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Examination of the endocrine-disrupting properties of "active chlorine generated from seawater by electrolysis" in response to the European Biocidal Products Regulation: current knowledge and methodological challenges

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Abstract

Background Currently, active chlorine is considered the most effective treatment for preventing biofouling of structures in contact with seawater. This compound falls under the scope of the EU Biocidal Products Regulation, which includes since 2018 a requirement to assess all active ingredients for their potential endocrine-disrupting properties on humans and non-target organisms. Therefore, this study examines the endocrine-disrupting (ED) potential of active chlorine based on the European Chemicals Agency and European Food Safety Authority guidance (ED TGD). It includes two approaches: (i) a systematic literature review using appropriate search terms and (ii) an in silico assessment, both supported by expert judgement. Finally, the feasibility and relevance of in vitro tests were examined by considering the stability of chlorine and the applicability domain of the recommended in vitro assays.

Results No significant adversity or endocrine activity based on EATS (estrogen, androgen, thyroid, and steroidogenesis)-modalities were evidenced based on the literature data. However, these modalities remain understudied and further datasets are needed for a comprehensive assessment. The in silico approach revealed a low probability of binding between active chlorine and a set of 14 human nuclear receptors, for both agonist and antagonist effects. This is not surprising given the great structural difference between active chlorine and natural ligands. The in vitro investigation of the ED potential of active chlorine raises several operational limits, including: (i) its instability ($t_{1/2} < 48$ h) which is incompatible with a reasonable time window between collection and ex situ analysis; (ii) its rapid and complete reaction with several essential nutrients in cell culture media; (iii) its documented cytotoxicity on various cell lines; and (iv) its exclusion from the scope of certain OECD guidelines.

Conclusions Overall, neither the in silico evaluation nor the systematic literature review performed indicates a significant adversity based on EATS-mediated parameters or EATS-related endocrine activities. This study highlights

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the challenges of performing a comprehensive ED assessment for a data-poor chemical and questions the relevance of transposing generic methodologies to the case of unstable and inorganic molecules.

Keywords Active chlorine, Biocidal products regulation, Endocrine disruptors, EFSA/ECHA TGD

Introduction

The Electricité de France (EDF) group operates four nuclear power plants (NPPs) located along the French coast, facing the English Channel: Flamanville, Paluel, Penly and Gravelines (see location in Additional file 1: Fig. S1). These NPPs use seawater as a coolant (in a once-through mode) to extract heat from their condensers and other auxiliary heat exchangers. Inevitably, many sedentary marine organisms (e.g., barnacles, mussels, algae and microbial slimes), upon being entrained in the cooling circuits, may settle in hospitable areas of these circuits and grow. This natural phenomenon, known as biological fouling or biofouling, affects a wide range of maritime industries, causing significant economic and safety repercussions [1–3]. In the case of coastal power plants, for example, biofouling reduces heat transfer efficiency, restricts water movement, increases corrosion–erosion rates and under extreme circumstances causes an "unplanned" shutdown of the installations for cleaning operations [4–8]. Mussels appear to be the most problematic fouling organisms in coastal power plant cooling systems around the world [9–12].

Different physicochemical methods have been examined to mitigate the deleterious effects of biofouling [7, 13, 14]. Among these techniques, chlorination is by far the most widely used chemical process due to its efficacy against micro- and macro-organisms, ease of application, reasonable cost and current environmental acceptability [7]. It is currently considered the best available technique in industrial cooling water systems [15]. EDF has adopted this approved procedure to prevent and control biofouling in its NPPs previously mentioned. The treatment implemented consists of a continuous or intermittent injection of low doses of chlorine (< 1 mg Cl_2 L^{-1}) as soon as water temperature approaches 10 °C. The chlorine used is produced on-site by electrolysis of seawater, considering the large quantities required and the hazards associated with the transport and storage of large quantities of this chemical. Electrolysis parameters are adjusted to produce nominal concentrations ranging from 0.5 to 2.0 g Cl_2 L^{-1} [16]. The chlorine solutions produced feed so-called "buffer" tanks before being distributed to the cooling circuits. The residence time in these tanks is a few minutes. Given that the pH of these solutions ranges from 8.3 to 9.7 and the pKa of hypochlorous acid (HOCl) is 7.5 at 25 °C, the chlorine generated is a mixture of approximately 20% HOCl and 80% hypochlorite anion ($^{-}$OCl) [17]. Typically, chlorination is practiced with applied doses of 0.5–1.5 mg Cl_2 L^{-1} and residual oxidant levels of 0.1 ± 0.2 mg Cl_2 L^{-1} in the cooling water [16].

Chlorine generated in EDF's NPPs falls under the scope of the Biocidal Products Regulation (BPR), which sets the rules for the approval and use of biocidal products on the European Union (EU) market [18]. The European Commission (EC) has classified biocidal products into 22 biocidal Product-Types (PT) grouped into four main areas of use: disinfectants, preservatives, pest control products and other biocidal products. Chlorine produced in EDF's NPPs was referenced by its common name of "active chlorine generated from seawater by electrolysis" and recorded in group 2 under PT 11 ("preservatives" for "liquid-cooling and processing systems").

Under BPR, biocidal products cannot be placed on the EU market or used without prior approval of the active substance(s) that they contain [18]. Approval is based on the evaluation of several intrinsic properties of the active substance with regard to safety data. These data, which must be generated by the manufacturers, are reviewed by one of the competent public authorities of the EU Member States. In the case of EDF, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) is the competent agency in charge of this examination. Since June 2018, biocides must also be evaluated for their potential endocrine-disrupting (ED) properties in accordance with the scientific criteria set out in the Commission Delegated Regulation (EU) No. 2017/2100 [19]. A Technical Guidance Document (TGD), developed jointly by the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA), hereinafter referred to as "ED TGD", provides a structured approach to carry out this assessment [20].

The purpose of this study was to review the endocrine-disrupting potential of active chlorine based on the process described in the ED TGD. It includes two approaches: (i) a systematic literature review using appropriate search terms and (ii) an in silico assessment, supported by expert judgement. Finally, the experimental feasibility and relevance of in vitro tests (level 2, ED TGD) were examined regarding the stability of chlorine and the applicability domain of the recommended in vitro assays (e.g., OECD TG 455, 456 and 458) [21–23].

Material and methods

Literature search approach

A review of the scientific literature on active chlorine was carried out according to the methodological instructions of the ED TGD. Recently, several studies have shown the application of this methodology to organic substances in the context of "test cases" [24–26]. Although the ED criteria may cover all endocrine-disrupting modes of action (MoAs), the ED TGD focused more on the effects related to Estrogen, Androgen, Thyroid, and Steroidogenic (EATS) modalities [20]. Indeed, these modalities are currently the best understood and are assessed by several standardized in vivo and in vitro tests with a broad scientific consensus. The global process follows five steps illustrated schematically in Additional file 1: Fig. S2 and presented briefly below:

1. Data collection: In this first step, all available scientific information published up to April 2023 was collected. This includes data generated using internationally agreed protocols as well as scientific data from the literature, databases, (Q)SAR, and read-across models selected using a systematic review methodology. The search was conducted considering three forms of active chlorine (^{-}OCl, HOCl, NaOCl) using IUPAC names and CAS numbers as search terms, combined using the Boolean operator "OR". The electronic databases and search terms used are summarized in Additional file 1: Table S1. After removing duplicates, studies in languages other than English, those containing no original data (e.g., literature reviews), and those for which the full text is not available were excluded. Subsequently, an initial selection of potentially relevant studies was performed by a rapid assessment based on the titles and/or abstracts. Those mentioning any potentially endocrine-related effect, activity or adversity, or which could not be considered as irrelevant after abstract analysis were retained for further analysis of their full text. The relevance of the data is assessed by examining the relevance of the experimental design (e.g., animal model, exposure, examinations, etc.). The studies deemed relevant were then assessed for their reliability according to the criteria proposed by Klimisch et al. [27] or based on expert judgment by considering the inherent quality of the publication/report and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings. Figure 1 summarizes schematically the overall process and its results. All studies included in the final dossier were coded with ID numbers.
2. Evidence assessment: In a second step, the information collected was assembled into lines of evidence for both endocrine activity and adversity. Parameters were grouped based on whether they were measured in vitro or in vivo and reflecting the fact that, based on OECD GD 150, some effects are considered indic-

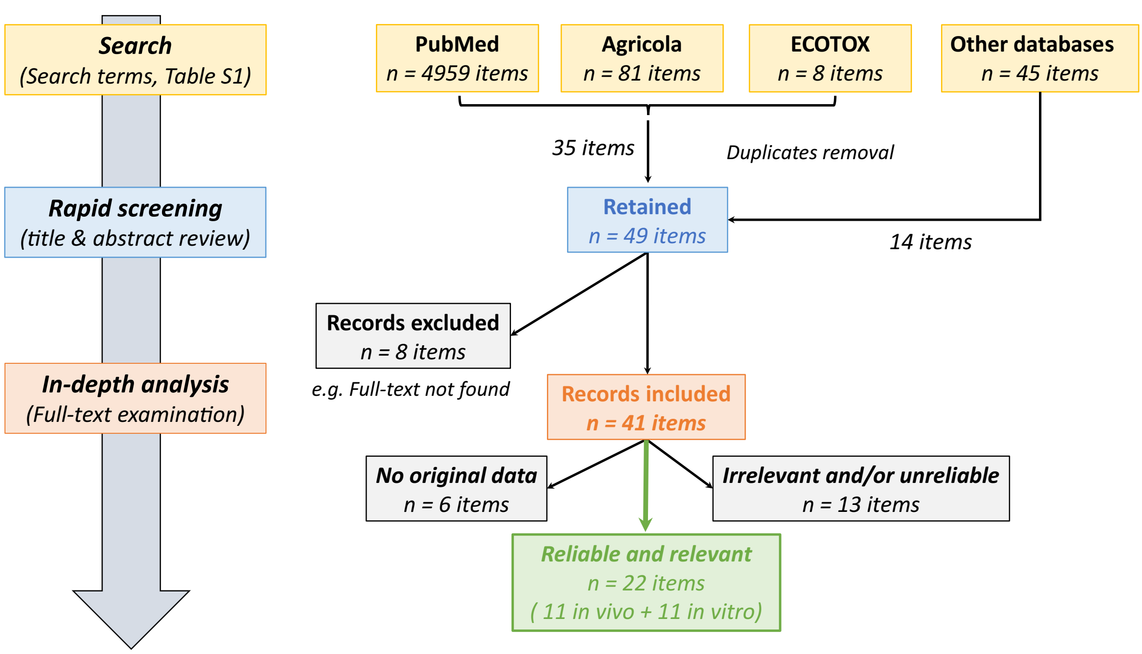

Fig. 1 Number of items remaining after each selection step

ative of an EATS mode of action (EATS-mediated), while others are considered to be potentially sensitive to, but not diagnostic of, EATS modalities.

3. Lines of evidence analysis: The third step was to evaluate if EATS-mediated adversity and endocrine activity have been sufficiently investigated. For this purpose, the results relating to all endpoints were grouped by modality to facilitate a weight-of-evidence analysis. ED TGD describes six different scenarios represented as a decision tree, based on the results of this step.
4. MoAs analysis: In the case of proven adversity and/or endocrine activity, a mode of action analysis should be conducted to establish if there is a biologically plausible link between endocrine activity and adverse effects.
5. Conclusion on the ED criteria: Finally, a general conclusion was given to determine if the endocrine disruptor criteria were met for the active chlorine, including remaining uncertainties or missing information, if any.

The searches and data analysis were performed by one reviewer. Uncertainties were resolved within a technical committee made up of colleagues with relevant and complementary expertise.

In silico molecular docking approach

To complete and support the data of the bibliographic approach, the endocrine-disrupting potential of active chlorine was evaluated in silico using a computational tool called "Endocrine Disruptome" developed by Kolšek et al. [28]. It is a user-friendly, open-source and web-based prediction tool (http://endocrinedisruptome.ki.si/) that uses the molecular docking approach to predict interactions between test substances and 14 distinct human nuclear receptors (NRs), including those of estrogens, androgens, and thyroid hormones. For four receptors (AR, ER_{α}, ER_{β}, and GR (glucocorticoid receptor)), both agonistic and antagonistic effects are predicted by this tool. More detailed information on this tool is provided in Kolšek et al. [28] and Vedani et al. [29]. After docking, the output results are the binding affinities of ligands (test substances) to nuclear receptors, presented as free energies (kcal mol^{-1}). This energy corresponds to the preferential position of the ligands within the targeted receptors. More negative energy values indicate a greater possibility for binding. The software presents the results in color code, divided into four probability binding classes, and based on three threshold values of sensitivity (SE): red ($SE < 0.25$), orange ($0.25 < SE < 0.50$), yellow ($0.5 < SE < 0.75$), and green ($SE > 0.75$) for high, moderately high, moderate, and low binding probability, respectively [27]. A compound with a high or moderately high receptor binding energy (red or orange colors) is considered a potential endocrine disruptor. Endocrine Disruptome has already been successfully employed to screen a wide range of chemicals for their endocrine-disrupting ability (e.g., pesticides, flame retardants, per- and polyfluoroalkyl substances, cosmetic ingredients, halogenated parabens, and plasticizers), helping to fill data gaps and prioritize chemicals for further experimentation [29–39]. The tool has been validated on small drug-like molecules. Chemicals with a molecular weight greater than 600 g mol^{-1}, those multi-charged and those containing boron are not covered by this tool [29]. It is therefore concluded that active chlorine falls within the applicability domain of the model.

Stability of active chlorine

The stability of active chlorine produced at EDF's NPPs has been assessed. The evolution of the chlorine concentration as a function of time was carried out by monitoring the residual concentration at several time points. Active chlorine samples were taken directly from the storage tanks downstream of the electrolysis cells of the Gravelines NPP where the expected nominal concentration is around 1.0 g Cl_2 L^{-1}. Additional file 1: Fig. S3 shows the sampling point. Collection was carried out in 250-mL amber borosilicate bottles that were closed with no headspace to avoid any loss of $HOCl/^{-}OCl$. Prior to sampling, all glassware and other routine equipment used were cleaned according to the procedure described in Kinani et al. [14] to ensure that no chlorine demand was present. Active chlorine decay was monitored by taking 0.1 mL samples periodically and measuring residual chlorine as described below. The first measurements were made on-site, immediately after sample collection to determine the initial concentration (C_0). Then, the samples were stored in the dark at 4 °C, first in a cooler filled with ice during sample transport and then in a laboratory refrigerator, to continue the measurement over time. Active chlorine measurements were carried out by the DPD (N,N-diethyl-p-phenylene-diamine) colorimetric method, following the procedure described in the standard method EN ISO 7393-2 [40]. The measurement was performed using DPD test kits (Hach # 1407028, Loveland, CO, USA) for free chlorine and a DR 1900 UV–visible spectrophotometer with a 2.5 cm path length quartz cell, set to method 80 (Hach, Loveland, CO, USA). The measured concentration represents free residual chlorine (mainly $HOCl + ^{-}OCl$). As active chlorine concentrations in NPP samples exceed the upper range of the instrument (2.00 mg Cl_2 L^{-1}), the samples were diluted 1000 times with ultrapure water (specific resistance, 18 MΩ cm^{-1} at 25 °C) produced by a PURELAB Chorus 1 water purification system (Veolia Water Technologies, Wissous, France). The protocol used consists of transferring 100 µL of sample solution into a 100-mL glass volumetric flask filled to the gauge line with water. Each analysis (dilution and measurement) was repeated three times, and the result is presented as the arithmetic mean.

Results

Literature search approach

Data collection

Results of the systematic literature review: As shown in Fig. 1, a total of 35 publications were retained out of 5048 references initially identified after evaluating titles and abstracts for their potential relevance. The full text of 7 of them was not available and one publication was in Japanese. Of the remaining 27 studies, 6 were literature reviews or reports not presenting original results. Detailed full-text examination of the other studies led to exclusion for 13 of them for irrelevance and/or unreliable (Additional file 1: Table S2). In addition to the studies selected by the search in the publication databases, 3 relevant studies were retrieved from the REACH registration dossier for sodium hypochlorite. Among the 11 included studies, summarized in Additional file 1: Table S3, five were reliable with restrictions and six were considered reliable without restrictions. Some studies outlined signs of systemic toxicity, including decreased body weight, decreased survival (in fish), target organ toxicity such as lymph node histopathology, as well as brain weight decrease or presence anomalies in embryos, which are sensitive to, but not diagnostic of EATS.

Results of the systematic review in databases of compiled data, (Q)SAR and read-across models: Databases of compiled data such as ToxCast, COSMOS DB and eChemPortal were scrutinized using the search terms specified in Additional file 1: Table S1. In ToxCast, the search for hypochlorous acid (CAS No. 7790-92-3) and hypochlorite ion (CAS No. 14380-61-1) did not yield any results, while in contrast 41 in vitro tests were found for sodium hypochlorite (CAS No. 7681-52-9). Eleven of these were found to be potentially relevant for assessing EATS modalities, including 4 for estrogen modality, 4 for androgen modality, 2 for thyroid modality and 1 for steroidogenesis modality. The OECD eChemPortal simultaneously queries multiple databases and retrieves data from reports prepared for government chemical review programs at national, regional, and international levels. Through a search on this database, 3 relevant studies were identified from REACH registration dossier for sodium hypochlorite, available on ECHA website (study ID numbers: 5, 8 and 9). No additional studies were included after searches in the COSMOS DB. In the OECD (Q)SAR Toolbox, the three forms of active chlorine (hypochlorous acid, hypochlorite ions or sodium hypochlorite) were predicted to be non-binders for the estrogen receptor. Searches in the ToxRefDB, EDKB, EADB, Danish (Q)SAR, and NURSA databases retrieved no results for any of the 3 chemical forms.

Presentation of global information: As requested in the ED TGD, all relevant and adequate information collected (such as mammalian, fish, amphibian, avian and in vitro assays) were reported in a summary table composed of 37 columns, detailing the type of study, the experimental conditions and the characterization of the effect observed. The table is filled according to the associated instructions; each row corresponding to one effect observed in one study. The information reported in the excel template was rearranged in the automatically built data matrix presented in Additional file 2: Table S4 (toxicity studies in mammals and wildlife) and in Additional file 1: Table S5 (ToxCast data). In this matrix, data are re-organized according to the species and the following information are extracted from the template: source, year, principle, and experimental conditions (species, life stage, doses, route of administration and exposure time). Moreover, studied and observed effects are grouped by endpoint (e.g., sensitive to, but not diagnostic of, EATS). For each study, observed effect(s) for each parameter (e.g., litter viability, fertility…) are summarized and represented in boxes of different colors according to the type of effect (e.g., no effect, decrease, increase, induction, etc.). Finally, for each endpoint, the number of studies reporting the presence of an effect or not is entered in Table 1.

Assessment of the evidence

A line of evidence is defined as a "set of relevant information of similar type grouped to assess a hypothesis" [20]. As requested in TGD ED and OECD TG 150, the relevant parameters for identification of the endocrine-disrupting potential of active chlorine were grouped into five distinct groups: (i) "in silico prediction", (ii) "in vitro mechanistic", (iii) "in vivo mechanistic", (iv) "EATS-mediated" and (v) "sensitive to, but not diagnostic of, EATS" [20, 41]. Lines of evidence for adversity and for endocrine activity were assembled and organized by each modality (Additional file 3: Table S6).

Overall, lines of evidence analysis did not conclude that active chlorine influences EATS-mediated parameters or parameters sensitive to, but not diagnostic of, EATS. Therefore, no adversity was evidenced. Likewise, no endocrine activity was evidenced, as no effect was observed on in vitro mechanistic parameters and no effect was predicted using in silico tools (Additional file 1: Tables S3, S5, Additional file 2: Table S4, Additional file 3: Table S6).

Table 1 Data matrix summarizing the collected information (toxicity studies in mammals and wildlife)

	In vitro mechanistic			*In vivo* mechanistic		EATS-mediated																				Sensitive to, but not diagnostic of, EATS															Target organ toxicity				Systemic toxicity			[Not in list]
	Androgen receptor	CYP19	Estrogen receptor	Thyroid receptor	T3 and T4 level	Age at Vaginal opening	Epididymis histopathology	Epididymis weight	Mammary gland histopathology (female)	Mammary gland histopathology (male)	Ovary histopathology	Ovary weight	Oviduct histopathology	Prostate histopathology (with seminal vesicles and coagulating glands)	Prostate weight	Seminal vesicles weight	Sperm morphology	Sperm motility	Sperm numbers	Testis histopathology	Testis weight	Thyroid histopathology	Uterus histopathology (with cervix)	Uterus weight (with cervix)	Vagina histopathology	Adrenals histopathology	Adrenals weight	Brain histopathology examination	Brain weight	Fertility (mammals)	Fetal development	Litter size	Litter viability	Litter/pup weight	Numbers of embryonic or foetal deaths and viable foetuses	Pituitary histopathology	Pituitary weight	Post implantation loss	Presence of anomalies (external, visceral, skeletal	Survival of embryos	Kidney histopathology	Liver histopathology	Liver weight	Lymph nodes histopathology	Body weight	Food consumption	Survival (fish)	Tumour incidence
Effect	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	1	2	8	0	1	0
No effect	4	1	4	2	2	1	3	1	6	6	7	4	1	7	1	1	1	1	1	7	4	7	7	1	2	7	4	1	2	1	1	1	1	1	1	7	2	1	1	1	1	1	5	0	6	5	0	3

EATS estrogen/androgen/thyroid/steroidogenesis; effect, number of studies reporting an effect on targeted parameter; no effect, number of studies in which no detectable effect was observed on the parameter studied; not in list, studies on parameters other than those presented in the colored boxes

Initial analysis of the evidence

This step involves assessing the sufficiency of the dataset for EATS-mediated adversity and EATS-related endocrine activity for humans, mammals and other non-target organisms. The goal is to check if there is enough evidence to reach a conclusion or if additional data are needed to support a conclusion. Since EAS- and T-mediated parameters require different data to be considered sufficiently investigated, they are discussed separately.

EATS-mediated adversity in humans and mammals: To consider an EAS-mediated adversity sufficiently investigated, it is necessary to include all EAS-mediated parameters investigated in either an extended one-generation reproductive toxicity study (OECD TG 443) or a two-generation reproductive toxicity study (OECD TG 416) [42, 43]. To best of our knowledge, neither of these two studies has been reported for active chlorine. However, several EAS-mediated parameters were covered in the studies retrieved from the systematic review, while others were not (information summarized in Additional file 1: Table S7). For T-mediated adversity to be considered sufficiently investigated, the ED TGD states that all thyroid parameters investigated in the OECD TG 407, 408, 409 (and/or the 1-year dog study, if available), 416 (or 443 if available) and 451-3 should been included [42–47]. Only some of the parameters were covered in the studies retrieved from the systematic review, although these were not carried out according to the requirements of the OECD TG. For example, the thyroid was histopathologically examined in several studies in adults, and one study measured thyroid hormone levels in the parent generation (without specifying which hormones) [48]. Concerning the evaluation of phytosanitary compounds, EFSA indicates that the absence of measurement of thyroid hormones may not constitute a criterion allowing the conclusion that the thyroid has not been sufficiently studied. However, the thyroid weight (P and F1 generations) and HDL/LDL (high-density *vs* low density lipoprotein) ratio were not measured, and histopathology of the thyroid in the F1 generation was not performed. Consequently, it was concluded that EATS-mediated adversity regarding humans and mammals has not been sufficiently studied for active chlorine.

EATS-mediated adversity with regard to other non-target organisms: As stated in the ED TGD, to consider EAS-mediated adversity as sufficiently investigated, it is necessary to measure the parameters investigated in the Medaka extended one-generation test (OECD TG 240) [49]. None of these EAS-mediated parameters has been investigated in the sole relevant study in fish retrieved from the systematic review [50]. To consider T-mediated adversity sufficiently studied, it is necessary to measure the parameters of the larval amphibian growth and development assay (LAGDA, OECD TG 241) or those of the amphibian metamorphosis assay (AMA, OECD TG 231) [51, 52]. Dang [53] concluded that performing the LAGDA in case of positive AMA results is not necessary given the similarity between the two tests in terms of endpoints, exposure, and sensitivity. None of these studies were performed and no relevant amphibian studies were retrieved in the systematic review. However, no effect on embryonic development (potentially interfered with by some thyroid-disrupting chemicals) in fish was observed in the study of Goodman et al. [50]. Therefore, according to the ED TGD, EATS-mediated adversity for non-target organisms other than mammals has not been sufficiently investigated for active chlorine.

EATS-related endocrine activity with regard to humans and mammals: As EATS-mediated adversity has not been sufficiently investigated for active chlorine, the ED TGD states that EATS-related endocrine activity should be further considered. This means that the results of the following in vitro and in vivo biological tests must be available: OECD TG 440, 441, 456, 407, 408, 409, 416 (or 443) and 451–3 and OPPTS 890.1200 [20, 42–47, 54–56]. However, except for some thyroid parameters detailed in the previous paragraph, data on most parameters are still lacking. Some ToxCast ER assays were performed for sodium hypochlorite, but the ToxCast ER bioactivity model (including 18 assays) is not available. It is therefore concluded that EATS-related endocrine activity is not sufficiently investigated for active chlorine.

EATS-related endocrine activity with regard to other non-target organisms: To consider the EAS-modalities sufficiently investigated, it is necessary to perform the fish short-term reproduction assay (OECD TG 229) or the 21-day fish assay (OECD TG 230) [57, 58]. Alternatively, other available data covering the mechanistic parameters investigated in these studies are acceptable. To consider the T-modality sufficiently investigated, it is necessary to include results of the amphibian metamorphosis assay (OECD TG 231) [53]. None of the studies retrieved from the systematic review report the use of the above-mentioned OECD tests. Consequently, EATS-related endocrine activity is considered insufficiently investigated.

Mode of action analysis

As no significant endocrine activity and no adversities were observed and/or identified, no mode of action could be defined.

Conclusion on the endocrine disruption criteria

Based on the available information collected from the scientific literature, databases and (Q)SAR models, neither significant adversity based on EATS-mediated parameters nor EATS-related endocrine activities have been evidenced for active chlorine. However, it appears that all EATS-mediated parameters or endocrine activity have not been sufficiently investigated for humans and mammals as well as non-target organisms other than mammals.

In silico molecular docking approach

To start the simulation, the active chlorine (HOCl and $^{-}$OCl) and sodium hypochlorite molecules were drawn in the software interface or introduced by means of their SMILES (Simplified Molecular-Input Line-Entry System) strings (OCl for HOCl, Cl[-O] for $^{-}$OCl and O(Cl)[Na] for NaOCl). The docking process took less than one minute to complete. The results obtained are presented in Table 2 as predicated binding affinities for each receptor, color-coded according to the binding probabilities presented above. Values represent predicted binding energies with individual NRs (kcal mol^{-1}). A more negative score means a greater possibility for binding. Applied threshold of binding free energies (in kcal mol^{-1}) for specific NRs is shown in Additional file 1: Table S8. A higher probability of binding represents a greater potential risk for interference with the endocrine system.

The predictions obtained using the Endocrine Disruptome tool are all colored green (values presented in italics in Table 2), indicating that active chlorine (HOCl and $^{-}$OCl) has a very low probability of binding to the 14 targeted nuclear receptors (NRs), either in their agonist or antagonist conformations. Similar results are observed for sodium hypochlorite. This suggests that active chlorine will not directly interact with NRs. This is not surprising given its low molecular weight, its chemical composition as well as the size of the ligand-binding pockets. Indeed, the active chlorine molecules does not share physicochemical and structural properties with known natural ligands, which are essential for the activation or inhibition of their targeted NRs. To illustrate this point, the binding of xenoestrogens to ER is discussed as an example. Numerous in vitro studies have shown that molecules acting as ER environmental ligands share certain structural characteristics with the endogenous ligand, 17β-estradiol (aromatic A ring and C3 phenolic group) [59–62]. In the study by Blair et al. [60] for example, 188 chemicals belonging to various chemical families were tested for their affinity with ER. They found that certain structural features, such as an overall ring structure, were important for ER binding. It has also been reported that the presence of a phenolic ring in chemicals is important for their binding to the estrogen receptor. The studies by Routledge and Sumpter [63] and Miller et al. [64] indicate that the size and degree of branching of the alkyl group, as well as its position relative to the hydroxyl group on the phenol ring, are also important features for estrogenic activity.

Methodological difficulties and challenges in data generation

One of the important issues when carrying out biological assays (whether in vitro or in vivo) is to ensure the "chemical integrity" of samples both before and during the tests. Performing tests on an unstable sample will inevitably lead to biased biological data. This is particularly important in situations where samples collected in the

Table 2 The binding affinity scores of hypochlorous acid (HOCl), hypochlorite anion ($^{-}$OCl) and sodium hypochlorite (NaOCl) with 18 human nuclear hormone receptor conformations (14 agonistic and 4 antagonistic conformations)

HOCl (SMILES: OCl)			
AR: – 2.1 (– 7.4)	*AR_{an}: – 2.1 (– 3.1)*		
ER(α): – 2.2 (– 8.2)	*ER(α)$_{an}$: – 2.2 (– 8.6)*	*ER(β): – 2.2 (– 8.0)*	*ER(β)$_{an}$: – 2.1 (– 8.3)*
GR: – 2.1 (– 7.3)	*GR_{an}: – 2.3 (– 8.5)*		
LXR(α): – 2.1 (– 9.8)	*LXR(β): – 2.1 (– 10.3)*		
MR: – 2.0 (– 6.8)			
PPAR(α): – 2.1 (– 8.9)	*PPAR(β): – 2.2 (– 9.6)*	*PPAR(γ): – 2.4 (8.9)*	
PR: – 1.1 (– 2.8)			
PXR(α): – 2.1 (– 10.0)			
TR(α): – 2.2 (7.2)	*TR(β): – 2.2 (– 7.8)*		
$^{-}$OCl (SMILES: Cl[O-])			
AR: – 2.0 (– 7.4)	*AR_{an}: – 1.7 (– 3.1)*		
ER(α): – 1.5 (– 8.2)	*ER(α)$_{an}$: – 1.5 (– 8.6)*	*ER(β): – 1.7 (– 8.0)*	*ER(β)$_{an}$: – 1.6 (– 8.3)*
GR: – 2.0 (– 7.3)	*GR_{an}: – 1.7 (– 8.5)*		
LXR(α): – 1.7 (– 9.8)	*LXR(β): – 1.6 (– 10.3)*		
MR: – 1.8 (– 6.8)			
PPAR(α): – 1.6 (– 8.9)	*PPAR(β): – 1.8 (– 9.6)*	*PPAR(γ): – 1.8 (8.9)*	
PR: – 0.9 (– 2.8)			
PXR(α): – 1.9 (– 10.0)			
TR(α): – 1.6 (7.2)	*TR(β): – 1.5 (– 7.8)*		
NaOCl (SMILES: O(Cl)[Na])			
AR: – 2.6 (– 7.4)	*AR_{an}: – 2.6 (– 3.1)*		
ER(α): – 2.5 (– 8.2)	*ER(α)$_{an}$: – 2.6 (– 8.6)*	*ER(β): – 2.4 (– 8.0)*	*ER(β)$_{an}$: – 2.5 (– 8.3)*
GR: – 2.8 (– 7.3)	*GR_{an}: – 2.6 (– 8.5)*		
LXR(α): – 2.4 (– 9.8)	*LXR(β): – 2.5 (– 10.3)*		
MR: – 2.5 (– 6.8)			
PPAR(α): – 2.4 (– 8.9)	*PPAR(β): – 2.5 (– 9.6)*	*PPAR(γ): – 2.6 (8.9)*	
PR: – 1.4 (– 2.8)			
PXR(α): – 2.6 (– 10.0)			
TR(α): – 2.6 (7.2)	*TR(β): – 2.7 (– 7.8)*		

For comparison purposes, the free energy values from which the ligand–receptor interaction can be considered moderately probable are provided in parentheses

AR, androgen receptor; ER (α,β), estrogen receptors; GR, glucocorticoid receptor; LXR (α,β), liver X receptors; MR, mineralocorticoid receptor; PPAR (α,β,γ), peroxisome proliferator-activated receptors; PR, progesterone receptor; RXR (α), retinoid X receptor; TR (α,β), thyroid receptors; an, antagonistic conformation

field (e.g., water treatment plants, distribution systems) cannot be analyzed immediately after collection. It is therefore essential that sample stability be demonstrated to ensure reliable and accurate results, adequately reflecting the case of a "freshly drawn" sample. Two notions of "analyte stability" can be distinguished: the first refers to the stability of analytes in their matrix from the time of sampling to the start of the bioassays, while the second relates to the stability of the analytes in the culture media during the exposure period. These two notions are developed and discussed in the following paragraphs.

Changes in active chlorine concentration over time

Given the logistical constraints of transporting samples from NPPs to experimental toxicology laboratories, it is impossible to analyze samples the day they are collected. A question therefore arises: how long can active chlorine samples from NPPs be stored before analysis? Numerous studies have been conducted to investigate active chlorine decay in the context of tap water treatment, in industrial cooling waters or in commercial bleach solutions [65–70]. To our knowledge, no studies to date have examined the stability of in situ electrogenerated chlorine.

Accordingly, a chlorine stability study was conducted in refrigerated amber glass bottles (at 4 °C). To this end, a total of 8 active chlorine samples were collected from the Gravelines NPP between November and December 2022 and analyzed according to the procedures specified above. Figure 2A presents the evolution of residual active chlorine concentrations in the 8 samples from the Gravelines NPP, and Fig. 2B displays their decrement ratios ((initial concentration—final concentration)/initial concentration). Each residual active chlorine value represents the arithmetic mean of three measurements.

Initial residual active chlorine concentrations are in the range of 1.06–1.19 g Cl_2 L^{-1}. According to Fig. 2A and B, all samples show a rapid decrease in active chlorine concentrations beginning almost instantaneously (i.e., without a lag phase) after sample collection. Up to 40% of the initial active chlorine was lost within the first 6 h and a consumption limit (concentrations reached a plateau) was achieved after approximately 10 h of storage time. Differences in the amount and rate of active chlorine consumption were observed among the tested samples. This difference in active chlorine decay rate may be related to the physicochemical composition of the input electrolyzed seawater and the pH of the samples. Hsu et al. [69, 70] carried out laboratory experiments to investigate the stability of active chlorine electrogenerated from deep ocean waters at concentrations much higher than those examined in the present study (0.6–9.0 g Cl_2 L^{-1}). The samples were stored in sealed brown bottles at room temperature. They found that up to 67% of the initial active chlorine was lost in the 3-week storage period. The change in the rate of decrease in active chlorine concentrations over time (Fig. 2) suggests that chlorine consumption in seawater occurs in two phases: an initial rapid decay phase, followed by a slow decay phase. This trend has been reported by several researchers and can be explained by differences in the types of reactions and the types of reactants involved. The disappearance of residual active chlorine may be due to two types of reactions: auto-decomposition and reactions with organic and inorganic substances.

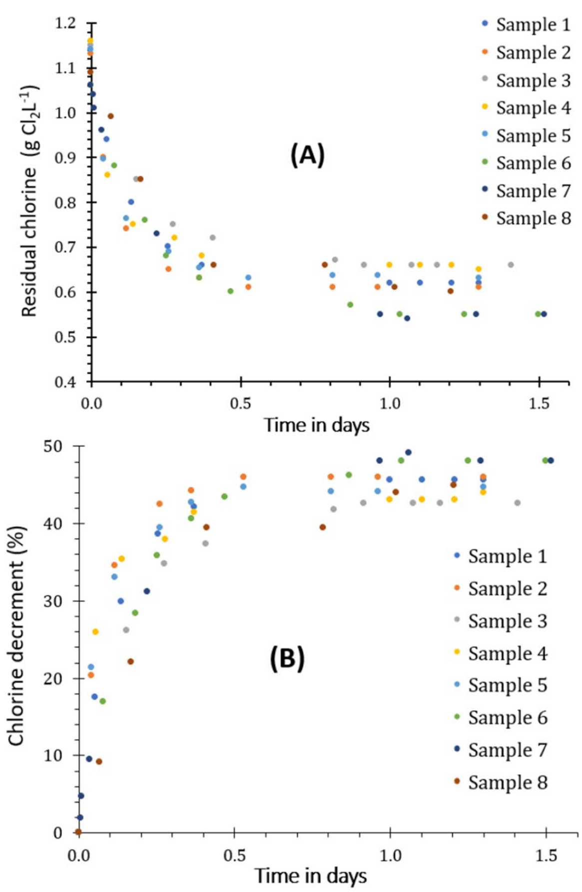

Fig. 2 Evolution of active chlorine concentrations in the collected samples ($n = 3$). **A** Presents the evolution of residual chlorine concentrations in the 8 samples from the Gravelines NPP, and **B** displays their decrement ratios ((initial concentration—final concentration)/initial concentration)

The distance between the sampling sites and toxicology laboratories as well as the limited capacities of these laboratories makes it impossible to treat samples within a few minutes after their sampling. It is also important to point out that in addition to a decrease in concentration, the consumption of active chlorine inevitably modifies the chemical composition of the samples with potential consequences for the bioassay results. This instability calls into question the relevance of the current approach which consists of carrying out biotests in the laboratory (off-site) on samples collected from NPPs.

Active chlorine behavior during in vitro bioassays

Another important aspect to be examined is the behavior of active chlorine under the conditions of in vitro bioassays. In these tests, cell cultures are exposed to the test substance before measuring their response after a predetermined exposure time (usually 16 to 24 h). The recommended exposure procedure takes place in three successive stages: first, dilution of the sample in an appropriate solvent (solution denoted S_1); second, dilution of S_1 in the culture medium (solution denoted S_2); and third, exposure of a volume of S_2 to the cell culture [21–23]. The maximum percentage of the sample in the final culture medium (S_2) should not exceed 1% (v/v). Phenol Red Free DMEM/F-12 (Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture) supplemented with 5% (v/v) fetal bovine serum (FBS) is the culture medium recommended in OECD Technical Guidelines 455 and 458. It contains nutrients for the growth of various mammalian cell lines, including essential and non-essential amino acids, vitamins and other organic and inorganic compounds. Additional file 1: Table S9 in the Supplementary material shows a typical composition of DMEM/F-12. It is interesting to note that the cumulative content of amino acids in the culture medium reaches concentrations on the order of grams per liter. It is therefore essential to ensure that these compounds do not interfere with active chlorine during the exposure period. It should be noted that OECD TG 455 and 458 as well as several authors [71, 72] recommend the use of charcoal treated-FBS. This treatment is known to eliminate free steroid hormones and various other substances naturally present in FBS but remains ineffective against more polar molecules, such as amino acids for example.

Amine-containing substances (i.e., amino acids and vitamins) are the most important constituents of the DMEM/F-12 medium with respect to interactions with active chlorine. It is well known that active chlorine reacts readily with nitrogen compounds (e.g., amines, amino acids, peptides, and proteins) to yield N-chloro or N,N-dichloro compounds depending on the dosage of the oxidant [14, 73–77]. For example, the reported rate constants for the reaction of active chlorine with amino acids range between 10^7 and 10^8 $M^{-1}{\cdot}s^{-1}$ [74–77]. A complete consumption of active chlorine is therefore expected during its dilution in the culture medium, since amino acids are present in large excess over $HOCl/^{-}OCl$. A detailed estimation is given in Additional file 1: Table S10. In addition to the interaction of active chlorine with nitrogen compounds, reactions with other constituents of the culture medium are possible. Reducing agents such as ferrous ion (Fe^{2+}) and organic carbon can react with active chlorine to produce chloride ion (Cl^-) and chloro-organics [78, 79]. It is likely for all these reasons that OECD TG 455 (for the evaluation of estrogenic activity) explicitly states the following: “this assay is applicable to a wide range of substances, provided they can be dissolved in dimethyl sulfoxide (DMS, CAS No. 67-68-5), do not react with DMSO or the cell culture medium, and are not cytotoxic at the concentrations being tested”.

The choice of dilution solvent (vehicle) is also of great importance. Whereas OECD TG 455 and 458 (for the evaluation of androgenic activity) propose water, ethanol (95% to 100% purity) and DMSO as appropriate vehicles, the OECD TG 456 (for the evaluation of steroidogenesis-disrupting activity) recommends DMSO [21–23]. However, these guidelines specify that for any vehicle, the maximum volume used must be demonstrated to be non-cytotoxic and not interfere with assay performance. DMSO solvent is known to be an excellent masking agent (scavenger) for active chlorine [80–82]. The study by Imaizumi et al. [81] showed that DMSO stoichiometrically reduces active chlorine to chloride ions. The authors found that DMSO could rapidly and completely mask chlorine under neutral and acidic conditions. As OECD TG 455, 458 and 456 must be performed with a large dilution step (e.g., 1:10) [21–23], it is therefore reasonable to expect complete active chlorine consumption if DMSO is the vehicle used.

The stability of the test substance in the test system is also a prerequisite for the performance of certain in vivo bioassays [57, 58]. For example, OECD TG 229, which describes an in vivo screening assay for fish reproduction, states the following: “Prior to initiation of the exposure period, proper function of the chemical delivery system should be ensured. All analytical methods needed should be established, including sufficient knowledge on the substance stability in the test system” [57]. Some characteristics of acceptable dilution water for in vitro tests are also specified in the OECD TG [57, 58]. For example, the OECD TG 230 assay, which covers oestrogenic and androgenic activity in addition to aromatase inhibition, requires the use of water with a residual chlorine content less than 10 μg L^{-1} [58]. This restriction is likely due to the interference of active chlorine with the test systems.

Discussion

Based on a systematic literature review, which includes a synthesis of all relevant information from scientific papers, databases and (Q)SAR models, no significant adversity based on EATS-mediated parameters nor EATS-related endocrine activities have been evidenced for active chlorine. To complete and support the data of the bibliographic approach, the endocrine-disrupting potential of active chlorine was evaluated in silico using Endocrine Disruptome tool. A very low probability of binding to the 14 targeted nuclear receptors was found, either in their agonist or antagonist conformations, confirming evidence from literature. However, the fact remains that not all EATS-mediated parameters or endocrine activity have been sufficiently investigated for humans and mammals as well as non-target organisms other than mammals. This result corresponds to scenario 2a (iii) discussed in Sect. 3.4.4 of the ECHA/EFSA TGD: : “no EATS-mediated adversity nor endocrine activity is observed, but both have not been sufficiently investigated”. In this case, the ED TGD suggests performing level 2 (in vitro) and level 3 (in vivo) tests using standardized procedures to generate the missing information [20]. The significant amount of toxicological data needed for ED assessment according to the requirements of the ED TGD raises two main questions: the first is related to the considerable effort to generate all the missing data in a relatively short time, while the second concerns the limitations associated with current methodologies.

From a general point of view, a recurrent question when performing bioassays is whether the analyte is stable before and during the assays. It is often assumed that the analyte concentration of the analyzed sample is representative of the analyte concentration at the collection site. The results obtained in the present study clearly indicate that active chlorine is unstable over time, with half-lives less of 48 h. This instability has already evidenced in various studies in the context of tap water treatment, in industrial cooling waters or in commercial bleach solutions [65–70]. In addition to a decrease in concentration, the degradation of active chlorine inevitably modifies the chemical composition of the samples (e.g., formation of active transformation products), which may lead to misinterpretation of bioassay results and consequently to erroneous conclusions. For example, dissipation of substances has been identified as a source of overestimation of biologically effective doses in several studies [83–85]. The active chlorine instability calls into question the relevance of the current approach which consists of carrying out biotests in the laboratory on samples taken in the field (e.g., NPPs). Performing bioassays on samples "immediately after collection" remains the best way to obtain the most accurate data possible. However, in practice, this approach is not technically feasible, unless a dedicated laboratory is installed on-site. Another alternative would be to carry out the tests on synthetic active chlorine, prepared on a laboratory scale. Different preparation protocols have been reported in the literature, mainly to investigate the chemical and biological aspects of chlorination at lab-scale, under controlled conditions [86–90]. To our knowledge, no study relating a synthesis of the existing protocols, nor their comparison was found in the literature whereas these protocols can strongly influence the chemical composition and therefore the biological response of the active chlorine prepared. For example, Sakcham et al. [91] recently carried out such study on monochloramine (NH_2Cl), a molecule analogous to active chlorine and falling within the scope of Biocidal Products Regulation (BPR). The authors compared four of the most frequently used protocols and found that the choice of protocol resulted in substantial differences in decay kinetics and disinfection efficacy of the prepared solution. They recommended the standardization of lab-based protocols as a solution to limit the discrepancies obtained by using different synthesis protocols. A major challenge would therefore be to obtain a synthetic active chlorine representative of that produced in situ by electrolysis of seawater in EDF's NPPs. Another aspect also of great importance is the behavior of active chlorine under the conditions for carrying out bioassays. This study suggests that active chlorine would also be unstable under the conditions prescribed by many OECD test guidelines, whereas the stability of the substance tested is a sine qua non condition for carrying out these tests. This highlights the limits of the transposition of generic methodologies, initially developed for organic molecules, to inorganic molecules such as active chlorine.

Conclusions

In this study, we applied the ED TGD methodology to examine the endocrine-disrupting potential of active chlorine under the requirements of the EU Biocidal Products Regulation. Overall, the available experimental data were insufficient to complete the ED identification process set out in the ED TGD and draw categorical conclusions about both EATS-mediated adversity and endocrine activity. However, neither the available data from the systematic literature review nor the results of the employed in silico method did reveal endocrine disrupting for active chlorine.

It is important to verify the reliability and toxicological relevance of the standardized bioassays regarding the specificities of the test chemicals before any experimental testing. In this sense, consultations and joint efforts between industrials, researchers and regulatory authorities are essential. If additional biotests were to be carried out, it is essential to find a reasoned solution to the instability of the in situ-generated active chlorine given the cost of these tests and to avoid obtaining unintelligible results. The use of in-lab synthetic solutions could be a useful approach. The appropriate protocol should be chosen based on clear scientific and technical objectives, previously discussed between the regulatory authorities and the producers concerned.

The results of the present study were presented to the public authority in charge of EDF's biocide file and exchanges took place to discuss potential solutions to the methodological difficulties highlighted. A more global reflection is currently underway at the EU Member States level to propose a harmonized position on similar substances.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00790-9.

Additional file 1: Figure S1. Location of EDF's NPPs in the English Channel (adapted from NordNordWest/Wikipedia, link to the original figure: //commons.wikimedia.org/wiki/File:English_Channel_location_map.svg). **Figure S2.** Flowchart illustrating the endocrine disruption assessment strategy as provided in the ECHA/EFSA Technical Guidance Document (2018). **Figure S3.** Electrochlorination cells (A) and chlorine storage tank (B) of one of the units od the Gravelines NPP, operated by EDF (the arrow to the right of Figure 2-B indicates the sampling point). **Table S1.** Results of the systematic literature review. **Table S2.** List of studies excluded for irrelevance after detailed assessment and reason(s) for exclusion. **Table S3.** List of experimental studies whose results were considered relevant and reliable. **Table S5.** Result of ToxCast Database. **Table S7.** EAS-mediated endpoints studied *vs* missing. **Table S8.** Threshold applied to receptor-ligand binding free energies (in kcal mol^{-1}) *vs* threshold values of sensitivity (SE). **Table S9.** Composition of DMEM/F-12 marketed by PAN Biotech. **Table S10.** Estimation of the chlorine to -NH_2 ratio in the exposure wells.

Additional file 2: Table S4. Data matrix summarizing the collected information (toxicity studies in mammals and wildlife) (given in excel format).

Additional file 3: Table S6. Lines of evidence for the E, A and S-modalities and for the T-modality (given in excel format).

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Author contributions

All authors contributed to the final manuscript. SK, SA, SR-P, APB and CG-L each wrote a specific part of the manuscript. IT, MW and FN contributed to the interpretation of the results and the revision of the manuscript. All listed authors have read and approved the final manuscript before submission.

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Competing interests

The authors declare that they have no competing interests.

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