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Assessment of Toxic Effects of 2-Ethylhexanoic Acid on Zebrafish Embryos Using an Automated Phenotyping Method

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This study uses an automated phenotyping method to investigate the toxic effects of 2-ethylhexanoic acid (2-EHA) on zebrafish (*Danio rerio*) embryos. Concerns have been raised over 2-EHA's possible developmental toxicity due to its widespread industrial use and structural similarities to the teratogenic substance valproic acid. Automated phenotyping method was used with FishInspector software to improve the consistency of toxicological assessment. Different concentrations of 2-EHA were administered to zebrafish embryos under OECD 236 Fish Embryo Acute Toxicity (FET) test guidelines and effects were observed on multiple developmental endpoints. The KNIME and R were used for statistical analysis of morphological data, to identify phenotypic patterns and responses. The exposure to 2-EHA changed the jaw-to-eye distance, decreased swim bladder inflation, and resulted in craniofacial abnormalities. The morphological changes observed were as expected, the valproic acid, also caused malformations in the same body parts and organs. These results support the theory of grouping compounds according to their phenotypic patterns of action and add to the increasing amount of data demonstrating the effectiveness of automated toxicological approaches, which may have potential applications for regulatory practices in assessing chemical safety.

Contents

1. INTRODUCTION

As industrial production increases each year, understanding the toxicological effects of chemical production becomes increasingly critical. The rising number of industrial chemicals and other hazardous substances has led to the development of comprehensive regulatory systems for evaluating their risks to human health and the environment (OECD, 2014; Scholz et al., 2013). One such chemical is 2-ethylhexanoic acid (2-EHA), which is used as an additive in the production of plasticizers, paint, and ink. Structurally related to valproic acid, a compound known for its teratogenic effects that cause developmental abnormalities, 2-EHA has been shown to have similar effects in previous mammalian studies.

However, there were concerns over animal welfare and the ethical considerations related to mammalian testing which have led to the development of different models in toxicology. In 2017, over 2.18 million animals, including 172,000 fish, were used in the European Union for regulatory toxicity assessments, raising ethical concerns (European Commission, 2020). The EU's REACH regulation, alongside animal welfare legislation, encourages the use of alternative methods based on the 3Rs principle—Replacement, Reduction, and Refinement (Russell and Burch, 1959; Brotzmann et al., 2020).

One alternative model to mammalian testing involves early developmental stages of lower vertebrates, such as fish, which are, until the stage of independent feeding, considered non-protected under current EU animal welfare regulations (EU 2010; Strähle et al., 2012). Over the past two decades, the zebrafish *(Danio rerio*) embryo has become one of the most promising models for both ecotoxicity and mammalian toxicology testing (Brotzmann et al., 2020). Zebrafish are small cyprinids that are cost-effective, easy to maintain and breed in large numbers. Their *ex-utero* developing, transparent embryos allow continuous observation of developmental disorders without the need for a protected model within a mammalian host (Braunbeck et al., 2014). Notably, zebrafish data from screening tests have shown an agreement of at least 80% with mammalian developmental toxicity results (Brannen et al., 2010; Nagel, 2002), as well as with rodent models and even human data (MacRae and Peterson, 2015). Approximately 84% of the genes linked to human diseases, along with many drug metabolism pathways, are shared between humans and zebrafish or have a zebrafish equivalent (Howe et al., 2013; MacRae and Peterson, 2015; Uechi and Kenmochi, 2019; Brotzmann et al., 2020). Additionally, around 70% of human genes have at

least one clear zebrafish orthologue (Howe et al., 2013). The effects observed in zebrafish often correspond to those seen in mammals, showing the value and effectiveness of the zebrafish embryo model in detecting potent mammalian toxicants (Brotzmann et al., 2020).

Traditionally, toxicological assessments in zebrafish depend on visual inspection and scoring of morphological changes by a human observer, a method that is subjective and prone to variability. Considering this limitation, in this study, we employed a 2D image-based method for detecting and quantifying morphological features in zebrafish embryos using an automated system to position the embryos in a capillary. Multiple morphological features were automatically extracted from the zebrafish images using custom MATLAB-based FishInspector software. The process was set up to automatically position objects in a capillary but can also be used for placing embryos manually as demonstrated in previous research (for example Peravali et al., 2011). Following that step is the use of the KNIME platform and R scripts, for analyzing shapes and quantities based on the positions of specific characteristics identified by FishInspector (Teixidó et al., 2019).

This study aims to assess an automated phenotyping method for identifying toxicological effects resulting from the interaction between 2-EHA and zebrafish embryos. The results of this investigation add to the increasing amount of data demonstrating the effectiveness of automated toxicological approaches, which may have potential applications for regulatory practices in assessing chemical safety, and environmental risks and hazards.

2. LITERATURE REVIEW

2.1 Chemical profile of 2-ethylhexanoic acid

2-Ethylhexanoic acid (2-EHA) is a widely used carboxylic acid in industrial processes, especially in the production of plasticizers, stabilizers, and paint. With its structural similarities to valproic acid, a known teratogen, concerns have been raised about its potential toxic effects, particularly regarding developmental toxicity and teratogenicity.

2.1.1 Physicochemical properties and its environmental fate

Depending on their physical and chemical characteristics, the chemicals are absorbed by organisms at varying rates. This results in variations in the time it takes for an organism's internal chemical concentration to reach its highest point or a stable, balanced state (equilibrium). The rate at which organisms absorb chemicals can also influence how they behave in the environment. A chemical may remain in the environment for a prolonged period and accumulate in ecosystems if it is absorbed or degrades slowly. The absorption rate and environmental persistence are important to understand how chemicals affect different environmental phases.

Table 1 shows the physical and chemical properties of 2-EHA, including its solubility, pK_a(acid dissociation constant), and log K_{ow} (octanal-water partition coefficient), which gives the data on its environmental fate and potential risks. The compound's low solubility in water suggests its limited mobility in aqueous environments, reducing the potential for groundwater contamination. Being soluble in dimethyl sulfoxide (DMSO) and other organic solvents, on the other hand, shows its capability to persist in organic phases.

When 2-EHA enters the terrestrial environment, it is expected to have low mobility in soils due to a K_{oc} (soil adsorption coefficient) value of 650, and pK_a 4.9 shows that it can exist in both ionized and neutral forms depending on environmental pH. The pK_a value influences bioavailability and interactions with biological systems. Under slightly acidic to neutral conditions, 2-EHA may partially ionize, reducing its ability to adsorb to soil organic matter. Because anions do not bind to soil as effectively as neutral molecules, soil organic carbon content may impact the partial ionization of 2-EHA (NCBI, 2024).

Due to its adsorption onto suspended solids and sediments, 2-EHA has limited mobility in aquatic environments. Volatilization from water is predicted to be a slow process, approximately 15 and

120 days. The log K_{ow} value of 2.64 indicates moderate hydrophobicity. 2-EHA has a low bioconcentration factor (BCF) of 3, indicating a limited potential for bioaccumulation. Yet 2-EHA can still build up in aquatic organisms' lipid-rich tissues, endangering bottom-feeding species and benthic ecosystems (NCBI, 2024).

If released into the atmosphere, 2-EHA may undergo photodegradation. However, its vapor pressure is low, thus limiting the distribution in the atmosphere. 2-EHA will only be in the form of vapor, which in further be degraded with hydroxyl radicals. The half-life of this photodegradation process lasts for 2 days. Particulate 2-EHA will be removed from the atmosphere through wet or dry deposition processes (NCBI, 2024).

Table 1. Physicochemical properties of 2-ethylhexanoic acid (National Center for Biotechnology Information, 2024).

Compound Name	Formula	Molecular Weight (g/mol)	CAS Number	SMILES Canonical	Solubility (DMSO 10 mmol/L)	Solubility (H_2O mg/L)	Density (kg/L)
2-Ethylhexanoic Acid	C8H16O2	144.214	149-57-5	CCCCC(CC)C(O)=O	Soluble	Insoluble	0.95

pK_a(Acid)	Neutral Form (pH 7.4)	Negative Form (pH 7.4)	Positive Form (pH 7.4)	log K_{ow} (L/L)	log D_{lipw} (lip/water, pH 7.4)	Zebrafish Baseline (µmol/L)
4.9	0	1	0	2.64	1.51	5310

2.1.2 Comparison to valproic acid

2-EHA and valproic acid (VPA) share several toxicological mechanisms due to their structural similarities (Figure 1), especially in their capacity to inhibit histone deacetylases (HDACs). This inhibition disrupts gene expression and causes developmental malformations. Due to its greater affinity for HDACs and a wider spectrum of effects on gene regulation, VPA has more severe teratogenic effects than 2-EHA, causing congenital abnormalities such as neural tube

malformations (Nau et al., 1991). In humans, the application of VPA as an anticonvulsant drug increases the risk of neural tube defects by a factor of 10–20 (Spiegelstein et al., 2003).

Figure 1. Chemical structure similarities between 2-ethylhexanoic acid, valproic acid

While both compounds have a teratogenicity effect through HDAC inhibition, 2-EHA has a narrower spectrum of developmental abnormalities, showing changes mainly in skeletal malformations. Valproic acid, on the other hand, has a much broader range of effects including neurological deformities (Brotzmann et al., 2020).

The study by Brotzmann et al (2020) assessed the neurotoxic effects of valproic acid and 9 analogs. One of the analogs was 2-ethylhexanoic acid. A comparison of the ten test substances based on their EC_{10} values (Figure 2) identified 2-n-propyl heptanoic acid, valproic acid, 2-ethylhexanoic acid, and 4-ene valproic acid as the most toxic to fish embryos. Notably, these four substances, which exhibited the highest levels of toxicity in zebrafish embryos, also caused exencephaly in mice.

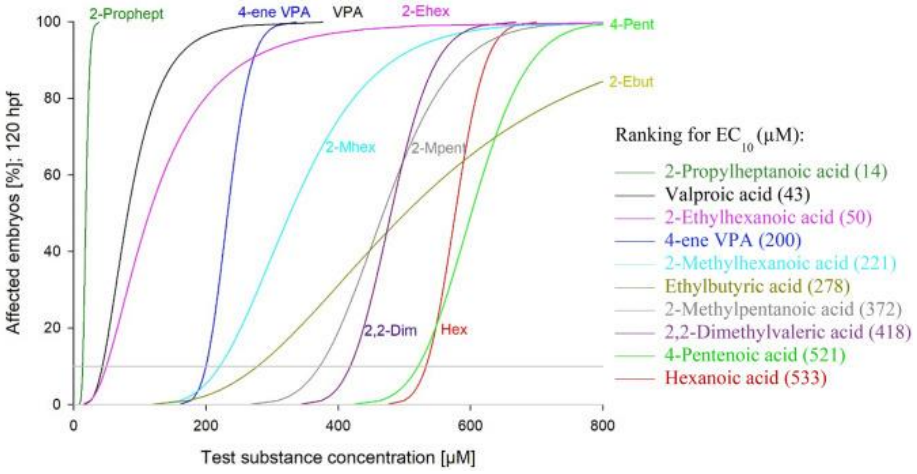

Figure 2. Cumulative percentage of zebrafish embryos (n = 20-40) showing lethal or sublethal effects after 120-hour exposure to valproic acid and its nine analogs, ranked in order of overall toxicity (Brotzmann et al., 2020).

2.1.3 Mechanisms of teratogenicity and metabolism

The main way that 2-EHA induces teratogenic effects is by inhibiting histone deacetylases (HDACs), which control the transcription of genes connected to development. Genes regulated by the transcription factor PPARδ (peroxisome proliferator-activated receptor delta) are derepressed when 2-EHA inhibits HDACs. Deregulation of this gene leads to developmental abnormalities and other harmful consequences. Skeletal abnormalities and other impairments have been associated with this mechanism in animal experiments, especially in the progeny of exposed individuals ("2-Ethylhexanoic Acid [MAK Value Documentation, 2002]", 2015).

In addition to HDAC inhibition, 2-EHA induces the production of metallothionein, a zinc-binding protein in the liver, which reduces the availability of zinc to the developing fetus. The teratogenic consequences seen in studies, such as delayed development and skeletal abnormalities, are exacerbated by this decrease in zinc levels (Bui et al., 1998).

Studies on rats have shown that within 24 hours, 20% of the prescribed dose of 2-EHA is eliminated as a glucuronic acid conjugation. The majority of the chemical is removed by urine, with minor amounts passing through feces (English et al., 1998). This metabolic profile demonstrates the compound's quick clearance and its tendency to build up in tissues.

2.1.4 Developmental and teratogenic effects

Studies conducted on Wistar rats show that 2-ethylhexanoic acid has embryotoxic qualities that result in reduced average fetal weight and an increase in skeletal abnormalities such as adactylia (missing digits), tail deformities, and abnormalities of the spinal column. Exposure to the substance resulted in a large increase in skeletal abnormalities, such as extra vertebrae, twisted necks, additional ribs, and delayed skeletal development, even if the increase in visceral malformations was not statistically significant ("2-Ethylhexanoic Acid [MAK Value Documentation, 2002]", 2015).

In mammalian research, 2-EHA has been shown to have teratogenic effects in zebrafish embryos, resulting in neurodevelopmental abnormalities like tremors, craniofacial deformities, and eye anomalies. Studies on zebrafish have shown a 75% concordance between the neurotoxic effects seen in fish embryos and those in mice and other mammalian models. Specifically, jitter/tremor and craniofacial deformities were observed in zebrafish embryos, which are analogous to neural tube defects found in mammals (Brotzmann et al., 2020).

2.1.5 Subchronic toxicity in mammals

Subchronic oral administration to 2-EHA had a significant effect on the liver and metabolic processes in rat and mouse investigations. Rats and mice were given meals containing varying amounts of 2-EHA for 13 weeks. Higher doses (1.5%) caused alterations in liver function, including increased liver weight and hepatocyte hypertrophy, but no mortality or substantial clinical symptoms of toxicity were observed in the trial. Both male and female rats exhibited elevated cholesterol levels, which returned to normal after a recovery period. Histopathological changes in the liver were reversible following the recovery phase (Juberg et al., 1998).

Similar effects were observed in mice, reduced triglyceride levels, and liver hypertrophy. Significant alterations happened at the highest doses, and these effects were dose-dependent. Male rats' no-observed-adverse-effect level (NOAEL) was 61 mg/kg/day, whereas female rats' NOAEL was 71 mg/kg/day, according to the study. Male and female mice had NOAELs of 180 and 205 mg/kg/day, respectively (Juberg et al., 1998).

2.1.6 Human occupational exposure

In industrial settings, 2-EHA exposure occurs mostly through skin contact or inhalation, especially in sawmills that use wood preservatives containing 2-EHA. The inhibition of citrulline synthesis in the urea cycle may be the reason for the higher urine excretions of arginine and ornithine among workers in Finnish sawmills that used Sinesto B, a wood preservative that contains 26% sodium salt of 2-ethylhexanoic acid (“2-Ethylhexanoic Acid [MAK Value Documentation, 2002]”, 2015). This result supports the possibility of the idea that 2-EHA exposure may cause biochemical disruptions in people. At a production facility, reports of acute dermal or inhalation exposure to 2-ethylhexanoic acid between 1989 and 1996 included cases of skin, eye, and airway irritation. There was one recorded case of corneal corrosion, but it was treated (McLaughlin, 1946).

2.2 Zebrafish as a model organism

Increasing emphasis on animal welfare in toxicity testing led to zebrafish (*Danio rerio*) becoming a widely favored organism. The 3R principles—refine, reduce, or replace animal testing—were first presented by Russell and Burch in 1959 as a foundation for creating replacement approaches, which aim to either completely replace animal tests, reduce the number of animals used, or refine tests to minimize animal suffering. The 3Rs have been incorporated into modern legislation and regulations. A major challenge with adopting alternative methods is producing results that are

comparable to animal tests, many of which lack proper validation and have inconsistent reproducibility. The main framework for animal welfare in research is Directive 2010/63/EU, which replaced Directive 86/609/EEC and emphasizes the 3Rs in scientific procedures across EU member states (Ban on Animal Testing, 2023). The REACH regulation (Registration, Evaluation, Authorization, and Restriction of Chemicals) (European Union, 2006) continues to require ecotoxicity data for chemical safety assessments, which encourages non-animal testing techniques. Indirectly influencing the development of alternatives in environmental testing. Later, the EU Cosmetics Directive's 7th Amendment banned vertebrate animal testing for cosmetics in 2013. These principles are further supported by several national legislations, such as the UK Animal Protection Act (DEFRA, 2006) and Germany's guidelines limiting animal use in wastewater effluent testing (DIN, 2001; ISO, 2007). Globally, initiatives such as Canada's Domestic Substance List (Canada Gazette, 1999), REACH, and the OECD/US EPA HPV Challenge (OECD, 2004) emphasize the growing demand for fish toxicity data.

In July 2013, the OECD introduced guideline 236, the "Fish Embryo Acute Toxicity Test (FET)" for chemical testing. Although this guideline was primarily designed for zebrafish (*Danio rerio*), it can also be used with other small laboratory fish species like medaka (*Oryzias latipes*) and fathead minnow (Pimephales promelas) (Braunbeck et al., 2005; Braunbeck et al., 2014). The Fish Embryo Test (FET) is now widely recognized as a reliable alternative for acute fish toxicity testing, particularly under Directive 2010/63/EU, which excludes non-feeding stage (instead of relying on nutrients from the yolk), which in zebrafish is considered to last until 120 hours' post-fertilization (hpf) (Strähle et al., 2012). Zebrafish, as predators, depend on active swimming, prey detection, and coordinated body movements to effectively feed on their own. While body contractions begin on the first day of development, free swimming behavior in tanks typically becomes noticeable only around 120 hpf (Braunbeck et al., 2014).

2.2.1 Benefits of using zebrafish in toxicity testing

The zebrafish, *Danio rerio* (Hamilton-Buchanan, 1822), indigenous to the Ganges River, Burma, the Malacca Peninsula, and Sumatra (Eaton and Farley, 1974; Talwar and Jhingran, 1991), is a small, benthopelagic cyprinid fish. Zebrafish complete their life cycle in three months and grow quickly in both soft and hard waters at 26°C. They are a cost-effective model organism for study because they are affordable, simple to care for, and, given the right circumstances, may lay vast

numbers of transparent eggs (Laale, 1977). Females, with enlarged bellies, can lay 50 to 200 eggs a day, while males are distinguished by their thinner bodies and the reddish tint to the silvery bands on their sides. Zebrafish are ideal models for researching toxicological effects on vertebrate development and disease because of their short life cycle and roughly 70% genetic resemblance to humans (Scholz et al., 2008).

The transparency of the egg envelope, known as the chorion, allows for microscopic observation of embryonic and organ development. According to multiple research, zebrafish embryos fully grow at 26°C in 96 hours (Hisaoka and Battle, 1958; Kimmel et al., 1995; Laale, 1977).

Figure 3 shows the different stages of zebrafish organ development. In the light boxes, the key organs, including the heart, liver, kidney, gills, and brain (cerebellum, olfactory bulb, and telencephalon), are targeted for toxicity screening, and the specific endpoints used to assess toxicity (shown in dark boxes). Each organ is linked to various endpoints, such as heart rate, liver necrosis, and renal clearance, making zebrafish an ideal model for studying normal development and the teratogenic or embryotoxic effects caused by chemical exposure. This makes it possible to study both normal development and the teratogenic or embryotoxic effects caused by chemicals.

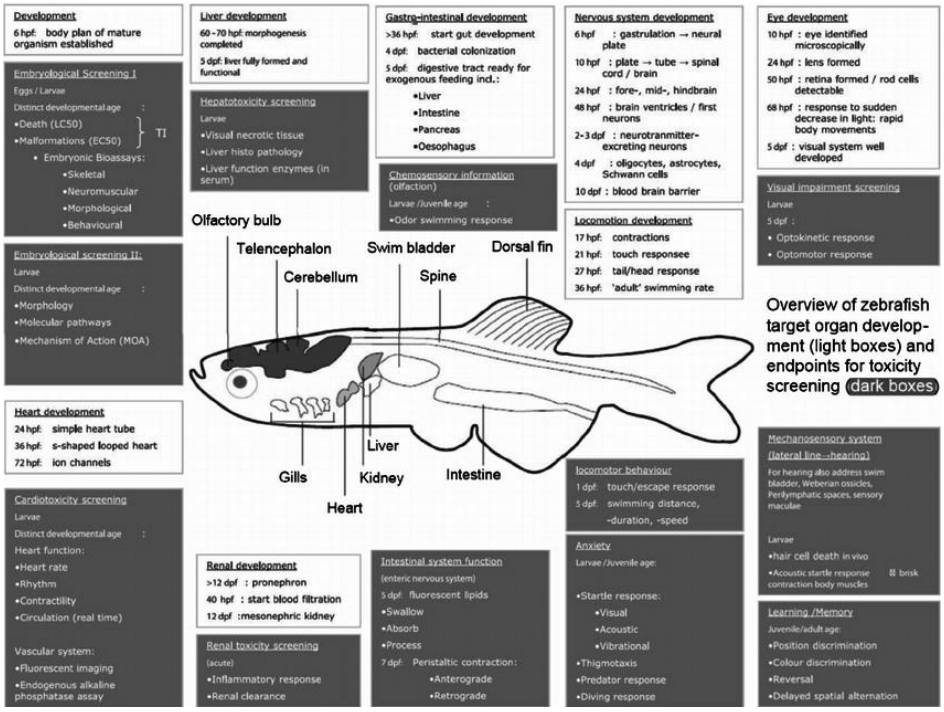

Figure 3. Zebrafish organ development and toxicity screening endpoints (TI: Teratogenic Index) (Esch et al., 2012).

Another advantage of zebrafish as a model organism is its ability to adapt to different environmental conditions, such as exposure to a medium with low oxygen tension and variable oxygen levels. Zebrafish embryos showed no signs of development retardation or deformity, even at concentrations as low as 2 mg/L. When testing sewage effluents under oxygen-depleted conditions, this adaptability is especially valuable. Their suitability for evaluating small quantities of test compounds, such as pesticide metabolites, underscores their versatility, as they can be assessed in minimal medium volumes. However, for highly lipophilic compounds, glassware may be required instead of microtiter plates to prevent absorption and ensure accurate measurements (Braunbeck et al., 2005).

Small-scale pilot studies have shown a strong agreement between zebrafish and mammalian developmental toxicity, with an overall concordance ranging from 72% to 92% (Brannen et al., 2010; Hermsen et al., 2011; Krupp, 2016; Selderslaghs et al., 2009; Van den Bulck et al., 2011; Teixidó et al., 2019). Brotzmann et al. (2020) compared the in vivo mouse potencies for exencephaly and effects observed in zebrafish embryos in Table 2, which showed the concordance between zebrafish and mammalian models by using valproic and its 9 analogs. Green-shaded fields show consistent trends between mouse and zebrafish data, red-shaded fields highlight conflicting trends, and grey-shaded fields represent substances for which mouse data are unavailable. "+" indicates the effect is present, while "- "indicates the effect is absent. 2-EHA showed consistent results for both mouse and zebrafish embryos.

Table 2. Comparison of in vivo mouse potencies for exencephaly with observed effects in zebrafish (*Danio rerio*) embryos (Brotzmann et al., 2020).

		Valproic acid	4-*ene* Valproic acid	2-Propyl heptanoic acid	2-Ethyl hexanoic acid	4-Pentenoic acid	2-Ethylbutyric acid	2,2-Dimethyl valeric acid	2-Methyl hexanoic acid	2-Methyl pentanoic acid	Hexanoic acid
Mouse	*In vivo* exencephaly	+	+	+	+	–	–	–	–	?	?
Zebrafish embryo	Small eyes	+	+	+	+	–	±[1]	+	–	–	–
	Jitter/tremor	+	+	+	+	–	–	±[2]	±[2]	–	±[3]
	Craniofacial deformation	+	+	+	+	+[4]	–	+	±[4]	+	+

Zebrafish have become a key model in neurobiology, toxicology, and molecular and developmental biology (Ekker and Akimenko, 1991; Goolish et al., 1999; Hisaoka and Battle, 1958; Kimmel et al., 1995; Nüsslein-Vollhard, 1994). Their ability to undergo transgenic modifications, and in vivo, genome editing using techniques like CRISPR/Cas9 (Hruscha et al., 2013; Hwang et al., 2013), makes zebrafish embryos a highly attractive alternative model for developmental toxicity testing (Sipes et al., 2011). Since understanding thalidomide's toxic mode of action (Ito & Handa, 2012), zebrafish have been considered a promising model for teratogenicity screening in both fish and mammals (Brannen et al., 2013; Busch et al., 2011).

While the Fish Embryo Test (FET) is primarily used for acute toxicity assessment, it is also effective in studies of chronic toxicity, endocrine disruption, genotoxicity, and immune modulation. These capabilities improve the zebrafish model’s value in comprehensive environmental risk assessments. For example, markers of endocrine disruption, such as vitellogenin gene expression, and genotoxicity assays, like the comet assay, demonstrate the ability of zebrafish embryos to detect DNA damage caused by chemical exposure (Scholz et al., 2008).

The use of zebrafish embryos in sediment toxicity and mutagenicity testing shows their adaptability for assessing environmental pollutants in a range of situations. Toxicogenomic techniques with zebrafish embryos enable the study of gene and protein expression patterns in response to chemical exposure, providing valuable insights into molecular responses and toxicological effects. This molecular-level data gives deeper insights into the causes of toxicity and potential long-term effects, going beyond traditional measures like lethality, offering up possibilities for applications in toxicokinetics and toxicodynamics, and exceeding conventional endpoints like lethality (Scholz et al., 2008).

2.2.2 Limitations and challenges of zebrafish embryo

The embryo's outer layer, the chorion, may develop into a barrier that stops the absorption of the larger molecules. To avoid polyspermy protecting the embryo, and decrease permeability, the chorion hardens in the first few hours after fertilization (hpf). This hardening has been shown to make embryos exposed from 0-1 hpf more sensitive than those exposed after 4 hpf (Gellert and Heinrichsdorff, 2001). Whether a molecule can pass through the chorion depends on its chemical and physical properties or size. Limited uptake has been observed for small molecules, heavy metals, highly lipophilic substances, and nanomaterials (Pelka, 2017). However, after three days

of exposure, the fish embryo begins direct contact with the chemical, bypassing the chorion barrier. Since our experimental period lasted five days, this effectively negates any limitations posed by the chorion.

The predictability of the zebrafish model could be improved by further refining the OECD test guideline 236 protocol. Stricter pH adjustment would be advantageous for compounds that affect pH, such as the acids in this investigation. Although the OECD (2013) states that zebrafish embryos can withstand pH values between 6.5 and 8.5, pH can have a substantial impact on the speciation and solubility of test chemicals by changing the ratio of ionized to non-ionized molecules, which affects the compounds' availability to the embryos. If pH adjustments were made, the EC_{10} values for VPA analogs would likely vary more, with negative analogs requiring higher concentrations to induce effects compared to positive ones, supporting the study's conclusions (Brotzmann et al., 2020).

2.3 Automated phenotyping methods

In concordance analyses research that looked at interlaboratory variability found inconsistencies (Ball et al., 2014; Gustafson et al., 2012). Only five out of twenty chemicals were classified similarly (as either teratogenic or nonteratogenic) across four different labs, with individual labs' consistency in identifying developmental toxicity or teratogenicity in comparison to mammalian data ranging from 60% to 70% (Gustafson et al., 2012). A 71% concordance for teratogen categorization was observed in a subsequent investigation that involved 37 substances and two labs (Ball et al., 2014). This variability may be due to the subjective nature of visual observations and classification of developmental changes by researchers, as well as limited standardization across labs. Therefore, observer accuracy and expertise have an impact on the traditional methods used to screen for developmental toxicity in zebrafish embryos.

In response to these inconsistencies, various approaches have been developed to improve objectivity in zebrafish embryo phenotyping. Many previous phenotypic image analyses have focused on fluorescent imaging to evaluate aspects such as cardiovascular development (Leet et al., 2014), cardiovascular function (Burns et al., 2005; Leet et al., 2014; Letamendia et al., 2012; Yozzo et al., 2013), and angiogenesis (Letamendia et al., 2012; Vogt et al., 2009). However, fewer studies have utilized automated phenotypic image analysis for brightfield microscope images without the need for fluorescent markers or staining. Certain traits, such as lethality, hatching

status, pigmentation changes, or the lack of eyes, have only been detected in a small number of these studies (Teixidó et al., 2019).

Deal et al. (2016) created a computational malformation index to improve objectivity by combining a quick human visual evaluation with quantifiable morphometric characteristics, such as convexity and total body area. This approach improved impartiality by measuring the overall severity of anomalies and basing the score on microscopic observations. Its limitations included the fact that some phenotypes, including edema or small eyes, were not addressed. Jeanray et al. (2015) employed supervised machine learning as a different method to identify developmental traits. This method begins with expert classification and involves several rounds of classification and learning to refine the model, allowing it to generate concentration-response curves for cumulative phenotypic assessment. Although this methodology provides a more systematic approach to examining developmental changes, its applicability may be limited because it requires the use of similar tools and settings across different labs.

As the need for reliable and consistent results grew, there was a push to reduce human subjectivity in assessing phenotypes. In this study, ensuring the correct orientation of zebrafish embryos was identified as essential for accurate and unbiased phenotypic analysis using 2D imaging. In 2D projections, the precision of morphological feature recognition may be impacted by slight variations in embryo orientation. Therefore, an image-based system was implemented to detect and quantify morphological features, with an automated capillary system positioning the embryos. The custom MATLAB-based FishInspector software was used to extract morphological data from the images. The extracted data is further processed by KNIME and R scripts for morphometric analysis and quantification. This workflow established a consistent and impartial method for phenotype assessment.

2.3.1 The Automated Imaging Robot (AIR)

The Automated Imaging Robot (AIR) uses a deep learning-based technology to handle, dispense, and image zebrafish embryos and other small aquatic organisms. A source plate containing the embryos to be imaged (in 1, 6, 12, 24, 48, or 96-well formats) is placed into a receptacle, along with a destination plate for dispensing the photographed samples. During programming, users can select one or more orientations (e.g., dorsal, ventral, lateral) and regions of interest for imaging. They can also choose the entire plate or specific wells for imaging, as well as the imaging modes.

The robot is equipped with two cameras for brightfield imaging: a telecentric camera with a liquid lens and a microscope camera that records a pre-set number of image stacks, which are then compressed to produce the highest quality images. For extra imaging capabilities, an optional perpendicular addition of an epi-fluorescent imaging module is also possible. Maximum resolution, brightness, and imaging speed are attained by using high numerical aperture (NA) water immersion with high magnification objectives.

Tomographic imaging is also available, allowing automated imaging from multiple rotational angles within a region of interest. This can overcome the limitations of background luminescence in epi-fluorescent microscopy for some applications or offer possibilities for 3D reconstruction of certain areas or organs. A short video of the embryos can also be recorded for the subsequent analysis of the cardiovascular system and heart rates.

Once the imaging parameters are specified, a single button starts the imaging process, quickly capturing all of the selected samples. The robot has a changeable polycarbonate tube and a high-speed delta robotic head. For imaging, this head collects up each embryo separately, applies specific fixation gel, and then lowers them into a glass cuvette that is filled with distilled water. After imaging, the embryos can either be transferred to the destination plate or returned to their original wells. The robot's internal hard drive stores images, which may be shared by a thumb drive or by linking the robot to the network.

2.3.2 The FishInspector

The FishInspector software, developed by Teixidó et al. (2019), a MATLAB-based tool for image analysis that automates the identification and measurement of key morphological features on 2D images of zebrafish embryos. FishInspector gives objective and quantitative assessments by automatically extracting features (body length, eye size, yolk sac size, and jaw position). This automation improves reproducibility and accuracy in developmental toxicity investigations by reducing the subjectivity and variability that are associated with manual phenotyping. A comparison between automated image-based analysis and conventional visual evaluations was used to evaluate the software, showing that FishInspector not only generated results that were comparable but also more sensitive in identifying specific chemical toxicities. The modularity of FishInspector allows users to adjust parameters for different image qualities and apply manual corrections when needed, enhancing its flexibility.

The study also showed how FishInspector can detect minor morphological abnormalities that the human eye cannot detect. For example, FishInspector was able to identify an EC_{50} (the concentration at which 50% of embryos are affected) based on certain morphological alterations, such as pericardial size, yolk sac size, and jaw position, whereas visual analysis was unable to determine an EC_{50} for dexamethasone. This shows the software's sensitivity in detecting minor phenotypic changes that are important for developmental toxicity assessment.

2.3.3 Grouping chemicals into mode of action classes

Classifying substances based on their phenotypic patterns is the main objective of automated phenotyping techniques. This classification is achieved by conducting toxicity tests on zebrafish embryos with known chemicals, recording the resulting phenotypic effects, and establishing a "toxicological fingerprint" of these chemicals. By using clustering methods, chemicals then could be assigned into mode of action (MoA) groups according to their fingerprint. These fingerprints then allow for the classification of unknown chemicals or even complex environmental samples according to their phenotypic effects. Automated, unbiased image analysis can link specific phenotype patterns to the chemical's mode of action. By combining multiple phenotypes, a chemical fingerprint can be used to predict mechanisms of action or group chemicals with similar biological activity.

Teixidó et al. (2022) used quantitative phenotypic analysis to investigate the suitability of the zebrafish embryo test in assessing the mode of action of 25 chemicals. In Figure 4, the heatmap shows the findings across various phenotypic and functional endpoints. The top row of the heatmap represents the lipid-water coefficient (log D_{lipw}) values, which indicate the hydrophobicity of each chemical at a specific pH, more polar compounds shown in white. The color intensity shifts to orange/brown. Toxic Ratio (TR) values which is calculated by comparing the observed toxicity of a chemical to its baseline toxicity. A TR above 10 indicates that the chemical's toxicity is due to a specific mode of action, rather than baseline toxicity. Each cell's color corresponds to the sensitivity ratios, denoted as $SR_{Lethality,}$ which reflects the chemical's effect on each endpoint. This was calculated by using EC_{50} or EC_{20} for some endpoints that didn't reach 50% effect before lethality.

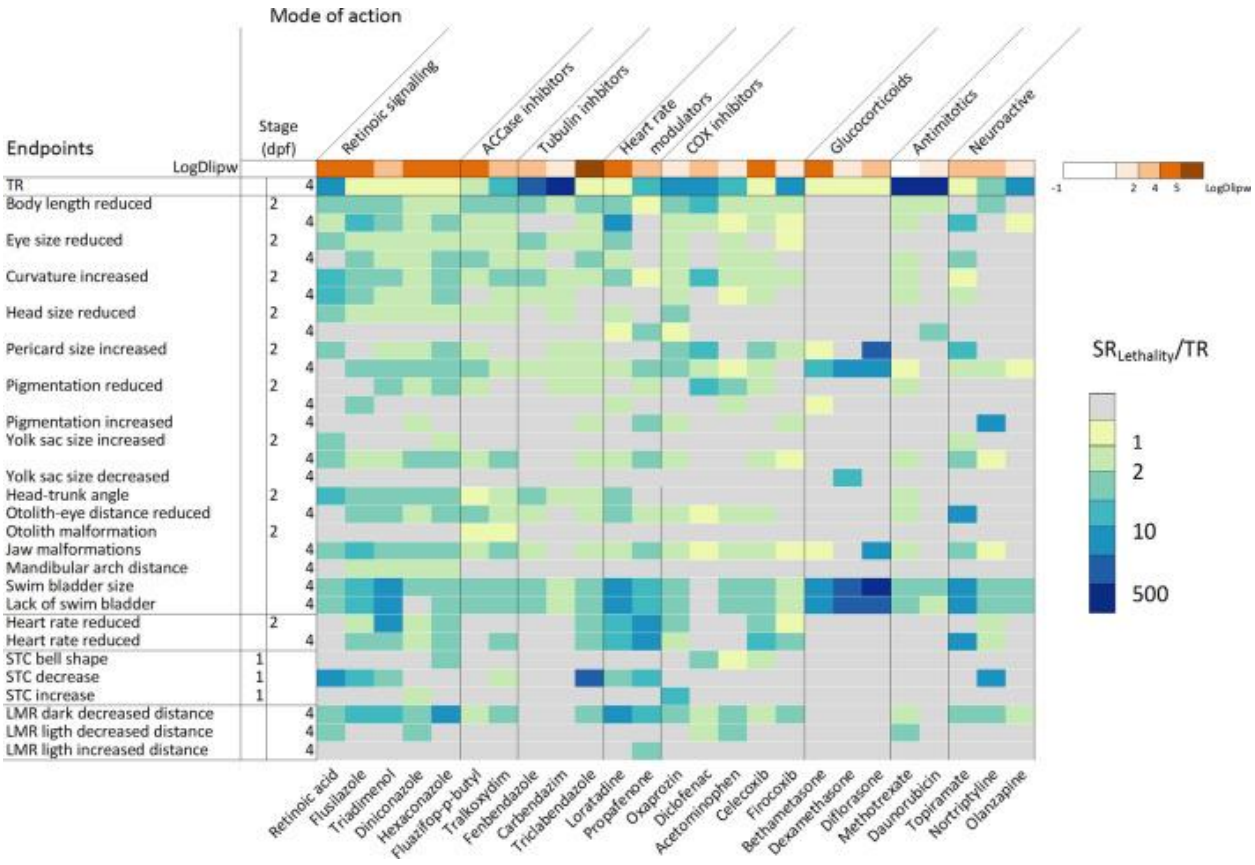

Figure 4. Toxicity profiles of 25 chemicals at 1, 2, and 4 days post-fertilization (dpf). The heatmap shows $SR_{Lethality}$, from no effect (gray) to strong effect (blue), with compound polarity in the top row (Teixidó et al., 2022).

2.4 KNIME statistical software

The KNIME or Konstanz Information Miner platform and R scripts were then used to handle the extracted data from the FishInspector for morphometric analysis and quantification. KNIME is an open-source platform for data mining visual processes and an effective tool for data science, statistical analysis, and life sciences research. Figure 5 shows the modular design of KNIME. This design helps users connect various processing nodes into a pipeline. Both beginners and experts can perform complex data manipulations without requiring programming knowledge. Users can visually build workflows, run data transformations, interactively review results while maintaining

transparency and reproducibility (Berthold et al., 2009).

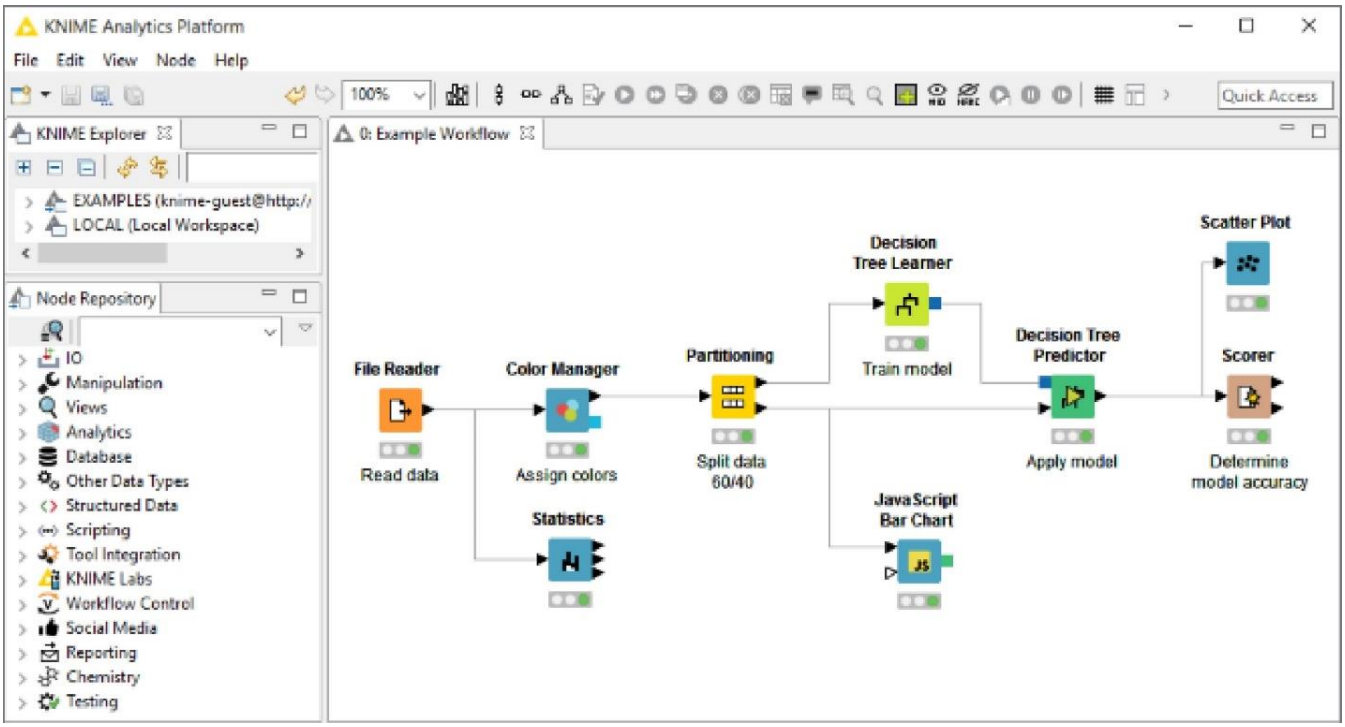

Figure 5. The KNIME user interface displays the workflow being constructed in the center of the screen, with the node and workflow repository views located on the right side (Fillbrunn et al., 2017).

KNIME has become an important tool in the life sciences for reproducible, cross-domain analysis. The platform's visual interface makes it easy to document and share workflows, reinforcing the core scientific principles of reproducibility and transparency. KNIME supports the integration of popular programming languages like R and Python, extending its functionality and enabling users to blend existing scripts with KNIME's visual workflow system. R offers an adaptable and universal package for dose-response analysis (Ritz et al., 2015). Statisticians frequently use R for analysis and visualization, while Python is a general-purpose programming language, making it highly versatile for developers involved in data analysis. KNIME supports interoperability between these two environments with multiple nodes, allowing users to incorporate existing R and Python scripts into KNIME's visual workflows. This allows seamless reuse of packages and scripts, enabling data to be processed and visualized in R while maintaining the results in a format that can be further manipulated using other KNIME nodes (Fillbrunn et al., 2017).

While traditional methods often rely on qualitative assessments, the platform's strength is further demonstrated by its quantitative capabilities, by the integration of FishInspector with KNIME, which generates detailed concentration-response curves, improving the precision of toxicity evaluations. KNIME allows for thorough statistical analysis that makes cross-correlation of developmental characteristics easier, it significantly enhances the reliability and depth of toxicity studies. It is also highly scalable, efficiently handling large datasets with a robust table-based format that caches results, making it suitable even for environments with limited computing resources. In bioinformatics and cheminformatics, where genomic sequencing and molecular modeling produce enormous volumes of data, this capacity to manage large-scale data processing is highly advantageous (Berthold et al., 2009).

Berthold et al. (2009) emphasize that KNIME's modular architecture and adaptability allow for the seamless integration of new algorithms and tools as customizable nodes, making it highly useful across various domains. Fillbrunn et al. (2017) noted KNIME's significance in complex life sciences experiments due to its capacity for handling data from multiple sources.

3. MATERIALS AND METHODOLOGY

3.1 Chemicals used

2-ethylhexanoic acid and tricaine-methanesulfonate (MS-222) were purchased from Sigma-Aldrich (Merck Hungary Ltd., Budapest, Hungary). Exposure concentrations were diluted by using E3 medium: 60x stock solution was prepared by dissolving 34.8g NaCl, 1.6g KCl, 5.8g CaCl2 x 2H2O, and 9.78g MgCl2 x 6H2O in 1.95L RO water, pH was adjusted to ~7.2 and autoclaved. To prepare 1xE3 embryo medium, 16.5ml 60x stock was diluted in 1L RO waster, and autoclaved. For automated imaging, embryos were anesthetized in 125mg/L MS-222 solution.

3.2 Zebrafish maintenance

Adult wild-type, laboratory-bred AB zebrafish line were maintained under standard conditions (25.5°C ± 0.5°C, 14h light/ 10h dark photoperiod) in a ZebTEC Multilinking recirculating fish housing system (Tecniplast S.p.a., Italy) with controlled water quality (pH 7.0±0.2, conductivity 550 ± 50µS) at the Institute of Aquaculture and Environmental Safety. Fish were fed twice daily with commercial, dry granulated zebrafish food (Zebrafeed 400-600µm, Sparos Lda., Portugal), supplemented once daily with freshly hatched live *Artemia salina* nauplii. For breeding, adult female and male zebrafish were placed separately into specially designed breeding tanks (Tecniplast S.p.a.) applied with a divider on the afternoon before the day of the experiments. The next morning, after the lights switched on, the dividing walls were taken out to allow the fish to spawn. Embryos were obtained by natural spawning and collected within 30 minutes, placed into 10 cm Petri dishes (JET Biofil, China) filled with E3 medium, and put into an incubator at 25.5°C ± 0.5°C until sorting. Following incubation, normally developing 4-16 cell stage embryos were sorted under a dissection microscope according to Kimmel et al. 1995 (Figure 6).

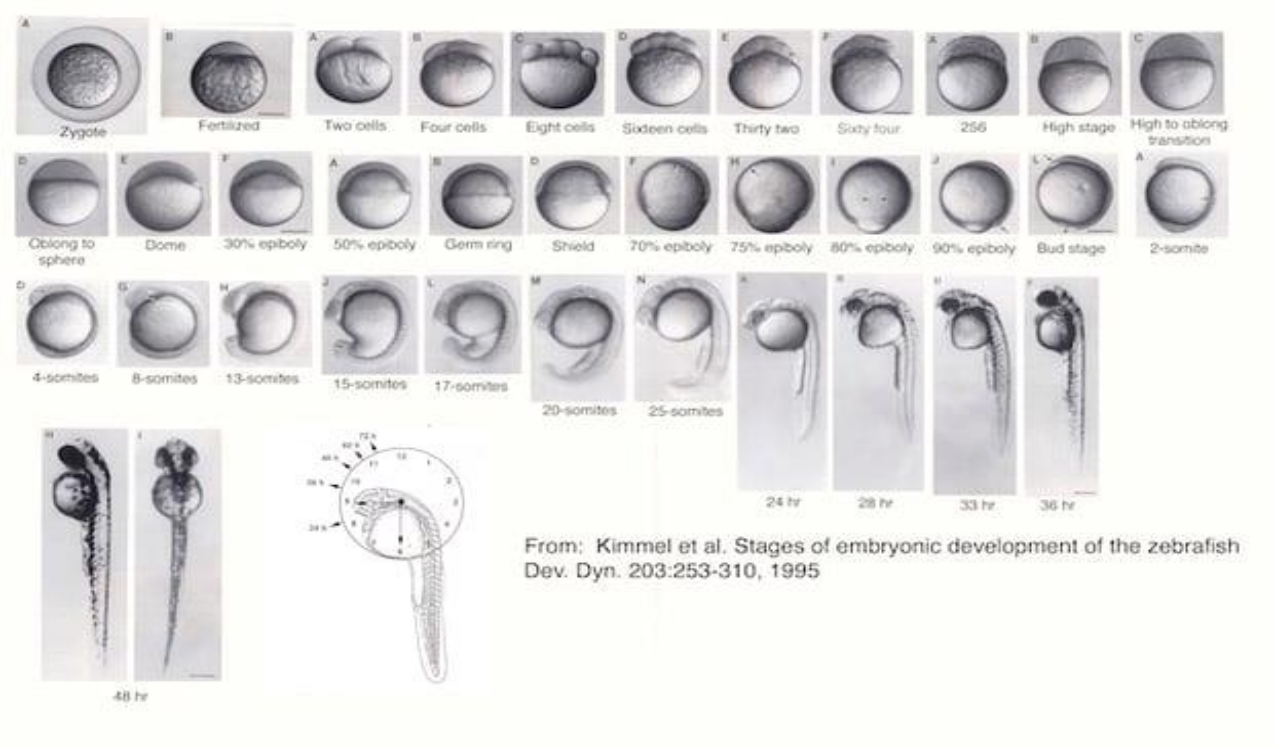

Figure 6. Development stages of zebrafish embryos from 0 to 48 hpf (Kimmel et al., 1995).

3.3 Chemical exposure

To test the toxic effects of 2-EHA, we performed a slightly modified OECD Fish Embryo Acute Toxicity (FET) test (OECD, 2013). Exposure solutions were prepared by diluting 2-EHA with an E3 medium. Exposure concentrations are shown in Table 3. Since we were interested in sublethal morphological effects, exposure concentrations were chosen carefully in order to avoid high mortalities in the tests. Experimental lethal concentration values lack in the case of 2-EHA so we used the QSAR calculated baseline toxicity value for zebrafish embryos as reference, that is ~3mM. The exposure test was carried out in two replicates. From each exposure solution 380µL was measured into a well of a 96-well microplate (Whatman UNIPLATE, Cytiva, USA) in 16 replicates (two columns). For negative control, the E3 medium was used in 16 replicates. The exposure solutions and controls were distributed in a way to avoid the plate effect (Table 4), Into each well one embryo was pipetted with 20µL E3 medium using a cut-down pipette tip (n=16). The plates were wrapped in Parafilm to avoid evaporation and placed into an incubator at 25.5°C ± 0.5°C for 5 days. After the exposure period had ended, mortalities were recorded, and surviving embryos were anesthetized with MS-222 (125 mg/L final concentration).

Table 3. Stock solution dilutions. The exposure concentrations were prepared as outlined below using two stock solutions of 500 µM and 300 µM with serial dilutions. The final concentrations and volumes of the stock solutions used are presented in this table.

a)

Concentrations	Final Concentration µM	Volume of stock solution (ml)	E3 (ml)
Concentration 1	500	15.00	0.00
Concentration 2	250.00	7.50	7.50
Concentration 3	125.00	3.75	11.25
Concentration 4	62.50	1.88	13.13
Concentration 5	31.25	0.94	14.06
Control	0	0.00	15.00

b)

Concentrations	Final Concentration µM	Volume of stock solution (ml)	E3 (ml)
Concentration 1	300	15.00	0.00
Concentration 2	150.00	7.50	7.50
Concentration 3	75.00	3.75	11.25
Concentration 4	37.50	1.88	13.13
Concentration 5	18.75	0.94	14.06
Control	0	0.00	15.00

Table 4. Experiment plate layout. The 96-well microplate was organized as shown with each row containing a control group and concentrations ranging from the highest (conc1) to the lowest (conc5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
B	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
C	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
D	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
E	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
F	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
G	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1
H	control	conc5	conc4	conc3	conc2	conc1	control	conc5	conc4	conc3	conc2	conc1

3.4 Automated imaging of zebrafish embryos

The microplates containing the anesthetized embryos were put into the receptacle of the AIR, and imaging parameters were programmed manually. A new capillary was inserted into the delta head and rinsed three times with E3 medium. Capillary length and rotation were calibrated automatically, and a 3D model of the capillary was also automatically recorded. For automated image analysis, only the brightfield microscope camera was utilized. The number of image stacks gained was set to 40, and the z-axis range was set to 80% to avoid recording the capillary itself.

The angle of imaging was set to 0° (lateral view). Videos of the embryos were not recorded for heart rate analysis, since 2-EHA was not expected to influence the heart rate according to literature data and unpublished results for its closely related compound, valproic acid. The imaging process of each plate did not take longer than 2 hours, and during this time the anesthesia did not cause any phenotypic changes that would have biased the test results. The recorded image stacks were compressed into a single tiff (tagged image file format) file and stored on the hard drive of the robot until image analysis

3.5 Automated image analysis of zebrafish embryos

The phenotypic feature detection and quantification process after image acquisition was conducted using the FishInspector 1.7 software (Helmholtz Centre for Environmental Research, Leipzig, Germany) and a customized KNIME workflow. The FishInspector software was developed within the MATLAB environment for feature detection. Lateral control images of embryos at 120 hpf were used for software development. Identifying certain features depends on previously detected features making the detection process hierarchical. Detecting the contour of the embryo is guided by the capillary boundaries, ensuring the embryo is located within the capillary. Subsequent features are identified step by step (Figure 7).

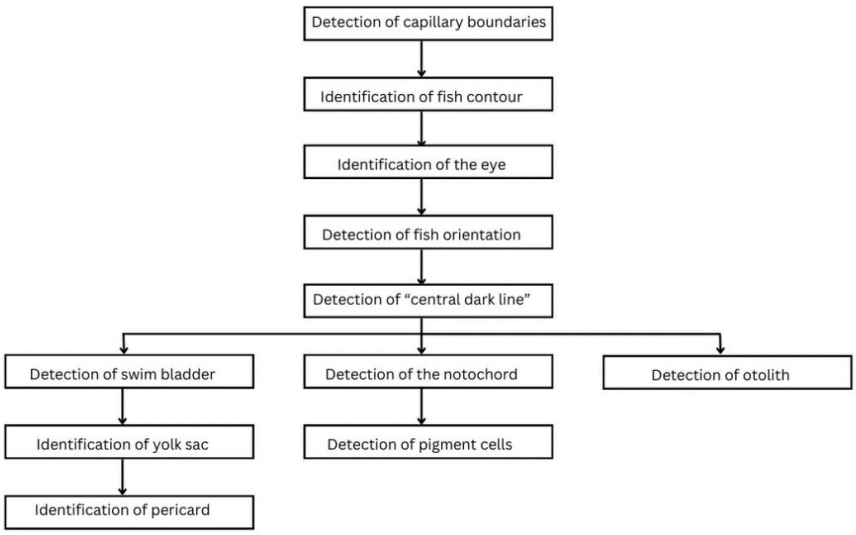

Figure 7. Step-by-step feature recognition by FishInspector. The "central dark line", starting from the fish's eye, marks high contrast between the upper and lower parts. Images without capillaries are automatically adjusted to fit the software (Teixidó et al., 2019).

The process of identifying morphological features is dependent on excluding regions that may interfere with the detection regions of interest that were identified through visual observation and measurement of general object properties. For instance, after localizing the fish contour, the eye was detected by searching for a dark object within the right or left half of the zebrafish. The detection algorithms were refined using images of embryos treated with all-trans retinoic acid, a positive control for body morphology changes. While achieving 100% automated feature detection is challenging, the software allows users to modify detection parameters and manually correct features that are not properly identified. The head-trunk angle was determined by drawing a line between the centers of the ear and eye and another line parallel to the notochord in the mid-trunk region, based on coordinates provided by the FishInspector software. Mandibular arch thickness was measured as the distance between the eye and the lower jaw contour, using the lowest Y contour value of the eye as the reference point (Teixidó et al., 2022).

The output generated by FishInspector consists of a set of XY coordinates representing the detected morphological features. A single JSON file, an open standard file format used for data communication between applications, contains the data for each analyzed image. The boundary coordinates of the various detected features are stored in this structured text format, enabling easy integration of FishInspector's output into custom post-processing algorithms, which can be written in any programming language. After the FishInspector annotates the morphological features, the resulting JSON files serve as input to a KNIME workflow that has embedded R scripts (R Core Team, 2014) to quantify the features (Table 5). Shape data, such as length and surface area, were extracted using the "Momocs" R package (Claude et al., 2008; Bonhomme et al., 2014).

Table 5. Morphological Features Measured in the Zebrafish Using the FishInspector Software.

Phenotypic feature	**Parameter or Metric**	**Data Exported as JSON Format**
Eye size	Surface area (mm2)	Eye XY coordinates
Body length	Length (mm)	Fish contour XY coordinates
Yolk sac size	Surface area (mm2)	Yolk sac contour XY coordinates
Otolith-eye distance	Length (mm)	Otolith XY centroid (saccule, the largest otolith)
Pericard size	Surface area (mm2)	Pericard contour XY coordinates
Tail malformations	Curvature	Notochord XY coordinates
Swim bladder inflation	Surface area (mm2)	Swim bladder contour XY coordinates

Head size	Surface area (mm2)	Fish contour XY coordinates, otolith, and eye centroid
Lower jaw position	Distance (mm)	Distance in the X coordinate between eye centroid and lower jaw tip
Head-trunk angle	Angle (degrees)	The angle between the ear-eye center line and a line parallel to the notochord in the mid-trunk
Mandibular arch thickness	Distance (mm)	Distance in the x coordinate between the eye and the lower jaw contour

Only the notochord coordinates from the fish's tail were used to determine its curvature. The curvature along the tail was determined by calculating the second derivative of the smoothed notochord line, focusing on points where the first derivative is zero. The analysis was conducted using the curvature value that was highest along the tail. Tail curvature was calculated in R using the "features" package (Varadhan et al., 2015) along with the "smooth.spline" function, applying a smoothing parameter (spar) set to 0.9 (Teixidó et al., 2019).

Head size was measured by drawing a line between the eye and the otolith centroid, followed by calculating angles from the otolith to the fish's upper contour and from the eye to the lower contour, enclosing the head region. The distance between the tip of the lower jaw and the eye centroid along the x-axis was measured in order to evaluate the effects (Teixidó et al., 2019). After the automatic recognition algorithm has run, detected features are shown according to Figure 8.

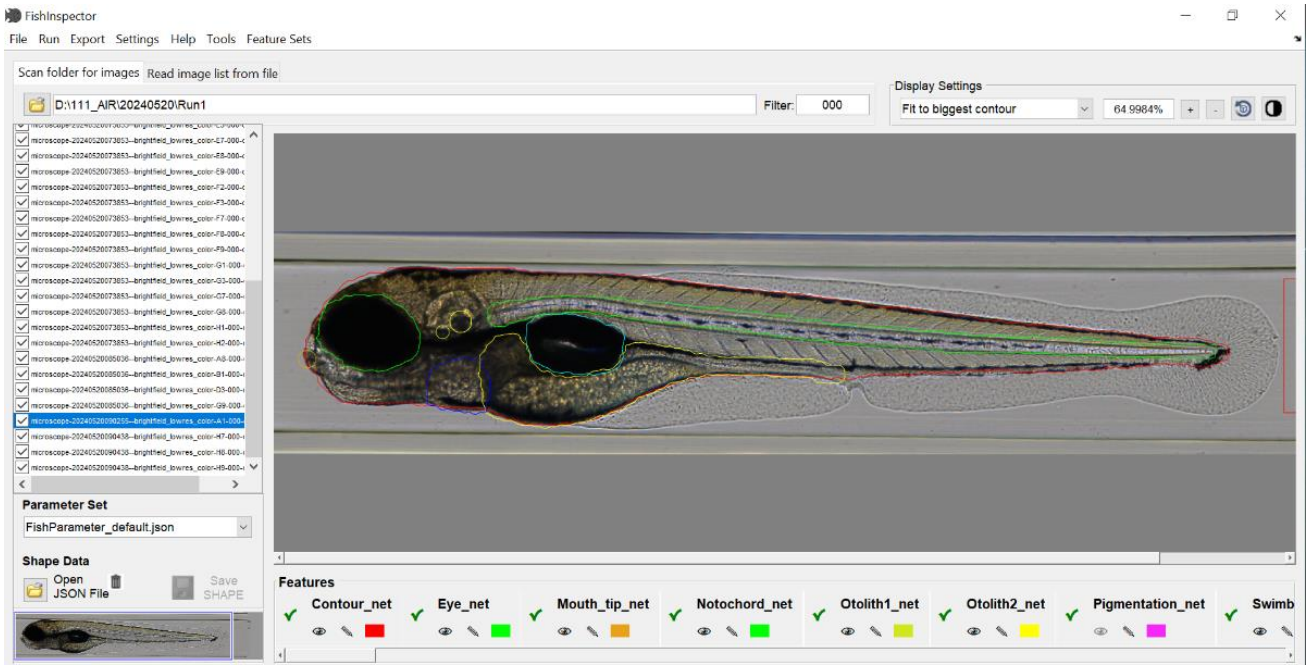

Figure 8. The main window of FishInspector 1.7 after automatic recognition of morphological features.

After feature detection and any manual corrections, a built-in tool, TableCreator is used to save all feature parameters into a single XLS file. From the feature coordinates, 46 different metrics (endpoints) were calculated that were distances (e.g. body length), areas (e.g. eye size), and angles (e.g. notochord curvature). Metadata from the plate layout files (well ID, concentration, chemical name, exposure time) were also integrated into the output table.

3.6 Statistical analysis

Concentration-response curves were fitted for morphological features. Lethality and morphological features were assessed based on the percentage of affected embryos. Morphological data were simplified by converting feature metrics into the percentage of embryos that differed from controls. For each endpoint, an embryo was considered to deviate if its value fell outside 1.5 times the standard deviation of the control group mean (Wilhelmi et al., 2023), which was determined from a pooled sample of 32 control embryos.

The experiments were designed to calculate 50% effective concentrations (EC_{50}) using modeled curve fits. Different concentrations were tested in two independent replicates. Each replicate's mean is based on the exposure of 16 embryos per concentration. Effect concentrations were modeled only when three conditions were met (Nöth et al., 2023):

1. The Tukey trend test (from the R package "tukeytrend") yielded a maximum p-value of 0.05.

2. The Akaike information criterion (AIC) for the log-logistic model was lower than for a linear model with a slope of 0 which indicates no trend in the data.

3. At least 30% of embryos were affected at one exposure concentration, or (for normalized data) a 30% increase or decrease was observed.

This approach accounted for variable features with 10-20% random effects in controls and avoided artifacts from extrapolating to EC_{50} concentrations when the maximum effect was below 50%. Visual inspection of concentration-response curves confirmed that these criteria led to automated modeling only when a clear relationship between concentration and response was present. For endpoints that didn’t meet the criteria, no effect was considered to occur (Nöth et al., 2023).

Output data that we obtained were dose-response curves, effective concentration values (EC_{50}), box plots, and heat maps which are included in the Annexes.

4. RESULTS

4.1 Validity of the toxicity assays

Our test results met the validity criteria of the standard OECD 236 FET test, namely:
a) The overall fertilization rate of all eggs collected was ≥ 70% in the batch tested.

b) The water temperature was maintained at 25 ± 0.5 °C in test chambers at any time during the test.

c) Overall survival of embryos in the negative (dilution-water) control was ≥ 90% until the end of the 120h exposure.

d) The hatching rate in the negative control was ≥ 80% at the end of 120 h exposure.

e) At the end of the 120 h exposure, the dissolved oxygen concentration in the negative control and highest test concentration was ≥80% of saturation.

4.2 Results of the toxicity assays with 2-EHA

The acidic property of 2-EHA caused significant embryo mortality in the initial experiment. In the subsequent experiments, pH was adjusted to ~7.5 by adding sodium hydroxide (NaOH) to the exposure solutions, which was sufficient to keep the pH constant during the test period. At 500 µM concentration mortality after 120h was 100%, therefore only concentration from 300 µM and below were included in the morphological assessment. At every other concentration, mortality did not exceed 10%.

Key endpoints were identified by the morphological examination of zebrafish subjected to 2-EHA. The KNIME analysis was used to determine these endpoints and the associated EC_{50} values. Out of 46 measurements conducted, seven endpoints were selected and their EC_{50} values were determined. These results are shown in Table 6. Feature names used by FishInspector were translated into onthologies using Ontobee (https://ontobee.org/), and the corresponding organ is also indicated.

Table 6. An overview of the selected features, their respective anatomical regions, and the calculated EC_{50} values.

Feature name	Organ	Translated feature name	EC_{50} (µM)
All malformations			210
EukledianDistance_eye_otolith2_red	embryonic head	Eye decreased distance to posterior otolith	348
Eye_regionpropsArea_red	embryonic head	Eye decreased size	508
AngleToX_mouth_tip_otolith2_inc	embryonic head	Mouth increased angle to posterior otolith	224
JawEyeOtolithAngle_inc	embryonic head	Mouth increased angle to eye posterior otolith	125
MandibularArchDistance_red	embryonic head	Eye decreased distance to the mandibular arch	305
Pericard_regionpropsArea_inc	pericardium	Pericardium increased size	302
Swimbladder_regionpropsArea[µm2]_missing	swim bladder	Swim bladder absent	113

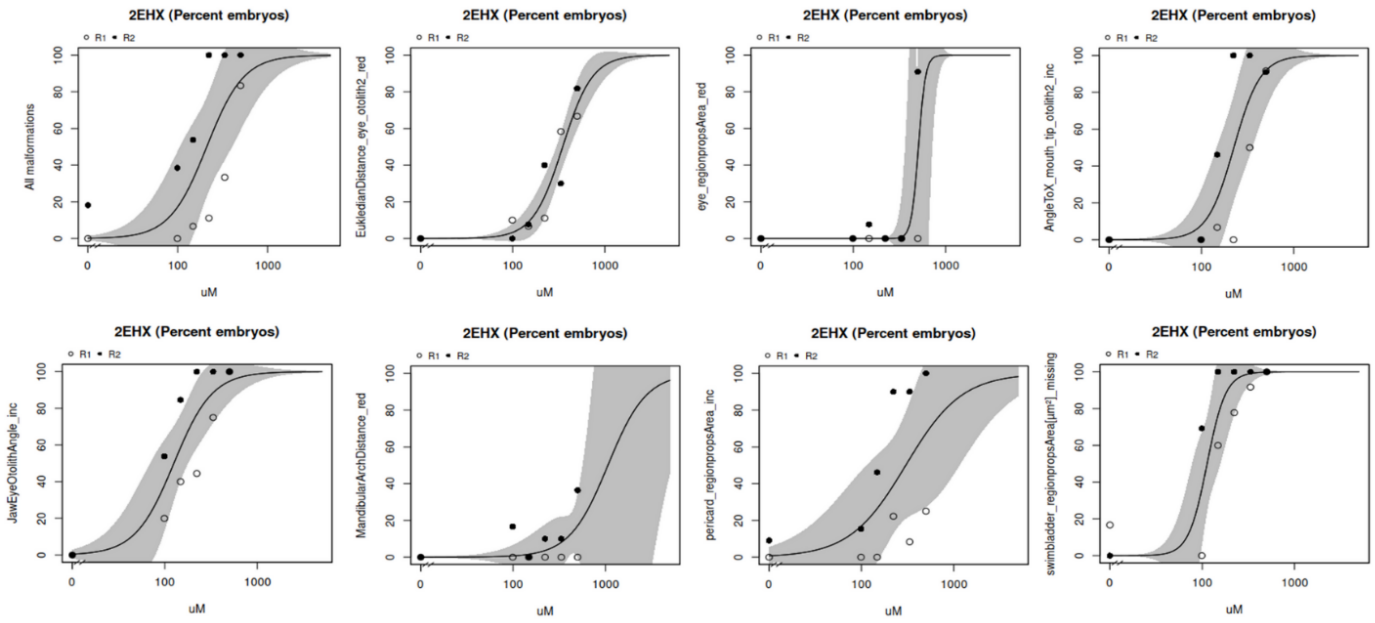

Figure 9. Dose-response relationship curves for seven selected morphological endpoints, along with the overall malformations observed in zebrafish embryos exposed to the test compound.

A dose-response relationship is represented by the sigmoidal form of the curve (shown in Figure 9), which shows that the percentage of affected embryos increases as the concentration of 2-EHA increases. The inflection point of the curve represents the EC_{50}, the concentration at which 50% of the embryos are affected. Confidence intervals are represented by grey-shaded areas, which demonstrate the reliability and variability of each dose-response relationship across different endpoints.

Identifying the most sensitive organs and assessing EC_{50} values for specific features can reveal how the substance disrupts zebrafish embryonic development. Multiple features in the head region showed developmental disturbances, features including eye, mouth, and otoliths. The compound significantly caused the eye size to decrease, with an EC_{50}= 508 µM and an increase in the mouth-to-otolith angle (EC_{50}= 224 µM) suggesting disruptions in the growth and spatial arrangement of head structures during development, likely disrupting neural crest migration or other craniofacial patterning processes.

The size of the pericardium was significantly affected, with an enlarged pericardium size observed at an EC_{50} of 302 µM. A common indicator of developmental stress in zebrafish embryos, pericardial edema (fluid buildup) may have direct effects on the formation of the heart. With an EC_{50} of 113 µM, the most sensitive endpoint was the absence of swim bladder inflation, suggesting that the compound significantly impairs swim bladder development. The absence of a swim bladder at lower concentrations could impair buoyancy and overall physiological functionality in the embryos. A wider disruption in organogenesis, which may impact gas exchange and endodermal differentiation in the early phases of embryogenesis, is suggested by interference with swim bladder development.

Shown effects in eye size, jaw-to-eye distances, and mouth-to-otolith angles point to interference with neural crest cell migration or craniofacial patterning. Induced at the neural plate border early in development, neural crest cells are initially immobile but then go through an epithelial-to-mesenchymal transition that allows them to migrate and differentiate into a variety of structures, such as the skeleton and craniofacial cartilage, as well as sensory neurons. This process plays a major role in the patterning of vertebrates' embryos (Hines & Taneyhill, 2019).

Typical visual morphological changes are shown on Figure 10.

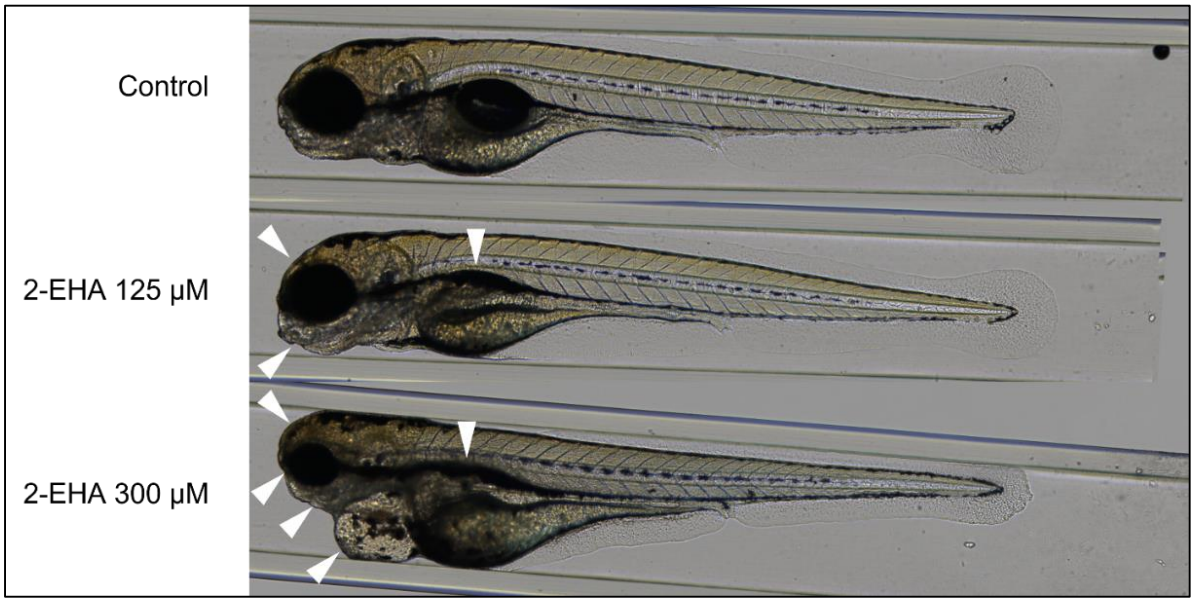

Figure 10. Typical morphological deformations caused by 2-EHA exposure after 120h. Arrows show the affected areas (head deformation, eye size decrease, mandibular deformation, pericardial edema, uninflated swim bladder).

A heatmap of all tested endpoints are shown on Figure 11, with the strength of effect at each individual endpoint.

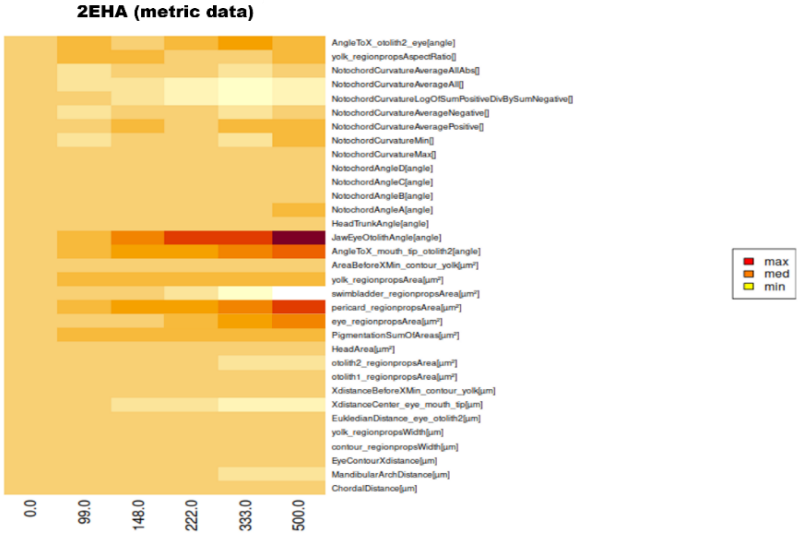

Figure 11. Heatmap of individual morphological endpoints, showing strength of effect at each point.

Overall, the morphological changes observed were as expected, as the most closely related compound, valproic acid, also caused malformations in the same body parts and organs, such as the embryonic head, mandibular arch, or pericardial area (unpublished internal results). These results support the theory of grouping compounds according to their phenotypic patterns of action and the possibility of using toxicological fingerprinting.

5. CONCLUSION

5.1 Comparison with other studies

Our findings are consistent with those of Brotzman et al. (2020), who observed similar morphological changes, which were reduced eye size and an increase in individuals showing tremors and craniofacial deformities with 2-EHA. In their study on valproic acid and its nine analogs, 2-EHA was identified as one of the most toxic compounds for fish embryos, showing consistent observations of development impairments.

Teixidó et al. (2022) used quantitative phenotypic analysis to investigate the suitability of the zebrafish embryo test in assessing the mode of action (MoA) of chemicals. In the study, which involved 25 different chemicals, it was found that failure to inflate the swim bladder is a key developmental endpoint that is strongly correlated with yolk sac size and heart rate. Based on this study and our findings, we can conclude that the swimbladder is the one of most sensitive endpoints in the zebrafish embryo toxicity test.

5.2 Advantages and limitations of automated imaging and analysis

The automated imaging and image processing used in our study offers several important advantages. Since this method is not susceptible to human observation, observer bias is eliminated. High-throughput, objective analysis makes it possible to detect small phenotypic changes that could have gone unnoticed. Automated methods offer consistency across large datasets, improving the accuracy of the results. However, there are certain restrictions. To fix misidentifications or mistakes, image recognition frequently needs manual corrections, which might take a lot of time and the processing of high-resolution images and large datasets demands great computational power. This need could limit its accessibility and efficiency in some circumstances.

5.3 Future applications of toxicological fingerprinting

Future toxicological research could greatly benefit from the toxicological fingerprinting method used in this work. With this method, based on the chemicals' mode of action, chemicals could be classified, and a comprehensive phenotype database for a wide range of chemicals can be built. A key aspect of our research was to obtain unbiased assessment data from automated image analysis, which allowed us to obtain reproducible concentration-response relationships for each phenotypic endpoint. This is highly important to predict and understand the toxic effects of structurally similar compounds, like valproic acid and 2-ethylhexanoic acid. Potential applications of the presented

approach include screening of compounds and assigning potential MoAs of developmental toxicity to unknown chemicals or complex environmental samples, and prioritization for further investigation. Further research is needed to evaluate the potential factors influencing the patterns of effects and to confirm whether differences between compounds likely to have similar MoAs are indeed caused by different MoAs.

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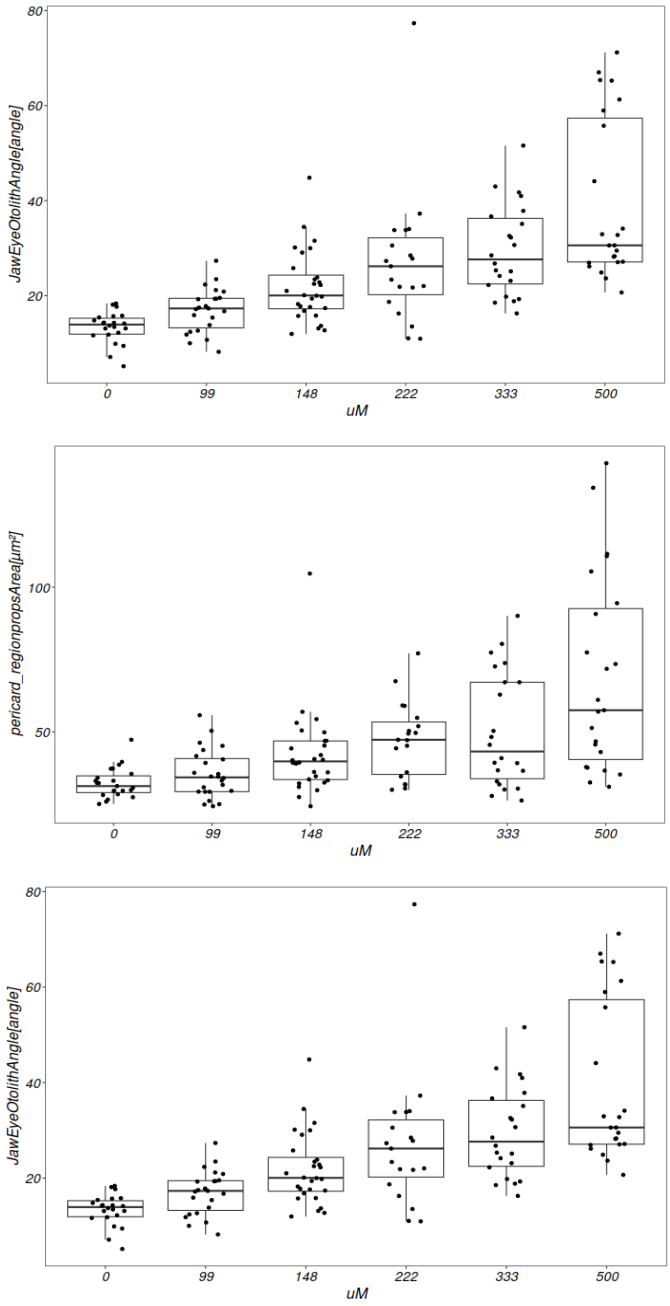
7. LIST OF TABLES AND FIGURES.

8. ANNEXES

A. Boxplot



9. ABBREVIATIONS

2-EHA: 2-Ethylhexanoic Acid

AIR: Automated Imaging Robot

CAS: Chemical Abstracts Service

dpf: days post fertilization

EC_{50}: Effective Concentration at which 50% of embryos are affected

FET: Fish Embryo Test

HDAC: Histone Deacetylase

hpf: hours post-fertilization

K_{oc}: Soil Adsorption Coefficient

log D_{lipw} : Lipid-Water Coefficient

log K_{ow}: Octanol-Water Partition Coefficient

MoA: Mode of Action

NA: Numerical Aperture

NOAEL: No-Observed-Adverse-Effect Level

OECD: Organisation for Economic Co-operation and Development

pK_a: Acid Dissociation Constant

PPARδ: Peroxisome Proliferator-Activated Receptor Delta

SMILES: Simplified Molecular Input Line Entry System

$SR_{Lethality}$: Sensitivity Ratio

TR : Toxic Ratio

VPA: Valproic Acid

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Sincerely,

Munkhtsatsral Bolortoli

DECLARATION

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