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Digesting the Indigestible: Microplastic Extraction From Prawn Digestive Tracts

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Microplastics (MPs) have become ubiquitous in the marine environment, and are likely ingested by a broad cross-section of marine life. The extent to which marine organisms ingest MPs is uncertain due to limitations in analytical methods. Effective identification and analysis of ingested MPs is a precursor to understand their impact on marine organisms and their human consumers. This is particularly challenging for crustaceans, due to the chitin present in their exoskeleton and digestive systems, which is resistant to chemical degradation. This study presents a novel application that can efficiently break down the stable organic tissue of banana prawns (*Penaeus merguiensis*), and subsequently isolate putative MP polymers from the digestive tract without damaging their integrity. Five treatments were examined for their capacity to break down chitin from the prawn digestive system; namely acid, alkaline, oxidant, enzyme and microwave assisted oxidant digestion. Gravimetric and image analysis revealed that the organic tissue of the prawn gastrointestinal tract can be effectively removed by acid, oxidant, and microwave assisted oxidant digestion methods. However, testing on seven reference polymers (polyamide (PA), polyethylene (PE), polyester (PES), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and rayon) revealed significant degradation when exposed to acid digestion. Overall, microwave assisted oxidant digestion achieved the best recovery rate of spiked MPs (> 90%) with minimal size, shape, and Fourier transform infrared (FTIR) spectral changes for all polymers except for rayon. These results highlight a new direction for tissue removal and MP extraction in crustacean ingestion studies.

Keywords: crustacean, plastic, microwave ovens, seafood, chitin

1 INTRODUCTION

Microplastics (MPs) are a heterogenous mixture of plastic polymers with a size less than 5 mm (Dawson et al., 2021), which have become ubiquitous in the marine environment due to extensive plastic pollution (Thompson et al., 2004). Presence of these particles in marine organisms raises increasing concerns over animal welfare and food safety, given that seafood compromises over 17% of animal protein consumption by humans globally (Murray and Cowie, 2011; FAO, 2018). Therefore, it is of extreme importance that the risk seafood presents to consumers, in terms of exposure and health effects, is accurately quantified. To do this comprehensively, not only should MP contamination be quantified across the breadth of globally consumed organisms, but the MP

FIGURE 1 | Prawn anatomy. **(A)** the digestive tract diagram of a penaeid prawn, adapted from Dall (1968), the orange shaded area is the digestive tract being preserved in all samples (A—anus, D—digestive gland or hepatopancreas, M—mouth, MG—midgut, O—oesophagus, P—proventriculus or foregut, R—rectum). **(B)** the remaining gut lining of the N. norvegicus after the application of the digestion protocol using potassium hydroxide (KOH) at 40°C (Hara et al., 2020).

concentration in both the edible and discarded tissues should be quantified separately, so that the risk of human exposure to MP contaminated seafood can be accurately assessed (Dawson et al., 2021). At this stage, little is known about MPs in large decapod crustaceans, such as prawns and crabs (de Sá et al., 2018), in part, due to a lack of efficient protocols to extract ingested MPs from the structurally sophisticated digestive tract of decapods (Enders et al., 2017).

The digestive tract of decapods is composed of the oesophagus, foregut (also termed proventriculus), digestive gland (also termed hepatopancreas or midgut gland), the midgut, and the hindgut (rectum, anus) (**Figure 1A**) (Felgenhauer, 1992; Hill et al., 2004), where all regions except the midgut and digestive gland are coated by complexes of chitin and protein (Ceccaldi, 1989; Felgenhauer, 1992). Since chitin is resistant to degradation by many chemicals including water, organic solvents, mild acidic or basic solutions (Roy et al., 2017), its presence within the digestive tract of decapods has previously been suggested to inhibit the extraction of MP from organic tissue (Cole et al., 2014). For example, potassium hydroxide (KOH) is a commonly used solution which effectively dissolves the organic tissue of most taxa for the extraction of MP (Dawson et al., 2020). However, when applied to decapod intestinal tracts, KOH has been shown to be ineffective, with intact gut linings visible amongst the filter retentate (**Figure 1B**) (Hara et al., 2020). Thus, there is scope to explore suitable methodologies to extract MP from the digestive tracts of large crustaceans.

Conventional MP extraction methodologies that could remove chitin should be considered in this particular scenario. Conventional treatments usually involve dissection alone or in combination with chemical (acids, oxidative, and alkaline reagents) or biological (enzyme) digestion (Thompson et al., 2004; Choy and Drazen, 2013; Lusher et al., 2017; Miller et al., 2017). Direct dissection (Welden and Cowie, 2016; Wójcik-Fudalewska et al., 2016) or acid digestion (Desforges et al., 2015; Devriese et al., 2015) have been effectively shown to remove chitin, allowing MP extraction. However, dissection without a tissue digestion step may miss MP, and acids are known to degrade some polymers. Further, specific chitinase enzymes are also available for chitin digestion (Roy et al., 2017). Microwave assisted digestion is used extensively in
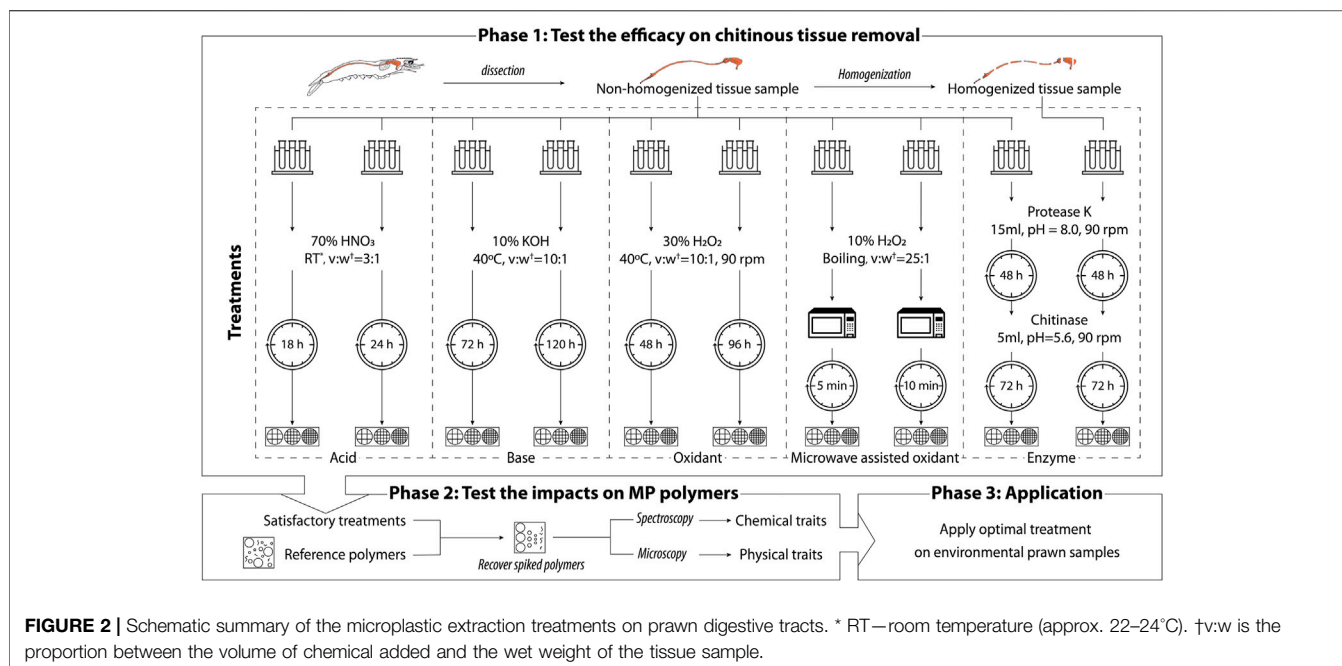
removal of organic matter to facilitate trace metal analysis (Kuss, 1992; Lamble and Hill, 1998; Ritter et al., 2004) and capable of degrading chitin to chitosan and glucosamine (Shao et al., 2003; Wojtasz-Pająk and Szumilewicz, 2007), but as of yet have not been used in MP research. However, the high temperature used in microwave ovens may be capable of degrading certain polymers, particularly when used in conjunction with harsh chemicals. (Castle et al., 1990; Sakurai et al., 2006; Karami et al., 2017). Few studies have validated MP extraction methods against reference polymers to ensure their integrity (Desforges et al., 2015; Abbasi et al., 2018; Cau et al., 2019; Hara et al., 2020). Even fewer have systematically tested the impact of digestion methods on MPs in large crustaceans.

Therefore, the goal of this study was to develop a suitable MP extraction method for decapod digestive tracts. Five treatment methods were adapted from the literature focusing on general organic tissue removal or chitin degradation (acid, base, oxidant, enzyme, and microwave assisted oxidant digestion) (Cole et al., 2014; Miller et al., 2017; Piarulli et al., 2019; Wojtasz-Pająk and Szumilewicz, 2007). The treatments were tested on the digestive tract of banana prawns (*Penaeus merguiensis*) since they are arguably the most commercially valuable species of penaeid prawns distributed accrosee tropical and sub-tropical regions. (Fischer and Bianchi, 1984; Göltenboth and Schoppe, 2006; Safaie, 2015; Vance and Rothlisberg, 2020). Specifically, this work will contribute to an efficient and effective digestion protocol for banana prawn digestive tracts by 1) comparing several common tissue digestion methods and assessing their ability to break down the chitinous tissue present within prawn digestive tract; and 2) evaluating the impacts of satisfactory treatments on the physical and chemical traits of known reference synthetic polymers.

2 MATERIALS AND EQUIPMENT

Banana prawns (*P. merguiensis*) were obtained from commercial sources. The oesophagus, foregut and mid gut (**Figure 1A**) of prawns were carefully dissected from surrounding tissue, weighed, and stored at −20°C in glass containers if not used immediately. The digestive gland was excluded from analysis as it does not contain any chitinous lining. Furthermore, the particles able to pass through the filter-press from the foregut are usually less than 1 µm (Wade et al., 2018) which is currently below the size detection limits for most MP analysis methods.

Nitric acid (70% HNO_3) (UN No. 2031), 30% hydrogen peroxide (H_2O_2) (CAS No. 7722-84-1), absolute ethanol (EtOH) AR grade (CAS No. 64-17-5) were all sourced from Thermo Fisher Scientific. 30% H_2O_2 and absolute EtOH were also diluted to 10% H_2O_2 and 70% EtOH with Milli-Q (Millipore) water (Milli-Q H_2O). Potassium Hydroxide (KOH) pellets (Thermo Fisher Scientific UNILAB CAS No. 1310-58-3) were dissolved with Milli-Q (Millipore Merck) water to form a 10% w/v aqueous solution. Protease K (Bioline/Meridian Bioscience Cat. No. BIO-37039, 40.8 U mg^{-1}, 20 mg ml^{-1}) and Chitinase (entochitinase, EC 3.2.1.14, ASA Spezialenzyme GmbH, Germany, 100 U ml^{-1}) was stored at −20°C as lyosphilized

FIGURE 2 | Schematic summary of the microplastic extraction treatments on prawn digestive tracts. * RT—room temperature (approx. 22–24°C). †v:w is the proportion between the volume of chemical added and the wet weight of the tissue sample.

powder and stable solution with 50% glycerin respectively. Homogenization solution for protease K was prepared with 400 mM Tris-HCl buffer, 60 mM EDTA, 105 mM NaCl and 1% Sodium Dodecyl Sulfate (SDS) to achieve pH 8.0. Citric acid buffer was prepared using 0.1 M Sodium citrate and 0.1 M citric acid buffer to achieve pH 5.6 for chitinase. A total of seven different synthetic polymers were selected based on the previously reported particles or fibers in crustaceans (Murray and Cowie, 2011; Cole et al., 2014; Brennecke et al., 2015; Wójcik-Fudalewska et al., 2016; Abbasi et al., 2018; Cau et al., 2019). These were: polyamide (PA, 1–2 mm), polyethylene (PE, < 500 µm), polyester (PES, 1–3 mm), polypropylene (PP, 235–500 µm), polystyrene (PS, 1–2 mm), polyvinylchloride (PVC, 1–2 mm), and rayon (2 cm). Detailed information on the reference polymers is presented in **Supplementary Table S1** and **Supplementary Figure S1**. A commercial domestic microwave oven (LG Microwave Oven MS4042GR, 1100 W) was used at relevant steps.

3 METHODS

3.1 Preventing Contamination
Strict procedures were followed in order to minimize MP contamination with the laboratory throughout analysis. A full-cotton (100%) lab coat dyed green was worn during all experimental procedures. All containers were made of glass and stainless steel and rinsed with tap water, followed by three rinses in reverse osmosis (RO) water. All equipment and samples were covered when not in use. The cover or caps of the containers were either aluminium foil or plastic with polytetrafluoroethylene (PTFE) lining depending on the compatibility of the chemicals used. Solutions and liquids were prefiltered to remove

contaminants with PTFE filters (0.45 µm pore size, 47 mm Ø diameter, Millipore). A stainless steel (316 grade) filtration system was used with sizes (263, 77 and 26 µm) to obtain post treatment residual. Three pore sizes (263, 77 and 26 µm) of stainless steel filters (19 mm Ø) were punched from woven mesh. All samples were prepared in laminar flow and were rinsed thoroughly with Milli-Q water before and after dissection. Digestion and filtration was carried out in either a fume hood (acid digestion) or laminar flow (base, oxidant digestion).

3.2 Method Development
Following the stepwise approach to select an optimum extraction treatment in fish (Karami et al., 2017), the experimental design of this study was divided into three phases (**Figure 2**).

Two variations (treatment duration or homogenization status of the tissue sample) were compared for each treatment according to preliminary visual assessments. The results of Phase 1 were assessed using gravimetric and image analysis to select satisfactory treatments. The satisfactory methods obtained in Phase 1 subsequently proceeded to Phase 2. Phase 2 evaluated the impact on reference polymers by analyzing the physical and chemical characteristics of polymers before and after treatment using microscopy image analysis and spectroscopy. Combining the result of Phase 1 and Phase 2, the optimal treatment is further applied to environmental sample captured in the wild. The results of Phase 3 are available in Dawson et al. (2022).

3.3 Tissue Sample Digestion Treatment in Phase 1
A total of five digestion methods with two variations of each were chosen based on preliminary tests via visual assessment of the

remaining chitinous gut tissue after treatment (**Figure 2**). Each method was tested in triplicate following the protocols outlined below. All treated samples were filtered sequentially onto pre-weighed filters. These were then rinsed with Milli-Q water followed by 70% EtOH using a PTFE wash bottle, to reduce the possibility of particulates adhering to the sides of the container and to remove any chemical residue. Filters and the filtration system were flushed at least three times or until there were no particulates adhering to the system. Filters were dried overnight (approx. 18–24 h) at room temperature (approx. 22–24°C) and stored in a desiccator. All treatments except microwave assisted oxidant treatment were conducted at 40°C or below to achieve maximum enzyme activity and reduce MP degradation at high temperature, as previous studies have thoroughly demonstrated MP degradation above 50°C (Karami et al., 2017; ?). Once the treated samples were completely dry and re-weighed, the remaining residue was stained with lactophenol cotton blue, a commonly used staining agent demonstrating an affinity for chitin (Leck, 1999). The staining agent gave chitin a blue colour that was used to visualize the chitinous residues remaining on the filter after digestion.

3.3.1 Acid Treatment
70% HNO$_3$ was added to the tissue samples at a ratio of 10:1 v:w, or until covering the sample. Samples were then covered with PTFE lined caps and stored at room temperature inside a fume hood during the treatment time (12 or 24 h). Before filtration, the solution was neutralized using 10% KOH in a ratio of 3:1 (v:v, 10% KOH: 70% HNO$_3$).

3.3.2 Base Treatment
10% KOH was added to the tissue samples at a ratio of 10:1 v:w or until the sample was submerged. Samples were incubated at 40°C in an oven during the treatment time (72 or 120 h).

3.3.3 Oxidant Treatment
30% H$_2$O$_2$ was added to the tissue samples at a ratio of 10:1 v:w or until the sample was submerged. Samples were incubated in a Thermoline incubator at 40°C and shaken at 90 rpm throughout the treatment time (48 or 96 h).

3.3.4 Enzyme Treatment
Each frozen tissue sample was homogenized using a glass rod. Both homogenized and non-homogenized tissue sample were digested in Proteinase-K in 15 ml homogenizing solution (pH = 8.0) for 48 h. Samples were then filtered and rinsed before adding chitinase with citric acid buffer solution (pH = 5.6) for a further 72 h. Protease digestion was maintained at 40°C and chitinase was maintained at 37°C in the incubator and shaken at 90 rpm throughout the treatment.

3.3.5 Microwave Assisted Oxidant Treatment
10% H$_2$O$_2$ was added to the tissue samples at a ratio of 25:1 v:w in a glass tube with a small glass beaker loosely covering the top to prevent a buildup of pressure within sample tubes. Samples were heated to the boiling state inside the microwave at half power (i.e., 550 W). Samples were closely monitored for throughout

digestion, with the heating briefly paused periodically to avoid overflow of the reaction agent. The microwave time, 5 or 10 min (**Figure 2**) was the run time only, without taking the cooling-off time into account.

3.4 Sample Digestion in Phase 2 With Spiking Reference Polymers
Based on Phase 1, the three most satisfactory digestion methods were selected to evaluate their impacts on seven different types of polymers: polyamide (PA), polyethylene (PE), polyester (PES), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), and rayon. Each sample was spiked with 10 replicates of each polymer type. Triplicates of spiked samples were subsequently digested using the same protocols outlined in **Subsection 3.3**.

3.5 Gravimetric Analysis (Phase 1)
Digestive efficiency is for each method was calculated as per Karami et al. (2017) using **Equation (1)**. Treatments resulting greater than 95% average digestive efficiency among all three replicates were considered efficiently digested.

$$\text{Digestive efficiency} \% = \frac{W_i - (W_a - W_b)}{W_i} \times 100 \qquad (1)$$

where W_i = Initial wet weight of tissue samples and spiked polymers; W_a = Weight of dry tissue and stainless steel filters after filtration; W_b = Weight of stainless steel filters before filtration.

3.6 Image Analysis (Phase 1 and 2)
Digestive efficiency alone has recently been suggested to be an inadequate metric to evaluate complete digestion (Dawson et al., 2020). Thus in Phase 1, image analysis was applied to examine the retention of chitinous tissue after treatment as visualized by lactophenol cotton blue stain: 1) overall digestion level, 2) normalized undigested residue coverage, and 3) average blue value. True colour, high resolution, RGB images were taken using a Leica Stereo microscope MZ16A. Overall digestion level was defined as a score (1–4, with higher scores relating to lower residual tissue) assigned to the status of remaining residues on 263 μm filters for each sample. The four digestion scores (**Figure 3**) corresponded to the following criteria:

- 1: very poor, i.e. the entire esophagus and proventriculus chambers intact;
- 2: poor, i.e. the esophagus and proventriculus chambers partially intact;
- 3: moderate, i.e. no obvious chitinous structure with many irregular particles;
- 4: good, i.e. no obvious chitinous structure with little irregular particles.

Both normalized undigested residue coverage and average blue value were computed using a computer vision image analysis algorithm (OpenCV library with Python 3.8) (Bradski, 2000). All images were taken under consistent brightness and nadir angle
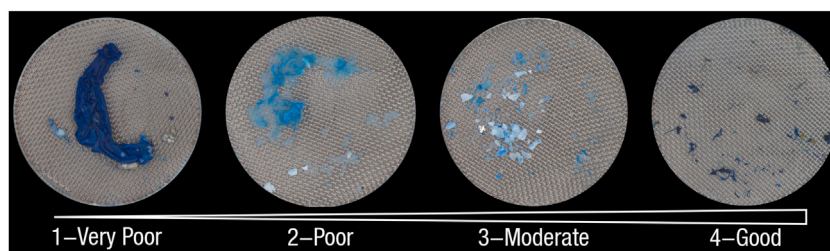
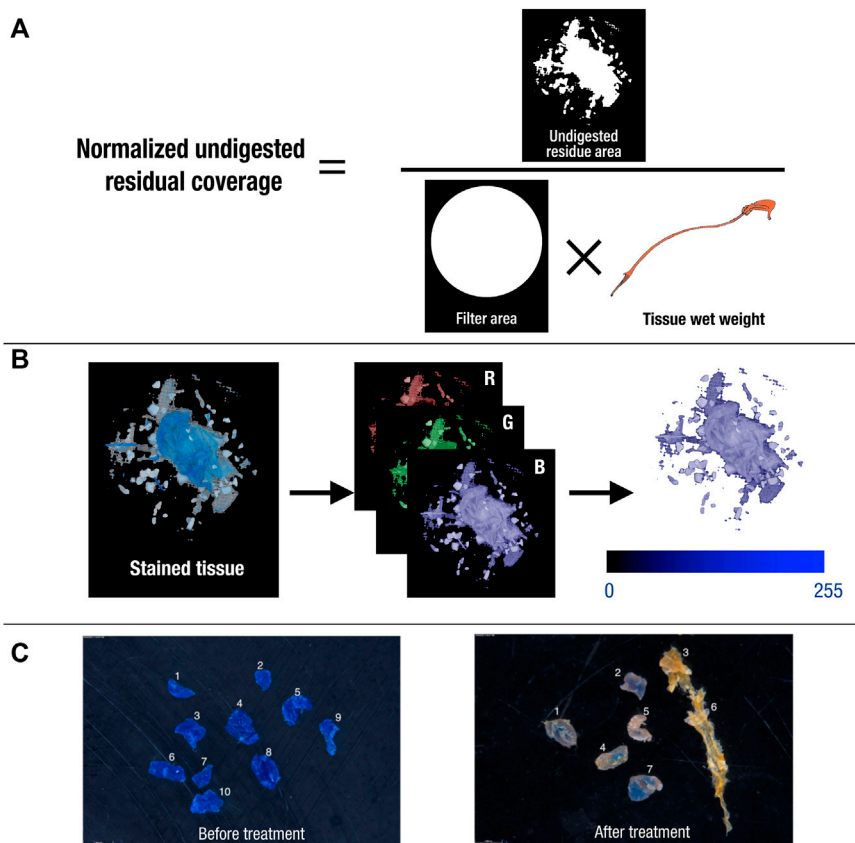**FIGURE 3 |** Illustration of overall digestion level scores.

FIGURE 4 | Illustration of image analysis protocol for **(A)** using masks of filter and residue and sample tissue wet weight to calculate "normalized undigested residue coverage", **(B)** extracting blue channel from RGB images of stained tissue to calculate "average blue value", and **(C)** an example of reference polymers (e.g. PE) comparison before and after treatment.

with a microscopic camera as 8-bit true colour RGB images with pixel having a value for the brightness of the red, blue, and green colour ranging from zero to 255 (with zero indicating no light and 255 indicating maximum light). The algorithm detected the area with remaining tissues and calculated the percentage coverage of undigested residual over the whole filter (**Figure 4A**) as well as the average value encoded in the blue channel of all pixels located in the detected area (**Figure 4B**). A black and white mask was created for both filter area and residue area ((**Figure 4A**) which were quantified by their pixel count, normalised by the wet weight

of the tissue measured during the sample preparation stage. The undigested residual coverage measures how widely the residue was spread over the filter per unit weight. I.e., a high normalised undigested residual value implies the tissue sample had been broken down into small pieces that are able to spread over the filter. The last criteria, average blue value, was used as an indicator to show how well chitin has been broken down due to the binding specificity of the colouring agent. A higher the value suggests that less colouring agent has combined with the residue, thus, more chitin has been digested.
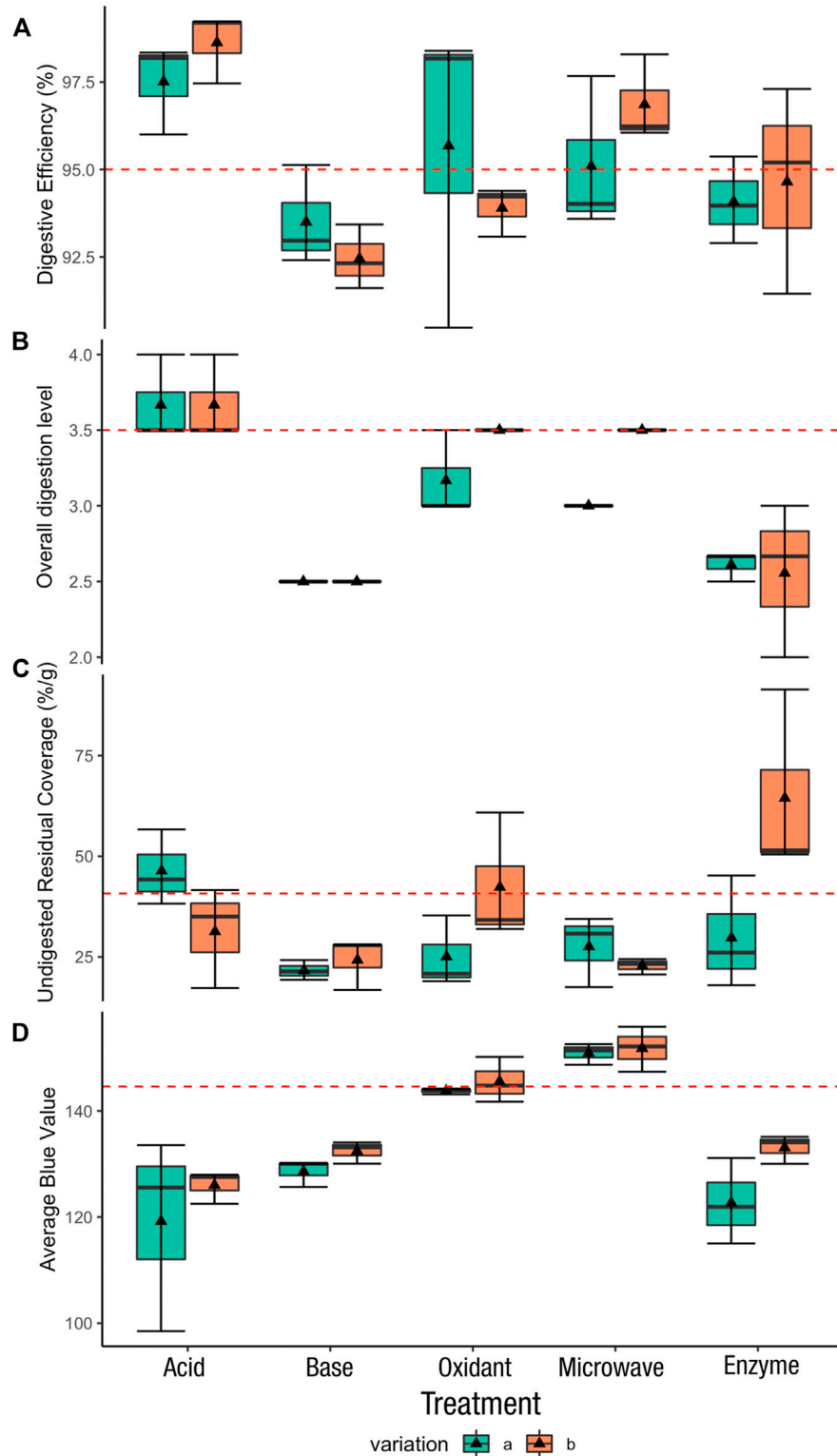
FIGURE 5 | Boxplot for all evaluated aspects. The triangle marker is the mean value of each treatment. The red dash line is the cut-off line: **(A)** digestive efficiency—95% adopted from (Karami et al., 2017), **(B)** normalized overall digestion level—Q3 = 3.5, **(C)** undigested residue coverage—Q3 = 40.7%, **(D)** average blue value—Q3 = 145.

Treatments achieving an average value equal or greater than the value of third quartile (Q3), i.e. top 25% of the values in the dataset, for each assessment were considered acceptable in Phase 1.

Image analysis was also performed in Phase 2 to evaluate the degradation of each treatment on the physical properties of each polymer. The size of spiked reference polymers was determined by the length of fibres or the Feret's diameter of particles, which were measured from microscopic images before the treatment (i.e. virgin polymers), and after the treatment (i.e. exposed polymers) in FIJI ImageJ (Schindelin et al., 2012) (**Figure 4C**). Polymer recovery rate was calculated using the equation below:

$$\text{Recovery rate} \% = \frac{N_v - N_e}{N_v} \times 100 \qquad (2)$$

where N_v = Counts of virgin reference polymers before treatment; N_e = Counts of exposed reference polymers after treatment.

3.7 Spectroscopic Analysis (Phase 2)

Reference polymers also had their chemical characteristics analysed using Fourier transform infrared (FTIR) spectroscopy with a PerkinElmer Spectrum 100 Spectrometer (16 scans at 4 cm^{-1} resolution, wavenumber range = 4,000-600 cm^{-1}). Spectra of virgin ($n = 3$) and exposed polymers ($n = 9$) were acquired measuring attenuated total reflectance (ATR-FTIR). Spectral signature of virgin and exposed polymers were correlated within the PerkinElmer Spectrum IR software, generating a coefficient of correlation (R) (Kroon et al., 2018). If exposed polymers obtained R that is greater than 0.9, they were considered as very similar to the virgin polymers.

3.8 Data Analysis

Tukey's boxplots were adopted to visualize the distribution of all collected data (Tukey, 1977). Mann-Whitney test (RStudio 1.4.1106) was performed to check whether there was a significant difference between the size of reference polymers before and after treatment in Phase 2.

4 RESULTS

4.1 Efficacy on the Chitinous Tissue Sample Removal

Acid, oxidant, and microwave assisted oxidant digestion methods resulted in digestive efficiency values greater than 95% (**Figure 5A**). Both variations of acid digestion resulted in digestive efficiency >97% (97.51 ± 0.76% and 98.63 ± 0.58% for short and long treatment duration, respectively). While both variations of microwave assisted oxidant gave a mean digestive efficiency higher than 95%, the longer treatment time resulted in lower variability between replicates (95.09 ± 1.29% and 96.86 ± 0.72% for short and long treatment duration, respectively) and the shorter treatment time resulted in more than half of the replicates failing to achieve a high digestive efficiency. The shorter treatment duration (95.68 ± 2.60%) of oxidant digestion resulted in a higher digestive efficiency than

the longer variation (93.90 ± 0.41%), however the data was more variable between replicates. For image analysis, the results varied with the efficacy of the chitinous lining degradation. Acid digestion with longer treatment duration resulted in the highest overall digestion level (**Figure 5B**). Enzyme treatment with homogenized tissue samples had particles spread across the largest area on the filters (**Figure 5C**). Both variations of microwave assisted oxidant treatment performed well when assessing the chitin digested level using average blue value (**Figure 5D**).

Overall, base digestion and enzyme digestion failed to satisfy most of the evaluation criteria (**Table 1**). Whereas acid, oxidant and microwave assisted oxidant digestion all had one variation each that met 3 of the 4 cut-off criteria. These were the 70% HNO$_3$ for 24 h, 30% H$_2$O$_2$ for 96 h, and 10% H$_2$O$_2$ 10 min microwave method. Thus, these three treatments proceeded to the next phase. The details of the performance of each treatment can be seen in supplementary material (**Supplementary Table S2**).

4.2 Impacts of Selected Treatments on Reference Polymers

Recovery rate, colour and spectra of MPs exposed acid, oxidant and microwave assisted treatments varied considerably, while, other than PA, the shape and size did not show significant changes for all recovered polymers. Rayon fibers were unable to be recovered in any treatment, and PVC fragments were fully recovered in all treatments. PE, PP, and PS were almost fully recovered in all treatments. PA and PES had very low recovery rate (0 and 36.7%, respectively) after being treated with 70% HNO$_3$ for 24 h (**Table 2**). Moreover, the image analysis further showed that PE fragments and PES fibers were discoloured, and the surface of some polymers were smoothed after the treatment (**Supplementary Figure S2**). Minor color and morphological changes occurred during oxidant and microwave assisted oxidant treatments, however some remaining tissue residue adhered to the surface of the polymers (**Supplementary Figures S3, S4**). The size distribution of recovered exposed polymers was very similar compared to virgin polymers (**Figure 6**). The exposed PE, PES, PP, PS, and PVC polymers show no significant difference to the virgin polymers (**Supplementary Table S3**). Only PA recovered from microwave assistant treatment had significant changes in size (Mann-Whitney test, $nv = 30$, $ne = 28$, $p = 0.0014$). The average length of PA fragments before and after the treatment was 1.71 ± 0.05 mm and 1.54 ± 0.04 mm respectively. Notwithstanding these significant differences, the changes in size were small and the polymers had no obvious shape and colour changes.
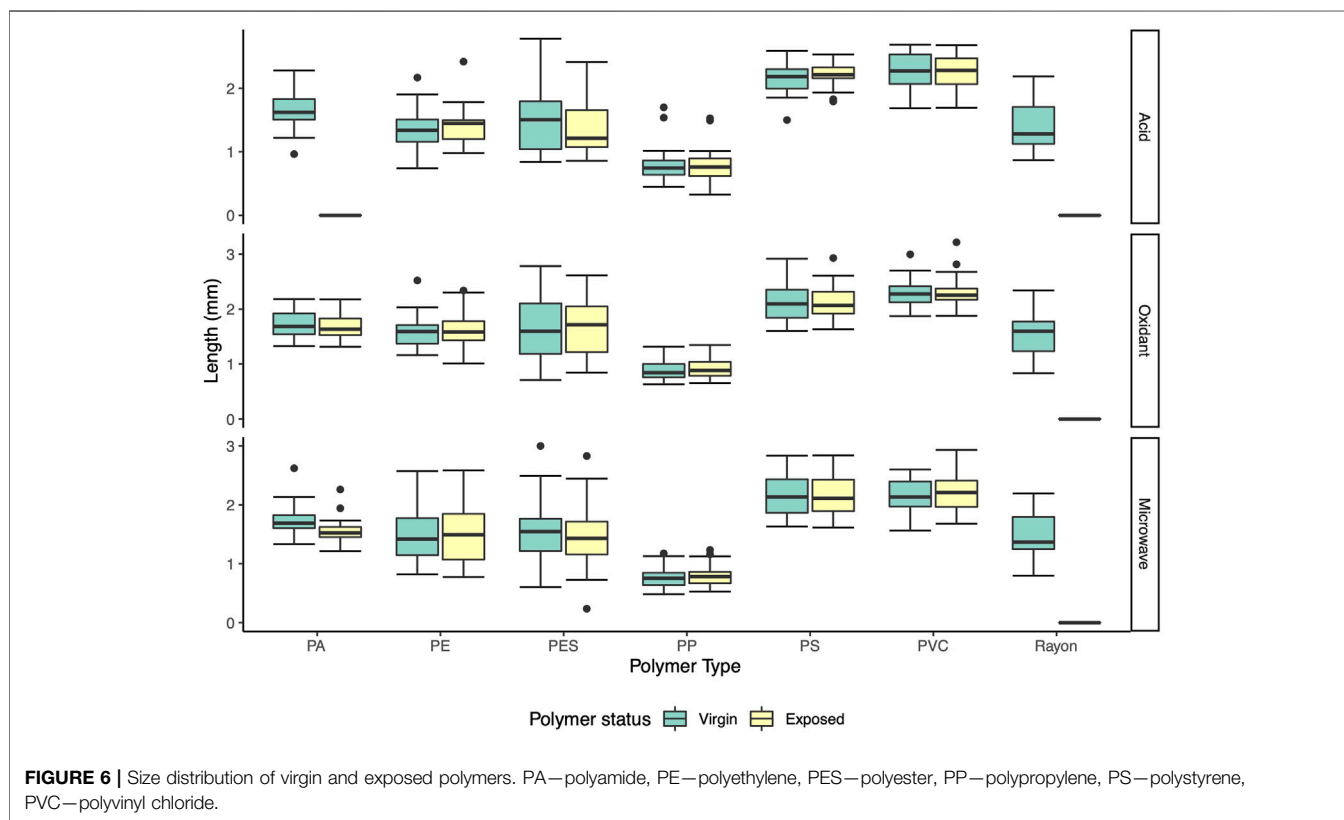
In terms of the chemical characteristic changes after each treatment, polymers recovered from microwave assisted oxidant digestion had very high FTIR spectra correlation scores ($R > 0.95$ for all nine polymer types on average) to their virgin polymer counterparts (**Figure 7**, **Supplementary Table S4**). After oxidant treatment, the correlation score of

TABLE 1 | Summary of gravimetric and image analysis results in an evaluation matrix, where a tick (✓) indicates that the cut-off criteria is met.

Treatment	Acid		Base		Oxidant		Microwave		Enzyme	
Variation	a	b	a	b	a	b	a	b	a	b
Digestive efficiency	✓	✓				✓		✓	✓	
Overall digestion level	✓	✓					✓		✓	
Undigested residual coverage	✓					✓				✓
Average blue value						✓	✓		✓	
Total no. of ✓	3	2	0	0	1	3	2	3	0	1

TABLE 2 | Recovery rate of reference polymers under acid, oxidant and microwave assisted oxidant treatment (PA—polyamide, PE—polyethylene, PES—polyester, PP—polypropylene, PS—polystyrene, PVC—polyvinyl chloride).

Treatment	PA	PE	PES	PP	PS	PVC	Rayon
Acid	0.0%	76.7%	36.7%	100.0%	96.7%	100.0%	0.0%
Oxidant	93.3%	93.3%	90.0%	100.0%	100.0%	100.0%	0.0%
Microwave	100.0%	100.0%	83.3%	96.7%	100.0%	100.0%	0.0%

FIGURE 6 | Size distribution of virgin and exposed polymers. PA—polyamide, PE—polyethylene, PES—polyester, PP—polypropylene, PS—polystyrene, PVC—polyvinyl chloride.
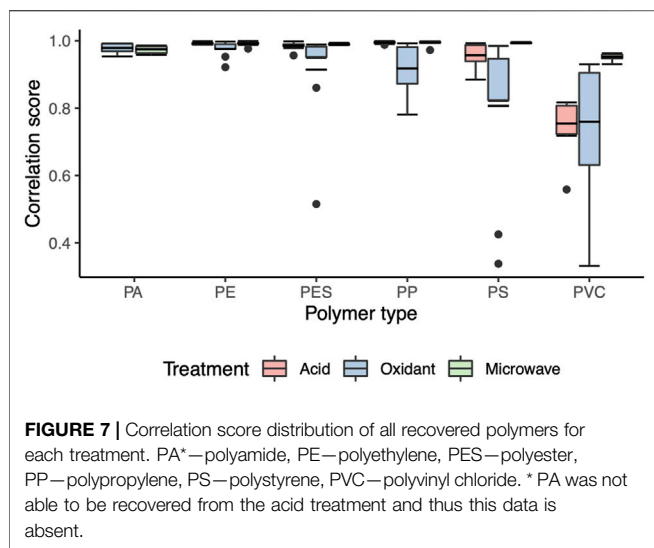
exposed polymers to the virgin polymer spectrum library showed that PA, PE, PES and PP had high average correlation scores for recovered polymers ($R > 0.9$) while PS ($R = 0.81 \pm 0.07$) and PVC ($R = 0.76 \pm 0.06$) had low scores. As for acid treatment, PA were unable to recovered with no correlation score available and the score of PVC polymer was the lowest ($R = 0.75 \pm 0.03$) amongst all polymers recovered.

5 DISCUSSION

5.1 Optimal Tissue Removal and MP Extraction Approach for Prawns

The oxidant treatment successfully removed the chitinous lining of the prawn digestive tract, however the addition of microwave radiation to this treatment resulted in more complete and efficient digestion while maintaining the integrity of the

FIGURE 7 | Correlation score distribution of all recovered polymers for each treatment. PA*—polyamide, PE—polyethylene, PES—polyester, PP—polypropylene, PS—polystyrene, PVC—polyvinyl chloride. * PA was not able to be recovered from the acid treatment and thus this data is absent.

polymer samples. Even though the solution was heated to the boiling point of the 10% H_2O_2 solution (approx. 100°C), the spectral signature of exposed polymers remained very similar to the virgin polymers. In contrast, colour change has previously been described in PA and PVC at temperatures of 50–60°C, which is likely a result of prolonged incubation (96 h) using traditional heating oven (Karami et al., 2017). Microwave heating converts electromagnetic energy to thermal energy by water molecule oscillation rather than relying on heat transfer from the ambient environment using ovens or incubators (Kuss, 1992; Sun et al., 2016). As a result, the materials in microwaves are heated with a uniform thermal field in much less time (Sun et al., 2016). The short reaction time may reduce the likelihood of MP degradation as the high temperature is only maintained very briefly compared to other digestion methods used in our study. Furthermore, the microwave energy creates a small but consistent vibration of the molecules throughout the solution. Mechanical movement facilitates the digestion process (Cole et al., 2011; Therien et al., 2019) making it likely that the molecular vibration imparted by microwaves would improve digestion of chitin. Therefore, in the absence of significant MP degradation, microwave assisted oxidant digestion presents a promising approach for a standardized method of MP extraction and quantification from crustacean gastrointestinal tracts. It is worth point out in this study that relatively large MPs were used for ease of manipulation and measurement. Although these particles are still within the size range of microplastics, further studies should verify that this method also applies to smaller MP (<1 mm). Recent method developments using very small immobilised MPs may be of use to validate this digestion method (?).

This study further confirmed that treating prawn digestive tracts with 70% HNO_3 at room temperature for 24 h could remove the majority of the tissue samples with very high digestive efficiency (>97% on average) but with an unacceptable level of damage to some MP polymers. Acid digestion, using HNO_3 or a mixture of inorganic acid such as HNO_3, HCl or $HClO_4$ is well known to degrade crustacean exoskeletons and chitinous digestive tract lining (Desforges et al., 2015; Devriese et al., 2015). However, HNO_3 was

very destructive to PA polymers. This is consistent with previous work, where Claessens et al. (2013) was unable to recover spiked nylon fibers after HNO_3 incubation. While HNO_3 treatment did not cause the loss of PVC particles or result in a colour change, it did reduce the clarity of the FTIR spectrum ($R = 0.75 \pm 0.03$). Thus polymers may not be identifiable from environmental samples if HNO_3 was used to digest the samples, as spectral signature is currently the optimal method to accurately identify a putative polymer from the environment (Miller et al., 2017).

In contrast to acid digestion, alkaline and enzyme treatments failed to break down the complex lining in the foregut, resulting in intact proventriculus in all replicates (**Supplementary Figure S5**). It is reasonable to suspect that any MP polymers present inside the proventriculus would not be isolated for further analysis, as isolation and identification of putative MP is based on visual inspection of the filter residue after digestion. KOH is one of the most widely used chemical digestion solutions for MP extraction, but extensive treatment with 10% KOH (40°C for 120 h) without degrading the lining could be a result of insolubility of chitin in mild basic solutions. The samples treated sequentially with protease and chitinase also failed to break down the tissue. This is consistent with a recent study by (Kallenbach et al., 2021), demonstrating that the chitinous exoskeleton of terrestrial isopods can resist broad-spectrum enzymatic digestion. A combination of pharmaceutical pancreatic enzyme mixes and chitinase was applied to the isopod for 48 h (24 h for pancreatic enzyme mix and 24 h for chitinase), yet the exoskeleton remained intact. On the other hand, the study claimed that a combination of 30% H_2O_2 and chitinase has removed the exoskeleton efficiently (Kallenbach et al., 2021). Since the experiment design did not compare this method with H_2O_2 treatment only, our current results suggest that the success of chitin degradation has little to do with chitinase, but mainly due to the addition of H_2O_2, which is same as the oxidant treatment used in this study. Chitinase is widely used to derive glucosamine from the α-chitin in shrimp and crab shells (Chen et al., 2010). Enzymatic hydrolysis the preferred method in industrial process due to its environmental friendliness comparing to other methods that tend to produce large quantities of chemical waste (Chen et al., 2010). Various chitinolytic enzymes exist in the wild including endochitinases (EC 3.2.1.14), exochitinases (EC 3.2.1.52), chitobiosidases (EC 3.2.1.30) and N-acetylglucosaminidases (NAGases) (EC 3.2.1.96) (Chen et al., 2010). This study tested endochitinases which would cleave chitin at internal sites on the glycosidic bonds randomly to generate the soluble oligosaccharides (Chen et al., 2010; Wang et al., 2019). There is little information about the chemical composition and structural difference amongst the chitinous tissues from the proventriculus and oesophagus, and that of the exoskeleton, which is widely used for glucosamine production. This knowledge gap may have hindered the use of targeted enzymes, thus leading to insufficient breakdown of the endogenous chitinous tissue in the prawns. It is also important to note that the aim of industrial chitinase utilization is yield of glucosamine rather than the complete removal of tissues to reveal their contents, so while an enzyme may be efficient for industrial aims, it is not applicable to MP research in this context.

5.2 Digestion Efficiency as an Indicator of Method Suitability

The five treatments were assessed by combining all the results into an evaluation matrix, in order to make an informed decision on the method suitability. In conventional MP extraction methodology development, the optimal treatment is typically selected based on one or more of the following criteria: digestive efficiency, weight changes of reference polymers, and FTIR spectral changes of the polymers (Cole et al., 2014; Desforges et al., 2015; Devriese et al., 2015; Karami et al., 2017; Hara et al., 2020). The results of this study clearly demonstrate that in cases involving crustacean digestive systems, digestive efficiency is not sufficient for method evaluation. For instance, the oxidant treatment (30% H_2O_2, 48 h) achieved a digestive efficiency of 95.68 ± 2.60%, but visual inspection showed that the undigested residuals were still clustering after filtration, making isolation of putative particles impossible (**Supplementary Figure S6**). The KOH treatment recommended by Hara et al. (2020) showed a similar pattern of results, with intact proventriculus material clearly visible despite a gravimetric digestive efficiency greater than 95% (**Figure 1B**). Furthermore, MPs can be too light to measure accurately, rendering gravimetric measures severely limited. Using larger particles that are able to be weighed is also problematic, as larger particles are more stable due to diminished surface to volume ratios. As demonstrated in this study, visual inspection of remaining residues could play a crucial role in verifying digestion methods.

It is worth noting that the evaluation matrix is not identical among all tissues. A selection of gravimetric analysis and image analysis will be case specific. In this study, digestive efficiency was the only conventional decision criterion in MP studies, while the three image analysis criteria were developed according to specific features of the prawn digestive tract. Overall digestion level is a straightforward visual cue to evaluate if the chitinous lining still exits after treatment, which may not apply to other biota or tissues that lack clear structures. Undigested residual coverage is highly related to the filtration technique. The rinsing and washing angle and force could influence the distribution of the particles. Thus, it is crucial to keep the operating procedure consistent throughout the experiment. It may also be desirable to increase the sample size in order to mitigate any potential artefacts. Additionally, the computer vision algorithm chosen to determine the particle distribution on filters also played an important role in the evaluation criteria. Since it is impossible to manually select all fine particles on the filters, the presence of distributed particles were approximately detected using the OpenCV library with the embedded Gaussian blur and binary threshold functions (Bradski, 2000). The optimization of the parameters for these functions could improve the detection accuracy in future research.

6 CONCLUSION

In conclusion, microwave assisted oxidant digestion outperformed the traditional acid, alkaline, oxidant and enzyme digestion treatments in MP extraction and quantification. The method achieved high digestion efficiency and satisfactory visual assessment. Thus, it was successfully applied to disintegrate the lining of *P. merguiensis* digestive tracts. Importantly, six out of seven types of reference polymers were recovered at a high rate with minimal shape, size, and spectral changes after treatment. This has highlighted a new method to efficiently remove difficult organic tissues and thereafter identify putative MP particles.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JL conceptualized the study, acquired funding, carried out the laboratory analysis, analysed the data, and wrote the original draft. JL and AD acquired funding for the project. AD and LN contributed to the conceptualization methodology, reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

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