Bioanalytical Tools in Advanced Water Treatment

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Abstract

Globally, today’s augmented water consumption increases the pressure on quantity and quality of resource water, resulting in water bodies containing adverse organic trace pollutants which can negatively influence reproduction and biodiversity in aquatic organisms.

Since wastewater treatment plants do not entirely remove them, these pollutants can affect humans by re-entering the urban water cycle after entering the aquatic environment.

The impact of organic trace matters can be assessed by bioassays and biological endpoints because these evaluate various substances that have the same effect on organisms. Therefore, application of labour-intensive and incomplete chemical analysis may be reduced.

The local importance is evident as the effluent of Vienna’s wastewater treatment plant discharges into the river Danube and is, downstream, used as a drinking water source by the city of Bratislava after riverbank filtration.

This paper explores the application of bioassays for toxicological investigations of wastewater treatment plants’ effluents.

Bioassays’ current use in the evaluation of wastewater treatment will be discussed and compared to chemical analysis. In Austria, several bioassays and biological endpoints could be available for testing the effect of organic pollutants. In advanced water treatment activated carbon adsorption and ozonation eventually reduce their effect.

Keywords

Oestrogenic substances, bioassays, advanced water treatment, ozonation, activated carbon filtration

Contents

1 Purpose of the research

In the course of time, hormones and hormonally active substances, especially oestrogens, have constituted an increasingly problematic factor in terms of adverse effects on aquatic organisms. Even traces of these substances are potentially perilous to both human beings and aquatic organisms.

In Austria, water quality has generally increased over the last few decades. Nevertheless, the problem of endocrine disruptors in water has become a more controversial topic. These hormonal substances appear in various compounds that can be detected in wastewater. Apart from endogenous steroid hormones of women and men, endocrine disruptors also appear in synthetic hormones, for instance medication and contraceptives, in agricultural pesticides, plants like soy or broccoli and industrial products. According to their mode of action they can be further classified into oestrogenic, antioestrogenic, androgenic and antiandrogenic substances.

A significant increase of the amount of oestrogenic compounds in water bodies over the last few decades led to an inadvertent feminising effect on fish population [1]. In male fish, the hormonal agents in question directly stimulate the synthesis of vitellogenin, a precursor protein of egg yolk, and has a negative impact on the procreation of these species. Some limit values regarding the relative virility have already been investigated. [2]

The upgrade of the biological wastewater treatment plant of Vienna's main wastewater treatment plant towards a system with full nitrification/denitrification and phosphorous removal has augmented not only the removal of nitrogen, but also of various organic trace compounds. Especially, oestrogenically active substances such as nonylphenol or oestrone belong to a substance group that could be removed better after the upgrade. Importance of this topic is given because the effluent of the Viennese plant discharges into the river Danube that is, about 50 kilometres downstream, after river bank filtration used as drinking water source by the city of Bratislava.

In order to meet the objective of further removal of organic trace pollutants in general and endocrine active substances in particular from water and secure high quality of the drinking water in Bratislava, research concerning a fourth (advanced) wastewater treatment step is conducted. In Austria, the combination of ozonation and a subsequent activated carbon filtration has already been investigated at a pilot plant. In this process, organic trace compounds can be removed from water, as ozone and emerging OH-radicals can degrade organic trace substances including hormones. Remaining organic trace compounds can be removed from the water in a subsequent activated carbon filtration step, where granules of carbon are used for adsorbing organic substances. In activated carbon adsorption, two different forms, either powdered or granulated active carbon, can be used. [3-6]

Considering that the concentrations of different oestrogenic substances in the water need to be assessed, bioassays can be used. These are methods of analysis using living cells or even organisms to measure the reaction of different biological endpoints on cells and hereby show the existence of the organic trace compounds in question. In comparison with the conventional chemical analysis to detect trace compounds of concern, bioassays work effect-based. In this sense, three different levels of tests can be used, namely *in vitro, in vivo* and *in situ* assays. [7]

This paper aims at describing the use of bioassays in the evaluation of the efficiency of wastewater treatment on the removal of organic trace pollutants. This is represented by the particular issue of oestrogenic hormonal agents, mainly found in natural female steroid hormones, and their environmental effects. Some examples of these endocrine disruptors are the female steroid hormone 17β-oestradiol and the synthetic steroid hormone 17α-ethinyl-oestradiol, commonly applied as a contraceptive. With a focus on *in vitro* assays, which already are used in the evaluation of advanced water treatment, other tools are described only briefly. Results from bioassays will be compared to chemical analysis. The results from bioassays in biological wastewater treatment will also be correlated to results from advanced wastewater treatment to evaluate the efficiency of ozonation and activated carbon adsorption. Data is taken from relevant literature or previous research projects and will be supported by an experiment self-conducted at a pilot plant for advanced wastewater treatment by ozonation and activated carbon filtration in Frauenkirchen, Austria.

2 Methods

2.1 Effects of oestrogenic substances in water

Natural and synthetic oestrogens, like oestradiol, oestrone and oestriol, enter the wastewater through human excretion. None of these substances are completely removed even by conventional wastewater treatment plants even applying the best technology available and commonly used (nitrogen removal). Hence, the hormones and other oestrogenic agents that are not removed can be found in the effluent, which further on directly influences aquatic organisms. In case of the female sexual hormone 17β-oestradiol, the oestrogenic receptor of the concerned cell is activated after binding with 17β-oestradiol. Together they build the oestrogen-receptor-ligand-complex, which activates the expression of numerous genes. This effect can be detected in the liver of fish and other affected vertebrates, such as birds, amphibians and reptiles. Within a progressive biochemical process, e.g. in fish the protein vitellogenin is synthesized. Accordingly, one possibility to examine the concentration of oestrogenic substances in the water is the determination of the vitellogenin concentration in aquatic animals, which act as biomarkers. [8] This protein is then modified into egg yolk and occurs in male fish too, leading to their feminisation. Direct consequences of this process are the disruption of the sexual determination, disturbance of the balance of steroid hormones and the imitation of female sexual hormones. Consecutively, this effect has a negative impact on the procreation of concerned species. [9]

2.2 Effect and employment of bioassays

To measure the potential biological effects of an environmental sample, bioassays are applied as analytical tools. This method employs living cells and organisms or investigates subcellular effects such as DNA damage and receptor activation. These tests can further scale the toxicity of a mixture of substances. This is of vital importance as in some cases, substances do not lead to any toxicological effects when they occur separately but have combinatory effects.

Offering a wide range of possible detection on various biological levels, bioassays are divided into three main groups. While *in vitro* assays examine subcellular or monocellular systems, *in vivo* assays work with organisms, for instance algae or fish. Both are applied in the laboratory, whereas *in situ* assays are used on site, using biological endpoints in aquatic organisms as indicators. [10, 11]

Related to oestrogenic substances, *in vitro* assays are mostly applied to detect the concentration of the concerned substances in water samples. Thereby, certain cellular mechanisms are used to measure the effects of substances with comparable mechanisms. Cell cultures, transgenic bacteria and genetically modified yeast cells are predominantly applied. [12]

Specific sample treatment is needed before the actual examination in ecotoxicological tests can be conducted. However, this pre-treatment needs to be implemented with care as it might cause serious alternations in the final composition and therefore could affect the results.

The assays monitor the receptor binding potential in terms of the potency of oestrogens. 17α-ethinyl-oestradiol (EE2), 17β-estradiol (E2) and estrone (E1 hereby are most relevant substances. The results derived are called "E2-equivalents" or EEQs. The potency of E2 lies between the less potent E1 and the more potent EE2.

$$RQ = \frac{MEC\ or\ EEQ}{QC} \quad (1)$$

The measured environmental concentration (MEC) or the concentration of E2 (EEQ) obtained from the tests then is compared to the quality criteria (QC) proposed by the European Union to determine the risk quotient (RQ), a boundary value for acceptable oestrogenic effects in Eq. (1).

Even small concentrations, in the range of nanograms per litre, have effects and therefore constitute a risk for aquatic organisms, which are persistently exposed to these hormonal substances.

Considering the small concentrations of oestrogenic substances in the water, a sensitive measurement tool is required. "In most cases, *in vitro* assays are considered sensitive, because they measure effects on a low organisational level." [13, 14]

2.3 Applied *in vitro* bioassays to detect oestrogenic substances

When selecting a suitable bioassay to detect a certain trace compound, specific criteria need to be taken into consideration. Beside technical requirements such as the adoptability, sensitivity, reproducibility and robustness, cost-efficient aspects as well as the effect-manifestation duration of the measurement process are important selection criteria. [15]

Two different *in vitro* assays are commonly referred to as approved tools to detect oestrogenic activity in the water. They are namely the Yeast Estrogen Screen (YES) assay and the HEP-Vtg assay.

At the moment, the YES assay is the most common one to monitor oestrogenic effects in wastewater treatment, as this test is comparably uncomplicated to administer and perform. Within this procedure, the genetically-modified yeast *saccharomyces cerevisiae* is used to achieve the necessary receptor binding. The test takes about 96 hours, which can be considered a long duration.

In contrast, hepatocytes of fish are used for the HEP-Vtg assay. By measuring the production of vitellogenin, the oestrogenic activity can be determined. A similar *in vivo* assay monitors the vitellogenin concentration by examining blood samples of fish. However, it has to be taken into consideration that this assay is more complex and time-consuming than the comparable *in vitro* assay.

Other generally approved and employed bioassays are the ER-CALUX and the T47D-Kbluc assay, which are more sensitive in comparison with the YES assay. The ER-CALUX® detects the luciferase activity to examine the induction of oestrogenic reporter genes for hERα and β. The CALUX® ERα is currently applied at the wastewater treatment plant in Frauenkirchen to determine the estrogenic effect before and after advanced wastewater treatment with ozonation and activated carbon. This assay measures the oestrogenic activity with the oestrogen receptor alpha by a process called a hormone-mediated mechanism of action. In general, luciferase assays as used in Frauenkirchen are highly sensitive. Through luminescence of the cells exposed to the investigated sample, the concentration of oestrogenic substances in the water sample can be measured. [16, 17, 18]

2.4 Status quo of bioassay application and regulatory aspects in the EU

In member states of the EU, surface water bodies need a good ecological status that results from a combination of several quality elements to fulfil the Water Framework Directive (WFD). [19] Additionally to the fact that there is a list of substances that are limited in surface waters and therefore would be needed to be removed from wastewater, EU's member states "are required to assess whether they or other potential pollutants are discharged in significant quantities into water bodies." The EU outlines the suitability of effect-based tools for investigative monitoring and recommends further studies for water safety.

Some member states have already implemented chemical approaches with bioassays on a national level in order to meet the EU's requirements or to attain national environmental objectives. Examples currently include aquatic hazard detection, surface water evaluation and the assessment of oestrogenic activity with *in vitro* assays (Czech Republic, Denmark, Ireland, Netherlands, Germany), various research projects or case studies as well as the use of other effect-based tools, especially *in vivo* assays with *Daphnia magna* and algae (Italy, Netherlands). Although there are examples of the implementation of effect-based tools in a wastewater treatment context, monitoring programs outside the scientific community seem to focus on general limnic, marine or sediment assessment. [20]

2.5 Ozonation and activated carbon adsorption as treatment strategies

With the help of advanced wastewater treatment, a more effective treatment method concerning organic trace compounds and especially hormonally active substances can be achieved. In this type of advanced water treatment, ozonation or activated carbon adsorption or – as in our case - a combination of ozone and activated carbon is used to further remove organic trace pollutants from wastewater. In addition to chemical analysis, this process is evaluated by bioassays.

Ozonation is the first stage of this type of advanced wastewater treatment. When implementing ozone, there are two main possibilities how a pollutant are removed.

Ozone can react directly with the pollutant forming an oxidation or transformation product. As most of the direct reactions with ozone run very slowly, the main factor is not the oxidation potential, which is indeed very high (+2,07 V), but rather the kinetics. The reaction does not only run slowly, but it also is highly selective for electron dense chemical bounds, which provides great benefits, but also disadvantages.

The second mechanism for removing pollutants is the reaction with OH-radicals which are formed during direct reactions of ozone with certain functional groups such as phenols and amines. As OH-radicals are highly reactive, they would react with almost every compound found in water, which is relevant for the elimination of ozone refractory substances.

Radicals easily accumulating oxygen will often form as an intermediate product (biradical). Those radicals will go on reacting or they will disproportionate. If they continue the reaction, substances like peroxides, aldehydes, acids or hydrogen peroxide might form. The actual end product cannot be predicted. As some of the products might have a toxicological impact, the conditions have to be adjusted so that the least toxic product is most likely to be formed within the process.

Moreover, OH-radicals hold another problem. As they react very unselectively, they might react with so-called scavenger substances, whose products behave unreactively towards ozone. The chain reaction will be stopped and the organic trace pollutants that need to be degraded will not be degraded as the ozone within the water is already consumed. Therefore, it is important to know the scavenger substances and their concentrations within the water to properly adjust the ozone concentration. [21]

In the post treatment stage, an activated carbon filter removes by-products such as peroxides, aldehydes, acids or hydrogen peroxide. Water is passed through a granulated activated carbon filter (GAC filter) which adsorbs biradicals and by-products. Due to its big surface, many molecules that are too small to be filtered otherwise can be adsorbed. After a certain time, the GAC load becomes a dynamic equilibrium between adsorbent and adsorbate, adsorbent loaded with adsorptive. This equilibrium is temperature-dependent.

$$X = \alpha \bullet C^{1/\beta} \qquad (2)$$

This balance can be expressed through the empirical formula by Freundlich in Eq. (2), X being the specific load in g/kg (e.g. a solid material), α being the empirical affinity coefficient (adsorptive/adsorbent), β the empirical parameter for saturation of the adsorbent surface and C the equilibrium concentration of the solution in mg/L representing the residual concentration. [22]

The biological degradation of potentially hazardous by-products also plays an important role in the post treatment stage, though it cannot be said whether the GAC filter or the biological degradation is the decisive removal mechanism.

2.6 Construction of an Ozonation and Activated Carbon Treatment Plant

A combination of ozonation and activated carbon adsorption is used at the pilot plant in Frauenkirchen, Austria, after the treatment stages of a conventional wastewater treatment plant (screen, the sand filter, primary sedimentation tank, aeration tank, secondary sedimentation tank). The advantage of this combination of procedures compared to a single-stage 4th treatment stage, for example only ozonation or only activated carbon filtration, is being a multi barrier system. [23]

As shown in Fig. 1, the setup of the pilot plant is the following:

The oxygen tank (feed gas supply for ozone production) and the ozone generator are located separately due to safety reasons. The ozone generator produces the ozone which is then transferred to the ozone reactors, in which the concentration of the ozone during the treatment is controlled, based on the DOC and nitrite load. The liquid ozone is transferred to the ozone reactors through injectors. [24, 25]

Before entering the activated carbon filter, the water flows through a post-reaction tank (N1). To generate a consistent flow increasing the reaction volume, the effluent from N1 is pumped to the activated carbon filters.

The activated carbon filters, normally granulated activated carbon (GAC) filters, are used to adsorb by-products of the ozonation which might be dangerous.

[26]

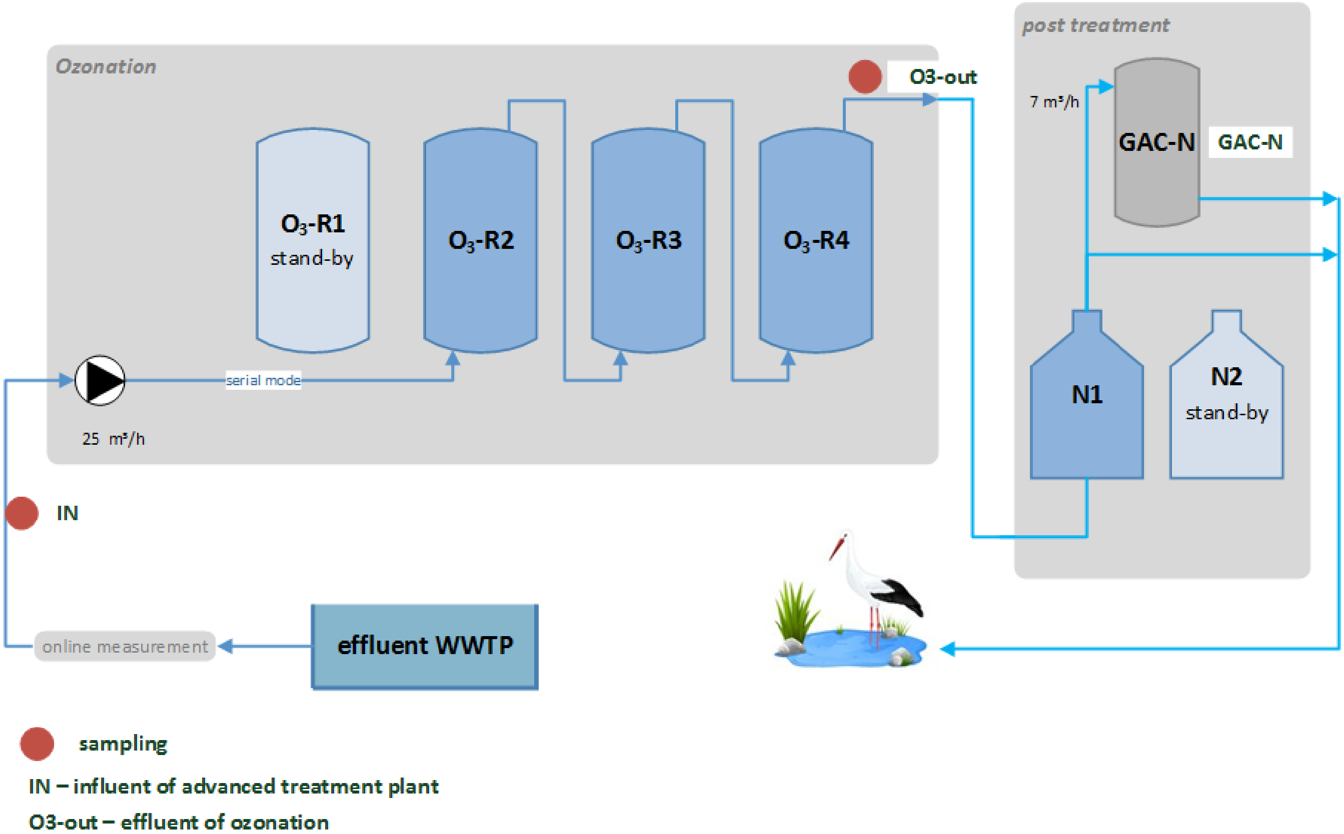

Figure 1: Flow scheme of the pilot plant

3 Results

3.1 Comparing effect-based tools to conventional chemical analysis

Chemical analysis on the one hand requires knowledge about substances in a sample in advance (target analysis). Bioassays, on the other hand, are able to detect the toxicity of unknown substances without any precedent analysis and are hence called effect-based. Additionally, cumulative effects that occur when an organism is simultaneously exposed to a mixture of pollutants, can be monitored. Cumulative effects often appear due to the large number of substances present in the aquatic environment. *In vitro* assays only require small amounts of samples but, nevertheless, react to a variety of substances with high sensitivity. For that, they are available at relatively low costs. These advantages might be crucial in wastewater treatment because of the wide range of trace pollutants that need to be removed. [27, 28]

Nevertheless, the duration of their effect-manifestation, minutes to days for *in vitro* tools, seem to be a slight disadvantage. If a hazard of biological magnification is provided, the effects cannot be monitored directly "for ethical, scientific, practical and economic reasons." However, cost effectiveness and monitoring efficiency are most likely to be reached through a combination of chemical and effect-based analysis, using the same sample for both procedures. [29]

3.2 Description of the conducted experiment

When implementing advanced treatment, the dose of ozone and activated carbon has to be adjusted to the wastewater entering the purification plant. In order to determine the ozone concentration needed for proper adjustment of the plant, the following examination was taken. First, the concentration of the dissolved organic carbon (DOC) within the influent of the pilot plant was determined. The DOC represents the organically bonded dissolved carbon that is typically determined by oxidising dissolved carbon, to carbon dioxide, and then measuring the concentration of the gas. [30]

In the illustrated case in Table 1, the determined DOC-concentration was 5mg/L. Afterwards, the concentration of NO_2-N was determined. Photometry was used to examine the concentration of 0.06 mg/L within the influent of the plant.

Figure 2: Photometer

Photometry is a spectroscopical method of analysis using the interaction between the electromagnetical radiation and the particles of the sample in order to determine its concentration. In the applied test the nitrite bonds with reagents and forms a colour This colour results in the adsorption of light depending on the concentration of the nitrite.

The total inflow volume of the pilot plant was set to 25 m^3/h. Thus, the DOC load is 125 and the NO_2-N load is 1.5 g/h. In addition to the DOC as the major consumer of ozone, nitrite can play an important role, depending on its concentration. The ozone consumption of NO_2-N is based on the stoichiometric reaction of ozone and nitrite, resulting in 3.43 g ozone consumed per g NO_2-N oxidized. For the investigated influent (concentrations, see Table 1) the ozone consumption of nitrite amounts to 5.1g O_3/h. A specific ozone of 0.6 g O_3/g DOC has proved to be adequate for reducing the concentration of many organic trace compounds, but it has to be NO_2-N compensated. The required ozone load for the given concentrations of DOC and NO_2-N is 80.1 g per hour, calculated by multiplying the specific ozone dose with the DOC concentration and adding the NO_2-N ozone consumption. As the gas flow is 3 m^3 per hour, the targeted ozone concentration equals 26.71 g/m^3.

Table 1: Calculation of the targeted ozone concentration in the feed gas

DOC concentration	5 mg/L
NO_2-N concentration	0.06 mg/L
Influx	25 m^3/h
DOC load	125 g/h
NO_2-N load	1.5 g/h
Ozone consumption NO_2-N	5. 1 g ozone/h
Specific ozone dose (NO_2-N compensated)	0.6 g ozone/g DOC
Ozone load	80.1 g ozone/h
Gas flow	3 m^3/h
Ozone concentration	26.71 g/m^3

These calculations have to be recalculated regularly as the DOC concentration and the NO_2-N concentrations may vary over time, therefore a continuous ozone dose control is required. As the water entering the purification plant exhibits a wide range of different origins and fluctuations over time and season, taking these measures is especially important in wastewater treatment in contrast to drinking water treatment.

Examining the DOC and NO_2-N concentrations is usually performed on site and is later re-evaluated in the laboratory. The accuracy of the figures determined on site is acceptable.

Samples were taken before and after ozonation within the pilot plant.

The sample that was taken before the ozonation had the typical yellowish colour due to the presence of humic substances, while the sample that was taken after treatment was much clearer. An olfactory difference from a mouldy scent to an ozone odour reminiscent of chlorine could also be noticed.

With UV measurement, the change in absorption by ozonation can be monitored. Fig. 4 demonstrates the effect of ozonation resulting from the oxidation of the organic matter absorbing in the UV range, mostly double bonds and aromatics that are well oxidized by ozone. The concentration of certain indicator substances, namely carbamazepine, diclofenac, benzotriazole and acesulfame K, was determined. While carbamazepine and diclofenac belong to highly ozone reactive substances, benzotriazole and acesulfame K react moderately with ozone. Oestrogenically active substances, such as natural

Figure 4: UV spectroscope

hormones, the synthetic hormone 17α-ethinyl-oestradiol or bisphenol A, belong to the same indicator group as the indicator substances carbamazepine and diclofenac with regard to their ozone reactivity. Therefore, the same behavior (removal) can be assumed during ozonation.

Since a removal of >99% (below the limit of detection) was determined for both carbamazepine and diclofenac, a reduction in the same order of magnitude can be estimated for the estrogens. The moderately reacting substances acesulfame K and benzotriazole were eliminated to a lower degree (33 and 34%).

The decline of the UV absorbance that can be seen at 254 nm correlates with the removal of organic trace pollutants.

At the time the samples were taken, activated carbon was not operated. It would have led to an even higher stage of removal of trace pollutants, which means that the removal efficiency through advanced water treatment is even higher than in the example above.

3.3 Possible consequences based on the interpretation of the results

Removing organic trace compounds does not only result in a better ecological status, but also provides the city of Bratislava with a safer drinking water source the Danube is used as. Considering the changes in colour, odour and the organic trace compounds, an effect can already be observed after ozonation treatment. Mainly through biological degradation, advanced wastewater treatment with a combination of ozonation and subsequent activated carbon also tackles potentially hazardous by-products. [31, 32]

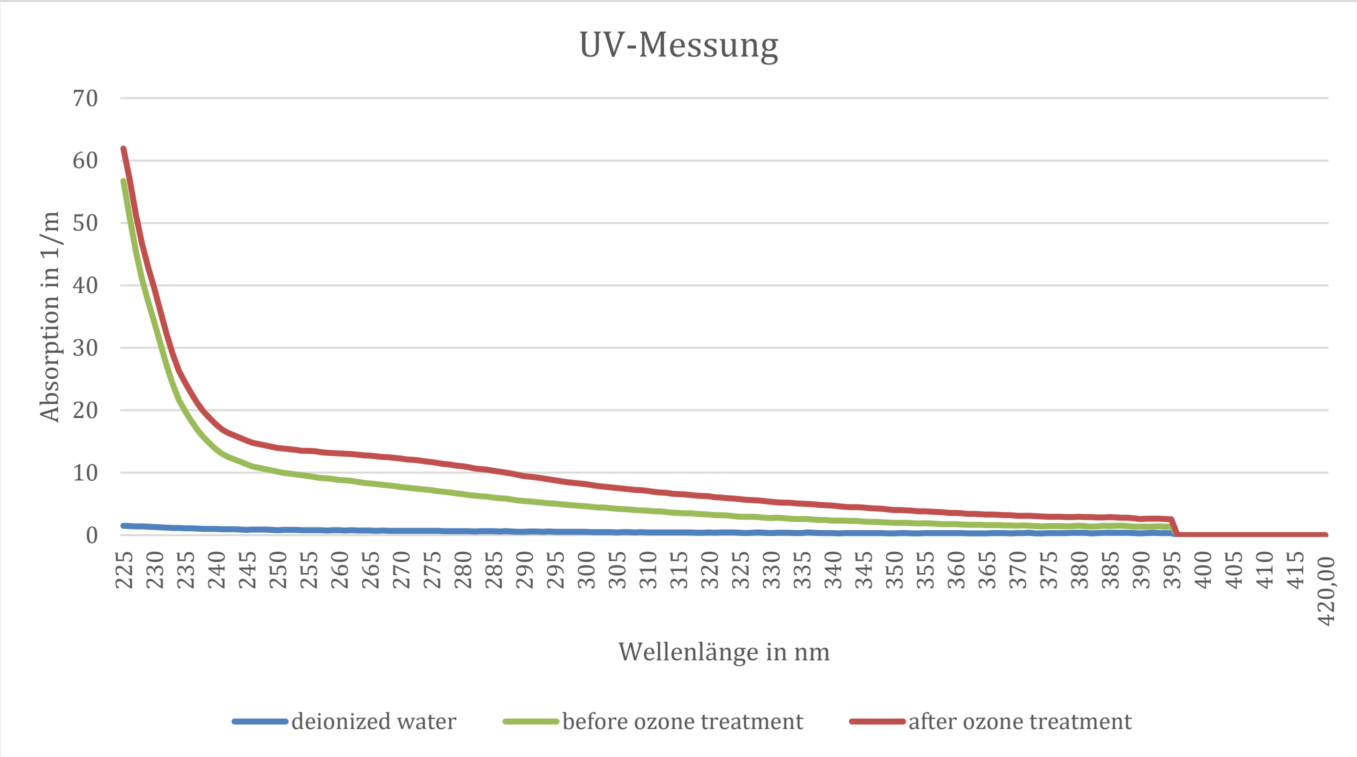

Figure 3: Determination of oxidized organic matter through UV measurement

4 Conclusion

For endocrine disrupting substances can negatively influence both animals and humans, an efficient wastewater treatment is inevitable as such substances easily enter the urban water cycle through wastewater. Advanced wastewater treatment with ozone and activated carbon offers an adequate multi-barrier approach to reduce these emissions to the aquatic environment. It enables the removal of endocrine disruptors and subsequently reduces their toxicological effect.

Bioassays help to assess the removal of toxicologically relevant substances and consequently improving them by evaluating their functioning.

Improving wastewater treatment plants will be especially relevant in the very near future, as the European Union might soon release new and stricter guidelines regarding the quality of effluents coming from wastewater treatment plants. In order to meet these guidelines, effect-based tests on water quality are required in addition to traditionally used chemical analysis methods.

Apart from governmental guidelines, the prioritized goal is to achieve a maximum removal efficiency for toxicological substances, especially endocrine disruptors, in order to preserve our environment and minimize health risks for both humans and animals.

Currently used assays are either expensive, though still comparatively cost-effective for monitoring, hard to produce, or the results need a long time to be available. Therefore, improving bioassays will be an important measure over the next decades.

5 Acknowledgements

We would like to thank the team of KomOzAk II at TU Wien, especially Dr. Norbert Kreuzinger and Dr. Heidi Schaar, for showing us the pilot plant in Frauenkirchen and providing us with relevant articles and papers. This project could not have been done without their great and continuous support.

Moreover, we want to thank to Viktoria Satzinger and Martin Windischhofer, our teachers, who introduced us to important topics related to water and guided us through the process of preparation.

Further, we wish to thank Julian Kalitzke, a friend who supported us in terms of layout and graphic design.

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