UNIVERSITI PUTRA MALAYSIA

DECIPHERING GUT PATHOLOGY, OXIDATIVE STRESS, METABOLOME AND MICROBIOME ALTERATIONS IN JAVANESE MEDAKA FISH (Oryzias javanicus Bleeker, 1854) EXPOSED TO POLYSTYRENE MICROPLASTICS

SUNUSI USMAN

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By

SUNUSI USMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

July 2022

DEDICATION

This thesis is dedicated to my lovely parents (Malam Sanusi Muhammad Tela and Hajiya Hinda Halliru), my darling wife (Dr. Ramla Muhammad Kamal) and my children (Muhammad Kamal and Muhammad Sunusi)

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DECIPHERING GUT PATHOLOGY, OXIDATIVE STRESS, METABOLOME AND MICROBIOME ALTERATIONS IN JAVANESE MEDAKA FISH (*Oryzias javanicus* Bleeker, 1854) EXPOSED TO POLYSTYRENE MICROPLASTICS

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Chairman : Ahmad Faizal bin Abdull Razis, PhD
Institute : Bioscience

Microplastics (MPs) defined as plastics of less than 5mm in size have become pollutants of concern due to their continuous and unregulated release into the environment, making them readily accessible to a wide range of aquatic organisms and easily transferred across the food web. Their ever-present nature has led to human exposure largely through food and drinking water, with unrevealed health implications. Animal studies, in most instances, heavily relate its effects to the mere accumulation and induction of oxidative stress and inflammation in the gut system with other organs not well studied. Recently, a few studies on the exposure effects of MPs on the gut have reported bacterial microbiome and metabolome perturbations, which need to be explored further. This study hypothesised that polystyrene microplastics (PS-MPs) exposure induces organs histological alterations, gut oxidative stress and increase permeability, brain oxidative stress, oxidant damage and neurotoxicity and gut microbiome and metabolome alterations in Javanese medaka fish. The goal of the study is to determine the exposure of effects of PS-MPs on organs histopathology, gut oxidative stress and permeability, brain oxidative stress, oxidant damage, and neurotoxicity, and gut microbiome and metabolome alterations. Javanese medaka fish were exposed to polystyrene microplastics (PS-MPs) suspensions for a period of 21 days at concentrations of 100µg/L (MP-LOW), 500µg/L (MP-MED) and 1000µg/L (MP-HIGH). The gut and other organs were evaluated for histological alterations, oxidative stress, permeability, and neurotoxicity. Furthermore, gut metabolome and microbiome alterations were assessed. Histological features of inflammation and tissue damage was found in the PS-MPs exposed groups, but none in the control, with significant difference (p-value < 0.05) found between the three exposure concentrations in the intestines [MP-HIGH (74 ± 6%), MP-MED (54 ± 6%) and MP-LOW (26 ± 5%)], Liver [MP-HIGH (86 ± 3%), MP-MED (60 ± 5%) and MP-LOW (46 ± 3%)] and the kidney [MP-HIGH (66 ± 4%), MP-MED (26 ± 5%) and MP-LOW (14 ± 4%)]. Intestinal permeability assessed by D-Lactate in nmol/mL [Control (38 ± 20), MP-LOW (60 ± 2), MP-MED (67 ± 2), MP-HIGH (78 ± 2)], and intestinal oxidative stress using catalase (CAT) in U/mg protein [Control (191 ± 22), MP-HIGH (29 ± 17)] and total superoxide dismutase (T-SOD) activity in U/mg of

protein [Control (61.8 ± 5), MP-HIGH (43 ± 4)] were found to be significantly increased. In the brain, a significant increase in oxidative stress [CAT activity, Control (16±3), MP-HIGH (6 ±1) and T-SOD activity (Control (67 ± 18), MP-HIGH (38 ± 5)], oxidant damage measured using MDA in ng/mL [Control (30.8 ± 2), MP-LOW(47 ± 2), MP-MED (55 ± 3), MP-HIGH (38.8 ± 50)], and neurotoxicity by inhibition of acetylcholinesterase in ng/mL [Control (8 ± 0.1), MP-MED (6 ± 1), MP-HIGH (5 ± 1)] was elicited. High throughput sequencing of the bacterial 16S rRNA gene V3-V4 region and fungal ITS2 region, revealed reduction in richness and diversity of the gut microbiome. The top 5 relative abundance of bacterial phyla showed increase in Proteobacteria from 65% in the control to 79% in the MP-LOW and MP-MED groups, and 88% observed in MP-HIGH. Conversely, Actinobacteriota showed a decline from 22% in the control group to 9%, 10% and 6% in the low, medium and highest PS-MPs exposed groups respectively. A total number of 7 bacterial biomarkers including g_Aeromonas as unique feature in the MP-HIGH group, and g_Ralstonia, g_Paraburkholderia, g_Pelmonas, g_Staphylococcus, g_Bradyrhizobium, and g_Pararhizobium were found as the unique features in the MP-LOW group. The top 5 fungal phyla relative abundance showed a reduction of Ascomycota and Chytridiomycota from 42% and 48% in the control to 30% and 40% respectively in MP-MED group, and 24% and 21% in MP-HIGH group. ^{1}H NMR metabolomics revealed 9 metabolites responsible for metabolomic alteration due to PS-MPs exposure including anserine, glucose, creatine, glucuronate, glutamate, alanine, lactate, valine, and 2-hydroxyvalerate. The glucose and lactate showed a statistically significant increase with glucose having more than a fourfold increase (Log 2 fold change >2) in all the PS-MPs exposed groups and lactate having more than twofold increase (Log 2 fold change >1) in MP-MED and MP-HIGH exposed groups. The metabolomic pathway analysis revealed the enriched metabolites to be related to energy metabolism via tricarboxylic acid cycle (TCA), creatine pathway and urea cycle. Furthermore, positive correlation was found between the genus Aeromonas and glucose, lactate and creatine metabolites. The results revealed that P S-MPs exposure causes histopathological alterations in the gut and other vital organs including the brain, it causes significant increase in gut oxidative stress and permeability, brain oxidative stress, oxidant damage, as well as neurotoxicity. In the same vein, PS-MPs exposures causes significant alterations in gut bacterial and fungal microbiome both in terms of relative abundance, reduction in species richness and diversity, and differential enrichment of certain clades of the gut microbiome. Furthermore, it led to the alteration of the gut metabolites, by upregulation of glucose, lactate and amino acids. The altered gut microbiome and metabolome are related to hypoxia, inflammation, tissue injury and metabolic disorders. This study have provided additional data on gut bacterial and fungal clades, as well as metabolites associated with MPs toxicity in aquatic organism, this will inevitably enable further exploration, identification of biomarkers, and future health risks associated with MPs exposure in aquatic organisms and possibly humans.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MENGHURAI PATOLOGI USUS, TEKANAN OKSIDATIF, PERUBAHAN METABOLOM DAN MIKROBIOM DALAM IKAN MEDAKA JAWA (*Oryzias javanicus* Bleeker, 1854) TERDEDAH KEPADA MIKROPLASTIK POLISTIRENA

Oleh

SUNUSI USMAN

Julai 2022

Pengerusi : Ahmad Faizal bin Abdull Razis, PhD
Institut : Biosains

Mikroplastik (MP) yang ditakrifkan sebagai plastik bersaiz kurang daripada 5mm telah menjadi bahan pencemar yang membimbangkan kerana pembebasannya yang berterusan dan tidak terkawal ke alam sekitar, menjadikannya mudah diakses oleh pelbagai organisma akuatik dan mudah dipindahkan merentasi siratan makanan. Sifat mereka yang sentiasa ada telah membawa kepada pendedahan manusia sebahagian besarnya melalui makanan dan air minuman, dengan implikasi kesihatan yang tidak dihebahkan. Kajian haiwan, dalam kebanyakan keadaan, banyak mengaitkan kesannya dengan pengumpulan dan induksi tekanan oksidatif dan keradangan dalam sistem usus dengan organ lain yang belum dikaji sepenuhnya. Baru-baru ini, beberapa kajian mengenai kesan pendedahan MP kepada usus telah dilaporkan terdapat gangguan mikrobiom bakteria dan metabolom, yang perlu diterokai lebih lanjut. Kajian ini membuat hipotesis bahawa pendedahan mikroplastik polistirena (PS-MPs) mendorong perubahan histologi organ, tekanan oksidatif usus dan meningkatkan kebolehtelapan, tekanan oksidatif otak, kerosakan oksidan dan neurotoksisiti serta mikrobiom usus dan perubahan metabolom dalam ikan medaka Jawa. Matlamat kajian adalah untuk menentukan pendedahan kesan PS-MP pada histopatologi organ, tekanan oksidatif usus dan kebolehtelapan, tekanan oksidatif otak, kerosakan oksidan, dan neurotoksisiti, dan mikrobiom usus dan perubahan metabolom. Ikan medaka Jawa telah didedahkan kepada ampaian polistirena mikroplastik (PS-MPs) untuk tempoh 21 hari pada kepekatan 100µg/L (MP-LOW), 500µg/L (MP-MED) dan 1000µg/L (MP-HIGH). Usus dan organ lain dinilai untuk perubahan histologi, tekanan oksidatif, kebolehtelapan, dan neurotoksisiti. Tambahan pula, perubahan pada metabolom usus dan mikrobiom dinilai. Kebolehtelapan usus dinilai oleh D-Laktat dalam nmol/mL [Kawalan (38 ± 2, MP-LOW (60 ± 2), MP-MED (67 ± 2) MP-HIGH (78 ± 2)] dan tekanan oksidatif usus menggunakan katalase (CAT) dalam protein U/mg [Kawalan (191 ± 22), MP-HIGH (29 ± 17)] dan jumlah aktiviti superoksida dismutase (T-SOD) dalam U/mg protein [Kawalan (61.8 ± 5), MP-HIGH (43 ± 4)] didapati meningkat dengan ketara. Di dalam otak, peningkatan ketara dalam tekanan oksidatif [aktiviti CAT (Kawalan (16±3), MP-HIGH (6 ±1) dan aktiviti T-SOD

(Kawalan (67 ± 18), MP-HIGH (38 ± 5)], kerosakan oksidan diukur menggunakan MDA dalam ng/mL [Control (30.8 ± 2), MP-LOW (47 ± 2), MP-MED (55 ± 3), MP-HIGH (38.8 ± 50)], dan neurotoksisiti oleh perencatan acetylcholinesterase dalam ng/mL [Kawalan (8 ± 0.1), MP-MED (6 ± 1), MP-HIGH (5 ± 1)] telah ditunjukkan. Penjujukan pemprosesan tertinggi bagi 16S rRNA bakteria gen V3-V4, ITS2 kulat, mendedahkan pengurangan bilangan dan kepelbagaian mikrobiom usus. Terdapat 5 limpahan relatif bakteria filum teratas menunjukkan peningkatan Proteobacteria daripada 65% dalam kawalan kepada 79% dalam kumpulan MP-LOW dan MP-MED, dan 88% diperhatikan dalam MP-HIGH. Sebaliknya, Actinobacteriota menunjukkan penurunan daripada 22% dalam kumpulan kawalan kepada 9%, 10% dan 6% masing-masing dalam kumpulan terdedah PS-MP rendah, sederhana dan tertinggi. Sejumlah 7 penanda bio bakteria termasuk g_Aeromonas sebagai ciri unik dalam kumpulan MP-HIGH, dan g_Ralstonia, g_ Paraburkholderia, g_Pelmonas, g_Staphylococcus, g_Bradyrhizobium, dan g_Pararhizobium ditemui sebagai ciri unik dalam kumpulan MP-LOW. Limpahan relatif filum kulat 5 teratas menunjukkan pengurangan Ascomycota dan Chytridiomycota daripada 42% dan 48% dalam kawalan kepada 30% dan 40% masing-masing dalam kumpulan MP-MED, dan 24% dan 21% dalam kumpulan MP-HIGH. Metabolomik 1H NMR mendedahkan 9 metabolit yang bertanggungjawab untuk perubahan metabolik akibat pendedahan PS-MP termasuk anserin, glukosa, kreatin, glukuronat, glutamat, alanin, laktat, valin, dan 2-hidroksivalerat. Glukosa dan laktat menunjukkan peningkatan yang ketara secara statistik dengan glukosa mempunyai peningkatan lebih daripada empat kali ganda (Log 2 kali ganda perubahan >2) dalam semua kumpulan PS-MP yang terdedah dan laktat mempunyai peningkatan lebih daripada 2 kali ganda (Log 2 kali ganda perubahan >1) dalam kumpulan terdedah MP-MED dan MP-HIGH. Analisis laluan mendedahkan metabolit yang diperkaya yang berkaitan dengan metabolisma tenaga melalui kitaran asid trikarboksilik (TCA), laluan kreatin dan kitaran urea. Tambahan pula, korelasi positif didapati antara genus Aeromonas dan metabolit glukosa, laktat dan kreatin. Keputusan mendedahkan bahawa pendedahan P S-MP menyebabkan perubahan histopatologi dalam usus dan organ penting lain termasuk otak, ia menyebabkan peningkatan ketara dalam tekanan oksidatif usus dan kebolehtelapan, tekanan oksidatif otak, kerosakan oksidan, serta neurotoksisiti. Dalam nada yang sama, pendedahan PS-MP menyebabkan perubahan ketara dalam mikrobiom bakteria dan kulat usus dari segi kelimpahan relatif, pengurangan kekayaan dan kepelbagaian spesies, dan pengayaan pembezaan klad tertentu mikrobiom usus. Tambahan pula, ia membawa kepada pengubahan metabolit usus, dengan penyelarasan glukosa, laktat dan asid amino. Mikrobiom dan metabolom usus yang diubah adalah berkaitan dengan hipoksia, keradangan, kecederaan tisu dan gangguan metabolik. Kajian ini telah menyediakan data tambahan tentang klad bakteria dan kulat usus, serta metabolit yang dikaitkan dengan ketoksikan MP dalam organisma akuatik, ini pasti akan membolehkan penerokaan lanjut, pengenalpastian biomarker, dan risiko kesihatan masa depan yang berkaitan dengan pendedahan mikroplastik dalam organisma akuatik dan mungkin manusia

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Ahmad Faizal bin Abdull Razis, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairperson)

Khozirah binti Shaari, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Mohd Zamri bin Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Mohammad Noor bin Azmai Amal, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Nurulfiza binti Mat Isa, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 October 2022

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Associate Professor Dr. Ahmad Faizal bin Abdull Razis

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Khozirah binti Shaari

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Mohd Zamri bin Saad

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Mohammad Noor bin Azmai Amal

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Nurulfiza binti Mat Isa

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1H-NMR	Proton Nuclear Magnetic Resonance
AC	Acrylate
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APV	Vanish
ATP	ATP; Adenosine Triphosphate
ATR	Attenuated Total Reflectance
BCA	Bicinchoninic Acid
BDE-209	Decabromodiphenyl Ether
BPA	Bisphenol A
CAT	Catalase
CP	Cellophane
CPMG	Carr-Purcell-Meiboom-Gill
EPDM	Propylene- Diene Rubber
EPM	Ethylene-Propylene Rubber
EU	European Union
EVA	Ethylene Vinyl Acetate
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography-Mass Spectrometry
GESAMP	Joint Group of Experts on The Scientific Aspects of Marine Environmental Protection
GSH-Px	Glutathione Peroxidase

HD	Huntington's Disease
HDPE	High Density Polyethylene
IFNa	Interferon Alpha
IL1a	Interleukin-1a
IL1b	Interleukin-1a
LDA	Linear Discriminative Analysis
LDH	Lactate Dehydrogenase
LDPE	Low Density Polyethylene
LEfSe	Linear Discriminative Analysis Coupled With Effect Size Measurements
MDA	Malondialdehyde
MPs	Microplastics
MVDA	Multivariate Data Analysis
NCBI	National Centre for Biotechnology Information
NMDS	Non-Metric Multi-Dimensional Scaling
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
OTU	Operational Taxonomic Unit
PA	Nylon
PA	Polyamide
PAN	Polyacrylonitrile
PAS	Polyarylsulfone
PB	Poly 1- Butene
PC	Principal Component
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction

PD	Parkinson's Disease
PE	Polyethylene
PES	Polyester
PET	Polyethylene Terephthalate
PMMA	Poly methyl methacrylate
PP	Polypropylene
ppm	Part Per Million
PS	Polystyrene
PS-MPs	Polystyrene Microplastics
PU	Polyurethane
PVA	Polyvinyl Alcohol
PVC	Polyvinylchloride
QIIME	Quantitative Insights into Microbial Ecology
SBR	Styrene-Butadiene-Rubber
SDGs	Sustainable Development Goals
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TED-GC-MS	Thermal Extraction/Desorption-Gas Chromatography-Mass Spectrometry
T-SOD	Total Superoxide Dismutase
UN	United Nation
UNEA	United Nations Environment Assembly
VC/E	Vinyl Chloride / Ethylene
VIP	Variable Importance in Projection
WHO	World Health Organisation
µm	Micrometre

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Plastics have become valuable commodities for our day-to-day activities, which has boosted their production following advancements in science and technology(Lambert & Wagner, 2018). Annual global plastic production has dramatically risen from 1.5 million MT in the 1950s to 359 million MT in 2018 (Statista: Plastic Waste Worldwide - Statistics & Facts, 2021), with present cumulative global production of above 8 billion MT expected to have a progressive increase in the coming decades (Geneveva Environment Network:Plastics and the Environment, 2021).

Microplastics are plastics of size 5um or less that have been estimated to pollute the oceans to the tune of 51 trillion (European Parliament:Microplastics: Sources, Effects and Solutions, 2018), with coastal countries alone contributing about 4.8 to 12.7 million MT (Jambeck et al., 2015). MPs are found in all seas (Yang et al., 2015) and other marine environments due to their indiscriminate disposal and intrinsic properties (Sharma & Chatterjee, 2017). They are categorised based on their sources into primary microplastics (15–35%) formed and released as small particulate matter into the environment, or secondary microplastics formed from the disintegration of larger plastics (69-81%) (European Parliament:Microplastics: Sources, Effects and Solutions, 2018).

Microplastics of size $\leq$ 5mm have been regarded as highly important because their small size allows them to be ingested by marine organisms (GESAMP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection, 2016), making them easily accessible and transferable across the food web(Sharma & Chatterjee, 2017). MPs have been found as polymers of polyethylene, polypropylene, polystyrene, polyethylene terephthalate, polyester, polyamide, nylon, polyacrylonitrile, poly 1-butene and cellophane, existing as mixture of various shape and sizes as fibres, pellets and fragments in a variety of seafood (Abidli et al., 2019; Bessa et al., 2018; Karbalaei et al., 2019; Li et al., 2015; Li, Green, et al., 2018); fruits (Oliveri Conti et al., 2020); milk and beverages (Diaz-Basantes et al., 2020); salt (Yang et al., 2015) (Karami, Golieskardi, Keong Choo, et al., 2017) and drinking water (Schymanski et al., 2018). The human exposure to MPs through various sources has been confirmed by the findings of MPs in the human stool (Luqman et al., 2021; Schwabl et al., 2019), colon (Ibrahim et al., 2021), and placenta (Ragusa et al., 2021).

The contamination of foods and drinking water by MPs findings has garnered much concern among policymakers and scientists. Among them is the call by World Health Organisation (WHO) for the assessment of MPs in the environment and their effects on human health (WHO, 2019). However much effort is needed in this respect as less than a quarter of the 192 countries in the world have conducted research related to microplastics (Ajith & Arumugam, 2020) and there is lack of knowledge till date on the

negative effects of consuming MPs containing organisms (Barboza et al., 2018) despite being a potential threat to food safety and security.

1.2 Problem Statement

The threat posed to the aqua-terrestrial ecosystem and the concomitant integration of microplastics into the food web, exposing humans to unknown health implications, has put microplastics under critical observation by policymakers and researchers in order to understand their effects and formulate a holistic approach to address the menace. Studies mostly focus of the effects of MPs on growth and reproduction (Cong et al., 2019; Li et al., 2020; Zhu et al., 2020) and the gut system, reporting the effect to mere accumulation and , to a lesser extent, oxidative stress and inflammation (Jabeen et al., 2018; Jin et al., 2019; Lei et al., 2018; Zhang et al., 2019; Zhu et al., 2020), thus requiring further investigation and expansion to include other organ systems. Recently, metagenomics has been recognised as a valuable tool in understanding the toxicity of environmental pollutants via gut microbiome alterations (Zhang et al., 2015; Zhao et al., 2016). External stressors influence the microbiome generating huge compounds that are vital in microbial selection and metabolic signalling, which in turn influences host metabolome and health (Vernocchi et al., 2016). However, there are limited studies that investigate the effects of MPs on organisms using metabolomic and metagenomic approaches (Qiao et al., 2019).

1.3 Research Justification

This study was conducted to evaluate the effects of MPs exposure on the gut and other organs in vitro. It also entails the use of high-throughput next-generation sequencing technology and NMR metabolomics to understand the gut microbiome-metabolome responses. This is the first research that deeply explored the effects of microplastics on different organ systems, including fungal and bacterial aspects of the microbiome and metabolome, utilising the Javanese medaka fish as a model not previously studied. It is hoped that the study will aid in a deeper understanding of the extent and toxicity mechanisms of MPs exposure, allow for biomarker identification, and provide insight as to the potential disease risk that may be incurred in the future. This is to provide a foundation for more arrears to be explored by researchers, allow consumers to make informed decisions about plastics and their handling, and finally, to provide convincing evidence that will shape policies that will address the menace.

1.4 Research hypothesis

Polystyrene microplastic exposure will cause histological alterations in multiple organs, increase gut oxidative stress and permeability, induce brain oxidative stress, oxidant damage and neurotoxicity, as well as gut microbiome and metabolome perturbations in Javanese medaka fish.

1.5 Main objective

The aim of the study is to decipher organs histological alterations, gut oxidative stress and permeability, brain oxidative stress, oxidant damage and neurotoxicity, and gut microbiome and metabolome alterations in Javanese medaka exposed to PS-MPs.

1.6 Specific objectives

The specific objectives of the study are:

1. To determine histopathological features and degree of alterations in the intestines, liver, kidney, and brain of Javanese medaka fish due to PS-MPs exposure.

2. To determine gut CAT and T-SOD activity, D-lactate level, brain CAT and T-SOD activity, MDA, AChE and ACh level as markers of gut oxidative stress and permeability, brain oxidative stress, oxidant damage and neurotoxicity following PS-MPs exposure.

3. To determine the changes in relative abundance, species richness and diversity, and enriched clades of the bacterial and fungal gut microbiome, as well as metabolome alterations, and the relationship between gut microbiome and metabolome due to MPs exposure.

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