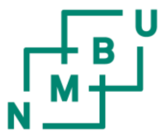

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Quantitative HPLC-MS/MS analysis of neurotransmitters in the zebrafish model

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Statements

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature, as well as those which were generated using artificial intelligence tools, are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

City, date Ås, December 15.2024 First name Even Nilsen Edvardsen

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Table of contents

Statements....i
Acknowledgments....ii
Table of contents....iii
Abstract....v
Sammendrag....vi
List of abbreviations....viii
Motivations....xiii
1. Background....14
1.1 Introduction....14
1.1.1 Neurotransmitters....14
1.1.2 Per- and polyfluoroalkyl substances (PFAS)....15
1.1.2.1 History and environmental occurrence of per- and polyfluoroalkyl substances (PFAS)....15
1.1.2.2 Per- and polyfluoroalkyl substances (PFAS) exposure, guidelines and neurotoxic effects. 16
1.1.2.3 Perfluoroalkyl acids (PFAAs) and "Precursors"....17
1.2 Chemical and physical properties of per- and polyfluoroalkyl substances (PFAS)....18
1.2.1 Perfluorooctanoic acid (PFOA)....20
1.2.2 Perfluorohexanesulphonic acid (PFHxS)....21
1.3 Acetylcholine, adrenaline and cortisol....21
1.3.1 Acetylcholine....21
1.3.2 Adrenaline....22
1.3.3 Cortisol....23
1.3.4 Analysis of neurotransmitters in zebrafish....25
1.3.5 Carbamazepine....25
1.3.6 Hydrocortisone....26
1.4 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS)....26
1.4.1 High performance liquid chromatography (HPLC)....26
1.4.1.1 Reversed phase liquid chromatography (RPLC)....27
1.4.1.2 Hydrophilic interaction chromatography (HILIC)....27
1.4.2 Tandem mass spectrometry (MS/MS)....28
1.4.2.1 Electrospray ionization (ESI)....29
1.4.2.2 Quadrupole(Q/q)....30
1.4.2.3 Electron multiplier (EM)....30
1.4.2.4 Scan modes....31
2. Hypothesis and aim....33
3. Methods and Materials....35
3.1 Zebrafish samples....35
3.1.1 Sampling data for 48 hours past fertilization (hpf) zebrafish embryos and 96 hours past fertilization(hpf) zebrafish larvae....36

3.2 Calibration curve and internal standards..38
3.3.1 Internal standards (IS)..38
3.3.2 Calibration curve..38
3.3 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) sample preparation..39
3.4 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) specifications..42
3.4.1 Mobile phases..42
3.4.2 High pressure liquid chromatography (HPLC)..42
3.4.3 Tandem mass spectrometry (MS/MS) Ion selection..43
3.5 Validation parameters..46
3.6 Statistical analysis..46
4. Results..48
4.1 Acetylcholine..48
4.2 Cortisol..50
4.3 Adrenaline..53
5. Discussion..56
5.1 Methodological consideration..56
5.1.1 Use of zebrafish model for neurotransmitter detection..58
5.2 Acetylcholine levels..60
5.3 Cortisol levels..61
5.4 Adrenaline levels..63
6. Conclusion..64
7. Future considerations..65
References..66
List of equipment..75
List of chemicals..77
List of tables / figures..81

Abstract

Neurotransmitters are signalling molecules that increase or inhibits signals between neurons. Furthermore, neurotransmitters can be classified by structure or activity.

Per- and polyfluoroalkyl substances (PFAS) are substances that have been used in industrial manufacturing since the 1950s. Further, PFAS substances are used for firefighting foams and household items like nonstick finishes, food packaging and water repellent fabrics. In addition, the PFAS substances are also used in aviation, aerospace and automotive industries.

The aim for this master thesis is to optimize the analysis of neurotransmitters in the zebrafish model to reduce the amount of tissue sample need. Furthermore, the objective is to test whether human relevant environmental concentrations of Perfluorooctanoic acid (PFOA) and Perfluorohexanesulphonic acid (PFHxS) could change the neurotransmitter profile (acetylcholine, adrenaline and cortisol) of zebrafish embryo and larvae.

The zebrafish were exposed to carbamazepine, hydrocortisone, PFOA, and PFHxS. Furthermore, RP-LC-ESI-MS/MS was used and optimized for quantifying neurotransmitter levels in zebrafish embryo (48 hpf) and larvae (96 hpf). All neurotransmitter levels were detected and quantified by use of the mentioned method. The results of exposures should be carefully interpreted due to small sample sizes. Zebrafish embryo and larvae exposed to PFOA and PFHxS showed concentration dependent effects on cortisol, adrenaline and acetylcholine. The effects of low concentrations had varying effects across developmental stages, while high concentrations generally caused deceasing trends or no impact on the cortisol, adrenaline and acetylcholine levels. The levels depended on the compound, concentration and life stage.

Sammendrag

Nevrotransmittere er signalmolekyler som øker eller hemmer signaler mellom nevroner. Videre kan nevrotransmittere klassifiseres etter struktur eller aktivitet.

Per- og polyfluoralkylstoffer (PFAS) er stoffer som har vært brukt i industriell produksjon siden 1950-tallet. Videre brukes PFAS-stoffer til brannslokkingsskum og husholdningsartikler som nonstick-finish, matemballasje og vannavstøtende stoffer. I tillegg brukes PFAS-stoffene også i luftfart-, romfart- og bilindustri.

Målet for denne masteroppgaven er å optimalisere analysen av nevrotransmittere i sebrafisk modellen for å redusere mengden vev i prøven. Videre er målet å teste om menneskelige relevante miljøkonsentrasjoner av perfluoroktansyre (PFOA) og perfluorheksansulfonsyre (PFHxS) kan endre nevrotransmitterprofilen (acetylkolin, adrenalin og kortisol) til sebrafisk embryoer og -larver.

Sebrafisken ble utsatt for karbamazepin, hydrokortison, PFOA og PFHxS. Videre ble RP-LC-ESI-MS/MS brukt og optimalisert for å kvantifisere nevrotransmitternivåer i sebrafisk embryo (48 hpf) og larver (96 hpf). Alle nevrotransmitternivåer ble påvist og kvantifisert ved bruk av den nevnte metoden. Resultatene av eksponeringer bør tolkes nøye på grunn av små prøvestørrelser. Sebrafisk embryo og larver eksponert for PFOA og PFHxS viste konsentrasjonsavhengige effekter på kortisol, adrenalin og acetylkolin. Effektene av lave konsentrasjoner hadde varierende effekter på tvers av utviklingsstadier, mens høye konsentrasjoner generelt forårsaket avtagende trender eller ingen innvirkning på kortisol-, adrenalin- og acetylkolinnivåene. Nivåene var avhengig av forbindelsen, konsentrasjonen og livsstadiet.

List of abbreviations

Abbreviation	English	Norwegian(norsk)
5-HIAA	5-hydroxyindoleacetic acid	5-hydroksyindoleddiksyre
ACh	Acetylcholine	Acetylkolin
ACN	Acetonitrile	Acetonitril
B	Magnetic sector	Magnetisk sektor
CAS	Chemical abstract service	Tjeneste for kjemisk sammendrag
CBC	Carbamazepine	Karbamazepin
CECs	Contaminants of emerging concern	Forurensinger av økende bekymring
ChAT	Choline acetyltransferase	Kolinacetyltransferase
CID	Collision induced dissociation	Kollisjonsindusert dissosiasjon
CRH	Corticotropin releasing hormone	Kortikotropinfrigjørende hormon
DC	Direct current	Likestrøm
DOPAC	dihydroxyphenyl acetic acid	Dihydroksyfenyleddiksyre
DFP	Days after fertilization	Dager etter fetilisering
E	Electric sector	Elektrisk sektor
EIA	Enzyme immunoassay	Enzym immunoanalyse
ELISA	Enzyme-linked-immunosorbent assay	Enzymkoblet immunosorbentanalyse
EM	Electron multiplier	Elektron multiplicator
ESI	Electrospray-ionization	Elektrospray-ionisering
H_2O	Water	Vann

HCOOH	Formic acid	Maursyre
HILIC	Hydrophilic interaction chromatography	Hydrofil interaksjonsvæskekromatografi
HCl	Hydrochloric acid	Saltsyre
HPF	Hours past ferilization	Timer etter befruktning
HPLC	High pressure liquid chromatography	Høytrykk væskekromatogrfi
HPLC-MS/MS	High pressure liquid chromatography-tandem mass spectrometry	Høytrykk væskekromatografi-tandem massespektrometri
IC	Ion chromatography	Ionekromatografi
IS	Internal standard	Intern standard
IUPAC	International Union of Pure and Applied Chemistry	Internasjonalt forbund for ren og anvendt kjemi
LoD	Limit of detection	Deteksjonsgrense
LoQ	Limit of quantification	Kvantifikasjonsgrense
NPLC	Normal phase liquid chromatography	Normalfase væskekromatogrfi
MeOH	Methanol	Metanol
MRM	Multiple reaction monitoring	Multippel reaksjonsmonitorering
MS/MS	Tandem mass spectrometry	Tandem massespektrometri
m/z	Mass to charge ratio	Masse til ladningsforhold
N_2	Nitrogen	Nitrogen
OECD	The organisation for economic co-operation and development	Organisasjonen for økonomisk samarbeid og utvikling

PFAS	Per- and polyfluoroalkyl substances	Per- og polyfluoralkylstoffer
PFHxA	Perfluorohexanoic acid	Perfluorheksansyre
PFHxS	Perfluorohexanesulphonic acid	Perfluorheksansulfonsyre
PFAAs	Perfluoroalkyl acids	Perfluroalkylsyrer
PFCAs	Perfluoroalkyl carboxylic acids	Perfluoralkylkarboksylsyrer
PFNA	Perfluorononanoic acid	Perfluornonansyre
PFOA	Perfluorooctanoic acid	Perfluoroktansyre
PFOS	Perfluorooctanesulfonic acid	Perfluoroktansulfonsyre
PFSAs	Perfluoroalkylsulfonic acids	Perfluroalkylsulfonsyrer
PP	Polypropylene	Polypropylen
QC	Quality control	Kvalitetskontroll
Q/q/Q	Quadrupole	Kvadrupol
RI	Refractive index	Refraksjonsindeks
RPLC	Reverse phase liquid chromatography	Omvendt fase væskekromatogrfi
(RP-LC-ESI-MS/MS)	Reverse phase-liquid chromatography-electrospray ionization- tandem mass spectrometry	Omvendt fase-væskekromatografi-elektrospray ionisasjon-tandem massespektrometri
SE	Standard error of the mean	Standardfeilen for gjennomsnittet
S/N	Signal to Noise ratio	Signal til støy forhold
SRM	Single reaction monitoring	Enkel reaksjonsmonitorering
SD	Standard deviation	Standardavvik
TIC	Total ion chromatogram	Totalt ionekromatogram

THF	Tetrahydrofuran	Tetrahydrofuran
TOF	Time of flight	Flygetid
TWI	Tolerable weekly intakes	Torerabelt ukentlig inntak
UHLPC	Ultra high-pressure liquid performance chromatography	Ultra-høytrykks væskekormatografi.
XIC	Extract ion chromatogram	Ekstrakt ionekromatogram
Zfe	Zebrafish embryo	Sebrafisk embryo

Motivations

I have always found the environment and how we contaminate the environment interesting. Especially organic pollutants like PFAS became interesting after learning about them at NMBU.

The strong motivator for this master thesis was the 3RS of animal research replacement, reduction and refinement. Furthermore, trying to reduce the amount of zebrafish tissue to lessen the use of too many zebrafish. Moreover, to see if it's possible to replace animals used in similar and other studies with zebrafish. Another strong motivator in this thesis was the possible effects PFOA and PFHxS could have on neurotransmitters in zebrafish embryo and larvae.

1. Background

1.1 Introduction

1.1.1 Neurotransmitters

The neurotransmitters in focus in this thesis are acetylcholine, adrenaline and cortisol. Furthermore, the reason these neurotransmitters are focused on in this thesis is that acetylcholine is one of the main neurotransmitters of the autonomous nervous system. When it comes to adrenaline and cortisol these neurotransmitters are important, because adrenaline and cortisol are part of the "fight or flight" system which is important to survival for all species on earth (Berg and Otterholt, 2020-06-30; Sam and Bordoni, 2023; Rogers, 2024).

Neurotransmitters are signalling molecules that are released from neurons over the synaptic cleft form the presynaptic neuron to the post synaptic neuron. After these molecules are released, they affect either other neurons, muscles, or glands. Neurotransmitters work by binding to cell receptors. The receptors that neurotransmitters bind to are G-coupled-receptors and ligand-gated ion channels. The signal received will either inhibit or increase the activity of the receiver cells (Hassel, 02-08-2023).

For a chemical compound to be called a neurotransmitter there are a few criteria that must be filled (Wright and O'Neill, 2012):

1. It must be synthesized (by enzymes) in the presynaptic neuron.
2. The neurotransmitter must be stored inactively in the presynaptic terminal.
3. When the presynaptic neuron depolarizes, the neurotransmitter is released from the presynaptic cell.
4. There must be a receptor in the postsynaptic neuron that gets activated and causes an effect.
5. The neurotransmitter must have a degrading enzyme in the synapse or reuptake mechanism in the presynaptic cell that inactivates the neurotransmitter.

If a chemical compound is released from neurons that affect other cells but fails at the mentioned criteria for being called a neurotransmitter, is called a neuromodulator. An example of a neuromodulator is nitrogen monoxide NO(g) (Hassel, 02-08-2023).

There are over 100 neurotransmitters, but the total number is unknown. Scientist have been able to detect over 50 neurotransmitters. Furthermore, neurotransmitters can be characterised by either chemical components/molecular structures or function. When it comes to function neurotransmitters can be classified into either inhibitors or exhibitors or having both functions (Purves D *et al.*, 2001; Moini, Gutierrez and Avgeropoulos, 2023/01/01).

When it comes to molecular structure neurotransmitters can be split into three groups amino acids, peptides and amines. Where Acetylcholine (ACH) is included in the amine group despite being an ester (Goodman, 2008).

1.1.2 Per- and polyfluoroalkyl substances (PFAS)

1.1.2.1 History and environmental occurrence of per- and polyfluoroalkyl substances (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are defined by OECD (the organisation for economic co-operation and development) as "fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom without any H/Br/Cl/I attached to it" (Schymanski *et al.*, October 23, 2023). There are over 7 million different PFAS in PubChem and the number is growing (Schymanski *et al.*, October 23, 2023). Moreover, PFAS were introduced in industrial manufacturing in the 1950s. Furthermore, PFASs with functional groups was made from at least the 1940s. Moreover, PFAS can be found in everything from household products like non-stick finishes, food packaging and water repellent fabrics, to firefighting foam and aviation, aerospace, and automotive industries. However, because of an international and expansive use of PFAS this has made PFAS universally exposable to humans (Paquette *et al.*, 2023-11-15; Gaines, 2023/05/01). The first time PFAS was found to be in nature was trough the identification of perfluoro octane sulfonic acid (PFOS). Moreover, PFAS was not detected in the environment before 1990 , then in low concentrations (Abunada *et al.*, 2020-12-21). Furthermore, PFAS was

measured for the first time in tissue in 2001 by Giesy and Kannan. Then as PFOS(Perfluorooctanoic acid) and PFOA in global wildlife (Giesy and Kannan, March 1, 2001).

PFAS have been found in different environmental compartments for example soil, air (dust), organisms (biota) and water (rivers, lakes, groundwater, snow, etc.) (Abunada *et al.*, 2020-12-21; Death *et al.*, 2021/06/20). A study of ground and surface water in the Turin metropolitan area found that PFAS was in 24% of the investigated deep and shallows aquifers (groundwater). Furthermore, PFAS was found in 96% of the sampling sites in streams(rivers). Additionally, the lake that was investigated had no PFAS detected (Randazzo *et al.*, 2025/01/01). Another study in north China did an ecological risk assessment for 69 different agricultural soils from Fuxin with a focus on 31 different PFASes. Furthermore, in these soil samples 26 of 31 PFASes were detected (Xing *et al.*, 2024/08/05). In addition, a study in northwestern Illinois sampled tissue from nine different fish species from 15 sampling locations from four targeted water ways. This was to evaluate the amount of 17 possible PFAS in the fish. Four PFASes (PFOA, PFOS, PFHxS and Perfluorohexaonic acid(PFHxA) was found at each sampling site (Sands *et al.*, 2024/06/01). A final study to look at was a study in North Carolina which tested for 26 possible PFASes in 11 homes (living room only) and right outside the home for 9 months. The result of this study was that 24 out of 26 PFAS was detected. And PFOA, PFOS and PFHxA was detected most regularly(Chang *et al.*, 2025).

1.1.2.2 Per- and polyfluoroalkyl substances (PFAS) exposure, guidelines and neurotoxic effects.

The source of most significant PFAS exposure to humans is trough drinking and eating, with some additional exposure through inhalation (dust). (Abunada *et al.*, 2020-12-21; Death *et al.*, 2021/06/20). Examples of species and refined food products that could be exposed to PFAS is freshwater fish, beef, dairy products, and vegetables. Moreover, the most produced and most frequently detected PFASes in the environment are PFOA and PFOS(Abunada *et al.*, 2020-12-21; Death *et al.*, 2021/06/20).

In 2020 European food safety authority (EFSA) set the Tolerable weekly intake (TWI) in Europe to 4.4 ng/kg bodyweight per week for the total intake of PFOA, PFOS, PFHxS and Perfluorononanoic acid (PFNA) (EFSA CONTAM Panel,2020). Furthermore, the European

commission set guidelines for different maximum levels of PFOA, PFNA, PFOS and PFHxS in µg/kg for the different foods like beef, fish, crustaceans and bivalve molluscs, eggs, game meat and bovine. For example the limits for PFOS, PFOA, PFNA and PFHxS in eggs is 1.0 µg/kg , 0.30 µg/kg, 0.70 µg/kg, 0.30 µg/kg and for all combined above mentioned PFAS 1,7 µg/kg. Furthermore the limits in for example flounder(fish) for PFOS,PFOA, PFNA and PFHxS is 7.0 µg/kg, 1.0 µg/kg, 2.5 µg/kg, 0.20 µg/kg and sum of all above mentioned PFAS 8,0 µg/kg (European Commission, 2023). Moreover, when it comes to guidelines for drinking water the limit of the the total concentration for all PFASes in water was set to 0,5 µg/L. Furthermore, the concentration of the 20 PFASes of concern in the C/2024/4910 european commission notice which had 4-13 carbon length that contains either a perfluoroalkyl moiety with 3 or more carbons or perfluoroalkyl ether moiety with 2 or more carbons. Have a guideline of 0,10 µg/L for their total concentration in water (European Commission, 7-8-2024).

The neurotoxic effects of PFAS exposure can cause cognitive and behaviour disorder, memory disfunction, intellectual disability, attention hyperactivity deficit disorder and predominantly inclusive of fetal palsy (Nannaware, Mayilswamy and Kandasubramanian, 2024-01-26). Furthermore, the potential ways PFAS effects the central nervous system is by eliciting a change in neurotransmitter levels, altering of synaptic and neuronal protein expression and function and dysfunction of the synaptic calcium homeostasis (Brown-Leung and Cannon, August 3, 2022). Brown- Leung and Cannon (2022) found that PFAS caused neurotoxicity through disruption og glutamate and dopamine in the brain. This was found to be the case when Atlantic cod was exposed to different PFASes in low and high doses. Furthermore, in higher doses of PFAS the dopamine levels where decreased(Khan *et al.*, May 15, 2019).

1.1.2.3 Perfluoroalkyl acids (PFAAs) and "Precursors"

The "Precursors" are PFAS substances that can degrade into perfluoroalkyl acids (PFAAs). Furthermore, the two main types of PFAA are perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs). Two examples of a PFAA would be PFOS and PFOA. Moreover, PFAAs and PFASes are also extremely resistant to different breakdown processes. Furthermore, a big problem with PFAAs and PFAS is that PFAAs and PFAS can be freely mobile between air, water, and soil. This is because of the many physicochemical interactions PFAA and

PFAS have with other co-contaminants and with various surfaces. Furthermore, these interactions are van der Waal forces, hydrogen bonds, ion bridging interactions with divalent cations, fluorophilic interactions, ionic exchange and π-π bonds. (Abunada *et al.*, 2020-12-21; Death *et al.*, 2021/06/20; Leung *et al.*, 2023/12/20; McDonough *et al.*, March 24, 2022).

1.2 Chemical and physical properties of per- and polyfluoroalkyl substances (PFAS)

Every PFAS species consists of two main components perfluorinated alkyl chain (tail) and a functional group (head) (Figure 1). The perfluorinated chain consist of multiple carbons with fluor attached to them instead of hydrogen in a chain, this chain will be nonpolar (hydrophobic). Furthermore, the functional group or the "functional head" (Figure 1) can be a sulfonate or a carboxylate. In addition, the functional group is polar(hydrophilic) since both the sulfonate and the carboxylate are acid groups. The highest constituent of PFAS substances is fluor atoms. This means that PFAS is highly affected by fluor's high electron negativity (which is the highest in the periodic table), low polarizability and high ionization potential. This is visible in that the chemical and physical properties of PFAS is highly influenced by fluorine and C-F bonds (Leung *et al.*, 2023/12/20).

When it comes to the chemical properties of PFAS. PFAS have a C-F bond which have an optimal overlap between fluorine 2s and 2p orbital which also overlaps with carbons orbitals. And this benefits PFAS by forming multiple resonance structures along the perfluoroalkyl chain. In addition, when there is more fluorine bonded to the central carbon the strength of the C-F bond increases. The electrons in the C-F bond which is shared between the fluor and carbon atom in the C-F bond are drawn to the F(δ–) atom because of the highly electropositive sp^3 carbon(δ+). Then with the low atomic radius of the fluor atoms, creates a very high polarization energy. Another thing the C-F bonds does is create an electrostatic and steric shield with the three lone ion pairs of the fluorine coupled with partial negative charge. Because of this the central carbon is shielded from any nucleophilic attack and this creates a kinetic stability. This one main contributor to PFAS chemical inertness or unreactiveness. Another thing that adds to the inertness of PFAS is the fluor's low polarization of the s and p electrons with makes fluor a poor hydrogen bond acceptor. Even if

fluor has high electron negativity and lone pairs. Furthermore, when fluorine binds to carbon, the bond becomes the strongest and most single inert bond found in organic compounds. The bond dissociation energy is up to 531,5 KJ/mol^{-1} (Leung *et al.*, 2023/12/20).

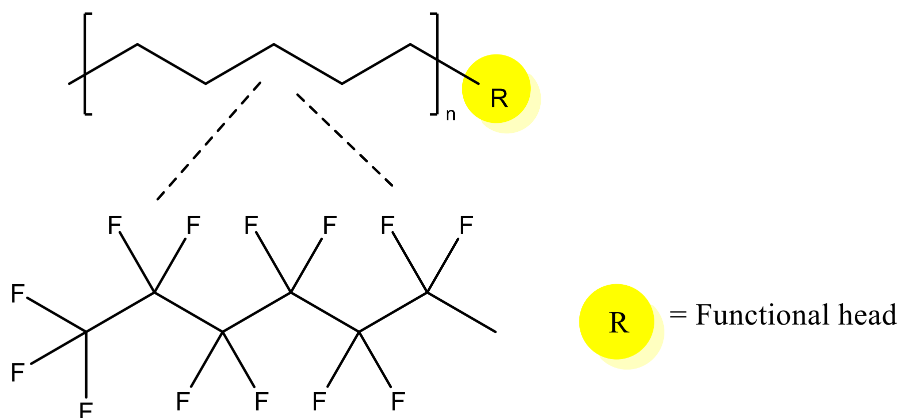


Figure 1: Basic PFAS structure for all PFAS species. Made in Chemdraw v.22.2.0.

When it comes to physical properties PFAS have weak intermolecular and intramolecular interactions. This is due to fluorine's low polarizability. This is prevalent because of PFAS lower boiling point and higher volatility compared to the hydrocarbons of similar molecular mass. PFAS have excellent surface wettability, this because of the weak intermolecular forces, which leads to exceptionally low surface tension. Since the van der Waals forces are weak because of an increased radius of van der Waals forces in the C-F bonds (1,47 $Å^3$) in comparison to hydrogen C-H bonds (1,20 $Å^3$). And because all hydrogen atoms have been swapped with fluor atoms a steric congestion is created. Moreover, this creates steric repulsion between the flour atoms coupled to the alkyl chain in the 1,3 positions. In addition, the steric repulsion coupled with the increased radius of the van der Waals forces creates a twisting and stretching of the alkyl chain. Further, the alkyl chain is twisted and stretched 12 degrees which forms a 15/7 helix. Additionally, this causes the right and left helices to have equal proportion. Furthermore, this is different to the hydrogen carbon helix which has a zigzag configuration. This makes PFAS with C/F bonds have lower conformational flexibility and PFAS gets more rigid than an alkyl chain with hydrogen carbon bonds. Moreover, this makes PFAS even more inert. Furthermore, since PFAS has an amphiphilic structure as well, it makes PFAS the perfect surfactant. If PFAS is mixed with water and oil, PFAS

will not dissolve in water nor in oil, PFAS will sit outside of them, this is because PFAS has amphiphilic properties because of the polar acid group and the nonpolar perfluorinated alkyl chain (Leung *et al.*, 2023/12/20).

PFAS has another important characteristic, which is the critical micelle concentration. The critical micelle concentration of PFAS is lower than its hydrocarbon counterparts. This because of the cancelling of dipole movements in the PFAS molecule. Furthermore, PFAS with CF_2 has 1,5 times as much micellization energy than CH_2. This means that PFAS will create micelles quicker than organic compounds with hydrocarbon bonds (CH_2). The micelles will also be smaller than organic compounds with hydrogen bonds (CH_2). This will again lead to higher solubility in water than for organic compounds with hydrocarbon bonds (CH_2). The solubility in water will increase with the length of the chain of CF_2. Meaning that PFAS with longer chains dissolve easier in water than shorter chained PFAS at lower concentrations (Leung *et al.*, 2023/12/20).

1.2.1 Perfluorooctanoic acid (PFOA)

International Union of Pure and Applied Chemistry (IUPAC): Pentadecafluorooctanoic acid (PFOA) has the chemical formula of $C_8HF_{15}O_2$, Chemical abstract service (CAS) number :335-67-1 and the molecular structure can be viewed in Figure 2.

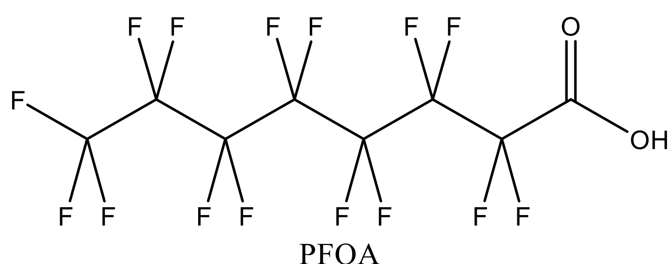


Figure 2: Structure of PFOA. Made in Chemdraw v.22.2.0.

1.2.2 Perfluorohexanesulphonic acid (PFHxS)

Perfluorohexanesulphonic acid (PFHxS) has the chemical structure of $C_6HF_{13}O_3S$, CAS number: 355-46 4, IUPAC: 1,1,2,2,3,3,4,4,5,5,6,6,6-tridecaflurohexane-1-sulfonic and the molecular structure can be viewed in Figure 3.

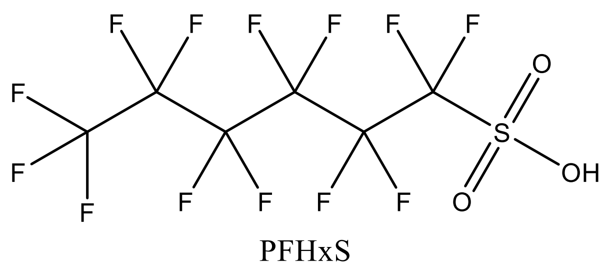


Figure 3: Structure of PFHxS**.** Made in Chemdraw v.22.2.0.

1.3 Acetylcholine, adrenaline and cortisol

1.3.1 Acetylcholine

Acetylcholine's molecular structure can be viewed in Figure 4 and the chemical formula is $C_7H_{16}NO_2+$ and has CAS number: 51-84-3. Acetylcholine (ACh) is an ester of choline and acetic acid. Within the central and peripheral nervous system, acetylcholine is used as a neurotransmitter. Acetylcholine can increase a signal as an excitor or block a signal as an inhibitor and binds to ionotropic nicotinic ACH receptors and G-protein-coupled muscarinic receptors within the synaptic cleft (Rico *et al.*, 2011/11/01; Sam and Bordoni, 2023).

Acetylcholine is the main neurotransmitter of the parasympathetic nervous system, which is a part of the autonomic nervous system. The autonomic nervous system controls the contraction of smooth muscles and blood dilation in the blood vessels, increase of bodily secretions and slowing of the heart rate. Acetylcholine is also known for having a role in learning and memory. Furthermore, ACh is a key modulator of glutamate and might be involved in olfactory mechanisms (Rico *et al.*, 2011/11/01; Sam and Bordoni, 2023).

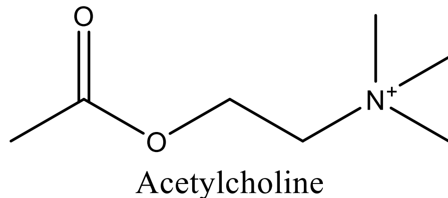


Figure 4: Molecular structure of Acetylcholine. Made in Chemdraw v.22.2.0.

In human`s acetylcholine is synthesized by acetylcholine producing neurons and stored in vesicles at the end of the acetylcholine producing neurons (Rico *et al.*, 2011/11/01; Sam and Bordoni, 2023). The main enzyme that synthesizes acetylcholine is choline acetyltransferase (ChAT). ChAT synthesizes acetylcholine from choline and acetate in the pre-synaptic neurons (Verma *et al.*, 2022/01/01). Furthermore, ChAT exist in zebrafish, Clemente, *et al.* (14-06-2004) analysed zebrafish brains for chat positive areas and fund that there where ChAT in multiple places in the zebrafish brain. This means that acetylcholine is synthesized similarly in zebrafish when compared to humans (Clemente *et al.*, 2004/06/14).

The cholinergic system made up of acetylcholine and its precursors and metabolites. Furthermore, acetylcholine is detectable at 9 hpf in the zebrafish embryo. The following is how the acetylcholine level in zebrafish decreases and increases from egg to larva during early development. First acetylcholine increases to 3dpf or 72 hpf, then a decrease to 96 hpf. Furthermore, after in the larval stage's acetylcholine increases more. (Tufi *et al.*, February 25, 2016).

1.3.2 Adrenaline

Adrenaline or epinephrine is a catecholamine and monoamine. The molecular structure of adrenaline is presented Figure 5 and chemical formula of adrenaline is $C_9H_{13}NO_3$ and has CAS number: 51-43-4. Adrenaline functions as a neurotransmitter and hormone. Furthermore, adrenaline is nearly identical to noradrenaline except for a methyl group on the nitrogen sidechain. Adrenaline is what makes animals ready to "fight or flight" in an acute stress situation. In a "flight or fight" situation adrenaline will constrict blood flow in smaller blood vessels. At the same time adrenaline will dilate the blood vessels in the skeletal muscles and liver. Moreover, the heart in a "fight or flight" situation will have an increased heart rate and have increased contractions.

Resulting in the heart pumping more blood, increasing blood pressure and blood output. In the liver the breakdown of glycogen to glucose increases. Furthermore, increasing glucose levels in the blood stream as well as the amount of circulating free fatty acids. The extra free fatty acids and glucose is used as fuel in acute stress situations and dangerous situations when extra alertness and exertion is needed (Rogers, 2024).

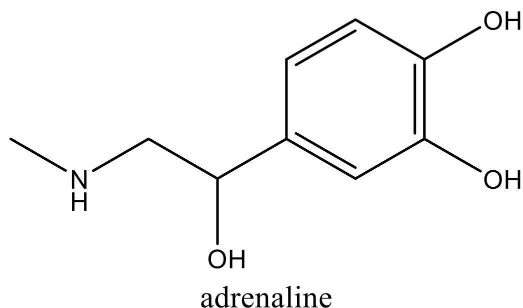


Figure 5: Molecular structure of adrenaline. Made in Chemdraw v.22.2.0**.**

In human's adrenaline is synthesized in the adrenal glands and some in the ends of the sympathetic nerves (Rogers, 2024). In zebrafish the adrenaline is synthesized in the interrenal gland in the head of the kidney. Moreover, the excretion/regulation of adrenaline is controlled by the hypothalamus. (Abreu *et al.*, 29-09-2021; Bacila, Cunliffe and Krone, 2021/09/15; Abreu *et al.*, 2021/11).

Adrenaline is detectable at 1hpf in zebrafish. Furthermore, the adrenaline level changes begins with a gradual increase to 48 hpf from 0 hpf to a little decrease to about 72 hpf. After 72 hpf the adrenaline levels increase a little to 120 hpf (Tufi *et al.*, February 25, 2016).

1.3.3 Cortisol

The molecular structure of cortisol is presented Figure 6, and the chemical formula is $C_{21}H_{30}O_5$ and has CAS number:73565-87-4. Cortisol is a glucocorticoid and a hormone. Like adrenaline, cortisol is a stress hormone. Furthermore, cortisol helps the body to cope with stress and helps by mobilising energy in and around the muscles. In addition, cortisol helps with other functions as well. These functions are inhibition of the immune system by reducing the response from the immune system, inhibition of production of bone tissue and calcium secretion through the kidneys

increase. Further effects are inhibition of connective tissue formation and a degrading effect on the skeletal muscles. Furthermore, cortisol will give a mineralocorticoid effect. Since cortisol is a lipophilic molecule, cortisol can cross the blood-brain barrier and can influence the cognitive function of the brain as well. Further, cortisol can help with formation of memories if the stress is acute. And If the stress is chronic, it can decrease the learning ability and memory formation (Berg and Otterholt, 2020-06-30).

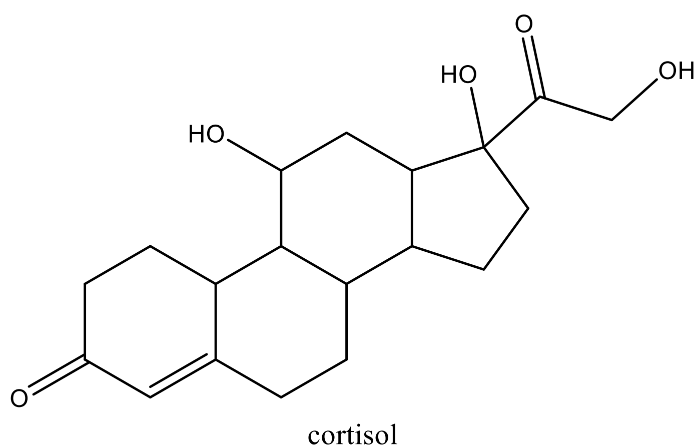


Figure 6: Molecular structure of cortisol. Made in chemdraw v.22.2.0.

In humans' cortisol is synthesized in the adrenal gland. However, in zebrafish cortisol is synthesized in the interrenal gland in the head of the kidney. Furthermore, cortisol excretion/regulation is controlled by the hypothalamus (Abreu *et al.*, 29-09-2021; Bacila, Cunliffe and Krone, 2021/09/15).

Corticotropin releasing hormone (CRH) is the hormone that regulates cortisol. In addition, the first CRH neurons are detected at 25 hpf in the hypothalamic region. Then at 28 hpf CRH neurons is detected in the telencephalon region (Chandrasekar, Lauter and Hauptmann, 2007/12/01). Development of cortisol level in zebrafish egg to larvae is the following. Cortisol decreases by about 70% from 1.5 hpf to 25 hpf and stays at this level until right after hatching (49 hpf). Then increases 27-fold by 146 hpf larvae (Alsop and Vijayan, 2008 Mar 01).

1.3.4 Analysis of neurotransmitters in zebrafish

One way to analyse neurotransmitters in zebrafish is with an immunoassay kit. Furthermore, this is what Eto, *et al.* (01-01-2014) did. In that experiment adrenaline and noradrenaline was analysed with an enzyme-linked immunosorbent assay (ELISA) kit while cortisol was analysed with an enzyme immunoassay kit (EIA). Other neurotransmitters are analysable, Dong *et al.* (01-09-2022) analysed acetylcholine, 5-hydroxyindoleacetic acid (5-HIAA), dopamine, 5-HT (serotonin) and dihydroxyphenyl acetic acid (DOPAC) with ELISA kits. I addition, 5-HT and 5-HIAA are not neurotransmitters but metabolites of serotonin. And DOPAC is a metabolite of dopamine (Dong *et al.*, 2022/09/01).

An ELISA kit or EIA kit works by using the catalytic properties of enzymes to quantify and detect immunologic reactions. This done on a solid phase that is either a microtiter well, a magnetic particle or plastic bead. Furthermore, where one of the reaction components are covalently bond or non-specifically adsorbed. This will then facilitate the separation of bound to free-labelled reactants (Alhajj, Zubair and Farhana, 2023).

Another way to analyse neurotransmitters in zebrafish would be with high pressure liquid chromatography (HPLC) or ultra high-pressure liquid chromatography (UHPLC), Santos-Fandila, *et al.* (15-09-2015) used UHPLC coupled with tandem mass spectrometry to detect and quantify 17 neurotransmitters, metabolites and precursors. Where acetylcholine and adrenaline(epinephrine) were analysed. Furthermore, HPLC will be explained in more detail in section 1.4.

1.3.5 Carbamazepine

Carbamazepine is a drug that is used as a mood stabilizer and additionally is also used to treat mania, epilepsy and neuropathic pain. Moreover, it`s also used as a maintenance treatment for bipolar disorder. Additionally, glutamate and GABA is the two neurotransmitters that carbamazepine is antagonistic against. Furthermore, the drug Carbamazepine can decrease the neurotransmitter levels of other neurotransmitters as well like norepinephrine (noradrenaline), serotonin and dopamine (Ayano, 2016).

1.3.6 Hydrocortisone

Hydrocortisone is a steroid drug that is used to treat some allergies, asthma, arthritis, skin disorders, blood disorders, a few cancer types, auto immune diseases, kidney disorders, thyroid problems and intestinal problems. Furthermore, hydrocortisone is used to treat different types of inflammation in or on the body. In addition, hydrocortisone can also be used by people that can`t produce a normal amount of cortisol in the body (Muhammad, 2024/03/27).

Hydrocortisone is the synthetic version of cortisol. Furthermore, adrenergic receptors are upregulated by hydrocortisone. Moreover, hydrocortisone decreases the breakdown of catecholamines and increases the calcium levels in the myocardial cells (Morris and Sharma, 2019/06/01).

1.4 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS)

HPLC-MS/MS or High-performance liquid chromatography tandem mass spectrometry is a way to separate and analyse different organic compounds with different chemical properties.

1.4.1 High performance liquid chromatography (HPLC)

In high performance liquid chromatography or HPLC the system is spilt into a pump, solvent reservoir, injector, column, detector and data system (PC) where data can be viewed. The HPLC columns (separation phases) are tightly packed, with particles that are uniform and about 1.7 to 10 µm particle size. Having a tightly packed column comes with some requirements such as a high-pressure pump that can reach the columns demand pressure up to 5000 psi. And a specialized injection device that will connect/transfer the sample in phase flow. The detector can be an ultraviolet detector, refractive index (RI), florescence detector, electrochemical detector, evaporative light-scattering detector or a mass spectrometer to name a few examples (Miller, 2005).

1.4.1.1 Reversed phase liquid chromatography (RPLC)

Reversed phase liquid chromatography or RPLC is a chromatographic analytical method that uses a polar mobile phase and a nonpolar stationary phase to separate different organic molecules based on how polar (hydrophilic) the molecules are. Furthermore, if the molecules are polar the molecules will pass through the column and not interact with the stationary phase and elute first. However, if the molecule is nonpolar (hydrophobic) the molecule will be adsorbed by the stationary phase and retained on the column and eluted last. Opposite of RPLC the normal phase liquid chromatography or NPLC, which uses a nonpolar mobile phase and a polar stationary phase to separate different organic molecules. The mobile phase can have an isocratic or gradient elution. Isocratic means that the mobile phase composition is constant throughout the analysis. Gradient means that the mobile phase is changed from a solvent with strong polarity to solvent with weaker polarity in the course of the analysis (Miller, 2005).

Stationary phases used for RPLC is one of the following ligands bonded with silica octadecyl ($C_{18}H_{37}$), octyl(C_8H_{17}), propyl (C_3H_7), phenyl (C_5H_6) and methoxy (CH_3O). However, octadecyl is the most popular stationary phase for RPLC, since octadecyl is most stable and, because with less carbon's hydrophobicity decreases. In addition, Octyl is often used as well (Miller, 2005).

Mobile phase setup used for RPLC are water with an organic modifier as one of the mobile phases with methanol, acetonitrile, or tetrahydrofuran (THF) as the other mobile phase. RPLC can use a third mobile phase, when it is necessary as a third phase could lead to a good advantage when used. However, this not common with RPLC. Furthermore, a buffer can be used if it is necessary to stabilise the mobile phase if the analyte/s have any acidic or basic properties. In addition, if a buffer is used pH should never be more than 2 pH unites away from any of the analyte/s pK_a value/s (Miller, 2005).

1.4.1.2 Hydrophilic interaction chromatography (HILIC)

Buszewski and Noga (21-08-2011, p.231) defines Hydrophilic interaction chromatography or HILIC as "an alternative HPLC mode for separating polar compounds". Historically HILIC has been described as a variant of NPLC. HILIC does have a more complicated separation mechanism than NPLC. HILIC is a chromatographic separation based on differences in analyte polarity. In contrast to RPLC, the strong solvent is water, and usually acetonitrile is the weak solvent. The

mechanisms of retention include partitioning into an adsorbed water layer and the stationary phase surface, adsorption, and ion exchange, in a complex interplay that depends on the eluent pH and buffer concentration. In HILIC, the stationary phase is polar, and the mobile phase is less polar than the stationary phase. Compounds with a wide range of polarities can be analysed by this technique. Furthermore, the compounds with high polar character such as neurotransmitters (adrenaline, acetylcholine for instance) are poorly retained on hydrophobic stationary phases, that is why HILIC is the most used analytical method to separate polar substances (Buszewski *et al.*, 2011-08-31).

1.4.2 Tandem mass spectrometry (MS/MS)

Hoffman and Stroobant (2007, p.189) define tandem mass spectrometry or MS/MS as "any general method involving at least two stages of mass analysis, either in conjunction with a dissociation process or in a chemical reaction that causes a change in the mass of an ion".

In MS/MS there are at least to analysers. The first analyser is used to isolate a precursor ion. Furthermore, this precursor ion will so undergo a spontaneous or by some activation a fragmentation that will yield a product ion and neutral loss fragments. In addition, the second analyser will analyse the product ions (Hoffman and Stroobant, 2007).

There is two ways to look at tandem mass spectrometry MS/MS. One way is to perform a tandem mass spectrometry in space by linking to two physically distinct instruments. The other way is to do it in time by performing an appropriate sequence of events in an ion storage device (Hoffman and Stroobant, 2007).

It is common for space instruments to have two mass analysers. This allows MS/MS experiments to be conducted. Furthermore, an instrument of this type frequently uses quadrupoles as analysers. Moreover, the configuration of the quadrupoles could be a $Q_1q_2Q_3$, which is used in this thesis. However, there are other configurations. In the QqQ configuration Q is a quadrupole and q are a quadrupole that operates in RF(Radiofrequency)-only mode. This makes q a type of lens for all the ions, it's also called a collision cell. However, in modern QqQ instruments the collision cell is a hexapole which have better focusing properties than a quadrupole. Furthermore, other instruments combine electric sectors and magnetic sectors (E and B), magnetic sectors(B), electric

sectors(E) and quadrupoles. In addition, another type of instrument used is a TOF or time of flight, where a reflectron or quadruples can be combined with the TOF instrument (Hoffman and Stroobant, 2007; Leiminger *et al.*, 2019/10/01).

1.4.2.1 Electrospray ionization (ESI)

Hoffman and Stroobant (2007, p.43) say that electrospray ionization or ESI “is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux (Normally 1-10μl min^{-1})”. This very similar to API but the difference is that API makes the ions in a gas phase instead of a liquid phase (Hoffman and Stroobant, 2007).

The way an ESI works is that first a liquid surface located at the end of a capillary will accumulate charge by an electric field. Moreover, the electric field is created by applying a difference of 3-6kW in the middle of a capillary and a counter-electrode. Furthermore, this charge will make a formation of highly charged droplets. Then a gas is injected in coaxially at a low flow rate to limit the dispersion of the spray. Furthermore, a heated curtain will be made of inert gas often nitrogen(N_2) where droplets will pass through. However, this could also be a heated capillary to remove the last of the solvent molecules. Additionally, the spray will not start before it reaches a certain “onset” voltage. The voltage is dependent on the surface tension of the solvent. Then a “Taylor cone” will be formed when the voltage reaches the required level, and small droplets will be released from the tip of the capillary. In these droplets the solvent evaporates. This makes the charge per volume to increase in the droplets and then the droplets shrink. Furthermore, a strong electric field causes the droplets to deform. Then the forces from the resulting accumulation of charge will elongate the droplets. This causes a new “Taylor cone” to form. This “Taylor cone” will emerge and release 20 smaller droplets. The process just described will continue until the surface ions on the droplets gets ejected into the gaseous phase because it is kinetically and energetically possible. The emitted ions get sampled by a sampling skimmer cone. Then in the end the ions get accelerated by the sampling skimmer cone into the mass analyser (Ho *et al.*, 2003; Hoffman and Stroobant, 2007).

1.4.2.2 Quadrupole(Q/q)

Hoffman and Stroobant(2007) defines a quadrupole analyser as “a device which uses the stability of the trajectories in oscillating electric fields to separate ions according to their mass to charge ratios (m/z)”. A quadrupole consists of 4 rods of circular or hyperbolic section. The rods are always perfectly parallel and equidistant from each other. There are two pair of rods one pair is positively charged, while the other pair is negatively charged. Between the two opposing rod pairs a radio frequency (RF) voltage with a direct current (DC) offset voltage is applied to them. Furthermore, this will make an electric oscillating field. Moreover, this field will be able to distinguish different ions form each other by charge and size(m/z). Additionally, the RF and DC voltage can be preset too desirable m/z ratios. This will make it so only ions with desirable m/z will go through the quadrupole. If the ion is to positively or negatively charged or too big or small, the ion will collide with the quadrupole. This will make it so that only the selected ions will make it through (Ho *et al.*, 2003; Hoffman and Stroobant, 2007)

1.4.2.3 Electron multiplier (EM)

EM or electron multiplier is one of the most used detector types in mass spectrometry. Furthermore, an electron multiplier or EM is a detector that accelerates ions to high velocities in order to enhance detection efficiency. The EM does this by having an electrode called a conversion dynode with a charge that is opposite of the detected ions. The conversion diode has a high potential from ±3 to± 30kv. When a negative ion or positive ion strikes the conversion dynode it causes emission of several secondary particles. Moreover, the secondary particles can be positive ions, electron, negative ions and neutrals. Furthermore, the conversion dynode can be either positive high voltage or negative high voltage. However, if a positive ion hits a negative high voltage conversion dynode the particles of interest will be electrons and negative ions. This is the opposite when a negative charged ion hits a positive charged high voltage conversion dynode. Then the particle of interest is positive ion. To produce a current in the electron multiplier the secondary particles are converted to electrons at the first dynode. The electrons will so be amplified by a cascade effect, to make the current. Furthermore, there are two classes of electron multipliers. The two classes of electron multipliers are discrete dynode type or continuous dynode type (Hoffman and Stroobant, 2007).

1.4.2.4 Scan modes

With a MS/MS instrument there are 4 main modes of scanning. Product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). Product ion scan (daughter scan) is a mode used to scan for a product ion from a selected precursor ion of chosen mass-to-charge ratio (m/z). This is then used to determine all the product ions as a result of CID or collision induced dissociation. If the only ions produced are fragment ions, the mode is also called "fragment ion scan" (Hoffman and Stroobant, 2007; Shi *et al.*, 2012/04).

A precursor ion scan (parent scan) determines precursor ions (parent ions) based on a chosen product ion or daughter ion. Furthermore, this method also uses a CID. This mode will scan for ions with the first spectrometer and selecting an ion with the second spectrometer. Then being the opposite of the product ion scan (Hoffman and Stroobant, 2007).

A Neutral loss scan works by selecting a neutral fragment. Then all the fragments leading to the loss of the neutral will be detected. This mode requires that both spectrometers are in scan mode and scanned together, but with a constant mass offset between the two. Fragmentation is achieved in the same as before with CID (Hoffman and Stroobant, 2007).

The final scan mode is selected reaction monitoring or SRM. This mode is also known as MRM or multiple reaction monitoring. In this mode instead of scanning, both spectrometers are focused on selected masses. This mode is then selecting a fragmentation reaction, instead of any fragment or ion. Furthermore, ions detected by the first mass analyser will only be detected if the ions produce a fragment from a selected reaction. Since there is no scanning, it is possible to monitor precursor and fragment ions over longer times. This increases the sensitivity of SRM and MRM. Furthermore, SRM becomes MRM when multiple selected fragments from multiple reactions are monitored at the same time from a complex mixture of analytes and other not wanted substances (Hoffman and Stroobant, 2007; Sherwood *et al.*, 2009 Jul; Shi *et al.*, 2012/04).

2. Hypothesis and aim

The aim of this work is to:

1. Optimize the method for neurotransmitter analysis by reducing the amount of zebrafish tissue needed to analyse neurotransmitter levels by optimising sample preparation, sampling and analysis method.
2. To test the hypothesis that environmentally relevant doses of PFOA and PFHxS will be taken up in the zebrafish embryo and larval organism in sufficient doses to induce negative or positive changes in the neurotransmitter profile, such as acetylcholine, adrenaline and cortisol levels

3. Methods and Materials

3.1 Zebrafish samples

The exposure experiment was performed in collaboration (Nilsen *et al.*, 2024/09/01) briefly. Fertilized zebrafish eggs were divided into petri dishes with 20 zebrafish eggs and exposed to either PFOA 1x, PFOA 1000x, PFHxS 1x, PFHxS 1000x, negative control (no exposure), carbamazepine or hydrocortisone (cortisol).

Fourteen empty cryo tubes were marked according to Table 1 and 15 according to Table 2, all tubes were weighed, and the weight of the tubes written down (Figure 7).

Figure 7: Marked cryo tubes. Cryo tubes marked for zebrafish embryos 48 hpf (14) according to table 1 and zebrafish larvae 96 hpf (15) marked accordingly to table 2.

Before sampling of zebrafish at 48 hpf and 96 hpf liquid nitrogen (N_2) (LINDE GAS, Dublin, Ireland) was poured into an insulated container and put in a fume hood.

Sampling and weighing of zebrafish were conducted as fast as possible, within minutes. This was to avoid any decomposition of the zebrafish and any change in the neurotransmitter amount in the zebrafish. Twenty zebrafish would be sampled into each of the weighed cryo tubes. The number of zebrafish in the petri dishes was counted multiple times before sampling. Under sampling the number of zebrafish was counted as well. This was to ensure the right number of zebrafish in each sample before weighing. Before weighing all water or as much water as possible had to be removed. After removal of water the cryo tube was weighed and weight denoted as in Table 1 and 2. Quickly after weighing the zebrafish was put into the liquid nitrogen(N_2).

After sampling the samples were quickly put in a marked box with name(s), date and contents. and in a -80°C freezer for further analysis.

3.1.1 Sampling data for 48 hours past fertilization (hpf) zebrafish embryos and 96 hours past fertilization(hpf) zebrafish larvae

Table 1: 48 hpf(hours past fertilization) zebrafish embryo(zfe) sample data. Zebrafish embryos was sampled 03.07.2024. The zebrafish was exposed to PFOA 1x (PF1.x) and 1000x (PF0.x), PFHxS 1x (PX1.x) and 1000x (PX0, x), Carbamazepine(B), Hydrocortisone(H) and one negative control(C). Every exposure had 2 replicates. Sample tube was weighed before and after sampling of zebrafish to find the weight of the zebrafish larvae.

No.	Hpf(hours past fertilization)	Sample ID	Weight when empty (g)	Weight with sample (g)	Sample weight (g)	Exposure group	Material sampled	Number of zebrafish sampled
1	48	C1	2,248	2,272	0,024	Control	Embryo	20
2	48	C2	2,262	2,290	0,028	Control	Embryo	20
3	48	B1	2,269	2,290	0,021	Carbamazepine	Embryo	20
4	48	B2	2,274	2,290	0,016	Carbamazepine	Embryo	20
5	48	H1	2,263	2,296	0,033	Hydrocortisone	Embryo	20
6	48	H2	2,243	2,265	0,022	Hydrocortisone	Embryo	20
7	48	PF1.1	2,279	2,300	0,021	PFOA (1x)	Embryo	20
8	48	PF1.2	2,251	2,272	0,021	PFOA (1x)	Embryo	20
9	48	PF0.1	2,254	2,346	0,092	PFOA (1000x)	Embryo	20
10	48	PF0.2	2,264	2,332	0,068	PFOA (1000x)	Embryo	20

11	48	PX1.1	2,252	2,260	0,008	PFHxS (1x)	Embryo	20
12	48	PX1.2	2,249	2,266	0,017	PFHxS (1x)	Embryo	20
13	48	PX0.1	2,246	2,301	0,055	PFHxS (1000x)	Embryo	20
14	48	PX0.2	2,275	2,320	0,045	PFHxS (1000x)	Embryo	20

Table 2: 96 hpf(hours past fertilization) zebrafish larvae sample data. Zebrafish larvae was sampled 05.07.2024. The zebrafish was exposed to PFOA 1x (PF1.x) and 1000x (PF0.x), PFHxS 1x (PX1.x) and 1000x (PX0,x), Carbamazepine(CBZ)(B), Hydrocortisonel(H) and one negative control(C). Every exposure had 2 replicates except PFOA 1x which had three. Sample tube was weighed before and after sampling of zebrafish to find the weight of the zebrafish larvae.

No.	Hpf(hours past fertilization)	Sample ID	Weight when empty (g)	Weight with sample (g)	Sample weight (g)	Exposure group	Material sampled	Number of zebrafish sampled
1	96	C1	2,260	2,269	0,009	Control	larvae	20
2	96	C2	2,253	2,262	0,009	Control	larvae	20
3	96	B1	2,254	2,263	0,009	Carbamazepine	larvae	20
4	96	B2	2,242	2,252	0,01	Carbamazepine	larvae	20
5	96	H1	2,257	2,266	0,009	Hydrocortisone	larvae	20
6	96	H2	2,258	2,271	0,013	Hydrocortisone	larvae	20
7	96	PF1.1	2,253	2,261	0,008	PFOA (1x)	larvae	20
8	96	PF1.2	2,252	2,261	0,009	PFOA (1x)	larvae	20
9	96	PF1.3	2,249	2,257	0,008	PFOA (1x)	larvae	20
10	96	PF0.1	2,255	2,265	0,01	PFOA (1000x)	larvae	20
11	96	PF0.2	2,253	2,263	0,01	PFOA (1000x)	larvae	20
12	96	PX1.1	2,267	2,278	0,011	PFHxS (1x)	larvae	20
13	96	PX1.2	2,256	2,264	0,008	PFHxS (1x)	larvae	20
14	96	PX0.1	2,257	2,267	0,01	PFHxS (1000x)	larvae	20
15	96	PX0.2	2,256	2,276	0,02	PFHxS (1000x)	larvae	20

3.2 Calibration curve and internal standards

3.3.1 Internal standards (IS)

The best internal standard (IS) is an isotopically labelled version of the compound we want to quantify. This will have a similar extraction recovery, ionization response in ESI mass spectrometry, and a similar chromatographic retention time.

The stock solutions of the internal standards were provided from Merck Sigma and LGC.

100µg/mL adrenline-d^6 in methanol with 5% 1MHCl

1mg/mL acetylcholine-d^4 in methanol with 5% 1M HCl

100µg/mL cortisol-$_{13}C^3$ in methanol

3.3.2 Calibration curve

Working solutions were prepared in dilution solution which consisted of 10% methanol /water(v/v) with 0.1% formic acid and 0.05% ascorbic acid.

External standard calibration curves were prepared in dilution solution as surrogate matrix.

The linear ranges were as follows: 0-2000 ng/g for Ach, 0-40ng/g for cortisol and 0-500ng/g for adrenaline. The calibration curves were constructed based on the peak area ratio of the analytes to the corresponding internal standards, versus the nominal concentration ratio. The concentration of each compound in each sample was calculated using the peak area ratio and linear regression analysis. The response for each compound was linear and gave correlation coefficients greater than 0.99. The limit of detection (LoD) was based on 3X signal to noise ratio, and the limit of quantification (LoQ) was determined as the lowest validated concentration. The validation study was conducted on 4 mg sample (ca 20zfe) spiked with the corresponding isotope labelled standard of all three compounds. LoD and LoQ for acetylcholine, cortisol and adrenaline is viewable in Table 3.

Table 3: Limits of detection and quantification for 4 mg sample spike –in experiment. Based on dry weight of zebrafish larvae tissue.

Compound	LoD(ng/g)	LoQ(ng/g)
Acetylcholine	5	15
Adrenaline	5	15
Cortisol	0.25	1

3.3 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) sample preparation

The sensitive and specific reverse phase-liquid chromatography-electrospray ionization- tandem mass spectrometry (RP-LC-ESI-MS/MS) for the identification and quantification of neurotransmitters (acetylcholine, adrenaline) and the steroid stress hormone cortisol in small amounts of zebrafish embryos (4 to 6 mg sample size) used here was based on the analytical method with some modifications to analyse 3 mg samples of 48 hpf zebrafish embryo tissue and 4 mg samples of 96 hpf zebrafish larval tissue of earlier LC-MS/MS analysis of neurotransmitters in brain tissues publication developed by Daniela Dulgheriu (C. Veit , J. Nordgreen , D. Dulgheriu and co, The effect of LPS and Ketoprofen on Cytokines, Brain Monoamines, and Social Behaviour in Group-Housed Pigs, Frontiers in Veterinary Science, Vol 7, Jan 2021, p.6) Frontiers | The Effect of LPS and Ketoprofen on Cytokines, Brain Monoamines, and Social Behaviour in Group-Housed Pigs (frontiersin.org).

Chemicals and stock solutions used in the sample preparation can be found in the section list of chemicals on page 77-79. List of equipment used in the sample prep is found in list of equipment on page 75-76.

Zebrafish samples at 48 hpf (Table 1) and 96 hpf (Table 2) were kept on ice during analysis. An internal standard solution mixture (1/10/100, $^{13}C_3$-cortisole/Adrenaline D_6/Acetylcholine-D_4

ng/ml) was previously prepared and kept in fridge under use. For information on internal standards used, look at the list of stock solutions on page 79.

One third of each sample was assumed to be composed of zebrafish tissue corresponding to 3 mg tissue for all 48 hpf zebrafish embryo samples. The 48 hpf and 96 hpf zebrafish samples were removed from the cryo tubes and transferred to the homogenization tubes and weighed. As much water as possible was removed before weighing. In the 48 hpf zebrafish samples water was not removed due to difficulties with removing the water from the zebrafish embryos without losing to much tissue. After this 400µl cooled, ACN was added to each homogenization tube (Figure 8).

Every homogenization tube with samples from 48 hpf and 96 hpf zebrafish samples was vortexed 30 sec with a vortex genie 2 (Thermo Fisher Scientific, Waltham, US) (Figure 8).

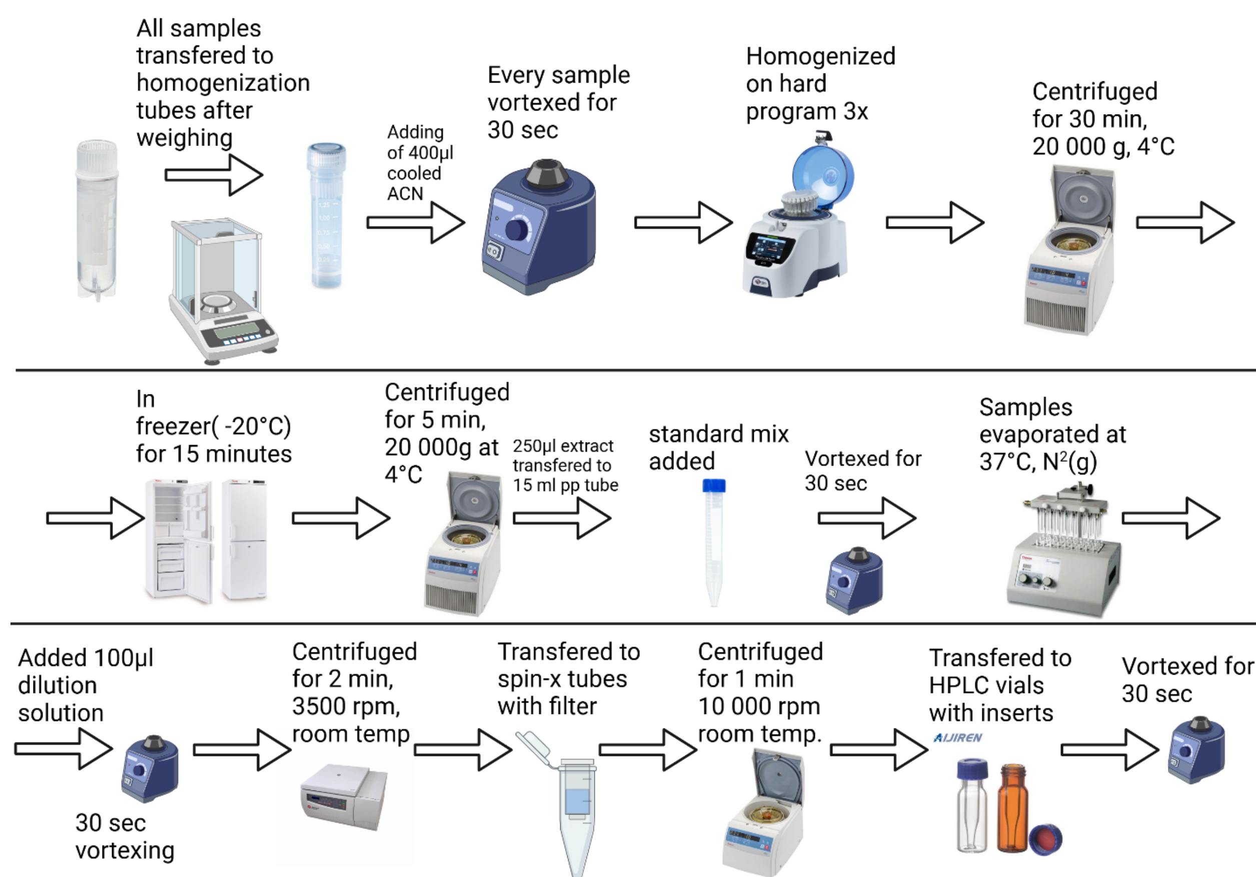


Figure 8: Illustration of the sampling preparation process for HPLC-MS/MS. Created in BioRender (https://BioRender.com).

After this, homogenization tubes were put into a Precellys 24 evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and homogenized 3 times with the hard program (Figure 8).

After homogenization, tubes were centrifuged on a Heraeus Fresco 21 centrifuge (Thermo Fisher Scientific, Waltham, US) for 30 minutes on 20000*g* at 4°C. While the centrifuge was ongoing 15 ml PP centrifuge tubes, spin-x centrifuge filter tubes and HPLC vials with inserts were marked and prepared for the next steps (Figure 8).

When the centrifuge was done the homogenization tubes were put in a freezer at -20°C for 15 minutes. The homogenization tubes were then centrifuged one more time on the Heraeus Fresco 21 centrifuge (Thermo Fisher Scientific, Waltham, US) for 5 minutes on 20000*g* at 4°C (Figure 8).

The extract (250µl) from each homogenization tube was transferred to a prepared 15 ml PP (polypropylene)centrifuge tube. Then the internal standard mix 1/10/100, $^{13}C_3$-cortisole/Adrenaline D_6/Acetylcholine-D_4 ng/ml was added to each sample (according to calculations based on the weight of each sample). Further each of the 15ml PP centrifuge tubes was vortexed 30 seconds on a vortex genie 2 (Thermo Fisher Scientific, Waltham, US) (Figure 8).

The extracts were then evaporated to dryness under N_2 (g) (LINDE GAS, Norway, 99.6 $\geq$ N_2(g)) gas stream at 37°C by using a Reacti-Vap Evaporator (Thermo Fisher Scientific, Waltham,US) After the samples evaporated and dried, the samples were dissolved in 100µl dilution solution (90% H_2O, 10% MeOH, 0,1% HCOOH, 0,05% ascorbic acid). The samples were vortexed 30 seconds on a vortex genie 2 (Thermo Fisher Scientific, Waltham, US) and then centrifuged on an Allegra X-12 R centrifuge (Beckman coulter, Brea, USA) 3500rpm for 2 minutes at room temperature, transferred to spin-x centrifuge filter tubes, with 0,22 µm filter (Costar,UT, USA), and centrifuged on a Heraceus Pico 21 centrifuge (Thermo Fisher Scientific, Waltham, US) 10000rpm for 1 minute at room temperature (Figure 8).

After this, the samples were transferred from the spin-x centrifuge filter tubes to HPLC vials with inserts (Agilent, Santa Clara, CA, USA). The HPLC vials were then vortexed for about 30 seconds on a vortex genie 2 (Thermo Fisher Scientific, Waltham, US) (Figure 8).

3.4 High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) specifications

3.4.1 Mobile phases

Two mobile phases were used in this HPLC-MS/MS set up. Mobile phase A was ultra-pure water (H_2O) ((Ultrapure water (18.2 Ω, TOC<1) was produced by Elga Ultrapure Laboratory water purification system (Lane End, UK)) with 0.1% Formic acid (HCOOH) (99%, Merck-Sigma-Aldrich, Burlington, USA). The mobile phase A was prepared in a 1000 ml measuring flask. The flask was nearly filled with H_2O to the 1000 ml line. Then 1 ml of Formic acid was added to the flask. The flask was then filled to the 1000 ml line with water. The flask was corked and mixed by hand. After this the solution was transferred to a 1000 ml reagent bottle. A lid was put on the bottle and hand shaken for about 30 seconds.

Mobile phase B were 50% methanol (CH_3OH) (≥99.9% HPLC quality, VWR chemicals, Radnor, USA) and 50% acetonitrile (ACN) (≥99,95%Maximum 0,003% H2O UPLC quality VWR chemicals, Radnor, USA) with 0,1% formic acid (HCOOH) (99%,Merck-Sigma-Aldrich ,Burlington, USA). Mobile phase B was prepared by adding formic acid 499,5 ml of methanol, 499,5 ml acetonitrile and 1 ml formic acid to a measuring flask. The solution was transferred to a 1000 ml reagent bottle with lid. Then hand shaken for 30 seconds

3.4.2 High pressure liquid chromatography (HPLC)

The HPLC-ESI-MS-MS system that preformed the analysis was an Agilent 1100 setup. The Agilent 1100 setup had a degasser, binary pump and auto sampler with thermostat (Agilent technologies, Santa Clara, CA, USA), coupled to an API 4000 triple-quadrupole spectrometer (AB Sciex, Ontario, Canada) equipped with a turbo ion spray. The temperature of the autosampler was set to 5°C. The column used for the chromatographic separation was a reverse phase Synergy-Fusion, 100x2.1mm, 2,5µm particles (Phenomenex, CA, USA), with a Fusion-RP guard column. The temperature of the column was 25°C. The injection volume was 30µl and flow rate was 0,25 ml/min.

Analyst version 1,7 (Sciex, Toronto, Canada) was the software used for controlling the equipment, acquiring and processing data.

3.4.3 Tandem mass spectrometry (MS/MS) Ion selection

Negative and positive ionization was used to detect the separated compounds. Furthermore, MRM was selected to detect the separated compounds in same run analysis. One precursor ion to two product ion transitions for each compound. In addition, Table 4 shows the data for the ion selection.

Figure 9-12 shows chromatograms for acetylcholine, acetylcholine-D^4, adrenaline, adrenaline-D^6, cortisol and cortisol $^{13}C_3$.

Table 4: Ion selection data. Acetylcholine, acetylcholine-D^4, adrenaline, adrenaline-D^6 was done in positive ionization and cortisol and cortisol-$^{13}C_3$ was done in negative ionization mode. Scan mode selected was MRM.

Compound	**Precursor ion (m/z)**	**Product ions (m/z)**	**Retention time (min)**
Acetylcholine	146	87 56	1,3
Acetylcholine-D^4	150	91 60	1,3
Adrenaline	186	168 118	1,2
Adrenaline-D^6	190	172 122	1,2
Cortisol	407.4	331 297	7,9
Cortisol-$^{13}C_3$	410.2	334 300	7,9

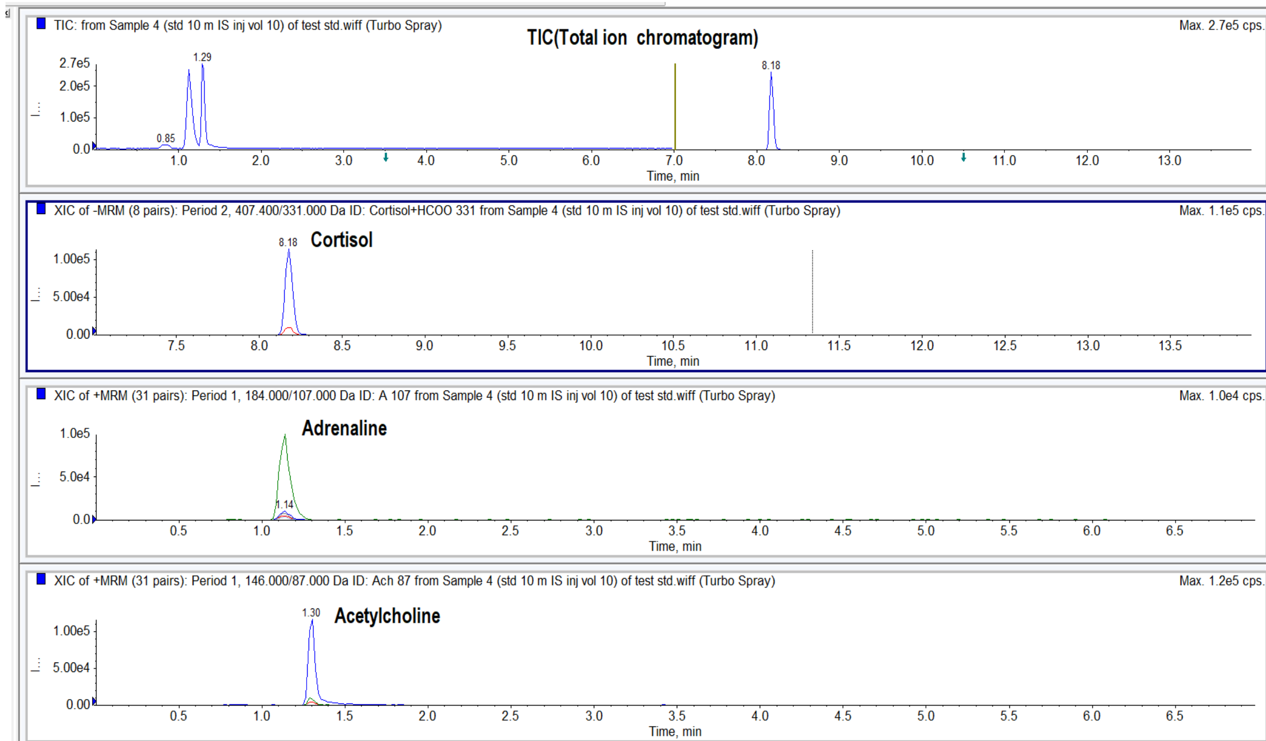


Figure 9: Chromatograms of cortisol, adrenaline and acetylcholine standards (10ppb) TIC (total ion chromatogram) and MRM for each compound

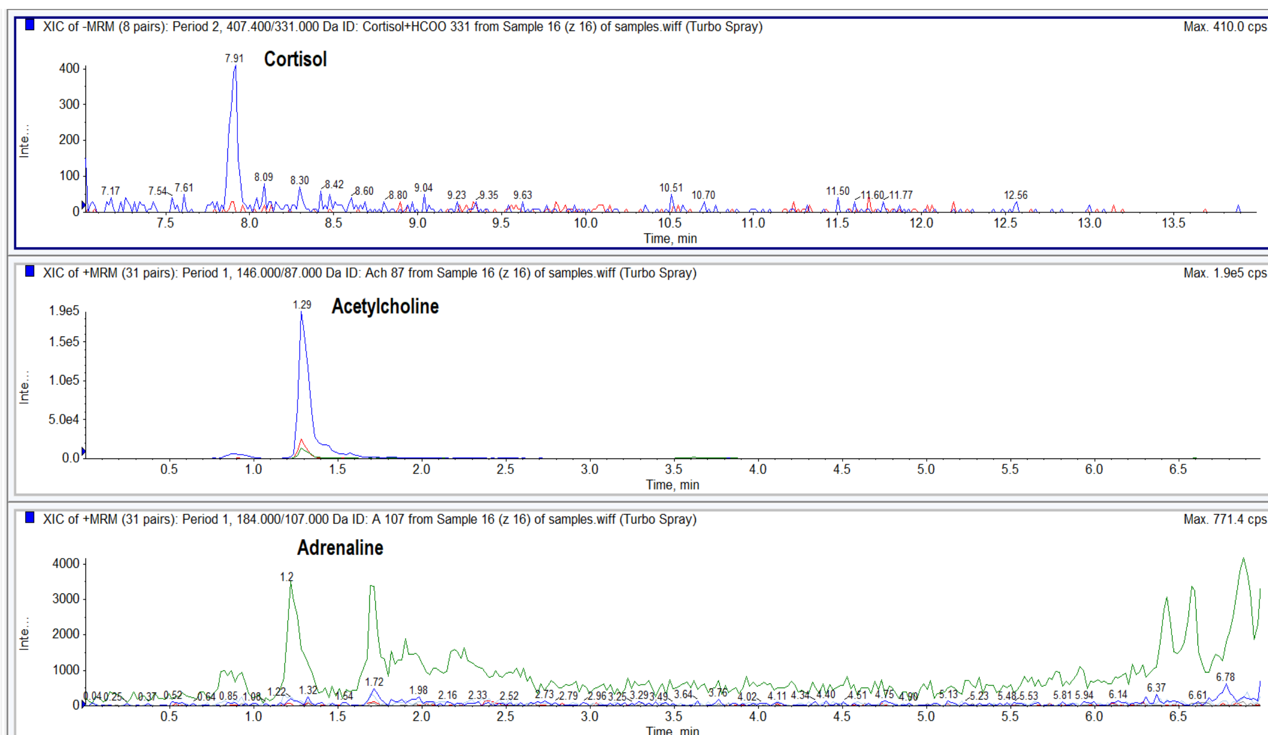


Figure 10: Extract ion chromatograms (XIC) of endogenous cortisol, acetylcholine and adrenaline in 3 mg zfe (48 hpf) sample

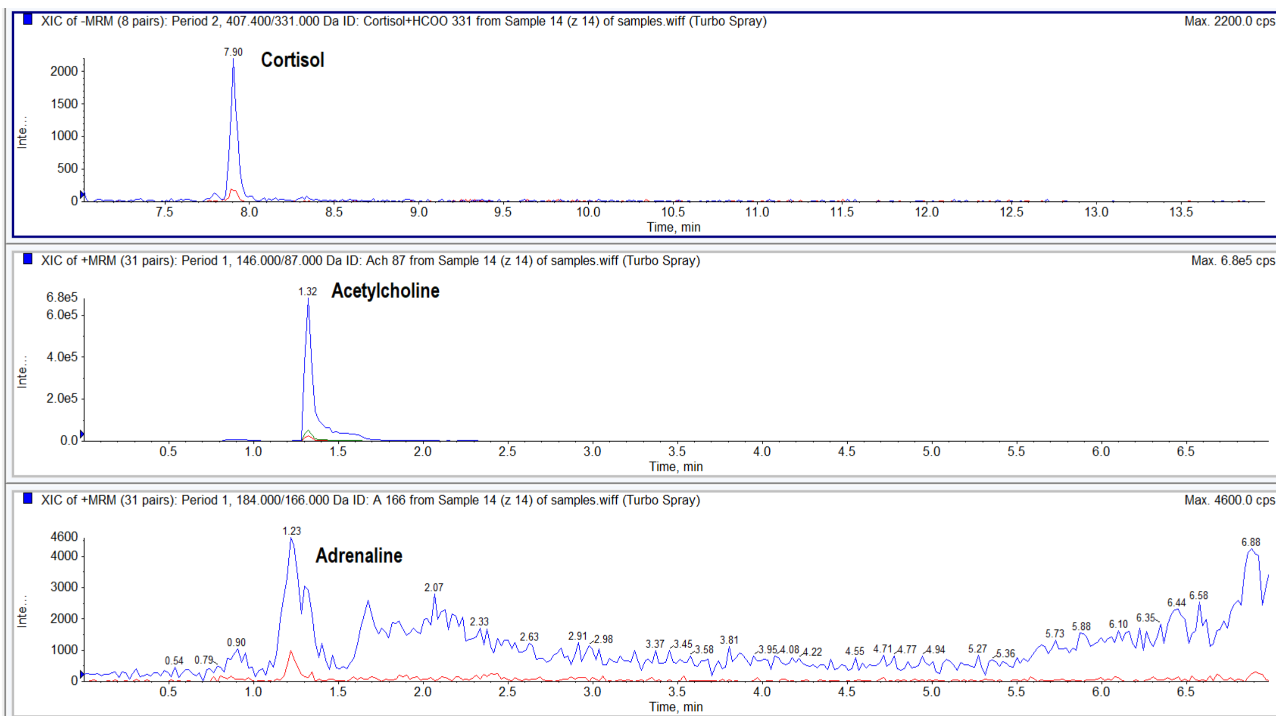


Figure 11: XIC of endogenous cortisol, acetylcholine and adrenaline in 4 mg zfe (96 hpf) sample.

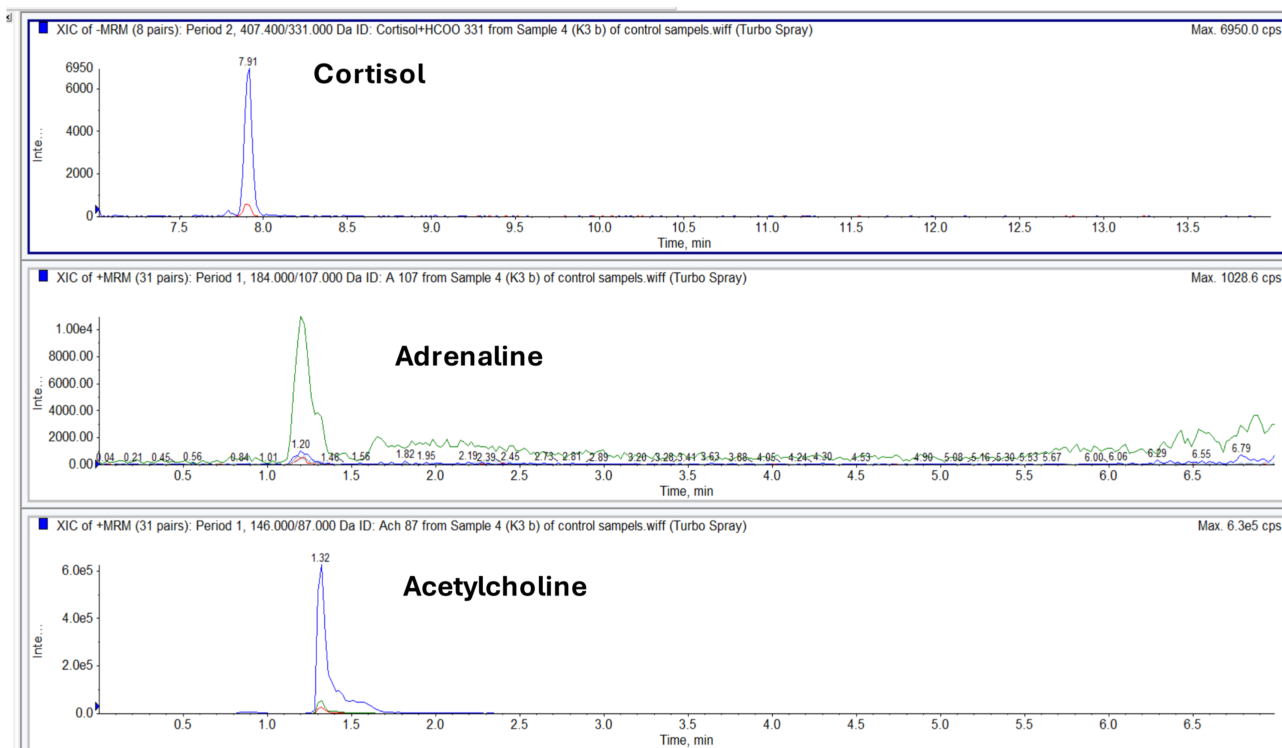


Figure 12: XIC of quality control (QC) spiked with 500 ng/g Acetylcholine, 50 ng/g adrenaline and 5 ng/g cortisol. With MRM.

3.5 Validation parameters

The validation parameters (detection limit, limit of quantification, linearity, precision, accuracy, recovery, and matrix effects) were determined by spiking the zfe homogenate with the corresponding stable isotope-labelled standard analogues of each compound as surrogate standard.

3.6 Statistical analysis

Data were collected from two replicates and three replicates where possible and six exposure groups. Initial data checks such as the mean, standard deviation (SD), Standard error of the mean (SE), fold change and fold change error were all calculated for all the data for zebrafish 48 hpf embryo and 96 hpf larvae.

All statistical testing was done in GraphPad Prism version 10.4.1. The groups represented different level of exposure and initial data checks were performed, but due to the small sample size due to

no resources or time left to repeat some of the samples. Assumptions of normality could not be reliably tested for all groups and the statical power is weak. However, for the groups with 3 samples, the assumption of normality was met. To provide a preliminary indication of difference between each exposure group and the control group one-way ANOVA test with Dunnett's multiple comparison test was used (to test all the 48 hpf embryo and 96 hpf larvae acetylcholine, cortisol and adrenaline levels (P-value $\leq$0,05) but should be interpreted with caution.

4. Results

All levels of adrenaline, acetylcholine and cortisol was detected and quantified in both zebrafish embryo (48 hpf) tissue and zebrafish larvae (96 hpf) tissue in all exposure groups (Figure 13-16). Furthermore, the exposure groups were carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1 x and PFHxS 1000x. Moreover, none of the samples were under the limits of quantification and the limits of detection (Table 3). The p-values of the one-way ANOVA and the multiple Dunnetts multiple comparison test trended toward lower p values for some acetylcholine levels and cortisol levels. Furthermore, the variability for the method was not high.

Figures 13-16 were made form the data found in Table L1 and L2.

4.1 Acetylcholine

Acetylcholine levels in zebrafish 48 hpf embryos exposed to various exposure groups (Figure 13A), shows that carbamazepine, hydrocortisone, PFOA 1x, PFHxS 1000x and PFOA 1000x exposures had a decremental fold change when compared to the control's acetylcholine level of 2075 ng/g. The fold change for carbamazepine was 0,745, hydrocortisone 0,549, PFOA 1x 0,882, PFOA 1000x 0,413 and PFHxS 1000x fold change was 0,875. Furthermore, the only exposure with a incremental fold change was PFHxS 1x, with a fold change of 1,046.

The levels of acetylcholine in zebrafish 96 hpf larvae after exposure to various exposure groups (Figure 13B), shows that exposure groups of carbamazepine, hydrocortisone, PFOA 1x and PFOA 1000x had decreamental fold change when compared to the control`s acetylcholine level of 1740 ng/g. Carbamazepine had a 0,670 fold change, hydrocortisone 0,802, PFHxS 1x 0,917 and PFHxS 1000x had a fold change of 0,810. Furthermore, PFOA 1x and PFOA 1000x had a incremental fold change with 1,054 and 1,096 respectively.

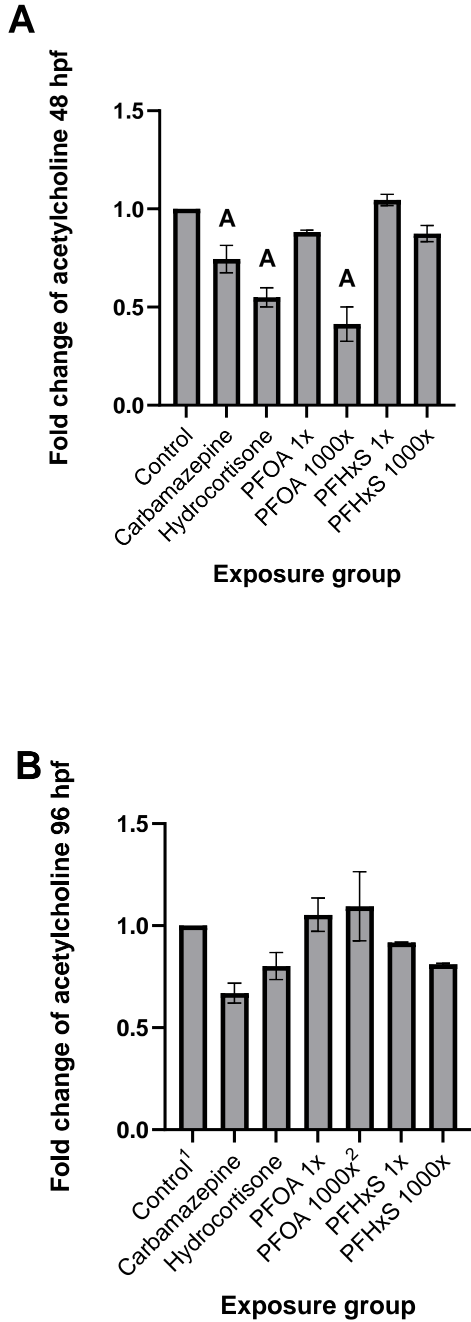
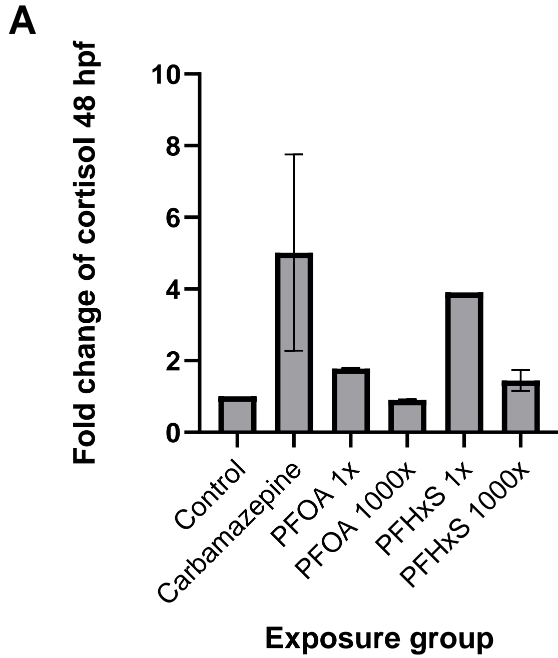


Figure 13: Fold change of acetylcholine levels in 48 hpf zebrafish embryo(A) and 96 hpf larvae(B) after exposure to control (no exposure), carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1x and PFHxS 1000x. There are two replicates of each exposure type except for PFOA 1x which had three replicates. There was n=20 zebrafish in each replicate. *Replicate one of the control (no exposure) exposure type was mostly lost during sample prep. ** Replicate one of the PFOA 1000x exposure had a vial break during sample prep and some sample was lost. [A]P-value≤ 0,05.

4.2 Cortisol

The cortisol level in zebrafish 48 hpf embryo after exposure to various exposure groups (Figure 14A and B) shows that the exposure group PFOA 1000x had a decremental fold change compared to the control's cortisol level of 2,115 ng/g. Furthermore, the fold change of PFOA 1000x was 0,905. Further cortisol levels show a incremental fold change in carbamazepine, hydrocortisone, PFOA 1x, PFHxS 1x and PFHxS 1000x. The fold changes were 5,02 (carbamazepine), 1,78 (PFOA 1x), 3,90 (PFHxS 1x) and 1,45 (PFHxS 1000x) respectively. Furthermore, hydrocortisone had a fold change of 761 when compared to the control. Because of that the hydrocortisone exposure has its own graph with the control (Figure 14B).

In zebrafish 96 hpf larvae the cortisol level after exposure to various exposure groups (Figure 15) shows that every exposure group except hydrocortisone had a decremental fold change compared to the control's cortisol level of 32,625 ng/g. The fold change for carbamazepine was 0,448, 3,934 for hydrocortisone, 0,519 for PFOA 1x, 0,385 for PFOA 1000x, 0,383 for PFHxS 1x and the fold change for PFHxS 1000x was 0,296.



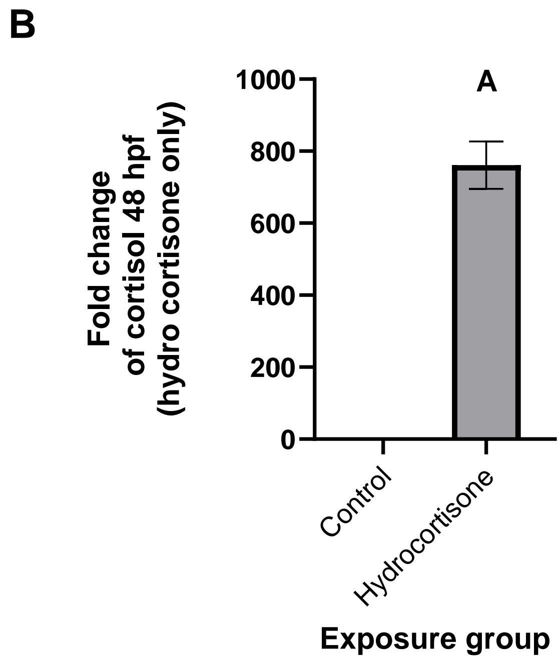


Figure 14: Fold change of cortisol levels of zebrafish 48 hpf embryo (A and B) after exposure to control (no exposure), carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1x and PFHxS 1000x. Hydrocortisone (B) exposure of 48 hpf zebrafish larvae led to a 761 fold change, which is way higher than the rest of the exposures. There were two replicates of each exposure. n=20 zebrafish in each replicate. [A]P-value≤0,05.

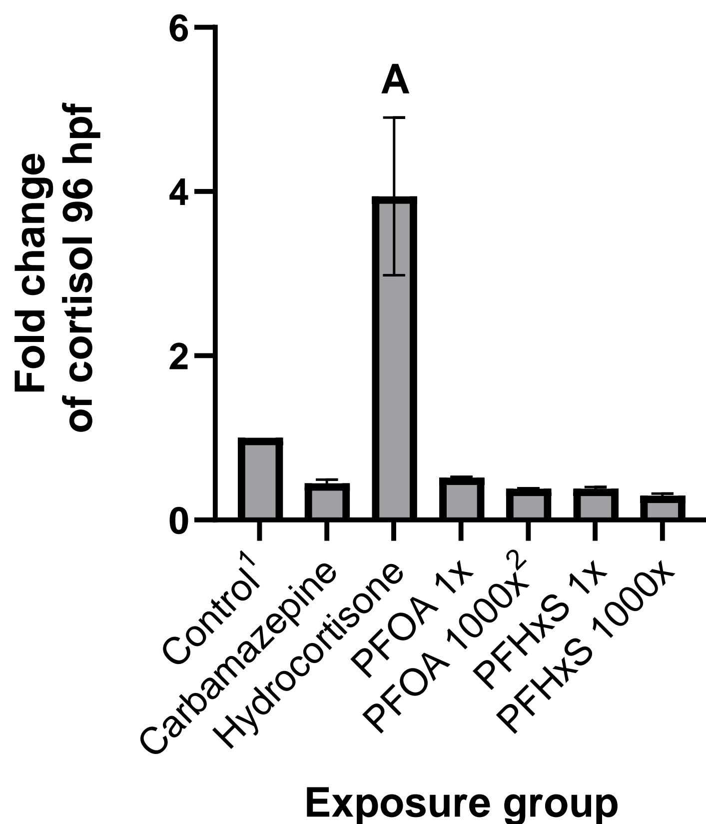
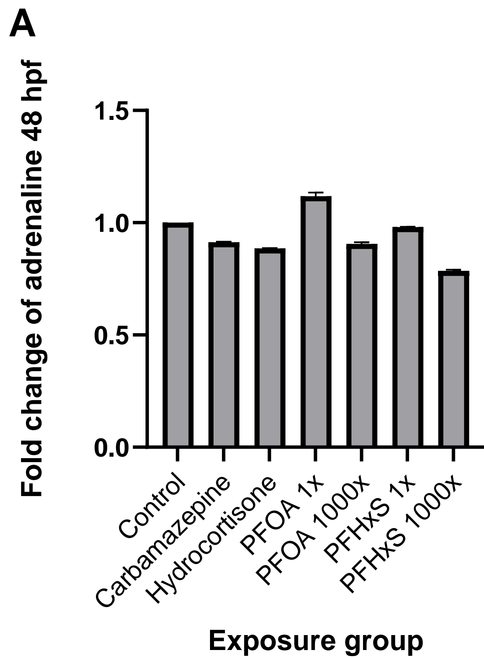


Figure 15: Fold change in cortisol levels in zebrafish 96 hpf after exposure to control (no exposure), carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1x and PFHxS 1000x. All exposure had two replicates except PFOA 1x which had three replicates. [1]Replicate 1 of the control (no exposure) exposure type was mostly lost during sample prep. [2]Replicate one of the PFOA 1000x exposure had a vial break during sample prep and some sample was lost. [A]P-value $\leq$0,05.

4.3 Adrenaline

Adrenaline levels in zebrafish 48 hpf embryo after exposure to various exposure groups (Figure 16A) shows that carbamazepine, hydrocortisone, PFOA 1000x, PFHxS 1x and PFHxS 1000x had a decremental fold change in comparison to the control`s adrenaline level of 38,1 ng/g. The fold changes were 0,913 (carbamazepine), 0,886 (hydrocortisone), 0,906 (PFOA 1000x), 0,980 (PFHxS 1x) and 0,786 (PFHxS 1000x). The only exposure group with a incremental fold change was PFOA 1x with a fold change of 1,118.

Adrenaline levels in zebrafish larvae 96 hpf after exposure to various exposure groups (Figure 16B) shows that hydrocortisone, PFOA 1x, PFHxS 1000x had a decremental fold change in comparison to the control's adrenaline level of 22,5 ng/g. The fold changes were 0,747 (hydrocortisone), 0,969 (PFOA 1x), 0,667 (PFHxS 1000x) respectively, while the exposure groups of carbamazepine, PFOA 1000x and PFHxS 1x had a incremental fold change of 1,13 (carbamazepine), 1,39 (PFOA 1000x) and 1,01 (PFHxS 1x).



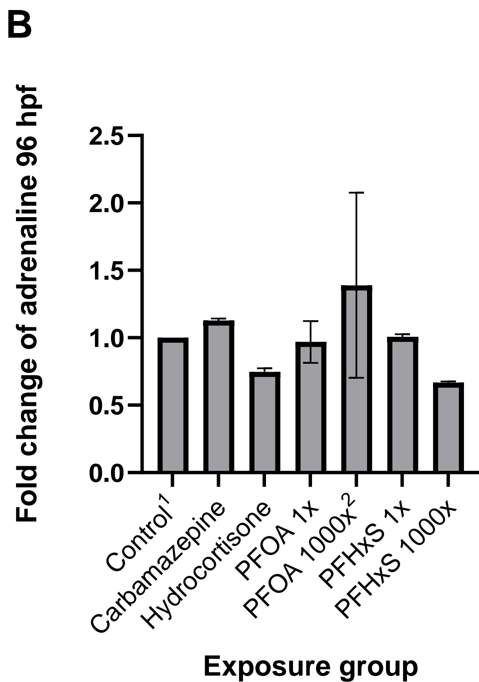


Figure 16: Fold changes of the adrenaline levels in zebrafish 48 hpf embryos(A) and 96 hpf larvae (B) after exposure to: Control (no exposure), carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1x and PFHxS 1000x. All exposure types had 2 replicates and n=20 zebrafish in each replicate. Except for PFOA 1x which had 3. [1]Replicate 1 of the control (no exposure) exposure type was mostly lost during sample prep. [2]Replicate 1 of the PFOA 1000x exposure had a vial break during sample prep and some sample was lost.

5. Discussion

5.1 Methodological consideration

All the neurotransmitter levels where detected and quantified. This means that samples of zebrafish embryo (n=20) and zebrafish larvae (n=20) can have the neurotransmitters acetylcholine, adrenaline and cortisol detected and quantified. The method had low standard error of the means for most exposure groups for all levels of adrenaline, cortisol and acetylcholine in 48 hpf zebrafish embryo and 96 hpf zebrafish larvae. sSome of the SE values were high due to lab error in form of lost sample or broken vial. The exposure groups in question were the 96 hpf control and 96 hpf PFOA 1000x. The 96 hpf control had a SE of ± 210 ng/g for acetylcholine, and ± 23,4 ng/g for cortisol. The 96 hpf adrenaline SE was low with a SE of ± 1,1 ng/g. The SE`s of the PFOA 1000x exposure group in 96 hpf of both acetylcholine and adrenaline were high (± 295 ng/g and ± 15,45 ng/g). The SE for the cortisol level of the same exposure was low with an SE of ± 0,05 ng/g. What can be said about these SE`s is that the errors in the lab had an impact on the result of the exposure groups just mentioned. Furthermore, there were some other high SE values who were high with no obvious reason. The SE that were high were the 48 hpf control for cortisol and adrenaline with SE values of ± 0,995 ng/g and ± 10,2 ng/g. The carbamazepine exposure of cortisol 48 hpf also had a high SE of ± 5,79 ng/g. The likely reason for these high SE values, is most likely the loss of zebrafish tissue during the transfer from cryo tubes to homogenization tube. The tissue lost from the transfer between the cryo tubes and the homogenization tubes during sample preparation was never more than a whole zebrafish larvae or embryo. One 48 hpf embryo was estimated to weigh 0,15 mg if 20 zebrafish embryos was estimated to be 3 mg total tissue. The 96 hpf larvae were estimated to be 0,2 mg since 20 zebrafish larvae were estimated to be 4 mg total tissue. This not a lot of tissue, but will get a lower adrenaline, cortisol and acetylcholine level than the other replicate of the same exposure. Furthermore, making a high SE for that exposure.

Since the removal of water was difficult to do without losing to much tissue in 48 hpf zebrafish larvae. What was done instead as stated in the methods was that every sample was counted as 3 mg zebrafish tissue. And cooled ACN was directly added to the Eppendorf tube and the sample transferred to a homogenization tube. This means that the water could contain excess hydrocortisone and make the tissue sample contain more cortisol than it should. This is likely

because the cortisol level of the hydrocortisone exposure group was 1610 ng/g when the control was 2,11 ng/g.

Tufi *et al.* (2016) used HPLC coupled to a triple quadrupole to analyse the zebrafish samples that had 20 zebrafish embryo or larvae in each sample. In that study, multiple neurotransmitters in zebrafish as a part of a study-to-study development in zebrafish and pesticide exposure effects zebrafish development were analysed. Furthermore, Tufi *et al.* (2016) analysed adrenaline (epinephrine) and acetylcholine as a part of the neurotransmitters analysed. These two neurotransmitters had low SE at 48 hpf and 96 hpf for both adrenaline and acetylcholine. Furthermore, none of the SE was higher than ± 0,5 pg/g. Even though the sample size is low, the low SE can also be seen with most of the exposure groups for adrenaline, cortisol and acetylcholine (Table L2) in this thesis. The only outliers are the ones already mentioned (Tufi *et al.*, February 25, 2016).

In another study by Santos-Fandilia *et al.* (2015) used a UHPLC coupled to a triple quadrupole mass spectrometer to analyse zebrafish samples with 5 zebrafish embryo or larvae. This is less zebrafish embryo/ larvae than was used in this thesis. Furthermore, Santos-Fandilia *et al.* (2015) analysed 17 different neurotransmitters, precursors and metabolites where acetylcholine and epinephrine (adrenaline) were two of them. Santos-Fandilia *et al.* (2015) got low SE for acetylcholine and an acetylcholine level of about 1 ng/g for 48 hpf and 1 ng/g 96 hpf. The SE for adrenaline was high with SE being about ± 0,5 ng/g for both 48 hpf and 96 hpf. Where adrenaline levels for 48 hpf and 96 hpf were not higher than 2 ng/g (Santos-Fandila *et al.*, 2015/09/15).

In a study by Eto *et al.* (2015) there was used an ELISA-kit to analyse adrenaline and cortisol levels in 48 and 97 hpf zebrafish after exposure to hand swirling and roller swirling. Eto *et al.* also had a control for both neurotransmitters at 48 and 97 hpf. In this study there were used 150 zebrafish embryo or larvae in each sample. The SE for the 97 hpf ca.0,650 ng/g cortisol control and the 48 hpf ca.0,50 ng/g cortisol control was low. The SE for the 48 hpf 0,010 ng/g adrenaline control and 97 hpf 0,080 ng/g adrenaline control was low. This shows that the colorimetric method is consistent, but the method needs more zebrafish per sample than HPLC which makes it a more expensive method to use (Eto *et al.*, 2014/01/01).

Acetylcholine was calorimetrically quantified in 120 hpf zebrafish in a study by Chen *et al* (2012), whereby, the quantification was done with a colorimetric assay kit. Chen *et al.* (2012) used 200

zebrafish larvae per replicate and 3 replicates. This study used more zebrafish larvae/embryo than the three other studies that were just discussed. The amount of acetylcholine was measured in nmol/min/mg protein and the acetylcholine concentration was found to be 800 to 810 nmol/min/mg protein. The SE in this study was low and was ±20-30 nmol/min/mg protein (Chen *et al.*, 2012/10/01).

By looking at the different studies the results from higher amounts of zebrafish tissue are more consistent and less variable than studies with low amounts of zebrafish. The amount of tissue used in this study can be reduced but that could lead to higher SE`s and variability between samples of the same exposure group. Furthermore, it is riskier if something happens to a sample with low amounts of tissue. Since the loss of too much tissue can give a more inaccurate result, which happened to some of the samples in this thesis previously mentioned. When the replicate size is 150 or 200 zebrafish as is the case for Chen *et al.* (2015) and Eto *et al.* (2014) it is possible to see that a sample size in the study of Tufi *et al.* (2014) can have up to 10 replicates for each replicate Chen *et al.* (2015) and Eto *et al.* (2014) had. Furthermore, the study of Santos-Fandilia *et al.* (2015) could have up to 40 replicates for each replicate Chen *et al.* (2015) and Eto *et al.* (2014) had. This means when it comes to resources a study that uses HPLC-MS/MS can have more replicates when compared to colorimetric methods and a lower use of resources.

5.1.1 Use of zebrafish model for neurotransmitter detection

The article written by Stråhle U., et al. (2012) about “zebrafish embryos as an alternative to animal experiments” highlights many points of interest. The article says that the zebrafish embryos is a choice for replacement and refinement of animal studies because the embryos are likely to experience less or no pain, distress, suffering and lasting harm. There are other benefits of using the zebrafish model. These benefits are a transparent embryo, which makes it easy to follow organ development. Easy maintenance, rapid development, small size and high fecundity to mention a few benefits. Zebrafish are independent up to 120hpf. After 120 hpf, they are no longer independent and need food. This means that the zebrafish are protected under the legislation of animal welfare (EU 2010/63/EU) after 120 hpf. Therefore, studies that use zebrafish embryo and/or larva up to 120 hpf are not under regulation. The effect of toxicity from carcinogenic substances on a human heart was more 80% successful in predicting the effect with the zebrafish

model. The successful predictability is showing in other studies with neurological toxicity, gastrointestinal toxicity, hepato toxicity and developmental toxicity (Strähle *et al.*, 2012/04/01).

In the human genome 71,4% of the genes that are protein coding have at least one ortholog in the zebrafish genome. Furthermore, in the zebrafish genome 69% of the protein coding genes have at least one ortholog in the human genome. When it comes to the orthologs in the human genome 41% of the genes have a one-to-one relationship with the zebrafish ortholog (Howe *et al.*, 2013). This means that zebrafish have similar proteins and enzymes to humans. When it comes to neurotransmitters zebrafish have high genetic similarity to humans in the way cholinergic transmission works, and the distribution of cholinergic cells (Verma *et al.*, 2022/01/01). Similarities can also be found for the neutrasnmitters adrenaline and cortisol. Furthermore, adrenaline and cortisol are released from the adrenal gland in humans and interrenal gland in zebrafish. Moreover, cortisol and adrenaline are released during stressful situations in zebrafish meaning zebrafish have a mechanism for coping with stress with is similar to the one that humans do. Additionally, adrenaline and cortisol have similar effects in zebrafish as in humans (Löhr *et al.*, 2011/03/17). Other neurotransmitters in zebrafish are also similar to neurotransmitters in humans (Verma *et al.*, 2022/01/01). Additionally, because of this similarity studies that uses zebrafish will have good indication of the outcome in humans. Something to take in mind is the zebrafishes high adaptability and regeneration capacity. Mammals for example humans have a limited regeneration capacity, where humans can regenerate skin, liver and (in a short time window after birth) the heart. Zebrafish or other vertebrae are different and have a high regeneration capacity. Further the zebrafish can regenerate many organs and tissues (Marques, Lupi and Mercader, 2019) . This high regeneration capacity and high adaptability could make the result in this thesis not accurate to the effect PFOA and PFHxS would have been in humans. Humans excrete adrenaline and cortisol in nearly the same way as zebrafish. Additionally, the excretion control is done by the hypothalamus in both zebrafish and humans. Moreover, acetylcholine is synthesized by the same enzyme ChAT in zebrafish and humans. This will mean that the results in this thesis could potentially not be far from the effect that PFOA and PFHxS would have had in humans.

5.2 Acetylcholine levels

The controls (no exposure) of both zebrafish embryo 48 hpf and zebrafish larvae 96 hpf shows a decrease in acetylcholine levels from 48 hpf to 96 hpf. this is similar to the trend that Tufi, *et al.* (2016) found when acetylcholine among other neurotransmitter and precursors were quantified in zebrafish ranging from 0-7 dpf (days past fertilization) or 0-168 hpf. The changes in acetylcholine level in the control exposure from 2075 ng/g in 48 hpf to 1740 ng/g 96 hpf (Figure 13A and B) is higher than Tufi, *et al.* (2016) changes from 3,0 pg/embryo 48 hpf to 2,9 pg/embryo 96 hpf. The change in Tufi *et al.* (2016) is 0,1 pg/embryo where the change in this thesis was 335 ng/g. Moreover, the study of Santos-Fandilia *et al.* (2015) shows a change from 48 hpf about 1 ng/g to 96 hpf about 0,9 ng/g. The change shown in Santos-Fandilia K *et al,* (2015) `s study as well is lower than here. This likely means that the change from 48 hpf to 96 hpf was too high. Furthermore, this could have occurred due to one of the control replicates from 96 hpf being mostly lost during sample preparation, because of a vial breakage during the homogenization stage of the sample prep. Moreover, this caused the replicate that had missing sample to have an acetylcholine level of 1950 ng/g against the other replicate that had acetylcholine level of 1530 ng/g. Additionally, this led to a high SE of ±209,9 ng/g. This however does not change the overall results for the acetylcholine levels of 96 hpf.

Carbamazepine had a clear decremental effect on the acetylcholine level in 48 hpf zebrafish embryo and 96 hpf zebrafish larvae. The acetylcholine level after carbamazepine exposure was 1545 ng/g in 48 hpf and in 96 hpf the acetylcholine level was 1165 ng/g . This signifies that the zebrafish could not recover the acetylcholine level between 48 hpf and 96 hpf. This is as expected since it is a mood stabiliser and is expected to have a negative effect or stabilizing effect on the neurotransmitters.

The effect of hydrocortisone on the acetylcholine levels in zebrafish embryo 48 hpf and zebrafish 96 hpf was decremental. The acetylcholine levels after hydrocortisone exposure was 1140 ng/g in 48 hpf , in 96 hpf hydrocortisones acetylcholine level was 1395ng/g. This is a slight recovery from 48 hpf to 96 hpf. Because of the high SE of the control and hydrocortisone in 96hpf the recovery can be higher than data (Table L2) shows.

Based on the results for the acetylcholine levels in zebrafish embryo 48 hpf and zebrafish larvae 96 hpf (Figure 13), PFOA 1x exposure had a decremental effect on the acetylcholine levels in 48

hpf zebrafish embryo. In 96 hpf zebrafish larvae acetylcholine level after PFOA 1x and PFOA 1000x exposure was similar compared to the acetylcholine level of the control of 1740 ng/g, which means that the zebrafish may have had recovered the acetylcholine level at 96 hpf.

The accuracy of the recovery is not great because of the lost sample. Furthermore, the PFOA 1000x exposure at 48 hpf had a high SE of ±182,5 ng/g. These results could be the result of the following: one of the replicates lost some zebrafish tissue when weighing before sample prep. Or some of the zebrafish died before getting deposited in liquid nitrogen after weighing, when the zebrafish was sampled. Dead zebrafish will have less neurotransmitters than live zebrafish. There is also the random variance between neurotransmitter levels in one zebrafish to another, including very slight variance of how far in the development the zebrafish are (breeding timespan 30 min to an hour). However, due to most of the SE being low, the effect of different growth is most likely not high enough to elicit much concern.

PFHxS 1x and 1000x had no significant effect on the acetylcholine levels at 48 and 96 hpf compared to the control. .

The acetylcholine levels after PFHxS 1000x exposure was 1815 ng/g at 48 hpf and at 96 hpf the acetylcholine levels were 1410 ng/g. Moreover, this signalises that PFHxS 1000x has a slight decremental effect on the acetylcholine levels in zebrafish embryo 48 hpf that the zebrafish could not recover from. This decremental effect is not as strong in the PFOA 1000x exposure at 48hpf.

5.3 Cortisol levels

The results for cortisol 48 hpf (Figure 14 A and B) and 96 hpf (Figure 15) shows a 15-fold increase of cortisol from 48 hpf to 96 hpf in control (2.11 ng/g and 32,6 ng/g , respectively). Most of the samples do have an increase in cortisol level from 48 hpf to 96 hpf. In Eto *et al.* (2014) study, the cortisol control had a significant increase from about 50 pg/g (48 hpf) to 650 pg/g (97 hpf).This is about a 13-fold change in cortisol level. This makes 15-fold change of cortisol level between 48 hpf and 96 hpf in this thesis possible. Further, Alsop and Vijayan (2008) found a 27-fold increase in cortisol level from 0,07 pg/µg protein in 49 hpf zebrafish embryo to 1,87 pg/ µg protein 146 hpf zebrafish larvae. This was assessed after using a colorimetric essay to measure cortisol levels with 25 zebrafish for each replicate. Further the change from 0,07 pg/µg protein in 49 hpf to about 0.7 pg/µg protein in 96 hpf makes a 10-fold increase in cortisol form 48 hpf to 96 hpf. The cortisol

level at 96 hpf in Alsop and Vijayan (2008) had a high SE of about ±0,3 pg/ µg protein. That means that normal fold change around 96 hpf zebrafish larvae falls around 10-13,5 based on the studys of Eto *et al.* (2016) and Alsop and Vijayan (2008).

The hydrocortisone exposure had decrease in cortisol level from 48 hpf and 96 hpf. The hydrocortisone exposures cortisol level from 48 hpf was 1610 ng/g when the control of 48 hpf was 2,1 ng/g. This gives the hydrocortisone exposure a fold change of 761.2 and a SE of ±140 ng/g. Hence hydrocortisone got its own graph (Figure 14B). The reason for this high level of cortisol at 48 hpf could be that the water was not removed from the zebrafish samples of 48 hpf zebrafish embryo before weighing and sample preparation (section 5.1). Another reason could be that zebrafish does not use cortisol before later in the development and therefore could have an excess of cortisol absorbed in the embryo. In the hydrocortisone exposure of 96 hpf zebrafish larvae the cortisol level is decreased to 128, 6 ng/g with a SE of 31.35 ng/g. This shows a significant reduction from the previous 1610 ng/g cortisol level of 48hpf.

Cortisol level in the carbamazepine exposure in 48 hpf was 10,6 ng/g in the 96 hpf the cortisol level was 14,6 ng/g. This is an increase of cortisol level form 48 hpf to 96 hpf. Furthermore, when the cortisol level in the carbamazepine exposure (48 hpf) was compared to the control (48 hpf) was found to have an incremental effect (5,02 fold change). Furthermore, the 96 hpf carbamazepine was found to have a decremental effect (0,448 fold change) when compared to the control of 96 hpf. The cortisol level in the carbamazepine exposure of 48hpf had a SE of ± 5,79 ng/g. Additionally this means that the cortisol level of the carbamazepine exposure of 48 hpf has a high SE. Furthermore, carbamazepine most likely has an incremental effect on the cortisol level in 48 hpf zebrafish larvae. But the incremental effect will not be as strong as the data implies.

PFOA 1x exposures effect on cortisol levels in 48 hpf zebrafish embryo is first incremental with a cortisol level of 3,760 ng/g and then decremental in 96 hpf zebrafish larvae with a cortisol level of 16,93 ng/g. This is also uncertain because of the control exposures high SE. The cortisol levels of PFOA 1x exposure of 48 and 96 hpf zebrafish embryo and larvae is most likely accurate. Furthermore, what is likely is that PFOA 1x exposure had an incremental effect (1,77 fold change) on zebrafish cortisol levels at 48 hpf. And at 96 hpf PFOA 1x had a decremental effect (0,519 fold change) on cortisol levels in 48 hpf zebrafish embryos. It is possible that PFOA 1x exposed zebrafish embryo 48 hpf was slightly stressed during sampling when compared to the control

exposed zebrafish at 48 hpf. Which caused the slight incremental effect on the cortisol levels. PFOA 1x exposure likely had an incremental effect on cortisol level in 48 hpf zebrafish embryo, then a decremental effect in 96 hpf zebrafish larvae.

PFOA 1, including PFHxS 1x and 1000 had a slight incremental effect on the cortisol level in 48 hpf zebrafish embryo, albeit not significant. In the 96 hpf zebrafish larvae the cortisol level was slightly decreased after exposure to these chemicals, but not significantly. Although studies in zebrafish embryos and larvae, are not available, laboratory fish exposures to PFOA in juvenile salmon have led to decreased plasma cortisol concentrations (Lemos *et al.*, 2023-06-27).

5.4 Adrenaline levels

There is a decrease in adrenaline levels in control from 48 hpf to 96 hpf by 15,6 ng/g. When adrenaline level trend in this thesis is compared to Tufi, *et al.* (2016) findings in their study show that epinephrine (adrenaline) levels slightly decreased from 37,4 pg/embryo in 48 hpf to 37,2 ng/embryo in 96 hpf.Another study by Santos-Fandila, *et al.* (2015) that found that epinephrine (adrenaline) slightly increased from 48 hpf to 96 hpf. Moreover, the increase that Santos-Fandila, *et al.* (2015) found was not more than 0,5 ng/embryo. Further the SE of the means of the 48 hpf and 96 hpf of Santos-Fandila *et al.* (2015) were high with a SE of ± about 0,5 ng/embryo. This aligns with the high SE found in the 48 hpf control in this thesis. Furthermore, Both of Tufi *et al* (2016) and Santos-Fandila *et al.* (2015) show either slight increase or slight decrease in adrenaline level. This means that the adrenaline level change between the controls is too high.

Neither of the exposures seem to have had a significant effect on the adrenaline levels of zebrafish embryos and larvae compared to control.

The PFOA 1000x exposure of 96 hpf zebrafish larvae had adrenaline level of 31,3 ng/g and a high SE of ±15,45 ng/g. This indicates an incremental effect of PFOA 1000x exposure. The reason why the SE of the PFOA 1000x is very high is because one of the replicates had a sample vial break in the homogenizer during sample preparation.

6. Conclusion

All zebrafish neurotransmitter levels were detected and quantified in all exposure samples, but the results of the exposures need to be carefully interpreted due to small sample sizes. PFOA was able to induce negative effects on acetylcholine levels of 48 hpf zebrafish embryo both in 1x concentration and 1000 x concentration. The negative effects from 1x and 1000x concentration were both recovered in 96 hpf zebrafish larvae. The effect on PFOA on cortisol levels of 48 hpf zebrafish was positive in PFOA 1x. The effect on cortisol levels turned negative in 96 hpf zebrafish. The cortisol level was not altered after an exposure to PFOA 1000x at 48 hpf, while the effect of the PFOA 1000x exposure on 96 hpf zebrafish larvae was negative. Adrenaline levels were not affected by the exposures. PFOA 1x exposure at 48 hpf in zebrafish larvae. Recovery of adrenaline levels happened in 96 hpf zebrafish larvae after PFOA 1x exposure. After exposure to PFOA 1000x adrenaline levels were affected negatively in zebrafish embryo 48 hpf and in zebrafish larvae 96 hpf.

PFHxS had no effect on acetylcholine levels in zebrafish embryo at low concentrations(1x). PFHxS had a slight decremental effect on zebrafish larvae. PFHxS had a slight decremental effect on acetylcholine levels in zebrafish in high concentrations(1000x). The effect of PFHxS on the cortisol levels of zebrafish is incremental in 48 hpf in zebrafish, then decremental at 96 hpf at any concentration. Low concentrations of PFHxS had no effect on adrenaline levels in zebrafish. At high concentrations PFHxS has a decremental effect on adrenaline levels in zebrafish.

7. Future considerations

For future considerations and analyses, using a better technique to remove water from 48 hpf zebrafish embryos before weighing for use in sample preparation would be recommended, where as little as possible zebrafish tissue would not be lost. This would lead to a much better accuracy when measuring neurotransmitter levels. More replicates of all the samples from the zebrafish embryo 48 hpf and larvae 96 hpf would allow for making better conclusions about the data and increasing the accuracy of the results. However, there was no time to redoing the analysis of some of the samples in the lab and this would have helped for the results as well.

The possibility to decrease the number of embryos per sample to a lower amount is possible. Furthermore, this will a have chance of increasing SE`s since the amount is lower than before. Further this what found when the number of zebrafish per sample was compared between studies. Considering the low amount of neurotransmitters in a zebrafish, lowering the amount of embryos per sample would also increase the chance of not detecting the the neutransmitters.

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List of equipment

Table E1: List of instruments and other equipment.

Type of equipment	Company	Country	City
Allegra X-12 R centrifuge	Beckman Coulter	USA	Brea
BioRender	BioRender	Canada	Toronto
Chemdraw v.22.2.0	PerkinElmer	USA	Waltham
Cryotubes	VWR international	USA	Radnor
GraphPad Prism v10.4.1	GraphPad Software	USA	La Jolla
Heraceus Fresco 21 centrifuge	Thermo Fisher Scientific	USA	Waltham
Heraceus Pico 21 centrifuge	Thermo Fisher Scientific	USA	Waltham
2 ml PP Homogenization tubes	VWR international	USA	Radnor
HPLC vials with inserts	Agilent	USA	Santa Clara
Balance RSX304 (Weight)	Mettler Toledo	USA	Columbus
Petri dish	Heger	Norway	Rjukan

15ml PP centrifuge tubes	VWR international	USA	Radnor
Precellys 24 evolution homogenizer	Bertin Technologies	France	Montigny-le-Bretonneux
Reacti-Vap Evaporator	Thermo Fisher Scientific	USA	Waltham
Spin-X centrifuge filter tubes	Costar	USA	Washington D.C
Synergy-Fusion RPLC column with 100x2.1mm, 2,5µm particles	Phenomenex	USA	California
Vortex genie 2	Thermo Fisher Scientific	USA	Waltham

List of chemicals

List of chemicals.

Type of chemical	Quality	Producer	Country	City	Product number	CAS number
Acetylcholine chloride	≥99% purity	Merck-Sigma-Aldrich	USA	Burlington	A2261	60-31-1
Acetylcholine-D_4 Chloride	95% purity (HPLC)	LGC/TRC	Germany	Luckenwalde	A172063	344298-94-8
Acetonitrile (ACN)	≥99,95% Maximum 0,003% H_2O UPLC quality	VWR chemicals	USA	Radnor	83639.320	75-05-8
Epinephrine (Adrenaline)	≥99% purity (HPLC)	Merck- sigma	USA	Burlington	E4250	51-43-4
Epinephrine-D_6 (Adrenaline-D_6)	100 µg/mL in methanol with 5% 1M HCl	Merck-Sigma-Aldrich/Cerillant	USA	Burlington	E-077	1219803-77-6
L-ascorbic acid($C_6H_8O_6$)	99% Quality level 200	Merck-Sigma-Aldrich	USA	Burlington	A92902	50-81-7
Cortisol	≥98% (HPLC)	Merck-Sigma-Aldrich	USA	Burlington	H4001	50-23-7

Cortisol-$^{13}C_3$	100µg/mL in methanol Quality level 300	Merck-Sigma-Aldrich/Cerillant	USA	Burlington	C216	1996649-39-8
Formic acid (HCOOH)	≥95% reagent grade quality level 100	Merck-Sigma-Aldrich	USA	Burlington	F0507	64-18-6
Hydrochloric acid (HCL)	37%	VWR chemicals	USA	Radnor	20252.335	7647-01-0
Liquid N_2		LINDE GAS	Ireland	Dublin		
Methanol (MeOH)	≥99.9% HPLC quality	VWR chemicals	USA	Radnor	85800.320	67-56-1

List of stock solutions. Dilution solution is 10% methanol/ water (w/w) with 0,1% formic acid and 0,05 ascorbic acid.

Standard	Producer	Prod nr.	Stock solution	Dilution
Epinephrine (Adrenaline)	Merck- Sigma-Aldrich	A2261	1 mg/mL I MeOH 5% 1M HCl	10 ng/mL in dilution solution
Epinephrine-D_6 (Adrenaline-D_6)	Merck-Sigma-Aldrich/Cerillant	A172063	100 µg/mL I MeOH 5% 1M HCl	10 ng/mL in dilution solution
Acetylcholine chloride	Merck- Sigma-Aldrich	E4250	1 mg/mL I MeOH 5% 1M HCl	100 ng/mL in dilution solution
Acetylcholine-D_4 Chloride	LGC/TRC	E-077	1 mg/mL I MeOH 5% 1M HCl	100 ng/mL in dilution solution
Cortisol	Merck- Sigma-Aldrich	H4001	1 mg/mL I MeOH	1 ng/mL in dilution solution
Cortisol-$^{13}C_3$	Merck- Sigma-Aldrich/ Cerillant	C216	100 µg/mL I MeOH 5% 1M HCl	1 ng/mL in dilution solution

List of tables / figures

Table L1: Neurotransmitter levels in 48 hpf zebrafish embryo and 96 hpf larvae after exposure to control (no exposure), carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x, PFHxS 1x, PFHxS 1000x . There was in total of 7 different exposures with 2 replicates each. Except for PFOA 1x which had n=3 replicates. Number of embryos in each sample was n=20. *One of the control samples was lost in homogenizer. ** One of the PFOA 1000x sample vial broke in the homogenizer.

HPF (hours past fertilization)	Sample ID	Exposure type	Acetylcholine (ng/g)	Cortisol (ng/g)	Adrenaline(ng/g)
48	C1	Control 1	2070	1,12	27,9
48	C2	Control 2	2080	3,11	48,3
48	B1	Carabamazepine 1	1400	4,82	34,9
48	B2	Carbamazepine 2	1690	16,4	34,7
48	H1	Hydrocortisone 1	1040	1750	33,8
48	H2	Hydrocortisone 2	1240	1470	33,7
48	PF 1.1	PFOA 1x 1	1850	3,79	43,2
48	PF 1.2	PFOA 1x 2	1810	3,73	42
48	PF 0.1	PFOA 1000x 1	675	1,91	34,8
48	PF 0.2	PFOA 1000x 2	1040	1,92	34,2
48	PX1.1	PFHxS 1x 1	2230	8,87	37,4
48	PX 1.2	PFHxS 1x 2	2110	7,64	37,3
48	PX 0.1	PFHxS 1000x 1	1900	3,09	29,8
48	PX 0.2	PFHxS 1000x 2	1730	3,03	30,1
96	C1*	Control 1*	1950	56	23,6
96	C2	Control 2	1530	9,25	21,4
96	B1	Carabamazepine 1	1080	13,2	25,1
96	B2	Carbamazepine 2	1250	16	25,7

96	H1	Hydrocortisone 1	1510	97,3	17,4
96	H2	Hydrocortisone 2	1280	160	16,2
96	PF 1.1	PFOA 1x 1	1960	17,3	22,9
96	PF 1.2	PFOA 1x 2	1990	16,9	26,1
96	PF 1.3	PFOA 1x 3	1550	16,6	16,4
96	PF 0.1**	PFOA 1000x 1**	2200	12,6	46,7
96	PF 0.2	PFOA 1000x 2	1610	12,5	15,8
96	PX 1.1	PFHxS 1x 1	1590	11,9	23,1
96	PX 1.2	PFHxS 1x 2	1600	13,1	22,2
96	PX 0.1	PFHxS 1000x 1	1420	10,5	15,2
96	PX 0.2	PFHxS 1000x 2	1400	8,82	14,8

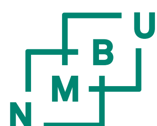
Table L2: Descriptive statistical information for neurotransmitter (acetylcholine, cortisol and arenaline) levels in 48 hpf zebrafish embryo and 96 hpf larvae. Number of replicates is n=2 for control, carbamazepine, hydrocortisone, PFOA 1x, PFOA 1000x exposure of 48 hpf embryo, PFHxS 1x and PFHxS 1000x. Number of replicates for each sample was 2. Except for PFOA 1x exposure of 96 hpf zebrafish larvae, number of replicates was n=3. Number of larvae of each sample is n=20. *One of the control samples was lost in homogenizer. ** One of the PFOA 1000x sample vial broke in the homogenizer.

Hours past fertlization (Hpf)	Neurotransmitter	Expsure group	Mean(ng/g)	SD(standarddeviation)	SEM(SE)	Fold change	Fold change error
48	Acetylcholine	Control	2075	7,07	5	1,0	0
48	Acetylcholine	Carbamazepine	1545	205	145	0,745	0,0699
48	Acetylcholine	Hydro-cortisone	1140	141	100	0,549	0,0482
48	Acetylcholine	PFOA 1x	1830	28,3	20	0,882	0,00964
48	Acetylcholine	PFOA 1000x	858	258	183	0,413	0,0880
48	Acetylcholine	PFHxS 1x	2170	84,9	60	1,046	0,0289
48	Acetylcholine	PFHxS 1000x	1815	120	85	0,875	0,0410
48	Cortisol	Control	2,12	1,41	0,995	1	0
48	Cortisol	Carbamazepine	10,6	8,19	5,79	5,02	2,74

48	Cortisol	Hydro-cortisone	1610	198	140	761	66,2
48	Cortisol	PFOA 1x	3,760	0,0424	0,0300	1,78	0,0142
48	Cortisol	PFOA 1000x	1,92	0,0071	0,005	0,905	0,0142
48	Cortisol	PFHxS 1x	8,26	0,8697	0,615	3,90	0,00236
48	Cortisol	PFHxS 1000x	3,060	0,0424	0,03	1,45	0,291
48	Adrenaline	Control	38,10	14,4	10,2	1	0
48	Adrenaline	Carbamazepine	34,80	0,141	0,100	0,913	0,00262
48	Adrenaline	Hydro-cortisone	33,8	0,0707	0,050	0,886	0,00131
48	Adrenaline	PFOA 1x	42,60	0,849	0,600	1,118	0,0157
48	Adrenaline	PFOA 1000x	34,50	0,424	0,300	0,906	0,00787
48	Adrenaline	PFHxS 1x	37,4	0,071	0,050	0,980	0,00131
48	Adrenaline	PFHxS 1000x	30	0,212	0,150	0,786	0,00394
96	Acetylcholine	Control*	1740	297	210	1	0
96	Acetylcholine	Carbamazepine	1165	120	85	0,670	0,0489

96	Acetylcholine	Hydro-cortisone	1395	163	115	0,802	0,0661
96	Acetylcholine	PFOA 1x	1833	246	142	1,054	0,0816
96	Acetylcholine	PFOA 1000x**	1905	417	295	1,095	0,170
96	Acetylcholine	PFHxS 1x	1595	7,07	5	0,917	0,00287
96	Acetylcholine	PFHxS 1000x	1410	14,1	10	0,810	0,00575
96	Cortisol	Control*	32,63	33,1	23,4	1	0
96	Cortisol	Carbamazepine	14,60	1,980	1,40	0,448	0,0429
96	Cortisol	Hydro-cortisone	128,7	44,3	31,4	3,943	0,961
96	Cortisol	PFOA 1x	16,93	0,351	0,203	0,519	0,00761
96	Cortisol	PFOA 1000x**	12,6	0,0707	0,0500	0,385	0,00153
96	Cortisol	PFHxS 1x	12,50	0,849	0,600	0,383	0,0184
96	Cortisol	PFHxS 1000x	9,660	1,20	0,850	0,296	0,0261
96	Adrenaline	Control*	22,50	1,56	1,100	1	0
96	Adrenaline	Carbamazepine	25,40	0,424	0,300	1,13	0,0133

96	Adrenaline	Hydro-cortisone	16,80	0,849	0,600	0,747	0,0267
96	Adrenaline	PFOA 1x	21,80	4,94	2,85	0,969	0,155
96	Adrenaline	PFOA 1000x**	31,3	21,8	15,4500	1,39	0,687
96	Adrenaline	PFHxS 1x	22,7	0,636	0,450	1,01	0,0200
96	Adrenaline	PFHxS 1000x	15,00	0,283	0,200	0,667	0,00889



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