# Identification of microalgae isolated from floating plastics found along Iloilo estuary and cultured in CM and F/2 media

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**Abstract**

Microalgae has been considered as a microorganism having potential to degrade plastic. This organism was able to attach itself to a substratum, forming a biofilm. It is vital to determine the microalgae present thriving in the surrounding environment, as the organism's characteristics contribute to its biofilm-formation properties. In this study, suspended plastics in the Iloilo river were collected. The microalgal organisms in the plastics were scraped, cultured, serially diluted, and identified. After identification, the genus-level taxonomic identification of isolates was analyzed, and the most common genus were named. From the data acquired, *Nitzschia sp.* was the most prevalent in all of the samples collected and was seen both in CM and F/2 media. Following *Nitzschia sp.* is *Navicula sp.*, with six (6) samples containing such. Most of the *Navicula sp.* were found in Conditioned media. *Anabaena sp.* were present in five of the samples identified.

**Keywords**: *plastic, biodegradation, microalgae, Nitzschia sp., Navicula sp.*

**Introduction.** Plastic pollution is a rampant problem in the Philippines due to improper plastic disposal and the country's lenient implementation of waste management laws. The Philippines is ranked as the 3rd worst plastic polluter of oceans just after China and India, with 1.88 million metric tons of mismanaged plastic waste generated annually [1]. Through the dissemination of mismanaged waste, plastic sheets enter through inland waterways, wastewater outflow, and transportation by the wind or tides [1]. It would take an approximate period of 400-1000 years to breakdown plastics, due to their stability and high durability. These materials tend to accumulate in the environment through time. Plastic bags, which are commonly used for packaging, are typically found afloat rivers, seas, and oceans and compose 60% of marine debris in the ocean [2]. Polymers which constitute plastics are harmless, but as they age in the ocean, they impose a danger to the marine fauna. The marine fauna is greatly impacted by the floating plastics adrift in bodies of water. Ingestion, entanglement to plastics, and exposure to chemicals coming from plastics are some of the commonly documented effects to marine animals.

Marine ecosystems are known to be polluted with significant amounts of plastic debris affecting various habitats including neustonic, pelagic, littoral, and benthic. Upon the exposure of plastic to the marine environment, a biofilm or a layer of organic and inorganic coating will be formed within minutes or hours. The formation of biofilm is mainly composed of a plethora of bacteria, algae, fungi, and protozoa species, and they are collectively called as microbial assemblage, biofouling community, or periphyton [3]. Biofilm formation which leads to biofouling is comprised of four distinct phases; adsorption of organic molecules, attachment of bacterial cells, attachment of unicellular eukaryotes (i.e. microalgae) and attachment of larvae and spores. Bacterial attachment is regulated through the production of extracellular polymers to form structured and complex matrices. These organisms, in which the first colonizers are microalgal diatoms, can influence in the process of degradation [4]. Microbial biofilms can later trigger the adherence of specific invertebrates and algae which increases the degree of biofouling [5].

Polyethylene, the common component of plastic bags, is known to be resistant to degradation [6]. Conventional methods of polyethylene degradation include incineration, landfill, and chemical treatment; however, even these methods pose a danger on the environment [7]. These processes aid in the degradation of polyethylene but cause more pollution in doing so. Without an efficient method of degrading these ethylene polymers, the plastic pollution and carbon footprints would retain, if not increase. Thus, biologically-friendly alternatives of plastic degradation are preferred, which are collectively called biodegradation. Biodegradation is a method of removing of organic pollutants, without any collateral destruction on the flora and fauna present in the same ecosystem [8].

Several research studies about plastic biodegradation utilizing bacteria and fungi have been previously conducted [9,10,11,12]. However, only a few have investigated the possible microalgal species that could cause biodegradation. There are two main mechanisms that would indicate that a microorganism can degrade plastics: (1) formation of biofilms, and (2) degradation of extracellular or intracellular enzymes. The biofilms can be seen in the surface of the plastics, which serves as its substratum, while the enzymatic degradation is suggested by the material's weight loss and changes in functional groups, both of which are exhibited by microalgae [12]. Microorganisms found in the marine environment include microalgae. It was previously reported that *Scenedesmus dimorphus, Anabaena species,* and *Navicula pupula,* the dominant microalgae which have biofilm formations with polyethylene plastic bags that were collected from three different rivers in Channai City, Tamil Nadu, India are potential microbes for biodegradation of polyethylene [7].

This study aimed to determine the microalgal species that are present in plastic samples floating along the stretch of Iloilo estuary (more commonly referred to as Iloilo river). With this, the study aimed to identify microalgal species which thrive in the Iloilo estuary, and determine the most common genus species from the collected plastic samples from the Iloilo estuary.

It specifically aims to:

(i) Determine microalgal species found in the plastic samples collected from Iloilo estuary

(ii) Determine the frequency of microalgae genera found in the plastic bags collected from Iloilo estuary

**Methods.** Twenty floating plastic bags were randomly collected among selected sites of the 11 km Iloilo River, along with water samples. The surface of the plastic samples was scraped, and the scraped material were cultured in two media: Conditioned Medium (CM) and F/2 media. The samples were left to bloom. After two weeks, the samples were serially diluted and identified. The dominant microalgal species were then determined.

*Collection of Samples.* Field sampling was conducted in the map shown in Figure 1 which represents Esplanade 1. Twenty plastic bags found floating along the stretch of Iloilo river were collected. Plastic bags can be found floating at or near the surface, but they can also be found in greater depths. Floating plastic bags were specifically targeted to isolate light dependent and aerobic algae avoiding anaerobic algae found at the bottom of the river. After the samples were collected, they were stored in separate plastic containers. Water samples were taken along with the plastic bags, for preservation. The plastic and water samples from each sampling site were kept inside containers, to preserve the samples.

The samples were transported to the Phycology Laboratory of Southeast Asian Fisheries Development Center - Aquaculture Department (SEAFDEC - AQD) in Tigbauan, Iloilo within 24 hours. and F/2 Media. However, only 28 were cultured further for identification because the remaining 12 samples were left dried. This is due to the personal error of the researchers since the level of medium were not monitored regularly, some of the samples dried up. The drying of samples may be attributed to the the samples being cultured in an outdoor laboratory with shade from direct sunlight.

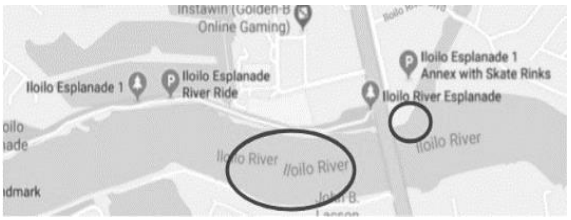


**Figure 1.** Map of Esplanade 1

For every plastic sample, the algal biomass on the surface was scraped off using a sterile blade. The scraped material was placed in each of the two media: Conditioned and F/2 Media. Conditioned Medium (CM) and F/2 media were bought from the Phycology Laboratory of SEAFDEC-AQD beforehand. There were twenty culture bottles containing CM, and another twenty with F/2 Media. Each of the culture bottles were filled with 150 ml of medium at 30 ppt [13]. An outdoor set-up with a fluorescent lighting, and aeration was used. The light source had an intensity 11.4 µmol photons $m^{-2}$ $s^{-1}$, or a 40W fluorescent lamp. The samples had constant aeration for two weeks and exposed to a light source with an intensity, at room temperature.

*Isolation of Microalgae.* Samples from respective CM and F/2 media were dispensed to 15 ml test tubes for serial dilution of $10^{-1}$ to $10^{-10}$. CM and F/2 Medium, depending on the growth medium of the samples, were used for the serial dilution process. An inoculum of 1 ml was added to 9 ml of the respective media, with CM for brown algae and F/2 media for green algae. After each dilution, pipette tips were changed, while the diluted sample were vortexed for 5 to 10 seconds. The same process was conducted until the $10^{th}$ dilution was done. This was conducted for every sample. The 10th and 9th inoculated samples were kept in capped test tubes to avoid contamination, and exposed to 40W fluorescent lamp, continuous lighting at room temperature, 25 C (± 2°C) for a minimum of one week.

*Identification of Microalgae.* Pure algal isolates from the final serial dilution were examined under a compound microscope under the low power objective (10x magnification) and high-power objective (40x magnification). The morphology of the suspected microalgae species was considered using several manuals [14,15,16,17] for the identification of the microalgal isolates. The preliminary identification, based on the image, measurements, and parameters of the microalgal growth, were verified by an expert.

*Safety Procedure.* Laboratory gowns and gloves were always worn when working in the laboratories. Glass wares were always handled carefully, to avoid breakage. An adult supervisor was always present, to guide in the laboratory work. Sampling was conducted with the supervision of an adult, to avoid any untoward incidents. When operating with hot surfaces, oven gloves were used. Spills were immediately wiped clean.

**Results and Discussion.** Out of the initial 40 cultured samples (20 from CM and 20 from F/2 Medium), the scraped of microalgae from the collected plastic samples were cultured in both CM and F/2 Media.

*Identification of Microalgal Isolates.* After three weeks of culture or when there is visible microalgal growth, 10 out of the 15 CM cultures were identified while the rest did not exhibit visible microalgal growth. Similarly, 9 out of the 13 F/2 cultures exhibited visible microalgal growth and were identified. Seven of the eight genera were identified in the F/2 cultures, while CM had cultured samples in each of the genera identified. Only those cultures with visible growth were viewed under the microscope and identified.

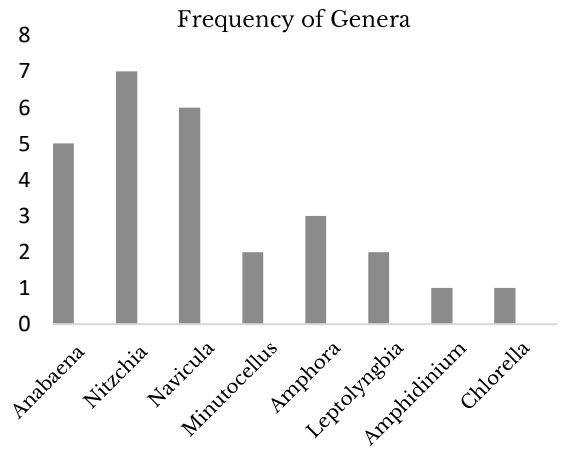


**Figure 2.** Frequency of microalgal species identified in the samples

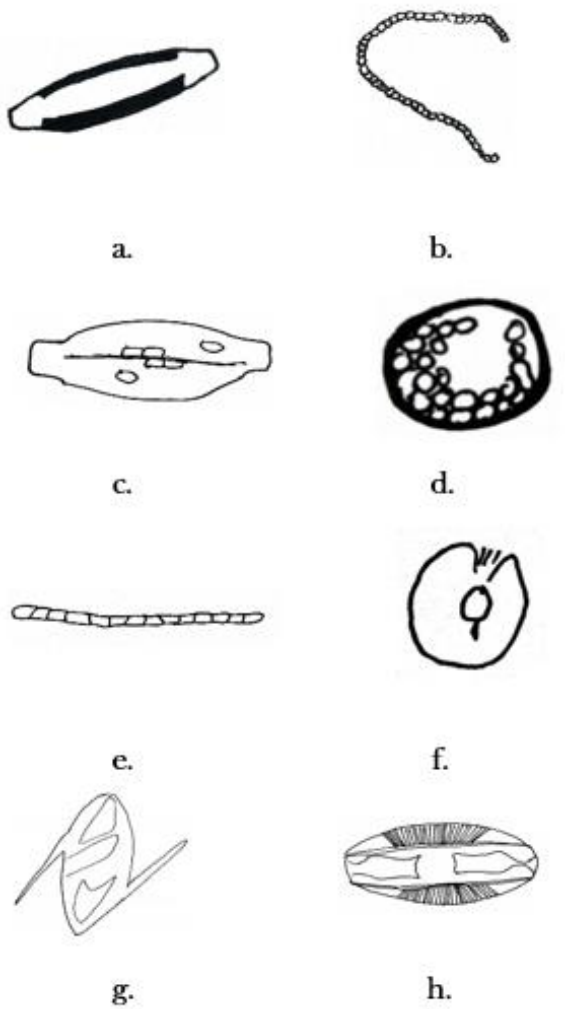


**Figure 3.** Identified microalgae based on their morphological features. A) *Nitzschia sp.* B) Anabaena *sp.* C.) *Navicula sp.* D) *Chlorella sp.* E) *Leptolyngbya sp.* F) *Amphidinium sp.* G) *Minutocellus sp.* H) *Amphora sp*

*Nitzschia sp.* are the most prevalent in all of the samples collected, with it being cultured in seven (7) samples. It was seen both in four samples with CM media and two samples containing F/2 media.

Following *Nitzschia sp.* is *Navicula sp.*, with six (6) samples containing such. Most of the *Navicula sp.* were found in Conditioned media. *Anabaena sp.* were present in five of the samples identified. It was found in three samples with CM media and 3 samples with F/2 media.

*Dominant Microalgae.* *Nitzschia sp.* is considered as a dominant form of microalgae during the early development of biofilms [18]. In addition, diatoms generally have higher growth rates, and cell density, compared to other major groups of microalgae [19]. *Nitzschia sp.* are also known to be the most abundant diatoms in oceans, and considering that the sampling site is an estuary, this is another factor that can be considered [19]. Furthermore, *Nitzschia sp.* has been found out to increase in abundance during the rainy season [20]. The Philippines' rainy season starts in June and lasts until October. This coincides with the study's sampling period, which was held on October 2018. *Nitzschia sp.* are more abundant in periods of lower temperature [21]. The dominance of *Nitzschia sp.,* therefore, can be attributed to the development stage of biofilms, its growth rate, the seasonal variances of the country, and periods of cooler temperatures.

Just like *Nitzschia sp., Navicula sp.'s* dominance also fluctuates due to seasonal variations. In a study conducted by Lohani and colleagues [22], *Navicula sp.* were dominant in Bhimtal Lake, India on the months of January, February, March, April and October. *Navicula sp.* also shows dominance in periods of low temperature, nearing December and January [21].
*Anabaena sp.*, the third most frequently-occurring species is considered as a colony-forming species of cyanobacteria [23]. They are especially prominent in freshwater systems, which can cause algal outbreaks. The sampling site is an estuary, which is a partially coastal body of water, with a river flowing into it. The site is similar to Mankyeong Estuary, Korea, where two strains of *Anabaena sp.* was also isolated [24]. *Anabaena sp.* have optimal growths at temperature between 28-32℃, under optimal nutrient conditions. Apart from the optimal temperature levels, however, it has a tolerance for wide ranges of temperature, salinity, pH, and irradiance [25]. Its adaptive tendencies support the occurrence of this species in the samples obtained. *Anabaena sp.* and *Navicula sp.* have been found to exhibit plastic-degrading capabilities as proven by Kumar et. al [4]. *Anabaena spiroides and Navicula pupula* were two of the three dominant microalgae isolated from polyethylene bags collected from three different sites in India. After using these species in treating low density polyethylene (LDPE) and high density polyethylene (HDPE), it was found that they both exhibit high growth especially in the setup with LDPE. Compared to *Navicula pupula*, *Anabaena spiroides* has shown high mass colonization over the surface of the LDPE sheet and has the highest percent of degradation of LDPE sheets. Scanning electron microscope images show that *Anabaena spiroides* were able to rupture the surface of LDPE sheets [4].

Following *Anabaena sp.* is *Amphora sp.*, which was seen in three of the cultured samples. A study by Eich and colleagues [26] found that Amphora sp., Nitzschia sp. and Navicula sp. were found in biofilm

**Table 1.** Isolated microalgae based on their morphological features

| Genus | Morphology | Culture Media of Isolates |
|---|---|---|
| *Nitzschia spp.* | unicellular, boat shaped (valve view), identical valves, raphe on the edge of cells | CM and F2 Media |
| *Anabaena spp.* | filamentous, unbranched, arranged in chains like a string of beads | CM and F2 Media |
| *Minutocellus spp.* | cells curve in girdle view (less in smaller cells) , growing in short chains, one plastid | CM |
| *Navicula spp.* | unicellular, boat shaped (valve view), with tapering ends, slit-like canal (raphe) running down the center of the cell surface | CM and F2 Media |
| *Amphora spp.* | rounded frustule, rounded caps at the ends | CM and F2 Media |
| *Chlorella spp.* | unicellular, cell outline is ellipsoidal, spherical, contains green chloroplasts | F2 Media |
| *Leptolyngbya spp.* | filamentous, long filaments which can be solitary or coiled into fine mats | F2 Media |
| *Amphidinium spp.* | epicone small, asymmetric, directed to left; crescent shaped in ventral view, button-shaped in lateral view. | F2 Media |

communities of plastic samples that were exposed to the marine environment of the Bay of Fetovaia at the Mediterranean Sea.

*Leptolyngbya sp.* is a genus that can be found in both terrestrial and freshwater environments [27]. *Amphidinium sp.* are commonly the most abundant dinoflagellate species in benthic zones of the bodies of water [28, 29]. However, the samples were taken in the pelagic zone, as they were near or on floating on the surface of the body of water. Since it was only found in one sample, it is possible that was only by chance that it was isolated, and it does not thrive in the Iloilo estuary.

*Chlorella sp.* are most often found in freshwater, however, some there are also marine species and estuarine species that exists. They are at high risk of sudden mortality at water temperatures that are over 30 C [30]. Upon testing the average water temperature during the preliminary sampling done, it was found that the average temperature of the sampling site was 32C. This could explain why *Chlorella sp.* were only found in one sample since they were not able to exhibit high growth because of the slightly higher water temperature from their threshold.

Biofilm formation occurs as soon as plastic surface is exposed to a marine environment. It is the initial step of plastic degradation. The biofilm surface is initially composed of unicellular and multicellular, photosynthetic and heterotrophic inhabitants, which includes cyanobacteria, microalgae, diatoms, bacteria, and fungi [16]. Diatoms are usually the first to colonize surfaces that are exposed to seawater and play an important role in the biofilm formation and overall biochemical activity in the biofilm. This can be related to the data gathered, three diatom species namely, *Navicula sp, Nitzschia sp, Minutocellus sp.* and *Amphora sp,* were the most abundant among the eight microalgae species when grouped according to their morphology.

The formation of biofilms has an order based on the number of days in the suspension of a substrate. Based on a study by Sekar et. al [18] the immersion in a freshwater cooling system is divided into three phases; first phase (1-4 days) dominated by green algae, second phase (5-7 days) by diatom, and third phase (10-15) days by cyanobacteria. This does not necessarily imply about the immersion age of the plastic, but it can be identified at what phase it currently is. The fouling of microorganisms on different surface tensions was more effective on hydrophobic surfaces like polyethylene than glass which is hydrophilic [31]. The order in which the formation of biofilms in the plastic samples that we have collected, however, is not verified because the kind of plastic collected were not verified upon the collection.

Out of the thirty samples, 10 were dominated by cyanobacteria, 18 by diatom, and 1 by green algae. It can be safely assumed that fouling of algae to the plastic surface is faster than the glass under similar immersion conditions because of the more applicable surface tension.

*Microalgae Media.* Condition media (CM) is a term pertaining to culture media in which cells have already been cultivated for some time. It contains a wide variety of organic compounds ranging from simple sugars to high molecular weight substances such as protein and polysaccharides that were secreted widely used general enriched seawater

medium in culturing marine algae used in phycological and aquaculture studies. In this study, the scraped microalgae were cultured in both CM and F/2 media upon the recommendation of Ma'am Annie Franco of SEAFDEC AQD. The purpose of growing them into two different media was to ensure that microalgal growth will occur, and to cover a wider range of microalgal species. Results have shown that some of the identified microalgae the lack of growth or the growth in the different media is depending on the nutrient threshold of the microalgal species and its ability to adapt to its environment. There is no literature which specifically compares the microalgal growth between CM and F/2 media so there is no known difference which can be discussed aside from their different concentrations of nitrate, phosphates, and vitamins. Conwy media was used as a composition of the CM media used in the study. There is no significant difference between the maximum concentration of microalgal genera when cultured in Conwy and F/2 media. This could mean that there is also no difference between the growth of microalgae cultured in CM media and F/2 media.

*Error Analysis.* Data gathered should not be used to represent the thriving microalgae found in the whole of Iloilo river since the sample site chosen was only one station of the river and only represented a small portion. The samples were identified through morphological analysis only thus, the certainty of the identification is until the genus level only. Interaction factors such as contaminants are also a factor which could have affected the cultures since no antibiotics were applied between bacteria and microalgae, could or could not promote growth. The length of the plastic submersion in water was not taken into consideration.

**Conclusion.** The study identified 8 microalgae genera that can be found in the surface of floating plastic bags in the Iloilo estuary. Diatoms under which are *Navicula sp, Nitzschia sp, Minutocellus sp.* and *Amphora sp,* are the frequently occurring group of microalgae having been found in 18 samples. The dominance of the microalgae were determined to be due to various factors, such as temperature, habitat, seasonal variations, salinity, and pH.

**Recommendations.** In this study, microalgal isolates were only identified morphologically. In order to determine the microalgal species accurately, the use of DNA identification is advised for succeeding research studies. Further testing is recommended in order to measure the potential of microalgae in biodegradation through biological treatments containing microalgae species and plastic sheets. In line with that, the tensile strength, total surface area, surface topography, chemical composition and weight loss of microalgal treated plastic are recommended to be studied. This study can be used as a basis for the microalgal species to be used for the biological treatment of plastics.

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