Master's Thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Neuro-transcriptomics and functional characterization of biological processes in female Atlantic Cod (*Gadus morhua*) after exposure to Polycyclic Aromatic Hydrocarbons and Perfluoroalkyl Substances.

Master's thesis in Biology (Cell and Molecular Biology)

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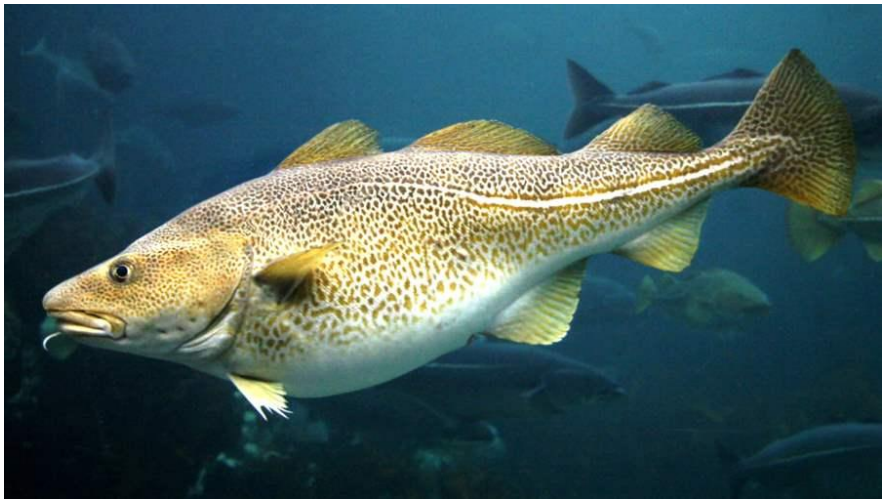

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1 Acknowledgement

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2 Abbreviations

PAH - Polycyclic aromatic hydrocarbon

PFAS - Per-fluoro alkyl substance

PFOS - Perfluorooctanesulfonate

PFTrA - Perfluorotetradecanoic acid

PFNA - Perfluorononanoic acid

PFOA - Perfluorooctanoic acid

PFAAs - Perfluoroalkyl acids

PFCs - Perfluorinated compounds

DEGs - Differentially expressed genes

GnRH - Gonadotropin-releasing hormone

PW - Produced water

POPs - Persistent Organic Pollutants

DDT - Dichlorodiphenyltrichloroethane

PCBs - Polychlorinated biphenyls

PFCs - Perfluoroalkyl chemicals

HMW PAHs - High molecular weight polycyclic aromatic hydrocarbon

LMW PAHs - Low molecular weight polycyclic aromatic hydrocarbon

ECM - Extra cellular matrix

DAVID - Database for Annotation, Visualization, and Integrated Discover

PBS - Phosphate buffered saline

MCODE - Molecular Complex Detection

CCO - Cellular component ontology

PPI - Protein-protein interaction

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3 Abstract

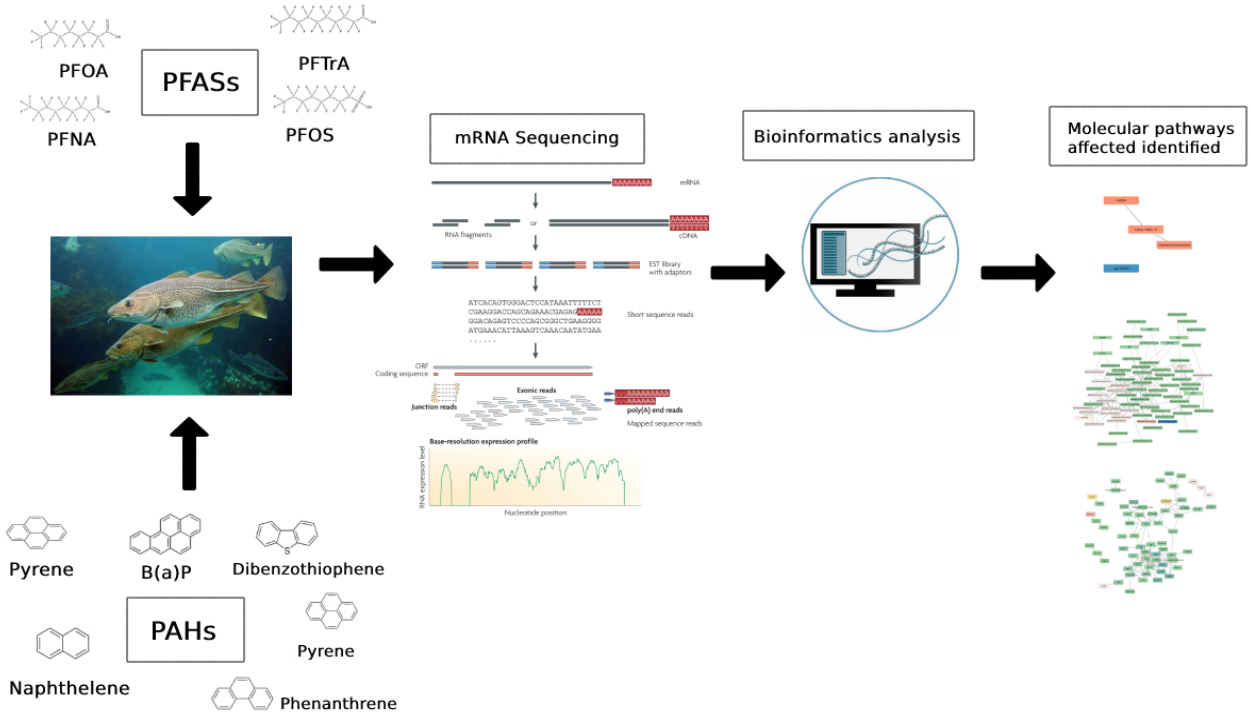


Figure 3.1: The graphical abstract which summarizes the contents of the article in a concise and pictorial form

The marine environment is under constant pollution pressure mainly due to anthropogenic activities in the sea and on land. Polycyclic aromatic hydrocarbons (PAHs) and Perfluoroalkyl substances (PFASs) are among the most common xenobiotics found in the aquatic environment and represent a significant threat to aquatic and terrestrial organisms. Recent studies have shown that PAHs and PFASs produce harmful effects on fish species, but a few studies have been conducted to explore genes and pathways affected with the use of high throughput transcriptomics approach. Like in other vertebrates, the fish brain is actively involved in stress response and is influenced by external factors. In the present study, the molecular changes (transcriptome profiling) in the brain of juvenile Atlantic cod after exposure to two different concentrations of PAHs and PFASs have been investigated. Both the PAHs and PFASs exposure groups contained different congeners. Low and high-dose PAHs exposure produced 131 and 5 deferentially expressed genes (DEGs), respectively. For the PFASs, a similar pattern with a large number of DEGs in the low-dose exposure group (94 DEGs), compared to a high dose with only one (1) DEG, was observed. Functional characterization of DEGs revealed pathways belonging to signal transduction such as neuron signalling, transmembrane signalling, biological adhesions and calcium signalling which were significantly affected in the brain of fish after PAHs and PFASs exposure. The GnRH signalling pathway was also enriched in both low PAH and PFASs exposure groups. Some enriched terms related to oxidative stress such as respiratory chain, sodium and electron transport, and oxidative phosphorylation were only affected in low PFASs exposure. High PAHs exposure increased the expression of genes functionally involved in

DNA -binding and DNA packaging. On the other hand, DEG belonging to high PFASs exposure were involved in signal transduction and chemical response. The findings of the study suggest that exposure of fish to an environmentally relevant concentration of PAHs and PFASs modulates brain cellular molecules to trigger the signalling cascades in the brain which activates the stress response that can ultimately have adverse effects on fish at the systems level. However, the high exposure doses may have caused exhaustion in stress response leading to a finite number of the differentially expressed gene when compared to low exposure groups. These effects need to be investigated further.

4 Introduction

Water is widely regarded as the most essential of natural resources. However, the freshwater systems are directly threatened by anthropogenic activities [1]. The usage of water resource for economic productivity is always accompanied by destruction to ecosystems and biodiversity, with potentially serious costs[1]. Acute spills of chemicals have been stable at 100 - 150 incidents per year on the Norwegian Continental Shelf (NCS) over the past decade along with chronic chemical spills in 2007, 2009 and 2010[2]. Globally, it appears that over 80% of wastewater is released to the environment with inadequate treatment[3]. The produced water (PW) discharges are of major concern due to its high discharge volumes, the complex content of hazardous chemicals, and the lack of information on their possible long term impact on ecosystem[4]. Polycyclic aromatic hydrocarbons (PAHs), and related heterocyclic aromatic compounds are considered major toxicants present in produced water[5],[6]. Water systems are transformed through urbanization, industrialization, changes in widespread land cover, and engineering schemes like reservoirs, irrigation and inter-basin transfers that maximize human access to water[1]. A major percent of Earth's accessible renewable freshwater (one-third) is used for agricultural, industrial, and domestic purposes which lead to water contamination with diverse synthetic and non-synthetic chemicals[7]. The chemical pollution of natural water has become a major public concern in almost all parts of the world[8].

The persistent Organic Pollutants (POPs) have been and continue to be a major environmental concern[8]. These pollutants includes diverse set of high-volume production compounds and their by-products which are formed from different combustion processes[8]. The most significant POPs primarily encompass highly chlorinated compounds [e.g., dichlorodiphenyltrichloroethane (DDT), PCBs and polychlorinated dioxins] and polycyclic aromatic hydrocarbons (PAHs). Due to their special properties and use in variety of industrial applications, the perfluoroalkyl chemicals (PFCs) are considered one of the emerging POPs[9] [10]. The majority of PFAS are highly persistent[11], and this has been seen as basis for managing them as a chemical class[12]. The persistence of xenobiotic compounds are usually considered as a less hazardous property when compared to the toxicity. However, persistence is the major factor that lets problems related to pollution become adverse[13]. This is because persistence enables chemicals to spread over large distances, causing long-term and even life-long exposure, and leads to increasing levels in the environment with high probability to lead to adverse effects[14].

4.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) consist of diverse group of hydrophobic organic compounds, which are found in freshwater and marine environments[15],[16],[17]. PAHs are ubiquitous environmental contaminants. Polycyclic aromatic hydrocarbons are mostly colourless, white, or pale-yellow solid compounds, which are composed of two or more fused aromatic rings of carbon and hydrogen atoms [18],[19]. The low molecular weight (LMW) PAHs includes, two or three fused rings, whereas the high molecular weight (HMW) PAHs includes four or more fused rings[20]. PAHs with increased molecular weight are more recalcitrant compared to the low molecular weight PAHs because of their decreased water solubility and increased lipophilicity[20][21]. Each additional ring added to PAHs decreases the aqueous solubility [22]. The persistence of PAHs in environmental matrices are dependent on different factors such as their chemical structure, environmental conditions,the bioavailability, and the concentration and dispersion of the PAHs[23]. There are four types of PAHs in the aquatic environment which includes the PAHs derived from fuels (petrogenic), derived from

incomplete combustion (pyrogenic), generated by organic metabolism (biogenic), and generated by the transformation process in sediment (diagenetic)[24]. The major contributors of environmental PAH pollution in aquatic ecosystems are the PAHs from petrogenic and pyrogenic sources which are mainly anthropogenic[18][25]. The oil spill accidents are among the most concerning exposure events regarding the PAHs pollution in aquatic environments[26]. The highest acceptance concentration for benzo(a)pyrene by WHO is 0.7 g/L, however the concentration of PAHs varies between 1 ng/L and 11 g/L in the drinking water[27].

The United States Environmental Protection Agency (US EPA) has designated 16 PAH compounds as priority pollutants [28], including the HMW PAHs (benzo(a)pyrene and dibenz(ah)anthracene among others) and the LMW PAHs (naphthalene, fluorene and phenanthrene among others), which are often monitored for measurement in environmental samples[20]. In this study, six different isomers of PAHs were included amongst which five are listed as priority pollutants by US EPA (Table 4.1).

Table 4.1: Properties of US EPA priority PAHs[20] which are included in the exposure group. Log Kow octanol – water partition coefficient, ND - not determined.

PAH	**Molecular weight**	**Melting point (°C)**	**Water solubility(mg/l)**	**Vapour pressure (mmHg)**	**Log Kow**
Naphthalene	128.2	79–82	320	ND	3.5
Fluorene	166.2	115–116	1.85	5.0×106	4.28
Phenanthrene	178.2	99	1.24	6.8×104	5.62
Pyrene	202.3	156	0.14	2.5×106	4.47
Benzo(a)pyrene	252.3	179	0.0038	5.6×109	5.74

The PAH pollution, affects the health of humans and other organisms across the planet, either directly or indirectly[29]. The pollution with PAH have been determined to be highly toxic, carcinogenic, mutagenic, immunotoxicogenic, and teratogenic to various organisms[30]. The PAHs pollution have also found to cause ecotoxic effects on aquatic life and birds[18]. Most of the aquatic vertebrates including fish can convert PAHs into water-soluble derivatives with their well-developed biotransformation systems[31]. However, these convertions can cause carcinogenesis, developmental effects, immunosuppression, mutagenesis, and reproductive effects by the production of electrophilic reactive metabolites which are toxic[32]. The reactive metabolites of some of the PAHs can often generate toxic effect as they have high potential to bind to cellular proteins and DNA[20]. An imbalance between the production of pro-oxidant compounds and cellular antioxidants can also be generated by the biotransformation of PAHs leading to oxidative stress[33]. The PAHs can also affect the fish growth by acting as an agonist to aryl hydrocarbon receptor (AhR)[34] and may form DNA adducts and neoplasia in fish liver through metabolic intermediates[35]. The ecotoxicological issues generated from exposure to PAHs has been investigated in several research papers which suggest that the PAHs may cause oxidative stress[36], cardiac function defects [37], DNA damage[38], or embryotoxicity[39]. The PAHs have been ranked on the U.S. Agency for Toxic Substances and Disease Registry priority list, which suggests that the PAHs pose risk to environmental integrity and human health[40].

4.2 Perfluoroalkyl substances (PFASs)

Perfluoroalkyl substances (PFASs) are organic compounds which are synthetic, and their properties (chemical and thermal) have led to extensive use in consumer products[41]. The PFASs have high economic importance as they are used in several commercial, residential, and industrial applications[42]. They have application in aqueous film-forming foams (AFFFs), textile, surfactants in fluoropolymer production, metal plating, paper, and household products[42]. The PFASs are persistent, mobile, and bio accumulative[43]. The PFASs are continuously emitted into the environment from sources such as industrial and municipal sewage treatment plants (point source), and atmospheric depositions (non-point source)[44]. Depending on the water usage in the community connected to the sewage treatment plant, the discharge of PFASs into the aquatic environment ranged between 10g d1 and 10000g d1[42]. The concentrations of single PFASs are considered low to cause adverse effects, however their mixtures are of significant environmental concern[42] . In this study, four different types of PFASs are included for elucidating the mixture effect, among which the PFOS is the most significant one which can cause acute and chronic effects at the individual, population, and community levels[42]. PFOS are very persistent and has a high bioaccumulation potential[42]. PFASs are widely detected in humans and animals, indicating that several of these substances are bioavailable[45][44].

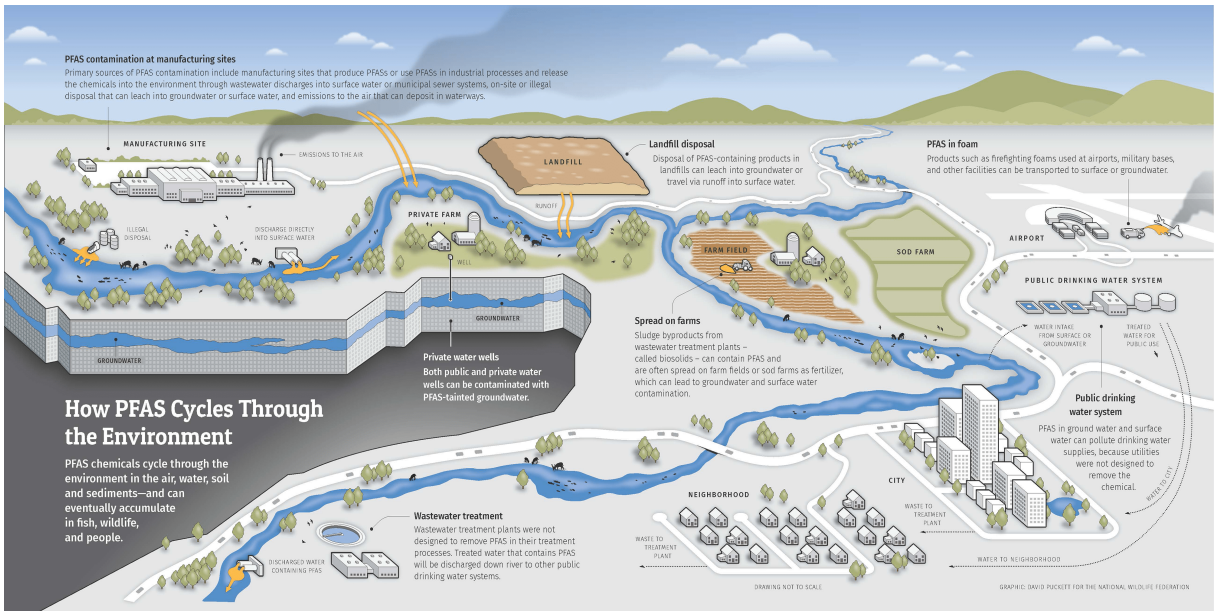


Figure 4.1: The National Wildlife Federation report, The Science and Policy of PFASs in the Great Lakes Region: A Road for Local, State and Federal Action, details the sources of PFASs in the Great Lakes[48].

The perfluoroalkyl substances (PFASs) are present in the environment, majorly in aquatic ecosystems, and continues to be a significant concern for environmental and human health. The PFOA and PFOS have biological half-lives on the order of year and are most frequently detected in human blood serum[46]. The exposure to PFAS has been associated with metabolic dysfunction and other numerous adverse outcomes in humans[47]. A study which considered both PFASs effects on liver biomarkers and histological data from rodent experimental studies have concluded that the PFOA and PFNA are hepatotoxic to humans[48]. The PFAS affects the functioning of hormones such as thyroid hormone and also affect functions of androgen, estrogen and aryl hydrocarbon receptor being potential endocrine disruptors in humans[49]. The exposure to PFAAs cause harmful effects in fish such as developmental toxicity, metabolic disruption, oxidative stress and reproductive toxicity[50]. A laboratory study of the aquatic toxicity of PFCs on zebrafish embryos showed that PFOS could cause abnormalities of larvae[51]. Another recent report also indicated that exposure of maternal zebrafish to low concentrations of PFOS could result in mortality and deformity of the offspring[52]. The study conducted by Megner.F et.al. on zebra fish found that the PFASs can also cause behavioural toxicity in fish[53]. Another study on Atlantic cod summarizes that PFAS exposure causes significant effects on transcriptomic responses in cod liver organ cultures,which includes the effects on pathways related to sterol metabolism, oxidative stress, and nuclear receptors[41]. The PFASs can also lead to trans-generational toxicity suggesting that their long-term consequences for aquatic ecosystems should become a great concern[54].

The PFOA and PFOS have been added to the Stockholm Convention and the PFOA use has been banned in the EU[55]. In 2020, a total ban of usage and production of all PFAS was proposed in five European countries, including Norway (Norwegian Environment Agency, 2020)[55]. However, PFASs are still being produced and released into the environment in other countries. Despite their high usage and ubiquitous environmental presence, scientific studies on toxicity of most of the PFASs are insufficient.

4.3 Bioaccumulation and Biomagnification potential of PAHs and PFASs

The bioaccumulation may be defined as the process where the pollutants are gradually accumulated in a living organisms whereas biomagnification is the phenomenon of increase in pollutants concentration along the trophic levels in aquatic and terrestrial food chain. The bioaccumulative property of a pollutant can aid for its biomagnification across the food web. Both PFASs and PAHs have the properties to bioaccumulate and biomagnify in organisms in the aquatic food chains. Seafood is one of the major food commodities recognised globally, conferring health benefits on consumers with their low-saturated-fat content, omega fatty acids, as well as for high-protein content[56]. Humans are exposed to PFASs (approx 60% of total exposure[57], and PAHs[20] mainly through food consumption. Several studies have documented the presence of PFASs and PAHs in fish and their concentration in fish were relatively high compared to the other foodstuffs analyzed, which implied that the intake of contaminated fish may be a significant source of PFASs in humans[20][57][58][59][60][61][62][63][64].

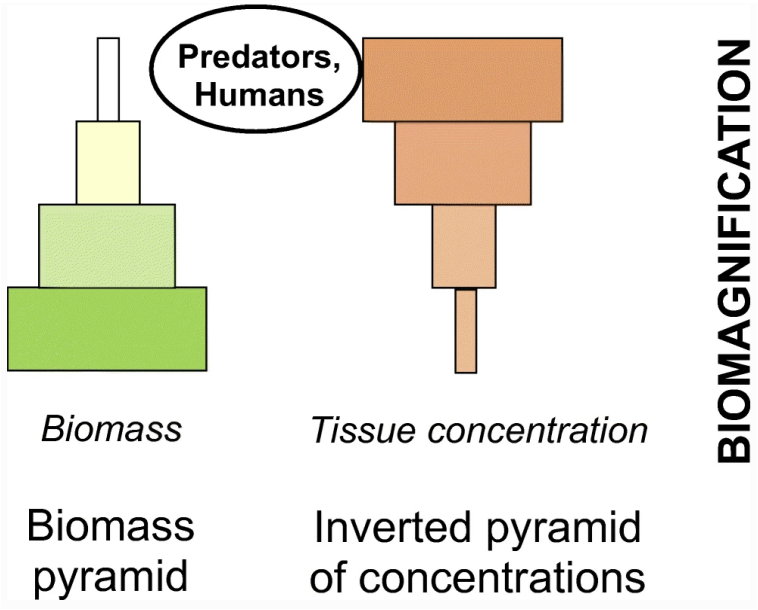


Figure 4.2: Schematic representation of the biomagnification process considering biomass and contaminant tissue concentrations (persistent bioaccumulative pollutants) across various trophic levels[65]

The schematic representation of the biomagnification of organic or inorganic persistent pollutants in food chains clearly shows that the residue concentrations increase when going up the food chain and reach very high concentration at the top trophic level of predators, such as fish-eating birds or humans[65].

PAH have been detected in significant concentrations in terrestrial and aquatic ecosystems, as well as in food commodities[20]. PAHs can bioaccumulate and biomagnify in organisms included in aquatic food chains, such as indigenous wildlife including fish and can have adverse effect on their growth and reproduction[58]. These potential negative effects can also be seen on the next trophic levels of food chain affecting the human health, particularly because of consumption of contaminated seafood[58]. Many studies have found varied and detectable concentrations of PAHs in Atlantic cod and other marine vertebrates worldwide[59][66][67][68][69] though the half-lives of PAHs in organisms are relatively short and are considered to be metabolized or excreted very quickly [26]. For example, the study conducted by Cheung et al. detected higher concentrations of PAHs in the carnivorous fish golden threadfin bream Nemipterus virgatus and catfish Clarias fuscus compared with herbivorous or omnivorous fish[59].

The occurrence of PFASs in the environment are widespread and their bioaccumulation in human and animals have been found since the early 2000s[70]. PFASs are persistently found in environment as complex mixtures that can affect autotrophic and heterotrophic food webs adversely[42]. The environmental cycling of PFASs depends on different factors such as their inherent physicochemical properties including chain length, and their functional groups and environmental conditions[71][72]. The short-chain PFASs are hydrophilic in nature and which make them more mobile in hydrosystems, whereas long-chain PFASs are hydrophobic, hence possess less mobility and tend to bind to particles, increasing its bioaccumulation potential[71]. The bioaccumulation potential of PFAS in biota is also related to their high affinity to serum albumin and fatty acid binding proteins[42]. In biota, the PFOS

(C8 fluorocarbon) is typically the most dominant PFAS, and the PFOS concentration increases along the food chain, showing its high bioaccumulation potential. In contrast, PFOA (C7 fluorocarbon) has a low bioaccumulation potential and is relatively similar among species from different trophic levels[73][74][75][76].

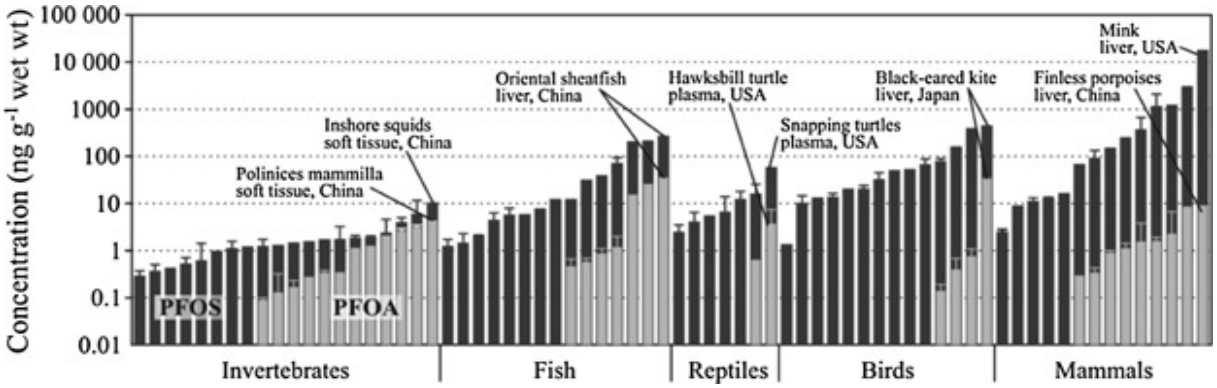


Figure 4.3: Average perfluorooctane sulfonate (PFOS; black bars) and perfluorooctane sulfonate (PFOA; grey bars) concentrations in wildlife from the aquatic environment including invertebrates (whole body), fish (liver), reptiles (plasma), birds (liver), and mammals (liver)[42]

4.4 Atlantic cod as a model species for environmental monitoring

Atlantic cod (*Gadus morhua*) is a commercially and ecologically important teleost species that are distributed majorly in the North Atlantic Ocean[77]. Atlantic cod is an attractive aquaculture species in a number of countries including Norway[77]. Norway has the wide-ranging production of farmed Atlantic cod and has the fast developing aquaculture industry[77]. The commercial landings of Atlantic cod amount to 1.6 million pounds in 2020, and were valued at more than $3.5 million, according to the NOAA Fisheries commercial fishing landings database[78].

Atlantic cod has been used as an indicator species in marine pollution monitoring programs, including water column monitoring of offshore petroleum activities in Norway, the Protection of the Marine Environment of the North-East Atlantic (OSPAR) convention, and in a waste dumping site outside the city of Bergen (Norway)[68][79][80][81]. The availability of the sequenced and annotated cod genome[82][83], has made Atlantic cod a common model organism in environmental toxicology studies, including the studies using omics-based approaches[84][85][86]. The habitat of Atlantic cod, including spawning and nursery grounds, are found in many studies to be the areas with off-shore petroleum activities, or coastal zones that are exposed to pollutants from runoff from roads, accidental oil spills, smelter industries, and sewage discharges[45]. Thus, this species is exposed to anthropogenic pollutants, including oil-related compounds such as polycyclic aromatic hydrocarbons (PAHs) and PFASs that have shown to produce significant impact on marine wildlife and ecosystems[87][88]. Atlantic cod is a major fisheries species which is very significant in costal as well as oceanic ecosystems and has been used as an indicator species as well as a model organism in environmental monitoring and toxicological studies[79][89][90]. Therefore, Atlantic cod is a valuable tool for ecotoxicological studies and risk assessment.

4.5 Influence of xenobiotics on gene expression profiles

The xenobiotic compounds, such as the PAHs and PFASs effects the gene expression profiles of an organism majorly through genetic and epigenetic mechanisms. The genetic mechanisms involves a direct interaction between a xenobiotic and DNA (genetic material)[91]. This interaction can lead to damages in DNA including mutations or DNA adduct formations which are capable of resulting in a sequence change in the DNA[91]. These damages to the DNA may influence the transcription machinery resulting in altered gene expression profile. The PAH-initiated carcinogenesis, where the formation of PAH-DNA adducts is the major step in the process, has been documented in human tissues and are studied in many experimental models[92]. Studies have also concluded that the metabolism of PAHs through different pathways results in formation of reactive metabolites which can produce DNA adducts, leading to mutations in DNA and ultimately affecting gene expression profiles[93]. PFASs can also cause DNA damage as documented in a study of different types of PFASs where the PFOA, PFOS, PFNA and PFHxS showed a dose dependent increase in DNA damage in the concentration range from 2×10^{-7}M to 2×10^{-5}M as determined by the comet assay[94](Figure 4.4).

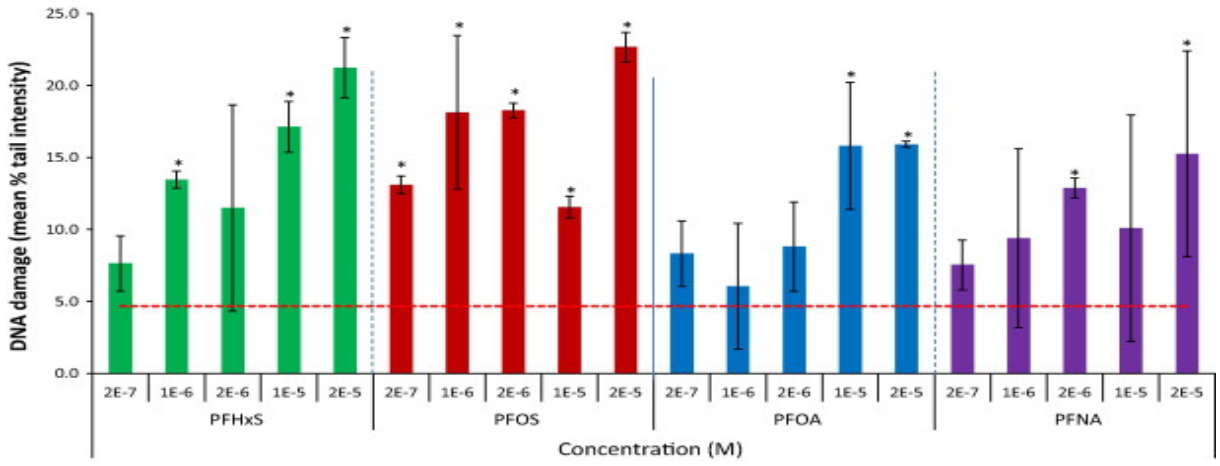


Figure 4.4: The level of DNA damage after PFAS exposure for 24 h[94]

The epigenetic mechanisms do not involve a direct interaction of the xenobiotic with DNA (genetic material)[91]. Here, the effect is mediated through the epigenome which results in gene expression changes[91]. The major epigenetic mechanisms affecting gene expression profile are histone modification, interaction with non-coding RNA, and DNA methylation which may alter the chromatin structure leading to gene expression changes[91]. The data derived from several in-vitro, in-vivo, and epidemiological studies have demonstrated the potential of various xenobiotic compounds, including PFASs and PAHs, to cause gene expression changes that are mediated through their effect on the epigenome [91][95]. The oxidative stress is a key mediator between epigenetic modification and health effects in PFASs[95]. The PAHs are associated with the epigenetic variations involving alterations in DNA methylation, micro RNA (miRNA) regulation, and histone modifications[96]. These changes are related to chronic diseases, such as cardiovascular disease, nervous system disorder, pulmonary disease, cancer, and endocrine disruption[96].

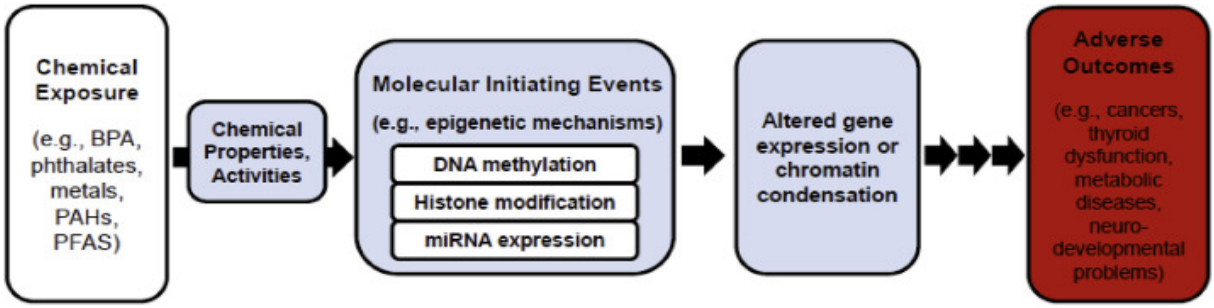


Figure 4.5: Potential mechanisms linking chemical exposure to adverse outcomes mediated by epigenetic alterations as molecular initiating events[95]

4.6 Gene expression profiling techniques

The most popular gene expression profiling techniques include qRT-PCR and next generation sequencing. qRT-PCR analysis is relatively a simple technique. This technique includes primarily the reverse transcription of mRNAs, which is then followed by PCR amplification of the cDNAs produced. The PCR amplification make use of the oligo-nucleotide primers which are specific for the gene of interest for amplification[91]. The final step includes the quantification of amount of the PCR gene products[91]. The qRT-PCR is able to determine accurately the expression profile of a limited number of transcripts or the expression of an individual gene transcript. The analysis of the expression profiles of the entire transcriptome in an organism which comprises large number of transcripts by qRT-PCR is not practical and economical[91].

The transcriptomics is an another major gene expression profiling technique which enables the detection and analysis of the entire transcriptome including the mRNAs, that are expressed in a biological system at a given point of time[91]. The most important aspect that is determined during the analysis of transcriptome is the differential expression profile of each transcript under variety of experimental conditions such as exposure to a xenobiotic compounds[97]. The transcriptome is highly dynamic in nature. As a result, the profiling of the differential expressions of transcriptome is a very sensitive indicator of toxicity resulting from exposure to the xenobiotic agents[91]. The differentially expressed genes can be used as biomarkers for detection of toxicity of the xenobiotic being investigated after appropriate validation. The bioinformatic analysis of the significantly differentially expressed transcripts provides valuable insight into the molecular function affected and the mechanism underlying the toxicity of the xenobiotic agent under investigation[91].

RNA sequencing (RNA-Seq) is a transcriptome profiling technique which uses deep-sequencing technologies. It provides more accurate measurement of transcripts levels compared to other profiling techniques. RNA sequencing can also reveal the exact location of the transcription boundaries up to a single-base resolution[98]. The RNA-seq method does not have an upper limit for quantification that is correlated with the largest number of sequences that can be obtained. As a result, the sequencing has a dynamic range of expression levels over which the transcripts can be detected[98]. RNA-Seq shows the highest levels of reproducibility, for both technical and biological replicates as the technique is highly accurate for quantifying expression levels when compared to expression profiles determined using quantitative PCR (qPCR)[98]. Considering these advantages, the RNA-Seq is the most significant sequencing-based method which permits the entire transcriptome to be surveyed in a very high-throughput and quantitative manner[98].

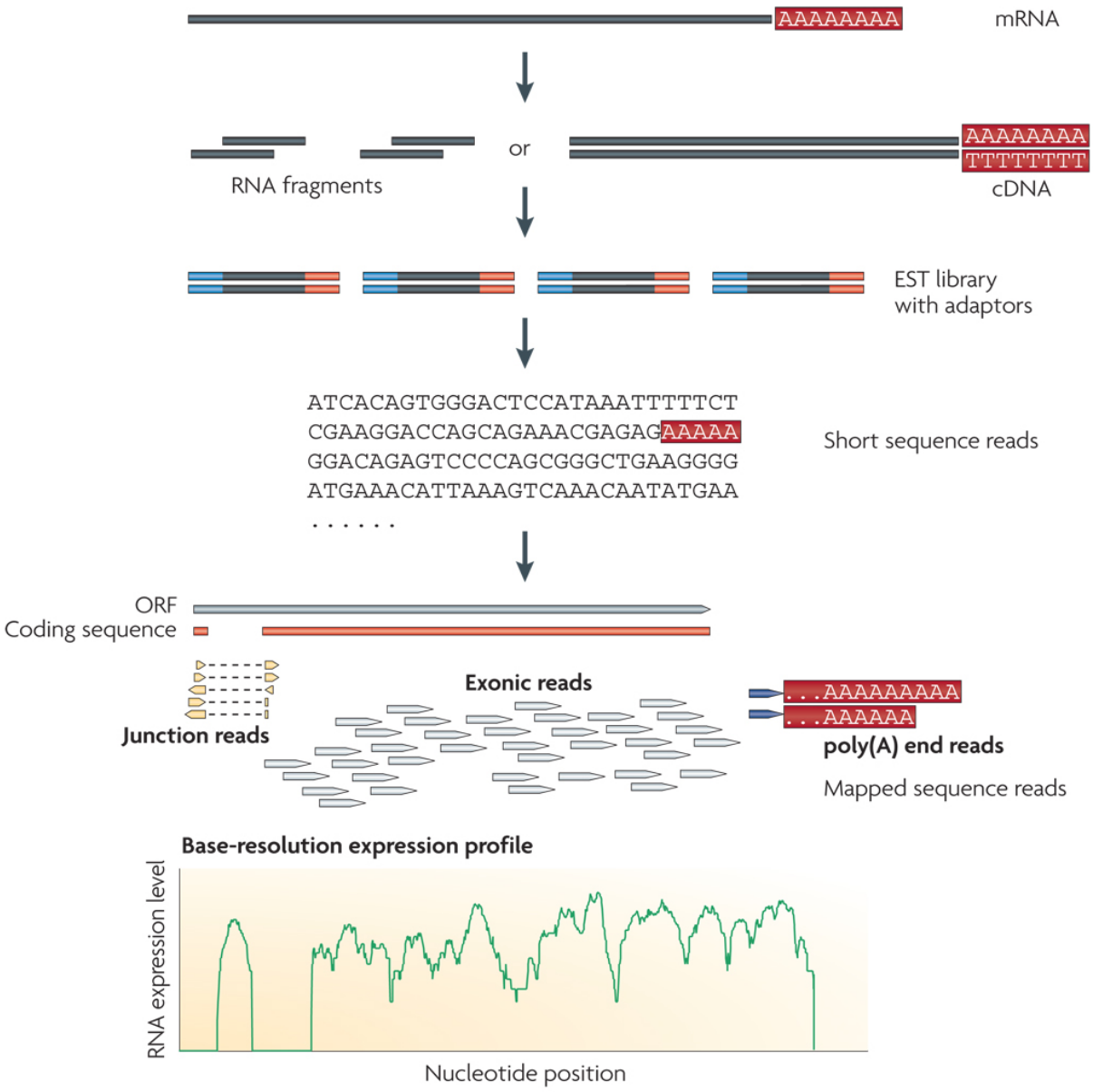


Figure 4.6: A typical RNA-Seq experiment: Initially, the long RNAs are converted into a library of cDNA fragments. Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a base-resolution expression profile for each gene[98]

4.7 Toxicogenomics

The rapid urbanization and industrialization have resulted in numerous anthropogenic activities, which dump variety of pollutants in to the environment, including polycyclic aromatic hydrocarbons (PAHs) and per-fluoro alkyl substances (PFAS)[17]. The further information about the ecotoxicological potential of PAHs and PFASs, among multiple generations, mixture toxicity, and species interactions, is fundamental to analyse the risks that affect the ecosystem structure and functions in the aquatic environment[42]. The toxicology field has kept pace with the advances in molecular studies. The discoveries in the molecular studies is also accompanied with the advancements in techniques and technologies to examine molecular events across species, within individuals, and across environmental and chemical exposures[99]. A significant growth and explosive understanding of the impact of the xenobiotic compounds in the environment to basic cellular and molecular machinery, has been recognized in the past 50 years. Toxicogenomics, which involves the application of advancements of molecular techniques in a whole-genomic capacity to study the effects of toxicants, is a significant milestone for toxicological research[99]. A typical toxicogenomics experiment follows the exposure of animals to a compound or environmental xenobiotic compound followed by tracking the changes in transcript profile across the genome and then the validation of the hypothesis that, these changes in transcripts can elucidate mechanistic insights which are previously unknown with the chemicals under investigation. This toxicogenomic approach also requires complex computational and bioinformatics approaches over the technical precision of experiments[99].

4.8 Signal transduction during stress response

The signal transduction is the process by which a signal, either chemical or physical is transmitted through a cell. The signals are transmitted across the cell as a series of molecular events. These signal transmissions generally results in a cellular response, including transcriptional or translational changes in genes[100][101]. The receptor proteins receives and detects the stimuli and the changes elicited by binding of specific ligands. The detection of stimuli by the receptor proteins give rise to a chain of biochemical events (biochemical cascade), which is defined as a signaling pathway[100]. The extracellular receptors, make up the majority of signalling receptors in a cell membrane and these are integral transmembrane proteins[102].

The transmembrane signaling is an important process involved in stress and chemical response in fish. The focal adhesion pathway and Extra Cellular Matrix (ECM) receptor pathway are the two significant pathways involved in the transmembrane signaling. The regulatory signals are transmitted between extracellular matrix and the interacting cell through large macromolecular assemblies called as focal adhesions[33]. The interacting cells adheres to specific ligands in the extracellular matrix (ECM) environment through integrin receptor proteins[33]. The integrins are transmembrane receptors and facilitates cell-extracellular matrix (ECM) and cell-cell adhesions. Thus the presence of integrins also allows for rapid responses to events happening at the surface of the cell[33]. The integrins activates several signal transduction pathways including intracellular cytoskeleton organization, movement of new receptors to the cell membrane and regulation of the cell cycle[33].

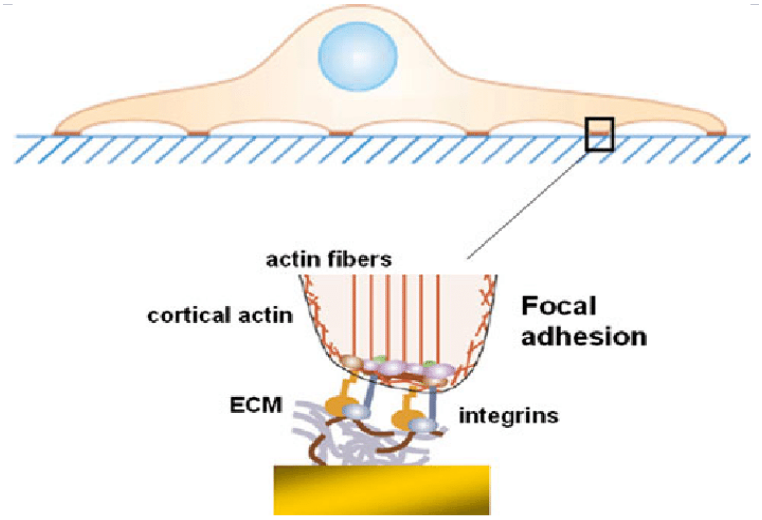


Figure 4.7: The focal adhesion assembly with cell adhesion to specific ligands in ECM through integrins.

The transmembrane signaling processes involves the detection and binding of an extracellular signal by an integral membrane receptor protein which leads to generation of intracellular signals through effector proteins[103]. On the basis of intermolecular or intramolecular interactions of the signal-receiving receptor to the intracellularly located effector proteins, the receptors can be divided into two groups[103].

4.8.1 I. Receptors that couple to an intramolecularly linked effector domain[103]

The category includes Enzyme linked receptors, which possess an extracellular domain with receptor function, a single transmembrane-spanning segment and an intracellular domain possessing catalytic activity. The largest family of enzyme linked receptors are Receptor tyrosine kinases, which plays a significant role in recruiting the intracellular signaling cascades and act as entry points for several extracellular cues. Another category includes Ligand gated ion channels, that consist of several identical or related subunits which together form a central pore acting as the regulated effector. Ligan gated ion receptors involves receptors that are connected to anion and cation channels.

4.8.2 II. Receptors associated with an effector system[103]

The major receptors included in this category are G-protein-coupled receptors, which regulate a variety of signal-generating enzymes and ion channels by interacting with and activating regulatory heterotrimeric G-proteins. These receptors represent the largest family of receptor proteins. Other major receptor types included in this category are Notch receptors and Cell adhesion molecules. Notch receptors are especially important during developmental stages by mediating lineage decision, lateral inhibition, and cellular segregation. Notch signalling involving notch receptors is a master regulator of neural stem cells (NSCs) and neural development. The cell adhesion molecules such as integrins, cadherins, and selectins do not only act as transmembrane proteins involved in cell–cell or cell–matrix interactions but are also involved in signal transduction processes.

4.9 Significance and objectives of study

The PAHs and PFASs are among the most threatening environmental pollutants which are ubiquitously present in natural environment and cause toxicological effects on different species of organisms. An understanding about the mechanisms involved in their toxicity is helpful to prevent or manage the potential adverse health effects. However, limited knowledge is available on the biological effects of PFASs and PAHs exposure on fish, especially with regards to their mixtures. The discovery of high throughput transcriptomics techniques has made it possible to determine the expression profile of the genes present in a biological system, following its exposure to xenobiotics[98]. Similarly, bioinformatic analysis of the gene expression data provides valuable information about the mechanisms underlying the toxicity of xenobiotics in a biological system[98].

In this study, the effects of two different concentrations of PAHs and PFASs on the gene expression profiles in the brain tissue of female Atlantic cod was investigated through RNA sequencing and transcriptome analysis. The juvenile Atlantic cod (*Gadus morhua*) were exposed to environmentally relevant concentrations of PAHs and PFASs [Low dose (1X) and High dose (20X)]. The PAHs and PFASs exposure groups contained different types of each compounds respectively to elucidate the mixture effect. Transcriptome sequencing were performed, followed by qRT-PCR analysis of selected genes to validate the results. The differentially expressed genes after each exposure were identified from the transcriptome sequencing data. These DEGs were analyzed using different bioinformatics tools and software's to understand the biological processes and pathways that are affected, to investigate the molecular functions that are altered at the cellular level.

The objective of the study includes;

- Detection of differentially expressed genes after exposure to environmentally relevant concentrations of PAHs and PFASs using transcriptome sequencing and to elucidate their effects on molecular level functioning through bioinformatics analysis in brain tissue of of Atlantic cod.

5 Methodology

5.1 Schematic representation of workflow

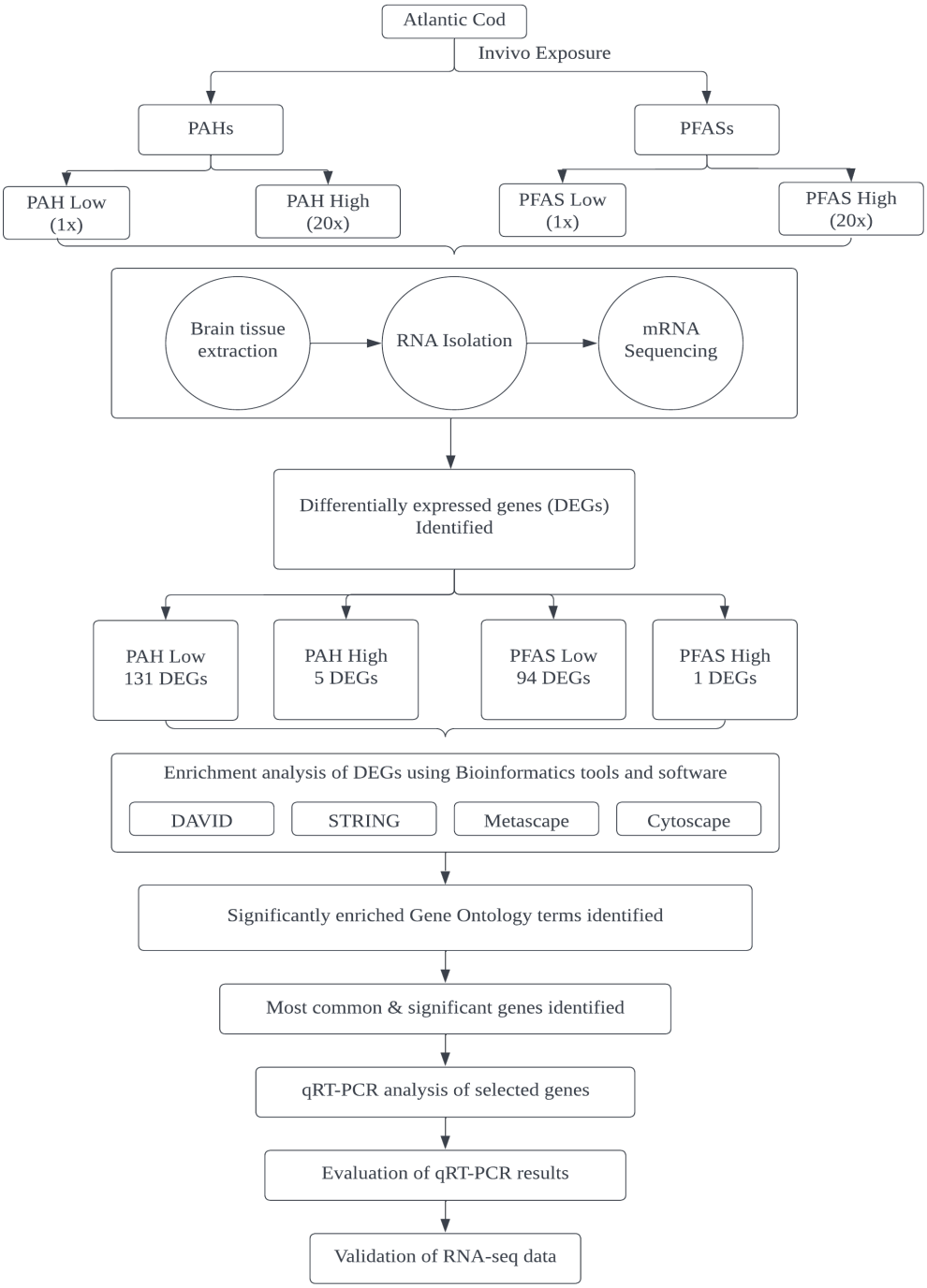


Figure 5.1: The schematic representation of the entire workflow

In molecular biology, the batch effect occurs when non-biological factors in an experiment cause changes in the data produced by the experiment[104]. The batch effect should be minimized during the experiment, because small and uncontrolled changes in an environment can result in detection of differentially expressed genes (DEGs) which are not related to the designed experiment[105] The sources of batch effect can occur during the experiment, during the RNA library preparation, or during the sequencing run[105].

5.2 Chemicals and reagents

The chemicals and reagents that were used in this study includes the exposure chemicals and the reagents used for qPCR validation analysis. The mRNA sequencing was done by contract with Genomics Core Facility at the University of Bergen. The reagents used for qPCR analysis included, LunaScript RT SuperMix cDNA Kit (BioNordica) and SYBR® Green I fluorescent dye.

5.2.1 Overview of exposure chemicals

The PAHs and PFASs exposure groups contained a mixture of specific congeners. The concentration of PAHs and PFASs congeners used for preparing exposure mixtures represent concentration levels that were quantified in cod liver from different stations of the North Sea and Norway's marine coastal environment[106]. The toxicities of these chemical exposures at cellular and molecular levels were studied at two different dose concentrations; a low dose (1X) and a high dose (20X). The concentrations of congeners used in the 1X exposure mixture of PAHs were chose based on the concentrations of different PAHs in a monitoring report by the Institute of Marine Research (IMR), where different PAHs were quantified in the cod liver from Egersund and Tampen stations of the North sea [107]. Whereas, the concentrations of the PFASs congeners used in 1X exposure mixture were chose based on the reported values in Atlantic cod samples from the Nordic environment and northern Norwegian mainland[108]. The different congener compounds used in PAHs and PFASs exposure group and their concentrations used in low and high dose are depicted in Table 5.1. The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Table 5.1: The chemical compounds included in PAH and PFAS groups used for in vivo exposure.

Exposure group	**Compounds**	**CAS No.**	**µg/kg**		**Percentage of total**
			Low Dose (1X)	**High Dose (20X)**	
PAHs	Naphthalene	50-32-8	12.64	252.8	31.6
	Phenanthrene	132-65-0	8.38	167.6	21.0
	Dibenzothiophene	86-73-7	0.58	11.6	1.4
	Pyrene	91-20-3	1.45	29.0	3.6
	B(a)P	85-01-8	1.93	38.5	4.8
	Fluorene	129-00-0	15.03	300.5	37.6
	Total dose		**40**	**800**	**100**
PFASs	PFOS	2795-39-3	25	500	48.3
	PFTrA	375-95-1	16.95	339	32.8
	PFNA	335-67-1	5.925	118.5	11.5
	PFOA	72629-94-8	3.825	76.5	7.4
	Total dose		**51.7**	**1034**	**100**

5.3 Model organism - Fish

The role of confounding sex-dependent parameters on the effects produced by different exposures were minimized by choosing juvenile Atlantic cods, rather than mature fish for the present study. The Juvenile Atlantic cod (*Gadus morhua*), which was approximately 5 months old were used for the study. The Atlantic cods for the study were were obtained from Havbruks stasjonen in Tromsø AS (Tromsø, Norway). These cods were reared in 500 L tanks supplied with seawater at 8 to 10 °C, 34 ppt salinity, at Industrilaboratoriet in Bergen (ILAB, Bergen, Norway). The fish were fed with a commercial marine diet (Amber Neptune, Skretting, Stavanger, Norway) and were held at a 12:12 h light/dark cycle. At the beginning of exposure, the cods were approximately 18 months old. The biometric data, which shows the total length, weight, and condition fact (k-factor) of the experimental fish was taken and shown in Table 5.2. The whole experimental setup was approved by the Norwegian Food Safety Authorities (FOTS 11730/ 17/18948) and performed accordingly.

Table 5.2: Biometric data of experimental fish used in the present study. Data are presented as mean (n = 6-9) ± standard error of the mean (SEM).

Exposure group	**Biometric data**		
	Fish weight (g)	Fish length (cm)	k-factor
Control	177.54 ± 5.56	26.84 ± 0.26	0.91 ± 0.02
Low PAH	167.27 ± 4.94	26.87 ± 0.21	0.85 ± 0.01
High PAH	174.18 ± 7.79	26.72 ± 0.36	0.90 ± 0.01
Low PFAS	180.81 ± 8.72	26.81 ± 0.32	0.92 ± 0.02
High PFAS	154.68 ± 6.27	25.93 ± 0.32	0.88 ± 0.03

5.4 Exposure and sampling

The in-vivo exposure of fish to the PAHs and PFASs at environmentally relevant concentrations provides an opportunity to better understand their mode of action[106]. In this present study, the juvenile Atlantic cods were exposed for two weeks with PAHs and PFASs exposure groups. The cods were injected intraperitoneally with two different doses: a low (1×) and a high (20×) dose of PAHs and PFAS, once per week (day 0 and day 7). The fish were injected with samples consisting of the following groups: a vehicle control, low PAH (1X), low PFAS (1X), high PAH (20X), and high PFAS (20X). The stock solution was prepared in a 1:1 (v/v) ratio mixture of rapeseed oil (Eldorado rapsolje) and phosphate buffered saline (PBS). The fish were injected with stock solutions at 1 mL/100 g fish. The control cod were injected with solvent vehicle (1:1 of oil and PBS). The fish were euthanized following the two weeks of exposure, and tissue samples were collected, which were frozen in liquid nitrogen before being transferred to 80 °C for downstream analyses.

5.5 RNA extraction and mRNA sequencing

The RNA samples were then extracted and submitted for mRNA sequencing to the Genomics Core Facility at the University of Bergen. The basic theory behind mRNA sequencing analysis is to capture the poly-A containing mRNA using poly-T oligos, followed by mRNA fragmentation, then cDNA synthesis and adapter ligation, followed in order by the generation of clusters and finally sequencing. The differential expression analysis was then performed after sequencing, where the sequenced reads were aligned against the available Atlantic cod genome that will generate a read

count. The reads of control and exposure groups were then compared to quantify the differential expression of genes. The differentially expressed genes (DEGs) were defined by a FDR value less than 0.05, that is the p-value<0.05 after adjustment using the Benjamini-Hochberg multiple testing correction. The FDR (False discovery rate) is used to measure the significance of gene expressions. The FDR values are more reliable over the simple P-value, as they are produced after adjustment using the Benjamini-Hotchberg multiple testing correction. The correction for multiple comparisons is very important as a typical RNA-sequencing data contains large number of genes. The genes with average counts per million/cpm > 1 in at least one of the control or treated groups and with fold changes > 1.5 (for up-regulated) or < 0.67 (for down regulated) were included in the final list of differentially expressed genes.

5.6 Bioinformatics analysis of DEGs

The bioinformatic analysis of the gene expression data obtained from a toxicity study is expected to provide information regarding the molecular mechanisms underlying the toxicity of the xenobiotic under investigation[91]. The differentially expressed genes after each exposure were identified and produced into an excel file with fold change (logFC), P Value and FDR. The Atlantic cod genes were also mapped to human and zebra fish orthologs for functional and pathway analysis. The orthologus genes can be defined as the genes which are present in different species and are originated by vertical descent from a single gene of the last common ancestor[109]. The orthologus genes maintain similar function to that of their ancestral gene from which they evolved[109]. The mapping of fish genes to mammalian orthologus genes enables the usage of well-annotated databases for mammalian model organisms[110]. The human and zebra fish orthologs of Atlantic cod genes were obtained using the BioMart tool in Ensembl genomes database. Different bioinformatics tools and software's were used for the enrichment analysis of the differentially expressed genes identified from the mRNA sequencing of the brain tissue samples from female Atlantic cod after each exposure. The initial assessment of mechanisms of tissue response to the exposures can be done by mapping of the expression changes in the DEGs to the annotated pathways using bioinformatics tools.

5.6.1 DAVID

The DAVID (Database for Annotation, Visualization and Integrated Discovery) is an online bioinformatic tool which was used for enrichment analysis of DEGs[111]. The zebra fish (*Danio rerio*) and human (Homo sapiens) orthologs were used for the analysis, as the Atlantic cod (*Gadus morhua*) gene set were not supported by DAVID. The significantly enriched pathways (KEGG pathway and Reactome pathways) and enriched terms (GO terms - cellular component, biological processes, molecular function and upregulated keywords) were produced by DAVID. The enriched protein domains by differential regulation of genes after each exposure were also provided by analysis in DAVID. The functional annotation clustering of DEGs were also performed where, the annotation terms were grouped along with the co-associated genes.

5.6.2 STRING

The systematic characterization of proteins provide more information in molecular systems biology, as the interactions between proteins are very significant in cellular processing[112]. The STRING database integrates and assess the protein–protein interactions which includes both physical (direct) and functional (indirect) associations between the proteins[112]. In this study, STRING was used

to produce protein-protein interaction network using the differentially expressed gene list for each exposure. The proteins coded by the DEGs were produced by the software, which was used for creating the interaction network. The zebra fish orthologs of the differentially expressed genes was used for creating the network in STRING. The network was produced with the low-confidence interaction parameter (0.150). The STRING interaction network shows the interconnected genes in the DEGs list at their functional protein level. The enrichment analysis of the protien-protein interaction network created, was performed in STRING which identifies the enriched pathways (KEGG and Reactome), enriched biological processes, enriched molecular functions, annotated keywords (UniProt), enriched protein domains (SMART) and other gene ontology (GO) terms like cellular component, and subcellular localization. The STRING software was also used to identify the predicted functional partners for the only differentially expressed gene in PFASs high exposure, followed by enrichment analysis.

5.6.3 Cytoscape

The visualization and further analysis of protein-protein interaction network constructed in STRING was performed using Cytoscape. Cytoscape is a free software package used for modelling, visualizing, and analyzing molecular and genetic interaction networks[113]. The interaction network produced from STRING was exported to Cytoscape software and the expression data was merged into the PPI network where, logFC values were used for continuous mapping. To analyse the network, different Cytoscape plugins like MCODE and ClueGO were used. The MCODE identified clusters (highly interconnected regions) in the protein - protein interaction network. The MCODE clusters represents the significant genes that are differentially expressed and can be part of pathways as they are interconnected. The MCODE clusters produced were then subjected to enrichment analysis using STRING to identify the enriched pathways and terms related to the cluster.

5.6.4 Metascape

Metascape is an online bioinformatics software which act as a comprehensive gene list annotation and analysis resource for experimental biologists[114]. The Metascape software were used for enrichment analysis of DEGs to find the significantly enriched terms. The zebra fish orthologs and human orthologs of DEGs were used for metascape analysis, as the software did not support for the *Gadus morhua* species. The user-provided gene identifiers are first converted into their corresponding *Danio rerio* Entrez gene IDs by the software. If multiple identifiers which corresponds to the same Entrez gene ID are present, they were considered as a single Entrez gene ID in downstream analyses.

The pathway and biological process enrichment analysis has been carried out with the following ontology sources: GO Biological processes, KEGG pathway, Reactome gene sets and WikiPathways. The entire set of genes in the genome have been used as the enrichment background. The enriched terms which are corresponding to a p-value less than 0.01, an enrichment factor greater than 1.5 and a minimum count of 3 were identified and grouped into clusters based on their similarities. The p-values were calculated based on the accumulative hypergeometric distribution, whereas the q-values were calculated using the Benjamini-Hochberg procedure which account for multiple testing. A bar graph of enriched terms across the input differentially expressed gene list, which were coloured by p-value was produced. The top-level gene ontology biological processes were also produced which were coloured by p-value. While performing hierarchical clustering on the enriched terms, the kappa scores were used as the similarity metric. The sub-trees which has a similarity greater than 0.3 were

considered a cluster. The most statistically significant enriched term within a cluster is chosen to represent the cluster. The top clusters within provided gene list along with their representative enriched terms were also produced in the analysis.

5.6.5 Enrichr

The gene enrichment analysis was also attempted using Enrichr, which is a comprehensive gene enrichment analysis web server[115]. The Ensembl BioMart was used to convert the provided Ensembl IDs of Atlantic cod to Entrez gene IDs as the Enrichr server only accepts Entrez NCBI gene ID. The BioMart only converted the gene stable IDs for only 91 genes from 131 input gene, with Atlantic cod species as the selection criteria. The converted list of genes didn't produce any enrichment result in Enrichr with *Gadus morhua* gene list and also within the human and zebra fish ortholog gene list.

5.7 RNA-seq validation with qRT-PCR

The mRNA sequencing provides more accurate measurements of transcripts in a sample. However, the factors such as the quality of cDNA and the differences in the starting concentration of the template may affect the quality of output data. As a result, it is recommended to validate the sequencing results using qPCR analysis, which is a sensitive method and relatively easy to perform. The drawbacks of qPCR analysis like high variance and less reproducibility could be easily mitigated by assessing the quality of qPCR components. Here, the RNA-seq data was verified by qRT-PCR of selected transcripts. The most significant and recurring genes which were involved in enriched pathways were selected from different exposure groups. A total of eight genes were selected which are, *adcy1, baiap2, camk2a, fos, grin2b, ptpn11, ndufa5, and tas2r* (ENSGMOG00000010248).

5.7.1 RNA quantity and quality

The quantity and quality check of the RNA samples used for qRT-PCR were done using NanoDrop-1000 spectrophotometer where, the concentration of total RNA in the samples were measured. The NanoDrop spectrophotometer contained default programs for RNA measurements that automatically read the absorbance [optical density (OD)] at wavelengths 230, 260, and 280 and calculate the concentration of the RNA[116]. The ratios A260/A280 and A260/A230 were calculated by the spectrophotometer, which was around 2 for all the samples that indicates the purity of RNA samples.

5.7.2 cDNA synthesis

The cDNA was synthesized from 1μg of each RNA sample in 20μL reactions using LunaScript RT SuperMix cDNA kit. The total RNA concentration produced from NanoDrop was used to calculate the volume required of each sample to make 1000ng in total 20μL. The cDNA synthesis reaction was performed following the LunaScript RT SuperMix protocol, where 4μL of RT supermix was added with calculated volume of RNA sample (to make 1μg/20μL) and the reaction volume was made to 20μL using nuclease-free water provided in the kit. The incubation steps for cDNA synthesis involved, the primer annealing at 25°C for 5 minutes, cDNA synthesis (reverse transcription) at 55°C for 10 minutes and heat inactivation at 95°C for 1 minute. The synthesized cDNA was diluted (1:6) using nuclease-free water and 5μL was used for qPCR reaction.

5.7.3 Primers for qRT-PCR

The primers (forward and reverse) for the selected genes were designed using the PrimerQuest™ Tool (Integrated DNA technologies-IDT), which is an online primer designing tool. The genes selected for qRT-PCR along with their forward and reverse primers, and amplicon size are shown in the Table 5.3. The sequences of designed primers were sent and synthesized at Merck Sigma Aldrich, Norway. The purchased primers were activated by mixing with water and vortexing. A mixture of forward and reverse primers (10nM concentration), along with the qPCR reaction mix were prepared for trial run for the primers. For the trial qRT-PCR run, a mixture of two samples were used as cDNA mix and the primers were loaded into separate wells with nuclease-free water and syber-green dye.

Table 5.3: The genes selected for qRT-PCR analysis along with the forward and reverse primers, and their amplicon size. Primer sequences are in 5' to 3' direction.

Target genes	Accession number	Forward primer	Reverse primer	Amplicon size (bp)
adcy1	ENSGMOG00000018341	AGAAGGTGAAGCTGGACAAC	CACTTGTGCGTAGGACTGATAG	122
baiap2	ENSGMOG00000009170	TACCAGACGGAGCACAAGA	GTTGCTGATAGCCTCCACATAC	144
camk2a	ENSGMOG00000012386	CAGTAGAGACCACCAGAAGTTG	TGGTGTCCTTCCTCTGAGATA	105
fos	ENSGMOG00000011046	GCCACCACCTTCACTTCTT	CTCAGGGAGTCTGAGGAATACT	119
grin2b	ENSGMOG00000005883	CGGCTTCGTCTTCTCCATTAG	TGTTGGAGTGGGTGTTGTT	122
ptpn11	ENSGMOG00000017724	CGGGCAGAGAACAAGAATAAGA	GCTCGGGCATGATGAGATTAG	133
ENSGMOG00000010248	ENSGMOG00000010248	TTCCTGTCCGGCTGAATATG	GGAGAAGAGGAAGAAGACCAAG	116
ndufa5	ENSGMOG00000006088	TAAGATCCTGGCCTCCCTT	CTGTCCACCGTTGATCTTCTT	142

5.7.4 qRT-PCR

The expression of each of the selected genes were quantified by performing qRT-PCR analysis. A 20μL qPCR reaction mix was prepared with the master mix for qRT-PCR (15μL) and cDNA samples (5μL). The master mix for qRT-PCR includes 2μL of primers (forward and reverse mix), 3μL of nuclease-free water and 10μL of syber green fluorescent dye. The program for qRT-PCR reaction was made using LightCycler® software, which includes 3 different steps with specific temperature profiles, the enzyme activation step at 95°C for 4 minutes, followed by 40 cycles of 95°C(15 seconds), 60°C(30 seconds) and 72°C(15 seconds), and the final step includes 95°C(60 seconds), 65°C(30 seconds) and 95°C(30 seconds). The expression of the genes were quantified by using absolute quantification using plasmids cloned with the gene of interest, which were used to generate a standard curve to quantify the initial levels of mRNA in the sample[117]. The equation produced from the standard curve was used for identifying the concentrations of unknown samples. The expression of selected genes from qRT-PCR were then compared to the RNA sequencing data for validation.

6 Results

6.1 Differentially expressed genes after exposures

6.1.1 Total number of DEGs in each exposures

The exposure of female Atlantic cod to different concentrations of PAHs and PFASs have caused differential expression of various genes. A total of 131 genes were differentially expressed after exposure to low concentration of PAHs, in which 118 genes were downregulated and 13 genes were upregulated whereas, only 5 genes were differentially expressed after PAHs high exposure in which 4 genes were upregulated and one gene was downregulated. The high exposure to PAHs resulted in differential expression of very few numbers of genes when compared to low exposure group. The PFASs exposure also showed a similar pattern in the count of differentially expressed genes after low and high exposures. A total of 94 genes were differentially expressed after exposure to PFASs low concentration, wherein 72 genes were downregulated and 22 genes where upregulated whereas, only one gene was downregulated in the PFASs high exposure group.

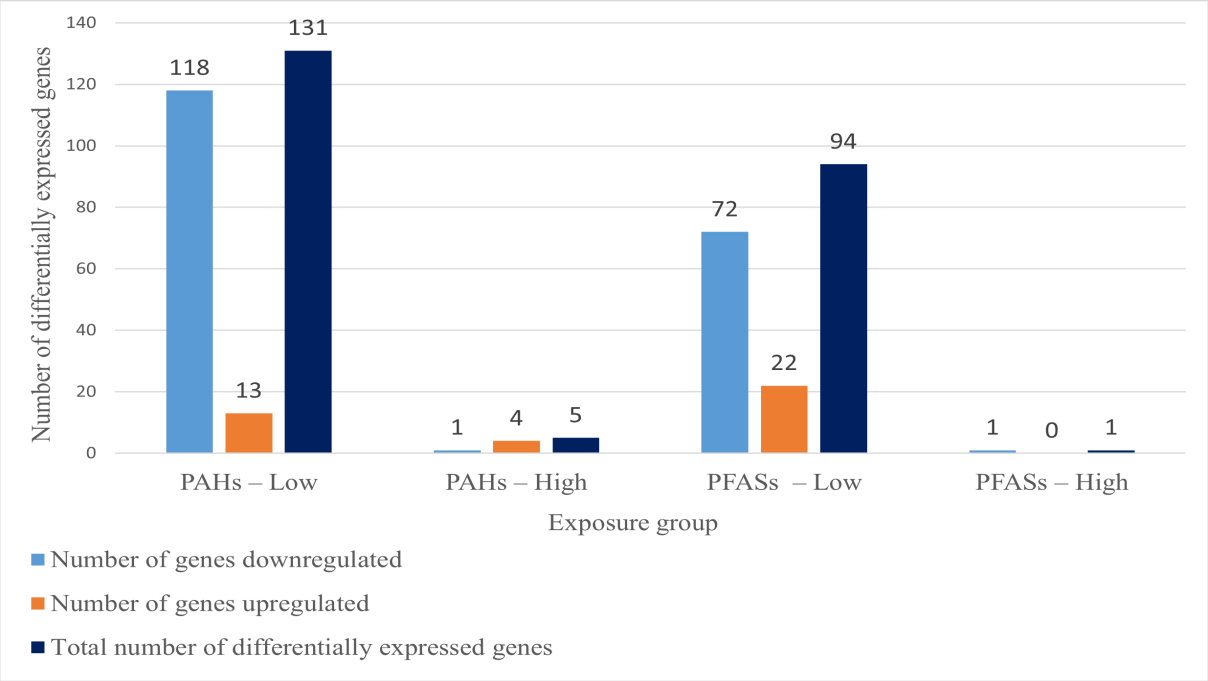


Figure 6.1: The number of differentially expressed genes after each exposure is represented by bar graph. The colour legend for downregulated, upregulated and total number of genes is shown in the graph.

6.1.2 Differentially expressed genes after PAHs low exposure

The low concentration exposure of PAHs caused differential expression of 131 gene. The logFC values represent the log 2 of fold change of each gene, in which a negative fold change depicts a downregulated gene and a positive fold change represents the upregulated genes. Majority of the differentially expressed gene were downregulated and very few were upregulated. The upregulated genes are highlighted with grey in the Table 6.1. The FDR values for all the differentially expressed genes is less than 0.05 (FDR < 0.05), which shows the significance of expression of genes.

Table 6.1: The differentially expressed genes after low exposure to PAHs, along with the fold change values (logFC) and False discovery rate (FDR). The genes that are upregulated are highlighted in grey in the table.

Gene ID	logFC	FDR	Gene ID	logFC	FDR
ENSGMOG00000000004	-0.63916	0.024382	ENSGMOG00000008244	-0.60028	0.04843
ENSGMOG00000000209	-0.37309	0.042079	ENSGMOG00000008252	-0.9492	0.02735
ENSGMOG00000000234	-0.54399	0.009377	ENSGMOG00000008304	-0.55722	0.012334
ENSGMOG00000000395	-0.50401	0.044985	ENSGMOG00000008751	-0.5628	0.017213
ENSGMOG00000000448	-0.70933	0.029456	ENSGMOG00000008807	-0.96375	0.004806
ENSGMOG00000000545	0.412212	0.034036	ENSGMOG00000008815	0.379651	0.047167
ENSGMOG00000000769	-0.79944	0.024327	ENSGMOG00000008826	4.619275	0.024382
ENSGMOG00000000886	-0.47461	0.012334	ENSGMOG00000009129	-0.49791	0.0042
ENSGMOG00000001139	-1.09402	0.034036	ENSGMOG00000009227	-0.55814	0.035861
ENSGMOG00000001318	-0.4989	0.020087	ENSGMOG00000009276	-0.57045	0.020432
ENSGMOG00000001832	-0.49103	0.029049	ENSGMOG00000009585	-0.53754	0.017213
ENSGMOG00000001883	-0.60395	0.032511	ENSGMOG00000009654	-0.37722	0.044758
ENSGMOG00000001941	-0.9317	0.012334	ENSGMOG00000009794	-0.81171	0.018376
ENSGMOG00000002161	-0.48277	0.009799	ENSGMOG00000009878	-0.69545	0.000905
ENSGMOG00000002178	-2.07942	0.034101	ENSGMOG00000009935	-0.51282	0.026936
ENSGMOG00000002178	-2.07942	0.034101	ENSGMOG00000010062	-0.61077	0.013835
ENSGMOG00000002178	-2.07942	0.034101	ENSGMOG00000010229	0.396876	0.020432
ENSGMOG00000002178	-2.07942	0.034101	ENSGMOG00000010229	0.396876	0.020432
ENSGMOG00000002178	-2.07942	0.034101	ENSGMOG00000010282	-0.52891	0.04491
ENSGMOG00000002803	-0.4337	0.024327	ENSGMOG00000010319	-1.46258	0.020544
ENSGMOG00000002809	-0.40379	0.033918	ENSGMOG00000010349	-1.13898	0.035861
ENSGMOG00000003140	-0.5676	0.04843	ENSGMOG00000010355	-0.7753	0.026815
ENSGMOG00000003199	-0.47961	0.012334	ENSGMOG00000010405	-0.45695	0.034036
ENSGMOG00000003468	-0.68838	0.043985	ENSGMOG00000010483	-0.57654	0.011764
ENSGMOG00000003525	-0.45368	0.012306	ENSGMOG00000010793	-0.58056	0.012334
ENSGMOG00000003625	-0.74393	0.012334	ENSGMOG00000010837	-0.52805	0.024327
ENSGMOG00000003711	-0.66584	0.005233	ENSGMOG00000011224	4.066137	0.023777
ENSGMOG00000004100	-0.52531	0.033496	ENSGMOG00000011924	0.947952	0.009377
ENSGMOG00000004117	-0.61002	0.000155	ENSGMOG00000012026	-1.33575	0.047699
ENSGMOG00000004352	-0.84669	0.0042	ENSGMOG00000012338	-0.48806	0.020544
ENSGMOG00000004518	-1.28398	0.000816	ENSGMOG00000012386	-0.96367	0.027045
ENSGMOG00000004524	-1.18652	0.0042	ENSGMOG00000012388	-0.94494	0.049452
ENSGMOG00000004625	-0.73993	0.043008	ENSGMOG00000012509	-0.77017	0.021535
ENSGMOG00000004817	-0.82941	0.012334	ENSGMOG00000012606	-0.67168	0.022334
ENSGMOG00000004887	-0.76274	0.023819	ENSGMOG00000012609	-0.77819	0.022334
ENSGMOG00000005018	-1.17043	0.024382	ENSGMOG00000012613	-0.56233	0.035385
ENSGMOG00000005131	-0.48993	0.024382	ENSGMOG00000012652	-0.41273	0.04654
ENSGMOG00000005267	-0.62637	0.012334	ENSGMOG00000012737	-0.45553	0.038402
ENSGMOG00000005354	0.518424	0.016342	ENSGMOG00000012833	-0.42024	0.029297
ENSGMOG00000005611	0.473278	0.026726	ENSGMOG00000012986	-0.59993	0.020362
ENSGMOG00000005783	0.475517	0.016342	ENSGMOG00000013635	-0.61893	0.025178
ENSGMOG00000005883	-0.70729	0.016342	ENSGMOG00000013797	-1.81418	0.04843

ENSGMOG00000006300	-0.48697	0.04843	ENSGMOG00000014031	-0.58674	0.000155
ENSGMOG00000006546	-0.41335	0.04491	ENSGMOG00000014371	-0.39688	0.030291
ENSGMOG00000007769	-0.71423	0.0042	ENSGMOG00000014477	-1.2805	0.022029
ENSGMOG00000008144	-0.40163	0.024327	ENSGMOG00000014490	-0.55899	0.016407
ENSGMOG00000008209	-0.44088	0.035861	ENSGMOG00000014906	-1.13618	0.03223
ENSGMOG00000015018	-0.4711	0.024327	ENSGMOG00000018220	-1.05868	0.000155
ENSGMOG00000015443	-0.55671	0.042472	ENSGMOG00000018268	-0.42264	0.04843
ENSGMOG00000015454	-0.61579	0.006583	ENSGMOG00000018346	-0.66837	0.036016
ENSGMOG00000015454	-0.61579	0.006583	ENSGMOG00000018360	-0.89834	0.039203
ENSGMOG00000015647	-0.66531	0.024327	ENSGMOG00000018672	-0.47521	0.049452
ENSGMOG00000015781	-0.86328	0.009377	ENSGMOG00000018917	-0.60646	0.005233
ENSGMOG00000015829	-0.51669	0.035861	ENSGMOG00000018958	-4.40304	0.042079
ENSGMOG00000016293	-0.54979	0.014753	ENSGMOG00000019174	-0.45261	0.022029
ENSGMOG00000016363	-1.17749	0.009377	ENSGMOG00000019244	-0.76265	0.012334
ENSGMOG00000016447	-1.18248	0.022029	ENSGMOG00000019277	-0.74382	0.0042
ENSGMOG00000016536	-0.47963	0.022029	ENSGMOG00000019285	-0.68299	0.013835
ENSGMOG00000016709	-0.6927	0.006823	ENSGMOG00000019318	-0.69159	0.043008
ENSGMOG00000016887	2.07579	0.04654	ENSGMOG00000019448	-0.54705	0.011654
ENSGMOG00000016922	-0.68591	0.042079	ENSGMOG00000019605	-0.96189	0.04654
ENSGMOG00000016956	0.384632	0.032511	ENSGMOG00000019665	-0.61179	0.023738
ENSGMOG00000017059	-0.41703	0.035861	ENSGMOG00000019790	-1.20564	0.020362
ENSGMOG00000017239	-0.58742	0.049452	ENSGMOG00000020025	-0.6537	0.047049
ENSGMOG00000018185	0.683091	0.012334	ENSGMOG00000020192	-0.56086	0.012334
ENSGMOG00000018214	-1.55333	0.014929			

6.1.3 Differentially expressed genes after PAHs high exposure

The exposure to high concentration of PAHs caused differential expression of 5 genes that are depicted in Table 6.2. Majority of genes were upregulated with a positive fold change value (logFC). All the genes showed a significant differential expression which was depicted with a false discovery rate less than 0.05 (FDR < 0.05).

Table 6.2: The differentially expressed genes after high exposure to PAHs, along with the fold change values (logFC) and False discovery rate (FDR). The genes that are upregulated are highlighted with grey in the table.

Gene ID	logFC	FDR
ENSGMOG00000009965	3.059820237	0.02976833
ENSGMOG00000009965	3.059820237	0.02976833
ENSGMOG00000017093	-0.717446273	0.009263109
ENSGMOG00000019708	3.197351835	0.037866212
ENSGMOG00000020050	3.014976618	0.02976833

6.1.4 Differentially expressed genes after PFASs low exposure

The differentially expressed genes after exposure to low concentration of PFASs is shown in the Table 6.3.

Table 6.3: The differentially expressed genes after low exposure to PFASs, along with the fold change values (logFC) and False discovery rate (FDR). The genes that are upregulated are highlighted with grey in the table.

Gene ID	logFC	FDR	Gene ID	logFC	FDR
ENSGMOG00000000139	-0.49642	0.03254	ENSGMOG00000010349	-1.19002	0.01632
ENSGMOG00000000209	-0.37560	0.03944	ENSGMOG00000010444	-0.57302	0.02147
ENSGMOG00000000788	-0.50179	0.03348	ENSGMOG00000010608	-0.39067	0.03036
ENSGMOG00000001116	-0.86865	0.03259	ENSGMOG00000010708	-0.40587	0.03259
ENSGMOG00000001502	-1.21731	0.00477	ENSGMOG00000010837	-0.60527	0.01632
ENSGMOG00000001941	-0.73521	0.03036	ENSGMOG00000010935	0.34098	0.04793
ENSGMOG00000002046	0.49876	0.03036	ENSGMOG00000011046	-1.27288	0.02709
ENSGMOG00000002161	-0.46182	0.01722	ENSGMOG00000011169	-0.53825	0.02040
ENSGMOG00000002305	-0.76840	0.04247	ENSGMOG00000011824	0.48210	0.03382
ENSGMOG00000002493	-0.68288	0.00583	ENSGMOG00000012343	-0.52298	0.04919
ENSGMOG00000002985	-0.46263	0.02709	ENSGMOG00000012388	-0.98237	0.03777
ENSGMOG00000003098	-1.08297	0.04776	ENSGMOG00000012489	-0.56054	0.01434
ENSGMOG00000003273	0.49549	0.01722	ENSGMOG00000012606	-0.57782	0.03036
ENSGMOG00000003350	-0.39731	0.02055	ENSGMOG00000012649	-5.23400	0.01036
ENSGMOG00000003540	-0.93316	0.01470	ENSGMOG00000012652	-0.44614	0.03197
ENSGMOG00000003580	-0.45310	0.03259	ENSGMOG00000012740	-0.61059	0.01272
ENSGMOG00000003838	0.40975	0.03259	ENSGMOG00000013034	-0.42638	0.04327
ENSGMOG00000004185	-6.60846	0.03986	ENSGMOG00000013148	-0.45392	0.04658
ENSGMOG00000004224	1.43672	0.03254	ENSGMOG00000013317	-0.59275	0.01632
ENSGMOG00000005131	-0.52685	0.01632	ENSGMOG00000013644	0.67807	0.03571
ENSGMOG00000005336	0.37400	0.04485	ENSGMOG00000013923	-1.60584	0.02040
ENSGMOG00000005347	0.43331	0.04593	ENSGMOG00000014031	-0.51441	0.01272
ENSGMOG00000005783	0.57178	0.03036	ENSGMOG00000014219	0.39666	0.03259
ENSGMOG00000005792	0.41585	0.04658	ENSGMOG00000014797	4.16687	0.02844
ENSGMOG00000005883	-0.62548	0.01549	ENSGMOG00000015231	-1.47868	0.02642
ENSGMOG00000005921	0.33308	0.04919	ENSGMOG00000015550	0.35918	0.04359
ENSGMOG00000005929	0.39761	0.03986	ENSGMOG00000015647	-0.57489	0.03259
ENSGMOG00000006088	0.40880	0.04302	ENSGMOG00000015829	-0.74015	0.01401
ENSGMOG00000006987	-0.47273	0.03254	ENSGMOG00000015964	-3.20476	0.03777
ENSGMOG00000007040	-0.45089	0.04506	ENSGMOG00000015997	-2.68335	0.00100
ENSGMOG00000007166	0.45893	0.03036	ENSGMOG00000016447	-0.97611	0.01036
ENSGMOG00000007434	1.67890	0.03259	ENSGMOG00000016645	-1.53773	0.01722
ENSGMOG00000007685	-0.54756	0.01470	ENSGMOG00000016709	-0.61240	0.01067
ENSGMOG00000008137	-0.38966	0.04776	ENSGMOG00000016776	-0.68878	0.02709
ENSGMOG00000008144	-0.35840	0.04919	ENSGMOG00000016869	-0.71469	0.03911
ENSGMOG00000009008	-0.43707	0.04919	ENSGMOG00000017093	-0.74945	0.00871
ENSGMOG00000009170	-0.51021	0.01902	ENSGMOG00000017305	-0.68021	0.01470
ENSGMOG00000009425	0.69203	0.01036	ENSGMOG00000017724	-0.39972	0.04830
ENSGMOG00000009794	-0.73214	0.03259	ENSGMOG00000017940	-0.55966	0.03259
ENSGMOG00000009878	-0.55576	0.02150	ENSGMOG00000018341	-0.71928	0.04593
ENSGMOG00000010062	-0.60776	0.01434	ENSGMOG00000018540	-0.34032	0.04485
ENSGMOG00000010190	-0.39959	0.04485	ENSGMOG00000018825	-0.41843	0.03036

ENSGMOG00000018905	-0.43192	0.04793	ENSGMOG00000019815	0.49406	0.027092
ENSGMOG00000019285	-0.60437	0.01036	ENSGMOG00000019861	0.67734	0.016321
ENSGMOG00000019391	-0.58969	0.01272	ENSGMOG00000019881	-0.81942	0.023880
ENSGMOG00000019448	-0.51159	0.01549	ENSGMOG00000020192	-0.55080	0.037467
ENSGMOG00000019665	-0.65834	0.02706	ENSGMOG00000020588	-1.02948	0.032587

The low exposure to PFASs cause more genes to get downregulated, shown by negative logFC values and less genes were upregulated, shown by a positive fold change. All of the genes are significantly differentially expressed with a FDR less than 0.05. The upregulated genes are highlighted by grey colour in the Table 6.3.

6.1.5 Differentially expressed genes after PFASs high exposure

The exposure to high concentration of PFASs lead to differential expression of a single gene, which was significantly downregulated with a logFC value of -8.61 and a very low FDR value (FDR=$6.62E^{-11}$).

Table 6.4: The differentially expressed gene after high exposure to PFASs, along with the fold change values (logFC) and False discovery rate (FDR).

Gene ID	**logFC**	**FDR**
ENSGMOG00000010248	-8.61	6.62E-11

6.2 Enriched biological processes after exposure to PAHs and PFASs

Table 6.5: The biological processes that are significantly enriched after exposure to high and low concentrations of Polycyclic aromatic hydrocarbons (PAHs) and Perfluoroalkyl Substances (PFASs).

ENRICHED BIOLOGICAL PROCESSES			
PAH Low	**PAH High**	**PFAS Low**	**PFAS High**
Cell adhesion	Cellular process	Regulation of cell adhesion mediated by integrin	G protein-coupled receptor signalling pathway
Extra cellular matrix organization	Negative regulation of DNA recombination	Transport	Sensory perception of taste
Signal transduction	Chromosome condensation	Intracellular signal transduction	
Chemical synaptic transmission	Negative regulation of chromatin silencing	Response to xenobiotic stimulus	
Developmental process	Reproductive process	Developmental process	
Vascular endothelial growth factor receptor signalling pathway	Nucleosome assembly	Cellular response to epidermal growth factor stimulus	
signalling	Nucleosome positioning	signalling	
Maintenance of permeability of the blood-brain barrier		Responses to xenobiotic stimulus	
Endodermal cell differentiation		Ephrin receptor signalling pathway	
Positive regulation of long-term neuronal plasticity		Mitochondrial electron transport, NADH to ubiquinone	
Biological adhesion		Mitochondrial respiratory chain complex I assembly	
Positive regulation of cell migration		MAPK Cascade	

The biological processes that are significantly enriched after exposure to low and high concentrations of PAHs and PFASs were produced using DAVID and Metascape software (Table 2). The biological processes related to signalling and signal transduction were enriched after exposure to low concentrations of PAHs and PFASs. Cell adhesion, signal transduction, developmental processes and signalling are the common biological processes that are enriched in both low PAHs and low PFASs. The biological processes related to cellular respiration such as mitochondrial electron transport and mitochondrial respiratory chain complex I assembly were also enriched after PFASs low exposure. Biological processes that are involved in DNA packaging such as nucleosome assembly and positioning, negative regulation of chromatin silencing, chromosome condensation and negative

regulation of DNA recombination were enriched after exposure to high concentration of PAHs. The biological processes enriched by the only differentially expressed gene after PFASs high exposure includes G protein-coupled receptor signalling pathway and sensory perception of taste.

6.3 Enriched pathways common in low exposure to PAHs and PFASs

Table 6.6: The enriched pathways that are common in both PAHs and PFASs low exposure groups. The common genes in both groups within the same pathway are highlighted in bold.

Enriched pathway	**Differentially expressed genes in pathway after PAHs low exposure**	**Differentially expressed genes in pathway after PFASs low exposure**
Long term potentiation	*camk2a, gria1,**grin2b**, grm5, **mapk1**, plcb1*	*adcy1, **grin2b, mapk1***
Rap1 signalling pathway	*kit, **grin2b, mapk1**, itgb1, sipa1l1, plcb1, tln2, vegfd*	*crk, adcy1, **grin2b, mapk1***
Glutamatergic synapse	***dlgap1**, **grin2b, mapk1**, grm5, gria1, plcb1*	***dlgap1**, **grin2b, mapk1** adcy1*
Circadian entrainment	*camk2a, **grin2b, mapk1**, gria1, plcb1*	*fos, adcy1, **grin2b, mapk1***
ErbB signalling pathway	*cbl, camk2a, **mapk1**, pak2*	***mapk1**, ptpn11*
Platelet activation	*itgb1, **mapk1**, plcb1, tln2*	*dgkh, ptpn11, **mapk1***
cAMP signalling pathway	*atp2a3, **grin2b, mapk1**, gria1, camk2a*	*fos, **grin2b, mapk1**, adcy1, sstr2*
Retrograde endocannabinoid signalling	*gria1, grm5, **mapk1**, plcb1*	*ndufa5, ndufb2, ndufs6, adcy1, **mapk1***
Phospholipase D signalling	*kit, grm5, **mapk1**, plcb1*	*adcy1, dgkh, **mapk1**, ptpn11*
Oxytocin signalling pathway	***camk2a, mapk1**, cacnb1, plcb1*	*fos, **camkk1, mapk1**, adcy1*

The pathways that were enriched after low exposure to PAHs involved, the pathways that are related to cellular signalling and pathways related to neuronal signalling (Appendix Table A.1), whereas the enriched pathways after low exposure to PFASs included pathways related to signalling, oxidative stress response and neuronal signal transmission (Appendix Table A.2). A number of pathways that are enriched after exposure was common in both PAHs and PFASs low exposure groups (Table 6.6). Most of them were signalling pathways such as the Rap1 signalling pathway, ErbB signalling pathway, cAMP signalling pathway, retrograde endocannabinoid signalling, phospholipase D signalling pathway, and oxytocin signalling pathway. Other common enriched pathways include long-term potentiation, glutamatergic synapse, circadian entrainment, and platelet activation. The genes *grin2b, mapk1, camk2a and dlgap1* were common in both exposure groups of different enriched pathways wherein the *mapk1* was present in all common enriched pathways. The reactome pathway, 'Transport of inorganic cations/anions' was significantly enriched after PAHs low exposure, which was found by STRING enrichment analysis of DEGs. The high exposure to PAHs and PFASs did not caused enrichment of any pathways due to very limited number of differentially expressed genes after exposure to high concentration of exposure chemicals.

6.4 Most common genes in enriched pathways after PAHs low Exposure

A considerable number of genes that are differentially expressed after exposure to low concentration of PAHs were involved in more than five of enriched pathways (Figure 6.2). *mapk1* is one of the significant gene which is present in 17 enriched pathways. Other important differentially expressed genes which are part of different enriched pathways includes *plcb1, camk2a, grin2b, itgb1, vegfd, gria1, pak2* and *grm5*.

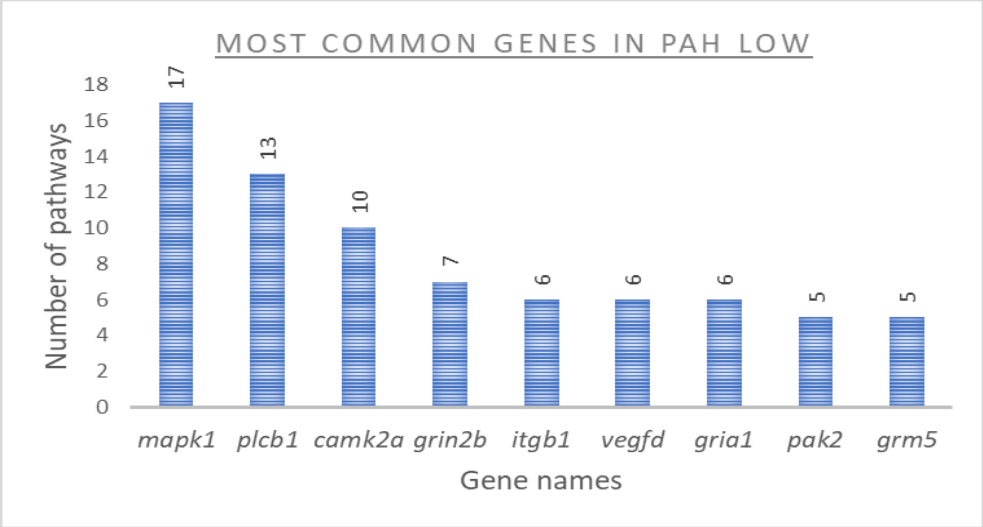


Figure 6.2: The most common genes that are involved in five and more enriched pathways after exposure to low concentration of PAHs.

6.5 Most common genes in enriched pathways after PFASs low Exposure

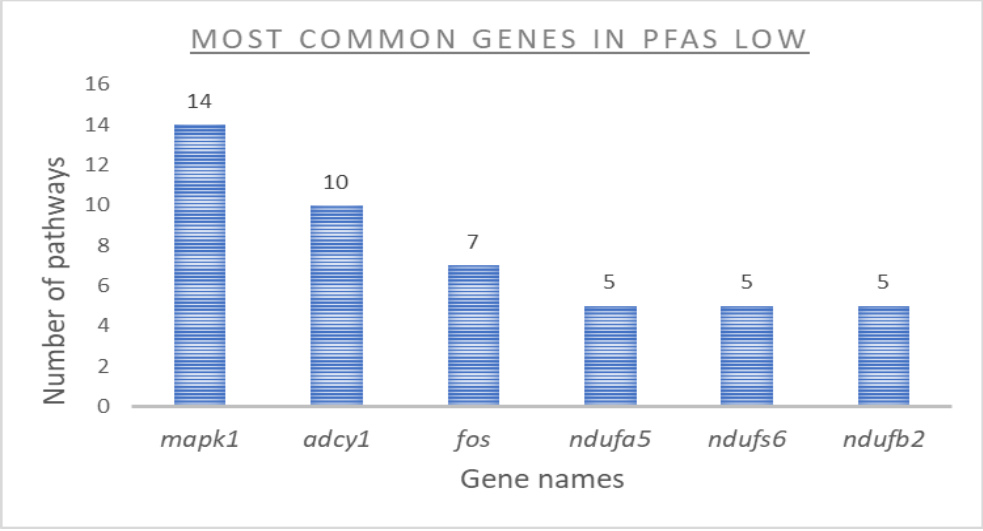
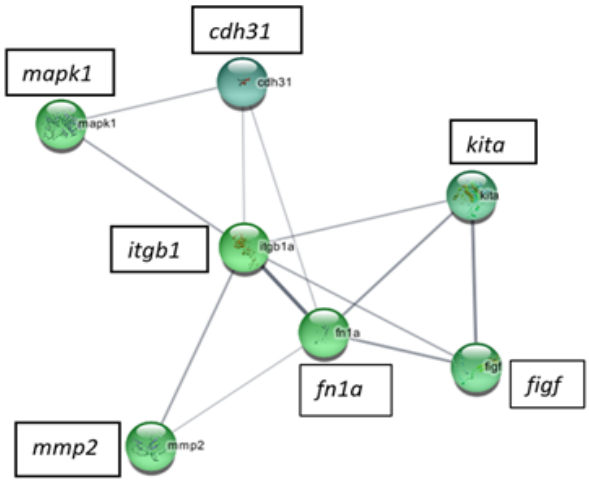


Figure 6.3: The most common genes that are involved in five and more enriched pathways after exposure to low concentration of PFASs.

A certain number of differentially expressed gene in low PFASs exposure group were involved in more than five of enriched pathways (Figure 6.3). Similar to PAHs low exposure group, the *mapk1* is one of the significant gene which is present in 14 enriched pathways in PFASs low exposure. Other important differentially expressed genes which are part of different enriched pathways includes *mapk1, adcy1, fos, ndufa5, ndufs6* and *ndufb2*.

6.6 MCODE cluster within DEGs after low PAHs exposure

Visualization and analysis of differentially expressed genes were done by creating a protein-protein interaction network of those genes in STRING software and then importing the network to Cytoscape software. A cluster (highly interconnected region of the protein-protein interaction network of differentially expressed genes) was produced by Cytoscape plugin, MCODE (Figure 6.4). The MCODE cluster consisted of seven highly interconnected genes, namely *mapk1, cdh31, kita, fn1a, figf, itgb1a and mmp2*, which are downregulated after exposure. The enrichment analysis done with STRING on the MCODE cluster, showed similar enriched pathways as that of the pathways enriched after analysis with the whole set of differentially expressed genes (Table 6.7). The common enriched pathways in MCODE cluster and in the whole DEGs set are focal adhesion, regulation of actin cytoskeleton, MAPK signalling, ECM-receptor interaction, adherens junction, GnRH signalling, cell adhesion molecules and apelin signalling pathway (Table 6.7).



Genes involved
mapk1
cdh31
kita
fn1a
figf
itgb1a
mmp2

Figure 6.4: The figure represents the MCODE cluster which depicts the most interconnected genes within the differentially expressed genes set after exposure to low concentration of PAHs.

Table 6.7: The result of enrichment analysis done by STRING on the MCODE cluster produced after exposure to low PAH exposure, which depicts enrichment in similar pathways (highlighted by bold) as that of the whole set of differentially expressed genes.

Category	Description	FDR value	Genes involved
KEGG Pathways	**Focal adhesion**	0.0004	*mapk1, itgb1a, fn1a,figf*
SMART Domains	Fibronectin type 2 domain	0.003	*mmp2, fn1a*
KEGG Pathways	**Regulation of actin cytoskeleton**	0.008	*mapk1, itgb1a, fn1a*
UniProt Keywords	Disulfide bond	0.01	*mmp2, itgb1a, kita, fn1a, figf*
KEGG Pathways	**MAPK signalling pathway**	0.014	*mapk1, kita, figf*
KEGG Pathways	**ECM-receptor interaction**	0.03	*itgb1a, fn1a*
KEGG Pathways	**Adherens junction**	0.034	*mapk1, cdh31*
KEGG Pathways	**GnRH signalling pathway**	0.034	*mmp2, mapk1*
KEGG Pathways	Melanogenesis	0.034	*mapk1, kita*
KEGG Pathways	**Cell adhesion molecules**	0.038	*itgb1a, cdh31*
KEGG Pathways	**Apelin signalling pathway**	0.04	*mapk1, cdh31*

6.7 Gene Ontology (GO) terms enriched after exposures

The enriched gene ontology terms (Cellular component and Molecular function) after each exposure were also identified during enrichment analysis (Table 6.8). The cellular component ontology (CCO) terms that are enriched after PAHs low exposure are related to membrane whereas the GO-CCO terms enriched after PAHs high exposure are related to nucleus and compartments within the nucleus. The GO-CCO terms enriched after PFASs high exposure is similar to PAHs low exposure group and is related to membrane whereas the CCO terms enriched after PFASs low exposure is linked to neuronal signal transduction. The molecular function ontology terms enriched in PAHs low exposure group are functions that are part of transmembrane transport whereas the molecular function enriched in PAHs high exposure includes DNA packaging and related functions. The exposure to PFASs low concentration has caused the enrichment of molecular functions that are significant for signal transduction along with NADH dehydrogenase (Ubiquinone) activity which is related to respiratory chain whereas the only differentially expressed gene in PFASs high exposure group is linked to the molecular function of G protein-coupled receptor activity.

Table 6.8: Gene ontology terms, [cellular component (Highlighted by grey) and molecular function] that are enriched due to differential expression of genes after PAHs low exposure.

ENRICHED GENE ONTOLOGY TERMS				
	PAH Low	**PAH High**	**PFAS Low**	**PFAS High**
Cellular component	Cell junction	Nucleosome	Synapse	Integral component of membrane
	Synapse	Chromosome	Somatodendritic compartment	
	Cell membrane	Nucleus		
	Membrane			
	Extracellular matrix			
	Secreted			
Molecular function	Amino acid transmembrane transporter activity	DNA binding	Cytoskeletal adaptor activity	G protein -coupled receptor activity
	Integrin binding	DNA packaging	Protein phosphatase 2A binding	
	Symporter activity	Nucleosomal DNA binding	Phosphotyrosine binding	
	Cadherin binding		NADH dehydrogenase (Ubiquinone) activity	
	Ephrin receptor binding		PDZ domain binding	
	Neurotransmitter receptor activity involved in the regulation of postsynaptic calcium ion concentration		Ligand-gated sodium channel activity	
	Extracellular matrix structural constituent		Kinase binding	
	Calcium ion binding			
	Collagen binding			
	Transmembrane transporter activity			
	Semaphorin receptor activity			
	Glutamate receptor activity			

6.8 Functional annotation clustering

Figure 6.5: The functional annotation cluster produced with DAVID software using DEGs from PAHs low exposure illustrates the grouped annotation terms along with the co-associated genes.

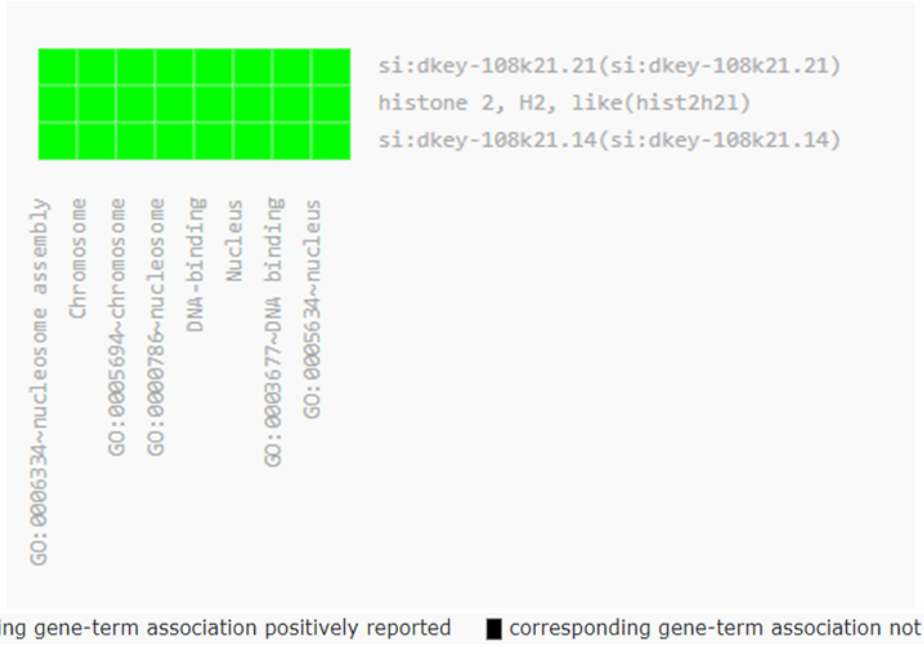


Figure 6.6: The functional annotation cluster produced with DAVID software using DEGs from PAHs high exposure illustrates the grouped annotation terms along with the co-associated genes.

Figure 6.7: The functional annotation cluster produced with DAVID software using DEGs from PFASs low exposure illustrates the grouped annotation terms along with the co-associated genes.

The functional annotation clustering was performed in different exposure groups with DAVID software which grouped the different redundant annotation terms together based on their co-association genes. The functional annotation cluster produced with the highest enrichment score in PAHs high exposure group showed annotation terms related to nucleus, and DNA binding and the co-associated genes includes *Hist2h2l, si:dkey-108k21.14,* and *si:dkey-108k21.21* (Figure 6.6). However, a large number of co-associated genes were found to be involved in functional annotation clustering in PAHs low exposure group, which are related to transmembrane (Figure 6.5). The functional annotation cluster produced within the PFASs low exposure group with the highest enrichment score showed the clustering of annotation terms related to respiratory chain and the co-associated genes includes *NDUFS6, NDUFA5* and *NDUFB2* (Figure 6.7) whereas the PFASs high exposure group with single differentially expressed gene did not produce any functional annotation cluster after analysis with DAVID software.

6.9 Protein-protein interaction network

The protein-protein interaction network of differentially expressed genes were created using STRING and were exported to Cytoscape for visualization. The expression data from differentially expressed genes were then merged in to the protein-protein interaction network created and the network was colour mapped with logFC values using continuous mapping. The nodes that are disconnected from network were removed. The network displays the downregulated and upregulated proteins in accordance with the colour legend used. The new proteins added into the network by the software were coloured yellow. The PPI network displays the highly interconnected genes at the protein level.

6.9.1 PPI network of differentially expressed genes - PAHs low exposure

The PPI network created from differentially expressed genes in PAHs low exposure produced a network with 63 nodes and 108 edges. The clustering coefficient of the network identified by network analyzer tool is 0.261. The colour legend used for continuous mapping of the PPI network with logFC values are provided in the figure 6.8. The minimum (-4.62) and maximum (4.62) values for continuous mapping were selected in accordance with the logFC values of DEGs produced by RNA sequencing.

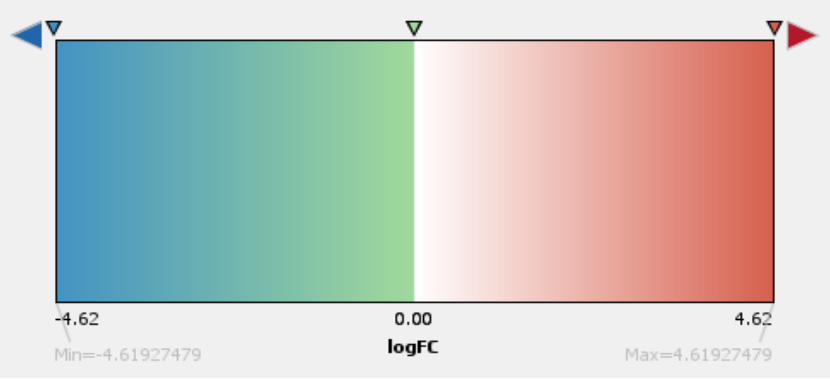


Figure 6.8: The colour legend used for the continuous mapping of the protein-protein interaction network of PAHs low exposure with logFC value.

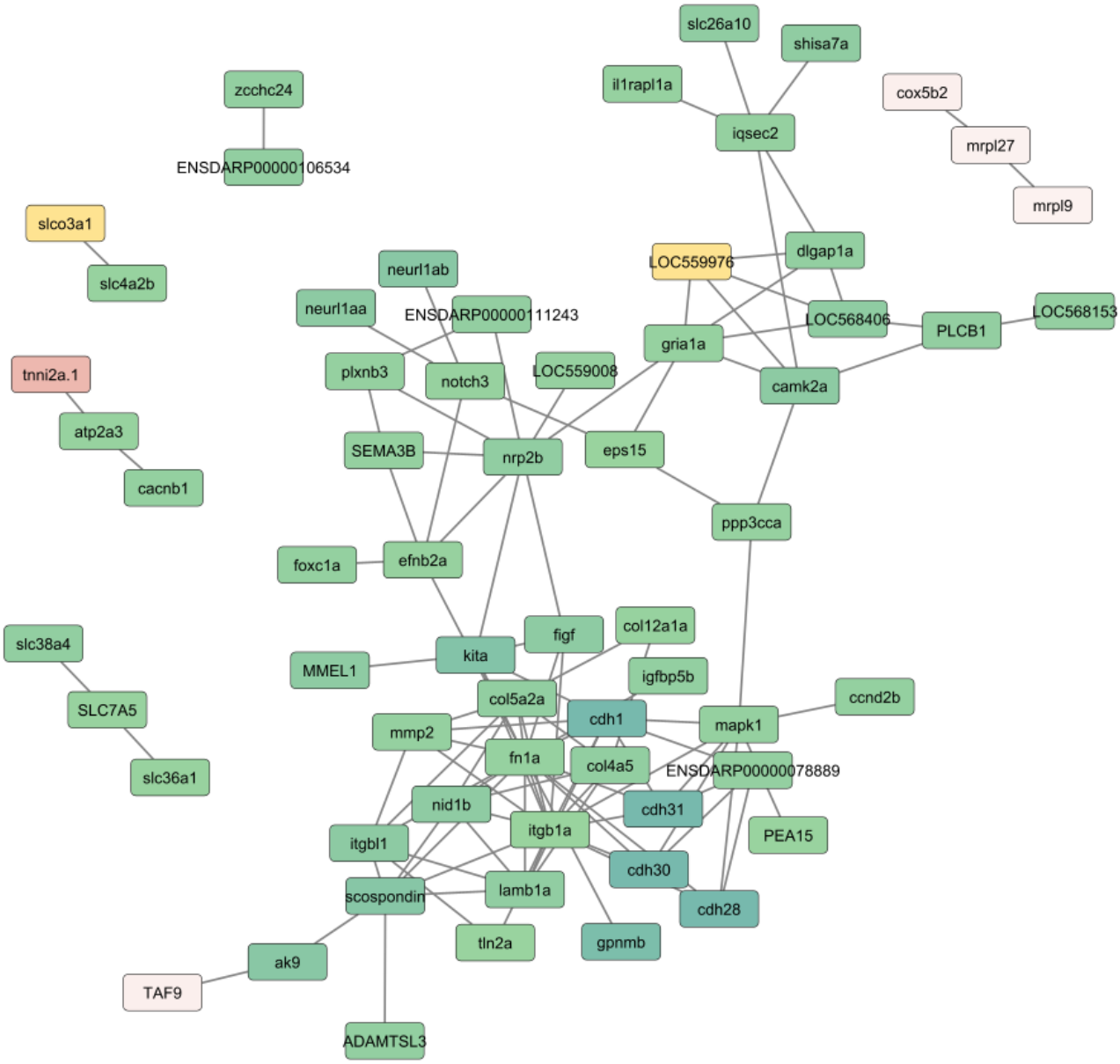


Figure 6.9: The protein-protein interaction network merged with expression data from DEGs in PAHs low exposure and colour mapped with logFC values.

6.9.2 ClueGO analysis on PPI from PAHs low exposure

The PPI network created in STRING was analysed using Cytoscape plugin ClueGO to identify clusters of proteins within the network through functional analysis. The ontologies included for analysis were biological processes, molecular function and KEGG pathways. Medium network specificity parameter was used with option to show only the significant pathways (P-Value less than 0.05). ClueGO reflects the relationships between the cluster terms based on the similarity of their associated genes. The major cluster terms involved are calcium dependent cell-cell adhesion via plasma membrane cell adhesion molecules, cell adhesion molecule binding, integrin binding,

semaphorin-plexin signalling pathway, ECM receptor interaction, epithelium migration, regulation of notch-signalling pathway and cell-cell adhesion mediated by cadherin (Figure 6.11).

Figure 6.10: The ClueGO clusters were produced from the PPI network of PAHs low exposure group.

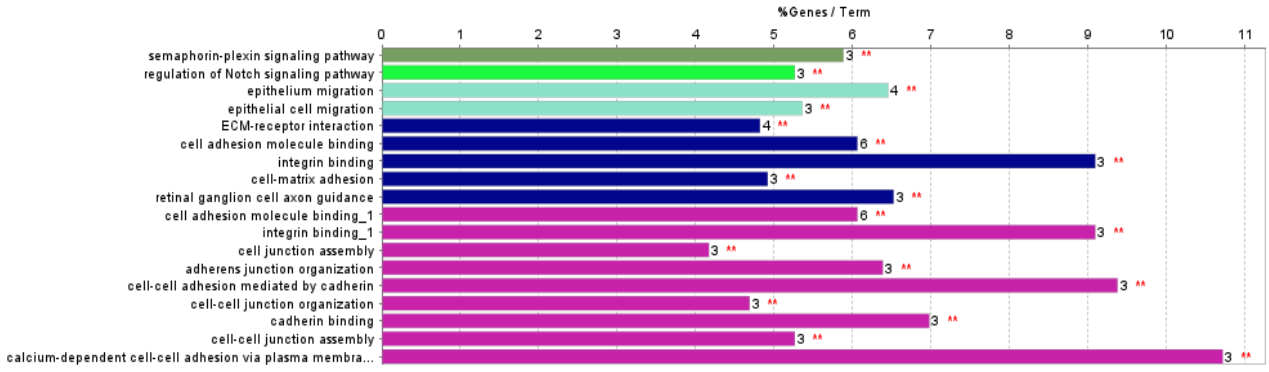


Figure 6.11: The ClueGO chart shows the different enriched terms to which the genes are clustered along with the percent of genes involved in each cluster.

6.9.3 PPI network of differentially expressed genes - PAHs high exposure

The PPI network created from differentially expressed genes in PAHs high exposure produced a network with 4 nodes and 2 edges. The clustering coefficient of the network identified by network analyzer tool is 0.00. No number of clusters could be produced from the limited number of DEGs in the network. The colour legend used for continuous mapping of the PPI network with logFC values are provided in the figure 6.13. The minimum (-0.72) and maximum (3.2) values for continuous mapping were selected in accordance with the logFC values of DEGs produced by RNA sequencing.

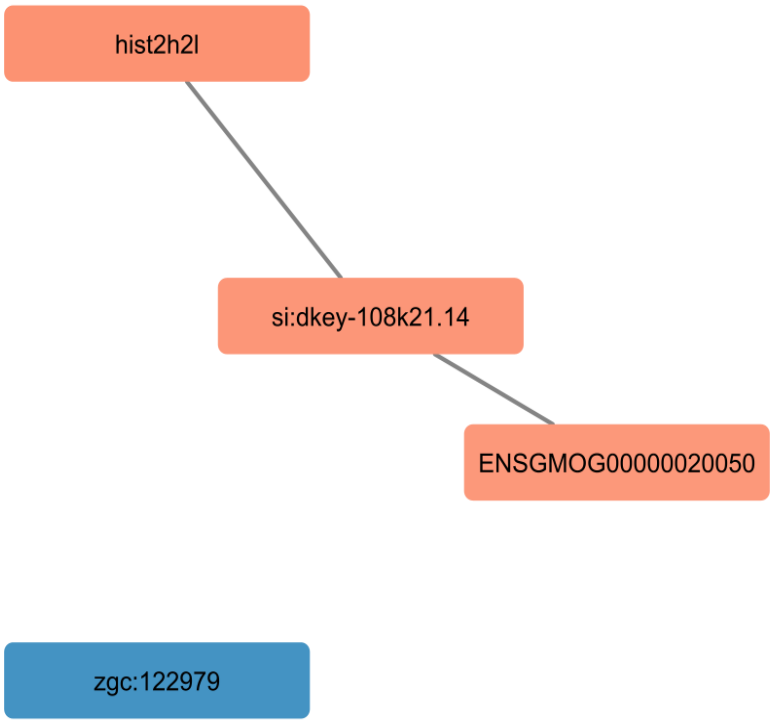


Figure 6.12: The protein-protein interaction network merged with expression data from DEGs in PAHs high exposure and colour mapped with logFC values.

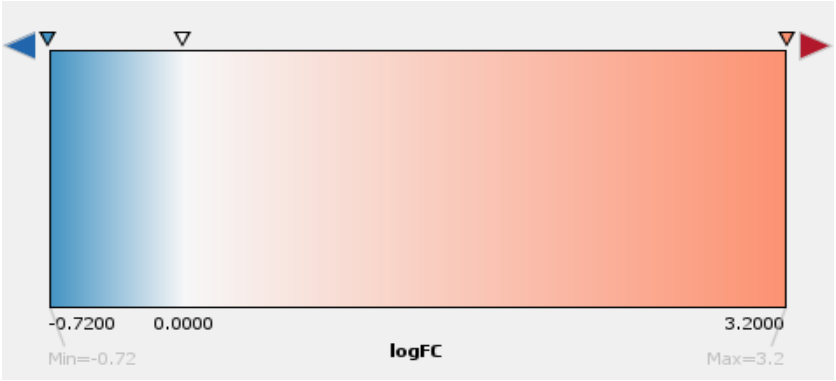


Figure 6.13: The colour legend used for the continuous mapping of the protein-protein interaction network of PAHs high exposure with logFC value.

6.9.4 PPI network of differentially expressed genes - PFASs low exposure

The PPI network created from differentially expressed genes in PFASs low exposure produced a network with 73 nodes and 177 edges. The clustering coefficient of the network identified by network analyzer tool is 0.257. The colour legend used for continuous mapping of the PPI network with logFC values are provided in the figure 6.15. The minimum (-6.61) and maximum (4.10) values for continuous mapping were selected in accordance with the logFC values of DEGs produced by RNA sequencing.

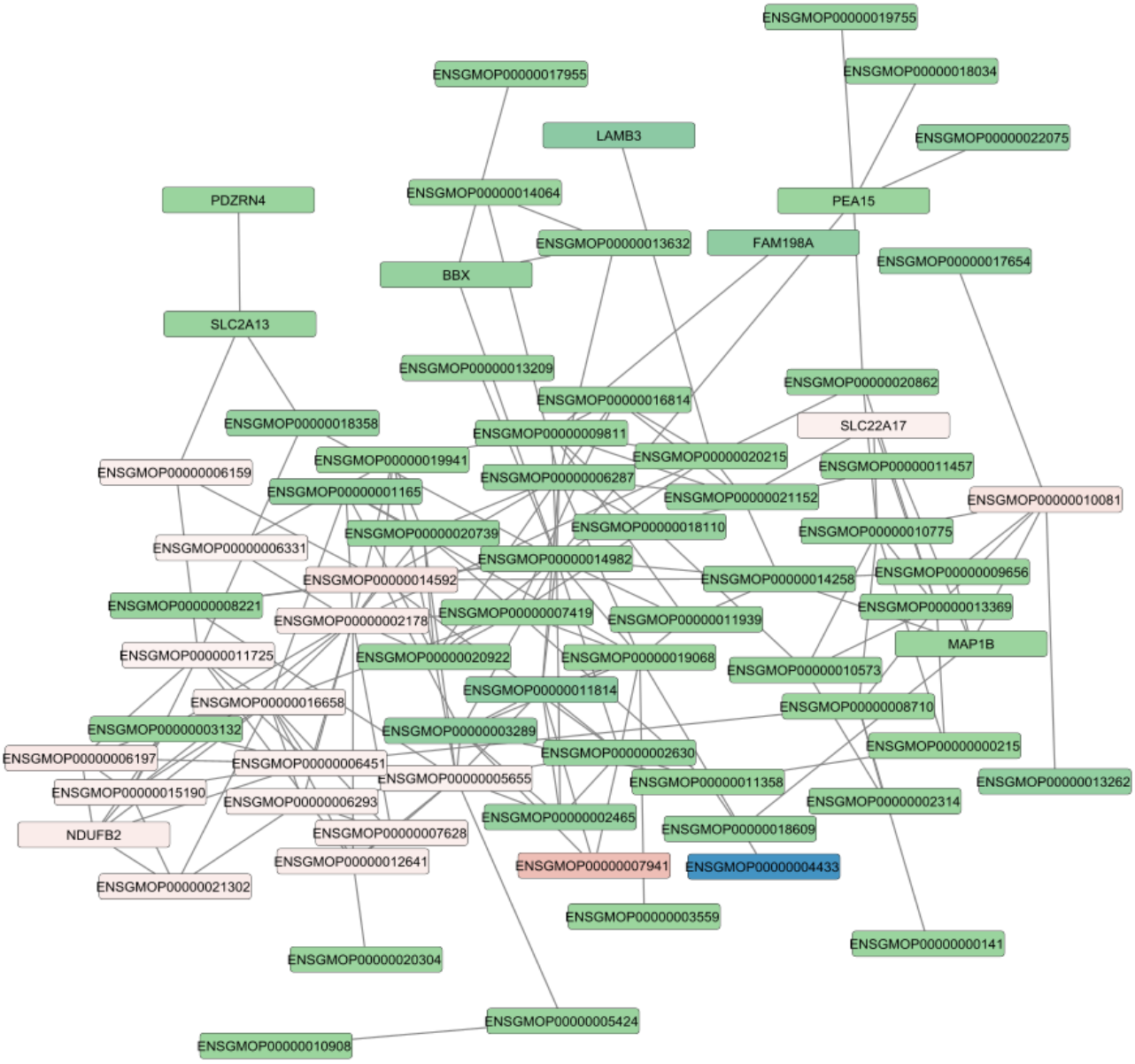


Figure 6.14: The protein-protein interaction network merged with expression data from DEGs in PFASs low exposure and colour mapped with logFC values.

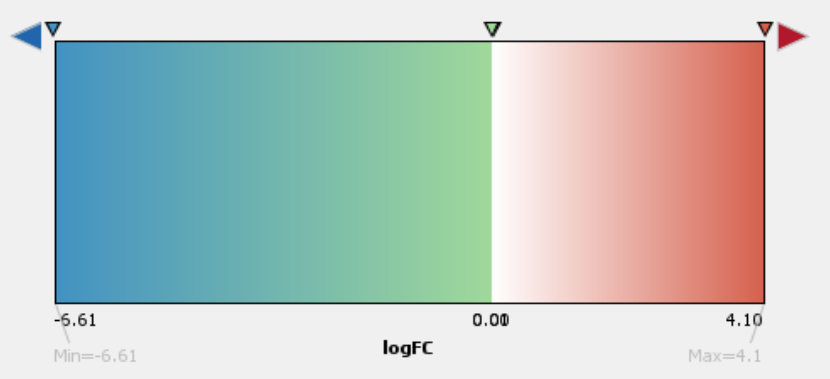


Figure 6.15: The colour legend used for the continuous mapping of the protein-protein interaction network of PFASs low exposure with logFC value.

6.10 RNA-seq validation by qRT-PCR

The gene concentrations of selected genes from PAHs and PFASs exposure were identified through qRT-PCR analysis. The concentration of each gene identified from qRT-PCR are represented as percentage of control. The concentration of all the selected genes in PAHs low exposure group is decreased when compared to the control which is in accordance with the RNA sequencing data where the genes are downregulated (Figure 6.16). Similarly, in the PFAS exposure groups, all the gene concentrations are decreased when compared to control (Figure 6.17. All the genes except for ndufa5 are in accordance with the RNA sequencing data in which the genes are downregulated. The ndufa5 gene is upregulated in the RNA sequencing data which is not in line with the qRT-PCR results produced.

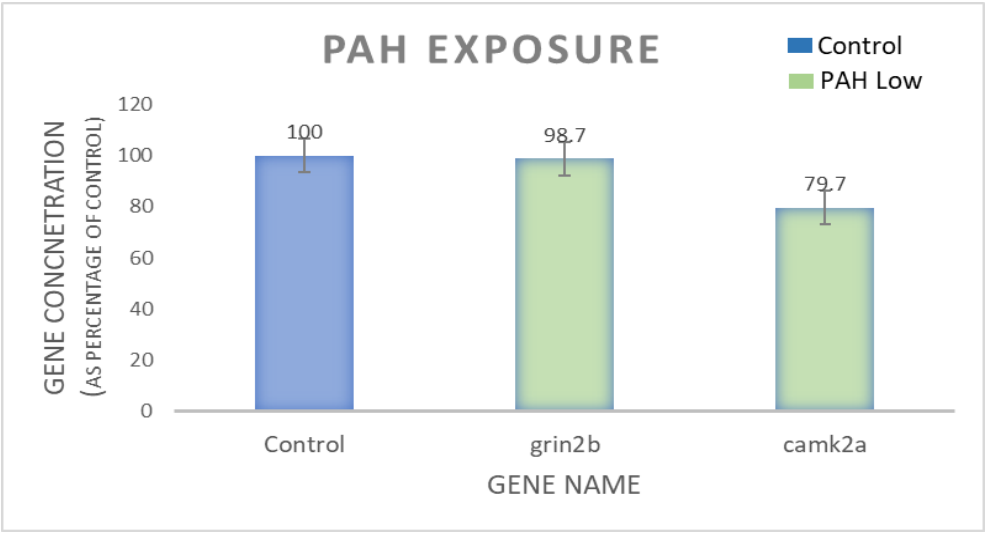


Figure 6.16: The gene concentrations of selected differentially expressed genes from PAHs exposure were represented as the percentage of control after qRT-PCR analysis.

Figure 6.17: The gene concentrations of selected differentially expressed genes from PFASs exposure were represented as the percentage of control after qRT-PCR analysis.

The correlation between the RNA-seq data and the qRT-PCR data produced was identified by creating a linear correlation graph between the fold change values (log2FC) for each gene produced by respective analysis. The correlation, denoted by r, is the measure of amount of linear association between two variables. The points in the graph are very close to the linear trend line with a $R^{[2]}$ value of 0.89 (Figure 6.18). The graph showed a large positive linear association between the data produced from RNA-seq and qRT-PCR analysis.

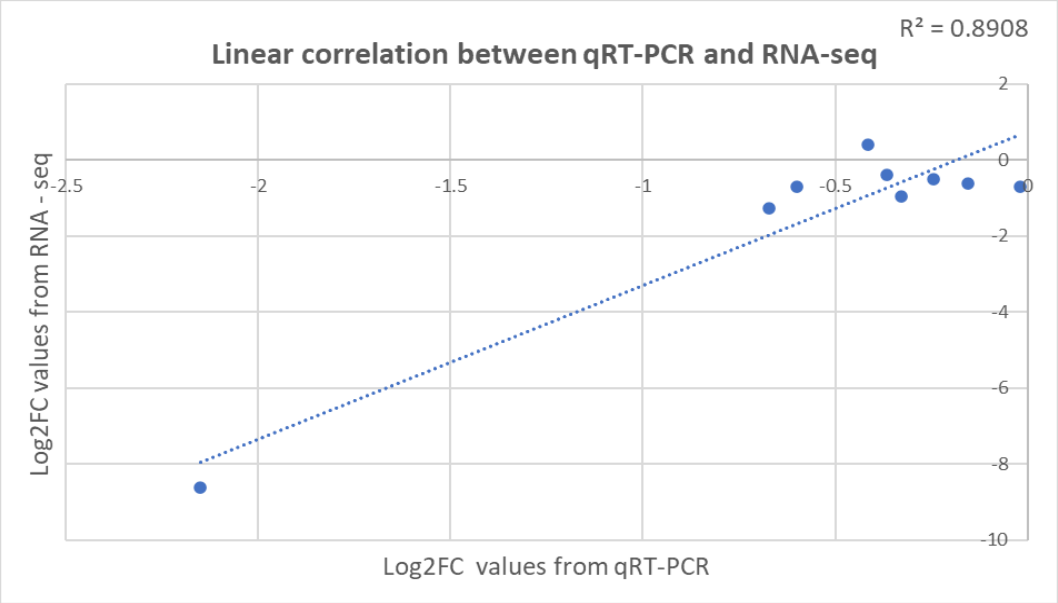


Figure 6.18: The linear correlation graph between qRT-PCR and RNA-seq data. The log2FC value is used as the comparison parameter.

7 Discussion

The degree of contamination of environmental matrices with polycyclic aromatic hydrocarbons (PAHs) and perfluoroalkyl substances (PFASs) has increased over the last several years due to anthropogenic activities and these chemicals pose a serious threat to the health of humans and ecosystems[20][118]. These chemicals are widely detected in the blood of human populations worldwide [20][119][120][121][122]. Understanding the molecular mechanisms underlying the toxicity of these harmful compounds is helpful in preventing the adverse health effects caused by the same. In this study, the juvenile female Atlantic cods (*Gadus morhua*) were exposed to environmentally relevant concentrations of polycyclic aromatic hydrocarbons (PAHs) and perfluoroalkyl substances (PFASs) to elucidate the effects of those xenobiotic compounds on the gene expression profiles in the brain tissue and to understand the effects of differential expression of genes at the systems level molecular functions.

7.1 Effects of congeners in exposure chemicals

The PAHs and PFASs exist in the environmental samples as complex mixtures[20]. Through various EU research activities, the mixture effects have received increased attention in the last few years[123]. Further research is required to understand the combined effects of contaminants on biological systems. In this study, both the PAHs and PFASs exposure group contained different congeners of respective compounds which are proved to be harmful compounds in the environment. Environmentally relevant concentrations of each of these congeners were used in the present study.

The congeners included in the PFASs exposure group are PFOS, PFTrA, PFNA, and PFOA. A study on whole blood, muscle, and liver samples of four freshwater fish species showed that the most predominant PFASs in the samples were, PFOS and perfluoroalkyl compounds with chain lengths from C10 to C14 such as PFTrDA[124]. The long-chain PFASs, such as PFOA and PFOS have high biological persistence and possess half-life of two years in humans[125][126][127]. Like the eight-carbon PFOA, the nine-carbon PFNA is a developmental toxicant and an immune system toxicant which act as an agonist of the nuclear receptors PPARα and PPARγ[128]. The PAHs exposure group contained Naphthalene, Phenanthrene, Dibenzothiophene, Pyrene, B(a)P and Fluorene. The best-known PAH compound is benzo[a]pyrene (B[a]P), which was classified among the highly genotoxic compounds in 2012 and according to the International Agency for Research on Cancer (IARC) it belongs to group 1—carcinogen in humans (IARC 2012)[129]. Literature studies have showed that all the other congeners of PAHs involved in the study are toxic and are found to be harmful to humans and environment[130][131][132][133][134].

7.2 Transcriptional responses to extracellular signals

The extracellular signals such as neurotransmitters, hormones, light, odorants, and other stimuli (physical or chemical) controls the cellular functions[103]. Only limited number of extracellular signals can cross the plasma membrane through diffusion in order to interact with their intracellular receptors. However, most regulatory factors are water-soluble. These water soluble regulatory factors interact with membrane receptors to induce a signal transduction process which leads to intracellular signal formation or activation and integration of signaling cascades[103]. The exposure to the different concentrations of PAHs and PFASs can act as external stimuli which affect the functioning of the fish at the molecular level. The changes in the functions at the molecular level are the result of differential expression, interactions and cross-talk between many genes[18]. The multi functionality of genes which is time and situation dependent and the equilibrium of genes

between many simultaneous pathways are other factors that affect the functioning at molecular level in the cell[18]. The gene expression regulation is very significant for the cell to adapt to a variable environment and external stimuli. The transcriptional responses to external signals can consist of many hundreds of genes that can be grouped into different categories based on cell-type, signal specificity, kinetics of induction, and duration of the response[135].

7.3 Signal transduction and stress response in fish

Signal transduction is an important process involved in stress and chemical responses in fish. The exposure to PAHs and PFASs can therefore affect biological processes and pathways that are related to signaling. This was observed in exposure group with low concentration of PAHs, where the pathways enriched were related to signal transduction. The enriched pathways were grouped in to two groups, which are pathways related to cellular signaling, and pathways related to neuronal signal transmission (Appendix table A.1). The focal adhesion pathway and extra cellular matrix (ECM) receptor interaction pathway were the most significantly enriched pathways which have vital role in transmembrane signaling (Appendix figure B.1 and figure B.2). The focal adhesion's serve as mechanical linkages to extra cellular matrix (ECM) and serve as biochemical signaling hub to concentrate and direct several signaling proteins at the integrin binding site[136]. The ECM receptor interaction and focal adhesion are interrelated processes with same genes from DEG data set involved in their pathways which includes *col4a5, col5a2a, fn1a, itgb1a,* and *lamb1a.* Focal adhesion pathway also includes *mapk1* gene in addition to the common genes. The *itgb1a* (Integrin beta1a) gene, which is involved in 6 enriched pathways has molecular function of collagen binding in cell matrix adhesion[137]. The integrin family receptors stimulate the cellular proliferation and migration through the focal adhesion pathway by the activation of PTK2, VASP and TSP1 proteins[138]. The ECM receptor interactions takes place through cell to cell adhesion's which are really important for transmembrane signaling.

The toxicity mechanism of PAHs are considered to be interference with the functioning of cellular membranes as well as with the enzyme systems which are related to the membrane[18]. The differentially expressed transcripts in PAHs low exposure also includes *itgb1a, cdh1, col4a5, col5a2a, lamb1a, tln2a and fn1* which codes integrin, cadherin, collagen, lamnin, talin and fibronectin respectively, which are structural and membrane proteins. The enriched molecular functions included integrin binding, cadherin binding, ephrin receptor binding, fibronectin binding, collagen binding and semaphorin receptor activity which propose that the functioning related to the integrity and transport across the membrane is affected after exposure to low concentration of PAHs. The enriched protein domains produced from SMART also included doamins related to membranes such as domains found in plexins, and integrins, cadherin predomain, and semaphorin domains. In a study conducted on Rainbow trout to explore the gene expressions in brain in response to handling stress, the expressions of transcripts of structural proteins, signal transduction and binding of metal ions were mainly affected[139].

7.4 Interaction between signaling pathways during stress response

The intracellular signaling is a complex process. Only limited number of signaling pathways act in isolation, and the interactions between pathways or molecular cross-talks are the important factors that plays critical role in coordination of signal transduction[140]. The pathways that are enriched in low PAHs exposure are inter connected to each other by activating common signaling pathways such

as MAPK1 signaling pathway, PI3-Akt signaling pathway, calcium signaling pathway, Ras signaling pathway, Wnt signaling pathway, cAMP signaling pathway, phosphatidyl inositol signaling pathway and regulation of actin cytoskeleton. Each of the enriched pathways in PAHs low exposure activates at least one of these common signaling pathways(Appendix figure B.1, B.2).

The calcium signaling has a significant role in activation of Ca2+ channels, and receptors in response to regulatory factors. These regulatory factors can be hormones, neurotransmitters, and growth factors[141]. An article about ´Calcium signaling and brain functions‘, reported that the functions of intracellular Ca2+ is mediated by CAMK, Ca2+/calmodulin-dependent protein kinases[141]. The *Camk2a* gene is one of the differentially expressed gene after PAHs low exposure which is involved in long term potentiation and is part of 10 enriched pathways. The calcium is involved in synaptic activity and participates in the depolarizing signal transduction[142]. As a result, calcium (Ca2+) is of critical importance to neurons. The term "Calcium" is one of the most significant annotated keywords identified by STRING to which 10 genes from the DEGs set were related. Several signal transduction pathways including the cAMP regulatory pathways and Erk/MAP kinase are induced by postsynaptic Ca2+ increase. The expression of genes which are required for late-phase long term potentiation (L-LTP) is increased by the convergence of these pathways at the level of the CREB/CRE transcriptional pathway (KEGG PATHWAY: hsa04720). The long term potentiation is another significant enriched pathway after exposure to low concentration of PAHs.

A number of common genes were identified within enriched pathways of low PAHs exposure, as these pathways are inter connected between each other (Figure 6.2). The most common gene from the DEG set which was present in 17 enriched pathways is *mapk1*. The gene expression of multiple cellular proteins are regulated by MAPK1 (Mitogen-activated protein kinase 1) by integrating the extracellular clues from cell surface receptors[143]. The MAPK signaling can affect both physiological and structural characteristics of neurons[144] and influences various neuronal properties including long-term potentiation in brain[144]. The long term potentiation pathway is enriched after exposure to low PAHs and requires modification of postsynaptic AMPA receptors and a increase in presynaptic neurotransmitter release for its maintenance[145]. The *gria1* gene, which encodes for a subunit of ionotropic glutamate AMPA receptors[146] is another important gene from DEG set which is involved in 6 enriched pathways. The functioning of glutamate AMPA receptors (AMPARs) are very important for excitatory synaptic transmission and these receptors act as major excitatory receptors in the central nervous system (CNS) of vertebrates[147]. The major regulator of integrated stress response (ISR) is the transcription factor cAMP response element binding protein (CREB). The expression of CREB gene is necessary for the specific maintenance of the GluA1 subunit coded by *gria1* gene[148][149].

7.5 Clustering of DEGs in PAHs low exposure with MCODE

The MCODE cluster produced from the protein-protein interaction network are often protein complexes and parts of pathways[150]. The MCODE cluster produced from DEGs of PAHs low exposure included genes that are important for transmembrane signal transduction. The information on various types of signals within the cell are transmitted by a large class of integral membrane proteins known as receptors. Receptor complexes, which are assemblages of receptors and their interacting proteins are considered as important units of signal transduction for several receptors including ligand-gated ion channel, G protein coupled, and receptor tyrosine kinase[151]. The *kita* gene encodes for tyrosine-protein kinase that acts as cell-surface receptor for the cytokine kitlg/scf whereas, the *fn1a* gene encodes fibronectin with molecular function of signaling receptor binding.

The *Vegfd* encodes for C-fos induced growth factor with vascular endothelial growth factor receptor binding activity. *Itgb1a* encodes integrin beta 1a with molecular function of collagen binding involved in cell-matrix adhesion however, *mmp2* encodes matrix metalloproteinases2, which is involved in collagen degradation. *Cdh31* encodes for cadherins, which are calcium dependent cell adhesion proteins involved in cell-cell adhesion and last member of cluster is *mapk1* which encodes mitogen activated protein kinase which has important function in cell surface receptor signaling pathway. The MCODE cluster represents the most significant genes from the DEG set as these genes possess the similar molecular functions as from the GO term category of molecular functions in the enrichment analysis of whole DEGs set of low PAHs exposure (Table 6.8). The STRING enrichment analysis on MCODE cluster also showed enrichment in similar pathways when compared to the enrichment analysis done on entire DEG set from PAHs low exposure (Table 6.7). The functional annotation clustering results (Figure 6.5) also highlighted the transmembrane as the most important annotation term.

7.6 Solute Carrier (SLC) superfamily transporters expression after PAH low exposure

The blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) are dynamic tissues in the brain which has a very significant role in central nervous system, wherein they regulate the influx of organic cations and anions into and out of the CNS compartment[152]. These functions are accomplished by the expression of transporter proteins representing numerous transporter families such as solute carrier (SLC) superfamily transporters[152]. The SLC transporters can interact with environmental toxins or toxicants[152]. The DEG data set from PAHs low exposure contains six transporters from SLC superfamily, such as, *slc7a3a*(Solute carrier family 7 member 3a), *slc38a4*(Solute carrier family 38 member 4), *slc7a5*(Solute carrier family 7 member 5), *slc26a10*(solute carrier family 26 member 1), *slc36a1*(solute carrier family 36 member 1), and *slc4a2b*(solute carrier family 4 member 2b). These genes encode for significant transporter proteins which act as the transport mechanisms that control the CNS penetration, disposition and clearance of xenobiotic compounds in order to regulate CNS homeostasis and impact neuronal health[153][154][155][156][157].

7.7 Chemical modification of DNA

The chemical modifications of DNA are induced by exposure to genotoxic chemicals and their reactive metabolites[158]. The ability of several xenobiotic compounds to be converted to reactive metabolites has been known for a long time[159]. Certain xenobiotic compounds or their metabolites, can interact and bind covalently to macromolecules such as DNA in a irreversible manner. PAHs are metabolized to reactive diol epoxide enantiomers which can bind covalently to DNA and form DNA adducts with different structures and motifs[160][161]. The DNA adduct formation by a reactive intermediate has been recognized to be an important step in carcinogenesis[159].The presence of adducts have been evaluated in marine and aquatic species as an indicator of environmental occurrence of PAHs[160][162]. It was observed in different studies that genotoxic effects of PAHs were dose-dependent, and the DNA adduct level increased with the increased B[a]P concentration[163][164][165]. This dose-dependency of genotoxicity can be a factor that DNA adducts may have been formed only after exposure to PAHs high exposure.

The GO terms (molecular function) related to DNA and nucleus were enriched after exposure to PAHs high exposure with differentially expressed genes that possess molecular functions such as DNA binding and DNA packaging, which is also evident in functional annotation clustering (Figure 6.6). The gene *hist2h2l* is one of the significantly differentially expressed gene in the PAHs low exposure which codes for Histone H2B3. The modification of histones determines whether DNA wrapped around histones can be transcribed and influences the rate of transcription, which ultimately affects the functioning at the molecular level[166]. The protein domains which are enriched in PAHs high exposure are also related to histones, which includes Histone H5, Histone H1/H5, H15, and Histone H2B. The STRING enrichment analysis shows that the biological process nucleosome assembly, and the annotated keywords chromosome, and nucleosome core were significantly enriched after exposure to high concentration of PAHs. The assembly and disassembly of nucleosome impact the regulation of nuclear gene functions, such as transcription, replication, repair, and recombination.

7.8 Common effects of low PAHs and low PFASs exposures

The exposure to low concentrations of PAHs and PFASs showed similar toxicity response, which is evident by the presence of common enriched pathways after exposure to each group. A considerable number of significantly enriched pathways (10 pathways) which are related to signal transduction were common in low exposure group of PAHs and PFASs (Table 6.6). The common genes that were involved in these enriched pathways are *grin2b, mapk1, dlgap1* and *camk2a*. The exposure to low concentration of PAHs and PFASs also lead to enrichment in biological processes related to reproduction and development process. GnRH signaling pathway which is related to reproduction were also significantly enriched in both PAHs and PFASs low exposures. Other pathways related to reproduction that are enriched in PAHs low exposure includes relaxin signaling pathway and oxytocin signaling pathway. The exposure to low concentration to PAHs and PFASs may affect the reproductive processes in fish as supported by previous studies, which have shown that exposure to PAHs and PFASs can cause developmental and reproductive toxicity in different fish[86][167][168].

7.9 Pathways enriched after PFAS low exposure

The low exposure to PFASs showed enrichment in pathways that are related to signal transduction and oxidative stress which were divided into 3 categories such as 1) Pathways related to signaling, 2) Pathways related to oxidative stress response, and 3) Pathways related to neuronal signal transmission (Appendix table A.2). In a study conducted previously, the comparison of expression patterns of PFOA, PFNA and PFOS, with the PFASs mixture shows that the expression pattern of the PFASs mixtures are similar to expression patterns produced by PFOS. This suggest that the effects induced by the PFASs congener mixtures is largely attributed to the PFOS[41]. The developmental toxicity was induced in zebrafish embryos by exposure to PFOS and they were also more potent in causing developmental toxicity when compared to PFNA and PFOA [169]. A report on chronic effects of waterborne PFOS exposure showed that exposure of maternal zebrafish to low concentrations of PFOS could result in deformity and mortality of the offspring[170]. Another laboratory study of the aquatic toxicity of perfluorooctane acid and perfluorooctyl sulfonates on zebrafish embryos showed that PFOS could cause larval abnormalities[171]. These studies elucidate the effect of low exposure of PFASs on developmental processes of fish. The biological processes such as xenobiotic response, signal transduction, processes related to respiratory chain and developmental processes were also enriched after low PFASs exposure. The Reactome pathway, 'Activation of AP-1 family of transcription factor' is significantly enriched after exposure to PFASs low concentration. The

Activator protein 1 (AP-1) is a family of bZIP transcription factors which regulates the neural gene expression by extracellular signals[172].

7.10 PFAS-induced oxidative stress response

The important genes that are differentially expressed after PFASs low exposure and related to signaling involved *mapk1* (involved in 12 pathways), *adcy1* (involved in 9 pathways) and *grin2b* (involved in 4 pathways). Most of the signaling pathways enriched after PFASs low exposure were similar to the enriched pathways after PAHs low exposure. However, the functional annotation clustering showed that the most significant differentially expressed genes in PFASs low exposure are *ndufa5, ndufb2,* and *ndufs6* which are related to respiratory electron transport. These genes encode for proteins that are subunits of NADH dehydrogenase (ubiquinone), which is the largest of the five complexes of the electron transport chain[173]. NADH dehydrogenase is located in the inner membrane of mitochondria[173]. The differential expression of these genes can affect the electron transport and mitochondrial-respiratory complex I assembly which can lead to mitochondrial dysfunction.

The alterations in NADH dehydrogenase can lead to mitochondrial dysfunction which is casually linked to oxidative stress-induced cellular injury and cytotoxic cell death[174]. In the present study, the pathways related to oxidative stress response such as oxidative stress induced senescence, reactive oxygen species, chemical carcinogenesis and oxidative phosphorylation were also enriched after low exposure to PFASs. A similar result was found in a study conducted on Atlantic cod, where the reactive oxygen species pathway were significantly enriched after exposure to PFOS and also for a mixture of PFAAs[41]. The oxidative stress is well-known as a non-organ directed toxicity following the exposure to PFAAs, and has been widely reported both in fish and other species[41]. The brain is considered highly vulnerable to oxidative damage because of its high consumption of oxygen, high content of easily peroxidizable polyunsaturated fatty acids (PUFA) and non-heme iron that contribute to substantial oxidative damage, and lower levels of antioxidant defenses[175]. A study on tissue distribution of perfluorodecanoic acid have showed that these chemicals can cross the blood–brain barrier[176]. The penetration of PFASs across the blood-cerebrospinal fluid barrier was also found in humans in a study conducted on hospital in-patients using UPLC/MS/MS[177].

7.11 PFAS high exposure

The exposure to PFASs high led to downregulation of Taste receptor, type 2 gene, coding for a taste receptor belonging to the TAS2R family of bitter receptors which is widely expressed in extra-oral tissues[178]. The receptor is an integral component to membrane having G protein-coupled receptor activity[179]. A study conducted on the TAS2R10 (Type 2 taste receptor 10) elucidate the biological roles of the gene beyond bitterness sensing which include 'cellular protein metabolic process', 'protein modification process', 'cellular protein modification process' and 'cellular component assembly'[180]. In the GO category, molecular function, TAS2R10 was categorized in to 'cytoskeletal adaptor activity', and 'cyclin binding'[180]. The functional partners of the gene were identified by using STRING software and the enrichment analysis were done on the whole network produced (Appendix Figure C.1, C.2). The analysis showed enrichment in biological processes and molecular function related to signaling and chemical response such as G-protein coupled receptor activity which points towards the role of gene in stress response (Appendix Table C.1). G-protein-coupled receptors (GPCRs) represent a major gateway through which cells convert external cues into intracellular

signals and respond with appropriate actions[181].

7.12 Exhaustion of stress response

The studies related to stressor exposure shows that, a unifying response in fish to exposure to stress is the activation of the hypothalamus-pituitary-inter-renal (HPI) axis, which leads to the release of cortisol into the circulation[182]. If an animal is exposed to very stressful environmental conditions, they can reach to a stage of exhaustion, which is characterized by decreased levels of cortisol and an inability to further mount a stress response[183]. A study conducted on toadfish glucocorticoid stress response to PAHs exposure showed that when the fish were subjected to additional crowding stress, they were no longer able to mount a stress response, suggesting an exhaustion of the stress response[184]. This reduction or exhaustion in stress response can be a reason that a smaller number of genes were differentially expressed after high exposure of PAHs and PFASs in female Atlantic cod when compared to the low exposure group of respective chemicals. These dissimilarities in transcriptomic effects between low and high concentrations of exposures may also be linked to differences either in chemical uptake or toxic potential.

8 Conclusion

The ubiquity of PAHs and PFASs in the natural environment and their wide range of biological toxicities on different species of organisms has made these compounds the most concerning organic pollutants. These pollutants are very persistent and the remediation of PAHs and PFASs from the environment has always been a global concern. The environmental systems are very complex containing mixtures of these contaminant compounds. The contaminant levels which are above the environmental quality standards (EQSs) can produce alterations and interfere with biological machinery and integrity. The suitability of water for many human usages are determined by the amount and characteristics of pollutants present in the freshwater aquatic systems. The habitat of fish and other aquatic wildlife is also effected by pollution of freshwater ecosystems by xenobiotic compounds. The proportional increase in human population, water use, species extinction, climate change, and development pressures along with pollution level propose that the aquatic systems will remain under anthropogenic threat to pollution in the future. The continuous monitoring of all the priority xenobiotic compounds like PAHs and PFASs are very significant in environmental samples since they have continued to be used in domestic activities and industrial applications. There is a lack of sufficient toxicological information on these group of xenobiotic chemicals which makes the toxicological assessments very significant.

The aim of the present study was to investigate the effects of PAHs and PFASs, both high and low concentrations, on the brain tissue of Atlantic cod using transcriptome sequencing and analysis of deferentially expressed genes with bioinformatics tools. In fish, during the stress response, a signaling cascade is activated by the simultaneous activation of neural circuits in the central nervous system. The hypothalamus is the central modulator of stress and leads to the production of essential neuroendocrine mediators required for this activation[185]. In this study, the female Atlantic cod exposed to chemical stress, through different concentrations of PAHs and PFASs showed differential expression in genes related to signal transduction which is necessary for stress response in brain. The exposure to PAHs low exposure and PFASs low exposure showed great similarity in response, with enrichment in identical signaling pathways sharing common genes. The exposure to low concentration of PFASs also caused differential expression of gene related to oxidative stress. As aforementioned, the differentially expressed genes in PFASs high exposure is involved in 'cytoskeletal adaptor activity', which is also categorized as the enriched molecular function in PFASs low exposure which shows the similarity in stress response in low and high concentrations of PFASs. The exposure to PAHs high concentration showed a dissimilar response with differential expression in genes related to DNA binding and packaging. The genotoxic effects of PAHs are dose-dependent and maybe the reason for the difference in stress response at different concentrations of PAHs exposure. The study conducted obtained results that explicate the mixture effect of different congeners of PAHs and PFASs exposures at molecular functional level, which highlighted the complexity of environmental systems where multiple PAHs and PFASs are present. This study concludes that the exposure to PAHs and PFASs, act as extracellular stimuli which signals the brain for downstream effector signalling to change the expression of different genes accordingly, which can affect the molecular function in the fish at the systems level.

9 Future perspectives

The studies suggest that the exposure to congeners of PAHs and PFASs can cause unpredictable, species-specific effects. However, the number of studies on the mixture effects of PAHs and PFASs are insufficient but very significant as both of these chemicals are present ubiquitously in the aquatic environment. Different studies have found that the Perfluoroalkyl acids can function as agonists or antagonists, in the presence of other chemicals resulting in different directions of combined toxicity. Therefore, the mixture toxicity produced with exposure to both PFASs and PAHs can be pursued as a further research opportunity. A systematic assessment of the potential implications of these mixtures are required to understand whether and how these compounds might jointly affect the integrity of aquatic ecosystems.

10 Bibliography

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Appendices

A Pathways enriched after exposure

A.1 Pathways enriched after exposure to low concentration of PAHs

Table A.1: Pathways that are enriched due to differential expression of genes after low exposure to Polycyclic aromatic hydrocarbons (PAHs), which are generated from DAVID and STRING software.

Pathway term – KEGG Pathways	**Genes from data set involved in pathway**	**P-value**
1. Pathways related to cellular signaling		
Focal adhesion	*ccnd2, fn1, itgb1, lamb1, mapk1, pak2, tln2, vegfd, col4a5, col5a2a*	2.1E-4
Rap1 signaling pathway	*kit, grin2b, itgb1, mapk1, plcb1, sipa1l1, tln2, vegfd.*	2.7E-4
ECM receptor interaction	*col4a5, col5a2a, fn1a, itgb1a, lamb1a*	1.5E-2
ErbB signaling pathway	*cbl, camk2a, mapk1, pak2*	1.5E-2
GnRH signaling pathway	*camk2a, mmp2, mapk1, plcb1*	1.9E-2
PI3K-Akt signaling pathway	*kit, ccnd2, fn1, itgb1, lamb1, mapk1, vegfd*	2.1E-2
Thyroid hormone signaling pathway	*atp2a3, mapk1, notch3, plcb1*	3.8E-2
Relaxin signaling pathway	*mmp2, mapk1, plcb1, vegfd*	4.5E-2
cAMP signaling pathway	*atp2a3, camk2a, gria1, grin2b, mapk1*	4.7E-2
Ras signaling pathway	*kit, grin2b, mapk1, pak2, vegfd*	5.4E-2
Calcium signaling pathway	*atp2a3, camk2a, grm5, plcb1, vegfd*	6.0E-2
Phospholipase D signaling pathway	*kit, grm5, mapk1, plcb1*	6.3E-2
Cell adhesion molecules	*cdh3, itgb1, jam3, nectin1*	6.4E-2
Oxytocin signaling pathway	*cacnb1, camk2a, mapk1, plcb1*	6.9E-2
2. Pathways related to neuronal signal transmission		
Long term potentiation	*camk2a, gria1, grin2b, grm5, mapk1, plcb1*	5.3E-5
Axon guidance	*camk2a, efnb2, itgb1, mapk1, pak2, plxnb3, sema3b, sema4g.*	1.1E-4
Glutamatergic synapse	*dlgap1, gria1, grin2b, grm5, mapk1, plcb1.*	6.4E-4
Circadian entrainment	*camk2a, gria1, grin2b, mapk1, plcb1.*	2.9E-3
Dopaminergic synapse	*camk2a, gria1, grin2b, plcb1*	5.4E-2
Retrograde endocannabinoid signalling	*gria1, grm5, mapk1, plcb1*	6.3E-2

A.2 Pathways enriched after exposure to low concentration of PFASs

Table A.2: Pathways (KEGG pathways and Reactome pathways) that are enriched due to differential expression of genes after low exposure to Perfluoroalkyl Substances (PFAS).

Pathway term (KEGG and Reactome)	**Genes from data set involved in pathway**	**P – value**
1. Pathways related to signaling		
Retrograde endocannabinoid signaling	*ndufa5, ndufb2, ndufs6, adcy1, mapk1*	3.1 E-3
cAMP signaling pathway	*fos, adcy1, grin2b, mapk1, sstr2*	1.3 E-2
Phospholipase D signaling pathway	*adcy1, dgkh, mapk1, ptpn11*	2.3 E-2
MAPK1 (ERK) activation	*mapk1, ptpn11*	3.5E-2
Oxytocin signaling pathway	*fos, adcy1, camkk1, mapk1*	5.2 E-2
Rap1 signaling pathway	*crk, adcy1, grin2b, mapk1*	5.5 E-2
Inflammatory mediator regulation of TRP channels	*asic1, asic4, adcy1*	6.2 E-2
Negative regulation of FGFR3 signaling	*mapk1, ptpn11*	8.4E-2
2. Pathways related to oxidative stress response		
Chemical carcinogenesis - reactive oxygen species	*fos, ndufa5, ndufb2, ndufs6, cox4i2, mapk1, ptpn11*	2.7 E-4
Oxidative phosphorylation	*ndufa5, ndufb2, ndufs6, cox4i1*	1.8 E-2
Complex 1 biogenesis	*ndufa5, ndufb2, ndufs6*	2.0E-2
Respiratory electron transport	*ndufa5, ndufb2, ndufs6*	4.4E-2
Oxidative stress induced senescence	*fos, h2afv, mapk1*	8.5E-2
3. Pathways related to neuronal signal transmission		
Circadian entrainment	*fos, adcy1, grin2b, mapk1*	7.4 E-3
Glutamatergic synapse	*dlgap1, adcy1, grin2b, mapk1*	1.1 E-2
Long – term potentiation	*adcy1, grin2b, mapk1*	3.1 E-2
Activation of the AP-1 family of transcription factors	*fos, mapk1*	3.9E-2
Cholinergic synapse	*fos, adcy1, mapk1*	7.9 E-2

B KEGG pathways- PAHs low exposure

B.1 Focal adhesion pathway

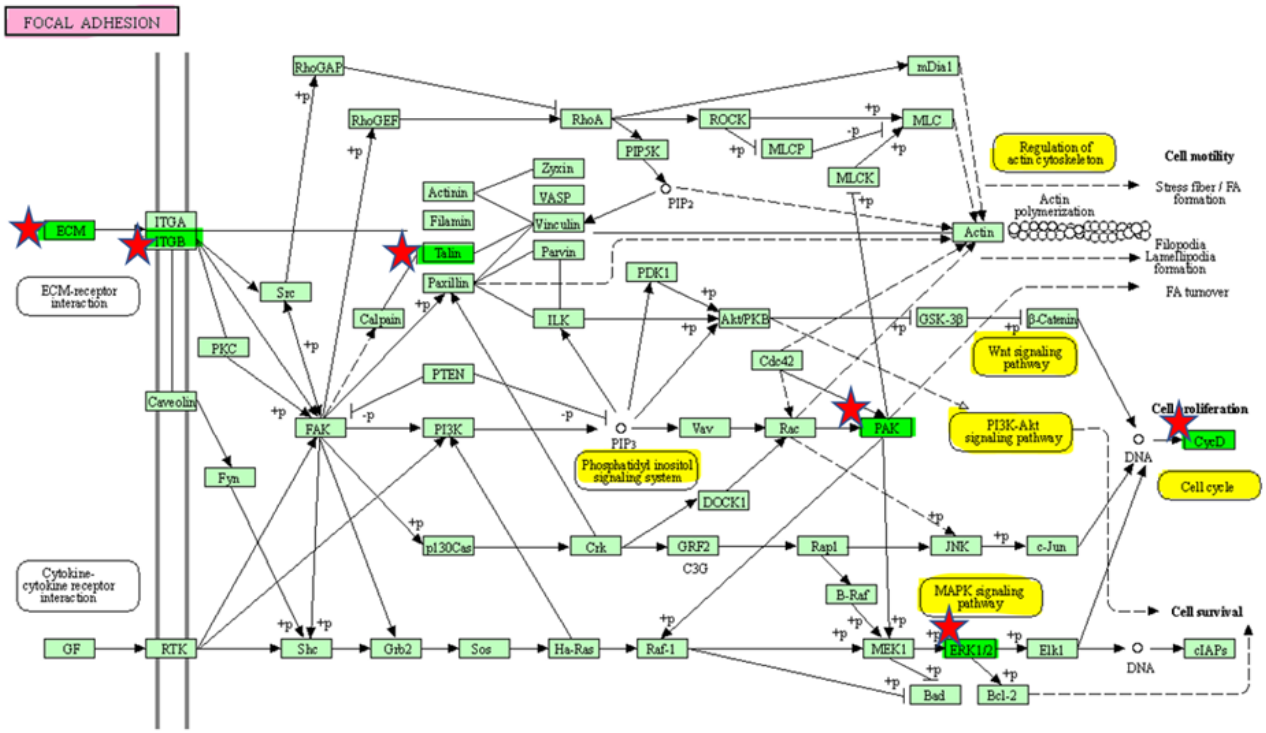


Figure B.1: The focal adhesion pathway showing interrelation to other pathways which are highlighted in yellow. The genes highlighted with red star are present in differentially expressed genes set after exposure to low concentration of PAHs

B.2 ECM receptor interaction pathway

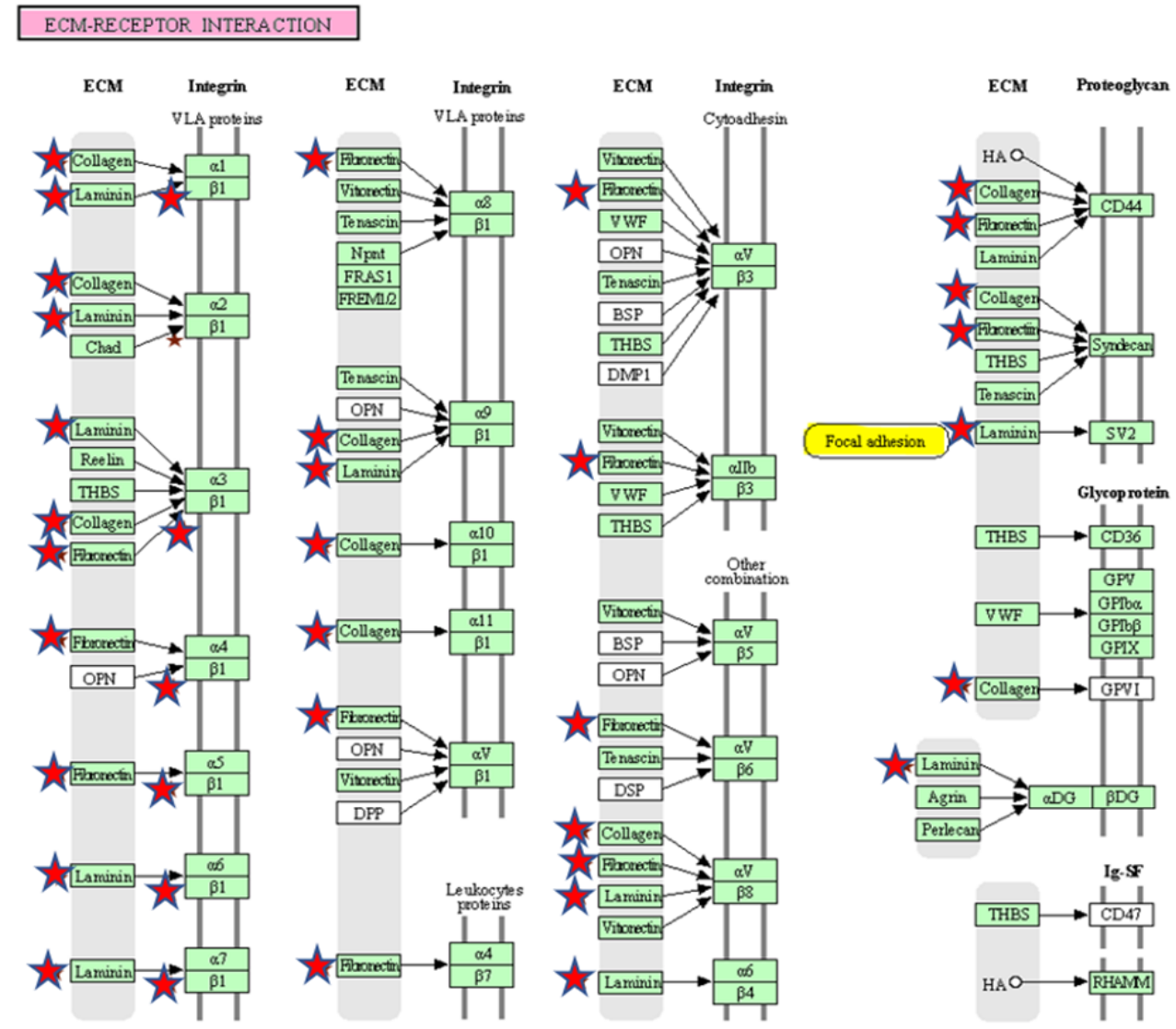


Figure B.2: The ECM receptor interaction pathway which is interrelated to focal adhesion pathway (Highlighted in yellow). The genes highlighted by red star are present in differentially expressed gene set after exposure to low concentration of PAHs

C STRING analysis on PFASs high exposure

C.1 Network with predicted functional partners of DEG in PFASs high exposure

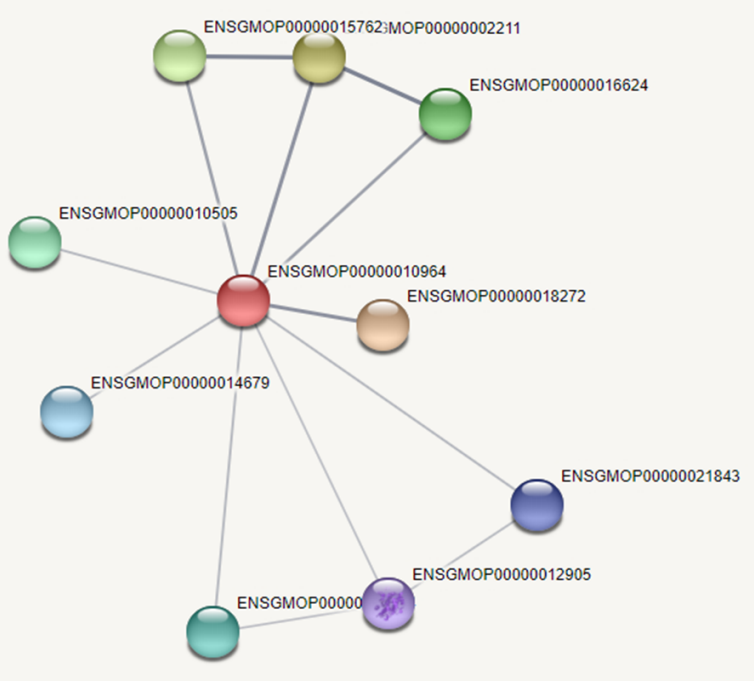


Figure C.1: The STRING network produced with the only differentially expressed gene in PFASs high exposure group (ENSGMOP00000010964 - STRING ID) and its predicted functional partners.

C.2 Predicted functional partners of DEG in PFASs high exposure

Predicted Functional Partners:

ENSGMOP00000018272	*Neurotrophin receptor associated death domain*
ENSGMOP00000002211	*Taste receptor, type 1, member 3*
ENSGMOP00000015762	*Taste receptor, type 1, member 1*
ENSGMOP00000016624	*Taste receptor, type 1, member 2, tandem duplicate 1*
ENSGMOP00000010505	*Purinergic receptor P2Y, G-protein coupled, 12*
ENSGMOP00000012264	*Odorant receptor, family E, subfamily 128, member 10*
ENSGMOP00000014679	*Purinergic receptor P2Y, G-protein coupled, 13*
ENSGMOP00000021843	*Odorant receptor, family F, subfamily 119, member 2*
ENSGMOP00000012905	*Guanine nucleotide binding protein (G protein), alpha 11b (Gq class)*

Figure C.2: The predicted functional partners of the DEG in PFASs high exposure (ENSGMOP00000010964 - STRING ID) by STRING software.

C.3 Enrichment analysis result of STRING network of DEG in PFASs high exposure

Table C.1: The enriched ontology terms (biological processes and molecular function respectively) after the enrichment analysis of the whole network which involve the differentially expressed gene (in PFASs high exposure) along with its predicted functional partners.

Biological processes	Molecular function
Detection of chemical stimulus involved in sensory perception	G protein-coupled purinergic nucleotide receptor activity
G protein-coupled receptor signaling pathway	G protein-coupled receptor activity
Response to chemicals	Transmembrane signaling receptor activity
Signal transduction	

D Metascape enrichment analysis results

D.1 Enriched terms in PAHs low exposure from metascape

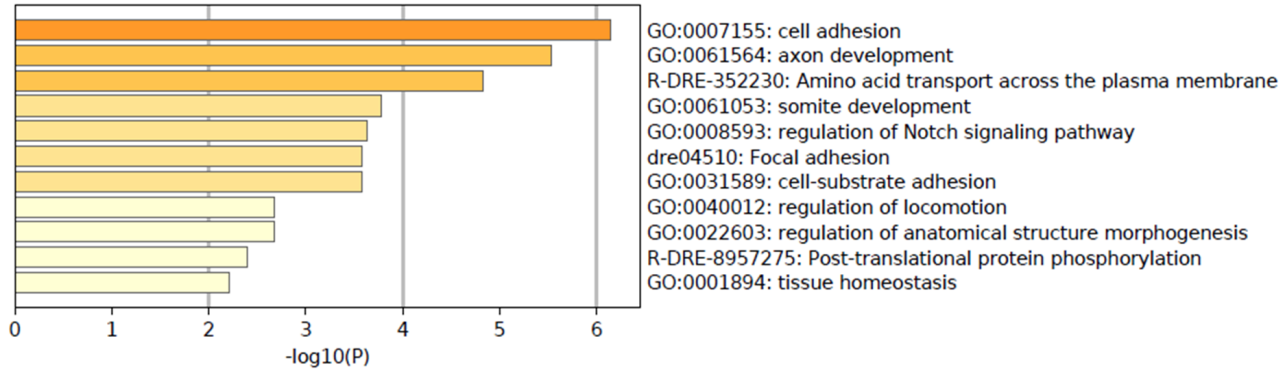


Figure D.1: Bargraph of enriched terms across input gene lists in PAHs low exposure, coloured by p-value.

D.2 Enriched Biological processes in PAHs low exposure from metascape

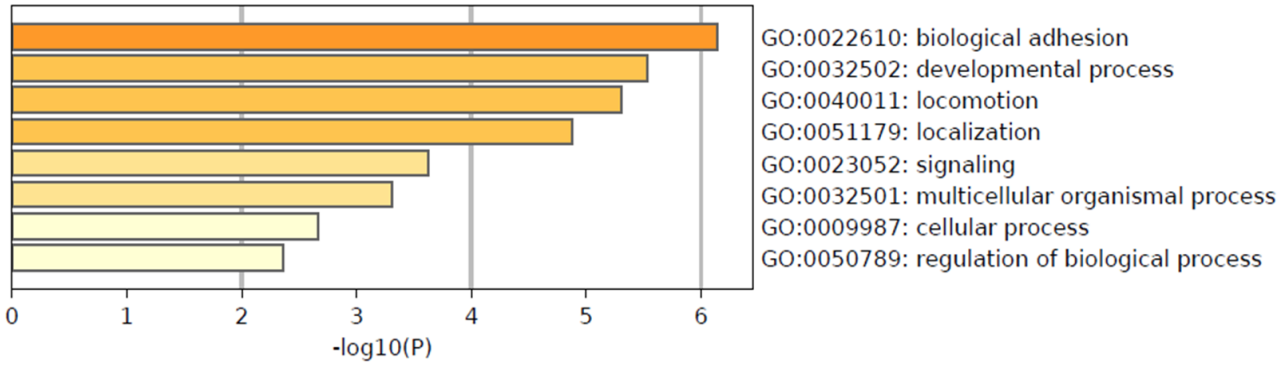


Figure D.2: The top level gene ontology biological process across input gene lists in PAHs low exposure, coloured by P-value.

D.3 Enriched terms in PAHs high exposure from metascape

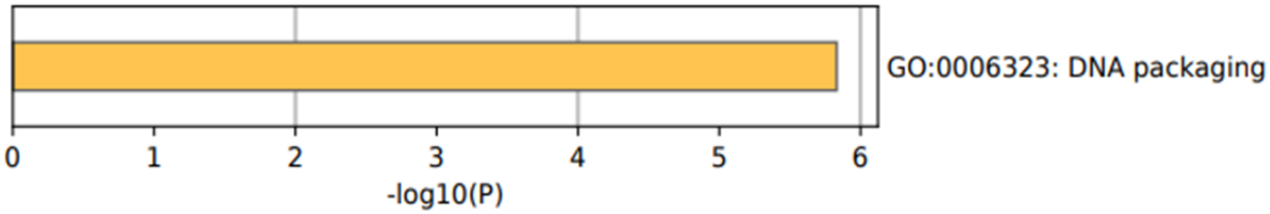


Figure D.3: Bar graph of enriched terms across input gene lists in PAHs high exposure, colored by p-values

D.4 Enriched biological processes in PAHs high exposure from metascape

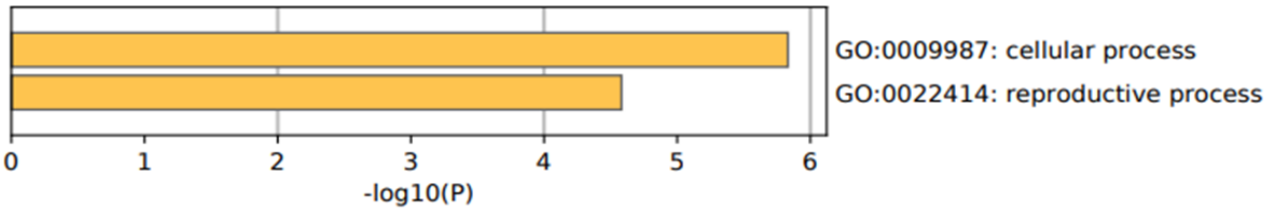


Figure D.4: The top level gene ontology biological process across input gene lists in PAHs high exposure, coloured by P-value.

D.5 Enriched terms in PFASs low exposure from metascape

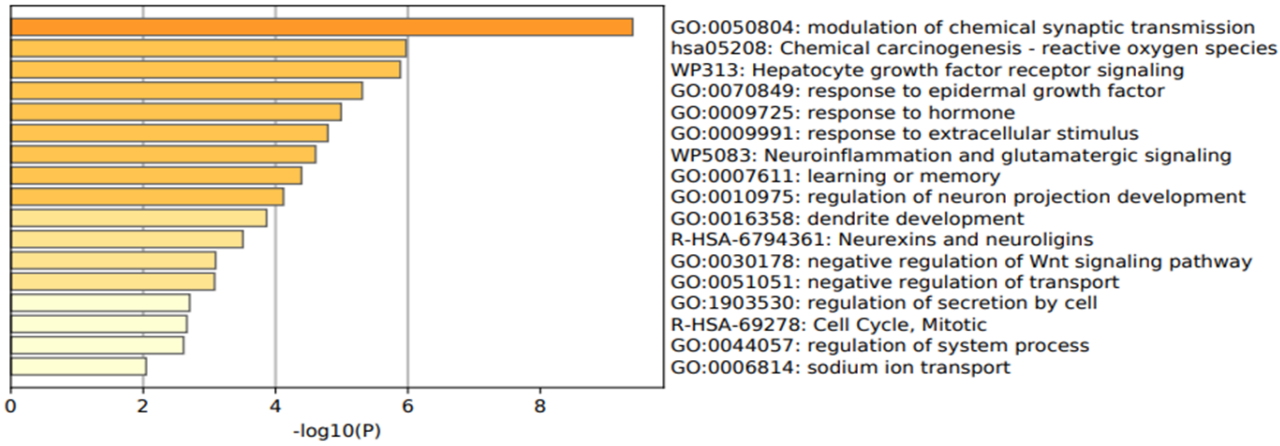


Figure D.5: Bar graph of enriched terms across input gene lists in PFASs low exposure, colored by p-values.

D.6 Enriched biological processes in PFASs low exposure from metascape

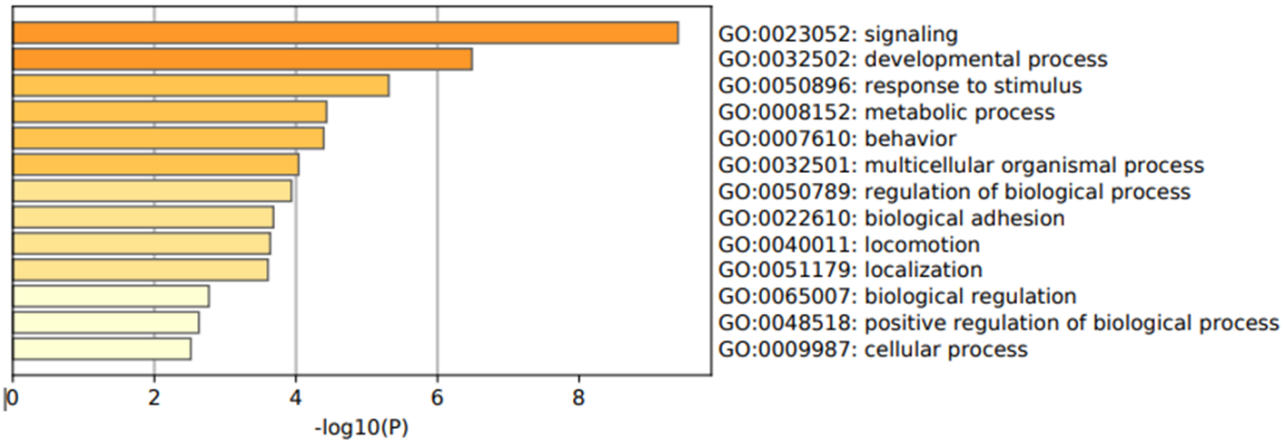
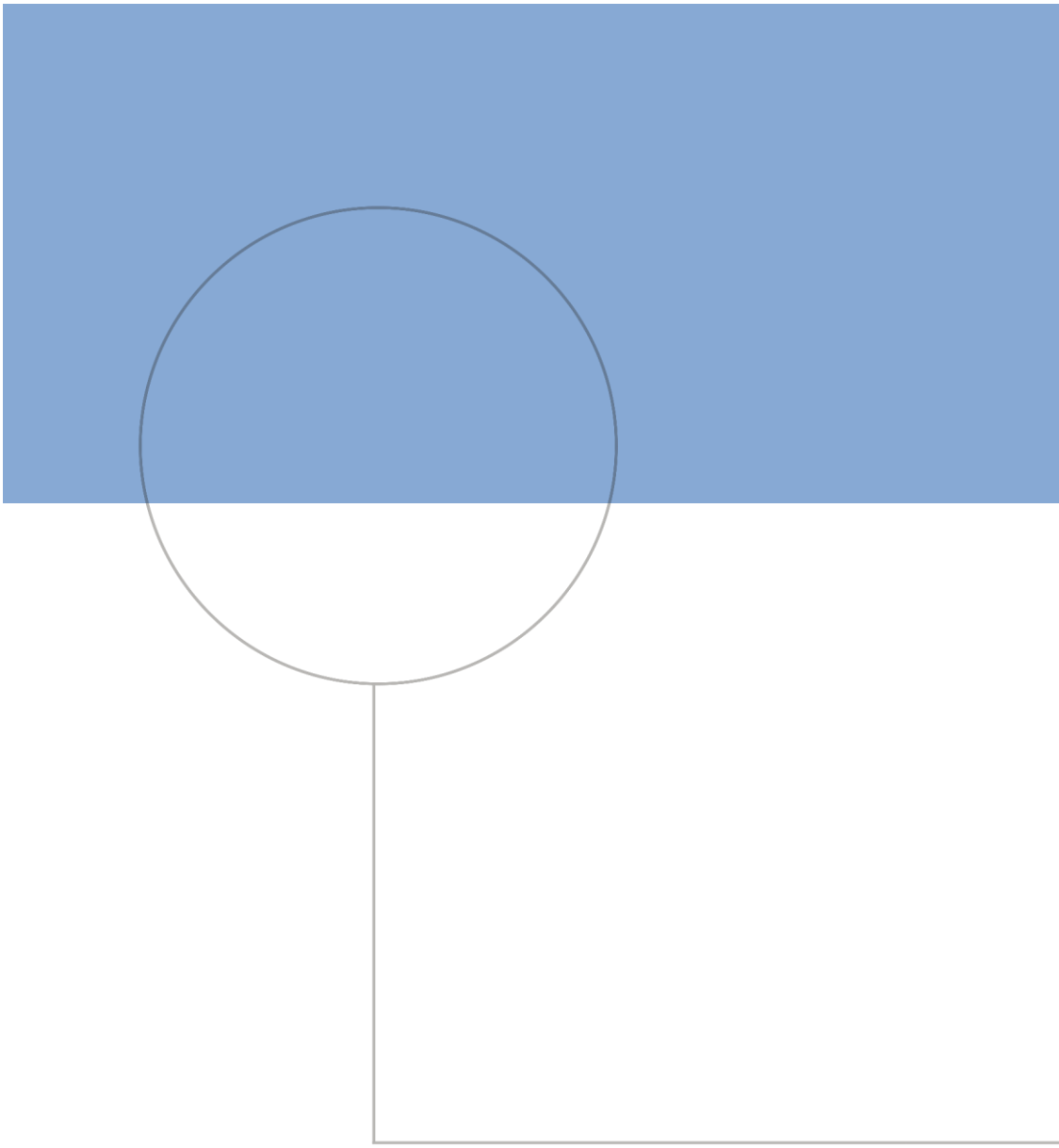


Figure D.6: The top level gene ontology biological process across input gene lists in PFASs low exposure, coloured by P-value.



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