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Review of ecologically relevant *in vitro* bioassays to supplement current *in vivo* tests for whole effluent toxicity testing - Part 1: Apical endpoints

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Review of ecologically relevant *in vitro* bioassays to supplement current *in vivo* tests for whole effluent toxicity testing- Part 1: Apical endpoints

Abstract

Whole effluent toxicity (WET) testing is commonly used to ensure that wastewater discharges do not pose an unacceptable risk to receiving environments. Traditional WET testing involves exposing animals to (waste)water samples to assess four major ecologically relevant apical endpoints: mortality, growth, development, and reproduction. Recently, with the widespread implementation of the 3Rs to replace, reduce and refine the use of animals in research and testing, there has been a global shift away from *in vivo* testing towards *in vitro* alternatives. However, prior to the inclusion of *in vitro* bioassays in regulatory frameworks, it is critical to establish their ecological relevance and technical suitability. This is part 1 of a two-part review that aims to identify *in vitro* bioassays that can be used in WET testing and relate them to ecologically relevant endpoints through toxicity pathways, providing the reader with a high-level overview of current capabilities. Part 1 of this review focuses on four apical endpoints currently included in WET testing: mortality, growth, development, and reproduction. For each endpoint, the link between responses at the molecular or cellular level, that can be measured *in vitro*, and the adverse outcome at the organism level were established through simplified toxicity pathways. Additionally, literature from 2015-2020 on the use of *in vitro* bioassays for water quality assessments was reviewed to identify a list of suitable bioassays for each endpoint. This review will enable the prioritisation of relevant endpoints and bioassays for incorporation into WET testing.

Keywords

Direct toxicity assessment; bioassay; wastewater; apical endpoints; toxicity pathway

1 Introduction

Wastewater from domestic and industrial sources contains thousands of chemicals, including pharmaceuticals, personal care products, metals, industrial chemicals, pesticides, and detergents. Wastewater is usually treated prior to discharge with varying treatment approaches available, each with its advantages and limitations and varying removal efficacy (Monarca et al. 2000, Melvin & Leusch 2016). With over 160 million chemicals registered by the Chemical Abstract Service and more than 350,000 registered for production and commercial use (Wang et al. 2020), it is not surprising that some chemicals are more efficiently removed by treatment than others. Thus, with or without treatment, wastewater effluent likely contains thousands of chemicals and their transformation products (Rogowska et al. 2020). This effluent is discharged into aquatic systems such as rivers, estuaries, and oceans, where chemicals can potentially cause environmental harm.

Wastewater treatment plants (WWTPs) in high income countries are typically required to ensure there is no unacceptable environmental risk associated with wastewater discharge. There are several approaches WWTPs can use to assess environmental risk of wastewater, including chemical analysis of effluent (and comparison against prescribed guidelines) and whole effluent toxicity (WET) testing (USEPA 1978, Prasse et al. 2015, Pérez et al. 2018). WET testing, also sometimes referred to as direct toxicity assessment (DTA), uses biological assays to test toxicity of wastewater samples or total pollutant loads in ambient waters, therefore focusing on toxicity of chemical mixtures rather than individual chemicals (ANZG 2018). Approaches to WET testing include *in situ* methods such as active biomonitoring, model ecosystems such as microcosms, and mesocosms, or single-species (*in vivo*) bioassays. Typically, *in vivo* bioassays focus on apical endpoints such as mortality, development, growth and reproduction (Prasse et al. 2015), which are considered ecologically relevant and have clear effects at the individual and population level. While whole animal bioassays

provide valuable information about toxicity at the individual level and above, they can be costly (both financially and ethically) and are often low throughput.

Over the past decade, there has been a global shift away from whole animal testing towards alternative methods. This is due, in part, to the ever expanding number of chemicals requiring assessment, and advances in bioanalytical techniques (Villeneuve & Garcia-Reyero 2011), but also due to widespread implementation of the 3Rs to replace, reduce and refine the use of animals in research and monitoring (Lillicrap et al. 2016, Norberg-King et al. 2018). The 3Rs, driven by ethical concerns over animal welfare, are aimed at reducing the number of animals used in toxicity testing, refining test procedures to reduce stress to animals and enhance the amount of data produced from each experiment, and replacing animals where possible. While the push for the 3Rs was originally aimed towards reduction of mammals in toxicity testing, it has since grown to include all species used in ecotoxicology testing such as amphibians and fish (Lillicrap et al. 2016). *In vitro* bioassays can provide a cost-effective, high throughput, ethical alternative to *in vivo* assays, with improved capacity for standardisation (Paparella et al. 2021). *In vitro* assays can reduce the use of animals by supplementing *in vivo* tests, or, in some cases, can replace the use of animals altogether. However, prior to inclusion of *in vitro* bioassays in regulatory frameworks, links between responses at the molecular or cellular level and adverse outcomes at the individual or population level must be established to demonstrate ecological relevance (Villeneuve & Garcia-Reyero 2011). This review will provide a systematic overview of *in vitro* bioassays that may provide more cost-effective, ethical and high throughput alternatives to single-species, *in vivo,* bioassays.

This is part 1 of a two-part review that aims to identify relevant *in vivo* toxic outcomes after exposure to wastewater, and the *in vitro* bioassays that are related to those effects through toxicity pathways and could therefore be used in WET testing. Part 1 addresses the four

apical *in vivo* endpoints of mortality, growth, development, and reproduction. Part 2 will address seven additional non-apical endpoints that were identified as ecologically relevant: endocrine disruption, xenobiotic metabolism, carcinogenicity, oxidative stress, inflammation, immunotoxicity and neurotoxicity. Critical issues for broader acceptance of *in vitro* bioassay toxicity testing, such as the regulatory context, the use of effect-based trigger values, the issue of sample preparation and sample sterilisation, as well as some recommendations for the development of test batteries are discussed in section 4.

2 Approach and methodology

The ecological relevance of the different toxic outcomes was assessed based on evidence of *in situ*, *in vivo* and *in vitro* effects of wastewater effluent established in the literature. Links between the *in vitro* and *in vivo* effects were then highlighted using toxicity pathways, which represent cellular response pathways that have the ability to result in adverse health effects (Ankley et al. 2010). For each endpoint, we present simplified amalgamations of published adverse outcome pathways (AOPs) as generalised toxicity pathways. AOPs were taken from the literature and databases such as AOPwiki, that contain much more detailed and sometimes quantitative AOPs from specific molecular events to *in vivo* outcomes. We have provided references to the original AOPs and encourage our readers to use these sources. Finally, a quantitative analysis was carried out to identify suitable *in vitro* assays for use with wastewater by sourcing relevant literature from 2015-2020 relating to water and *in vitro* assays. The search was limited to this period to identify recent popularity and trends in assay use. Google Scholar was used to search for "water" or "wastewater" in combination with "*in vitro* bioassay", "effect-based analysis", or "cell-based bioassay". Only primary research that applied water samples to *in vitro* bioassays were included (n=101). For the purposes of this review, embryonic models such as the fish embryo toxicity (FET) test or the *Xenopus* embryo toxicity assay (XETA) were not included. While embryonic models are often not legally

considered laboratory animals, there may still be ethical concerns (Scott & Minghetti 2020). More details on high-throughput fish embryo toxicity (FET) assays are available in Su et al. (2021) and Capela et al. (2020). While this was not an exhaustive search of the literature, patterns of assay use were evident early within search results. Additionally, previously published reviews were used to encompass a more complete list of assay options, and older literature was sourced for discussion, though these were not included in the quantitative analysis. The percentage of total studies that investigated each toxic outcome was calculated, and the number of studies that utilised a specific assay was also calculated for each toxic outcome. Some studies included more than one endpoint, resulting in percentages that do not necessarily add up to 100%. Similarly, some studies included more than one assay, resulting in percentages for each toxic outcome that also do not necessarily add up to 100%.

3 Literature review

3.1 Acute toxicity (mortality)

3.1.1 Whole animal effects

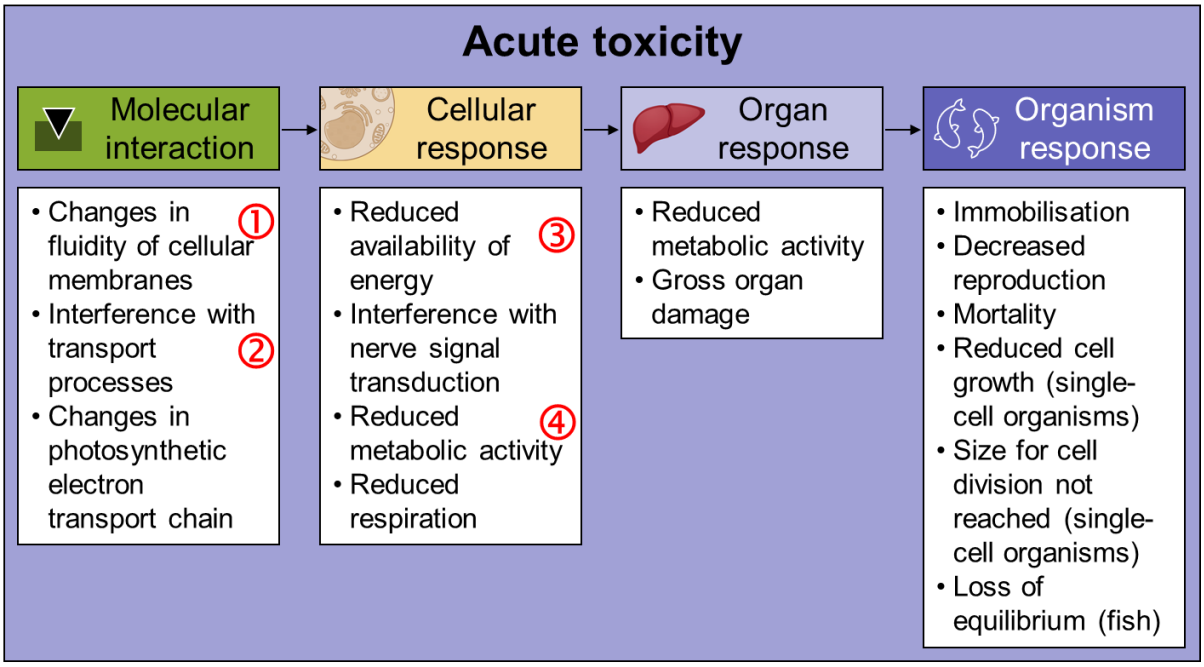
In some cases, the effect of exposure to effluent can lead to acute toxicity (and death). Increased mortality *in situ* has been found in invertebrates such as amphipods (*Gammarus fossarum*; Harth et al. 2018), and fish such as the brown trout (*Salmo trutta fario*; Escher et al. 1999) and streaked prochilod (*Prochilodus lineatus*; Cazenave et al. 2014). These effects are supported by *in vivo* bioassays that illustrated mortality in *Daphnia* sp. (Cao et al. 2009, Magdeburg et al. 2012), rainbow trout (*Oncorhynchus mykiss*; Vosylienė et al. 2010) and green chromide (*Etroplus suratensis*; Taju et al. 2012) exposed to wastewater effluent. Acute toxicity of wastewater effluent is also commonly detected *in vitro* using single celled organisms like bacteria (*e.g.* Monarca et al. 2000, Harth et al. 2018) or algae (Jia et al. 2015, Yu et al. 2019). Similarly, fish or human cell cultures exposed to wastewater effluent have also exhibited significant cytotoxicity (Taju et al. 2012, Escher et al. 2014, Yu et al. 2019).

Acute toxicity and mortality can lead to significant effects in the ecosystem, such as decreases in population numbers, altered community structures, altered decomposition rates, and altered primary production (Bundschuh & Schulz 2011).

3.1.2 Toxicity pathway context

In vitro cytotoxicity (cell viability or growth inhibition) can, in many cases, be used to predict *in vivo* mortality. Cytotoxicity and mortality can be linked through a toxicity pathway, where reduced cell viability and/or cell growth lead to dysfunction at the organ-level, and ultimately mortality, which negatively affects population numbers and structure (Figure 1). Strong correlations have been established between *in vitro* EC_{50} values in human and fish cells and *in vivo* LD_{50} values in humans (Ekwall 1999), rodents (Konsoula & Barile 2005, Payne et al. 2015) and several species of fish including rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Castano et al. 2003, Gülden & Seibert 2005, Tanneberger et al. 2013, Fischer et al. 2019, Scott et al. 2021). Similarly, bacterial *in vitro* EC_{50} values show reasonably good correlations with LD_{50} values of aquatic organisms (R^2 ranging from 0.46 to 0.83), such as invertebrates and fish, for a wide range of contaminants (Kaiser 1998). However, these relationships are not always straightforward, often resulting in good relative agreement (correlation) of *in vitro* and *in vivo* results, but low absolute agreement with cell lines typically less sensitive than *in vivo* fish tests for individual compounds as well as wastewater (Schirmer 2006, Rehberger et al. 2018). Significant differences can occur when toxicokinetics play a key role in modulating toxicity. Discrepancies can also occur due to binding of contaminants with media constituents, and better absolute agreement between *in vivo* and *in vitro* results can be achieved by calculating the bioavailable concentration of the chemical in the assay (Gülden & Seibert 2005, Henneberger et al. 2019, Proença et al. 2021), particularly for hydrophobic or volatile chemicals (Kramer et al. 2009). While these approaches produce better absolute

agreement for individual compounds, it is currently unclear how to translate them to complex mixtures of known and unknown compounds such as those found in wastewater. Regardless, cytotoxicity provides an important measure of non-specific basal toxicity, which can be induced by many compounds. Effects of wastewater on cell viability and/or cell growth should always be determined prior to the use of more specific *in vitro* bioassays to reduce cytotoxic interference and ensure quality assurance (Escher & Leusch 2012).



① Membrane integrity (*e.g.*, LDH); ② Lysosome function (*e.g.*, NRU); ③ ATP content (*e.g.*, ATP); ④ Reduced metabolic activity (*e.g.*, MTT, MTS, resazurin)

Figure 1 Toxicity pathway for narcosis in single- and multicellular organisms. Adapted from Escher et al. (2021). Assays used in water studies (2015-2020) have been added in red to demonstrate how they fit in the pathway.

3.1.3 Relevant in vitro bioassays - vertebrates

There are a plethora of *in vitro* assays that can be used to measure cytotoxicity, many of which have been applied to wastewater or water samples. These methods typically involve dyes, such as cellular metabolic activity (tetrazolium salts (*e.g.* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)), crystal violet, resazurin/Alamar blue), lysosome function (neutral red uptake (NRU)), membrane integrity (trypan blue, lactate dehydrogenase (LDH) leakage, CFDA-AM (5-Carboxyflourescein diacetate acetoxymethyl ester), ATP (CellTiter-Glo®)) or protein content (sulforhodamine B (SRB), Coomassie blue) (Escher & Leusch 2012, Leusch & Snyder 2015). These types of assays are relatively cheap, high throughput and can be applied to any cell type, and can therefore be used to assess cytotoxicity across a range of taxa or target organs.

Cytotoxicity is a commonly used endpoint in water related studies, though it is often also measured as a form of quality assurance and quality control in other assays, ensuring no cytotoxic interference with the specific measured endpoint. As a result, cytotoxicity was measured in 59% (n=60) of studies between 2015 and 2020. Of these studies, MTT was one of the most commonly used cytotoxicity assays (22%; Supplementary Table S1), although it may not be useful for wastewater effluents with high amounts of dissolved organic matter (Yang et al. 2015). Resazurin was the second most common cytotoxicity assay (13%), followed by NRU (10%), MTS (8%), and ATP (8%; Supplementary Table S1). There are many more assays available, though use of each was ≤3%.

Many studies have shown highly comparable results between different cytotoxicity assays within the same cell line (Wang et al. 2010, Abdul Majeed et al. 2013, Taju et al. 2017). Sensitivity, however, will vary depending on the cell type (Tong et al. 2016, Finlayson et al. 2019). The most common cell type used to assess cytotoxicity in recent literature was human liver cancer cells (HepG2), used in 22% (n=13) of cytotoxicity studies (Supplementary Table S2). The next most common cell type was human colon cells (Caco-2), which were used in 5% (n=3) of cytotoxicity studies (Supplementary Table S2). However, human cell cultures may be more relevant for human risk assessments than ecological risk assessments.

More relevant to ecological risk assessment, there are commercially available fish cell cultures from rainbow trout gill or liver (RTgill-W1, RTL-W1) and clearfin livebearer hepatocellular carcinoma (*Poeciliopsis lucida*, PLHC-1), each of which has been used ≤3% in studies published in the last five years (Supplementary Table S2). Recently, a protocol to assess basal toxicity in fish using RTgill-W1 has been validated by the International Organisation of Standardisation (ISO) (ISO: 21115) and the Organisation for Economic Co-operation and Development (OECD) (OECD 2021). This assay employs the use of a combination of three cytotoxicity assays – resazurin, CFDA-AM and NRU, which are performed concurrently in the same plate (Tanneberger et al. 2013, Fischer et al. 2019). In this manner, several different measures of cytotoxicity can be obtained simultaneously, thus accounting for any potential variation between assays. Additionally, this assay has a much better agreement with *in vivo* data, compared to previously used fish or mammalian cell cytotoxicity assays (Tanneberger et al. 2013).

RTgill-W1 cells and HepG2 cells have been validated for use with whole water samples by dissolving simple media constituents in the water samples (Dayeh et al. 2002, Schirmer et al. 2004, Niss et al. 2018). The use of whole water samples may be compatible with other dye-based assays, though not necessarily with cell lines that require extensive media supplementation. There are other issues regarding the compatibility of whole water samples for use in *in vitro* bioassays, such as sample sterility, which is discussed in more detail in section 4.2.

3.1.4 Relevant in vitro bioassays – other taxa

While tests on bacteria and algae are technically *in vivo*, we have included them here due to their single-celled nature and high throughput capacity. There are a range of bacterial toxicity bioassays using luminescent strains such as *Aliivibrio fischeri* (previously *Vibrio* or *Photobacterium fischeri* (Urbanczyk et al. 2007)), *Photobacterium phosphoreum*, *Vibrio*

harveyi, *Photobacterium leiognathi* and *Pseudomonas fluorescens* (Ulitzur et al. 2002, Parvez et al. 2006, Escher et al. 2014). Bacterial assays are highly relevant in environmental risk assessments, as bacterial communities play important roles in the ecosystem. Growth inhibition assays with bacteria are available; however, they are much less sensitive than those based on bioluminescence (Gellert 2000). Bioluminescent assays are based on a reduction of luminescence from bioluminescent bacteria, which is directly proportional to metabolic activity (Parvez et al. 2006). The most commonly used of these is the Microtox assay (using the marine bacteria *A. fischeri*), appearing in 25% (n=15) of bacterial toxicity studies (*e.g.* Punzi et al. 2015, Becker et al. 2016, Kudłak et al. 2016, Paździor et al. 2017). This assay has been validated by the ISO (ISO: 11348), and has been miniaturized to support a high throughput format in a 96-well plate (Escher et al. 2008). A similar assay, ToxScreen, uses the bacteria *P. leiognathi*, which is more sensitive to a wide range of pollutants than the Microtox assay (Ulitzur et al. 2002). Both the Microtox and ToxScreen assays require ongoing purchase of bacteria cultures, and require specialised equipment (van de Merwe & Leusch 2015). Recently, the BLT-screen has been optimised to use *P. leiognathi* in a high throughput format, with comparable results to the Microtox and ToxScreen assays (van de Merwe & Leusch 2015), and is increasingly being applied to assess cytotoxicity in water samples (5%, n=3; Supplementary Table S2). Bacterial luminescence assays are highly sensitive, and are typically more sensitive than mammalian cells (Escher et al. 2014).

Algae growth inhibition can also be used to assess basal toxicity. Algae are primary producers that play a critical role in the ecosystem, thus these assays have high ecological relevance. Algal assays may also be more sensitive than other assays (Geis et al. 2000). Protocols for freshwater algae growth inhibition assays have been approved by both the OECD (OECD 2011) and the ISO (ISO: 8692). These assays are carried out in Erlenmeyer flasks, though have since been miniaturised to a 96-well plate using *Desmodesmus*

subspicatus (Eisentraeger et al. 2003) and *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*; Geis et al. 2000). The ISO also has approved a protocol to measure growth inhibition of unicellular marine algae *Skeletonema* sp. and *Phaeodactylum tricornutum* (ISO: 10253). Currently, *R. subcapitata* is the most commonly used species of algae, used in 10% (n= 6) of algal cytotoxicity studies of water samples.

More recently, algae growth inhibition assays with *R. subcapitata* have been carried out in a high-throughput format in conjunction with a photosynthesis inhibition assay for a more comprehensive assessment of phytotoxicity, termed "combined I-PAM" (Escher et al. 2008, Escher et al. 2014, Tang & Escher 2014, Jia et al. 2015, Neale et al. 2017). In the combined I-PAM assay, photosynthesis inhibition is measured at 30 min for acute herbicidal activity, and growth rate measured at 24 h for non-specific basal toxicity (Escher et al. 2008). Growth inhibition of *R. subcapitata* shows good agreement with other algae inhibition assays across a range of species, as well as with the bacteria *V. fisheri* (Escher et al. 2008).

3.2 Reproduction

3.2.1 Whole animal effects

More commonly, *in situ* studies detect sub-lethal effects from exposure to wastewater effluent. Many studies have found direct effects on reproduction such as a decrease in brooding behaviour displayed by female amphipods (*Gammarus* sp.; Harth et al. 2018), a decrease in the number of embryos in New Zealand mudsnails (*Potamopyrgus antipodarum*; Gust et al. 2010), delayed spawning and altered gonadal development in fathead minnows (*Pimephales promelas*) and brook sticklebacks (*Culaea inconstans*; Tetreault et al. 2012), altered egg production in fathead minnows (Cavallin et al. 2016), and reduced spermatogenesis in walleye (*Stizostedion vitreum*; Folmar et al. 2001). These results are validated by a large number of *in vivo* bioassays finding reduced egg production in fathead minnows (Thorpe et al. 2009, Filby et al. 2010), decreased embryo production and increased

atretic oocytes in zebrafish (Galus et al. 2013), and decreased hatch success in Japanese medaka (*Oryzias latipes*; Cao et al. 2009). These results are mirrored by *in vivo* studies on invertebrate species finding reduced reproductive output in New Zealand mudsnails (Stalter et al. 2010, Magdeburg et al. 2012), and alterations to emergence time, resulting in a shortening of the breeding season, reported in midges (*Chironomus riparius*; Magdeburg et al. 2012). Conversely, an increase in reproductive output was observed in amphipods (*Gammarus pulex*) exposed to wastewater effluent (Schneider et al. 2015). Similarly, an increase in neonates has been observed in daphnia (*Daphnia magna*) exposed to wastewater (Magdeburg et al. 2012), though this may be the result of hormesis and followed by a lower number of neonates at higher concentrations of effluent (Rodriguez et al. 2006).

There are also many incidences of altered sex ratios leading to feminisation of populations following *in situ* exposure to wastewater effluent in eastern elliptio mussels (*Elliptio complanate*; Gagné et al. 2011), pale chub (*Zacco platypus*; Yeom et al. 2007), zebrafish (Nakari 2004), and *in vivo* in amphipods (*G. pulex*; Schneider et al. 2015). Masculinisation of females and feminisation of males has been detected in mosquitofish (*Gambusia holbrooki* and *G. affinis*; Leusch et al. 2006, Huang et al. 2016), and a significant decrease in the number of embryos has been found in masculinised female mosquitofish (Orlando et al. 2007). Altered gonadal development and increased incidence of intersex following exposure to wastewater has been detected *in situ* in walleye (Folmar et al. 2001) and gudgeon (*Gobio gobio*; Sanchez et al. 2011) and both *in situ* (Jobling et al. 2002, Jobling et al. 2006) and *in vivo* in roach (*Rutilus rutilus*; Liney et al. 2006, Lange et al. 2011).

Indirect effects on reproduction have also been reported through effects on the endocrine system. While this is technically endocrine disruption, which will be discussed in more detail in Part 2 of this review, we have included effects on the hypothalamo-pituitary-gonadal axis here due to the involvement of this axis in reproduction, and focus on the three classes of

hormones primarily involved in reproduction: estrogens, androgens and progestogens. Altered sex steroids have been found following exposure to wastewater effluent including a decrease in testosterone in carp (*Cyprinus carpio*; Folmar et al. 1996) and walleye (Folmar et al. 2001). Increases in testosterone have been detected in male longear sunfish (*Lepomis megalotis*; Porter & Janz 2003), and female and intersex roach (Jobling et al. 2002). Similarly, an increase in expression of the androgen receptor (AR) have also been detected in exposed mosquitofish (Huang et al. 2016), and male fathead minnows (Werner et al. 2010). In male fathead minnows, increases in AR expression occurred concomitantly with increases in expression of the estrogen receptor beta (ERβ), while expression of the estrogen receptor alpha (ERα) occurred with induction of vitellogenin (VTG; Werner et al. 2010, Cavallin et al. 2014). Vitellogenin (VTG), a precursor to egg yolk, is commonly expressed in male fish exposed to estrogen-like compounds *in situ* such as walleye (Folmar et al. 2001), gudgeon (Sanchez et al. 2011) and longear sunfish (Porter & Janz 2003), and *in vivo* such as rainbow trout (Nakari 2004), roach (Liney et al. 2006), mosquitofish and rainbowfish (Scott et al. 2017), and fathead minnow (Thorpe et al. 2009, Filby et al. 2010) to name a few. Increased VTG-like proteins have also been detected in eastern elliptio mussels exposed to wastewater effluent (Gagné et al. 2007, Gagné et al. 2011), among others (Tran et al. 2019). Increased VTG expression may occur with an increase in expression of ERα (Huang et al. 2016) or levels of plasma estradiol-17β (E2; Folmar et al. 2001). Similarly, induction of VTG is often associated with a higher incidence of intersex or feminisation (Jobling et al. 2002, Nakari 2004, Gagné et al. 2011, Sanchez et al. 2011).

3.2.2 Toxicity pathway context

As reproduction processes involve intercellular communication and, in most cases, interactions between two different organisms, direct toxic effects on reproduction at the individual level are difficult, if not impossible, to model and measure *in vitro*. However, it is

possible to detect changes to sex steroids and interference with endocrine signalling. Changes at the molecular and physiological level may provide an early warning of exposure to compounds that can impair reproductive function, as well as provide insight into the types of compounds that are responsible. For estrogenic compounds, the links from the key initiating event such as interaction with the estrogen receptor to effects at the population level have been well defined (Figure 2). An AOP with strong evidence has been developed for oviparous vertebrates linking ER agonism to reproductive dysfunction (AOPwiki AOP: 29). These relationships have been demonstrated in the literature linking *in vivo* ERα expression to VTG expression (Huang et al. 2016), and VTG expression associated with increased feminisation or intersex (Jobling et al. 2002, Nakari 2004, Gagné et al. 2011, Sanchez et al. 2011). There is evidence that using *in vitro* methods for VTG detection shows strong agreement with *in vivo* sexual differentiation in African clawed frogs (*Xenopus laevis*) following exposure to estrogenic compounds (Kloas et al. 1999). Significant correlations have been found between *in vitro* assays (ER-GeneBLAzer and MELN) and expression of brain aromatase (cyp19a1b) in zebrafish embryos following *in vivo* exposure to estrogenic compounds (Brion et al. 2019). Further, *in vitro* reporter gene assays (ERα CALUX and CHO-hERα) and an ERα receptor binding assay (MCF-7 cells) for estrogenicity show a strong correlation ($r^2 = 0.87$) with *in vivo* endocrine responses in rats (Sonneveld et al. 2006). Similarly, an AOP has been developed linking antagonism of the estrogen receptor with reduced fecundity in repeat-spawning fish (AOPwiki AOP: 30).

While the pathway for androgenicity is less clearly defined, changes to sex steroid ratios leading to masculinisation can result in reduced reproductive output in females (Orlando et al. 2007, Huang et al. 2016). Similarly, exposure to compounds that act on the AR can affect secondary sex characteristics in males (Margiotta-Casaluci et al. 2016). Androgen receptor agonism has been linked through the AOP to male biased sex ratios (AOPwiki AOP: 376).

For species of fish with repeat spawning, an OECD endorsed AOP links AR receptor binding to reduced spawning and fecundity in females (AOPwiki AOP: 23). *In vitro* reporter gene assays (AR-CALUX and CHO-hAR) and an AR receptor binding assay (MCF7-cells) for androgenicity have also shown moderate correlations (R^2 = 0.46) with *in vivo* endocrine responses in rats; however, the authors suggest that this is related to the insensitivity of the *in vivo* Hershberger assay used for this comparison rather than a deficiency of the *in vitro* test system (Sonneveld et al. 2006).

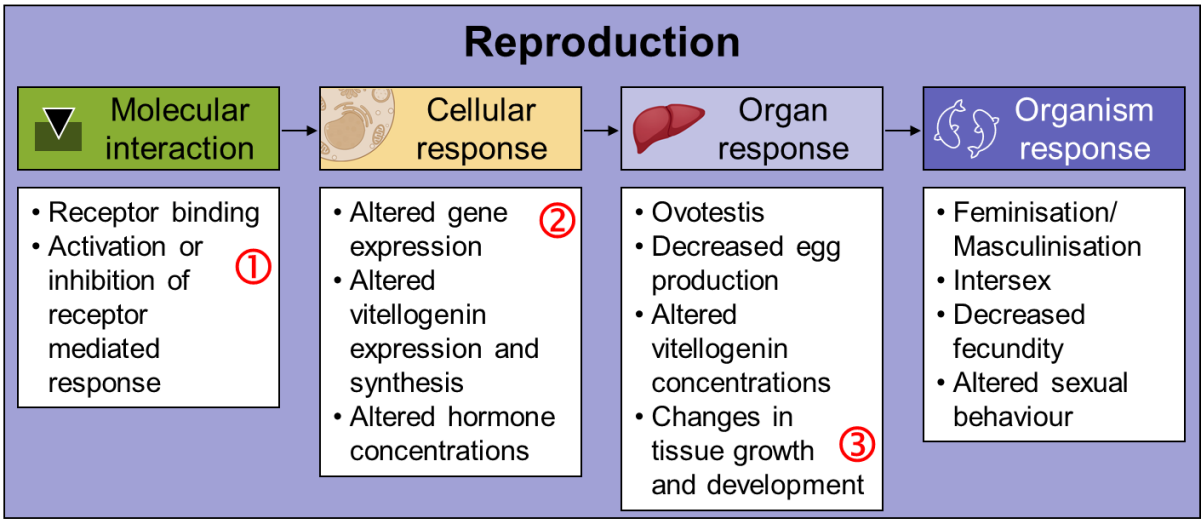


Figure 2 Toxicity pathway for reproduction. Adapted from Knapen et al. (2015) and Escher et al. (2021). Assays used in water studies (2015-2020) have been added in red to demonstrate how they fit in the pathway.

In addition to estrogens and androgens, progestogens (such as progesterone, P4) are another class of steroid hormones that play an important reproductive role in vertebrates (DeQuattro et al. 2012). Exposure to progestogens may lead to altered sex hormones, decreases in egg numbers, decreased fertilisation, and altered sex ratios (DeQuattro et al. 2012, Liang et al. 2015). Therefore, exposure to progestogens could be responsible for some of the reproductive effects observed from wastewater effluent exposure. *In vitro* reporter gene assays (PR-CALUX and CHOhPR-MMTV-LUC) and a PR receptor binding assay (MCF-7 cells) show

strong correlations with *in vivo* progestogenic responses in rabbits (Sonneveld et al. 2011), and may therefore provide another useful option for assessing effects of reproduction *in vitro*.

3.2.3 Relevant in vitro bioassays – Estrogen receptor

3.2.3.1 *Agonist mode.* Considering the attention on fish feminisation after exposure to wastewater, it is not surprising that estrogenic activity is a very common endpoint applied in water research, investigated in 62% (n=63) of recent studies. There are a wide variety of assays available including breast cancer cell proliferation assays, receptor binding assays, and reporter gene assays, with either mammalian, fish or yeast cells (Wangmo et al. 2018).
Cell proliferation for estrogenicity, known as the E-SCREEN, is usually based on the human breast carcinoma cell line MCF-7, typically using subclones such as MCF-7 BUS (Spina et al. 2015) or MCF 7-BOS (Hamilton et al. 2016). The E-SCREEN is highly sensitive (Soto et al. 2006, Leusch et al. 2017). However, it has only recently been used in a small number of studies investigating estrogenicity (6%, n=4), which may be due to the low throughput of the assay, which can take from 5-7 d to produce results (Wangmo et al. 2018) compared to 1-2 d for most other assays.

Receptor binding assays measure competitive binding to a receptor using radio-labelled steroids. While the estrogen receptor binding assay (ERBA) shows strong correlations with mammalian reporter gene assays (Sonneveld et al. 2006), it is generally less sensitive, by up to an order of magnitude, compared to other estrogenic assays (Leusch et al. 2017). Despite previous high popularity (Leusch et al. 2017), the ERBA has not been used frequently in recent years to investigate estrogenicity (2%, n=1). Decreased popularity may be due to expensive radio-labelled components, the need for specialised equipment (*i.e.* scintillation counter) and the potential interference from natural organic matter in the water extract, which can interfere with receptor binding in "naked" assays (*i.e.*, assays without a cell membrane acting as a barrier).

There are two Factorial gene expression assays to measure estrogenic activity, the ERE cis-Factorial and the ERα trans-Factorial. The Factorial gene expression assays were developed by Attagene (NC, USA), under contract to the US EPA (Martin et al. 2010). The cis-Factorial assay simultaneously measures 48 multiplexed human transcription factor DNA binding sites, while the trans-Factorial-2 assay simultaneously measures 48 multiplexed human nuclear receptors, using combined libraries of *cis*- or *trans*-regulated transcription factor reported constructs (Martin et al. 2010, Blackwell et al. 2019). The Factorial assays require the transient transfection of cells and can therefore be used with many different cell cultures although HepG2 is recommended. RNA is extracted from cells and amplified with reverse transcription polymerase chain reaction (PCR), a process which can be costly and labour intensive. The Factorial assays have thus far not been widely applied, particularly with receptors such as the ER that have many more available options, thus the cis- and trans-Factorial assays have only been applied once in recent water studies (2% each).

There are a plethora of reporter gene assays available for measuring estrogenic activity using yeast, fish or mammalian cells. The Yeast Estrogen Screen (YES) assay employs *Saccharomyces cerevisiae* containing the human estrogen receptor (hER) and the gene *lac-Z*, which synthesises the enzyme β-galactosidase and is used to measure the receptors activity (Routledge & Sumpter 1996). There are also many alternatives to the YES assay such as the yeast two-hybrid assay (Nishikawa et al. 1999), planar YES (pYES, Könemann et al. 2018), lyticase-assisted YES (LYES), firefly luciferase YES (FFluc-YES), the bioluminescent YES (BLYES), the chemiluminescent YES with either human or medaka ERα (CLYES-hERα, CLYES-medERα), and the A-YES based on *Arxula adeninivorans* (Wangmo et al. 2018). Both the YES (ISO: 19040-1:2018) and A-YES (ISO: 19040-2:2018) assays have recently been validated by the ISO. However, yeast reporter gene assays are typically less sensitive than mammalian reporter gene assays (Jia et al. 2015, Leusch et al. 2017, Wangmo et al.

2018). Regardless, the classical YES remains one of the most popular assays for estrogenicity, used in 27% of studies (n= 17; Supplementary Table S3), likely due to the ease of use and affordability (Wangmo et al. 2018). In addition, wastewater usually contains high levels of estrogenic activity, and its comparably lower sensitivity is perfectly adequate for wastewater testing (Leusch et al. 2010b).

Vertebrate reporter gene assays are commonly used to detect estrogenic activity, and there are a number of different cells that can be used to do so (Supplementary Table S3). Some cell cultures contain endogenous ER and activation is expressed through a reporter gene, while others have been transfected with hER and a corresponding luciferase reporter gene (Wangmo et al. 2018). Cell cultures that contain endogenous ER include those established from the breast cancer cell lines MCF-7 (MELN, MVLN, VM7Luc4E2 (formerly BG1luc4E2, NIEHS 2016) and T47D (T47D-luc, T47D-KBluc), or transiently transfected endometrial adenocarcinoma Ishikawa cells (Escher & Leusch 2012, Wangmo et al. 2018). Transiently transfected cells require repeated transfection and may have high inter-assay variability due to differences in transfection efficiency (Wangmo et al. 2018). VM7Luc4E2 and T47D-KBluc have been optimised for use of whole water samples (Wehmas et al. 2011, Niss et al. 2018). Those that have been transfected with the ER and a luciferase reporter gene include the ER-CALUX, HeLa-9903, HELN-ERα, HELN-ERβ and HGELN (Escher & Leusch 2012, Wangmo et al. 2018), while the ERα-GeneBLAzer uses beta-lactamase as a reporter gene instead. These cell cultures are desirable as potential interactions with other steroid receptors are reduced. Mammalian ER reporter gene assays are highly sensitive, and results often correlate well with each other (Könemann et al. 2018). However, there are some differences in robustness, reproducibility and repeatability resulting in different strengths and weaknesses for each assay (Leusch et al. 2010a, Kunz et al. 2017, Leusch et al. 2017). The commercially available assays (*e.g.* ER-CALUX, ERα-GeneBLAzer) require the purchase of

cell lines or licenses that limit the number of assays that can be performed, while assays such as VM7Luc4E2 and MELN are more affordable options. ISO has validated the ER-CALUX assay (ISO: 19040-3:2018), while the OECD has validated the use of HeLa-9903 cells (OECD 2009). Of the mammalian reporter gene assays, the ER-CALUX (17%, n=11), ERα-GeneBLAzer (16%, n=10) and MELN (14%, n= 9) were most commonly used to assess estrogenicity in water samples in the last five years (Supplementary Table S3).

There are, additionally, zebrafish reporter gene assays developed from a liver cell line (ZELN) transfected with zebrafish ER subtypes generating three cell lines, ZELH-zfERα, ZELH-zfERβ1, ZELH-zfERβ2 (Cosnefroy et al. 2012). The ZELH-zfERβ2 cell line exhibited higher sensitivity than the MELN human reporter gene assay, which highlights the importance of species specificity, as this subtype is not present in humans (Sonavane et al. 2016). These assays have only recently been developed, and therefore have only been used in a small number of studies (5%, n=3; Supplementary Table S3), though offer a promising model for environmental risk assessments.

3.2.3.2 *Antagonist mode.* Many of the assays for estrogenicity can also be used to detect anti-estrogenicity, though in recent years only 14 (14%) studies have investigated anti-estrogenic effects. Some assay types, like receptor binding assays, are not able to differentiate between agonists and antagonists (Soto et al. 2006, Leusch et al. 2017). Anti-estrogenicity can be detected in the E-SCREEN; however, it requires modifications to discriminate between cytotoxicity and anti-estrogenicity (Soto et al. 2006). The E-SCREEN remains the most sensitive assay to anti-estrogenicity (Leusch et al. 2017), though has not been used in recent years. The yeast anti-estrogenic screen (YAES) was the most common assay for anti-estrogenicity (64%, n=9; Supplementary Table S4), while the anti-A-YES (7%, n=1) and human reporter gene assays such as the anti-ER-CALUX (14%, n=2), anti-ER-GeneBLAzer

(7%, n=1), anti-VM7Luc4E2 (7%, n=1) and Ishiwaka cells (7%, n=1) have been used a small number of times (Supplementary Table S4).

3.2.4 Relevant in vitro bioassays – Androgen receptor

3.2.4.1 *Agonist mode.* Androgenic activity is another commonly assessed endpoint for water samples, investigated in 41% (n=41) of studies. There are many of the same options available for assessing androgenic activity as there were for estrogenic activity, such as cell proliferation, receptor binding assays and reporter gene assays with either mammalian cells or yeast cells.

Cell proliferation for androgenicity (A-SCREEN) uses proliferation inhibition of the stably transfected MCF7-AR1 cells to measure androgenicity (Szelei et al. 1997). The A-SCREEN shares the high sensitivity of the E-SCREEN assay (Soto et al. 2006, Leusch et al. 2017), although this assay has not been used for water assessments in recent years.

The androgen receptor binding assay (ARBA) shares the weaknesses of the ERBA, including low sensitivity and the use of expensive components and specialised equipment. While the ARBA has previously been popular (Leusch et al. 2017), it has not been used in recent years.

Reporter gene assays using yeast cells are a common method for assessing androgenicity. The Yeast Androgen Screen (YAS), based on *S. cerevisiae*, functions in a similar way to the YES assay by using synthesis of β-galactosidase to measure activation of the human androgen receptor (hAR; Gaido et al. 1997, Sohoni & Sumpter 1998). There are some alternatives to the YAS assay, such as a bioluminescent yeast (Lum-YAS), yeast two-hybrid and the A-YAS based on *A. adeninivorans* (Leusch et al. 2017, Wangmo et al. 2018). These assays share strengths and weaknesses with the yeast estrogenic assays, however the sensitivity of the YAS is more comparable to mammalian reporter gene assays than the YES (Leusch et al.

2017). The YAS remains popular, used in 27% (n=11) of recent studies (Supplementary Table S5).

Mammalian reporter gene assays are the most frequently used to detect androgenicity, and just like for estrogenic activity, there are a number of different cell lines available. Transiently transfected cell cultures such as CHO or Ishikawa cells have been used to test androgenicity (Kassotis et al. 2016, Wangmo et al. 2018). Stably transfected options include commercially available cell lines like the AR-CALUX (Sonneveld et al. 2004), AR-GeneBLAzer and AR-EcoScreen (Satoh et al. 2004), as well as unlicensed options such as PALM (Térouanne et al. 2000), TARM-luc (Willemsen et al. 2004) and MDA-kb2 (Wilson et al. 2002). Of these, TARM-luc and MDA-kb2 express endogenous AR. MDA-kb2 also expresses the glucocorticoid receptor (GR), which can activate the luciferase reporter (Wilson et al. 2002). This therefore requires co-exposure of an anti-androgen to differentiate the effects (Wilson et al. 2002). The MDA-kb2 assay has been widely applied in recent years to detect androgenicity (24%, n=10; Supplementary Table S5), despite lower sensitivity compared to other mammalian AR reporter gene assays (Leusch et al. 2017). The commercially available assays have also been widely used to detect androgenic activity, with AR-CALUX used in 20% (n=8) of studies, AR-GeneBLAzer used in 15% (n=6) of studies and the AR-EcoScreen used in 12% (n=5) of studies (Supplementary Table S5).

There is an AR trans-Factorial assay available for androgenic activity, which has the same limitations as the estrogenic Factorial assays. This assay has only been applied once in recent water studies (2%).

3.2.4.2 *Antagonist mode.* Compared to anti-estrogenic activity, anti-androgenic activity has been more commonly investigated (21%, n=21). In some cases, androgenic activity is only investigated in antagonistic mode (Pieterse et al. 2015, Alygizakis et al. 2019, De Baat et al.

2019). The yeast anti-androgen assay (YAAS) was commonly used to detect anti-androgenicity (33%, n=7; Supplementary Table S6). The anti-AR-CALUX was also commonly used in 33% (n=7) of anti-androgenic studies. Other commercial cell lines have also been used with some frequency, with the anti-AR-EcoScreen used in 19% (n=4) of studies and the anti-AR-GeneBLAzer used in 10% (n=2) of anti-androgenic studies (Supplementary Table S6). The MDA-kb2 assay and the transiently transfected Ishikawa cells have also each been used in antagonist mode in 5% (n=1) of studies each (Supplementary Table S6).

3.2.5 Relevant in vitro bioassays – Progesterone receptor

3.2.5.1 Agonist mode. Progestogenic activity is often not considered in water samples, investigated in only 12% (n=12) of recent water sample studies. Despite this, there are still a number of assays to detect progestogenic activity such as progesterone receptor binding assays and yeast or mammalian reporter gene assays.

Progesterone receptor binding assays (PRBA), like the ERBA and ARBA, usually have low sensitivity (Leusch et al. 2017), and have not been used in recent years for progestogenic activity in water samples.

There are several options of yeast-based reporter gene assays for detecting progestogenic activity. The yeast progesterone screen (YPS; Gaido et al. 1997) and YPH500-hPR-Gal assay (Li et al. 2008) are both based on *S. cerevisiae*, while the A-YPS is based on *A. adeninivorans* (Wangmo et al. 2018). The YPH500-hPR-Gal assay has higher sensitivity than mammalian reporter gene alternatives (Leusch et al. 2017). However, in recent years, no studies have employed yeast-based assays for progestogenic activity.

There are also a number of mammalian reporter gene options for detecting progestogenic activity. Transiently transfected Ishikawa cells have been developed and recently used with

water samples (Kassotis et al. 2016). Stably transfected options include CHO hPR-B luc (Dijkema et al. 1998) and HG_5LN-Gal4-PR (Molina-Molina et al. 2006), while commercially available options include the PR-GeneBLAzer and PR-CALUX (Sonneveld et al. 2011). There is some evidence that the PR-GeneBLAzer is a more sensitive option than the PR-CALUX (Leusch et al. 2017). Of the available mammalian reporter gene options, the PR-GeneBLAzer (50%, n=6) and PR-CALUX (42%, n=5) were the most commonly used progestogenic assays (Supplementary Table S7).

There is also a PR trans-Factorial assay, though it has only been applied once in recent water studies (8%).

3.2.5.2 *Antagonist mode.* Anti-progestogenic activity has not been widely investigated, used in only 7 (7%) recent studies. Similar to antiandrogenic assays, sometimes PR activity is only investigated in antagonist mode (Pieterse et al. 2015, Alygizakis et al. 2019). The anti-PR-CALUX has been used most frequently (57%, n=4), followed by the anti-PR-GeneBLAzer (29%, n=2), and Ishikawa cells (14%, n=1; Supplementary Table S8).

3.3 Growth

3.3.1 Whole animal effects

Effects of exposure to wastewater effluent on growth of aquatic organisms have been widely documented. Condition factor, a ratio of weight to length, is commonly used as an indicator of growth. Wild fish populations exposed to effluent often show an increase in condition factor (Porter & Janz 2003, Yeom et al. 2007, Tetreault et al. 2012), which is often attributed to high nutrient levels in wastewater effluent (Porter & Janz 2003, Yeom et al. 2007). Additionally, an increase in condition factor may be a result of a reduced population size, through mechanisms such as reduced reproduction or recruitment, and therefore an increase in food availability (Marshall Adams et al. 1992, Yeom et al. 2007). An increase in condition

factor has also been found in mussels exposed to effluent (Turja et al. 2015), although a reduced cellular energy budget, as a result of decreased energy reserves and increased energy consumption, indicated a lower scope for growth in exposed mussels (Smolders et al. 2004). Some effects of wastewater effluent on plants have also been documented, including growth inhibition (Magdeburg et al. 2012), reduced number of fronds and overall biomass (Stalter et al. 2010), and reduced root growth and mitotic index (Radić et al. 2010, Özkara et al. 2011). Similarly, growth inhibition of unicellular algae has been found following exposure to wastewater effluent (Escher et al. 2009, Tang & Escher 2014, Jia et al. 2015). Conversely, increased nutrient levels associated with wastewater input can increase algal growth, leading to eutrophication (Powley et al. 2016, Preisner et al. 2020).

3.3.2 Toxicity pathway context

Effects of chemicals on the growth of bacteria and algae can be easily measured *in vitro* with growth inhibition assays. Since bacteria and algae are unicellular organisms, these assays can function as both *in vivo*, whole organism, and *in vitro*, cell-based assays. These assays therefore provide a clear link between the effect at the cellular/organism level and effects at the population level.

Effects on growth in higher organisms is more difficult to measure *in vitro*. Growth is an apical endpoint, and thus impaired growth can result from a myriad of molecular, cellular and physiological dysfunctions (Groh et al. 2015). Cellular events such as ion imbalances, oxidative stress, membrane depolarisation, apoptosis and mitochondrial dysfunction have been linked through the AOP to effects at the individual level such as reduced appetite, locomotion impairment, increased metabolic demands and reallocation of energy resources, which can all result in decreased growth (Figure 3; Groh et al. 2015, Bolser et al. 2018). Several hormones and endocrine glands are also involved in control of metabolism, and glucocorticoid and thyroid hormones will be discussed in Part 2 of this review. Pathways

involved in growth are complex, and *in vitro* assays targeting any one of these cellular events could theoretically be used to measure growth with significant validation, though more likely a suite of bioassays would be required due to the complexity of the pathways involved in growth. Alternatively, cell population growth, the sum of cell death and proliferation, in rainbow trout gill (RTgill-W1) cells has been proposed as an *in vitro* measure of fish growth (Stadnicka-Michalak et al. 2015). Strong correlations were found between *in vitro* cell population growth and *in vivo* rainbow trout and fathead minnow fish mass following exposure to two fungicides (Stadnicka-Michalak et al. 2015). However, these correlations were obtained with the use of toxicokinetic modelling to predict the internal concentrations of chemicals in fish as well as toxicodynamic modelling to predict cell survival under longer exposure periods, and as mentioned above, were only carried out for two chemicals (Stadnicka-Michalak et al. 2015). This modelling can only be performed for individual chemicals, and adaptation to chemical mixtures in wastewater effluent may not be possible.

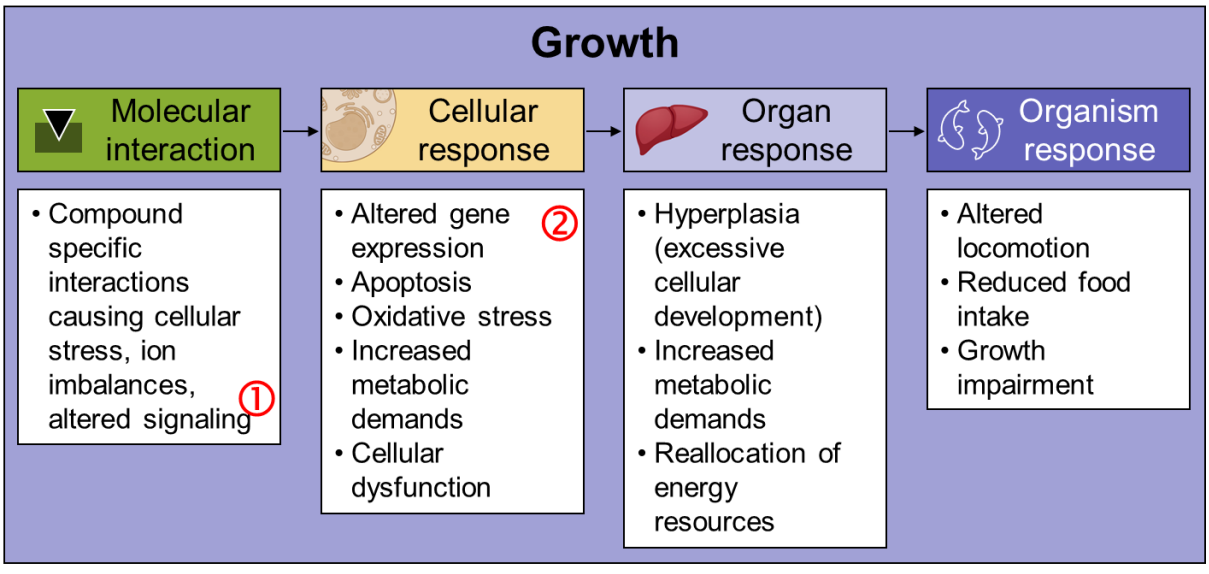


Figure 3 Toxicity pathway for growth. Adapted from Assays used in water studies (2015-2020) have been added in red to demonstrate how they fit in the pathway.

3.3.3 Relevant in vitro bioassays

As mentioned above, fish cell population growth can be used to assess growth inhibition in fish (Stadnicka-Michalak et al. 2015). While this assay presents a promising avenue, it requires the use of chemical-specific toxicokinetic and toxicodynamic modelling. Furthermore, it has not yet been validated with chemical mixtures, such as those present in wastewater.

3.4 Development

3.4.1 Whole animal effects

Development is currently considered one of the ecologically relevant endpoints in WET testing, and is closely tied to endocrine disruption and reproductive toxicity. Increases in developmental abnormalities have been observed across many taxa exposed to wastewater effluent. Embryo abnormalities have been reported in medaka, including extruding vesicles, internal haemorrhages, abnormal hatch patterns (*e.g.* head first pattern instead of tail first), and cranial herniation, which result in decreased hatch success and survival (Cao et al. 2009, Maya et al. 2018). Similarly, a wide range of embryo abnormalities have been reported in zebrafish exposed to wastewater effluent (Jiang et al. 2013, Babić et al. 2017). Developmental abnormalities, such as head malformation, eye dysplasia, ventral blisters, abnormal gut coiling and tail dysplasia have also been reported in Oriental fire-bellied toads (*Bombina orientalis*) following exposure to treated wastewater (Park et al. 2014), and significant effects on development have also been observed in sea urchins (*Paracentrotus lividus*; Meriç et al. 2005) and amphipods (Wigh et al. 2017). The range and severity of observed developmental effects is largely dependent on the wastewater sample and type of treatment (Meriç et al. 2005, Cao et al. 2009, Park et al. 2014).

3.4.2 Toxicity pathway context

Developmental toxicity can manifest in a number of different ways, and thus, key initiation events must be defined for each potential outcome. So far as many as 17 signalling pathways have been identified in vertebrate development, and disruption to any of these pathways is likely to have effects on development (Figure 4, Villeneuve et al. 2014). For fish, an AOP has been hypothesized for impaired swim bladder inflation following exposure to the flame retardant, tris(2,3-dibromopropyl)isocyanurate, or glycogen synthase kinase 3β inhibition (Villeneuve et al. 2014). An AOP has also been hypothesized for abnormal cardiac development following exposure to PAHs (Incardona & Scholz 2016). Spontaneous tail contractions following exposure to paraoxon in fish have also been linked through an AOP (Yozzo et al. 2013). An AOP has been developed linking AhR-mediated developmental toxicity following exposure to dioxins in fish (King-Heiden et al. 2012). *In vitro* reporter assays using teleost-specific AhR isoforms have been suggested to predict AhR-mediated developmental toxicity, though the assays require development (Volz et al. 2011). The extensive involvement of retinoic acid in embryo development is well documented and the AOP from altered retinoic acid homeostasis to developmental toxicity has been developed in vertebrates (Tonk et al. 2015, Piersma et al. 2017). Furthermore, *in vitro* assays based on the retinoic acid receptor (RAR) were found to be the most significant predictors of rat developmental toxicity (Sipes et al. 2011). This suggests that the RAR, its heterodimer partner the retinoid X receptor (RXR), and other related receptors could be used to measure disruption to development *in vitro*.

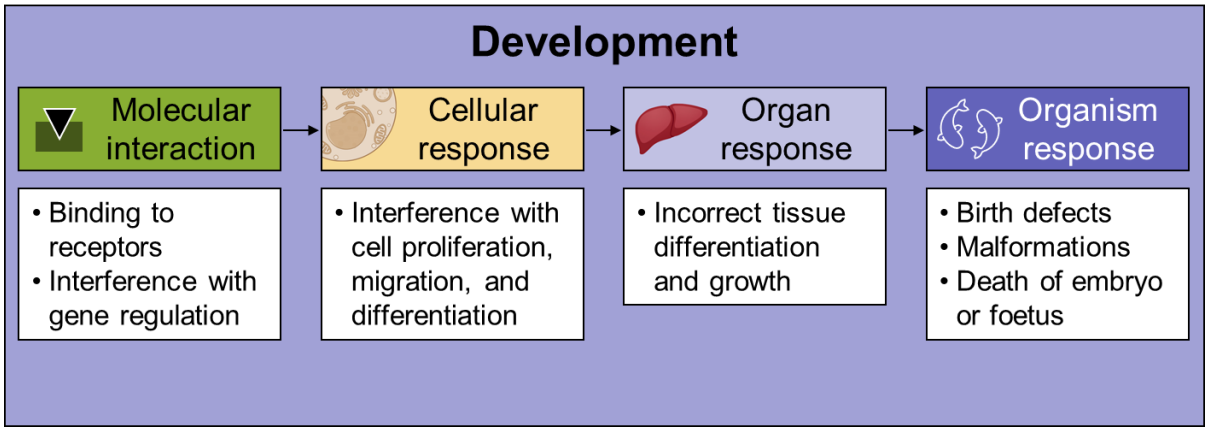


Figure 4 Toxicity pathway for development. Adapted from Escher et al. (2021) and Tonk et al. (2015).

3.4.3 Relevant in vitro bioassays

As mentioned, to measure effects on development *in vitro*, any number of genes and pathways involved in the developmental toxicity AOPs could be targeted, though many would require validation and more likely a suite of bioassays would be required to avoid false negatives. While the AhR pathway can be involved in developmental toxicity, it is also heavily involved in xenobiotic metabolism and thus covered in Part 2 of this review.

Assays related to the RAR, the RXR and the RAR related orphan receptor (ROR) have only been used in 5% (n=5) of all recent studies on the effects of wastewater. This may be due to the small number of available assays for these receptors. In many studies, both the RAR and RXR are measured, likely due to their heterodimeric relationship, meaning they form complexes and regulate gene expression of the retinoic acid response element as a functional unit (Minucci et al. 1997, Le Maire et al. 2019). There is a yeast-two hybrid assay for both the RAR and RXR (Inoue et al. 2009), both of which have been used in two recent studies (40% of studies that included development as an endpoint; Supplementary Table S9). A small number of mammalian reporter gene assays are also available such as the RAR-GeneBLAzer, the RXR-GeneBLAzer, the RXR-CALUX, and the HELN-RARα-RXR (Supplementary Table S9). The GeneBLAzer assays have low repeatability; however, this could be a result of

the instability of the reference compounds (König et al. 2017). HELN-RARα-RXR simultaneously measures both RAR and RXR activity and can be run both agonistically and antagonistically. Finally, there is the Factorial assay for RAR, RXR and ROR. Similar to other receptors, the Factorial assays have not been widely applied.

4 Conclusion

4.1 Regulatory context

Before they can be adopted for broader use, *in vitro* methods must first evidence reliability, ecological relevance and regulatory acceptance (Lillicrap et al. 2016). There have been many studies on reproducibility and repeatability of *in vitro* bioassays, demonstrating that most assays can, with proper laboratory protocols and quality assurance, produce reliable results (Escher et al. 2021). Environmental regulations are often based on the concept of "adversity", and demonstrating using toxicity pathways or more complex AOPs how adverse ecological outcomes are related to *in vitro* responses is critical to raise regulator support. One of main issues inhibiting the incorporation of *in vitro* assays into regulatory frameworks for WET testing is the incongruity between *in vitro* and *in vivo* results, discussed in section 3.1.2. For example, while there is often a good correlation between *in vitro* and *in vivo* effects, absolute agreement can be low, with *in vitro* assays for some effects often less sensitive than *in vivo* (Rehberger et al. 2018). Thus, simply using water quality guideline values based on whole animal toxicity data without *in vitro* adjustment is inappropriate. Over the past decade, there has been significant effort into the development of *in vitro* based trigger values (often called effect-based trigger (EBT) values) that establish a threshold for an *in vitro* response that is protective of individual or population-level effect. Significant progress has been made towards establishing EBTs for some assays and endpoints, such as basal toxicity and endocrine disruption (Tang et al. 2013, Jarošová et al. 2014, Escher et al. 2018, Been et al.

2021, Escher & Neale 2021). The establishment of EBTs would provide a significant step toward the implementation of *in vitro* bioassays in regulatory risk assessments.

4.2 Other considerations

The majority of studies on water toxicity use extraction methods to clean and concentrate samples for *in vitro* bioassays (Escher et al. 2021).Water samples can be extracted using techniques such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE). Extraction enables bioassays to focus on organic micropollutants by removing matrix interferences associated with organic matter, inorganics, metals, etc. (Escher et al. 2021). Extraction also allows for the concentration of extracts so that subtle effects can be better quantified. However, comparison with conventional WET testing methods may require the use of whole water samples, which may allow for better agreement between *in vivo* and *in vitro* effects.

When using whole water samples, the presence of pathogens can cause issues in *in vitro* bioassays, particularly with exposure periods longer than a couple hours. Arguably, water samples can be sterilised by filtration, autoclaving, chlorination, or UV exposure, but each of these methods could also impact on water quality, *e.g.* by creating disinfection by-products. The most common approach is filtration (e.g. Dayeh et al. 2002, Schirmer et al. 2004, Niss et al. 2018), though the removal of contaminants bound to suspended particulate matter should be considered, requiring separate extraction of the residue on the filter to account for full effects in the sample.

Many compounds require metabolic activation, thus the use of metabolically active S9 fractions may also need to be considered depending on the assay. Part 2, section 3.3.3 of this review addresses the addition of S9 in more detail, including S9 alternatives.

4.3 Recommendations

This review has identified *in vitro* bioassays that can supplement *in vivo* WET testing for apical endpoints including mortality, reproduction, development and growth. Some of these endpoints have clear AOPs linking key initiating events, which can be measured *in vitro,* with apical effects at the organism level, with some AOPs supported by strong *in vitro* and *in vivo* correlations (*e.g.* acute toxicity and reproduction). Additionally, some of the *in vitro* assays identified here can provide insight into the underlying mechanisms leading to toxicity and could act as an early warning, supporting the addition of these endpoints in future WET testing. As evidenced in this review, *in vitro* bioassays for mortality and reproduction are the most advanced on the path towards replacement, with a much larger selection of bioassays used extensively to assess toxicity of water samples with demonstrated reproducibility and sensitivity. Growth and development have fewer assays available or fewer assays applied to wastewater assessments, therefore requiring more validation.

Ideally, a battery would include many different *in vitro* assays, covering different endpoints. Of course, the cost of a bioassay battery is heavily influenced by the number of assays it includes, and a balance needs to be found between false negatives and excess monitoring costs. The inclusion of an acute bacterial (*i.e.* Microtox) and an algal growth inhibition assay should be paramount, considering the importance of these species in ecosystem functioning, the well-standardised protocols available from the OECD and ISO, and high sensitivity of these assays. The inclusion of cytotoxicity to fish cells would be a good addition to a battery of assays (including the Microtox and algal assays mentioned above) to measure acute toxicity. Estrogenicity is a commonly detected effect in wastewater, and should also be included in bioassay batteries to assess potential wastewater toxicity. Mammalian ER reporter gene assays are reproductible and highly sensitive (Leusch et al. 2010b) and considerable work has gone into the derivation of trigger values for these assays (Escher et al. 2018),

which may ease their adoption into regulatory frameworks. Note that it is critical to concurrently assess cytotoxicity when using reporter gene assays, as cytotoxicity can interfere with assessment of antagonistic activity. Androgenic and progestogenic activity are typically low in water samples (Escher et al. 2014), thus estrogenicity should take priority when resources are limited. Other effects are commonly detected in wastewater and should be included in a robust bioassay battery - these are discussed in Part 2 of this review.

Studies focusing on specific effects rather than an overall toxicity assessment should include more than one assay to get a more comprehensive assessment of effects leading to that toxic outcome. The inclusion of assays to measure androgenic activity and progestogenic activity should be included in studies focusing on reproductive effects. Similar to the recommendation for estrogenicity, the mammalian reporter gene assays represent a good option for these endocrine responses. The inclusion of other forms of assays, such as gene expression, receptor binding or steroidogenesis assays would also enhance the comprehensiveness of such a study.

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KF: Conceptualisation, writing – original draft

FL: Supervision, writing – review & editing

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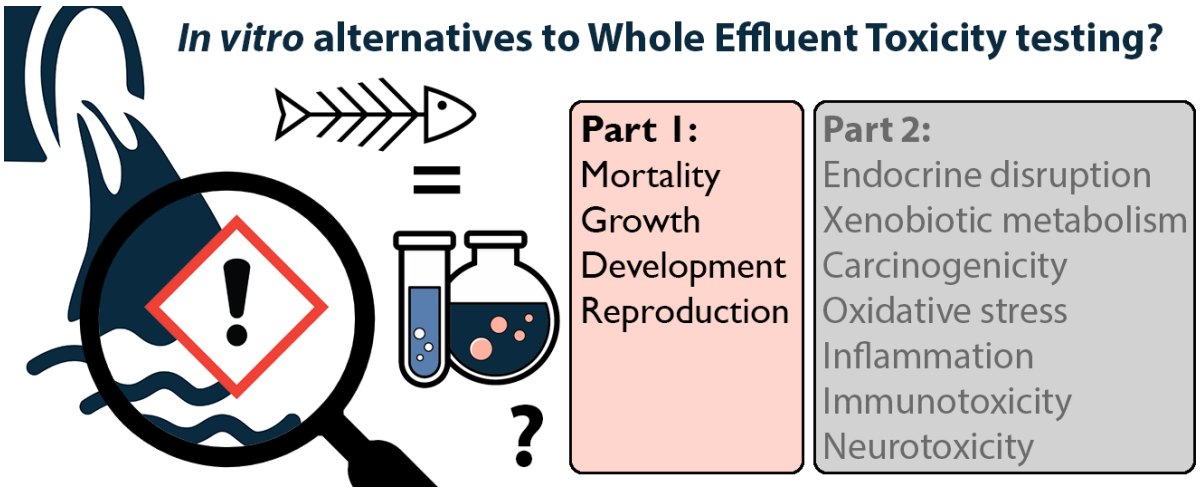
Graphical Abstract



Highlights:

- Relevant endpoints were identified from *in vivo* effects of wastewater
- Apical endpoints of mortality, growth, development and reproduction are covered
- Mortality and reproduction have strong *in vitro* to *in vivo* correlations
- Growth and development require further assay development