

Combining Fluorescence Microscope and FTIR to Investigate Microplastics in Sand at Vung Tau beaches

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Abstract

Microplastic pollution (plastic debris < 5 mm) has become a global environmental problem due to the accumulation of plastic debris in water bodies over the decades. The present study was conducted to determine the abundance, shape, color, size, and composition of microplastics in the sand collected at recreational beaches in Vung Tau city of Vietnam. Ten sand samples were collected, five from Front Beach and five from Back Beach. These samples were extracted and analyzed for microplastics with a dissecting microscope, Nile red staining and the Fourier Transform Infrared (FTIR) technique. Results showed that microplastics were present in all study areas, with concentrations ranging from 5 to 27 MPs/100 g of dry sand. Colored and fibers dominated in both beaches (70-80%), of which fibers shorter than 0.5 mm were commonly found. Many detected microplastics were black and blue with a similar percentage, 32.55 % and 34.66 %. The predominant microplastics were polyethylene (PE) and polypropylene (PP). The study proved the efficacy of Nile Red with FTIR in quantifying microplastics in the sand samples. The results alert the wide spreading of plastic debris along the beaches, which poses many risks to human entertainment activities and local ecosystems.

Keywords: Microplastics (MPs); Nile Red; FTIR; sand; beach; Vung Tau; Vietnam.

1. Introduction

Plastic is a commonly used material on the market due to its durability and lightweight. However, the increased usage of plastic products and improper waste management will lead to the discharge of a huge amount of plastic debris into the environment, especially the marine ecosystems (Auta *et al.*, 2017; Lebreton *et al.*, 2018). The annual global plastic production exceeded 320 million tons, of which 40% are single-use plastics (Waring *et al.*, 2018). Plastic particles were first found in the sea in 1972, and their number has rapidly increased due to disposing of plastic waste worldwide (Mai *et al.*, 2018). Microplastics (MPs) are tiny pieces

(smaller than 5 mm) and are classified into primary and secondary. Primary microplastics come from plastic materials in the industry and enter the environment through wastewater treatment plants and industrial drainage systems (Napper *et al.*, 2015). Secondary microplastics result from the breakdown of larger pieces of plastics, commonly due to long-term exposure to solar ultraviolet radiation (photodegradation or mechanical breakdown) (Andrady, 2011). Currently, microplastics have been found at beaches, saltwater (Khuyen *et al.*, 2021a), sands, and freshwater bodies in many parts of the world and Vietnam (Khuyen, 2022). In addition,

tiny microplastics are mistaken for food and consumed by aquatic organisms, including economically valuable fish and invertebrates, affecting the ecosystem and human health (Khuyen, 2022). The problems of microplastics in the environment have attracted many scientists due to their long-term accumulation, especially in the beach sand - where many tourism and fishing activities are gathered.

Infrared spectroscopy is considered the most reliable method to determine the chemical composition of microplastics (Veerasingam *et al.*, 2020). There have been several studies on microplastic pollution in sandy beaches, and most of them applied Fourier-transform infrared spectroscopy with the support of a dissecting microscope, for example, the study in beaches of Dubai UAE (Aslam *et al.*, 2020), Belgium (Claessens *et al.*, 2011), Bohai Sea (Yu *et al.*, 2016), Hong Kong (Lo *et al.*, 2018). The fluorescent technique is selected as a rapid detection for microplastics; however, this method is usually used as a single method rather than in combination with chemical spectroscopy. Different dyes such as Rose Bengal (Kosuth *et al.*, 2018), Oil red EGN, Eosin B, Rose Bengal, Hostasol Yellow 3G, and Nile Red (Khuyen, 2022) have been stained on the suspected pieces and plastic-like particles. Among these, Nile Red was chosen due to its high adsorption and fluorescence intensity when contacting with polymer in all organic solvents, commonly acetone, dimethyl sulfoxide (DMSO) 5 to 25 %, ethylene glycol (EG) 0.1 to 0.125 mg/ml at a temperature range of 30 to 40 °C (Alemán-Nava *et al.*, 2016). Nile Red is a sensitive method for screening tiny microplastics, up to 20 µm on black PC filter paper from beach sand (Shim *et al.*, 2016), and surface water (Erni-Cassola *et al.*, 2017). One recent research in Mumbai Tuticorin Dhanushkodi (India) Tiwari *et al.* (2019) replaced the stereomicroscope with a fluorescent microscope to identify microplastics accumulating at 3 to 4 centimeters of sand layers. With this combined method, popular plastic types could be detected, including PE, PET, PS, PP, PVC, and other unimportant types.

Microplastics is currently an emerging pollutant in Vietnam and the studies on its accumulation in the sand are still not many.

These studies used different analytical methods for microplastics distributed in famous areas of Vietnam. In Vietnam, microplastics were determined in sand at Đa Nang beaches with Raman microscope (Tran *et al.*, 2020), Tien Giang beaches with FTIR (To *et al.*, 2020), Can Gio beaches with Raman (Khuyen *et al.*, 2021a) and FTIR (Nhon *et al.*, 2022). Vung Tau city is located in the southern economic region of Vietnam and is one of the most popular entertainment places in the country. Since the late 70s, the rapid industrial and urban development of Vung Tau has grown tremendously in the oil and gas industries, tourism, sand mining, et cetera. Moreover, the white sand beach stretching along the coastal regions in Vung Tau City has attracted not only local people and national tourists but also international visitors in recreational activities and food consumption on the beaches. Tourism services have brought huge profits for the locals, but the daily use of plastic containers and utensils has contributed to plastic pollution along the beaches. The most crucial step in monitoring pollution is the determination of the parameter of interest. Despite many efforts and long-time research, the profile of microplastic accumulation on Vietnamese beaches has not been well established. There were only two studies on microplastic accumulation in Vung Tau - in seawater and salt with Raman microscope (Khuyen *et al.*, 2021b), and in sand (To *et al.*, 2020). This study tried to develop an optimal procedure of using simultaneous Nile Red and FT-IR to observe the morphology and call the polymer type of plastic debris extracted from the sand of two famous tourism beaches (Front Beach and Back Beach in Vung Tau). The results provide helpful information to propose strategies for protecting the coastal beaches of Vietnam.

2. Materials and methods

2.1 Study area and sampling strategies

Sand samples were collected at the Front Beach (from 10°19'41.00" north latitude 107°5'17.35" east longitude to 10°20'33.52" north latitude 107°5'44.26" east longitude) and the Back Beach (from 10°20'39.5" north latitude 107°04'26.4" east longitude to

10°21'10.91" north latitude 107°03'48.44" east longitude) (Figure 1). The samples were taken at high-tide currents, where debris was strongly washed away. Then, five positions were randomly selected on the Front and five on the Back beach of Vung Tau Beach (Figure 1) at the other tidal lines and standard plots. The large debris in the sand was removed, and 2 kg of the sand on top of 2 – 3 cm layers was taken with a shovel or stainless steel spoon. Samples were labeled and stored in sealed plastic bags at room temperature.

2.2 Microplastic extraction

Sand samples were transported directly to the laboratory for microplastic extraction. Firstly, the samples were dried for 24 hours at 90 °C (Cocca et al., 2018) and sieved through a 0.5 mm sieve to remove large pieces. Next, a saturated saline solution was prepared by adding 73 g of NaCl (Sigma-Aldrich, ReagentPlus®, ≥ 99%, USA) to 200 mL of distilled water. The solution was stirred well at room temperature, then filtered and stored until usage. The prepared saline solution was poured gradually into 100 g of the dried sand sample. The mixture was stirred for 5-10 minutes, and allowed to settle for 15-20 minutes. A 0.5 mm filter was used to filter out all solids, which were then dried at 90 °C for 24 hours.

Wet oxidation that combined 20 mL of 0.05 M Fe(II) and 20 mL of 30% H₂O₂ was applied to remove organic substances in the sample. The mixture was heated to 75 °C, prior to the floatation with saturated sodium chloride. The whole process was repeated several times until no organic matter remained observed. Finally, the samples were filtered with the support of a vacuum pump and all the debris including potential microplastics was deposited on the filter papers (cellulose acetate 0.45 μm, 47mm diameter, Whatman, UK) which were stored in glass Petri dishes at the room temperature.

2.3 Microplastic analysis

The materials on the filter paper were stained with 1 mL Nile Red (C₂₀H₁₈N₂O₂) of 5 μg/mL in acetone 95 %. The filter paper was examined under an Olympus BX53 fluorescence microscope at 20X magnification, at three wavelength ranges: DAPI (430 – 470 nm), FITC (515 – 560 nm) và SPO (650 – 670 nm) as described in previous publications (Anh et al., 2021; Khuyen, 2022).

The chemical composition of suspected microplastics was determined with a FTIR. The machine used a single beam percentage transmission technique that performs four



Figure 1. Sampling map at Vung Tau beach, Vietnam

scans per sample at a resolution of 4.0 cm⁻¹ and a wavelength range of 4000 – 500 cm⁻¹. The FTIR spectrum was compared with a reference database to confirm the plastic type.

These two methods can be used separately as a single method to determine the microplastics. However, the results will not describe comprehensively the properties of microplastics. In particular, FTIR only gives the spectra to identify the polymer type. Therefore, after FTIR recording, the samples were stained and screened under the fluorescence microscope.

2.4 Statistical analyses

Microplastic abundance in the sand samples was expressed as the number of microplastics per unit of dry sand (MPs/100g dry sand or MPs/kg dry sand). The collected data during the experiment were descriptive statistics using Microsoft Excel 2016 software, OriginPro 8.5.1, and Sigmaplot 14.0.

3. Results and Discussion

3.1 Microplastic abundance

Microplastics were found in all sampling locations with varied concentrations, which was explained by the differences in human activity. The average concentration at the Front Beach was 16 ± 8 MPs/100 g and 12 ± 7 MPs/100 g of dry sand at the Back Beach (Table 1). Compared with other overseas beaches, the amount of microplastics in Vung Tau beaches were higher than in Portugal beaches (10 MPs/kg sand) (Frias *et al.*, 2016), but still lower than in many beaches such as Belgium (97.2 MPs/kg dry sand) (Claessens *et al.*, 2011), German Baltic beaches (14 to 532 MPs/kg dry sands) (Stolte *et al.*, 2015), and Slovenia beaches (up to 177,8 MPs/dry sand) (Laglbauer *et al.*, 2014).

The study proves that microplastics were commodiously distributed on Vung Tau beaches, which was also reported in the previous study by To *et al.* (2020). The research group of To *et al.* also investigated the microplastics at the Back Beach, but used only stereo-microscope and FTIT-ATR. As a result, 0 to 295 microplastics were found in 200 g of analyzed sand, which is relevant to 147.5 g in 100 g of sand (2020). This finding was higher than our results, dated 2022. As the sampling time is different, it is challenging to compare the two studies, if Nile Red can replace the stereomicroscope completely in the visual step of the microplastic analysis procedure. In theory, Nile Red is a susceptible and precise method to distinguish very tiny microplastics (up to 20 µm) (Shim *et al.*, 2016, Erni-Cassola *et al.*, 2017) from non-plastic micro-particles if the organic matter, especially lipid and micro-organisms like microalgae (Alemán-Nava *et al.*, 2016), phytoplankton (Natumen *et al.*, 2015) are completely removed from the sample matrix. In practice, our study shows that the number of particles, especially black particles observed under the stereomicroscope was sometimes higher than the counting results under the fluorescence microscope. Then, the suspected microplastics were indeed plastics based on their IR spectra. Therefore, the Nile Red method is an effective scanning method for microplastic detection.

In other words, the microplastics in Vung Tau beaches which are among the top popular beaches for tourists in Southern Vietnam were surprisingly more minor than those a recreational beach of a UNESCO Biosphere Reserve in Vietnam (Can Gio), which varied from 31.99 to 92.56 MPs/g sand (Khuyen *et al.*, 2021). Microplastics were believed to be discharged from activities of the tourists, such as swimming, using plastic utensils on the beach (Khuyen *et al.*, 2021; Khuyen, 2022). The frequency of

Table 1. Microplastic amount in the sand at Vung Tau beaches

Location	Max (MPs/100 g)	Min (MPs/100 g)	Mean ± SD (MPs/100 g)
Front Beach (n = 5)	27	6	16 ± 8
Back Beach (n = 5)	24	5	12 ± 7

tourists at Can Gio beach is always lower than at Vung Tau beaches. However, the microplastic pollution at these beaches does not positively correlate with the number of tourists. This is because plastic wastes are broken down into secondary microplastics. However under the action of environmental forces such as ocean currents, tides, and human activities on the beach, microplastics are moved and spread widely on the beach and under different sand layers. Moreover, the analytical method is different, particularly the limit of microplastic size detection, which leads to the difference in microplastic abundance reported in the studies. The findings draw a conclusion that the inter-study comparison of microplastic distribution is based on not only sampling factors (time, weather factors etc.) and pollution sources but also analytical methods especially limit of detection.

3.2 The morphological properties of microplastics

3.2.1 Shape

The characterization of microplastics (shape, colour and size) is usually used to

determine the origin, chemical composition, and decomposition status of plastic debris in the natural environment (Botterell *et al.*, 2018). The pieces were firstly observed under the dissecting microscope, and plastic-like items were stained with Nile Red. The fluorescence were more clearly seen at 515 – 560 nm wavelengths, and their size was determined. The shapes of microplastics at Vung Tau beaches are described in Figure 2.

The abundance of microplastics classified by shapes was converted into percentage in Front and Back Beach and drawn in Figure 3. Fibers were the predominant shape of all microplastics found in the sand of two beaches, followed by particles and fragments. In particular, no fragments were found in two locations at the Front Beach, and only fibrous microplastics were encountered in two locations at the Back Beach (Figure 4). Fiber was the most easily and observed shape in dissecting and fluorescent microscopes. This form was also the most popular (51 %) at Mumbai Tuticorin Dhanushkodi (India) beaches, followed by granules (40 %) (Tiwari *et al.*, 2019), at Dubai (63.9 %), followed by fragments (20.5 %) (Aslam *et al.*, 2020).

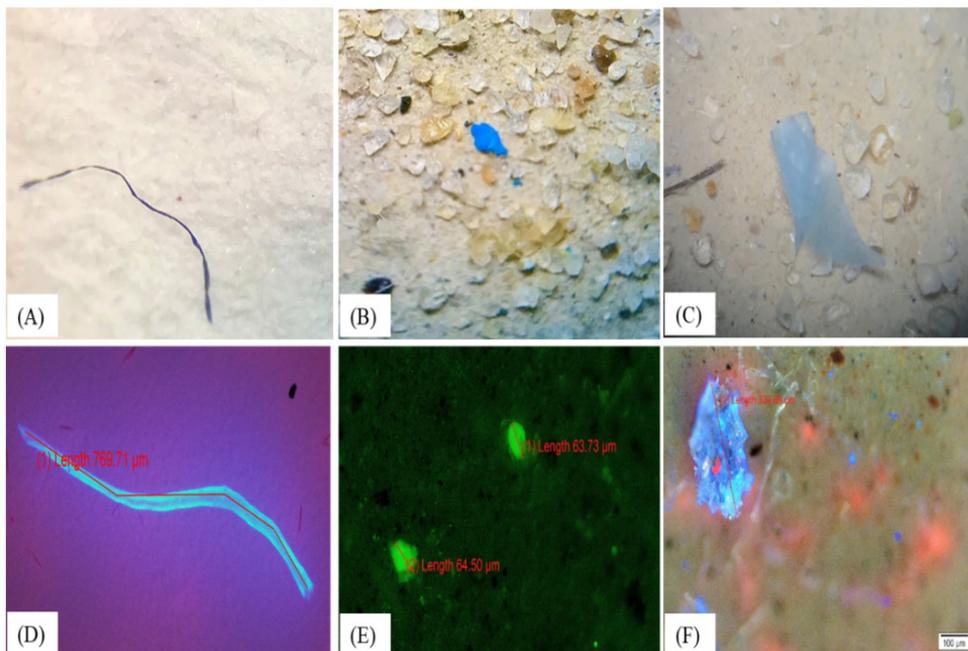


Figure 2. The image of microplastics on sandy beach under stereomicroscope (A, B, C), and fluorescence microscopy (D, E, F) (scale bar: 100 μm for all images)

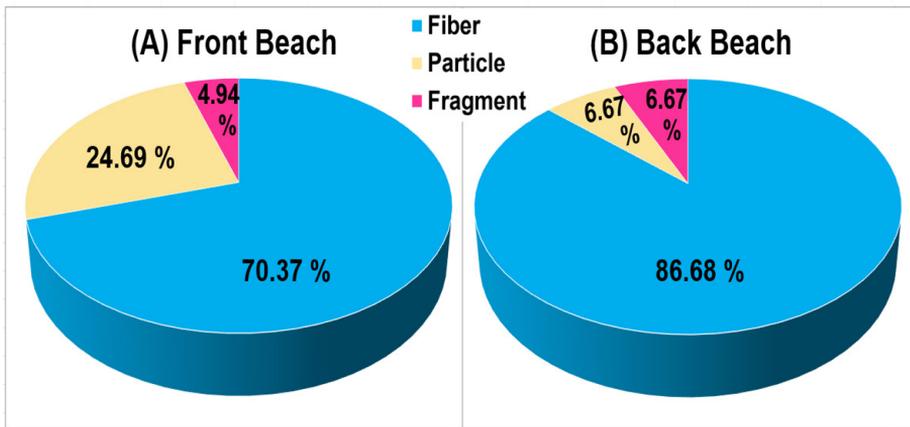


Figure 3. Microplastic distribution by shape at the Front Beach (A) and Back Beach (B)

The high abundance of fibrous microplastics in the sandy beaches can be attributed to clothing waste (Browne *et al.*, 2011; Henry *et al.*, 2019) and high population densities (Browne *et al.*, 2011). Along with the global trend, microfibers dominated in Vung Tau beach sands, which is similar to the reports of beaches in Belgium (Cauwenberghe *et al.*, 2013), France (Bosker *et al.*, 2018), Germany (Stolte *et al.*, 2015; Hengstmann *et al.*, 2018), Iran (Naji *et al.*, 2016), Mexico (Pinon-Colin *et al.*, 2018), Poland (Graca *et al.*, 2017), USA (Yu *et al.*, 2018), Da Nang – Vietnam (Tran *et al.*, 2020), and Tien Giang – Vietnam (To *et al.*, 2020).

3.2.2 Color

The real colour could not be recognized under the fluorescence microscope, thereby a dissecting microscope was used instead. The colour was proven to be a valuable tool to predict the origin of microplastics in the environment (Gallagher *et al.*, 2016). The variation in colour showed that microplastics came from different sources of emissions (Li *et al.*, 2019). Black, red, blue, yellow, purple and white colours were seen in sand samples. As seen in Figure 5, blue microplastics accounted for the highest percentage (38.95 %) at the Front Beach while black dominated at the Back Beach (33.93 % of the total microplastics). In comparison, purple microplastics accounted for the lowest proportion at Front Beach (1.30 %) and Back Beach (12.5 %). Yellow and white were not

detected in all samples at the Back Beach. The detailed comparisons of microplastics' color between the 5 locations of the two beaches are illustrated in Figure 6.

There were more fishing activities and docking boats at the Front Beach, therefore, more plastic pieces were discharged from fishing nets and fishing lines. The Back Beach is only for tourists' activities, so it is predicted that white fibers were not from the fishing net. Vung Tau City is a commercial, entertainment, and tourist city, and a huge amount of plastic waste is disposed of daily, which results in a variety of colors.

Similar to the study of Lots *et al.* (2017), blue/black was the predominant colour that accounted for 78 to 92 %, while red microfibers were not popular (about 5 to 12 %) at two French beaches. In the beach sands of Da Nang - Vietnam, blue (59.9 %) and white (22.9 %) were the most common colours of microfibers (Tran *et al.*, 2020). Besides, blue fragments have also been found in beach sands around the world (Stolte *et al.*, 2015; Graca *et al.*, 2017).

3.2.3 Size

Most of the plastic pieces separated in the samples were tiny microplastics (smaller than 0.5 mm) (Figure 7). The detailed comparisons of microplastics' color between the 5 locations of the two beaches is illustrated in Figure 8. The secondary microplastics are caused by the fragmentation of larger plastic items in the environment and accumulation in coastal

sands globally (Barnes et al., 2009). Abrasion is the main factor causing plastic fragmentation (Arthur et al., 2009). However, the primary mechanism considered for photodegradation is driven by sunlight. The breaking of chemical bonds results from the oxidation of polymeric

structures caused by direct exposure to the sun's ultraviolet radiation (Barnes et al., 2009). In addition, plastic debris is susceptible to fragmentation due to a combination of mechanical forces, including wave action and disturbance (Barnes et al., 2009).

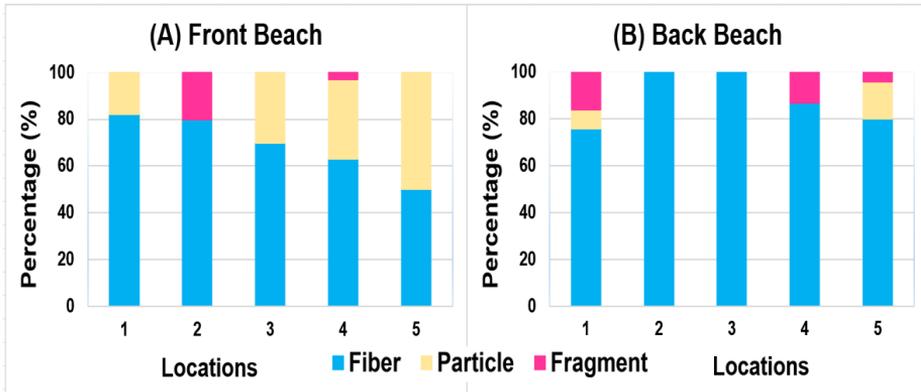


Figure 4. Microplastic distribution by shape at every location at the Front Beach (A) and Back Beach (B)

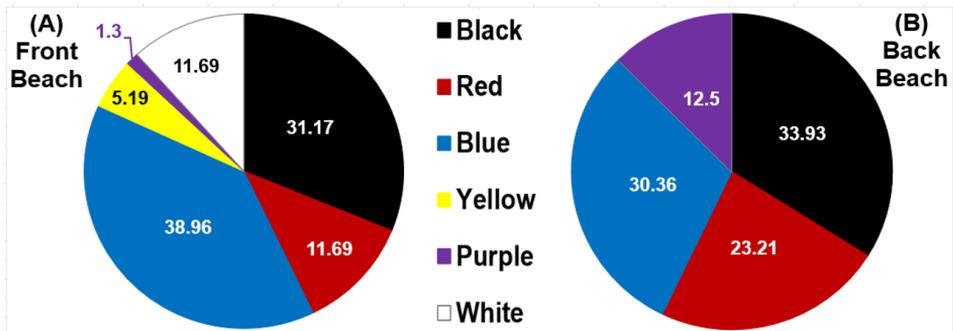


Figure 5. Microplastic distribution by colour at the Front Beach (A) and Back Beach (B)

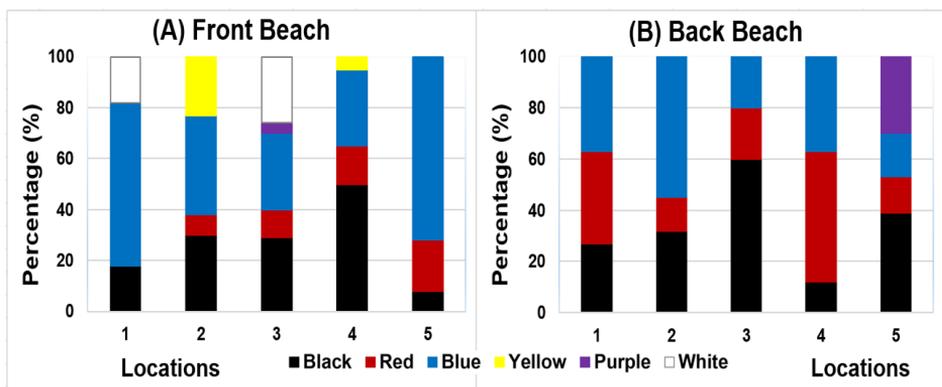


Figure 6. Microplastic distribution by colour at every location at the Front Beach (A) and Back Beach (B)

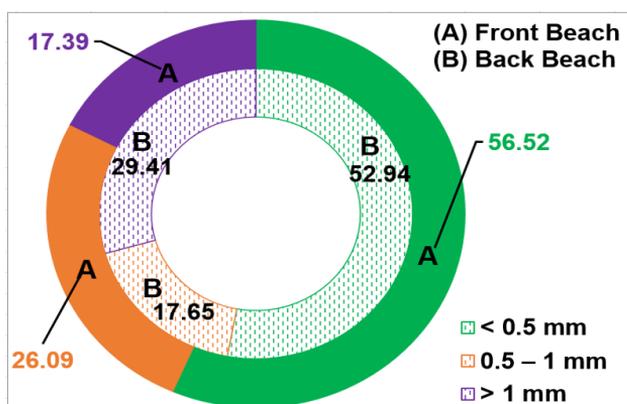


Figure 7. Microplastic distribution (%) by size at the Front Beach (A) and Back Beach (B)

The size of microplastics was categorized into three classes < 0.5 mm, 0.5 – 1 mm, and > 1 mm. Among them, microplastics smaller than 0.5 mm were dominant on both beaches (56.52 % at the Front Beach) and (52.94 % on the Back Beach). Due to the impact of natural factors such as water currents, and temperature, plastic tends to be split into small pieces. Specifically, the ratio of microplastics to size at each sampling location is shown in Figure 7. Tiny microplastics predominated, except for location 2 (at the Front Beach) and position 3 (at the Back Beach).

In practice, microplastics look like food in size and shape and, thereby, are easily consumed by various organisms by chance (Auta *et al.*, 2017), and act as toxic agents in ecosystems (Klein *et al.*, 2015). According to Rochman *et al.* (2013), small-sized from 2 – 5 mm microplastics might take a long time to pass the organism's stomach and be trapped in the digestive system, and cause an increase in the contact time with the absorbed toxins. Meanwhile, microplastics smaller than 0.5 mm were dominant at both Vung Tau beaches, which is reasonable to assume that microplastics in the study area carry a severe risk to the beach ecosystem.

3.3 The chemical determination of microplastics

Some clear-shape particles on the filter paper were observed under the stereomicroscope. The analysis was then processed with fluorescence and FTIR. Fluorescence technique could distinguish the microplastics from stone and salt particles.

Without the fluorescence screening, the image could be seen under a stereomicroscope, but a melting test should be used additionally. In other words, organic matter which was remained after sample treatment would be able to give fluorescence signals (Khuyen, 2022). In these cases, the pieces gathering around the fluorescence positions were then examined with a spectrometer. They were finally concluded plastics or non-plastics based on their IR spectra. Therefore, combination of Nile Red fluorescence with IR spectroscopy was able to provide a full set of information of microplastics including abundance, shape, colour, and size of microplastics, and plastic composition, which is essential to determine the origin of plastic debris.

FTIR has been considered a reliable method to determine microplastic composition without altering sample characteristics (Veerasingam *et al.*, 2020). The analysis results show that PE (in black particles) and PP (in blue fibers) were predominant at both Vung Tau beaches (Figure 9). The polyethylene gave long and strong peaks at 2820 – 2900 cm^{-1} , which were assigned to aliphatic C-H bonds of $-\text{CH}_3$ (Khuyen *et al.*, 2022). Moreover, polypropylene has the four highest peaks in the range of 2830 – 2970 cm^{-1} , and two other peaks in the range of 1400 – 1480 cm^{-1} (Atas, 2019). Environmental conditions such as UV radiation, water, oxygen, and temperature can affect the spectrophotometric results, and therefore, there would be a slight difference from the standard spectra in the references.

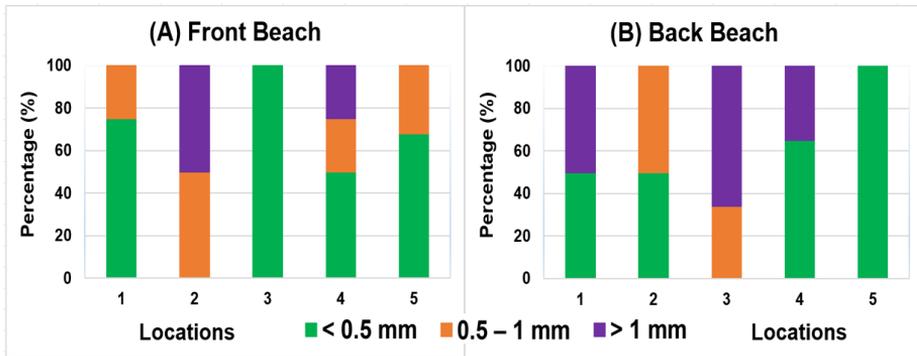


Figure 8. Microplastic distribution by size at every location at the Front Beach (A) and Back Beach (B)

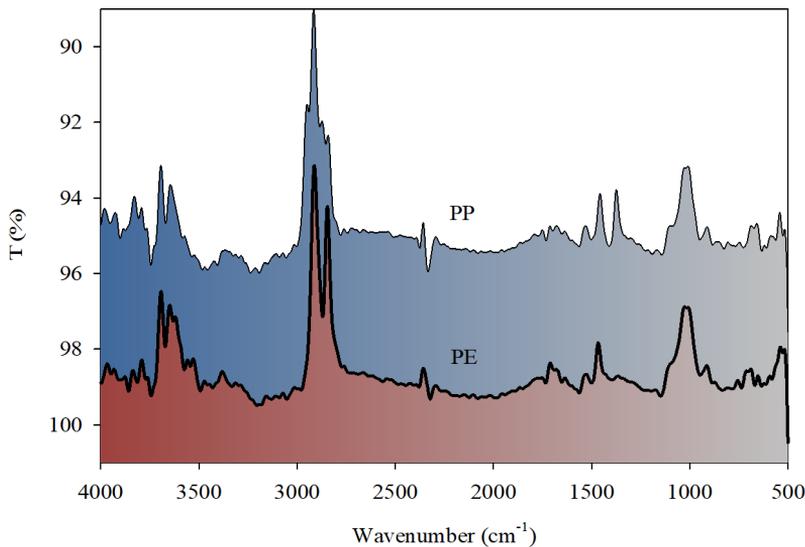


Figure 9. FTIR spectra of PE and PP microplastics separated from sand samples in Vung Tau beaches

The findings of this study are similar to most other research results. Polyethylene and polypropylene are usually predominant types in beach sands in Hong Kong (Lo *et al.*, 2018), northern France (Doyen *et al.*, 2019), Mumbai Tuticorin Dhanushkodi (Tiwari *et al.*, 2019), Dubai coast (Aslam *et al.*, 2020), Tien Giang – Vietnam (To *et al.*, 2020). The reason for the predominance of PE and PP in sands on beaches is the decomposition of plastic bags from the littering activities of locals and tourists.

4. Conclusion

This study was conducted to determine the abundance, morphological and chemical characteristics of microplastics in the sand at

two popular beaches in Vung Tau, Vietnam. Nile Red method is a supplement to a conventional FTIR to give a firmly reliable result on microplastics characteristics. In other words, it can be an alternative to FTIR, especially for pieces < 5 μm that cannot be detected exactly with a conventional FTIR (without microscope). The results show that the microplastic abundance ranged from 5 to 27 MPs/100g of dry sand taken at Vung Tau beaches. Furthermore, fiber was the dominant conformation in all collected microplastics. More than 50% of the microplastics obtained were black and blue smaller than 0.5 mm. In addition, the results of FTIR analysis identified PE and PP were the two most abundant resins in the sand samples at the Front and Back beaches. Different factors

such as population density, aquacultural activities of the local people, and recreation of the tourists have made the microplastics distribute widely at an uncontrollable degree in the study area. Thus, local authorities need to hold educational programs for the residents and tourists to reduce plastic pollution in this area, expand investment in infrastructure to improve the waste collection service and management. The on-time actions and programs will not only solve the problem of plastic pollution in the area but also protect marine and beach ecosystems and eventually increase the quality of human life.

Conflicts of interest

The authors declare no conflict of interest.

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