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OECD TG 249 as an In Vitro Alternative to Traditional Fish Acute Toxicity Tests

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Abstract:

The OECD Test Guideline (TG) 249, Fish Cell Line Acute Toxicity: RTgill-W1 Assay, provides an ethical, fast, and reliable in vitro alternative to conventional fish acute toxicity testing (TG 203). It uses the RTgill-W1 cell line from the gill epithelium of rainbow trout (Oncorhynchus mykiss), grown in a medium free from proteins and animal components (L-15/ex). Confluent monolayers are exposed to test substances for 24 hours. Cell viability is assessed with three fluorescent dyes: resazurin (for metabolic activity), CFDA-AM (for plasma membrane integrity), and neutral red (for lysosomal integrity). This method produces concentration-response curves to calculate EC_{50} and other endpoints. Interlaboratory validation has shown strong agreement between in vitro EC_{50} and in vivo LC_{50} values for different chemicals. TG 249 is useful for chemical pre-screening, hazard ranking, and application in Integrated Testing Strategies (ITS) and Integrated Approaches to Testing and Assessment (IATA). Benefits include a short duration, no need for live fish, cost savings, and reproducibility. Limitations include lower sensitivity for toxicants that depend on metabolism and difficulty with volatile or very hydrophobic substances. Adopted in June 2021, TG 249 supports the 3Rs principles (Replacement, Reduction, Refinement) and is gaining recognition in regulatory ecotoxicology. Future efforts will aim to improve predictive capabilities and integrate with mechanistic and high-throughput testing methods.

Keywords: TG 249, RTgill-W1, cell line, alternative testing, 3Rs, integrated testing strategies

1. Introduction

Aquatic toxicology studies how chemicals and environmental pollutants affect aquatic organisms. It often uses standardized tests to evaluate deadly and non-deadly responses over short exposure times, usually between 24 to 96 hours [1]. These tests play a key role in assessing ecological risks and making regulatory decisions. The OECD Test Guideline (TG) 203, known as the Fish Acute Toxicity Test, is one of the most established methods. It exposes young fish to various concentrations of a substance for 96 hours to find median lethal concentrations (LC_{50}) [2,3]. However, TG 203 has significant drawbacks. It relies on lethality as the only measure, lacks complete validation, shows variability in tested species and conditions, and raises ethical concerns about using live animals [4,5]. These issues can result in inconsistent outcomes and lead to doubts about its effectiveness for predicting

environmental hazards. Motivated by the 3Rs principles—Replacement, Reduction, Refinement—there is growing interest in alternative methods that are quick, affordable, and do not involve animals [6,7]. In vitro tests using fish cell lines, like the RTgill-W1 cell line from rainbow trout gill tissue, have shown promise. The OECD TG 249 was adopted in June 2021 after thorough interlaboratory validation [8,9].

2. OECD TG 249: Fish Cell Line Acute Toxicity

The OECD TG 249, Fish Cell Line Acute Toxicity: The *RTgill-W1* Cell Line Assay, offers an ethical, fast, and solid in vitro alternative to traditional fish acute toxicity testing, like TG 203 [10]. The assay uses the *RTgill-W1* cell line, which comes from the gill epithelium of rainbow trout (Oncorhynchus mykiss) and is grown in protein- and animal-component-free medium (*L-15/ex*) [11]. Researchers expose confluent monolayers to test substances for 24 hours. They assess cell viability using three fluorescent dyes: resazurin (metabolic activity), CFDA-AM (plasma membrane integrity), and neutral red (lysosomal membrane integrity) [12]. The method creates concentration-response curves to calculate EC_{50} and other toxicity endpoints. Interlaboratory validation showed strong correlations between in vitro EC_{50} and in vivo LC_{50} values across various chemical classes [13]. TG 249 supports pre-screening, hazard prioritization, and integration into Integrated Testing Strategies (ITS) and Integrated Approaches to Testing and Assessment (IATA) [14]. Key advantages include a shorter duration (24 hours), no use of live fish, cost-effectiveness, and high reproducibility. Limitations include less sensitivity for metabolism-dependent toxicants and difficulties with volatile or highly hydrophobic chemicals [15]. Adopted by the OECD in June 2021, TG 249 follows the 3Rs principles and is gaining recognition in regulatory ecotoxicology [16]. Ongoing research aims to broaden its predictive range and merge it with mechanistic and high-throughput methods [17].

2.1 Background

The *RTgill-W1* cell line was created in 1994 from the gill epithelium of rainbow trout (*Oncorhynchus mykiss*) by Bols and colleagues [18]. These cells are known for their epithelial shape, tight junctions, and functional similarity to intact fish gill tissue, making them useful in toxicological and physiological studies [19]. The gill epithelium is a significant target in aquatic toxicology since it is the main site of contact with waterborne pollutants and is crucial for respiration, osmoregulation, and uptake of harmful substances [20]. Initially, *RTgill-W1* cells were mainly used in academic settings to study cellular responses to metals, organic pollutants, and environmental stressors [21]. Early research showed their stability, ease of culture in Leibovitz's L-15 medium without CO_2, and suitability for high-throughput toxicity screening [22]. Their strong growth at 18–20 °C, along with the ability to grow in protein- and animal-component-free medium (*L-15/ex*), added to their importance for standardized testing [23]. Over time, interest grew in using *RTgill-W1* assays for regulatory purposes as part of the global movement to replace live animal tests in ecotoxicology [24]. Collaborative efforts under the OECD Test Guidelines Programme led to thorough interlaboratory validation from 2016 to 2020, confirming the assay's reproducibility, predictive ability, and applicability to various chemical classes [25]. These studies established strong quantitative links between in vitro median effect concentrations (EC_{50}) and in vivo median lethal concentrations (LC_{50}), especially for non-volatile, water-soluble substances [26]. The OECD's adoption of TG 249 in 2021 marked the first regulatory

acceptance of a fish cell line-based acute toxicity assay. This was an important step in integrating animal-free methods into global chemical hazard assessment frameworks [27].

Table 1 – Key Features of OECD TG 249 vs OECD TG 203

Parameter	OECD TG 249	OECD TG 203
Test System	In vitro (RTgill-W1 fish gill ce line)	In vivo (live fish)
Species Origin	Rainbow trout (*Oncorhynchu mykiss*)	Varies (commonly *O. mykiss*, zebrafis fathead minnow)
Exposure Duration	24 hours	96 hours
Endpoints	EC_{50} (cell viability via resazuri CFDA-AM, neutral red)	LC_{50} (mortality)
Ethical Considerations	No animal use; aligns with 3Rs	Requires use of live animals
Cost & Tim Efficiency	Low cost, rapid	Higher cost, longer duration
Regulatory Status	OECD adopted (2021)	OECD adopted (1992)

Table 2 – Common Positive Controls & Reference Chemicals in RTgill-W1 Assay

Positive Control Chemical	Chemical Type	EC_{50} Range (mg/L)	Purpose in Assay
3,4-Dichloroaniline (3,4-DCA)	Aromatic amine	2–4 mg/L	Benchmark control for assay sensitivity
Sodium Lauryl Sulfate (SLS)	Surfactant	5–10 mg/L	Membrane disruption control
Copper (II) sulfate ($CuSO_4$)	Heavy metal salt	0.5–2 mg/L	Metal toxicity reference
DMSO (vehicle)	Solvent control	≤0.1% (v/v)	Solvent tolerance verification

3. Test Principle

The OECD TG 249 "Fish Cell Line Acute Toxicity: *The RTgill-W1* Cell Line Assay" uses a standard 24-well plate format. In this setup, confluent monolayers of *RTgill-W1* cells are prepared to ensure even cell density and surface coverage [28]. Each well receives a specific volume of exposure medium,

and test substances are applied under closely controlled conditions [29]. Exposure lasts for 24 hours in L-15/ex medium, which is a modified Leibovitz's L-15 medium that is free of proteins and animal components. This makes it suitable for chemical testing and prevents interference from serum proteins [30]. The assay is kept at 19 ± 1 °C without CO_2 supplementation, creating optimal conditions for RTgill-W1 cells [31], after exposure in the OECD TG 249 assay, three complementary cytotoxicity endpoints are measured to evaluate cell viability accurately. These endpoints focus on different aspects of cellular health, giving a broad view of toxic effects:

3.1 Metabolic activity – This is measured using the resazurin reduction assay. Resazurin is a blue, non-fluorescent dye that enters viable cells and is converted by mitochondrial and other cellular oxidoreductases to resorufin, a pink, highly fluorescent compound. The strength of the fluorescence directly relates to metabolic activity and cell viability. A decrease in signal indicates problems with energy metabolism or a drop in cell numbers, typically happening early in the cytotoxic process [32].

3.2 Cell membrane integrity – This is assessed using 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). This dye can enter cells but is non-fluorescent until it is broken down by intracellular esterases into carboxyfluorescein, which is fluorescent and stays within cells that have intact membranes. A loss of fluorescence indicates compromised plasma membrane integrity, which is a sign of necrotic or late apoptotic cell death [33].

3.3 Lysosomal membrane integrity– This is determined through neutral red (NR) uptake. Neutral red is a weak cationic dye that passively enters cells and accumulates in lysosomes due to proton trapping. Healthy cells hold onto the dye, but if lysosomes are damaged or the pH is disrupted, this accumulation decreases, leading to lower optical density readings. This endpoint is particularly effective for detecting early sub-lethal toxicity that affects the endo-lysosomal system [34].

3.4 Combination– of these endpoints, the assay enhances reliability and lowers the chances of false negatives. Each parameter focuses on different aspects of cytotoxicity—energy metabolism, membrane permeability, and lysosomal function—thereby identifying various modes of action [35]. From the resulting concentration–response curves, key toxicity values are established, particularly the median effect concentration (EC_{50}). This parameter indicates the concentration of a test substance that reduces cell viability by 50% compared to controls. When compared with in vivo median lethal concentration (LC_{50}) data, EC_{50} values provide valuable insights into potential environmental risks, enabling researchers and regulators to predict acute toxicity without relying on live fish testing [36].

4. Data Generation & Analysis

4.1 The OECD TG 249 protocol produces concentration response curves by exposing *RTgill-W1* cell monolayers to a series of test chemical concentrations. These concentrations typically follow a geometric progression to ensure coverage of both sub-lethal and clearly toxic exposure ranges [37]. After a 24-hour incubation, viability is measured using three complementary endpoints: metabolic activity, cell membrane integrity, and lysosomal membrane integrity. Fluorescence or absorbance data from the resazurin reduction assay, CFDA-AM assay, and neutral red uptake assay are normalized to solvent controls. This gives the percentage cell viability for each tested

concentration [38]. Normalization helps the results account for baseline variability and solvent effects, improving the reliability of toxicity threshold estimation. By plotting the normalized viability data against the test concentrations, researchers establish a concentration response relationship. This forms the basis for determining key toxicological parameters, such as EC_{50}, LOEC, and NOEC values.

4.2 In the assay, researchers evaluate metabolic activity using the resazurin assay. In this method, viable cells convert resazurin, a non-fluorescent blue dye, to resorufin, a pink fluorescent product. The intensity of the pink color is proportional to metabolic function [32]. Cell membrane integrity is assessed with CFDA-AM, a non-fluorescent compound that enters cells and is enzymatically broken down by intracellular esterases to produce fluorescent carboxyfluorescein. This compound remains trapped in cells with intact membranes [33]. Researchers measure lysosomal membrane integrity through neutral red uptake. Living cells actively gather the dye in lysosomes, so a reduction in dye uptake suggests lysosomal damage or compromised membrane stability [34]. Evaluating these endpoints simultaneously captures various modes of cytotoxic action. This reduces the risk of false negatives and increases the assay's predictive reliability [35]. Ultimately, the multi-endpoint data enable the calculation of EC_{50} values, which can be compared directly with in vivo LC_{50} data. This strengthens the assay's usefulness for hazard classification and regulatory decision-making [36].

4.3 To ensure reproducibility, TG 249 requires at least three independent biological replicates, each with technical duplicates or triplicates, under the same exposure conditions [42]. Researchers use 3,4-dichloroaniline (3,4-DCA) as a positive control. The acceptance criteria state that EC_{50} values must fall within the historical range of 2 to 10 mg/L for the assay to be valid [43]. Data analysis usually employs non-linear regression models, such as a four-parameter logistic fit, to derive EC_{50} values and associated confidence intervals [44]. The OECD guideline offers specific guidance for data interpretation. It emphasizes comparing in vitro EC_{50} results with in vivo LC_{50} values, classifying according to chemical hazard categories, and integrating into Integrated Approaches to Testing and Assessment (IATA) [45].

5. Applications

The OECD TG 249 assay acts as an effective pre-screening tool. It helps identify and prioritize chemicals before investing in resource-heavy in vivo testing. This process significantly reduces the use of live fish in line with the 3Rs (Replacement, Reduction, Refinement) [46,49]. Its quick execution and high reproducibility make it a helpful resource for early-stage hazard identification in chemical safety evaluations [47]. In Integrated Testing Strategies (ITS) and Integrated Approaches to Testing and Assessment (IATA), TG 249 works alongside in vitro, in silico, and existing data sources. This collaboration strengthens weight-of-evidence decision-making. It allows regulators to make informed assessments while minimizing animal testing [48]. This approach improves the efficiency and ethical standards of regulatory toxicology workflows. The RTgill-W1 cell line assay goes beyond single-chemical evaluations. It can also test complex environmental samples like industrial effluents, wastewater discharges, and surface water extracts

to detect acute cytotoxicity [50]. These applications are especially valuable in environmental monitoring programs, where the composition of samples may vary or remain unknown [51].
From a regulatory perspective, TG 249 data provide acute toxicity endpoints (EC_{50}). These can be integrated into chemical hazard classification systems, such as the EU CLP Regulation and the UN Globally Harmonized System (GHS) [52]. Furthermore, when combined with mechanistic in vitro assays, TG 249 results can guide the development of safer chemical alternatives. This supports proactive risk management and sustainable chemical design, following green chemistry principles [53]

6. Advantages & Limitations

The Test Guideline has several benefits over traditional fish acute toxicity tests. It avoids the use of live fish, which follows ethical guidelines under the 3Rs principle [54]. The assay is quick, taking only 24 hours, and it is cheap and efficient, needing less test material than OECD TG 203 [55]. It also shows a strong link between in vitro EC_{50} values and in vivo LC_{50} results across various chemical types, which supports its relevance for predictions [56]. Its fit with high-throughput screening platforms improves its usefulness for large-scale chemical evaluations [57]. However, there are still some limitations. The assay might miss toxicity for certain compounds, especially those that depend on metabolism or are neurotoxic, because the RTgill-W1 cell line has a limited ability to transform substances [58]. Testing volatile or very hydrophobic substances requires special care to avoid loss or adsorption [59]. Also, while the model simulates exposure of gill epithelial cells, it can't completely represent the complex interactions within a whole organism, which could impact hazard predictions in some cases [60].

Table 3 – Major Limitations of OECD TG 249 and Mitigation Strategies

Limitation	Impact	Possible Mitigation
Low metabolic capability	Underestimates toxicity of pro-toxicants	Integrate with S9 liver fraction or metabolic co-cultures
Difficulty testing volatile chemicals	Loss of analyte during exposure	Use sealed exposure systems
Difficulty with highly hydrophobic chemicals	Poor bioavailability in aqueous medium	Use passive dosing (silicone O-rings) or solvent carriers
No systemic organism-level effects	Cannot detect behavioral/neurotoxic impacts	Use as part of Integrated Testing Strategies (ITS)

7. Validation & Regulatory Acceptance

Extensive interlaboratory validation of the RTgill-W1 assay, coordinated under the OECD framework, demonstrated high reproducibility across participating laboratories and strong correlation between in vitro EC_{50} values and in vivo LC_{50} data for a wide range of chemicals [61]. Validation exercises involved diverse chemical classes, including industrial chemicals, pesticides, and biocides, confirming

the method's robustness and predictive capacity [62]. Following these results, the OECD formally adopted Test Guideline 249 in June 2021, marking a pivotal milestone in replacing traditional OECD TG 203 fish acute toxicity tests for certain applications [63]. The guideline is now recognized as a regulatory screening tool within the European Union's REACH framework, and it is gaining traction in North America, Japan, and other jurisdictions committed to the 3Rs principles [64], [65]. Its acceptance within global regulatory contexts supports harmonized chemical safety evaluations, enabling data generated under TG 249 to be used in Integrated Testing Strategies (ITS) and Integrated Approaches to Testing and Assessment (IATA), thus reducing reliance on live fish testing without compromising environmental protection goals [66].

8. Future Perspectives

The future development of TG 249 lies in its integration with metabolic activation systems, such as incorporating fish or mammalian liver S9 fractions, to address the current limitations in detecting metabolism-dependent toxicants [67]. Coupling the *RTgill-W1* assay with omics technologies—including transcriptomics, proteomics, and metabolomics—can provide deeper mechanistic insights into toxicant modes of action, enabling linkage of molecular changes to apical toxicity endpoints [68]. Additionally, TG 249 is expected to play a pivotal role in advancing the 3Rs principles in ecotoxicology by further reducing or replacing the need for live fish in acute toxicity testing while maintaining regulatory relevance [69]. As part of Integrated Testing Strategies (ITS) and Integrated Approaches to Testing and Assessment (IATA), the assay can contribute to tiered hazard assessment frameworks, serving as a first-line screening method before more resource-intensive tests are considered [70]. Ongoing international collaborations aim to harmonize assay protocols, expand its applicability domain to cover emerging contaminants, and enhance compatibility with high-throughput screening platforms, supporting broader adoption in regulatory and research contexts worldwide [71].

9. Conclusion

OECD TG 249 marks a significant step in moving toward ethical, efficient, and scientifically sound methods in aquatic toxicology. By using the *RTgill-W1* cell line assay instead of live fish, it follows the principles of the 3Rs while still effectively predicting acute toxicity outcomes. Its fast 24-hour testing format, affordability, and reliability make it ideal for pre-screening chemicals, prioritizing hazard assessments, and aiding regulatory decisions within Integrated Testing Strategies and IATA frameworks. Some limitations still exist, including lower sensitivity to metabolism-dependent toxicants and specific physicochemical challenges. However, ongoing improvements, like integrating metabolic activation systems and omics technologies, promise to broaden its use. As global regulatory acceptance increases, TG 249 is set to become a standard part of modern ecotoxicological assessment, benefiting both environmental protection and animal welfare.

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