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Research proposal: Decrease of microplastic trophic transfer by transferring gut bacteria from *Tenebrio molitor* to *Danio rerio*.

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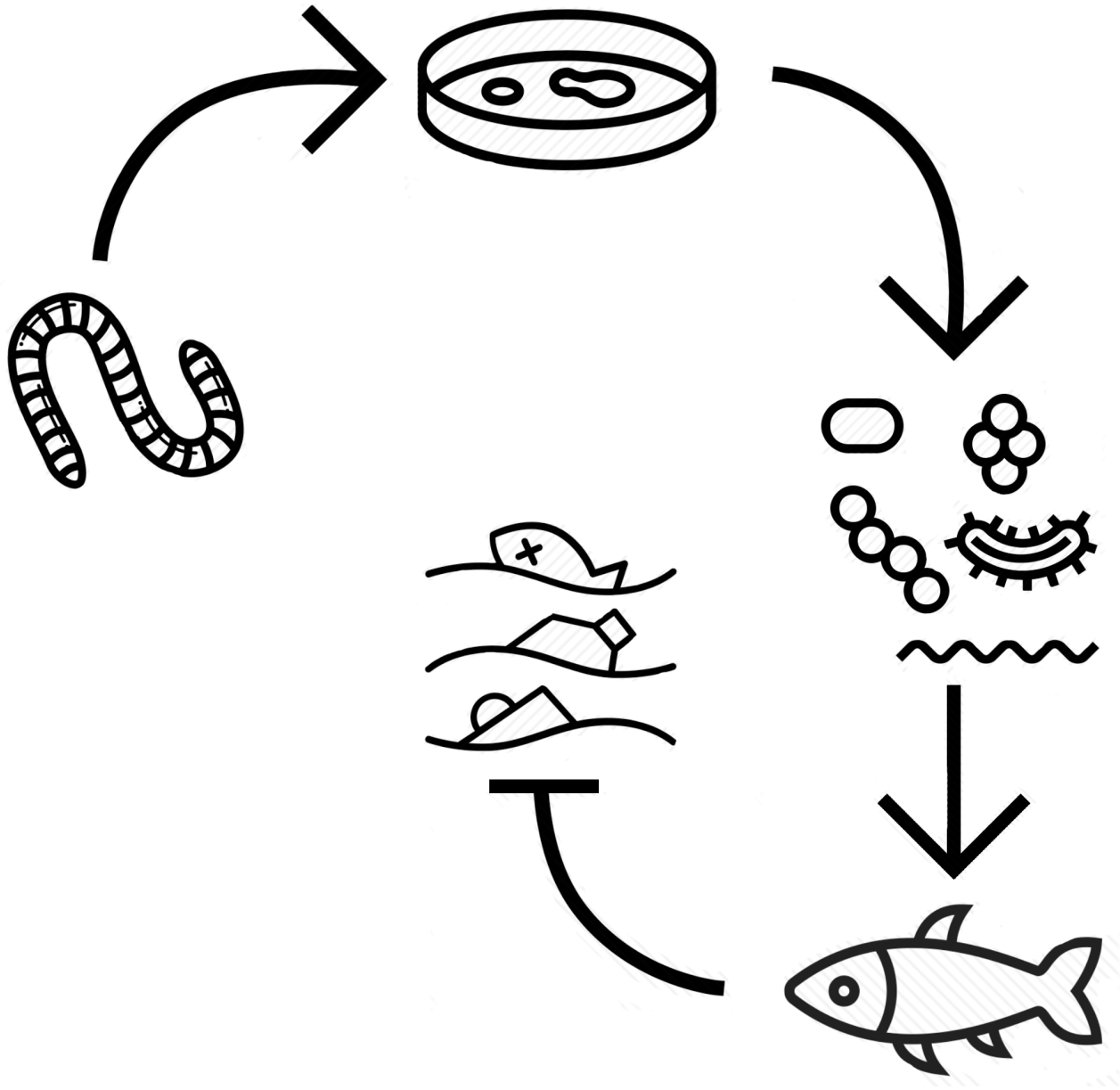


Abstract.
Plastic production has increased dramatically the past century; the ubiquitous use of plastics in products ranging from plastic bags to toothpaste causes global contamination. Nowhere is this as apparent as in the world's oceans, where not only a large patch of debris spanning 700.000 square kilometres is observed, but also the extensive spread of microplastics, which have been found as remotely as the Arctic polar waters. Ingestion of these microplastics have been observed in a variety of species and can potentially lead to bioaccumulation due to trophic transfer. To combat this threat, we propose the research of transferring plastic degrading bacteria found in the mealworm *Tenebrio molitor* to the gut of zebrafish (*Danio rerio*).

Problem definition

Plastic pollution is a serious problem brought about by the extensive production, use and lack of proper recycling. By current estimates, a total 8300 million metric ton (Mt) of virgin plastic was produced, generating 6300Mt of plastic waste up to 2015[2]. To make matters worse, a majority of these plastics, such as ethylene, propylene and polystyrene, are derived from fossil hydrocarbons, which already put a strain on the environment. Furthermore, none of these commonly used plastics are biodegradable. As a consequence, they accumulate onto landfills or the natural environment[4]. Indeed, severe plastic contamination of natural waters is ubiquitous throughout all sub-tropical gyres[5]. The large amount plastic circulating the planet's oceans has a profound effect on marine fauna, ranging from entanglement, smothering to ingestion and the subsequent release of chemicals in marine fauna[6]. Contributing to bioavailability of plastics are the so-called "microplastics", defined as microscopic plastic particles ranging from 0.1mm to 5mm[7]. These can be further divided into primary and secondary microplastics. Whereas primary microplastics are produced for certain applications, for example facial scrubbers, cosmetics, air-blasting media and drug vectors, secondary microplastics are fragmented macroplastics caused by a variety of physical, biological and chemical processes'[7]. These particles cannot only transfer waterborne toxic pollutants, but also leaching of plasticisers unto the base food chain, potentially leading to bioaccumulation[8]. Indeed, trophic transfer of microplastics in marine fauna has been observed, even subsequent ingestions by humans (see Fig.1)[9,10]. However, the exact danger of microplastics is not yet properly assessed[3,11].

However, not all hope is lost. Bacteria capable of degrading plastic marine debris have been found, living in their own specialized microbial community[12]. However, closer inspection shows that these bacteria are potential opportunistic pathogens and their mechanisms of degrading plastics have not yet been properly characterised[12]. A more promising study concerning the gut microbiota of several plastic eating caterpillars and mealworms show better results[13-15]. Not only showing plastic degradation and conversion into CO_2 and biomass, but also decreasing hydrophobicity. It was also observed that the isolated bacteria, *Exiguobacterium* sp. strain YT2, performed worse in degrading plastic outside of the host organism and that treatment with antibiotics severely impaired plastic processing by the

worm, implicating that the worms gut plays the role of bioreactor in plastic degradation[14]. In this research proposal, we look for a potential application of these bacteria by transferring the microbiota of the mealworm *Tenebrio molitor* to the gut of gnotobiotic zebrafish' *Danio rerio* and subsequent effects on microplastic degradation.

Approach

Enrichment of plastic degrading bacteria isolated from the gut of *Tenebrio molitor.*

These methods are adapted from Yang Et al (2015)[13,14]. First, to ensure enrichment of plastic degrading bacteria, it is required that the sole carbon source of these bacteria is derived from plastic. For this purpose, polystyrene (PS) is selected due to previous success by Yang Et al. As an added benefit, both α 13 C-labelled and β 13 C-labelled PS is available from Sigma-Aldrich, St. Louis, MO, this facilitates subsequent tracking of the possible degradation products from PS by combustion–isotope ratio mass spectrometry (GC–C–irMS). Next is to prepare a PS film for microbial degradation and enrichment. To do this, a PS sample is dissolved in xylene solvent (0.03g/ml). This solution is then spread on a glass plate and left for 5 hours. Following, the films are then removed from the glass plate and left to fix in a hood at room temperature (RT) for 3 days. Consequently, films were was in methanol solvent, followed by demineralized water and left to dry. The resulting PS film can then be used for the growth of bacterial biofilm on agar plates, or fragmented for a bacterial suspension. Liquid carbon-free basal medium (LCFBM) is then used to ensure that the PS film is the sole carbon source required for bacterial growth[13,14].

Mealworms put on a diet exclusively consisting of PS for 2 weeks are used to prepare a gut cell suspension to be used as an inoculum of PS degrading bacteria. Furthermore, this suspension can be transferred to an Erlenmeyer containing the LCFBM and PS suspension to selectively enrich PS degrading bacteria.

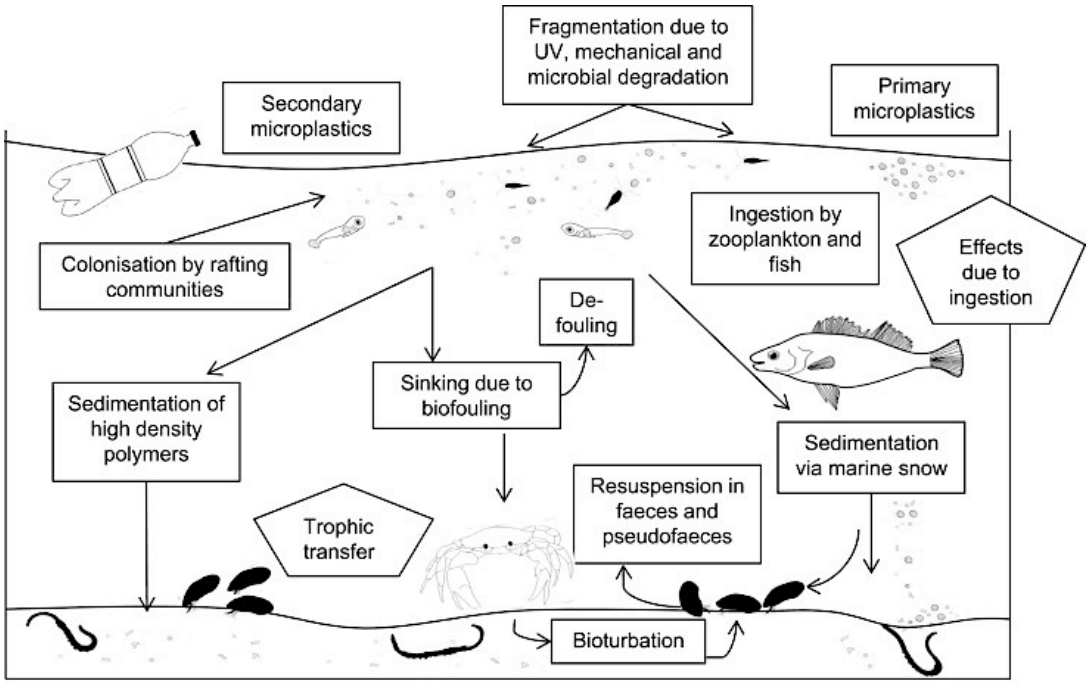
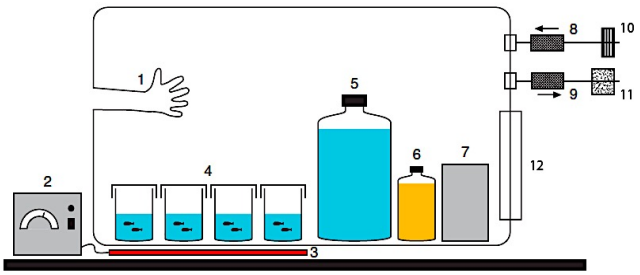


Figure 1. Summary of microplastics transport and potential biological interaction. Adapted from Wright Et al (2013)[3].

After which it is plated on regular LB agar plates and characterised by 16S rRNA sequencing.

Colonization of gnotobiotic *Dania rerio*.
The options for generating and colonising gnotobiotic zebrafish are numerous and well defined. The most viable methods which also has a low microbial burden consists of a process called "squeezing"[1]. This method consists of manually excreting the gametes and ejecting them through the cloaca, where they are subsequently submerged in antibiotic media to minimize microbial burden. The zebrafish can then be inoculated with our previously acquired PS degrading bacteria as specified by Pham Et al (2008). To determine succesfull colonization, feces of zebrafish can be isolated and be grown on enrichment media and conventional LB medium. Consequently, zebrafish should be housed in an isolated (see Fig. 2), this setup adapted by Pham Et al has been supplemented with additional CO_2 trapping systems to measure PS degradation into CO_2 as previously reported[13,14]. Furthermore, gnotobiotic zebrafish medium (GZM) will be supplemented with α13 C-labeled and β13 C-labeled PS microplastic particles.

Microplastics collection and measurements.
Observation of plastics and determining potential degradation products in fish is a difficult task. Several potential options are suggested in this research proposal. Firstly, to determine if 13C labelled CO_2 was formed due to microbial degradation of 13C labelled PS, CO_2 is first captured with 1M NaOH and subsequently precipitated from $BaCl_2$ to $BaCO_3$. Subsequently, the isotopic composition can be determined through the use of isotope ratio mass spectrometry. Secondly, microplastics in



1. Attached gloves
2. Heated water pump
3. Circulating warm water pad
4. Foil-covered beakers containing zebrafish in GZM
5. Bottles of sterile GZM
6. Bottles of sterile zebrafish food
7. Supplies
8. HEPA-filtered forced air supply
9. HEPA-filtered air exhaust
10. Pre-CO2 removal
11. Sequential CO2 trapping system
12. Sealed port for transfer of materials to/from isolator

Figure 2. Schematic diaphragm for the housing of gnotobiotic zebrafish. Adapted from Pham Et al (2008)[1].

suspension can be acquired through means of a filter, whereafter the acquired plastic particles can be weighed, characterised by pyrolysis gas chromatography mass spectrometry (Pyr-gc/ms), analysis of degradation products by fourier transform infrared spectroscopy (FTIR) and potential observation of change in the topography of PS by Atomic Force Microscopy (AFM)[13-15]. Microplastic particles ingested by zebrafish can also be isolated through the means of a 24h digestion of zebrafish with a solution of 10% KOH and subsequent filtration[16]. Admittedly, isolation and characterization of small microplastics from fish are notoriously difficult and prone to error.

Application

In this research proposal we suggest the transfer of PS degrading bacteria isolated from the gut of *Tenebrio molitor* to gnotobiotic *Danio rerio* as a proof of concept that plastic degrading bacteria can contribute to potentially decrease trophic transfer of microplastics.

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