UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO

DIVISIÓN ACADÉMICA DE CIENCIAS BIOLÓGICAS

EFECTOS DE LA INGESTA DE MICROPLÁSTICOS SOBRE LA FISIOLOGÍA DIGESTIVA, CRECIMIENTO Y SOBREVIVENCIA DE PEJELAGARTOS DE CULTIVO (*Atractosteus tropicus*)

TESIS PARA OBTENER EL GRADO DE:

MAESTRA EN CIENCIAS AMBIENTALES

PRESENTA:

BIÓL. ALEJANDRA PÉREZ LÓPEZ

BAJO LA DIRECCIÓN DE:

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EN CODIRECCIÓN DE:

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VILLAHERMOSA, TABASCO, MÉXICO. NOVIEMBRE 2024.

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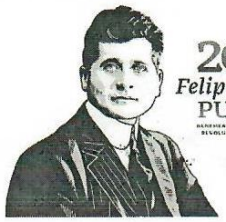

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Con amor, para mi familia:

Mi hijo Yosef M. Gallegos Pérez, por ser la luz de mi vida.

Mis padres, Sra. Carmela López Torres y Sr. Alejandro Pérez Hernández, por creer en mí.

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Así como a cada uno de los organismos sacrificados para esta investigación.

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RESUMEN. Los microplásticos (partículas plásticas con dimensiones entre 1 y 1000 µm) están presentes en ecosistemas acuáticos de todo el mundo donde son ingeridos por los organismos que allí habitan, ocasionando alteraciones en la fisiología digestiva. Este estudio evaluó los efectos de microplásticos de polimetilmetacrilato (MP-PMMA) en el crecimiento, sobrevivencia, enzimas digestivas y microbiota intestinal de pejelagartos *Atractosteus tropicus* alimentados con dietas enriquecidas con 0.00, 0.25, 0.50, 0.75 y 1.00% MP-PMMA, por 60 días. El peso final de los peces fue significativamente menor en el tratamiento con 0.25% comparado al de 1.00% MP-PMMA (23.21 ± 8.33 y 30.33 ± 12.14 g, respectivamente) (p=0.02). Los índices de crecimiento y factor de condición no mostraron diferencias significativas entre los tratamientos (p>0.05, respectivamente). La actividad enzimática de proteasas ácidas y alcalinas, tripsina, quimotripsina, L-aminopeptidasa y α-amilasa mostraron diferencias significativas entre los tratamientos (p = 0.012, 0.012, 0.012, 0.003, 0.011 y 0.004, respectivamente); excepto lipasas (p=0.83). Los índices de diversidad α (Chao, Shannon, Simpson y Equitatividad) de la microbiota intestinal, mostraron tendencia a disminuir en los grupos con 0.25, 0.75 y 0.50% MP-PMMA, en cuyos tratamientos la abundancia relativa del género *Mycoplasma* mostró la mayor cobertura (con 90, 67 y 88%, respectivamente) en comparación con los grupos 0.00 y 1.00% MP-PMMA (con 23 y 13%, respectivamente). El dendograma del índice de disimilitud Eucledian (coeficiente de correlación de 0.83), mostró agrupación entre los grupos 0.00 y 1.00% de MP-PMMA. Los MP-PMMA causaron alteraciones en la actividad enzimática y la microbiota intestinal, incluso con bajos porcentajes ingeridos.

ABSTRACT. Microplastics (plastic particles with dimensions between 1 and 1000 µm) are present in aquatic ecosystems around the world, where they are ingested by the organisms that inhabit them, causing alterations in digestive physiology. This study evaluated the effects of polymethylmethacrylate microplastics (MP-PMMA) on the growth, survival, digestive enzymes and intestinal microbiota of tropical gar *Atractosteus tropicus* fed diets enriched with 0.00, 0.25, 0.50, 0.75 and 1.00% MP-PMMA for 60 days. The final weight of the fish was significantly lower in the 0.25% treatment compared to the 1.00% MP-PMMA (23.21 ± 8.33 and 30.33 ± 12.14 g, respectively) (p=0.02). Growth rates and condition factors showed no significant differences between treatments (p>0.05, respectively). Enzymatic activity of acid and alkaline proteases, trypsin, chymotrypsin, L-aminopeptidase and α-amylase showed significant differences among treatments (p=0.012, 0.012, 0.012, 0.012, 0.003, 0.011 and 0.004, respectively); except lipases (p=0.83). The α-diversity indices (Chao, Shannon, Simpson and Equitability) of the intestinal microbiota showed a tendency to decrease in the groups with 0.25, 0.75 and 0.50% MP-PMMA, in which treatments the relative abundance of the genus *Mycoplasma* showed the highest coverage (with 90, 67 and 88%, respectively) compared to the 0.00 and 1.00% MP-PMMA groups (with 23 and 13%, respectively). The Eucledian dissimilarity index dendrogram (correlation coefficient of 0.83) showed clustering between the 0.00 and 1.00% MP-PMMA groups. MP-PMMA caused alterations in enzyme activity and intestinal microbiota, even at low percentages ingested.

PALABRAS CLAVE: Microbiota intestinal, actividad enzimática digestiva, pez dulceacuícola, *Atractosteus tropicus*, efectos de los microplásticos.

KEYWORDS: Intestinal microbiota, digestive enzymatic activity, freshwater fish, *Atractosteus tropicus*, microplastics effects.

INTRODUCCIÓN

En las últimas décadas, el uso excesivo y la fabricación masiva de materiales plásticos ha generado una gran cantidad de desechos, los cuales, debido al mal o inexistente manejo de residuos, llegan a los ambientes acuáticos de todo el mundo de manera directa o indirecta (Andrady, 2017).

Las partículas plásticas con dimensiones entre 1 y 1000 µm se denominan microplásticos (MP), de acuerdo con la Organización Internacional de Estandarización (ISO, *por sus siglas en inglés*, 2020). Los MP pueden generarse derivados de piezas de mayor tamaño, esto debido a que se desgastan o fragmentan por efecto de factores como la luz ultravioleta, la temperatura, la abrasión, el viento y el movimiento del agua, generando pequeñas partículas que llegan a alcanzar tamaños micrométricos (MP secundarios); o bien, los MP pueden ser fabricados para diversos usos a esta escala (MP primarios) gracias al avance de la tecnología. En cualquiera de los casos, los plásticos pueden tardar cientos y hasta miles de años en degradarse completamente permaneciendo en los ambientes acuáticos, fragmentándose cada vez más y liberando sustancias tóxicas al ambiente (Andrady, 2017; EPA, 2023).

Se estima que un mínimo de 5.25 trillones de partículas (268,940 toneladas) se encuentran flotando en los océanos de todo el mundo (Jambeck et al., 2015), con un aporte anual de 11 millones de toneladas métricas (ONU, 2023). Los MP presentes en ambientes acuáticos han demostrado importantes efectos adversos en la biota, ya que, al quedar expuestos, son ingeridos por diversos organismos entre los que se encuentran peces de importancia ecológica, pesquera y alimenticia para el ser humano (Cox et al., 2019; FAO, 2017; Phillips y Bonner, 2015).

Ejemplo de lo anterior es la ingesta de microplásticos en tilapias (*Oreochromis niloticus* y *Oreochromis aureus*) y pluma campechana (*Calamus campechanus*), evidenciada por los análisis de contenidos estomacales, en los que se encontraron polimetilmetacrilato (PMMA, acrílico), polietileno (PE), polipropileno (PP) y poliestireno (PS), entre muchos otros tipos de polímeros (Borges-Ramírez et al., 2020; Liu et al., 2023; Phillips y Bonner, 2015).

Por lo anterior, es necesario conocer las afectaciones potenciales de la ingesta de MP para crear un marco de información y reconocer los riesgos asociados. En ese sentido, se han realizado pruebas de toxicidad (bioensayos) para la evaluación de la exposición a diversos tipos de MP en peces, como los reportados con PE, PP y PS en pez cebra (*Danio rerio*), la dorada (*Sparus aurata*) y pez millón (*Poecilia reticulata*), en los que provocaron alteraciones en la actividad enzimática digestiva y cambios en la microbiota intestinal, entre otras afectaciones (Huang et al., 2020; Qiao

et al., 2019; Varó et al., 2021), comprometiendo la cantidad y calidad de los bienes y servicios ambientales que proporcionan estos organismos.

No obstante, el PMMA ha sido poco estudiado en comparación a polímeros como PS o PE, cuya demanda aumentó por su uso como barrera de protección contra la propagación del SARS-CoV 2 en la pandemia de COVID-19, además de su éxito como sustituto del vidrio, potencial antimicrobiano y antifúngico, es usado en diversos sectores industriales, médicos, farmacológicos y cosmetológicos (Bacali et al., 2020; Koike y Koike, 2012; Petrović et al., 2018).

En este contexto, se propone al pejelagarto (*Atractosteus tropicus*) como modelo biológico para la evaluación de los posibles efectos derivados de la ingesta de MP de PMMA (MP-PMMA), dada su importancia ecológica, alimenticia y cultural para el ser humano, además de ser una especie que existe desde hace aproximadamente 70 millones de años (especie pancrónica), por lo que cuenta con una fisiología (incluyendo la digestiva) conservada y exitosa desde el periodo Cretácico, de la era Mesozoica (Márquez-Couturier y Vázquez-Navarrete, 2015).

Por ello, el objetivo del presente estudio fue evaluar los efectos de la ingesta de MP-PMMA vía dieta sobre la fisiología digestiva y desarrollo del pejelagarto (*A. tropicus*) en etapa juvenil, mediante el análisis de la composición y estructura de la microbiota intestinal, actividad de las principales enzimas digestivas, e índices de crecimiento y sobrevivencia.

ESTADO DEL ARTE

Plásticos

El término plástico proviene del griego “plastikos” (πλαστικός) que significa apto para moldeo y “plastos” (πλαστός) moldeados, este concepto se utiliza comúnmente para englobar una gran gama de materiales poliméricos que tienen como principal característica que, en algún momento de su transformación en productos acabados, pueden ser fácilmente moldeables a temperatura y presión específicas (FAO, 2017; ISO, 2020).

El éxito de estos materiales radica en que son versátiles (por lo que pueden tomar casi cualquier forma), fáciles de fabricar, durables, de bajo costo, y pueden fabricarse en masa, por lo que han desplazado a materiales como la madera, papel, metal y algodón (FAO, 2017).

La fabricación de plástico a gran escala comenzó en la década de 1950 con una producción mundial de 2 millones de toneladas (Mt) (FAO, 2017; PlasticsEurope, 2020), desde entonces hasta el 2017, se estima que se han producido 8,300 Mt de plásticos de primera fabricación (no reciclados) (Geyer et al., 2017). Solo en el 2022, 400.3 Mt fueron producidos a nivel mundial, y se calcula que en el 2050 se podrían alcanzar las 1,000 Mt (FAO, 2017; PlasticsEurope, 2023).

Entre los compuestos poliméricos más comunes y de mayor producción en el mundo se encuentran el PP, PE, polietileno tereftalato (PET), policloruro de vinilo (PVC) y el PMMA (PlasticsEurope, 2020).

A nivel nacional, en el año 2017, el consumo de plásticos alcanzó las 7.1 Mt (con un crecimiento estimado anual del 4 %). En ese mismo año, la producción mexicana se estimó con un valor de 40 mil millones de dólares anuales (AmbientePlástico, 2019).

Entre los sectores industriales con mayor demanda de plásticos se encuentran (de manera descendente en orden de importancia): empaquetado de alimentos y bebidas, construcción, automotriz, eléctrica y electrónica, hogar, entretenimiento, deportes y agricultura (PlasticsEurope, 2020).

Microplásticos

La Organización Internacional de Estandarización (ISO, *por sus siglas en inglés*, 2020) define microplástico (MP) como cualquier partícula de plástico sólida e insoluble en agua con dimensiones entre 1 µm (0.001 mm) y 1000 µm (1 mm), haciendo la distinción con el concepto “microplástico grande/extenso” (*por su traducción del inglés* “large microplastic”) para referirse a

las partículas de plástico con tamaños entre 1 mm y 5 mm. Siendo esta definición de MP la que utilizaremos en el presente estudio, es decir, partículas entre 1 y 1000 µm.

Sin embargo, la Organización de las Naciones para la Agricultura y Alimentación (FAO, por sus siglas en inglés, 2017) denomina MP a partículas de plástico entre 0.1 µm y 5000 µm, y la Agencia de Protección Ambiental de los Estados Unidos (EPA, *por sus siglas en inglés,* 2023), así como diversa literatura científica, han adoptado el término MP para referirse a partículas de plástico de tamaños menores a 5000 µm (5 mm) (Andrady, 2017; Liu et al., 2023).

Como se mencionó anteriormente, existen MP primarios y secundarios. Los MP primarios son los que se han creado gracias al avance de la tecnología con la fabricación de partículas plásticas a micro y nanoescala para usarse, por ejemplo, en cosméticos, textiles, productos de limpieza, biomedicina y farmacología, o bien, para la fabricación de artículos de mayor tamaño (EPA, 2023; FAO, 2017). Por otra parte, los MP secundarios son los que se forman por el desgaste y/o fragmentación de plásticos más grandes, generados principalmente por la exposición a la luz ultravioleta, la temperatura y la abrasión, la acción del viento y el movimiento del agua cuando se encuentran en ríos, lagos o mares (Andrady, 2017; FAO, 2017).

La gran durabilidad y flexibilidad de estos materiales, que como ya se mencionó, los hace tener gran éxito en el mercado, también los convierte en un peligro para los seres vivos, incluido el ser humano (Lara et al., 2020; Ragusa et al., 2021). Debido a que, la eliminación completa de los polímeros implica la degradación total o mineralización en compuestos o elementos químicos naturales, es decir, la descomposición completa en agua, dióxido de carbono, metano y otras moléculas no sintéticas, lo cual podría implicar varios cientos de años en llevarse a cabo (Andrady, 2017; FAO, 2017; Lara et al., 2020).

Contaminación por microplásticos

Los MP se encuentran en ecosistemas acuáticos de todo el mundo, y las cifras son alarmantes. Estimaciones realizadas por Eriksen et al. (2014) mencionan que un mínimo de 5.25 trillones de partículas (268,940 toneladas) se encuentran flotando en los océanos; con un aporte entre 4.8 Mt a 12.7 Mt de desechos plásticos, solo en el 2010 (FAO, 2017; Jambeck et al., 2015); en ese respecto, se estima que aproximadamente el 90% de los plásticos en el medio marino pelágico son menores a 5 mm (EPA, 2023). En el Golfo de México y en las costas del Pacífico mexicano se calculan más de 10,000 piezas de MP/km^2 (Eriksen et al., 2014).

El uso excesivo y desmedido de los materiales plásticos aunado a la fabricación masiva de estos productos, ha causado una gran cantidad de residuos, de modo que, el aumento de la

contaminación plástica incrementa exponencialmente con el aumento de la población humana (Andrady, 2017; EPA, 2023).

El aporte de microplásticos primarios y secundarios a los ambientes acuáticos está relacionado con actividades antropogénicas que van desde la fabricación, manufactura y uso de estos materiales, aunado a una mala cultura de manejo de residuos, mal o falta de tratamiento de aguas residuales, infraestructura inadecuada y vertimiento directo en ambientes acuáticos (Andrady, 2017; Du et al., 2020). Así también, por actividades como el transporte terrestre (generados por el desgaste de neumáticos), las actividades agrícolas, las artes de pesca y la acuicultura (FAO, 2017), por mencionar algunas de las actividades que liberan importantes cantidades de plásticos al ambiente.

Los MP se encuentran en toda la columna de agua. El destino y comportamiento de los MP dependerá de su densidad relativa en agua, resistencia a la intemperie dictada por su estructura química, aditivos incorporados en la formulación plástica y cristalinidad (Andrady, 2017; FAO, 2017).

Resultado de la presencia de MP en ambientes acuáticos es la interacción con la biota presente, ya que queda expuesta a estas partículas y pueden captarlas por diversas vías (FAO, 2017). La ingesta es la interacción más común, debido a que muchas veces los organismos confunden los MP con alimento, o bien, porque estos materiales se encuentran entre el alimento, incorporándolos de esta manera a la cadena trófica (Roch et al., 2020).

Ingesta de microplásticos por peces

La ingesta de MP ha sido ampliamente evidenciada, incluso en especies de peces de importancia alimenticia para el ser humano. Diversas investigaciones se han dirigido al monitoreo de este fenómeno en especies de vida libre. Algunos reportes se mencionan a continuación.

Se han realizado monitoreos de MP en contenidos estomacales de especies de peces marinos, de ríos y lagos de varias partes del mundo. Algunos de estos estudios se han llevado a cabo en Australia (n=180; Su et al., 2019), China (n=147; Chan et al., 2019), Japón (n=64; Tanaka & Takada, 2016), Escocia (n=212; Murphy et al., 2017), Nigeria (n=109; Adeogun et al., 2020), Estados Unidos de América (n=700; McNeish et al., 2018, Phillips & Bonner, 2015), reportando desde 0.70 % hasta 85 % de presencia de MP en contenidos estomacales e intestinales de los organismos analizados.

En México existen estudios recientes como el realizado por Borges-Ramírez et al. (2020) en la bahía de Campeche, Golfo de México. En este estudio, se encontraron 316 partículas de MP en

el tracto gastrointestinal de 240 individuos (6 especies de peces diferentes), en forma de fibras, fragmentos y pellets. El análisis de las partículas encontradas reportó más de 24 compuestos químicos poliméricos, entre ellos el PMMA, PE y PS, por mencionar algunos.

En la zona metropolitana de México, en la cuenca del río Atayac (Puebla), fueron encontradas 139 fibras en el tracto gastrointestinal de 15 individuos de tilapia *O. niloticus*, especie de gran importancia alimenticia; él análisis de composición confirmó que se trataban de materiales plásticos de poliamida (PA), poliéster y celulosa (sintética y natural) (Martinez-Tavera et al., 2020).

Aunado a lo anterior, estudios realizados en sistemas de cultivo cerrado de *Mugil cephalus* reportaron MP en el 16 % (n=30) de los contenidos estomacales analizados (Cheung et al., 2018). Así también, 58 % (n=26) de frecuencia de aparición en *Lutujanus stellatus* de sistemas de cultivo en redes sobre aguas oceánicas (Chan et al., 2019).

Toxicidad de los microplásticos

Una herramienta para la investigación de los efectos adversos de los contaminantes sobre los seres vivos, son las pruebas o bioensayos de toxicidad, para establecer causa-efecto por medio del análisis de diversos indicadores (morfológicos, bioquímicos o genéticos, entre otros) en entornos controlados. Además, se reconoce su utilidad en programas de monitoreo de la calidad del ambiente y para el establecimiento de normas y leyes (Ramírez-Romero y Mendoza-Cantú, 2008).

En ese sentido, se han realizado exposiciones vía acuosa (suspendidos en agua) a MP de PS y PE con peces cebra (*D. rerio*) (Jin et al., 2018; Limonta et al., 2019; Lu et al., 2016; Qiao et al., 2019) y pez guppy (*P. reticulata*) (Huang et al., 2020), por mencionar algunos. Así también, bioensayos con MP de PE incorporados en el alimento (vía dieta) en el pez dorado (*Carassius auratus*) y *S. aurata* (Jabeen et al., 2018; Varó et al., 2021).

En los estudios anteriores, las alteraciones reportadas coinciden en que los MP que son captados por los organismos se acumulan principalmente en los intestinos, seguido de branquias e hígado; causando daños en la mucosa intestinal, inflamación, bloqueos y aumento de la permeabilidad en el tracto digestivo y disbiosis intestinal; además, alteración del sistema inmunológico y del metabolismo de lípidos en el hígado, y estrés oxidativo. Lo anterior deriva en la reducción del potencial de defensa de los organismos frente a patógenos y utilización diferente de reservas de energía, comprometiendo el crecimiento, la supervivencia, los mecanismos de adaptación y reproducción de las poblaciones expuestas (Brandts et al., 2018, 2021; Huang et al., 2020; Jabeen et al., 2018; Qiao et al., 2019; Varó et al., 2021).

Acrílico (polimetilmetacrilato, PMMA)

El PMMA es un termoplástico amorfo de la familia de lo acrilatos, transparente, incoloro y resistente a la intemperie, lo que le ha dado gran éxito en el mercado como sustituto del vidrio en diversos sectores industriales. Así también, tuvo gran auge en la fabricación de barreras protectoras anticontagios en la reciente pandemia de COVID-19, para frenar la transmisión del SARS-CoV 2. Además, el PMMA cuenta con propiedades antibacterianas y antifúngicas, por lo que es utilizado en medicina, farmacología y cosmetología, incluso en escalas micro y nanométricas (Ali et al., 2015; Bacali et al., 2020; Koike y Koike, 2012; Petrović et al., 2018).

Aunque se ha reportado la presencia de PMMA en ambientes acuáticos, así como su ingesta por peces de consumo humano (Borges-Ramírez et al., 2020; Liu et al., 2023), pocos son los estudios realizados para conocer los efectos sobre estos organismos. Ejemplo de lo anterior son los estudios realizados con peces dorada (*S. aurata*) y lubina (*Dicentrarchus labrax*) expuestos a partículas nanométricas de PMMA, en los que aumentó el estado oxidativo y respuesta antioxidante, así como alteraciones de las vías de señalización relacionadas con el metabolismo de los lípidos (Brandts et al., 2018, 2021). Sin embargo, no existen estudios realizados con MP de PMMA que evalúen los efectos sobre la fisiología digestiva de los peces.

Modelo biológico: Pejelagarto (*Atractosteus tropicus*)

El pejelagarto *A. tropicus* es un pez omnívoro (preferentemente carnívoro), de agua dulce (aunque también llega a ingresar en sitios salobres), perteneciente a la familia Lepisosteidae (Rush-Miller et al., 2009). Cuenta con una distribución en México desde la planicie costera del sur de Veracruz, Oaxaca, Tabasco, Campeche y Chiapas, y en América Central se encuentra en Guatemala, Belice, El Salvador, Nicaragua y Costa Rica (Instituto Nacional de Pesca, 2018; Rush-Miller et al., 2009).

La pesquería de esta especie es muy importante para la región, ya que solo en Tabasco la producción alcanzó 400 toneladas por año en el 2006 y 2007; sin embargo, la sobreexplotación de esta especie y otras actividades antropogénicas han disminuido sus poblaciones, por lo que en años recientes muchos esfuerzos se han encaminado a la construcción de una acuicultura sustentable (Márquez-Couturier y Vázquez-Navarrete, 2015; Instituto Nacional de Pesca, 2018).

Como resultado de años de investigación, las técnicas y condiciones de cultivo de *A. tropicus* en laboratorio se han perfeccionado y actualmente son relativamente fáciles y de bajo costo (Maldonado et al., 2020; Márquez-Couturier y Vázquez-Navarrete, 2015). Existe amplio conocimiento del ciclo de vida: procesos reproductivos, desove, etapa embrionaria, larval, juvenil

y adulta. Destacando que, en el estado juvenil (el cual inicia aproximadamente a los 20 días después de la eclosión), el sistema digestivo se encuentra totalmente desarrollado y la microbiota intestinal se ha constituido desde la etapa larval, además de que el dimorfismo sexual propio de la especie aún no se expresa (Frías-Quintana et al., 2015; Márquez-Couturier y Vázquez-Navarrete, 2015). Con base en lo anterior, *A. tropicus* en etapa juvenil cuenta con características de modelo biológico de evaluación de sustancias como los MP-PMMA, y análisis de la respuesta a nivel fisiológico digestivo, de crecimiento y sobrevivencia (Ramírez-Romero y Mendoza-Cantú, 2008).

Así mismo, existen estudios previos que nos proporcionan un marco de referencia sobre nutrición y actividad enzimática digestiva durante las diferentes etapas de desarrollo de *A. tropicus;* además, la composición de las comunidades microbianas intestinales, considerada un componente esencial debido a las funciones que realiza en la modulación del sistema inmunitario, la proliferación del epitelio intestinal y la regulación de la ingesta energética de la dieta, ya ha sido descrita anteriormente (Frías-Quintana et al., 2015, 2016; Guerrero-Zárate et al., 2019; Méndez-Pérez et al., 2020).

OBJETIVOS

General

Evaluar los efectos de la ingesta de microplásticos de polimetilmetacrilato (MP-PMMA) sobre la microbiota intestinal, actividad de las principales enzimas digestivas, crecimiento y sobrevivencia de pejelagartos (*Atractosteus tropicus*) juveniles de sistemas de cultivo.

Particulares

1. Caracterizar el agente a evaluar (acrílico comercial - PMMA) mediante el análisis de sus propiedades físicas (tamaño y forma) y composición química.

2. Diseñar y preparar dietas que incluyan cinco porcentajes distintos de MP-PMMA, para realizar bioensayos de exposición con *A. tropicus* juveniles por 60 días.

3. Analizar el efecto de la ingesta de MP-PMMA sobre índices de crecimiento y sobrevivencia de *A. tropicus*.

4. Determinar los efectos de la ingesta de MP-PMMA sobre la actividad de las principales enzimas digestivas de *A. tropicus*.

5. Determinar los efectos de la ingesta de MP-PMMA sobre la composición de la microbiota intestinal (a nivel de *phylum*, clase, orden y género) de *A. tropicus*.

HIPÓTESIS

La exposición de pejelagartos (*A. tropicus*) juveniles a MP-PMMA a través de la dieta, en distintos porcentajes, ocasionará disminución de la actividad de las principales enzimas digestivas y alteración de la composición y estructura de la microbiota intestinal después de 60 días de exposición, lo cual repercutirá en su nutrición y, por ende, en un menor crecimiento y sobrevivencia de los organismos expuestos.

JUSTIFICACIÓN

Existen cada vez más evidencias de la ingesta de MP por diferentes especies de peces, entre los que se incluyen de importancia alimenticia para el ser humano (Adeogun et al., 2020; Phillips y Bonner, 2015). Entre los compuestos MP que se han encontrado en contenidos estomacales se encuentran las partículas de PMMA conocidas comúnmente como acrílico (Borges-Ramírez et al., 2020; Liu et al., 2023).

En este contexto, los peces que ingieren estos materiales pueden presentar efectos adversos en su fisiología, comprometiendo su calidad alimenticia (Brandts et al., 2018, 2021; Huang et al., 2020; Jabeen et al., 2018). Por esta situación, es importante conocer los efectos que la ingesta de MP puede ocasionar en la fisiología de los peces y asegurar un consumo humano seguro y de calidad (FAO, 2017).

En ese sentido, los bioensayos de toxicidad son una herramienta para cuantificar niveles de concentraciones de agentes o sustancias que puedan causar algún efecto nocivo y establecer comparaciones de sensibilidad entre especies, aportan a un marco de información para la evaluación del estado de salud de los ecosistemas y ayudan a establecer normas y leyes (OECD, 2012; Ramírez-Romero y Mendoza-Cantú, 2008). De esta manera se han podido evidenciar alteraciones en la fisiología digestiva de peces ocasionadas por la ingesta de MP (Brandts et al., 2018, 2021; Huang et al., 2020; Jabeen et al., 2018).

A pesar de lo anterior, en México, no existen reportes de los efectos potencialmente tóxicos en peces autóctonos que representen, además, nuestras condiciones ambientales. Incluso, tampoco hay información sobre los efectos de MP-PMMA en peces.

En ese contexto, la presente investigación pone al pejelagarto *Atractosteus tropicus* como modelo de evaluación de los efectos de la ingesta de MP-PMMA sobre la fisiología digestiva, crecimiento y sobrevivencia en etapa juvenil.

La exposición vía dieta es recomendada para evaluar la toxicidad de sustancias insolubles, en la cual la sustancia es incorporada al alimento garantizando la interacción sustancia-organismo (OECD, 2012), por lo que en el presente estudio se opta por esta vía, anulando de esta manera, la posible interacción de los MP por otras vías de exposición (ej. exposición branquial).

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CAPÍTULO 1

Artículo científico:

POLYMETHYLMETHACRYLATE-MICROPLASTICS EFFECTS ON THE DIGESTIVE PHYSIOLOGY, GROWTH AND SURVIVAL OF CULTURED TROPICAL GAR (*Atractosteus tropicus*).

Autores:

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Resumen:

This study evaluated the effects of polymethylmethacrylate microplastics (PMMA- MPs) on the growth, survival, digestive enzyme activity, and intestinal microbiota of tropical gar (*Atractosteus tropicus*) fed diets enriched with different percentages of PMMA-MPs (0.0, 0.25, 0.50, 0.75, and 1.00%) for 60 days. Fish growth showed a significantly lower weight at 0.25% compared to 1.00% PMMA-MP treatments (23.21 ± 8.33 and 30.33 ± 12.14 g, respectively; $p = 0.02$), however, without differences to the control group ($p = 0.44$). The enzymatic activity of acid proteases, alkaline proteases, trypsin, chymotrypsin, L-aminopeptidase, and α-amylase showed significant differences among treatments ($p < 0.05$), except in lipases ($p = 0.83$). The intestinal microbiota showed higher values of the α diversity indexes (Chao, Shannon, Simpson, and Evenness) in the control groups and 1.00% PMMA-MPs and lower at 0.25, 0.75 and 0.50% PMMA-MPs. The relative abundance was represented mainly by Firmicutes in the groups with 0.25, 0.50, and 0.75 % PMMA-MPs (between 67 and 88% coverage) compared to controls and 1.00% PMMA-MPs (23 and 13%, respectively). PMMA-MP exposure could affect the bioavailability and assimilation of nutrients, compromising nutrition and, therefore, fish health.

Palabras clave:

Intestinal microbiota, digestive enzymatic activity, freshwater fish.

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Artículo científico

POLYMETHYLMETHACRYLATE-MICROPLASTICS EFFECTS ON THE DIGESTIVE PHYSIOLOGY, GROWTH AND SURVIVAL OF CULTURED TROPICAL GAR (*Atractosteus tropicus*).

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Abstract: This study evaluated the effects of polymethylmethacrylate microplastics (PMMA- MPs) on the growth, survival, digestive enzyme activity, and intestinal microbiota of tropical gar (*Atractosteus tropicus*) fed diets enriched with different percentages of PMMA-MPs (0.0, 0.25, 0.50, 0.75, and 1.00%) for 60 days. Fish growth showed a significantly lower weight at 0.25% compared to 1.00% PMMA-MP treatments (23.21 ± 8.33 and 30.33 ± 12.14 g, respectively; $p = 0.02$), however, without differences to the control group ($p = 0.44$). The enzymatic activity of acid proteases, alkaline proteases, trypsin, chymotrypsin, L-aminopeptidase, and α-amylase showed significant differences among treatments ($p < 0.05$), except in lipases ($p = 0.83$). The intestinal microbiota showed higher values of the α diversity indexes (Chao, Shannon, Simpson, and Evenness) in the control groups and 1.00% PMMA-MPs and lower at 0.25, 0.75 and 0.50% PMMA-MPs. The relative abundance was represented mainly by Firmicutes in the groups with 0.25, 0.50, and 0.75% PMMA-MPs (between 67 and 88% coverage) compared to controls and 1.00% PMMA-MPs (23 and 13%, respectively). PMMA-MP exposure could affect the bioavailability and assimilation of nutrients, compromising nutrition and, therefore, fish health.

Keywords: Intestinal microbiota, digestive enzymatic activity, freshwater fish.

Environmental implications

Food is a route for the acquisition of microplastics in fish. The impact of microplastics on digestive physiology includes histological damage, decreased assimilation, malnutrition, and microbiome changes in fish from various environments. However, there are fewer studies on tropical species, especially on panchronic organisms such as the tropical gar, including acrylic tests. This study demonstrates that environmentally realistic concentrations, which could represent 1% or less of the equivalent of indigestible fibre in cultured fish feed, can alter the production of various enzymes and the presence of certain bacteria in the microbiome despite not changing the growth of the fish.

Introduction

Microplastics (MPs) are plastic particles with dimensions between 1 and 1000 µm, according to the International Organization for Standardization (ISO, 2020). Plastics encompass a wide range of polymeric materials whose main characteristic is that, at some point in their transformation into finished products, they can be easily molded at specific temperatures and pressures; among the most common are acrylic (polymethylmethacrylate, PMMA), polystyrene (PS) and polyethylene (PE), to mention a few (FAO, 2017; ISO, 2020).

Plastic commercial success is mainly due to its durability, making it a potential environmental risk since it can take hundreds or even thousands of years to degrade completely. Industrial development allowed the massive manufacture of plastic products worldwide, bringing large amounts of waste and poor waste management, causing it to reach aquatic ecosystems worldwide directly and indirectly (Andrady, 2017; Tanaka & Takada, 2016). Eriksen et al. (2014) estimate that a minimum of 5.25 trillion plastic particles (268,940 tons) are floating in the oceans worldwide. An estimated 11 million metric tons of plastic enter the oceans yearly, a trend that could triple by 2050 (ONU, 2023). Based on the above, approximately 90% of the plastics in the pelagic marine environment are smaller than 5 mm (EPA, 2023).

As a result, microplastics are of secondary origin (particles derived from the fragmentation or wear of larger plastics) due to factors such as ultraviolet light, temperature, abrasion, wind, and water movement; and from the primary origin (particles manufactured on a micrometer scale) (Andrady, 2017; EPA, 2023).

Consequently, MPs are ingested by the organisms that inhabit these aquatic ecosystems, including individuals from culture systems, as demonstrated by stomach contents analyses

(Adeogun et al., 2020; Cheung et al., 2018; Phillips & Bonner, 2015). In this context, the need to analyze the impact of MP intake on fish physiology and survival has arisen and requires urgent attention.

Recent studies of exposure to MPs such as PE and PS in zebrafish (*Danio rerio*) and guppy (*Poecilia reticulata*) have been carried out, in which affectations at the digestive physiological level, such as intestinal dysbiosis and decreased digestive enzyme activity have been observed (Huang et al., 2020; Jin et al., 2018; Limonta et al., 2019). The digestive physiological alterations can compromise health since the adequate activity of the set of digestive enzymes is essential for the disintegration and assimilation of food and the generation of bioavailable nutrients for the construction of molecules and structures, as well as to provide the energy required for the functioning of the digestive system (Kennelly & Rodwell, 2013a).

Dysbiosis, understood as the imbalance of the intestinal microbial community that favors the proliferation of pathogenic bacteria, could have negative consequences since it plays an essential role in the health of individuals, contributing to the metabolism of the generation of nutrients derived from food and promoting intestinal health, they also create a protective barrier against the proliferation of pathogenic microorganisms, maintaining immune homeostasis, modulating important functions such as the antioxidant response, hepatic metabolism and lipid, and intestinal inflammatory responses, maintaining the balance, health and growth of the host (Huang et al., 2021; Talwar et al., 2018).

Recent studies have reported acrylic (PMMA) as a microplastic identified in the stomach contents of fish for human consumption (Borges-Ramírez et al., 2020; Liu et al., 2023). PMMA is widely used as a substitute for glass due to its excellent durability and transparency. It increased its use after the COVID-19 pandemic due to the mass manufacturing of face protective masks and anti-contagion barriers worldwide. In addition, PMMA has antifungal and antibacterial properties that are useful in pharmacology, medicine, and dentistry (Bacali et al., 2020; Bhosale et al., 2020). However, there are no evaluations of the impact that they could be causing the PMMA microplastic (PMMA-MPs) on the digestive physiology of fishes, so studies are required in different species and experimental conditions as a part of the risk assessment framework of MP exposures.

Therefore, in the present study, tropical gar (*Atractosteus tropicus*) was used as a biological model for assessing the risk of ingestion of PMMA-MPs because it has been shown to be sensitive to the components of the diet provided (Guerrero-Zárate et al., 2019; Nájera-Arzola et al.,2018), there is extensive knowledge of its biology, breeding and culture, it is representative of tropical freshwater ecosystems, it is use for human consumption, and recognized as a panchronic species

and regional cultural symbol in the state of Tabasco, Mexico (Frías-Quintana et al., 2016, 2017; Maldonado et al., 2020; Márquez-Couturier y Vázquez-Navarrete, 2015; Ramírez-Romero y Mendoza-Cantú, 2008). As a result, this study aimed to evaluate the effect of ingesting different percentages of PMMA-MPs in the diet of juvenile tropical gar (*A. tropicus*) on growth, survival, and activity of the main digestive enzymes and intestinal microbiota, resulting in the first study of this kind in a panchronic fish on the best of our knowledge for PMMA-MPs effects.

Materials and methods

Microplastics (MPs)

The PMMA particles were obtained from a fluorescent acrylic sheet (Acriplas®), which was pulverized and subsequently sieved to obtain particles smaller than 250 µm. The MPs obtained were washed with 30% hydrogen peroxide and subjected to density separation with NaCl (6 g/20 mL) to remove organic matter and impurities, according to the United States National Oceanic and Atmospheric Administration method (NOAA) (Masura et al., 2015).

The physical characteristics (size and shape) of the PMMA-MPs were analyzed through field emission scanning electron microscopy (FE-SEM, JEOL® JSM 7601F). The chemical composition was determined through Fourier Transform Infrared Spectroscopy (FTIR, Perkin Elmer® Frontier MIR/NIR) for the analysis of the functional groups $(C_8H_8)n$, carried out at the National Nano and Biomaterials Laboratory (LANNBIO) CINVESTAV-Mérida (Mexico).

Experimental organisms

Tropical gar (*A. tropicus)* larvae were obtained from the Aquatic Resources Physiology Laboratory (LAFIRA) of the Biological Sciences Academic Division of the Universidad Juárez Autónoma de Tabasco (DACBIOL-UJAT). After hatching, the larvae were maintained in a closed recirculating system in 70-L tanks and fed on their yolk sac until mouth opening, three days post-hatch (DPH), at which point exogenous feeding began.

The organisms were fed five times a day (at 8, 11, 13, 15, and 18 h) with live food (*Artemia* nauplii) from 3 to 18 DPH and simultaneously with frozen biomass biscuits (adult *Artemia*) from 9 to 18 DPH. The particulate feed was provided from 5 DPH until the bioassay started and consisted of trout feed (Silver Cup, 45% protein). The bioassay began with 80 DPH organisms (juvenile stage). Those with a size between 11 and 13 cm and in good health were selected (FAO, 2011). The population chosen obtained an average (± standard deviation [SD]) initial weight of 4.80 ± 1.18 g and a total length of 11.51 ± 0.89 cm, respectively.

Experimental design

Bioassays of exposure to PMMA-MPs incorporated into the food were carried out, following the Organization for Economic Cooperation and Development (OECD, 2012) recommendations, to evaluate chemical substances fish exposure through the diet. *A. tropicus* juveniles were fed five times a day (at 8, 11, 13, 15, and 18 h) with diets added with different PMMA-MPs percentages (0.25, 0.5, 0.75 and 1.00%), in addition to a control treatment (free of PMMA-MPs), for 60 days. All treatments were carried out in triplicate, using 70-L tanks, randomly placing 12 fish each in a closed recirculation system with 10% partial water changes daily.

Experimental diets

The diets were formulated based on the nutritional requirements of juvenile *A. tropicus*, modified from Frías-Quintana et al. (2017). The percentages of added PMMA-MPs replaced the wheat fraction (considered indigestible fiber) in each treatment (Supplementary material, Table S1) without nutrition changes in the feed supplied.

The diets were prepared by mixing the macro ingredients (soybean, starch, wheat, pork, poultry, and fish meal) in a CRT global® industrial mixer. The micro-ingredients (soybean lecithin, grenetine, vitamin C and vitamins and minerals mixture) were previously mixed with the corresponding amount of PMMA-MPs in powder from each treatment (except in the control diet), and water was added until a homogeneous and consistent mixture was obtained (approx. 400 mL/Kg). Subsequently, the pellets were made in a mill (Torrey®) and dried in a SanSon® oven at 60°C for 5 hours (Álvarez-González et al., 2001).

Sampling

For analysis of growth and survival, data on fresh weight, total length, standard length, and number of fish were taken at the beginning and every 15 days until reaching 60 days. At the end of the bioassay, the stomach and intestine were extracted from one random specimen of each replicate (3 samples per tissue per treatment) for enzyme activity analysis and stored at -80 °C in Eppendorf tubes. For the metagenomic analysis of the intestinal microbiota, intestines were extracted from 3 random specimens per treatment (from different replicates), washed on the surface with sterile water, and stored in DNase- and RNAse-free Eppendorf tubes at -80 °C. The previous was carried out following the "*Animal Handling Manual for Research and Teaching Purposes*" of the Universidad Juárez Autónoma de Tabasco (AILAD, 2010).

Growth and survival assessment

Growth and survival were evaluated through the following indexes (Guerrero-Zárate et al., 2019; Márquez-Couturier et al., 2006; OECD, 2012):

Survival: $S = \left(\frac{\text{Final number of fish}}{\text{Initial number of fish}}\right) \times 100$ (Eq. 1)

Absolute weight gain: $AWG = (\text{Final mean body weight} - \text{Initial mean body weight})$ (Eq. 2)

Weight gain: $WG = \left(\frac{\text{Final mean body weight} - \text{Initial mean body weight}}{\text{Initial mean body weight}}\right) \times 100$ (Eq. 3)

Specific growth rate: $SGR = \left(\frac{\text{Ln final mean body weight} - \text{Ln initial mean body weight}}{\text{days}}\right) \times 100$ (Eq. 4)

Condition factor: $CF = \left(\frac{\text{Final mean body weight}}{(\text{Final standard length mean})^3}\right) \times 100$ (Eq. 5)

Where Ln is the natural logarithm.

Digestive enzyme activity

Multienzyme extracts (ME) of the stomach (in 100 mM glycine-HCl buffer, pH 2, 1:10 w/v) and intestine (in 30 Mm Tris-HCl and 12.5 mM $CaCl_2$ buffer, pH 7.5, 1:10 w/v) were prepared separately of each treatment. The samples were macerated in an ultrasonic processor (20 pulses per minute, 70% amplitude) until a homogeneous mixture was obtained. Subsequently, they were centrifuged at 16,000 *g* at 4 °C for 15 min; the supernatant was taken and stored in Eppendorf tubes at -80 °C until analysis.

The soluble protein of the ME of the stomachs and intestines of each treatment was calculated through the Bradford (1976) technique, using a concentrated Bovine Serum Albumin (BSA, 600 mg/mL) standard. Their absorbances were determined by spectrophotometry at 595 nm.

The acid protease activity was determined according to Walter (1984) and Anson (1938), using haemoglobin (0.25%) as substrate in 100 mM glycine-HCl buffer at pH 2. It was allowed to react with stomach ME for 15 min at 37 °C; the reaction was stopped with 10% TCA (trichloroacetic acid). Subsequently, it was centrifuged at 17,500 *g* at 4 °C for 15 min, and the supernatant was read at 280 nm. The activity of total alkaline proteases was quantified by the method of Kunitz (1946) modified by Walter (1984), using casein (0.25%) as substrate in 100 mM Tris and 10 mM $CaCl_2$ buffer at pH 9, with the intestine ME, following the same procedure as acid proteases. Each unit of enzyme activity was defined as 1 µg of tyrosine released per minute, based on a Molar Extinction Coefficient (MEC) of 0.005 (mL μg^{-1} cm^{-1}).

Trypsin activity was analyzed with modifications of the method of Erlanger et al. (1961) using BAPNA substrate (N-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride, 3.5 mM) previously diluted in DMSO (dimethylsulfoxide), in 50 mM Tris-HCl buffer at pH 9. Chymotrypsin activity was analyzed according to DelMar et al. (1979) using SAAPpNA (N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide, 1.5 mM) previously diluted in DMSO, in 50 mM Tris-HCl buffer and $CaCl_2$ as substrate; 100 mM at pH 7.8. Leucine aminopeptidase (L-aminopeptidase) activity was determined using 4 mM L-Leucine-p-nitroanilide (previously diluted in DMSO) in 50 mM pH 7.2 sodium phosphate buffer, based on Maroux et al. (1973). The different reactions were carried out with intestine ME for 30 min at 37 °C, with a MEC of 8.8 (mL μg^{-1} cm^{-1}) at 410 nm.

The α-amylase activity was determined based on the Somogy-Nelson reagent Robyt & Whelan (1968) described. A starch solution (2%) was used in dibasic sodium phosphate buffer and 100 mM sodium citrate dihydrate, 50 mM $NaCl_2$, pH 7.5, incubated for 30 min at 37 °C with ME of intestines. Somogy-Nelson reagent 4 was added, boiled for 20 min in a water bath, and cooled to room temperature. Somogy-Nelson reagent 3 was added, vortexed, added water and read at 600 nm. MEC of 3.40 (mL μg^{-1} cm^{-1}).

Lipase activity was analyzed according to Versaw et al. (1989). A mixture of 50 mM Tris-HCl buffer with 100 mM sodium taurocholate was allowed to incubate for 5 min at 37 °C with ME of intestines. Subsequently, it was set for 30 min at 37 °C with 100 mM β-naphthyl acetate as substrate. 100 mM Fast Blue BB was added as an activator, and the reaction was quenched with 10% TCA. Finally, ethyl acetate (1:1 ethanol) was added and read at 540 nm. A MEC of 0.02 (M^{-1} cm^{-1}) was used.

The enzyme activity was reported in U/mg of protein using the following equations:

$$\frac{\text{Units}}{\text{mL}} = \frac{\Delta\text{Abs} \times \text{final reaction volume (mL)}}{\text{MEC} \times \text{time (min)} \times \text{ ME volume (mL)}} \quad \text{(Eq. 6)}$$

$$\frac{\text{Units}}{\text{mg protein}} = \frac{\text{Units/mL}}{\text{mg soluble protein/mL}} \quad \text{(Eq. 7)}$$

Where ΔAbs is the increase in absorbance, MEC is the Molar Extinction Coefficient, and ME is a multienzyme extract.

Gut microbiota analysis

Extraction of DNA.

A total of 15 random samples (3 per treatment) were sent to the Microbial Genomics Laboratory of the Food and Development Research Center (CIAD, Mazatlán, Mexico) for DNA extraction, DNA library preparation, and sequencing.

To determine the microbiota present in the samples, the V3 region of the 16S rRNA gene was amplified by PCR using universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') with Illumina adapters. The final products were indexed according to the standard protocol recommended by Illumina. Finally, the samples were quantified in Qubit and mixed in an equimolar pool to be sequenced in the Illumina Miniseq equipment under standard conditions (300 cycles, 2 x 150 pair-end).*Sequence analysis.*

The minimum length of the sequences considered was 149 bp (87% of the total sequences). Low-quality adapters and bases were removed with Trimmomatic 0.36 (Bolger et al., 2014). Then, the sequence analysis of the 16S rRNA amplicon was processed using the Quantitative Insights Into Microbial Ecology (QIIME2) software, implemented in Microbiome Helper (Douglas et al., 2018) following for filtering, assembling and removing chimera sequences using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016). Sequence analysis for clustering into Operational Taxonomic Units (OTUs) was assigned to taxonomic levels using the SILVA rRNA database v138 (https://www.arb-silva.de/) as a reference with 97% similarity. Artefacts corresponding to chloroplasts and mitochondria were removed during the bioinformatic process.

Diversity analysis.

The α diversity was determined utilizing the relative abundance at the phylum, class and genus level in the Excel v2207 software and through the Chao richness index (Chao-1), Shannon diversity (Shannon_H), Simpson uniformity (Simpson 1-D) and Evenness (Evenness J) in the Past v4.03 software. Beta diversity was analyzed with the Eucledian dissimilarity index employing clustering analysis (UPGMA, bootstrop N 9999) (Past v4.03).

Statistical analysis

The final weight and length results (transformed to LogN), as well as the growth indices (AWG, WG, SGR and CF), were analyzed by the parametric method based on compliance with the postulates of normality (Kolmogorov-Smirnov) and homoscedasticity (Levene test), applying the one-way ANOVA test and *a posteriori* HSD Tukey test. To analyze the survival rate and the digestive enzyme activity, non-parametric Kruskal-Wallis and *a posteriori* Nemennyi tests were used. The Statistica v.7 software was used for all the analyses with an α of 0.05.

Results

Microplastics (MPs)

SEM micrographs show particles with irregular shapes and sizes between 15 – 290 µm, some forming agglomerates and clusters (Fig. 1A). The FTIR analysis (Fig. 1B) confirmed the fingerprint zone from PMMA with the vibration bands among 2993 to 750 cm^{-1} according to other published studies (Table S1) (Duan et al., 2008; Sugumaran et al., 2017; Vijayakumari et al., 2013). The representative peaks at 2993 and 2949 cm^{-1}, corresponding to the stretching vibration of the C-H bond of CH_3 and CH_2, respectively (Duan et al., 2008). The peaks at 1721, 1238, 1189 and 1142 cm^{-1} correspond to the stretching vibration of the C=O and C-O bonds of the esther, respectively (Sugumaran et al., 2017; Vijayakumari et al., 2013). The α-methyl group was represented by the 1387 and 750 cm^{-1}; similarly, the peaks at 986 and 750 cm^{-1} peaks represent PMMA (Duan et al., 2008).

Growth and survival

The analysis of the fish's final weight suggests significant differences among treatments ($p = 0.04$). The post hoc test showed that the groups fed with 0.25% PMMA-MPs obtained significantly lower mean (± SD) weight at the end of exposure than the groups provided with 1.00% PMMA-MPs (23.21 ± 8.33 and 30.33 ± 12.14 g, respectively) ($p = 0.02$); however, they did not show differences to the control groups (25.24 ± 7.60 g) (Table 1). The fish total length was not statistically different among the treatments ($p = 0.11$) (Table 1). The mean (± SD) of the total length of all organisms was 18.17 ± 1.71 cm at the end of the experiment.

In general, the survival of the fish was not significantly different among treatments ($p = 0.44$), obtaining 94 % survival of the total population evaluated. The growth indexes (AWG, WG, SGR, CF) did not show significant differences between the treatments ($p = 0.15$, 0.21, 0.20, and 0.22, respectively); however, in all cases, the treatment with 0.25% PMMA-MPs had the lowest values, influenced by the lowest final weight obtained (Table 1).

Digestive enzyme activity

The enzymatic activity of acid proteases significantly differed among treatments ($p = 0.012$). Their activity increased with the presence of PMMA-MPs in the groups fed with 0.25% and 0.50% (median ± IQR, 15.34 ± 1.62 and 22.78 ± 2.57 U/mg protein, respectively) compared to controls (9.27 ± 0.35 U/mg protein). However, when the percentage of MPs was increased to 0.75%, the activity decreased to 6.55 ± 0.15 U/mg protein, significantly lower than the groups with 0.50% MPs ($p = 0.01$). The groups fed with 1.00% PMMA-MPs again increased enzymatic activity (14.71 ± 2.91 U/mg protein), although negligible (Fig. 2A).

The enzymatic activity of total alkaline proteases was significantly different among treatments (p = 0.012). Their activity decreased with the increase in PMMA-MPs in the diet. The treatment with the highest percentage of PMMA-MPs was significantly lower (4.71 ± 0.10 U/mg protein) compared to the control groups (57.90 ± 0.26 U/mg protein) (p = 0.01; Fig. 2B).

Trypsin, chymotrypsin, and L-aminopeptidase's enzymatic activity significantly differed among treatments (p = 0.012, 0.003, and 0.011, respectively) (Fig. 2C-E). In the case of trypsin, the treatments that included 0.50% and 0.75% PMMA-MPs showed significantly lower enzymatic activity (0.00038 ± 0.00004 and 0.00036 ± 0.00009 U/mg protein, respectively) compared to the control groups (0.0030 ± 0.0003 U/mg protein) (p = 0.047 and 0.035, respectively) (Fig. 2C).

Chymotrypsin activity decreased considerably in the groups with the highest percentage of PMMA-MPs, being significantly lower in those that included 0.50% and 1.00% PMMA-MPs (0.0087 ± 0.0002 and 0.0029 ± 0.0001 U/mg protein, respectively) compared to the controls (0.1112 ± 0.0102 U/mg protein) (p = 0.048 and 0.003, respectively) (Fig. 2D). L-aminopeptidase activity significantly decreased in the treatment with 0.75% PMMA-MPs (0.0041 ± 0.0001 U/mg protein) compared to the control (0.0107 ± 0.0002 U/mg protein) (p = 0.019) (Fig. 2E).

The α-amylase enzyme activity significantly differed among treatments (p = 0.004). Their action also decreased in the groups fed with PMMA-MPs, being significantly lower in the treatment with the highest percentage of MPs (0.005 ± 0.004 U/mg protein) compared to the control (0.032 ± 0.035 U/mg protein) (p = 0.026; Fig. 2F).

On the other hand, the enzymatic activity of lipases did not show statistically significant differences with the presence of PMMA-MPs in the diets (p = 0.83), showing a general range of 6.61 to 24.00 U/mg protein in all treatments.

Gut microbiota analysis

Sequence analysis

A total of 15 amplicon libraries were obtained (3 per treatment); however, one replicate of each treatment showed different and unusual behaviour compared to the other two replicates, so it was discarded. Therefore, ten libraries were analyzed (two per treatment), of which 253,689 sequences were derived from all the analyzed samples, with a length equal to or greater than 149 bp (87 % of the total sequences). The mean (± SD, n = 2) of sequences for control treatment and 0.25, 0.50, 0.75 and 1.00 % PMMA-MPs was 16718 ± 5169, 25752 ± 13618, 29862 ± 5766, 38105 ± 362 and 16409 ± 3522, respectively. The arrangements were generally distributed in 417 OTUs, 28 phyla, 64 classes and 112 orders, with 97 % similarity.

Microbiota composition

The relative abundance at the phylum level was represented in all groups, mainly by Firmicutes, Proteobacteria, Chloroflexi and Actinobacteriota; in the groups fed with 0.25, 0.50 and 0.75% PMMA-MPs, the phylum Firmicutes considerably increased their coverage percentage (90, 67 and 88%, respectively) compared to the control groups and 1.00 % PMMA-MPs (23 and 13 %, respectively) (Fig. 3). The increase in Firmicutes had an impact on the decrease of the Proteobacteria group, which represented a coverage of 37 and 35% in the control treatment and 1.00% PMMA-MPs (respectively), decreasing to 17, 5 and 4% in 0.50, 0.75 and 0.25% PMMA-MPs treatments, respectively (Fig. 3).

The other phyla (Chloroflexi, Actinobacteriota, Deinococcota, Bacteroidota, Gemmatimonadota, Myxococcota, Acidobacteriota) showed greater abundance in the control groups and in those feds with the highest percentage of PMMA-MPs, decreasing in the groups with 0.50, followed by 0.25 and 0.75% PMMA-MPs, where a more drastic decrease was observed (Fig. 3). This trend was reflected in the classes and orders belonging to each phylum (Supplementary material, Fig. S1-S4).

At the class level, the relative abundance was represented mainly by Bacilli (phylum Firmicutes), Alphaproteobacteria and Gammaproteobacteria (phylum Proteobacteria), Anaerolineae and Chloroflexia (phylum Chloroflexi), Acidimicrobiia and Actinobacteria (phylum Actinobacteriota), in general (Fig. 4).

The Bacilli class increased its abundance drastically in the groups fed with 0.25, 0.50 and 0.75% PMMA-MPs with coverage of 90, 66 and 87%, respectively, compared to the control groups and 1.00% PMMA-MPs with 21 and 11% of coverage. With the increase of Bacilli, the decrease in the relative abundance of classes such as Alphaproteobacteria, Gammaproteobacteria, Anaerolineae, Chloroflexia, and Acidimicrobiia was evident (Fig. 4).

Since the Firmicutes and Proteobacteria groups obtained the most representative abundances in all the treatments, it was decided to analyze the genera of these groups, managing to identify the most abundant ones. Therefore, we can observe that the genus *Mycoplasma* represents the highest abundance in the microbiota of tropical gar fed with 0.25, 0.75 and 0.50% PMMA-MPs, with coverage of 64, 87 and 90%, respectively, compared to the control groups and the fed with the highest percentage of PMMA-MPs with 16 and 4% coverage, respectively (Fig. 5). *Clostridium* also presented a different response, with relative abundances <1% in the groups without MPs

and those fed with 0.25 and 1.0 % PMMA-MPs, observing an increase in the intermediate groups with 0.50 and 0.75% of PMMA-MPs (with 2 and 1% coverage) (Fig. 5).

Plesiomonas decreased abundance in the groups fed with PMMA-MPs compared to the control groups. It is worth mentioning that the groups fed with 0.25% PMMA-MPs showed the greatest decrease, followed by the groups with 0.75 and 1.0% PMMA-MPs (Fig. 6).

Diversity

Alpha diversity

The Chao, Shannon, Simpson and Evenness indexes generally tended to have higher values in control and those with the highest percentage of PMMA-MPs (1.00%). On the contrary, the values decreased in the groups with 0.50% PMMA-MPs, followed by 0.25 and 0.75% PMMA-MPs, mainly in the Shannon and Evenness indexes. The Chao index showed higher species richness in the groups of 1.0% PMMA-MPs, followed by the control groups. Richness decreased in the groups with 0.50% PMMA-MPs, followed by 0.25 and 0.75% PM-PMMA (Table 2).

Beta diversity

The dendrogram derived from the Eucledian dissimilarity index shows three main clusters with a correlation coefficient 0.83. In the first grouping, the control groups and those fed with 1.00% PMMA-MPs can be observed; in the second, there are the groups with 0.50%, as well as one of the replicates of the treatment with 0.25%; in the third group, there are the groups of 0.75% PMMA-MPs, and another of the treatment replicates with 0.25% PMMA-MPs (Fig. 7).

Discussion

Microplastics (MPs)

The characterization of the plastic material used in this study showed coincidences in the FTIR analysis with that reported by Petrović et al. (2018), corresponding to PMMA, with transmittance peaks at the same wavenumber. However, the SEM micrographs presented by Petrović et al. (2018) show particles with a smooth surface and protuberances, unlike the particles in the present study in which a very irregular surface is observed in the form of layers or strata caused by the acrylic pulverization in the process of obtaining microparticles in our study.

Growth and survival

In the present study, PMMA-MPs did not affect the growth and survival of tropical gar (*A. tropicus*) in our experimental condition. All treatments showed higher AWG, WG and SGR values than

those reported in previous studies with *A. tropicus* of similar ages (Nájera-Arzola et al., 2018), guaranteeing the viability of the food as an appropriate diet for the juvenile phase of this species.

Like that reported in *S. aurata* fed for 45 days with diets enriched with PVC (polyvinyl chloride; high and low molecular weight), PE (medium and high molecular weight), PS and PA (polyamide) MPs, at 0.1 mg/kg of MPs per body weight of the fish, in this study also no effects on growth were observed (Jovanović et al., 2018).

In invertebrates, similar studies with PMMA (1 – 100 µm) added to the diet of the marine isopod (*Idotea emarginata*, 40 particles/mg of food) did not show any effects on the organisms under quality food conditions and on free demand as well as regarding the body mass and feed consumption rates (Korez et al., 2019).

It has even been reported that PMMA-MPs (10.0 – 400.0 µm) as well as PVC (0.1 – 100 µm), behaved similarly to natural red clay microparticles (0.1 – 100 µm) and diatom shells (4.0 – 400.0 µm) in mussels of the Mytilidae family (6 species from temperate, tropical and subtropical zones) exposed to concentrations of 1.5, 15.0 and 150.0 mg/L, in which no affectations were observed in the condition index of the organisms after six weeks of exposure (Hamm et al., 2022).

The trend of more growth was observed in the groups fed with 1.00% PMMA-MPs compared to the groups that did not ingest plastics (controls) (Table 1), which may be because, from the random distribution of the organisms in the tanks at the beginning of the experiment, the size and weight of the fish in these groups were slightly larger than the controls. Two of the smallest fish in the control groups were left behind, possibly due to natural feeding competition within the aquariums, probably affecting an individual's biological variability.

However, similar results have been reported in another study with *S. aurata*, which were fed for three months with diets enriched with 10% low-density PE (LDPE) (200 - 500 µm in size), showing significantly greater size and higher condition factor than their respective controls (Alomar et al., 2021).

Digestive enzyme activity

The control groups obtained digestive enzyme activity (acid proteases, total alkaline proteases and α-amylase) similar to what was previously reported for the species at similar ages (Nájera-Arzola et al., 2018), as expected. In the case of the increased acid enzymatic activity in the groups fed 0.25 and 0.50% PMMA-MPs (Fig. 2A), it could be considered as a response to try to increase the catalysis of dietary elements due to pepsin which is responsible for the hydrolysis of proteins in the stomach. They are activated with the acidic pH provided by HCl in the presence of food.

High activity is characteristic of monogastric fish, as the beginning of a high acid digestion to improve the hydrolysis of proteins and carbohydrates in the intestine (Frías-Quintana et al., 2017). The above is similar to what has been pointed out by Romano et al. (2018) with the increase of trypsin and chymotrypsin in *Barbodes genionotus* fish exposed to PVC-MPs (40 – 230 µm in size at 0.05 and 1.0 mg/L). However, increasing the percentage of PMMA could cause mucosal or stomach gland damage, which could have decreased enzyme activity in the 0.75 and 1.0% groups (Kennelly & Rodwell, 2013b).

The alkaline proteases include trypsin, chymotrypsin and L-aminopeptidase (Fig. 2B-E); these proteases hydrolyze dietary proteins at a basic pH in the intestine (Bender & Mayes, 2013). The decrease of alkaline proteases in the treatments with PMMA-MPs could have an impact on the catalytic capacity of digestive enzymes and the efficiency in the bioavailability of amino acids to be assimilated by enterocytes, which compromises the adequate nutrition of the animals (Bender & Mayes, 2013; Kennelly & Rodwell, 2013a).

Particularly, trypsin is important since it is an endopeptidase in charge of the hydrolysis of internal bonds of protein chains on the carboxyl-terminal side of basic amino acids. In addition to its role as a zymogen activator, it activates other digestive enzymes. Interference with proteins responsible for rate-limiting reactions of metabolic pathways, such as zymogen-activating trypsin, can reduce the entire pathway's metabolite flux (Kennelly & Rodwell, 2013b). Chymotrypsin is an endopeptidase responsible for unfolding peptide bonds on the carboxyl-terminal side of large hydrophobic amino acids (Kennelly & Rodwell, 2013b).

The decrease in trypsin and chymotrypsin enzyme activity observed in the PMMA-MPs treatments of the present study has also been observed in other studies in fishes by exposure to different types of MPs. As occurs in *P. reticulata* exposed for 28 days to PS (32 – 40 µm at 100 and 1000 µg/L), showing a significant decrease in the enzymatic activity of trypsin, chymotrypsin, α-amylase and lipase with increasing MPs concentration. This decrease is attributed to the feeling of not wanting to eat caused by the permanence of MPs in the intestinal tract (Huang et al., 2020).

In the discus fish (*Symphysodon aequifasciatus*) exposed to PE (70 - 80 µm, at 200 µg/L) for 30 days, at different temperatures (28 and 31 °C), a significant decrease in trypsin was also observed. In the case of α-amylase, the activity changed as a function of temperature, showing a significant increase in enzymatic activity at 28 °C in the groups exposed to MPs, compared to the controls; however, at 31 °C the control groups increased their activity in response to the increase in temperature, while that of the groups exposed to MPs remained at the same level, now being significantly lower than the controls (Wen et al., 2018).

Similar responses were observed in invertebrates such as *A. japonicus*, where trypsin and lipase activity decreased significantly in groups exposed to diets enriched with PS (20 µm and 100 nm at 10%). In contrast, α-amylase activity was notably reduced only in the 100 nm PS groups (Liu et al., 2022). Likewise, in the Mediterranean mussel (*Mytilus galloprovincialis*) exposed to PS (20 µm) and PE (20 and 70 µm), it was observed that the enzymatic activity of α-amylase was lower depending on the decrease in size of MPs and type of polymer, being significantly lower in the groups exposed to 20 µm PS particles compared to the rest of the treatments (Trestrail et al., 2021).

α-amylase is responsible for the breakdown of carbohydrates. The decreased activity by increasing PMMA-MPs in the diets could alter the glucose levels adequate for a balanced glycogen/lipid metabolism, affecting the ability to digest certain levels of carbohydrates to cover demand and maintain the reserve energy of the metabolism (Frías-Quintana et al., 2016, 2017).

In general, the decrease in enzymatic activity can also be attributed to possible damage generated in the secretory glands and blockages of the enzyme-substrate union due to inhibition or inactivation of the enzyme, causing a decrease in the catalytic capacity of the molecule (Kennelly & Rodwell, 2013b). Trestrail et al. (2021) suggests that the enzyme inhibitors that could be acting are MPs and their leached chemicals, causing a decrease in the digestive capacity of organisms.

Because additives used in the manufacture of plastic materials, such as phthalates and persistent organic pollutants (POPs) or polybrominated diphenyl ethers (DEPB), are found in a large percentage and are not chemically bound to the polymers, they can be released and leach from the particles remaining free in the medium (FAO, 2017). It has been documented that POPs such as benzo[a]pyrene associated with MPs can be shed in the intestine of fish and interact with the intestinal epithelium (Batel et al., 2016).

Contrary to the above, an increase in enzymatic activity has been reported in *B. gonionotus* fish exposed to PVC-MPs (0.2, 0.5 and 1.0 mg/L, with 90 % of the particles < 230 µm) for 94 h, where the trypsin and chymotrypsin activity was analyzed in the whole body. Trypsin showed higher enzymatic activity at the highest concentration of PVC-MPs (1.0 mg/L) and chymotrypsin in the groups with 0.5 mg/L PVC-MPs, probably due to an attempt to improve digestion because of the thickening of the intestinal epithelium caused by the presence of MPs (Romano et al., 2018).

Similar to the previously mentioned, *M. galloprovincialis* invertebrates increased the digestive enzymatic activity of non-specific proteases (intra and extracellular) in the groups exposed to 75

μm PE particles compared to the rest of the treatments. However, these increases are attributed to intracellular proteases derived from tissue damage (generating apoptosis and stimulating hemocytes to engulf damaged cells and repair tissue) (Trestrail et al., 2021).

In the study with PMMA-MPs in *I. emarginata* fed with natural diets (*Fucus vesiculosus* brown algae) enriched with 1 – 100 μm MPs (40 particles/mg of food), a decrease in the enzymatic activity of endopeptidases and exopeptidases was detected in the hindgut, and of esterase and lipases in the midgut. However, when isopods were fed lower-quality diets (frozen algae powder) added with PMMA-MPs at the same concentration, enzyme activity increased in the hindgut (endopeptidases and phosphatases) and midgut (esterase and phosphatases and endopeptidases); this increases is attributed to a response by the organism to increase the catalytic capacity and, therefore, the extraction and assimilation of nutrients from a poor diet and a fast-food passage through the digestive tract (Korez et al., 2019).

In summary, fluctuations in digestive enzyme activity seem to be associated with the individual's response to the presence of MPs since these could decrease activity due to a lack of appetite caused by feelings of satiety or discomfort or, instead, promote enzyme activity to increase digestive capacity (Huang et al., 2020; Korez et al., 2019; Romano et al., 2018). Damage to the intestinal epithelium and secretory glands can decrease the activity of digestive enzymes, although in some cases, they seem to reflect an increase in enzymatic activity. These responses have been seen in analyses of intracellular and extracellular, or non-specific, proteases that do not necessarily reflect an increase in the digestive capacity of the individual (Romano et al., 2018; Trestrail et al., 2021).

Generally, it can be deduced that the exposure dose, type of polymer and particle size, environmental conditions and the biology of the exposed organisms influence the enzymatic activity in the presence of MPs. The fluctuation in the levels of digestive enzymes affects the nutrition of organisms since they are biological catalysts responsible for the disintegration of proteins, carbohydrates and lipids into simpler molecules (amino acids, monosaccharides and fatty acids) that can be assimilated by intestinal cells (enterocytes) and distributed through the bloodstream (Bender & Mayes, 2013; Bone & Moore, 2008).

Gut microbiota analysis

Diversity

The set of microorganisms present in the intestine of living beings, called intestinal microbiota, colonizes the mucosa and the epithelium successively during the congenital development and

growth of the host, giving rise to a bacterial consortium with which it is in symbiosis and is host-specific (Biavati & Mattarelli, 2006; Kim et al., 2021; Marchesi & Ravel, 2015; Talwar et al., 2018). Environmental and habitat characteristics influence this bacterial consortium in fish, based on the ability to adapt to the environment, drives the selection of the intestinal microbiota according to the microbial functional profiles, creating a host-microbes-environment relationship (Kim et al., 2021; Talwar et al., 2018). As can be observed in the present study, in which Firmicutes, Proteobacteria and Actinobacteria (or Actinobacteriota, Nouioui et al., 2018) were representative of all treatments, similar to those recorded to juveniles *A. tropicus* from culture systems (Méndez-Pérez et al., 2020).

Therefore, we can deduce that the changes in the composition and structure of the intestinal microbiota of the groups fed with 0.25, 0.50 and 0.75% PMMA-MPs are because the diets have a long-term influence on the abundance of their constituents since these microorganisms feed on the ingested components, epithelial cells, and intestinal mucosa (Talwar et al., 2018). The increase in Firmicutes and decrease in Proteobacteria of tropical gars exposed to 0.25, 0.50 and 0.75% PMMA-MPs has been indicated as dysbiosis in *D. rerio* larvae and adults exposed to PS microspheres (0.5 to 50 µm), in which the same pattern was observed (Jin et al., 2018; Wan et al., 2019). Similar results were even reported in juvenile large yellow croaker (*Larymichthys crocea*) also exposed to PS spheres although in nanoparticles (100 nm) (Gu et al., 2020b).

Similar results have been observed in other studies carried out with PS-MPs, such as the increase in the abundance of Firmicutes in adults the marine medaka (*Oryzias melastigma*) exposed to 200 µm particles (Zhang et al., 2021) and the decrease of Proteobacteria in Grass carp (*Ctenopharyngodon idella*) exposed to spherical particles of 32 – 40 µm. However, in the latter, the abundance of Actinobacteria increased (Jia et al., 2022).

Interestingly, the opposite was observed in *P. reticulata* exposed to similar particles to the above (32 – 40 µm PS spheres) since the abundance of Proteobacteria increased, and Actinobacteria decreased (Huang et al., 2020). In addition to the above, adults *D. rerio* exposed to beads and PS fragments (10 - 40 µm) and PP fibres (20 - 250 µm) obtained a similar response to *P. reticulata*, in which Proteobacteria increased, and Actinobacteria decreased (Qiao et al., 2019).

Studies with PE have shown similar responses, as in *S. aurata* fed with diets that included 0.12% LDPE (200 – 500 µm), which showed a decrease in the abundance of Actinobacteria. However, Firmicutes and Proteobacteria remained without significant changes (Varó et al., 2021). In *D. rerio* larvae, exposure to PE decreased the abundance of Proteobacteria and Actinobacteria, and a decrease in Firmicutes was reported (Kurchaba et al., 2020; Zhao et al., 2021).

At the phylum level, Proteobacteria and Firmicutes are characteristic and commonly dominant in the gut microbiota of freshwater fish, followed by Actinobacteria (Kim et al., 2021). The Firmicutes group comprises microorganisms that can produce endospores resistant to extreme environments (Galperin, 2013). This feature could have favoured the proliferation observed in the present study in an environment possibly disturbed by MPs. Firmicutes have been pointed out as a beneficial phylum in fish, with crucial functions such as immune regulation, promotion of digestion and the fight against inflammation through the production of short-chain fatty acids, particularly butyrate, a product of fibre fermentation (Liu et al., 2023; Montero et al., 2022). However, in *D. rerio* larvae, intestinal dysbiosis related to the increase in Firmicutes and the increase of Proteobacteria in *P. reticulata* due to exposure to PS-MPs had repercussions on digestive physiology and anatomy (Jin et al., 2018; Wan et al., 2019).

In the foregoing, we can see that Proteobacteria, Firmicutes and Actinobacteria are commonly altered by exposure to various MPs. However, the fluctuation patterns of their abundances are not always similar. This difference could be understood due to the diversity of microorganisms housed at the phylum level since the lower taxonomic groups, such as class, order, and genus that proliferate within the intestinal bacterial community, are not always the same, as described below.

The abundance of the phylum Proteobacteria in the present study was covered mainly by the classes ɣ-Proteobacteria and α-Proteobacteria, which decreased in the treatments that included 0.25 and 0.75%, followed by 0.50% PMMA, similar results were observed in *D. rerio* larvae and adults exposed to PS (0.5, 5.0 and 50 µm) and PE (1 – 4 µm) in which the abundance of ɣ-Proteobacteria also decreased (Jin et al., 2018; Wan et al., 2019; Zhao et al., 2021).

ɣ-Proteobacteria was formed by the orders Enterobacterales and Pseudomonadales in the present study; Enterobacterales, covered mainly by *Plesiomonas* at the genus level, showed a tendency to decrease with increasing percentage of PMMA in the diet (Fig. 6). This pattern is similar to what was reported at the order level in *S. aurata* in which LDPE (200 - 500 µm) was included in the diet, as well as in *O. melastigma* exposed to PS (200 µm), in which it was observed decrease of Enterobacteriales, however, in *S. aurata* the genus that decreased was *Proteus,* while in *O. melastigma* was *Vibrio* (Varó et al., 2021; Zhang et al., 2021).

In the case of Pseudomonadales, this was mainly covered by the genus *Pseudomonas*, which decreased in the treatments that included 0.25 and 0.75%, followed by 0.50% PMMA (Fig. 6). This change was also observed in *S. aurata*, where Pseudomonadales decreased, although the *Acitenobacter* genus was affected in individuals exposed to LDPE of 500 to 1000 µm (Varó et al.,

2021). In contrast, exposures to PS of 200 µm with *D. rerio* and *O. melastigma* (Gu et al., 2020a; Zhang et al., 2021) and of 100 nm with *L. crocea* (Gu et al., 2020b) reported an increase in the genus *Pseudomonas*.

Clostridia class tended to increase in the treatments with 0.50 and 0.75% PMMA in the present study (Fig. 5), as was observed in *O. melastigma* exposed to PS (Zhang et al., 2021). The fluctuation of *Clostridium* in the present study was similar to that reported in *S. aurata* exposed to LDPE, as a function of particle size, increasing the abundance of *Clostridium* against particles from 200 to 500 µm and decreasing when particles of 500 to 1000 µm were evaluated (Varó et al., 2021).

In Bacilli class, Lactobacillales, represented by the *Lactobacillus* genus, decreased in the groups fed with 0.25, 0.50 and 0.75% PMMA-MPs (Fig. 5), contrary to what was observed in exposures to PS of *O. melastigma* to 2 µm particles (Zhang et al., 2021) and *L. crocea* at NP (100 nm) (Gu et al., 2020b), in which *Lactobacillus* increased.

The increase in *Mycoplasma* in the groups fed with 0.25, 0.50 and 0.75% PMMA-MPs in the present study could be a bacterial infection because in adults *D. rerio* exposed to PS (100 nm) particles, the increase of *Mycoplasma* caused the host response against the proliferation of pathogenic bacteria with increased expression of bacterial response genes (*Jak1*), NOD-like receptor signalling pathways (*Cxcl11.5*) and the metabolic processes of reactive oxygen species (*Hbba1* and *Hbaa1*) (Gu et al., 2020a; Gu et al., 2020b). In addition, in the treatments where *Mycoplasma* showed the highest relative abundance, the increase in *Clostridium* and the decrease in *Lactobacillus* coincided, and whose pattern has been positively correlated with stress biomarkers (SOD, MDA and GSH) in rainbow trout (*Oncorhynchus mykiss*) caused by heat stress, and were related to affectations anatomic and physiology digestive such as the decrease in the height of the villi, the thickness of the epithelial cells of the intestine and the thickness of the muscle, as well as the increase in the number of cells goblet cells and the inflammatory response, leading to severe tissue damage (Zhou et al., 2022).

Alterations in the intestinal microbiota can also be reflected in the alpha diversity indexes of *D. rerio* and *P. reticulata* exposed to PE and PS, in which richness (Chao), diversity (Shannon) and uniformity (Simpson) of the microorganisms in the intestine decreased (Huang et al., 2020; Wan et al., 2019; Zhao et al., 2021). However, in some cases, the increase in the diversity of microorganisms after exposure of *D. rerio* to PE at certain stages of ontogenetic development has also been reported (Kurchaba et al., 2020).

In the above, we can observe that the response in the structure and composition of the intestinal microbiota seems to be different, even in the same fish species exposed in various studies to the same type of polymer (e.g. *D. rerio* to PS) (Jin et al., 2018; Qiao et al., 2019); in the same species (*D. rerio*) exposed to different types of polymers (PS and PE) (Kurchaba et al., 2020; Qiao et al., 2019) or different sizes of the same polymer (*S. aurata* at LDPE of 200 - 500 and 500 - 1000 µm) (Varó et al., 2021); or where appropriate, using the same polymer (PS) in different fish species (*C. idella* and *P. reticulata*) (Huang et al., 2020; Jia et al., 2022). However, although it could be considered different responses, the other bacterial groups that proliferate could have similar functions within the bacterial community, as well as for the host, through the production of the same enzymes and nutrients, in what is known as functional redundancy (Adamovsky et al., 2018).

In addition, the fluctuations in the abundance of certain groups of the intestinal microbiota could respond depending on the components of the host diet associated with pro-inflammatory and anti-inflammatory responses in the intestine due to exposure to MPs, and the knowledge of these groups provides us with information on the response at the community level to exposure to pollutants (Bolte et al., 2021). It is also important to consider the functional profile of the microbiota linked to the composition of the community since microbial end products derived from metabolic processes (metabolites) regulate the host's physiology (Adamovsky et al., 2018). To broaden the panorama of the response of the microbiome functionality with the presence of MPs and the effects that could be deriving on the health of the host based on the changes in the production of metabolites, it is advisable to develop metabolic prediction analyses.

MPs ingested also influence the composition of the intestinal microbiota because they can be colonized by microorganisms that form biofilms attached to their surface (biofilm), which comprise various colonies that proliferate in relation to the characteristics of the substrate, reflecting a functional correspondence between both communities, depending on the needs of the host (FAO, 2017; Kim et al., 2021; Liu et al., 2023). For example, PMMA has been linked to the amount of *Candida albicans* that can harbor depending on surface characteristics, as colonies were found to lodge in the surface voids or valleys of the particles (Petrović et al., 2018), so we could speculate that the irregular shapes of our acrylic MPs could have formed biofilms with microorganisms affine to their structure in the intestinal microbiome of *A. tropicus*. In addition, if we add the potential antimicrobial effects of PMMA (Bacali et al., 2020), it could act as a selection artefact for microorganisms in the intestinal microbiota, which might result in serious problems for the host.

The *Mycoplasma's* proliferation of 0.25, 0.50 and 0.75% PMMA-MPs groups was probably due to greater adhesion of the microorganisms to the intestinal epithelium or the surface of the MPs in the present study (Rose & Pirt, 1981; Talwar et al., 2018). This proliferation might have been taken advantage of if the particles extended the intestinal transit time, leading to a longer MPs-intestine interaction time, as with *G. fossarum* fed PMMA-enriched diets (NIH, 2022; Straub et al., 2017). The groups with the highest percentage of microplastics, in which *Mycoplasma* did not proliferate, was possibly due to an effect of the MPs similar to that of indigestible fibre from regular food, which, although not metabolized, provides a mechanical effect that gives volume in the intestinal lumen, facilitating the transit and excretion of faeces, which would not allow to a prolonged MPs-food-intestine interaction (Bender & Mayes, 2013; Korez et al., 2019; NIH, 2022). In this sense, it would be advisable to carry out ingestion/excretion studies to know the behaviour of the particles as they pass through the intestine, and it would be pertinent to investigate whether *Mycoplasma* has an affinity or disparity with PMMA that implies a "selection" effect on the members of the microbial community.

Conclusions

In the present study, we confirmed the characteristic functional groups of PMMA, the micrometric scale of the particles, and the shapes obtained after the pulverization process implemented in the MPs used, ensuring acrylic fragments between 15 – 290 micrometres are suitable enough for microplastic bioassay with fish.

The diet designed for *A. tropicus* (control diet) provided optimal growth and survival for the culture of juvenile organisms. In our experimental conditions, the percentages of PMMA added to the diets did not affect the growth and survival indexes of juvenile *A. tropicus* fed for 60 days with respect to the control.

The digestive enzymatic activity of acid proteases tended to increase the activity in the treatments with a lower percentage of PMMA (0.25 and 0.50%); however, when it was increased to 0.75% PMMA-MPs, the activity decreased significantly. In the case of alkaline proteases (trypsin, chymotrypsin and L-aminopeptidase) and α-amylase decreased with the presence and increase of PMMA in the diets, showing significant changes in comparison to the groups that were not administered plastic (controls).

The microbiota's structure and composition were altered with *Mycoplasma*'s evident proliferation in the intermediate treatments (0.25 and 0.75, followed by 0.50%). The *Mycoplasma* dominance caused the loss of uniformity, richness (Chao) and decreased diversity indexes of the microbial

groups. These results indicate that the dose-response relationship is not necessarily proportional regarding the increase in the concentration of MPs. We can consider that the PMMA-MPs of the present study acted as artefacts that promoted the proliferation of these potentially pathogenic microorganisms, causing an imbalance in the microbial community.

In this context, we can conclude that PMMA-MPs caused alterations in digestive physiology by decreasing enzymatic activity and altering the composition and structure of the intestinal microbiota of *A. tropicus* juveniles, which, although not reflected in the growth of the organisms, could be affecting the bioavailability and assimilation of nutrients, compromising the nutrition, and, therefore, the health of the fish.

CRediT authorship contribution statement Alejandra Pérez-López: Conceptualization, Data curation, Formal analysis, Funding acquisition (scholarship), Investigation, Methodology, Validation, Writing – original draft, review & editing. **Gabriel Núñez-Nogueira and Carlos A. Álvarez-González:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Funding, Resources, Supervision. **Melina C. Uribe-López and Rafael Martínez-García:** Formal and chemical analyses, Methodologies, Supervision,Validation, Review & editing.**:** **CAAG**: Project administration. **Joan S. Salas-Leiva:** Metagenomic analyses, Data curation, Methodology, Supervision, Review & editing**.**

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Data will be made available on request.

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Tables and figures

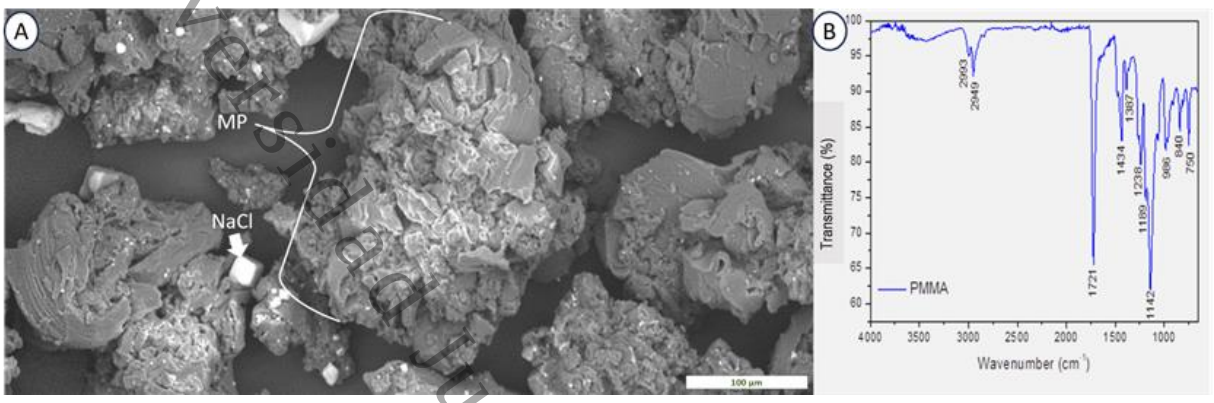


Fig. 1. Microplastics characterization: A) Scanning Electron Microscopy (SEM) micrographs (MP: microplastic, NaCl: salt cubes); and B) Infrared spectrum by Fourier Transform (FTIR) of polymethylmethacrylate (PMMA) microplastics incorporated into the diet of juvenil gar *(A. tropicus).*

Table 1. Weight and length (n=>32), growth and survival indices (n=3) of *A. tropicus* exposed to different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) incorporated into the food for 60 days. Mean ± SD.

	% PMMA-MPs				
	0.00	**0.25**	**0.50**	**0.75**	**1.00**
Initial weight	4.83 ± 1.19	4.76 ± 1.07	4.77 ± 1.32	4.92 ± 1.18	4.97 ± 1.12
Final weight	25.24 ± 7.60^{ab}	23.21 ± 833^{a}	26.69 ± 9.77^{ab}	25.96 ± 6.47^{ab}	30.33 ± 12.14^{b}
Inicial TL	11.35 ± 0.98	11.46 ± 0.84	11.46 ± 0.94	11.58 ± 0.89	11.75 ± 0.77
Final TL	17.77 ± 1.69	17.76 ± 1.73	18.37 ±1.97	18.24 ± 1.16	18.70 ± 1.78
AWG (g)	20.69 ± 2.65	18.53 ± 1.84	21.86 ± 2.68	21.13 ± 1.22	25.26 ± 2.90
WG (%)	454.79 ± 77.19	388.24 ± 15.37	458.10 ± 42.37	429.54 ± 24.63	506.80 ± 44.13
SGR (%/day)	2.84 ± 0.23	2.64 ± 0.05	2.86 ± 0.13	2.78 ± 0.08	3.00 ± 0.12
CF (%)	0.83 ± 0.11	0.71 ± 0.01	0.73 ± 0.00	0.72 ± 0.01	0.79 ± 0.05
S (%)	97.22 ± 9.93	94.44 ± 7.86	88.89 ± 3.93	91.67 ± 6.80	97.22 ± 3.93

TL=Total length; AWG=Absolute weight gain; WG=Weight gain; SGR=Specific growth rate; CF=Condition factor; S=Survival.

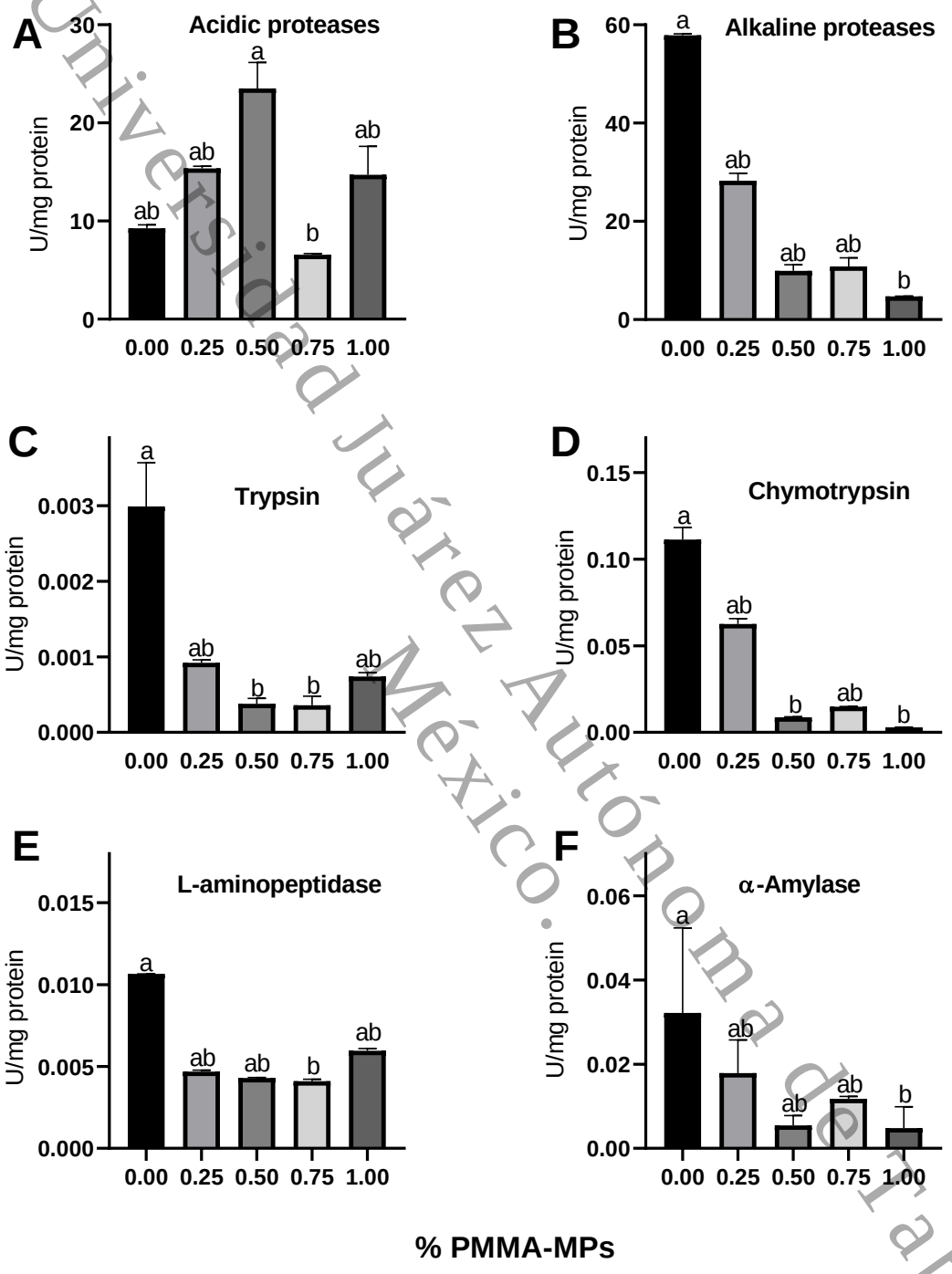


Fig. 2. The digestive enzyme activities of *A. tropicus* exposed to different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) incorporated into the food for 60 days. Median ± IQR, n=3. Different letters indicate significant differences between treatments ($p<0.05$).

Table 2. Alpha diversity indices of *A. tropicus* exposed to different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) incorporated into the food for 60 days (mean ± SD, n = 2).

% PMMA-MPs	Chao	Simpson	Shannon	Evenness
0.00	227.50 ± 40.31	0.93 ± 0.04	3.97 ± 0.20	0.244 ± 0.090
0.25	85.50 ± 14.85	0.25 ± 0.12	0.89 ± 0.46	0.029 ± 0.001
0.50	183.50 ± 0.71	0.61 ± 0.05	2.13 ± 0.002	0.046 ± 0.000
0.75	79 ± 16.97	0.29 ± 0.15	0.86 ± 0.28	0.032 ± 0.015
1.00	272.50 ± 24.75	0.973 ± 0.002	4.48 ± 0.04	0.324 ± 0.018

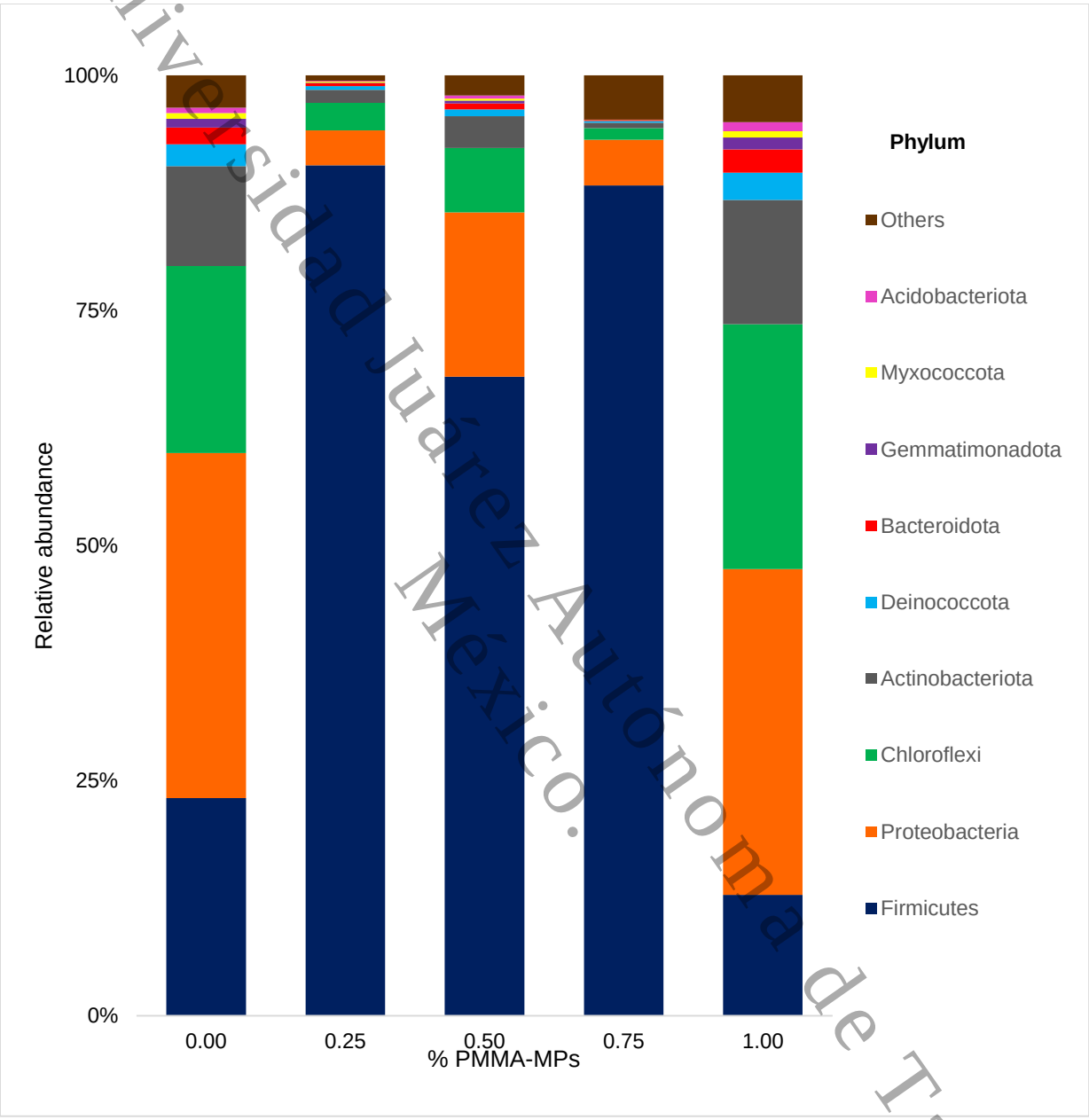


Fig. 3. Relative abundance of the main phyla of the intestinal microbiota of juvenile *Atractosteus tropicus* fed different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

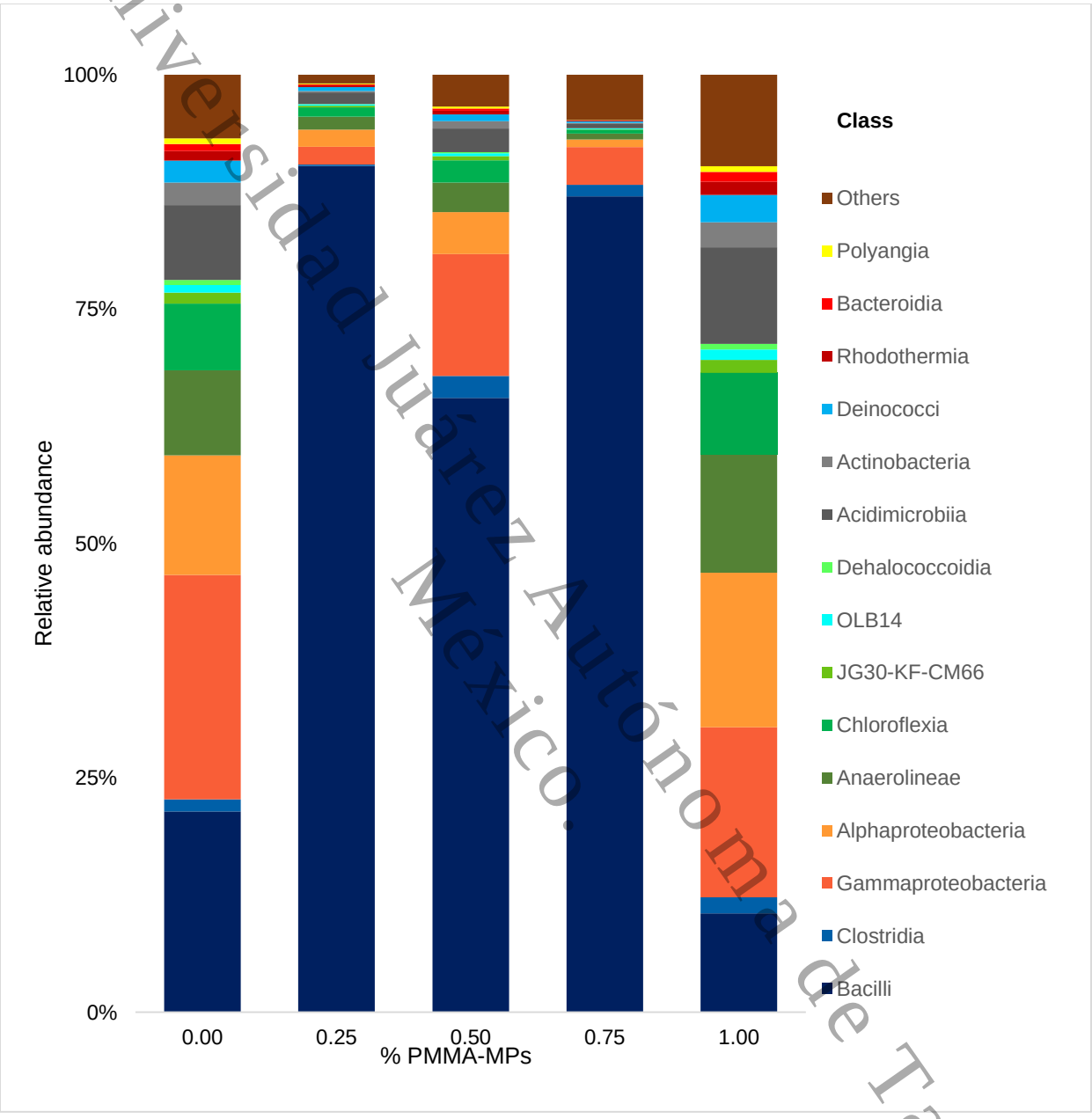


Fig. 4. Relative abundance of the main classes of the intestinal microbiota of juvenile *Atractosteus tropicus* fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

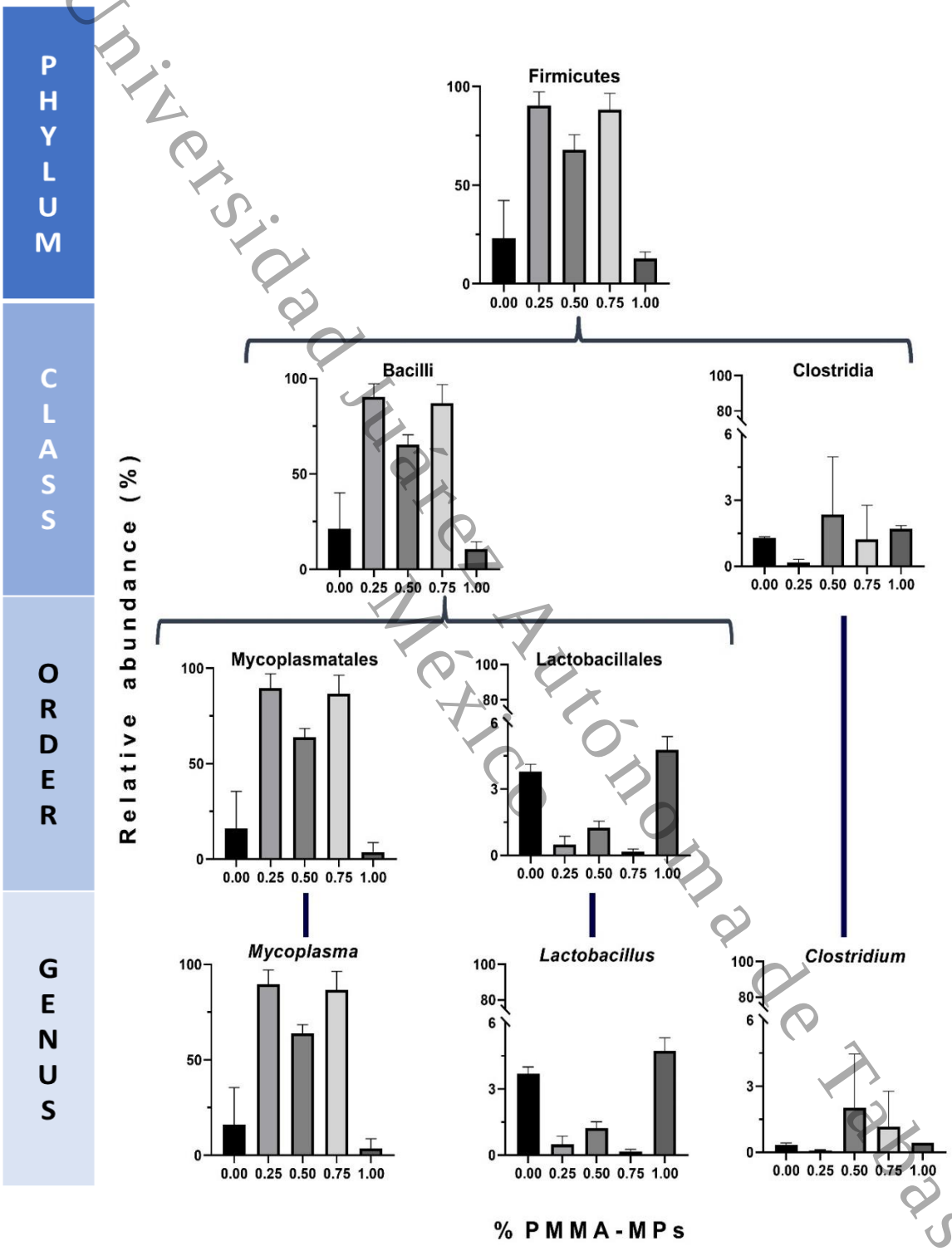


Fig. 5. Relative abundance of the most representative groups of the phylum Firmicutes of the intestinal microbiota of *Atractosteus tropicus* fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

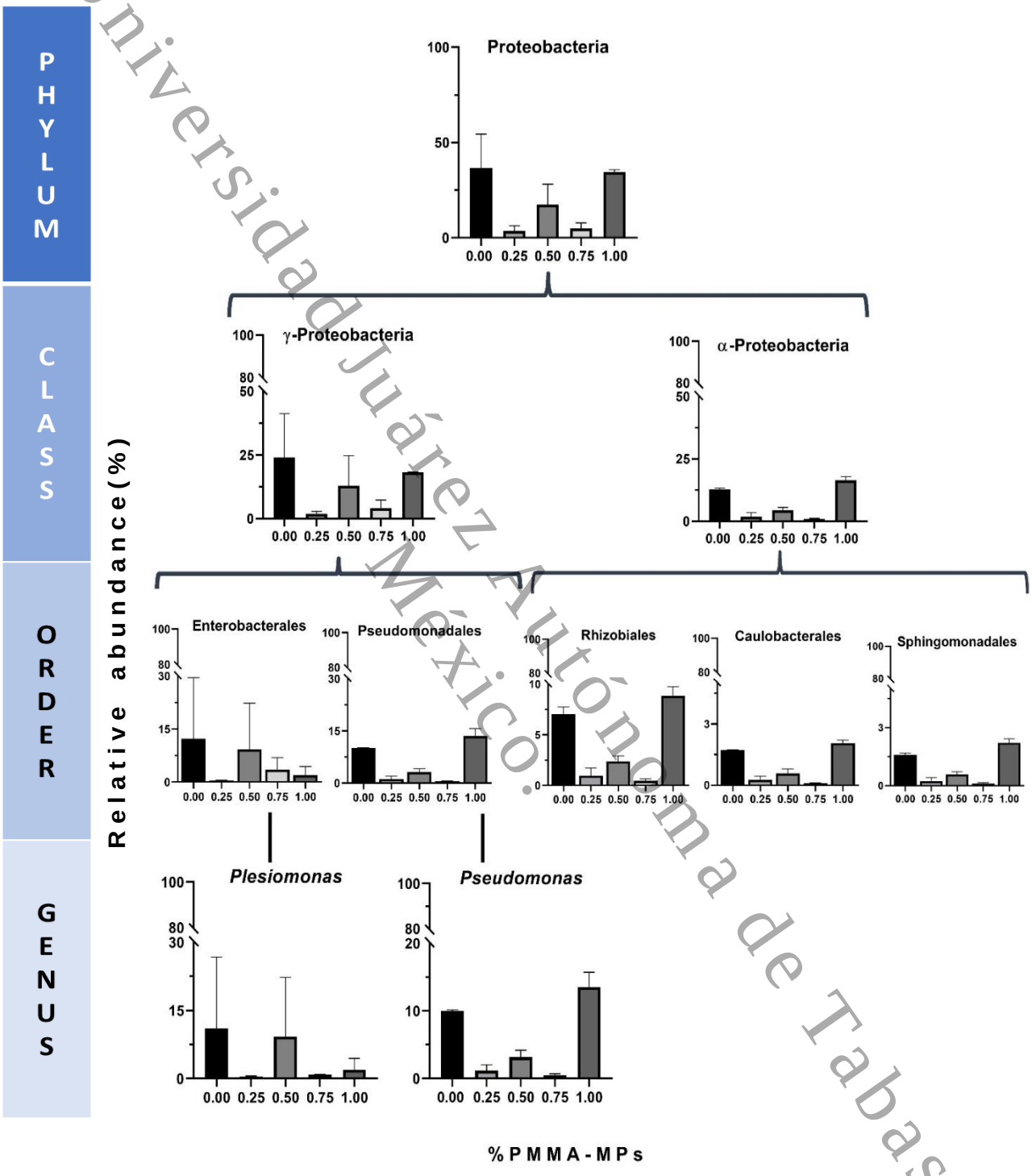


Fig. 6. Relative abundance of the most representative groups of the phylum Proteobacteria of the intestinal microbiota of *Atractosteus tropicus* fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

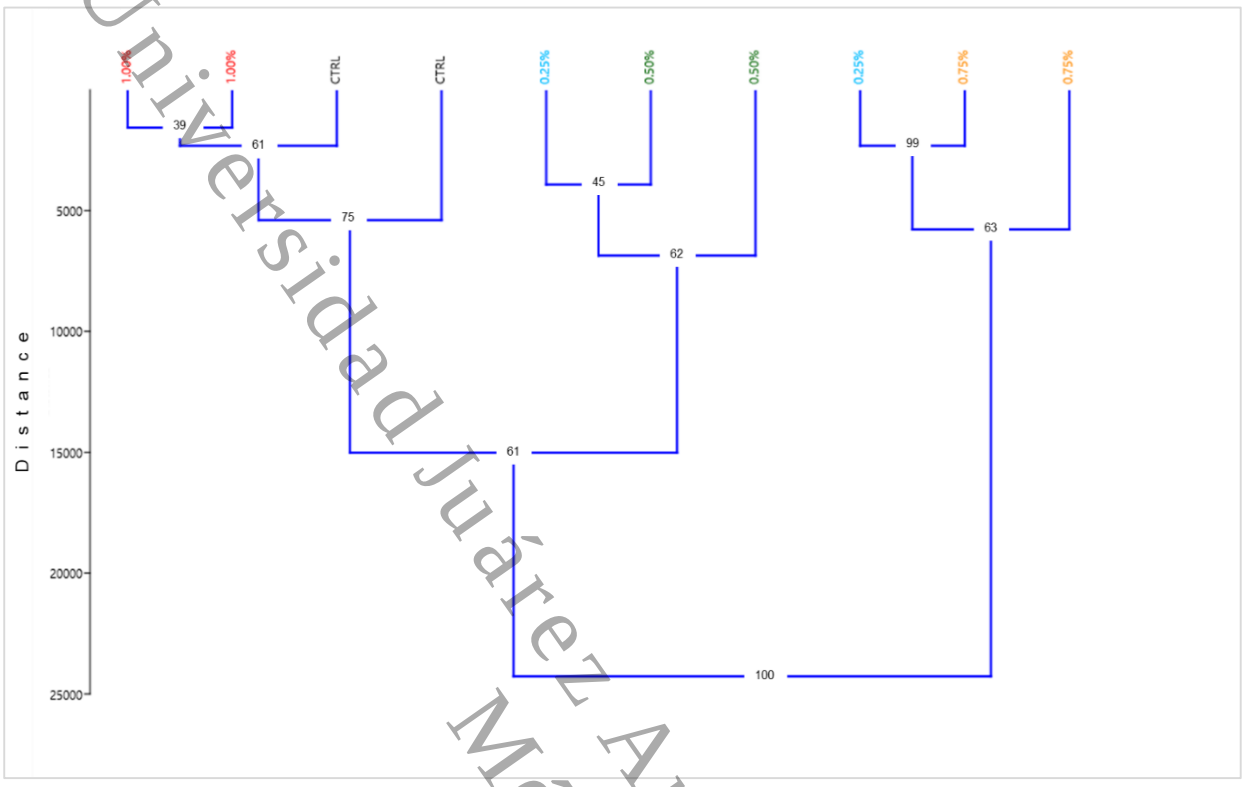


Fig. 7. A dendrogram derived from the Eucledian dissimilarity index (bootstrop 9999) shows three main groupings with a correlation coefficient of 0.83 of the intestinal microbiota of *Atractosteus tropicus* fed with different percentages of polymethylmethacrylate microplastics in the diet.

CONCLUSIONES GENERALES

En el presente estudio, confirmamos los grupos funcionales característicos de PMMA, así como la escala micrométrica de las partículas y las formas obtenidas del proceso de pulverización implementado en los MP utilizados, confirmando fragmentos de entre 15 – 290 µm.

La dieta diseñada para *A. tropicus* (dieta control) proporcionó crecimiento y sobrevivencia óptimos para el cultivo de organismos juveniles. En cuanto a los porcentajes de PMMA adicionados a las dietas, en nuestras condiciones experimentales, no mostraron afectaciones sobre los índices de crecimiento y sobrevivencia de los juveniles *A. tropicus* alimentados por 60 días, con respecto al control; sin embargo, se observó una tendencia de menor crecimiento en el tratamiento con menor porcentaje de PMMA (0.25 %) respecto al mayor (1.0 % PMMA).

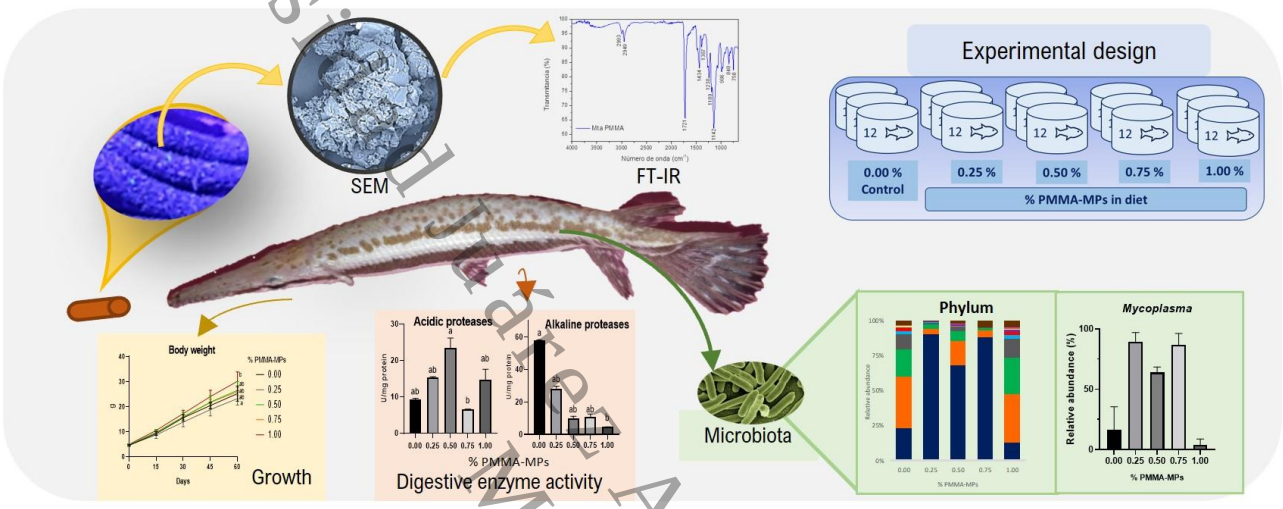
La actividad enzimática digestiva de las proteasas ácidas mostró tendencia a aumentar en los peces alimentados con 0.25 y 0.50 % MP-PMMA, probablemente para aumentar la capacidad digestiva en el estómago; sin embargo, cuando se aumentó a 0.75 % MP-PMMA, la actividad disminuyó significativamente, posiblemente derivado de afectaciones a las glándulas digestivas. En cambio, las proteasas alcalinas (tripsina, quimotripsina y L-aminopeptidasa) y α-amilasa, disminuyeron significativamente con la presencia e incremento de PMMA en las dietas.

Por su parte, la estructura y composición de la microbiota se alteró con la evidente proliferación de *Mycoplasma* en los tratamientos intermedios (0.25, 0.75, y 0.50 %). Por lo que podemos considerar que los MP-PMMA del presente estudio actuaron como artefactos que promovieron la proliferación de estos microorganismos potencialmente patógenos, causando desequilibrio en la comunidad microbiana de dichos tratamientos. Sin embargo, en el tratamiento con 1.0 % PMMA la microbiota fue similar a los controles, por lo que podemos observar que la relación dosis-respuesta no es necesariamente proporcional en cuanto al aumento de la concentración de MP.

En este contexto, los MP-PMMA causaron alteraciones en la fisiología digestiva disminuyendo la actividad enzimática y alterando la composición y estructura de la microbiota intestinal de los juveniles *A. tropicus*, incluso con bajos porcentajes ingeridos, lo cual, aunque no se vea reflejado en el crecimiento de los organismos, podría estar afectando la biodisponibilidad y asimilación de nutrientes, comprometiendo la nutrición, y, por ende, la salud de los peces.

ANEXOS

Graphical abstract



Supplementary material

PMMA-MICROPLASTICS EFFECTS ON THE DIGESTIVE PHYSIOLOGY, GROWTH AND SURVIVAL OF CULTURED TROPICAL GAR (*Atractosteus tropicus*).

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SUPPLEMENTARY MATERIAL

Table S 1. Formulation and design of diets for juvenile tropical gar (*Atractosteus tropicus*) with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) incorporated.

	% PMMA-MPs				
Ingredients g/kg	**0.00 (control)**	**0.25**	**0.50**	**0.75**	**1.00**
Pork meal	250.0	250.0	250.0	250.0	250.0
Poultry meal	218.4	218.4	218.4	218.4	218.4
Soybean meal	161.1	161.1	161.1	161.1	161.1
Potato starch	150.0	150.0	150.0	150.0	150.0
Fish meal	100.0	100.0	100.0	100.0	100.0
Wheat flour	**55.5**	**53.0**	**50.5**	**48.0**	**45.5**
PMMA-MPs	**0.0**	**2.5**	**5.0**	**7.5**	**10.0**
Soybean lecithin	30.0	30.0	30.0	30.0	30.0
Gelatin	20.0	20.0	20.0	20.0	20.0
Vit-min premix	10.0	10.0	10.0	10.0	10.0
Vitamin C	5.0	5.0	5.0	5.0	5.0

Table S 2. The wavenumbers identifications of the infrared spectrum by Fourier Transform of the polymethylmethacrylate microplastics (PMMA-MPs) incorporated in the food of *Atractosteus tropicus*.

Wavenumber (cm^{-1})		Vibration	Functional groups	References
2993	Stretching	Of the C-H bond of the $-CH_3$		(Duan et al., 2008)
2949	Stretching	Of the C-H bond of the $-CH_2$		
1721	Stretching	Of the carbonyl group (C=O) of the ester		(Sugumaran et al., 2017; Vijayakumari et al., 2013)
1434	Bending	Of the C-H bond of the $-CH_3$		(Duan et al., 2008)
1387		α-metil		
1238	Stretching	Of the C-O bond of the ester		(Vijayakumari et al., 2013)
1189				
1142				
986	Absorption	characteristic of PMMA		(Duan et al., 2008)
840	Absorption			
750		α-metil		

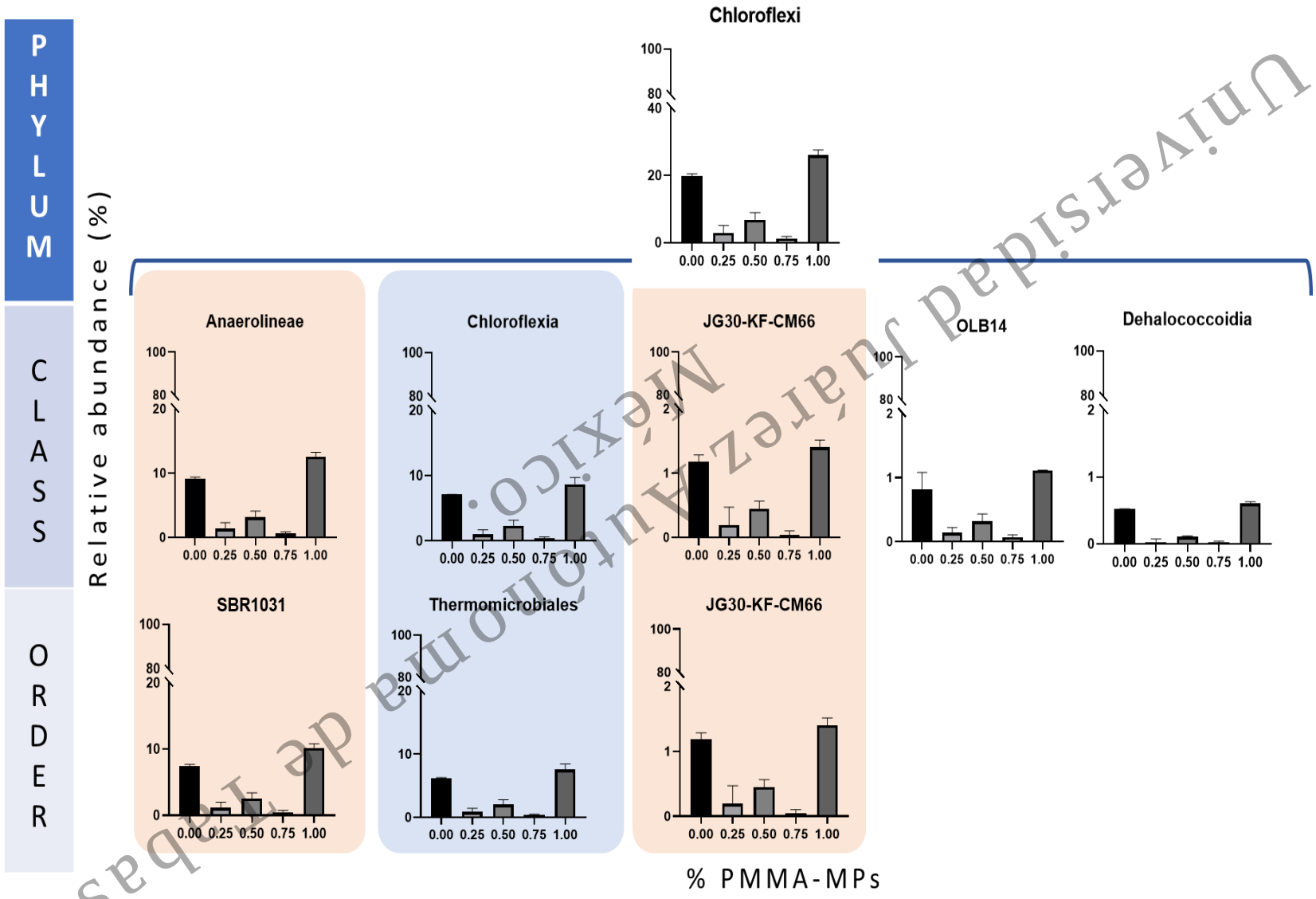

Fig. S 1. Relative abundance of the most representative groups of the phylum Chloroflexi of the intestinal microbiota of juvenile tropical gar (*Atractosteus tropicus*) fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet.

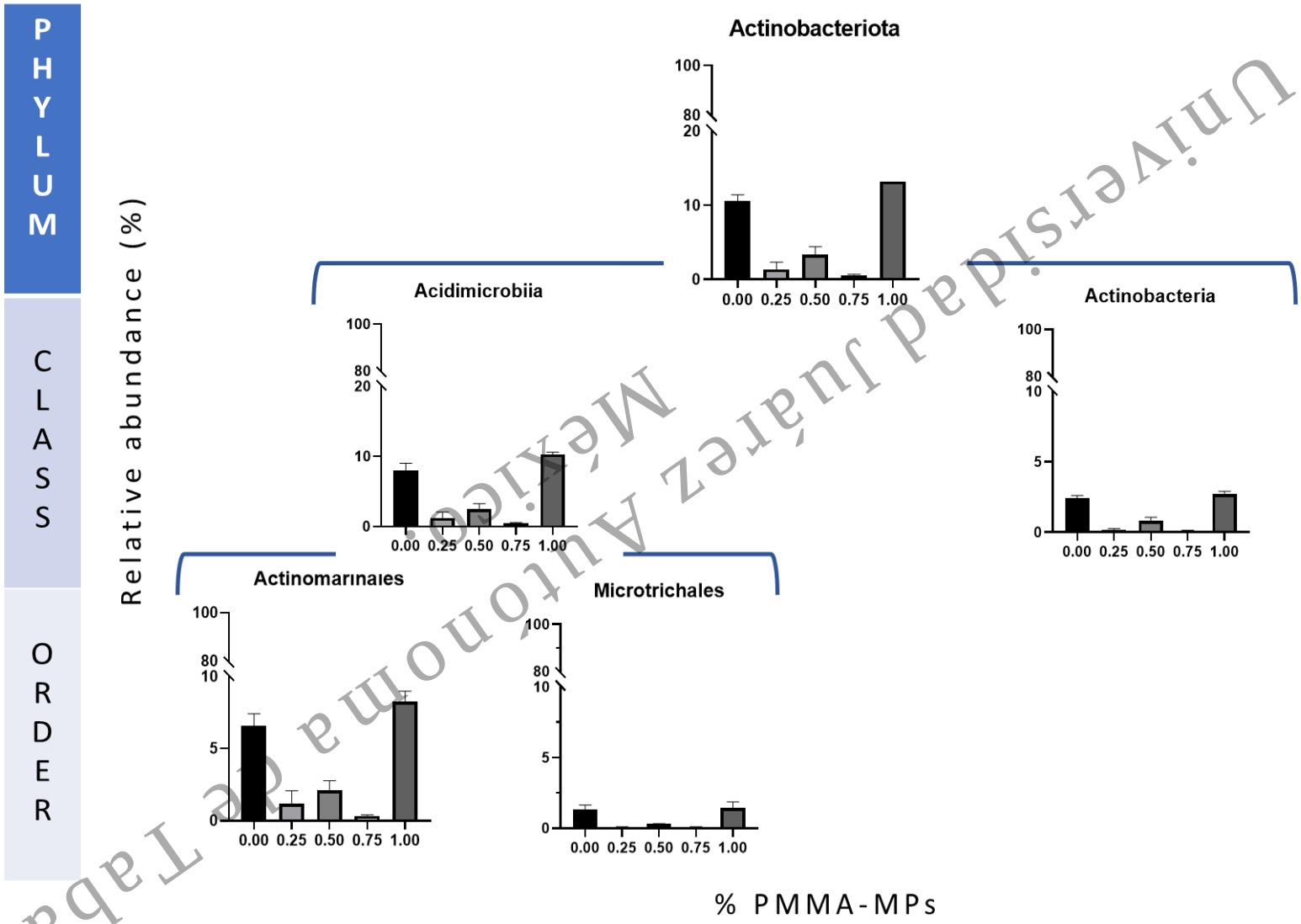

Fig. S 2. Relative abundance of the most representative groups of the phylum Actinobacteriota of the intestinal microbiota of juvenile tropical gar (*Atractosteus tropicus*) fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

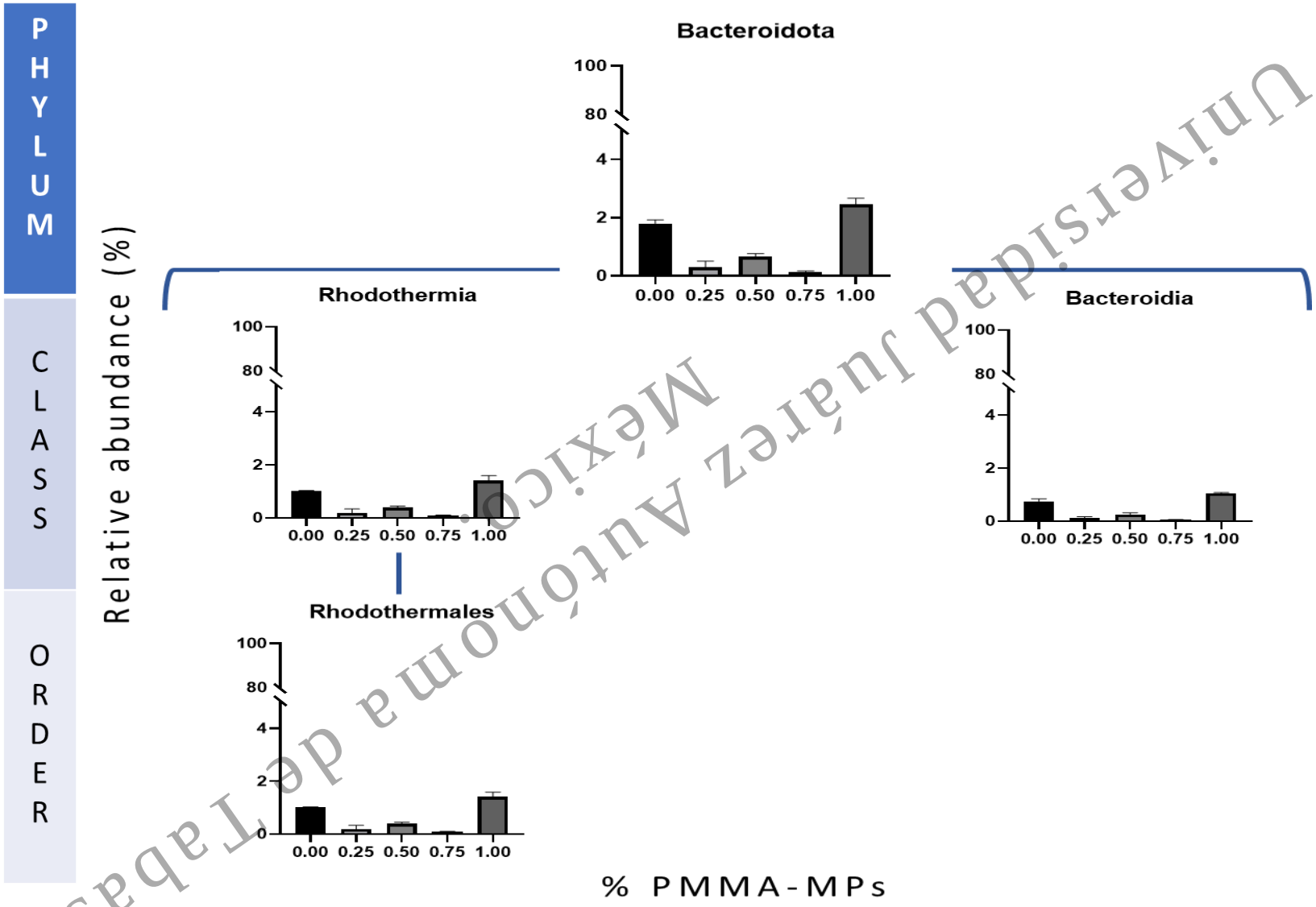

Fig. S 3. Relative abundance of the most representative groups of the phylum Bacteroidota of the intestinal microbiota of juvenile tropical gar (*Atractosteus tropicus*) fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

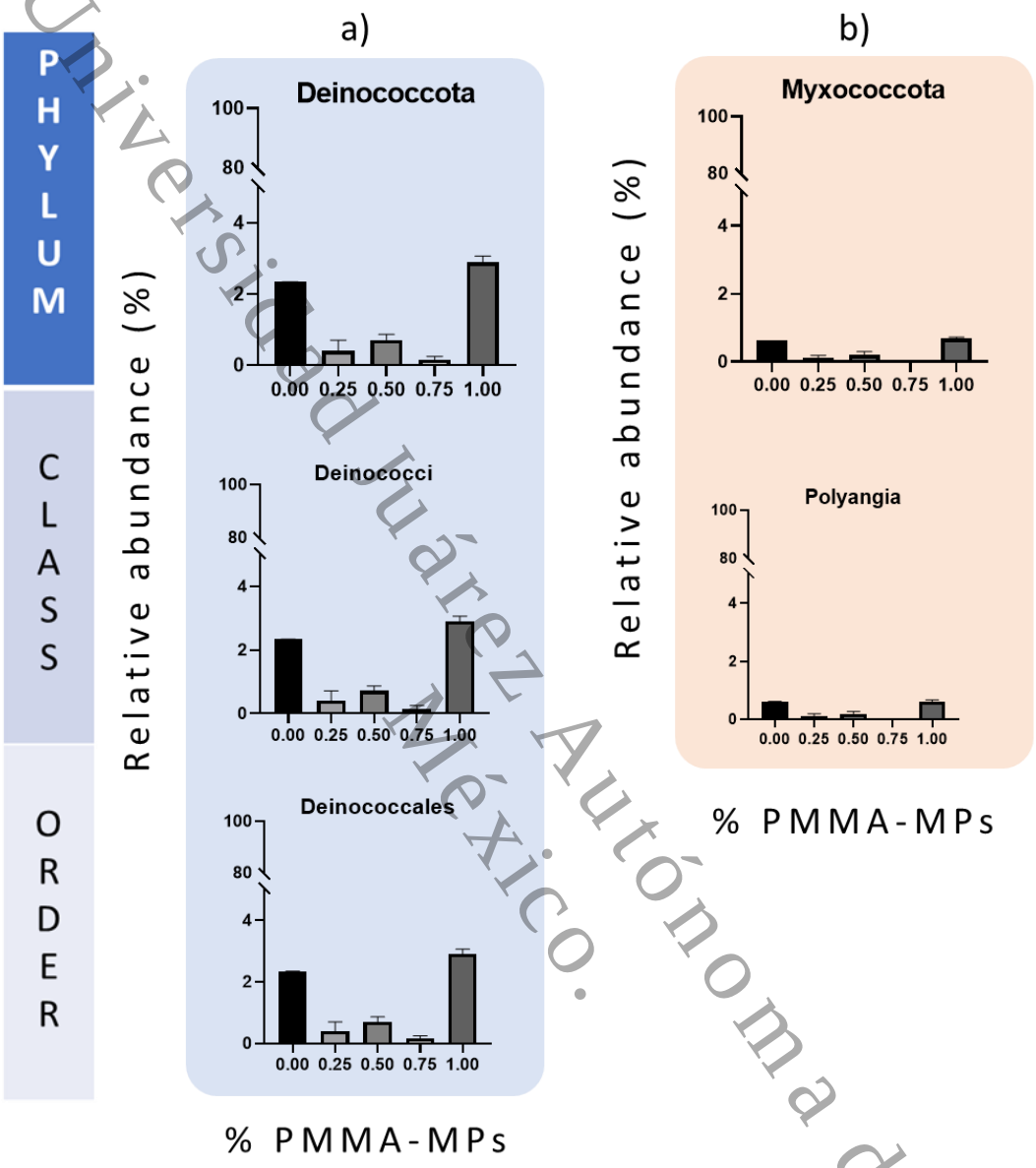

Fig. S 4. Relative abundance of the most representative groups of the phylum Deinococcota (a) and Myxococcota (b) of the intestinal microbiota of juvenile tropical gar (*Atractosteus tropicus*) fed with different percentages of polymethylmethacrylate microplastics (% PMMA-MPs) in the diet for 60 days.

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Vijayakumari, G., Selvakumar, N., Jeyasubramanian, K., & Mala, R. (2013). Investigation on the electrical properties of polymer metal nanocomposites for physiological sensing applications. *Physics Procedia*, *49*, 67–78. https://doi.org/10.1016/j.phpro.2013.10.012

Principios Bioéticos

Este estudio involucró el uso de seres vivos con fines observacionales, experimentales y de investigación, y se llevó a cabo siguiendo estrictamente el protocolo aprobado por la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (**NOM-062-ZOO-1999, 22 de agosto de 2001**) para la obtención, traslado y mantenimiento de los organismos (peces), con personal profesional, técnico y estudiantil, capacitado para realizar los procedimientos con animales de laboratorio.

La investigación con animales es crucial para comprender los mecanismos biológicos subyacentes al impacto de la contaminación del agua, y para el desarrollo de tratamientos efectivos para su mitigación. Durante el estudio, se garantizó que los peces utilizados fueran tratados con dignidad y respeto, minimizando el sufrimiento y asegurando su bienestar, en línea con el **Código Institucional de Ética para la Investigación (2019)** y con la evaluación en proceso de la **Comisión Institucional de Ética en Investigación (CIEI; folio UJAT-CIEI-2024-089)** y en apego a las recomendaciones de la aprobación previa **CIEI-Folio 1174** para bioensayos con peces**,** de la Universidad Juárez Autónoma de Tabasco, considerando el uso de compuestos químicos y biológicos con los estándares éticos más altos.

Se han implementado medidas rigurosas para el manejo de materiales potencialmente peligrosos, asegurando que todos los residuos sean manejados de manera responsable, de acuerdo con la normativa de manejo de residuos peligrosos biológicos e infecciosos (**NOM-087-ECOL-1995**) para la separación, envasado, almacenamiento, recolección, transporte, tratamiento y disposición final.

En conclusión, la investigación se ha llevado a cabo con un firme compromiso con los principios éticos, asegurando la protección del bienestar animal y el respeto por la vida, al tiempo que se promueve el avance científico y se minimiza el impacto ambiental.

Alojamiento de la Tesis en el Repositorio Institucional	
Título de Tesis:	Efectos de la ingesta de microplásticos sobre la fisiología digestiva, crecimiento y sobrevivencia de pejelagartos de cultivo (*Atractosteus tropicus*)
Autor(a) o autores(ras) de la Tesis:	Alejandra Pérez López
ORCID:	https://orcid.org/0000-0001-9671-4902
Resumen de la Tesis:	Los microplásticos (partículas plásticas con dimensiones entre 1 y 1000 µm) están presentes en ecosistemas acuáticos de todo el mundo donde son ingeridos por los organismos que allí habitan, ocasionando alteraciones en la fisiología digestiva. Este estudio evaluó los efectos de microplásticos de polimetilmetacrilato (MP-PMMA) en el crecimiento, sobrevivencia, enzimas digestivas y microbiota intestinal de pejelagartos *Atractosteus tropicus* alimentados con dietas enriquecidas con 0.00, 0.25, 0.50, 0.75 y 1.00% MP-PMMA, por 60 días. El peso final de los peces fue significativamente menor en el tratamiento con 0.25% comparado al de 1.00% MP-PMMA (23.21 ± 8.33 y 30.33 ± 12.14 g, respectivamente) (p=0.02). Los índices de crecimiento y factor de condición no mostraron diferencias significativas entre los tratamientos (p>0.05, respectivamente). La actividad enzimática de proteasas ácidas y alcalinas, tripsina, quimotripsina, L-aminopeptidasa y α-amilasa mostraron diferencias significativas entre los tratamientos (p = 0.012, 0.012, 0.012, 0.003, 0.011 y 0.004, respectivamente); excepto lipasas (p=0.83). Los índices de diversidad α (Chao, Shannon, Simpson y Equitatividad) de la microbiota intestinal, mostraron tendencia a disminuir en los grupos con 0.25, 0.75 y 0.50% MP-PMMA, en cuyos tratamientos la abundancia relativa del género *Mycoplasma* mostró la mayor cobertura (con 90, 67 y 88%, respectivamente) en comparación con los grupos 0.00 y 1.00% MP-PMMA (con 23 y 13%, respectivamente). El dendograma del índice de disimilitud Euclidian (coeficiente de correlación de 0.83), mostró agrupación entre los grupos 0.00 y 1.00% de MP-PMMA. Los MP-PMMA causaron alteraciones en la actividad enzimática y la microbiota intestinal, incluso con bajos porcentajes ingeridos.

Palabras claves de la Tesis:	Microbiota intestinal, actividad enzimática digestiva, pez dulceacuícola, *Atractosteus tropicus*, efectos de los microplásticos.
Referencias citadas:	**Borges-Ramírez, M. M., Mendoza-Franco, E. F., Escalona-Segura, G., y Osten, J. R. von. (2020).** Plastic density as a key factor in the presence of microplastic in the gastrointestinal tract of commercial fishes from Campeche Bay, Mexico. Environmental Pollution, 267. https://doi.org/10.1016/j.envpol.2020.115659 **Huang, J. N., Wen, B., Zhu, J. G., Zhang, Y. S., Gao, J. Z., y Chen, Z. Z. (2020).** Exposure to microplastics impairs digestive performance, stimulates immune response and induces microbiota dysbiosis in the gut of juvenile guppy (Poecilia reticulata). Science of the Total Environment, 733. https://doi.org/10.1016/j.scitotenv.2020.138929 **Jambeck, J. R., Geyer, R., Wilcox, C. Siegler, T. R., Perryman, M., Andrady, A., Narayan, R. y Lavender Law, K. (2015).** Plastic waste inputs from land into the ocean. Science, 347(6223), 764 – 768. https://www.science.org/doi/10.1126/science.1260352 **Méndez-Pérez, R., García-López, R., Bautista-López, J. S. B.-L., Vázquez-Castellanos, J., Alvarez-González, C., Peña-Marín, E., Baltierra-Trejo, E., Adams-Schroeder, R., Domínguez-Rodríguez, V., Melgar-Valdés, C., Martínez-García, R., Moya, A. y Gómez-Cruz, R. (2020).** High-throughput sequencing of the 16S rRNA gene to analyze the gut microbiome in juvenile and adult tropical gar (*Atractosteus tropicus*). Latin American Journal of Aquatic Research, 48(3), 456–479. https://doi.org/10.3856/vol48-issue3-fulltext-2419