Application of fluorescent microscopy and Fourier-transform infrared spectroscopy for analysis of microplastics in Can Gio seawater

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Abstract

Microplastics (MPs) pollution has become a global challenge due to their persistent properties and harm to nature and human health. Observation of MPs pollution, especially in marine environments, is a critical concern in environmental analysis. In this work, we validate the method for detecting the presence of MPs in seawater by using fluorescence microscopy with Nile Red dye combined with Attenuated Total Reflectance Fourier-transform infrared spectroscopy (ATC-FTIR). The specificity and accuracy of this method were evaluated by analyzing the standard spiked samples. Five standard MPs and chitin samples used in this study were polyamide (PA), polyethylene (PE), polyethylene terephthalate (PET), poly(methyl methacrylate) (PMMA), and polyvinyl chloride (PVC) with a diameter from 5 to 300µm. The obtained recovery was 88.3-96.7%, with the related standard deviation (RSD%) in the range of 4.6-11.0%. This method was applied to determine microplastics in 27 seawater samples at three locations in Can Gio Sea, Ho Chi Minh City. The results showed the pollution level of each type of MP was significantly varied, with PE and PET being the highest, while there was almost no PMMA in seawater samples.

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1 Introduction

Plastics are synthetic polymers used as the primary raw material for manufacturers of various household and industrial products. These products are present in almost every aspect of life because of their low cost, lightweight, flexibility, strength, and corrosion resistance [1]. Products made from plastic are increasingly used worldwide. It has been estimated that more than 320 million tons of plastic are used in a year. Plastic waste causes a tremendous source of microplastic pollution, especially in the marine environment, and has become a global problem [2]. Plastic debris found in the marine environment is broken into pieces by physical impact and biochemical digestion under environmental conditions. Microplastics (MP) are defined as particles with a size of less than 5 mm and are classified into two main subgroups: prominent MP (size 1–5 mm) and small MP (size 0.1–1 mm) [3]. MPs in seawater are derived from land-based and industrial activities at sea (e.g., domestic waste, tourism, sea transportation, and fisheries) [4, 5]. It is estimated that over 500 billion plastic objects, which is equivalent to about 250,000 tons of plastic waste, are discarded into the ocean [6]. MP in seawater cause a

direct impact on the marine ecosystem. They can be consumed by different marine species, including corals, plankton, invertebrates, and fish, which are eventually moved along the food chain [7]. Due to their large surface area, MPs readily absorb hydrophobic environmental pollutants such as pesticides, grease, and other organic toxins. Therefore, in addition to direct effects, MPs are also known to have indirect effects as a carrier of marine pollutants on biological populations [8]. Many studies that evaluate MPs pollution in the marine environment and organisms have been published [9-11]. These studies used analytical techniques for the qualitative and quantitative determination of MPs such as stereo-microscopy [6, 12, 13], surface scanning electron microscopy (SEM) [14], fluorescence microscopy [15-17], X-ray spectroscopy (EDS) [18], Attenuated Total Reflectance – Fourier transform infrared spectroscopy (ATR-FTIR) [19, 20], and Raman spectroscopy [21, 22]. Despite the success of these methods, the accuracy of sample handling and analysis procedures in these studies is limited. Therefore, this study aims to validate the specificity, recovery, and repeatability of the MPs analysis method in seawater by utilizing fluorescence microscopy combined with ATR-FTIR on standard MPs samples. The method was then applied to investigate the level of seawater pollution at some locations in Can Gio Sea, Ho Chi Minh City.

2 Materials and methods

2.1 Materials and reagents

All standard MPs powder were purchased from Goodfellow: low-density polyethylene (PE) with a particle size of smaller than 300 microns; polyamide – nylon 6 (PA) with an average particle size from 15 – 20 microns; polyethylene terephthalate (PET) with a particle size less than 300 microns; polyvinyl chloride (PVC) with a particle size less than 250 microns; polymethylmethacrylate (PMMA) with an average particle size of 85 microns. Chitin from shrimp and shellfish (Poly-(1→4)-β-N-acetyl-D-glucosamine) and Nile red fluorescent dye were supplied by Sigma-Aldrich. Acetone (>99%) from Scharlau. H_2O_2 30%, H_2SO_4 98%, and $FeSO_4$ (99.5 – 102%) were purchased from Merck. The water used in the analysis is ultrapure water with a resistance >18.3 MΩ·cm. The image of microplastic particles was analyzed on an Olympus BX53 fluorescence microscope (USA). Identification of microplastics by Fourier Thermo Scientific™ Nicolet™ iS50 FTIR Spectrometer (USA).

2.2 Sample collection

Seawater samples were collected in the Can Gio Sea at three locations. The coordinates of Location 1 at 10°22'07.8"N and 106°53'25.5"E; Location 2 at 10°22'09.8"N and 106°56'33.2"E, Location 3 at 10°24'43.8"N and 106°58'49.1"E. Nine seawater samples (1L per sample) for 9 replicates analyses in each location were taken, then stored in a glass bottle at 4 oC for further analysis. In addition, one glass bottle containing 1L deionized water (opening) was placed at each seawater sampling site during the sampling collection as a field control sample.

2.3 Sample preparation

Filter 1 L of seawater sample through a 5 mm stainless steel sieve to remove solid residues with a size larger than 1 mm. The obtained solution was continuously filtered through a cellulose acetate filter with 47 mm diameter and 0.45 µm pore size [23]. The solids remained on the filter paper were rinsed several times with 50 mL of distilled water, and the mixture was recovered into a 250 mL beaker. The mixture was allowed to evaporate at 75 °C until 10 mL were obtained. The organic compounds in the mixture were digested by 40 mL of Fenton reagent (containing 20 mL of 30% H2O2 and 20 mL of 0.05 M FeSO4) at 75 °C for 30 min [24]. The treated mixture was cooled and made up to the 100 mL mark.

Fluorescent staining of the MPs in the sample was performed by adding 1 mL of Nile Red (5 mg/L), and the solution was stored in the dark for 30 mins [15]. The solution was filtered through a glass fiber membrane filter (GF/C code number: 1822-047, diameter 47 mm, filter size 1.2 µm), rinsed the beaker three times with 50 mL of distilled water, and then transfer all MPs to the filter surface. The filter was carefully transferred to a glass Petri dish, covered with a lid then dried at 105 °C for 4 h. MPs on the surface of glass filter membranes were identified under fluorescence microscopy at an excitation wavelength of 515 nm, with emission wavelength of 563 nm [15], followed by direct ATR-FTIR identification.

2.4 Method verification

The specificity, recovery, and repeatability of the method were evaluated based on MPs standard samples. Standard samples were formed by spiking certain MPs to a blank sample (seawater without MPs).

Blank samples contained seawater that were pre-filtered through a 0.45-micron filter membrane with the support of a vacuum filtration system. The images and FTIR spectral of MPs in spike samples were carried out to verify the specificity of the analytical method. Method recovery was calculated from the number of particles counted by fluorescence microscopy images. The recovery for all types of MPs was the ratio between MPs number and the spiked number. The relative standard deviation (RSD%) of six replicates at each concentration level showed repeatability for different types of standard MPs.

The results of the actual sample analysis were calculated and evaluated for statistical homogeneity by analysis of variance (ANOVA) and multiple compared by mean by Tukey method (Post-hoc test) at 95% confidence level (p-value = 0.05), error bars used in MPs composition were the standard error of nine replicates.

3 Results and discussion

3.1 The specificity of the method

The characteristics of MPs were observed by fluorescence microscopy at the excitation wavelength range of 515 and emission wavelength of 563 nm. The infrared spectroscopy experiment to identify standard MPs was conducted after fluorescence imaging with wavenumbers from 4000 cm^{-1} to 500 cm^{-1}. The obtained results are shown in Figure 1.

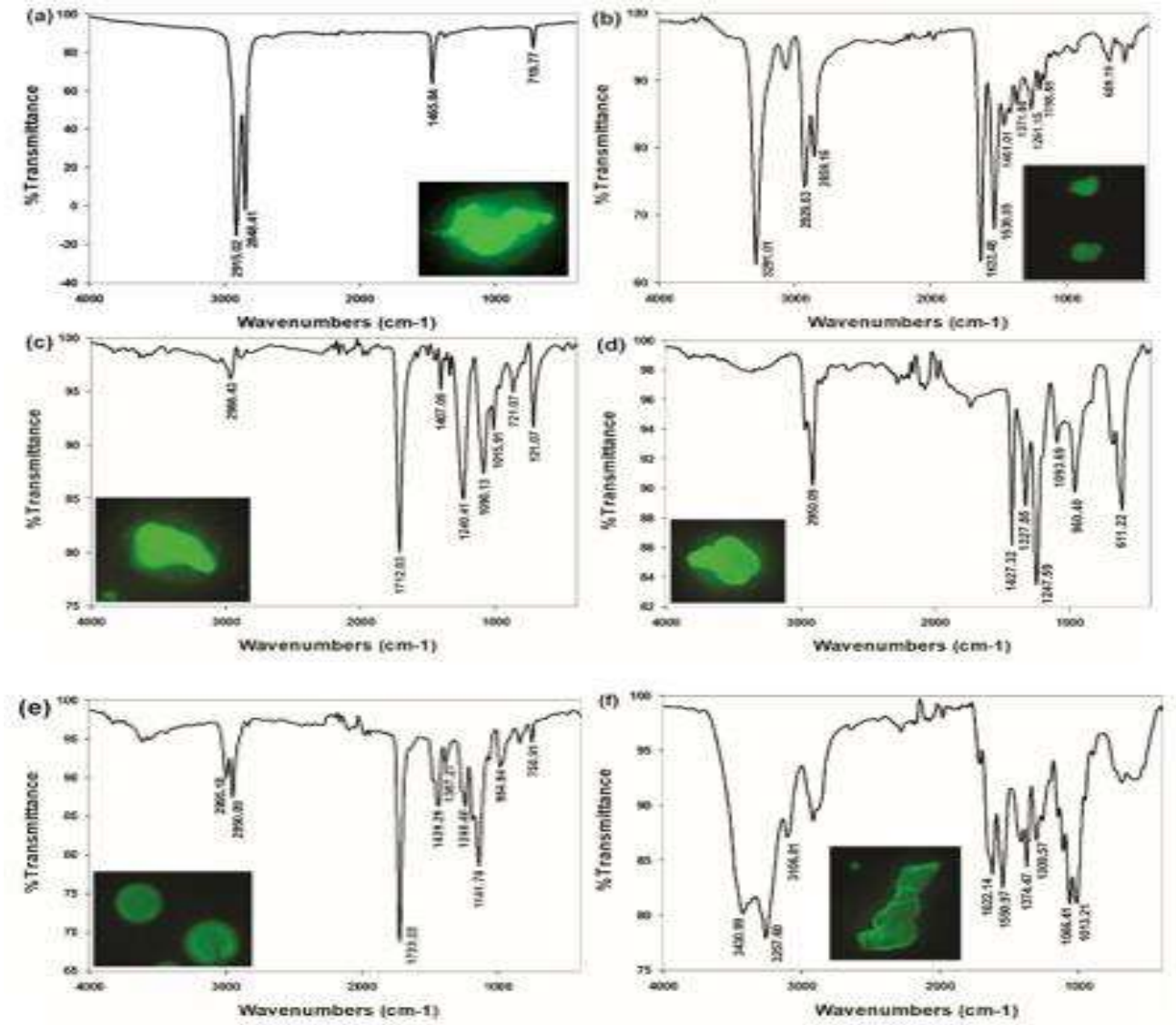

Fig. 1 Fluorescent microscopy images and FTIR spectral of standard microplastics in measurement specificity: (a) polyethylene; (b) polyamides; (c) polyethylene terephthalate, (d) polyvinyl chloride, (e) poly(methyl methacrylate), and (f) chitin.

All the images of MPs obtained from fluorescence microscopy were green, with a clear boundary line of the shapes. These proved that fluorescence microscopy with Nile red dye gives high reliability when quantifying by imaging. The obtained FTIR spectral with the specific vibration wavenumbers of each type of microplastics. The value of the wavenumbers for each MP is showed in Table 1.

Table 1 FTIR spectral of microplastics

Microplastics type	Absorption bands (cm^{-1})	Assignment
LDPE	2915.02	C – H stretching
	2848.41	C – H stretching
	1465.84	CH_2 bending
	719.77	CH_2 rocking
PA	3291.01	N – H stretching
	2929.63	C – H stretching
	2859.16	C – H stretching
	1633.48	C = O stretching
	1536.05	N – H bending
	1461.01	CH_2 bending
	1371.86	CH_2 bending
	1261.15	N – H bending
	1198.85	CH_2 bending
	689.19	N – H bending
PET	1712.03	C = O stretching
	1240.41	C = O stretching
	1090.13	C = O stretching
	721.07	Aromatic CH bending
PVC	1427.32	CH_2 bending
	1327.85	CH_2 bending
	1247.59	CH_2 bending
	1093.69	C – C stretching
	960.40	CH_2 rocking
	611.22	C – Cl stretching
PMMA	2995.18	C – H stretching
	2950.09	C – H stretching
	1723.29	C = O stretching
	1439.29	CH_2 bending
	1387.37	CH_3 bending
	1240.40	C – O stretching
	1141.78	C – O stretching
	984.84	CH_3 rocking
	750.91	CH_2 rocking
Chitin	3259.19	N – H stretching
	3105.46	N – H stretching
	2919.28	CH_3 stretching
	1622.76	Amide C = O stretching
	1549.22	N – H bending
	1414.86	CH_2 bending
	1375.62	CH bending
	1306.33	CH_2 wagging
	1065.59	C – O – C stretching
	1015.47	C – O stretching

The results of IR spectroscopy to identify MPs are entirely consistent with the structure of standard plastics [25, 26], and are compatible with previously published works [27, 28]. The results prove that the ATR-FTIR method can be used to identify the composition of standard plastics used in this work and can also be used for other types of MPs according to their FTIR spectral. The analysis of natural polymers in seawater such as chitin gives a clear fluorescence image with a difference in transparency compared to synthetic polymers (Figure 1f). These results confirm the specificity and reality of analyzing MPs in seawater by fluorescence microscopy with Nile red dye combined with ATR-FTIR.

3.2 The method accuracy

The recovery was calculated to evaluate the loss of MPs particles during the process of sample preparation. The number of each standard MPs type was spiked at three levels of 3, 5, and 10 particles in 1 L of blank seawater sample. These samples were analyzed by the procedure described in section 2.2. The obtained recovery was ranged from 88.3-96.7%. The repeatability of the analysis with six repetitive experiments had a relative standard deviation (RSD%) in the range of 4.6-11.0%. Although specific regulation on technical requirements for microplastic analysis remains lacking, the obtained parameters of the method in terms of specificity and accuracy confirm the high accuracy of the analytical method.

3.3 Determination of MPs in seawater samples

The validated method was applied to analyze twenty-seven seawater samples collected at three positions on Can Gio Sea. The results are shown in Table 2.

Table 2 The method application to analysis Can Gio seawater*

Type MPs	Position 1	Position 2	Position 3	Average
LDPE	5.0 ± 1.2 (31.6%)	3.3 ± 0.9 (22.8%)	3.8 ± 0.7 (34.2%)	4.0 ± 0.6 (28.4%)
PA	1.9 ± 0.5 (12%)	2.0 ± 0.8 (13.8%)	2.0 ± 0.8 (18.0%)	2.0 ± 0.4 (14.2%)
PET	2.9 ± 0.7 (18.4%)	5.4 ± 1.3 (37.2%)	2.2 ± 0.7 (18.8%)	3.5 ± 0.6 (24.8%)
PVC	1.8 ± 0.7 (11.4%)	1.4 ± 0.5 (9.7%)	0.4 ± 0.2 (3.6%)	1.2 ± 0.3 (8.5%)

PMMA	0.3 ± 0.2 (1.9%)	0.1 ± 0.1 (0.7%)	0.1 ± 0.1 (0.9%)	0.2 ± 0.1 (1.4%)
PS	1.9 ± 0.8 (12.0%)	0.9 ± 0.3 (6.2%)	0.5 ± 0.3 (4.5%)	1.1 ± 0.3 (7.8%)
PP	2.0 ± 0.4 (12/7%)	1.5 ± 0.4 (10.3%)	2.1 ± 0.7 (18.9%)	2.1 ± 0.3 (14.9%)
Total MPs	15.8 ± 4.4	14.5 ± 4.3	11.9 ± 3.7	14.1 ± 2.5

*The concentration of MPs was (particles ± std. error) /L with the correspondence percentage (%).

The analysis results showed that two types of MPs, PE and PET, contained the highest components with an average of 3.3 – 5.0 MPs/L for PE and 2.2 – 5.4 MPs/L for PET with the average percentage were 28.4% and 24.8%, respectively. The four types of plastics with medium concentration were PA, PVC, PS and PP, with an average of 0.5 – 2.1 MPs/L (7.8 – 14.9%). PMMA resin had the smallest attention and was rarely found in seawater samples (1.4%). These results are consistent with the main plastic components used in the actual products. The average total concentration of MPs was 11.4 MPs/L (Table 1).

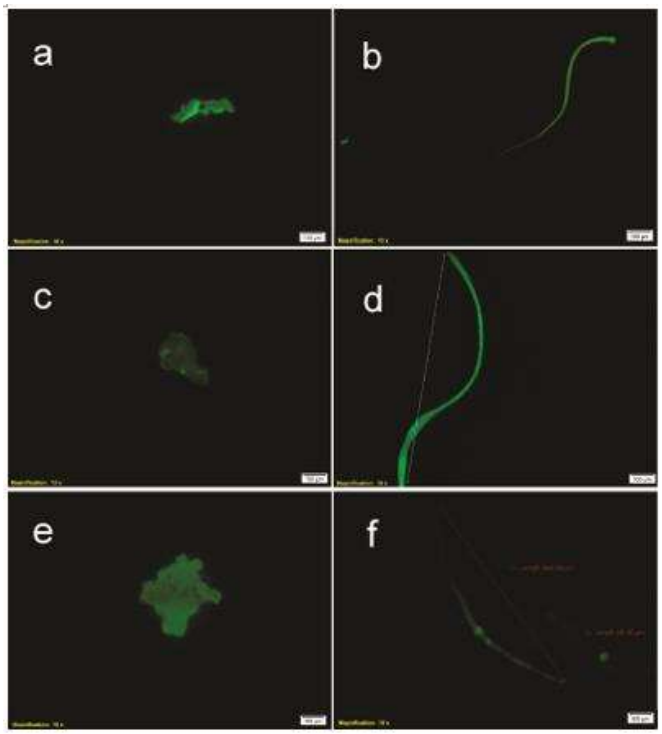

Fig. 2 Fluorescent microscopy images of microplastics in seawater: (a) PE fragment, (b) PA fiber, (c) PP fragment, (d) PET fiber, (e) PVC fragment, and (f) PMMA fiber

On the morphological side, fibers and fragments were the dominant shapes of MPs, accounting for 35.8 % and 25.4% because of their low density, which is difficult to settle and easily transported via waterways. Other fiber sizes ranging from 26 – 640 µm may derive from textile or fishing nets, commonly produced from PA, PP, PET and PP, indicating that the origin of nylon fibers is from cables used in fishery activities [29, 30]. Some MPs fibers and fragments were found in seawater as presented in Figure 2.

Using ANOVA combined with Tukey's Honest Significant Difference, the concentration of PE, PA, PVC and PMMA at three different positions was statistically consistent at the 95% confidence level (α=0.05). Particularly, the composition of PET plastic has a difference between Position 2 and Position 3 (P-value = 0.05). Because the sampling locations are located not far apart, leading to a high homogeneity in the composition of resins in the seawater sample.

4 Conclusion

An analytical method has been evaluated for analyzing MP particles in seawater samples by fluorescence microscopy combined with infrared spectroscopy. After dying with Nile red, MPs produced clear and distinguishable images compared to natural polymers such as chitin. The method recovery at 3, 5, and 10 MPs/L for all standard resins used in the study ranged from 88.3-96.7%. The repeatability with a relative standard deviation of six experiments was from 4.6-11.0%. The method was applied to analyse 27 water samples at three different locations in the Can Gio Sea. The MP sample was significantly contaminated, with the highest concentrations for PE and PET, while the PMMA resin content is shallow and almost absent in the analysed samples. The method of fluorescence microscopy combined with infrared spectroscopy allows the analysis of MPs in seawater easily and accurately. It can be applied in practice to control the MP pollution in seawater or can be developed for MPs analysis in other matrices.

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Ứng dụng phương pháp kính hiển vi huỳnh quang và quang phổ hồng ngoại biến đổi Fourier phân tích ô nhiễm vi nhựa trong nước biển Cần Giờ

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Tóm tắt Ô nhiễm hạt vi nhựa (MPs) trở thành một thách thức toàn cầu do tính chất khó phân hủy, khả năng gây hại cho sinh vật và sức khỏe con người. Đánh giá mức độ ô nhiễm MPs, đặc biệt là ô nhiễm trong môi trường biển được quan tâm trong phân tích môi trường. Trong nghiên cứu này, chúng tôi tiến hành thẩm định phương pháp phân tích MP trong nước biển bằng kính hiển vi huỳnh quang với thuốc nhuộm Nile Red kết hợp với quang phổ hồng ngoại biến đổi Fourier. Độ đặc hiệu và độ chính xác của phương pháp được đánh giá bằng cách phân tích trên các mẫu hạt vi nhựa chuẩn. Kính hiển vi huỳnh quang cho phép xác định số lượng hạt vi nhưa trong khi FTIR dùng để định tính thành phần của MPs. Năm loại MP tiêu chuẩn được sử dụng trong nghiên cứu này là polyamit (PA), polyetylen (PE), polyetylen terephthalate (PET), poly(metyl metacryit) (PMMA) và polyvinyl clorua (PVC) với đường kính từ 5 đến 300 µm. Độ thu hồi của phương phương pháp định lượng có giá trị từ 88,3-96,7 %, với độ lệch chuẩn tương đối (RSD%) trong khoảng 4,6-11,0 %. Phương pháp này được áp dụng để xác định hạt vi nhựa trong 27 mẫu nước biển tại 3 địa điểm thuộc biển Cần Giờ, Thành phố Hồ Chí Minh. Kết quả cho thấy mức độ ô nhiễm của từng loại là đáng kể, trong đó PE và PET chiếm ưu thế và hầu như trong các mẫu nước biển không chứa hạt vi nhựa loại PMMA.

Từ khóa Vi nhựa, Ô nhiễm môi trường biển, Biển Cần Giờ, Nile Red, FTIR