 2 in each step.

A blank sample was prepared by extraction of 5 L Millipore water on the same solid phase extraction system as described above. This sample was then analyzed for bioactivity in a single concentration factor of REF 50.

Cell culture

VDR-UAS-bla HEK 293 T were purchased from ThermoFisher Scientific (Stockholm, Sweden). This cell line, based on human embryonic kidney cell line HEK 293, contains a human vitamin D receptor (VDR) ligand-binding domain and a Gal4 DNA binding domain as well as a beta-lactamase reporter gene under the control of a UAS response element. Upon ligand binding to the VDR, the expression of the beta-lactamase reporter gene will increase (see Sect. "Cell based evaluation of VDR antagonism").

Cells were subcultured twice a week. Culture flasks were coated with 1×Matrigel matrix (Gibco, Invitrogen) in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (Lonza) and incubated for 15 min in a humidified incubator. After coating and incubating, the matrigel medium was aspirated and cells were seeded into the flasks.

This cell line was routinely cultured in growth medium consisting of DMEM with GlutaMAX™ (Gibco, Invitrogen) supplemented with 10% dialyzed fetal bovine serum (Gibco, Invitrogen), 0.1 mM non-essential amino acids (Gibco, Invitrogen), 25 mM HEPES buffer (Gibco, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen), and 80 µg/mL, respectively, of Hygromycin (InvivoGen) and Zeocin (Gibco, Invitrogen) for selection purposes. To assay VDR antagonistic effects, cells were plated in assay medium with phenol red-free DMEM (Lonza) medium supplemented with 2% charcoal-stripped FBS (Gibco, Invitrogen), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, Invitrogen), 0.1 mM non-essential amino acids (Gibco, Invitrogen) and 1 mM sodium pyruvate (Gibco, Invitrogen). Cells were cultured in an incubator with humidified atmosphere at 37°C containing 95% air and 5% CO₂. Medium was changed every 2–3 days. Trypsin–EDTA (Gibco, Invitrogen) was used for sub-culturing of cells.

Evaluation of cytotoxicity

To ensure that the specific biological effects were analyzed under non-cytotoxic conditions, the cytotoxicity of the water samples were analyzed using three different methods; MTS reduction capacity, ATP amount and LDH release. MTS reduction capacity was assayed using the colorimetric Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA). ATP level was analyzed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and LDH release was measured with CytoTox One™ (Promega).

Cells were seeded at a density of 56,000 cells per well in transparent 96-well plates (Costar® Corning, USA) for MTS and white-walled 96-well plates with transparent bottom for ATPase assay. The cells were exposed to the concentrated water samples and incubated for 24 h. At experiment termination the cytotoxicity was evaluated in accordance with the manufacturer's instructions for each assay. Absorbance for MTS was measured on a Wallac Victor 1420 microplate reader (PerkinElmer, USA). The ATP amount and LDH release was measured using an Infinite M1000 plate reader (Tecan).

A decrease in viability of $\geq 20\%$ compared to vehicle control (0.8 fold change) for the MTS and ATP, and a LDH release of $\geq 40\%$ (0.4 fold change) of the positive control for the LDH assay, was considered as cytotoxic effects.

Cell-based evaluation of VDR antagonism

The VDR antagonist reporter assay was performed by harvesting the VDR-UAS-bla HEK 293 T cells from routine cultures of 70–90% confluency. Cells were seeded into 384-well black walled, clear-bottom plates at a density of 20,000 per well and treated with 0.05 nM 1 α , 25-dihydroxyvitamin D₃, corresponding to a VDR activating effect of approximately 80% of the assay maximum effect. The cells were then exposed to water samples or vehicle controls. Cell-free control wells, unstimulated control wells and stimulated control wells were included for each plate. Vehicle controls (ethanol) and 1 α , 25-dihydroxyvitamin D₃ stimulated controls were tested in 8 replicates, while water samples were analyzed in 4 replicates. After 24 h of exposure, reporter activity was measured by adding LiveBLAzer™-FRET B/G substrate mixture (Invitrogen). Fluorescence was measured in the blue channel with excitation filter 409/20, emission filter 460/40 nm and in the green channel with excitation filter 409/20, emission filter 530/30 nm, using an Infinite M1000 plate reader (Tecan). The cell-free control wells were used for background subtraction. The average blue background was subtracted from all blue emission data and average green background from all green emission data. The

blue/green emission ratio for each well was calculated and normalized with vehicle control.

Effects of natural organic matter and cadmium on VDR antagonism

To test if presence of natural organic matter (NOM) in the water samples could cause a false positive response in the VDR antagonist assay, e.g., by binding the 1α , 25-dihydroxyvitamin D_3 , two mixtures of NOM were tested in this assay. Nordic Aquatic Fulvic Acid (NA-FA) (1R105F) and Nordic Reservoir Natural Organic Matter (NR-NOM) (1R108N) were purchased from the International Humic Substance Society (St Paul, USA). The powder was stored in darkness at room temperature. Stocks were prepared in a concentration of 60 g L^{-1} in 0.05 M sodium hydroxide (NaOH) and stored in darkness at $4 \text{ }^\circ\text{C}$. The VDR antagonistic activity was assayed as described in Sect. "Cell based evaluation of VDR antagonism", in cells treated with a combination of 0.05 nM 1α , 25-dihydroxyvitamin D_3 and 20–200 mg L^{-1} of NA-FA or NR-NOM, respectively. Furthermore, cadmium acetate dihydrate (Fluka, Sigma-Aldrich) was tested for VDR antagonistic effects. Cadmium acetate dihydrate was dissolved in water and tested for VDR antagonism in cells co-exposed to 0.05 nM 1α , 25-dihydroxyvitamin D_3 and cadmium (60 or 190 nM).

Data analysis

Concentrated water samples were analyzed in a range of REF values from 50 to 6.25 (for cytotoxicity) or 3.125 (for VDR antagonistic effects). The limit of detection (LOD) for VDR antagonistic effects was defined as one minus three times the standard deviation of the negative control. The LOD was calculated to 0.85. The cut-off for antagonistic effect was set at 0.80. EC_{80} values, representing the concentration needed to decrease the VDR activity to 0.80 fold change as compared to the 1α , 25-dihydroxyvitamin D_3 stimulated control, were calculated using GraphPad Prism 7. Linear regression analysis was performed in GraphPad Prism 7.

Results

Evaluation of cytotoxicity

Cell viability and cytotoxicity following exposure to the concentrated water samples was evaluated with three different methods, to ensure the correct interpretation of the VDR activity. Using the MTS test to measure cell viability, no sample showed cytotoxicity in the REF range studied (Fig. 1). In the LDH release assay, where cytotoxicity is measured as an increase in the LDH release, cytotoxic effects were observed at REF 50 for most of the influent water samples (WW1, WW5, WW9) and at all studied REFs in one influent sample (WW7) (Fig. 2). For

the effluent samples, no cytotoxicity was observed with the LDH assay. The ATP assay was the most sensitive method of the three, and cytotoxicity was observed for all influent water samples at all REFs evaluated (WW1, WW3, WW5, WW7, WW9) (Fig. 3). For the effluent samples, cytotoxicity was observed at REF 50 for most samples (WW2, WW4, WW8, WW10), but most samples at $REF \leq 25$ were found to be non-cytotoxic or of borderline cytotoxicity.

Evaluation of VDR antagonistic activity

To assay VDR antagonistic activity, cells were co-exposed with concentrated water sample and a concentration of 1α , 25-dihydroxyvitamin D_3 that activated the receptor activity to 80% of the maximum assay response. An antagonistic response can then be observed as a decreased reporter gene activity as compared to the 1α , 25-dihydroxyvitamin D_3 -treated control. The results are presented in Fig. 4.

For the influent water samples, we observed a dose-dependent decrease in the 1α , 25-dihydroxyvitamin D_3 -induced VDR activity for four out of five samples (WW1, WW3, WW5, WW9). For WW7, the results were ambiguous. For these influent water samples, it should, however, be noted that we observed cytotoxic effects in the ATP assay for all studied REF values, meaning that the observed antagonistic effects should be interpreted with care.

For the effluent water samples, we observed a dose-dependent decrease in the 1α , 25-dihydroxyvitamin D_3 -induced VDR activity for all five samples. The EC_{80} values (Table 2) were in the range of REF 6–20. Cytotoxicity was observed only at REF 50 for four effluent samples, but not at lower concentrations. Hence, we were able to detect a decrease in the VDR signaling after exposure to non-cytotoxic concentrations of effluent water samples from wastewater treatment plants.

A blank sample, consisting of Millipore water concentrated in the SPE system, was analyzed at REF 50. The blank sample did not show bioactivity (VDR activity of 0.89 ± 0.03 as compared to vehicle control, cutoff for activity 0.8), showing that the observed VDR antagonism in the water samples is not caused by release of compounds from the SPE system.

Impact of natural organic matter and cadmium on VDR antagonistic activity

It has been reported that natural organic matter in environmental water samples can cause false positives in cell-based in vitro assays, especially when measuring antagonistic responses [23, 24, 29, 30]. This can, for example, happen if the ligand used to stimulate the assay, in this case 1α , 25-dihydroxyvitamin D_3 , is

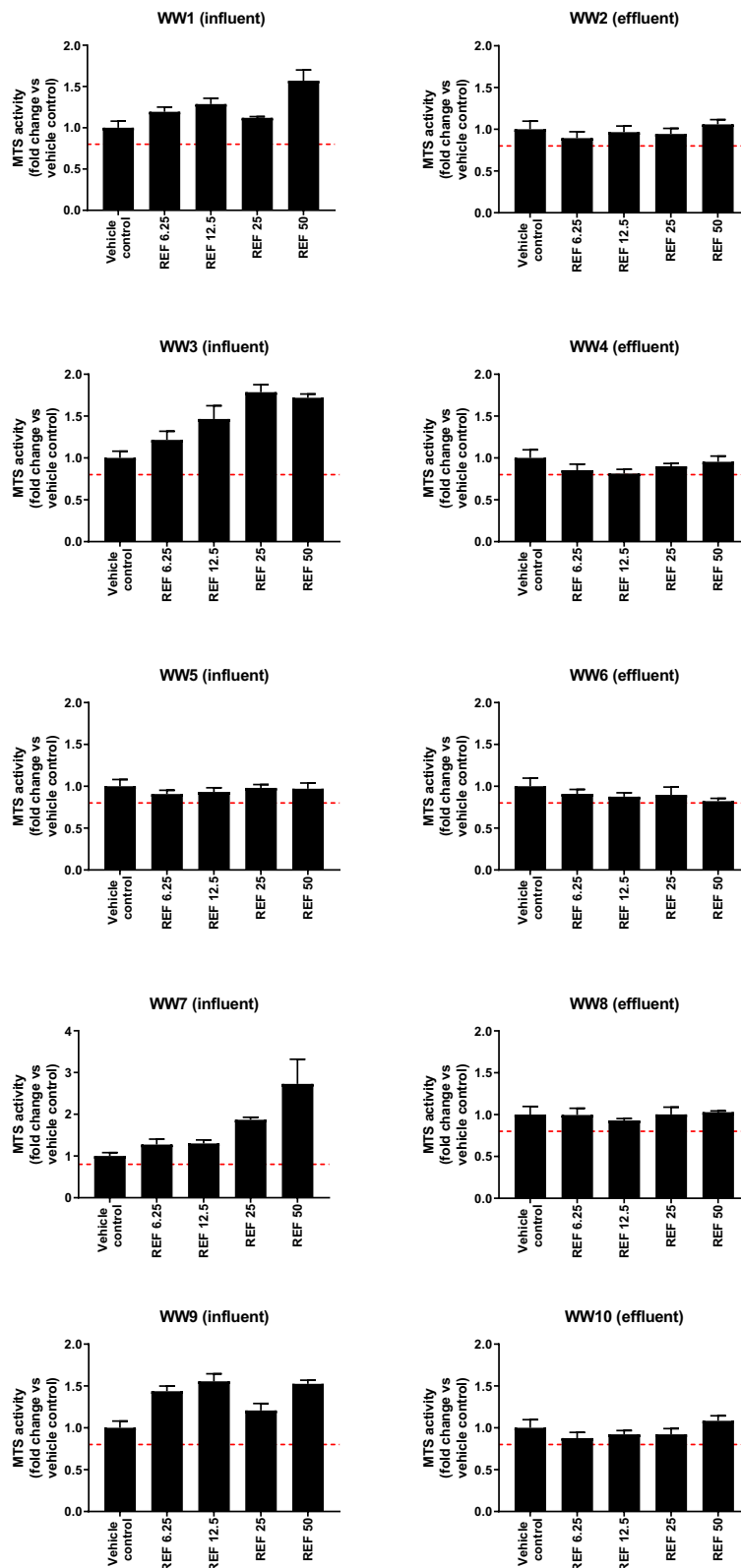


Fig. 1 Cytotoxicity of concentrated water samples as measured by MTS assay in VDR-UAS-bla HEK 293 T cells. Bars show mean \pm standard deviation ($n=4$). Cytotoxicity was defined as a decrease in cell viability of $>20\%$ as compared to the vehicle control (cutoff for cytotoxicity shown as dotted line)

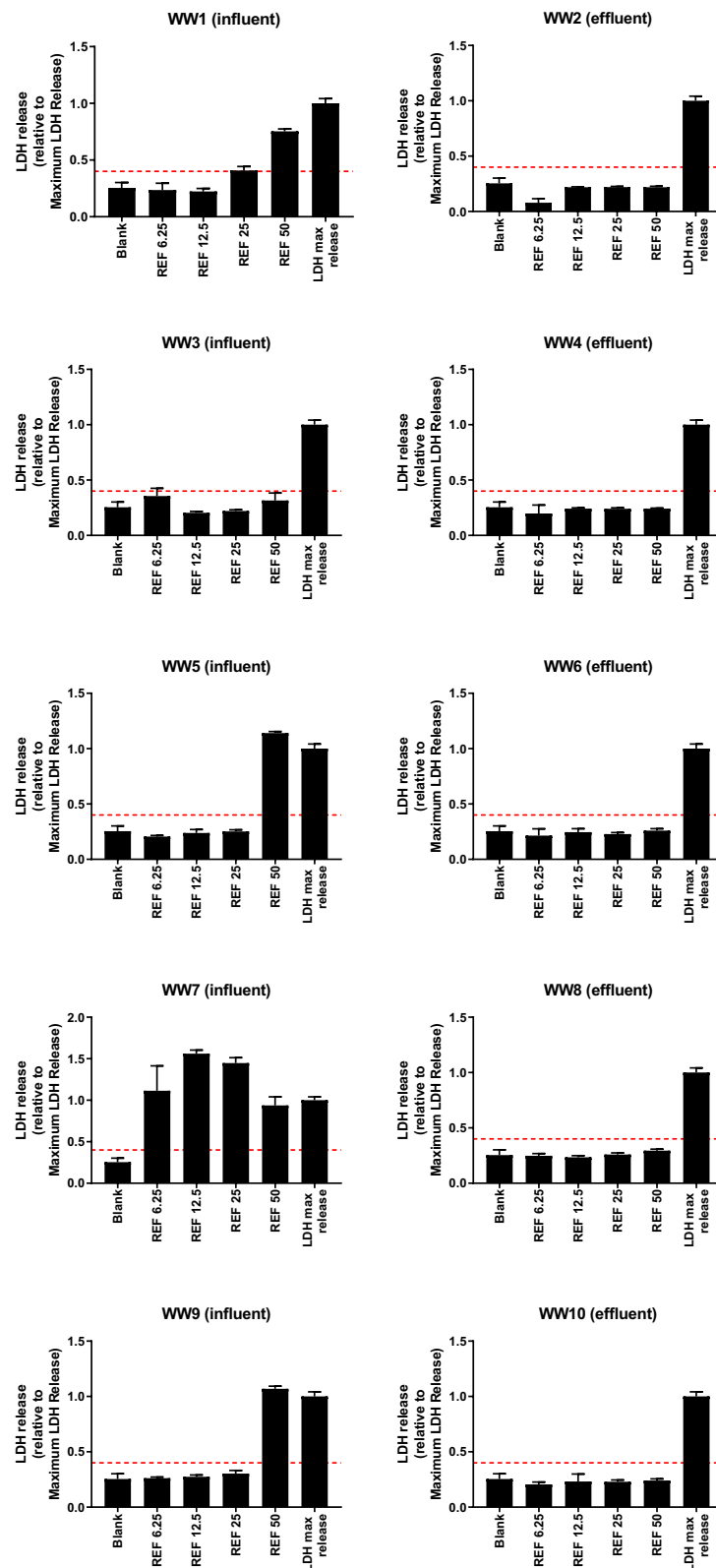


Fig. 2 Cytotoxicity of concentrated water samples as measured by LDH assay in VDR-UAS-bla HEK 293 T cells. Bars show mean \pm standard deviation ($n = 4$). Cytotoxicity was defined as an increase in LDH level of $\geq 40\%$ of the positive control (cutoff for cytotoxicity shown as red dotted line)

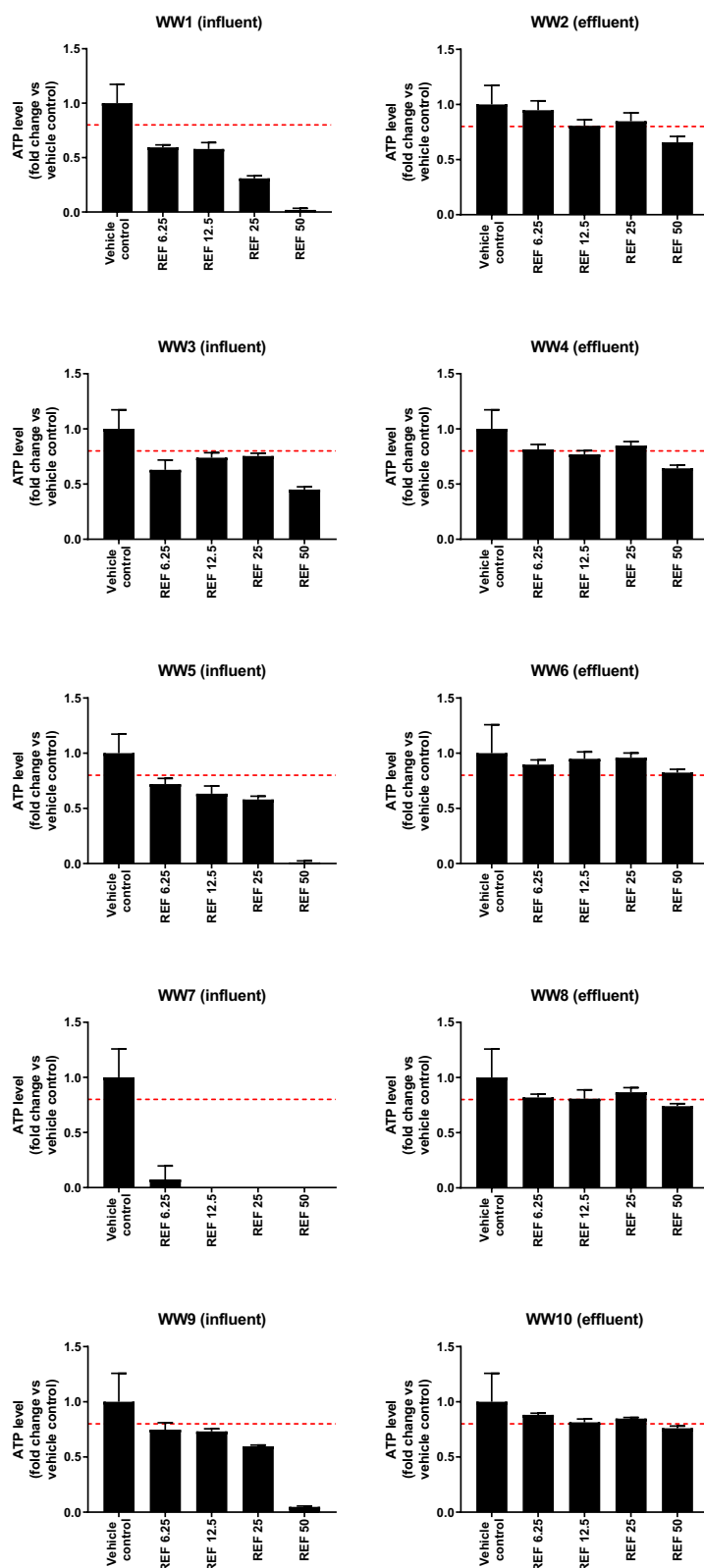


Fig. 3 Cytotoxicity of concentrated water samples as measured by ATP assay in VDR-UAS-bla HEK 293 T cells. Bars show mean \pm standard deviation ($n=4$). Cytotoxicity was defined as a decrease in cell viability of $>20\%$ as compared to the vehicle control (cutoff for cytotoxicity shown as red dotted line)

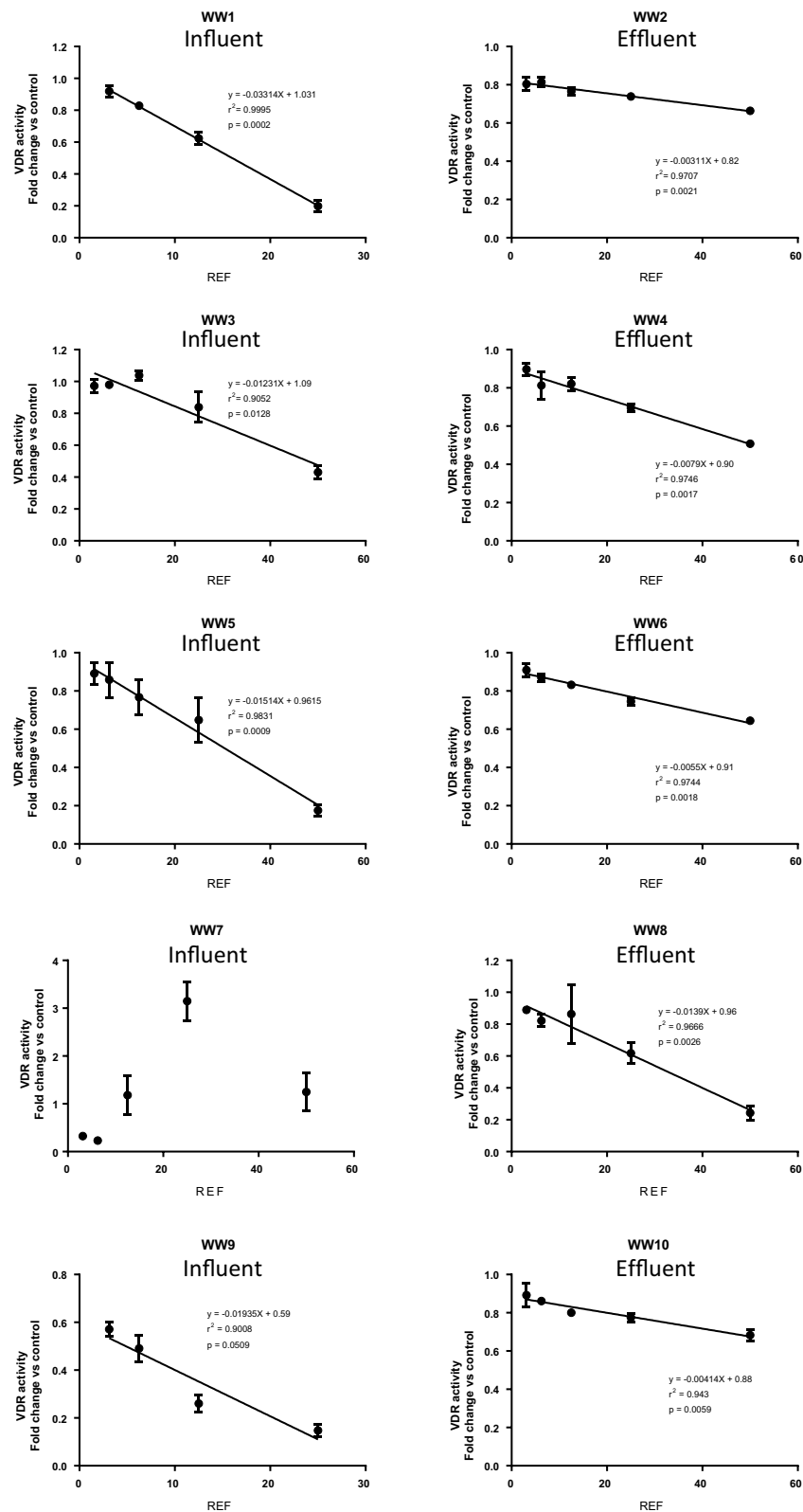


Fig. 4 Dose response of VDR antagonistic effects measured in VDR-UAS-bla HEK 293 T cells. Cells were treated with 0.05 nM $1\alpha, 25$ -dihydroxyvitamin D_3 before addition of the concentrated water samples, to activate the VDR signaling. Linear regression analysis was performed in GraphPad Prism 7. Data is presented as mean \pm standard deviation ($n = 4$)

Table 2 Effect-concentration values for VDR antagonistic effects in effluent samples

Sample	EC ₈₀ (REF)
WW2	6
WW4	13
WW6	19
WW8	11
WW10	20

deactivated by sorption to NOM. To ensure that the VDR antagonistic effects observed in this study is not due to binding of $1\alpha, 25$ -dihydroxyvitamin D₃ to NOM, we tested two mixtures of NOM in the VDR antagonistic assay. We found (Fig. 5A) that neither the Nordic Aquatic Fulvic Acid mixture (NA-FA) nor the Nordic Reservoir Natural Organic Matter (NR-NOM) mixture caused VDR antagonistic effects in this assay.

Wastewater is often contaminated with cadmium, which is proposed to have VDR antagonistic properties [7]. To investigate if cadmium could cause the observed VDR antagonistic effects, we tested cadmium acetate dehydrate for VDR antagonism. We found that cadmium did not interfere with $1\alpha, 25$ -dihydroxyvitamin D₃-induced VDR activity in the studied concentrations (Fig. 5C).

Discussion

$1\alpha, 25$ -dihydroxyvitamin D₃ is de novo synthesized in humans and regulates a broad range of physiological processes, and VDR signaling is thereby part of the endocrine system. The research field of EDCs has to a large extent been focused on effects on the estrogen, androgen, thyroid and glucocorticoid receptors. Effects of environmental pollutants on the vitamin D signaling system have been proposed, but has mainly been focusing on epidemiological studies and animal studies investigating the links between exposure to environmental pollutants and serum levels of 25-hydroxyvitamin D₃

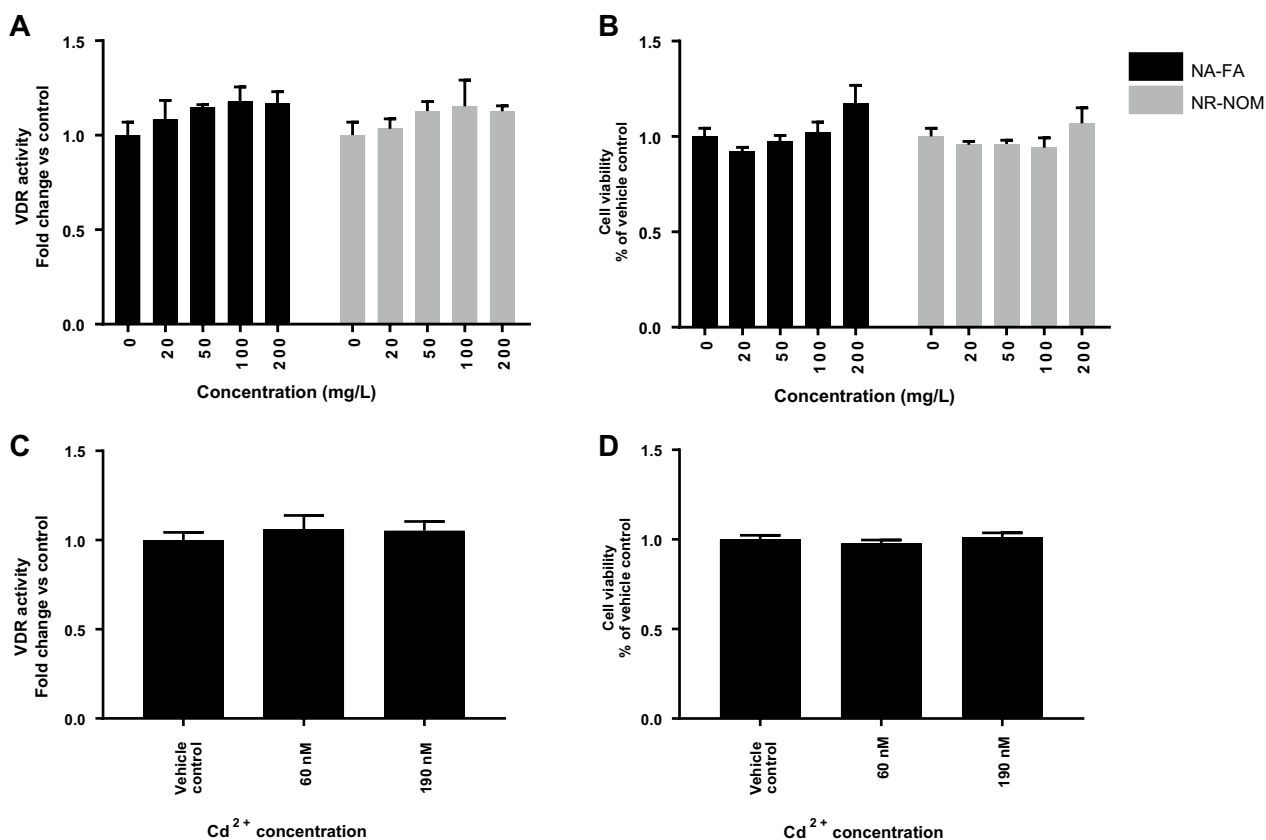


Fig. 5 VDR antagonistic effects of NOM and cadmium, measured in VDR-UAS-bla HEK 293 T cells. Cells were treated with 0.05 nM $1\alpha, 25$ -dihydroxyvitamin D₃ before addition of 20–200 mg/L of NOM mixtures Nordic Aquatic Fulvic Acid mixture (NA-FA) and the Nordic Reservoir Natural Organic Matter (NR-NOM) (A) or 60 and 190 nM cadmium acetate (C). Cell viability was monitored by MTS test (B, D). Data is presented as mean \pm standard deviation ($n=4$)

and 1α , 25-dihydroxyvitamin D_3 [22]. Experiments in rats have shown that exposure to cadmium and lead can decrease serum level of both 25-hydroxyvitamin D_3 and 1α , 25-dihydroxyvitamin D_3 [5, 28, 32, 36] and exposure of rats to uranium altered the 1α , 25-dihydroxyvitamin D_3 plasma level as well as the expression of VDR and a number of CYP enzymes involved in biosynthesis and metabolism of vitamin D [34, 35]. Exposure to polychlorinated biphenyls (PCBs) have been shown to decrease the mRNA level of VDR in zebrafish [15] and reduced serum levels of 25-hydroxyvitamin D_3 and 1α , 25-dihydroxyvitamin D_3 in rats [17]. Rats exposed to a high dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been reported to have decreased levels of 25-hydroxyvitamin D_3 [13].

The US EPA ToxCast program [7] and a follow-up study [19] has identified a number of compounds with the potential to interact with VDR in cell-based models. For example, Di Nisio et al. [6] reported inhibition of vitamin D-induced expression of genes in two cell lines after treatment with 400 ng/ml of perfluoro-octanoic acid (PFOA). However, to date and to the best of our knowledge, there has been no reports investigating the actual presence of VDR antagonistic activity in environmental samples.

The potential effects of a disruption of the vitamin D signaling pathway has been discussed in an OECD report, reviewing the state of the science for endocrine disruptors [26]. The report concludes that a disruption of the vitamin D signaling pathway could be associated with severe health consequences, but the analysis is mainly based on animal experiments where VDR has been knocked out. VDR knock-out experiments have shown that a disruption of the vitamin D signaling pathway could be associated with hypocalcemia, hyperparathyroidism, decreased bone mineralization and impaired bone health. Furthermore, VDR knock-out animals has been shown to have impaired neural development and cognition as well as dysfunctions in the immune system. It is reasonable to think that a disruption of the VDR signaling by environmental pollutants could cause similar physiological effects as a VDR knock-out, although less severe if the VDR antagonism is only partial. In a review by Manibusan and Touart [20], it is concluded that interference with the vitamin D pathway may disrupt developmental-, growth-, and reproduction-regulated processes. A study have reported that vitamin D deficiency disrupted learning and brain connectivity in mice [2] and the effects of vitamin D deficiency on brain development and function has been reviewed by Mayne and Burne [21].

In this study, we report vitamin D receptor antagonistic activity in environmental matrices, indicating the

presence of compounds that can disrupt the vitamin D signaling pathway by acting as VDR antagonists. This is a novel finding that constitutes a new class of endocrine disrupting effects in the environment. Previous studies have investigated the presence of vitamin D receptor agonists in the environment, but has been unable to detect any such compounds [1, 3, 9].

It has been reported that natural organic matter (NOM), such as humic and fulvic acids, can cause false positive results when assessing antagonism in vitro [23, 24, 29, 30] by sorption of the stimulating hormone (in this case 1α , 25-dihydroxyvitamin D_3). It has also been reported that NOM can be co-extracted when samples are concentrated by SPE [25]. To investigate if NOM could cause false positive results when assessing VDR antagonism in vitro, we tested two NOM mixtures in the VDR antagonism assay and found that neither Nordic Aquatic Fulvic Acid mixture (NA-FA) nor the Nordic Reservoir Natural Organic Matter (NR-NOM), previously demonstrated to inhibit androgen receptor activity [29, 30], interfered with the 1α , 25-dihydroxyvitamin D_3 -induced VDR activity. This lends support to the conclusion that the VDR antagonistic activities observed in wastewater samples are caused by presence of VDR antagonists in the sample and not due to 1α , 25-dihydroxyvitamin D_3 -binding compounds.

Contamination of wastewater with cadmium is a known problem. It has been reported in the ToxCast database [7] that cadmium can interfere with VDR signaling. In general, metals are not expected to remain in a sample concentrated with SPE. However, it has been reported that cadmium can bind to humic and fulvic acids [14], which can retain in the sample after SPE concentration, at least to some extent [25]. All the wastewater treatment plants included in this study monitor cadmium concentration in the effluent water, as part of their normal quality control, and report annual mean cadmium concentrations of 0.25–1 nM or lower (personal communication). We tested if cadmium could interfere with 1α , 25-dihydroxyvitamin D_3 -induced VDR signaling in concentrations of 60 and 190 nM. The concentration of 60 nM corresponds to the concentration range of cadmium expected to be found at REF 50 if 100% of the cadmium in a water sample with a concentration of around 1 nM would retain after the SPE extraction. Cadmium was unable to interfere with the VDR signaling at 60 nM and also at the three times higher concentration of 190 nM. We conclude from this experiment, that the VDR antagonistic effects that we observe in wastewater effluent samples in this study is not the result of potential cadmium contamination of the samples.

The high-throughput screening project ToxCast [7] has tested 7521 compounds for VDR antagonism and

classified 567 compounds as active for this bioactivity. It should, however, be noted that many of these compounds interfere with VDR with relatively high EC_{50} values (30–100 μM) and the effect is often overlapping with cytotoxicity. Mahapatra et al. [19] have performed a study where putative VDR antagonists identified in a high-throughput screening were tested for reproducibility in a low throughput system. The authors were able to identify 19 active VDR antagonists with this approach, including both metal containing compounds and non-metal containing compounds. The most potent VDR antagonists identified by Mahapatra et al. were triphenyltin hydroxide, potassium dicyanoaurate, phenylarsine oxide, chlorambucil, all with EC_{50} values of less than 0.1 μM .

We observed an antagonistic effect towards VDR signaling in both influent and effluent water samples from five wastewater treatment plants. However, the antagonistic effects caused by the influent water samples coincided with cytotoxicity, which can cause false positive results when measuring an antagonistic effect. It should be noted that the three methods used to monitor cytotoxicity showed a large degree of disparity or difference in sensitivity in the detected cytotoxicity. For example, all influent samples were considered cytotoxic in all studied concentrations when monitored with the ATP level assay, while no influent samples were considered cytotoxic when monitored with the MTS assay. The results for the influent samples should, therefore, be interpreted with care. For the effluent samples, we observed antagonistic effects towards the VDR also in concentrations of water samples that were far below the concentrations where we observed cytotoxicity by any of the three cytotoxicity assays.

Environmental VDR antagonists cause concern both from a human health perspective, e.g., exposure via drinking water, and for effects on other vertebrates, since all vertebrates possess the vitamin D endocrine axis. It should be noted that most of the wastewater treatment plants included in this study discharge the effluent water into Lake Mälaren, which is the source for drinking water production for approximately 2 million people in the Stockholm area. Current drinking water treatment processes are generally not designed to target organic micropollutants in the water and we have in a previous study shown that bioactive compounds, activating other toxicity pathways, present in the inlet water to a drinking water treatment plant are also present in the treated drinking water that is distributed for consumption [27, 29, 30, 37]. Hence, the risk of exposure via drinking water is not insignificant. With an average consumption of drinking water of 2–2.5 L per person and day [8], even very low levels of contaminants in drinking water

can be a health risk due to the large intake and life-long exposure.

The findings presented in this study are novel, causes concern for both human health and aquatic toxicity, and prompts further research in the field of environmental vitamin D signaling pathway disruptors. Future research is needed to investigate the presence in such compounds in other water systems and in other matrices (such as food, soil, sediment), identification of the causative compound(s) by an effect-directed analysis approach and, when the causative compound(s) have been identified, in-depth studies on the exposure to these compounds and the physiological consequences of such exposure.

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Author contributions

J.L. planned the study. G.M. performed all laboratory work. J.L. performed all data analysis. J.L. and A.O. evaluated the data. J.L. wrote the manuscript. A.O. and G.M. provided critical feedback and helped shape the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets generated in this study are in major parts included in this publication. Any additional data is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no actual or potential competing financial interests.

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