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Rapid detection of microplastic contamination using Nile red fluorescent tagging

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Abstract

This study delves into the evaluation of fluorescent lights staining microscopy and its efficiency in cross validation by comparison with light microscopy. Rapid detection of microplastics of various sizes can be distinguished in assessing coastal marine sediment. A development of a novel approach in rapid detection is employed for analysis of coastal marine sediment microplastic contamination, based on fluorescent tagging using Nile Red (NR), separated by density-based extraction using Zinc Chloride ($ZnCl_2$) and filtration. The fluorescent staining tags onto microplastic to fluorescent, aides with excitation of blue light and color filters. Fluorescence excitation is detected using simple smartphone photography through a polarizer filter. Rapid detection using light microscopy allows fluorescent particles to be identified and counted in image-analysis. The study used a paired sample t-test to compare particle counts across five mesh sizes, revealing minimal too little to no significant differences between fluoresced and suspected MPs particles, indicating a novel detection process with greater selectivity and fluorescence intensity.

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Introduction

The Philippines is an archipelagic country which consists of 7,641 islands and has an extensive and diverse coastline. It boasts as one of the longest coastlines in the world with a measure of approximately 36,000 kilometers (22,370 miles). This coastline encompasses a wide variety of features ranging from pristine beaches to rugged cliffs and rocky shores. These coastal areas play a crucial role in the country's culture, economy, and ecology as they provide habitat to numerous fish species, marine life, and migratory birds. It also serves as hubs for trade, tourism, and agriculture. However, with high level of tourism and recreational activities in the area such as fishing, swimming, sailing, and snorkeling may lead to have larger amount of plastic waste that can pollute and contaminate the marine environment (Chaisanguansuk *et al.*, 2023)

Plastics and other synthetic, non-biodegradable pollutants, which are often referred to as "marine debris," have been contaminating and polluting the world's enclosed seas, coastal waters, and the wider open oceans for the past five or six decades (Gregory, 2009). The hazard posed by plastic waste is significant because it starves and suffocates wildlife, distributes invasive and possibly dangerous species, absorbs toxic chemicals, and breaks down into microplastics that can be ingested (Barnes *et al.*, 2009). These microscopic particles, also known as microplastic which are smaller than 5 mm in size, are present in many different environments. It poses a threat to the ecosystem due to their small size (millimeters or less), it is accessible to a variety of organisms with the ability to cause both physical and toxicological harm (Law and Thompson, 2014). Microplastics can be swallowed by low-trophic feeders, filters, and deposits, as well as by detritivores and plankton-eating organisms. As a result, they can build up inside organisms and cause physical damage, like internal abrasions or blockages. Aside from the physical damage, microplastics can also leach into the environment, where they can cause cancer or endocrine disruption (Wright, 2013).

Even though microplastic contamination affects biota, the environment, and public health significantly, it is a difficult problem to solve since it is so pervasive, and the specific adverse consequences of both long-term and short-term exposure are unknown (Savuca, 2022). Because of the growing worries about the amount of marine plastic waste and the effects it has had on marine ecosystems, marine plastic debris pollution has been identified as a global concern (Mu *et al.*, 2019).

This study serves as baseline studies in microplastic contamination in the coastal environment in sediments of Anda, Northwestern Pangasinan. This study generally aimed to identify the presence of microplastic contamination along the coastlines in sediments of Anda, Northwestern Pangasinan. Specifically, it aimed to detect microplastic contamination using Nile Red fluorescence along the coastlines in sediments of Anda, Northwestern Pangasinan and to assess the efficacy of Nile Red fluorescence in staining for rapid detection of microplastics along the coastline of Anda, Northwestern Pangasinan.

Materials and methods

Sediment sampling

The sediment samples were collected from the three sampling stations namely Tondol Beach (16°19'9.46"N, 120° 1'30.86"E), Tanduyong Island (16°19'39.57"N, 120° 1'51.02"E), and Cory Island (16°18'51.90"N , 120° 0'54.95"E) with three sampling points for each area with 100 meters distance to one another. At each sampling point, three samples of sediments (0-5cm from the sediment surface) weighing 1 kilogram each were collected. Sediments samples were stored in a canister or metal container and labeled properly.

Sediment pretreatment

The sediment samples were rinsed with distilled water before being placed in a glass container. The sediments are then agitated with either a metal spoon or a glass rod and covered with aluminum foil. The samples were stored in the freezer of a Laboratory Biomes RET to allow for drying.

A volume of 100 ml of a 35% H_2O_2 solution was added to the sediment, the solution was mixed with a metal spoon or a glass rod for 1 minute and allowed to sit for 24 hours in a fume hood, covered with aluminum foil. The reaction will degrade organic matter, creating bubbles and foam. When the reaction stops, the sediment needs to be thoroughly washed with distilled water until it has no more bubbles, and then rinsed through a metal sieve with a mesh size of 105 µm and placed in an individual petri dish (Frias *et al.*, 2018).

Microplastic separation

The samples were stored in aluminum trays, and each sample was transferred to individual labelled 1L glass bottles and covered with aluminum foil to allow for decontamination. The sediment and the 1500g of $ZnCl_2$ with 1L of distilled water solution was stirred and mixed with a metal spoon, and the sediment was stored overnight to settle. The aqueous component of the sample was filtered and decanted. The filter paper was placed in the designated petri dish and dried with a closed glass dehydrator. Silica gel may be added to the solution to further improve the drying process. Following the method based on the standardized protocol for monitoring microplastic in sediments with slight modification (Frias *et al.*, 2018).

Microplastic analysis

Microscopic analysis was used to observe the fully dried sample to identify the microplastics. Nile Red fluorescence staining was used for the rapid detection of microplastic as well as the physical characteristics of the microplastics. The samples were examined using polarization and fluorescence in search of microplastics. The samples were observed under the stereomicroscope followed by crossed polarizers in which the polarizing film was placed under the petri dish and the second film will rest on top of the dish. Excitation of Nile Red fluorescence with blue LED blue lights 365*nm* flashlight was used to allow the excitation beam to illuminate the microplastic.

Results and discussion

Geomorphological characteristics of Anda, northwester Pangasinan

In Anda, located at the tip Northwestern Pangasinan, the intertidal flats of Tondol, Cory, and Tanduyong serve as an exceptional and crucial habitat for a diverse range of organisms. The most significant geomorphological feature of Anda is its intertidal flat is its long, shallow nature. With less people residing in the study's area, which suggests that anthropogenic influences on the natural geomorphology may be minimal. Since coastal ecosystems and landforms are less likely to be altered or disrupted, this can help preserve their integrity of the coastal area and its actual multifaceted blend of materials with various dimensions.

Sediment characterization

Sediment compositions are a key parameter in assessing the health of coastal ecosystem, acts as both a substrate and a product of the environment, as it is closely related to the amount of organic matter and nutrients present in the sediments, also a product of the physical and biological processes in the area. A visual estimation of grain size centered on sand classes to differentiate the complex mixture of substances with various dimensions. In general, sediment classification can be divided into broad categories from pebbles (64mm) to very fine sand (0.125mm).

The Table 1 highlights the dry weight of sediments from different collection sites and mesh diameters. Sediment samples was measured in grams (g) and separated into each micron specifically, 2000 µm, 1680 µm, 1000 µm, 400 µm, and 105 µm.

Wentworth scale sediment classification (Wentworth, 1922) on Table 1 shows the dry weight and mesh sizes of sediments ranged from 2000µm to 105µm. The highest average dry weight is measured in 105µm mesh diameter with 678g in Tondol Island, and the lowest dry weight recorded in 2000µm mesh diameter with 5g. Similar with, Tanduyong Island sediment sample significantly shows high in fine sand in 400µm with 317g. The highest average dry weight is observed in Cory Island with 2000µm mesh size recorded with 323g, indicating that sediments with granule particles were predominant in this area.

Table 1. Wentworth scale for grain size analysis

Locations	Size of mesh net per micron				
	2000 µm (g)	1680 µm (g)	1000 µm (g)	400 µm (g)	105 µm(g)
Tondol Beach	5	2	8	20	678
Tanduyong Island	155	101	302	317	41
Cory Island	323	128	201	128	118

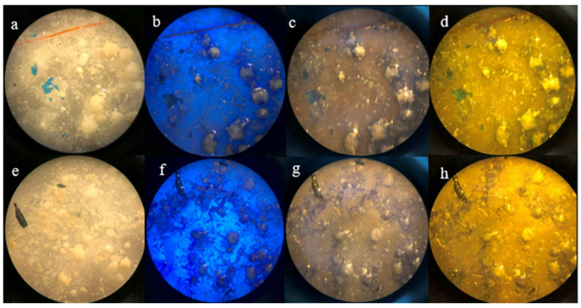

Fig. 1. Microscope image 40x magnification of the processed and stained coastal sediment samples from Tondol Beach: (a,e) normal photo, (b,f) fluorescent image, (c,g) orange filter, (d,h) yellow filter

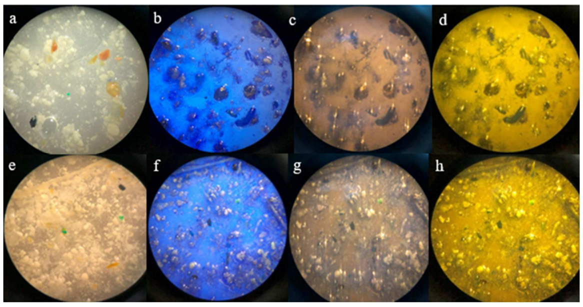

Fig. 2. Microscope image 40x magnification of the processed and stained coastal sediment samples from Tanduyong Island: (a,e) normal photo, (b,f) fluorescent image, (c,g) orange filter, (d,h) yellow filter

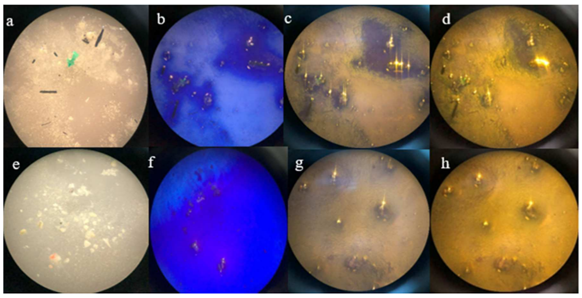

Fig. 3. Microscope image 40x magnification of the processed and stained coastal sediment samples from Cory Island: (a,e) normal photo, (b,f) fluorescent image, (c,g) orange filter, (d,h) yellow filter

Coastal sediment sizes provide insight into the distribution and depiction of wave conditions, highlighting the oceanic processes that mold the shoreline in a certain region. Coastal regions are complex and highly variable environments, longer wavelengths of high-energy input waves typically result in particle classification with a homogeneous, or comparable, variable of grain sizes. Smaller wavelengths of lower energy waves archetypally produce sediments with a more varied, or heterogeneous, classification of grain sizes. Larger sediments are often found on beaches exposed to high-energy waves than those exposed to lower-energy waves (Klemm *et al.*, 1990).

Microplastic rapid detection using Nile red fluorescence imaging

To assess spatial and temporal trends and distribution pattern of microplastics, new approach, standard protocols and cost-effective, capable of efficiently evaluation of microplastic thru fluorescent lights staining microscopy and its efficiency in validation by comparison. A development of a novel approach in rapid detection is employed for analysis of coastal marine sediment microplastic contamination, based on fluorescent tagging using Nile Red (NR), separated by density-based extraction using Zinc Chloride ($ZnCl_2$) and filtration.

Using $ZnCl_2$ as salt solutions density-based (2.90 g/cm^3) extraction method, microplastics had a range of (0.88–1.01 g/cm^{-3}), which is less than the density of sea water, float and can be separated, filtered and analyzed. Microscopic analysis presented of staining spiked particles of in coarse and fine marine sediments are shown in Fig. 1 to 3. The results presented the efficiency and show the new approach on counter staining with Nile-Red and detection through photography as rapid detection is employed for analysis of coastal marine sediment microplastic contamination.

The images were taken and processed using mage J to calculate fragment length from the image regions with the presence of microplastic particles.

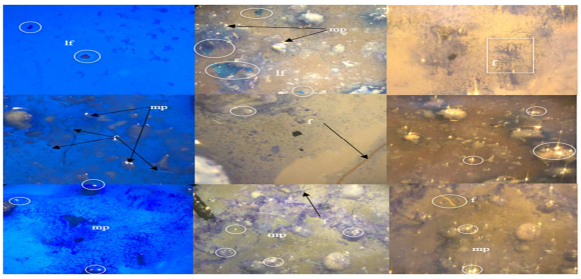

Fig. 4. Fluorescent images 40x magnification of microplastics and natural particles stained with NR (Nile Red) taken with polarized modification of the microscope obtained from Tondol Beach (f.) fiber, (mp.) microplastic, (lf,) large fragments

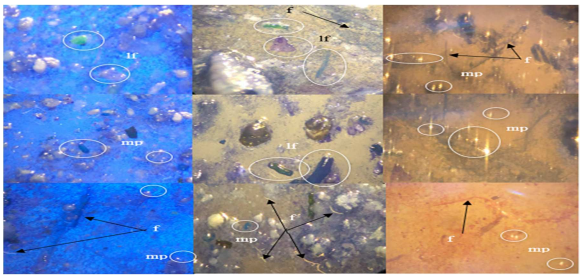

Fig. 5. Fluorescent images 40x magnification of microplastics and natural particles stained with NR (Nile Red) taken with polarized modification of the microscope obtained from Tanduyong Island (f.) fiber, (mp.) microplastic, (lf,) large fragments

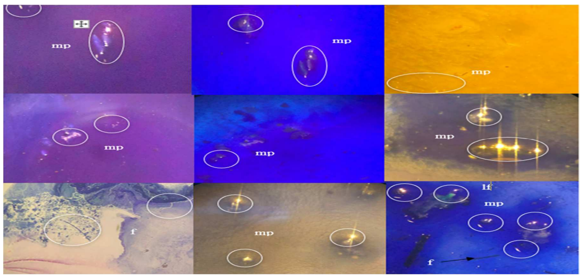

Fig. 6. Fluorescent images 40x magnification of microplastics and natural particles stained with NR (Nile Red) taken with polarized modification of the microscope obtained from Cory Island (f.) fiber, (mp.) microplastic, (lf,) large fragments

These results are displayed against published values for static contact angle measurements for different polymers, where photos with original color seen are inserted for reference. This highlights clear leaning, the relationship for rapid distinction for general detection and categorization of polymer surface polarity and NR fluorescent color.

This approach for individual classification of polymer is still unlikely for rapid detection, but still offers promising with further validation categorization. Cross validation of different polymer fragments of plastics, which need to be identified and counted. Visual sorting and through mesh size differentiation larger than 2000µm is still the most common method utilized for classification of microplastics.

Using the blue light excitation 365*nm* result the light image highlights particles on the Nile-Red surface, while polarized fluorescence image with filter reveals that the sample contain a few large fluorescent fragments.

Due to high sensitivity and salvotochromic range of Nile-Red and in addition to visual quantification, NR microplastic determination also subject for risk of false positive (Vianello *et al.*, 2013). To fully utilize the potential application of NR for microplastic detection and quantification, recent studies suggested that the application of applied chemical and physical characterization (Hidalgo-ruz *et al.*, 2012) vibrational spectroscopy (Tagg *et al.*, 2012) or electron microscopy (Fries *et al.*, 2013) that limit the margin of error or misidentification,

In addition, the integration of using a automating IR-microscopy procedures for microplastic identification and Fourier transform infrared (FTIR) to measure and identify the fragment polymer down to smaller µm (Löder *et al.*, 2015). Significant use of spectroscopic and mass spectrometry techniques for the identification and quantification of microplastics (Xu *et al.*, 2019). Recent advancement in monitoring and quantification have been utilized to assess spatial and temporal trends and distribution pattern of microplastics, new approach, standard protocols and cost-effective, capable of efficiently evaluation of microplastic.

Fluorescent images aide with excitation of blue light (365*nm*) of sediment samples after counter stained with Nile-Red (NR) and with new polarized image with yellow and orange filter are cross validated.

Table 2. Detection of microplastics from sediment samples by direct counting of Nile Red (NR) stained fragments.

Location	Mesh size	Microplastics		
		Particle that fluorescent under Nile red	Suspected MPs (Particles that only fluorescence with Nile red)	*p-value*
Tondol Beach	2000 µm	5	4	
	1680 µm	2	1	
	1000 µm	2	2	0.1778
	400 µm	2	2	
	105 µm	4	4	
Tanduyong Island	2000 µm	2	2	
	1680 µm	7	6	
	1000 µm	5	4	0.0341
	400 µm	6	5	
	105 µm	9	7	
Cory Island	2000 µm	2	2	
	1680 µm	2	2	
	1000 µm	5	5	0.3739
	400 µm	7	7	
	105 µm	3	2	

Significantly, the pretreatment of the H_2O_2 to dissolve organic matter and $ZnCl_2$ as salt solutions density-based (2.90 g/cm^3) extraction method, is visualized in Fig. 4 to 6. The results highlight the fluorescent images to differentiate and rapid detection of microplastics with new approach, standard protocols and cost-effective, capable of efficient evaluation.

The variation of different polymers in a single particle also added with different fluorescence white image intensity even within single particles poses an additional challenge, this causes inconsistent fluorescence emission and adds to the difficulty of determining appropriate thresholds (Nel *et al.*, 2021). Presence of fiber, large fragments of microplastics were recorded in all station for sediment samples were collected.

Efficacy of Nile red fluorescent tagging

Number of particles that fluoresced with Nile Red at 40x magnification across five mesh sizes and Suspected MPs (particles that only fluoresce with Nile Red) with two sets of polarized filter area respectively is presented in Table 2.

Significance was calculated using a paired sample t-test across five mesh sizes at 40x magnification, in which the mean particle counts across microns was compared for particles that fluoresced with Nile Red, and suspected particles that fluoresced with Nile Red. The paired samples T test calculated shows least to no significant differences between particle that fluorescent under Nile Red and suspected MPs particles that only fluoresce with Nile Red. These prove that the detection process, novel approach, this results in greater selectivity in microplastic staining and higher fluorescence intensity.

References

Barnes DK, Galgani F, Thompson RC, Barlaz M. 2009. Accumulation and fragmentation of plastic debris in global environments. Philosophical Transactions of the Royal Society B: Biological Sciences **364** (1526), 1985-1998.

Chaisanguansuk P, Ploenbuppa S, Assawincharoenkij T, Phantuwongraj S, Jiraphinyakul A. 2023. Microplastic Contamination in the Coastal Environment: A Case Study from the Mae Klong River, Samut Songkhram. Applied Environmental Study **45** (2). https://doi.org/10.35762/AER.2023009

Frias J, Pagter E, Nash R, O'Connor I, Carretero O, Filgueiras A, Gerdts G. 2018. Standardised protocol for monitoring microplastics in sediments. Deliverable 4.2.

Fries E, Dekiff JH, Willmeyer J, Nuelle M T, Ebert M, Remy D. 2013. Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environmental Science: Processes & Impacts **15**(10), 1949-1956.

Gregory MR. 2009. Environmental implications of plastic debris in marine settings—entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. Philosophical Transactions of the Royal Society B: Biological Sciences **364**(1526), 2013-2025. https://doi.org/10.1098/rstb.2008.0265

Hidalgo-Ruz V, Gutow L, Thompson RC, Thiel M. 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. Environmental Science & Technology **46**(6), 3060-3075. https://doi.org/10.1021/es2031505

Law KL, Thompson RC. 2014. Microplastics in the seas. Science **345**(6193), 144-145.

Klemm EB, Pottenger FM, Speitel TW, Reed SA, Coopersmith FA. 1990. The Fluid Earth. Honolulu. Curriculum Study & Development Group. https://manoa.hawaii.edu/exploringourfluidearth/physical/coastalinteractions/beaches-and-sand.

Löder MG, Gerdts G. 2015. Methodology used for the detection and identification of microplastics—a critical appraisal. Marine Anthropogenic Litter, 201-227. https://doi.org/10.1007/978-3-319-16510-3_8

Mu J, Qu L, Jin F, Zhang S, Fang C, Ma X, Wang J. 2019. Abundance and distribution of microplastics in the surface sediments from the northern Bering and Chukchi Seas. Environmental Pollution **245**, 122-130. https://doi.org/10.1016/j.envpol.2018.10.097

Nel HA, Chetwynd AJ, Kelleher L, Lynch I, Mansfield I, Margenat H, Onoja S, Goldberg Oppenheimer P, Sambrook Smith GH, Krause S. 2021. Detection limits are central to improve reporting standards when using Nile Red for microplastic quantification. Chemosphere **263**, 127953.

Savuca A, Nicoara MN, Faggio C. 2022. Comprehensive review regarding the profile of the microplastic pollution in the coastal area of the Black Sea. Sustainability **14** (21), 14376.

Tagg AS, Sapp M, Harrison JP, Ojeda JJ. 2015. Identification and quantification of microplastics in wastewater using focal plane array-based reflectance micro-FT-IR imaging. Analytical Chemistry **87**(12), 6032-6040.

Vianello A, Boldrin A, Guerriero P, Moschino, V, Rella R, Sturaro A, Da Ros L. 2013. Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence, spatial patterns and identification. Estuarine, Coastal and Shelf Science **130**, 54-61.

Wentworth CK. 1922. A scale of grade and class terms for clastic sediments. The Journal of Geology **30**(5), 377-392.

Wright SL, Thompson RC, Galloway TS. 2013. The physical impacts of microplastics on marine organisms: A review. Environmental Pollution **178**, 483-492. https://doi.org/10.1016/j.envpol.2013.02.031

Xu JL, Thomas KV, Luo Z, Gowen AA. 2019. FTIR and Raman imaging for microplastics analysis: State of the art, challenges and prospects. TrAC Trends in Analytical Chemistry **119**, 115.