Is Fish Embryo Test (FET) According to OECD 236 Sensible Enough for Delivering Quality Data for Effluent Risk Assessment?

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Abstract: Over the past few years, the fish embryo test (FET) has become widely accepted as an animal-friendly protocol for ecotoxicological research. As Organisation for Economic Co-operation and Development (OECD) 236, the FET has been widely applied for simple mixture exposures under the Registration, Evaluation, Authorisation, and Restriction of Chemicals regulation of the European Union; and now its use is spreading worldwide as a supposedly reliable whole-effluent test (i.e., the testing of complex mixture exposures). However, comparative peer-reviewed data regarding the FET’s efficiency for whole-effluent tests are virtually nonexistent. The primary objective of the present study was to make the first comparative test between the FET according to OECD 236 and other standard and slightly modified standard fish protocols used worldwide for whole-effluent tests. For that, we used an untreated hospital effluent considered to be highly toxic but disposed of in municipal sewerage. The base methods were OECD 203 (juvenile), US Environmental Protection Agency method 2000.0 (larvae), and OECD 236 (embryo). We also evaluated the addition of 3 virtually costless sublethal metrics (immobility, nonhatching, and pericardial edema) that could enhance the sensitivity of OECD 236. We observed acute toxicity in all 8 methodologies tested, with a clear escalation in sensitivity (larvae > juvenile > embryo). Larvae were the most sensitive life stage for whole-effluent tests. The addition of sublethal metrics to OECD 236 enhanced its previous sensitivity in over 30%. Thus we conclude that OECD 236 acts below its potential and that the embryonic stage (as used in the FET) may not be the most sensitive life stage for whole-effluent tests. Environ Toxicol Chem 2018;9999:1–8. © 2018 SETAC

Keywords: Hazard/risk assessment; Whole effluent toxicity testing; Freshwater toxicology; Fish embryo test; Danio rerio; Ecotoxicological test guidelines

INTRODUCTION

Within the past few years, the substitution of standard ecotoxicological protocols by fairly new animal-friendly methods has been the predominant topic in terms of fish usage for risk assessment. The European Union, Directive 86/609/ECC (European Commission 1986), followed by Directive 2010/63/EU (European Commission 2010), restricted the use of vertebrates for research, triggering a search for new feasible methods to produce ecotoxicological data for vertebrates (Fent 2001; Nagel 2002; Strmac et al. 2002; Castaño et al. 2003). Thus the fish embryo test (FET) has become a candidate for further development. At the moment, and fostered by the 2013 release of Organisation for Economic Co-operation and Development (OECD) test guideline 236 (Organisation for Economic Co-operation and Development 2013), the use of the FET has been spreading worldwide as a reliable method for effluent risk assessment. We have observed an international movement to use the FET as a substitute for previous fish tests with larvae and juveniles, not only in the assessment of chemicals but also for environmental samples and wastewaters. Embryo tests have been widely applied according to the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation of the European Union (European Commission 2006), with the majority of the published data coming from simple mixture exposures (Busquet et al. 2014; Braunbeck et al. 2015; Scholz et al. 2016). In Germany, however,
the FET became mandatory for effluent analysis after 2005 (German Standardization Organization 2001). Nevertheless, more than a decade later, we still lack international peer-reviewed scientific information regarding the FET’s efficiency and equivalence with other fish tests for complex mixtures and effluents.

Unlike simple mixtures, the toxicity of a complex mixture depends on its unpredictable chemical speciation followed by the formation of new compounds (Stewart and Carter 2009), in addition to its additive effect, synergism, and bioavailable fraction. Thus it is expected and understandable that methodologies designed for simple mixture exposures (i.e., OECD 236) may not perform the same for complex mixtures (i.e., effluents). The same holds true for the standard ecotoxicological tests. Hence water managers are lacking comparative quality data for deliberations on effluent assessment and disposal. At the same time, the use of the FET as a whole-effluent test is being increasingly accepted as an improvement in the environmental toolbox for policy-making and environmental protection.

The use of doubtful whole-effluent test data presents a number of risks for environmental management and the assurance of ecosystem services. In many watersheds for which knowledge of the total biodiversity of fish has not yet been established, a less sensitive test may provide doubtful information, which is then used to steer protective measures, policymaking, biomonitoring, and conservation, resulting in insufficient protection for biodiversity in its genetic, organismal, and ecosystemic aspects (Hamilton et al. 2016).

Logically, the use of one less-sensitive test will not be responsible for an ecosystem collapse. However, the failure of environmental projects and policies arises from a pool of tiny unfortunate choices (Fisher 2013). Furthermore, the gap between laboratory results and the real-life effect of complex mixtures on the environment is well known (Heugens et al. 2001; Landis et al. 2013). In terms of the uncertainties surrounding effluent risk assessment, the use of refined tools is a must whenever possible. Thus, based on a precautionary approach to effluent risk assessment, it is strongly recommended to use the most sensitive methods available (Schwarzenbach et al. 2006). Even though a single most suitable test for all effluents is unlikely to exist, much of the issue rests on the lack of direct comparative data for protocols, organisms, and life stages. Thus, an agreement of the most suitable ecotoxicological acute fish test might never be a consensus, and so the critical review of standard methodologies become a constant need to guarantee the best usage of risk assessment tools and defining their most fit scenarios.

Considering the lack of peer-reviewed studies showing the efficiency of the FET for whole-effluent tests, the aim of the present study was to make the first comparative test between the FET (according to OECD 236) and other standard and slightly modified standard fish protocols used worldwide for whole-effluent tests. For that we used a hospital effluent that is directly disposed of into the municipal sewerage system with no further treatment. First we compared the sensitivity of OECD 236 with 7 other fish toxicity protocols in such a way as to provide a comprehensive overview of available tests for whole-effluent tests. Second, we evaluated the addition of 3 virtually costless sublethal metrics that could enhance OECD 236 sensitivity. Finally, we checked the validity of the sublethal metrics and recovery rate by observing the organisms over 72 h after the end of the exposure time. It is important to emphasize that our intention was to compare the response of similar fish tests, and thus a single batch of effluent was used. Thus our results represent an initial step toward understanding the response of the FET for whole-effluent tests, and generalizations must be taken with caution.

MATERIALS AND METHODS

Effluent

The effluent used for our comparative study came from a large hospital complex located in Porto Alegre (RS, Brazil). The hospital complex has an area of 128,339 m² with 843 beds, and produces 830 m³/d of effluent from all its sectors (surgery, wash, toilets, wards, and ambulatory and diagnostic sections). The effluent is considered to be a highly complex mixture with traces of medicines, chemicals, organic pollutants, domestic sewerage, washing products, and their combined byproducts. A complete description of the sample, which was analyzed for the presence of 82 target pharmaceutical compounds, can be found in Souza et al. (2017). Samples were taken from a single batch of 70 L collected directly from the hospital duct before its release into the urban sewage system. Prior to storage, the effluent was homogenized and aliquoted into flasks of 500 mL or 1 L depending on the intended use. Homogeneity of the aliquots was checked by conductivity measurements (using a WTW LF 197 condutivimeter).

A physicochemical fingerprint of the effluent was created just after sampling. We quantified 19 key properties including 4 metals, 5 physical parameters, 4 measures of organic content, and 6 measures of inorganic content (Table 1). Due to the complexity of the sample, targeting of specific compounds proved to be unrealistically expensive for whole-effluent tests. For the ecotoxicological tests, samples were frozen at −27 ± 2°C until the morning of the tests, when they were defrosted and homogenized at room temperature.

Dilution water and tested concentrations

Dilution water was made according to International Organization for Standardization (ISO; 1999) standard 12890 and adjusted to a final hardness of 40 to 47 mg Ca²⁺ L⁻¹ and pH 7.4 to 7.5.

Tests were performed using effluent concentration of 100, 75, 50, 25, 12.5, and 6.25%; negative controls (dilution water); and positive controls with 4 mg L⁻¹ of 3,4-dichloroaniline (Sigma-Aldrich) when requested by norm. Cross-checks of concentrations were made through conductivity measurements (WTW LF 197 condutivimeter).

Cultivation and production of embryos

Mature wild-type zebrafish (Danio rerio) from the Laboratory of Ecotoxicology (Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil) were maintained in mechanically and biologically filtered water at 27 ± 2 °C with a controlled photoperiod
TABLE 1: Physicochemical parameters evaluated for generating a hospital-based untreated effluent fingerprint (example of a complex mixture)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.6</td>
<td>—</td>
<td>Potentiometric</td>
<td>Brazilian Association of Technical Standards 13736 (1996)</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>125</td>
<td>mg CaCO₃ L⁻¹</td>
<td>Titration</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>2.5</td>
<td>mg O₂ L⁻¹</td>
<td>Winkler’s method</td>
<td>Brazilian Association of Technical Standards 11265 (1990)</td>
</tr>
<tr>
<td>Temperature</td>
<td>20.5</td>
<td>°C</td>
<td>Thermometric</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Turbidity</td>
<td>201</td>
<td>NTU</td>
<td>Nephelometric</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>BOD</td>
<td>140</td>
<td>mg O₂ L⁻¹</td>
<td>Winkler’s method</td>
<td>Brazilian Association of Technical Standards 11265 (1990)</td>
</tr>
<tr>
<td>COD</td>
<td>448</td>
<td>mg O₂ L⁻¹</td>
<td>Open reflux</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>TOC</td>
<td>154.4</td>
<td>mg CL⁻¹</td>
<td>Combustion</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>TS</td>
<td>488</td>
<td>mg L⁻¹</td>
<td>Gravimetric 105°C</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Ammoniacal nitrogen</td>
<td>35.6</td>
<td>mg NH₃-N L⁻¹</td>
<td>Nesslerization</td>
<td>Brazilian Association of Technical Standards 105601 (1988)</td>
</tr>
<tr>
<td>Chlorite</td>
<td>42.5</td>
<td>mg Cl⁻ L⁻¹</td>
<td>Titration</td>
<td>Brazilian Association of Technical Standards (1997) 13797</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>3.94</td>
<td>mg PO₄-P L⁻¹</td>
<td>Ion selective electrode</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.61</td>
<td>mg F L⁻¹</td>
<td>Ascorbic acid</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Sulfate</td>
<td>&lt;0.1</td>
<td>mg S⁻² L⁻¹</td>
<td>Electrothermal AAS</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Chromium (total)</td>
<td>15.1</td>
<td>µg Cr L⁻¹</td>
<td>Electrothermal AAS</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Iron (total)</td>
<td>0.803</td>
<td>mg Fe L⁻¹</td>
<td>Total solids</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Lead</td>
<td>7.14</td>
<td>µg Pb L⁻¹</td>
<td>Electrothermal AAS</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.474</td>
<td>µg Hg L⁻¹</td>
<td>FIA–Hg–AAS</td>
<td>American Public Health Association 2005</td>
</tr>
<tr>
<td>Surfactants</td>
<td>4.96</td>
<td>mg MBAS L⁻¹</td>
<td>Methylene blue</td>
<td>American Public Health Association 2005</td>
</tr>
</tbody>
</table>

BOD = biological oxygen demand; COD = chemical oxygen demand; TOC = total organic carbon; TS = total solids; NTU = nephelometric turbidity unit; AAS = atomic absorption spectroscopy; FIA = flow injection analysis; MBAS = methylene blue active substances.

(16:8-h light:dark cycle). Hardness (100–150 mg CaCO₃ L⁻¹), pH (7.4 ± 0.2), and NH₃-N (virtually absent) were checked weekly and corrected whenever necessary. Fish daily diet was adult frozen Artemia and freshly hatched Artemia nauplii ad libitum, 6 d/wk. A maximum proportion of 1 g fish/L water was maintained. The fish were kept in the laboratory for >5 yr free of macroscopically discernible symptoms of infection and disease, and they have never been exposed to any pharmaceutical or other toxicant.

For reproduction, the procedure adopted was the spawning groups as described in OECD 236 (Organisation for Economic Co-operation and Development 2013). Three reproduction events were performed along the comparative study, resulting in three replicates of each test performed. Each replicate (batches A, B, and C) had enough eggs to perform all the comparative tests. Eggs were microscopically checked for fertility (overall fertility >90%) and viability. For the FET, embryos were used up to 90 min post fertilization, and for other comparative methods the organisms were nurtured until they had reached the appropriate age/size for testing following Freiry et al. (2014). For all replicates, mortality rates of spawning events were <20% up to the latest life stage tested (juveniles).

Pimephales promelas (fathead minnow) cultivation and egg production were conducted by Ecotox Analysis and Consultancy (Porto Alegre - RS, Brazil), an external laboratory with a history of partnership with UFRGS and with ISO/International Electrotechnical Commission (IEC) 17025 certification (International Organization for Standardization 2005).

Ecotoxicological tests

So as to compare the different standard methods, the ecotoxicological tests were executed strictly as described in their official protocols. The proposed modifications are described below, and any other test characteristic not mentioned shall be considered in full accordance with the respective base method. A summary of the tests is presented in Table 2. Tests were repeated 3 times (batches A, B, and C), except for the US Environmental Protection Agency (USEPA) method 2000.0 test that strictly followed the standard protocol (US Environmental Protection Agency 2002), which was performed once. The latter method (USEPA 2000.0) uses fathead minnow as test organism, and an external specialized laboratory with a history of partnership and ISO/IEC 17025 certification performed the test. All procedures performed were in compliance with institutional guidelines for research on animals.

OECD 236

The standard method was performed (termed OECD 236 from now on) in addition to 2 modified protocols: the inclusion of sublethal metrics (termed FET 96 h sublethal from now on), and a reduction in exposure time (termed FET 48 h from now on). Immobility, nonhatching, and pericardial edema were used as the sublethal metrics, which were chosen because they are easy to observe, associated extra costs are virtually absent, and little time is consumed. It is important to state that sublethal metrics were only measured when lethal metrics were not observed. Lethal metrics were considered exclusive once an animal was considered dead. When both metrics were present, sublethal effects were disregarded (following the deterministic logic that sensitivity based on sublethal metrics could not be evaluated in a dead animal).

Organisms presenting sublethal effects (FET 96 h sublethal) were observed for another 72 h after the end of the exposure time. Embryos were transferred to Petri dishes containing dilution water and checked daily for recovery signs. Recovery rate was calculated based on the simple ratio between the number of organisms showing sublethal effects before and after recovery time (results in percentage).
OECD 203

The OECD 203 protocol (Organisation for Economic Co-operation and Development 1992) says that the preferred exposure time is 96 h but requests that records be taken every 24 h, providing the freedom to choose when the test shall be finished. Thus the standard method was used over 2 distinct final exposure times (48 and 96 h for Juvenile 48 h and OECD 203, respectively). When the exposure time was defined as 96 h, the test solution was completely renewed 48 h after the beginning of exposure.

USEPA 2000.0

Like OECD 203, USEPA 2000.0 provides flexible exposure times depending on the objectives of the test and the requirements of regulatory authorities (US Environmental Protection Agency 2002). Therefore, we performed the standard test exposing P. promelas for 48 h (USEPA 2000.0), along with 2 variations. Both modifications were executed by changing P. promelas for D. rerio larvae aged 10 ± 2 d. Variations were performed for 2 distinct exposure times (48 and 96 h, termed Larvae 48 h and Larvae 96 h, respectively). Again, when the exposure time was 96 h, the test solution was completely renewed 48 h after the beginning of exposure.

Statistical evaluation

The median lethal concentration (LC50) values were calculated using the probit method (Finney 1978), and the 95% confidence intervals were calculated by the inverse prediction method (Kutner et al. 2004). The LC50 values are presented in terms of the ratio of effluent to dilution water (i.e., LC50 = 30% means a concentration of 30% of effluent and 70% of water section).

Significant differences between LC50 values were determined by comparison of the relative median potency values using SPSS Ver 18.0.3 software. If the 95% confidence interval of the relative median potency between 2 groups was different from 1, then the LC50s were statistically significant (p ≤ 0.05).

RESULTS

Overall comparison of the protocols tested

Acute toxicity was observed in all methodologies tested, with comparative results shown in Figure 1. All juvenile and embryo protocols were less sensitive, and larval tests were the most sensitive. Eight methodologies were analyzed by the overlap of confidence intervals method resulting in 5 distinct sensitivity groups. A clear escalation in sensitivity was observed as follows: FET 48 h (LC50 = 56.5%) ≤ OECD 236 (LC50 = 53.5%) ≤ Juvenile 48 h (LC50 = 48.8%) ≤ OECD 203 (LC50 = 48.2%) < FET 96 h Sublethal (LC50 = 37.3%) < Larvae 48 h (LC50 = 27.0%) ≤ USEPA 2000.0 (LC50 = 18.6%) ≤ Larvae 96 h (LC50 = 10.7%; Table 3 and Figure 1). The LC50 variation within the replicates was very low (coefficient of variation < 10%), except for Larvae 96 h (coefficient of variation < 24.6%). These overall results highlight the robustness of the data obtained.

Comparison within fish embryo tests

All the test repetitions (replicates) from FET 48 h had lower sensitivity than other embryo tests (Table 3). The LC50 values from OECD 236 were consistently lower (more sensitive) than those from FET 48 h; nevertheless, there was no statistical difference between the 2, and their mean LC50 value differed by only 3%. The FET 96 h Sublethal test was significantly more sensitive than other FETs.

The inclusion of very simple and nearly costless sublethal metrics (pericardial edema, nonhitching, and immobility) reduced the LC50 from 53.5% in OECD 236 to 37.3% in FET 96 h Sublethal, translating to an increment of >30% in sensitivity (Table 3). Sublethal metrics were observed only in the 50% concentration in all 3 tests performed (batches A, B, and C). The presence of sublethal metrics overlapped lethal metrics at higher concentrations and was not observed at lower concentrations. Of the 60 organisms evaluated at the 50% concentration (Table 2), 28 had a sublethal effect. This approach was responsible for adding 46% of effect to the previous approach, when only lethal effects were considered. It is important to recall that the presence of lethal endpoints excluded the possibility of evaluating sublethal effects, so this result was exclusively due to the additional effect of sublethal metrics.

Sublethal metrics were evaluated for 72 h after exposure ended to assess recovery rates. From those 28 embryos showing sublethal metrics, only 1 was able to recover (nonhatching), representing a recovery rate of <4%. Thus the sublethal metrics could be considered indicators of acute toxicity (i.e., lethal metrics) once it was shown that recovery was unlikely to happen.

Comparison between fish embryo tests (OECD 236 based) and juvenile tests (OECD 203 based)

No significant difference was observed when FET 48 h, OECD 236, Juvenile 48 h, and OECD 203 were compared. These 4 protocols belonged to the same sensitivity group, and it was impossible to distinguish one from another based on their LC50 values (Figure 1). The FET 96 h Sublethal test was the only group that was detached from all comparisons involving OECD 203 and 236. As described in the previous section, FET 96 h Sublethal was significantly more sensitive than the other juvenile and embryo toxicity tests.

DISCUSSION

The possible equivalence of results between OECD 203 and 236 protocols for simple mixtures has been widely documented in the literature (Braunbeck et al. 2005; Lammer et al. 2009; Belanger et al. 2013). On the other hand, Scholz et al. (2016) showed an average lower sensitivity of the FET compared with juveniles after evaluating 123 chemicals. Our results were on the edge of these 2 scenarios. Although we could not show statistical differences between the methods, the FETs were consistently less sensitive than juveniles. Thus we observed that the use of OECD 236 as a possible equivalent for OECD 203 has a background noise that remains for complex mixtures and should be studied in more detail.
Surprisingly, the comparative sensitivity of the OECD protocols studied was shown to be a secondary issue. The primary issue was related to the overall sensitivity of both tests. Lethal FETs and juveniles were the less sensitive tests evaluated, and their use for effluent risk assessment add up uncertainty to a field surrounded by unknown factors such as the unpredictability of speciation forms, environmental representativeness, and toxicokinetics (Heugens et al. 2001). The positive side is that the addition of sublethal metrics resulted in a remarkable increase in sensitivity. Although the addition of 3 extra sublethal endpoints brought the FET closer to the most sensitive protocols studied, it did not bridge the gap between lethal FETs and larvae tests. Larvae were shown to be the most sensitive life stage for the effluent selected.

### TABLE 2: Summary of the comparative acute toxicity studies performed with a complex mixture at different life stages in Danio rerio and Pimephales promelas

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Embryo</th>
<th>Larva</th>
<th>Juvenile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FET 48 h</strong></td>
<td>OECD 236</td>
<td>US EPA 2000.0</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>OECD 236</strong></td>
<td>OECD 236</td>
<td>US EPA 2000.0</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>FET 96 h Sublethal</strong></td>
<td>OECD 236</td>
<td>US EPA 2000.0</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>Larvae 48 h</strong></td>
<td>OECD 236</td>
<td>OECD 236</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>Larvae 96 h</strong></td>
<td>OECD 236</td>
<td>OECD 236</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>USEPA 2000.0</strong></td>
<td>OECD 236</td>
<td>OECD 236</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>Juvenile 48 h</strong></td>
<td>OECD 236</td>
<td>OECD 236</td>
<td>OECD 203</td>
</tr>
<tr>
<td><strong>Juvenile 96 h</strong></td>
<td>OECD 236</td>
<td>OECD 236</td>
<td>OECD 203</td>
</tr>
</tbody>
</table>

a Tests were performed based on standard toxicity protocols (OECD 236, OECD 203, and USEPA 2000.0) and minor modifications of them using untreated hospital-based effluent.

b USEPA 2000.0 allows organism age between 1 and 14 d, so age was not considered a modification of the method.

c Modification of the base method described in the standard method. When no modification is described, it strictly followed the base protocol.

d USEPA 2000.0 allows P. promelas to be from 1 to 14 d; however, within the same test all organisms should have the same starting age.

e This method allows static or semistatic renewal, so renewal was not considered a modification of the method.

f A negative control was provided in all the tests. A positive control was provided in all the FETs. Controls are not quantified as concentrations in the table. Concentrations were 100, 75, 50, 25, 12.5, and 6.25%.

g Values in parentheses refer to the no. of vials × number of organisms.

h Coagulation, absence of somites, lack of heartbeat, and no tail detachment.

i Nonhatching, pericardial edema, and immobility.

FET = fish embryo test; OECD = Organisation for Economic Co-operation and Development; USEPA = US Environmental Protection Agency.

Surprisingly, the comparative sensitivity of the OECD protocols studied was shown to be a secondary issue. The primary issue was related to the overall sensitivity of both tests. Lethal FETs and juveniles were the less sensitive tests evaluated, and their use for effluent risk assessment add up uncertainty to a field surrounded by unknown factors such as the unpredictability of speciation forms, environmental representativeness, and toxicokinetics (Heugens et al. 2001). The positive side is that the addition of sublethal metrics resulted in a remarkable increase in sensitivity. Although the addition of 3 extra sublethal endpoints brought the FET closer to the most sensitive protocols studied, it did not bridge the gap between lethal FETs and larvae tests. Larvae were shown to be the most sensitive life stage for the effluent selected.

![FIGURE 1: Results of multiple comparisons (confidence interval [CI] = 95%) from the median lethal concentration (LC50) CIs for each of the tested batches (A, B, and C). Different lower case letters and colors represent different sensitivity groups according to the estimation of CIs of the relative median potency (p ≤ 0.05). The y-axis represents the different protocols used for acute toxicity tests. USEPA = US Environmental Protection Agency; FET = fish embryo test; Sub = sublethal; OECD = Organisation for Economic Co-operation and Development.](image-url)
TABLE 3: The LC50 values of the comparative acute toxicity tests performed with a complex mixture at 3 life stages

<table>
<thead>
<tr>
<th>Process</th>
<th>Embryo</th>
<th>Larva</th>
<th>Juvenile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detoxification by gills</td>
<td>–/–</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Chorion protective effect</td>
<td>–</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Intestine tube uptake</td>
<td>+</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Liver formation and functionality</td>
<td>–/+</td>
<td>Partially</td>
<td>Fully</td>
</tr>
<tr>
<td>Free swimming and feeding</td>
<td>+</td>
<td>Static</td>
<td>Functional</td>
</tr>
<tr>
<td>Volume (biomass): toxicant ratio</td>
<td>–</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

a Data are based on a literature review. + indicates that sensitivity is enhanced, and – indicates that sensitivity is reduced.

The tests we compared have remarkable dissimilarities in terms of developmental stages, physiological capacity, and behavior, all of which could interfere with the sensitivity trends observed (Table 4). By day 8 of life (the approximate age used for the larval tests) D. rerio and P. promelas are free-swimmers, and actively seek their own food in later life stages. In the effluent sample studied, in which the amounts of dissolved carbon and suspended solids were high, foraging is likely to be a major exposure route for toxicants through ingestion. Even though some FETs allow use of the posthatch larval stage at 48 to 72 h, larvae are mainly dependent on the vitelline sac until day 5 post fertilization (Scholz et al. 2008). Thus ingestion and foraging may not be a relevant exposure route for early-stage larvae, at which point the intestinal duct is not fully developed (Le Fol et al. 2017). Another difference is that the liver in embryos is not yet fully developed (Tao and Peng 2009). However, it is unclear (and also relative to the sample used) whether biotransformation capacity will increase or decrease overall toxicity. Another important toxicity driver in fish is the detoxification route by transport through the gills. Le Fol et al. (2017) described this route as not yet functional within 96 h post fertilization, so only larvae and adults would be able to take advantage of this physiological process. However, the absence of this mechanism in embryos did not result in an increase in their overall sensitivity. In juveniles and larvae, there is a clear difference in the ratio between the body’s volume and its surface (Hutchinson et al. 1998). This allometric relation between volume (biomass in L³) and surface (uptake in L²) gives juveniles a higher tolerance to toxicants compared with larvae (Le Fol et al. 2017). The combination of both metrics proportionally diminishes the concentration of toxicant per weight (volume) and reduces its relative uptake per area (surface). The differential sum of the processes described above may have determined the differences in sensitivity among larvae, embryo, and juveniles. However, the relative importance of those processes and how strongly they interact in terms of determining fish sensitivity at different life stages have not been the focus of extensive research. Although advanced state-of-the-art research is increasing rapidly (e.g., proteomics and gene expression), the overall ecophysiological trade-off between relevant processes in terms of systemic toxicity and their ecological outcomes is still unknown.

In addition to the processes described, embryos may have the protective effect of the chorion against chemicals, which has been the topic of exhaustive research (Ozoh 1980; Braunbeck et al. 2005; Henn and Braunbeck, 2011; Klüver et al. 2014). An attempt to overcome this protective effect observed on FET was extension of exposure time from 48 to 96 h, allowing the posthatch larvae to enter into contact with the test solution (Braunbeck et al. 2015). However, as described above, the ecophysiological processes expected in FET 96 h are different from the ones observed in larvae and juveniles. In the present study we used an untreated hospital effluent that could be classified as having a highly toxicological potential considering the expected chemical composition, origin, and complexity of the mixture (Verlicchi et al. 2015). Our data show that the posthatch exposure time provided by the 96-h exposure FET was not long enough to compare the sensitivity observed in
larvae or to differentiate 96- from 48-h exposure FET. Nevertheless, the increased exposure time allowed us to use the sublethal metrics, which would have been impossible to observe in the first 48 h. Thus, for the effluent we used, the increase in FET exposure time was an advantageous strategy only if sublethal metrics would be taken into account. Our results corroborated the idea that the chorion may have an important protective effect and that the extension of FET to the posthatch period may not be enough to increase its sensitivity. On the other hand, sublethal metrics were an important upgrade for increasing the sensitivity of FET based on OECD 236.

In the past decade, the European Union has tried to reduce the number of chordates sacrificed for research (Directive 2010/63; European Commission 2010) by changing the previous toxicity tests (e.g. OECD 203) to more animal-friendly protocols (e.g. OECD 236). The results from both of these standard methods were similar (Braunbeck et al. 2005; Lammer et al. 2009; Belanger et al. 2013), and after minor improvements, it would be possible to exchange one for another. However, in the present study, neither of them delivered the most reliable data for effluent assessment. The observed differences in sensitivity between larval tests and OECD 236 were shown to be as high as 5-fold, and juvenile testing following OECD 203 was shown to be 4-fold less sensitive than larval testing. Regardless of the origin, physicochemical composition, and possible synergism present in the effluent, a biased result giving an LC50 value 4- to 5-fold higher than it could be for the same test species (only at a different life stage) has the potential to ecologically, economically, and socially harm integrated water management plans.

Before the present study, researchers mainly called attention to the weak predictive effect of the FET for neurotoxic and narcotic substances (Scholz et al. 2016), for molecules of >3 kDa (Organisation for Economic Co-operation and Development 2013), and even for hydrophilic substances in some instances (Braunbeck et al. 2005). The remaining question is which classes of substances would we expect to encounter in a complex mixture. From a practical perspective, it is not possible to determine which fraction of the effluent is responsible for generating toxicity. Based on the studies published to date, the universalization of the FET for whole-effluent tests does not seem to be a prudent decision.

We added a behavioral (immobility), a morphological (formation of edema), and a physiological sublethal metric (nonhatching) that would not involve major modifications in terms of time, cost, or logistics needed for FET assessments. By so doing, we were able to observe an increase in sensitivity of >30%. Other studies have reported relevant increases in sensitivity after addition of molecular (Hermsen et al. 2012; Beasley et al. 2014; Jeffries et al. 2015), morphological (Lammer et al. 2009; Brannen et al. 2010; Di Paolo et al. 2015), and behavioral endpoints to the FET (Di Paolo et al. 2015), and thus this could be a fruitful solution to the problem of overcoming OECD 236 sensitivity issues. In addition to comparing LC50 values across fish tests for a specific effluent, we have shown ways to improve the sensitivity of the FET without extra costs or administrative burdens. The modifications of OECD 236 proposed in the present study (addition of sublethal endpoints) would be feasible worldwide for any country already using FETs for whole-effluent tests.

CONCLUSIONS

We conclude that for the effluent tested, embryos may not represent the most sensitive approach for whole-effluent test. Our results raise an alert in terms of OECD 236 use for complex mixtures and shine a light on the urgent need for good-quality data that would help us to understand the capabilities of the FET compared with other methodologies and other effluent classes. We argue that the use of OECD 236 as a possible equivalent for OECD 203 may not fulfill the ecotoxicological assumption of representing the most sensitive phase of the organism. We have presented an initial study regarding the use of the FET for whole-effluent tests, and additional studies should be encouraged.

Our focus was not to criticize the use of the FET or to generalize results based on a single effluent. The FET evolved to solve an ethical issue regarding animal use for scientific proposes and has been shown to have the potential to do it well. When different acute fish tests in a real-life effluent were compared, our complex sample indicated a need to complement the FET according to OECD 236 with sublethal metrics in a way that would increase its protective effect. Our results suggest that OECD 236 as it presently stands may be acting below its potential for environmental samples; however, this statement must be validated by further studies using other effluents.

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