Oekotoxzentrum
Eawag
Überlandstrasse 133
Postfach 611
CH-8600 Dübendorf

T +41 (0)44 823 55 62
F +41 (0)44 823 58 63
info@oekotoxzentrum.ch
www.oekotoxzentrum.ch

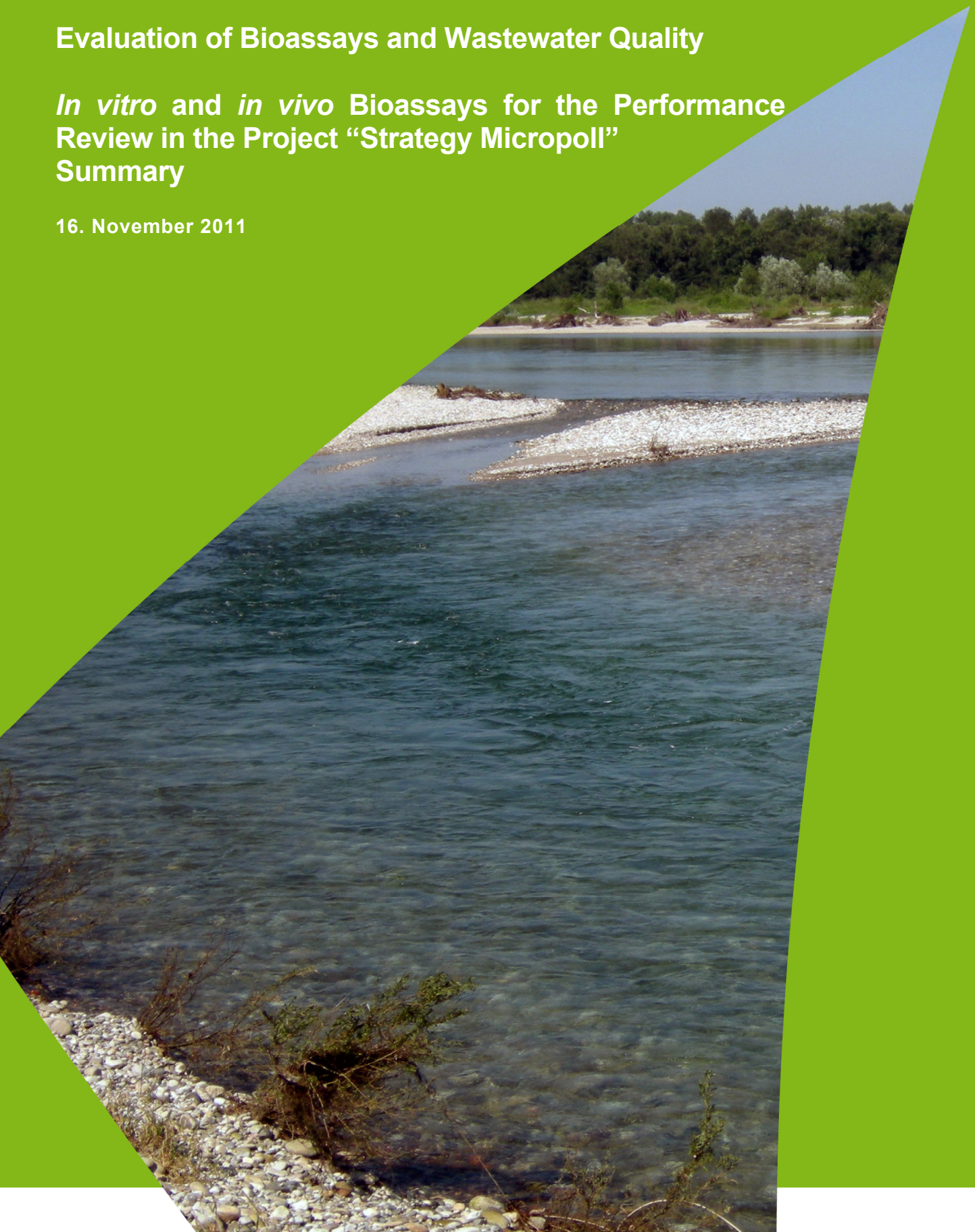
oekotoxzentrum
centre ecotox

Schweizerisches Zentrum für angewandte
Oekotoxikologie | Eawag-EPFL

Evaluation of Bioassays and Wastewater Quality

In vitro and *in vivo* Bioassays for the Performance Review in the Project "Strategy Micropoll" Summary

16. November 2011



Imprint

Publisher

Swiss Centre for Applied Ecotoxicology (Ecotox Centre), Eawag-EPFL, 8600 Dübendorf, Switzerland

Authors

Swiss Centre for Applied Ecotoxicology, Eawag/EPFL	Cornelia Kienle, Robert Kase, Inge Werner

Acknowledgements

The initiators of the project as well as the authors would like to thank the following persons for their valuable comments and contributions:

Federal Office for the Environment	Michael Schärer, Sebastién Lehmann, Bettina Hitzfeld
WWTP Vidy Lausanne	Denis Thonney, Anoÿs Magnet
Expert group ecotoxicology within the project “Strategy Micropoll”	Pius Niederhauser (AWEL ZH), Andreas Häner (BMG Engineering), Sergio Santiago (Soluval Santiago), Christoph Hafner (Hydrotox), Tilman Floehr (RWTH Aachen), Mirco Bundschuh, Ralf Schulz (University of Landau), Mirco Weil (ECT), Helmut Segner (University of Bern), Matthias Liess (Helmholtz-Zentrum für Umweltforschung Leipzig-Halle), Phillipe Vioget (Canton de Vaud), Jörg Oehlmann (University of Frankfurt), Jeanne Garric, Benoit Ferrari (Cemagref)
AWEL Zürich	Barbara Känel
BMG Engineering	René Gälli
Eawag	Christian Abegglen, Roman Ashauer, Rik Eggen, Juliane Hollender, Kristin Schirmer
University of Lausanne	Nathalie Chèvre
EPF Lausanne	Jonas Margot
Biodetection Systems	Peter Behnisch, Harrie Besselink, Sander van der Linden,
RWTH Aachen	Henner Hollert, Sibylle Maletz, Tilman Floehr
Entrix Inc., CA	Markus Hecker, Eric Highley
Swiss Centre for Applied Ecotoxicology, Eawag/EPFL	Marion Junghans, Petra Kunz, Tamas Mengesha, Rebecca Flück

Note

This study was carried out on behalf of the Federal Office for the Environment (FOEN) of the Federal Department of the Environment, Transport, Energy and Communications (DETEC) within the project “Strategy Micropoll” and was co-funded by the Swiss Centre for Applied Ecotoxicology. The publisher bears sole responsibility for the content.

Contact

Cornelia Kienle cornelia.kienle@oekotoxzentrum.ch, +41(0)58 765 5563

Robert Kase robert.kase@oekotoxzentrum.ch, +41(0)58 765 5197

Citation Proposal

Kienle, C., Kase, R., Werner, I. 2011. Evaluation of bioassays and wastewater quality: *In vitro* and *in vivo* bioassays for the performance review in the Project "Strategy MicroPoll". Swiss Centre for Applied Ecotoxicology, Eawag-EPFL, Duebendorf.

This study was initiated by the Swiss Federal Office for the Environment.

Dübendorf, Swiss Centre for Applied Ecotoxicology, Eawag/EPFL, 16.11.2011

Cover picture: Andri Bryner, Eawag

Summary

The goal of the FOEN project “Strategy Micropoll“ was to develop a strategy regarding micropollutants originating from wastewater. Besides an analysis of water quality in Switzerland and other aspects, this included the evaluation of the efficiency of complementary wastewater treatments such as ozonation followed by sand filtration (ozonation-SF) and different processes including powdered activated carbon addition (PAC) in eliminating micropollutants from wastewater treatment effluent. Two large-scale pilot studies were conducted at the wastewater treatment plants (WWTP) Wüeri in Regensdorf (ozonation followed by sand-filtration) and Vidy in Lausanne (ozonation followed by sand-filtration and PAC followed by ultrafiltration). The studies were done in close collaboration with experts from research, practice and with personnel and financial support of cantonal water protection agencies (Gewässerschutzfachstellen) and the operators of the WWTPs.

In both studies, samples before and after various treatment steps were analysed for micropollutants and ecotoxicological effects, in order to get insight into the efficiency of the different treatment steps. The focus laid on studying the effects of the advanced treatment technologies, ozonation and PAC, on the removal efficiency for polar, persistent and bioactive substances as well as possible side products and their biological effects.

This report focuses on the study performed at the WWTP Vidy in Lausanne from April 2009 to July 2010 with four major measurement campaigns being conducted. Detailed information on the Regensdorf study can be found in Abegglen et al. (2009). The Lausanne study is described in Margot et al. (2011).

Results at the WWTP Vidy showed that both ozonation-SF and PAC-UF treatments are useful measures to reduce the concentration and biological effects of micropollutants in treated wastewater and therefore in surface water bodies. Total elimination efficiency with regard to the measured specific effects was generally above 80 %. Similarly, advanced treatment led to lowered risk quotients, i.e. the relationship between measured concentrations and environmental quality standards, as well as reduced toxicity in bioassays, and thus lowered risk of adverse effects. There was no evidence of toxic effects due to the formation of stable by-products during ozonation (i.e. by-products still present after the final filtration step). A final filtration step with biological activity (such as a sand filter) after ozonation is recommended, in order to minimize the risk of reactive and potentially toxic ozonation by-products being released in waterbodies.

Overall, the application of bioassays for comparing the performance of advanced wastewater treatment methods has proven to be relevant and useful. In general, *in vitro* bioassays were deemed most promising for the routine monitoring of the performance of advanced treatment in WWTPs, however only specific cellular effects are assessed in those tests. Certain *in vivo* bioassays also showed the beneficial effects of ozonation-SF and PAC-UF treatment such as the fish early life stage test with *Oncorhynchus mykiss*.

Main conclusions

- A broad range of micropollutants and their effects were eliminated by more than 80% after the advanced treatments.
- There was no evidence for a toxicity increase due to a constant formation of stable toxic ozonation by-products.
- An ozonation should be followed by a final filtration step with biological activity.
- Quality of treated effluent was significantly improved, leading to improved surface water quality.

Table of contents

Abbreviations

AA-EQS	Annual average environmental quality standard
AR CALUX	Androgen receptor CALUX
BOD	Biochemical oxygen demand
CAG	Effluent carbon filter (sortie charbon actif granulé)
CALUX	Chemically Activated LUciferase gene EXpression
COD	Chemical oxygen demand
CI	Change Index
DEQ	Diuron equivalent concentration
DOC	Dissolved organic carbon
EC_x	Effect concentration at which x % of the test organisms show a defined effect in a concentration response curve
EEQ	Estradiol equivalent concentration
EN	WWTP Influent (Entrée STEP)
EQS	Environmental quality standard
ERα CALUX	Estrogen receptor α CALUX
FELST	Fish Early Life Stage Test
GR CALUX	Glucocorticoid receptor CALUX
ISO	International Organisation for Standardisation
LF	Effluent moving bed biology (sortie Lit fluidisé)
LOEC	Lowest observed effect concentration
MAC-EQS	Maximum acceptable concentration EQS
MC	Measurement campaign
MEC	Measured environmental concentration
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
OZ	Effluent ozonation, Sortie O_3
PAC-UF	Effluent powdered activated carbon treatment followed by ultrafiltration (sortie charbon actif en poudre – UF)
PEC	Predicted environmental concentration
PPARg1 CALUX	Peroxisome proliferator activated receptor g1 CALUX
PR CALUX	Progesterone receptor CALUX
Q_{347}	Low tide discharge

RQ	Risk quotient
SB	Effluent biological treatment, "old" biology (sortie biologie)
SF	Effluent sand filtration (sortie filtre à sable)
SOP	Standard operation procedure
STEP	Station d'épuration des eaux usées
TEQ	Toxic equivalent concentration
TRβ CALUX	Thyroxin receptor β CALUX
WWTP	Wastewater treatment plant
YES	Yeast Estrogen Screen

1. Introduction

1.1. Project Strategy Micropoll

The aim of the project “Strategy Micropoll” of the Swiss Federal Office for the Environment (FOEN) was to develop a strategy regarding micropollutants originating from municipal wastewater.

In the frame of this project, a situation analysis was conducted in order to assess the contamination of Swiss surface waters with micropollutants (Gälli et al., 2009), and an evaluation concept for Swiss specific micropollutants was developed (Götz et al., 2010; Götz et al., 2011). A further step of the project consisted of a performance review aiming to define suitable indicators and methods to detect organic micropollutants in surface water and wastewater, which should be used to assess the efficiency of the applied advanced treatment methods. Additionally, possibilities for financing measures for advanced wastewater treatment were evaluated.

In order to evaluate possible technical treatments to reduce the concentrations and effects of organic micropollutants in Swiss surface waters, two large-scale pilot studies were conducted, one at the WWTP Wüeri in Regensdorf and the other at the WWTP Vidy in Lausanne. In both studies the efficiency of complementary wastewater treatment for the elimination of micropollutants from wastewater treatment effluent was assessed, such as ozonation followed by sand filtration (ozonation-SF) and different processes including powdered activated carbon addition (PAC). Technical aspects as well as a performance review regarding the elimination of micropollutants using chemical measurements and ecotoxicological test systems were included. Regarding the ecotoxicological methods, the Federal Office for the Environment was advised by a board of international experts within the project “Strategy Micropoll”.

The present report is focused on the bioassay results of the pilot study at the WWTP Vidy in Lausanne. The results for the pilot study at the WWTP Wüeri in Regensdorf are reported in Abegglen et al. (2009).

1.2. Aims of the performance review at the WWTP Vidy, Lausanne

The aim of the performance review on the WWTP Vidy in Lausanne was to gain knowledge from trace analytics and ecotoxicological test systems regarding the effects of complementary treatments on the contamination of wastewater with organic micropollutants.

The first part of the performance review focused on the effects of the advanced treatment technologies ozonation followed by sand filtration (ozonation-SF) and powdered activated carbon treatment followed by ultrafiltration (PAC-UF) on the removal efficiency of polar, persistent and bioactive substances as well as possible by-products. In a second part, the wastewater quality was investigated regarding micropollutants and their effects, followed by a discussion on the relevance of the observed reduction in micropollutant concentrations and effects.

As shown in earlier studies, micropollutants can seriously affect the aquatic ecosystem, e.g. influence the macrozoobenthos community (Ashauer, in preparation; Bundschuh et al., 2011a) or influence the reproduction of fish (e.g. estrogens) (Jobling et al., 2006; Kidd et al., 2007; Sumpter, 1998).

1.3. Questions to be addressed by the performance review

The following central questions were to be answered through the evaluation and comparison of bioassays in the performance review:

- How effective is ozonation-SF or PAC-UF treatment for the removal of micropollutants and their biological effects? What is the expected effect on aquatic organisms?
- What is the significance/informative power of the tests regarding elimination efficiency? Are the tests able to detect differences between the different treatment steps?
- If a test shows no effect of WWTP influent, does that mean that the wastewater is unproblematic?
- How reproducible are the bioassay results? If a test shows variable effects, can this variability be explained? Do similarly treated or untreated samples cause similar effects (e.g. WWTP influent samples, ozonated samples etc.)?
- How relevant are the various assays for a monitoring programme (performance assessment of advanced treatment steps)? Can bioassays be applied for this purpose?

2. Executive Summary

In the pilot study at the WWTP Vidy, Lausanne, a variety of parameters were measured, including classical water quality parameters (DOC, COD, BOD, pH, conductivity, nutrients etc.), aqueous concentrations of 58 micropollutants (EPF Lausanne) and 120 other micropollutants, as well the formation of the by-products nitrosamines and bromates (Eawag, Dübendorf). Additionally a biological effect assessment of the different treatment steps was made with 16 *in vitro*- and 9 *in vivo*-assays to cover a broad range of ecotoxicological effects.

2.1. Research Questions Addressed

In the following section, the questions addressed in chapter 1.3 are answered based on the results obtained during the pilot study. Details on the used approach and the results can be found in the chapters 3 to 5.

How effective is ozonation-SF or PAC-UF treatment for the removal of micropollutants and their biological effects? What is the expected effect on aquatic organisms?

Various studies showed that a considerable number of micropollutants are not or incompletely removed during biological treatment (e.g. Abegglen et al., 2009; Gälli et al., 2009; Götz et al., 2010; Schärer et al., 2010). Additional treatment by ozonation-SF or PAC-UF increased the removal efficiency of most of those substances significantly. These techniques were able to eliminate the majority of the specifically acting substance classes (see chapter 4.4) as well as most of the toxicity observed in *in vivo* assays (see chapter 4.3.2 and 4.3.3).

Ozonation-SF and PAC-UF treatments proved to be very effective in the removal of micropollutants. Chemical analyses showed improved elimination efficiency for most of the micropollutants compared to the biological treatment. For both advanced treatments the overall elimination efficiency of micropollutants ranged from ~75 to 90 % (Margot et al., 2011).

Most mechanism-oriented, cellular *in vitro* bioassays and one integrative *in vivo* bioassay (Fish Early Life Stage Test) showed a significant reduction in toxicity through ozonation-SF and PAC-UF treatment. Importantly, there was no consistent increase in toxicity due to the additional treatments.

What is the significance/informative power of the tests with regard to elimination efficiency? Are the tests able to detect differences between the different treatment steps?

The evaluation of the elimination efficiency of different substance classes is only possible with mechanism-oriented cellular *in vitro* bioassays with sample enrichment, as they refer to toxic equivalent concentrations (see chapters 4.1 and 4.4).

Generally, it has to be kept in mind that the enrichment of samples eliminates matrix components such as salts (e.g. nutrients, which might mask the toxicity of the samples) as well as metals (Macova et al., 2010). Enrichment of water samples also enables a detection of low concentrations of substances using short-term bioassays. However, the sample composition may be altered and substances might be lost during the enrichment procedure. Such issues

have to be assessed thoroughly before an extraction procedure is selected, and for more information on this topic refer to Escher et al. (2005), Escher et al. (2008b). In native wastewater samples the undisturbed sample is measured, however toxic effects might be masked by positive effects of nutrients, e.g. growth promotion due to nutrients in algae assays.

In the Lausanne pilot study, the *in vitro* bioassays with sample enrichment showed a decrease of specific substance classes both after ozonation-SF and PAC-UF treatment. The overall reduction of specific activities was above 80% in most cases. One *in vivo* bioassay was able to detect the decrease in micropollutant concentrations as well (by indicating decreasing effects after ozonation-SF and PAC-UF treatments compared to the biological treatment), the Fish Early Life Stage Test with rainbow trout, *Oncorhynchus mykiss*. This assay is especially relevant as *O. mykiss* is a representative of sensitive cold water fish species, and therefore relevant for Swiss surface waters. Also a decrease in trout catches of more than 60% since the early 80s indicates its sensitivity for multiple stressors which are not yet identified (Burkhardt-Holm et al., 2005).

A decrease in toxicity following biological treatment was observed in most of the applied *in vivo* bioassays when the WWTP influent was toxic. However, most *in vivo* assays were unable to detect any additional reduction in toxicity following the ozonation-SF or PAC-UF treatment steps. This could be due to the lack of sensitivity of the respective bioassay endpoints (e.g. mortality, growth) for micropollutants (for details see chapter 4.3).

If a test shows no effect of WWTP influent, does that mean that the wastewater is unproblematic?

This question can clearly be answered: No.

There is evidence in the literature that there are effects in ecosystems (e.g. Ashauer, in preparation; Bundschuh et al., 2011a), even when some tests show no toxicity. There are several factors which may play a role for the toxicity of a wastewater sample, such as:

- Test duration (e.g. acute, chronic, whole-life-cycle)
- Test organism
- Sensitivity of the test regarding the specific type of wastewater
- Sensitivity and selectivity of the chosen endpoint

Many standard test organisms are not the most sensitive species of the respective taxonomic group, due to e.g. difficulties with lab cultivation or longer life cycles. At the same time, some standard test organisms are sensitive to other types of wastewater e.g. landfill leachate, industrial waste water, but are apparently not that suitable to detect effects of micropollutants in urban wastewater. However, as organisms integrate the effects of all potentially toxic substances in an environmental sample it cannot be concluded for sure that the observed effects result from micropollutants.

How reproducible are the bioassay results? If a test shows variable effects, can this variability be explained? Do similarly treated or untreated samples cause similar effects (e.g. WWTP influent samples, ozonated samples etc.)?

Differences in the results of the different measurement campaigns were observed. Due to differences in wastewater composition and in changes of process parameters like dosage of

ozone or PAC, it is difficult to assess the reproducibility of the assays from the Lausanne pilot study. Indeed this variability was reflected in the chemical analyses as well (Margot et al., 2011).

Variability in the bioassays performed in this study might reflect differences in the wastewater composition during different sampling campaigns, changes of process parameters during treatment, differences in sample handling between measurement campaigns and in the laboratories, and the variability of the respective test procedure itself.

Nevertheless a reduction in toxicity for the different treatment steps was detected by:

- mechanism-oriented cellular *in vitro* bioassays after sample enrichment for all measurement campaigns,
- integrative *in vivo* bioassays for selected measurement campaigns.

Additionally, the sampling procedure and the sample handling are important factors which might cause differences in the test results. Therefore these procedures must be determined in advance considering the analyses to be made (e.g. what sampling devices and material to use, which filtration technique, how should the samples be stored etc.), and integrated in the study design in order to enable good comparability of the results and to eliminate the possibility that measured effects may be based on different sample handling and storage conditions.

2.2. Summary

In the pilot study at the WWTP Vidy, Lausanne, it was demonstrated that ozonation-SF and PAC-UF treatment are both useful measures to reduce the concentrations and biological effects of micropollutants in waterbodies. The overall elimination efficiency regarding specific effects was generally above 80% (see chapters 4.3 and 4.4).

There was no evidence for a toxicity increase due to a constant formation of stable toxic by-products in the ozonation, i.e. by-products still present after the final filtration step (see chapters 4.3 and 4.6).

In order to reduce the risk of reactive and potentially toxic ozonation by-products being released in waterbodies, a final filtration step with biological activity after ozonation is recommended (for example sand filtration).

Overall the advanced treatments led to strongly lowered risk quotients (between 6 and 13 times) as well as to a reduction in toxicity in bioassays compared to the effluent of the biological treatment, and thus a lowered risk of adverse effects for aquatic organisms in receiving waterbodies (see chapter 5).

The application of bioassays for the performance review of advanced wastewater treatment has proven to be relevant and useful. In general, mechanism-oriented, cellular *in vitro* bioassays were deemed most promising for a routine monitoring of the performance of advanced treatment in WWTPs, however only specific effects are assessed in these tests. Certain *in vivo* bioassays could also show the beneficial effect of ozonation and PAC-UF treatment, such as the Fish Early Life Stage Test with rainbow trout (*Oncorhynchus mykiss*).

It can be concluded that the quality of the treated effluent was significantly improved, leading to improved surface water quality.

3. Approach

3.1. Bioassays

A broad range of biotests for the evaluation of water and wastewater quality is available. An important goal of this project was to identify appropriate bioassays sensitive enough to detect the effects of micropollutants. The selection of ecotoxicological test systems was based on preliminary studies conducted before the first pilot study at the WWTP Wüeri Regensdorf, and on surveys performed during the Regensdorf pilot study with 17 bioassays, including tests for measuring specific cellular effects, as well as integrative tests with whole organisms (Abegglen et al., 2009). Based on the results of these studies, and on the input from an international expert group on ecotoxicology, the most suitable tests as well as several additional mechanism-oriented *in vitro* assays were chosen for the Lausanne pilot study. A set of 16 *in vitro* and 9 *in vivo* bioassays was selected based on one or more of the following selection criteria (Abegglen et al., 2009):

- Test sensitivity is high enough to detect contaminant effects in treated wastewater (WWTP effluent) in the preliminary studies
- Standardised test methods are available (OECD-, DIN or ISO certification)
- Consideration of different trophic levels (bacteria, algae, macrophytes, invertebrates, vertebrates)
- Application of different types of sample processing and test systems:
    a) assessment of enriched wastewater samples
    b) assessment of wastewater samples without sample enrichment
    c) effect measurements with organisms in flow-through systems (channels, microcosms)

Two types of bioassays were used:

- *In vitro* bioassays based on specific cellular mechanisms measure cellular effects specific to groups of toxicants with similar modes of action. These assays use cell cultures or transgenic bacteria or yeast to detect changes in receptor activation or enzyme function, e.g. endocrine, genotoxic or mutagenic effects; or inhibition of signal transduction.
- For a better evaluation of integrative effects on whole organisms, validated and standardised *in vivo* assays with test species from different trophic levels (algae, macrophytes, invertebrates and fish) are applied. These assays measure effects on parameters such as growth, reproduction, feeding activity and mortality, as well as effects based on more specific biochemical endpoints, e.g. vitellogenin concentration in the fish early life stage assay.

In this report, the following assays were referred to as *in vitro* bioassays based on the specific endpoints measured: Ames, micronucleus and umuC assay, YES and CALUX assays, H295R assay and combined algae assay, similar to Ratte and Ratte (2009). The term i*n vivo* bioassays was used for assays with bacteria, algae, duckweed, aquatic crustaceans, oligochaetes, snails and fish.

It is important to note that both test types, *in vitro* and *in vivo* bioassays, answer qualitatively different questions. *In vitro* bioassays based on specific cellular mechanisms, often combined with sample enrichment, enable a highly sensitive detection of certain chemical substance classes such as estrogens or herbicides, which are also relevant as micropollutants. However, interpretation of the results in an ecological context and extrapolation to potential consequences for whole organisms is difficult.

On the other hand, integrative *in vivo* bioassays without the need for sample enrichment provide conclusive information about biological and potentially ecological effects such as growth, reproduction, and mortality. They integrate the effects of all substances in a wastewater sample such as chemicals, nutrients etc., but, depending on the used assays they give none or only limited information about the causative substance classes or relevant molecular processes. Another important point is that, depending on the endpoints used in the assay, the sensitivity of the frequently applied test systems is often too low for the evaluation of the contamination with micropollutants. This may be due in part to the limited exposure time or the lower sensitivity of the standard bioassay species when compared to native species, as well as to the lack of sensitivity of the investigated endpoints.

Figure 1 and Table 1 provide an overview of the tests performed in this study. The appropriate bioassays selection aimed to cover relevant modes of action, such as mutagenicity, genotoxicity, endocrine disruption (e.g. androgen, estrogen, and glucocorticoid receptor activation, modulation of steroidogenesis), and herbicidal effects. Integrative *in vivo* bioassays were chosen to detect general toxicity of the wastewater to whole organisms from different trophic levels, as well as effects based on more specific endpoints, such as vitellogenin induction and number of offspring.

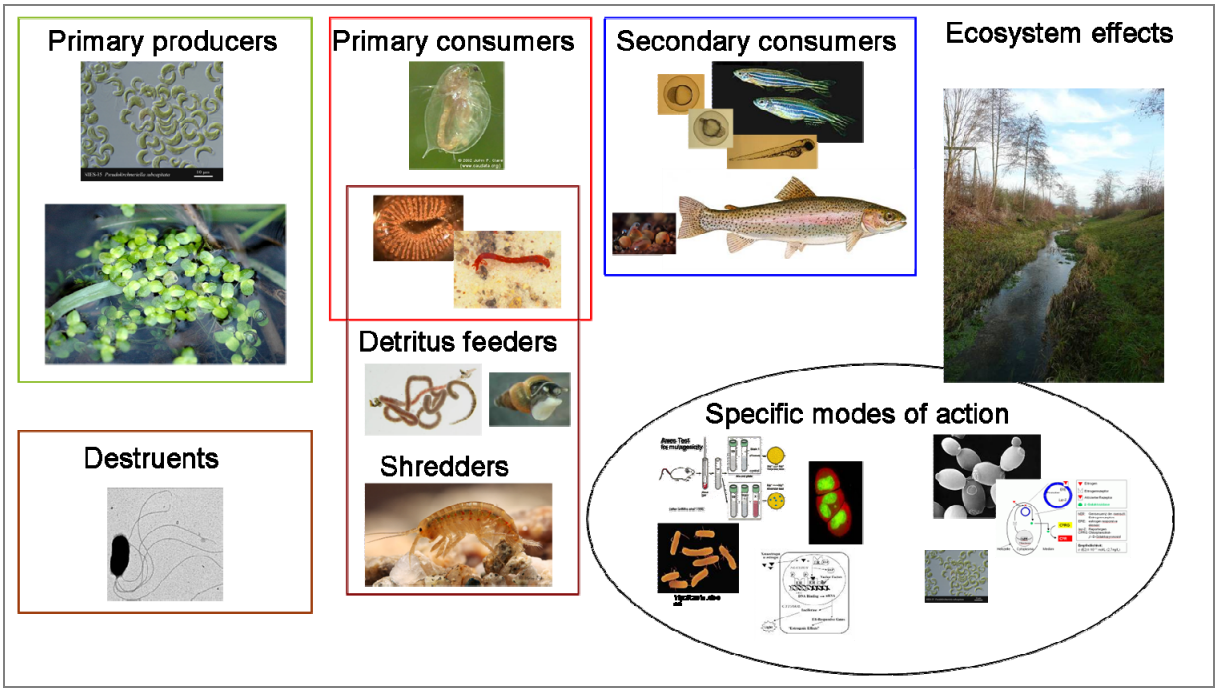


Figure 1: Overview of test organisms and test systems used in the two pilot studies Regensdorf and Lausanne

Table 1: Overview of the applied test systems, test organisms and detectable effects.

Test	Organism	Detectable Effect (*endpoint,* test duration)	Performing Laboratory
Test systems based on specific cellular mechanisms (without sample enrichment) / *in vitro* – assays			
Ames test according to ISO 16240 *(International Organization for Standardization, 2005b)*	*Salmonella typhimurium*	mutagenicity *(number of revertant/mutated colonies)*	Hydrotox (D)
Micronucleus assay according to ISO 21427-2 *(International Organization for Standardization, 2006)*	cell line VC79 of the Chinese hamster	genotoxicity *(formation of micronuclei as indication of DNA damage)*	Hydrotox (D)
Test systems based on specific cellular mechanisms (with sample enrichment) / *in vitro* – assays			
UmuC assay according to ISO 13829 *(International Organization for Standardization, 2000)*	*Salmonella typhimurium*	genotoxicity *(induction of the SOS response of the cell)*	Ecotox Centre,(CH), Hydrotox (D)
Yeast Estrogen Screen (YES) *(Routledge and Sumpter, 1996)*	*Saccharomyces cerevisiae*	estrogenic effects *(receptor binding)*	Ecotox Centre (CH)
CALUX10-Panel (ERα- + Anti-ERα- CALUX, AR- + Anti-AR-CALUX, GR- + Anti-GR- CALUX, PR- + Anti-PR- CALUX, PPARg1-+ Anti-PPARg1-CALUX, TRβ- CALUX) *(Van der Linden et al., 2008)*	human cell line	effects on various hormone receptors *(receptor binding)*	BDS (Biodetection Systems) (NL)
H295R Steroidogenesis Assay *(Gracia et al., 2006)*	human cell line of adenocarcinoma cells	effects on the genesis of steroidal hormones *(production of estradiol and testosterone)*	RWTH Aachen (D) Entrix Inc. (CA)
Combined algae assay (Chlorophyll fluorescence/ algae growth) *(Quayle et al., 2008)*	*Pseudokirchneriella subcapitata*	a) Inhibition of photosynthesis after 2h (herbicide action) *(chlorophyll fluorescence)* b) unspecific growth inhibition after 24 h (OD)	Ecotox centre (CH)
Standardised test systems without enrichment of the wastewater samples / *in vivo* – assays conducted in the laboratory			
Bacteria luminescence inhibition assay according to ISO 11348-3 *(International Organization for Standardization, 2007b)*	*Vibrio fischeri*	disturbance of ATP synthesis, (*inhibition of bioluminescence after 30 min)*	Soluval Santiago (CH)
Green algae growth assay according to ISO 8692 *(International Organization for Standardization, 2004)*	*Pseudokirchneriella subcapitata*	growth *(cell number* after 72 h)	Soluval Santiago (CH)

Lemna minor growth assay according to ISO 20079 *(International Organization for Standardization, 2005c)*	*Lemna minor*	growth *(frond number and biomass* after 7 d)	Soluval Santiago (CH)
Chronic daphnia reproduction assay according to ISO 20665 *(International Organization for Standardization, 2005a)*	*Ceriodaphnia dubia*	reproduction, mortality (after 7/8 d)	Soluval Santiago (CH)
Investigations on amphipods *(Bundschuh et al., 2011b)*	*Gammarus fossarum*	feeding activity, mortality (after 7 d)	Institute for Environmental Sciences, University of Landau (D)
Snail reproduction assay *(Duft et al., 2002)*	*Potamopyrgus antipodarum*	reproduction, mortality (after 28 and 56 d) endocrine disruption	RWTH Aachen (D)
Fish egg assay according to DIN 38415-6/ ISO 15088:2007 *(Deutsches Institut für Normung, 2003; International Organization for Standardization, 2007a)*	*Danio rerio*	mortality, lethal endpoints (48 h)	ECT (D)
Standardised test systems without enrichment of the wastewater samples / *in vivo* – assays conducted in flow through systems at the WWTP Vidy			
Lumbriculus-Reproduction assay according to OECD Guideline 225 *(OECD, 2007)*	*Lumbriculus variegatus*	reproduction, biomass (28 d)	ECT (D)
Fish Early Life Stage Test (FELST) according to OECD Guideline 210 *(OECD, 1992)*	*Oncorhynchus mykiss*	hatching rate, mortality, deformations, behavioural disturbances (swim up of larvae), growth (length and weight), vitellogenin concentration (endpoint for endocrine disruption) (69 d)	ECT (D)

Background information on effects and respective bioassays used in this study is provided below.

Test systems based on specific cellular mechanisms, *in vitro* – assays

DNA Damage: In order to address the possible formation of reactive ozonation by-products, which are known to damage DNA (for review see Victorin, 1992, 1996), in this study, genotoxicity as well as mutagenicity were measured using the micronucleus and umuC assay (genotoxicity) and the Ames assay (mutagenicity).

Genotoxicity describes any damage to the genome (Williams, 1989), and when genotoxic substances act on the cell, they can elicit DNA strand breaks, the insertion or deletion of bases, and shifts of the DNA reading frame. The majority of those changes is detected and reversed by the cellular repair system, however if repair is not possible the changes are passed on during cell

division. This event is referred to as mutagenicity, the heritable, irreparable consequences of genotoxicity.

Hormonal Effects: WWTPs are the main source of hormonally active chemicals, and discharge their effluent directly into surface waters. Estrogenic substances have a high risk potential in susceptible waterbodies (NFP 50, 2008), being especially important as they have a wide range of implications on organisms and ecosystems (e.g. Kidd et al., 2007) and may affect the health and reproduction of wildlife in very low concentrations (e.g. Burkhardt-Holm et al., 2005; NFP 50, 2008). Therefore, special attention to those substances was paid in the performance assessment.

Estrogenicity was measured using receptor-based *in vitro* systems. The binding of estrogenic substances to the human estrogen receptor (hER) was assessed using the YES (Routledge and Sumpter, 1996) and the ER CALUX assays (Van der Linden et al., 2008).

Apart from estrogenic substances, various other hormonally active substances can be present in the water which may exert effects on organisms. To test if those substances can also be removed by advanced wastewater treatment, the effects on various hormone receptors (such as the androgen, glucocorticoid, progesterone and thyroid hormone receptor) were assessed with several CALUX assays (Van der Linden et al., 2008).

The cellular effects of hormonally active substances on hormone production and metabolism was measured by quantifying cellular hormone levels through the H295R steroidogenesis assay, which uses a human cell line of adenocarcinoma cells able to produce steroid hormones (such as testosterone or estradiol) and integrates effects on all relevant metabolism pathways leading to the production of those hormones (Gracia et al., 2006).

Herbicidal Effects: To assess specific effects of herbicides, in particular inhibitors of the photosystem II, the combined algae assay with the green algae *Pseudokirchneriella subcapitata* was performed. It detects specific effects on the photosynthesis of unicellular green algae as well as unspecific effects on cell growth (Schreiber et al., 2002).

Most samples used for the bioassays described above were tested in enriched form, except for the micronucleus and the Ames assay, where native wastewater samples were assessed. In both variations different dilution steps were tested.

Standardised whole organism tests at different trophic levels, *in vivo* –assays

Primary Producers: To assess effects of municipal wastewater on primary producers, growth tests were performed with the single-celled green algae *P. subcapitata* (using native wastewater samples, and growth as sole endpoint), and with the duckweed *Lemna minor* as representatives of higher water plants or macrophytes. Both organisms play a central role in the aquatic ecosystem. They serve as food source for organisms of higher trophic levels, such as crustaceans, mussels and other filtering and detritus feeding invertebrates or vertebrates. Additionally, macrophytes play an important role for the structure of a waterbody and serve as habitat for numerous species.

Primary Consumers: The water flea *Ceriodaphnia dubia* was chosen as primary consumer and filter feeder. They are amongst others feeding on green algae, and serve as an important food source for larval fish and other aquatic species. Standardised tests with *C. dubia* are one of the

most widespread bioassays used for evaluating the quality of surface water and effluent samples (e.g. U.S. Environmental Protection Agency, 2002).

Among sediment feeders, aquatic oligochaetes, such as *Lumbriculus variegatus* play an important ecological role and serve as food source for benthivorous fish. Sediment feeding and inhabiting (endobenthic) organisms are directly exposed to toxic substances. They can alter the bioavailability of substances bound to the sediment to other organisms (OECD, 2007; Phipps et al., 1993) as they mix sediments (bioturbation) and bioaccumulate chemicals.

The New Zealand mud snail *Potamopyrgus antipodarum* belongs to the group of detritus feeders, living as grazer on stones and water plants. Original from New Zealand, the species is now abundant in Switzerland as well. *P. antipodarum* reproduces parthenogenetically and is viviparous, its fecundity being a sensitive indicator for exposure to estrogenic or androgenic active substances (Duft et al., 2002). Therefore, this test organism was included in the set of biotests.

The freshwater amphipod *Gammarus fossarum* is a key species in stream ecosystems. It shreds organic material, thus assisting in the recycling of nutrients and organic carbon, and is an important prey organism e.g. for fish (Karaman and Pinkster, 1977; Kunz et al., 2010). Laboratory and *in situ* tests with these organisms can provide valuable information on the effects of pollutants on important ecological functions in aquatic ecosystems (e.g. Bundschuh and Schulz, 2011; Kunz et al., 2010).

Secondary Consumers: Fish are the most important secondary consumers in aquatic ecosystems. As embryos and larvae are considered especially sensitive to pollutants (e.g. Pascoe and Shazili, 1986); effects of wastewater were assessed using two standardized early life stage tests:

- the zebrafish (*Danio rerio*) embryo assay was used to assess mortality and lethal endpoints in fish embryos. It is a test which is often used for the toxicity assessment of wastewater samples and replaces the acute fish test with adults in the German wastewater regulation (Deutsches Institut für Normung, 2003; Nagel, 2002).
- the fish early life stage test (FELST) with rainbow trout (*Oncorhynchus mykiss)*, a cold water fish species and therefore especially relevant for Swiss waterbodies. In previous studies, effects of estrogenic substances (vitellogenin induction) on this fish species were detected (e.g. Pawlowski et al., 2003; Stalter et al., 2010b). Vitellogenin is a precursor protein of egg yolk normally only found in female fish. Induction of vitellogenin concentration in fish was used as an indicator for estrogenic activity elicited by the test medium (Weil, 2010). In addition, hatching rate, mortality, deformations, behavioural disturbances (swim up of larvae) and growth (length and weight) were assessed.

A number of *in vivo* bioassays were found not to be sensitive enough to assess the elimination efficiency of the complementary wastewater treatments studied here: the bioassays based on ISO 11348-3 (V. *fischeri*), ISO 8692 *(P. subcapitata)*, ISO 20079 (*L. minor*), ISO 20665 (*C. dubia*) and DIN 38415-6 (*D. rerio*) (see Abegglen et al., 2009). Nevertheless, these assays as well as the bioassays measuring genotoxicity and mutagenicity were used in this study in order to detect potential adverse effects of ozonation by-products. Additionally, they are capable of detecting toxicity of untreated wastewater samples (without enrichment) and more polluted effluents, e.g. industrial wastewater (e.g. Cordova Rosa et al., 2001; Tchounwou et al., 2001).

Samples used for *in vivo* bioassays, were tested in different dilution steps, except for the assays with *P. antipodarum*, *L. variegatus, G. fossarum* and *O. mykiss*, which were exposed only to undiluted wastewater samples.

3.2. Sample Collection and Preparation

Four large and several small measurement campaigns (MC) were carried out to assess the performance of the complementary treatments, ozonation and PAC-UF on the WWTP Vidy in Lausanne, which is built to treat municipal wastewater of 220'000 person equivalents. More specific information on technical details of the WWTP and the sampling campaigns can be found in the final report on the pilot study (Margot et al., 2011). The technical procedures were optimized in the 4th measurement campaign.

Table 2 lists the sampling dates and technical information on the respective ozone and powdered activated carbon treatments.

Table 2: Ozone concentrations and powdered activated carbon types and concentrations applied in the four measurement campaigns (according to Margot et al., 2011).

Sampling date	Ozone dose (mg O_3/g DOC)	Powdered activated carbon dose (mg PAC/L wastewater)
20.-27.07.09	0.5	No PAC treatment
30.10.-05.11.09	0.7	10 (Norit)
10.-17.03.10	0.8	12 (Sorbopor)
26.05.-02.06.10	1.1	20 (Sorbopor)

Samples were collected time-proportionaly using automated sampling devices for chemical analysis of micropollutants, and ecotoxicological tests described in chapter 3.1 and Table 1. Additionally classical water quality parameters were assessed: dissolved organic carbon (DOC), biological oxygen demand (BOD), chemical oxygen demand (COD), pH, nutrients. Details regarding the selection and analysis of micropollutants can be found in Margot et al. (2011).

The samples were collected at the following points (see Figure 2):

- WWTP influent (Entrée STEP, EN)
- Effluent biological treatment ("old" biology, sortie biologie, SB) (1st measurement campaign)
- Effluent moving bed biology (Sortie lit fluidisé, LF) (2nd - 4th measurement campaign)
- Effluent ozonation (Sortie O_3, OZ)
- Effluent carbon filter (Sortie charbon actif granulé, CAG) (1st and 2nd measurement campaign)
- Effluent sand filter (Sortie filtre à sable, SF) (3rd and 4th measurement campaign)
- Effluent powdered activated carbon treatment followed by ultrafiltration (Sortie charbon actif en poudre - UF, PAC-UF) (2nd - 4th measurement campaign)

Figure 2 gives an overview of the different treatment steps and sampling points.

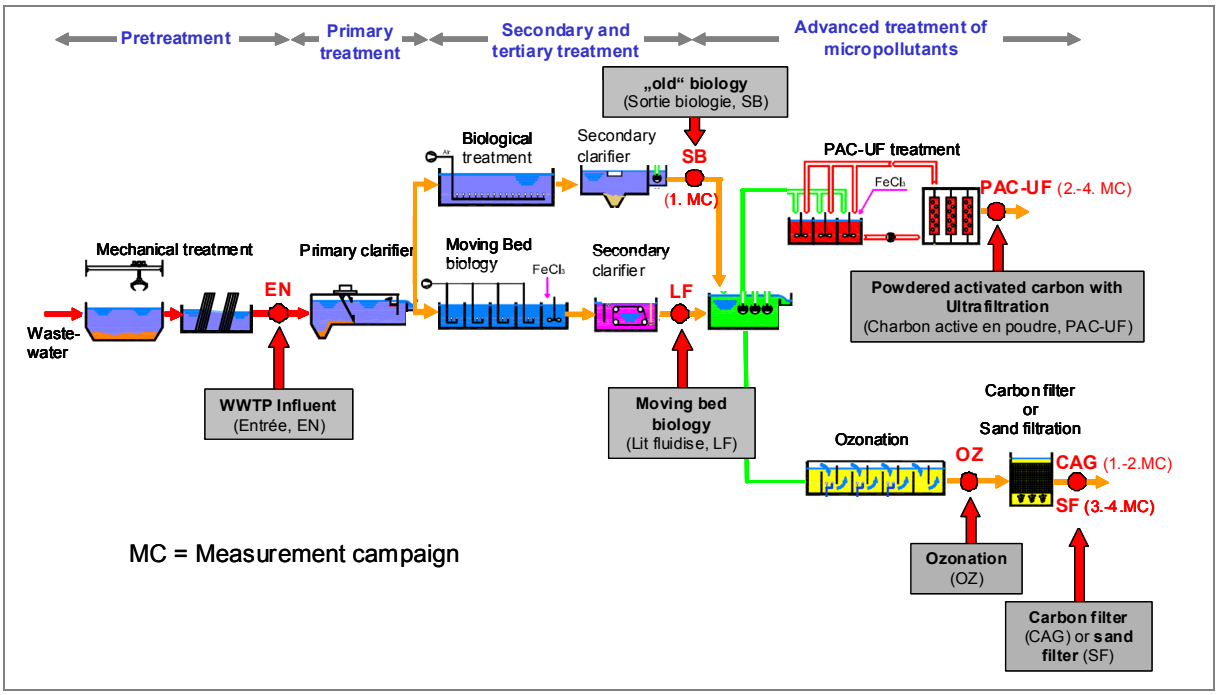


Figure 2: Overview of the sewage treatment plant Lausanne and the different sampling points.

The classical water quality parameters were assessed in the daily composite samples as soon as possible after collecting, usually on the same day. For bioassays, daily composite samples were collected during one week, filled in conditioned glass bottles, filtered through a glass fibre filter (1 µm, Millipore, type APFD 09050) and stored at 4°C. These samples were then proportionally combined to obtain 2, 3 or 7-day-composite samples, which were submitted to analytical chemistry and used in bioassays. Samples were transported to the responsible laboratories cooled on ice or frozen.

For most *in vitro* bioassays focusing on specific cellular mechanisms, 7-day composite samples were enriched using solid phase extraction (SPE) at the Swiss Centre for Applied Ecotoxicology, Dübendorf, CH (see Table 1). Following standard operating procedures (Eawag, 2007), 250 ml (influent samples) or 500 mL (all others) were filtered using C18/EN cartridges (Supelco, Sigma-Aldrich, USA) (Escher et al., 2008b); 500 mL of Millipore water served as a blank. After extraction using 4*1 mL of Acetone and 1 mL of Methanol, 250 times (influent) or 500 times (all other sampling points) enriched samples were stored in 1 mL of a solvent mixture (~50% ethanol, ~50% acetone and methanol) at -20°C until being transported on dry ice for analysis.

4. Performance Analysis of Advanced Wastewater Treatment

4.1. Toxicity Parameters

Biological parameters measured in bioassays, such as mortality, number of offspring, cell number, weight, and cellular receptor activity, are commonly referred to as 'endpoints'. The term 'toxicity parameter' refers to effect values, which are calculated using statistical or mathematical methods (see also Figure 3). They are defined as follows:

EC_x The EC_x is the effective concentration (or % test/effluent sample) at which x % (e.g. 10, 20 or 50 %) of its maximal effect is reached, e.g. 50 % of the test organisms show a defined effect. The calculation is done by regression analysis and gives, additionally to the respective derived toxicity parameter, a confidence interval (usually 95% confidence interval), which stands for the concentration range in which the 'real' value lays with a probability of 95 %.

The lower the EC_x-value, the more toxic the evaluated substance or sample is.

NOEC The no observed effect concentration (NOEC) is the highest tested concentration that does not yet cause a statistically significant effect compared to the control.

LOEC The lowest observed effect concentration (LOEC) is the lowest tested concentration that elicits a statistically significant effect compared to the control.

TEQ The toxic equivalent concentration (TEQ) is defined as the concentration of a reference substance, which would have the same effect as the environmental sample (see e.g. Escher et al., 2008a). The reference substances vary depending on the type of the measured specific endpoint. The TEQ allows to express toxic potency (or toxic quantity) of a mixture as concentration of a reference chemical and integrates the effects of all substances with the same mode of action.

The higher the TEQ value, the more toxic the evaluated sample is.

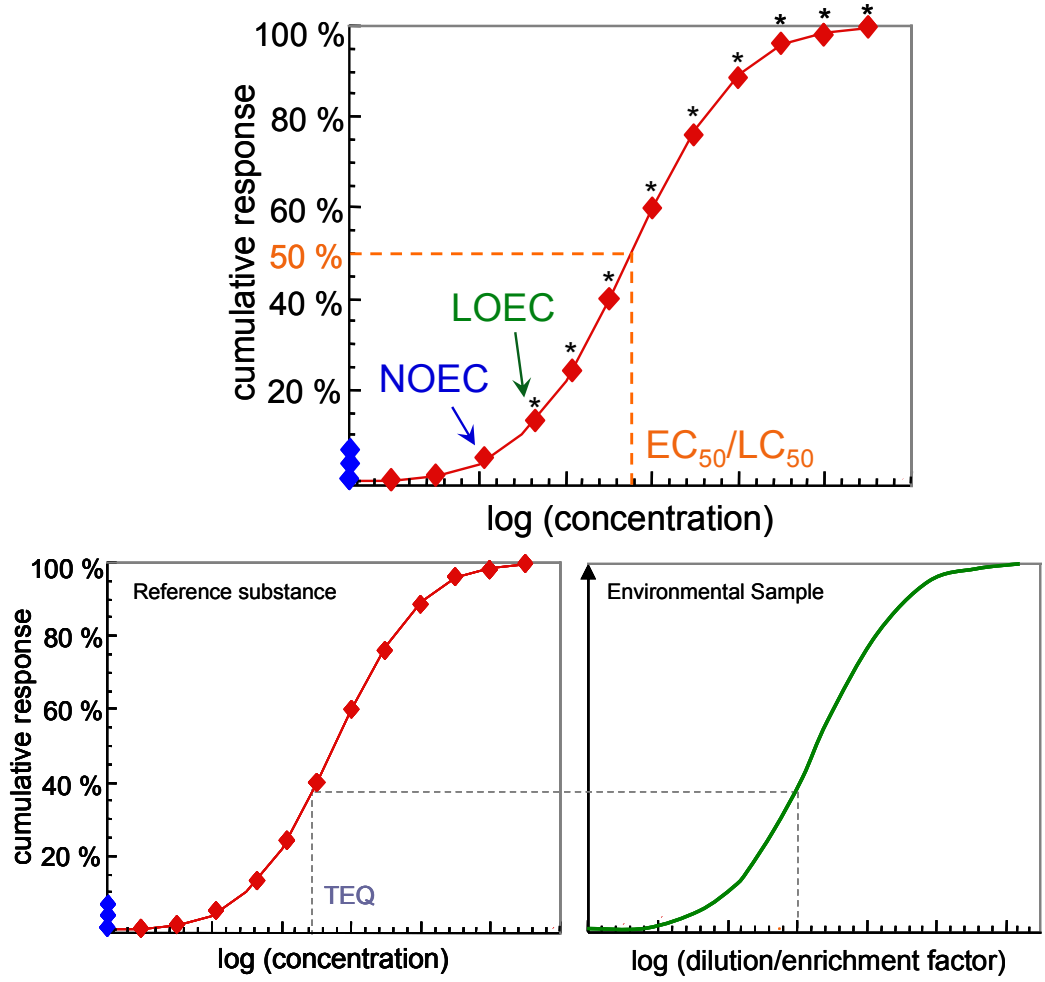


Figure 3: Example for a dose response curve with the toxicity parameters NOEC, LOEC and EC_{50}. ◊ control, ◊ treatment, * significant difference to the control (top graph). The graphs below show the derivation of TEQs by comparing the effect concentration of an environmental sample with the effect concentration of a reference substance.

4.2. Change Index

For the efficiency evaluation of the different sewage treatment steps, the bioassay results and calculated toxicity parameters provided a broad database. For better comparison between toxicity detected using different bioassays and native wastewater samples or enriched samples, a common parameter was used, the 'Change Index (CI)'. The CI describes the relative change in toxicity after individual sewage treatment steps (Ratte and Ratte, 2009). It allows a direct comparison of tests with and without sample enrichment and therefore also of *in vitro* and *in vivo* bioassays.

Use of the CI provides the following advantages:

1) The CI enables a conclusion if and to which extent the toxicity is changed by the observed treatment step independent of the respective absolute value of a toxicity parameter.

2) The CI enables a direct comparison between mechanism-oriented cellular *in vitro* bioassays and integrative *in vivo* bioassays, as it is independent of the definition/calculation of the respective toxicity parameter.

The CI is analogous to elimination efficiency, and is calculated as follows:

$$(1)\ CI = \frac{\text{Toxicity value after treatment}}{\text{Toxicity value before treatment}}$$

$$(2)\ CI_{TEQ} = \left(\frac{\text{Toxicity value after treatment}}{\text{Toxicity value before treatment}} \right)^{-1}$$

Equation 1 is used for EC- and NOEC / LOEC values: Here the higher the EC_x or NOEC / LOEC values, the more the toxicity of the sample is decreasing (and CI increases).

Equation 2 is used for toxic equivalent concentrations (TEQs). The TEQ is the concentration of a reference substance that would have the same effect as the environmental sample. The higher the TEQ, the more toxic is the substance or environmental sample. With decreasing toxicity the TEQ decreases. Therefore the CI_{TEQ} is the inverse of equation 1.

In summary, the CIs for the toxicity parameters EC_x, TEQ and for values significantly different to the control indicate:

Change Index CI > 1 decreased toxicity

Change Index CI ~1 equal toxicity (range: 0.75 < CI < 1.25)

Change Index CI < 1 increased toxicity

A deviation of equal to or more than 25 % from CI = 1, i.e. CI ≥1.25 or CI≤1.25, was considered respectively a significant reduction or increase of toxicity due to a specific treatment step. This rather high tolerance level was set arbitrarily in order to address the variability of bioassay data in a common way.

The following CIs were calculated for comparing the results of the bioassays:

$CI_{LF/EN}$ or $CI_{SB/EN}$ Effect biological treatment

$CI_{OZ/LF}$ or $CI_{OZ/SB}$ Effect ozonation

$CI_{SF/OZ}$ Effect sand filtration (3^{rd} + 4^{th} MC)

$CI_{SF/LF}$ Effect ozonation + sand filtration (3^{rd} + 4^{th} MC)

$CI_{PAC/LF}$ Effect PAC-UF treatment (2^{nd} to 4^{th} MC)

For four bioassays (*L. variegatus*, *O. mykiss*, *P. antipodarum*, *G. fossarum*) it was not possible to calculate toxicity parameters, because the water samples were not tested as dilution series. For these, the CI was derived by statistical comparison of effect data (e.g. number of hatched fish) for organisms exposed to undiluted water samples before and after each treatment step. Students t-test (GraphPad Prism 5, GraphPad Software Inc., CA, USA) followed by a Bonferroni-

Holm correction (Holm, 1979) to account for multiple comparisons was used to determine significant differences.

In the subsequent chapter, the indicator value of the different bioassays will be compared using the CI.

4.3. Comparison of Bioassay Results

The following tables (Table 3 and Table 4) give an overview of the bioassay results using the CI to display the changes in toxicity following each wastewater treatment step. A quantitative analysis for the different steps is presented in chapter 4.4. More detailed results for each toxicity parameter, the CIs and elimination efficiencies can be found in the appendix (Table 7 to Table 26).

4.3.1. In vitro Bioassays

Table 3 shows the changes in specific effects based on change indices (CI_{TEQ}) resulting from *in vitro* bioassays.

Table 3: Change indices (CI_{TEQ}) for bioassays, based on specific cellular mechanisms / *in vitro* bioassays with sample enrichment.

Red arrows pointing up (↑, in red) mean CI_{TEQ} < 1 = increasing effects, a tilde (~, in grey) means CI_{TEQ} ~ 1 = equal effects and a green arrow pointing down (↓, in green) means CI_{TEQ} > 1 = decreasing effects in most of the measurement campaigns (≥ 3). Var. marks varying results between different measurement campaigns (in grey). Crossed out fields mean that no influent samples have been measured and therefore no effect of the biological treatment could be assessed.

Bioassay	Substance classes (effect parameter)	Effect Biological treatment ($CI_{LF/EN}$)	Effect Ozonation ($CI_{OZ/LF}$)	Effect Sand filtration (3.+4. MC) ($CI_{SF/OZ}$)	Effect Ozonation + Sand filtration (3.+4. MC) ($CI_{SF/LF}$)	Effect Powdered activated carbon – UF ($CI_{PAC/LF}$)
YES assay	Estrogens (Estradiol equivalents, ng/L)	↓	↓	var.	↓	↓
ER CALUX	Estrogens (Estradiol equivalents, ng/L)	↓	↓	var.	var.	↓
AR CALUX	Androgens (Dihydrotestosterone equivalents, ng/L)	↓	↓	var.	↓	↓
GR CALUX	Glucocorticoids (Dexmethason equivalents, ng/L)	~	↓	↓	↓	↓
PR CALUX	Progesterones (Org-2058 equivalents, ng/L)	↑	↓	↑	↓	↓
PPARg1 CALUX	Peroxisome proliferator like acting substances (Rosiglitasone equivalents, ng/L)	↓	↓	var.	var.	↓
H295R Assay	Estradiol induction		↓	~	↓	↓
	Testosterone induction		~	~	~	~
Green algae	Herbicides (Diuron equivalents, µg/L) (Photosynthesis inhibition)	var.	↓	~	↓	↓
	General Toxicity (baseline toxic equivalent conc., mg/L) (Growth inhibition)	↓	↓	↓	↓	↓

Biological wastewater treatment presented decreased effects as indicated by most *in vitro* bioassays measuring effects on specific cellular mechanisms. Exceptions were progesterone-like activity, which increased. An explanation might be that this substance class was insufficiently removed during biological treatment, or that breakdown products contributed to this effect. In general, the measured concentrations of progesterone-like acting substances (e.g. progesterone, a steroid hormone involved in the menstruation cycle, pregnancy and embryogenesis of humans and other species, and medroxyprogesterone or levonorgestrel, synthetic progestogens similar to progesterone) were low and ranged from 0.11 ng/L (EN, 2nd MC) to 2.6 ng/L (SB, 1st MC) (see Table **13**, Appendix). Those concentrations were in the same range as data from earlier studies on effluent samples and surface waters (Kolodziej and Sedlak, 2007; Van der Linden et al., 2008). Known effect concentration data of these substances for aquatic organisms are rare, and ranged from 156 ng/L for the western clawed frog (*Xenopus tropicalis*) (Kvarnryd et al., 2011) up to 5000 µg/L for adult rainbow trout (Billard et al., 1981). Therefore, these data are difficult to interpret, and further investigation is needed before the environmental relevance of this finding can be established. The influent samples were cytotoxic to cell cultures used in the H295R steroidogenesis assay .

Glucocorticoid like acting substances (i.e. steroid hormones affecting the metabolism of glucose and reducing immune activity, e.g. cortisol, corticosterone) were unaffected by biological treatment. Concentrations of those substances (expressed as dexmethasone equivalent concentrations) ranged from < 4 ng/L (PAC-UF, 2nd-4th MC) to 140 ng/L (LF, 2nd MC) (see Table 12, Appendix). The lowest effect concentration for aquatic organisms found in the literature was 3.46 µg/L for the African clawed frog (*Xenopus laevis*) exposed to corticosterone (Lorenz et al., 2009), which is 24 times higher than the highest measured value in the pilot study. However as toxicity data regarding this substance class are still rare no final conclusion on the environmental relevance of the measured concentrations can be drawn.

The effect of biological treatment on photosystem II inhibiting herbicides showed variable results.

Ozonation and PAC-UF decreased effects as indicated by all *in vitro* bioassays used, with one exception. In the H295R steroidogenesis assay, the advanced treatment processes were not able to reduce the slight inhibition of testosterone production, which was present in biologically treated wastewater.

Sand filtration by itself showed variable results. These results are discussed in more detail in chapter 4.3.4.

The net effect of ozonation plus sand filtration was a decrease in toxicity for most *in vitro* bioassays.

4.3.2. Laboratory In Vivo Bioassays

Based on the results of the laboratory *in vivo* bioassays, it can be summarized that no consistent toxicity increase due to ozonation by-products was found.

Table 4 shows the changes in toxicity determined by *in vivo* bioassays performed in the laboratory.

Table 4: Change indices (CI) for *in vivo* bioassays performed in the laboratory with wastewater in a dilution series. Red arrows pointing up (↑, in red) mean CI < 1 = increasing toxicity, a tilde (~, in grey) means CI ~ 1 = equal toxicity and a green arrow pointing down (↓, in green) means CI > 1 = decreasing toxicity in most of the measurement campaigns (≥ 3). Var. marks varying results between different measurement campaigns (in grey) and n.t. means not toxic (in white). Crossed out fields mean that no influent samples have been measured and therefore no effect of the biological treatment could be assessed. For *Gammarus fossarum* only the effect of ozonation + sand filtration and of PAC-UF was measured.

Test organism	Endpoint (Toxicity parameter)	Effect Biological treatment ($CI_{LF/EN}$)	Effect Ozonation ($CI_{OZ/LF}$)	Effect Sand filtration (3.+4. MC) ($CI_{SF/OZ}$)	Effect Ozonation + Sand filtration (3.+4. MC) ($CI_{SF/LF}$)	Effect Powdered activated carbon – UF ($CI_{PAC/LF}$)
Vibrio fischeri	Inhibition of Luminescence (EC_{20})	↓	n.t.	n.t.	n.t.	n.t.
Pseudokirchneriella subcapitata (without nutrient addition)	Cell number (EC_{20})	~	~	var.	var.	~
	(EC_{50})	~	~	~	~	~
Pseudokirchneriella subcapitata (with nutrient addition)	Cell number (EC_{20})	↓	n.t.	var.	var.	n.t.
	(EC_{50})	↓	n.t.	n.t.	n.t.	n.t.
Lemna minor	Frond number (EC_{20})	↓	var.	var.	var.	n.t.
	(EC_{50})	var.	n.t.	~	~	n.t.
Ceriodaphnia dubia	Number of offspring (EC_{20})	↓	var.	var.	↑	n.t.
	(EC_{50})	↓	n.t.	var.	var.	n.t.
	Mortality (EC_{50})	var.	n.t.	~	~	n.t.
Gammarus fossarum	Feeding rate				~	~
Potamopyrgus antipodarum	Mortality		~	~	~	↓
Danio rerio	Mortality	↓	n.t.	n.t.	n.t.	n.t.

Biological Treatment: Most *in vivo* bioassays performed in the lab showed a decrease in toxicity after biological treatment compared to influent samples. The resulting toxicity level was mostly non-toxic or slightly toxic.

Ozonation and PAC-UF: If toxicity was still present after biological treatment, there was no additional reduction by advanced treatment. No increase or decrease in toxicity was evident after ozonation and PAC-UF. The toxicity remained on a mostly low level showing rather varying results or no changes at all. The chronic bioassay with *C. dubia* indicated an increase in toxicity (EC_{20} and fecundity) after ozonation plus sand filtration treatments. A possible explanation for

that might be toxic substances originating from the pilot sand filter. As the 3rd measurement campaign took place soon after the installation of the pilot sand filter, it is possible that the effects were caused by this filter. However, such effects have not been observed in common sand filters. In the other *in vivo* bioassays no such effects were observed.

The snail reproduction test with *P. antipodarum* did not yield reliable results due to high mortality in some samples (Maletz and Hollert, 2010). No mortality was observed in wastewater samples after PAC-UF treatment. Increased mortality after biological treatment, ozonation and sand filtration may have been due to increased growth of green and brown algae during the test, and/or toxic substances still present there, which were removed by PAC-UF treatment only. No parasites were detected. At this point, no definite explanation for the increased mortality of *P. antipodarum* can be provided. In a similar study at the WWTP Neuss, Germany, no mortality of *P. antipodarum* in wastewater from the different cleaning steps was reported (Stalter et al., 2010a).

The feeding assay with *G. fossarum* did not indicate a change in wastewater toxicity due to ozonation (Schulz and Bundschuh, 2010). However, if wastewater was treated with slightly higher ozone concentrations in the laboratory the feeding rates increased, suggesting that the ozone concentrations applied at the WWTP were too low to cause a shift in toxicity detectable for these organisms. Similarly, on-site PAC treatment did not alter feeding activity This may be due to the PAC-mediated removal of trace elements (Filby et al., 2010) and a subsequent decline in activity and health of the test organisms.

4.3.3. *In Situ Bioassays*

Table 5 shows the changes in toxicity resulting from *in vivo* bioassays performed at the WWTP Vidy in flow-through systems under *in situ* conditions.

Table 5: Change indices (CI) for *in vivo* bioassays performed at the WWTP Vidy with undiluted wastewater
Red arrows pointing up (↑, in red) mean CI < 1 = increasing toxicity, a tilde (~, in grey) means CI ~ 1 = equal toxicity and a green arrow pointing down (↓, in green) means CI > 1 = decreasing toxicity. No influent samples have been measured and therefore no effect of the biological treatment could be assessed.

Test organism	Endpoint	Effect Ozonation ($CI_{OZ/LF}$)	Effect Sand filtration ($CI_{SF/OZ}$)	Effect Ozonation + Sand filtration ($CI_{SF/LF}$)	Effect Powdered activated carbon – UF ($CI_{PAC/LF}$)
Lumbriculus variegatus	Reproduction	~	~	~	~
	Biomass	↑	~	~	↑
Oncorhynchus mykiss	Overall Survival	↓	~	↓	↓
	Survival of embryos	~	~	~	↓
	Survival of larvae and juveniles	↓	~	↓	↓
	Hatching rate	~	~	~	↓
	Swim-up of hatched larvae	↓	~	↓	↓
	Fresh weight of larvae at end of test	↓	~	↓	↓
	Length of larvae at end of test	~			↓
	Vitellogenin concentration	↓	~	↓	↓

Biological treatment: *In vivo* bioassays performed in flow through systems showed an increase in *L. variegatus* biomass after biological treatment compared to the control treatment (water of Lake Geneva) (Weil, 2010), suggesting better than normal conditions for this species.

The effluent from the biological treatment step negatively affected all developmental stages of trout, i.e. delayed hatching of more than 6 days, 40-50 % of overall mortality, diminished swimming ability and a significantly elevated vitellogenin concentration compared to the controls (63.1 ng/mL, measured in 69 days old larvae via ELISA (Knacker et al., 2010)). Results indicate that toxic amounts of substances that cause deleterious developmental effects and endocrine disruptors were present in biologically treated effluent.

Ozonation and PAC-UF: Biomass of *L. variegatus* was significantly decreased after both ozonation and PAC-UF suggesting increased toxicity. For PAC-UF treatment, it is possible that the effect was caused by the reduced availability of food and nutrients in the treated wastewater. Negative effects of ozonation may be caused by reactive toxic ozonation by-products that were eliminated in the sand filtration. Similar observations were made by Stalter et al. (2010a), which prompted the recommendation to include a final filtration step with biological/bacterial activity

such as a sand filter after ozonation. In Lausanne however a tendency for a decrease was observed in the reproduction of *L. variegatus* exposed to the sand filtered water as well as to water from PAC-UF, but results were not significant with Bonferroni-Holm adjustment. This effect could be due to a reduced availability of nutrients and suspended matter in the effluent of those treatments compared to the biological treatment and ozonation (Weil, 2010). Similar results have been obtained in other studies (Stalter et al., 2010a).

The Fish Early Life Stage test with *O. mykiss* showed decreased toxic effects for 5 out of 8 observed endpoints after ozonation. After ozonation and sand filtration treatment however weight and length of the fish were significantly reduced compared to the controls, as was also observed by Stalter et al. (2010b). Treatment with PAC-UF led to decreased toxicity compared to biologically treated wastewater for all the observed endpoints. Overall, organism performance after exposure to PAC-UF treated effluent was similar to controls. Both advanced treatment systems were able to decrease vitellogenin concentration in fish to control levels (10.6 ng/mL), indicating good elimination of estrogenic active substances. These effects were also observed in the YES and ER CALUX assays, where the EEQs ranged from 0.9 - 3 ng/L in the effluent of biological treatment and around 0.3 ng/L in the effluent of the advanced treatments. These values are in the same range as measurements at the WWTP Wüeri (effluent ozonation-SF: 0.05 - 0.33 ng/L EEQ), and up to 5 ng/L EEQ have been measured in Swiss rivers downstream of WWTPs (e.g. Burkhardt-Holm et al., 2005).

It has also to be kept in mind that early life stages of salmonids are very sensitive to ammonia (e.g. U.S. Environmental Protection Agency, 2009). The measured values of 5 mg/L NH_4-N (at 9°C) in the test correspond to approximately 0.005 to 0.04 mg/L NH_3-N for pH values between 7.0 and 8.0 (Weil, 2010). In rainbow trout ammonia concentrations higher than 0.04 mg/L elicited histopathological effects after long term exposure of 5 years (Thurston et al., 1984) and the EC_{20} for effects on growth of rainbow trout larvae and fish during a 90 day exposure was 7.72 mg/L NH_3-N (Brinkman et al., 2009). Derived from all available toxicity data for aquatic freshwater organisms, the US EPA recommends a chronic quality criterion for ammonium in freshwater at pH 8 and 14°C of either 0.521 or 3.74 mg NH_4-N/L, depending on whether freshwater mussels and early life stages of fish are present or absent respectively (U.S. Environmental Protection Agency, 2009). Overall, based on these data, it is unlikely that direct effects of periodically elevated ammonium concentrations on the survival and development of the fish are occurring.

4.3.4. Assessment of different filter types

Based on the results obtained from bioassays, a final filtration step with biological/microbial activity after ozone treatment was recommended to prevent effects of reactive and possibly toxic by-products resulting from ozonation. At WWTP Vidy, two different filter types were assessed for this purpose: a carbon filter and a sand filter.

Carbon filter (1st and 2nd MC)

With the carbon filter, which has already been used in the WWTP for several years, increased toxicity was observed in most bioassays, in agreement with the results of the chemical analyses, which showed higher concentrations of micropollutants after passage through the filter. This was probably due to desorption of substances from this filter. Desorption results from both backwash with physico-chemically treated wastewater (not suited for micropollutant removal) and long-term operation of this filter. Therefore the continued use of this carbon filter after complementary treatment was not recommended.

Sand filter (3rd and 4th MC)

After passage through the sand filter (mobile sand filter device for pilot studies) in the 3rd MC, an increase in estrogenic and progesterone-like activity was detected. In the 4th MC no increased effects could be observed in most *in vitro* bioassays, but a slightly increased toxicity was observed in some laboratory *in vivo* bioassays.

In the 3rd measurement campaign, a slight increase in concentrations of bisphenol A and estrone was detected by the chemical analysis, which might have been washed out from the plastics around the sand filter and may have played a role in the toxicity increase. In the 4th measurement campaign no obvious reason for the slight toxicity increase in some laboratory *in vivo* bioassays was found by comparison with the results of the chemical analysis as well as with the physico-chemical parameters measured. The toxicity increase might be related to differences in wastewater composition, which might not necessarily be due to micropollutants.

Overall no evidence for a toxicity increase after sand filtration was observed.

4.4. Elimination efficiency of treatment steps

The following figures (Figures 4 and 5) show the mean elimination efficiencies for the different treatment steps as well as the mean overall elimination efficiencies. Separate elimination efficiencies for the four measurement campaigns as well as a comparison of elimination efficiencies for the "old" biology and the moving bed biology can be found in the appendix (Figure 11 - 18).

Biological treatment, ozonation and sand filtration

The mean elimination efficiencies for biological treatment, ozonation and sand filtration are displayed in Figure 4. Only the elimination efficiencies for the 2nd to 4th MCs are displayed, as in the 1st MC another biological treatment was applied (see Figure 2, "old" biology, SB).

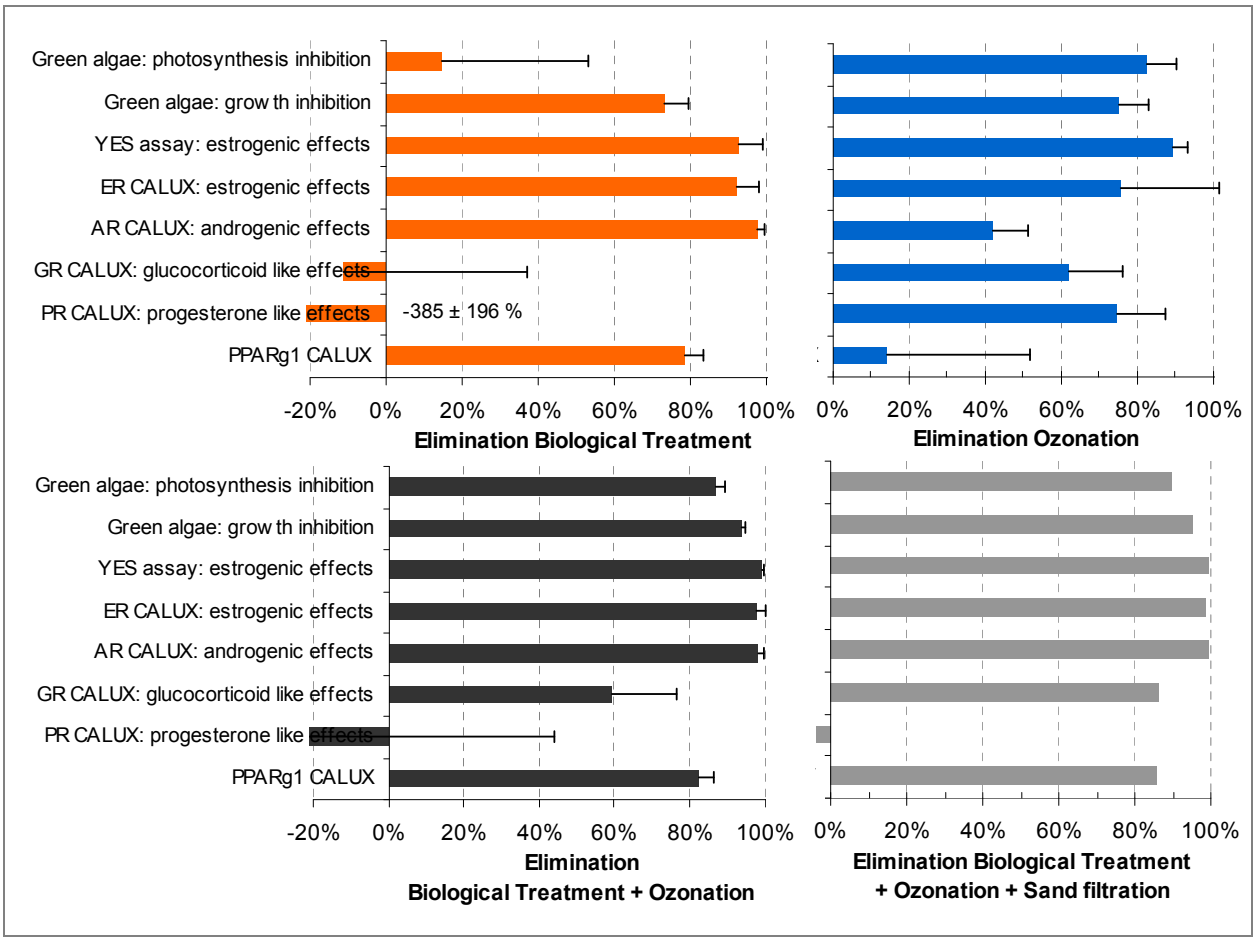


Figure 4: Mean elimination efficiency for specific effects measured in *in vitro* bioassays in the different treatment steps: biological treatment, ozonation, biological treatment + ozonation (mean ± SD of 2nd to 4th measurement campaign, 1st MC excluded due to differences in biological treatment, see chapter 3.2) and biological treatment plus ozonation followed by sand filtration (elimination efficiency of 4th measurement campaign, only this MC used as here technical procedures were optimized).

In the biological treatment, only 3 to 4 out of 8 specifically acting substance classes were eliminated by more than 80%. Herbicides, glucocorticoid and progesterone-like acting

substances were not removed at all or only to a small extent. General toxic substances were eliminated by less than 80%.

Ozonation additionally removed 14 to 87 % of the remaining specifically acting substances. Elimination efficiency of ozonation varied depending on the biological treatment, ozone concentration and substance classes.

Biological treatment combined with ozonation removed 82 to 99 % (mean of 3 MCs) of most of the specifically acting substances with exception of progesterone and glucocorticoid like acting substances. Removal of those substances was less efficient (59 ± 18 % and -24 ± 68 % respectively) with biological treatment and ozonation. For progesterone, a good degradation due to ozonation was shown in other studies (Barron et al., 2006; Labadie and Budzinski, 2005), however this was strongly dependent on the respective ozone concentrations (Barron et al., 2006). For glucocorticoid-like acting substances, no information regarding ozonation was available.

For the 4th MC the elimination efficiencies of the combination of biological treatment, ozonation and sand filtration was evaluated. The presentation of results including the sand filter is restricted to the 4th MC as the technical procedures were optimized at this point.

The overall elimination efficiencies for the 4th measurement campaign ranged from 86 to 99 % for most specific effects. Elimination efficiency with the sand filter was slightly improved compared to the efficiency after ozonation. Only progesterone-like effects could not be reduced with the overall treatment, however as mentioned in chapter 4.3.1 the values were generally low, i.e. in the range of 0.11 to 2.6 ng/L.

Biological and powdered activated carbon –UF treatment

Treatment of the wastewater with PAC-UF led to an additional 61 to 100 % elimination of the remaining specifically-acting substances when compared to the removal in the biological treatment (Figure 5).

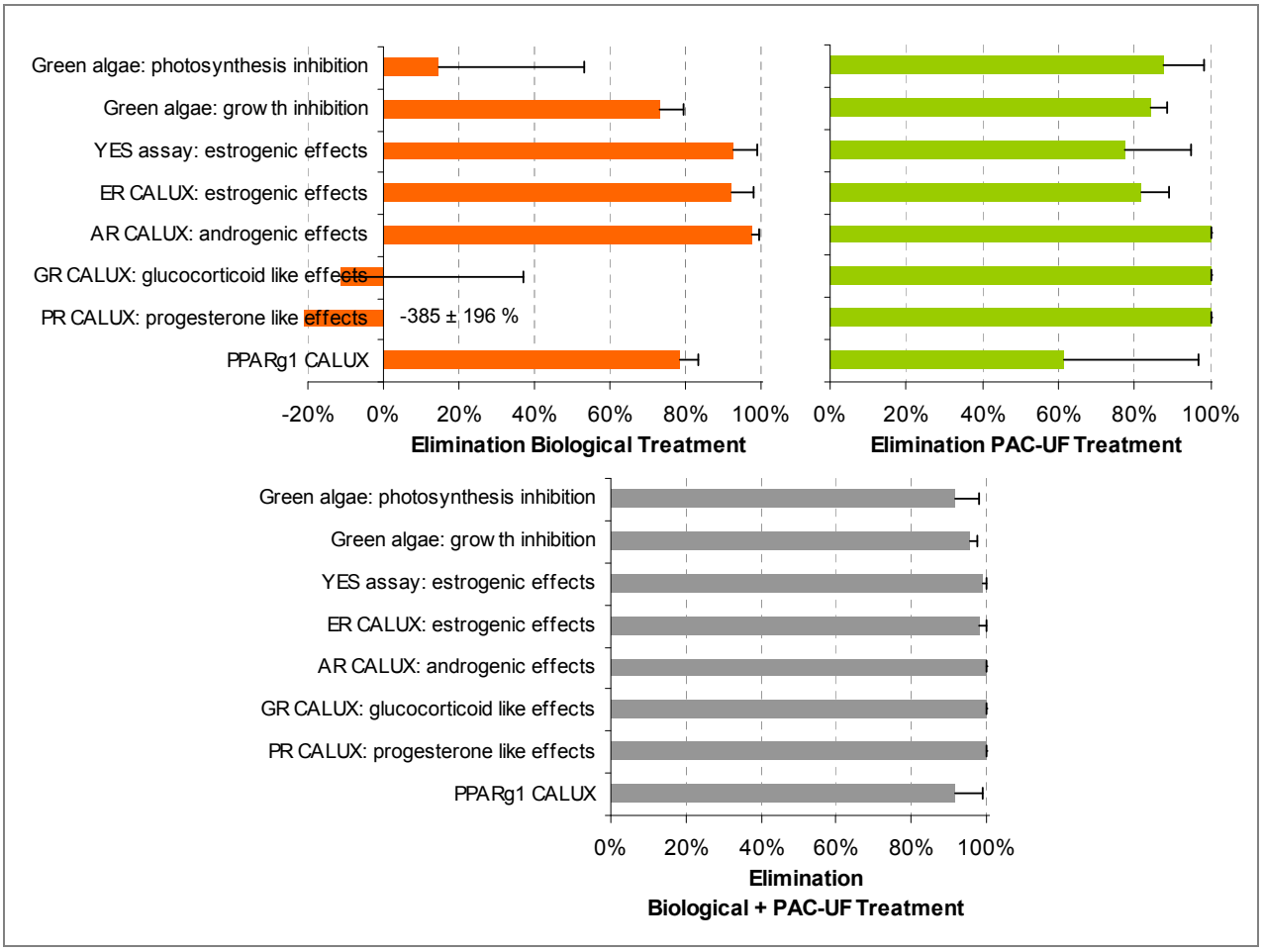


Figure 5: Mean elimination efficiency for specific effects measured in *in vitro* bioassays in the different treatment steps: biological, PAC-UF and biological + PAC-UF treatment (mean ± SD of 2nd to 4th measurement campaign).

The combination of biological and PAC-UF treatments removed all of the specifically-acting substances to more than 80 % (elimination efficiencies ranged from 92 to 100 %).

4.5. Comparison of bioassays with chemical measurements

To compare the results of the chemical measurements with the bioassay results, the mean elimination efficiencies, calculated from the combined algae assay for all measured herbicides, were compared between the different sampling campaigns (Figure 6).

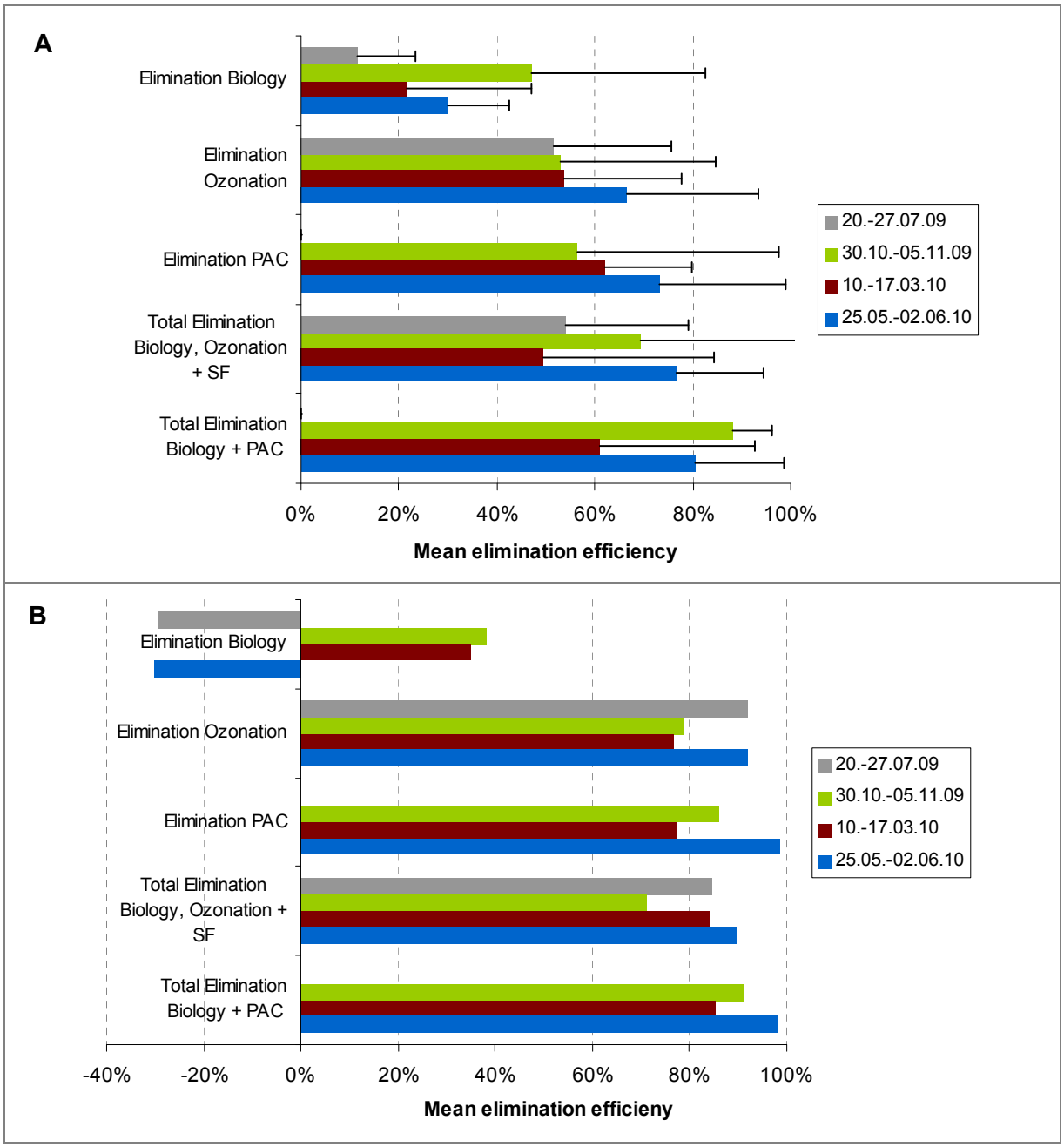


Figure 6: Mean elimination efficiencies in the different treatment steps for each of the four measurement campaigns for A: based on concentrations of herbicides from chemical analysis (mean ± SD of four 1-2 day composite samples over 7 days) and B: based on diuron equivalent concentrations (DEQs) (µg/L) in the algae assay focusing on inhibition of photosynthesis (mean elimination efficiency from one composite sample over 7 days based on the mean TEQ value of three replicates in the experiment). In the 4th measurement campaign (25.05.-02.06.10) the technical procedures were optimised.

Compared with chemical analytics, results from the combined algae assay indicated higher elimination efficiency for herbicides acting as photosystem II inhibitors. This may be explained by the presence of additional herbicides with this mode of action, which were not included in the analytical measurements.

4.6. Mutagenicity and genotoxicity

No mutagenicity (Ames assay, strains TA98 and TA100) (Ames et al., 1975) (ISO 16240, 2005) and no genotoxicity (micronucleus assay, with and without S9 activation) (Reifferscheid et al., 2008) (ISO 21427-2, 2006) were detected. In the umuC assay (ISO 13829, 2000, with S9 activation), genotoxic effects in the influent and after biological treatment have been detected only in highly enriched samples (EN ≥14.8 fold, LF ≥ 37 fold). In one MC slight genotoxic effects in PAC-UF (≥ 74 fold enrichment) were detected.

Overall no change in genotoxicity and mutagenicity was measured following advanced treatments. This was observed in other studies as well for PAC treatment (e.g. Stalter et al., 2010a) and ozonation (e.g. Mišík et al., 2011; Petala et al., 2006; Reungoat et al., 2010; Takanashi et al., 2002). In selected studies genotoxic effects were detected after ozonation (e.g. Stalter et al., 2010a); however they were eliminated by subsequent sand filtration.

We conclude that there is no evidence of higher toxicity due to the formation of stable ozonation by-products. It is, however, possible that the tests used were not sufficiently sensitive to these compounds. Because formation of reactive ozonation by-products cannot be excluded based on our results, the installation of a final filtration step with biological activity is recommended.

4.7. Conclusions from the performance review

A clear reduction of wastewater toxicity due to ozonation and powdered activated carbon treatment followed by ultrafiltration was demonstrated with most *in vitro* bioassays and with one *in vivo* bioassay (Fish Early Life Stage test with *O. mykiss*). The other applied bioassays showed either no toxicity or no change in toxicity in the course of wastewater treatment. The overall elimination efficiency for most of the assessed specific substance classes was more than 80%. However, there were differences in toxicity for different sampling time points, which presumably resulted from differences in wastewater composition. There was no evidence of increased toxicity due to a constant formation of ozonation by-products. Using *in vivo* bioassays performed in the lab, a slight toxicity of raw wastewater was detected. This was mostly eliminated after biological treatment. The toxicity of the wastewater observed in those *in vivo* assays was generally low.

In general, the application of bioassays for a performance assessment of advanced wastewater treatment technologies proved to be relevant and useful, as shown in this pilot study as well as in the previous pilot study at the WWTP Wüeri in Regensdorf (Abegglen et al., 2009).

However, it is important to note, that a single general bioassay for the overall assessment of the toxicity of a wastewater sample does not exist. No single bioassay covers the whole range of different toxic effects in wastewater. Therefore, a set of bioassays has to be used to evaluate the performance of wastewater treatment plants.

To evaluate the efficiency of advanced treatment steps in reducing the biological effects of micropollutants with specific modes of action, the use of *in vitro* bioassays with sample enrichment is highly promising. However, some of the available test protocols are not certified. Therefore it should be aimed for a standardization and certification of those tests. Additionally, a cost efficiency analysis should be performed.

For an effect assessment for whole organisms, chronic *in vivo* bioassays are desirable. Here is a need for the development of tests especially with sensitive test organisms. For those assays the costs are often much higher than for small-scale / *in vitro* bioassays. Furthermore, the interpretation of observed effects in *in vivo* bioassays generally is more difficult, especially with regard to micropollutants. Regarding bioassays with vertebrates there is also a need to reduce the use of animals in experimentation.

Overall the performance review with bioassays has shown that ozonation-SF and PAC-UF treatment are useful measures to reduce the effects of micropollutants in waterbodies. A broad range of micropollutants and their effects were eliminated by more than 80%, and there was no evidence for a toxicity increase due to a constant formation of stable toxic ozonation by-products. *In vitro* bioassays based on specific effects with sample enrichment were generally deemed more suitable for the performance assessment of advanced wastewater treatments than integrative *in vivo* bioassays without sample enrichment.

5. Evaluation of wastewater quality regarding micropollutants and their effects

In environmental risk assessment, risk quotients are used to evaluate whether measured concentrations of environmental chemicals pose a risk to the environment. Those quotients are calculated by dividing the MEC (measured environmental concentration) by the respective quality criterion (QC) (usually the annual average environmental quality standard, AA-EQS or the MAC-EQS), which takes into account ecotoxicological effect data for the respective substance (see below).

$$\text{Risk quotient (RQ)} = \frac{\text{MEC}}{\text{QC}} = ?$$

<1 tolerable risk

>1 intolerable risk

A risk quotient lower than 1 indicates a tolerable risk, whereas a risk quotient higher than 1 indicates an intolerable risk. The Annual Average-EQS (AA-EQS) is derived as a protection against the effects of long-term exposure, and the Maximum Acceptable Concentration EQS (MAC-EQS) against the effects of short-term exposure.

For an assessment of the wastewater quality, risk quotients were calculated for the three priority substances diclofenac, carbamazepin and clarithromycin using the MECs (usually the 90th percentile) and the derived EQS (Götz et al., 2010). More detailed information on the derivation of water quality criteria as well as on the classification of risk quotients for this purpose according to Götz et al. (2010) can be found in the appendix.

5.1. Risk quotients of three relevant micropollutants during wastewater treatment

Figure 7 shows results of the pilot study at the WWTP Vidy Lausanne for the three priority substances. The change of the risk quotients and quality classes during wastewater treatment for the 3rd MC is shown. The respective quality criterion (AA-EQS) for the pain killer diclofenac is 50 ng/L, for the anticonvulsant carbamazepin 500 ng/L and for the antibiotic clarithromycin 60 ng/L (Götz et al., 2010).

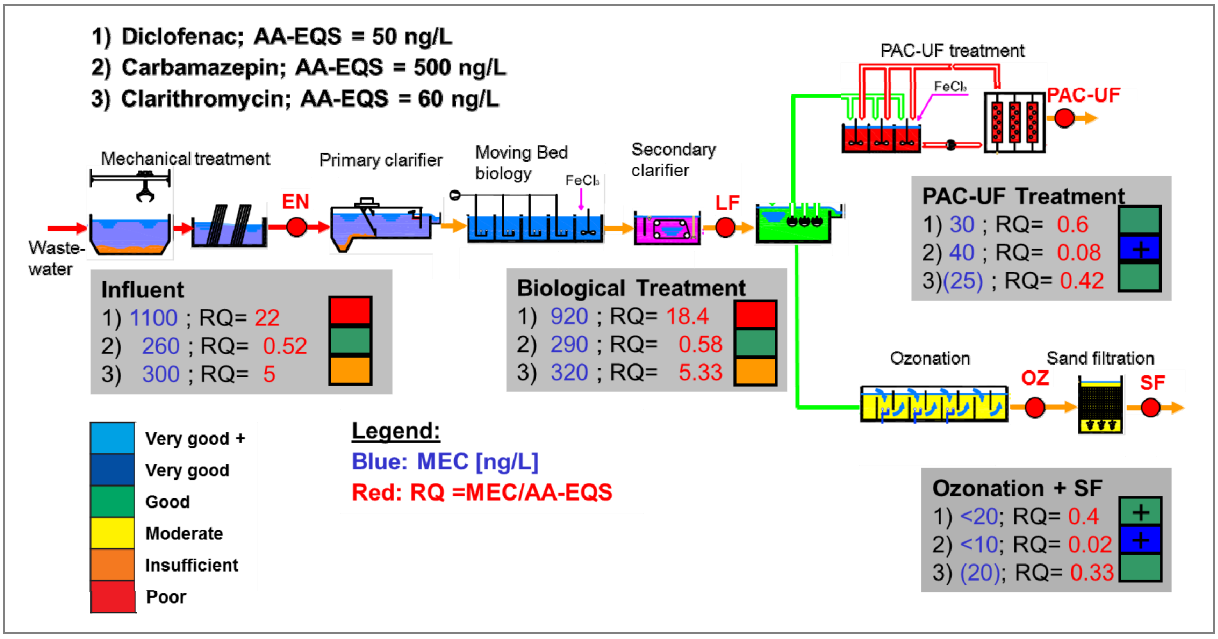


Figure 7: Evolution of risk quotients during wastewater treatment: measured environmental concentrations (MECs) from the screening of 120 substances at the Eawag and risk quotients (RQs) of three selected micropollutants during wastewater treatment in the 3rd measurement campaign (MC).

During wastewater treatment, the water quality regarding the three substances increased significantly, from poor water quality up to good quality for diclofenac; from good to very good water quality for carbamazepin; and from insufficient to good water quality for clarithromycin in undiluted wastewater. During biological treatment the respective substance concentrations decreased between 0.9 and 1.2 fold compared to the influent, and during PAC-UF or ozonation-SF treatment the concentrations decreased additionally 7 to 46 fold compared to the biological treatment. At the Regensdorf pilot study a similar improvement of water quality was observed.

In the adjacent watercourses, those values will be even lower due to the additional dilution. For rivers usually a dilution factor of 10 is assumed, and for lake Geneva an estimated 50 fold dilution showed the best correlation with the actually measured concentrations (Morasch et al., 2010).

5.2. Estrogenicity during wastewater treatment

When looking at the improvement of wastewater quality regarding estrogenic substances (Figure 8), a strong decrease in estrogenicity could be detected with the Yeast Estrogen Screen (YES) and the ER-CALUX assay at the Lausanne pilot study based on 17β-estradiol-equivalents (EEQ).

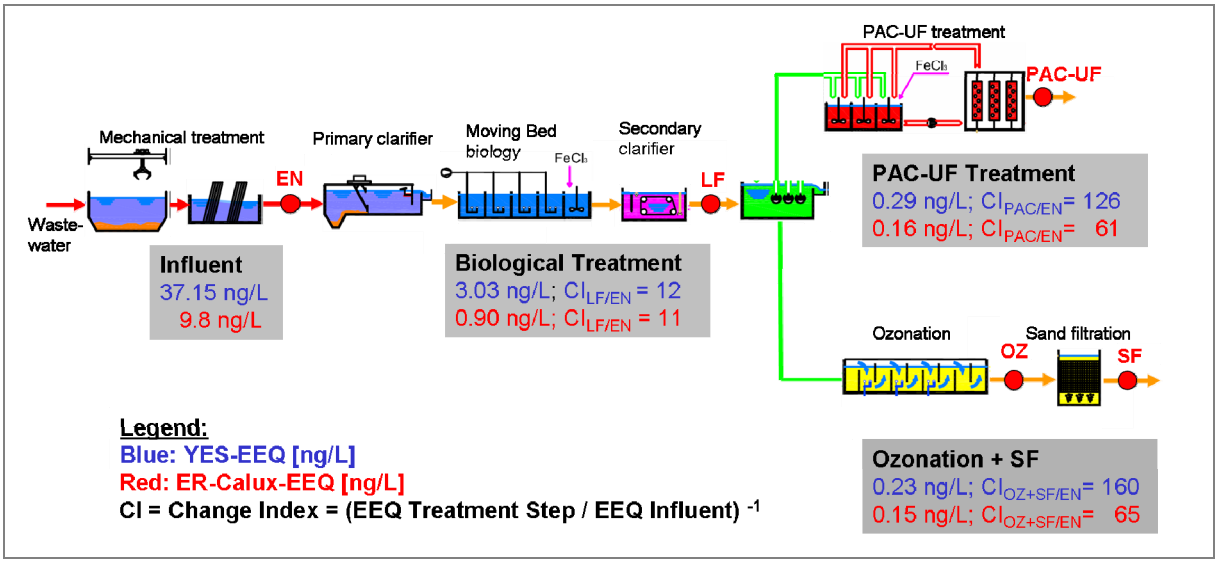


Figure 8: Evolution of estrogenicity during wastewater treatment steps: Estradiol equivalent concentrations [ng/L] and change indices (CI_{TEQ}) from the YES and the ER-CALUX assay for the 4th MC. $CI_{LF/EN}$ Effect biological treatment, $CI_{PAC/EN}$ Overall effect biological treatment and PAC-UF treatment, $CI_{OZ/EN}$ Overall effect biological treatment and ozonation, $CI_{OZ+SF/EN}$ Overall effect biological treatment and ozonation+ sand filtration.

During biological treatment, estrogenicity decreased between 11 and 12 fold compared to the influent, and during PAC-UF treatment or ozonation the concentrations decreased additionally 6 to 13 fold compared to the biological treatment.

5.3. Conclusions for the evaluation of wastewater quality regarding micropollutants and their effects

The advanced treatment steps PAC-UF and ozonation led to a substantially lowered risk potential for diclofenac, carbamazepin and clarithromycin. Additionally both treatments lowered the estrogenicity between two or three orders of magnitude in comparison to the influent. PAC-UF treatment and ozonation led to a 6 to 13 times lower estrogenicity compared to the biological treatment.

In combination with the results of the performance analysis (chapter 4), for micropollutants and estrogenic substances a significant reduction of risks can be expected with advanced wastewater treatment.

6. Main conclusions

From the results of the performance review (chapter 4) and the investigation of wastewater quality (chapter 5) several main conclusions were drawn and approved by the international expert group on ecotoxicology accompanying the pilot studies.

Main conclusions from the pilot studies in the project “Strategy Micropoll” are:

- Due to the advanced treatments in the pilot studies a broad range of micropollutants and their effects were eliminated by more than 80%, as detectable in most *in vitro* bioassays and with chemical analytics.
- There was no evidence for a toxicity increase due to a constant formation of stable toxic ozonation by-products (i.e. by-products still present after sand filtration) in any test.
- Ozonation should be followed by a final filtration step with biological activity in order to reduce the risk of potentially toxic, reactive transformation products being released in waterbodies. The occurrence of such products and their potential to elicit effects was shown in both pilot studies in selected *in vivo* bioassays (Lumbriculus reproduction assay in both pilot studies, Fish Early Life Stage assay in Regensdorf).
- Overall, the quality of treated effluent was significantly improved, leading to improved surface water quality.

7. References

Abegglen C, Escher B, Hollender J, Koepke S, Ort C, Peter A, Siegrist H, von Gunten U, Zimmermann S, Koch M, Niederhauser P, Schärer M, Braun C, Gälli R, Junghans M, Brocker S, Moser R, Rensch D, 2009. Ozonung von gereinigtem Abwasser Schlussbericht Pilotversuch Regensdorf. Eawag, Dübendorf. p. 80.

Ames BN, McCann J, Yamasaki E, 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Research 31:347-364.

Ashauer, in preparation. Post-ozonation in a municipal wastewater treatment plant improves water quality in receiving stream as indicated by the abundance of species at risk.

Barron E, Deborde M, Rabouan S, Mazellier P, Legube B, 2006. Kinetic and mechanistic investigations of progesterone reaction with ozone. Water Research 40:2181-2189.

Billard R, Breton B, Richard M, 1981. On the Inhibitory Effect of Some Steroids on Spermatogenesis in Adult Rainbow Trout (*Salmo gairdneri*). Canadian Journal of Zoology 59:1479-1487.

Brinkman SF, Woodling JD, Vajda AM, Norris DO, 2009. Chronic toxicity of ammonia to early life stage rainbow trout. Transactions of the American Fisheries Society 138:433-440.

Bundschuh M, Pierstorf R, Schreiber WH, Schulz R, 2011a. Positive effects of wastewater ozonation displayed by *in situ* bioassays in the receiving stream. Environmental Science and Technology 45:3774-3780.

Bundschuh M, Schulz R, 2011. Population response to ozone application in wastewater: an on-site microcosm study with *Gammarus fossarum* (Crustacea: Amphipoda). Ecotoxicology 20:466-473.

Bundschuh M, Zubrod JP, Schulz R, 2011b. The functional and physiological status of *Gammarus fossarum* (Crustacea; Amphipoda) exposed to secondary treated wastewater. Environmental Pollution 159:244-249.

Burkhardt-Holm P, Giger W, Guttinger H, Ochsenbein U, Peter A, Scheurer K, Segner H, Staub E, Suter MJF, 2005. Where have all the fish gone? Environmental Science & Technology 39:441A-447A.

Cordova Rosa EV, Simionatto EL, Sierra MMDS, Bertoli SL, Radetski CM, 2001. Toxicity-based criteria for the evaluation of textile wastewater treatment efficiency. Environmental Toxicology and Chemistry 20:839-845.

Deutsches Institut für Normung, 2003. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung - Suborganismische Testverfahren (Gruppe T) - Teil 6: (Fischeitest). DIN 38415-6.

Duft M, Tillmann M, Schulte-Oehlmann U, Markert B, Oehlmann J, 2002. Entwicklung eines Sedimentbiotests mit der Zwergdeckelschnecke *Potamopyrgus antipodarum* (Gastropoda: Prosobranchia). Umweltwissenschaften und Schadstoff-Forschung 14:12-17.

Eawag, 2007. Festphasenextraktion mit C18/EN von Supelco.

Escher BI, Bramaz N, Maurer M, Richter M, Sutter D, Von Känel C, Zschokke M, 2005. Screening test battery for pharmaceuticals in urine and wastewater. Environmental Toxicology and Chemistry 24:750-758.

Escher BI, Bramaz N, Mueller JF, Quayle P, Rutishauser S, Vermeirssen ELM, 2008a. Toxic equivalent concentrations (TEQs) for baseline toxicity and specific modes of action as a tool to improve interpretation of ecotoxicity testing of environmental samples. Journal of Environmental Monitoring 10:612-621.

Escher BI, Bramaz N, Quayle P, Rutishauser S, Vermeirssen ELM, 2008b. Monitoring of the ecotoxicological hazard potential by polar organic micropollutants in sewage treatment plants and surface waters using a mode-of-action based test battery. Journal of Environmental Monitoring 10:622-631.

EU, 2010. Chemicals and the Water Framework Directive: Technical Guidance for Deriving Environmental Quality Standards. Draft 2010. Draft version 6.0, 23 February 2010.

Filby AL, Shears JA, Drage BE, Churchley JH, Tyler CR, 2010. Effects of advanced treatments of wastewater effluents on estrogenic and reproductive health impacts in fish. Environmental Science and Technology 44:4348-4354.

Gälli R, Ort C, Schärer M, 2009. Mikroverunreinigungen in den Gewässern. Bewertung und Reduktion der Schadstoffbelastung aus der Siedlungsentwässerung. Umwelt-Wissen. Bundesamt für Umwelt, Bern. p. 103.

Götz CW, Kase R, Hollender J, 2011. Mikroverunreinigungen - Beurteilungskonzept für organische Spurenstoffe aus kommunalem Abwasser. Studie im Auftrag des BAFU. Eawag, Dübendorf. p. 108.

Götz CW, Kase R, Kienle C, Hollender J, 2010. Mikroverunreinigungen aus kommunalem Abwasser - Kombination von Expositions- und ökotoxikologischen Effektdaten. Gas, Wasser, Abwasser 7:575-585.

Gracia T, Hilscherova K, Jones PD, Newsted JL, Zhang X, Hecker M, Higley EB, Sanderson JT, Yu RMK, Wu RSS, Giesy JP, 2006. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicology and Environmental Safety 65:293-305.

Holm S, 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6:65-75.

International Organization for Standardization, 2000. Water quality -- Determination of the genotoxicity of water and waste water using the umu-test. EN ISO 13829 (2000) and 38415-3 (1996).

International Organization for Standardization, 2004. Water quality -- Freshwater algal growth inhibition test with unicellular green algae. ISO 8692:2004.

International Organization for Standardization, 2005a. Water quality -- Determination of chronic toxicity to *Ceriodaphnia dubia.* ISO 20665:2008.

International Organization for Standardization, 2005b. Water quality -- Determination of the genotoxicity of water and waste water -- Salmonella/microsome test (Ames test). ISO 16240:2005.

International Organization for Standardization, 2005c. Water quality -- Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) -- Duckweed growth inhibition test. ISO 20079:2005.

International Organization for Standardization, 2006. Water quality -- Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 2: Mixed population method using the cell line V79. ISO 21427-2:2006.

International Organization for Standardization, 2007a. Water quality -- Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). ISO 15088:2007.

International Organization for Standardization, 2007b. Water quality -- Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) -- Part 3: Method using freeze-dried bacteria. ISO 11348-3.

Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, Tyler CR, van Aerle R, Santos E, Brighty G, 2006. Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. Environmental health perspectives 114 Suppl 1:32-39.

Karaman G, Pinkster S, 1977. Freshwater *Gammarus* species from Europe, North Africa and adjacent regions of Asia (Crustacea, Amphipoda). I. *Gammarus pulex* group and related species. Bijdragen tot de Dierkunde 47:1-97.

Kase R, Eggen RIL, Junghans M, Götz C, Hollender J, 2011. Assessment of micropollutants from municipal wastewater - Combination of exposure and ecotoxicological effect data for

Switzerland. In: Einschlag FSG, editor. Waste Water - Evaluation and Management: InTech - Open Access Publisher.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW, 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of the United States of America 104:8897-8901.

Klimisch HJ, Andreae M, Tillmann U, 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regulatory Toxicology and Pharmacology 25:1-5.

Knacker T, Boettcher M, Frische T, Rufli H, Stolzenberg HC, Teigeler M, Zok S, Braunbeck T, Schäfers C, 2010. Environmental effect assessment for sexual endocrine-disrupting chemicals: Fish testing strategy. Integrated Environmental Assessment and Management 6:653-662.

Kolodziej EP, Sedlak DL, 2007. Rangeland grazing as a source of steroid hormones to surface waters. Environmental Science and Technology 41:3514-3520.

Kunz PY, Kienle C, Gerhardt A, 2010. *Gammarus* spp. in Aquatic Ecotoxicology and Water Quality Assessment: Toward Integrated Multilevel Tests. In: Albert LA, Gerba CP, Giesy J, Hutzinger O, Knaak JB, Stevens JT, Tjeerdema RS, Voogt P, Ware GW, editors. Reviews of Environmental Contamination and Toxicology: Springer New York. p. 1-76.

Kvarnryd M, Grabic R, Brandt I, Berg C, 2011. Early life progestin exposure causes arrested oocyte development, oviductal agenesis and sterility in adult *Xenopus tropicalis* frogs. Aquatic Toxicology 103:18-24.

Labadie P, Budzinski H, 2005. Determination of steroidal hormone profiles along the Jalle d'Eysines River (near Bordeaux, France). Environmental Science and Technology 39:5113-5120.

Liechti P, 2010. Methoden zur Untersuchung und Beurteilung der Fliessgewässer. Chemisch-Physikalische Erhebungen, Nährstoffe. Umwelt-Vollzug. Bundesamt für Umwelt (BAFU), Bern. p. 44.

Lorenz C, Opitz R, Lutz I, Kloas W, 2009. Corticosteroids disrupt amphibian metamorphosis by complex modes of action including increased prolactin expression. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 150:314-321.

Macova M, Escher BI, Reungoat J, Carswell S, Chue KL, Keller J, Mueller JF, 2010. Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. Water Research 44:477-492.

Maletz S, Hollert H, 2010. Untersuchung nativer Abwasserproben aus dem MicroPoll-Projekt im Reproduktionstest mit *Potamopyrgus antipodarum*. Abschlussbericht. Institut für Umweltforschung, RWTH Aachen.

Margot J, Magnet A, Thonney D, Chèvre N, De Alencastro LF, Rossi L, 2011. Traitement des micropolluants dans les eaux usées - Rapport final sur les essais pilotes à la STEP de Vidy (Lausanne). Ville de Lausanne.

Matthiessen P, Babut M, Batley G, Douglas M, Fawell J, Hommen U, Hutchinson TH, Janssen M, Maycock D, Reiley M, Schneider U, Weltje L, 2009. Water and Sediment EQS Derivation and Application. In: Crane M, Matthiessen P, Maycock DS, Merrington G, Whitehouse P, editors. Derivation and Use of Environmental Quality and Human Health Standards for Chemical Substances in Water and Soil: CRC Press, Boca Raton. FL., USA. p. 47-103.

Mišík M, Knasmueller S, Ferk F, Cichna-Markl M, Grummt T, Schaar H, Kreuzinger N, 2011. Impact of ozonation on the genotoxic activity of tertiary treated municipal wastewater. Water Research 45:3681-3691.

Morasch B, Bonvin F, Reiser H, Grandjean D, De Alencastro LF, Perazzolo C, Chèvre N, Kohn T, 2010. Occurrence and fate of micropollutants in the Vidy Bay of Lake Geneva, Switzerland. Part II: Micropollutant removal between wastewater and raw drinking water. Environmental Toxicology and Chemistry 29:1658-1668.

Nagel R, 2002. DarT: The Embryo Test with the Zebrafish *Danio rerio* - a General Model in Ecotoxicology and Toxicology. Alternatives to Animal Experimentation - ALTEX 19:38-48.

NFP 50, 2008. Nationales Forschungsprogramm 50 - „Hormonaktive Stoffe“. Konsensplattform „Hormonaktive Stoffe in Abwasser und Gewässern“ - Schlussdokument.

OECD, 1992. OECD Guideline for testing of chemicals 210: Fish, Early-life Stage Toxicity Test.

OECD, 2007. OECD Guidelines for the testing of chemicals 225: Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment.

Ort C, Siegrist H, Hosbach H, Studer C, Morf L, Scheringer M, 2007. Mikroverunreinigungen. Nationales Stoffflussmodell. Gas, Wasser, Abwasser 87:853-859.

Pascoe D, Shazili NAM, 1986. Episodic pollution - A comparison of brief and continuous exposure of rainbow trout to cadmium. Ecotoxicology and Environmental Safety 12:189-198.

Pawlowski S, Ternes T, Bonerz M, Kluczka T, van der Burg B, Nau H, Erdinger L, Braunbeck T, 2003. Combined *in Situ* and *in Vitro* Assessment of the Estrogenic Activity of Sewage and Surface Water Samples. Toxicological Sciences 75:57-65.

Petala M, Samaras P, Zouboulis A, Kungolos A, Sakellaropoulos G, 2006. Ecotoxicological properties of wastewater treated using tertiary methods. Environmental Toxicology 21:417-424.

Phipps GL, Ankley GT, Benoit DA, Mattson VR, 1993. Use of the aquatic oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminants. Environmental Toxicology and Chemistry 12:269-279.

Quayle P, Rutishauser S, Escher B, 2008. Standard Operation Procedure (SOP): Combined Algae Test, Determination of photosynthesis inhibition by means of algae chlorophyll fluorescence with the Maxi-Imaging-PAM and growth inhibition by means of OD measurement in a microtiter plate. Eawag, Dübendorf.

Ratte M, Ratte HT, 2009. Beurteilung des Effektes von Abwasserbehandlung mittels Ozonung anhand von Toxizitätsparametern aus *in vitro*- und *in vivo*-Biotests. Daten aus Projekt „MicroPoll“ (Ozonung von gereinigtem Abwasser), Pilotversuch Regensdorf, Teil: „Effektbasierte Testsysteme mit Aufkonzentrierung“ und „Standardisierte Testverfahren“.

Reifferscheid G, Ziemann C, Fieblinger D, Dill F, Gminski R, Grummt H-J, Hafner C, Hollert H, Kunz S, Rodrigo G, Stopper H, Selke D, 2008. Measurement of genotoxicity in wastewater samples with the in vitro micronucleus test--Results of a round-robin study in the context of standardisation according to ISO. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 649:15-27.

Reungoat J, Macova M, Escher BI, Carswell S, Mueller JF, Keller J, 2010. Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Research 44:625-637.

Routledge EJ, Sumpter JP, 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 15:241-248.

Schärer M, Sieber U, Müller S, 2010. Weitere Massnahmen gegen Mikroverunreinigungen - Situation in der Schweiz. Gas, Wasser, Abwasser 7:563-568.

Schreiber U, Müller J, Haugg A, Gademann R, 2002. New type of dual-channel PAM chlorophyll fluorometer for highly sensitive water toxicity biotests. Photosynthesis Research 74:317-330.

Schulz R, Bundschuh M, 2010. Pilot experiments STEP Vidy – investigations using amphipods. Final report, to the attention of FOEN, Strategy MicroPoll. Institute for Environmental Sciences, Landau, Germany.

Stalter D, Magdeburg A, Oehlmann J, 2010a. Comparative toxicity assessment of ozone and activated carbon treated sewage effluents using an *in vivo* test battery. Water Research 44:2610-2620.

Stalter D, Magdeburg A, Weil M, Knacker T, Oehlmann J, 2010b. Toxication or detoxication? *In vivo* toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. Water Research 44:439-448.

Sumpter JP, 1998. Xenoendocrine disrupters - Environmental impacts. Toxicology Letters 102-103:337-342.

Takanashi H, Mayumi M, Kato M, Hirata M, Hano T, 2002. Removal of mutagen precursor from wastewater by activated sludge and oxidation treatment. p. 389-394.

Tchounwou PB, Wilson BA, Ishaque AB, Schneider J, 2001. Toxicity tests to assess pollutants removal during wastewater treatment and the quality of receiving waters in Argentina. Environmental Toxicology 16:217-224.

Thurston RV, Russo RC, Luedtke RJ, 1984. Chronic toxicity of ammonia to rainbow trout. Transactions of the American Fisheries Society 113:56-73.

U.S. Environmental Protection Agency, 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition ed. Washington, DC.

U.S. Environmental Protection Agency, 2009. Draft 2009 Update Aquatic Life Ambient Water Quality Criteria for Ammonia - Freshwater. Washington, DC.

Van der Linden SC, Heringa MB, Man HY, Sonneveld E, Puijker LM, Brouwer A, Van der Burg B, 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environmental Science and Technology 42:5814-5820.

Victorin K, 1992. Review of the genotoxicity of ozone. Mutation Research - Reviews in Genetic Toxicology 277:221-238.

Victorin K, 1996. Genotoxicity and carcinogenicity of ozone. Scandinavian Journal of Work, Environment and Health 22:42-51.

Weil M, 2010. Final report - Ecotoxicological evaluation of waste waters from different treatments with two chronic test systems. Floersheim/Main: ECT Oekotoxikologie GmbH.

Williams GM, 1989. Methods for evaluating chemical genotoxicity. Annual Review of Pharmacology and Toxicology 29:189-211.

8. Figures

Tables

9. Appendix

Appendix for chapter 5

Derivation of water quality criteria

The quality criteria for the prioritized substances were derived from ecotoxicological effect data as shown in Figure 9.

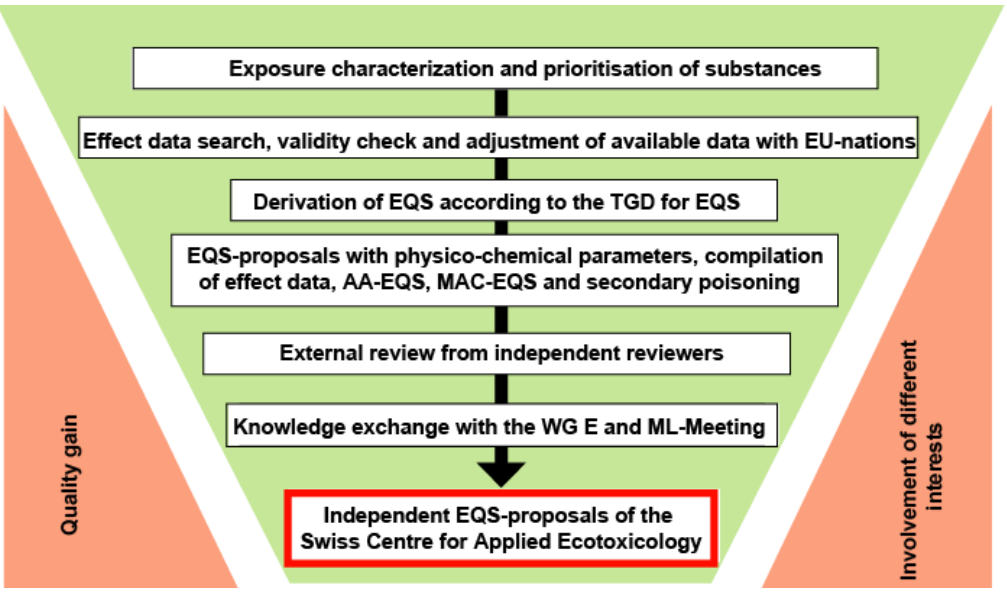


Figure 9: Steps of the development of an EQS-proposal at the Swiss Centre for Applied Ecotoxicology (Kase et al., 2011).

For substances for which EQS exist or existed in EU countries, only an assessment of the appropriateness of the EQS is made with respect to current ecotoxicity data. For substances for which EQS are developed at the same time in the EU and in Switzerland, the EQS are derived in close collaboration with the appropriate experts. All effect data which were not assessed for their quality by other institutions before are assessed for their validity (Klimisch et al., 1997; Matthiessen et al., 2009) and relevance. The proposals put forward are still provisional and will undergo a further phase before they are finalized.

Classification of risk quotients for the evaluation of water quality regarding priority substances

In the pilot study at the WWTP Vidy Lausanne the risk quotients were classified according to a classification system proposed by Götz et al. (2010) (Table 6).

Table 6: Proposed classification system for risk assessment to assess the water quality regarding selected chemical substances (adapted from Götz et al., 2010; Kase et al., 2011). EC environmental concentration, AA-EQS Annual Average EQS, RQ risk quotient.

Evaluation		Condition/description		Compliance with quality criterion
	very good	The environmental concentration (EC) is 100 times smaller than the quality criterion (AA-EQS)	RQ < 0.01	AA-EQS passed
		The environmental concentration (EC) is 10 times smaller than the quality criterion (AA-EQS)	0.01 ≤ RQ < 0.1	
	good	The environmental concentration (EC) is smaller than the quality criterion (AA-EQS)	0.1 ≤ RQ < 1	
	moderate	The environmental concentration (EC) is smaller than the double quality criterion (AA-EQS)	1 ≤ RQ < 2	AA-EQS exceeded
	insufficient	The environmental concentration (EC) is smaller than the tenfold quality criterion (AA-EQS)	2 ≤ RQ < 10	
	poor	The environmental concentration (EC) is the same or greater than the tenfold quality criterion (AA-EQS)	RQ > 10	

The evaluation of the water quality regarding micropollutants originating from municipal wastewater was adapted according to the module 'Physicochemical Water Quality' (Liechti, 2010) of the modular stepwise procedure for Switzerland (www.modul-stufen-konzept.ch). This classification system reflects the water quality and not the ecological quality. However, in the European Union (EU) a connection between the ecological status and the chemical status is made in the context of the Water Framework Directive (WFD): an exceedance of the AA-EQS for one substance leads to the surpass of a good ecological status in the context of the EU-WFD (EU, 2010), as very sensitive species are possibly impacted.

During the evaluation of micropollutants according to the classification based on single substances described above, it was noticed that the detection and quantification limit for the respective substances could influence the outcome. When the analytical limit of quantification for a substance is above a category limit (see Table 6), the classification results as a minimum category. For example if a substance is not detectable and if the limit of quantification is between the AA-EQS/10 and the AA-EQS, then the minimum category can be designated "good+" since the actual exposure concentration can be classified as "good" or "very good".

Götz et al. (2010) have evaluated effects on the concentrations of selected micropollutants in Swiss rivers due to wastewater effluents. The water quality regarding those substances was assessed using the predicted environmental concentrations (PEC) from the exposure model of (Ort et al., 2007) assuming low flow conditions (Q_{347}) and compared with the respective AA-EQS (Figure 10).

Based on this assessment scheme a moderate to poor water quality regarding three of the six selected micropollutants (diclofenac, carbamazepin and clarithromycin) was detected in 14 % of the assessed 543 water courses.

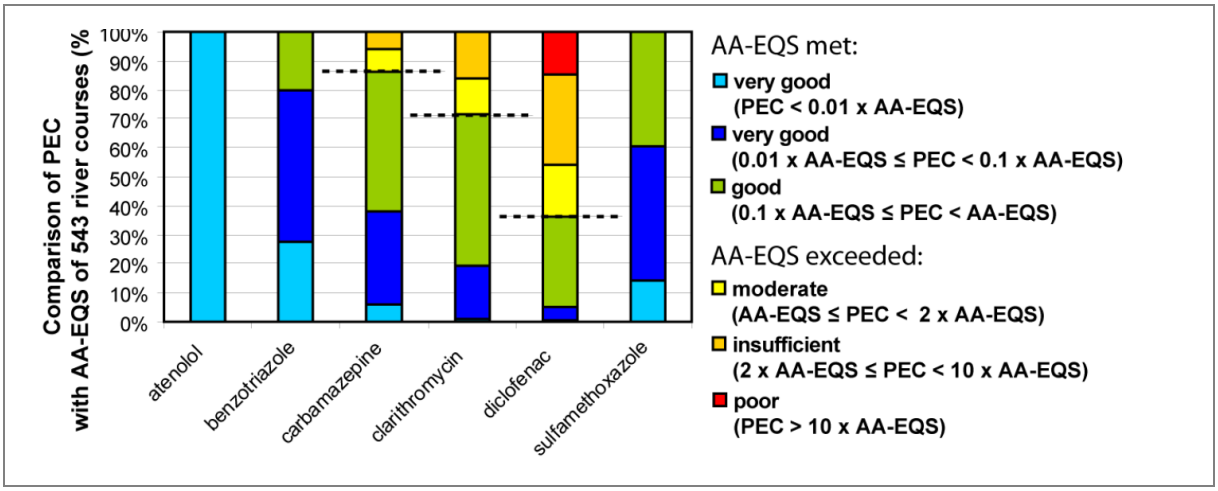


Figure 10: Comparison of predicted environmental concentrations (PEC) with annual average EQS of 543 river courses (%) (from (Götz et al., 2010).

For diclofenac, carbamazepin and clarithromycin risk quotients were derived using the quality criteria as well as the 90th percentile of the measured concentrations (see chapter 5.1).

Figures

Biological treatment

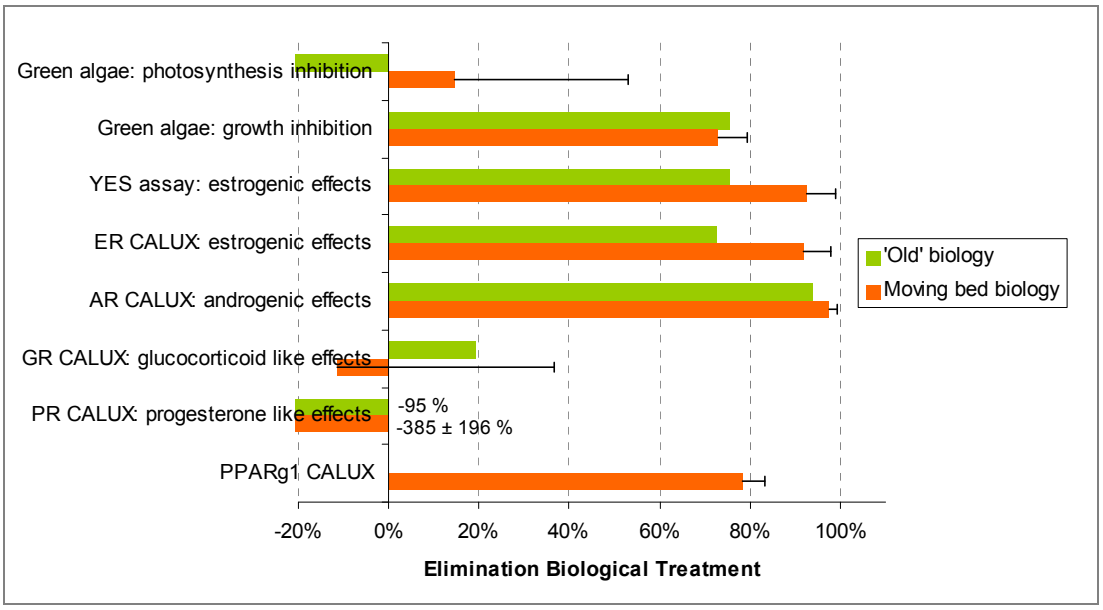


Figure 11: Elimination efficiencies (%) for the "old" biology (SB) (1st MC) and mean elimination efficiencies (%) for the moving bed biology (LF) (2nd to 4th MC, mean ± SD) for specific effects measured in *in vitro* bioassays.

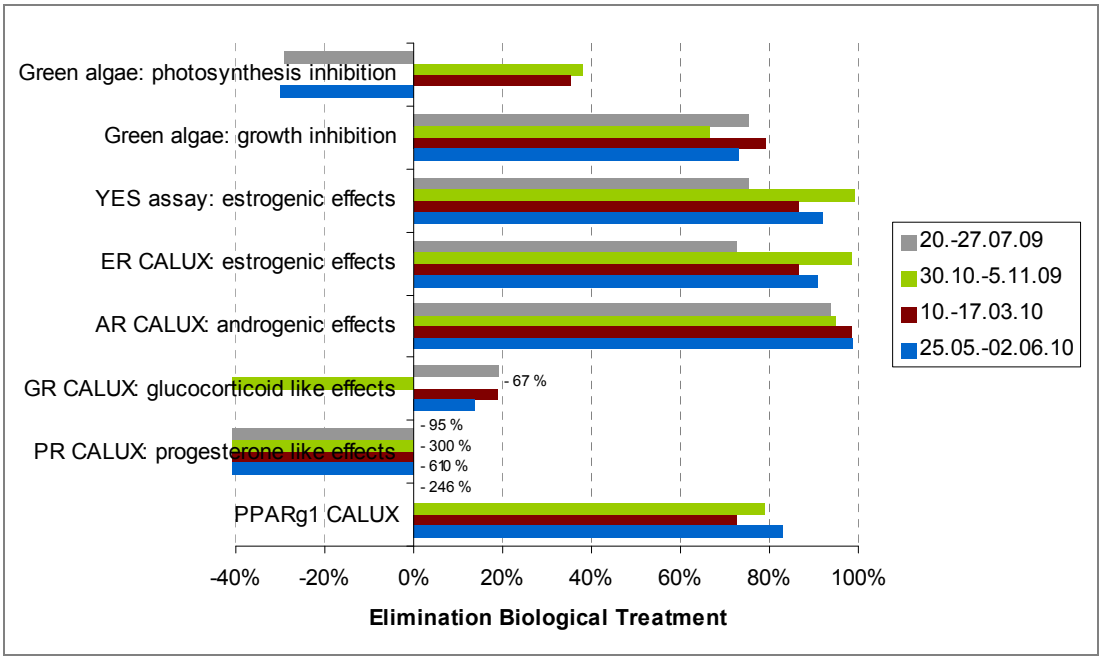


Figure 12: Elimination efficiencies (%) in the biological treatments for each of the four measurement campaigns regarding specific effects measured in *in vitro* bioassays.

Biological treatment combined with ozonation

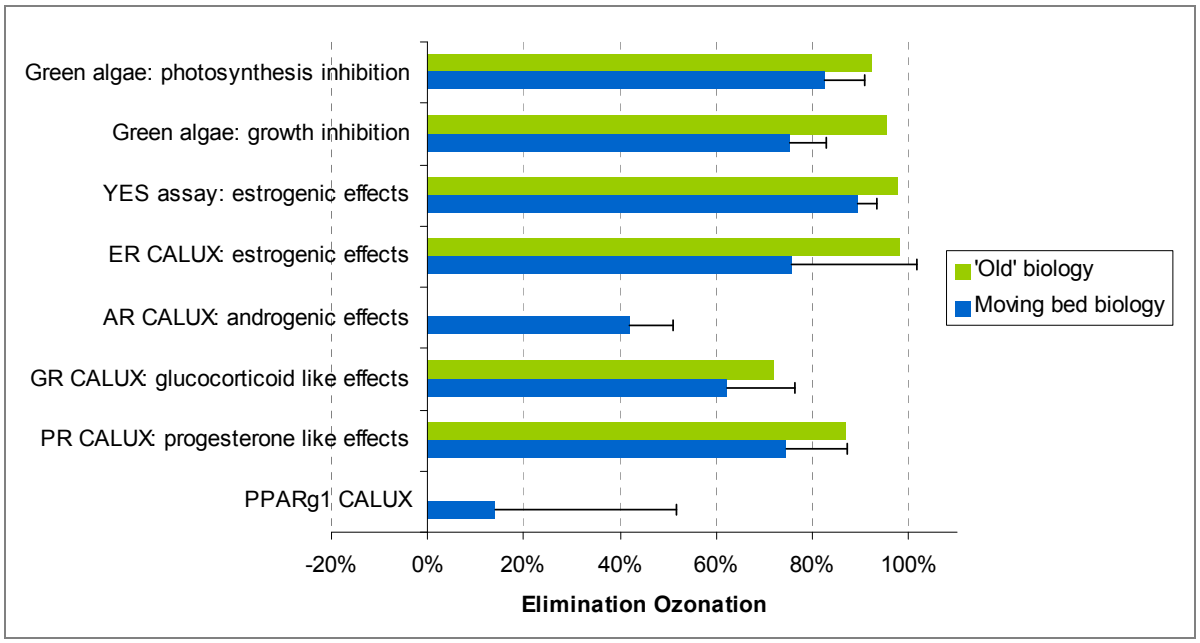


Figure 13: Elimination efficiencies (%) in the ozonation treatment with wastewater from the "old" biology (SB) (1st MC) and from the moving bed biology (LF) (2nd to 4th MC, mean ± SD) for specific effects measured in *in vitro* bioassays.

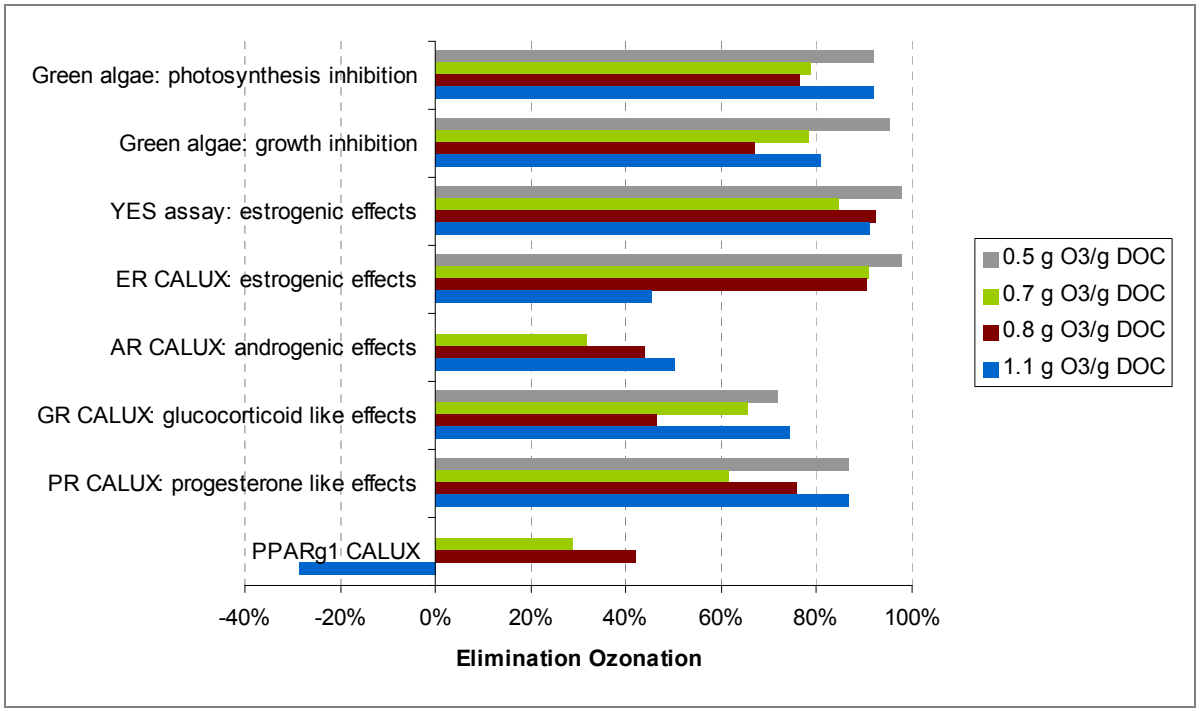


Figure 14: Elimination efficiencies (%) in the ozonation treatments for each of the four measurement campaigns regarding specific effects measured in *in vitro* bioassays.

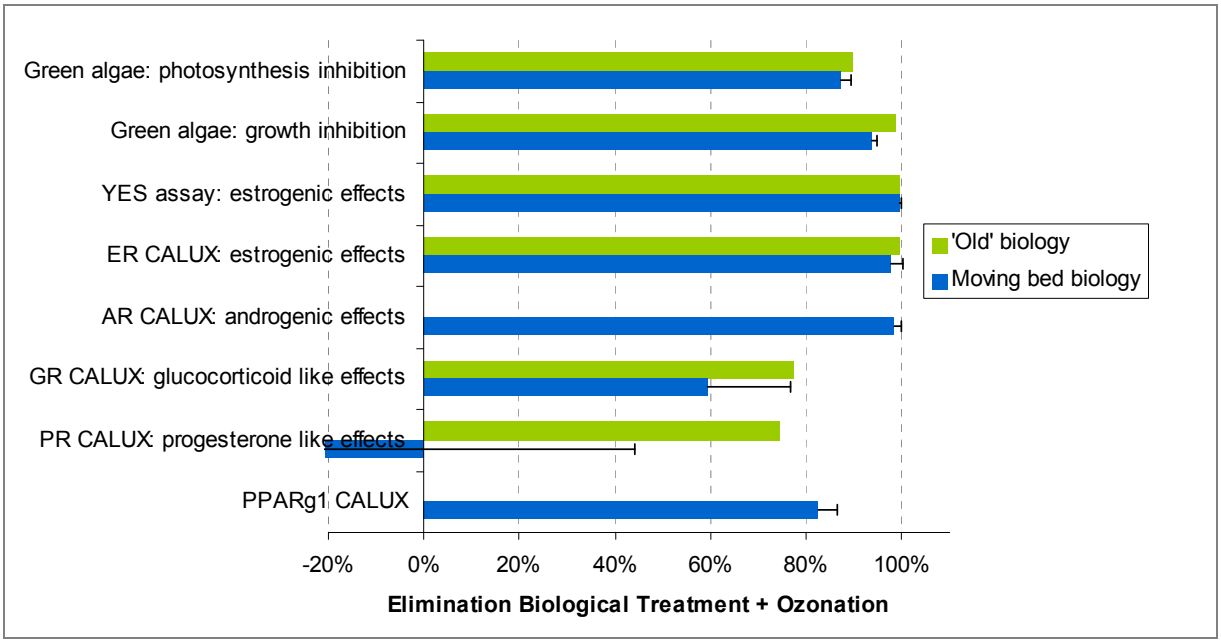


Figure 15: Elimination efficiencies (%) in the biological treatment with ozonation with wastewater from the "old" biology (SB) (1st MC) and wastewater from the moving bed biology (LF) (2nd to 4th MC, mean ± SD) for specific effects measured in *in vitro* bioassays.

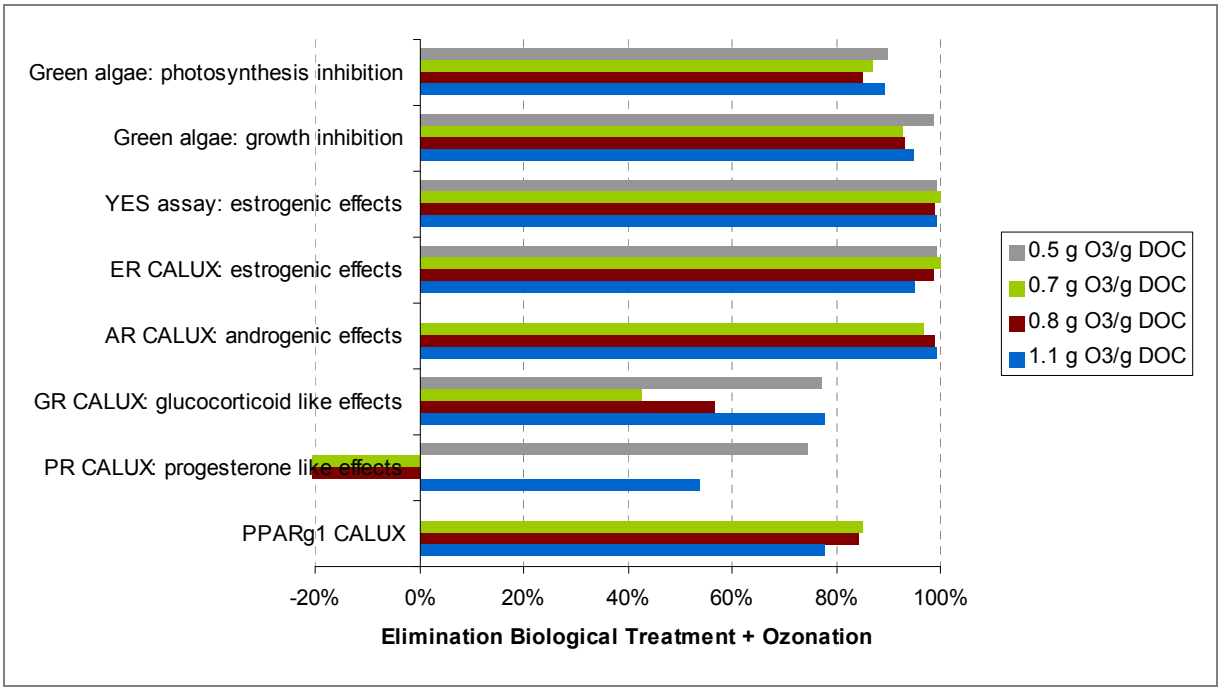


Figure 16: Elimination efficiencies (%) in the biological treatment + ozonation for each of the four measurement campaigns regarding specific effects measured in *in vitro* bioassays.

Biological treatment combined with powdered activated carbon –UF treatment

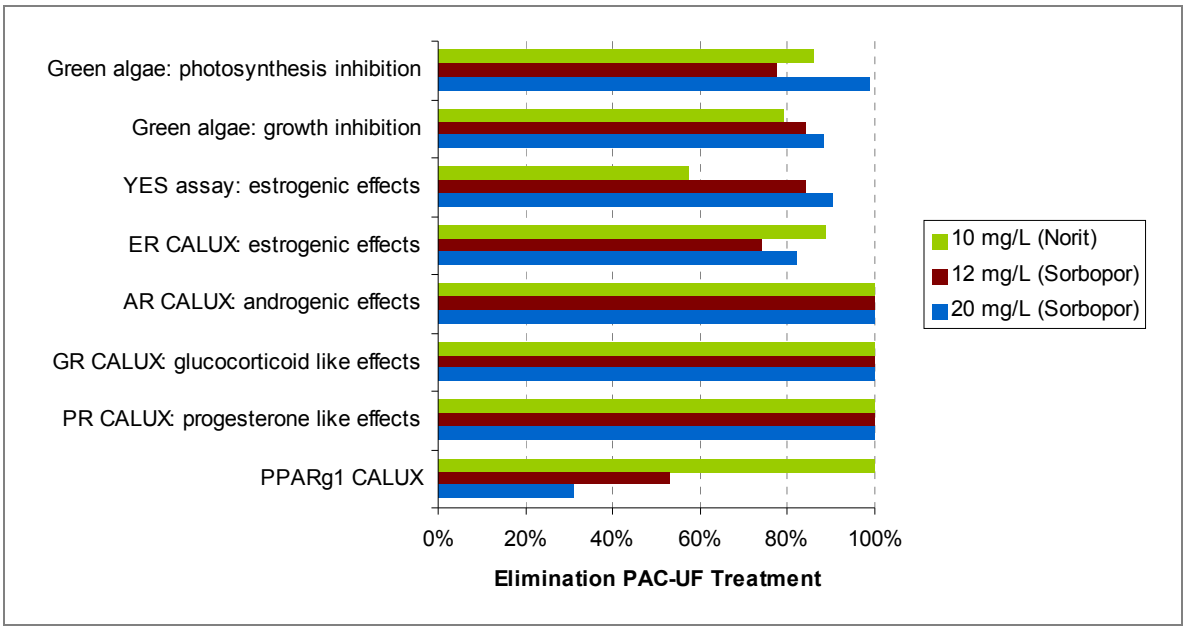


Figure 17: Elimination efficiencies (%) in the PAC-UF treatment for each of the four measurement campaigns regarding specific effects measured in *in vitro* bioassays.

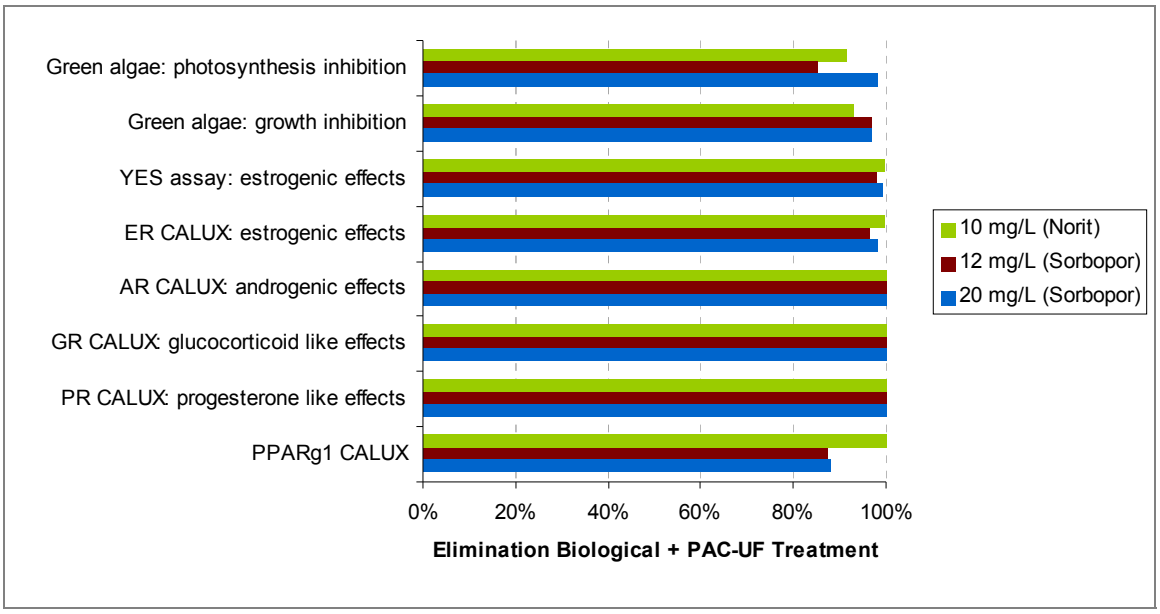


Figure 18: Elimination efficiencies (%) in the biological and PAC-UF treatment for each of the four measurement campaigns regarding specific effects measured in *in vitro* bioassays.

Tables

Table 7: *Pseudokirchneriella subcapitata*, photosynthesis inhibition after 2h: Diuron equivalent concentrations, DEQ [µg/L] (SD in brackets), elimination efficiency [%] and change index for the respective treatment steps. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**
20.-27.07.09	0.5	No PAC	0.193	0.250	-29%	0.77	0.020	92%	12.67	0.029	88%	8.53	n.m.	-	-	90%	-
			(0.031)	(0.002)			(0.004)			(0.004)							
30.10.-05.11.09	0.7	10 (Norit)	0.306	0.189	38%	1.62	0.040	79%	4.70	0.088	53%	2.14	0.026	86%	7.22	87%	91%
			(0.007)	(0.005)			(0)			(0.011)			(0.001)				
10.-17.03.10	0.8	12 (Sorbopor)	0.148	0.096	35%	1.54	0.022	77%	4.30	0.024	75%	4.06	0.022	77%	4.43	85%	85%
			(0.002)	(0.004)			(0)			(0)			(0)				
25.05.-02.06.10	0.11	20 (Sorbopor)	0.305	0.398	-30%	0.77	0.032	92%	12.31	0.031	92%	12.73	0.005	99%	77.23	89%	98%
			(0.006)	(0.027)			(0)			(0.001)			(0.001)				

Table 8: *Pseudokirchneriella subcapitata*, growth inhibition after 24h: Toxicity equivalent concentrations, TEQ [mg/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**
20.-27.07.09	0.5	No PAC	16.72	4.12	75%	4.06	0.19	95%	21.63	1.06	74%	3.88	n.m.	-	-	99%	-
			(3.78)	(0.56)			(0.27)			(0.14)							
30.10.-05.11.09	0.7	10 (Norit)	18.09	6.05	67%	2.99	1.30	78%	4.64	4.14	32%	1.46	1.26	79%	4.81	93%	93%
			(6.84)	(1.55)			(0.15)			(0.89)			(0.06)				
10.-17.03.10	0.8	12 (Sorbopor)	31.43	6.55	79%	4.80	2.17	67%	3.02	1.17	82%	5.58	1.03	84%	6.37	93%	97%
			(1.10)	(0.30)			(0.05)			(0.13)			(0.04)				
25.05.-02.06.10	0.11	20 (Sorbopor)	29.88	8.06	73%	3.71	1.54	81%	5.23	1.39	83%	5.78	0.93	88%	8.63	95%	97%
			(2.06)	(0.21)			(0.09)			(0.10)			(0.04)				

Table 9: *Saccharomyces cerevisiae*, estrogen receptor binding: Estradiol equivalent concentration, EEQ [ng/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2rd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**
20.-27.07.09	0.5	No PAC	48.68	12.00	75%	4.06	0.27	98%	44.87	3.08	74%	3.89	n.m.	-	-	99%	-
			(4.25)	(0.88)			(0.09)			(0.59)							
30.10.-05.11.09	0.7	10 (Norit)	100.55	0.68	99%	147.04	0.10	85%	6.53	11.43	-1571%	0.06	0.29	58%	2.35	100%	100%
			(12.03)	(0.12)			(0.05)			(0.56)			(0.09)				
10.-17.03.10	0.8	12 (Sorbopor)	61.68	8.34	86%	7.39	0.65	92%	12.77	3.26	61%	2.56	1.32	84%	6.31	99%	98%
			(6.82)	(0.47)			(0.12)			(0.33)			(0.28)				
25.05.-02.06.10	0.11	20 (Sorbopor)	37.15	3.03	92%	12.25	0.27	91%	11.34	0.23	92%	13.04	0.29	90%	10.32	99%	99%
			(2.42)	(0.51)			(0.01)			(0.08)			(0.08)				

Table 10: ER CALUX, estrogen receptor binding: Estradiol equivalent concentration, EEQ [ng/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. LOD agonism = 0.03 ng 17ß-estradiol equivalents/l water. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Eliminatio n OZ (%)	Effect OZ	CAG SF	Eliminatio n OZ + SF (%)	Effect OZ + SF	PAC -UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**	**Blank**
20.-27.07.09	0.5	No PAC	24.50	6.70	73%	3.66	0.14	98%	47.86	1.45	78%	4.62	n.m.	-	-	99%	-	<LOD
			(4.95)							(0.07)								
30.10.-05.11.09	0.7	10 (Norit)	81.00	1.40	98%	57.86	0.13	91%	10.77	5.60	-300%	0.25	0.16	89%	8.75	100%	100%	<LOD
10.-17.03.10	0.8	12 (Sorbopor)	22.00	3.00	86%	7.33	0.29	90%	10.34	2.60	13%	1.15	0.78	74%	3.85	99%	96%	<LOD
25.05.-02.06.10	0.11	20 (Sorbopor)	9.80	0.90	91%	10.89	0.49	46%	1.84	0.15	83%	6.00	0.16	82%	5.63	95%	98%	<LOD

Table 11: AR CALUX, androgen receptor binding: dihydrotestosterone (DHT) equivalent concentration [ng/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. LOD agonism = 0.1 ng DHT equivalents/l water. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**	**Blank**
20.-27.07.09	0.5	No PAC	11.00 (1.41)	0.67	94%	16.42	< LOD	-	-	< LOD	-	-	n.m.	-	-	-	-	<LOD
30.10.-05.11.09	0.7	10 (Norit)	4.50	0.22	95%	20.45	0.15	32%	1.47	0.67	-205%	0.33	< LOD	100%	-	97%	100%	<LOD
10.-17.03.10	0.8	12 (Sorbopor)	28.00	0.48	98%	58.33	0.27	44%	1.78	0.17	65%	2.82	< LOD	100%	-	99%	100%	<LOD
25.05.-02.06.10	0.11	20 (Sorbopor)	18.00	0.20	99%	90.00	0.10	50%	2.00	0.13	35%	1.54	< LOD	100%	-	99%	100%	<LOD

Table 12: GR CALUX, glucocorticoid receptor binding: dexmethasone (dex) equivalent concentrations [ng/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. LOD agonism = 4.0 ng dex equivalents/l water. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**	**Blank**
20.-27.07.09	0.5	No PAC	70.50 (7.78)	57.00	19%	1.24	16.00	72%	3.56	12.50 (0.71)	78%	4.56	n.m.	-	-	77%	-	<LOD
30.10.-05.11.09	0.7	10 (Norit)	84.00	140.00	-67%	0.60	48.00	66%	2.92	66.00	53%	2.12	< LOD	100%	-	43%	100%	<LOD
10.-17.03.10	0.8	12 (Sorbopor)	74.00	60.00	19%	1.23	32.00	47%	1.88	22.00	63%	2.73	< LOD	100%	-	57%	100%	<LOD
25.05.-02.06.10	0.11	20 (Sorbopor)	72.00	62.00	14%	1.16	16.00	74%	3.88	10.00	84%	6.20	< LOD	100%	-	78%	100%	<LOD

Table 13: PR CALUX, progesterone receptor binding: Org-2058 equivalent concentration [ng/L] (SD in brackets), elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. LOD agonism = 0.10 ng Org-2058 equivalents/l water. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**	**Blank**
20.-27.07.09	0.5	No PAC	1.34 (0.66)	2.60	-95%	0.51	0.34	87%	7.65	0.37 (0.01)	86%	7.03	n.m.	-	-	75%	-	<LOD
30.10.-05.11.09	0.7	10 (Norit)	0.11	0.44	-300%	0.25	0.17	61%	2.59	0.14	68%	3.14	< LOD	100%	-	-55%	100%	<LOD
10.-17.03.10	0.8	12 (Sorbopor)	0.21	1.49	-610%	0.14	0.36	76%	4.14	0.72	52%	2.07	< LOD	100%	-	-71%	100%	<LOD
25.05.-02.06.10	0.11	20 (Sorbopor)	0.26	0.90	-246%	0.29	0.12	87%	7.50	0.27	70%	3.33	< LOD	100%	-	54%	100%	<LOD

Table 14: PPARg1 CALUX, peroxisome proliferator like receptor binding: Rosiglitasone equivalents [ng/L], elimination efficiency [%] and change index (CI_{TEQ}) for the respective treatment steps. LOD agonism = 0.10 ng Org-2058 equivalents/l water PPARg1 CALUX, LOD agonism = 25 ng Rosiglitasone equivalents/l water. EN Entrée STEP, SB Sortie biologie (1st MC), LF Lit fluidisé (2nd- 4th MC), OZ Sortie O_3, CAG Sortie charbon actif granulé (1st- 2nd MC), SF Sortie filtre à sable (3rd- 4th MC), PAC-UF Sortie charbon actif en poudre - Ultrafiltration (2nd- 4th MC). n.m. not measured.

Date	Ozone dosage (mg_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB LF	Elimination Biology (%)	Effect Biology	OZ	Elimination OZ (%)	Effect OZ	CAG SF	Elimination OZ + SF (%)	Effect OZ + SF	PAC-UF	Elimination PAC-UF (%)	Effect PAC-UF	**Overall Elimination Ozonation**	**Overall Elimination PAC-UF**	**Blank**
20.-27.07.09	0.5	No PAC	< LOD	< LOD	-	-	< LOD	-	-	< LOD	-	-	n.m.	-	-	-	-	<LOD
30.10.-05.11.09	0.7	10 (Norit)	1010	212	79%	4.76	151	29%	1.40	214	-1%	0.99	< LOD	-	-	85%	-	<LOD
10.-17.03.10	0.8	12 (Sorbopor)	368	100	73%	3.68	58	42%	1.72	67	33%	1.49	47	53%	2.13	84%	87%	<LOD
25.05.-02.06.10	0.11	20 (Sorbopor)	491	84	83%	5.85	108	-29%	0.78	69	18%	1.22	58	31%	1.45	78%	88%	<LOD

Table 15: *Vibrio fischeri* luminescence inhibition after 30 min: EC_{20} [%] after 30 minutes (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O_3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{20} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\,g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	42 (30.3-58.2)	n.t.	2.62	n.t.	-	n.t.	-	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	100	n.t.	1.10	n.t.	-	n.t.	-	n.t.	-
10.-17.03.10	0.8	12 (Sorbopor)	75.4 (60.8-93.5)	n.t.	1.46	n.t.	-	n.t.	-	n.t.	-
26.05.-02.06.10	0.11	20 (Sorbopor)	74.9 (50.7-93.4)	n.t.	1.47	n.t.	-	n.t.	-	n.t.	-

Table 16: *Pseudokirchneriella subcapitata* growth inhibition without nutrient addition after 3 d: EC_{20} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O_3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{20} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\,g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	71.1 (43.4-85.2)	67.1 (59.2-74.3)	0.94	63 (56.6-69.4)	0.94	58.6 (54.2-62.8)	0.87	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	39.7 (25.5-51.4)	32.3 (29.2-36.2)	0.81	38.9 (34.4-41.9)	1.20	47.9 (43.5-53.1)	1.48	32.4 (27.2-39.6)	1.00
10.-17.03.10	0.8	12 (Sorbopor)	56.38 (42.2-59.9)	54.57 (51.8-57.0)	0.97	52.21 (50.1-57.7)	0.96	57.16 (54.4-59.7)	1.05	53.7 (52.0-55.1)	0.98
26.05.-02.06.10	0.11	20 (Sorbopor)	35.23 (16.5-79.8)	84.23 (70.5-93.6)	2.39	67.82 (59.6-75.9)	0.81	32.38 (23.1-77.6)	0.38	33.23 (26.7-47.2)	0.39

Table 17: *Pseudokirchneriella subcapitata* growth inhibition without nutrient addition after 3 d: EC_{50} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O_3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{50} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage (g_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	84	85.4	1.02	79.3	0.93	77.7	0.91	n.m.	-
			(71.8-142)	(81-92.3)		(75.2-83.8)		(74.5-81.4)			
30.10.-05.11.09	0.7	10 (Norit)	73.9	57.6	0.78	57.8	1.00	63.9	1.11	53.7	0.93
			(61.2-81.5)	(54.3-60)		(54.6-60.2)		(61.8-65.9)		(51.7-55.4)	
10.-17.03.10	0.8	12 (Sorbopor)	72.47	79.96	1.10	77.63	0.97	78.43	0.98	72.19	0.90
			(65.0-75.9)	(79.0-80.9)		(73.9-79.5)		(76.8-79.9)		(70.8-73.6)	
26.05.-02.06.10	0.11	20 (Sorbopor)	82.62	91.5	1.11	86.77	0.95	81.6345	0.89	69.87	0.76
			(62.6->125)	(87.3-163)		(80.8-96.1)		(74.1-84.6)		(64.6-74.0)	

Table 18: *Pseudokirchneriella subcapitata* growth inhibition with nutrient addition after 3 d: EC_{20} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O_3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{20} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage (g_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	76.7	n.t.	1.43	n.t.	-	89.5	0.81	n.m.	-
			(71.2-83.7)					(85.9-115)			
30.10.-05.11.09	0.7	10 (Norit)	52.7	71.7	1.36	n.t.	1.53	n.t.	1.53	100	1.39
			(26.3-59.3)	(61-83)							
10.-17.03.10	0.8	12 (Sorbopor)	48.06	n.t.	2.29	n.t.	-	n.t.	-	n.t.	-
			(18.9-60.4)								
26.05.-02.06.10	0.11	20 (Sorbopor)	62.98	n.t.	1.75	n.t.	-	87.93	0.80	n.t.	-
			(47.5-76.7)					(66.5->125)			

Table 19: *Pseudokirchneriella subcapitata* growth inhibition with nutrient addition after 3 d: EC_{50} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O_3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{50} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage (g_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	86.3 (84.2-89.6)	n.t.	1.27	n.t.	-	n.t.	-	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	78.8 (75.2-83.1)	n.t.	1.40	n.t.	-	n.t.	-	n.t.	-
10.-17.03.10	0.8	12 (Sorbopor)	62.23 (65.3-72.3)	n.t.	1.77	n.t.	-	n.t.	-	n.t.	-
26.05.-02.06.10	0.11	20 (Sorbopor)	80.19 (70.8-99.8)	n.t.	1.37	n.t.	-	n.t.	-	n.t.	-

Table 20: *Lemna minor* growth rate fronds after 7 d: EC_{20} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{20} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage (g_{O3} g_{DOC}^{-1})	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	20.5 (6.7-35)	36.1 (11.8-50.4)	1.76	53.5 (40.9-63.8)	1.48	64.7 (55.2-73.4)	1.79	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	100	n.t.	1.10	n.t.	-	n.t.	1.00	n.t.	-
10.-17.03.10	0.8	12 (Sorbopor)	47.9 (24.7-67.7)	95	1.98	86.92 (52.1-151)	0.91	72.42 (42.8-95.4)	0.76	100	1.1
26.05.-02.06.10	0.11	20 (Sorbopor)	74.94 (50.7-93.4)	n.t.	1.47	n.t.	-	72.11 (61.7-80.5)	0.66	n.t.	-

Table 21: *Lemna minor* growth rate fronds after 7 d: EC_{50} [%] (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{50} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\ g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	92.7 (68-170)	74.4 (60.6-95.9)	0.80	80.3 (71.7-90.7)	1.08	83.1 (76.1-90.4)	1.12	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	n.t.	n.t.	-	n.t.	-	n.t.	-	n.t.	-
10.-17.03.10	0.8	12 (Sorbopor)	77.22 (61.7-100)	n.t.	1.42	n.t.	-	94.27 (81.9-123)	0.86	n.t.	-
26.05.-02.06.10	0.11	20 (Sorbopor)	100 (92-133)	n.t.	1.10	n.t.	-	92.16 (85.8-98.09	0.84	n.t.	-

Table 22: *Ceriodaphnia dubia* mortality after 7 d: LC_{50} [%] for number of offspring (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{50} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\ g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	90	54.6 (41.9-73.6)	0.61	83.1 (60-100)	1.52	73.5 (60-90)	1.35	n.m.	-
30.10.-05.11.09	0.7	10 (Norit)	90	n.t.	1.22	90	0.82	n.t.	-	n.t	-
10.-17.03.10	0.8	12 (Sorbopor)	70.8 (60-90)	n.t.	1.55	n.t.	-	90 (45->100)	0.82	n.t.	-
26.05.-02.06.10	0.11	20 (Sorbopor)	95.8 (95.1-97)	n.t.	1.15	n.t.	-	97.3 (94.1-102)	0.88	n.t.	-

Table 23: *Ceriodaphnia dubia* reproduction after 7 d: EC_{20} [%] for number of offspring (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{20} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\,g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	54.6	50.1	0.92	62.8	1.25	58.9	1.18	n.m.	-
				(44.8-56.1)		(56.2-79.5)		(54.9-60.8)			
30.10.-05.11.09	0.7	10 (Norit)	55.4	n.t.	1.99	n.t.	-	n.t.	-	n.t.	-
10.-17.03.10	0.8	12 (Sorbopor)	50.246	n.t.	2.19	n.t.	-	71.699	0.65	n.t.	-
			(44.7-58.31)					(31.6-88.1)			
26.05.-02.06.10	0.11	20 (Sorbopor)	58.199	n.t.	1.89	30	0.27	48.01	0.44	n.t.	-
			(49.6-59.4)					(42.9-54.6)			

Table 24: *Ceriodaphnia dubia* reproduction after 7 d: EC_{50} [%] for number of offspring (95% confidence interval in brackets) and change index (CI) for the respective treatment steps.

EN Entrée STEP, SB Sortie biologie, LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration. n.t. not toxic, n.m. not measured. If e.g. only the influent sample was toxic, a surrogate EC_{50} of 110% (as the "real" value is >100%) was used for the not toxic samples in order to be able to calculate a change index.

Date	Ozone dosage ($g_{O3}\,g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)	EN	SB (1st MC) LF (2nd - 4th MC)	Effect Biology	OZ	Effect OZ	CAG (1st, 2nd MC) SF (3rd,4th MC	Effect OZ + SF	PAC-UF	Effect PAC-UF
20.-27.07.09	0.5	No PAC	67.2	63.5	0.94	71.1	1.12	62.6	0.99	n.m.	-
			(60.2-76.2)	(59.2-68.3)		(63.1-82.5)		(61.0-66.2)			
30.10.-05.11.09	0.7	10 (Norit)	67.3	n.t.	1.63	n.t.	-	n.t.	-	n.t.	-
			(58.6-78.2)								
10.-17.03.10	0.8	12 (Sorbopor)	58.38	n.t.	1.88	n.t.	-	90.97	0.83	n.t.	-
			(54.62-61.76)					(75.2->125)			
26.05.-02.06.10	0.11	20 (Sorbopor)	60.37	n.t.	1.82	n.t.	-	60.446	0.55	n.t.	-
			(58.6-62.4)					(56.4-65.0)			

Table 25: *Lumbriculus variegatus* reproduction and biomass after 28 d: Mean (SD in brackets) and change index (CI) for the respective treatment steps. LF Lit fluidisé, OZ Sortie O3, CAG Sortie charbon actif granulé, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration.

Date	Ozone dosage ($g_{O3}\ g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)			Control	LF	OZ	Effect OZ	SF	Effect OZ + SF	PAC-UF	Effect PAC-UF
02.04.-30.04.10	0.8	12 (Sorbopor)	Reproduction (mean number of worms per treatment)	[n]	38.9	35.5	38.5	1.08	27.5	0.77	24	0.68
			SD		6.31	6.81	2.08		6.45		5.89	
				[%]	100	91.26	98.97		70.69		61.70	
02.04.-30.04.10	0.8	12 (Sorbopor)	Mean individual biomass per treatment	[mg]	0.71	0.912	0.557	0.61	0.882	0.97	0.59	0.65
			SD		0.12	0.03	0.17		0.18		0.11	
				[%]	100	128.45	78.45		124.23		82.96	

Table 26: *Oncorhynchus mykiss* survival and developmental parameters: Mean (± SD in brackets) and change index for the respective treatment steps. LF Lit fluidisé, OZ Sortie O3, SF Sortie filtre à sable, PAC-UF Sortie charbon actif en poudre - Ultrafiltration.

Date	Ozone dosage ($g_{O3}\ g_{DOC}^{-1}$)	PAC conc. (mg PAC/L wastewater)			Control	LF	OZ	Effect OZ	SF	Effect OZ + SF	PAC-UF	Effect PAC-UF
11.03.-19.05.10	0.8	12 (Sorbopor)	Overall survival rate	[n]	152	69	102	1.48	103	1.49	112	1.62
				[% of eggs at test start]	95	57.5	85		85.8		100	
11.03.-19.05.10	0.8	12 (Sorbopor)	Survival pre-hatch	[n]	160	96	116	1.21	118	1.23	120	1.25
				[% of eggs at test start]	100	80	96.7		98.3		100	
11.03.-19.05.10	0.8	12 (Sorbopor)	Hatch	[n]	160	96.0	116	1.21	118	1.23	120	1.25
				[% of eggs at test start]	100	80	96.7		98.3		100	
11.03.-19.05.10	0.8	12 (Sorbopor)	Survival post-hatch	[n]	152	69	102	1.48	103	1.49	112	1.62
				[% of eggs at test start]	95	71.8	88.2		87.2		93.3	
11.03.-19.05.10	0.8	12 (Sorbopor)	Swim-up	[n]	149	43	105	2.44	107	2.49	112	2.60
				[% of hatched]	93.1	45	90.7		90.5		93.3	
11.03.-19.05.10	0.8	12 (Sorbopor)	Mean fresh weight of larvae at test end	[mg]	337.6	158.6	273.3	1.72	267.1	1.68	332.6	2.10
				[%]	100.0	47.0	81.0		79.1		98.5	
11.03.-19.05.10	0.8	12 (Sorbopor)	Mean length of larvae at test end	[mm]	30.2	22.6	28.0	1.24	27.9	1.23	29.8	1.32
				[%]	100.0	74.8	92.7		92.4		98.7	
11.03.-19.05.10	0.8	12 (Sorbopor)	Vitellogenin concentration	[ng/mL]	10.6	63.1	9.9	6.37	14.1	4.49	10.2	6.18
			SD		(4.7)	(33.2)	(7.1)		(9.1)		(5.8)	
				[%]	100.0	208.8	32.8		46.5		33.8	