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Swiss Centre for Applied
Ecotoxicology | Eawag-EPFL

Evaluation of bioassays and wastewater quality

In vitro and *in vivo* bioassays for the performance review in the Project **“Strategy MicroPoll”**

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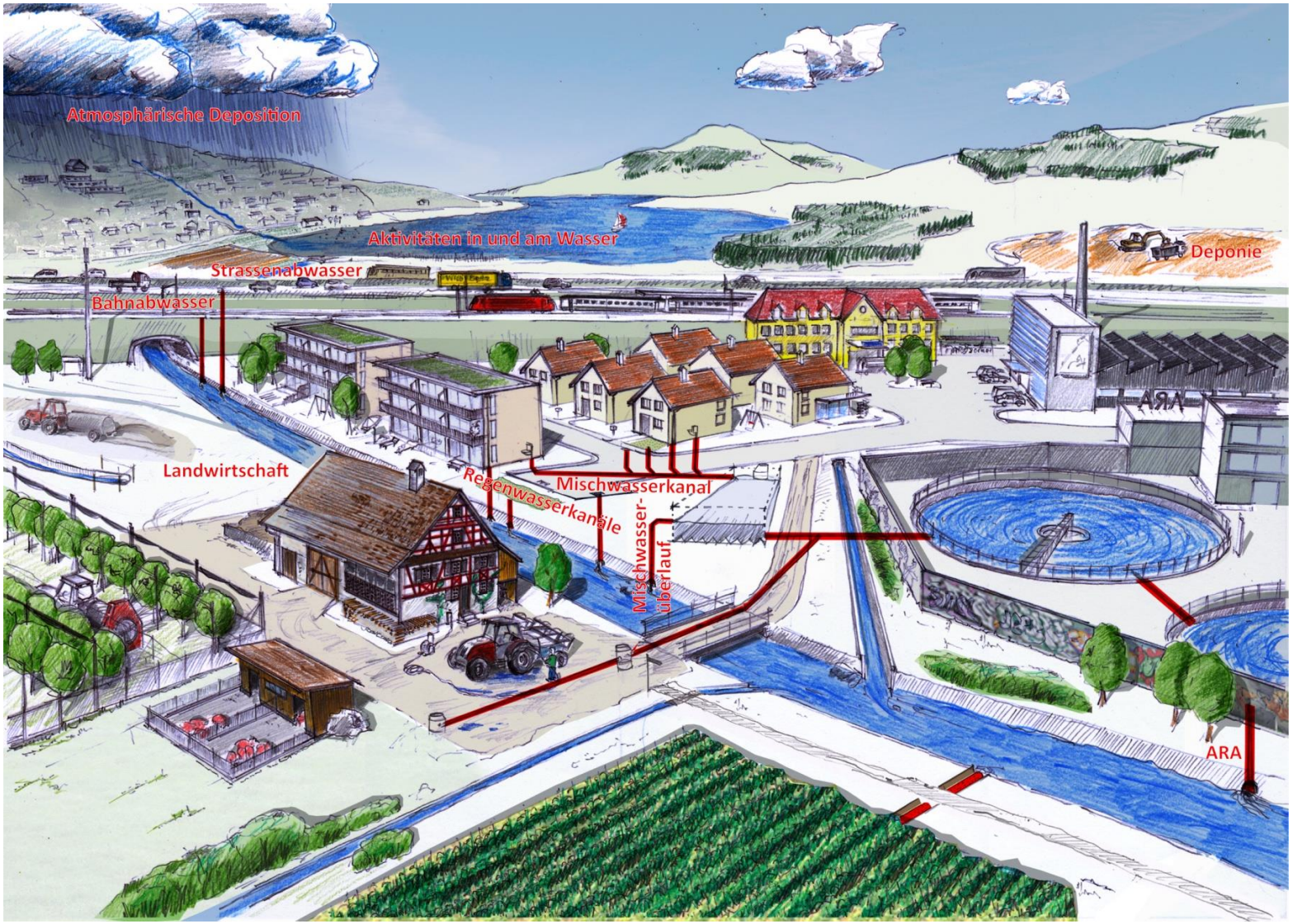
What are micropollutants?

von Darren Hester

Von capl@washjeff.edu

von Anna Warren

How can micropollutants reach surface water bodies?

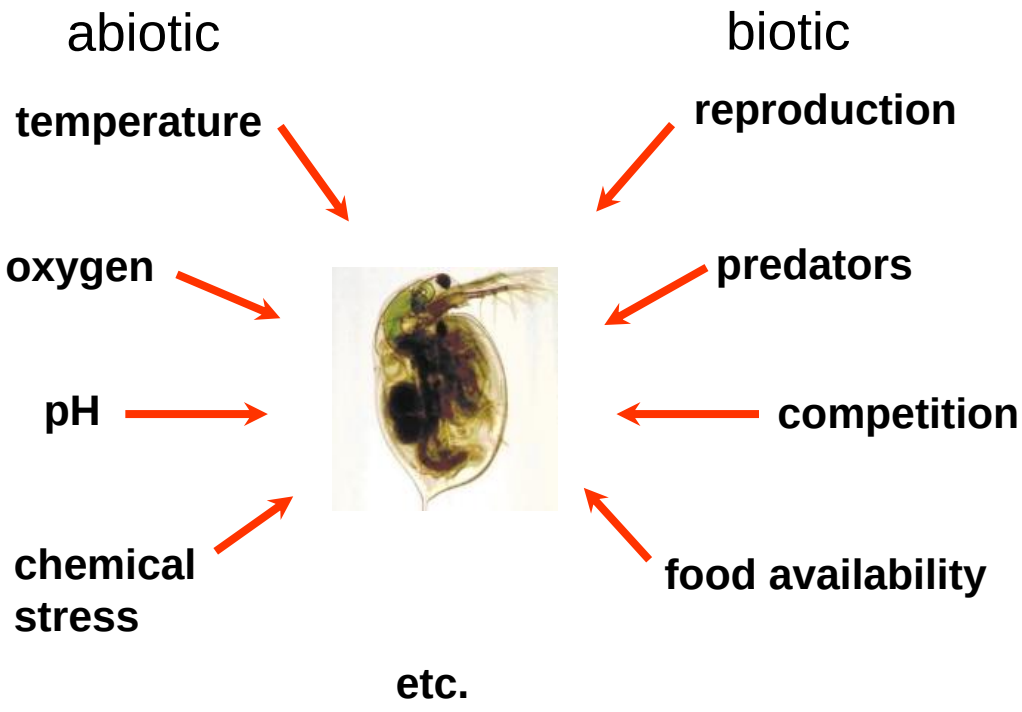


Abegglen et al. 2011, Umweltwissen, BAFU

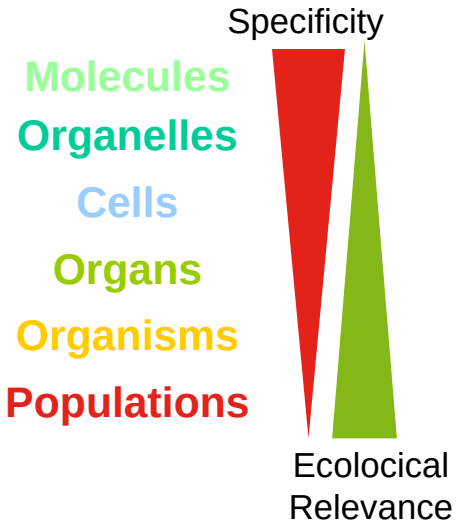
Aquatic Ecosystems and Food Webs

Aquatic Ecosystems and Food Webs

→Organisms living there are exposed to various factors



→ and show reactions on various levels.

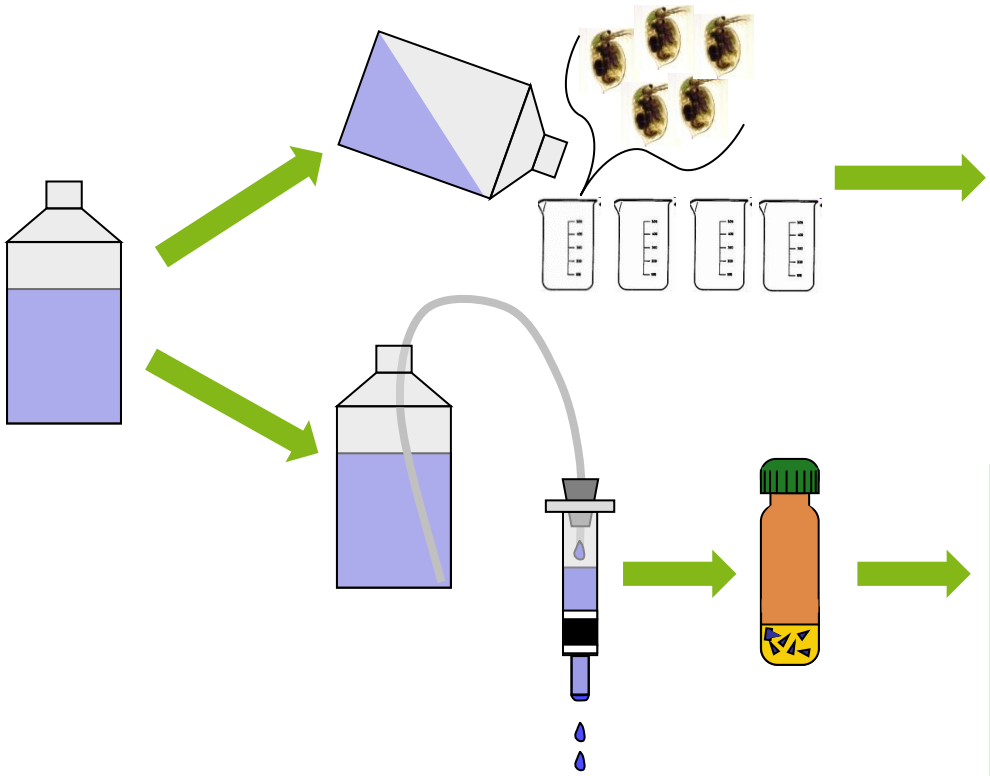


→ Bioassays are applied to detect those reactions.

Bioassays

Bioassay definition:

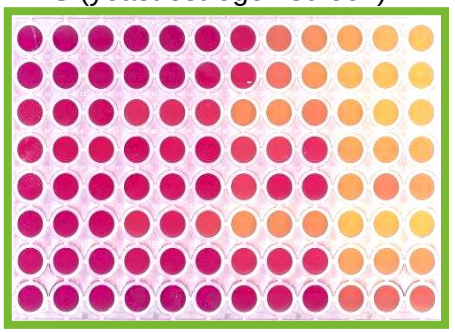
"Standardized" process of an experiment with defined procedure and evaluation



Assay with water flea (Daphnids)

e.g. according to OECD guideline 202

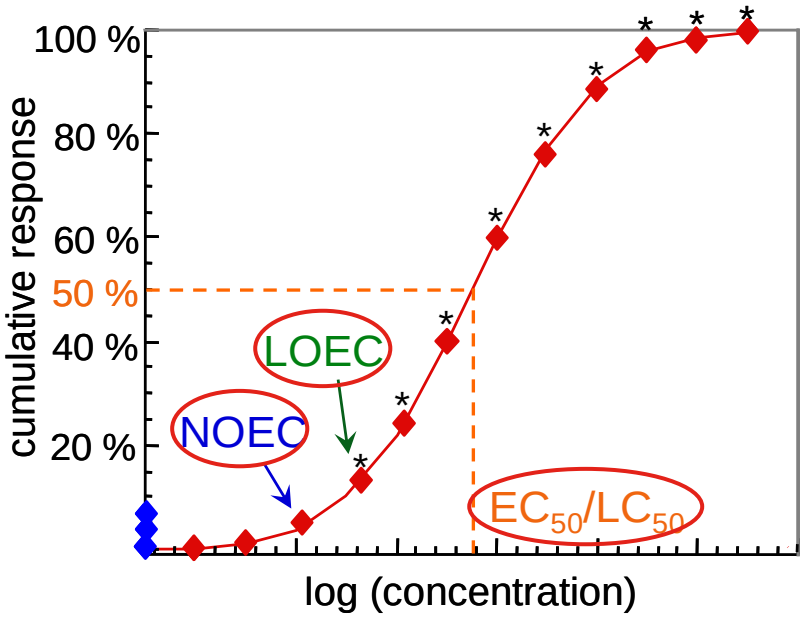
YES (yeast estrogen screen)

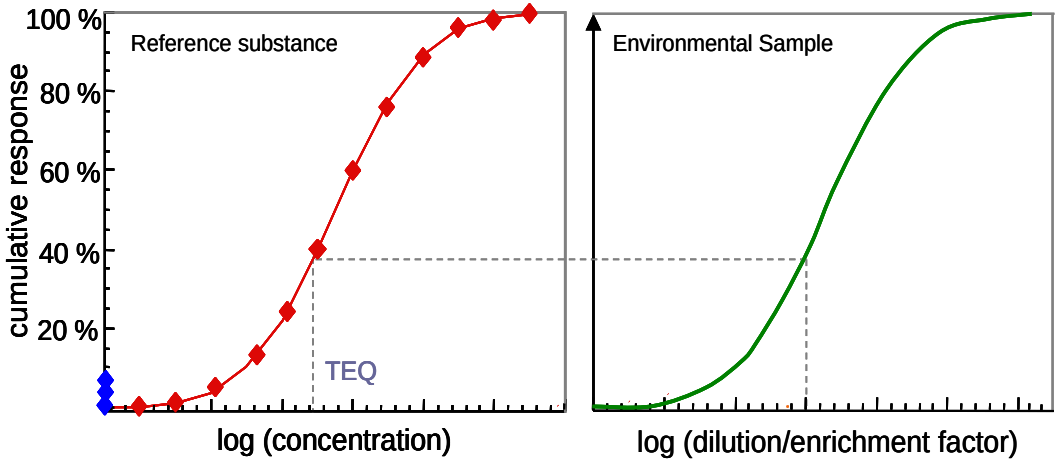


Solid Phase Extraction (SPE)

Evaluation of Bioassay Results

Toxicity parameters:





TEQ (toxic equivalent concentration)

Why Using Bioassays?

- For an **ecotoxicological performance review**
- **With chemical analytics**:
  - Determination of **substance concentration possible**
  - But: **only limited conclusions** about **ecotoxicological consequences** possible
- Hence: bioassays as **reasonable amendment of chemical analytics**
- Various **types of bioassays:** ***in vitro*** and ***in vivo***

- Sensitive detection of effects specific to groups of toxicants with similar modes of action,
- Extrapolation on possible consequences for organisms more difficult.

- Integrate effects of all substances in a water sample,
- Allow conclusions on biological/ ecological effects,
- Give limited information about responsible substance classes.

How can the effects of micropollutants in surface waters be reduced?

Micropollutants in Surface Water - Strategy Micropoll

Project «Strategy Micropoll» of the Swiss Federal Office for the Environment (FOEN)

- **Aim: Development of a strategy regarding micropollutants from urban wastewater**
- **Two large-scale pilot studies at:**

WWTP Wüeri in Regensdorf

(Abegglen et al. 2009, Eawag)

WWTP Vidy in Lausanne

(Margot et al. 2011, Ville de Lausanne)

- in close collaboration with experts from research, practice, and with personal and financial support of the cantonal environmental protection agencies and the operators of the WWTPs

Aims for the Pilot Studies

- **To gain knowledge from trace analytics and ecotoxicological test systems regarding the contamination of wastewater with organic micropollutants**.
- **Evaluation of the advanced treatments ozonation + sand filtration and activated carbon treatment + ultrafiltration (PAC-UF) regarding:**
  - Elimination efficiency for the removal of micropollutants with focus on polar persistent bioactive substances
  - Evaluation of possible side products
- **Investigation of wastewater quality regarding micropollutants and their effects** → Relevance of observed reduction

Pilot Studies for the Evaluation of Advanced Wastewater Treatment

- **Sampling campaigns**: - longer campaigns over one week

  - shorter campaigns over 1-2 days

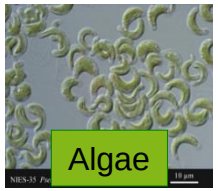
- **Composite samples** (1-7d) collected **before and after each treatment step**

- **Analysis of >50 selected chemicals**

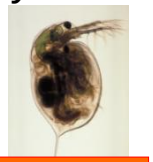
- A range of ***in vitro*- and *in vivo*-bioassays** performed using:
  - **enriched wastewater samples** (using solid phase extraction, most *in vitro* bioassays) (effect parameter: TEQ (toxic equivalent concentration))
  - **native wastewater samples** (assays for mutagenicity and genotoxicity, all *in vivo* bioassays) (toxicity parameter: e.g. EC_{20}, EC_{50})

Which bioassays were performed? – Different trophic levels and modes of action

Primary producers

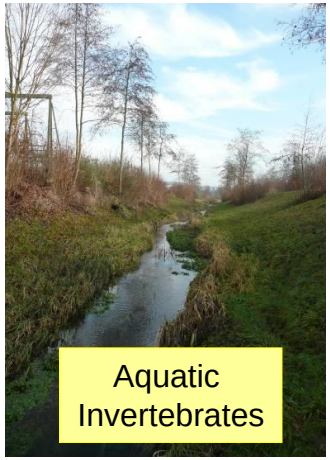


Primary consumers



Secondary consumers

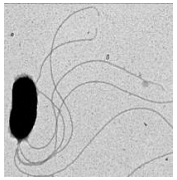
Ecosystem effects



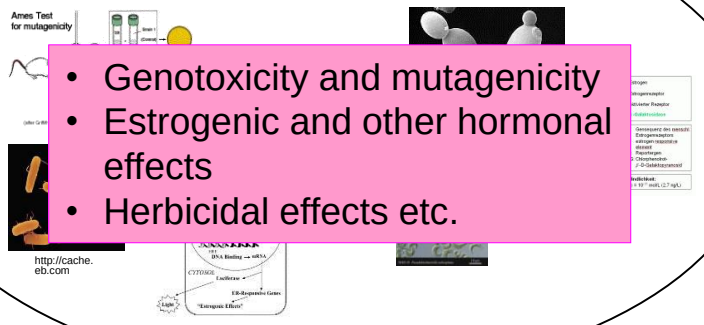
Detritus feeders

Shredders

Destruents



Specific modes of action



- Genotoxicity and mutagenicity
- Estrogenic and other hormonal effects
- Herbicidal effects etc.

PART 1

Performance Analysis of Advanced Wastewater Treatment

or

What can we learn from bioassays about the performance of advanced wastewater treatment?

Comparison of Bioassay Results

Change Index (CI):

- **An indicator for relative changes over sewage treatment steps.**
- Allows a direct comparison of tests with and without sample enrichment and therefore also of *in vitro* and *in vivo* bioassays.

Calculation

$$\mathbf{CI} = \frac{\text{Toxicity value after treatment}}{\text{Toxicity value before treatment}}$$

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

Change Index CI > 1 decreasing toxicity

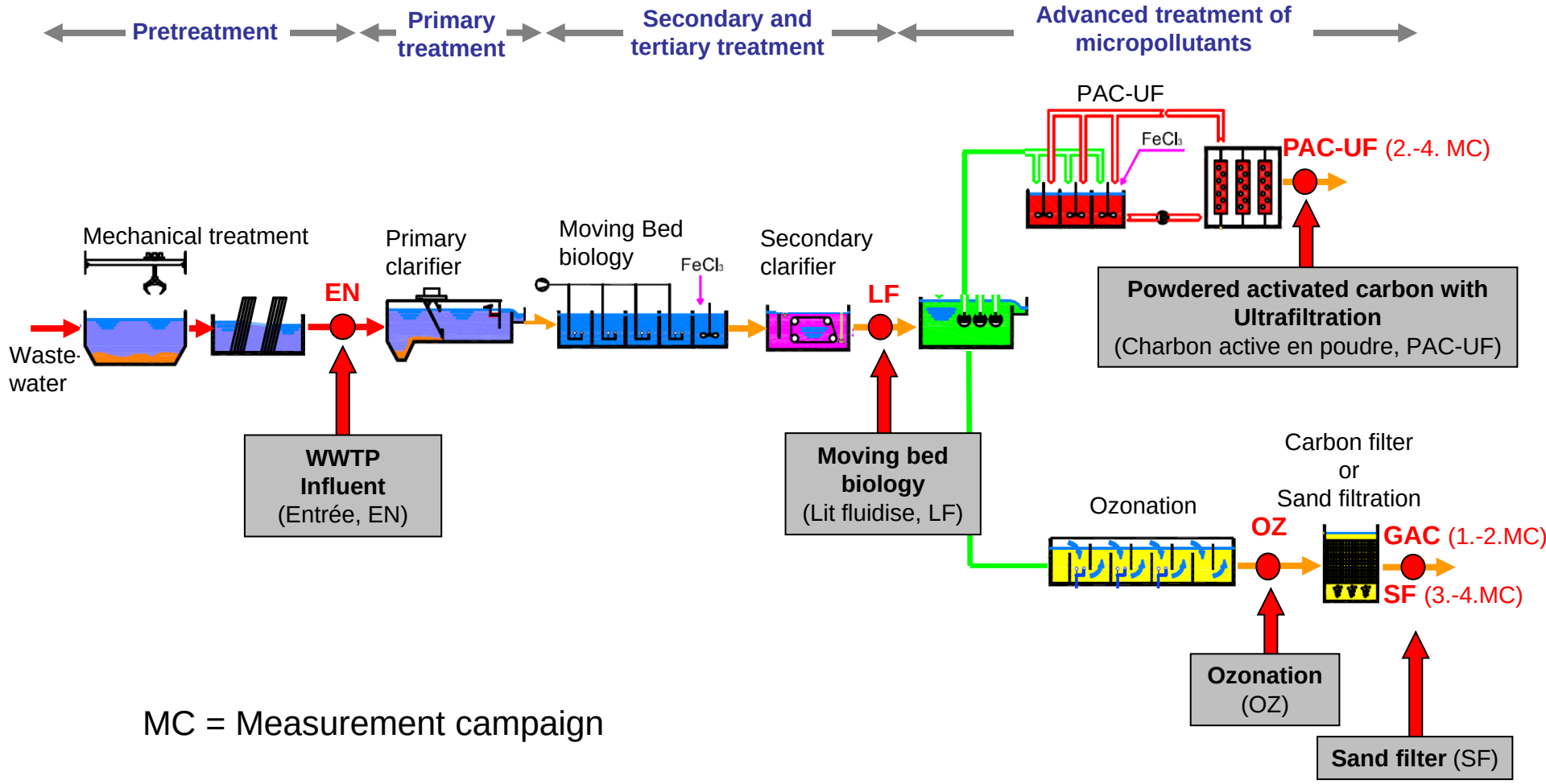
Change Index CI ~1 equal toxicity (range: 0.75<CI<1.25)

Change Index CI < 1 increasing toxicity

→ Deviation of ≥ 25% from CI = 1 considered as a significant effect

Ratte & Ratte, 2009

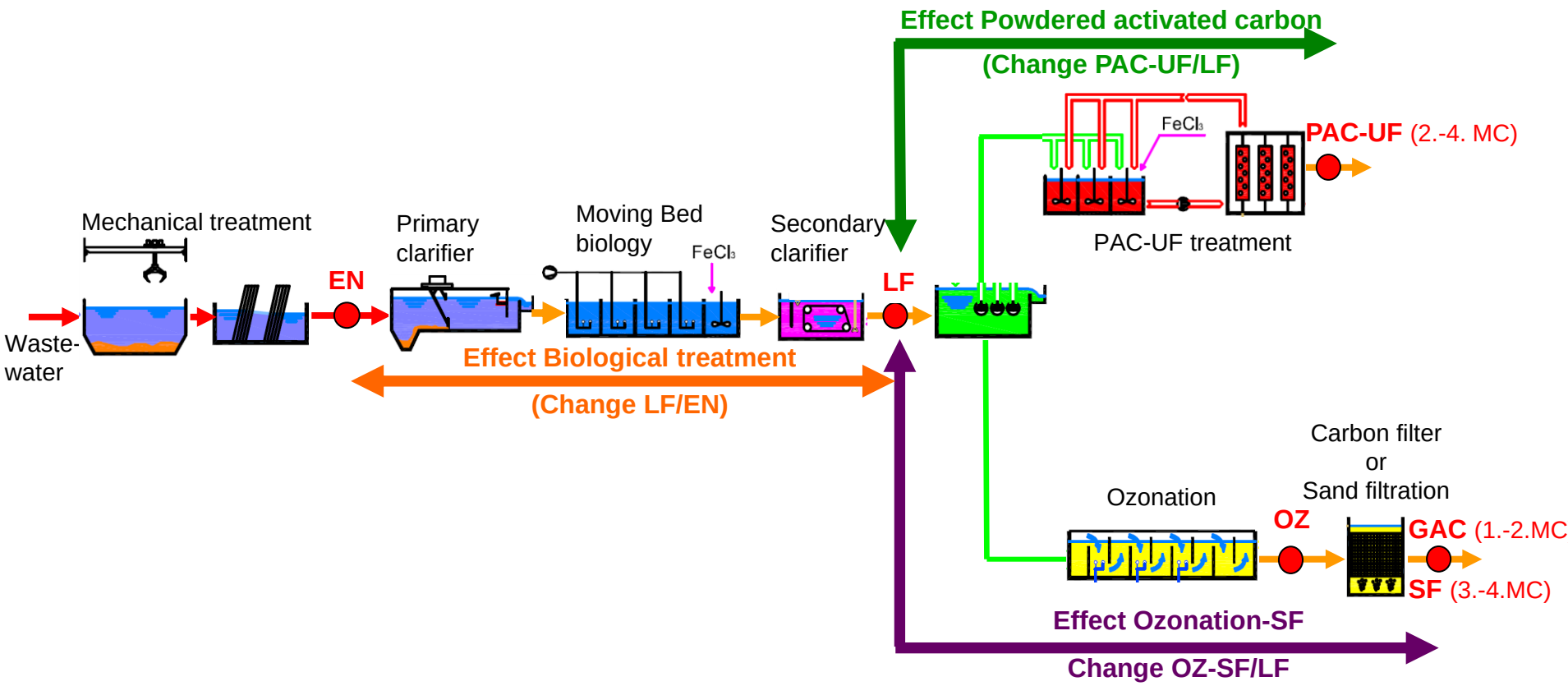
Overview over sewage treatment steps (WWTP Vidy, Lausanne)



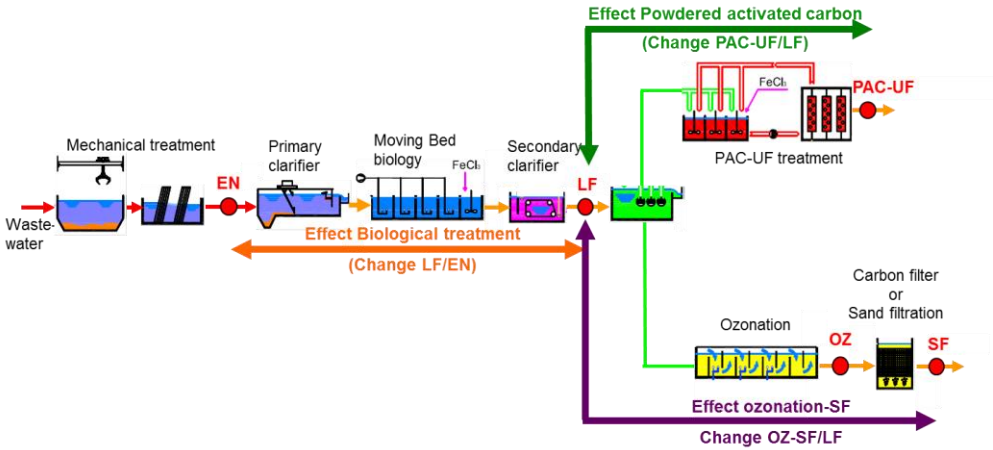
MC = Measurement campaign

according to Margot et al. 2011

Overview over sewage treatment steps and change indices



Example Change Index



Change Index (CI)

> 1 decreasing toxicity

~ 1 equal toxicity

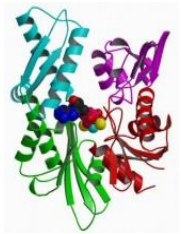
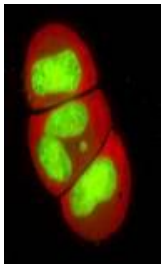
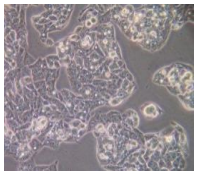
< 1 increasing toxicity

Example EC_{50} $CI = \frac{80\ \%}{40\ \%} = 2$ → CI > 1→ decreasing toxicity

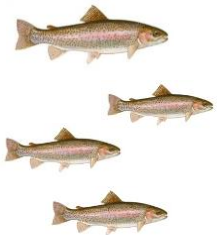
Example TEQ $CI_{TEQ} = \left(\frac{5\ ng/L}{10\ ng/L}\right)^{-1} = 2$ → CI > 1→ decreasing toxicity

Different Levels and Mechanisms of Effect

Molecules Organelles Cells Organs Organisms Populations Ecosystem






Genotoxicity:	Micronucleus assay
Mutagenicity:	Ames assay
Hormonal effects:	YES, ER-, AR-, GR-, PR- and PPAR-CALUX, H295R

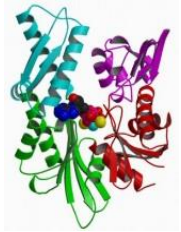
General toxicity:	Bacteria, Algae, Trout
Herbicidal effects:	Algae
Hormonal effects:	Vitellogenin in Trout

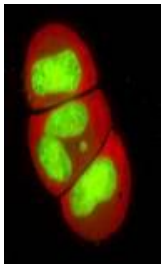
Specificity

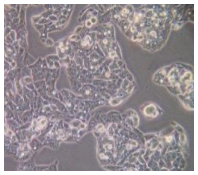
Ecological Relevance

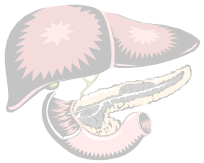
Different Levels and Mechanisms of Effect

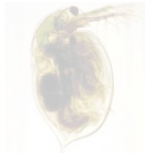
Molecules Organelles Cells Organs Organisms Populations Ecosystem

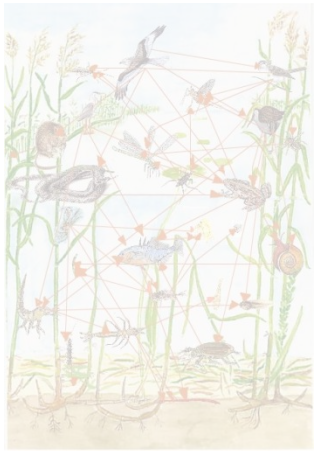












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Specificity

Ecological Relevance

Genotoxic and Mutagenic Effects

Genotoxic and Mutagenic Effects

Definitions

Genotoxicity
denotes any damage to the genetic apparatus, the genome.
(Williams 1989, Annu. Rev. Pharmacol. Toxicol.)

Repair possible

If damages are passed on

Mutagenicity
describes the irreparable, hereditary consequences of genotoxicity.

Repair impossible

Responsible: various physical (e.g. radioactivity, UV radiation) and chemical (e.g. (oxygen) radicals, PAHs, hormones, benzo-a-pyrene,…

Hoeijmakers 2001, Nature 411:366-374

Genotoxic or Mutagenic Effects of Micropollutants?

In Pilot Studies Regensdorf and Lausanne:

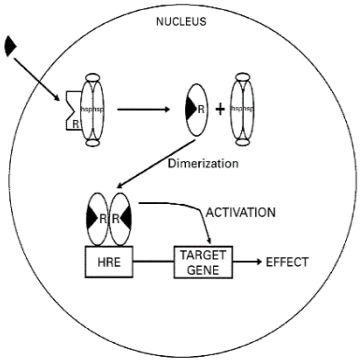
- **No genotoxic or mutagenic effects detected in ozone treated wastewater**
- **No evidence for a toxicity increase due to a continuous formation of stable ozonation by-products** (i.e. still present after sand filtration)
- Similar results observed in various other studies (e.g. Petala et al. 2008 Environ. Toxicol., Reungoat et al. 2010 Water Res., Mišík et al. 2011 Water Res., Takanashi et al. 2002 Water Sci.Technol.)
- In single studies effects after ozonation observed (e.g. Stalter et al. 2010 Water Res.) → <u>But:</u> Elimination of effects through sand filtration
- However, as a formation of reactive ozonation by-products cannot be excluded → **Additional filter with biological/microbial activity recommended**

Hormonal Effects

Bioassays for the Evaluation of Hormonal Effects

YES ER-Calux	♣ (Anti-)Estrogens
AR-Calux	♣ (Anti-)Androgens
PR-Calux	♣ Progesterones
PPAR-Calux	♣ PPAR (Peroxisome-proliferator activated receptor) like substances
GR-Calux	♣ Glucocorticoids
H295R	♣ Production of endogenous hormones
Rainbow trout	♣ FELST with embryos and larvae of rainbow trout ♣ Vitellogenin induction ♣ Estrogenic- und anti-androgenic effects

In vitro
receptor activation



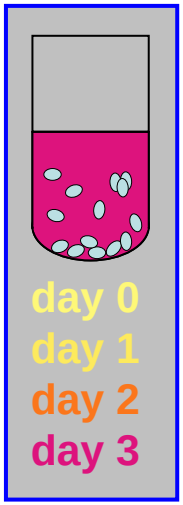
(Jacobs und Lewis 2002 P. Nutr. Soc.)

In vitro
steroidogenesis

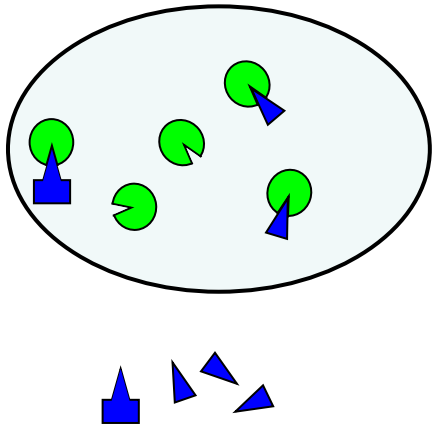
In vivo

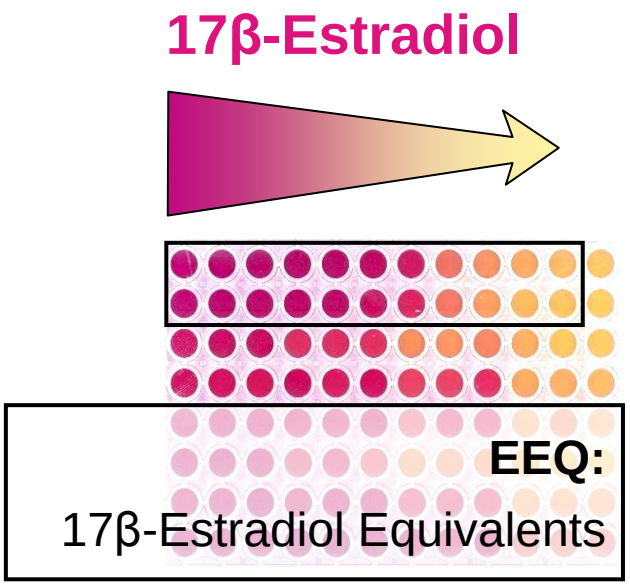
Receptor Activation Assays to Assess Specific Effects

- **Yeast Estrogen Screen (YES)** (acc. to Routledge und Sumpter 1996)
  - **Test organism**: yeast (*Saccharomyces cerevisiae*)
  - **Principle**



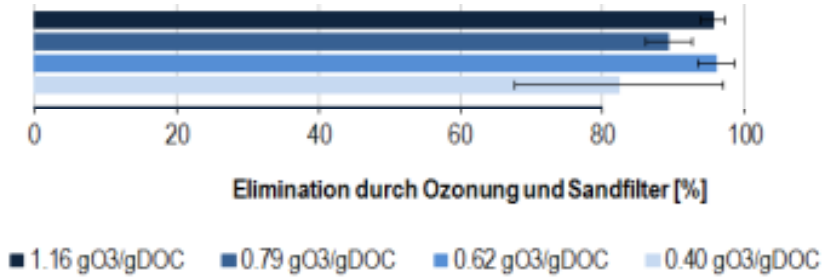
Genetically modified yeast in yellow medium





Comparison of Bioassay Results: Hormonal Effects

Regensdorf:



(Abegglen et al. 2009, Eawag)

Lausanne:

Bioassay	Substance group (effect parameter)	Effect Biological Treatment	Effect Ozonation	Effect Powdered activated carbon - UF
YES	**Estrogens** (Estradiol equivalents, ng/L)	↓	89% → 77% → ↓	↓
ER-CALUX	**Estrogens** (Estradiol equivalents, ng/L	↓	76% → 82% → ↓	↓
H295R	Estradiol production		↓	↓

↓ CI_{TEQ} >1 decreasing effects, ~ CI_{TEQ}~1 equal effects ↑ CI_{TEQ} <1 increasing effects

→ Significant reduction of estrogenic activities due to ozonation an PAC-UF treatment

→ No anti-estrogenic effects detected.

Comparison of Bioassay Results (WWTP Vidy, Lausanne)

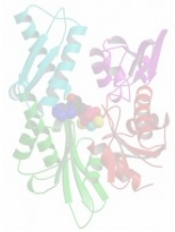
Change Indices for test systems based on specific cellular mechanisms / *in vitro*

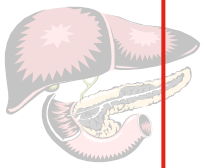
↓ decreasing effects, ~ equal effects ↑ increasing effects **var.** variable effects

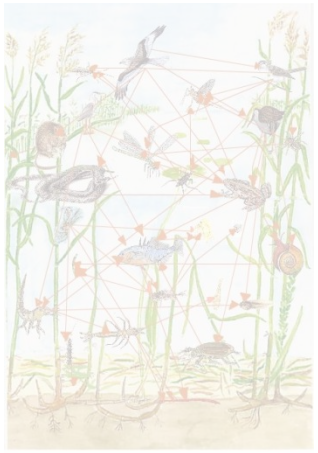
Bioassay	Substance group (effect parameter)	Effect Biological treatment	Effect Ozonation	Effect Ozonation + Sand filtration (3.+4. MC)	Effect Powdered activated carbon - UF
YES	**Estrogens** (Estradiol equivalents, ng/L)	↓	↓	↓	↓
ER CALUX	**Estrogens** (Estradiol equivalents, ng/L)	↓	↓	var.	↓
AR CALUX	**Androgens** (Dihydrotestosterone equivalents, ng/L)	↓	↓	↓	↓
GR CALUX	**Glucocorticoids** (Dexmethason equivalents, ng/L)	~	↓	↓	↓
PR CALUX	**Progesterons** (Org-2058 equivalents, ng/L)	↑	↓	↓	↓
PPARg1 CALUX	**Peroxisome proliferator like acting substances** (Rosiglitasone equivalents, ng/L)	↓	↓	var.	↓
H295R	Estradiol production		↓	↓	
	Testosterone production		~	~	~
Green algae	**Herbicides** (Diuron equivalents, µg/L) (Photosynthesis inhibition)	var.	↓	↓	↓
	General Toxicity (baseline toxic equivalent conc., mg/L) (Growth inhibition)	↓	↓	↓	↓

Different Levels and Mechanisms of Effect

Molecules Organelles Cells Organs **Organisms** Populations Ecosystem





Genotoxicity: Micronucleus assay

Mutagenität: Ames assay

Hormonelle Wirkungen: YES, ER-, AR-, GR-, PR- and PPAR-CALUX, H295R

General Toxicity: Bacteria, Algae, Trout

Herbicidal Effects: Algae

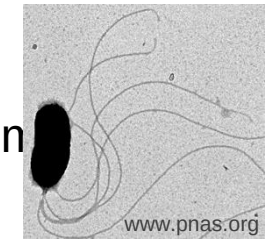
Hormonal Effects: Vitellogenin in Trout

Specificity

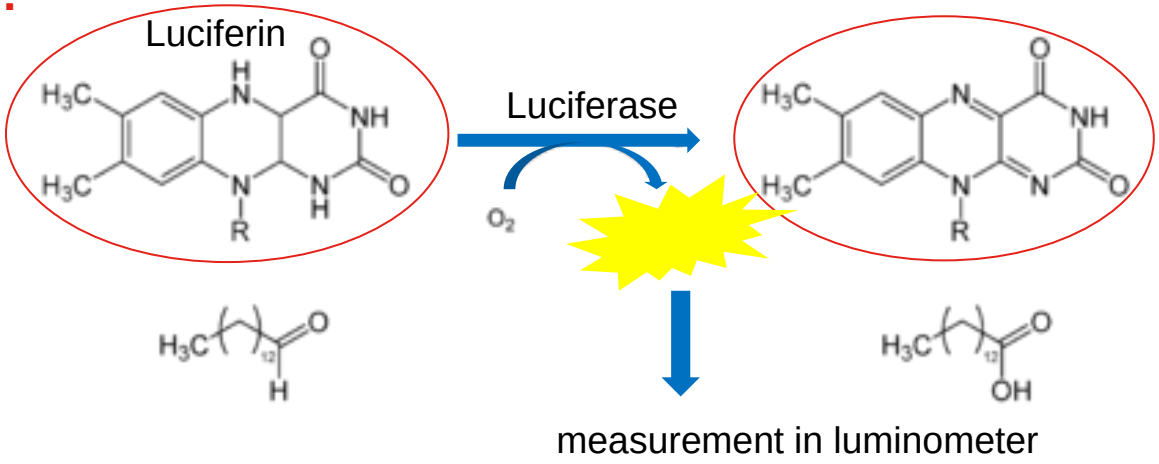
Ecological Relevance

Bioassays with Bacteria

Bakteria – Luminescence Inhibition Assay (ISO 11348-3, 2007)



- **Test organism:** *Aliivibrio fischeri* (marine luminescence bacterium)
- **Principle:**

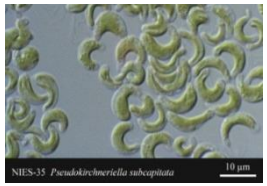


- **Endpoints:** Inhibition of luminescence (%)
- **Toxicity parameter:** EC_x
- **Methodology** for 96 well microplates (Richter et al. 2008)

Bioassays with Primary Producers – Green Algae

Algae 72h Growth Inhibition Assay (OECD 201, ISO 8692, 2004)

Combined Algae Assay (Quayle et al, 2008, Eawag)



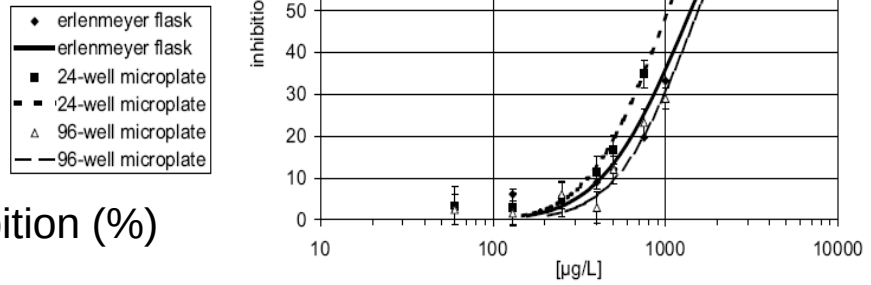
www.nies.go.jp

- **Test organism:** single-celled freshwater green algae (e.g. *Pseudokirchneriella subcapitata)*
- **Principle:**

  Determination of effects on

  1) **Photosynthesis activity** and/or
  2) **Growth** of algae
- **Duration:** 24 -72 h

B:
- variation of incubation vessels and test volumina
- evaluation parameter: growth rate



Eisenträger et al. 2008, Ecotox. Environ. Saf.

- **Endpoints** Photosynthesis-, growth inhibition (%)
- **Toxicity parameter:** ECx, Diuron Equivalent Concentration (DEQ)

Comparison of Bioassay Results (WWTP Vidy, Lausanne)

Change Indices for integrative *in vivo* bioassays performed in the laboratory

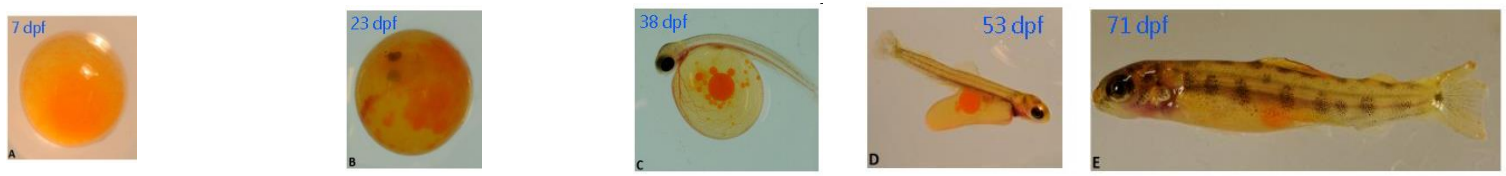
↓ CI >1 decreasing effects, ~ CI~1 equal effects ↑ CI <1 increasing effects **var.** variable effects **n.t.** not toxic

Test organism	Endpoint (toxicity parameter)	Effect Biological treatment	Effect Ozonation	Effect Ozonation + Sand filtration (3.+4. MC)	Effect Powdered activated carbon - UF
Vibrio fischeri (bacteria)	Inhibition of Luminescence (EC20)	↓	n.t.	n.t.	n.t.
Pseudokirchneriella subcapitata (alga)	Cell number (EC20)	~	~	var.	~
	(EC50)	~	~	~	~
Lemna minor (duckweed)	Frond number (EC20)	↓	var.	var.	n.t.
	(EC50)	var.	n.t.	~	n.t.
Ceriodaphnia dubia (crustacean)	Number of offspring (EC20)	↓	var.	↑	n.t.
	(EC50)	↓	n.t.	var.	n.t.
	Mortality (EC50)	var.	n.t.	~	n.t.
Gammarus fossarum (crustacean)	Feeding rate			~	~
Potamopyrgus antipodarum (snail)	Mortality		~	~	↓
Danio rerio (fish)	Mortality	↓	n.t.	n.t.	n.t.

Bioassays with Fish

Fish Early Life Stage Test (FELST, OECD 210)

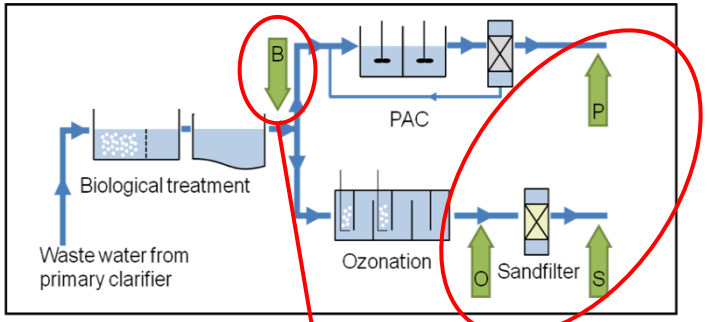
- **Test organism: Rainbow trout (*Oncorhynchus mykiss*)**
- **Principle:** Observation of fish embryo and larval development over 69 days in flow-through systems
- **Endpoints:**

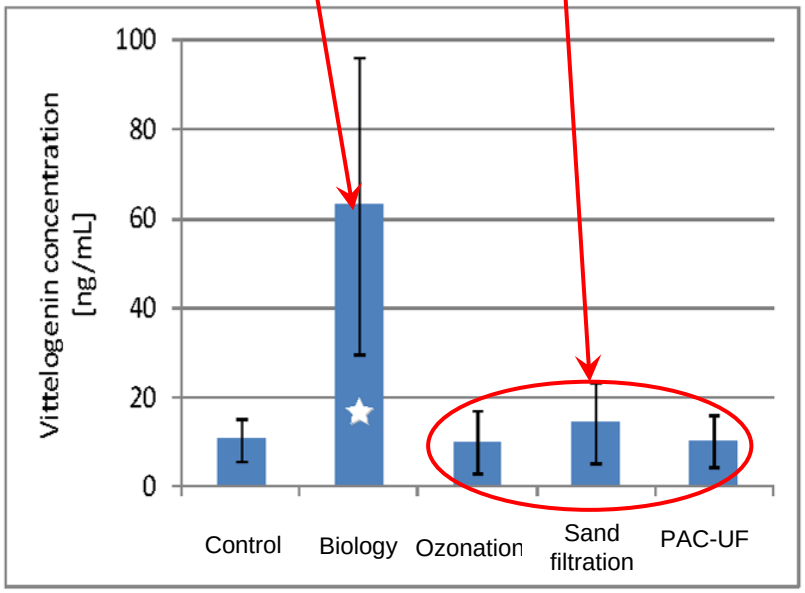


Exposure of embryos **Hatching** **Swim up**

0-----5----10-----15-----20-----25-----30-----35-----40-----45-----50-----55-----60-----65----69 days

Example: FELST with Wastewater, WWTP Vidy Lausanne



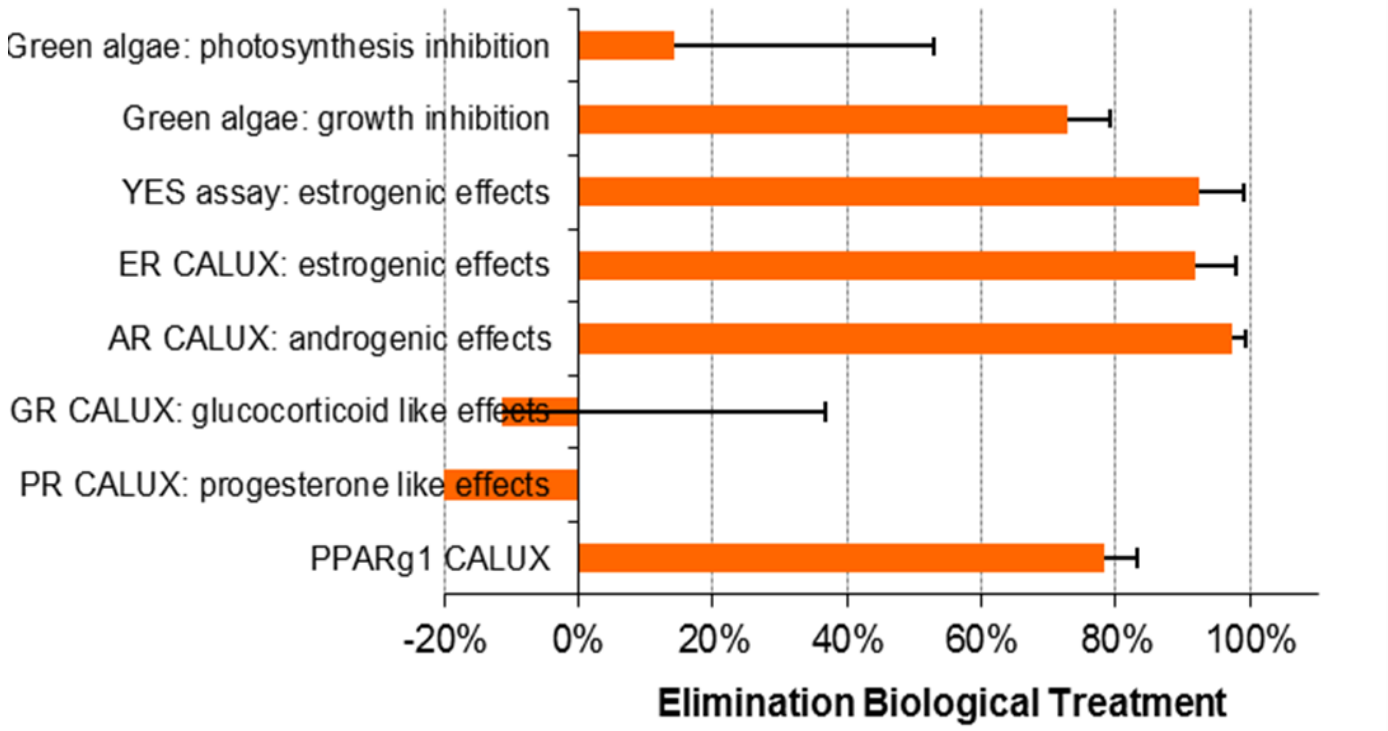


Endpoint	B	O	SF	PAC-UF
Overall survival rate		↓	↓	↓
Suvival of embryos		~	~	↓
Survival of larvae and juveniles		↓	↓	↓
Hatching rate		~	~	↓
Swim up of hatched larvae		↓	↓	↓
Fresh weight of larvae at test end		↓	↓	↓
Length of larvae at test end		~	~	↓

↓ **decreasing toxicity** ~ equal toxicity ↑ increasing toxicity

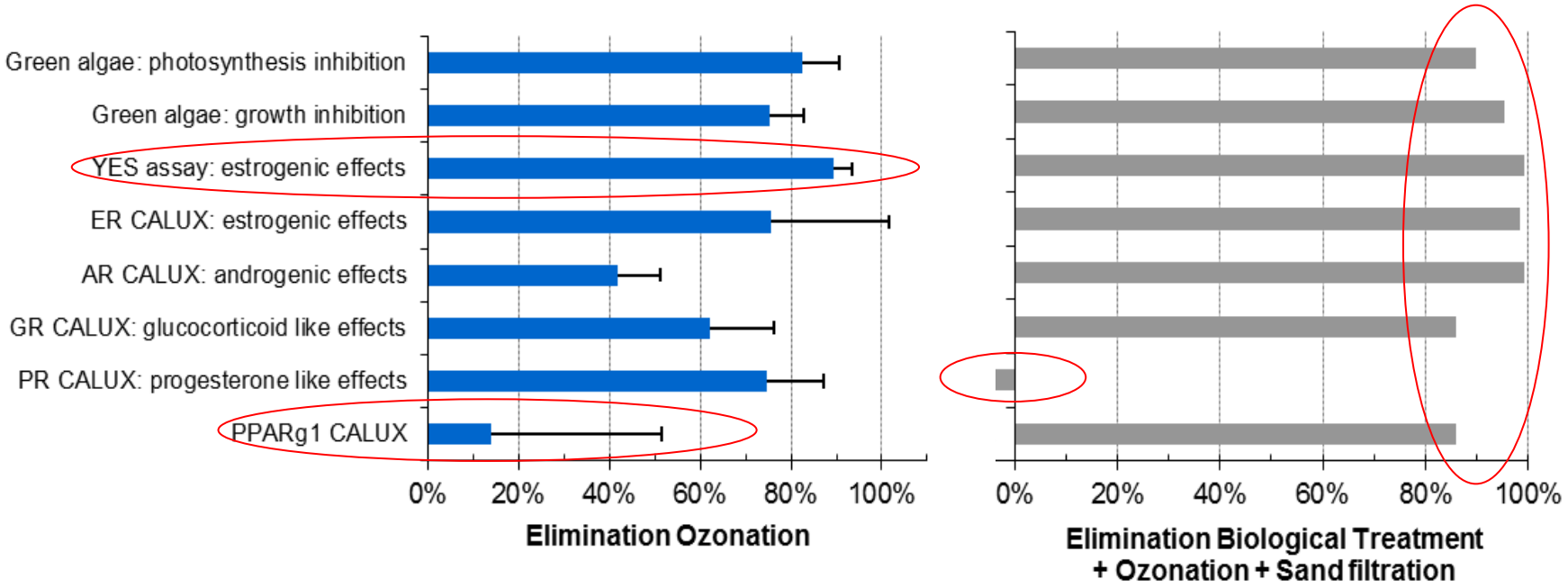
Weil 2010 ECT Oekotoxikologie GmbH

Elimination Efficiency of Biological Treatment



→ Specific effects were partly eliminated in biological treatment

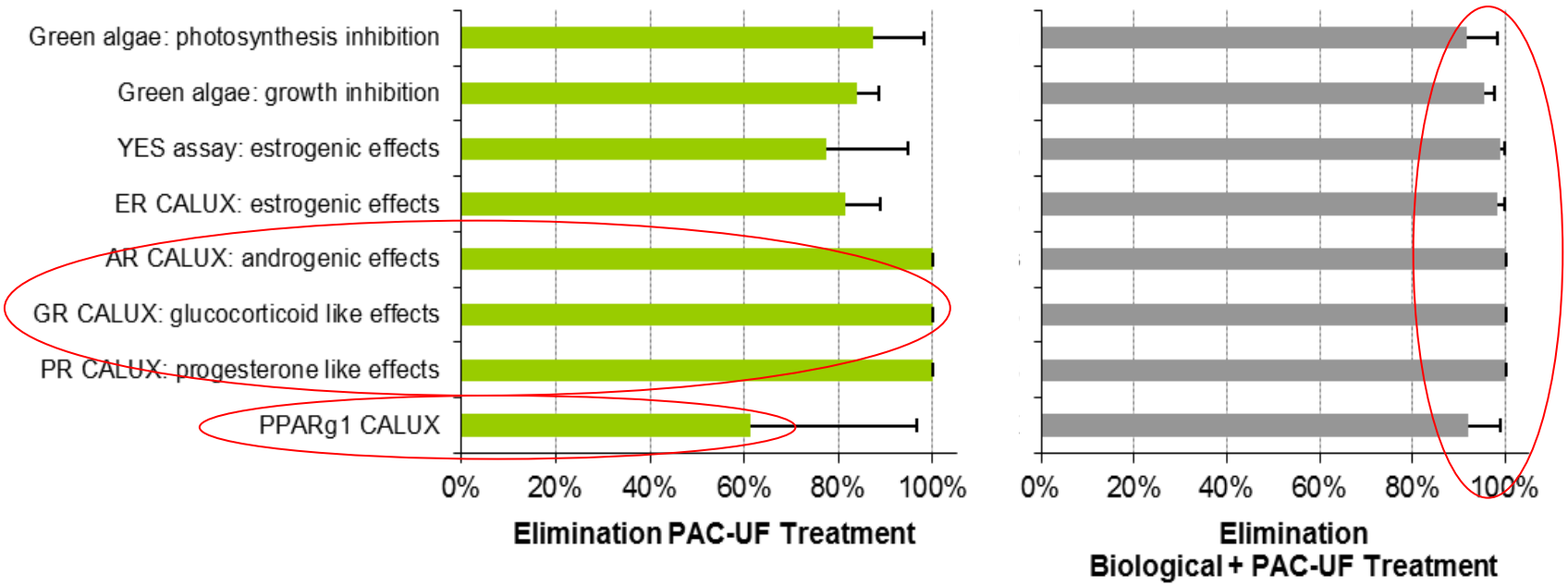
Elimination Efficiency of Biological and Ozonation Treatment



→ Additional 14 – 87% elimination by ozonation

→ Total elimination in WWTP of 86 – 99% (4th MC) (exception: progesterone like acting substances)

Elimination Efficiency of Biological and PAC-UF Treatment

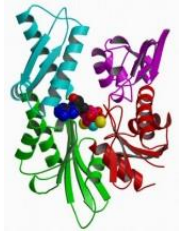
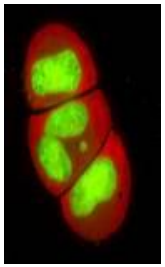
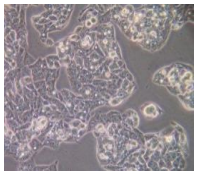


→ Additional 61 – 100% elimination by PAC-UF

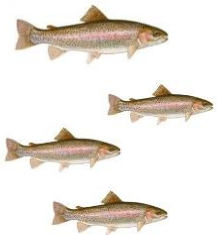
→ Total elimination in WWTP of 92 – 100%

Different Levels and Mechanisms of Effect

Molecules Organelles Cells Organs Organisms Populations Ecosystem






Relevance of the results from the project «Strategy Micropoll» for populations and ecosystems?

Ecological Relevance

Specificity

“Adverse Effect Pathways”: Field Monitoring

Example: Estrogenicity

Mode of action:
e.g. activation of the estrogen receptor

Increased vitellogenin production

Feminisation and Intersex in ♂

Retardation of egg production in ♀

Collaps of fish population (5-6 ng/L EE2, 2 years)

Kidd et al. 2007, Proc. Natl. Acad. Sci. USA 104 (21): 8897–8901

E2 Equivalents

Part 1: Conclusions from the Performance Review (WWTP Vidy, Lausanne)

- An **application of bioassays for** the **performance review** has proven to be **relevant and useful**.
- **Clear reduction of biological effects due to Ozonation-SF** and **PAC-UF treatment** shown with **most *in vitro* – bioassays** and with **selected *in vivo* – bioassays** (Fish Early Life Stage test).
- ***In vitro* bioassays** seem to be **most promising for** a **routine monitoring** of WWTPs.
- **No evidence of higher toxicity by a constant formation of stable ozonation by-products.**

Ozonation and **PAC treatment** are **useful measures** to **reduce the biological effects of micropollutants**

PART 2

Investigation of Waste Water Quality regarding Micropollutants and their Effects

Or

How can Environmental Quality Standards (EQS) and risk quotients tell us something about the water quality during waste water treatment?

Risk Assessment = Exposure Assessment / Hazard Assessment

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

<1 tolerable risk

>1 intolerable risk

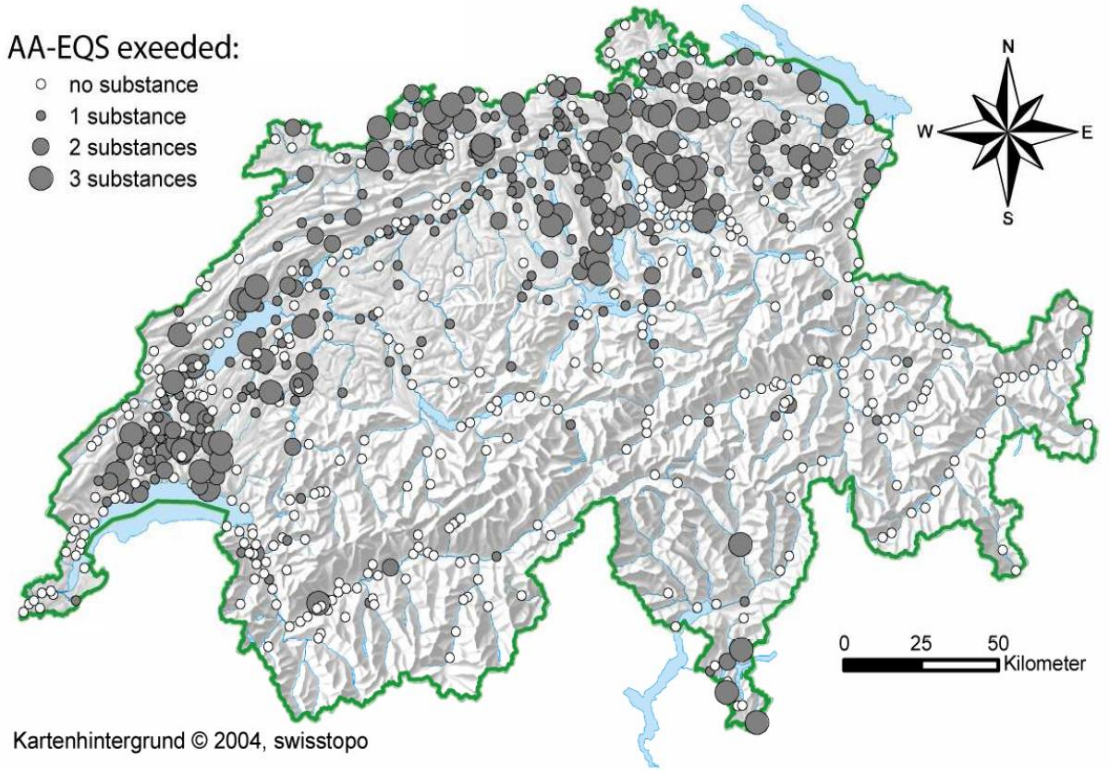
MEC= Measured environmental concentration (in usual the 90th percentile)

QC= Quality criteria (in usual the AA-EQS)

AA-EQS (Annual Average-Environmental Quality Standard) derived as **protection against effects of long-term exposure**.

Where and how often are EQS exceeded?

Swiss-wide risk assessment:
Evaluation of 543 water sections downstream of WWTP



Evaluated substances:

- **Atenolol**
- **Benzotriazole**
- **Carbamazepine**
- **Clarithromycin**
- **Diclofenac**
- **Sulfamethoxazole**

Source: Combination of exposure and ecotoxicological effect data for Switzerland (Kase et al. 2011)

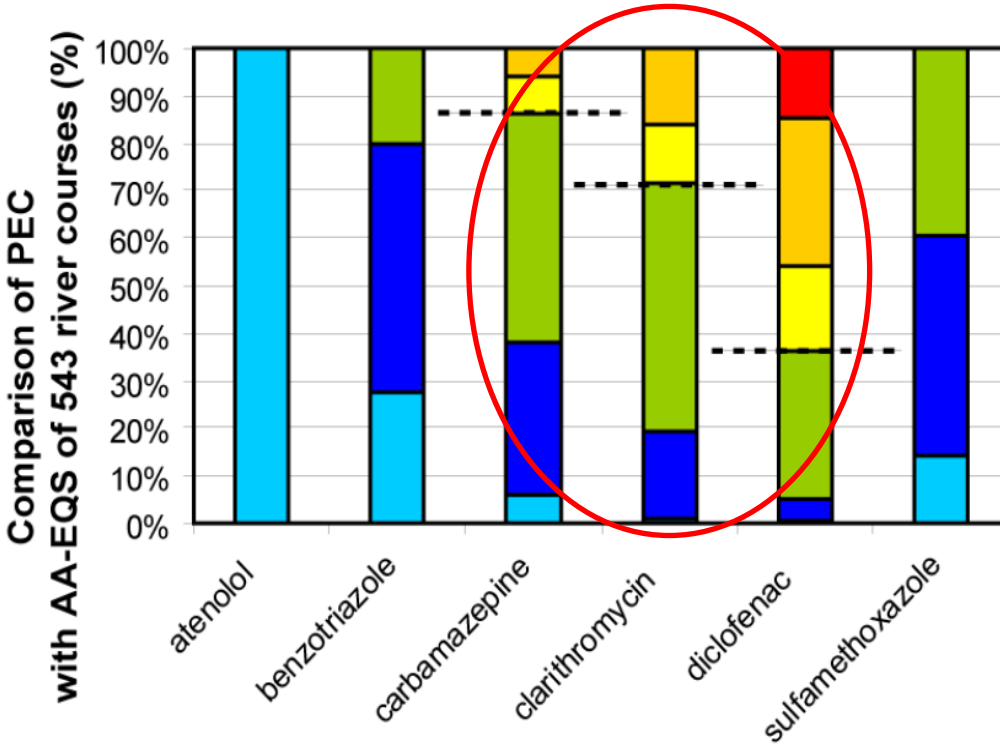
Proposed Chemical Classification System for Micropollutants

Evaluation		Condition/description		Compliance
	very good	EC < 1/100 AA-EQS	RQ < 0.01	AA-EQS passed
		1/100 AA-EQS ≤ EC < 1/10 AA-EQS	0.01≤ RQ < 0.1	
	good	1/10 AA-EQS ≤ EC < AA-EQS	0.1≤ RQ < 1	
	moderate	AA-EQS ≤ EC < 2 AA-EQS	1≤ RQ < 2	AA-EQS exceeded
	insufficient	2 AA-EQS ≤ EC < 10 AA-EQS	2≤ RQ < 10	
	poor	EC ≥ 10 AA-EQS	RQ > 10	

Kase et al. 2011, InTech

Is this classification system able to identify environmental risks and safety regarding the chemical quality?

Which exceedances of EQS would be expected?



Conclusion:

With quality criteria risky and non-risky substances can be identified

Risk-potential :

Diclofenac: fish-toxicity (under discussion)

Clarithromycin: blue algae-toxicity

Carbamazepin: daphnia-toxicity and new fish toxicity study (Gallus et al. 2013)

Single- and combination effects cannot be excluded

Identification of three „risky“ substances using the exposure model PECs from Ort et al. 2009 assuming low flow(Q347) and compared with AA-EQS.
For sulfamethoxazole a 5 times lower EQS of 0.12µg/l was used.

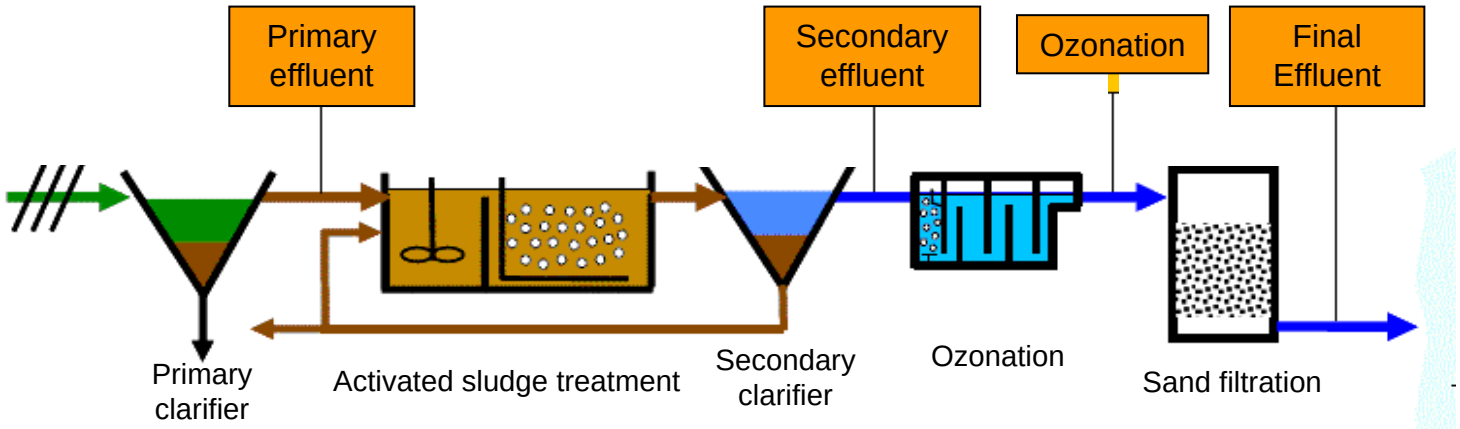
Risk quotients of three micropollutants during wastewater treatment

→ WWTP Wüeri, Regensdorf

Results with 0.62 ± 0.15 g_{O3}/kg_{DOC} for n = 6 measurement campaigns (Abegglen et al. 2009)

Environmental Quality Standards:

1) Diclofenac; AA-EQS = 50 ng/L
2) Carbamazepin; AA-EQS = 500 ng/L
3) Clarithromycin; AA-EQS = 60 ng/L



Primary effluent
1)1840; RQ = 36.8
2) 792; RQ = 1.6
3) 664; RQ = 11.1

Secondary effluent
1)1465; RQ= 29.3
2) 693; RQ= 1.4
3) 316; RQ= 5.3

Ozonation
1)<20; RQ< 0.4
2) 6; RQ= 0.012
3) 20; RQ= 0.33

Final Effluent
1)<20; RQ< 0.4
2) 1; RQ= 0.002
3) 1.5;RQ= 0.025

Very good +
Very good
Good
Moderate
Insufficient
Poor

Legend:
Blue: 90th percentile of MEC [ng/L]
Red: RQ =MEC/AA-EQS

Reduction of risk quotients (OZ-SF) compared to secondary clarifier:

1) Diclofenac > 73.25
2) Carbamazepin = 700
3) Clarithromycin = 212

Part 2: Conclusion for risk quotients in both pilot studies

→ **WWTP Vidy, Lausanne:**

Similar reduction of risk quotients due to PAC-UF and ozonation-SF treatment observed

Ozonation-SF and PAC-UF led to a **lowered risk potential for Diclofenac, Carbamazepine and Clarithromycin**

Main conclusions for both pilot studies

- A broad range of micropollutants and their effects were eliminated by more than 80%.
- 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.

Outlook – Amendment of the Swiss Water Protection Law

- **100 of 700 Swiss WWTPs** will be **upgraded** with an **advanced treatment step for micropollutant removal by 2040** (Overall costs: 1.2 Mrd. Swiss Francs).
- **To finance** this upgrade, the Swiss National Council has decided at the 03.03.14 on an **amendment of the Swiss Water Protection Law**.
- The **waste water fees** will be **increased Swiss-wide by max. 9 CHF/inhabitant and year**→ 75% of the costs can be covered by this fee.
- In order to give WWTP operators **an appeal for the upgrading**, the **fee will be ceased as soon as the WWTP is upgraded**.

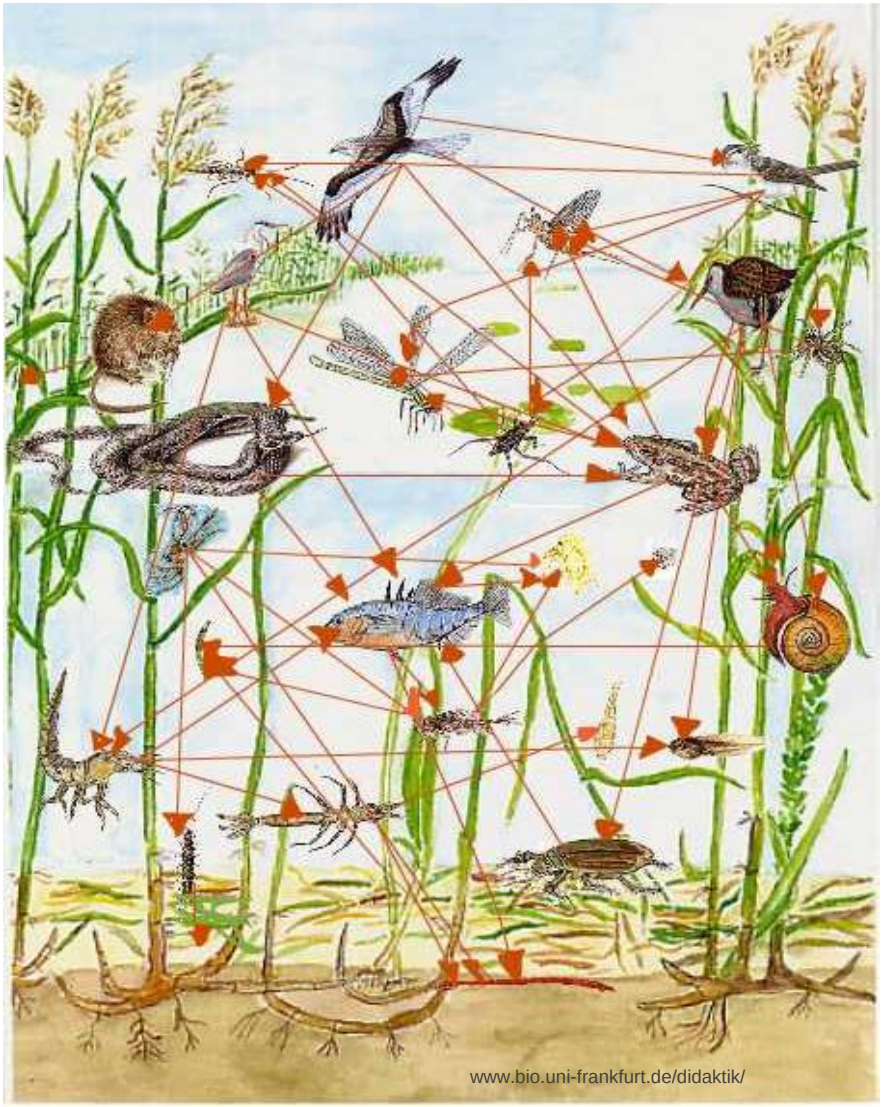
→ «An amendment, which represents a **milestone in the history of water protection**» (Citation Beat Jans, Nationalrat Basel-Stadt)

→ Measure leads to an **improvement of the water quality in Switzerland**

TAKE HOME MESSAGES

- Micropollutants can elicit negative effects on aquatic organisms.
- Bioassays are suitable for detecting those effects.
- Effects on aquatic organisms can effectively be reduced with advanced wastewater treatment.

→ Improved protection of aquatic ecosystems


www.bio.uni-frankfurt.de/didaktik/

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the members of the different accompanying groups,

my colleagues from the ecotox centre,

…

and you for your attention!

Contact: cornelia.kienle@oekotoxzentrum.ch

Selected References

Results for the pilot study in Lausanne are available in:

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". Summary.* Dubendorf : Ecotox Centre Eawag/EPFL.

Additional references:

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Ratte M, Ratte HT, 2009. *Report, 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“ (Studie der Eawag, CH-Dübendorf).