UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

ECOLOGÍA

TRANSFERENCIA DE MICROPLÁSTICOS A TRAVÉS DE LA RED TRÓFICA: ZOOPLANCTON - ANFIBIO URODELO *AMBYSTOMA MEXICANUM* (ENDÉMICO DEL LAGO DE XOCHIMILCO, CIUDAD DE MÉXICO)

TESIS

POR ARTÍCULO CIENTÍFICO

MICROPLASTICS ARE TRANSFERRED IN A TROPHIC WEB BETWEEN ZOOPLANKTON AND THE AMPHIBIAN AXOLOTL (*AMBYSTOMA MEXICANUM*): EFFECTS ON THEIR FEEDING BEHAVIOR

QUE PARA OPTAR POR EL GRADO DE:

MAESTRA EN CIENCIAS BIOLÓGICAS

PRESENTA:

DIANA LAURA MANRÍQUEZ GUZMÁN

TUTOR PRINCIPAL DE TESIS: DR. DIEGO DE JÉSUS CHAPARRO HERRERA
FES IZTACALA, UNAM
COMITÉ TUTOR: DR. PEDRO RAMÍREZ GARCÍA
FES IZTACALA, UNAM
DR. FAUSTO ROBERTO MÉNDEZ DE LA CRUZ
INSTITUTO DE BIOLOGÍA, UNAM

LOS REYES IZTACALA, TLANEPANTLA, ESTADO DE MEXICO, OCTUBRE 2023

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Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **19 de junio de 2023** se aprobó el siguiente jurado para el examen de grado de **MAESTRA EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Ecología** de la alumna **MANRÍQUEZ GUZMÁN DIANA LAURA** con número de cuenta **311235090** por la modalidad de graduación de **tesis por artículo científico** titulado: **“Microplastics are transferred in a trophic web between zooplankton and the amphibian Axolotl (*Ambystoma mexicanum*): Effects on their feeding behavior”,** que es producto del proyecto realizado en la maestría que lleva por título: **“Transferencia de microplásticos a través de la red trófica: zooplancton-anfibio urodelo *Ambystoma mexicanum* (endémico del Lago de Xochimilco).”,** ambos realizados bajo la dirección del **DR. DIEGO DE JESÚS CHAPARRO HERRERA,** quedando integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
“POR MI RAZA HABLARÁ EL ESPÍRITU”
Ciudad Universitaria, Cd. Mx., a 17 de agosto de 2023

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Dedicado a mi padre Uriel Garibay León y

a nuestra perrita amada Peluchina quienes están en el Cielo

ÍNDICE

RESUMEN

Los microplásticos por su ubicuidad son un contaminante emergente que se ingiere, bioacumula, transfiere a través de las redes tróficas y son una amenaza para la salud de los seres vivos principalmente acuáticos. El zooplancton es particularmente susceptible a la ingestión de microplásticos a causa de su alimentación por filtración; y debido a que forma parte de la base de las redes alimentarias se considera como uno de los vectores que transfiere microplásticos a los siguientes niveles tróficos.

En particular, los cladóceros son dieta fundamental de larvas de anfibios. Los anfibios son animales que se han extinguido alrededor del mundo en un 48% debido a su sensibilidad a la contaminación; a pesar de esta situación aún es escasa la investigación sobre la interacción que tienen los anfibios con los microplásticos y su posible ingesta a través de la depredación y los efectos que tienen en su ecología alimentaria.

El objetivo de este proyecto fue determinar la transferencia de microplásticos de zooplancton a larvas del anfibio urodelo *Ambystoma mexicanum* (conocido como ajolote), para lo cual evaluamos los posibles efectos en su alimentación y examinamos sus heces. Para ello, expusimos cladóceros de cuatro especies diferentes a una dieta de fitoplancton y microplásticos en un rango de medidas de 6 a 300 µm. Posteriormente, los cladóceros se utilizaron para alimentar a los ajolotes a partir de los 12 días de eclosión, edad en la que consumieron completamente su vitelo y comenzaron a depredar.

Cada semana cinco ajolotes se separaron en viales de 30 ml y se alimentaron con distinta disponibilidad de zooplancton con y sin microplásticos (6, 12, 24, 48 y 96 cladóceros por larva). Cada vial tuvo cuatro repeticiones. Después de alimentarse se contó el número de presas consumidas. Después de 24 horas, las heces de los ajolotes fueron examinadas para determinar la presencia o ausencia de microplásticos.

Nosotros demostramos que hubo un efecto negativo de las presas con microplásticos en la alimentación de los ajolotes, ya que las larvas alimentadas con zooplancton con microplásticos se alimentaron menos que los ajolotes del grupo control. Además, observamos que desde la

primera semana hasta que terminó el experimento están presentes los microplásticos en el 78% de las heces egestadas y que su presencia aumenta conforme avanza la edad de la larva.

Esta investigación evidencia que existe transferencia de microplásticos de zooplancton al ajolote *A. mexicanum* y que son afectados negativamente en su alimentación. Es importante mencionar que este anfibio es endémico de México y requiere atención especial porque se encuentra en peligro crítico de extinción; por lo tanto, si queremos conservar la especie debemos solucionar esta problemática que limita su alimentación en las etapas larvas, la cual es una edad vulnerable debido a su baja supervivencia.

ABSTRACT

Due to their ubiquity, microplastics are an emerging pollutant that is ingested, bioaccumulates, is transferred through trophic webs, and is a threat to the health of living beings, mainly aquatic. Zooplankton is susceptible to ingesting microplastics due to their filter feeding, and is part of the base of trophic webs, as consequence is considered as one of the vectors that transfers microplastics to the following trophic levels.

In particular, cladocerans are an important part of the diet of amphibian larvae. Amphibians are animals that have become extinct around the world by 48% due to their sensitivity to pollution; despite this situation, there is little research about the interaction that amphibians have with microplastics and about their possible ingestion through predation and effects they have on their feeding ecology.

The objective of this project was to determine the transfer of microplastics of zooplankton to larvae of the urodele amphibian *Ambystoma mexicanum* (known as axolotl), for which we evaluated the possible effects on their diet and examined their feces. To achieve our purpose, we exposed cladocerans of four different species to a diet of phytoplankton and microplastics of six to 300 µm. Subsequently, the cladocerans were used to feed the axolotls from 12 days after hatching, the age at which they completely consumed their yolk and began to prey.

Every week five axolotls were separated into vials (30 ml) and fed with different availability of zooplankton with and without microplastics (6, 12, 24, 48 and 96 cladocerans per larva). Each vial had four replicates. After feeding, the number of preys consumed was counted. After 24 hours, the feces of the axolotls were examined to determine the presence or absence of microplastics.

We demonstrated that microplastics were transferred from the cladocerans to *A. mexicanum* and that microplastics negatively affected the larval feeding behavior since the axolotls fed zooplankton exposed to microplastics consumed less prey than those of the control group. We also observed that microplastics were present in 78% of the feces and that their presence increases as the age of the larva advances.

This research shows that there is a transfer of microplastics from zooplankton to the amphibian axolotl *A. mexicanum* and that they are negatively affected in their diet. It is important to mention that this amphibian is endemic to Mexico and requires special attention because it is in critical danger of extinction; therefore, if we want to conserve the species, we must solve this problem that limits its feeding in the larval stages, which is a vulnerable age due to its low survival.

INTRODUCCIÓN

Actualmente, uno de los problemas ambientales más graves que enfrenta la humanidad es la contaminación por plástico en los cuerpos de agua. Los plásticos que miden de 1 µm a 5 mm se denominan microplásticos (Frias & Nash, 2019). De acuerdo con el tipo de polímero y las condiciones ambientales, los microplásticos pueden pasar por procesos mecánicos, químicos y biológicos que los fragmentan en partículas cada vez más pequeñas, de tal forma que, se convertirse en piezas imperceptibles y difíciles de retirar de la naturaleza (Corcoran, 2020). En 1972 se reportó por primera vez la presencia de microplásticos en el mar (Carpenter & Smith, 1972), sin embargo, por su reciente ubicuidad, desde apenas hace una década ha aumentado la investigación sobre microplásticos y actualmente son reconocidos como contaminante emergente que afecta principalmente a los ecosistemas acuáticos (Turan et al., 2021). Uno de los componentes de los ecosistemas acuáticos afectados por los microplásticos son los seres vivos; por ejemplo, en el mar organismos como las tortugas marinas (Duncan et al. 2019), las aves (Masía et al. 2019), los peces (David da Costa et al. 2023) y los mamíferos marinos (Moore et al. 2020) son claros testigos de la ingestión de microplásticos.

En 1973, Rothstein informó por primera vez la presencia de microplásticos en los estómagos de las aves marinas (Rothstein, 1973). Este estudio desencadenó investigaciones que comprobaron que los microplásticos interactúan con los seres vivos de tal forma que son ingeridos (Cole et al., 2013), bioacumulados (Whatts et al., 2014) y transferidos a través de las redes tróficas (Farrel & Nelson, 2013). La interacción e ingestión de microplásticos con la biota puede causar daños por obstrucción (Cole et al., 2013) o tener efectos adversos, desde el nivel celular hasta el nivel ecosistema (Galloway et al., 2017). Estos daños podrían deberse al hecho de que los microplásticos pueden adsorber contaminantes del medio ambiente, como los metales pesados (Holmes et al., 2012; Cao et al., 2021), pesticidas (Wang et al., 2020) y contaminantes lipofílicos. Los contaminantes lipofílicos son compuestos persistentes, bioacumulables y tóxicos, incluidos los compuestos bifenilos policlorados (Wang et al., 2015; PNUMA, 2016).

En los ecosistemas de agua dulce, los microplásticos pueden ser ingeridos por una alta biodiversidad, que van desde invertebrados como crustáceos, anélidos y moluscos hasta organismos vertebrados como peces, anfibios, aves y mamíferos (Scherer et al., 2018; Azevedo-Santos et al., 2021). El zooplancton es particularmente susceptible a la ingestión de microplásticos debido a su alimentación por filtración, esto se ha reportado en diferentes grupos de agua dulce como rotíferos (por ejemplo*, Brachionus calyciflorus* (Pallas, 1766); Xue et al. 2021), anfípodos (por ejemplo*, Gammarus duebeni* (Lilljeborg, 1852); Mateos-Cárdenas et al. 2020) y cladóceros (por ejemplo*, Daphnia magna* (Straus, 1820); Jemec et al. 2016; Scherer et al., 2017). Existe evidencia de que los microplásticos pueden ingresar a las redes tróficas dulceacuícolas a través del zooplancton (Elizalde-Velázquez et al., 2020).

El zooplancton es un alimento fundamental de algunas larvas de anfibios (Holomuzky et al 1994; Jacobson et al. 2017). Los anfibios sufren altas tasas de extinción, en parte debido a su sensibilidad a la contaminación (Hayes et al., 2010). Sin embargo, es escaso el conocimiento sobre la ingestión de microplásticos por parte de los anfibios a través del consumo de sus presas. Hay estudios que principalmente se centran en las larvas y estos revelan que los anfibios pueden ingerir microplásticos (Hu et al 2018; Kolenda et al 2020). La ingestión de microplásticos puede tener consecuencias negativas como daño hepático (Araújo et al., 2019), aumento de infecciones por hongos (Bosch et al., 2021), malformaciones nucleares y disminución del tamaño corporal (Araújo et al., 2020).

Una revisión reciente reveló que dos especies de anuros son los anfibios más estudiados en relación con los efectos de los microplásticos (Araújo et al., 2021). Sin embargo, hasta donde sabemos, la ingestión de microplásticos solo se ha estudiado en una especie de anfibio urodelo *(Triturus carnifex*) (Iannella et al., 2020).

*Ambystoma mexicanum (*Shaw & Nooder, 1978), conocido como ajolote, es un anfibio urodelo endémico del lago Xochimilco en la Ciudad de México, México, que se encuentra en peligro crítico de extinción (Contreras et al. 2009; IUCN, 2019). Las larvas de ajolote se alimentan del zooplancton en las primeras ocho semanas de desarrollo (Chaparro-Herrera et al., 2011). A medida que avanza el desarrollo de las larvas, se producen cambios en el comportamiento de alimentación, que pueden analizarse con base en experimentos de respuesta funcional (Chaparro-Herrera et al., 2011). Los modelos de respuesta funcional ayudan a probar los efectos que pueden ocurrir en la alimentación a medida que cambian las condiciones de los depredadores o de las presas. Se desconoce si el anfibio urodelo ajolote es propenso a ingerir microplásticos a través del consumo de zooplancton y los efectos que esta ingestión podría tener en su comportamiento alimentario en etapas larvales.

Nuestro objetivo fue determinar si ocurre la transferencia de microplásticos de zooplancton a larvas de *A. mexicanum*, para lo cual evaluamos dos respuestas a lo largo de las primeras semanas de desarrollo: (1) la respuesta funcional de las larvas, es decir, la relación que existió cada semana entre el número de presas consumidas y la disponibilidad de zooplancton, y (2) la presencia de microplásticos en las heces. La información obtenida en este estudio ayudará a tomar medidas para favorecer la conservación de esta especie.

MANUSCRIPT SUBMISSION

Food Webs

Microplastics are transferred in a trophic web between zooplankton and the amphibian Axolotl (Ambystoma mexicanum): Effects on their feeding behavior

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First Author:	Diana L Manríquez-Gúzman, Biol.
Order of Authors:	Diana L Manríquez-Gúzman, Biol.
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Suggested Reviewers:	Adriana Aranguiz-Acuña, Ph. D research professor, University of Tarapaca aaranguiza@academicos.uta.cl Her lines of research focus on Aquatic Ecology and Ecotoxicology.
	Mathieu DENOËL, Ph. D research professor, University of Liege Mathieu.Denoel@ulg.ac.be for his work in ecology and amphibian conservation
	Claudia A. Ponce de Leon Hill, Ph. D research professor, National Autonomous University of Mexico caplh@ciencias.unam.mx Research in water quality analysis in aquatic systems, as well as the analysis of emerging pollutants such as heavy metals and microplastics.
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Conflict of Interest

Microplastics are transferred in a trophic web between zooplankton and the amphibian Axolotl (*Ambystoma mexicanum*): Effects on their feeding behavior

Diana L. Manríquez-Guzmán[a] , Diego de Jesús Chaparro-Herrera[*b], Pedro Ramírez-García [b]

[a] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México.

[b] División de Investigación y Posgrado, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Laboratorio de Bacteriología, UIICSE.

Corresponding author: Chaparro-Herrera Diego de Jesús, Phone number: +52 55 9163 0267., Email: diego.chaparro.herrera@gmail.com

Postal address:

Facultad de Estudios Superiores Iztacala, UNAM. Avenida de los Barrios 1, Los Reyes Iztacala, Tlalnepantla de Baz, cp. 54090, Estado de México, México.

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.

Microplastics are transferred in a trophic web between zooplankton and the amphibian Axolotl (*Ambystoma mexicanum*): Effects on their feeding behavior

Diana L. Manríquez-Guzmán[a], Diego de Jesús Chaparro-Herrera[*b], Pedro Ramírez-García[b]

[a] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México.

[b] División de Investigación y Posgrado, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Laboratorio de Bacteriología, UIICSE.

Corresponding author: Chaparro-Herrera Diego de Jesús., Phone number: +52 55 91630267., Email: diego.chaparro.herrera@gmail.com

Postal address:

Facultad de Estudios Superiores Iztacala, UNAM. Avenida de los Barrios 1, Los Reyes Iztacala, Tlalnepantla de Baz, cp. 54090, Estado de México, México.

E-mail addresses of co-authors: Diana Laura Manríquez Guzmán, dlmanriquez@ciencias.unam.mx., Pedro Ramírez García, micro@unam.mx.

Abstract

Microplastics are contaminants that are often ingested, bioaccumulated, and transferred through food chain, affecting aquatic ecosystems. Zooplankton is susceptible to ingesting microplastics, so it is probably a vector that transfers microplastics to higher trophic levels. Cladocerans are a diet of amphibian larvae. Amphibians' survival is under threat worldwide due to their sensitivity to pollution. This work aimed to determine the transfer of zooplankton microplastics to *Ambystoma mexicanum* larvae (known as axolotl), for which we evaluated two responses in the first five weeks of development: 1) functional response of the larvae and 2) examination of their feces. Cladocerans were exposed to a diet of phytoplankton and microplastics to use them as food for axolotl larvae. Five axolotl larvae were randomly selected each week, each fed with a different concentration of cladocerans, and prey consumption was recorded for each larva. Finally, all the feces of the axolotls expelled after feeding were digested and examined under a light microscope. We found that microplastics were transferred from the cladocerans to *A. mexicanum*. Microplastics negatively affected the larval feeding behavior since the axolotls fed zooplankton exposed to microplastics consumed less prey than those of the control group. We also observed that microplastics were present in 78% of the feces. Microplastics reduce the feeding of juvenile *A. mexicanum*, an endemic amphibian of Mexico, in critical danger of extinction.

Keywords: *Ambystoma mexicanum*, amphibian, axolotl, functional response, microplastics, predator-prey, transfer, trophic web, zooplankton.

1. Introduction

Currently, one of the most severe environmental problems facing humanity is plastic pollution in aquatic ecosystems. Plastics that measure from 1 µm to 5 mm are called microplastics (Frias & Nash, 2019). Depending on the polymer and environmental conditions, microplastics can go through mechanical, chemical, and biological processes that break them into smaller particles until they become imperceptible pieces that are difficult to remove from nature (Corcoran, 2020). In 1972 the presence of microplastics in the water column in the Atlantic Sea was reported for the first time (Carpenter & Smith, 1972); however, due to their recent ubiquity, research on microplastics has increased in just a decade and is currently recognized as an emerging pollutant that mainly affects aquatic ecosystems (Turan et al., 2021). One of the components of the aquatic ecosystems affected by microplastics is living beings; for example, in the sea, organisms such as sea turtles (Duncan et al., 2019), birds (Masía et al., 2019), fishes (David da Costa et al., 2023), and marine mammals (Moore et al., 2020) are clear witnesses of the ingestion of microplastic waste.

Ingestion of microplastics was first reported in 1973 in the stomachs of seabirds (Rothstein, 1973). This study triggered investigations that verified that microplastics interact with living beings in such a way that they are ingested (Cole et al., 2013), bioaccumulate (Whatts et al., 2014), and are transferred through food webs (Farrel & Nelson, 2013). The interaction and ingestion of microplastics with biota may cause damage by obstruction (Cole et al., 2013) or have various adverse effects from the cellular to the ecosystem level (Galloway et al., 2017). These damages could be because microplastics can adsorb environmental pollutants, such as heavy metals (Holmes et al., 2012; Cao et al., 2021), pesticides (Wang et al., 2020), and lipophilic pollutants. Lipophilic contaminants are persistent, bioaccumulative, and toxic, including polychlorinated biphenyl compounds (Wang et al., 2015; UNEP, 2016).

These threats are also present in freshwater ecosystems; microplastics can be ingested by high biodiversity, ranging from invertebrates such as crustaceans, annelids, and mollusks to vertebrate organisms such as fishes, amphibians, birds, and mammals (Scherer et al., 2018; Azevedo-Santos et al., 2021). Among the freshwater diversity, zooplankton is particularly susceptible to the ingestion of microplastics due to their filter feeding; this has been reported in different freshwater groups such as rotifers (e.g., *Brachionus calyciflorus* (Pallas, 1766); Xue et al., 2021), amphipods (e.g., *Gammarus duebeni* (Lilljeborg, 1852); Mateos-Cárdenas et al., 2020) and cladocerans (e.g., *Daphnia magna* (Straus, 1820); Jemec et al., 2016; Scherer et al., 2017). Zooplankton is the base of food webs (Setälä et al., 2014); this means that it is possible that when they ingest microplastics they transfer them to the following trophic levels (Elizalde-Velazquez et al., 2020).

Zooplankton is a fundamental food of some amphibian larvae (Holomuzky et al., 1994; Jacobson et al., 2017). Amphibians suffer high extinction rates, partly due to their sensitivity to pollution (Hayes et al., 2010). Despite this, there is little knowledge about the ingestion of microplastics by amphibians through their prey consumption. The existing studies focus on amphibian larvae and reveal that they can ingest microplastics (Hu et al., 2018; Kolenda et al., 2020). The ingestion of microplastics may have negative consequences for amphibians, such as hepatic damage (Araújo et al., 2019), increased infection by fungus (Bosch et al., 2021), nuclear malformations, and decreased body size (Araújo et al., 2020).

A recent review revealed that two species of anurans are the most studied amphibians concerning the effects of microplastics (Araújo et al., 2021). However, as far as we know, the ingestion of microplastics has only been studied in one species of urodele amphibian (*Triturus carnifex*) (Iannella et al., 2020).

Ambystoma mexicanum (Shaw & Nooder, 1978), known as the axolotl, is a urodele amphibian endemic to Lake Xochimilco in Mexico City, Mexico, which is in critical danger of extinction (Contreras et al., 2009; IUCN, 2019). Axolotl larvae prey on zooplankton in the first eight weeks of development (Chaparro-Herrera et al., 2011). As larval development progresses, changes in feeding behavior occur, which can be analyzed based on functional response experiments (Chaparro-Herrera et al., 2011). Functional response models help test the effects that may occur in feeding as predator or prey conditions change. It is unknown whether the urodele axolotl amphibian is prone to ingesting microplastics through the consumption of zooplankton and the effects this ingestion could have on its feeding behavior in larval stages.

Our objective was to determine if the transfer of microplastics ingested by zooplankton to *A. mexicanum* larvae occurs. We evaluated two responses throughout the first weeks of development: (1) the functional response of the larvae, that is, the relationship that existed each week between the number of prey consumed and the availability of zooplankton, and (2) the presence of microplastics in the *feces.* The information obtained in this study will help to take action to favor the conservation of this species.

2. Materials and methods

2.1. Maintenance of Ambystoma mexicanum *larvae*

A hundred eggs were used for the experiment. These came from the same pair of parents that live in an authorized center for the reproduction of wild species at risk according to the IUCN (registration number: SEMARNAT DGVS-PIMVS-2045-MEX/22 in Tepotzotlán, Mexico state). The eggs, and later the larvae were kept in the laboratory, in shallow 40 × 30 cm plastic containers, at room temperature, photoperiod 12:12 h, shaded, and in running water that was replaced every 48 hours to remove waste urine and feces and prevent bacterial growth. Once all 100 eggs hatched, the larvae were divided into groups of 15 individuals to avoid crowding. Approximately 12 days after hatching, once the larvae had completely consumed their yolk sac, they were fed daily zooplankton *ad libitum*.

2.2. Maintenance of zooplankton and phytoplankton

As zooplankton prey of *A. mexicanum*, we selected four species of cladocerans *Daphnia pulex* (Linnaeus, 1758), *Alona glabra* (Sars, 1901), *Simocephalus vetulus* (Müller, 1776) and *Ceriodaphnia sp*. Cladocerans were chosen because are the preferred choice of *A. mexicanum* larvae (Chaparro-Herrera et al., 2011). Likewise, the cladocerans we use co-occur with *A. mexicanum*. Cladocerans were cultivated in medium EPA medium ([Environmental Protection Agency medium]) (EPA, 1985). Zooplankton were maintained at room temperature, with a 12:12 h photoperiod, replacement of EPA medium every 48 h, and feeding with *Chlorella vulgaris* phytoplankton at a density of 1.0 × 10^6 ml^{-1} cells. *C. vulgaris* is a green microalgae commonly used as food for zooplankton (Ruangsomboon & Wongrat, 2006; Sarma et al., 2001). The microalgae were cultivated for ten days in Bold Basal medium (Borowitzka & Borowitzka, 1988) supplemented with ~1 g of sodium bicarbonate per liter every 48 h, pH 7.5, at room temperature, constant white light, and aeration (Morales-Ventura et al., 2004).

2.3. Microplastics

We used plastic of the Acrylonitrile Butadiene Styrene (ABS) type. Studies reveal that ABS microplastics are present in freshwater ecosystems (Sighicelli et al., 2018; Fan et al., 2019) and wastewater (Sun et al., 2019). Likewise, although it is not the most abundant plastic in freshwater ecosystems, ABS is the ninth most produced and discarded polymer in the world (Yuan et al., 2022). Particularly, Lake Xochimilco is artificially supplied with treated water effluents and receives wastewater from urbanization (Bojórquez et al., 2017); for this reason, it is very likely that ABS is present in this ecosystem. In addition, ABS is considered one of the polymers with the greatest potential for toxicity to living beings (Lithner et al., 2011), and it is difficult to biodegrade (Lusher et al., 2017), so we believe that it is persistent and will interact with *A mexicanum* and other living beings for many years.

The ABS came from an industrial mill in the State of Mexico, and we crushed it into plastic powder (particles from 24 to 300 µm long and from six to 260 µm wide; Fig. 1a). The microplastics obtained are within the dimensions that naturally exist in the habitat of axolotls (Fig. 1b and 1c).

2.4. Transfer of microplastics in the trophic web.

2.4.1. Exposure of zooplankton to microplastics.

After fasting for 24 hours, we placed a mixture of the four cladocerans species, in 1 L of EPA medium, with *C. vulgaris* (1×10^6 cells/ml) and ABS microplastics (200 mg/L). The mixture oscillated at 80 rpm to keep the particles suspended for three hours. We chose this exposure time because cladocerans consume most microplastics in the first three hours of contact (Elizalde-Velazquez et al., 2020).

2.4.2. Functional response

We analyzed the effect of microplastics every week through the functional response of the larvae. The experiment began with 12-day-old larvae (when the yolk was completely consumed). The experimental design consisted of two factors: 1) type of food with two levels: cladocerans without microplastic (control) and cladocerans with microplastic, and 2) availability of prey with five levels: 6, 12, 24, 48, and 96 cladocerans offered per larva. Each treatment and control had four replicates. Larvae that consumed cladocerans without microplastics (control) were kept separated from the larvae fed with cladocerans exposed to microplastics (Fig. 2).

Fifty larvae were used as control and fifty larvae as treatment. All larvae in the control and treatment containers were starved for 24 hours to ensure prey consumption. Twenty larvae were randomly selected from each container (control and treatment), and each larva was placed separately in a 30 ml glass vial with EPA medium. Each vial contained a known number of cladocerans (6, 12, 24, 48, and 96). The five vials with larvae and their prey had four replicates each. Finally, the larvae were kept in contact with their prey for two hours; this time was sufficient to observe a functional response (Morales-Ventura et al., 2004). After this, we enumerated the number of prey consumed. For the functional response, the consumption data were transformed using the Michaelis-Menten constant, where the line is a hyperbola similar to the consumption of *A. mexicanum* (Chaparro-Herrera et al., 2013). At the end of the experiment, the larvae returned to the container they came from (control or treatment).

The experiment was repeated once a week, randomly selecting 20 larvae from each container each week. It is essential to mention that we intended to experiment during the first eight weeks of development because axolotls prey on zooplankton within this period. However, the larvae in our experiment with microplastics died in the fifth week of development.

2.4.3. Statistical Analyses

The data on consumed prey were transformed using the Michaelis-Menten constant, where the line is a hyperbola similar to the consumption of *A. mexicanum*. The data obtained were analyzed by three-way ANOVA; the variables were: 1) cladocerans without and with microplastics cladocerans without and with microplastics, 2) number of zooplankton offered per larva and 3) larval development time (week one-five). We performed a test post hoc Tukey's. All analyses were carried out in the SigmaPlot®11 software (Systat Software, Inc., San Jose, CA, EE.UU., https://systatsoftware.com/).

2.5. A Feces in the Ambystoma mexicanum analysis

Larval feces were analyzed to visualize the transfer of microplastics. Immediately after the experiment described above, each axolotl was isolated in another 30 ml glass vial with

EPA medium. After 24 hours, all the expelled feces were collected with a pipette and placed on slides. Between five and seven drops of 30% H_2O_2 were added to digest the organic matter for two hours at room temperature. Subsequently, the samples were observed in the inverted light microscope (Nikon eclipse TS100), and the presence or absence of microplastics was recorded (Fig 2).

2.6. Ethical considerations

This study was performed with axolotl larvae in captivity. The larvae come from an authorized center called PIMVS (Predios o Instalaciones que Manejan Vida Silvestre), which is a specialized place for the management and breeding of wildlife in captive (license number SGPA/DGVS/03520/22). The PIMVS has the ideal environmental conditions to maintain a viable and sustainable population of axolotls. The axolotls were kept in the laboratory, with the necessary conditions following the manual for their care in captivity (González & Zamora, 2014), with as little manipulation as possible, except when they were selected for the experiment. The research was performed with the acceptance of the Ethics Committee of the Facultad de Estudios Superiores Iztacala, UNAM (official number CE/FESI/072022/1543).

3. Results

3.1 Transfer of microplastics in the trophic webs

3.1.1. Exposure of zooplankton to microplastics.

We observed microplastics inside the cladocerans (Fig. 3).

3.1.2. Functional response

To determine the effect of microplastics on the axolotl's prey consumption, we performed functional response tests according to prey availability, recording the number of zooplankton preyed on by *A. mexicanum* larvae per week. Functional response graphs showed changes in the feeding behavior of *A. mexicanum* larvae when the prey was exposed to microplastics (Fig. 4).

Both the control group and treatment larvae constantly increased their prey consumption as their age increased from week 1 to week 4. However, prey consumption was different for the control and treatment.

The asymptotes of the control charts are greater than the asymptotes of the treatment. This is because the control group axolotls had a minimum average consumption of 2 cladocerans and a maximum consumption of 27 cladocerans per week. As for the axolotls in the treatment, the minimum average consumption of prey was 1 cladoceran and a maximum consumption of 17 cladocerans per week. This means the asymptotes, the satiation points of the control and treatment larvae, have an average difference of 10 prey.

We observed that, in the control group and in the treatment, at week 5 the consumption of prey decreased. It is important to mention that the larvae that consumed prey with microplastics died at the end of week 5.

The consumption of prey with microplastics was significantly lower than that of prey without microplastics from the second week of the experiment until the fifth week (ANOVA: $F_{4,96} = 2.77$, $P < 0, 05$).

3.2.3 Feces in the Ambystoma mexicanum analysis

To visually determine the transfer of microplastics from zooplankton to the axolotl, we observed the feces of the treatment larvae. Of the 85 expelled feces, 66 had microplastics (78%), and 19 not (22%). Microplastics were present from the first week of the experiment (Fig. 5). In addition, as the larvae grew from week 1 to week 4, they consumed more prey and, therefore, egested more feces with microplastics. In week 5, prey consumption was lower; therefore, the amount of feces with microplastics decreased (Fig. 6).

4. Discussion

This study evaluated the threat microplastics pose to the urodele amphibian, *A. mexicanum*, the axolotl, a critically endangered endemic species of Mexico. We determined that microplastics are transferred between zooplankton and axolotl larvae in a trophic web. We found microplastics in the feces of axolotl larvae and showed that their feeding behavior is reduced when their preys contain microplastics. This information supports the conservation of the axolotl.

The ingestion of microplastics through prey has also been reported for freshwater fish (Justino et al., 2023); Likewise, it has been documented that these organisms ingest more microplastics through predation than through the water column (Athey et al., 2020; Hasegawa & Nakaoka, 2021; D'Avignon et al., 2023). Ferreira et al. (2018) demonstrated that half of 551 adult fish contained microplastics; the authors indicate that they were actively ingested through the consumption of zooplankton when they were juveniles and subadults. Hasegawa & Nakaoka (2021) state that in freshwater systems, trophic transfer increases exposure to microplastics because predators demand more food to nourish themselves and because prey, due to their size, can consume smaller fragments, therefore, predators may be more likely to bioaccumulate them, translocate them to other organs, and suffer negative effects.

In our study, the ingestion of microplastics through zooplankton decreased the number of prey consumed by *A. mexicanum* larvae, so it caused the death of the axolotls at the end of the fifth week. Although mortality is not a variable we measure, it is important to mention that it was a secondary effect we did not observe in the control group. We think that the decrease in feeding, and the death of all larvae may be due, in part, to the ingestion of ABS microplastic, a polymer with a high risk to the health of living beings (Lithner et al., 2011). For this reason, it is important to consider the toxicity of microplastics and determine the possible risks they represent for amphibians and other living beings.

Some studies have indicated that microplastics can cause damage to the health of amphibians when they ingest them from their environment (Araújo et al., 2020; Araújo & Malafaia et al., 2020). For example, Boyero et al. (2020) reported that feeding rates, survival, and growth decrease when midwife toad (*Alytes obstetricans*) tadpoles are exposed to polystyrene microplastics. Likewise, Pannetier et al. (2020) mention that microplastics have a negative impact at the cellular level in aquatic organisms, inducing oxidative stress, changes in metabolic parameters, reduced enzymatic activity, and cell necrosis.

In addition to the negative effects of direct consumption of microplastics, it has also been shown that this contaminant and its attached toxics are transferred from prey to predator in aquatic systems. Athey et al. (2020) observed that fish larvae ingested more toxics through their prey with microplastics than by directly ingesting the water column; in addition, larvae that consumed prey with microplastics developed less biomass and length. Additionally, Uy & Johnson et al. (2022) found that there was a transfer of microplastics from zooplankton to fish larvae, and, consequently, their growth and survival decreased. Therefore, it is likely that the larvae of *A. mexicanum* in their natural environment are facing health problems due to the consumption of microplastics and their associated toxics through their prey. It is necessary to evaluate the possible effects *in situ* since its habitat is highly contaminated (Zambrano et al., 2003).

Based on our observations, we propose that there are several mechanisms through which microplastics within zooplankton could cause decreased feeding by axolotl larvae. We observed that, on occasions, microplastics blocked the feeding canals and appendages of cladocerans; which caused the swimming of cladocerans to be erratic, slow, or even nonexistent; this phenomenon affected the ability of axolotl larvae to capture their prey since, in part, they perceive their prey through their mechanosensory organs that allow them to feel vibrations (Münz et al., 1984). Obstruction of the digestive system and immobilization by microplastics in cladocerans seems to be recurrent (Rehse et al., 2016; Frydkjær et al., 2017; De Felice et al., 2019).

Likewise, it is important to mention that cladocerans are likely to transfer microplastics to predators since it has been shown that after exposing *D. magna* to microplastics, its heart rate is constantly reduced, resulting in a blocked digestive tract and prevents the expulsion of microplastics (Pan et al., 2022).

A second mechanism is that the ABS microplastic, due to its low density (1.02–1.08 g cm−3), functions as a float that keeps the cladocerans on the surface; this phenomenon decreases the probability of contact with their prey since the axolotl generally has a benthic behavior and swims to the surface on certain occasions, in addition, axolotls are passive hunters, that is, they only wait for the zooplankton to be close to open their mouth and swallow the whole prey; therefore, if the prey is far away, it is unlikely that will capture it. Floating microplastics have previously been reported to affect the ability of fish larvae to detect and consume zooplankton (Uy & Johnson et al., 2022).

A third mechanism is that microplastics can alter feeding rates due to the false satiation effect. Hu et al. (2016) found that tadpoles of *Xenopus tropicalis* frogs that consume more microplastics tend to eat less and less because they bioaccumulate microplastics in the digestive system, leading to a feeling of satiety. However, in our work, we had a microplastic transfer vector, that is, zooplankton (cladocerans), so it must be considered that the bioaccumulation of microplastics in axolotls will depend on how much they consume cladocerans.

Finally, with the presence of microplastics in the feces of the *A. mexicanum* larvae, we verified that there was ingestion of microplastics by the cladocerans (Fig. 3), this explains their decrease in swimming speed and buoyancy. Confirming the presence of microplastics in feces is an important step; only in this way can we be sure that this contaminant can be ingested, bioaccumulated over time, and excreted and have contact with the digestive system of living beings. Here we show, through observing feces, that microplastics transfer from zooplankton to the axolotl larvae. Previous research found microplastics in the feces of anuran tadpoles, but in this case, the ingestion of microplastics was direct from the environment since anuran tadpoles are filter feeders (Hu et al., 2016; Boyero et al., 2020).

Microplastics are stressors for axolotl larvae when they consume zooplankton previously exposed to microplastics. We suggest future studies to find the reasons for the alteration in the feeding behavior of axolotls. More research is also needed on the ingestion of microplastics by amphibians through water, their food, and its effects on their first weeks of life (Venâncio et al., 2022). In addition, we need to answer other questions in the future to understand better the risk that microplastics pose to axolotls: Are survival, development, and growth affected by the presence of microplastics? Are swimming and mobility reduced when the larvae are exposed to this contaminant? How can microplastics influence the health of these animals at the tissue and organ level? Solving these questions will help us understand how microplastics threaten axolotls and, in turn, will allow us to find solutions for their conservation.

Author´s contributions

Design of the work and provide background theory and intellectual by Diego de Jesús Chaparro Herrera and Pedro Ramirez Garcia. Data collection and analysis were performed by Diana Laura Manríquez Guzmán. The first draft of the manuscript was written by Diana Laura Manríquez Guzmán and all authors revised subsequent versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The raw data for this study are available as Supplementary material.

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.

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Figures

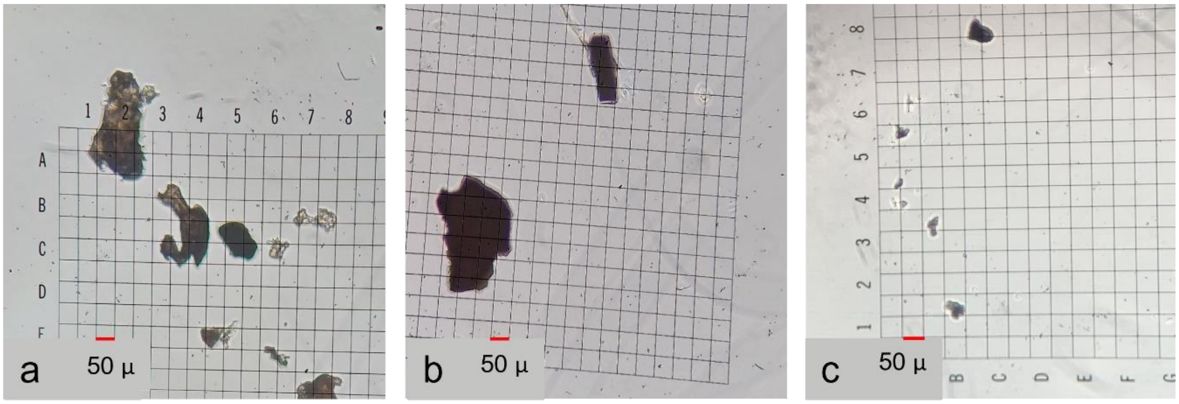


Figure 1. Comparison of the Acrylonitrile Butadiene Styrene (ABS) microplastic that we used as food for zooplankton (a) and the microplastics that could find naturally in the water of Lake Xochimilco, Mexico City, which is the habitat of the axolotl *Ambystoma mexicanum* (b and c). Inverted optical microscope at 10×. (2 column).

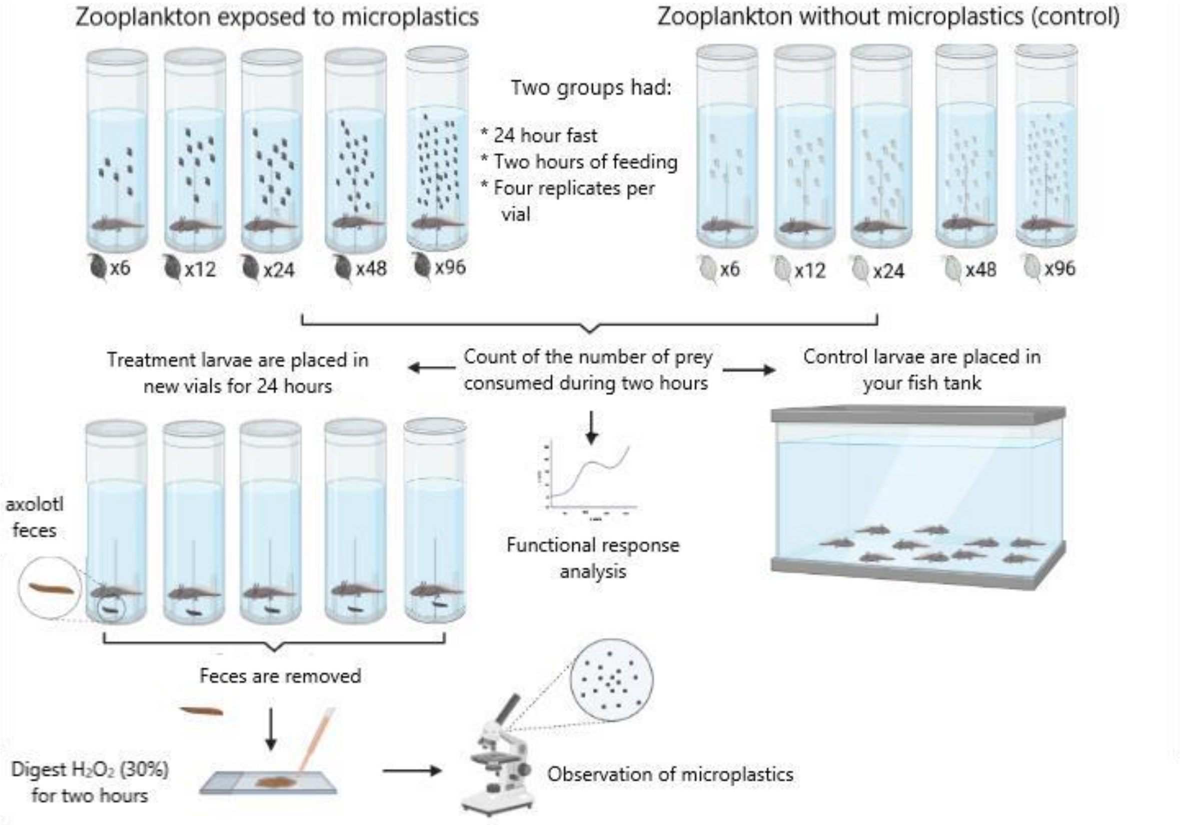


Figure 2. Experimental design. (2-column)

Figure 3. Cladoceran (*Daphnia pulex*) with Acrylonitrile Butadiene Styrene (ABS) microplastic among its valves (red arrow) (1-column)

544

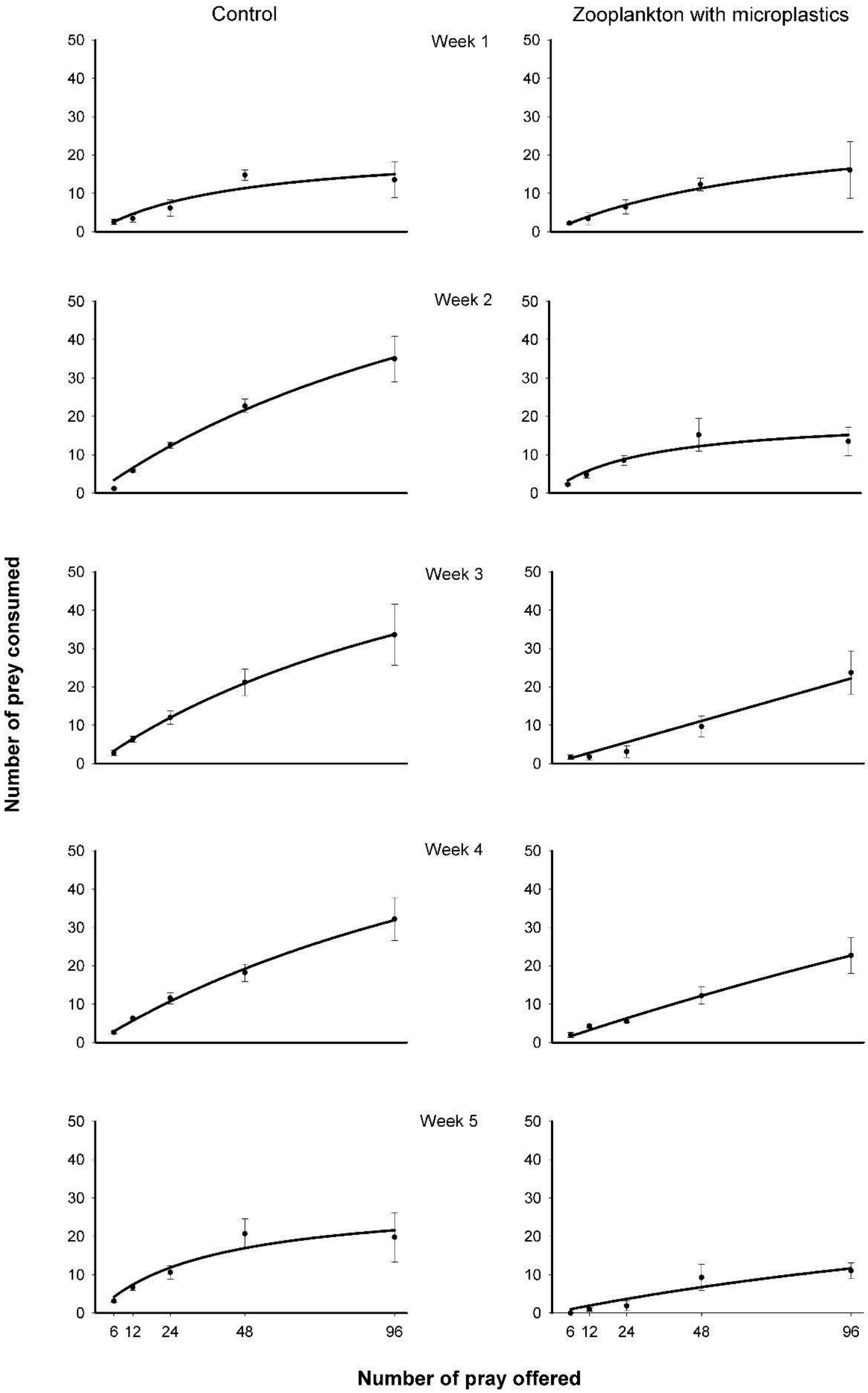
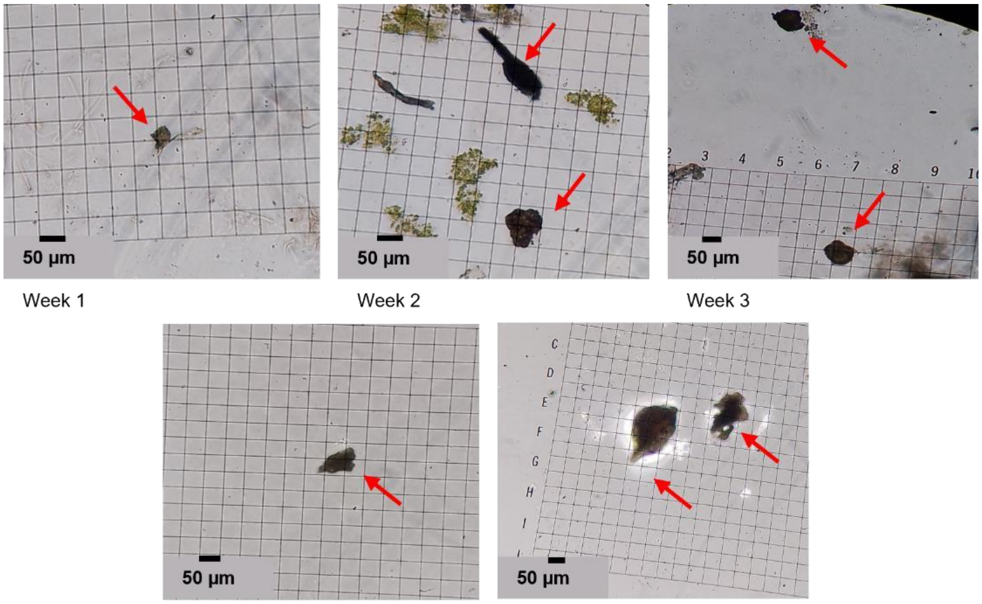

Control
Zooplankton with microplastics
Week 1
Week 2
Week 3
Week 4
Week 5
0
10
20
30
40
50
6
12
24
48
96
Number of prey consumed
Number of pray offered

Figure 4. Functional responses of *Ambystoma mexicanum* larvae when fed with zooplankton exposed to microplastics and not exposed (control) throughout five weeks of development. The number of prey items available were 6, 12, 24, 48 and 96 cladocerans. Values are expressed as means ± standard error based on four replicates. (2-column)



Week 1 Week 2 Week 3

Week 4 Week 5

Figure 5. Acrylonitrile Butadiene Styrene (ABS) microplastics in the feces of *Ambystoma mexicanum* larvae that were fed with zooplankton exposed to this contaminant. Inverted optical microscope at 10×. (2-column).

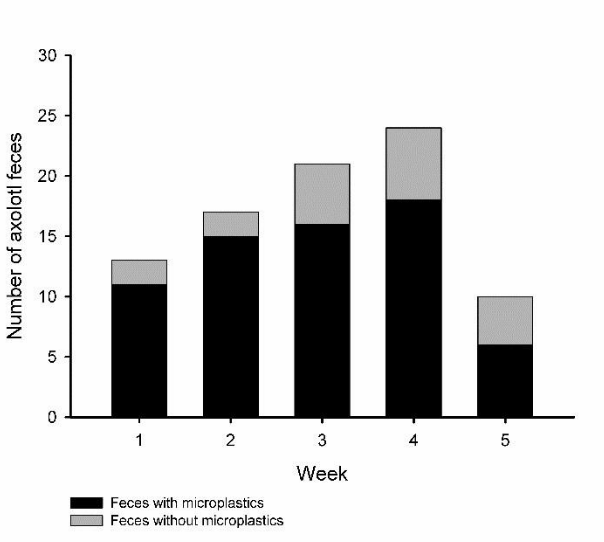


Figure 6. Amount of feces of the amphibian Axolotl *Ambystoma mexicanum* with presence and absence of microplastics. (1-column)

DISCUSIÓN Y CONCLUSIONES

Este estudio evaluó la amenaza que representan los microplásticos para el anfibio urodelo, *A. mexicanum*, el ajolote, una especie endémica de México en peligro crítico de extinción. Determinamos que los microplásticos se transfieren entre las larvas de zooplancton y ajolote en una red trófica. Encontramos microplásticos en las heces de las larvas de ajolote y demostramos que su comportamiento alimentario se reduce cuando sus presas contienen microplásticos. Esta información apoya la conservación del ajolote.

También se ha informado la ingestión de microplásticos a través de sus presas en peces de agua dulce (Justino et al., 2023); asimismo, se ha documentado que estos organismos ingieren más microplásticos a través de la depredación que a través de la columna de agua (Athey et al., 2020; Hasegawa & Nakaoka, 2021; D'Avignon et al., 2023). Ferreira et al. (2018) demostraron que la mitad de 551 peces adultos contenían microplásticos; los autores indican que fueron ingeridos activamente mediante el consumo de zooplancton cuando eran juveniles y subadultos. Hasegawa & Nakaoka (2021) afirman que en los sistemas de agua dulce la transferencia trófica aumenta la exposición a los microplásticos porque los depredadores exigen más alimento para nutrirse y porque las presas, por su tamaño, pueden consumir fragmentos más pequeños, por lo tanto, los depredadores pueden tener más probabilidades de bioacumularlos, trasladarlos a otros órganos y sufrir efectos negativos.

En nuestro estudio, la ingestión de microplásticos a través del zooplancton disminuyó el número de presas consumidas por las larvas de *A. mexicanum*, por lo que provocó la muerte de los ajolotes al final de la quinta semana. Aunque la mortalidad no es una variable que medimos, es importante mencionar que fue un efecto secundario que no observamos en el grupo control. Pensamos que la disminución de la alimentación y la muerte de todas las larvas puede deberse, en parte, a la ingestión de microplástico ABS, un polímero con un alto riesgo para la salud de los seres vivos (Lithner et al., 2011). Por ello, es importante considerar la toxicidad de los microplásticos y determinar los posibles riesgos que representan para los anfibios y otros seres vivos.

Algunos estudios han indicado que los microplásticos pueden causar daños a la salud de los anfibios cuando los ingieren de su entorno (Araújo et al., 2020; Araújo & Malafaia et al., 2020). Por ejemplo, Boyero et al. (2020) informaron que las tasas de alimentación, la supervivencia y el crecimiento disminuyen cuando los renacuajos del sapo partero (*Alytes obstetricans*) se exponen a microplásticos de poliestireno. Asimismo, Pannetier et al. (2020) mencionan que los microplásticos tienen un impacto negativo a nivel celular en organismos acuáticos, induciendo estrés oxidativo, cambios en los parámetros metabólicos, reducción de la actividad enzimática y necrosis celular.

Además de los efectos negativos del consumo directo de microplásticos, también se ha demostrado que este contaminante y sus tóxicos adjuntos se transfieren de la presa al depredador en los sistemas acuáticos. Athey et al. (2020) observaron que las larvas de peces ingerían más tóxicos a través de sus presas con microplásticos que al ingerirlos directamente de la columna de agua; además, las larvas que consumieron presas con microplásticos desarrollaron menos biomasa y longitud. Además, Uy & Johnson et al. (2022) encontraron que hubo una transferencia de microplásticos del zooplancton a las larvas de peces y, en consecuencia, disminuyó su crecimiento y supervivencia. Por lo tanto, es probable que las larvas de *A. mexicanum* en su ambiente natural estén enfrentando problemas de salud debido al consumo de microplásticos y sus tóxicos asociados a través de sus presas. Es necesario evaluar los posibles efectos *in situ* ya que su hábitat se encuentra altamente contaminado (Zambrano et al., 2003).

Con base en nuestras observaciones, proponemos que existen varios mecanismos a través de los cuales los microplásticos dentro del zooplancton podrían causar una disminución de la alimentación de las larvas de ajolote. Observamos que, en ocasiones, los microplásticos bloqueaban los canales de alimentación y apéndices de los cladóceros; lo que provocó que la natación de los cladóceros fuera errática, lenta o incluso inexistente; este fenómeno afectó la capacidad de las larvas de ajolote para capturar a sus presas ya que, en parte, perciben a sus presas a través de sus órganos mecanosensoriales que les permiten sentir vibraciones (Münz et al., 1984). La obstrucción del sistema digestivo y la inmovilización por microplásticos en cladóceros parece ser recurrente (Rehse et al., 2016; Frydkjær et al., 2017; De Felice et al., 2019).

Asimismo, es importante mencionar que los cladóceros son propensos a transferir microplásticos a los depredadores, ya que se ha demostrado que luego de exponer *D. magna* a los microplásticos, su ritmo cardíaco se reduce constantemente, lo que resulta en un bloqueo del tracto digestivo e impide la expulsión de los microplásticos (Pan et al., 2022).

Un segundo mecanismo es que el microplástico ABS, debido a su baja densidad (1,02–1,08 g cm^{-3}), funciona como un flotador que mantiene a los cladóceros en la superficie; este fenómeno disminuye la probabilidad de contacto con sus presas ya que el ajolote generalmente tiene un comportamiento bentónico y nada hacia la superficie en ciertas ocasiones, además, los ajolotes son cazadores pasivos, es decir, solo esperan que el zooplancton esté cerca para abrir su boca y tragar toda la presa; por lo tanto, si la presa está lejos, es poco probable que la capture. Se ha informado anteriormente que los microplásticos flotantes afectan la capacidad de las larvas de peces para detectar y consumir zooplancton (Uy & Johnson et al., 2022).

Un tercer mecanismo es que los microplásticos pueden alterar las tasas de alimentación debido al falso efecto de saciedad. Hu et al. (2016) encontraron que los renacuajos de ranas *Xenopus tropicalis* que consumen más microplásticos tienden a comer cada vez menos porque bioacumulan microplásticos en el sistema digestivo, lo que genera una sensación de saciedad. Sin embargo, en nuestro trabajo contamos con un vector de transferencia de microplásticos, es decir, el zooplancton (cladóceros), por lo que hay que considerar que la bioacumulación de microplásticos en los ajolotes dependerá de cuánto consuman cladóceros.

Finalmente, con la presencia de microplásticos en las heces de las larvas de *A. mexicanum*, verificamos que hubo ingestión de microplásticos por parte de los cladóceros (Fig. 3), esto explica su disminución en la velocidad de nado y flotabilidad. Confirmar la presencia de microplásticos en las heces es un paso importante; sólo así podremos estar seguros de que este contaminante puede ser ingerido, bioacumulado en el tiempo, excretado y tener contacto con el sistema digestivo de los seres vivos. Aquí mostramos, mediante la observación de heces, que los microplásticos se transfieren del zooplancton a las larvas de ajolote. Investigaciones anteriores encontraron microplásticos en las heces de renacuajos anuros, pero en este caso, la ingestión de microplásticos fue directa del medio ambiente, ya que los renacuajos anuros se alimentan por filtración (Hu et al., 2016; Boyero et al., 2020).

Los microplásticos son estresantes para las larvas de ajolote cuando consumen zooplancton previamente expuesto a microplásticos. Sugerimos futuros estudios para encontrar las razones de la alteración en el comportamiento alimentario de los ajolotes. También se necesitan más investigaciones sobre la ingestión de microplásticos por parte de los anfibios a través del agua, su alimentación y sus efectos en sus primeras semanas de vida (Venâncio et al., 2022). Además, necesitamos responder otras preguntas en el futuro para comprender mejor el riesgo que los microplásticos representan para los ajolotes: ¿La presencia de microplásticos afecta la supervivencia, el desarrollo y el crecimiento? ¿Se reduce la natación y la movilidad cuando las larvas están expuestas a este contaminante? ¿Cómo pueden influir los microplásticos en la salud de estos animales a nivel de tejidos y órganos? Resolver estas preguntas nos ayudará a comprender cómo los microplásticos amenazan a los ajolotes y, a su vez, nos permitirá encontrar soluciones para su conservación.

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