

# Toxicology of Water

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**Abstract** To protect the quality of water from toxic pollutants for the health of humans and the environment, two approaches are generally applied in the field of toxicology to predict the effects of pollutants and to monitor the toxic pollutants in water. Here we provide our perspective on state-of-the-art methods to develop water quality criteria and the use of molecular techniques for monitoring water quality. Emphasized is the recent development and application of cell-based assays and small fish model in toxicology research of water.

**Keywords** Alternative methods for toxicity testing · Bioanalytical method · Bioassay · Cell-based assay · HPG axis · *In vitro* bioassay · Small fish model · Species sensitivity distribution · Water quality criteria

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## **Prediction of the Effects of Pollutants: Development of Water Quality Criteria for the Protection of Aquatic Organisms and Humans**

The goal of establishing water quality criteria (WQC) for the protection of aquatic life is the protection of the structure and function of the ecosystem from long-term exposures. The methods to conduct these assessments vary among jurisdictions but in general have the same elements and suffer from the same sorts of limitations and uncertainties. The WQC are often structured to protect organisms that might accumulate sufficient exposure through trophic transfer as well as from direct exposure of aquatic organisms. For instance, the WQC should protect animals such as predatory fish, birds, and humans that might eat aquatic organisms such as plankton that would be expected to come to a steady-state distribution with contaminants in water from direct exposures. This can be done through the application of bioaccumulation factors (BAFs) for uptake from water into organisms and biomagnification factors (BMFs) for accumulation through the diet via trophic transfer. These two pathways of exposure are often combined into overall bioaccumulation factors (BAFs). Due to bioaccumulation, the WQC to protect predatory animals from the effects of bioaccumulative compounds (with  $\log K_{ow} \geq 3.0$ ), depending on relative sensitivities, are likely to be less than that to protect aquatic animals from direct exposures. Thus, either one comprehensive WQC value can be derived to be protective of both situations or, as is done by some jurisdictions, two separate WQC values could be derived.

### ***Water Quality Criteria for Protection of Aquatic Ecosystems***

In some cases, it might be important to protect specific species which might exhibit unique sensitivities or might be of special functional, cultural, or economic importance. In that case, testing of those species might be appropriate. But the many types of organisms that might be critical to the functioning of the ecosystem fall into many different families and orders which might have varying sensitivities to the compounds of interest, and it would be impossible to test all of the individual species. Thus, to be protective, the WQC must take into consideration this variation in sensitivities. There are several approaches to address this issue. One is to select model or sentinel species that, based on experience, have proven to be relatively more sensitive to toxicants. Then, “an application,” “an assessment,” or “a safety” factor can be applied to increase the probability of the WQC being sufficiently protective. There is some information that has been developed that gives some guidance as to the level of uncertainty factors to be applied, depending on how much information is available and what uncertainties remain (Table 1). For instance, if little data are available or the data are only for acute exposures, then a larger safety factor might be warranted than if a great deal of information is available from chronic exposures, especially with species that are generally known to be sensitive.

**Table 1** Comparison of assessment factors in current use (adapted from [1])

Regulatory authority	Dataset <sup>a</sup>	Assessment factor	Notes <sup>b</sup>
Canada <sup>c</sup>	Chronic LOEC	10	3 Species of fish, 2 invertebrates, and algae (or freshwater vascular plant)
Australia	Lowest of $\geq 5$ species chronic NOECs	10	If necessary, NOECs are estimated from other data as follows: MATC/2 LOEC/2.5 LC(EC)50/5
	Lowest of $\geq 5$ species acute LC(EC)50	100 or $10 \times$ acute-to-chronic ratio	Applied to the lowest LC(EC) 50 value
EU (freshwater)	Acute LC(EC)50	1,000	
	Chronic NOEC	10–100	
EU (marine)	Acute LC(EC)50	1,000–10,000	
	Chronic NOEC	10–1,000	
OECD	Chronic NOEC	10–100	

<sup>a</sup>LOEC = lowest observed effect concentration; NOEC = no observed effect concentration; LC(EC)50 = median lethal or effect concentration

<sup>b</sup>MATC = maximum acceptable toxicant concentration = (NOEC + LOEC)/2

<sup>c</sup>CCME (Canadian Council of Ministers of the Environment) 2007 (draft new protocol)

## Water Quality Zones

Some jurisdictions have multiple WQC, depending on the classification of waters, or to what use they are to be put. For instance, a waterway might be classified to be used only for industrial or transportation purposes. In such cases, the WQC might be different if the water is classified for recreational use or for aquaculture production. Even other areas might be defined to be kept as pristine areas where the magnitude of a potential stressor would not be allowed to vary from what has been determined to be natural ranges or at least within ranges appropriate to protect very sensitive components of the ecosystem, such as endemic and/or endangered species.

## Toxicity Data Requirements

In the absence of toxicity information for compounds of interest, there are several possible ways to predict the potential toxicity. Some of these methods require the collection of actual toxicity information, while others rely on existing information to make extrapolations. For instance, the toxicity of a chemical of concern can be predicted from information on the toxicity to other species. The use of species sensitivity distributions (SSDs) allows for estimation of the probability of protecting a particular proportion of a population. In some situations, the toxicity of a chemical can be inferred from the toxicity of structurally similar compounds. This approach, referred to as the quantitative structure activity relationship (QSAR), can use

relationships, referred to as linear free energy relationships (LFERs), to predict the toxicity of a chemical of concern from information on the toxicity of similar compounds. Similarly, the potential of chemicals to cause certain types of effects can be inferred from similar relationships where the particular response is predicted from structural and/or functional properties of compounds. Thus, if the structure of a chemical is known, some inferences about its toxicity can be predicted. Also, it might be possible to use information collected on freshwater organisms if it is for the protection of marine ecosystems [2]. However, there is no generalization that can be made on whether marine organisms are more or less sensitive to particular contaminants.

Sometimes, partial information is available for a compound or species of interest. For instance, acute toxicity might be available for several species such that a WQC that would be expected to be protective of most species could be predicted from an appropriate acute-to-chronic ratio (ACR) based on the class of chemical. The US Environmental Protection Agency (US-EPA) has developed an acute-to-chronic estimation (ACE v 2.0) model based on time–concentration–effect models (ACE) [3] that allows for this sort of prediction to be made for freshwater species. The software developed uses three different methods to make predictions. These include (1) accelerated life testing (ALT), (2) multifactor probit analysis (MPA), and (3) two-stage linear regression analysis (LRA). Of the three, the US-EPA suggests that the method of choice is ALT.

If toxicity information is available, there are basically two types of models that are used: time to effect or concentration to cause a defined effect in a specified time. In one, the duration of exposure is set at a fixed period of time (e.g., 24 h, 7 days, or 21 days), and the magnitude of the parameter or concentration of chemical required to elicit a particular level of effect is determined. In this type of study, the endpoint might be a quantal effect such as lethality or it might be a continuous variable such as growth or reproduction. The level of effect, such as the median effect concentration (EC50), which would be the concentration to cause adverse effect to 50% of the population, would be selected. The level of effect can be chosen as appropriate for any level of protection desired. Then by analyzing data from a range of concentrations, a function could be fit to the data to interpolate the desired effect level for a specified duration of exposure. In the second approach, the concentration is fixed and the duration of time to cause the specified level of effect is determined by observing the responses of organisms exposed to different concentrations for different periods of time. It is suggested that both methods have advantages, that both types of data be collected, and that toxicity curves, incorporating both the duration and magnitude of exposure, be developed [4, 5]. In this way, a reciprocity relationship can be developed that allows determination of both the incipient (threshold) duration and threshold concentration for effect. In fact, by developing the toxicity curve, the greatest amount of data can be extracted from the dose–response relationships.

*Data Collection*

Toxicity data can be obtained from searches of the most updated US-EPA ECOTOXicology database (ECOTOX). ECOTOX integrates three previously independent databases—AQUIRE, PHYTOTOX, and TERRETOX—into a unique system, which includes toxicity data derived predominately from the peer-reviewed literature, for aquatic life, terrestrial plants, and terrestrial wildlife, respectively. The current information in the ECOTOX (Version 4) database now contains a total of 285,798 records, with the majority of the literature reviewed from 1972 to 2008. The number of references, species, and chemicals included in these data are 18,831, 4,999, and 7,630, respectively. The quality of the toxicity data obtained from ECOTOX (2009) has been assessed by the US-EPA to determine how the toxicity data were generated using a number of criteria. Data not meeting these criteria were excluded from the ECOTOX databases.

Once the quality of the toxicity data has been determined, the aqueous solubility of the compounds needs be considered. There can be considerable error in determining aqueous solubility particularly for hydrophobic chemicals. Therefore, data that reported toxic effects at concentrations greater than twice the aqueous solubility should be removed. Also, the toxicity data will be screened in terms of the endpoints that they measured. Only toxicity data that measured lethality, growth, immobilization, photosynthesis, and reproduction should be considered as being environmentally relevant [6]. In particular, biochemical and behavioral endpoints should be excluded because of their doubtful ecological significance [7, 8].

In order to ascertain the likelihood of differential chemical sensitivity among different groups of species, the dataset should consist of acceptable acute (or chronic) test results of the key taxonomic groups of aquatic species (Table 2). To be protective of animals from long-term exposures to toxicants, the WQC should be based on chronic toxicity. However, it is also useful to have a WQC for short-term or spill type situations. This value, termed the maximum acceptable

**Table 2** Data requirements for the modified Great Lakes Initiative (GLI) Tiered Method proposed for the derivation of water quality criteria (WQC) for protecting saltwater ecosystems such as Hong Kong coastal marine waters

Data type	Required data for saltwater (SW) systems
A. Results of acceptable acute (or chronic) tests for:	1. One SW fish 2. One SW algae/cyanobacterium/fungus 3. One SW mollusk 4. One SW crustacean 5. One SW polychaete 6. One other SW invertebrate (e.g., echinoderm)
B. Acute-chronic ratios with data for at least:	1. One SW fish 2. One SW invertebrate 3. One other SW or freshwater species (if data not available, then the other two may be freshwater species)
C. Data for at least:	1. One SW algae or vascular plant (if data not available, then freshwater algae or vascular plant)

toxicant concentration (MATC) is based on the short-term or acute toxicity information.

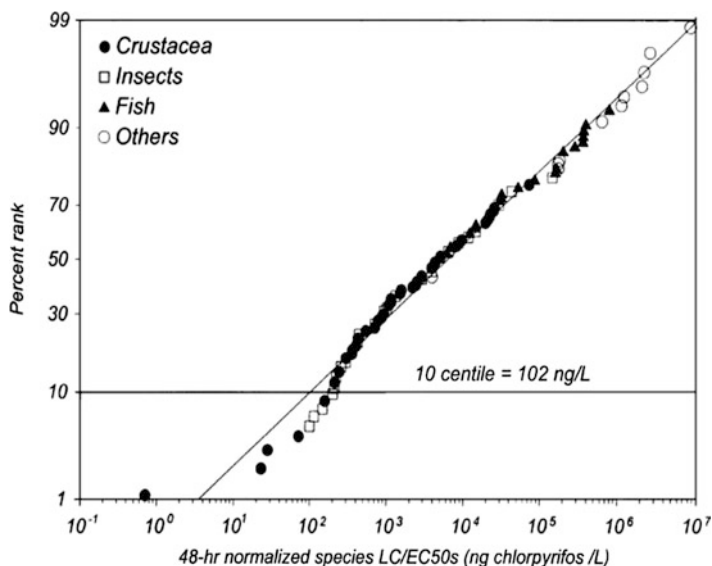
Since there is often more acute toxicity information available, usually acute lethality data are used to predict the concentration of a chemical to which animals can be exposed for longer periods of time by use of the ACR. This ratio is used to estimate allowable chronic exposure concentrations for other species for which little chronic toxicity data exist. The ACR is derived by dividing a compound's acute LC<sub>50</sub> value by the chronic nonobservable effect concentration (NOEC) for the same species. One limitation to this approach is that biological endpoints/responses are sometimes not comparable between acute and chronic studies. Acute studies involve lethality as an endpoint, while a chronic MATC is often derived from an endpoint other than lethality (growth, reproductive ability, *etc.*). Although the mode of action for lethality is assumed to be the same under acute and chronic exposures, the mode of action may not be the same for different toxicity endpoints. For a variety of organisms and chemicals, the ACR has been found to be approximately 10 [9]. This value is supported by QSARs for some chemical groups, in which acute and chronic regressions are separated by about an order of magnitude [10–12].

### ***Derivation of Water Quality Criteria***

Once the toxicity information has been assembled, there are basically two approaches that can be taken. The first approach, termed the assessment factor (AF or application factor) method, which is used by the USA, EU, Australia, and South Africa, is where data on the NOEC from toxicity tests are divided by an arbitrary factor (10 to 1,000) to account for uncertainties, such as among-species differences in toxicity, a lack of data on some types of species, or tests of sufficient duration or of the most sensitive life stages. The AF is essentially a safety factor meant to protect species and/or ecosystem integrity instead of predicting potential for effects. The other approach, which is applied when more information is available, is the statistical extrapolation approach (i.e., probabilistic approach). In this approach, statistical approaches are used to relate the concentration and duration of exposure to the level of effect. Both methods are based on acute or chronic toxicity test results with either surrogate or local species.

### ***Species Sensitivity Distribution Approach***

Probabilistic approaches have been used to describe the among-species sensitivity [13] (Fig. 1). In the SSD approach, the probability of a threshold for effect for either acute or chronic exposures being exceeded is developed. For instance, in this approach, the probability of a particular proportion of individuals of a particular proportion of species can be estimated. This approach requires data on the dose–response relationships for a relatively large number of species. As an

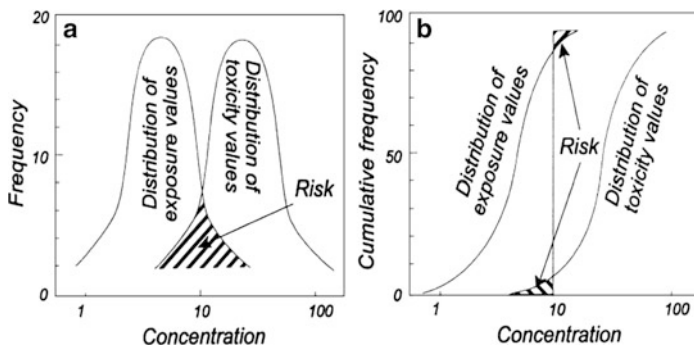


**Fig. 1** An example showing the species sensitivity distribution of the organophosphorus insecticide chlorpyrifos for freshwater aquatic species (adapted from [13])

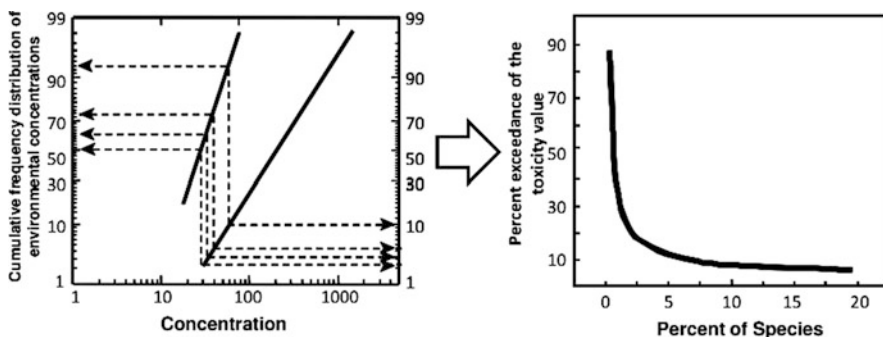
example, if data for only two species were available, each species would represent 50% of the frequency distribution and resolution or prediction would be poor. As a rule of thumb, it is suggested that to use the SSD approach, data for a minimum of 20 species should be available. In this case, each species would represent 5% of the total, and the resolution of the predictive power of the analysis would be 5%. The SSD approach assumes that the species that have been tested represent a random selection of all the possible sensitivities. If, for instance, the data available were for similar species with similar sensitivities that did not represent the entire range of possible sensitivities, then the predicted probabilities would not be accurate.

In addition to determining the probability of effect, one can also determine the probability that a particular exposure concentration, associated with a defined probability of effect can be determined (Figs. 2 and 3) [5, 13]. In this analysis, a relatively large number of measurements of the concentration of a contaminant are required. Therefore, the SSD approach will enable probabilistic ecological risk assessments of toxic chemicals in the marine environment.

The SSD approach is the preferred method by many developed countries, such as the Netherlands, EU, and Australia, although a variety of models to fit the SSD have been used. These models include parametric models (log-normal and log-logistic models), semiparametric models (i.e., bootstrap regressions) and nonparametric bootstrap model as well as Burr type III models. Different models can generate very different WQC values at the same level of protection (e.g., 95%) [14]. Thus, the best model fit should be carefully selected to generate the most accurate WQC value. For instance, such comparisons can be easily made using the Burr distribution software developed by CSIRO of Australian Government, which allows



**Fig. 2** Relationship between effect and exposure concentration distributions expressed as log-normal distributions (a) and cumulative log-normal distributions (b) (adapted from [13]). The effect concentration distribution (i.e., distribution of toxicity values) is represented by a species sensitivity distribution



**Fig. 3** Illustration of the derivation of a joint probability curve/exceedance profile from exposure and toxicity probability functions (adapted from [5, 13]). The dashed line and solid line on the left subfigure represent the linearized probability distributions of exposure and toxicology data

simultaneous comparison of various model fits for a SSD and is freely available for download from the CSIRO website (<http://www.cmis.csiro.au/envir/burrlioz/>).

The SSD probabilistic approach is useful if there are sufficient data and the implicit assumptions of the SSD method are met. In particular, the SSD method is useful in determining the types of organisms that are the most sensitive. It is also data intensive. To generate useful predictions, toxicity information is required for a large number of species and those species need to be distributed among representatives of different groups that might have different sensitivities or might have different exposures. Also, because it is a probabilistic approach, there are no values for zero or 100%. This is important because when representing the procedures used to derive WQC to the public, it is difficult to portray the risk. In general, the public does not want to accept any risk, but by definition, when applying the SSD approach, some proportion of the individuals of some proportion of the species will be affected some

proportion of the time. Our experience has led us to understand that exposures in the environment are generally less than those under controlled laboratory testing and that there is a certain amount of redundancy in the functions of environments that leads to a certain level of ecosystem resiliency. For this reason, setting the WQC based on the 5th centile is probably protective of most individuals of most species most of the time. In fact, when exposures set at the 5th centile have been compared to the results of multispecies mesocosm studies, the 5th centile has not resulted in measurable responses in populations or ecosystem function. But to the public, it appears that the regulations will allow for 5% of all species to be adversely affected. Thus, from a policy perspective, it becomes difficult to explain, let alone defend regulations based on this approach. In the following section, we present an analysis comparing the results of the SSD and GLI-type approaches. We present the relative protection afforded by each of the approaches.

### ***Great Lakes Initiative Approach***

One method developed by the US-EPA for use in developing WQC for the Great Lakes [5] is a semiprobabilistic approach that specified the types of data that are required and depending on the quality and quantity of toxicity data available assign assessment factors to correct the data to account for these uncertainties. This approach, referred to as the Great Lakes Initiative (GLI), allows development of WQC for the protection of aquatic organisms. In addition, BCFs and BMFs can be used to derive BAFs that can be applied to infer if the WQC are sufficient to protect higher trophic level organisms such as humans that might eat aquatic organisms.

While these are large bodies of water, the Great Lakes are freshwater. Thus, the WQC values that have been developed based on this method would not be directly applicable to other aquatic environment WQC, such as marine. However, it is suggested that the method is appropriate to be used to generate toxicity information for local organisms [5]. This method is a semiprobabilistic approach that specifies a required minimum dataset. By specifying data requirements for different classes, families, and genera, the method maximizes the potential for including a species of a type that would be expected to be sensitive to the stressor of concern. For instance, because a plant species (e.g., microalgae or macroalgae) is required in the dataset, there would be a species sensitive to the effects of herbicides. The GLI methodology is useful, because it allows for different levels of completeness in the datasets and specifies uncertainty factors to apply when the datasets are deemed to be insufficient. Two levels of WQC can be derived using this methodology. The first or Tier I value is calculated when all of the necessary information is available and is the value for which there is greater confidence. If less data are available or the data are of lesser quality, then a Tier II value can be calculated by the use of assessment factors. In this way, there is a built-in mechanism to facilitate collection of additional data because the additional data reduce uncertainty and in doing so can result in greater values for the WQC. This gives the regulatory community

incentive to provide the resources needed to conduct the testing. The method also has the advantage that it takes into account multiple datasets. It is suggested that a listing of specified species and or classes and families appropriate to capturing likely sensitive local species be developed to make a GLI-like approach workable for the aquatic system of concern. Finally, the GLI-type approach considers both acute and chronic toxicity information and allows prediction of one from the other, by use of an ACR, which might be derived as a species-specific factor or derived from among stressor comparisons.

In addition to the development of WQC for the protection of aquatic life, the GLI presents methodologies to develop WQC for the protection of higher trophic levels from potential effects of bioaccumulated chemicals, and biota–sediment accumulation factors can be used to calculate sediment quality objectives to protect both aquatic life and higher trophic levels. We favor the use of this GLI-like approach because it is flexible and a probabilistic approach. Because specific types of organisms are required for the analysis, it does not suffer from the limitations of as SSD approach alone. In addition, the GLI approach allows for the calculation of WQC for compounds with differing amounts of toxicity data. The GLI approach takes into account the amount and quality of data available and uses AFs to correct the WQC. The use of the GLI-type approach also allows the risk assessor the opportunity to review the data. In doing so, the risk assessor can assess the quantity and quality of data available and determine which groups would be most at risk and apply safety factors where necessary to protect ecologically or economically valuable species or maintain overall ecosystem structure and/or ecological functions. The GLI approach also allows the use of multiple testing with the same species without weighting each test as would be done if each test were given as a separate test result or losing an estimate of the variability in the data as it would be in a strict SSD. This approach rewards having more data and encourages the development of additional data to reduce uncertainty. Finally, the flexibility of the approach allows risk managers to apply more or less stringent protection criteria in a risk–benefit approach.

The GLI-type approach avoids some of the limitations of the standard SSD approach. In the SSD approach, there are some implicit assumptions that apply. It is assumed that the species for which toxicity data are available are a fair representation of the population of possible sensitivities. For instance, an SSD can be developed from data for 50 species, and a probability of effect, such as the 5th centile, can be derived. However, if all of these species were closely related, they could not represent a true measure of the entire range of sensitivities. For this reason, a straight SSD approach can give a false sense of accuracy and precision.

### ***Issues Related to Chemical Mixtures***

Chemicals of environmental concern often occur as a mixture such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), both of

which are currently listed as priority toxic chemicals by some jurisdictions. The presence of different compounds in a mixture presents a challenge to WQC derivation. For instance, different isomers and congeners of PCBs are known to have different physicochemical properties (e.g., various persistency) and, hence, different toxicities (i.e., different potencies) to animals. Due to variation of pollution sources and weathering, the composition of PCBs can vary considerably in water samples among different sites. Therefore, this raises a fundamental question whether or not the WQC derived for a mixture, like total PCBs, is protective of marine organisms and human. At present, there are two main approaches for derivation of WQC for a mixture such as PCBs, namely, the total mixture method (identical to that of single compound) and the toxicity equivalence quotient (TEQ) method. Therefore, our review will also address the relative merits of these two approaches for developing WQC and conducting risk assessments.

Here we present total PCBs as an example. PCBs are members of the group of halogenated aromatic hydrocarbons and consist of 209 isomers and congeners with different numbers and positions of chlorine atoms substituted on the biphenyl moiety. Individual PCB congeners exhibit different physicochemical properties which results in different profiles for environmental distribution and toxicity. PCBs have low water solubility, which decreases with increasing degree of chlorination. For example, the water solubilities of monochlorobiphenyl congeners are in the range of 1–5 g/L but that of decachlorobiphenyl is only 0.015 mg/L [15].

The advantages of total PCB-based WQC derivation and/or risk assessment include its simplicity by being used as a conventional method. This approach also incorporates risks due to metabolites and interactions among congeners. The ability of animals to metabolize PCBs does not necessarily imply that the metabolites can be excreted and therefore that risk can be minimal. The potential adverse effects of PCBs on marine organisms are dependent on several factors including the overall concentrations of PCBs to which they are exposed and the relative toxic potencies of the individual congeners present in the mixture and their interactive effects. Due to the limitations of the total PCB-based approach in risk assessment, application of congener specific risk assessment methods has been suggested.

Alternatively, the TEQ approach allows the expression of the toxic potential of a complex mixture of individual congeners as one integrated parameter, the toxic equivalency value, in which the toxic potency of the mixture corresponds to the potency of the most toxic congener of PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). If relative potencies can be derived for PCB congeners for a few endpoints and species that are found to be intercorrelated and if congeners can be established to have the same rank order among endpoints and species, the relative potencies can be used to develop a toxic equivalency factor (TEF) for each congener. As an example of the technique, if the ED<sub>50</sub> values for immunosuppressive activity of TCDD and 1,2,3,7,8-penta-CDD were 1.0 and 2.0 µg/kg, respectively, then the TEF for the latter compound would be the ratio of ED<sub>50</sub>(TCDD)/ED<sub>50</sub>(1,2,3,7,8-pentaCDD), or 0.5. TEF values have been determined for several different aryl hydrocarbon receptor (AhR)-mediated responses. However, for every PCB congener tested, the TEF values are response and species dependent [16]. As an example, TEFs for

TCDD obtained from *in vivo* and *in vitro* studies varied from 0.17 to 0.016 and 0.43 to 0.006, respectively [17]. Regulatory agencies have chosen consensus TEF values for individual congeners. Selection criteria have been based on the importance of data obtained for specific responses (e.g., carcinogenicity, reproductive, and developmental toxicity).

The TEF approach was first utilized to assess the risks associated with air emissions of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) formed during high-temperature incineration of industrial and municipal waste [18, 19]. Subsequently, the US-EPA proposed interim criteria for estimating risks associated with mixtures of PCDDs and PCDFs for other media as well [20]. Several international agencies have also adopted the TEF approach for the risk assessment of PCDDs and PCDFs [21–24]. The mechanistic considerations for the development of TEFs for the risk assessment of PCBs have been described elsewhere [17, 25]. TEF values have been proposed by the WHO for mammals, birds, and fish [26, 27]. We will briefly review the development of TEFs using mammalian models [17] and the recent progress in studies relating to fish- and bird-specific TEFs for PCBs. Such techniques may be extended to other mixture chemicals.

### ***Approach for Deriving Chemical Water Quality Criteria for the Protection of Wildlife***

Because the water not only supports numerous human activities and provides habitat for aquatic organisms, but also sustains viable mammalian and avian wildlife populations, chemical WQC need to be derived for the protection of populations of these predatory animals. The water quality objectives for mammalian and avian wildlife are surface water concentrations of toxicants that will cause no significant reduction in the viability or usefulness (in a commercial or recreational sense) of a population of exposed animals utilizing target waters as a drinking and/or foraging source over several generations. The application of the proposed approach requires the acquisition of BCFs and BAFs (trophiic transfer coefficients) as well as the development of toxicity reference values (TRVs) for each of the priority pollutants. Furthermore, appropriate application factors need to be derived depending on the quality and quantity of data available. In general, the number of studies available for the higher trophiic level species is less, and some studies do not consider an entire life cycle or the most sensitive and ecologically relevant measurement endpoints. For this reason, safety factors are normally applied. The most appropriate set of safety factors are those given in the GLI methodology (cf. above). The procedure for selecting the overall safety factors is well described and results in transparent safety factors so that the risk assessor can understand where the uncertainties lie and the magnitude of uncertainty factors that have been applied.

### ***Calculation of Tier I Wildlife Objectives and Tier II Wildlife Values***

The equation used to calculate wildlife values (WV) has both an effect and an exposure component. The effect component is defined as the test dose (TD) which is either a lowest observed adverse effect level (LOAEL) or a no observable adverse effect level (NOAEL) for milligrams of substance per kilogram body weight per day (mg/kg BW/day). The exposure routes considered in this derivation are food and water ingestion, and because the intake level is dependent on organism size, it is scaled to body weight. The total toxicant intake through these exposure routes is determined and then set equal to the TD (1) and (2):

$$\text{Toxicant intake through drinking water} = (WV \times W)/Wt \quad (1)$$

$$\text{Toxicant intake through food} = [WV \times \Sigma(F_{TLi} \times BAF_{TLi})]/Wt \quad (2)$$

where: WV = Species-specific wildlife value in milligrams of substance per liter (mg/L).  $W$  = Average daily volume of water consumed in liters per day (L/day) by the representative species.  $F_{TLi}$  = Average daily amount of food consumed from trophic level in kilograms per day (kg/day) by the representative species.  $BAF_{TLi}$  = Bioaccumulation factor for wildlife food in trophic level in liters per kilogram (L/kg). For consumption of piscivorous birds by other birds, the BAF is derived by multiplying the trophic level three BAF for fish by a BMF for biomagnification of the chemical from fish to birds that consume these fish.  $Wt$  = Average weight in kilograms (kg) for the representative species.

Equations (1) and (2) are combined to yield (3):

$$TD > (WV \times W)/Wt + [WV \times \Sigma(F_{TLi} \times BAF_{TLi})]/Wt \quad (3)$$

where TD = Test dose in milligrams of substance per kilogram body weight per day (mg/kg BW/day) for the test species (either a NOAEL or LOAEL derived from mammalian or avian toxicity studies).

To account for differences in toxicity among species and uncertainties in LOAEL to NOAEL extrapolations and subchronic to chronic extrapolations, the TD is divided by three uncertainty factors:  $UF_A$ ,  $UF_s$ , and  $UF_L$ ,

where:

$UF_A$  = Uncertainty factor for extrapolating toxicity data across species (unitless). A species-specific uncertainty factor shall be selected for each representative species.

$UF_s$  = Uncertainty factor for extrapolating from subchronic to chronic exposures (unitless).

$UF_L$  = Uncertainty factor for LOAEL to NOAEL extrapolations (unitless).

The final equation of the WV therefore is (4):

$$WV = \frac{\frac{TD}{UF_A \times UF_s \times UF_L} \times Wt}{W + \sum [FT_{Li} \times BAF_{TLi}]} \quad (4)$$

### ***Derivation of the Final Tier I Wildlife Objective***

The wildlife values specific for each taxonomic class are derived by taking the geometric mean of the wildlife values across all of the representative species within each taxonomic class (5):

$$WV_{(taxonomic\ class)} = \text{Exp}[\sum \ln WV_{(representative\ species)}/n] \quad (5)$$

where  $n$  = The number of representative species in a given taxonomic class for which species-specific wildlife values were calculated.

The water quality objective is then set equal to the lower of the two taxonomic class-specific wildlife values.

### ***Derivation of a Tier II Wildlife Value***

The equation to derive a Tier II wildlife value is the same as that presented above to derive the taxonomic class-specific Tier I wildlife values which are then used to determine the Tier I wildlife criterion. One of the major differences in the derivation of a Tier I wildlife value and a Tier II wildlife value is that for a Tier I wildlife objective, a taxonomic class-specific wildlife value is derived for both taxonomic classes, such as *Aves* and *Mammalia*, while a Tier II wildlife value can be determined when a taxonomic class-specific wildlife value is available for only one taxonomic class.

## **Monitoring of Toxic Chemicals**

Development of WQC provides criteria for efficient monitoring and management of a number of prioritized toxicants in aquatic environments by relying on a large amount of toxicity data. There are thousands of chemicals including industrial and agricultural chemicals that could be spilled or discharged into aquatic systems and lead to human and wildlife exposures; however, most of these chemicals have very limited toxicity information [28]. This is primarily due to the high cost and time required to conduct conventional toxicity testing with many species.

Also, animals are more often exposed to mixtures of chemicals rather than a single chemical. Therefore, what is needed are alternative toxicity testing methods that can be used to efficiently evaluate the adverse effects of mixtures of environmental chemicals or effluents. The recent development of cell-based *in vitro* bioassays and small fish models in toxicological research of water is reviewed here.

### ***Cell-Based In Vitro Bioassays***

Cells used in *in vitro* bioassays can be permanently established eukaryotic cell cultures that are used to measure specific toxic effects or to detect the presence of specific classes of toxic chemicals in samples related to aquatic systems (water, sediment, suspended matter, biota). Cell-based *in vitro* bioassays can be classified based on whether the cell line is an untransformed wild type or has been genetically modified. Each type of cells has advantages and disadvantages for identifying a specific mechanism of toxic action or exposure to a certain group of chemicals. Cell lines are generally derived from tumor cells due to their proliferative properties that are suppressed in healthy tissues. These cell lines are then immortalized with the aim to maintain their particular properties that enable the detection of chemicals or potential of environmental samples to interact with specific biological pathways. For example, since it expresses all the key steroidogenic enzymes and produces most of the steroid hormones, such as mineralocorticoids (aldosterone), glucocorticoids (cortisol), and sexual hormones (estradiol and testosterone), the human H295R adrenocortical carcinoma cell line has been widely used to assess chemical-induced effects on steroidogenesis [29–33]. Chemical agents that alter production of steroids at the cellular level have the potential to disrupt the endocrine system in living organisms. Examination of the expression of different steroidogenic enzymes provides mechanistic information on the molecular basis of the altered hormone biosynthesis by chemical exposure. In the H295R assay, cells are exposed to different concentrations of a chemical to assess the presence or potential of compounds that modulate steroid hormone synthesis. Biological effects can then be measured at different organizational levels such as gene expression, enzyme activity, or hormone production as desired [31, 33].

Sometimes, wild-type cell lines are genetically modified to express favorable attributes that allow the detection of certain classes of chemicals. They often use different combinations of endogenously expressed elements and exogenous factors that are artificially introduced into the cell system. For example, the H4IIE-*luc* is a stably transfected cell line used in a transactivation assay to detect dioxin-like chemicals [34]. In this cell line, the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator protein (ARNT) are endogenous, but an exogenous dioxin-responsive element (DRE) and the reporter gene (luciferase) were stably transfected into the cells as a construct. Once incorporated into the genome of the cells, the induction of the introduced luciferase gene as measured by light emission is proportional to exposure to AhR agonists. In fact, in this particular

example, the amount of light produced is proportional to the potency of mixtures of AhR agonists and is proportional to the toxicity of the mixture. Similarly, transcriptional assays have been developed for a number of other endpoints such as the characterization of the potential of chemicals to agonistically or antagonistically bind to the estrogen or androgen receptor.

Besides well established mammalian cell lines, there are increasing numbers of nonmammalian cells, particularly those isolated from aquatic species, such as fish and amphibians, applied in the toxicological research of water. For example, a rainbow trout (*Oncorhynchus mykiss*) fibroblastic cell line, RTG-2, has been applied to assess chemical-caused genotoxicity using random amplified polymorphic DNA (RAPDs) analysis [35] or micronuclei (MN) estimations by means of flow cytometry [36]. An embryonic fibroblast-derived cell line XTC-2, which was derived from the African clawed frog (*Xenopus laevis*), was used to investigate exposure to environmentally relevant concentrations of the bactericidal agent triclosan by quantifying effects on thyroid hormone associated gene expression [37]. Recently, a gill epithelial cell line from seawater-adapted tilapia (*Oreochromis niloticus*) has been developed to assess exposure and potential effects of toxicants in marine water [38]. As an example of a genetically modified fish cell line, SJD.1 is a zebra fish (*Danio rerio*) caudal fin cell line transformed with a metallothionein (MT) reporter construct, which can be used as to assess exposure to heavy metals [39].

## Application

Cell-based *in vitro* bioassays have been widely used for chemical screening and prioritizing approaches such as effect-directed analysis (EDA) or toxicity identification and evaluation (TIE) and the study of chemical-induced molecular mechanisms of toxicity. Assessment of cytotoxicity, e.g., by means of the MTT or Live/Dead<sup>®</sup> assay, is used to determine the general toxic potential of chemicals or environmental samples that can kill cells either directly or indirectly through the inhibition of cellular metabolic pathways. Genotoxicity tests are used to examine harmful effects induced by chemicals on genetic materials in cells, such as DNA strand breakage or base oxidation [39, 63]. Furthermore, cell lines have been applied in functional *in vitro* assays. For example, the H4IIE-*luc* reporter gene assay has been used to assess the potency of individual AhR active compounds or the overall potency of complex planar halogenated hydrocarbon (PHH) mixture in environment samples [34, 40, 41]. In these examples, chemical or environmental samples can be ranked by their differences in potency using the same cell system.

In addition to their utilization in research on the interactions of chemicals with certain biological pathways, cell lines can be used as bioanalytical tools in environmental diagnostics such as the above described EDA or TIE approaches. EDA is based on a combination of fractionation procedures, biotesting, and subsequent chemical analyses to aid in the characterization of exposure to pollutants in

complex environmental samples [42, 43]. Particularly, the use of *in vitro* bioassays as part of EDA has been shown to be a powerful tool in support of the exposure characterization step in environmental risk assessments and already is routinely utilized in environmental monitoring programs [44]. The vulnerability or sensitivity of cellular components or pathways to exposure with certain chemicals, such as ligand-induced receptor-mediated responses, renders them useful tools to detect the presence of pollutants in aquatic media such as surface water, sediments, or suspended particulate matter. This is particularly true in environments that are characterized by exposure to chemical mixtures. In such situations, the sole use of classic chemical-analytical techniques is not suitable for characterizing exposure due to extreme cost and limits in the available analytical methodologies for many chemicals, especially as often no *a priori* knowledge of the chemicals present in the sample exists. As a consequence, there is an increasing trend of supplementing chemical analysis with bioanalytical techniques that make use of the specific properties of certain groups of chemicals to interfere with specific biological processes. For example, the gene expression of cytochrome P4501A (CYP1A) in hepatic cells and the luciferase activity in H4IIE-*luc* cells can be used to characterize the exposure to dioxin-like compounds [34]. MVLN cells, in which the luciferase gene is under the control of an estrogen receptor-responsive element, and H295R cells have been used to detect the presence of endocrine-disrupting chemicals in water and sediment samples [45, 46]. Under circumstances where there is little information on the identities of the chemicals in a solution, for example, in an effluent discharged into an aquatic system, nontarget screening approaches using a battery of cell lines with different diagnostic properties (e.g., AhR, endocrine disruptor, genotoxicity) can be employed to aid in the identification of the biologically active components.

Beyond their utility as screening assays, cell lines are useful to identify mechanisms of toxicity because the initiating events and any subsequent interactions of a chemical with an organism occur at the cellular or subcellular level (e.g., alteration of the transcriptome). Mechanistic studies using cell lines include but are not limited to examining chemical-caused oxidative stress, altered cellular signaling pathways, and modulated responses at the genomic, proteomic, and/or metabolomic level. For example, simultaneously examination of chemical-induced effects at transcriptional, enzymatic, and metabolite levels in the H295R cells has been used to characterize the disruption of steroid hormone production by endocrine active chemicals [30, 31, 33]. A recent study demonstrated that the strategy of measuring multiple endpoints is effective to differentiate between chemical-caused direct inhibition of aromatase activity from indirect inhibition (e.g., by altering transcriptional expression) [47].

Recently, with the increasing development of *in vitro* assays and application of high-throughput technologies in toxicity testing, there is a demand for a data-driven and science-based system that can classify chemicals based on toxic mechanisms and prioritize chemicals for animal testing. These new techniques are increasingly being used in priority chemical screening programs such as Tier 1 of the Endocrine Disruptor Screening Program of the US-EPA [48]. During these screening

initiatives, large amounts of multidimensional data (e.g., gene transcripts, proteins, and enzyme products) are collected for various concentrations of each chemical analyzed. Concentration (dose)–response relationships of endpoints at the molecular and cellular level provide essential mechanistic information for the toxicity of each tested chemical. To efficiently manage and interpret these large datasets, a novel computational toxicology program, ToxClust, was developed which can cluster chemicals based on concentration–response data derived with single or multiple endpoints [49].

As an alternative approach to *in vivo* animal testing methods, cell-based *in vitro* assays used in aquatic toxicology have advantages, which include (1) cost-effectiveness, (2) short testing time, (3) representing the primary targets of chemical-induced effects, (4) increasing the number of chemicals to be tested by amenability to high throughput, and (5) obviation of the need for the use of whole animals. In order to completely replace animal model-based testing by cell-based *in vitro* assays, much work is underway to address the limitations such as low metabolic capability. One common approach to circumvent this issue is to introduce a metabolism step by supplementing the assay with S9 fraction or microsome treatment. Other well-known limitations of *in vitro* approaches include their abnormal biology, lack of tissue/organ organization, and limited kinetic and dynamic extrapolation [50]. However, increased reliance on the use of cell-based approaches is expected for toxicology studies. With proper application and data interpretation, cell-based *in vitro* bioassays will play an important role in risk identification and prioritization of chemical testing.

### ***Small Fish Models***

There are two major challenges which must be met in order to effectively address the toxicology of water. First, methods to efficiently evaluate the toxicity of the large number of chemicals that could enter aquatic environments must be developed. Second, methods to predict chemical-induced toxicities among large number of species are required. Coinciding with the increased reliance on the use of cell-based approaches for toxicology studies, recent development in small fish models, such as the Japanese medaka (*Oryzias latipes*), zebra fish, and fathead minnow (*Pimephales promelas*), have gained much popularity in toxicity testing and risk assessment. The development of small fish models is attractive not only because these *in vivo* models carry normal metabolic capability, relevant organ/tissue organization, and regular growth states but also because the data generated from *in vivo* studies are ready to inform decisions by risk assessors. Fish acute and chronic tests are key components in ecotoxicity testing for quantitatively evaluating the potential adverse effects of substances and effluent discharges.

Compared to large fish models and other mammalian toxicological models, small body size fish models have advantages favoring toxicity evaluation of a large number of chemicals. First, their small body size and ease of culture (breeding

and cultivating) under common laboratory condition make them useful for studies with reduced cost. Secondly, small fish models share many biological similarities with human and other mammalian species, which make them an ideal testing model to assess chemical-induced neurotoxicity, developmental toxicity, hepatotoxicity, and reproductive toxicity. Third, a plethora of literature regarding the physiological, embryological, genetic, genomic, and toxicological knowledge about these small fishes is readily available [51, 52]. Specially, the available genomic information of these small fish models allow the application of toxicogenomics to evaluate toxicity of different chemicals across the genomes of these fish and extrapolate the observed toxicity in these models to humans and other species.

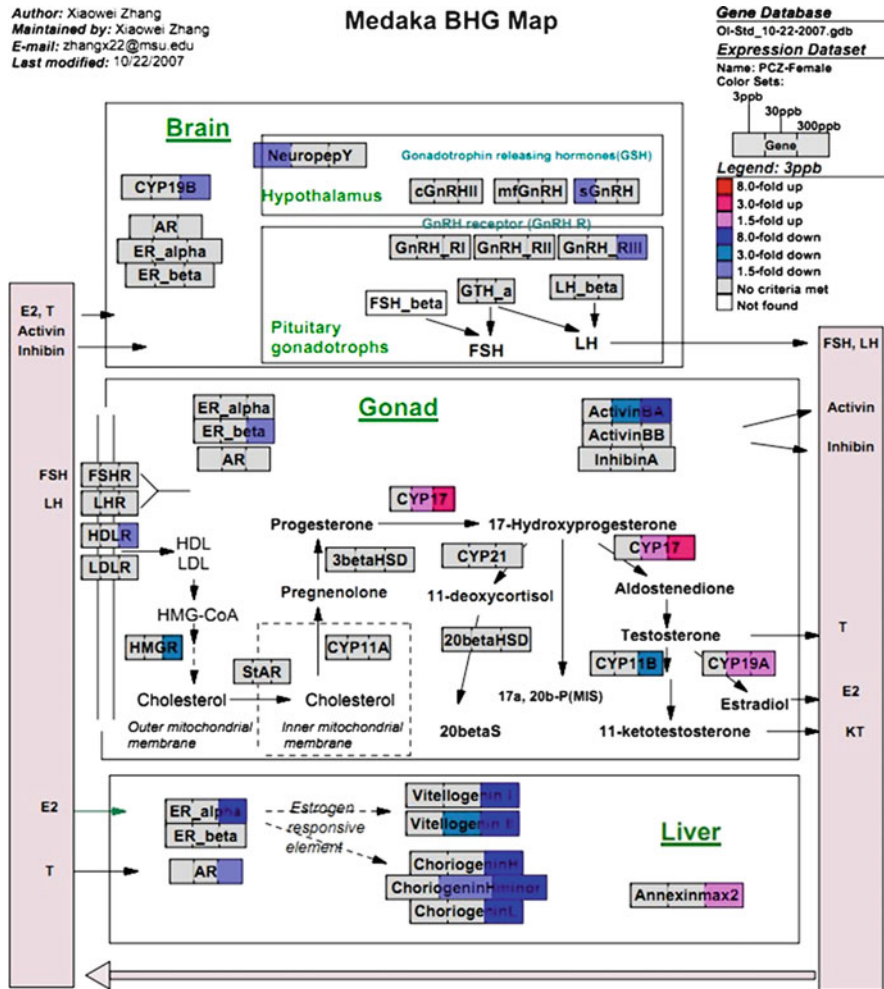
### ***Medaka Hypothalamic–Pituitary–Gonadal Axis Model***

Mechanistic information derived at biochemical and molecular levels could provide essential clues to predict effects among species and chemicals [52–54]. To illustrate the recent development of small fish models in chemical toxicity evaluation, the Japanese medaka was used as an example in the testing and evaluation of endocrine-disrupting chemicals [54]. The hypothalamic–pituitary–gonadal (HPG) axis plays a critical role in the development and regulation of the reproductive system in vertebrates. The significant conservation in the basic aspects of the HPG axis across vertebrates makes the use of small fish species for identifying and assessing the effects of EDCs possible [55]. Recently, a HPG-PCR array system has been developed to study effects of chemicals on the HPG axis of the Japanese medaka. This Japanese medaka HPG-PCR array carries the quantitative performance of SYBR Green-based real-time PCR and the multiple gene profiling capabilities of a microarray when examining expression profiles of 36 genes associated with endocrine pathways in the brain, liver, and gonad (Fig. 4). The key signaling pathways and functional processes within the brain (including hypothalamus and pituitary), gonad, and liver of Japanese medaka were included in the HPG transcriptional model. The selected genes consisted of receptors (including steroid receptors, peptide receptors, and lipid receptors), peptide hormones, steroidogenic enzymes, and other key receptor-responsive genes. A pathway-based approach using modified GenMAPP software was implemented to analyze and visualize concentration- and time-dependent gene expression in the HPG axis of Japanese medaka exposed to environmental chemicals. Furthermore, phenotypic anchoring strategies were applied by intercorrelating the gene expression data with physiological alterations and reproductive performance, including fertility and fecundity observed during exposure [56].

The Japanese medaka HPG-PCR array has potential not only as a screening tool of potential endocrine-disrupting chemicals but also in elucidating mechanisms of action. For example, both the anabolic androgen 17 $\beta$ -trenbolone (TRB) and the aromatase inhibitor fadrozole (FAD) can cause decreased plasma concentrations of 17 $\beta$ -estradiol (E2) and reduce fecundity of fish. The mechanisms of the reduced

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### Medaka BHG Map



**Fig. 4** Medaka hypothalamic–pituitary–gonadal (HPG) axis model and striped view of concentration-dependent response profiles in prochloraz (PCZ) exposed female Japanese medaka (adapted from [56]). Gene expression data from medaka treated with 3.0, 30, and 300  $\mu\text{g}$  PCZ/L are shown as striped color sets on the selected endocrine pathways along the medaka HPG axis. The legend listed in the upper right corner of the graph describes the order of the three PCZ concentrations and the eight colors designating different fold thresholds. Abbreviations: LH luteinizing hormone, FSH follicle-stimulating hormone, E2 17 $\beta$ -estradiol, T testosterone, HDL high-density lipoproteins, LDL low-density lipoproteins

fecundity by TRB and FAD were differentiated by a time-course exposure study aided with the Japanese medaka HPG-PCR array [57]. Both TRB and FAD caused lesser mRNA expression of vitellogenin and choriogenin (CHG) in the liver of females. Exposure to FAD for 8 h resulted in an eightfold and 71-fold down-regulation of expression of estrogen receptor  $\alpha$  (ER $\alpha$ ) and choriogenin L (CHG L),

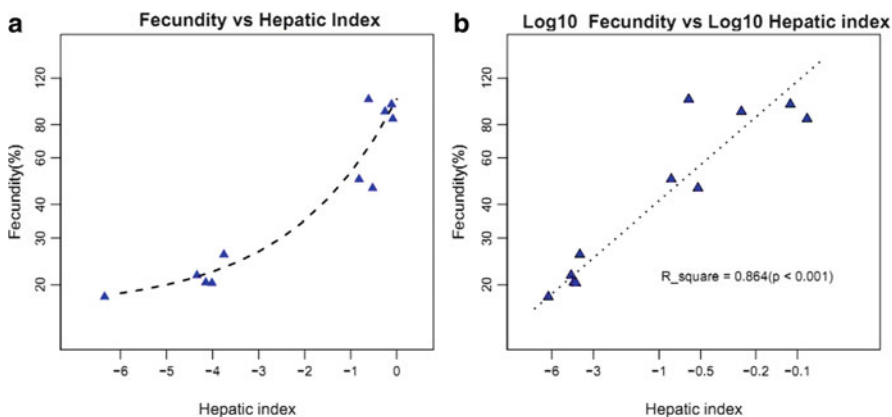
respectively, in female liver. The downregulation of estrogen-related genes was not observed until 32 h of TRB exposure. These results support the hypothesis that FAD reduces plasma E2 more quickly by inhibiting aromatase enzyme activity than does TRB, which inhibits the production of the E2 precursor testosterone [57].

### ***In Situ Hybridization***

In addition to the Japanese medaka real-time PCR array system, a whole-animal tissue section *in situ* hybridization (ISH) system using radiolabeled probes was developed to detect differential gene expression among tissues of Japanese medaka by Tompsett *et al.* [58]. The ISH method not only allows the measurement of tissue-specific gene expression in a whole-animal model but also provides cellular morphological information in the same organism. Furthermore, a fluorescence *in situ* hybridization (FISH) methodology using fluorescence-labeled riboprobes was developed by Park *et al.* [59] to evaluate gene expression profiles simultaneously in multiple target tissues of Japanese medaka. In this optimized FISH method, confocal fluorescence microscopy was optimized to reduce the autofluorescence signal. Using the aromatase inhibitor fadrozole as a model chemical and gonadal aromatase (CYP19a) as a model gene, the optimized FISH method revealed tissue-specific expression of the CYP19a gene and differentiated the abundance of CYP19a mRNA among cell types. Expression of CYP19a was found to be primarily associated with early stage oocytes, and expression gradually decreased with increasing maturation. In females exposed to 500 ng 17 $\alpha$ -ethinylestradiol (EE2)/L, the downregulation of CYP19a expression in ovary was found to be a result of tissue degeneration, specifically a decrease in the number of cells (previtellogenic oocytes) where CYP19a mRNAs are primarily transcribed, rather than to a decrease in expression per cell [60]. These examples suggested that FISH combined with histology enables elucidation of molecular effects of chemicals by associating changes in gene expression with histological effects at tissues and/or cellular level.

### ***Hepatic Transcript Index***

The large dimensional dataset of transcriptional profiles generated from toxicogenomics studies can not only be useful in revealing toxic mechanisms or describing pollutant-specific molecular fingerprints but can also be utilized to assess the risk of pollution. To develop the environmental threshold of toxicants, conventional ecological risk assessment theories usually employ toxicity endpoints of mortality, growth, and reproduction, which are directly relevant to the ecological outcomes. To reduce the dimension of gene expression data, a new concept of hepatic transcript index (HTI) was developed to facilitate the application of toxicogenomic data in risk assessment. After investigating a group of chemical-induced effects at



**Fig. 5** Relationship between fecundity and gene expression in livers of female Japanese medaka (adapted from [56]). (a) Fecundity vs. hepatic transcript index (HTI), the broken line shows the trend of data. (b) Simple linear regression of  $\log_{10}$ -transformed fecundity and HTI. The functions describing the relationship are hepatic index =  $0.236 * \log_{10}(\text{ER}\alpha) + 0.326 * \log_{10}(\text{VTG I}) + 0.537 * \log_{10}(\text{VTG II}) + 0.472 * \log_{10}(\text{CHG L}) + 0.343 * \log_{10}(\text{CHG H}) + 0.457 * \log_{10}(\text{CHG HM})$ . The formula for the regression model was  $\log_{10}(\text{fecundity}) = 1.616 - 0.4493 * \log_{10}(-\text{HTI})$

the Japanese medaka HPG axis, which included ketoconazole, prochloraz, EE2, TRB, and FAD, HTI in females was found to display a significant linear relationship with fish fecundity [56]. In this analysis, six hepatic genes were observed to be closely correlated at the mRNA expression level across different treatment, which included ER $\alpha$ , VTG I, VTG II, CHG L, CHG H, and CHG HM. Principal component analysis on the mRNA expression of the selected hepatic genes among chemical treatments revealed that the first principle component (PC1) explained 96.3% of the variance among the six genes. The HTI is a sum of log-transformed expression levels of the six hepatic genes weighted by the PC1 factor, which represents the overall expression level of this cluster of gene (Fig. 5). The significant linear relationship between log-HTI and log-fecundity ( $r^2 = 0.864$ ) suggested that the HTI within the HPG axis could be a good indicator of adverse effects at ecological fitness and has potential to be incorporated into ecological risk assessment and regulatory framework.

## Application

Cell-based assays and small fish models are useful tools to assess the toxicity and risk(s) of large numbers of pollutants in aquatic systems, such as environmental pharmaceuticals. Pharmaceuticals in the aquatic environment have gained increasing public concern for their potential consequences on human and ecosystem health. These chemicals, which have the potential to alter the endocrine system in

humans or wildlife, could eventually lead to changes in reproductive fitness. Using the H295R cell line and the small fish model medaka, it has been shown that certain pharmaceuticals used in Korea could affect the steroidogenic pathway and alter sex hormone balance although the current concentrations of these pharmaceuticals that occur in Korean rivers are much less than the thresholds for effects on the endpoints [61, 62].

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