Improving water and sediment quality assessment using adaptive stress response assays

and implementing more efficient methodologies

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ABSTRACT

In vitro cell based bioassays are commonly used in water and sediment quality assessment. Such assays can be a valuable tool in assessing general toxicity endpoints including cell viability and genotoxicity as well as specific endpoints including adaptive stress response activation. Adaptive stress responses include the cellular reactions that occur to protect the cell, tissue or organism following among others genotoxic damage, oxidative stress and inflammation. Measuring these endpoints can be useful in screening chemicals and environmental samples because such effects occur prior to general toxicity. This review is focused on describing possible improvements to the current bioassay based water and sediment quality assessment. To improve the water and sediment quality assessment developing, validating and implementing headspace-free setups in bioassays is suggested. Measuring several adaptive stress response endpoints for samples is recommended for screening purposes, either in a test battery or multiplex setup. The development of a high-throughput screening multiplex method for adaptive stress responses would provide valuable data whilst saving time and valuable samples. Targets of interest for screening purposes include p53, NF-κB , Nrf2, XBP-1 and GSH, which should be measured simultaneously with general markers of cell viability and mitochondrial activity. Although many specific single assays measuring adaptive stress response endpoints exist improvements can be made by improving efficiency of the methods and the quality of data.

ABBREVIATIONS

BFR	Brominated flame retardant
DBP	Disinfection by-product
EDC	Endocrine disrupting chemical
EPA	U.S. Environmental Protection Agency
ER	Endoplasmatic reticulum
HAA	Haloacetic acid
HSF	Headspace free
HTS	High-throughput screening
MOA	Mode of action
MT	Metallothioneins
OCP	Organochloride pesticide
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
PCP	Personal care product
POP	Persistent organic pollutant
QSAR	Quantitative structure–activity relationship
ROS	Reactive oxygen species
THM	Trihalomethane
WWTP	Wastewater treatment plant

INTRODUCTION

Water quality has been an important topic in environmental sciences due to the ecological and human health impacts caused by pollutants. Pollutants in drinking and surface water include pesticides, heavy metals, phenols and disinfection by-products (DBPs) (Escher *et al.*, 2013; Wasi *et al.*, 2013). These pollutants are found as complex mixtures in water. The types and quantity of each of these pollutants are often unknown. Indeed, Escher *et al.* found that the toxicological effects of detected chemicals in water samples from treated effluent and drinking water could explain less than 0.1% of the overall observed effects in the samples, affirming the need to use effect-based as opposed to chemical concentration based toxicity values for assessing whether a water sample is safe for human consumption (Escher*, et al.*, 2013). Polluted water and sediment can cause adverse health effects. Therefore, the assessment of water quality is of utmost importance in preventing chemical-induced adverse effects in humans. The current risk assessment paradigm is in part focused on monitoring the concentrations of single chemicals in water samples and comparing these concentrations to 'safe' concentration limits (Dellarco *et al.*, 2010; Escher and Leusch, 2012). Effect-based monitoring of water samples complements chemical monitoring studies. Traditional ecotoxicological tests in effect-based monitoring studies include exposing fish and aquatic invertebrates to water samples and assessing mortality, growth and feeding responses (Fent *et al.*, 2006; Handy, 1994). *In vitro* bioassays are considered valuable alternatives or additions to traditional effect-based monitoring studies (Farré and Barceló, 2003). In these bioassays, the cellular response to chemical exposure is often measured in fluorescence, luminescence or absorbance after conversion of a substrate. Endpoints measured with *in vitro* bioassay include cell viability, production of reactive oxygen species (ROS) and genotoxic potency.

Genotoxicity is often described as "potentially harmful effects on genetic material", which also includes mutagenicity, "the induction of permanent transmissible changes in the amount or structure of the genetic material" (Van Leeuwen and Vermeire, 2007). Chemicals found in environmental samples could cause a variety of effects, such as genotoxicity. Reactions to the DNA causing genotoxicity include alkylation, single and double strand breaks, base damage, binding of bulky molecules and cross links within the DNA or with proteins (Klaassen and Watkins, 2003). The cellular reactions following damage induced by the aforementioned stressors are part of the so called "adaptive stress response". These reactions include cell cycle arrest, cellular senescence, DNA repair and in some cases apoptosis (Hussain and Harris, 2006). Other adaptive stress response pathways exist besides the DNA damage response, such as oxidative stress, heat shock, hypoxia, endoplasmatic reticulum (ER) stress, metal stress, osmotic stress and inflammation responses (Simmons *et al.*, 2009). *In vitro* bioassays specifically measuring adaptive stress response activation can be a valuable tool in the water and sediment quality assessment. Such methods are relevant since activation of adaptive stress responses can be an early indicator of general adverse effects such as genotoxicity or cytotoxicity.

The aim of this thesis is to identify ways to improve the current water and sediment quality assessment with bioassay measuring the adaptive stress response. The current water quality assessment strategies is discussed (Chapter 1) to elucidate their strengths and weaknesses. Pathways and genes involved with the cellular adaptive stress responses will be discussed thereafter and presented in a comprehensive

figure (Chapter 2). To answer the question how current water and sediment quality assessment may be improved, two different approaches to the problem are discussed. First, the implementation of existing bioassays not currently used in environmental research will be considered. Furthermore, novel targets related to cellular adaptive stress responses will be identified as possible additions to the current test battery (Chapter 3). The second approach is focused on improving the methodology of bioassays. A transition from measuring single endpoints towards complete test batteries or multiplex bioassays should be made (Chapter 4). Finally, concluding remarks answering the research questions and recommendations for future water quality assessment will be given (Chapter 5).

CHAPTER 1: Water quality assessment: now and in the future

The newly devised risk assessment paradigm is based on problem formulations by taking exposure scenarios and multiple endpoints into account (Dellarco*, et al.*, 2010; Krewski *et al.*, 2010). A connection is made between the source, such as chemicals, and adverse effects using a broad range of methods. Quantitative structure–activity relationship (QSAR) analysis is a valuable tool used to efficiently screen large libraries of chemicals for structural similarities to known toxicants to identify and prioritize potential toxicological targets (Dellarco*, et al.*, 2010). *In vitro* studies are to form the next step in the risk assessment. They are used to identify specific targets of and effects caused by chemicals. Such methods can identify chemicals causing basal cytotoxicity or activating adaptive stress response pathways. Adaptive cellular stress response pathways play a key role in maintaining cell homeostasis and/or for repairing damage by transcriptional activation of cytoprotective genes. Activation and detection of adaptive stress response pathways is typically more sensitive than cytotoxicity and other measures of cellular damage and thus provide early warning signals of cellular exposure to chemicals. *In vitro* methods can be used for high-throughput screening (HTS) in which many chemicals can be tested simultaneously in a short period of time. A common disadvantage of most *in vitro* methods is that the effects on a single (or few) cell types can be determined but interactions occurring at a whole tissue or organism scale are not taken into account. *In vivo* methods are used to determine adverse effects by chemicals on tissues or whole organs and organisms. These *in vivo* methods however are more expensive, time consuming and ethically questionable. Finally, long term epidemiologic population data can be studies establishing a correlation between a stressor and an adverse outcome in (human) populations (Dellarco*, et al.*, 2010). Detection of chemicals in complex water samples is commonly performed by gas and liquid chromatography and mass spectrometry (Rebe Raz and Haasnoot, 2011). The performance of the chemical detection methods is a crucial step in the risk assessment of environmental samples as it can link effects to specific chemicals.

The U.S. Environmental Protection Agency (EPA) has started the ToxCast program to screen hundreds of environmentally relevant chemicals with a large number of bioassays (Kavlock *et al.*, 2012). The goal of the program is increasing the knowledge of the mode of action (MOA) of the test chemicals and finding their target toxicity pathways (Kavlock*, et al.*, 2012). Such knowledge can improve risk assessment through prioritizing chemicals based on their suspected MOA as well as predicting adverse effects (Kavlock*, et al.*, 2012). A review by Connon *et al.* states the need for environmental risk assessment to shift the focus from finding the most potent or dangerous chemicals towards analyzing the effects of

(less potent) chemicals in mixtures and their interactions (Connon *et al.*, 2012). Water and sediment quality assessment focuses on traditional pollutants as well as emerging pollutants. Traditional pollutants include pesticides, persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs) (Ratola *et al.*, 2012). Pesticides have long been studied and are known to cause a variety of effects (carcinogenicity, endocrine disruption, reproductive, developmental and acute toxicity) (Damalas and Eleftherohorinos, 2011). The term POP is used to describe chemicals such as organochlorine pesticides (OCPs), brominated flame retardants (BFRs), dioxins and polychlorinated biphenyls (PCBs) (Ratola*, et al.*, 2012). Due to the chemicals properties of these POPs sorption to particles in the water and sediment phase these chemicals are removed from the surface water (Ratola*, et al.*, 2012). Perfluorinated compounds are rarely removed during water treatment and are more commonly found in surface water (Ratola*, et al.*, 2012).

More interesting than traditional pollutants are emerging pollutants such as pharmaceutical residues, personal care products (PCPs), endocrine disrupting chemicals (EDCs) and disinfection by-products (DBPs) (Jiang *et al.*, 2013). Although the their human health effects have not been fully elucidated, there is increasing evidence that many of these pollutants are endocrine disruptors. Pharmaceuticals can be grouped as antibiotics, anti-inflammatories, betablockers, cytostatics, tranquilizers and lipid regulators (Jiang*, et al.*, 2013). PCPs include pharmaceuticals for human and veterinary use and ingredients of personal care products (Jiang*, et al.*, 2013). EDCs are "the natural and/or synthetic compounds which would affect endocrine systems", often causing cumulative and persistent effects (Jiang*, et al.*, 2013). DBPs are a group of over 600 chemicals often formed during water treatment with chemical disinfectants in the presence of organic or inorganic matter (Simmons *et al.*, 2002). Novel DBPs can be occur in treated water due to changes in disinfection methods.

The problem with many of the emerging pollutants is that their risk to human health, as well as their level of occurrence and source remain are largely unknown (Deblonde *et al.*, 2011). Adverse health effects due to the exposure to these chemicals through water consumption are, however, likely given that many of these chemicals, particularly pharmaceuticals, are designed to be biologically active, water soluble and not readily degradable (Jiang *et al.*, 2013). A recent review analyzing data on the occurrence of drugs in surface water of 14 countries found that the pharmaceuticals ibuprofen, naproxen, erythromycin and roxithromycin were most commonly detected due to their widespread use (Jiang*, et al.*, 2013). The concentrations of pharmaceuticals and PCPs are known to vary significantly between the pg/L and µg/L range due to differences in their availability between countries (Ratola*, et al.*, 2012). Antibiotics and anti-inflammatory drugs were measured with the highest concentrations (Heberer, 2002; Jiang*, et al.*, 2013). From the EDCs, bisphenol A, nonylphenols, octylphenols and estrogens were most abundant in sewage and surface water (Jiang*, et al.*, 2013). Jiang *et al.* note that the traditional water treatment processes are insufficiently effective in the removal of emerging water pollutants, leading to contamination of surface water (Hernando *et al.*, 2006; Jiang*, et al.*, 2013). Among the DBPs the trihalomethanes (THMs) and Haloacetic acids (HAAs) were the most abundant groups of chemicals present in treated water after disinfection in several wastewater treatment plants (WWTP) (Krasner *et al.*, 2006). DBPs are known to be or become electrophilic after metabolism allowing them to potentially react with DNA, proteins, cystein groups and peptides (Plewa *et al.*, 2004).

CHAPTER 2: Adaptive stress response pathways

Adaptive stress responses include the cellular reactions that occur after exposure to various stressors. These responses include among others DNA repair, antioxidant reactions, apoptosis, and cell cycle arrest. Transcription factors are activated after cellular damage occurs, these transcription factors directly or indirectly cause the aforementioned responses. Such adaptive stress responses occur before the general endpoints such as cytotoxicity or genotoxicity can be measured (Christmann and Kaina, 2013) and are therefore especially relevant when testing complex mixtures of chemicals at poorly analytically detectable concentrations in water samples. Several pathways and their related stressors and TFs are shown in table 1 and described in the paragraphs below. First the pathways are described and cross-linking genes mentioned.

The DNA damage response pathway is mainly regulated by tumor protein 53, also called p53. This protein is involved in processes such as apoptosis, DNA repair, cell cycle arrest and senescence. p53 is activated by ATM, ATR, JNK, Chk1 and Chk2 mainly through phosphorylation and acetylation of the p53 protein (Mahadevan *et al.*, 2011). Carcinogenic compounds can affect growth factors and receptors and indirectly activate the transducer mitogen-activated protein kinase (MAPK) which is involved in multiple adaptive stress response pathways (Leonard *et al.*, 2004). The breast cancer associated gene 1 (BCRA1) is an upstream regulator of many genotoxicity related genes, including p53 (Deng and Wang, 2003). MDM2 is a negative regulator which promotes p53 breakdown in unstressed cells, this however is not possible with phosphorylated p53 (Mahadevan*, et al.*, 2011). Apoptosis is induced by p53 through activation/promotion of the apoptosis-related genes BAX and BCL12 (Mahadevan*, et al.*, 2011).

Proteins are commonly folded or modified within the ER by complex pathways and various genes. Any disruption of ER functions by stressors is called ER stress, this leads to accumulation of unfolded proteins within the cell and possibly apoptosis (Kadowaki and Nishitoh, 2013). The adaptive stress response pathway related to ER stress is the unfolded protein response (Kadowaki and Nishitoh, 2013). Accumulation of unfolded proteins leads to dissociation of immunoglobin-binding protein (BiP) from ATF6 and is followed by the upregulation of inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1) and X-box-binding protein 1 (XBP1) (Kadowaki and Nishitoh, 2013). Upregulation of XBP1 decreases protein synthesis and increases the cell's chance of survival. Alternatively, in cases of prolonged ER stress and unfolded protein accumulation IRE1 activates apoptosis through Bax and Bak, as well as activates the NF-κB response through IKK (Kadowaki and Nishitoh, 2013). Furthermore, JNK and p38 are activated in the ER stress pathway.

Heat shock proteins can be induced by cellular and oxidative stress and are involved in the negative regulation of apoptosis (Benarroch, 2011). Among the heat shock proteins are Hsp90, Hsp70 and Hsp27, which are most active in processes such as protein degradation and apoptosis (Benarroch, 2011). Hsp70 inhibits JNK activity while Hsp90 is known to activate JNK, NF-κB and tumor necrosis factor α receptor (TNFR) (Benarroch, 2011). These opposing effects should be taken into account when measuring transcription factors related to the Hsp70 and Hsp90.

Table 1: Adaptive stress response pathways with related stressors and genes

Adaptive stress response pathway	Transcription Factor	Sensor	Transducer	Activated gene promotors	Effect
DNA damage	p53, AP-1[a], BCRA1[a], (NF-κB)[a]	MDM2	ATM, ATR[a] , JNK, Chk1, Chk2, MAPK[a,] Erk[c]	CDKNIA, GADD45A, MDM2, BCL12, TP53, BAX[c]	DNA repair, apoptosis[a,] senescence[c], cell cycle arrest[c]
ER stress	XBP-1, ATF4, ATF6	BiP	IRE1α, S2P, PERK[b]	HSP90B1, HSPA5, DNAJB9	apoptosis, lipid synthesis, amino acid metabolism[b]
Heat shock	HSF-1	Hsp90, Hsp70, Hsp27	CaMK2, CK2	HSPA6	apoptosis, protein degradation
Hypoxia	HIF-1	VHL	p38, PI3K	VEGF, TF, EPO	apoptosis, cell proliferation, migration[e]
Inflammation	NK-kB, (AP-1)	IkB	IKK	IL1A, TNFA	apoptosis
Metal Stress	MTF-1	-	PKC, CKII, TKs	MT1 E, MT2 A	Decreasing cellular metal concentrations
Osmotic stress	NFAT5	-	p38, ATM, PKA	AKR1B1, SLC6A12, SLC5A3	Synthesis and transport of osmolytes[f]
Oxidative stress	Nrf2	Keap1	MAPK, ERK, p38, PKC, ARE[d]	HMOX1, NQO1, GST2A, GCLC[d], GCLM[d]	antioxidant reaction, increased GSH levels

* Adapted from (Simmons*, et al.*, 2009) and [a] (Christmann and Kaina, 2013) [b] (Hetz *et al.*, 2013) [c] (Whibley *et al.*, 2009) [d] (Johnson *et al.*, 2008) [e] (Harris, 2002) [f] (Macián *et al.*, 2001)

Hypoxia is a cellular state of reduced or depleted oxygen levels which leads to adverse effects. The hypoxia-inducible transcription factor 1 (HIF-1) binds to hypoxia-response elements when oxygen levels decrease (Harris, 2002). Through interactions with the Von Hippel-Lindau (VHL) proteins expression of genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) is activated (Harris, 2002). These gene interactions lead to cellular stress responses including apoptosis, cell proliferation and migration (Harris, 2002).

Other bioassay biomarkers for activation of adaptive stress responses in cells may be found in the signaling pathway induced by metal transcription factor-1 (MTF-1). This protein senses an increase in concentrations of labile and potentially toxic metals as zinc, copper, silver and cadmium. In the presence of metals, MTF-1 induces the expression of metallothioneins (MTs), cysteine-rich proteins that have the capacity to bind (heavy) metals and thus regulate metal homeostasis. Metals are known to cause production of oxidative species though reactions with H2O2 and could therefore activate the oxidative stress pathway (Leonard*, et al.*, 2004). .

Osmotic stress is caused by elevated concentration of sodium chloride (NaCl) or urea and can lead to effects including the production of ROS, damaging DNA and proteins and disrupting mitochondrial functioning (Burg *et al.*, 2007). Therefore it is not surprising that the osmotic stress pathway is influenced by genes involved in DNA damage, oxidative stress and heat shock adaptive stress responses. Activation of the osmotic stress pathways leads to apoptosis, cell cycle arrest, DNA repair and production of antioxidant enzymes (Burg*, et al.*, 2007). The transcription factor nuclear factor of activated T-cells 5 (NFAT5) is known to be involved in regulating genes related to synthesis or transport of osmolytes that prevent osmotic stress (Macián*, et al.*, 2001). It should be noted that this pathway is less relevant for aquatic environmental screening and emerging pollutants and will not be further discussed as a potential target in water and sediment screening assays.

Oxidative stress is often described as a disturbance in the balance between ROS and the antioxidant reaction which can be caused by free radicals and a wide variety of chemicals (Leonard*, et al.*, 2004). The main transcription factor involved in the oxidative stress response pathway is NF-E2 related factor 2 (Nrf2) which is mediated by Kelch-like ECH-associated protein 1 (Keap1) and activates the antioxidant response element (ARE) (Escher*, et al.*, 2013). Activation of this pathway leads to increased production of antioxidant proteins such as glutathione (GSH) through increased production of glutamate-cysteine ligase catalytic (GCLC) and glutamate-cysteine ligase modifier (GCLM) subunits (Johnson*, et al.*, 2008). The pregnane-X receptor (PXR) is involved in regulating the expression of glutathione-S transferase.

Some of the genes mentioned in the pathways above act on multiple stress pathways. The c-Jun N-terminal kinase (JNK) is a transducer central in many adaptive stress responses. JNK is known to be activated by many stressors including DNA damage, heat shock and inflammation (Guma and Firestein, 2012). After activation JNK can phosphorylate and thus alter functioning substrates such as p53 and Bcl, explaining its functions in apoptosis and cell proliferation (Guma and Firestein, 2012). NF-κB is shown to be active in the inflammation, ER stress and DNA damage responses. Inflammation related genes interleukin-1 (IL1) and tumor necrosis factor α (TNF-α) activate NF-κB , however, inflammation is also known to enhance tumour development (Karin, 2006). The IKK complex and therefore NF-κB are shown

to be activated following genotoxic damage (Christmann and Kaina, 2013). NF-κB and Gadd45β are shown to indirectly inhibit JNK functioning (Guma and Firestein, 2012). Mitogen-activated protein kinase (MAPK) is not only relevant in oxidative stress responses but is also shown to activate AP-1 following genotoxic damage (Christmann and Kaina, 2013).

Figure 1 shows a simplified overview of the pathways related to adaptive stress responses as well as the transducers, sensors, transcription factors and effects.

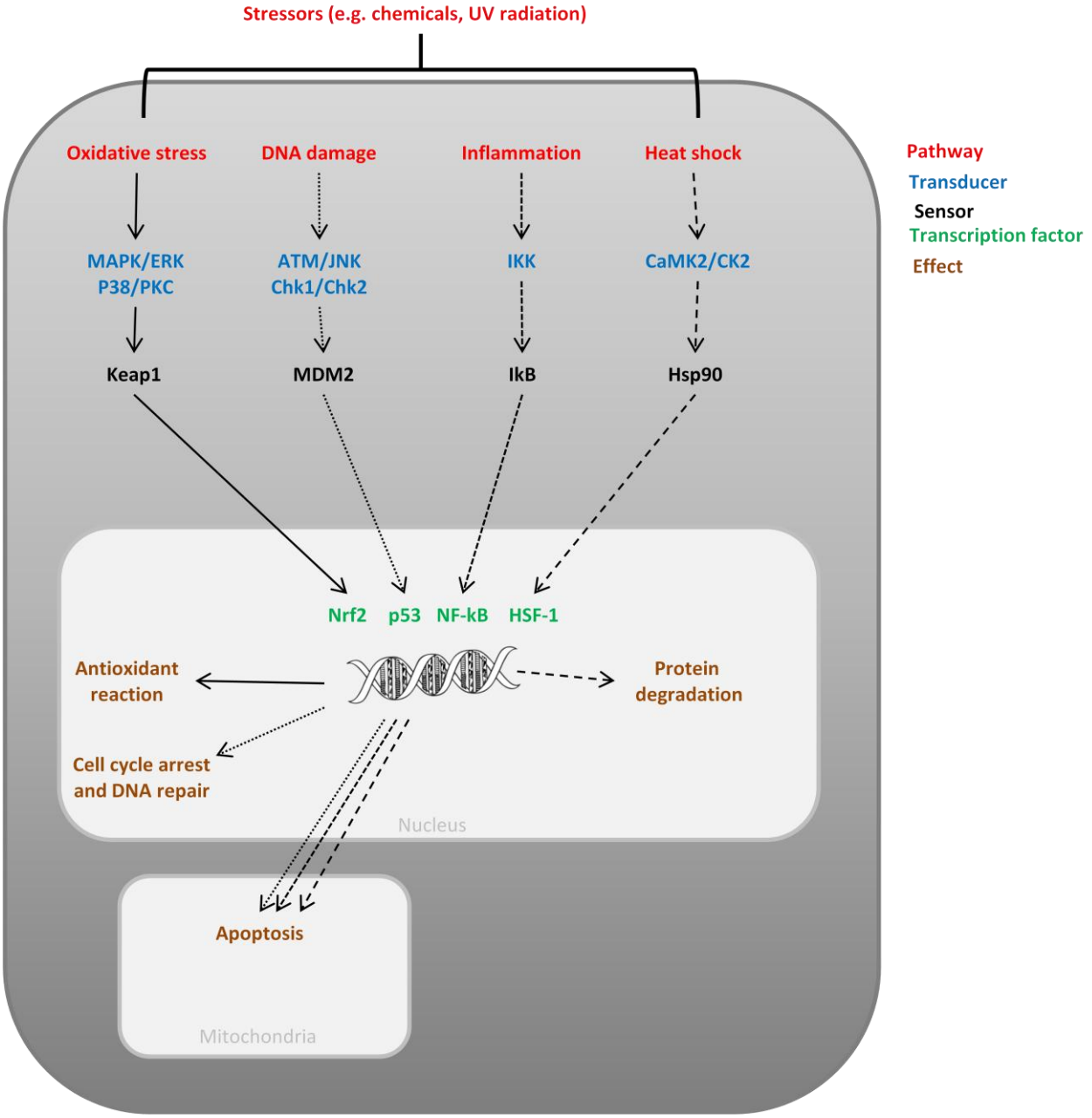

Figure 1: Cellular adaptive stress response pathways (Simmons, *et al.*, 2009) (Hetz, *et al.*, 2013) Coloured text is used to differentiate between pathways and stressors (red), transducers (blue), sensors (black), transcription factors (green) and effects (orange). Different lines are used to separate the pathways.

CHAPTER 3: Adaptive stress response in water quality assessment: now and in the future

A valuable tool in the water quality assessment are *in vitro* bioassays which are often cell based methods used to measure general endpoints such as cytotoxicity, ROS production and genotoxicity or sensitive endpoints such as the activation of adaptive stress related genes. Several bioassays based on adaptive stress response pathways are currently in use to assess water quality.

Several requirements for the use of bioassays in environmental quality assessment exist. Most importantly, the results of a bioassay should be reliable and reproducible to ensure comparisons of data between different experiments and laboratories is possible. Furthermore, an assay should be sufficiently sensitive to pick up effects of single compounds as well as effects caused by complex mixtures as the concentrations of individual chemicals can be low. A high sensitivity is needed for environmental screening because the factor by which environmental samples can be enriched is limited by extraction methods, solubility of the compounds and the solvents. Commonly used solvents such as DMSO, methanol or MTBE may cause toxicity at low concentrations, depending on the cell line. Often solvent concentrations between 0.1 and 1% of the exposure sample volume are used. Preferably, cytotoxicity should be measured in addition to adaptive stress response pathways as a decrease in cell viability could influence the results of other assays (Hallis *et al.*, 2007). Although enriching environmental samples can significantly increase final chemical concentrations in the bioassay the raw sample volume needed can be very high. Besides the solubility of mixture chemicals after enrichment, another factor to take into account is evaporation of volatile compounds when using methods such as solid-phase extraction (SPE). Evaporation of mixture chemicals can cause an underestimation of the toxic potency of the mixture, this issue is further addressed in chapter 4.

The ease of use and time required to run the bioassays for screening purposes is important. Assays with short incubation times, exposure periods, and fast growing cells that easily adhere to the test matrix are preferred. Cells should adhere strongly to ensure that washing and exposure procedures during the experiment do not reduce the cell number on the test surface. A commonly used test format in bioassays are multiwell plates which often contain 24, 48, 96 or even 384 separate wells in which cells can be cultured. The use of a large number of low-volume wells decreases the sample and reagent volumes needed, yet generates great amounts of data per experiment. Factors influencing the choice of multiwell plates include the cell adherence and available equipment in laboratories.

The effects of chemicals on adaptive stress responses can be measured based on multiple targets and genes in each pathway. For example, activation of the oxidative stress response can be assessed by assays focused on upstream genes in the pathways such as Keap, Nrf2 and ARE or specific targets such as GSH (Koenig and Solé, 2012). NF-κB is interesting due to its functions in the transcription of DNA. Inducers of NF-κB include ROS and TNFα. Therefore NF-κB is used in bioassays as a sensitive measurement of several stressors. Bioassays measuring the activation of p53, AP-1 and BCRA1 are interesting due to the functions of these targets related to genotoxicity. The advantages and disadvantages of using bioassays based on (upstream) genes active in multiple adaptive stress pathways should be taken into account for water quality assessment. Upstream genes would likely be activated by a broader range of chemicals which can be an advantage in chemical screening. However, this would be less useful in specifying the MOA of the test chemicals due to lower specificity.

A bioassay test battery measuring the adaptive stress response activation in environmental samples should be focused on multiple pathways instead of a single pathway. Such a broad system could elucidate the MOA of tested single compounds or the constituents of a complex mixture, this is discussed in chapter 4.

Reporter gene assays are commonly used methods with which the expression of a specific gene is measured. Such methods work by linking reporter genes to the promoter gene regulating the gene of interest. Following activation of the promoter by stressors reporter gene proteins are produced and can be measured by fluorescence, luminescence or absorbance. Two different bioassay test systems used for the quality assessment of environmental samples will be described below.

The CellSensor® test system based on Invitrogen GeneBLAzer® technology has been used in the HTS of single compounds, as well as complex water mixtures (Hanson, 2006). Reporter gene methods such as those using the GeneBLAzer® system, which measures the production of the bacterial β-lactamase enzyme in mammalian cells, are shown to be very robust (Hanson, 2006). The β-lactamase is produced as a result of activating specific receptors by chemicals and other stressors. GeneBLAzer® assays measuring several adaptive stress response related genes such as p53, NF-κB , ARE, ATF6, HSF-1 and HIF-1 exist.

Another test system commonly used in water quality assessment is the CALUX reporter gene bioassay. This system is based on U2OS human bone cancer cells transfected with a luciferase gene under control of a endocrine receptor such as estrogen, androgen, glucocorticoid or progesterone (Brand *et al.*, 2013). The application of a test battery of four CALUX assays with the aforementioned endocrine receptors was used in this study, however, its usability could not be confirmed due to the low endocrine activity in the tested samples (Brand*, et al.*, 2013). The combined endocrine activity of all mixed chemicals in a water sample was measured instead of single compounds. Table 2 shows an overview of the bioassays currently in use to determine water and sediment quality.

Table 2: Current bioassays and their uses

Pathway	Gene	Bioassay	Used for
DNA damage	p53	CellSensor p53RE-bla	ToxCast
		Greenscreen p53	ToxCast
		p53 Calux	Water quality assessment
		Attagene Factorial p53	ToxCast
		Cellumen p53	ToxCast
		NCGC p53	ToxCast
	AP-1	Attagene Factorial AP1	ToxCast
	BCRA-1	-	-
ER stress	ATF4	-	-
	ATF6	CellSensor ESRE-bla	-
	XBP-1	Attagene Factorial XBP1	Toxcast
Heat shock	HSF-1, -2, -4	CellSensor HSE-bla	-
	HSF-1	Attagene Factorial HSE	ToxCast
Hypoxia	HIF-1	CellSensor HRE-bla	-
	HIF-1a	Attagene Factorial HIF1a	ToxCast
Inflammation	NF-κB	CellSensor NFkB-bla	-
	NF-κB	NF-κB Calux	Water quality assessment
	NF-κB	Attagene Factorial NF-κB	ToxCast
Metal Stress	MTF-1	-	-
	MTF-2	Attagene Factorial MRE	ToxCast
Osmotic stress	NFAT5	-	-
Oxidative stress	Nrf2	AREc32 assay	Water quality assessment
	Nrf2	Attagene Factorial NRF2/ARE	ToxCast
	ARE	CellSensor ARE-bla	-
	PXR	Attagene Factorial PXR	ToxCast
	GSH	Promega GSH-Glo Glutathione	Drugs of abuse

(Antolino-Lobo *et al.*, 2011; EPA, 2013; Escher *et al.*, 2012; Kavlock*, et al.*, 2012; Knight *et al.*, 2009; Martin *et al.*, 2010; Xiu *et al.*, 2006)

The assays shown in table 2 are focused mainly on the transcription factors of each pathway and the related sensors. ATF4 has recently been measured in a transiently transfected luciferase based assay to further elucidate the ER stress pathway (B'Chir *et al.*, 2013). Surprisingly, no bioassays measuring the activation of BCRA1 for environmental screening purposes were found. This gene could be measured in addition to p53 and AP-1 to improve DNA damage related screenings. To our knowledge MTF-1 and NFAT5 are not routinely measured in water and sediment quality assessments.

Assays based on upstream transducers of the pathways are rarely used (in water and sediment quality assessment) since activation of a transducer is not always specific for a single pathway. Bioassay for transducers such as PXR and JNK exist, however, these targets are represented in many pathways, making results less useful. The ER stress response pathway leads to various effects depending on the severity and duration of the stress, which could work towards cell survival or apoptosis (Cawley *et al.*, 2011). In case of the ER stress pathway measuring early markers would be more relevant than those related to apoptosis such as XBP1. Another example is p53, which is involved in apoptosis in the DNA damage pathway. Although such transcription factors can be measured as in indirect marker of activation of the involved pathways the data acquired may not be useful. Often chemical induced apoptosis (though p53 or similar genes) occurs at concentrations within one order of magnitude from the concentration causing general cytotoxicity. As such, measuring apoptosis via specific transcription factors could sometimes be replaced by less expensive general assays. Furthermore, Simmons *et al.* point out that some genes related to adaptive stress responses are activated at relatively low concentrations and may prevent toxic effects (Simmons*, et al.*, 2009). Therefore activation of such genes by chemicals may overestimate the toxic potential in bioassays, therefore the role of genes used for environmental screening should be clear (Simmons*, et al.*, 2009).

CHAPTER 4: Improvements in methodology bioassays for risk assessment

Although a variety of bioassays measuring adaptive stress responses exist improvements can still be made. It is important to take the purpose of bioassays and environmental quality assessment into account. Different requirements exist for the outcome and analysis of bioassays used in simple screenings compared to methods also taking exposure data into account. For screening purposes the ranking of toxicity of large groups of chemicals is the main goal and could be done by calculating a toxicity equivalent concentration compared to a reference compound. Bioassays should be robust, reproducible, sensitive and efficient in the sample volume and time needed during experiments. Preferably the assays should be possible to perform in most laboratories with common equipment such as cell culture facilities and plate readers to measure fluorescence, absorbance and luminescence.

An adaptation to current bioassays that is rarely used are headspace free (HSF) setups in which wells are completely filled with medium and solutions before sealing to minimize a gas phase above the liquid (Stalter *et al.*, 2013). This can be useful since some groups of chemicals (such as DBPs) are known to evaporate from the exposure medium and therefore reducing the concentration to which the cells are exposed (Stalter*, et al.*, 2013). For environmental samples this can prevent underestimation of toxicity by loss of chemicals during the experiment or extraction and enriching of samples. A disadvantage of such an adaptation is the increased use of solutions and medium to completely fill the wells. Furthermore, additional liquid removal procedures during the experiment could influence the results if cell adhesion to the wells is insufficient.

Samples used in environmental monitoring could be tested for a single endpoint or multiple endpoints simultaneously, which is called multiplex analysis (Zhao *et al.*, 2011). Multiplex methods can be divided in assays using a separate label for each endpoint or position specific measurements (Roda *et al.*, 2012). Such multiplex methods have been used in PCR to simultaneously measure the expression of multiple genes after exposing cells to chemicals. The use of multiplex assays would significantly decrease the sample volumes used in environmental monitoring of water, such water samples can be valuable or limited.

A form of multiplexing bioassays includes the in tandem measurements of different endpoints in the same cells. Maintaining cells in optimum conditions for HTS can be costly, therefore measuring multiple endpoints before discarding the cells can save time and money (Hanson, 2006). Hanson *et al.* co-cultured CHO-K1 and Jurkat cells and were able to successfully measure GeneBLAzer® signals and calcium flux (Hanson, 2006). In this study two similar endpoints were measured with the different multiplexed assays, making identification of false negative reactions possible (Hanson, 2006). To reach this goal several adaptations to the original methods had to be made to solve problems in compatibility between the assay. Due to the use of different cell lines adaptations had to be made in the growing and assay conditions of the cells. The growing medium of either cell should not include chemicals influencing the endpoint measurements. Furthermore, substrates and dyes shown in the assay must not interfere.

If the same cell line and test system are used in multiplex assays to measure different endpoints less problems with compatibility will occur. Multiplex assays have been developed in which a large number of

labeled dyes (present in the suspension or on the surface area) can be measured within individual wells on a multiwell plate (Zhao*, et al.*, 2011). Zhao *et al.* note that fluorescent dyes can be used in multiplex bioassays if the fluorescent wavelengths do not interfere (Zhao*, et al.*, 2011). Fluorescent dyes however tend to have wide emission ranges rather than a single wavelength, therefore the number of simultaneously measured endpoints can be limited by the dyes (Zhao*, et al.*, 2011). Recent developments have made the labeling of nanobarcodes and nanostrings possible for the use in multiplex assays (Zhao*, et al.*, 2011). Selection of a cell line used for multiplex screening purposes should be based on cell type, metabolism and expression of adaptive stress response pathways.

The Attagene Factorial test system is used to measure the activity of transcription factors, including those related to adaptive stress responses (Romanov *et al.*, 2008). This assay uses nearly identical reporter transcripts which can be processed and differentiated by electrophoresis (Romanov*, et al.*, 2008). Despite some limitations this method is promising because of the simultaneous measurement of many transcription factors (Romanov*, et al.*, 2008).

The novel Luminex suspension bead assay is based on labeled microspheres which can be differentiated by the ratio of different fluophores on the surface (Wang *et al.*, 2012). This multiplex method has been compared with single endpoint clinical assays used to measure concentrations of tumor markers in clinical samples (Wang*, et al.*, 2012). The Luminex method showed results with similar a similar sensitivity and accuracy as the conventional methods for the tested tumor markers (Wang*, et al.*, 2012). This system however is used to detect samples with flow cytometry, not in a multiwell bioassay.

Data obtained from multiplex or a test battery of methods should be comparable and methods should be performed with well defined parameters and as similar as possible. A point of critique and a general consideration in chemical risk assessment is that the nominal chemical concentrations used to analyze data from bioassays could lead to under- or overestimation of the toxic potencies of compounds (Blaauboer *et al.*, 2012; Wetmore *et al.*, 2013; Wetmore *et al.*, 2012). Many factors can influence the chemical concentrations to which cells are exposed including binding to serum proteins or plastic, variable uptake, metabolism and excretion by cells and cell density.

When measuring different adaptive stress response endpoints the use of one cell line and similar growth and assay conditions is preferable. Data obtained from analyzing different adaptive stress response pathways is not suited for direct comparisons, however, complimentary data can be useful for screening and regulatory purposes. To rank the toxicity of chemicals the relative potency compared to a environmentally relevant reference compound can be made (Escher and Leusch, 2012). Such a concept can be useful in predicting the potency of a complex mixture based on chemical analysis and bioanalytical data of the constituents (Escher*, et al.*, 2013; Tang *et al.*, 2013). For each endpoint in a bioassay the induction ratio (compared to the controls) that represent an effect should be defined and can be calculated based on the variation in control wells (Escher and Leusch, 2012).

CHAPTER 5: Discussion

The need for specific and early markers of cellular damage such as adaptive stress responses as opposed to general endpoints like cytotoxicity or genotoxicity has become apparent in recent years. Adaptive stress response related bioassays can be a valuable tool in the shift of environmental risk assessment towards analyzing complex mixtures and less potent chemicals. Bioassays detecting adaptive stress responses upon exposure to chemicals exist, although few have been used for water and sediment quality assessment so far. Targets that are most often measured for this purpose include the transcription factors p53, NF-κB and Nrf2 (Martin*, et al.*, 2010). Genes not commonly analyzed in water quality assessment include upstream transducers of each pathway, due to the low specificity towards a single endpoint. Examples include JNK and PXR; which are activated by various stressors and consequently regulate many downstream genes. In contrast, genes that are activated and function at the end of a pathway are very specific and are influenced by smaller groups of chemicals. Such targets could provide valuable information on the MOA of test single chemicals and environmental samples.

Assays used for routine water quality assessment should be robust, fast and possible to be performed in most laboratories. A multiplex test system for the aforementioned targets would be efficient and require smaller volumes of samples. Ideally, a HSF setup should be used for environmental mixtures and volatile (emerging) compounds such as DBPs.

Routine water monitoring would benefit from a multiplex assay or test battery of adaptive stress response bioassays rather than a single endpoint to elucidate the chemical MOA. The effects of genes involved in multiple pathways should be taken into account before developing a novel multiplex test system. This is needed to prevent measuring genes causing similar effects that are activated by the same chemicals. The targets measured in screening should include specific markers from different pathways as well as more general toxicity endpoints including cytotoxicity as data from multiple related pathways could help explaining the observed effects. Measuring GSH concentrations for example could elucidate the relation between oxidative stress and cell viability.

If bioassays in water and sediment quality assessment are to be used to determine the quantitative toxicity rather than as a screening tool a thorough understanding of the gene effects is required (Blaauboer*, et al.*, 2012). Careful selection of the appropriate dose metric as well as defining the *in vitro* effects that cause observable toxicity effects in the population is needed (Groothuis *et al.*, 2013).

Concluding, the current environmental quality assessment gain from simultaneous measurements of various adaptive stress response related endpoint and developing a system for volatile compounds. Although many bioassays measuring the adaptive stress response related endpoints exist there is still room for changes. Implementation of a multiplex system based on a single cell line would improve comparability between results and lead to a more efficient methodology.

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