

Point of View

It takes a school [of fish] to avoid an environmental collapse: Zebrafish as an outstanding alternative animal model in chemical toxicity screening

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How come the world became a filthier place?

“To waste, to destroy our natural resources, to skin and exhaust the land instead of using it so as to increase its usefulness, will result in undermining in the days of our children the very prosperity which we ought by right to hand down to them amplified and developed.” [Theodore Roosevelt]

In the early 20th century, when Roosevelt wrote the Seventh Annual Message, from which the above excerpt was extracted, his omen was a call for the perverse consequences the economic development at any cost would represent to the natural resources and reinforced the responsibility one generation has with those to come. By this time, the industrial revolution was at full throttle for way more than one century, and its consequences could be seen worldwide. Undoubtedly the transition to new manufacturing processes and the increasing mechanized factory system was a great advancement, providing many technological and architectural innovations, better conditions to produce different goods in an easier and faster way, and an extraordinary increase in agricultural productivity. However, the industrial revolution also led to a considerable rise in the rate of population growth. The associated consequences were an increase in the process of urbanization, characterized by poor working and living

conditions, low wages, child labor, and unprecedented levels of pollution.

The technological explosion of agribusiness and industrial trades consequently led to a tremendous increase in the number of chemical compounds manufactured. In fact, the emergence of the chemical industry as an independent branch is associated with the industrial revolution. For example, the development of the textile and glass industries prompted the initiation of soda production. Also, in the mid-19th century, artificial fertilizer plants appeared in Europe. The chemical industry did not begin on a massive scale until the 19th century when advancements in organic chemistry made the production of a wide variety of compounds possible.

The outcomes of the chemical innovations can be witnessed by checking the Chemical Abstracts Service (CAS) collection, which has a registry system for all completely identified compounds or substances since the early 1800s (<https://www.cas.org/>). The CAS Registry displays 197 million organic and inorganic substances with a daily update of about 15,000 entries. Because of the promiscuous use of chemicals, in industry and agriculture, environmental contamination has become a remarkable global problem. The risk we face from pollutants increases as their levels rise and become part of the exposome.

This is of primary concern in low- and middle-income countries, like Brazil, where public policies continue to stimulate the practice of basing some foreign company's

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processes or services overseas to take advantage of local natural resources and lower costs (low foreign taxes and wages) with minor responsibility to the environmental liabilities. Among other reasons, this is why offshoring has acquired such a bad reputation.

Take the case of Brazilian industrial agriculture to illustrate this phenomenon. Historically Brazil has had an agricultural-based economic development. However, as a post-colonial territory, the main activities are characterized by large-scale monoculture associated with heavy use of chemical fertilizers and pesticides based on a few crops that overwhelmingly end up as animal food or biofuels later exported. This highly chemically dependent model dramatically impacts the natural environments and contaminates water, soil, and air. Brazil is not only one of the main pesticides consumers but also the largest buyer of Highly Hazardous Pesticides (HHPs), which contain active ingredients with extremely acute toxicity, persisting in the environment and organisms, with chronic adverse effects on human health and the environment even with very low levels of exposure. The approval of pesticides in the country has been increasing since 2016. Brazil ended 2021 with 562 new pesticides approved, the highest number in the historical series started in 2000 by the Ministry of Agriculture (<https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/agrotoxicos/informacoes-tecnicas>). Furthermore, many of the pesticides accredited in the last three years are banned in Europe and the United States.

Why do we need finer and more reliable alternative animal models for high-throughput chemical toxicity screening?

The use of experimental animal models in research has changed greatly in the last decades. The demand for animal welfare and practices based on the 3 Rs principles in animal experimentation has positively shifted the course of 21st-century science. The 3 Rs stand for Replacement, Reduction, and Refinement, and that means scientists should avoid or replace the use of animals when possible or make the most to reduce their number and distress during the tests. This is somewhat eased by the increase in the bioinformatics and data analysis approaches that allow early investigations that minimize the use of animals as well as all the range of *in vitro* methods using cultured cells, which can be both in the traditional monolayer growth or in novel 3D models like organoids and tissue-like compositions seeking to better resemble realistic scenarios. Such changes are extremely critical, although they impose some challenges in toxicity investigations since research involving animals is required to secure and enhance human and animal health and safeguard the environment.

In the unavailability of human data, research with experimental animals is the most reliable manner of identifying key toxic properties of chemical substances and estimating

risks to human and environmental health. Additionally, tests with whole organisms are required in ecological health studies since they are way more focused on the one health perspective and environmental risk assessment or when non-humans are the target of investigations. Scientists need to study natural conditions to understand biological processes at a cellular and molecular level and assess how adding a specific substance can change living systems. Since the chemical reactions run all life processes, when a substance is introduced into an animal, it can interact in many places throughout the whole body, and effects upon one process can cause unexpected consequences in others. Therefore, using animals in experiments is critical because such complexity cannot be explored through *in vitro*, *in silico*, or *in chemico* means.

So, if you are still wondering, “Will we need experimental animals in the future?” the answer is “Yes,” but to a less extent and in the last stages of research when the repertoire of putative negative effects has already been compiled by other platforms, notably New Approach Methods (NAMs). At the very least, whole animal testing will still be needed to validate the results of methods that do not use whole animals and as a last protective step before exposure of humans and animals to potentially dangerous substances. Besides, they will continue to be important agents in ecotoxicology studies as new chemicals and combinations are studied and complex environmental conditions are taken in account. However, there might be a tendency to use models that enable fast and high-throughput screening since the number of molecules to be tested tends to expand continuously.

Why is zebrafish one of the frontrunners when it comes to the future of toxicology?

Zebrafish (*Danio rerio*) is a teleost fish from the Cyprinidae family, originally found in Southeast Asia. Adult zebrafish typically range between 2 and 3 cm in length and are well known to be highly prolific. In their natural habitats, this species exhibits clear reproductive seasonality. However, they reproduce all year round under controlled conditions. Each female can produce hundreds of eggs per week. Such a high fecundity has made zebrafish an excellent model for studying toxicology since it can provide scientists with a huge number of embryos to test many chemicals. Once there is an ever-expanding demand for toxicity screening due to the novel chemicals released every day, on the other hand, it is also necessary an experimental model in an equivalent number to work at the same pace. That is why we used the collective noun for a group of fish in the title, as a reference to the high fecundity of this species and its applicability in (eco)toxicological research to create scientific-based evidence to support the regulation and control of the chemical contamination.

The zebrafish embryogenesis happens *ex vivo* and rapidly. The entire body plan is established 24 hours post-fertilization (hpf), hatching occurs after around 50 hpf, and

most internal organs like the heart, liver, kidney, and intestine are developed by 72 hpf. Moreover, the larvae begin to feed on day 5. That is why according to specific regulations, such as Directive 2010/63/EU, zebrafish is not considered an animal before 5 days old, and no legislation governing animal testing applies in this phase. Due to the optical transparency of the embryo-larval stages, it is possible to visualize organs, cells, and tissues in real-time and follow the development by identifying normal or altered phenotypic characteristics during screening and assessment of toxicity endpoints. Furthermore, zebrafish screenings reveal crucial information about absorption, distribution, metabolism, excretion, and toxicity of compounds, as zebrafish larvae have functional liver, kidney, and blood-brain and blood-retinal barriers.

Due partly to the ease of husbandry, reduced housing costs, and all the marvelous features abovementioned, the zebrafish has been actively studied for a century in life science research. It was recognized as a tool for developmental biologists in the 1970s. Since 1980, the zebrafish has become a popular model organism to study many aspects of human behavior, development, and diseases. Then, in the 1990s, zebrafish were used for the first vertebrate large-scale mutagenesis screen, yielding thousands of mutations, some of which recapitulated human diseases and started to be a common model in ecotoxicology. One of the first scientists to give zebrafish the spotlight was George Streisinger (1927-1984) that used diploid homozygote fish for the genetic analysis of development.

For the future, much more is yet to come, mainly because there is a massive demand for alternative models in animal experimentation, and zebrafish is considered a bridge between *in vitro* and *in vivo* models since it can be raised in a lab plate but at the same time present all the features and complexity of a whole vertebrate organism. Additionally, after the endeavor to publish the zebrafish reference genome in 2013 (Nature 496, p. 498), zebrafish became even more powerful as a translational model since the genome is strikingly similar to the human.

The knowledge gained from the molecular studies revealed that zebrafish possess 26,206 protein-coding genes, and a direct comparison with humans shows that 71.4% of human genes have at least one zebrafish orthologue. Reciprocally, 69% of zebrafish genes have at least one human orthologue, representing a 70% homology between species. The genetic homology is lower than between humans and mice (85%); however, zebrafish have cardiovascular, nervous, and digestive systems, and a repertoire of inflammatory cells, mediators, and receptors like those in mammals, including humans, besides other advantages that compensate for the lower genetic homology. On top of that, 84% of genes that cause human genetic diseases have a zebrafish counterpart, making it an excellent model for studying human gene

functions on diseases, including some multifactorial ones which can be triggered by environmental pollutants like metabolic diseases, gastrointestinal illness, reproductive problems, and neurological disorders.

Zebrafish is amenable to molecular analysis through genetic manipulation and genome editing. In the last few years, the use of tools such as Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 (CRISPR/Cas9), RNA interference (RNAi), Zinc-finger nucleases (ZFNs), and antisense oligonucleotide morpholinos for the determination of temporal and spatial gene expression, label different cell types by fluorescent markers, examination of specific gene function by transgenic development, gene knockdown, and through large-scale mutagenesis has increase dramatically representing an enormous leap in knowledge.

Another thing that must be considered during toxicity screening is the amount of residues generated. In that regard, the diminutive size of zebrafish embryos and larvae minimizes the use of test solutions or environmental samples to low amounts, as less as 50 microliter volumes, thus creating limited volumes of waste for disposal. Furthermore, it allows multiple samples on a large scale to be tested in parallel using a single cell culture dish or a series of Petri dishes to provide multiple experimental replicates simultaneously quantified by high-level automated tools. Automation facilitates the toxicity screening of a high number of compounds, which enables whole libraries to be prescreened for potential toxicity.

Fish are one of the main allies in ecotoxicology, and zebrafish can contribute greatly to the advance in the knowledge of hazards and risks of chemicals present in ecosystems, novel substances reaching the environment, and their combination. However, when it comes to ecological assessments, it still might be challenging to extrapolate results considering zebrafish is not a native species. Also, its use is more recommended for laboratory screening and early prediction rather than higher ecological levels evaluations.

The study of the harmful effects of chemicals on living organisms is part of the strategy to ensure the conservation of natural environments. It requires that a continuous monitoring program provide information on the toxicity of environmental pollutants, identifying dose-response relationships and mechanisms of toxicity. This process can be catalyzed by a unique combination of strengths provided by zebrafish and its numerous attributes. Of course, that might be facilitated by computational technology and data science. Still, emerging models such as zebrafish will help avoid environmental collapses by improving the knowledge of the toxicity of hazardous environmental contaminants. How we respond now will certainly determine the future and the long-term benefit to people and all life on Earth.

