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Effects of bifenthrin on neurological development, behaviour and the expression of biomarkers associated with Alzheimer's disease in *Nothobranchius furzeri*

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Attention: This thesis is presented in the form of a scientific article rather than a conventional thesis. It therefore consists of a main article, supplemented by an appendix containing additional information.

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Abstract

Neurodegenerative diseases such as Alzheimer's are influenced by various environmental factors, including exposure to neurotoxic pesticides. In this context, *Nothobranchius furzeri*, which is characterised by an accelerated ageing process, represents a model of choice for studying the effects of these substances on neuronal ageing, due to its similarities with mammals. The specific aim of this study was to assess the effects of bifenthrin, a neurotoxic pesticide widely used in the agri-food industry, on the behaviour, neurological development and gene expression of this model fish. In this study, larvae were exposed for 7 days, from day 7 post-hatching, to two concentrations of bifenthrin (5 μg/L and 10 μg/L) and compared with a control group. We assessed behavioural effects using light-dark and tapping tests on days 14 (2 weeks) to evaluate the immediate effects of exposure. To complete these tests, we selected genes involved in Alzheimer's disease-related syndromes, including *appa, psen1, syn2a, manf, gfap, slc6a4a and elavl3*. These genes were analysed by qPCR to identify any changes in their expression associated with exposure to the pollutant. Our results will contribute to understanding the mechanisms by which bifenthrin affects brain functions and associated behaviours, and to the assessment of its potential implications in neurodegenerative disorders.

Keywords: *Nothobranchius furzeri*, Neurodegenerative disease, Bifenthrin, Aging

1. Introduction

Neurodegenerative diseases represent a major global health challenge due to their rising prevalence. These diseases are characterized by the loss and degeneration of neurons within the nervous system, affecting millions worldwide (Agnello and Ciaccio 2022). In 2019, Alzheimer's disease (AD) and dementia were identified as the seventh leading cause of death globally, accounting for 1.6 million deaths (WHO et al., 2020). Currently, over 55 million people are affected by dementia, with projections indicating that this number will double every 20 years, reaching 139 million by 2050 ('ADI - Dementia Statistics', n.d.). The ageing population, a major risk factor for these diseases, is particularly impactful in low and middle-income countries where the elderly population is growing the fastest (Chávez-Fumagalli et al. 2021; Syam et al. 2023).

Understanding the aging process is essential to elucidate the mechanisms underlying neurodegenerative diseases. Ageing, a complex and multi-component phenomenon, is often defined as the progressive decline of biological functions or the accumulation of cellular damage over time, which probably includes elements of both (Keshavarz et al. 2023). It involves a decline in repair mechanisms and increased exposure to damaging factors, increasing the risk of serious diseases and contributing to mortality. This process is intrinsically linked to senescence, a natural cellular response to stressors such as telomere attrition, genomic instability or oxidative stress (McHugh and Gil 2018). By stopping cell proliferation without inducing apoptosis, senescent cells accumulate age-related changes that can disrupt tissue function over time and ultimately contributing to diseases such as dementia and cancer (Fridlyanskaya et al. 2015; Zhang et al. 2022).

While age is a major factor, genetic predispositions and environmental influences also play crucial roles in disease progression. Environmental factors, including exposure to pollutants, stressors and microbial agents, can have a detrimental effect on brain health (Ayeni et al. 2022). More specifically, pollutants such as heavy metals and pesticides have been associated with the onset of neurodegenerative diseases (Jaishankar et al. 2014; Engwa et al. 2019; Arsuffi-Marcon et al. 2024). For example, pyrethroid pesticides are known to produce neurotoxic effects by interfering with the function of calcium channels, excessively activating glutamatergic receptors and inducing high oxidative stress levels (Arsuffi-Marcon et al. 2024).

This category includes bifenthrin, the pesticide used in this experiment, a third-generation synthetic pyrethroid. Bifenthrin is mainly used as a powerful insecticide in agriculture and for the prevention of diseases caused by pests such as termites, flies and cockroaches (Eghan et al. 2023). It affects the sensitivity of sodium ion channels, causing neurotoxic effects and increasing oxidative stress throughout the mammalian brain (Park et al. 2020). This pollutant interacts with the alpha subunits of voltage-gated sodium transporters, holding them open. This causes a continuous flow of sodium into the nerve cells, leading to persistent depolarisation of the neurons (Hołyńska-Iwan and Szewczyk-Golec 2020).

Bifenthrin was detected in the environment at concentrations ranging from nanograms per liter to micrograms per liter, with an average concentration of 5 μg/L. Levels of 437 ng/g were measured in suspended sediments, while concentrations of 3.79 μg/L to 5.3 μg/L were reported in surface water (Pennington et al. 2014; Sung et al. 2023; Eghan et al. 2023). Studies have also revealed concentrations of as high as 6.12 μg/L in water from residential areas (Beggel et al. 2011).

Research has highlighted the toxicological effects of bifenthrin on vertebrates and mammals, such as oxidative stress, immunotoxicity and tumorigenicity (Jin et al., 2014). For example, in *Danio rerio*, bifenthrin disrupts embryonic cell cycles, increases ROS levels, decreases heart rate and has anti-angiogenic effects (Park et al., 2020). Other effects include hepatotoxicity, endocrine disruption and impaired swimming performance (Eghan et al., 2023). In *Hypomesus transpacificus*, bifenthrin concentrations as low as 2 ng/L induced hyperexcitability, increasing larval motility and potentially reducing predator escape and feeding efficiency (Abrahams 2005; Mundy et al. 2020). Given the known neurotoxic effects of certain pollutants released into the environment, such as bifenthrin, and the progression of AD, which is now affecting more and more people, it is important to study and understand the impact of the environment on the health of the populations exposed to it.

AD is a neurodegenerative disorder characterised mainly by memory loss and cognitive impairment. It affects most people over the age of 65, although cognitive changes can occur long before the first symptoms appear. These disorders are mainly characterised by the aggregation of β-amyloid plaques and tau proteins, leading to the cessation and degradation of neuronal activity (Scheltens et al. 2021; Knopman et al. 2021). From a genetic point of view, certain genes such as amyloid precursor protein (*appa*), presenilin 1 (*psen1*) and presenilin 2

(*psen2*) are closely associated with the development of AD (Xiao et al. 2023). However, despite a great deal of research and the complexity of the lab models available, no curative treatment has yet been discovered (Drummond and Wisniewski 2017; Passeri et al. 2022).

In this study, *Nothobranchius furzeri*, a fish known to be the most rapidly ageing vertebrate in the laboratory, was used as a model to assess the potential neurotoxic impact of bifenthrin at environmentally relevant concentrations. This species, native to south-east Africa, has an average lifespan of 3 to 7 months and is widely recognised in ageing research (Platzer and Englert 2016; Hu and Brunet 2018). Its unique reproductive strategy, involving periods of diapause (**Appendix 1**) (Bartáková et al. 2013; Poeschla and Valenzano 2020), makes it a promising model for studying the factors that influence ageing and, by extension, the development of neurodegenerative diseases such as AD. One of the main reasons for the growing use of this model in the laboratory, apart from its ability to be easily bred in captivity, is the presence of many ageing markers like those found in mammals (Platzer and Englert 2016). These similarities include telomere reduction, the length of which in *N. furzeri* (5 to 7 kb) is comparable with the human length (5 to 10 kb), as well as altered mitochondrial function and epigenetic modifications (Hartmann et al. 2009; 2011; Baumgart et al. 2014; Platzer and Englert 2016).

Even though some studies have already explored the effects of bifenthrin on cognitive disorders and the development of neurodegenerative diseases, such as Parkinson's symptoms in rodent or cytotoxic effects in microglial cell cultures (Gargouri et al. 2018; Y. Zhang and Zhang 2024), no study to date has assessed the toxic effects of this pollutant on *N. furzeri*, and even less with the aim of identifying syndromes similar to those of AD.

In this context, the aim of this study was to demonstrate the potential effects of bifenthrin on neurodevelopment, neurobehaviour and gene expression in *N. furzeri*. To do so, this experiment was carried out on three groups of 14 days post hatching (dpf) larvae exposed to bifenthrin for one week from the 7dpf. The concentrations selected were based on the averages found in the environment, which is 5 μg/L, as well as twice of this concentration and a DMSO control group.

Behavioural tests, such as the visual motor response (VMR) and the vibrational startle response (VSR), were performed on each individual at the end of the 14dpf. The heads of these individuals were then recovered for gene expression analyses using RT-qPCR on their brains.

Eight genes known to be involved in neurodevelopment, neurobehaviour and the potential development of AD were tested: *appa, psen1, psen2, slc6a4a, syn2a, gfap, manf and elavl3*. The goal was to identify any changes in the behaviour of individuals and in gene expression following exposure to bifenthrin, in order to determine whether this pollutant can induce AD-like syndromes. Our results will contribute to understanding the mechanisms by which bifenthrin affects neuronal development and associated behaviours, as well as assessing its potential implications in neurodegenerative disorders.

2. Material and methods

All this experimentation was carried out in accordance with the ethical protocol ETHICFORM2024 24/424 KE Killi bifenthrin (UNamur).

2.1 *Nothobranchius furzeri* husbandry

All experiments were conducted on the MZCS222 strain of *Nothobranchius furzeri*, present in the laboratory since February 2024. The fish were maintained at a water temperature of 27°C ± 1°C, with a conductivity ranging from 600 to 650 µS cm^{-1}, a pH between 7.0 and 7.6, and subjected to a 12/12 h photoperiodic light/dark cycle. All physico-chemical parameters, including pH, temperature and conductivity, were measured daily. The fish were fed *ad libitum* once a day with live *Artemia nauplii* produced on site.

The adults, whose eggs were used to produce the individuals used in the experiments, were kept in 9L aquariums integrated with a continuous water circulation system. These individuals were reared in harem conditions, with a ratio of one male to three to four females. A sandbox has been placed in each aquarium to encourage the females to lay their eggs there, preventing them from at the bottom of the aquarium, where they could be predated.

2.2 Collection and hatching of eggs

The eggs were collected and sorted using a sieve before being examined under an electronic microscope to select only fertilised and viable individuals. After sorting, the eggs were successively exposed for 30 seconds to a 0.5% hydrogen peroxide solution, autoclaved system water (SW) and a 1% methylene blue solution. The aim of this procedure was to remove as many impurities as possible from the eggs. After treatment, the eggs were placed on coconut fiber in Petri dishes and incubated at 28°C for 20-30 days until the golden eye stage was reached.

Once this stage had been reached, it indicated that the eggs were prepared for hatching. They were then exposed to a 1 g/L humic acid solution mixed with 2 cm of SW at 28°C in 1 L oxygenated tanks using a bubbler and left to hatch naturally at room temperature (20°C). After hatching, the newly emerged larvae were transferred individually to 12-well plates, each containing 4 ml of SW.

2.3 Bifenthrin exposure

Bifenthrin (CAS No. 99267-18-2, purity ≥ 98.0%, typically 99.0% according to the certificate of analysis) was purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. Freshly hatched larvae, placed individually in 12-well plates, were placed for 7 days in SW. From the 7th day post-hatching, they were subjected to different experimental conditions for 7 days: control group (DMSO 0.01%), 5 μg/L bifenthrin and 10 μg/L bifenthrin (N=28 per condition). The solutions in the wells were renewed at 75% every 24 hours, by removing 3 mL of solution, after which the larvae were fed *ad libitum* with *Artemia salina nauplii*. On the day 14 post hatching all the wells were refilled with SW before behavioural assays.

2.4 Measurements of body length and weight

During the experiment, daily photos of each 12-well plate in the presence of a graph paper standard were taken using an iPhone 11. These images were then analysed using ImageJ software to assess the possible impact of bifenthrin on the growth of the individuals.

2.5 Behavioural analysis

All behaviour tests were measured in 12-well plates at 14 days post-hatch (14dph) using the DanioVision video tracking system (Noldus, Netherlands). The data obtained were then processed and analysed using EthoVision XT 15 (Noldus, Netherlands) and R software.

- Visual motor response

The visual motor response (VMR) or light/dark test is designed to assess an individual's ability to adapt to a change in environment, while measuring their anxiety level and stress tolerance in response to these changes (Burton et al. 2017). In this test, individual larvae placed in 12-well plates identical to those in which they were exposed underwent 10 minutes of acclimation in the dark before being exposed successively to 10 minutes of light and 10 minutes of darkness for 2 cycles. The total distance moved (TDM) during each minute of the test was recorded for statistical analysis.

- Vibrational startle response

The vibrational startle response test (VSR) was designed to assess the ability of individuals to react to a sudden stimulus, such as a threat from a predator, as well as their ability to escape and habituate to repeated stimuli (Faria et al. 2019). This test was performed after the VMR test, with a 10-minute acclimation period in between. At the start of the recording, a delay of 15 seconds was allowed before the first impact was applied to the plate. Thereafter, an impact was applied every second for a total of 40 seconds. After the series of 40 impacts, a further period of 15 seconds was recorded. The intensity of the impacts applied to the plate was defined by a force of 8/8.

2.6 Euthanasia

After behavioural analyses, all larvae were euthanised by overdosing with MS-222 at a concentration of 200 mg/L. Each larva was weighed individually before being dissected to remove the heads for RT-qPCR analyses targeting the brains. The heads were pooled into 8 pools of 3 and 2 pools of 2 for each experimental condition (10 pools of samples per experimental condition), then rapidly frozen in liquid nitrogen to preserve RNA integrity.

2.7 Biomarker genes

- *Amyloid beta precursor protein a* (*appa*): is one of the most implicated genes in the development of AD, due to its role in the production of amyloid peptides, responsible for the formation of β-amyloid plaques. The aggregation of these plaques forms neurotoxic structures, triggering inflammatory reactions and altering neuronal transmission (Dey et al. 2024);
- *Presenilin 1 and 2* (*psen1 & psen2*): multitransmembrane proteins forming the catalytic subunit of the γ-secretase enzyme complex, which is involved in the cleavage of amyloid peptides, thus preventing the formation of β-amyloid plaques. A mutation in these presenilins is thought to be largely responsible for many cases of AD (Newman et al. 2020);
- *Solute carrier family 6 member 4a* (*slc6a4a*): gene coding for a serotonin transporter, which plays a fundamental role in the modulation of the serotonin system and is an important marker of neuronal development and anxiety levels (Lamb et al. 2020);
- *Synapsin IIa* (*syn2a*): codes for a protein involved in regulating the release of key neurotransmitters such as glutamate and GABA. Any disruption to this process can

lead to neurological disorders, particularly in response to nerve damage (Pullaguri et al. 2021; H. Hu et al. 2022);

- *Glial Fibrillary Acidic Protein* (*gfap*) and *Mesencephalic Astrocyte-Derived Neurotrophic Factor* (*manf*): these two genes are of interest for the neurological development of individuals. The first is expressed in astrocytes and maintains their structure and plasticity. The second is involved in the survival and development of neurons (Zhu et al. 2020; Ivantsova et al. 2023);
- *ELAV like neuron-specific RNA binding protein 3* (*elavl3*): codes for a neuron-specific RNA-binding protein (Huc) and serves as a biomarker of early neuronal development. In zebrafish, Elavl3 is expressed very early in embryogenesis and persists throughout neuronal development, but it remains to be seen whether this model also applies to *N. furzeri* (S. Wang et al. 2022; Eghan et al. 2023);

All these genes were normalized using the housekeeping gene (**Appendix 7**) encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), commonly used as a reference in brain research. Essential for glycolysis and involved in functions such as membrane transport and microtubule assembly, this gene is conserved and expressed in all cells (Zamani et al. 2020).

2.8 Gene expression quantification

In preparation for the gene expression assay, RNA extraction and isolation were performed using the TRI Reagent (TRIzol) method. The sample pools, consisting of 10 samples per experimental condition, were mixed with 500 μL of TRIzol, then homogenised using the 'Bullet Storm 24' apparatus in a cold chamber (2 cycles of 2 minutes at a speed of 12). Next, 500 μL of TRIzol was added to reach a total volume of 1 mL, followed by the addition of 100 μL (10% of the total volume) of 1-bromo-3-chloropropane (BCP). The samples were then vigorously mixed, incubated for 10 minutes at room temperature (RT) and centrifuged for 15 minutes at 12 000 X *g* at 4°C using a model 5810R centrifuge.

After centrifugation, the RNA-containing supernatant was collected in a new Eppendorf PCR tube for each sample and mixed with 500 μL of 2-propanol. The samples were incubated at RT for 10 minutes. Centrifugation was repeated at 4°C for 10 minutes, followed by removal of the supernatants by inversion to preserve the sedimented pellets. The pellets were washed twice with 75% ethanol, each wash being followed by a 5-minute centrifugation at 4°C. The pellets were air-dried until visible disappearance, then resuspended in 25 μL of nuclease-free water.

This was followed by measurement of the RNA concentrations of the sample using Nanodrop. Before being subjected to DNase treatment (DNA-free™ Kit, DNase Treatment and Removal Reagents, #AM1906), all samples were adjusted to an equivalent RNA concentration by dilution with nuclease-free water, based on the results obtained by Nanodrop (**Appendix 6**).

Before qPCR amplification, all samples were subjected to a reverse transcription step using the Thermo Scientific RevertAid RT Kit (#K1691) according to the manufacturer's protocol. This reaction was completed by a sequential incubation of 5 minutes at 25°C, followed by 60 minutes at 45°C, and a final step at 70°C for 5 minutes, allowing the synthesis of complementary strands of DNA (cDNA) from the RNA sequences.

qPCR (ThermoFisher QuantStudio 5 Real-Time PCR) was performed in 384-well plates. For each gene studied, a Reverse Transcription minus (RT-) control was performed, containing 1 μL of each of our samples and allowing the generation of 5 standards (1x, 5x, 25x, 125x and 625x), in addition to a No Template Control (NTC). In the plates, 2.5 μL of samples diluted to 25x were added to 2.5 μL of primers mastermix, supplemented with 5 μL of SYBR Green. All samples were loaded onto the plate in triplicates. qPCR conditions: Initial incubation at 95°C for 15 seconds, followed by 45 cycles of amplification comprising 10 seconds at 95°C, 20 seconds at 60°C and a three-step melting curve: 15 seconds at 95°C, 1 minute at 60°C and 15 seconds at 95°C.

2.9 Statistical analysis

Statistical analyses of the behaviour were carried out using RStudio software, version 4.3.1. Firstly, the VMR test was analysed using a linear mixed model (Distance ~ Condition * Cycle + (1 | Arena) + (1 | Time)), considering a repeated day-night cycle. This model evaluates the impact of the experimental condition, light-dark cycles and their interaction on the distance travelled, with 'Arena' and 'Time' as random effects to consider the variability between arenas and the correlation of observations over time. Conditions were compared using post-hoc tests within each cycle. For the VSR test, the data were analysed using a repeated measures ANOVA to evaluate the potential effect of experimental condition and time on distance travelled. RT-qPCR analyses were performed using Excel software version 16.89.1 using the delta delta Ct method. Graphs and two-way ANOVA analyses were then performed using GraphPad Prism software version 10.4.1.

To compare the growth of individuals during exposure to bifenthrin, daily averages of fish size for each experimental condition were calculated to reduce the margin of error associated with the quality of the photos and the use of ImageJ software to perform the measurements. Changes in fish growth between the different experimental conditions over time were compared using a repeated measures ANOVA.

3. Results

3.1 Larval growth

Comparison of weights between the different experimental conditions revealed a small significant difference only between the 5 μg/L and 10 μg/L groups ($p = 0.049$). The average weights observed for each condition were as follows: Control: 3.98 mg, 5 μg/L: 4.34 mg, and 10 μg/L: 3.91 mg. In terms of body length, no significant differences were observed between the different experimental conditions.

3.2 Behavioural tests

- Visual Motor Response (VMR)

All the individuals tested showed significantly greater nocturnal activity than in the presence of light (**Figure 1**). The group exposed to 10μg/L bifenthrin showed a significant reduction in exploratory behaviour in the dark, measured by distance travelled. Indeed, p-values of 0.0050 and 0.0062 were obtained respectively for the comparison between 10μg/L and 5μg/L, and between 10μg/L and the control group. No significant difference was observed between the 5μg/L group and the control group (p-value: 0.9903). In comparison, the control and 5μg/L groups moved an average of 78608 mm and 76761 mm respectively over the test, while the 10μg/L group moved an average of 60235 mm, a reduction of over 20%. In the presence of light, no significant difference was observed between the different groups.

- Vibrational Startle Response (VSR)

The VSR test did not show any significant difference between the different experimental conditions, despite a small apparent decrease in the reaction level of individuals exposed to bifenthrin in the 5μg/L and 10μg/L concentrations (**Figure 2**). The experimental condition variable showed no significant difference in the statistical model used to compare the different conditions, with a p-value of 0.197.

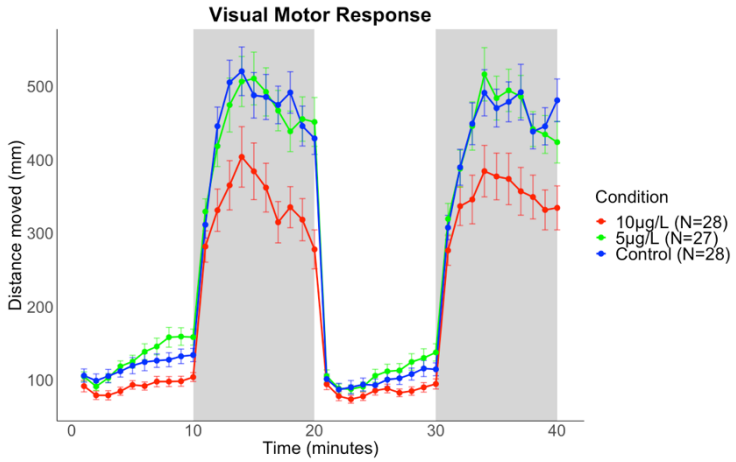

Figure 1: The visual motor response test was performed on 14dpf larvae of *Nothobranchius furzeri* exposed to three experimental conditions: a DMSO control group and concentrations of 5 µg/L and 10 µg/L bifenthrin. The test consisted of two day-night cycles, each cycle consisting of 10 minutes of light (white zone) followed by 10 minutes of darkness (grey zone). Data are presented as means for each condition and for each minute of the test, with the standard error of the mean (SEM).

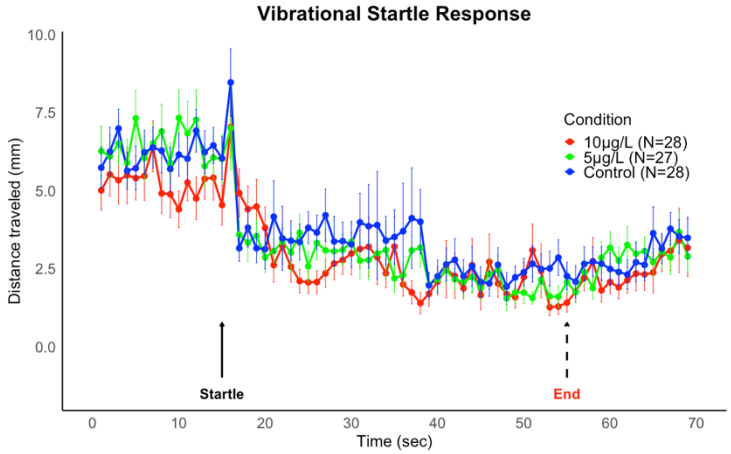

Figure 2: The vibrational startle response test was performed on 14dpf larvae of *Nothobranchius furzeri* exposed to three experimental conditions: a DMSO control group and concentrations of 5 µg/L and 10 µg/L bifenthrin. The test took place entirely in the dark, after a 10-minute acclimatisation period. Fifteen seconds after the start of recording, the first tap was applied to the plate. Thereafter, a tap was applied every second for 40 seconds. Once this period was over, the recording continued for a further 15 seconds. Data are presented as means ± SEM for each condition and each second of the test.

3.3 Gene expression

A pattern of increased gene expression was observed for all the genes tested in this study, except for the elavl3 gene, whose expression remained stable across the different experimental conditions. From a statistical point of view, ANOVA tests revealed three significant differences, observed for the *appa* (p-value: 0.0027), *gfap* (p-value: 0.0114), and *syn2a* (p-value: 0.0026) genes, each time between the control condition and the highest concentration (**Figure 3**). The increases in expression for these three genes were 59.4%, 51.3% and 59.4% respectively.

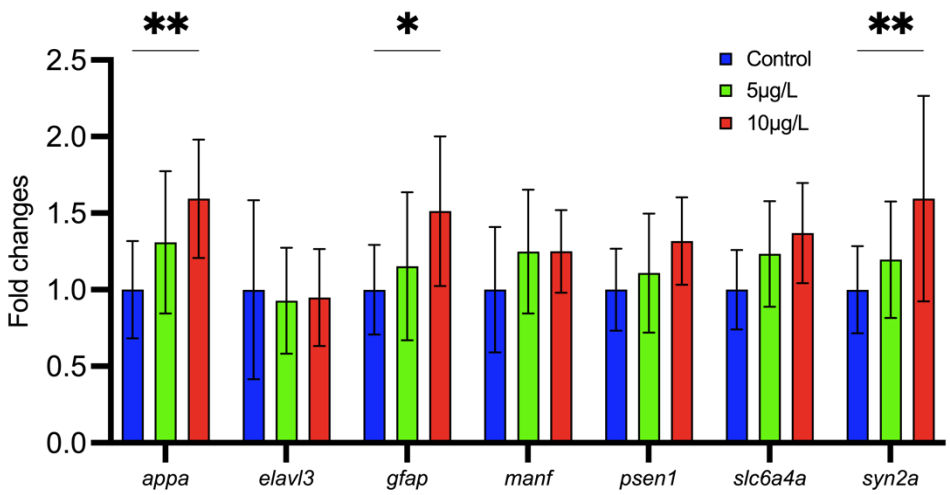

Figure 3: Gene expression changes in 14dpf *Nothobranchius furzeri* larval heads exposed to different experimental conditions: DMSO control group, 5μg/L bifenthrin and 10μg/L bifenthrin. The genes selected above are involved in the neurodevelopment and neurobehaviour of individuals. For each gene, the results show the mean gene expression and standard error (SD). Significant differences (p-value < 0.05) are illustrated by an asterisk with a bar linking significantly different conditions.

4. Discussion

The results highlight the behavioural effects induced by exposure to bifenthrin at environmental relevant concentrations. As demonstrated by the VMR and VSR tests, fish of the species *N. furzeri* exposed to environmentally relevant concentrations of bifenthrin showed notable behavioural changes. These changes were characterised by a reduction in the total distance travelled and the appearance of erratic movements in certain larvae during the exposure period. Indeed, during this experiment, some larvae exposed to the highest concentration remained immobile for long periods before showing occasional erratic movements. In addition, analysis of data from the VMR test showed that the nocturnal activity of the *N. furzeri* species was significantly reduced when exposed to a concentration of 10μg/L. These observations, based on the total distance moved, are good indicators of the neurotoxic effects of this pollutant on the nervous system and can also illustrate a certain neurodegeneration in muscle performance (Sztal et al. 2016; Fitzgerald et al. 2019).

The observed reduction in activity, demonstrated by the decrease in distance travelled in the group exposed to 10 μg/L, is consistent with studies showing that dietary exposure to bifenthrin reduces swimming behaviour in juvenile *Oncorhynchus tshawytscha* (Magnuson et al. 2022). Furthermore, impaired cardiac function is also known to negatively affect the swimming

behaviour of fish. Bifenthrin, which alters voltage-dependent sodium channels, probably impacts the cardiac muscle function of exposed individuals and, consequently, their swimming performance (Claireaux et al. 2005). This impact on sodium channels is also probably responsible for the erratic movements observed in exposed fish, and these cases do not appear to be isolated, as shown by studies on zebrafish (DeMicco et al. 2010; Eghan et al. 2023).

The effects of hypoactivity observed in exposed individuals appear to be supported by other studies conducted on the larval stage. Indeed, signs of hypoactivity were also reported following exposure to bifenthrin in larval *Spirinchus thaleichthys* specie. However, these effects dissipated between 72 and 96 hours after exposure (Mauduit et al. 2023). This is consistent with the observations made during this experiment, where, during the daily renewal of the bifenthrin solutions, the larvae appeared to be severely affected, showing signs of immobility and spasms. However, the following day, all seemed to have returned to their normal form.

These observations are aligned with the known effects of bifenthrin, which causes tremors, convulsions and muscle contractions in exposed individuals (Gammon et al. 2019; Buchweitz et al. 2019). By disrupting sodium channels, bifenthrin promotes a significant increase in the entry of this ion, leading to an intensification of multiple nerve impulses. Other studies suggested that this phenomenon could be linked, among other things, to an alteration in the activity of acetylcholine esterase, which was not evaluated in this study. Inhibition of this enzyme would lead to a significant accumulation of acetylcholine neurotransmitters, contributing to significant neurotoxic effects (Ullah et al. 2019; 2022).

Under natural conditions, this type of behavioural alteration poses a risk to the integrity of exposed individuals. This is why the VSR test was developed, to assess the ability of larvae to escape from a negative stimulus, such as a predator, but also to observe their ability to accommodate to a repeated stimulus (Faria et al. 2019). Larvae exposed to bifenthrin showed no significant difference from the control group, but nevertheless appeared less responsive to the first tapping stimulus at 15 seconds. Thereafter, the trend observed for the three conditions was relatively similar, with a search for habituation up to around 40 seconds, before reaching a similar habituation pattern in all groups. The distance covered was also lower in the presence of the tappings than before the first stimulus. However, the exposed groups showed a tendency towards reduced reactivity to the first stimulus, which could indicate the potential neurotoxic effects of the pollutant studied, notably on the serotonin system (Faria et al. 2019).

The RT-qPCR results show a significant increase in the expression of the *syn2a*, *gfap* and *appa* genes, suggesting neuronal dysfunction, accompanied by an inflammatory reaction and possibly oxidative stress. Increased expression of the *gfap* gene could indicate a response by glial cells, particularly astrocytes, to bifenthrin-induced neuroinflammation. In many neurodegenerative diseases, such as AD, the activation of microglia plays a crucial role in their development. This is because stimulation of glial cells is often associated with excessive production of inflammatory cytokines, leading to significant neuronal damage (Gargouri et al. 2018). Concerning *appa* and *syn2a*, these two genes are involved in synapses and their function, but at different levels. The first, *appa*, is associated with neuronal differentiation and proliferation, contributing to the formation of synapses (Kreis et al. 2021; Orobets and Karamyshev 2023). In contrast, *syn2a* is mainly involved in synaptic function, regulating the transmission of GABA and glutamate (Guo et al. 2023). Alterations in these different genes could suggest impairment of synaptic transmission and cognitive decline. Although the other genes studied showed no significant difference, it is interesting to note an upward trend for three of them: *manf*, *slc6a4a*, and *psen1*. This observation supports the interpretations described above.

The findings obtained in this research reinforce the relevance of using *N. furzeri* in the laboratory as a model for studying AD. Indeed, it has now been shown that the fully sequenced genome of this fish contains numerous genes paralogous to those involved in the genesis of AD in humans, such as *appa* and *appb* (paralogs of *APP*), *psen1* and *psen2* (subunits of γ-secretase), and *bace1* and *bace2* (β-secretase) (De Bakker et al. 2024).

In addition to its biological specificities, *N. furzeri* naturally exhibits several characteristics associated with cerebral ageing, such as a reduction in the number of dopaminergic and adrenergic neurons, an increase in microglia, the presence of insoluble aggregates, and a significant reduction in learning capacity (de Bakker and Valenzano 2023). However, despite the presence of these manifestations, no typical symptoms, such as the appearance of β-amyloid plaques or dementia, have yet been clearly observed in species other than humans (Walker and Jucker 2017).

Nevertheless, amyloidosis remains a key factor in the development of neurodegenerative diseases, the absence of natural models of age-related brain degeneration is a major limitation to the study of the causal mechanisms of these diseases. This underlines the importance of *N. furzeri* as an interesting biological model for exploring the genetic and environmental factors

involved in the development of these diseases (Walker and Jucker 2017; de Bakker and Valenzano 2023).

Instead, it is essential to consider the fundamental differences between the brains of *N. furzeri* and those of other species, such as humans, with which comparisons are made. In addition to the significant differences in size and number of neurons, these brains have very different architectures. For example, in humans, the functions linked to memory and learning are located in the cortex, whereas in this species of fish, these functions reside in the telencephalon. It is therefore crucial to explore in detail the anatomical and functional analogies between the species before drawing any conclusions. Despite these differences, the *Nothobranchius furzeri* laboratory model is proving particularly promising as a tool for studying neurodegenerative diseases such as AD.

5. Conclusion

Exposure to environmentally relevant concentrations of bifenthrin appears to induce behavioural alterations in *Nothobranchius furzeri* larvae, resulting in a marked reduction in swimming activity and a tendency to reduce reactivity to a sudden stimulus. In addition, RT-qPCR results revealed significant alterations in genes associated with neurological behaviour and development, as well as a significant increase in the expression of a gene similar to those involved in the development of Alzheimer's disease in humans (*appa*).

However, it is important to stress that the results of this study are not intended to identify behaviours specific to Alzheimer's disease, as observed in humans. Rather, the main objective was to examine the potential neurotoxic effects of bifenthrin on the development of biomarkers associated with Alzheimer's-like syndromes. As the individuals used in this research were at the larval stage, it would be relevant to study a later point in the life cycle of *N. furzeri*. This would make it possible to determine whether this pollutant can indeed induce Alzheimer's-like syndromes at a more mature stage of life.

N. furzeri is therefore a promising model, particularly from a genetic point of view, for studying brain degeneration and the potential impact of pollutants. Its many advantages, such as brain phenotypes associated with ageing, facilitate longitudinal research (de Bakker and Valenzano 2023).

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Appendixes

Appendix 1: State of the art with additional information

Neurodegenerative diseases

Neurodegenerative diseases pose a significant global health challenge due to their rising incidence. They cover a variety of complex diseases characterised by the loss and degeneration of neurons within the nervous system, affecting individuals worldwide (Agnello and Ciaccio 2022). In 2019, according to the Global status report on the public health response to dementia, Alzheimer's disease and dementia have been identified as the seventh leading cause of death worldwide, accounting for 1.6 million deaths (Organization W.H. and others 2020). Currently, more than 55 million people are affected by dementia, and this figure will double every 20 years over the next few decades, reaching 139 million by 2050 ('ADI - Dementia Statistics', n.d.). With the ageing population process being a major risk factor for numerous neurodegenerative diseases, particularly in low and middle income developing countries, where the elderly population is growing fastest and life expectancy is longest (Chávez-Fumagalli et al. 2021; Syam et al. 2023).

Neurodegenerative diseases cover a wide range of pathologies, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). Their increasing prevalence represents an unprecedented challenge for healthcare systems and society. In addition to their impact on individual health, neurodegenerative diseases also exert a considerable social and economic burden. The costs of medical care, the challenges for family carers and the loss of productivity associated with these conditions represent major challenges for modern societies (Huang et al. 2023; Tay et al. 2024).

It is widely acknowledged that neurodegenerative diseases are associated with ageing. However, beyond age, genetic predispositions and environmental influences exert significant influence on disease progression. Environmental factors include a range of variables, including exposure to pollutants, environmental stresses, and microbial agents, all of which can have a direct impact on brain well-being. For example, the prenatal environment represents a critical window during which foetuses can be exposed to stressors such as hypoxia or hormonal imbalances, which ultimately influence the trajectory of mental development later in life (Ayeni

et al. 2022). Umbilical cord occlusion (UCO) can also lead to synaptic dysfunction in newborns and neurodegeneration in later life (Hyun et al. 2008; Ayeni et al. 2022). This is due to the phenomenon of hypoxia and severe asphyxia caused by UCO following a rearrangement of ventricular output towards organs of interest such as the brain. Prolonged exposure to hypoxia can cause significant brain damage due to overexcitation of N-methyl-D-aspartate receptors, leading to inflammation and apoptosis of the tissues (Zarate et al. 2018).

From an environmental perspective, there is evidence suggesting that various pollutants such as heavy metals or pyrethroid pesticides can have an impact and contribute to the onset of neurodegenerative conditions (Jaishankar et al. 2014; Engwa et al. 2019; Arsuffi-Marcon et al. 2024). However, further research is needed because of the inconsistency of the results. Some studies establish a correlation between high levels of aluminium in drinking water and the development of Alzheimer's disease, but not all agree on this (Ayeni et al. 2022). In addition, pesticides such as pyrethroids are known to have significant neurotoxic effects by altering the function of calcium channels, overstimulating glutamatergic receptors and inducing significant oxidative stress. These combined stresses have a significant impact on neurogenesis, which can lead to changes in memory mechanisms (Arsuffi-Marcon et al. 2024).

This research focuses on evaluating the impact of ageing on the emergence and progression of neurodegenerative diseases. As the principal parameter in the onset of these conditions, an in-depth understanding of the ageing process and its involvement in triggering these diseases is essential.

Ageing process

Defining ageing with scientific precision is challenging, as the meaning attributed to 'ageing' varies considerably. Some define it as the progressive decline of basic biological functions, while others see it as the accumulation of harmful damage over time. It is likely that ageing encompasses aspects of both definitions, involving altered or totally lost repair mechanisms and increased exposure to damage. These alterations not only increase the likelihood of developing serious diseases, but can also contribute to individual mortality (Keshavarz et al. 2023).

Ageing is linked to the natural process of senescence which is a physiological process of cellular response aimed at limiting the development of aged or damaged cells. It is a stress response

triggered by biological signs of ageing such as telomere attrition, genomic instability or oxidative stress (McHugh and Gil 2018). Senescence is characterized by the arrest and permanent exit of proliferative cells from the cell cycle without their entering apoptosis. These cells accumulate age-related changes and build up in the tissues over time. This natural process can lead to the development of diseases associated with ageing, such as dementia and cancer (Fridlyanskaya et al. 2015; L. Zhang et al. 2022).

Ageing therefore results from natural processes such as programmed senescence, but also from changes accumulated throughout an organism's life due to exposure to a variable environment, inducing phenotypic alterations. These phenotypic changes that organisms undergo may be species-specific or genetically determined, but they also depend on the environment to which they are exposed: *« [phenotype] = [genotype] + [(diet, lifestyleandenvironment)] »* (da Costa et al. 2016; Keshavarz et al. 2023). It should be noted that certain phenotypic changes can be completely benign and have no negative consequences on individuals, such as wrinkles (da Costa et al. 2016).

Although ageing is characterized in the majority of species by genomic and epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence or altered stem cell function and cellular communication (Hou et al. 2019). However, many ageing mechanisms are specific to certain taxa or environments (Wensink and Cohen 2022). For example, certain species such as crocodiles, sharks and turtles do not age in the same way as mammals or birds (Jones et al. 2014). In fish, some species such as ray-finned fish show no signs of ageing, while others such as *Nothobranchius furzeri* undergo very accelerated ageing process (Wensink and Cohen 2022). These interspecific variabilities appear to be driven by adaptation mechanisms and mutations specific to the taxon. Each species faces taxon-specific constraints that influence the ageing process. For example, high levels of glucose are toxic and contribute to ageing in mammals, whereas they are essential for flight metabolism in birds (Holmes et al. 2001). Specialists call this the Danaid theory, which proposes that ageing is the result of taxon-specific constraints (Wensink and Cohen 2022).

However, there are other more general theories on the ageing process. Among these are the theory of programmed ageing and the theory based on the absence of natural selection after the reproductive period of life (da Costa et al. 2016). For the first, the ageing process is orchestrated by evolutionary mechanisms. Ageing is perceived as a decline, but one that brings evolutionary

benefits. Indeed, some studies have suggested that the phenomenon of programmed ageing reflects a self-regulating mechanism within populations. The aim of this programmed ageing is to give populations the capacity to adapt more effectively to environmental changes through reproduction and natural selection, by making younger individuals reproduce while eliminating older ones. Eliminating post-reproductive individuals would prevent overpopulation and competition for resources (Longo et al. 2005; Kirkwood and Melov 2011).

If this theory proves correct, the biochemical pathways and genes specific to ageing should be found in higher eukaryotes, as is the case in yeast, mice and salmon (Longo et al. 2005). The genetic implication of this theory of programmed ageing is not entirely illusory, since studies conducted on the *Caenorhabditis elegans* model have shown that mutations in genes involved in the insulin/IGF pathway significantly increase the longevity of these organisms (Berdichevsky et al. 2006). However, drawing conclusions applicable to vertebrates from these results is not relevant, given that these models are very different from vertebrates (da Costa et al. 2016).

Another theory of ageing, known as the damage theory, postulates that the ageing process results primarily from the progressive accumulation of cellular and molecular damage over time. This damage is caused by a variety of factors, including environmental conditions, oxidative stress and exposure to reactive oxygen species (ROS), which cause significant damage to proteins, lipids and DNA inside cells (da Costa et al. 2016). Contrary to the idea that ROS themselves directly cause age-related changes, research suggests that it is the disruption of signaling pathways involving ROS that plays a crucial role. This disruption leads to cellular dysfunction and contributes to the ageing phenotype observed in organisms (De Magalhães 2013).

Furthermore, ageing is associated with an increased incidence of neurodegenerative diseases, partly due to the accumulation of oxidized proteins (David 2012). With age, the activity of the proteasome, responsible for degrading damaged proteins, decreases, disrupting the homeostasis of proteins in cells. Studies indicate that increasing proteasome activity in cell models can extend lifespan by up to 20%, underlining the crucial importance of protein renewal in the ageing process (da Costa et al. 2016). Consequently, the damage theory of ageing emphasizes that the progressive accumulation of cellular and molecular damage, exacerbated by oxidative

stress and altered proteostasis, contributes significantly to the ageing process and the development of age-related diseases.

Despite a better understanding of the main biomarkers of ageing, there is still a need to quantify this complex process more precisely. A better understanding of the specific correlations between these biomarkers and ageing is crucial to progress in this area of research (Hou et al. 2019). In this context, while the animal models traditionally used in this field include transgenic mice, *Drosophila melanogaster*, *Caenorhabditis elegans*, and certain vertebrates such as *Danio rerio* (Folch et al. 2018), a new animal model has emerged in recent years as a valuable resource for laboratory research: the turquoise killifish (*Nothobranchius furzeri*).

Previous models used to study ageing were limited by their long lifespan and high rearing costs, making the research uneconomical. In contrast, the turquoise killifish has emerged as a promising new model with a very rapid life cycle (Brunet 2020). It shares many ageing markers with mammals, including humans, thereby facilitating a deeper understanding of the ageing process and the diseases associated with it (Platzer and Englert 2016).

Nothobranchius furzeri

For some years now, the *Nothobranchius furzeri* species has gained prominence in ageing research. These fish have a median lifespan of 3 to 7 months, age rapidly, achieve sexual maturity within 3 to 4 weeks, and exhibit ageing markers akin to those observed in mammals. This similarity makes *N. furzeri* particularly valuable for comparative studies with humans in the context of ageing and age-related diseases (Platzer and Englert 2016; C. Hu and Brunet 2018). This species of fish is particularly interesting compared to other species used in the laboratory, such as *Danio rerio* and *Oryzias latipes*. Its ability to be reared in the laboratory and its short lifespan make it a model of choice for research that needs to be carried out over short periods of time (Polačik et al. 2016).

Indigenous to the southeastern region of Africa, the turquoise killifish, a species dwelling in freshwater environments, exclusively inhabits the temporary pools of the African savannahs (Reichard et al. 2015; Cellerino et al. 2016). In the wild, this fish species only thrives during the rainy season. Consequently, *N. furzeri* has evolved a specific adaptation enabling it to survive dry seasons and the disappearance of its habitat zones. To achieve this, the fish species

undergoes what is known as diapause at the egg stage (Bartáková et al. 2013). Indeed, each year during the dry season, all adults of the species perish, but the eggs enter a dormant state buried in the soil, awaiting favourable conditions for hatching during the subsequent rainy season (**Figure 4**) (Cellerino et al. 2016; Terzibasi Tozzini and Cellerino 2020).

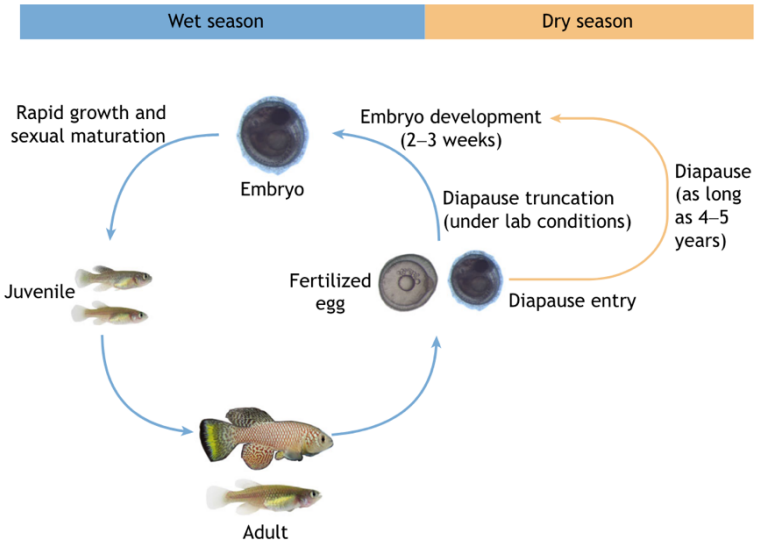


Figure 4: Figure illustrating the life cycle of *Nothobranchius furzeri*. After hatching, individuals develop and reach sexual maturity very rapidly. Fertilized eggs are capable of developing quickly within a few weeks or entering diapause, which can last for several years (Poeschla and Valenzano 2020).

Within this species, the diapause process is delineated into three distinct phases: diapause I begins when environmental conditions become challenging, initiating prior to gastrulation while all embryos develop synchronously. Diapause II initiates during mid-somitogenesis and is characterized by decreased protein synthesis, cell cycle arrest, and a reorganization of energy metabolism. During diapause II, embryos exhibit maximal resistance to extreme conditions. Finally, diapause III occurs towards the end of embryonic development, preparing embryos for hatching (Bulavkina et al. 2022; Furness et al. 2015).

During this diapause process, the embryos do not undergo the same ageing process as adult individuals. In fact, diapause can sometimes last up to several years if environmental conditions are not conducive to egg hatching. It should be noted, however, that not all individuals at the embryonic stage systematically enter diapause and that some may bypass this process, especially diapause II, and have a continuous development (**Figure 5**) (Furness et al. 2015; Hu and Brunet 2018).

The ability of this species to complete a continuous life cycle and enter diapause at the same time makes it a remarkable example of a species capable of both adaptive phenotypic plasticity and bet-hedging (Simons 2011). Bet-hedging is an evolutionary strategy in which populations randomly produce a diversity of phenotypes. This stochastic production of phenotypes enables populations to increase their chances of achieving the best fitness by eliminating phenotypes that are poorly adapted to their environment. This strategy confers a significant selective advantage in environments subject to unpredictable fluctuations (Morawska et al. 2022).

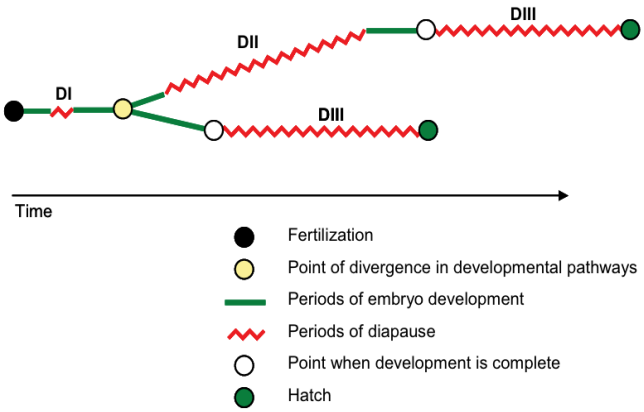


Figure 5: The figure depicts various developmental pathways of *N. furzeri*. Both pathways initiate diapause I, but the divergence between them occurs thereafter. The direct development pathway is characterized by the absence of diapause II (upper pathway in the figure), whereas the diapause-induced development pathway exhibits all three phases of diapause (lower pathway in the figure) (Furness et al. 2015).

The importance of balancing these two adaptive processes lies in the ability to cope with the uncertainty of future environmental conditions. In fact, adaptive phenotypic plasticity is advantageous when the species can produce the most appropriate adaptive phenotype based on precise indices of future environmental conditions. Otherwise, bet-hedging is a more favourable strategy (Furness et al. 2015).

Since 2015, the turquoise killifish genome has been fully sequenced, opening up new avenues for research into evolutionary and developmental biology (Valenzano et al. 2015; Reichwald et al. 2015). This fish species offers a set of standardised strains for laboratory experiments, including GRZ (Gonarezhou Park in Zimbabwe), MZMO403, MZM0410, ZMZ1001 to ZMZ1007, all collected in Mozambique and Zimbabwe (C. Hu and Brunet 2018). These strains have different lifespans, with GRZ having a median lifespan of 2 to 3 months, while the others have a median lifespan of around 6 months (Anagnostopoulos et al. 2021). In this research, the

strain used is MZCS222, originating from the Chigamane and Chefu region of Mozambique (Cellerino et al. 2016; 'Nothobranchius furzeri - la Killi-fiche du KCF', n.d.).

The *Nothobranchius furzeri* demonstrates notable adaptability for controlled laboratory breeding, owing to its capacity to thrive across diverse environmental parameters, notably encompassing variations in temperature and salinity (Harel et al. 2016; Hu and Brunet 2018). Morphologically, sexual dimorphism is evident within this species, characterized by the larger size and vividly colored fins exhibited by males (**Figure 6**) (C. Hu and Brunet 2018).

Figure 6: Apparent sexual dimorphism of the species *Nothobranchius furzeri*. In the foreground of the photo is a very colourful, large male, while in the background is a smaller, less colourful female. Both individuals are sexually mature. Picture: Nadine Grimm (Terzibasi Tozzini and Cellerino 2020).

Regarding their social behaviour, both sexes can be co-reared in laboratory settings and reproduce continuously. However, it is noteworthy that the eggs produced necessitate specific environmental conditions for hatching. Additionally, it is imperative to emphasize that males often exhibit pronounced territorial behaviours, which may lead to aggressive interactions among individuals, potentially resulting in mortality. Thus, it is recommended to maintain males separately to mitigate any risk of aggressive behaviour (C. Hu and Brunet 2018).

Despite the growing number of studies using this model to understand the mechanisms underlying ageing and associated pathologies, few results have been published specifically on Alzheimer's disease. Nevertheless, this remains an important area of research, as demonstrated by the support given to eminent researchers such as Dr Vasanta Subramanian from the University of Bath (UK) (in Alzheimer's Research UK, n.d.) and Dr Melissa Page from the Catholic University of Louvain (Belgium) ('Alzheimer Standard Grant for Melissa Page', n.d.) carrying out their research on the *N. furzeri* model.

Alzheimer

In a world where people are living longer, the emergence of neurodegenerative diseases has become a major issue. Among them, Alzheimer's disease stands out as the most common form of dementia worldwide and is ranked by the World Health Organization (WHO) as the seventh most deadly disease (WHO - 'Dementia', n.d.).

Discovered in 1906, Alzheimer's disease is a pathology primarily affecting the brain, characterized by memory loss, cognitive impairments, while even the simplest tasks become challenging. This disease primarily manifests in individuals over the age of 65. Sometimes cerebral alterations specific to Alzheimer's disease can develop well before the onset of initial symptoms. Among these symptoms, the foremost is the appearance of protein aggregates known as β-amyloid plaques and tau proteins, which induce neuronal activity cessation and consequently their degradation (Scheltens et al. 2021; Knopman et al. 2021).

Recent studies demonstrate a significant interaction between tau proteins and β-amyloid plaques in the development of Alzheimer's disease. In particular, the increase in β-amyloid plaques in the cortex promotes the spread of tau proteins beyond the entorhinal cortex, a key region involved in memory and spatial navigation (Adams et al. 2019). The migration of this tau-β-amyloid interaction to the posterior cingulate cortex, an important region for episodic memory and emotional regulation, leads to significant memory alterations. This tau-β-amyloid interaction induces cognitive alterations even in individuals without Alzheimer's disease (Sperling et al. 2019).

Moreover, post-mortem analyses have revealed that individuals suffering from Alzheimer's disease present a high concentration of β-amyloid plaques, in association with hyperphosphorylated high molecular weight tau proteins, favoring intercellular propagation (**Figure 7**). However, this phenomenon is not observed in elderly people, whose tau proteins are only associated with the ageing process (Takeda et al. 2015; Busche and Hyman 2020).

In addition to their combined impact on cognitive functions, tau proteins and β-amyloid plaques appear to exert antagonistic effects on neuronal activation once separated. Indeed, the presence of β-amyloid plaques alone induces marked neuronal hyperactivity, which is attenuated or even suppressed in the presence of tau proteins (**Figure 8**) (Busche and Hyman 2020).

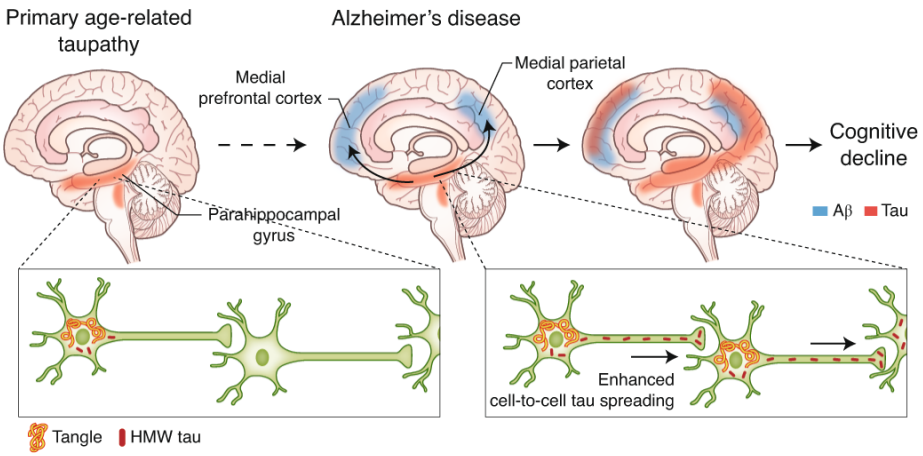


Figure 7: Top left, a cognitively healthy elderly person shows age-related tau proteins in the parahippocampal gyrus (in red). In Alzheimer's disease, β-amyloid plaques (in blue) promote the spread of tau proteins into neocortical areas, leading to cognitive decline. The lower part of the figure contrasts an elderly person in good cognitive health with the presence of a little tau protein with a person suffering from Alzheimer's disease with high molecular weight tau proteins favoring their propagation from one cell to another (Busche and Hyman 2020).

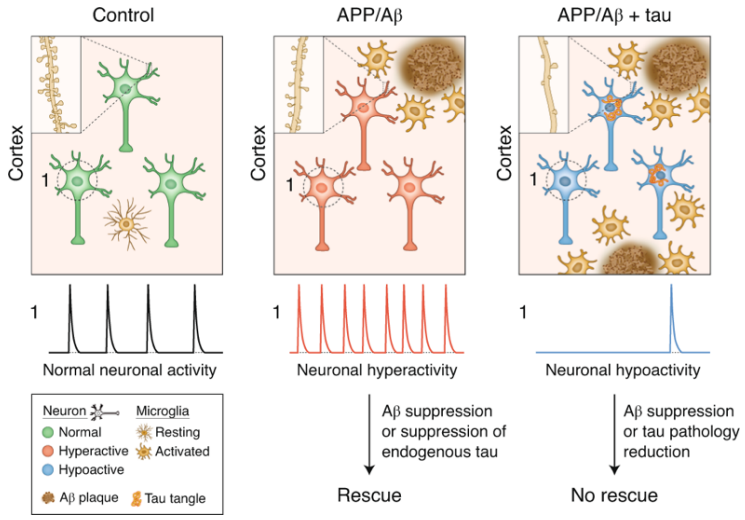


Figure 8: Compared with the control brain, the middle brain with β-amyloid plaques, microglia activation and slight loss of dendritic spines shows significant neuronal hyperactivity. This reaction is reversible following the elimination of endogenous tau proteins or β-amyloid plaques. In the third scenario, the brain shows the tau-β-amyloid interaction, characterized by almost complete loss of dendritic spines, marked activation of microglia and suppression of neuronal activity. Suppression of β-amyloid plaques or tau proteins alone is not sufficient to correct these alterations (Busche and Hyman 2020).

Alzheimer's split into three different stages: preclinical, prodromal and dementia. During the preclinical phase, certain abnormal markers like β-amyloid clusters or neurofibrillary tangles (NFTs) start to appear, but without apparent cognitive impairment. As episodic memory deficits start to show, it transitions into the prodromal stage, characterized by mild cognitive impairment (MCI). Finally, the dementia stage is the most common form known by the general public and is marked by abnormal biomarkers and clear cognitive functioning impairment (Rosin et al. 2020).

Our understanding of Alzheimer's disease has advanced considerably in recent years, but there are still significant gaps. This complex pathology is the result of an interaction between different factors, both genetic and environmental. At the genetic level, several genes have been identified as contributing to the development of the disease, with complex interactions between these genes, lifestyle, and the individual's environment (X.-X. Zhang et al. 2021; Knopman et al. 2021; Scheltens et al. 2021). Of the genes involved, three are currently recognized as major determinants of the disease: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Xiao et al. 2023). However, it is essential to note that other genetic variants, such as the apolipoprotein E (APOE) gene, also play a crucial role. In particular, the APOEε4 variant is associated with a twelve-fold increased risk of developing the disease, while the APOEε2 form appears to offer a degree of protection of 60 to 85% (Serrano-Pozo et al. 2021).

It should be noted, however, that this disease develops more prevalently in women. Initially, this finding was justified by the fact that women live longer than men. However, after further studies, scientists found that women of a younger age were less likely to develop Alzheimer's disease than men of the same age, but this trend reversed after the menopause (Beam et al. 2018). This difference is linked to a neuro-immune response by microglia that differs according to sex. The development of Alzheimer's disease is associated with a microglial response that appears to be abnormally greater in women than in men. This alteration is thought to stem from a group of microglia that favour the presentation of self-antigens on major histocompatibility complex II (MHCII) and the binding of amyloid-β. All this is combined with a greater APOEε4 genotype in women (Wu et al. 2024).

There is currently no cure for Alzheimer's disease, but several preventive measures aimed at slowing the development of the disease are currently being used. One of the problems limiting curative treatment of this disease is finding a way for the drugs to reach the nervous system and the brain. Several strategies are currently being studied, including the use of nanoparticles to increase the bioavailability of the molecules used. New routes of administration are being evaluated, such as liposomes, exosomes and the intranasal route, which has been shown to improve absorption in the brain (Passeri et al. 2022).

In addition, the animal models used in this area of research are physiologically different from humans, which can lead to certain problems (Drummond and Wisniewski 2017; Folch et al.

2018). For example, transgenic mice are the most commonly used models for studying Alzheimer's disease during the ageing process (Puzzo et al. 2015). They make it possible to reproduce various phenotypes of the disease by expressing human genes. Despite numerous positive preclinical trials on animal models, the treatments developed on the basis of these studies show a relatively high failure rate in clinical trials (Banik et al. 2015; Drummond and Wisniewski 2017). This is partly because these animals only develop certain specific characteristics of the disease without developing it fully. For example, although these animal models show an accumulation of amyloid plaques characteristic of Alzheimer's disease and associated with memory disorders, they do not develop other features typical of the disease, such as neurofibrillary tangles (Banik et al. 2015).

At the same time, research has focused on the impact of the environment and lifestyle on the development of Alzheimer's disease. Studies are examining the potential links between cardiovascular disease, metabolic disorders, and various aspects of lifestyle, such as eating habits, physical activity, and social interactions. These environmental factors may act in synergy with genetic predisposition, modulating the risk of developing the disease (Jia et al. 2019; X.-X. Zhang et al. 2021; Stefaniak et al. 2022). Furthermore, it is widely recognized that exposure to environmental stressors plays a crucial role in the development of neurodegenerative diseases. For example, exposure to pharmaceutical products or various environmental pollutants is known to have an impact on the neurological development, neurotransmission and behaviour of exposed individuals (Chin-Chan et al. 2015). Thus, as part of this study aimed at identifying the environmental factors involved in neurodegeneration in the *N. furzeri* model, bifenthrin, a pyrethroid, was chosen as the pollutant for this experiment.

Bifenthrin

Bifenthrin, a third-generation synthetic pyrethroid, is mainly used as a powerful insecticide in agriculture and for the prevention of diseases caused by pests such as termites, flies and cockroaches. (Eghan et al. 2023). Naturally derived from *Chrysanthemum cinerariaefolium* (Dalmatian pyrethrum), bifenthrin disrupts the sensitivity of sodium ion channels, resulting in neurotoxic effects in exposed individuals, accompanied by increased levels of oxidative stress in all regions of the mammalian brain. (Park et al. 2020). This third generation of pyrethroids has been developed with the aim of improving the photostability and general stability of these

compounds to prevent them from degrading and releasing by-products that are all the more toxic for the environment and exposed mammals (Hodoșan et al. 2023).

Pyrethroids have been used as insecticides since 1945, particularly in agriculture and agroforestry to combat pests. The WHO also recommends their use against disease-carrying insects such as mosquitoes, to combat malaria, for example ('Global Technical Strategy for Malaria 2016-2030, 2021 Update', n.d.). Pyrethroids are highly fat-soluble chrysanthemic acid esters, which means they are easily absorbed through mucous membranes (Hughes and Edwards 2010).

These molecules are present in the form of two chiral enantiomers, cis and trans, acting directly on membrane potential-dependent sodium channels. They interact with the alpha subunits of sodium channels, keeping them open and causing a continuous flow of sodium ions into nerve cells, thereby maintaining constant depolarisation. Pyrethroids also reduce acetylcholinesterase activity by modifying the active substrate-binding site and altering cytochrome P450 activity (Hołyńska-Iwan and Szewczyk-Golec 2020).

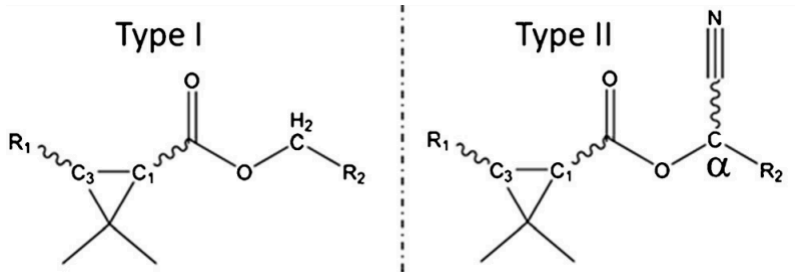


Figure 9: Comparison of the molecular structures of type I and type II pyrethroids. Identification of the alpha-cyano group in type II pyrethroids (Corcellas et al. 2015).

Classified by the WHO as class II toxicity, which makes it a moderately dangerous pesticide, the use of bifenthrin has grown rapidly over the decades. For instance, its application surged by over 53% in the last decade (Eghan et al. 2023). There are two types in the pyrethroid family (**Figure 9**): type I pyrethroids are distinguished by the absence of an alpha-cyano group within the molecule and are more aggressive, producing reflex hyperexcitability and tremors, known as T-syndrome. Type II pyrethroids have an alpha-cyano group and induce salivation, gross tremors, and scratching, in this case referred to the choreoathetosis–salivation (CS) syndrome. However, the particularity of bifenthrin lies in the fact that it belongs to type I pyrethroids while

inducing type CS syndromes, i.e. those specific to type II pyrethroids (Syed et al. 2017; Ramchandra et al. 2019).

A study carried out on sugar cane farms reported that the use of bifenthrin at the recommended doses (100g/ha) shows that its persistence in soils. This molecule has a half-life ranging from 16 to 25 days and persists in soils for up to 75 days after application of the insecticide (Ramasubramanian and Paramasivam 2021). This molecule exhibits a propensity for facile sorption onto organic substrates within terrestrial ecosystems, consequently facilitating its transport into aquatic environments, thereby engendering pervasive ecological perturbations.

Due to its extensive usage, bifenthrin has been detected in sediment samples globally. Across various locations where sediment sampling has been conducted, bifenthrin has been identified at a prevalence rate of 78%. Moreover, in California, 79% of water samples have exhibited the presence of bifenthrin, with concentrations peaking at 106 ng/L in urban runoff areas, 133 ng/L following stormwater runoff events, and with concentrations varying from 0.005 to 5.3 µg/L in the Sacramento-San Joaquin Delta (Magnuson et al. 2021; Eghan et al. 2023).

Research has extensively investigated the potential toxicological effects of bifenthrin on vertebrates and mammals. Findings indicate that this compound elicits oxidative stress, immunotoxicity, and tumorigenicity. Notably, investigations conducted on *Danio rerio* have revealed that bifenthrin can disrupt the cell cycle during embryogenesis, elevate reactive oxygen species (ROS) levels, decrease heart rate, and exert anti-angiogenic effects (Park et al. 2020). Indeed, beyond the mentioned effects, bifenthrin is associated with a spectrum of additional toxicological outcomes. These include hepatotoxicity, impairment of swimming performance, and endocrine disruption among others (Eghan et al. 2023).

Furthermore, bifenthrin exposure can also lead to behavioral alterations. A study on Delta smelt (*Hypomesus transpacificus*) showed that exposure to bifenthrin had significant effects on larval motility. The larvae of this species naturally show a preference for moving in response to light. However, exposure to bifenthrin concentrations as low as 2 ng/L induced hyperexcitability in the larvae, resulting in a significant increase in distance travelled (Mundy et al. 2020). This behavioral response could compromise their ability to avoid predators and feed effectively in their natural environment (Abrahams 2005). In addition, these results underline the negative

impact of bifenthrin at concentrations naturally found in their habitat, highlighting the significant neurological disturbance that this pollutant can cause.

In view of the well-documented effects of pyrethroids such as bifenthrin on fish models (Farag et al. 2021; Y. Wang et al. 2022), it is interesting to identify the genetic and behavioural biomarkers likely to be affected in *N. furzeri* fish model. These biomarkers were selected based on their correlation with neurodegenerative diseases, particularly Alzheimer's disease. According to the National Institute of Environmental Health Sciences a biomarker is "*…an objective measure that captures what is happening in a cell or an organism at a given moment. Biomarkers can serve as early warning systems for your health.*"('Biomarkers', n.d.). Using biomarkers allows for precise targeting of the genetic factors involved in Alzheimer's disease development and observing how pyrethroids influence the development of the nervous system and the expression of these target genes during the turquoise killifish's development.

Additional protocol information

Appendix 2: Experimental timeline design

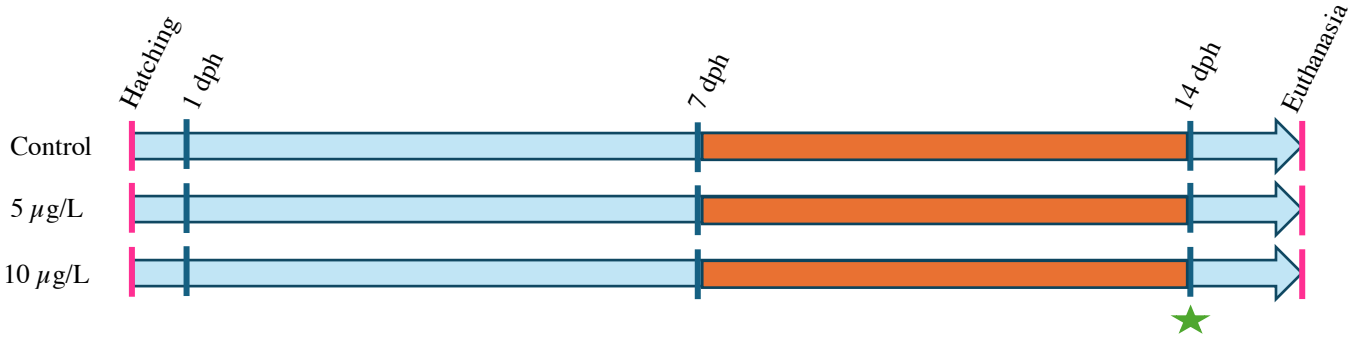


Figure 10: Representation of the experimental design used for the main experiment. The three experimental conditions are illustrated, with the period of exposure to bifenthrin indicated in orange. The time of behavioural analyses, followed by molecular and morphological analyses, is marked by a green star. The abbreviation 'dph' stands for 'days post hatching'.

Appendix 3: Pre-test to determine a sublethal concentration

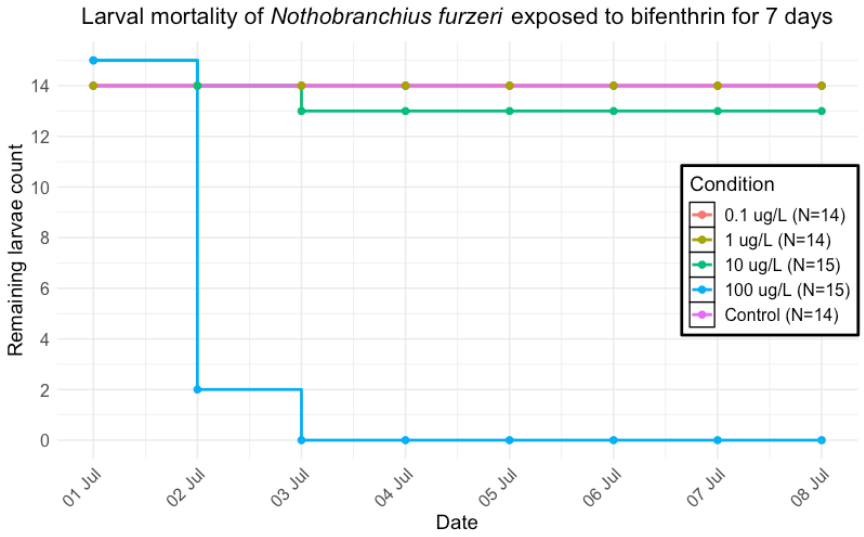


Figure 11: Graphical representation of the pre-test to determine the sublethal concentration of bifenthrin for the *Nothobranchius furzeri* model. The figure illustrates the mortality of larvae exposed to five different experimental conditions (DMSO control group, 0.1 µg/L, 1 µg/L, 10 µg/L and 100 µg/L). After two days, 100% of the larvae exposed to 100 µg/L died, as did two larvae exposed to 10 µg/L. The other larvae survived to the end of the experiment.

Appendix 4: Pictures of 12-Well Plates for experimental length measurements

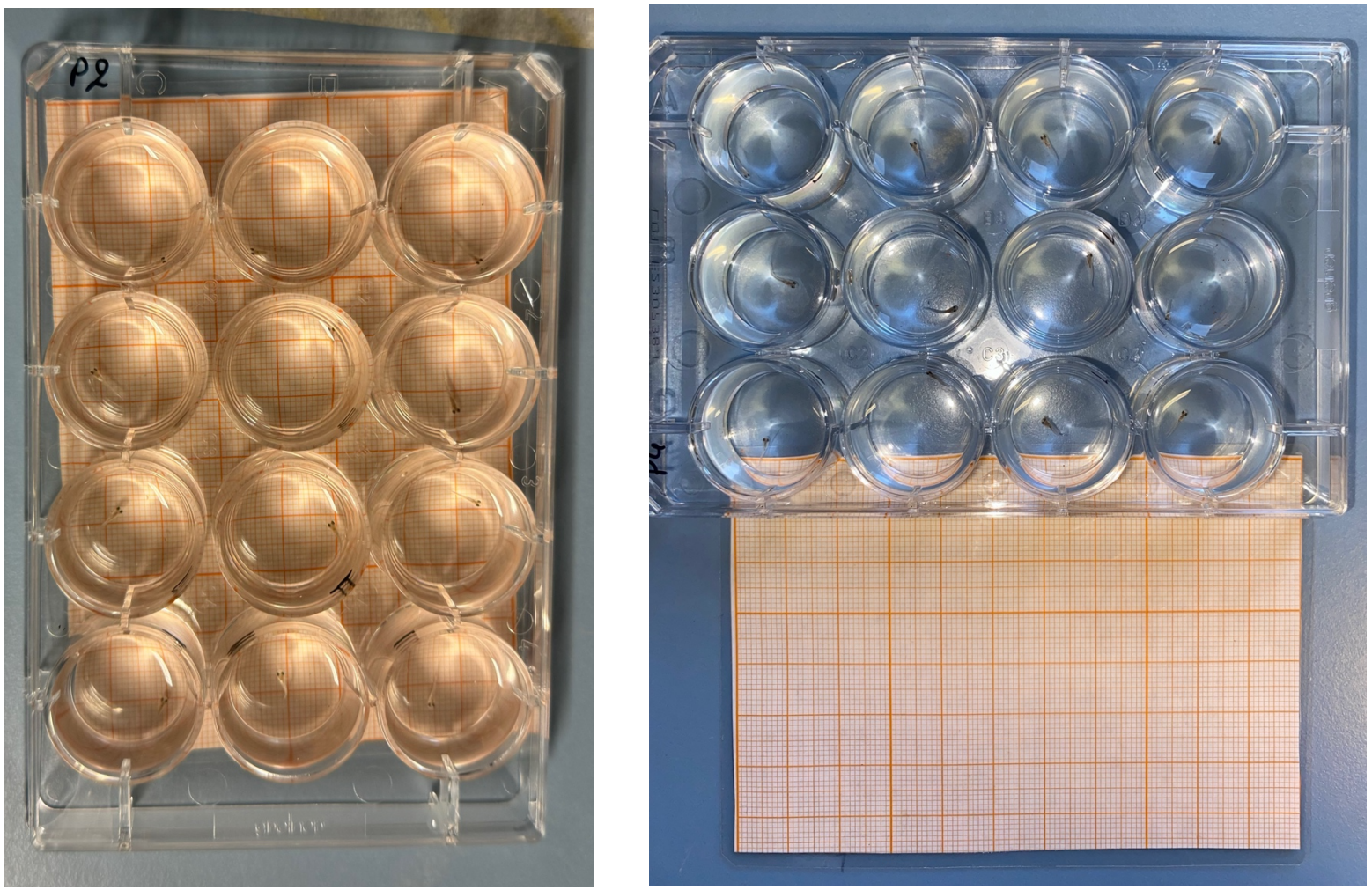


Figure 12: Photographs of the 12-well plates taken during the experiment with a template. These pictures were taken to measure the length of the larvae and to evaluate the potential effects of bifenthrin on the larval growth of *Nothobranchius furzeri*. The photos were captured using an iPhone 11 and then analysed using ImageJ software.

Additional data

Appendix 5: Bifenthrin properties

Table 1: Main chemical characteristics of the bifenthrin used in this experiment.

Chemical	Formula	Purity	LogKow	Molecular weight	Solvent	CAS Number
Bifenthrin	$C_{23}H_{22}ClF_3O_2$	99.0 %	6.00	422.87	DMSO	99267-18-2

Appendix 6: Calculation of RNA dilution quantities

Table 2: Table used to standardise the amount of RNA between samples. The lowest concentration measured at Nanodrop was multiplied by 20 to define the target threshold. The other samples were diluted with RNAse-free water to reach this quantity.

Sample	*Concentration (ng/µL)*	*RNA Quantity (ng))*	*Sample volume (µL)*	*RNase-free water volume (µL)*
CTL3	199,2	3984	20,0	1,5
CTL1	283,5	3984	14,1	7,4
10ug 2	321,9	3984	12,4	9,1
CTL 10	349,7	3984	11,4	10,1
5ug 2	353,3	3984	11,3	10,2
CTL 9	371,3	3984	10,7	10,8
5ug 10	388,9	3984	10,2	11,3
5ug 9	395,4	3984	10,1	11,4
10ug 9	423,1	3984	9,4	12,1
10ug 5	434	3984	9,2	12,3
10ug 6	447,4	3984	8,9	12,6
10ug 10	456,3	3984	8,7	12,8
5ug 1	458,1	3984	8,7	12,8
10ug 7	463,5	3984	8,6	12,9
5ug 6	473,2	3984	8,4	13,1
10ug 3	483,6	3984	8,2	13,3
5ug 7	496,8	3984	8,0	13,5
10ug 4	515	3984	7,7	13,8
5ug 5	515,7	3984	7,7	13,8
10ug 1	552,3	3984	7,2	14,3
5ug 4	563,2	3984	7,1	14,4
10ug 8	579,1	3984	6,9	14,6
5ug 8	580,7	3984	6,9	14,6
CTL 8	597,6	3984	6,7	14,8
5ug 3	660,8	3984	6,0	15,5
CTL 6	707,9	3984	5,6	15,9
CTL 5	1635,4	3984	2,4	19,1
CTL7	2618	3984	1,5	20,0
CTL 4	3046,5	3984	1,3	20,2

Appendix 7: Primer list for RT-qPCR

Table 3: List of primers used for RT-qPCR, with their forward and reverse sequences. The efficiency of each pair is also indicated. Note that the *psen2* genes and the *actin* housekeeping gene were not used due to poor quality results after RT-qPCR.

Gene	*Accession No.*	*Forward primer*	*Reverse primer*	*Efficiency*
appa	XM_015952596.2	GACGACGTCATCGACATGCT	TGGCGGTGGGTACTCTAACC	98.034%
manf	XM_015958303.2	GCGTTTAATTGGGCTATCGGT	GAAAGAGACGCACACTTCAC	108.027%
psen1	XM_015969322.2	ACGATGGGCAAAGACTGATTT	GTTGACAGCTCTCTGCGCTA	101.775%
psen2	XM_054743864.1	TACTTCTCTGCACAGCCATGAA	AGAACGGAACCGGAGGATTC	Plate contamination: NOT USED
syn2a	XM_015968151.2	TTCAACCCTCACAGGGGACT	GACGCCGTTACAGTCCAGAA	111.117%
slc6a4a	XM_054738535.1	TTGTGTCTGCTACCTTGGGG	ACAGAAACGGTTGGTACCGTAG	110.446%
gfap	XM_015948793.2	CAGGAGGCCAATGACTACCG	CCAGAGACTCGTTTGTGCCA	107.589%
elavl3	XM_015948534.2	TCAGCGTTTCAGACTCGACA	GCTTCGGGTGACAGGTTGTA	96.157%
gapdh	XM_015950201.2	TCGGCAAGGTCATTCCTGAG	TCGTCGTACTTGGCTGGTTT	99.258%
actin	XM_015960326.2	GACTCACCTGAACAGACATGGA	CGGCCTTGCACATACCAGAA	Didn't work properly

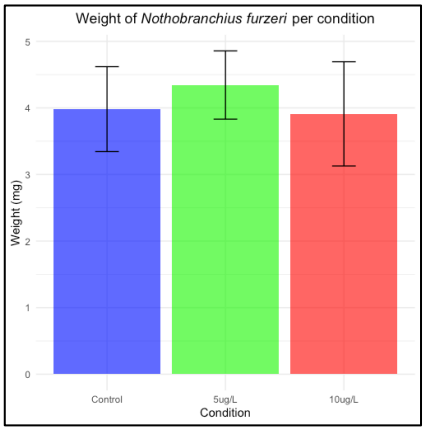
Appendix 8: Means and SDs of gene expression after normalization

Table 4: Summary of the means and standard deviations (SD) of the expression of genes according to the different experimental conditions (control, 5μg/L and 10μg/L). These data are obtained after normalisation, which is why all the means for the control condition are 1.

Gene	***Control***	***5 µg/L***	***10 µg/L***
appa	*Mean = 1.000* *SD = 0.317*	*Mean = 1.309* *SD = 0.465*	*Mean = 1.594* *SD = 0.387*
elavl3	*Mean = 1.000* *SD = 0.585*	*Mean = 0.928* *SD = 0.346*	*Mean = 0.949* *SD = 0.316*
gfap	*Mean = 1.000* *SD = 0.293*	*Mean = 1.154* *SD = 0.483*	*Mean = 1.513* *SD = 0.489*
manf	*Mean = 1.000* *SD = 0.409*	*Mean = 1.249* *SD = 0.405*	*Mean = 1.250* *SD = 0.270*
psen1	*Mean = 1.000* *SD = 0.268*	*Mean = 1.109* *SD = 0.388*	*Mean = 1.318* *SD = 0.286*
slc6a4a	*Mean = 1.000* *SD = 0.260*	*Mean = 1.234* *SD = 0.344*	*Mean = 1.370* *SD = 0.327*
syn2a	*Mean = 1.000* *SD = 0.284*	*Mean =1.196* *SD = 0.380*	*Mean = 1.594* *SD = 0.672*

Additional results

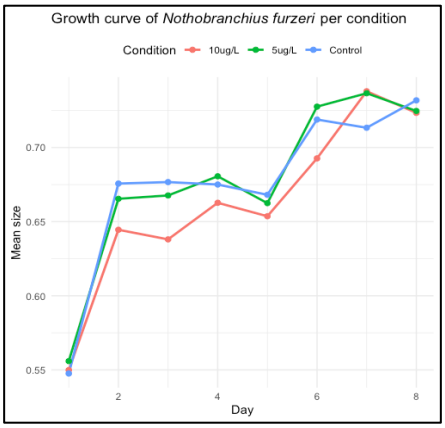
Appendix 9: Weight of *N. furzeri* larvae after bifenthrin exposure



	Condition	Mean	SD	Median
1	Control	3.982143	0.6377353	4.1
2	5ug/L	4.344444	0.5124101	4.2
3	10ug/L	3.910714	0.7828435	4.1

Figure 13: Graphical representation of the weight of *Nothobranchius furzeri* larvae after 7 days of exposure to bifenthrin under different experimental conditions. Below are the values with the mean, standard deviation and median for each condition.

Appendix 10: Growth of *N. furzeri* larvae during bifenthrin exposure



	Condition	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	10ug/L	0.550	0.645	0.638	0.663	0.654	0.693	0.738	0.723
2	5ug/L	0.556	0.665	0.668	0.681	0.662	0.728	0.737	0.725
3	Control	0.548	0.676	0.677	0.675	0.668	0.719	0.713	0.732

Figure 14: Growth curve for *Nothobranchius furzeri* larvae exposed to different concentrations of bifenthrin. A potential effect of bifenthrin on larval size is observed at 10 μg/L. However, the analysis method used raises concerns, as the daily mean sizes decrease inconsistently (see values), probably due to the quality of the photos, the accuracy of the ImageJ software and the precision of the experimenter when measuring each larva.

Appendix 11: The Benelux Zoology Congress 2024

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Effects of bifenthrin on neurological development, behaviour and the expression of biomarkers associated with Alzheimer's disease in *Nothobranchius furzeri*?

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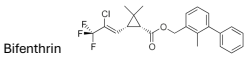
Introduction

Neurodegenerative diseases like **Alzheimer's** are influenced by environmental factors, including neurotoxic pesticides. In this context, ***Nothobranchius furzeri***, with its accelerated ageing process and genetic characteristics similar to those of mammals, is a useful model for studying the effects of such substances on neuronal ageing.

The pollutant studied is **bifenthrin**, a neurotoxic pesticide widely used in agriculture to control pests. It enters the environment via agricultural run-off, accumulating in water systems and posing risks to aquatic ecosystems. Commonly detected at concentrations averaging around **5 µg/L**, bifenthrin's presence highlights the need to assess its ecological impacts.

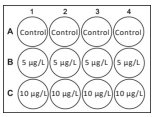


Objectives

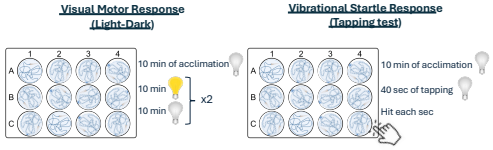
- Assess the effects of bifenthrin on the **behaviour** and **neuronal development** of *Nothobranchius furzeri*.
- Evaluate immediate behavioural changes following exposure to two concentrations of bifenthrin (5 µg/L and 10 µg/L) **at 2 weeks post-hatching**.
- Analyse changes in the **expression of genes associated with Alzheimer's disease syndromes** after bifenthrin exposure.

Methodology

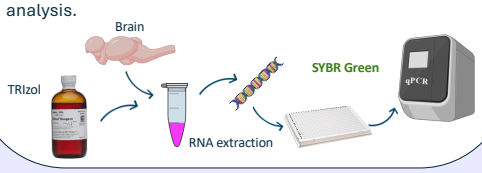
Larvae were exposed to bifenthrin at concentrations of **5 µg/L and 10 µg/L for 7 days**, starting at day 7 post-hatching. In each 12-well plate, 1 larva was placed per well, with **4 replicates per condition** (control, 5 µg/L, and 10 µg/L).



Behavioural effects were evaluated using **light-dark** and **vibrational startle response tests** on day 14 (2 weeks).

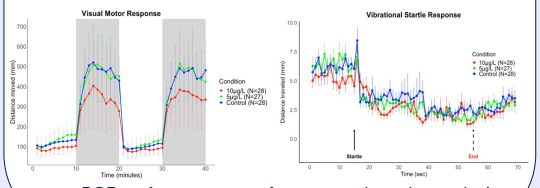


To complete these tests, genes involved in Alzheimer's disease-related syndromes were selected, including **APP, PSEN1, PSEN2, SYN2A, MANF, HTR1B, GFAP, SLC6A4A, and ELAVL3.** RNA was extracted using TRIzol prior to qPCR analysis.



Results

- **Visual Motor Response:** Exposure to 5 µg/L of bifenthrin seems to have no significant effect on the behaviour of *Nothobranchius furzeri* at 2 wph. However, exposure to **10 µg/L resulted in a very clear reduction** in the distance travelled.
- **Vibrational Startle Response:** No significant differences were observed in this test. However, a trend suggests that individuals exposed to bifenthrin seem to react less strongly to vibrations.



- **qPCR and gene expression:** currently under analysis

Conclusions

Bifenthrin appears to **affect the behaviour** of individuals exposed to **environmentally relevant concentrations**. Gene expression analyses have been carried out and are currently being studied to determine whether this neurotoxic compound influences the development of Alzheimer's disease.

These results will help to clarify the mechanisms by which bifenthrin affects neuronal development and behaviour, and to understand its potential role in neurodegenerative disorders.

References

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Figure 15: During this master thesis year, participation in The Benelux Zoology Congress 2024, took place in Mons, Belgium, from 12 to 13 November 2024, included the presentation of this poster.