

Assimilation of Microplastics and Bacteria Associated with the Organs of Wild Mullet Harvested from an Estuary in Rivers State, Nigeria

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Abstract: This research investigates the assimilation of microplastics (MPs) and bacteria associated with internal organs of wild Mullet (*Liza grandisquamis*) harvested from Ohiakwo estuary. The bacterial quality was evaluated using standard microbiological and Fourier transform infrared (FTIR) spectroscopy analyses. The adult mullet samples used have supposedly acclimatized to the biogeochemical, stress and plastsphere ecology of the aquatic ecosystems. Bacterial group counts (BGCs) among the organs (gill, intestine and tissue) was high (10^7 - 10^8 CFU/g) but varied slightly with the highest being total coliform counts, 2.33×10^8 CFU/g. The bacteria associated were identified from culture-dependent molecular techniques with accession numbers as *Staphylococcus epidermidis* (AE015931), *Pseudomonas xiamenensis* (MH734834), *Bacillus licheniformis* (AF478085), *Klebsiella pneumoniae* (MH021669), *Bacillus lentus* (AB021189), *Escherichia coli* (KX609714) and *Vibrio alginolyticus* (MH734825) respectively. FTIR spectroscopy and other contemporary analyses revealed the percentage occurrence of the synthetic polymers/MPs in the following order; sediment (S) > surface water (SW) > gill (G) > intestine (I) > tissue (T) during the dry season. Whereas in the rainy season the order was; S > G > SW > I > T. Further analyses of the MP particles in the gill identified the components as 92.00% polypropylene (PP), 3.11% polyester, 1.90% polyvinyl chloride (PVC), 1.21% polyurethane (PU), 1.14% cellulose, 0.37% polyamide, 0.15% polystyrene (PS) and 0.01% low density polyethylene (LDPE) respectively. However, the presence of MPs in the aquatic environment and in mullet internal organs poses a serious threat to food security and public health. Further research, however, should focus on methodologies or technologies that will monitor and eliminate MPs and their toxicity along the food supply chain.

Keywords: Microplastic, accession numbers, mullet, FTIR spectroscopy, food security

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I. Introduction

Plastics are ubiquitous and one of the most common and persistent organic pollutants (POPs) in modern days^{1,2} and biodegradation is often hampered by the durability, stability, crystallinity and macroscopic structure of the polymers^{3,4}. Their visibility in litters of many terrestrial and aquatic environments poses a great challenge for environmentalists, microbiologists and the global community. Plastic debris are categorised as macroplastics (particle diameter > 25mm), microplastics (< 5mm) or mesoplastics (5-25mm)^{5,6,7}. MPs are defined as synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1µ-5mm, of either primary or secondary manufacturing origin, which are insoluble in water⁸. Primary MPs originate from manufactured beads of microscopic size, e.g., preproduction resin pellets, microbeads in cosmetics, toothpaste and blasting, powders for textile coatings, and drug delivery media⁹, whereas the secondary MPs are derived from the degradation/weathering of larger (i.e., meso- and macro-) plastics¹⁰ due to photolytic, mechanical fragmentation and biodegradation¹¹.

The occurrence of MPs in different species at various trophic levels such as phytoplankton¹², zooplankton¹³ to the highest levels in both invertebrates and vertebrates is well documented¹⁴⁻¹⁷ but most of the studies have no report on gut microbiome which may act mutualistically as biodegraders *in vivo* and/or *in vitro*. However, one of the primary environmental risks associated with MPs is their availability for aquatic organisms^{18,19}, small size and spatial distribution which renders them susceptible to ingestion, particularly by fish^{20,21}. Despite the available scientific literatures on MPs, their effects on human health are poorly understood and controversial. Different workers have reported that they have many detrimental consequences both physically and physiologically such as direct mortality from entanglement and choking, sublethal effects; compromised feeding, digestion and reproduction as well as translocation of non-native species^{22,23}. MPs have been reported to accumulate in gills, liver and gut, causing inflammation, immunotoxic responses, lipid accumulation in the liver and oxidative stress instauration evidenced by the increase in superoxide dismutase and catalase enzymes

^{24,25}. They have been detected in human stool, suggesting ingestion from different sources with PE, PP and PET being the most abundant ²⁶. Consumption of whole or complete organism present higher risk than eviscerated ones ²⁷ and such evidence should not preclude further investigations in fish, foodstuffs and impacts on human health.

Mullet (*Liza grandisquamis*) the principal specimen for study is the most widespread species among the family Mugilidae, comprising a total of 20 genera and 70 valid species ²⁸. It inhabits the fresh, brackish, hyposaline lagoons and coastal marine waters of shallow depth of less than 20m ²⁹ and have significant economic value in the tropical regions ³⁰. As an omnivorous, detritivorous and benthic forager, the rate of anthropogenic discharges and the risk of MP ingestion from contaminated aquatic environment has resulted in fluctuations of production statistics ^{31,32}. Despite its economic value, there is dearth of scientific literatures on the occurrence of MPs and microbial ecology of the fisheries of Niger Delta estuarine ecosystem. This research was therefore, designed to investigate the assimilation of MPs and bacteria associated with the internal organs of wild Mullet, *L. grandisquamis* harvested from Ohiakwu estuary.

II. Materials and Methods

Study area and collection of Mullet (Fish) sample

A total of 40 wild grey mullet (*Liza grandisquamis*) samples were collected for five months during wet and dry seasons from fisher men from Ohiakwu Estuary which has a common boundary with the Rivers State University, Agip Base landing Jetty and Eagle Island, Nkpolu-Oroworukwo, Port Harcourt. It is a brackish water ecosystem influenced by tidal fluxes with mangrove vegetation cover interspersed in some areas with Nypap plants and lies within the rectangular coordinates of N4°46'50", N4°48'10" and E6°57'10", E6°57'30", Figure no1. Physical characteristics of the mullet samples were used to determine their status, Plate no 1.

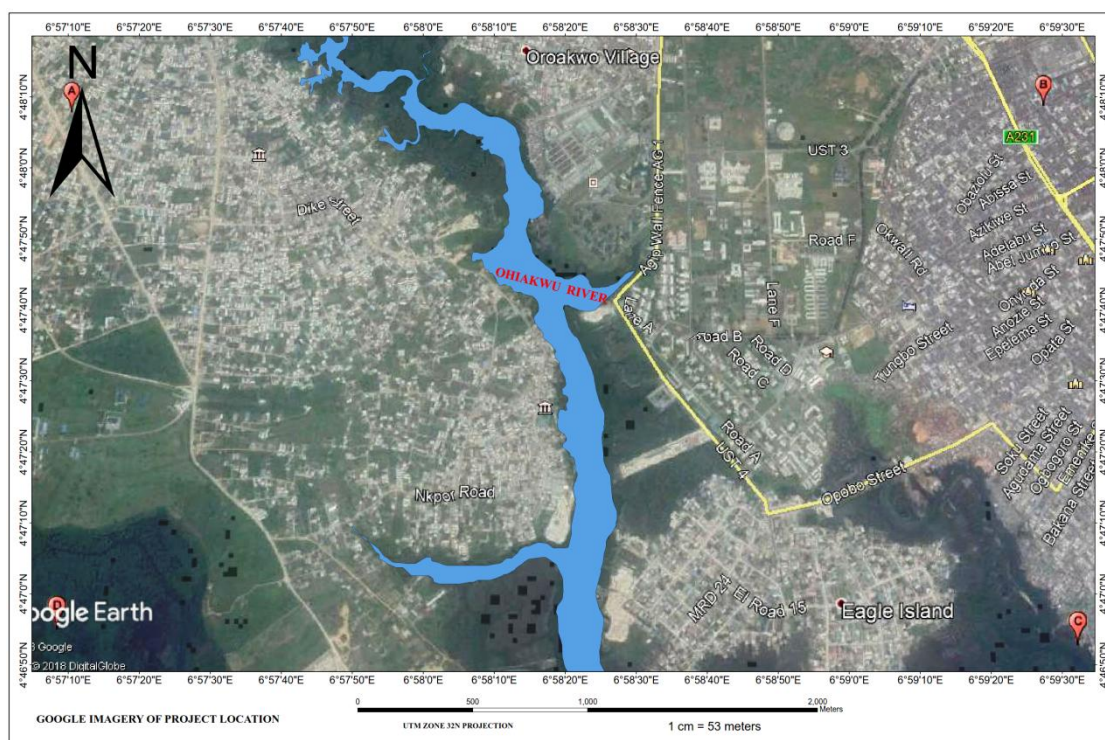


Figure no 1. Satellite imagery of study area.



Plate no 1. Flathead grey wild Mullet harvested from Ohiakwu Estuary.

Bacteriological analysis

Total heterotrophic count (THC) was determined on sterilized surface-dried nutrient agar (NA) using spread plate method, aliquots (0.1mL) of appropriate decimal dilutions were inoculated onto the solidified NA in duplicate plates and incubated at 37°C for 24h. MacConkey agar was used to determine total coliform bacteria whereas mineral salt agar (MSA) was used for hydrocarbon utilizing bacteria. Discrete colonies were counted as colony forming units (CFUs) from plates with colonies ranging from 30-300 and representative colonies were sub-cultured onto sterile NA plates to obtain pure cultures which were preserved in slants overlaid with 10% glycerol for further identification tests. Isolated bacterial were subjected to cultural, morphological, biochemical protocols were purified³³⁻³⁶ followed by molecular identification.

DNA extraction and quantification

Culture-dependent molecular identification protocols was followed after characterization of organisms from the various samples to ascertain the bacterial species. DNA extraction from pure bacterial culture was performed using the boiling method. This was achieved by centrifuging five millilitres (5mL) of overnight broth culture of the isolates grown in Luria Bertani (LB) broth and spun at a speed of 14000rpm for 3minutes. This was followed by re-suspension of the cells in 500µl of normal saline and subjected to heat to a temperature of 95°C for 20 min and thereafter allowed to cool on ice for 10min and spun for 3 min at 14000rpm. The DNA suspension was decanted into a 1.5ml volume micro-centrifuge tube and stored at -20°C for further downstream reactions. The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. Two micro-litres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button and read via computer device attached to the system.

16S rRNA amplification

The amplification of the gene was achieved by polymerase chain reaction (PCR) method. The 16S rRNA region of the genes of the isolates were amplified using the 27F and 1492R primers (16SF: GTGCCAGCAGCCGCGCTAA and 16SR: AGACCCGGAACGTATTAC) on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5µM and extracted DNA as a template. The gene amplification was performed using a set of conditions which involved initial denaturation of DNA at a temperature of 95 °C for 5 minutes and at the same temperature for 30 seconds. The next was a 35 cycle series of annealing at 52°C and extension at 72°C, for 30 seconds each, except for the final extension that was maintained for a period of 5 minutes.

The final PCR product was electrophoresed at 130V for 30minutes using 1% agarose gel concentration and DNA bands were visualized on a UV trans-illuminator.

DNA sequencing

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer (Inqaba Biotechnological, Pretoria South Africa) and maintained at a final volume of 10µl. The components included 0.25 µl Big Dye® terminator v1.1/v3.1, 2.25 µl of 5 x Big Dye sequencing buffer, 10µM Primer PCR primer and 2-10ng PCR template per 100bp. The sequencing conditions involved 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 minutes.

Phylogenetic analysis

The sequences obtained from isolates were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. Then, aligned using ClustalX and Neighbor-Joining method in MEGA 6.0 followed with bootstrap consensus tree from 500 replicates to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method, Figure no 2.

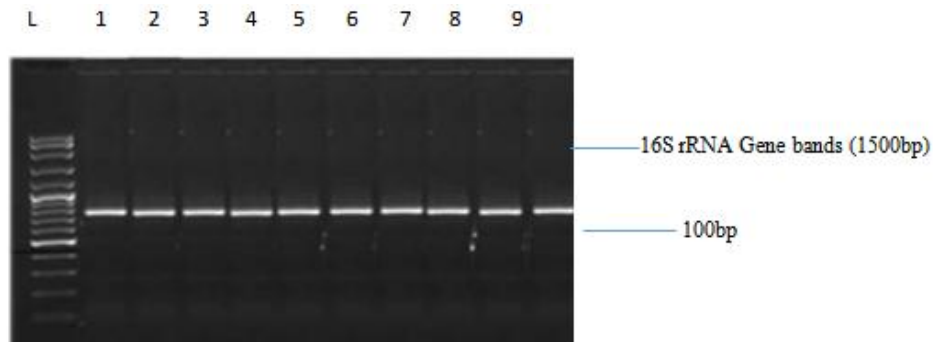


Plate no2. Agarose gel electrophoresis of the amplified 16S rRNA Gene Bands. Lanes 1-9 indicates band at 1500bp whereas Lane L represents the 100bp molecular ladder.

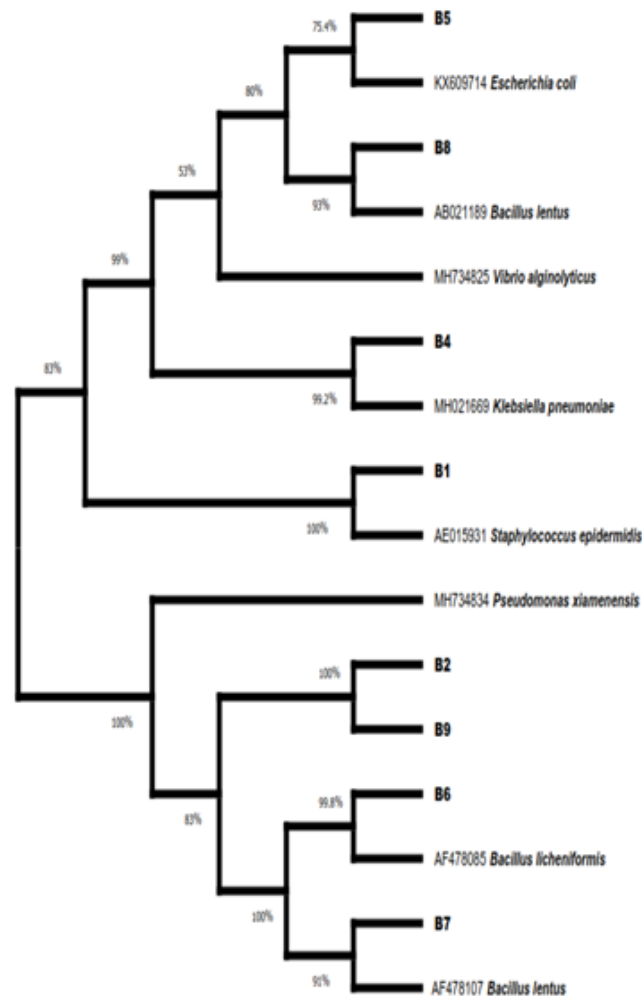


Figure no 2. Phylogenetic tree of bacterial isolates.

Digestion and extraction of MP

Hydrogen peroxide (H_2O_2) digestion was carried out as described by³⁷. Various fish components such as intestine, gill and muscle samples were placed into clean beakers of 1-litre capacity and digested to separate the plastic from the tissue. Approximately 400 mL of 30% hydrogen peroxide was added to digest the organic matter, and beakers covered with sterile ceramic tiles and placed on a hotplate stirrer at approximately 60 °C for 24 h. The dissolved solution was retained upon completion of the digestion. Filtration and density separation of plastic particles was achieved by saline (NaCl) solution, followed by the classical setup methods adopted by^{38,39}. Approximately 800 mL of 1.2 g NaCl solution (w/v) was added to the beaker for separation of MP via floatation

by increasing the density of the solution. The solution was mixed and kept overnight and the overlying water was directly filtered with 11µm pore size Whatman Grade 1 filter paper. Then filters were placed into clean Petri dishes and covered until further analysis.

Identification of MP

The filters were subjected to virtual inspection and larger MPs were sorted out whereas smaller MP particles were sorted under a dissection microscope fitted with ocular lens and finally identified under a light microscope. Microplastics were selected for polymer identification using Fourier transform infrared (FTIR) spectroscopy with a single-element MCT detector. Cary 620–670 FTIR microscope, equipped with a GeATR crystal (Agilent Technologies) was used for the analysis of MPs and recorded directly with a spectral resolution of 8cm⁻¹, in the 4000–650 cm⁻¹ spectral range.

Statistical analysis

All the experiments were carried out in duplicates (n = 2) and the results are presented in mean value with standard deviation (Mean ±SD). Duncan multiple test, two-way ANOVA and SPSS version 22 were used to check the significance of the various data and mean separation.

III. Results

The physical characteristics of the mullet caught during the rainy/wet and dry seasons are presented in Table 1. The mean values of fish weight, length and width, tissue and intestinal weight were higher during the dry season than at the wet season whereas only the gill weight (bold) was higher during the wet season (Table no 1).

Table 1. Physical characteristics of adult mullet (n = 40) harvested during rainy and dry seasons

Mullet parameter	Rainy season (mean ±SD)	Dry season (mean ±SD)
Length (cm)	32.50±8.62	39.58±3.54
Width (cm)	9.28±1.51	9.59±1.33
Weight (g)	177.75±57.59	250.81±42.34
Tissue weight (g)	23.62±8.45	23.75±3.08
Gill weight (g)	11.11±0.98	10.73±0.90
Intestine weight (g)	21.60±0.33	22.53±2.95

Mean bacterial group counts (CFU/g) in different mullet organs during rainy and dry seasons are displayed in Table no 2. The highest mean total heterotrophic bacterial (THB) group counts ranged from 7.30-7.14x10⁷ in the gill during the rainy and dry seasons and marginal differences between the intestine and muscle/tissue. Mean total coliform count (TCC) of 2.33x10⁸ was recorded in the gill being the highest in both seasons and the least count of 1.38x10⁸ occurred in the intestine during the rainy season. Highest mean total hydrocarbon utilizing bacterial (HUB) group counts of 2.21x10⁷ and 1.95x10⁷ were obtained in the intestine in both seasons followed by the tissue whereas the least counts occurred in the gill during both seasons.

Table 2. Total bacterial group counts (CFU/g) in Mullet organs during rainy and dry seasons

BGC (CFU/g)	Mullet organs/Seasons					
	Gill		Intestine		Muscle (Tissue)	
	<i>Rainy</i>	<i>Dry</i>	<i>Rainy</i>	<i>Dry</i>	<i>Rainy</i>	<i>Dry</i>
THB	7.30x10 ⁷	7.14x10 ⁷	3.34x10 ⁷	3.96x10 ⁷	3.71x10 ⁷	3.47x10 ⁷
TCC	2.33x10 ⁸	1.50x10 ⁸	1.38x10 ⁸	1.85x10 ⁸	1.86x10 ⁸	1.82x10 ⁸
HUB	1.35x10 ⁷	1.38x10 ⁷	1.95x10 ⁷	2.21x10 ⁷	1.56x10 ⁷	1.91x10 ⁷

Legend: BGC = Bacterial group count

The amplified 16S rRNA of the bacteria isolated highlighted by the bands and lane of the genes are presented in Plate 1. Lanes 1-9 showed the bands at 1500 base pair while the L Lane represents the 100 base pair molecular ladder. The culture-dependent molecular identified recurrent bacterial isolates with their GenBank accession numbers indicate that the 16S rRNA of the isolates had similarities with *Staphylococcus epidermidis* (AE015931), *Pseudomonas xiamenensis* (MH734834), *Bacillus licheniformis* (AF478085),

Klebsiella pneumoniae (MH021669), *Bacillus lentus* (AB021189), *E. coli* (KX609714) and *Vibrio alginolyticus* (MH734825) respectively. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16s rDNA of the isolates (Figure no 2).

Percentage occurrence of MPs in Mullet’s habitat and internal organs during wet and dry seasons are shown in Figure no 3. The sediment had the highest MPs, followed by gill and intestine with the least percentage occurrence in the tissue during the dry season. During the wet season, the sediment had the highest MPs followed by surface water and gill whereas the least was the tissue. Further study on MPs was focused on the Mullet (fish) since it has direct consequence on the food chain, ecosystem as well as human health.

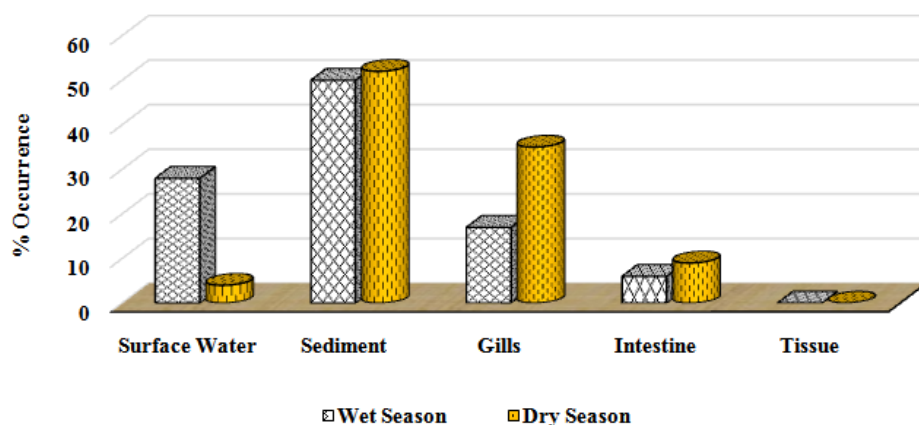


Figure no 3. Occurrence of MPs in Mullet’s habitat and internal organs during wet and dry seasons.

Figure no 4. Percentage composition of identified microplastic particles by mass and abundance in the gills of Mullet as depicted by FTIR spectroscopy analysis. FTIR spectra revealed that the samples contained MPs in the following proportions 92.00% polypropylene, 3.11% polyester, 1.90% polyvinyl chloride, 1.21% polyurethane, 1.14% cellulose, 0.37% polyamide, 0.15% polystyrene, 0.11% protein and 0.01% low density polyethylene (LDPE) respectively. Although, there are synthetic MPs in other parts of the Mullet but the most abundant were polypropylene, polyester, polyvinyl chloride and polyurethane, Figure nos 3 and 4.

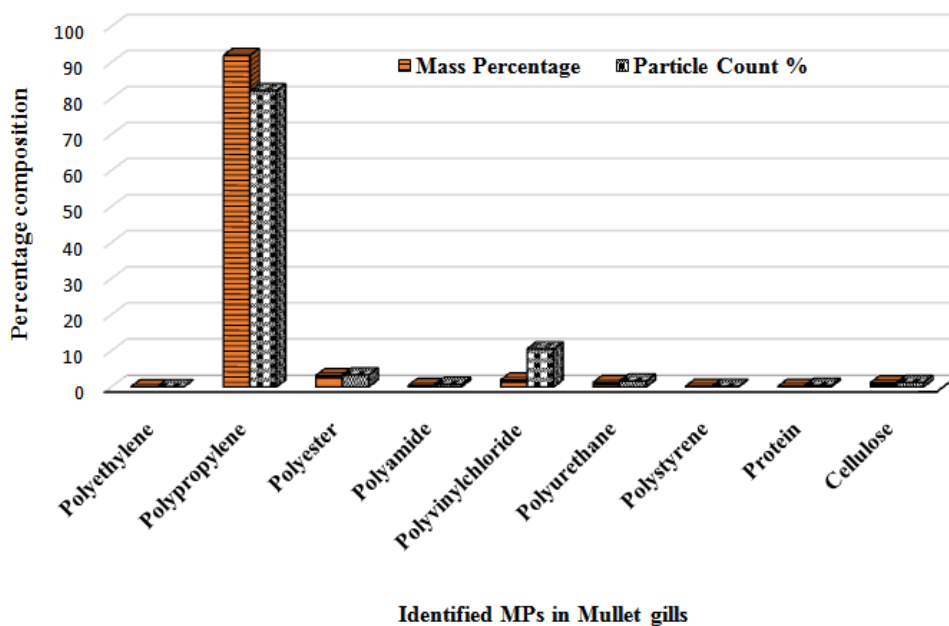


Figure no 4. Percentage composition of different plastic particles identified in the gills.

IV. Discussion

The use of Mullet at a size of about 30 cm suggests that the specimen has attained sexual maturity^{40,41} and supposedly have had proper adaptation to the biogeochemistry, stress and plastisphere of the aquatic ecosystem. The longer the lifespan of the mullet the more likelihood of bioavailability of microplastics and microorganisms as they feed and perform other normal life functions in the environment. The presence of MPs in the water column and sediment validates that they were ingested during feeding which corroborates several reports in literature^{20,21,42,43}.

The incidence of high bacterial group counts and diversity in mullet's organs provide useful basis for assessing the extent of contamination of coastal waters as well as the potential health risks posed to seafood consumers. The bacteria isolated from the organs of mullet include *Staphylococcus epidermidis*, *Pseudomonas xiamenensis*, *Bacillus licheniformis*, *Klebsiella pneumoniae*, *Bacillus lentus*, *Escherichia coli* and *Vibrio alginolyticus* respectively. Such magnitude of bacterial community composition may not be unconnected with favourable adaptation in the organs, high gut microbiome metabolism, and accentuated anthropogenic inputs in the ecosystem⁴⁴⁻⁴⁶. However, organ microbiota seems to reflect on the microplastic composition in the mullet as well.

Microplastic particles such as PE and PP are the two most abundantly produced globally^{47,48}. MPs have myriad of applications such as in polymer blending processes, food packaging materials, medical, beverage and automobile industries and are widely distributed^{8,48,49,50}. This could also explain for the abundance of PP, polyester and PVC in the organs of mullet in the study area and corroborates earlier reports of ingestion of MPs by fish^{20,21,51}. However, the preference or affinity of the gill for PP is not yet understood but different authors have reported the bioavailability of MPs in the gills, stomach, intestine and liver^{24,25} with elevated levels in benthopelagic fish⁵². The levels of occurrence of PP and microbes in fish may vary due to contamination, geographical location, affinity to different organs, differences between MPs or microbial community types as well as extraction/isolation methods and techniques.

V. Conclusions

There was assimilation of MPs in the organs of adult Mullet's (*Liza grandisquamis*) which was a reflection of those present in the aquatic environment. The most abundant MPs was PP extracted from the gill. The bacteria associated with *in vivo* plastisphere of the mullet organs were *B. lentus*, *B. licheniformis* and *Staphylococcus epidermidis*. Further research is needed to understand the toxicity of PP and its affinity for gills and other organs in mullet. However, the negative impact of increasing plastic pollution in our coastal environments such as poor aesthetic scenarios, entanglement and choking of aquatic forms amongst others demand urgent mitigation and actionable decisions from state, federal and non-governmental organisations.

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