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WASTEWATER TREATMENT AND MANAGEMENT

Testing the incorporation of bioassays into life cycle assessment: A case study on advanced wastewater treatment

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Abstract

Purpose Advanced wastewater treatment (AWT) methods, such as the use of ozone (O_3) and granular activated carbon (GAC), have been shown to mitigate toxic impacts on receiving waters. However, life cycle assessments (LCAs) on AWT technologies commonly evaluate toxicity through chemical analysis and conclude that the increased toxicity caused by operational inputs exceeds the reduction in toxicity via micropollutant removal from effluent. Here, a novel approach of incorporating bioassays for assessing the toxicity of wastewater effluent within the LCA framework was tested.

Methods To evaluate this recently developed method in a Swedish context, an LCA case study was performed comparing two treatment trains with ozonation and post-treatment—ozonation followed by either a moving bed biofilm reactor (O_3-MBBR) or GAC (O_3-GAC)—using data derived from Swedish pilot studies. A suite of in vitro bioassays was utilised to detect estrogenicity, activation of the aryl hydrocarbon receptor, and oxidative stress. Reference substances were employed to calculate the bioequivalent concentration of the samples for each bioassay. Characterisation factors for the reference substances were taken from USEtox2.14 and used to convert the assay results into LCIA results with the rest of the system modelled in LCA for Experts using EF3.1.

Results and discussion The results revealed a markedly disparate outcome compared with the traditional approach of using chemical analysis data as LCA input: the AWT benefits outweighed other toxicological impacts. Notably, oxidative stress emerged as the primary contributor to freshwater ecotoxicity, whereas aryl hydrocarbon receptor activation was the predominant contributor to human toxicity, including cancer risks. The normalised and weighted results showed the same pattern—the benefits of AWT, when based on bioassays, outweighed the environmental impacts considerably. Using different reference substances altered the magnitude of the AWT benefit, although still resulting in a net reduction of toxicological impacts. Regarding the case study, technological considerations, such as ozone source and GAC-filter lifespan, showed to be as important for the result as the choice of AWT technology.

Conclusions and recommendations The coupling of bioassays and LCA thus shows promise as a complementary evaluation to conventional toxicity assessment within LCA, and future research should help establish guidelines on which biological endpoints, bioassays, reference substances, and characterisation factors should be incorporated.

Keywords Effect-based analysis · Environmental assessment · Toxicity · Wastewater treatment · Ozonation · Activated carbon · Moving bed biofilm reactor · Quaternary treatment

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Abbreviations

AhR	Aryl hydrocarbon receptor
AWT	Advanced wastewater treatment
BEQ	Bioanalytical equivalent concentration
BV	Bed volume
E2	Ethinylestradiol
EBT	Effect-based trigger value
EBPR	Enhanced biological phosphorus removal
ER	Estrogen receptor
FETP	Freshwater ecotoxicity potential
GAC	Granulated activated carbon

HTPc	Human toxicity potential, cancer
HTPnc	Human toxicity potential, non-cancer
MBBR	Moving bed biofilm reactor
O_3	Ozone
Ox. stress	Oxidative stress
PE	Population equivalents
tBHQ	Tert-butylhydroquinone
TCDD	2,3,7,8-Tetrachlorodibenzodioxin;
WWTP	Wastewater treatment plant

1 Introduction

The removal of organic micropollutants, including pharmaceuticals, personal care products, and pesticides, has been a significant research focus in recent decades. Advanced wastewater treatment (AWT) methods, such as the use of ozone (O_3) and activated carbon (AC), are commonly explored for their effectiveness in abating these pollutants (Hollender et al. 2009; Eggen et al. 2014; Bourgin et al. 2018), and their implementation is increasing (Kosek et al. 2020). Despite the broad introduction of conventional wastewater treatment, European wastewater treatment plants (WWTPs) are estimated to release micropollutants in quantities that are comparable to untreated wastewater from 160 million population equivalents (PE), corresponding to approximately 30% of all wastewater pollution in Europe (Pistocchi et al. 2022). Thus, conventional treatment alone cannot sufficiently mitigate the unwanted biological effects of released wastewater. Fortunately, implementing AWT can significantly reduce these impacts on aquatic organisms and generate water that meets established safety thresholds (Völker et al. 2019; Näslund et al. 2022; Enault et al. 2023).

The European Urban Wastewater Treatment Directive (UWWTD) has been updated with stricter effluent requirements to protect the aquatic environment, including the removal of micropollutants. There is also a requirement to mitigate the impacts on climate change (Council of the EU 2024). An understanding of how best to manage the inevitable trade-offs between these objectives—given the increasing requirements of energy and other resources by more advanced treatment methods (Rodríguez et al. 2012)—requires systems thinking. To this end, life cycle assessment (LCA) is a commonly used tool for environmental systems analysis.

Most LCAs of AWT methods consistently show that the toxicity benefits that are gained from reducing micropollutant concentrations are outweighed by the environmental burdens that arise from increased energy and chemical use (e.g. Igos et al. 2021; Pesqueira et al. 2020; Risch et al. 2022; Surra et al. 2024). However, most of these LCAs rely on chemical analyses of a limited number of compounds: in the review by Pesqueira et al. (2020), only four of 18 studies examined more than 12 compounds, which may have constrained the assessment by failing to capture a larger spectrum of potential toxicological effects. Even when a broader range of compounds is considered—as in Arzate et al. (2019) (25 pollutants), Surra et al. (2024) (27 pollutants), Rahman et al. (2018) (35 pollutants), Risch et al. (2022) (65 pollutants), and Li et al. (2019) (126 pollutants)—the impacts of increased energy and chemical consumption predominate. The inherent shortcomings of chemical analysis include its inability to account for the effects of unknown substances or mixtures. The limited range of characterisation factors in current life cycle impact assessment (LCIA) methods also challenges the ability to perform fair assessments (Rashid and Liu 2021; Menghini et al. 2023).

Surface waters in Europe contain tens of thousands of human-made chemicals, but only a small fraction of them is routinely monitored (Brack et al. 2019; Undeman et al. 2022). Current regulations rely on targeted chemical analysis of individual substances, neglecting unmeasured compounds and mixture toxicity. Consequently, they merely capture the "tip of the iceberg". In contrast, bioassays evaluate the overall biological effects of an entire sample, providing a more comprehensive assessment of potential toxicity and representing a promising complementary approach. Bioassays are analytical methods that quantify the biological responses of cells (in vitro) or organisms (in vivo) that come into contact with the sample under study. These methods are more sensitive than chemical analysis and naturally encompass the effects of mixtures and unknown substances—i.e. the "entire iceberg" (Tang et al. 2013; Simon et al. 2022). A common critique, however, is that these tests present challenges in terms of experimental repeatability as the responses of the tested organism can vary between trials, even under the same test conditions (Menghini et al. 2023). This is contradicted by in vitro tests and well-plate-based in vivo that suggest the method is precise and robust, and many in vitro tests have a variability similar to that of targeted chemical analysis methods (Neale et al. 2023).

Initial attempts by a research group in Italy to include bioassays for estimating human toxicity and ecotoxicity in an LCA have generated promising results (Pedrazzani et al. 2018; Menghini et al. 2023). In this approach, bioassay results are translated into bioanalytical equivalent concentrations (BEQs) using dose–response curves of reference substances. Dose–response curves are created from bioassay results of different concentrations of the reference substance. The concentration at which the reference substance exerts the same toxicity as the sample in question is the BEQ. The BEQ values are then multiplied by the corresponding characterisation factors within the LCIA toxicity modelling framework and integrated into the LCA (Pedrazzani et al. 2020). The research group showed that although chemical analysis indicated improvements in water quality, the bioassays still detected toxic activity after conventional treatment (Bertanza et al. 2021) and AWT (Papa et al. 2021).

In vivo bioassays are limited by their high cost and ethical concerns with regard to animal testing. As a result, there has been a shift toward in vitro assays, which are faster, more sensitive, and show less variability (Neale et al. 2023). However, challenges remain in extrapolating the findings from in vitro studies to generate conclusions that apply at the organismal and ecosystem levels. Although there are standards for certain in vitro bioassays (such as OECD Test No. 455 for estrogenicity), many other bioassays lack formal OECD or ISO standardisation and harmonised protocols, and the standardisation on the overall data evaluation processes is lacking. Nevertheless, reference substances can be used to estimate BEQs (Völker et al. 2019).

In a systematic review of toxicity removal by ozonation and AC, Völker et al. (2019) analysed 46 studies, comprising 100 in vitro assays and 20 in vivo species tests. The authors found that whereas conventional treatment reduces toxicity efficiently, toxic effects that exceed effect-based trigger values (EBTs) persist after treatment. However, AWT methods, such as ozonation and AC, reduce residual toxicity well. Both approaches achieve reductions to a comparable extent, highlighting their potential for mitigating toxic effects in treated water. But ozonation can also induce toxicity through oxidation of target compounds and constituents of the water matrix by generating ozonation transformation products and oxidation by-products, respectively. Due to the potential toxicity of some such products, ozonation should be followed by biological post-treatment (Wunderlin et al. 2017).

Several studies have investigated various types of post-treatment following ozonation, but general treatment requirements for this treatment step remain lacking, preventing a clear consensus on which post-treatment step should be implemented (Bourgin et al. 2018; Völker et al. 2019; Stapf et al. 2020; Kienle et al. 2022; Sauter et al. 2024). Although the combination of ozonation and filtration with granular AC (O_3-GAC) has been suggested to be the most efficient regimen in removing pollutants and reducing toxicity, at least when the lifespan of GAC is low (Bourgin et al. 2018; Kienle et al. 2022), an environmental assessment of ozonation and post-treatments is warranted.

To the best of our knowledge, no LCA comparing various post-treatments after ozonation for AWT has been published—a gap that this study aims to fill. Furthermore, we will evaluate the approach of coupling bioassays and LCA, as initiated by Pedrazzani et al. (2018); implement it in a practical and Swedish setting; and discuss its applicability with regard to data availability and relevance. The results of this study will be pertinent to LCA practitioners and researchers in terms of its methodological implications and can contribute to refining environmental monitoring practices and improving water quality assessments.

2 Methods

To contextualize the evaluation of AWT, the study was set in Lidköping, Sweden, due to the availability of data and the city's need for guidance regarding future implementation of AWT. A new, medium-sized WWTP (45,000 PE, 14,900 m^3/day) is planned, which will include an AWT process that involves ozonation (Dahlberg 2019). Either a moving bed biofilm reactor (MBBR) or a GAC filter has been suggested as a post-treatment step to ozonation to remove potentially toxic oxidation by-products and transformation products. An LCA that compared O_3–MBBR and O_3–GAC using bioassays was therefore conducted. The water quality of the influent and effluent of the current WWTP was assessed by chemical analysis and in vitro bioassays, using composite samples (Holm and Önnby 2022).

With regard to measuring toxicity reduction and micropollutant removal, pilot data, representative for the yet non-existent plant in Lidköping, were used, comprising identical bioassays and chemical analysis of micropollutants for an O_3-MBBR pilot (Holm and Önnby 2022) and an O_3-GAC pilot (Baresel et al. 2024), allowing for direct comparison. To validate and evaluate their reliability, the pilot data were compared with toxicity reduction data in the literature (Völker et al. 2019; Stapf et al. 2020; Itzel et al. 2020; Kienle et al. 2022) (Sect. 3.5).

2.1 Scope of LCA

The methodological starting point of the LCA was ISO 14040:2006. The main function of the two configurations that were considered was treatment of wastewater to remove a large spectrum of micropollutants—i.e. quaternary treatment (EU Directive 2024/3019). The degree of treatment differed between configurations, which was accounted for in the LCIA by including the impacts of the differing effluents on the receiving water, i.e. the recipient. The functional unit was defined as "quaternary treatment of 1 m^3 secondary effluent".

Figure 1 shows the two treatment configurations. For the O_3-MBBR setup, the biologically treated, secondary effluent enters the ozonation reactor, where ozone is added, and is then led to an MBBR for degradation of oxidised organic matter. In the Lidköping design, the MBBRs are also intended for post-denitrification with the addition of an external carbon source. However, this approach is not common practice and does not fall within the defined function of the system (micropollutant removal); thus, the post-denitrification component of the MBBR was considered beyond the scope of the study and excluded. Subsequently, post-precipitation of phosphorus

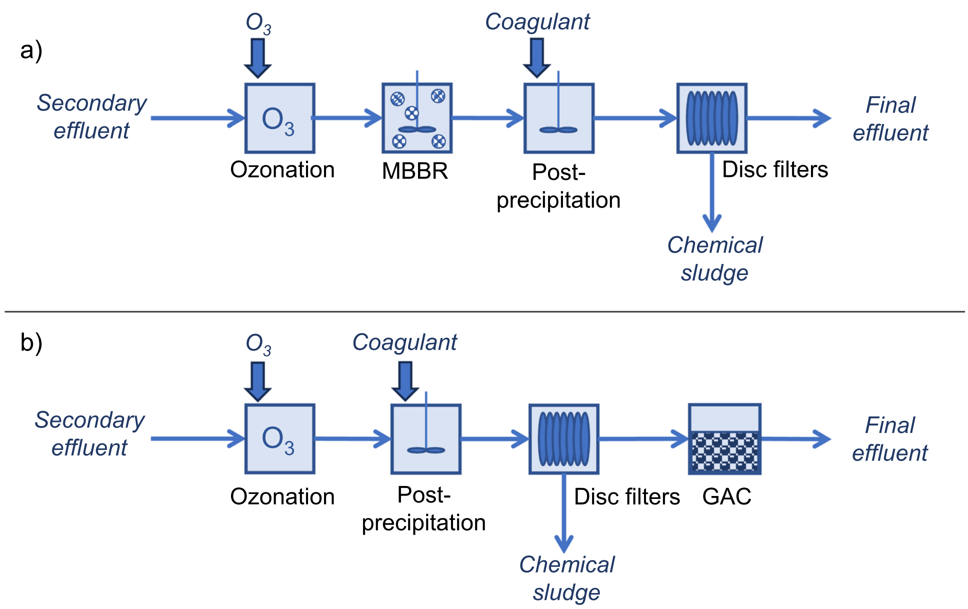

Fig. 1 Process flow diagrams of **a** the O_3-MBBR and **b** the O_3-GAC configurations

with poly-aluminium chloride and final polishing in disc filters are performed before the treated, final effluent is discharged to the recipient. Conversely, for the O_3-GAC setup, the water flows from the ozonation reactor to the post-precipitation and disc filters, before entering the GAC filtration tank. Because both configurations include post-precipitation and disc filtration, their construction and operation have been excluded from the LCA calculations.

The life cycle begins with the input of biologically treated, secondary effluent and ends with the release of the final effluent to the recipient (Fig. 2). Construction and operation of ozonation, MBBR and GAC are included, as is the impact of the effluent on the recipient in terms of heavy metals and additional benefits from AWT (i.e. toxicity reduction from removal of organic micropollutants). Inclusion of construction-related impacts in these types of LCAs, however, is uncommon (Pesqueira et al. 2020). Disregarding construction in wastewater treatment LCAs has long been considered a legitimate action due to the long lifespans of the infrastructure (Lundie et al. 2004). Recent studies, however, recommend including construction in the inventory, especially for non-intensive technologies and decarbonised energy systems (Morera et al. 2017; Corominas et al. 2020). Thus, construction was included in this study.

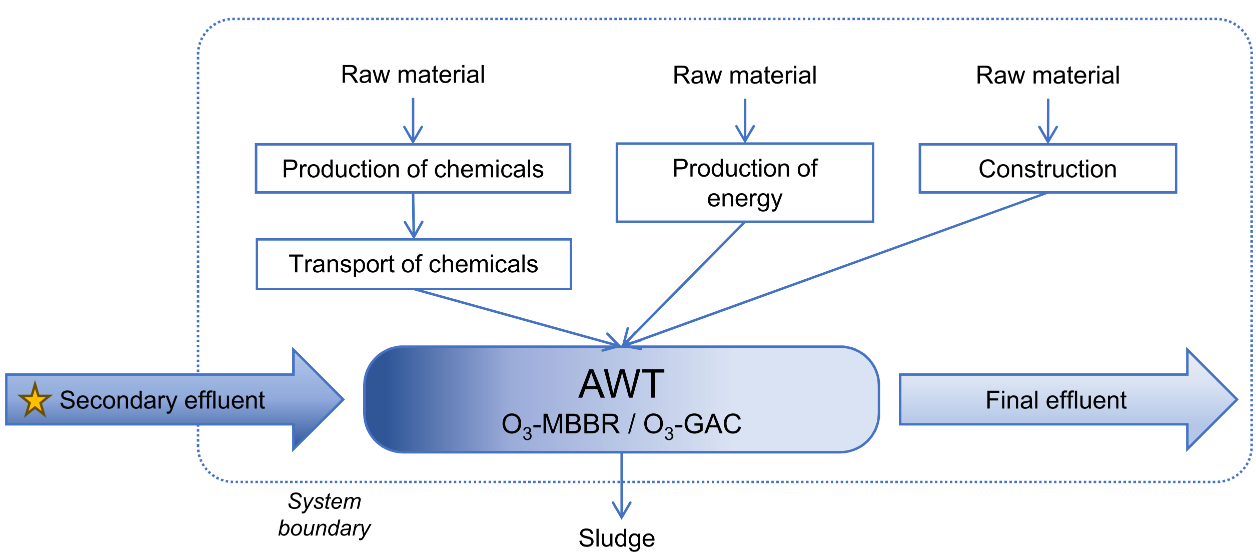

Fig. 2 System boundaries of the two AWT systems. The life cycle begins with biologically treated, secondary effluent entering the AWT and ends when the final effluent is released to the recipient. Reference flow (denoted with the star) is 1 m^3 of incoming secondary effluent

Impacts of nutrients in the final effluent are also excluded from the scope as any difference between the two scenarios is deemed minor. Regarding phosphorus, the chemical precipitation and subsequent disc filtration will ensure robust removal of dissolved and particulate phosphorus so that the GAC filters will not markedly reduce the levels further. There may be limited biological assimilation of phosphorus in the MBBRs, but the resulting final effluent will be the same in both configurations due to the precipitation and disc filtration. Regarding nitrogen, a recent pilot study of O_3-MBBR noted no removal of nitrogen in the MBBRs, merely conversion from ammonium to nitrite and nitrate (Hey et al. 2022). Nor will there be removal of nitrogen in the GAC filters to any greater extent. Therefore, the choice of excluding nutrient impact in the effluent may be assumed reasonable.

Whereas sludge disposal is important due to the affinity of sludge for heavy metals (Rashid and Liu 2021), we excluded further management of chemical sludge, because the differences between configurations were considered minor. The main difference between configurations regarding heavy metals stems from the GAC filtration process, which resides after the disc filters and the sludge outlet (Fig. 1). We have thus assumed any difference in heavy metal content of the sludges between configurations to be minor, although the heavy metal loads in the final effluent may differ, because part of the metal content can be removed in the GAC filter.

The geographical setting of the study in Sweden primarily affects the average electricity mix: 40% nuclear, 40% hydro, 11% wind, 6% biomass, and 3% miscellaneous (Sphera 2024). The Swedish context is also reflected in the choice of bioassay data from two Swedish pilot plants following the recent increased implementation of AWT in Sweden. For reasons of availability, the data on chemical production were instead based on European averages.

Regarding the timeline, the WWTP is assumed to be built within a few years. Major changes in energy or transport systems are thus not expected within this short timeframe, and the current systems are assumed to be relevant. Although there may be changes over the lifetime of the completed WWTP, they are outside the scope of this study.

2.2 Life cycle inventory

In this study, the foreground data were based largely on Högstrand et al. (2024), Holm and Önnby (2022), Baresel et al. (2024), and other literature, as outlined in the following. Background data were obtained primarily from the LCA for Experts version 10.9 (formerly GaBi) (Sphera 2024) and ecoinvent 3.9.1 (ecoinvent 2023) databases. The datasets that were used are listed in Table S1.

2.2.1 Construction

The construction inventory of the ozonation and GAC reactors was based on proposed designs for a Swedish WWTP of 971,000 PE with an assumed lifespan of 50 years (Ernst et al. 2020; Neth et al. 2023). The GAC reactor in these designs was intended to be incorporated as a separate treatment, without a prior ozonation step. Thus, the GAC is likely over-dimensioned in our study, rendering it risk-averse from an environmental perspective.

There is an apparent lack of LCAs on MBBRs that cover construction and demolition of real, full-scale installations. Previous literature on LCAs of MBBRs has focused primarily on the operational phase and excludes the construction and demolition stages (e.g. Bui et al. 2022; Singh et al. 2018, 2016; Starkl et al. 2018; Teow et al. 2021; Yang et al. 2020). When construction has been included, it has been on a pilot scale using plastic reactors (Masmoudi Jabri et al. 2020), included in the cost assessment without detailed specifications (Ćetković et al. 2023), or lumped with the construction inventory for the entire WWTP (Awad et al. 2024). For this study, the data from Awad et al. (2024) were considered to be the best available, although the equipment is too large for our case (i.e. the data refer to a full WWTP of 240,000 m^3/day, 30-year lifespan).

In our study, the construction data were thus scaled down to 45,000 PE using a power-law function to account for nonlinearity according to Gallego-Schmid et al. (2019), where *M* is the amount of material, *cap* is the treatment plant capacity (size), *f* is the economy of scale factor, and *ref* denotes the reference plant (Eq. 1). The economy-of-scale factor is often 0.6–0.8 for wastewater treatment processes (Remer and Chai 1993); thus, a factor of 0.7 was chosen (Arzate et al. 2019; Risch et al. 2022; Maniakova et al. 2023). To obtain the values per functional unit (m^3), the material requirements were then related to the total volume of treated wastewater, based on a daily flow of 14,900 m^3/day (Dahlberg 2019) (see Tables S2–S3).

$$M = M_{ref} \bullet \left(\frac{cap}{cap_{ref}} \right)^f \quad (1)$$

However, the amount of plastic that was suggested for the moving bed media by scaling Awad's numbers was unrealistic and would have resulted in blockage of the reactor, possibly an error stemming from scaling based solely on flows rather than PE because of the lack of this information (Awad et al. 2024). Instead, we calculated the required mass of plastic carriers based on the effective volume, filling ratio, plastic material, and bulk density (Table S4), yielding a value that was approximately one-tenth of the scaled values in Awad et al. (2024), which was used for the LCA.

The concrete and steel for MBBR were left as they were, although it is possible that they were also over-dimensioned.

2.2.2 Operation of ozonation

Ozone may be generated on-site from air or liquid oxygen (LOX). With the latter, the energy requirement may be 25% less than with the former (Choubert et al. 2017), although it is accompanied by impacts that are related to off-site production of LOX and transport to the WWTP. Here, ozone generation from both air and LOX has been included for comparison, assuming an electricity requirement of 18.8 kWh/kg O_3 for generation from air (Risch et al. 2022) and 14.1 kWh/kg O_3 and 8.76 kg O_2/kg O_3 for generation from LOX (Choubert et al. 2017; Risch et al. 2022). A transport distance of 300 km by truck without a return trip was assumed, as in Högstrand et al. (2024). The Swedish average electricity mix (40% nuclear, 40% hydro, 11% wind, 6% biomass, and 3% miscellaneous (Sphera 2024)) was assumed for foreground system electrical demand.

Ozone dosage is generally related to the amount of dissolved organic carbon (DOC) in wastewater. The secondary effluent from the dynamic simulation model of the planned WWTP in Lidköping had an average annual COD content of 35.4 mg COD/L (Högstrand et al. 2024), corresponding to 13.6 mg DOC/L, assuming a conversion factor of 2.6 (Juárez et al. 2021). An ozone dose of 0.6 g O_3/g DOC is assumed, consistent with previous studies (von Sonntag and von Gunten 2012; Bourgin et al. 2018), corresponding to 8.2 g O_3/m^3.

2.2.3 Operation of MBBR

Energy consumption for pumping and mixing in the MBBR was estimated to be 0.025 kWh/m^3 (Zkeri et al. 2021), corresponding to roughly half of the mixing energy required for the entire WWTP in the EBPR model in Högstrand et al. (2024). This assumption is reasonable, because no aeration of the MBBRs is necessary due to the high dissolved oxygen concentration from the previous ozonation step.

2.2.4 Operation of GAC

The lifespan of a GAC filter is usually given in bed volumes (BV)—the volume of water per volume of filter (m$^3{}_{\text{wastewater}}$/m$^3{}_{\text{filter}}$). For a conventional GAC filter design, a lifespan of 20,000 BV is a general operating assumption, although there may be a wide span in BVs at which breakthrough of pollutants occurs, depending on how well targeted compounds adsorb to the carbon. As a result, BV can be < 5000 for poorly adsorbing compounds and > 30,000 for well-adsorbing compounds (Altmann et al. 2016; Boehler et al. 2020) if only one GAC filter is present. It is reasonable to assume that longer filter lifespans are achieved when implementing pre-ozonation (Boehler et al. 2020; Schollée et al. 2021); 40,000 BV has been suggested for the O_3-GAC configuration (Baresel et al. 2024). In the present study, the standard lifespan of 20,000 BV and a longer lifespan of 40,000 BV were selected and contrasted in two scenarios. Assuming FiltraSorb 400 as the carbon type (as used in Baresel et al. (2024)), with a density of 540 kg/m$^3{}_{\text{filter}}$, the amount of carbon required results in 13.5 g/m$^3{}_{\text{wastewater}}$ at 40,000 BV and 27 g/m$^3{}_{\text{wastewater}}$ at 20,000 BV.

In Nordic countries, no activated carbon is produced, and most regeneration is performed abroad. Thus, a transport distance to Belgium of 1250 km was assumed for the new GAC, and twice this distance (round trip) for the regenerated GAC (Ernst et al. 2020). The density of wet GAC varies widely, depending on particle and pore sizes (Yiğit Hunce et al. 2016; Kramer et al. 2021; Novia et al. 2022). A density of 1200 kg/m^3 was assumed for the transport of gravity-dewatered, wet GAC to the site of regeneration. During regeneration of GAC, 10% is assumed to be lost and must be replaced by fresh GAC. Thus, of the 13.5 g/FU of GAC that is used for 40,000 BV, 1.35 g was modelled as new (1250 km, dry weight), and 12.15 g was modelled as regenerated GAC (1250 km, wet weight plus 1250 km, dry weight).

The electricity that was needed for the operation of the GAC filter was estimated to be 0.0268 kWh/m^3 (Risch et al. 2022).

2.2.5 Heavy metals

The heavy metal concentration in the final effluent after the O_3-MBBR was based on Högstrand et al. (2024). For O_3-GAC, further metal removal is expected, and effluent concentrations were calculated based on the removal rates in Mailler et al. (2024) (Table S5) and the O_3-MBBR effluent (Table 1). No removal rates were obtained for mercury;

Table 1 Heavy metal concentrations in the final effluent

Parameter	Unit	Cd	Cr	Cu	Hg	Ni	Pb	Zn	Reference
O_3-MBBR	mg/m^3	0.024	0.25	4.06	0.022	1.82	0.59	10.86	Högstrand et al. (2024)
O_3-GAC	mg/m^3	0.002	0.025	0.771	0.022	0.510	0.094	5.213	Högstrand et al. (2024); Mailler et al. (2024)

thus, the same mercury concentration was assumed for both configurations, to remain conservative. As seen in Sects. 3.1.2 and 3.1.3, this assumption did not alter the outcome notably, although the benefit of GAC might be slightly underestimated.

2.2.6 Organic micropollutants

The impact of using bioassays to estimate the toxicity in the final effluent was compared with that of the more conventional LCA method of chemical analysis of organic micropollutants. The selection of micropollutants was based on available data on current secondary effluent concentrations from Lidköping WWTP (102 compounds) and available removal rates using AWT (Holm and Önnby 2022; Baresel et al. 2024) (23 and 31 compounds, respectively), in combination with available characterisation factors in USEtox (version 2.14). After matching concentrations, removal rates, and characterisation factors, 14 substances remained: carbamazepine, diclofenac, furosemide, imidacloprid, ibuprofen, metoprolol, oxazepam, paracetamol, benzotriazole, erythromycin, sulfamethoxazole, trimethoprim, bisphenol A, and estrone (Tables S6–S7). This panel of compounds is consistent with or broader than those in most studies in the review by Pesqueira et al. (2020).

2.2.7 Bioassays

The biological endpoints that were assessed by Holm and Önnby (2022) and Baresel et al. (2024) included estrogenicity (ER), androgenicity (AR), anti-androgenicity (anti-AR), effects from aryl hydrocarbon receptor activity (AhR), effects from oxidative stress (Ox. stress), and genotoxicity by micronuclear test. ER, AhR, and Ox. stress have been recommended for monitoring the water quality of wastewaters (Escher et al. 2021) and were thus selected for further assessment of toxicity. Table 2 lists the biological endpoints, type of bioassays, the reference substances that were used and the influent and secondary effluent levels of Lidköping WWTP. The WWTP secondary effluent samples were 24-h composite samples from three occasions (February, March and June, 2022), whereas the O_3-MBBR results were based on duplicate grab samples from two occasions (April and May, 2022) (Holm and Önnby 2022) and O_3-GAC results are based on time-proportional weekly samples for six non-consecutive weeks (November, 2022, January, March, April, July, and August, 2023) (Baresel et al. 2024). ER activity was analysed with a cell-based reporter assay based on the human breast carcinoma MCF7 cell line (VM7Luc4E2), stably transfected with an ER-responsive luciferase reporter. AhR activity was analysed through transiently transfected human hepatocarcinoma cells (HepG2) with a luciferase reporter plasmid. Ox. stress was assessed as Nrf2 activity in stably transfected HepG2 cells with a luciferase plasmid (see, e.g. Rosenmai et al. 2018; Lundqvist et al. 2019; Oskarsson et al. 2021). Characterisation factors for the reference substances are given in Table S8. Mean and median toxicity reduction rates are shown in Table 3; further details are presented in Tables S9–S11. Due to the occurrence of negative data points (i.e. toxicity formation), we decided to use the median rather than the mean for further toxicity calculations.

The choice of reference substance may have a considerable impact on the results due to the varying characteristics of the USEtox characterisation factors. As a baseline, we decided to use the same reference substances that the bioassay results were already expressed in (Holm and Önnby 2022; Baresel et al. 2024). The reference substance for AhR

Table 3 Mean and median toxicity reduction rates based on Holm and Önnby (2022) and Baresel et al. (2024)

	ER	AhR	Ox. stress
O_3-MBBR			
Mean ± standard deviation (%)	98.6 ± 0.28	83.4 ± 5.79	39.0 ± 31.8
Median (%)	98.6	83.4	39.0
Number of calculations	2	2	2
O_3-GAC			
Mean ± standard deviation (%)	88.0 ± 11.7	52.0 ± 56.7	38.6 ± 33.1
Median (%)	94.6	82.7	48.7
Number of calculations	8	8	4

Table 2 Biological endpoints used in the study, their respective bioassay, reference substance, and limit of detection as well as influent and secondary effluent activity levels from the current Lidköping WWTP expressed in BEQs. The activity levels are averages of three sampling occasions using 24-h, composite samples (Holm and Önnby 2022)

Biological endpoint	ER	AhR	Ox. stress
Type of assay	VM7Luc4E2	Transiently transfected HepG2 cells	Activity of Nrf2 in stably transfected HepG2 cells
BEQ reference substance	Ethinylestradiol [E2]	2,3,7,8-tetraCDD [TCDD]	Tert-butylhydroquinone [tBHQ]
Limit of detection	14.2 pg E2-eq./L	0.0688 ng TCDD-eq./L	6.41 µg tBHQ-eq./L
Influent	68 533 pg E2-eq./L	7.21 ng TCDD-eq./L	992.7 µg tBHQ-eq./L
Secondary effluent	8423 pg E2-eq./L	1.903 ng TCDD-eq./L	33.4 µg tBHQ-eq./L

activation, i.e. TCDD, is a dioxin that is highly bio-accumulative, environmentally persistent, and toxic, characteristics which are reflected in a high USEtox characterisation factor (Fantke et al. 2015). While in soil and sediment, hydrophobic dioxin-like chemicals will explain most of the AhR activity, but in water, many chemicals can contribute, with only a small fraction of the effect explained (e.g. Lee et al. 2022). To test the implications of using a different reference substance, the Lidköping secondary effluent levels expressed in TCDD-eq. were also converted to bisphenol A equivalents (B(a)P-eq.) as well as carbaryl equivalents, assuming conversion factors of 1416 and 303,000, respectively (Enault et al. 2023). USEtox characterisation factors for B(a)P and carbaryl differ not only in size but also regarding which impact categories are covered compared with those for TCDD (Table S8), as further discussed in Sect. 3.3.

2.3 Life cycle impact assessment

Toxicity-related impact categories—freshwater ecotoxicity potential (FETP) and human toxicity potential, cancer (HTPc)—are the main priority in this LCA. Global warming potential (GWP) is a frequently used impact category within LCA—and a major focus in the UWWTD—and was thus also studied in detail to explore trade-offs.

Several impact assessment methods are available for evaluating toxicity. Environmental Footprint (EF) 3.1 is recommended by the European Commission (European Commission 2021) and bases toxicity modelling on USEtox (Rosenbaum et al. 2008), as supported by UNEP and SETAC. However, the version of USEtox in EF3.1 (version 2.1) is not the most recently released. The LCA software LCA for Experts incorporates USEtox version 2.12 (Sphera 2024), whereas USEtox 2.14 and a beta version of USEtox 3.0 are accessible on the USEtox webpage (USEtox 2024). However, at the time of writing, version 3.0 is for demonstration purposes only and does not contain any substances that are relevant to this study. Thus, we decided to base estimations of the benefits of AWT (i.e. toxicity reduction, as shown by bioassay or chemical analysis of organic substances) on USEtox 2.14 (i.e. by multiplying the concentrations of reference substances with the corresponding characterisation factors for the freshwater compartment), whereas the rest of the system was modelled in LCA for Experts according to EF3.1.

Finally, to evaluate toxicity in the context of the total impact, all 16 impact categories in EF3.1 were normalised and weighted according to Zampori and Pant (2019), based on normalisation (Andreasi Bassi et al. 2023) and weighting factors (Sala et al. 2018). It was assumed that these normalisation and weighting factors were also applicable for the AWT benefits calculations performed using USEtox 2.14.

2.4 Interpretation

The sensitivity of the results was determined by varying technical and methodological factors. Technical variations included the type of configuration, the source of ozone, and the GAC filter lifespan. Methodological variations concerned the use of chemical analysis (conventional method) or bioassays (novel method) for assessing the toxicological benefits of implementing AWT. Additionally, the sensitivity of using different reference substances for bioassay conversion was tested.

By normalising and weighting the results, the relative impact of all EF3.1 impact categories was obtained to aid in the interpretation of the data. Furthermore, the reliability of the toxicity reduction, calculated based on the pilot data, was determined by comparison with data in the literature.

3 Results and discussion of life cycle impact assessment (LCIA)

3.1 Characterised results

3.1.1 Climate change

The climate impact of the treatment processes is shown in Fig. 3. The results indicate that the technical considerations within the configurations, such as ozone source and GAC lifespan, are significant decision parameters. However, it is also clear that compared with MBBR, GAC has a substantially higher climate impact.

For O_3-GAC, the most important factor was the production and regeneration of activated carbon, as noted also by Pesqueira et al. (2020). A longer lifespan with a high BV has the potential to decrease the climate impact substantially. As stated in Sect. 2.2.4, the lifespan of GAC may vary, depending on such factors as the substances to mitigate. For example, if perfluorooctanesulfonic acid (PFOS) is targeted for removal, a lifespan as short as 5000 BV could be recommended (Edefell et al. 2022), resulting in a climate impact from the GAC operation that is seven times higher versus a scenario in which the lifespan is 40,000 BV. Conversely, there are indications that lifespans over 40,000 BV are possible when the filter is placed after an ozonation reactor. In general, the more extensive a pre-treatment is, the more effective the micropollutant removal in the GAC is (Betsholtz 2023) and, consequently, the greater the potential for a longer lifespan will be.

The impact of the ozonation operation was approximately four times larger when using LOX compared with on-site production from air, which can be explained by the nearly fossil-free energy mix in Sweden. Similar results were obtained by Risch et al. (2022), who used

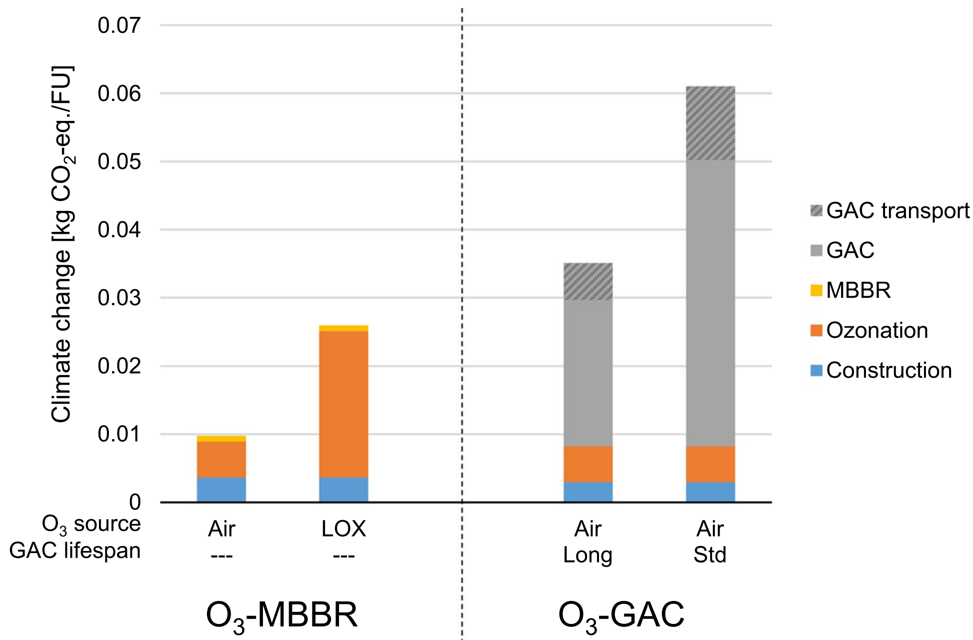

Fig. 3 Contributions to climate change in kg CO_2-eq./FU. The bars represent different technological variations such as ozone source (air or LOX) and GAC filter lifespan ("long" = 40,000 BV and "Std" (standard) = 20,000 BV). "Construction" entails construction impacts for all processes, whereas the operational parameters are divided into "ozonation", "MBBR", and "GAC". For GAC, the transport-related impacts are highlighted specifically

nuclear-based French electricity on-site and European oxygen production. It is evident, however, that increased reliance on fossil-based electricity will significantly elevate the operational impact from ozonation, as also suggested by Risch et al. (2022). In fact, in many AWT applications, the electricity consumption that is associated with ozonation emerges as the primary contributor to climate change due to the prevailing fossil-based electricity sources worldwide (Pesqueira et al. 2020).

The impact of construction was minor for both configurations and all scenarios, although their relative impacts varied widely (~4–37%), with air-based O_3-MBBR having the largest share. These values are comparable with those of other studies. Risch et al. (2022) found that construction constituted 7–20% of the total climate impact for an ozonation process without post-treatment, versus 2% for only AC. Of the nine studies that were reviewed by Pesqueira et al. (2020) that included construction, three showed that the impact of construction was negligible (Tarpani and Azapagic 2018; Rahman et al. 2018; Li et al. 2019), four studies reported that the contribution ranged from 2 to 30% of GWP (Ioannou-Ttofa et al. 2017); Foteinis et al. 2018; Gallego-Schmid et al. 2019; Arzate et al. 2019), and two studies did not show any data nor discussed them in the results (Wenzel et al. 2008; Høibye et al. 2008). These findings demonstrate that although impacts of construction are often minor, they are not necessarily negligible—especially for less energy-intensive technologies or decarbonised energy systems, consistent with previous studies (Corominas et al. 2020). But, to facilitate comparison between studies, guidelines on the aspects of construction that should be included are warranted. Currently, construction data in LCA studies are often limited—especially for MBBR facilities—and a more detailed inventory would increase precision and accuracy.

3.1.2 Freshwater ecotoxicity potential (FETP)

Concerning freshwater ecotoxicity potential (FETP), Fig. 4 shows that when the benefits of AWT were based on bioassays, the impact on the toxicity related to material and energy supplies was outweighed by the reduction in final effluent toxicity. The largest contributor to this category was the reduction of Ox. stress, whereas the reductions of ER and AhR did not contribute to the same extent. This finding can be explained by the fact that the effects of Ox. stress are influenced by a plethora of substances, rendering it a more general indicator of ecosystem health (Hayes et al. 2009). When the benefits were instead based on chemical analysis of the 14 organic micropollutants, the reduction in toxicity was negligible, consistent with the literature, wherein toxicity benefits were negligible or, at best, minor—also for studies that included more micropollutants (e.g. Pesqueira et al. 2020; Risch et al. 2022; Tarpani and Azapagic 2018). Thus, incorporating bioassays may provide a completely different view of toxicity in LCA. However, the toxicity of the remainder of the system, such as emissions to freshwater from the production of LOX or GAC, remained based on chemical analysis, and implementing bioassays for the final effluent alone might still fail to capture the entire toxicological impact.

As shown in Fig. 4, the largest toxicity impacts for O_3-MBBR stemmed from LOX production and transport as well as the remaining heavy metals in the effluent. For O_3-GAC, the main impact came from GAC operation, of

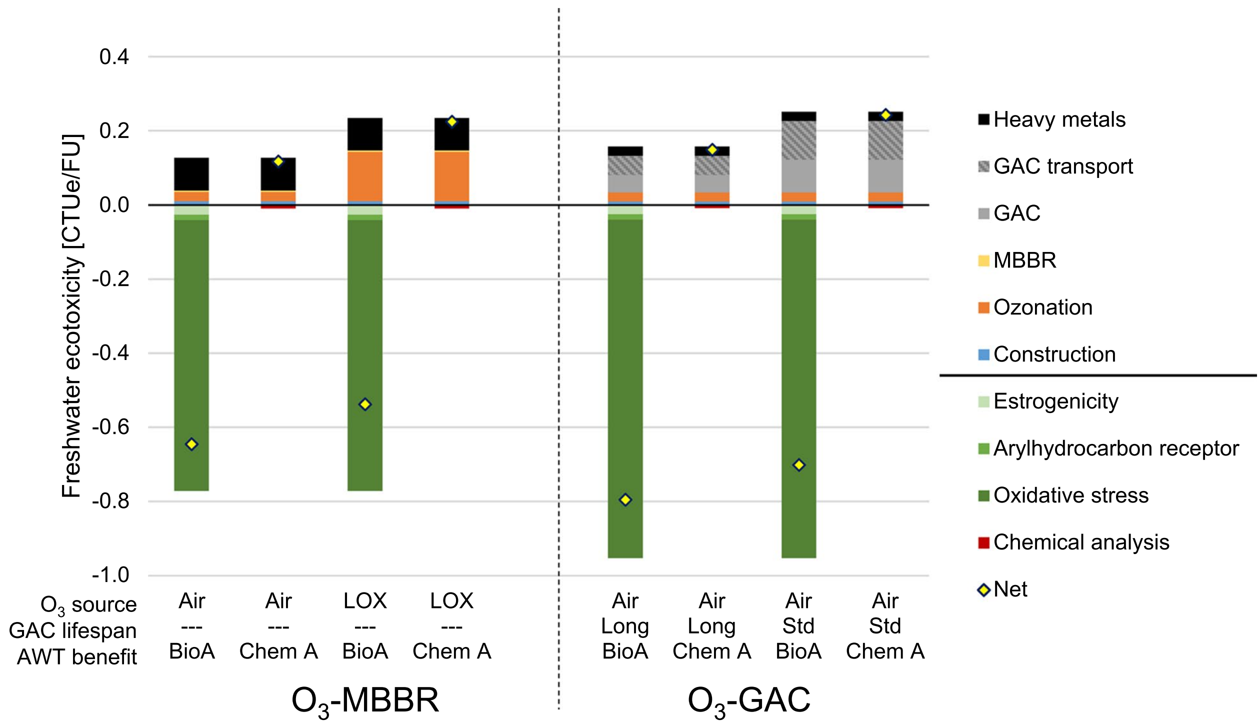

Fig. 4 Freshwater ecotoxicity potential in CTUe/FU. The bars represent different technological and methodological variations such as ozone source (air or LOX), GAC filter lifespan ("long" = 40,000 BV and "Std" (standard) = 20,000 BV), and AWT benefit ("BioA" = bioassay, "Chem A" = chemical analysis). "Construction" entails construction impacts for all processes, whereas the operational parameters are divided into "ozonation", "MBBR", and "GAC". For GAC, the transport-related impacts are highlighted specifically. "Heavy metals" shows the remaining concentrations of heavy metals in the effluent, whereas the removal of organic micropollutants is shown through AWT benefits

which 53% was due to transport, 25% was due to production of activated carbon, and 18% was attributed to regeneration. Halving the GAC lifespan would double the ecotoxicological impact of GAC operation. The literature on LCA does not frequently report ranges in GAC lifespan. Instead, technical variations often entail the extent of regeneration (Pesqueira et al. 2020; Risch et al. 2022; de Boer et al. 2022) or choice of carbon source (Igos et al. 2021; de Boer et al. 2022). Although these factors are important, our findings suggest that extending the GAC lifespan is a straightforward and effective strategy for reducing toxicity. These results also raise the questions of whether not only draining but also thermal drying of GAC before transportation is merited, or developing strategies to decrease transportation distance—e.g. by facilitating large-scale GAC regeneration in Sweden.

3.1.3 Human toxicity potential, cancer (HTPc)

Figure 5 shows the importance of remaining heavy metals in the effluent to the human toxicity potential, cancer (HTPc), but these effects are nevertheless several orders of magnitude smaller than the benefits that are portrayed by bioassays. The benefits of AWT consisted primarily of reductions in AhR, with no substantial difference between configurations. This finding can be attributed to the handling of AhR-related toxicity by USEtox, which reflects the reference compound's (TCDD) high bioaccumulation potential, extreme environmental persistence, and significant toxicity through AhR activation, often associated with cancerogenic risks (Fantke et al. 2015). These results thus support the recommendation to incorporate AhR activation into water quality monitoring (Escher et al. 2021) and underscore its critical nature in comprehensive wastewater LCAs. However, the importance of dioxin-like chemicals such as TCDD in water samples may be questioned (Lee et al. 2022), and evaluations with other reference substances are therefore warranted, which is further discussed in Sect. 3.3.

Remaining heavy metals contributed considerably to the overall toxicity, most notably in the O_3-MBBR versus O_3-GAC configuration, despite the final effluent concentrations for both configurations remaining markedly below Swedish national averages for heavy metals in wastewater effluents (Villner and Myhr 2022). Also, the construction impacts of O_3-MBBR were nearly four times greater compared with O_3-GAC, of which 88% was attributed to the production of plastic for the carriers. Nonetheless, when the benefits were evaluated based on bioassay data, the differences in toxicity impact between configurations became

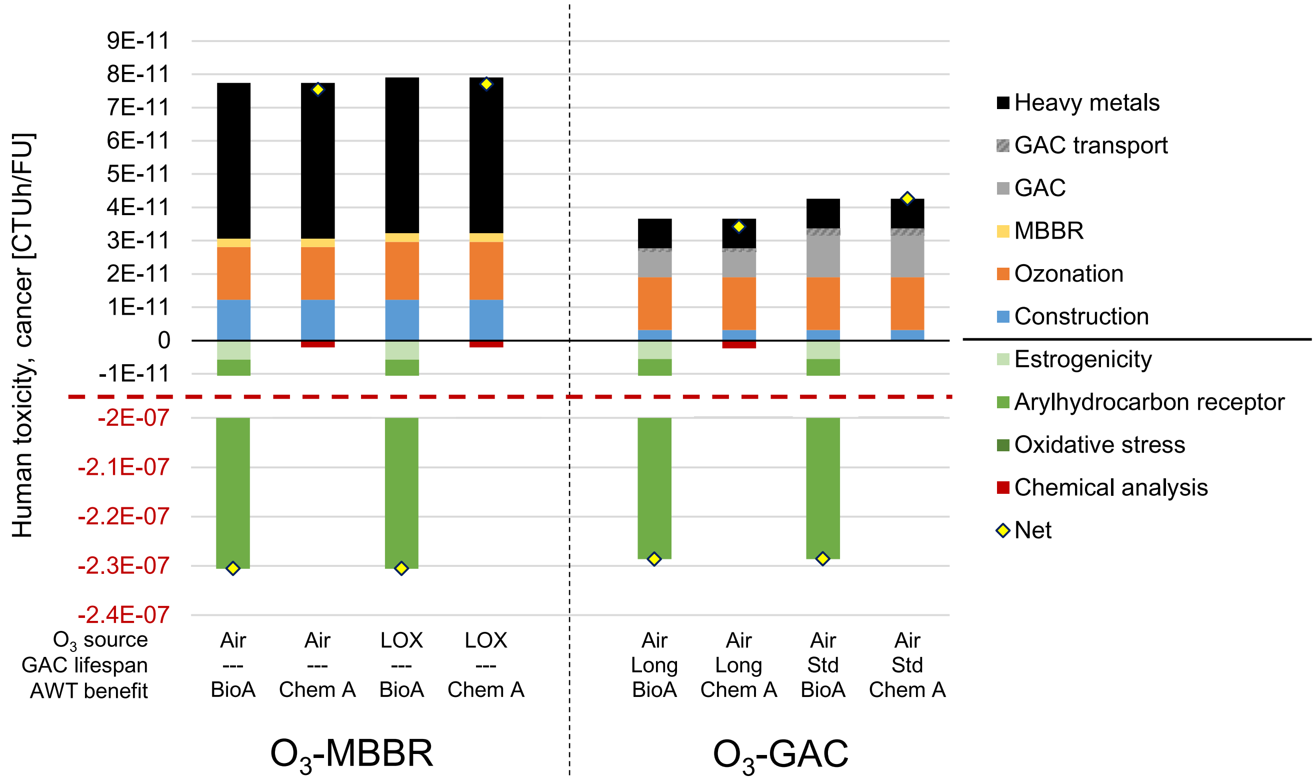

Fig. 5 Human toxicity potential, cancer, in CTUh/FU. The bars represent different technological and methodological variations such as ozone source (air or LOX), GAC filter lifespan ("long" = 40,000 BV and "Std" (standard) = 20,000 BV), and AWT benefit ("BioA" = bioassay, "Chem A" = chemical analysis). "Construction" entails construction impacts for all processes, whereas the operational parameters are divided into "ozonation", "MBBR", and "GAC". For GAC, the transport-related impacts are highlighted specifically. "Heavy metals" shows the remaining concentrations of heavy metals in the effluent, whereas the removal of organic micropollutants is shown through the AWT benefits. Note the broken *y*-axis and differing scales

negligible, suggesting that the configurations are equivalent from this perspective.

3.2 Normalised and weighted results

To put these results in perspective, all 16 impact categories of EF3.1 were normalised and weighted (Fig. 6). Notably, the differences due to technological variations within each configuration had as extensive an impact as the choice of configuration (Fig. 6a). When benefits were assessed solely by chemical analysis, their influence was minor. However, with bioassays, the benefits dominated the toxicity categories and outweighed all other impacts by several orders of magnitude. These results were based on the assumption that the normalisation and weighting factors were applicable to the AWT benefits that were calculated based on the characterisation factors in USEtox 2.14. However, the weighting factors of the toxicity categories are the lowest among all the weighting factors for the impact categories, to reflect the higher uncertainty and lower robustness of the toxicity estimates. Nevertheless, the toxicity benefits of AWT, based on bioassays, dominated the results.

To further contextualize the results, the benefits of AWT based on bioassays were compared with the impacts of the planned full WWTP in Lidköping (biological phosphorus removal with post-precipitation and AWT), based on a published LCA that included O$_3$-MBBR (Högstrand et al. 2024). As shown in Fig. 6b, although the impact of the full WWTP was roughly 5–10 times greater than that of AWT alone, the benefits of AWT still outweighed its associated impact. The overall benefits of the full WWTP, particularly in terms of toxicity reduction from influent to final effluent, are likely to be considerably greater than shown in the figure (i.e. toxicity reduction only from secondary to final effluent).

Moreover, as mentioned in the introduction, most LCAs on AWT that base toxicity estimations on chemical analysis note that the impacts generated by the AWT are larger than the estimated benefits. Igos et al. (2021) reported that the (eco)toxicological benefits were around 30–80 times lower than the impacts. Rahman et al. (2018) noted benefits 25–100,000 times lower than the impacts. Also, Risch et al. (2022), Arzate et al. (2019), and Surra et al. (2024) report a net increase in impacts. This could be compared with our results, where in the freshwater ecotoxicity category, AWT

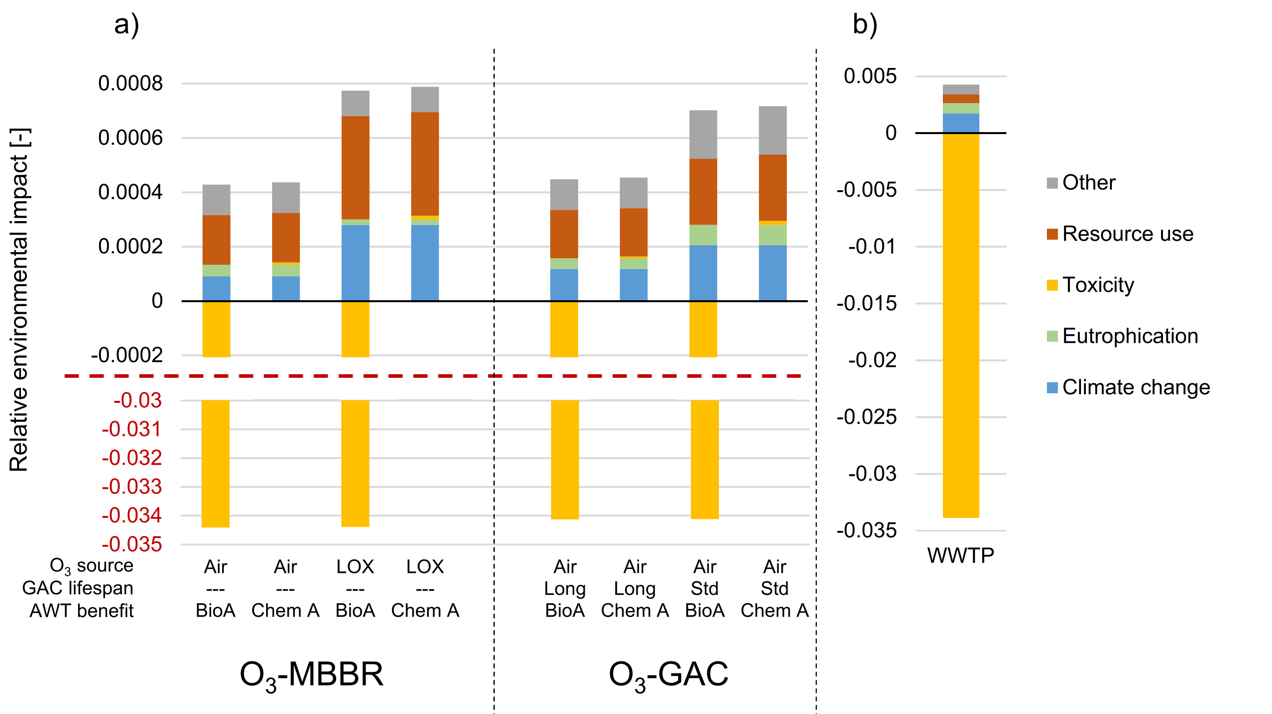

Fig. 6 **a** and **b** Normalised and weighted net results showing the dimensionless, relative impact of each category and scenario with AWT benefits. **b** The WWTP scenario shows the impacts from a full-scale WWTP with biological phosphorus removal, post-precipitation, and O_3-MBBR, wherein the AWT benefits were based on bioassays. The bars represent different technological and methodological variations such as ozone source (air or LOX), GAC filter lifespan ("long" = 40,000 BV and "Std" (standard) = 20,000BV), and AWT benefit ("BioA" = bioassay, "Chem A" = chemical analysis). "Eutrophication" includes freshwater, marine, and terrestrial eutrophication; "Toxicity" includes ecotoxicity, human toxicity carcinogenic, and human toxicity non-carcinogenic; "Resource use" includes fossil and mineral resource use, as well as land and water use; and "Other" consists of the following categories: acidification, ozone depletion, particulate matter, photochemical ozone formation, and ionising radiation. Note the broken *y*-axis and differing scales

benefits are 13 times lower than impacts if estimated with chemical analysis, but 6 times *higher* if estimated through bioassays (O_3-MBBR scenario with air as ozone source). For human toxicity, cancer, the results for the same scenario are even more striking: the benefits of AWT are 38 times lower with chemical analysis but 3000 times *higher* with bioassays.

3.3 Influence of reference substance

As mentioned in Sect. 2.2.7, dioxins (such as TCDD) may not be solely responsible for AhR activation in water. The AhR is involved in xenobiotic metabolism and is a fairly promiscuous receptor, with previous studies showing that many chemicals (both toxic and non-toxic) can activate the receptor (NWRI 2020). This means that the risk to human health may be overestimated. A sensitivity analysis was therefore performed where the AhR activity expressed in TCDD-eq. was converted to B(a)P-eq. and carbaryl-eq. with significant influence on the results, especially since B(a)P does not have a characterisation factor for HTPc. Figure S1-S3 show how the AWT benefit is strongly affected by the choice of reference substance for the three toxicity-related impact categories at midpoint (FETP, HTPc, and HTPnc). Comparison of the overall, weighted result is shown in Fig. 7. It is clear that the results may vary substantially when selecting different reference substances. Nevertheless, from Fig. 7, it can be seen that for all three reference substances, the toxicity net impact (yellow) is negative, indicating a toxicological benefit from AWT—although of varying size—when based on bioassays compared with when based on chemical analysis, where the net toxicity is positive (i.e. net increase).

3.4 Comparison with effect-based trigger values (EBTs)

Extrapolating in vitro results to ecosystems is challenging, because bioassays do not capture the complex ecological interactions (e.g. competition, predation, and symbiosis), nor the processes of bioaccumulation and biomagnification across trophic levels (Escher et al. 2020, 2021). In addition, chemical transformation may render metabolites with different toxicity profiles (Wilk-Zasadna et al. 2015). This means that in vitro bioassays may underestimate or mis-represent

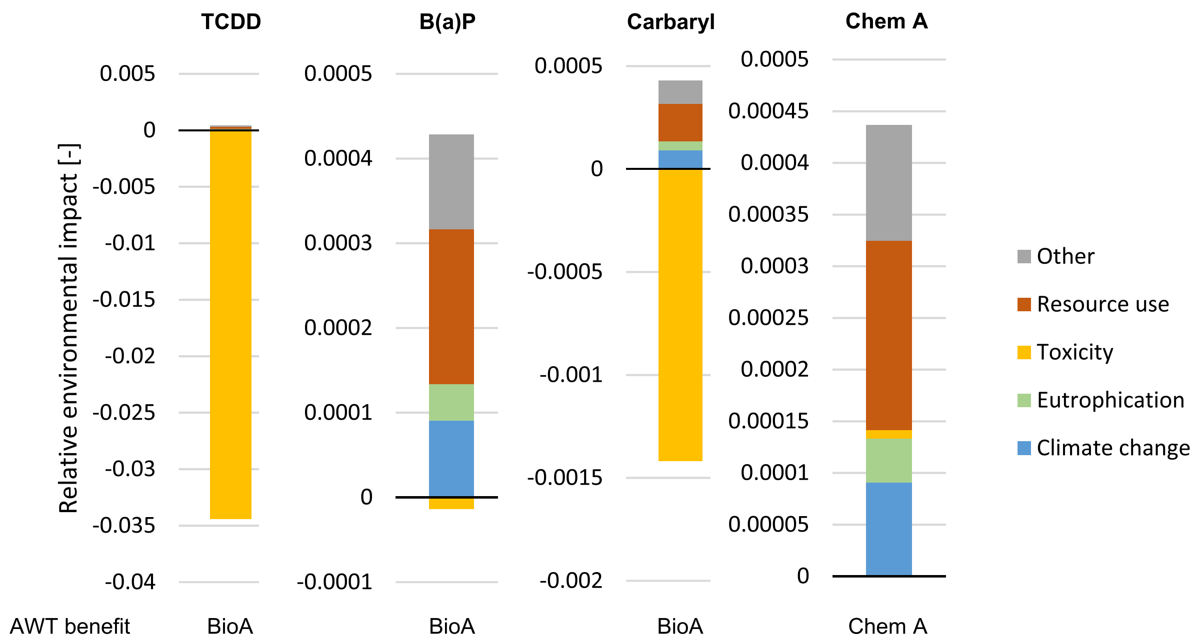

Fig. 7 Influence of choice of AhR-reference substance (TCDD, B(a)P or carbaryl) on net normalised and weighted results compared to when the AWT benefit is based on chemical analysis for the O_3-MBBR scenario with ozone sourced from air. "BioA" = bioassay, "Chem A" = chemical analysis. "Eutrophication" includes freshwater, marine, and terrestrial eutrophication; "Toxicity" includes ecotoxicity, human toxicity carcinogenic, and human toxicity non-carcinogenic; "Resource use" includes fossil and mineral resource use, as well as land and water use; and "Other" consists of the following categories: acidification, ozone depletion, particulate matter, photochemical ozone formation, and ionising radiation

ecological risks. Nonetheless, effect-based trigger values (EBTs) serve as critical benchmarks for translating in vitro findings into water quality assessments by enabling comparisons to be made between biologically equivalent concentrations (BEQs) and environmental protection thresholds (Escher et al. 2021). These thresholds—tailored to the sensitivity of specific bioassays—are used to evaluate such endpoints as ER, Ox. Stress, and AhR activation (Völker et al. 2019; Escher et al. 2021).

Holm and Önnby (2022) demonstrated that ER activity (8.42 ng E2-eq./L) and Ox. stress (33.4 µg tBHQ/L) exceeded ecological EBTs (0.1–2.2 ng E2-eq./L and 6.7–35.9 µg tBHQ-eq./L, respectively (Enault et al. 2023)) after conventional treatment at the current WWTP in Lidköping. By applying reduction rates for toxicity removal after AWT from the pilot processes in that study and that in Baresel et al. (2024), both ER and Ox. stress activities were successfully brought below the EBT (0.11–0.46 ng E2-eq./L and 17.1–20.4 µg tBHQ/L, respectively). However, the reported AhR value of 1.9 ng TCDD-eq./L after conventional treatment, measured through transiently transfected HepG2 cells, cannot be compared directly to ecological EBTs that are derived from the H4L1.1c4 AhR assay (0.003 ng TCDD/L Escher et al. 2021; Enault et al. 2023)) due to differences in assay sensitivity and metabolic capacity. After AWT, the estimated AhR activation decreased to 0.32–0.33 ng TCDD-eq./L—still 100 times higher than the EBT—emphasising the nuances of bioassay variability and the potential toxicological risks.

This analysis aligns with reviews by Völker et al. (2019) and Enault et al. (2023), which show that conventional treatment often fails to meet ecological EBTs for ER, despite high removal rates. Advanced technologies like ozonation and GAC improve compliance but still leave residual activity for sensitive targets like AhR. However, dilution in receiving waters is an important parameter to include that may mitigate risks further (Völker et al. 2019; Enault et al. 2023). In the Lidköping case, the effluent will be discharged into a river (117 times dilution) that leads to a large lake a couple of kilometres downstream (> 1000 times dilution) (Styf and Magnusson 2020). That would result in a level just below the ecological EBT in the river and well below it in the lake. Nevertheless, in many cases, the proposed EBT for the AhR-CALUX assay coincides with the limit of detection, indicating that residual AhR-active compounds persist even after advanced treatment. Furthermore, a Danish study reported negative toxicity reduction (− 15 to − 199%), suggesting that some AhR-active compounds resist breakdown or form as toxic byproducts during treatment (Castro et al. 2021). These findings underscore the challenges of properly managing AhR-active compounds in wastewater treatment.

3.5 Data quality and availability

As indicated in the previous section, the AhR values in the final effluent in this study were several times higher than the ecological EBT (if excluding dilution). The higher sensitivity of the applied bioassay compared with other AhR assays may explain this finding, but the high AhR values may also be attributed to influent variations and the calculation method. The conclusions regarding the dominant impact of AhR on the HTPc category, however, would remain even if the AhR results decreased 1000-fold (Fig. S4). Regarding influent variations, AhR results on secondary effluent from 11 WWTPs were collected and compared, showing a wide variation between sampling locations and over time with those from Lidköping WWTP among the highest (Fig. S5). This may affect the toxicity calculations as they are based on a percentage reduction, which means that the resulting effluent activities depend on the characteristics of the influent. In fact, Holm and Önnby (2022) noted that the O_3-MBBR pilot reduced AhR to below detection limits. But because this location also showed low AhR activity in the secondary effluent, the calculated reduction in toxicity might not be representative of other settings. Thus, we must understand how AhR is affected by various AWT technologies.

It should also be noted that the number of data points of bioassay results from the underlying references (Holm and Önnby 2022; Baresel et al. 2024) is low which can be a factor limiting the interpretation. To that end, a detailed inventory of available toxicity reduction rates was compiled (Tables S12-S13, Fig. S6) by reviewing existing literature (Reungoat et al. 2012, 2010; Macova et al. 2010; Escher et al. 2012, 2014; Kienle et al. 2013, 2022; Maier et al. 2016; Ternes et al. 2017; Itzel et al. 2017, 2020; Giebner et al. 2018; Stapf et al. 2020). As shown in Fig. S6, the available data were unevenly distributed across biological endpoints, with a considerably higher number of studies on estrogenic effects than oxidative stress. Moreover, as anticipated, there were more studies on O_3-GAC treatment systems than O_3-MBBR configurations. There was a difference between the literature and pilot results regarding O_3-MBBR removal of estrogenicity, as the pilot median exceeded the literature values. However, the available data were insufficient to definitively determine which configuration reduces toxicity better—both configurations seemed to perform similarly in this regard. Additional measurements of these biological endpoints, especially AhR, are warranted to improve the reliability of the results. Nevertheless, whereas the results may depend on the data source (e.g. literature versus pilot studies), the overarching conclusion—that incorporating bioassays into LCA may significantly enhance the perceived benefits of AWT—is robust.

3.6 Method evaluation

We attempted to integrate bioassays into LCA, as proposed by Pedrazzani et al. (2018). In short, the method was straightforward to implement, particularly when employing established in vitro assays that were accompanied by reference substances for which characterisation factors are available. However, several methodological challenges arose: determining which bioassays should be incorporated, identifying the most suitable reference substances, and establishing reliable methods for data collection, even prior to AWT construction and commissioning. Furthermore, a critical issue that remains concerns the interpretation of the results when bioassays are used in only some foreground parts of the system and thereby generates a difference in perspectives for different parts of the system in the same study. These challenges are elaborated on in the following.

Based on recommendations for wastewater monitoring (Escher et al. 2021), we selected three biological endpoints with one bioassay and one reference substance each and that were related to two impact categories (FETP and HTPc). However, as some substances may contribute to several biological endpoints, the risk of double-counting cannot be excluded. Nevertheless, even if assuming 100% double-counting, that would still not alter the conclusions, as for each impact category, one biological endpoint contributed substantially more than the others. Based on our results, this selection of biological endpoints had a profound impact on the toxicity evaluation—especially Ox. stress on FETP and AhR on HTPc, albeit depending on the reference substance.

Other biological endpoints are considered in the literature. Pedrazzani et al. (2018) evaluated three biological endpoints (mutagenicity, estrogenicity, and baseline toxicity) using four bioassays with three reference substances for three impact categories (HTPc, HTPnc, and FETP). Each endpoint was related to a specific impact category—in contrast to our study, in which all biological endpoints could affect both categories. Their selection of bioassays was based on feasibility, the existence of standardised protocols, and whether they could be linked to a specific biological endpoint, rather than ensuring that the bioassays covered a wide range of substances in wastewater, as in our case.

In a more recent study, Menghini et al. (2023) used three biological endpoints (cytotoxicity, genotoxicity, and carcinogenicity), eight bioassays, eight reference substances (more than one per bioassay), and two impact categories (FETP and HTPc). The bioassays were selected to encompass various modes of action and test species from several trophic levels. They concluded that a battery of assays was necessary over a single assay and recommended the use of several reference substances for each impact category. It has also been suggested that if several bioassays are available for the same biological endpoint, the results on the greatest effect should be used to perform a risk-averse assessment (Pedrazzani et al. 2020). However, in our study, we connected the bioassays to all toxicity-related impact categories and had only one reference substance per biological endpoint. Thus, more data and guidelines are needed regarding the choice of endpoints, bioassays, reference substances, and corresponding impact categories, along with the development of characterisation factors to match suggested reference substances.

Yet, the question remains on how the results should be interpreted when there are only bioassays in the foreground system. The intent of an LCA is to assess all of the main impacts of a technical system meaningfully (ISO14040). For a technical system that treats large flows of wastewater of complex composition, evaluating the impacts of direct release to the recipient *as thoroughly as possible* increases the meaningfulness of the assessment. Bioassays allow for unknown contaminants and mixture effects to be included and hence contribute with another level of thoroughness compared with chemical analysis. Ideally, all points of toxicant emissions in the upstream system will also be analysed in the same manner, but such an endeavour is currently unfeasible. In this study, we attempted to avoid a known data gap—i.e. the effects of mixtures and unknown substances in the effluent—through an alternative form of LCIA. This raises the question of whether introducing a bias towards a better description of a significant emission is better than continuing with a poorer description of the impacts of all emissions. There are precedents in the LCA literature that favour the former type of inconsistency in LCIA in exploring method development (e.g. Holmquist et al. 2021).

Considering the main function of the system (i.e. wastewater treatment) and its main flow (i.e. treated wastewater to the recipient), the employed approach might be justifiable. Previous research has indicated that contaminants in discharged wastewater can dominate the aquatic ecotoxicity potential of water utilities (Lundie et al. 2004), so if focus is to be laid somewhere first, wastewater quality is a reasonable target. Nevertheless, if other points of emission in the upstream systems are also subject to bioassay evaluations, the relative significance of the wastewater discharge can be expected to decrease, and thus, our conclusions should be considered preliminary. Nonetheless, we use this point as a call to better characterise all emissions from the technosphere.

4 Conclusions

This study applied a recently developed method for using bioassays as a basis for toxicity evaluation in LCAs to assess AWT in a Swedish setting. Two configurations of ozonation with post-treatment were compared: biological treatment with MBBRs and adsorption to GAC filters. The main findings are as follows:

- Incorporation of bioassay data into LCA may reveal a markedly greater benefit from implementing AWT at WWTPs compared with assessments that relied solely on chemical analysis, although the magnitude of the increased benefit is highly sensitive to the choice of reference substance. Nevertheless, this approach holds promise for capturing the influence of unknown contaminants, trace concentrations, and mixture effects.
- Overall, technical variations within each configuration, such as the source of ozone and the lifespan of activated carbon, were at least as significant as the choice of configuration itself with regard to environmental impact. In the Swedish context, in which electricity is predominantly fossil-free, the principal environmental trade-offs between configurations were observed between the impacts of GAC production and regeneration (across all impact categories) versus heavy metal removal (notably, human toxicity, cancer).
- Based on bioassay data, both configurations performed comparably in terms of toxicity reduction; however, additional data—particularly from site-specific and full-scale installations and concerning AhR activation—are needed to substantiate these findings.
- The biological endpoints that represented oxidative stress and AhR activation emerged as the most important indicators for freshwater ecotoxicity and human toxicity, cancer, respectively. The normalised and weighted results indicate that the reduction in AhR activity was the primary driver of the reduction in impact, even when considered in the context of the overall environmental footprint of the entire WWTP. However, using a different AhR reference substance alters the overall AWT benefit.
- Guidelines should be established on the selection of biological endpoints, bioassays, reference substances, and impact categories. Also, the development of characterisation factors and the expansion of relevant databases are necessary to support more robust assessments. Moreover, further exploration of the interpretation of LCIA results is necessary, especially when bioassays can be used only in parts of the system. Finally, testing the approach in varied contexts to validate applicability and efficacy is essential.

In the long term, incorporating bioassays within LCA will facilitate more comprehensive and robust evaluations, identifying the WWTPs that would generate significant benefits from the implementation of AWT. In this manner, the net environmental benefits can be weighed rigorously against the broader impacts that are associated with quaternary treatment—as mandated by the revised UWWTD—thus guiding informed prioritisation and decision-making on whether to implement AWT technologies.

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Author contribution Sofia Högstrand: conceptualisation, data curation, investigation, formal analysis, methodology, visualisation, writing—original draft, writing—review and editing. Greg Peters: conceptualisation, methodology, writing—review and editing. Magdalena Svanström: conceptualisation, methodology, supervision, writing—review and editing. Linda Önnby: conceptualisation, methodology, writing—review and editing.

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Data availability Data will be made available upon request.

Declarations

Competing interests The authors declare no competing interests.

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