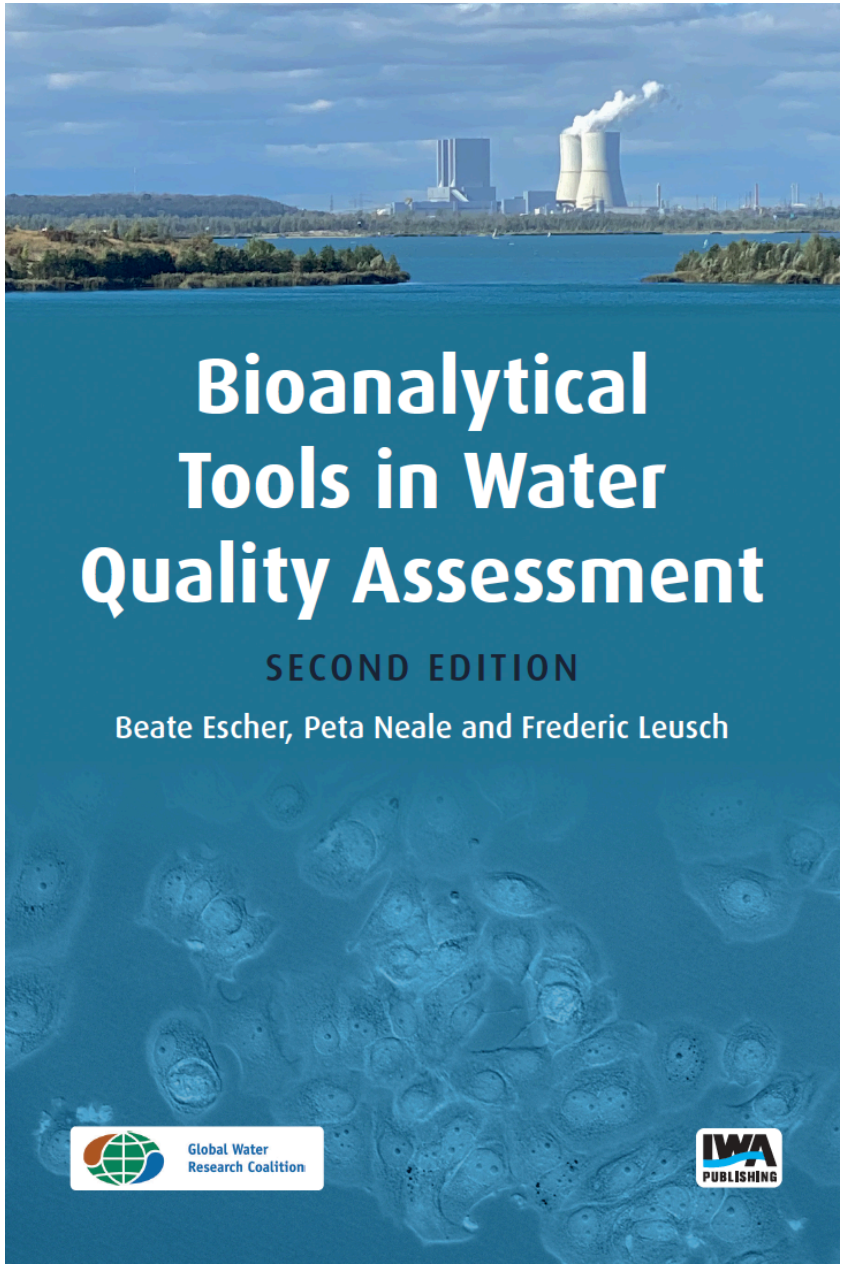
Chapter 10
Current bioanalytical tools for water quality assessment



This presentation accompanies Chapter 10 of "Bioanalytical Tools in Water Quality Assessment" https://www.iwapublishing.com/books/9781789061970/bioanalytical-tools-water-quality-assessment-2nd-edition

Exercises and more material can be found at www.ufz.de/bioanalytical-tools.

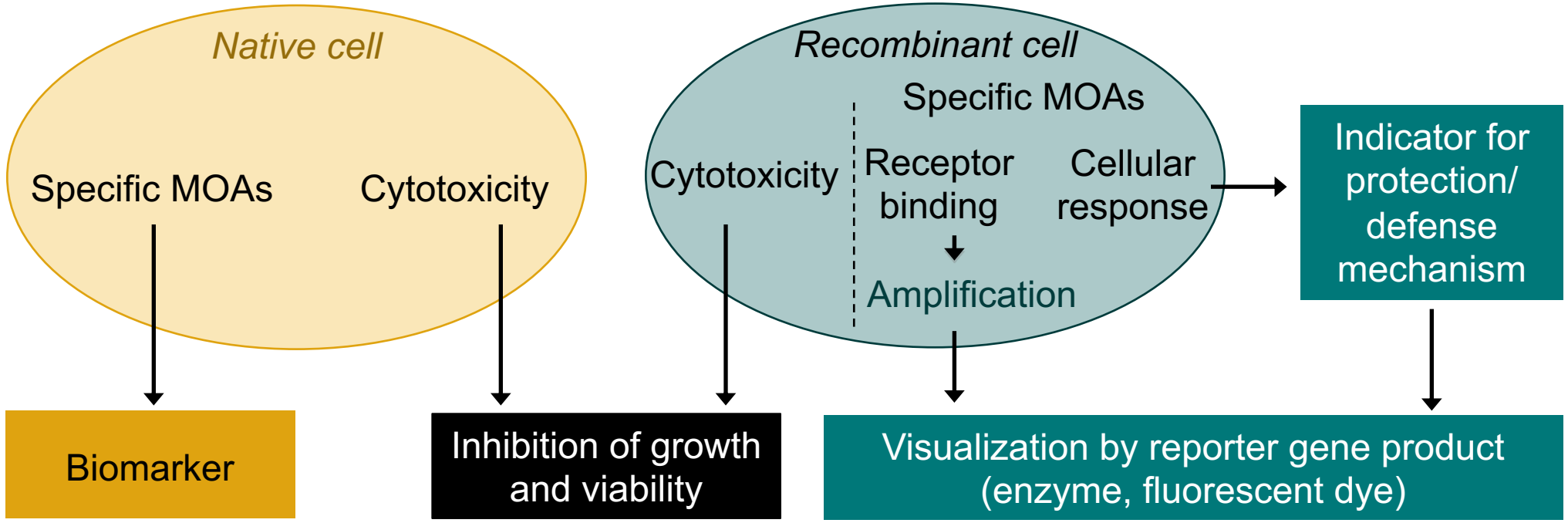
For questions please send e-mail to bioanalytical-tools@ufz.de

Learning goals

- To know, which *in vitro* assays are suitable and often applied for water quality monitoring and which gaps future research has to fill
- To understand the principles underlying reporter gene assays and other *in vitro* bioassays
- How cytotoxicity can be measured and why it should always accompany quantification of reporter gene activation

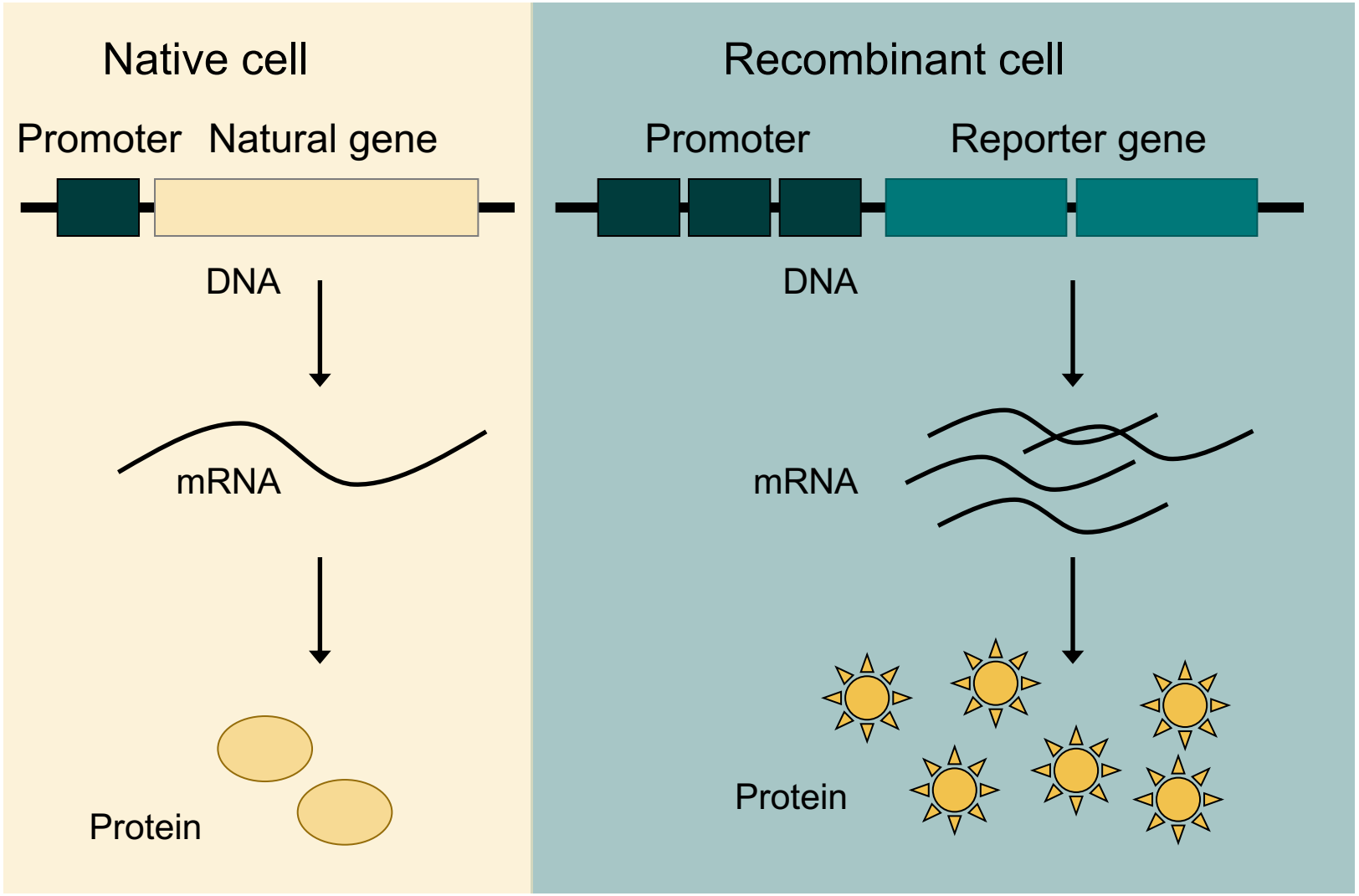
Principles of cell-based bioassays

- primary cells – the REAL thing but limited lifespan *in vitro*, high variability
- immortalised cell lines: proliferate indefinitely (but mutations, so keep >40-80 passages)
- Cytotoxicity and specific effects: biomarkers or reporter genes (genetic engineering used to enhance visualisation of the cellular response)



Principles of reporter gene assays

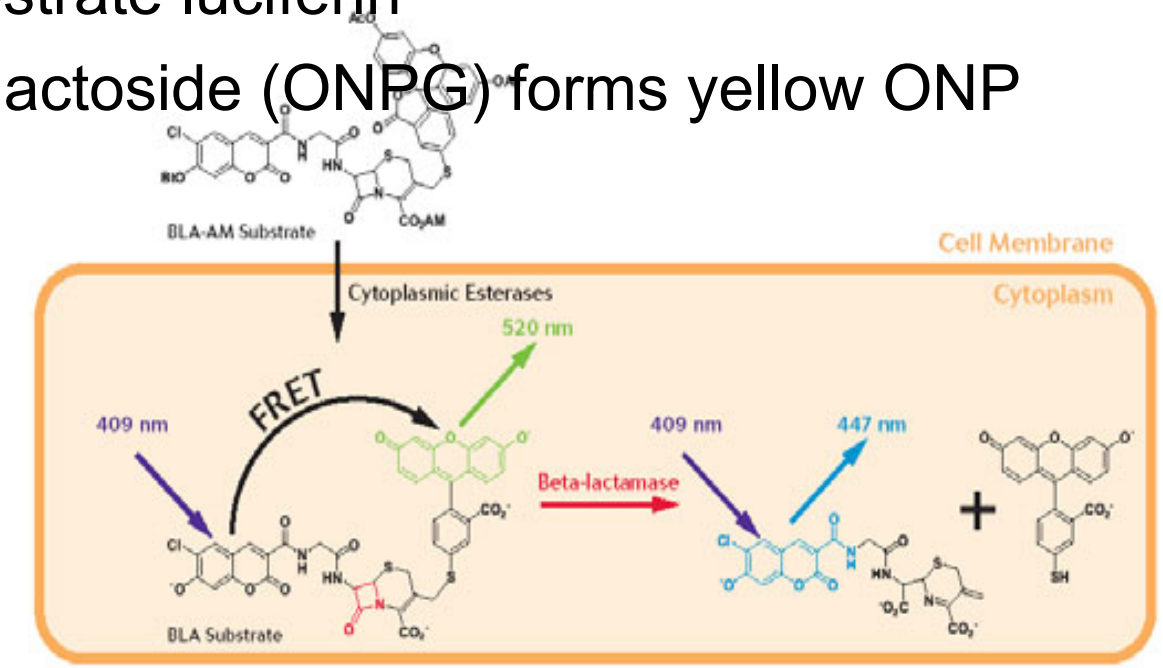
- Reporter gene encodes for an easily detectable product such as a fluorescent protein or enzyme (e.g., luciferase or β-galactosidase)
- Multiple copies of promoter and reporter gene to enhance the signal



Widely used reporter genes, their products and detection

Goal: reporter gene encodes for a reporter protein that can be easily measured

- enhanced green fluorescent protein, EGFP
- Luciferase: only energy (ATP) and substrate luciferin
- β-galactosidase: o-nitrophenyl-β-D-galactoside (ONPG) forms yellow ONP
- β-lactamase: BLA-AM substrate

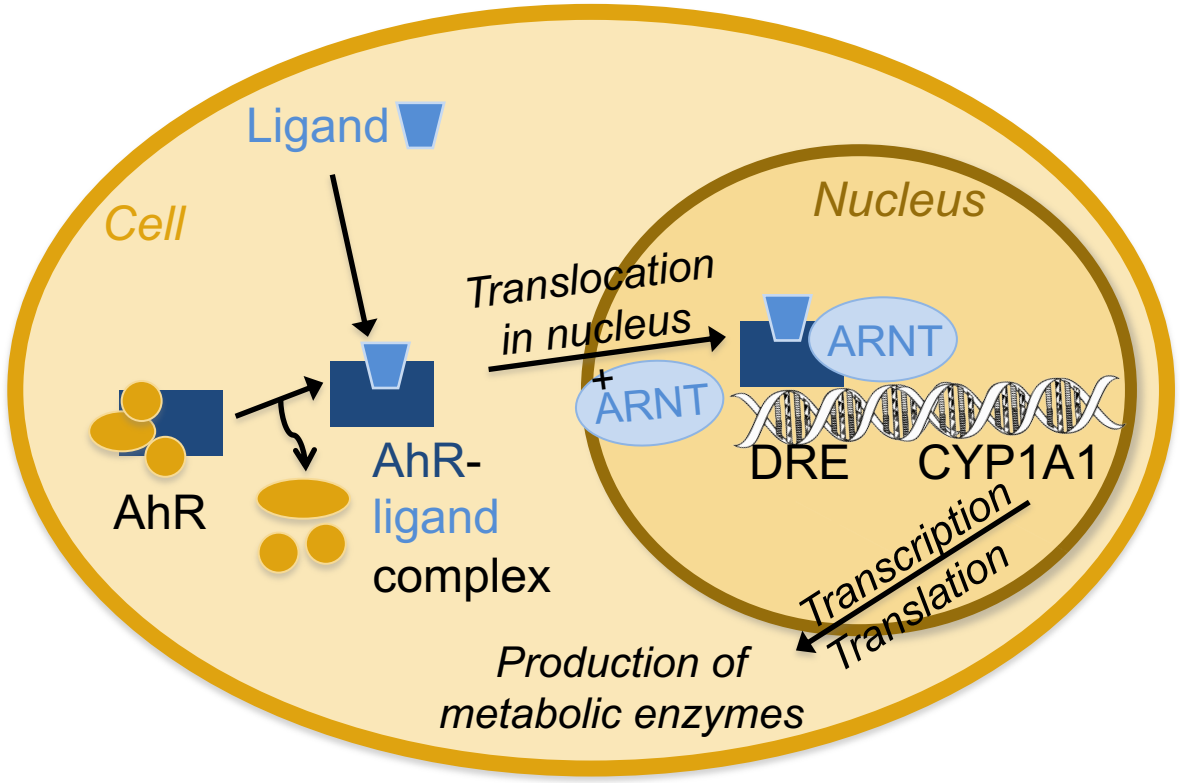


Bioassays indicative of xenobiotic metabolism

Xenobiotic metabolism receptors

- Arylhydrocarbon receptor (AhR)
- peroxisome proliferator-activated receptor (PPAR)
- pregnane X receptor (PXR)
- constitutive androstane receptor (CAR)

Activation of xenobiotic metabolism receptors indicates presence of chemicals that can induce biotransformation processes in cells to metabolise, detoxify or in some case bioactivate chemicals but is not a toxicity per se.



Activation of the arylhydrocarbon receptor

- Activates target genes encoding for the metabolic enzymes CYP1A1, CYP1B1 and NADPH-quinone oxidoreductase (NQO1)
- Reporter gene assays or native metabolic enzyme activation quantified with ethoxyresorufin-O-deethylase (EROD) assay
- Relevant for dioxin-like chemicals, PAH (specific PAH CALUX)
- Very responsive in many water types

Assay	Cell line	Detection method	TCDD EC_{10} (M)	TCDD EC_{10} (ng/L)	EC reference
AhR CAFLUX	H1.G1.1c3	Fluorescence	6.50×10^{-13}	0.21	(Jia et al., 2015)
AhR CAFLUX	H4.G1.1c2	Fluorescence	6.87×10^{-13}	0.22	(Neale et al., 2015; König et al., 2017)
AhR CALUX	H4L1.1c4	Luminescence	5.92×10^{-13}	0.19	(Nivala et al., 2018)
AhR HepG2	HepG2	Luminescence	6.22×10^{-11}	20	(Rosenmai, 2018)
H4IIE-luc	H4IIE	Luminescence	1.60×10^{-13}	0.05	(Lee et al., 2015)

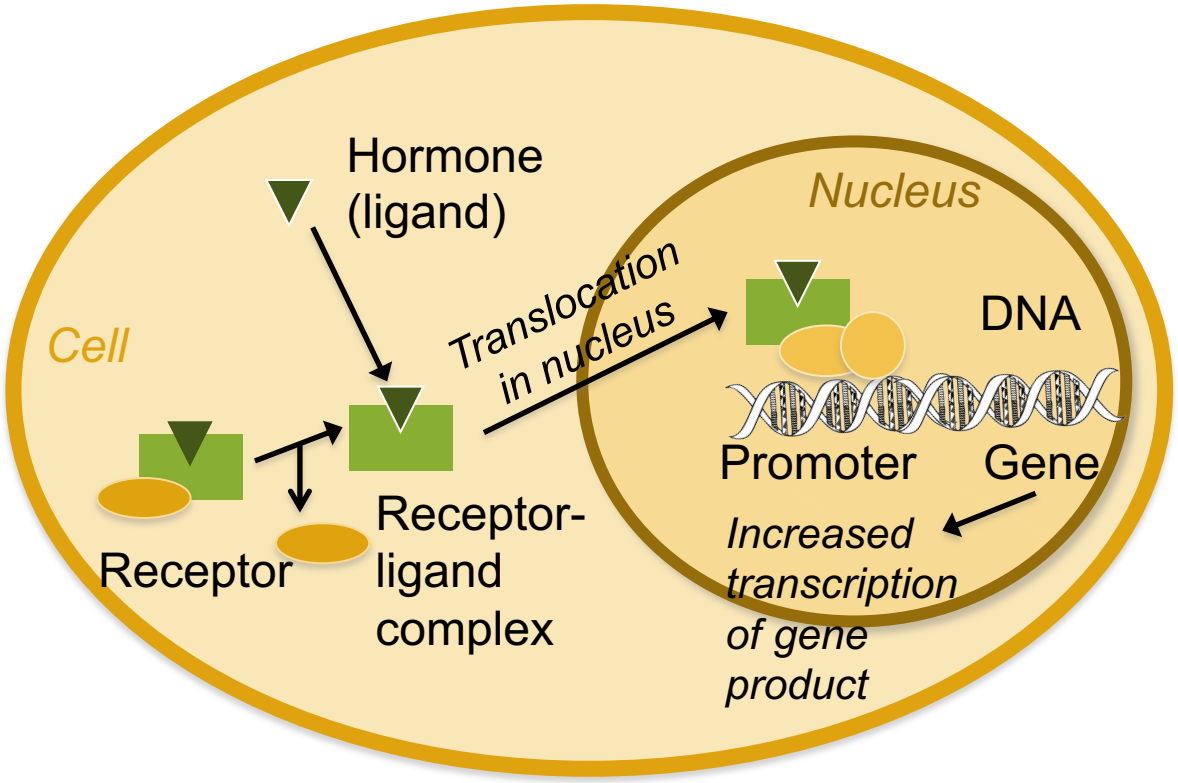
Activation of the peroxisome proliferator-activated receptor (PPARγ)

- PPAR is a transcription factor that belongs to the superfamily of nuclear receptors and is involved in the regulation of glucose and lipid metabolism
- delivery of peroxisomes, which are important for fatty acid oxidation and thus relevant for lipid metabolism
- Responds to “obesogens”- often carboxylic acids

Assay	Cell line	Detection method	Rosiglitazone EC_{10} (M)	Rosiglitazone EC_{10} (ng/L)	EC reference
PPARγ CALUX	U2OS	Luminescence	1.00×10^{-8}	3,600	(Gijsbers *et al.*, 2011)
PPARγ GeneBLAzer	HEK 293	Fluorescence	3.30×10^{-10}	118	(Jia *et al.*, 2015)

Activation of hormone receptors

	Endpoint	Reference compound
AR	Androgenicity	Dihydrotestosterone
ERα	Estrogenicity	17-ß-Estradiol
GR	Glucocorticoid activity	Dexamethasone
PR	Progesterone activity	Levonogestrel
RAR α	Retinoic acid receptor activity	ATRA (all-*trans* retinoic acid)
RXR	Retinoid X receptor activity	9-*cis*-RA
TRβ	Thyroid hormone activity	T3 hormone (Triiodothyronine)
MCR	Mineralocorticoid receptor	Aldosterone



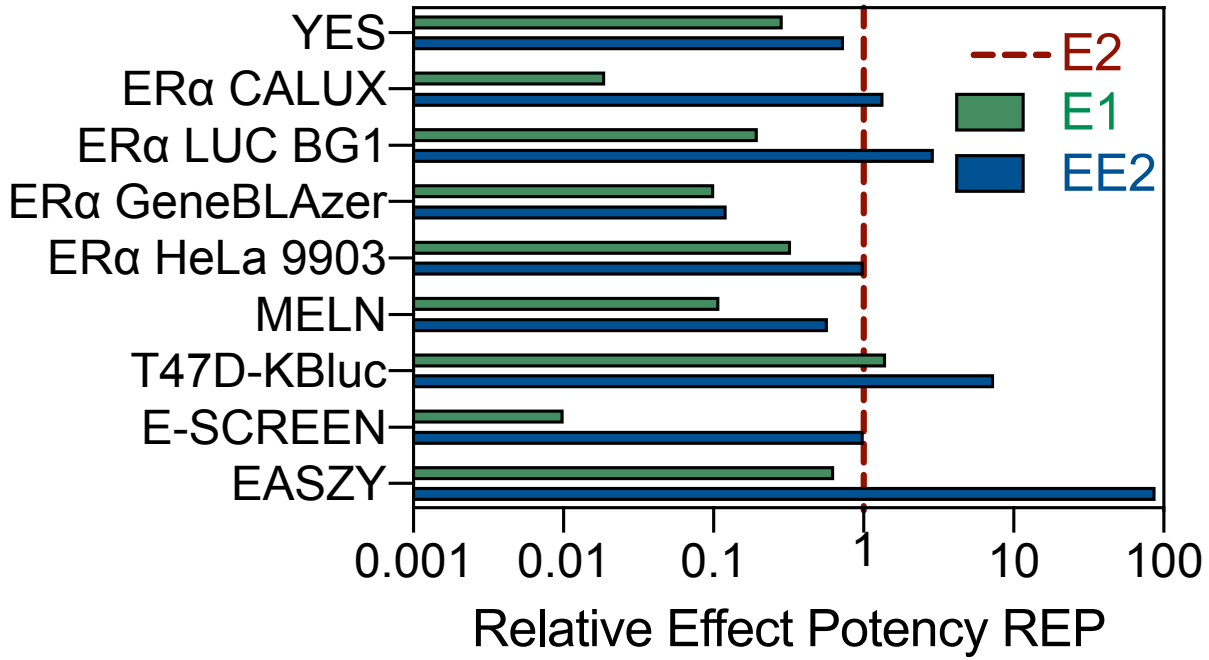
Activation of hormone receptors by water samples

	Endpoint	Reference compound	Applications for water quality assessment
AR	Androgenicity	Dihydrotestosterone	Well-removed in WWTP: only low activity in surface water 0.25-12 ng/L DHT EQ
ERα	Estrogenicity	17-ß-Estradiol	Well-removed in WWTP: only low activity in surface water but problematic for fish reproduction
GR	Glucocorticoid activity	Dexamethasone	Poorer removal in WWTP, often detected in surface water
PR	Progesterone activity	Levonogestrel	Well removed by WWTP, often below detection in surface water
RARα	Retinoic acid receptor activity	ATRA (all-*trans* retinoic acid)	Activity found in supface water after relatively high enrichment (>40)
RXR	Retinoid X receptor activity	9-*cis*-RA	Not widely applied, so far no responses detected
TRβ	Thyroid hormone activity	T3 hormone (Triiodothyronine)	Higher Reporter gene assays only activated in WWTP influent, activity in XETA with frog embryos
MCR	Mineralocorticoid rec.	Aldosterone	No activity in water

Reporter gene assays for the estrogen receptor differ in relative sensitivity

- EE2 is more potent *in vivo* (EASZY)
- E1 has a relatively low potency in ER CALUX and E-SCREEN
- Only compare responses of water samples within the same assay

$$REP_i = \frac{EC_{10}(E2)}{EC_{10}(i)}$$



Antagonistic effect on hormone receptors

	Endpoint	Reference	Occurence in water
Anti-AR	Anti-androgenicity	Flutamide	High in effluent up to 360 ng/L Flutamide EQ
Anti-ERα	Anti-estrogenicity	Tamoxifen	Rarely observed in water, often masked by cytotoxicity
Anti-GR	Anti-glucocorticoid	Mifepristone	Rarely observed in water, often masked by cytotoxicity
Anti-PR	Anti-progestogenic	Mifepristone	Rarely observed in water, often masked by cytotoxicity
Anti-TRβ	Anti-thyroid	Amiodarone	Rarely observed in water, often masked by cytotoxicity
MCR	Anti MCR	Spironolactone	Observed, few data

Reactive toxicity

Genotoxicity

- SOS response, e.g. umuC, SOS chromotest
- Comet Assay or single cell gel electrophoresis

Mutagenicity:

- AMES assay

DNA repair

- Green Screen

Genotoxicity

Often active only after activation with S9

Assay for DNA damage	Cell type	Endpoint
SOS response assays: umuC assay (also called umu and SOS/umu), umu microtest and SOS Chromotest.	Bacterium *Salmonella typhimurium* TA 1535/pSK1002	Induction of the umu operon (SOS response) activates β-galactosidase, which can metabolise the substrate to a coloured product for colorimetric measurement
Cytotoxicity in SOS defective E. coli	Bacterium *E. coli* (several K12 AB and KL strains)	Colony formation
Vitotox assay (kit for detection of SOS response)	Bacteria genetically modified *S. typhimurium* (TA 104 recN2-4 strain)	SOS response, which induces luminescence (the TA 104 pr1 strain, which constantly ex-presses lux genes is used as positive control)
Comet assay (also known as single cell gel electrophoresis (SCGE) assay)	A variety of mammalian (incl. human) cells, also fish liver cells (zebrafish Danio renio and rainbow trout Oncorhynchus mykiss RTL-W1, RTH-149)	Measures DNA double strand breaks in single cells (single strand break in some variants). Staining technique, fluorescence. Image analysis results in an output image resembling a comet. The body of the comet represents undamaged cells and the tail, the damaged cells
Alkaline yeast comet/SCGE	Yeast *Saccharomyces cerevisiae* DLH3	Same as normal comet but appears to be more sensitive than mammalian cell line
Micronucleus formation measured by flow cytometry (FCMN or FCMMN assay)	Non-secreting human lymphoblast (WIL2-NS)	Micronucleus formation, measured by flow cytometry
Propidium iodide (PI) staining and flow cytometry	Mammalian and human cell lines can be used	PI is fluorogenic and binds stoichiometrically to nucleic acid. DNA content can be quantified via fluorescence
GreenScreen EM (yeast reporter gene assay)	*Yeast S. cerevisiae* transfected with a plasmid incorporating γEGFP3	DNA damage, or rather the resulting DNA repair, which induces the green fluorescent protein (GFP)
Sister chromatid exchange (SCE) induction	Chinese hamster lung (CHL) cells	SCE is measured by a fluorescence staining technique

Mutagenicity

- Few quantitative studies- most yes/no

Assay for mutagenicity	Cell type	Endpoint
Ames test (and modified Ames test)	*Bacterium S. typhimurium* (many strains incl. TA98, TA100 and 98NR)	Number of histidine revertants
Mutatox assay	Bacterium *Aliivbrio fischeri*	Genotoxic damage such as frame-shift mutations or base-substitution point mutations and more, which induce a dark variant of *Aliivibrio fischeri* to regain its luminescence
Alternative mutagenicity test	Yeast *S. cerevisiae* D7 diploid strain	Formation of ‘mutagen-specific’ colonies on selective media

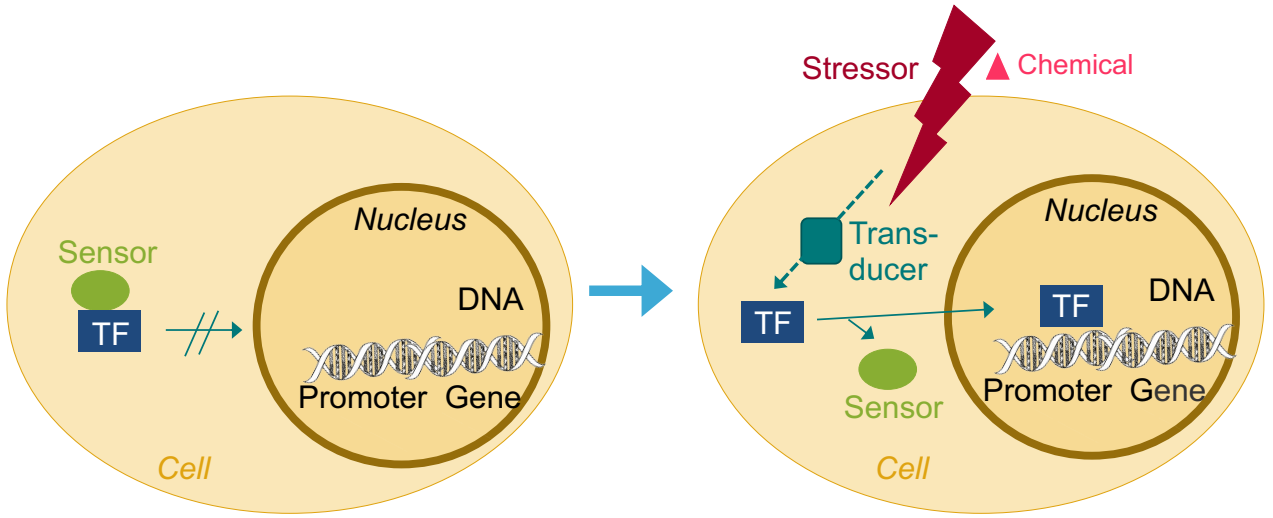
Neurotoxicity

- Mainly only acetylcholinesterase inhibition assays with isolated enzyme
  - False positives in presence of dissolved organic matter (2 mg_C/L), which isüpartially coextracted in SPE

- Mammalian neuronal and glial cell viability assays using SK-N-SH (and derivatives, such as SH-SY5Y cells) and C6 cells
  - still to be validated for water quality assessment

Activation of adaptive stress response pathways

Pathway	Sensor	Transcription factor	Inducing Chemicals
Heat shock response	Hsp90,Hsp70	HSF-1	Temperature, metals
Hypoxia	VHL	HIF-1	Oxygen depletion (can be caused by metals)
Metal stress	None	MTF-1	Heavy metals
Endoplasmic reticulum stress	BiP	XBP-1, ATF6, ATF4	Tunicamycin, thapsigargin, caplain, brefeldin A
Osmotic stress	None	NFAT5	High salt, glycol
Inflammation	IkB	NF-κB	Metals, PCBs, smoke, particles
Oxidative Stress	Keap1	Nrf2	Chemicals that produce reactive oxygen species
DNA damage	MDM2	p53	Electrophilic chemicals, UV radiation



Oxidative stress response

- Chemicals that produce reactive oxygen species (ROS) and electrophilic chemicals can induce the oxidative stress response, in particular the antioxidant defence pathway Keap1-Nrf2
- Often highly responsive in surface water, but also in drinking water if chlorinated

Matrix	Assay	$EC_{IR1.5}$ (REF)
Wastewater influent	AREc32	0.28 - 4.7
	Nrf2 reporter gene assay	8.1 - 30
Wastewater effluent	AREc32	1.5 - 22
	ARE GeneBLAzer	8.9 - 17
	Nrf2-CALUX	4.8
	Nrf2 reporter gene assay	47 - >50
	Nrf2-MDA-MB	>10
Recycled water	AREc32	4.2 - 94
	Nrf2-CALUX	4.8 - >30
	Nrf2-MDA-MB	>10 - >20
Surface water	AREc32	0.6 - >100
	ARE GeneBLAzer	6.9 - >490
	Nrf2-CALUX	6.9
	Nrf2 reporter gene assay	22
	Nrf2-MDA-MB	>20
Drinking water	AREc32	2.5 - >150
	Nrf2-CALUX	2.9
	Nrf2 reporter gene assay	21 - 25
	Nrf2-MDA-MB	>20

Apical effects and cytotoxicity

- Bacterial cytotoxicity tests (Microtox or BLT screen with *Aliivibrio fischeri* or *Photobacterium phosphoreum*) reduction of natural bioluminescence
- Mammalian cell lines: functional assays
  - mitochondrial activity with MTT or Alamar blue
  - Neutral red uptake
  - lactate dehydrogenase (LDH) assay
  - Cell staining
- Fish cell lines: same functional assays as above
- Whole organisms
  - Algal growth rate, Daphnia magna immobilisation, fish embryo toxicity

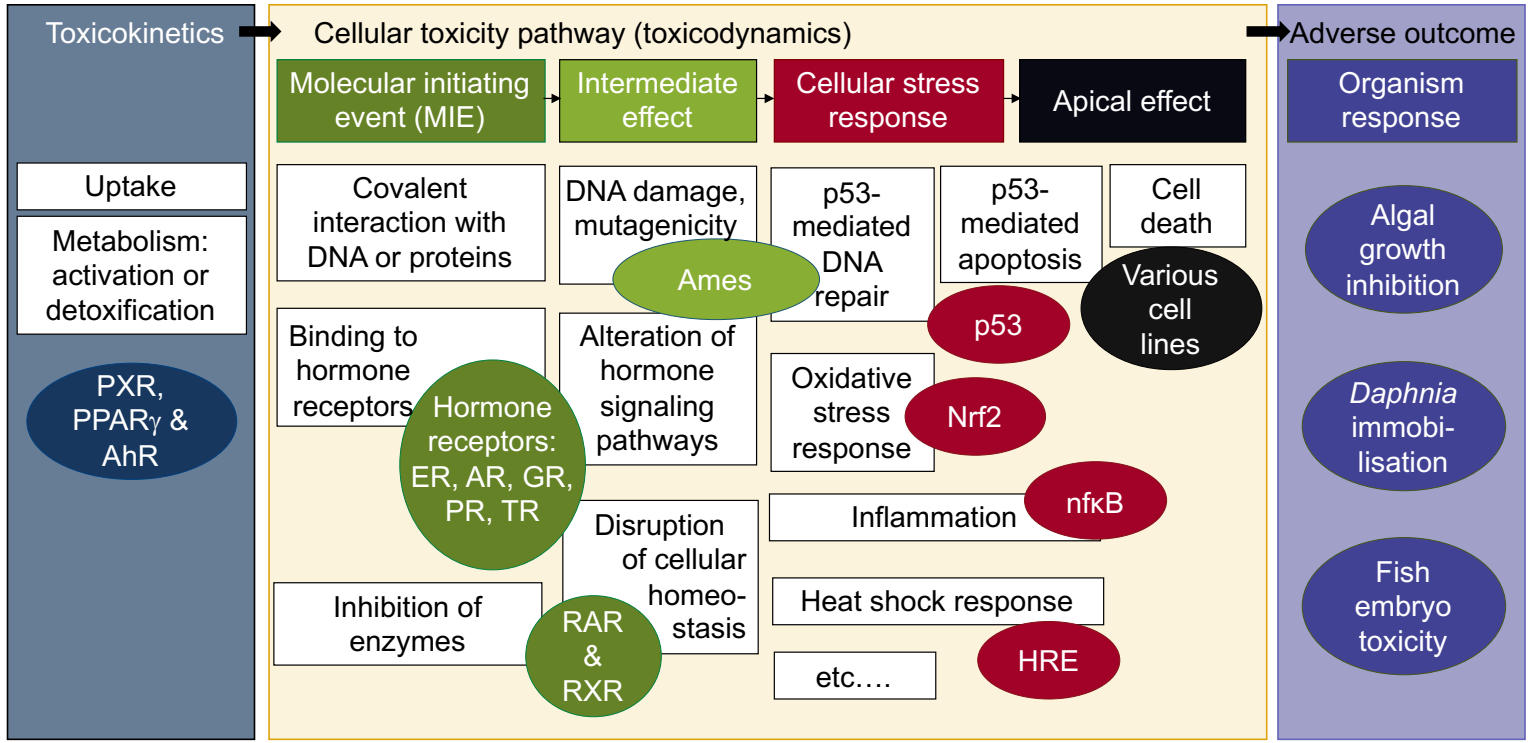
Mammalian cell cytotoxicity

Assay	Cell type	Endpoint/Principle
MTT	Various human (e.g., Hep-G2, MELN, HG5LN-hPXR (transfected HeLa cells)) and other mammalian cells (e.g., mouse lymphoma cells EL_4.3)	Colorimetric measurement of live cells, tetrazolium is reduced to formazan by the mitochondrial enzyme succinate dehydrogenase
Alamar Blue	Human kidney cells (HK2) also known as resazurin reduction assay)	Resazurin(Alamar Blue) is reduced to resorufin by mitochondrial enzymes substrate by live cells yields fluorescent product
Caco2-NRU	Human epithelial colorectal adenocarcinoma cells (Caco-2)	Cell viability measured by neutral red uptake (viable cells neutral red via active transport and incorporate the dye into their lysosomes but non-viable cells cannot not take up this chromophore
NRU	Human breast cancer cells (MCF-7)	Cell viability (measured by NRU test)
LDH leakage, e.g., CytoTox 96®	Human liver cells (HepG2)	Colorimetric measurement of lactate dehydrogenase (LDH) leakage from lysed cells
Mammalian cell cytotoxicity	Chinese hamster ovary cells (CHO AS52)	Cells stained with crystal violet and quantified by absorbance at 595 nm
SRB assay	Mouse neuroblastoma cells (neuro-2A); human fetal lung cells (MRC-5)	SRB is a bright-pink aminoxanthene dye that binds stoichiometrically to basic amino-acid residues –the amount of dye extracted from stained cells is directly proportional cell mass
CFDA-AM	Any cell, often used for fish cell lines	cell-permeant esterase substrate that is cleaved by intracellular esterases to a fluorescent carboxyfluorescein
Cell viability and growth	Any adherent cell	Confluency assessed via imaging analysis with Incucyte S3

Conclusion

- Diverse types of *in vitro* bioassays have been applied for testing of water quality
- Typically not single assays applied but rather test batteries (more in Chapter 13)

- Bioassays classified according to position in cellular toxicity pathway and AOP
  - also helps to link to *in vivo* effects and adverse outcomes
- Indicator assays are representative of crucial MIE and KE and very responsive to certain water types
  - ER, AhR, genotoxicity
  - always include cytotoxicity



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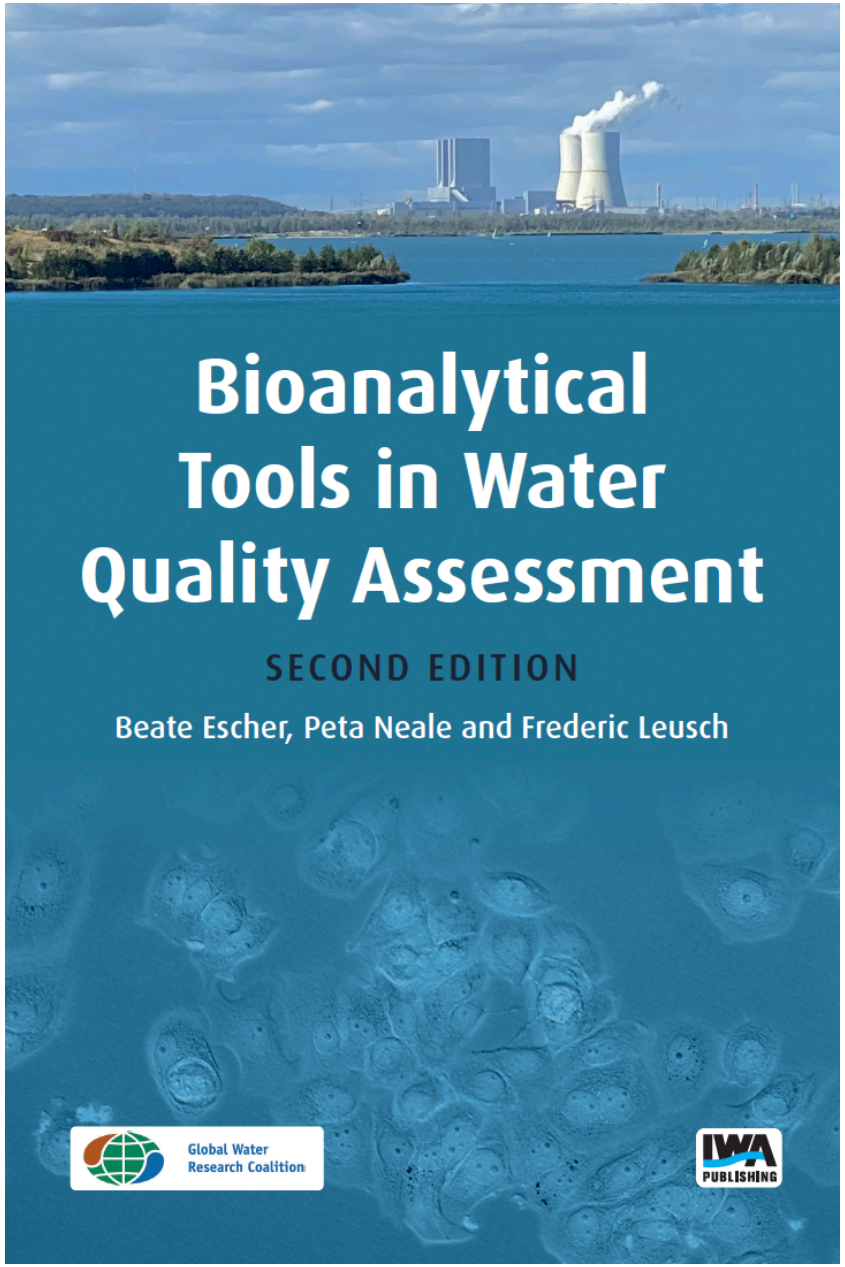

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