Isolation of microplastics in biota-rich seawater samples and marine organisms

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Microplastic litter is a pervasive pollutant present in aquatic systems across the globe. A range of marine organisms have the capacity to ingest microplastics, resulting in adverse health effects. Developing methods to accurately quantify microplastics in productive marine waters, and those internalized by marine organisms, is of growing importance. Here we investigate the efficacy of using acid, alkaline and enzymatic digestion techniques in mineralizing biological material from marine surface trawls to reveal any microplastics present. Our optimized enzymatic protocol can digest >97% (by weight) of the material present in plankton-rich seawater samples without destroying any microplastic debris present. In applying the method to replicate marine samples from the western English Channel, we identified 0.27 microplastics m⁻². The protocol was further used to extract microplastics ingested by marine zooplankton under laboratory conditions. Our findings illustrate that enzymatic digestion can aid the detection of microplastic debris within seawater samples and marine biota.
One of the main environmental risks associated with microplastics is their bioavailability to marine organisms. A number of studies have shown that microplastics can be ingested by marine biota under laboratory conditions; however, there is limited data relating to the biological uptake of microplastics in situ. When studying larger field-collected organisms, including fish and crustaceans, the intestinal tract can be dissected and any internalized microplastics enumerated. Van Franeker et al. have routinely applied this method with ocean-foraging Fulmars, providing important, novel data relating to microplastic concentrations at sea. However, dissection is less applicable for animals such as zooplankton and mussels, which can consume extremely small (2–31 μm diameter) microplastics7–9 that are not visible to the naked eye. Bio-imaging techniques including coherent anti-Stokes Raman scattering (CARS) microscopy7 have previously been employed to visualize the distribution of tiny microplastics internalized by zooplankton, but this method is time-intensive and can be hindered by algal pigments in natural specimens. Recently, Claessens et al.10 trialled the use of nitric acid to digest mussels to reveal ingested microplastics but found this caustic treatment destroyed some pH-sensitive polymers. As such, an alternate method is needed if a greater range of field-collected organisms are to be comprehensively bio-monitored for microplastic contamination.

In this study, we developed, optimized and validated a rapid and efficient protocol for digesting biological material without destroying microplastics. We compared the use of acid (HCl), alkaline (NaOH) and enzymatic (Proteinase-K) digestion treatments on plankton-rich seawater samples and a range of microplastics. We applied the optimized enzymatic protocol to replicate sub-surface seawater samples and microplastics internalized by zooplankton, but this method is time-intensive and can be hindered by algal pigments in natural specimens. Recently, Claessens et al.10 trialled the use of nitric acid to digest mussels to reveal ingested microplastics but found this caustic treatment destroyed some pH-sensitive polymers. As such, an alternate method is needed if a greater range of field-collected organisms are to be comprehensively bio-monitored for microplastic contamination.

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**Results**

**Mitigating contamination.** Procedural blanks used during method development highlighted potential sources of contamination, including residue from aluminium foil lids used during the 60°C NaOH exposure, and plastic shavings following physical homogenization of samples within universal tubes. These sources of error were subsequently eliminated. Following method optimization, the microscopic analysis of filters retained from procedural blanks showed no evidence of microplastic contamination.

**Digestion efficacies.** The digestion efficacies of the treatments ranged from 54.0 to 97.7% (Figure 3). The least effective treatment was hydrochloric acid, with 1 M HCl (Figure 3a) and 2 M HCl (data not shown) resulting in digestion efficacies of 82.6 ± 3.7% and 72.1 ± 9.2% respectively. Comparatively, 1 M NaOH (Figure 3a) and 2 M NaOH (data not shown) digested the samples by 90.0 ± 2.9% and 85.0 ± 5.0% respectively. The optimized alkaline digestion protocol (10 M NaOH at 60°C) had a digestion efficacy of 91.3 ± 0.4% (Figure 1b; Figure 3a). Application of the original enzymatic protocol resulted in a digestion efficacy of 88.9 ± 1.5%, while, the optimized Proteinase-K treatment digested >97% of the samples and was significantly more effective than other treatments (Figure 1c; Figure 3a; ANOVA with Tukey post-hoc analysis, P < 0.05).

**Ultrasonication.** Post-digestive ultrasonication was equally or less effective than using digestive treatments alone (Figure 3b). Use of an ultrasonication bath on 5 M and 10 M NaOH digested samples resulted in digestion efficacies of 90.9 ± 2.3% and 87.3 ± 1.0% respectively (Figure 3b). With samples digested using 10 M NaOH at 60°C, direct sonication increased digestion efficiency by just 0.3% (Figure 3b). Meanwhile, with enzymatically digested samples, direct sonication proved significantly less effective than using Proteinase-K alone (Figure 3b; t-test, P < 0.05).

**Filtering.** Visually, the optimized alkaline (Figure 1b) and enzymatic (Figure 1c) protocols were effective in reducing the biological material present within the marine samples. With both treatments, the undigested residue consisted of a thin, transparent film of glutinous biological material, in which microplastics (Figure 4) and small fragments of shell, wood and carapace were clearly visible. In comparing filters following optimized enzymatic digestion, we found 0.02 μm GF/Fs clogged quickly and were therefore inappropriate for this task; while 50 μm mesh-filters had the fastest filtration rates, 20 μm mesh-filters will theoretically capture smaller microplastics without significantly impacting upon digestion efficacy (50 μm filter: 97.3%; 20 μm filter: 97.1%; t-test, P = 0.67).

**Impact of optimized digestion protocols on microplastics.** The optimized enzymatic protocol showed no visible impact on any of...
the microplastics undergoing treatment (Figure 5). Conversely, the optimized alkaline treatment resulted in the partial destruction of Nylon fibres, melding of polyethylene fragments, and a yellowing of uPVC granules (Figure 5); further, several polyester fibres were lost in applying this protocol (data not shown).

Validation of the enzymatic digestion with in-situ microplastic debris. In all, 162 suspected plastics were isolated in four trawl samples taken in the western English Channel, of which 96% were classified as 'microplastic' (Figure 6). Concentrations of suspected microplastics averaged 0.26 items m\(^{-3}\) at Penlee, and 0.31 items m\(^{-3}\) at L4 (SI Table 2). FT-IR analysis revealed the majority of this material was plastic, primarily consisting of polyamide (e.g. Nylon) or polypropylene (Figure 6b). Notably, two of these 40 (5%) items were identified as leather (data not shown). The majority of the isolated micro-debris was fibrous (61%; Figure 6a), with widths varying between 6 and 175 \(\mu\)m and lengths \(>250 \mu\)m (Figure 6c). Non-fibrous microplastics, including granular or planar fragments (36%) and beads (3%), were predominantly <250 \(\mu\)m in diameter (Figure 6d). Fibres were most commonly found to be black, blue or red (Figure 6e), while non-fibrous plastics had a more even spread of colours (Figure 6f).

Detecting microplastics ingested by copepods. Microplastics were ingested by 11 of the 18 (77%) T. longicornis; fluorescent beads were clearly visible in the hindgut, but obscured by the opaque, pigmented carapace when in the fore-gut (Figure 7a). The enzymatic protocol digested the copepod tissue and the microplastics were retained on a GF/F (Figure 7b). With fluorescence enabled, microplastics were successfully enumerated, indicating an average load of 10.7 ± 2.5 beads per copepod (at the point at which the experiment was terminated) (SI Table 3).
While microplastic pollution in oceanic gyres microplastic abundance and distribution be monitored within MSFD (Descriptor 10.1.3: Marine Litter) requires that trends in widely consider microplastics to be a marine pollutant. In the EU, the method’s potential application for detecting whether marine organisms are ingesting microplastic debris in the wild. The ubiquity of microplastics in sea-surface, water-column and sediment samples from across the globe has highlighted the prevalence of this contaminant within our oceans. Microplastics can be ingested by a range of marine biota, including shellfish and fish fit for human consumption, and can result in adverse health impacts to these organisms. As such, national, regional and global bodies now consider microplastics to be a marine pollutant. In the EU, the MSFD (Descriptor 10.1.3: Marine Litter) requires that trends in microplastic abundance and distribution be monitored within European waters. While microplastic pollution in oceanic gyres and coastal sediments has been well reported in recent years, there has been limited focus on microplastics in near-shore waters. Coastlines are prone to fresh inputs of anthropogenic litter from run-off, urban waterways and sewage outfall but are also highly productive marine habitats. As microplastics pose a major threat to marine biota, providing data on bioavailable microplastics in coastal waters is of growing importance.

Traditionally, acid digestion might be used to remove biological material, such as plankton, present in marine samples (e.g. for analysis of trace metals); this method typically uses strong mineral acids (e.g. H₂SO₄, HNO₃), either in open or closed systems in conjunction with high temperature and pressure, which can oxidize compounds, causing molecular cleavage. However, these oxidizing acids can also destroy or damage polymers with a low pH tolerance (e.g. polyamide, polystyrene), thus the application of acid digestion in the analysis of microplastics is limited. As an alternative, we used low concentrations of the non-oxidizing, mineral acid HCl at room temperature. In digesting plankton, HCl proved inconsistent and inefficient, with large quantities of material remaining on the filters post-digestion. A viable alternative is alkaline hydrolysis, in which strong bases (e.g. NaOH, KOH) are used to denature proteins and hydrolyse compounds. Use of 1 M NaOH at room-temperature proved 90% effective in digesting 0.2 g DW marine samples; by increasing molarity and experimental temperature, digestion efficiencies were both increased and stabilized. The procedural blanks used with this protocol showed that aluminium foil lids (used to prevent airborne contamination) were degenerating from the high pH with higher temperature incubations, and as such were soiling the samples; this issue was rectified by replacing the aluminium lids with glass caps. Recently Nuelle, et al. have demonstrated that hydrogen peroxide (H₂O₂) is a more effective agent for removing biogenic material from sediment samples than HCl or NaOH. However, use of 35% H₂O₂ over a relatively long exposure period of 7 days resulted in the complete removal of just 25% of the biological material (<1 mm) present. Remaining biogenic material was bleached, an issue which the authors note may interfere with the isolation of microplastics from the samples.

Ultrasoundication can be used to disintegrate, fragment or solubilize solutes by using high-frequency sound waves to cavitate media, creating high shear forces. In the preparation of sewage sludge, Jin et al. found that using a combination of alkaline hydrolysis and ultrasoundication increased digestion efficacy by an average of 7.7% compared with using alkaline hydrolysis or ultrasoundication alone. However, our data showed that neither the use of an ultrasoundication bath or probe was any more effective than using NaOH alone. While the optimized alkaline digestion method was suitable for digesting plankton, it also proved damaging to microplastics: exposed to 10 M NaOH at 60°C, three of the five polymers (Nylon, polyethylene and uPVC) were damaged or discoloured. When applied to marine samples, use of alkaline hydrolysis could therefore result in an under-representation of pH-sensitive polymers or miscataloging of microplastic by colour, and as such cannot be recommended for this purpose. Similarly, while many plastics are resistant to hydrogen peroxide, Nuelle, et al. recently demonstrated that 35% H₂O₂ can result in significant (15.9 – 17.2%) size losses in exposed polyethylene and polypropylene microplastics.

The proteolytic enzyme Proteinase-K has previously been used to extract DNA from zooplankton specimens prior to molecular analysis. In using this protocol we attained a digestion efficacy of 88%, which was increased to >97% by increasing the concentration of Proteinase-K used, raising the active temperature to 50°C and prolonging the incubation period. Use of procedural blanks highlighted that by physically homogenizing our samples within plastic universal tubes we were introducing plastic shavings to the sample, which was rectified by switching to acid-washed glass containers. Undigested material consisted of fragments of shell, carapace, wood and anthropogenic litter embedded within a thin film of a clear, glutinous biological material. Although this biological material was not further analysed, we hypothesize it consisted of chitin, an insoluble, polysaccharide-based biopolymer present in zooplankton car apaces. Microplastic litter within the samples could be identified and extracted from this film without issue. However, if this chitin were problematic in future applications of this protocol then the enzyme chitinase could be applied to break down this residue. Bermejo et al. demonstrated that proportionate ultrasoundication efficiency was increased to 97% by increasing the concentration of alkaline hydrolysis from 0.5 M NaOH to 1 M NaOH. In our work, the optimized alkaline digestion method was suitable for digesting plankton, it also proved damaging to microplastics: exposed to 10 M NaOH at 60°C, three of the five polymers (Nylon, polyethylene and uPVC) were damaged or discoloured. When applied to marine samples, use of alkaline hydrolysis could therefore result in an under-representation of pH-sensitive polymers or miscataloging of microplastic by colour, and as such cannot be recommended for this purpose. Similarly, while many plastics are resistant to hydrogen peroxide, Nuelle, et al. recently demonstrated that 35% H₂O₂ can result in significant (15.9 – 17.2%) size losses in exposed polyethylene and polypropylene microplastics.

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increases the specific activity of enzymes in solution. However, in ultrasonicallying our enzymatically digested marine samples, we noted a substantial decrease in digestion efficacy compared with using enzymatic digestion alone. This likely resulted from the formation of protein precipitates within the digestion media, formed as a by-product of intense cavitation37, which were then unable to pass through the mesh-filters post-treatment. In contrast with the acid and alkaline digestion, enzymatic digestion is conducted at a neutral pH and moderate temperature and has biological specificity36. It was therefore of little surprise that the enzymatic protocol had no discernible effect on any of the five microplastics exposed. We would therefore consider enzymatic digestion to be highly applicable in removing biological material from biota-rich marine samples to elucidate microplastic pollution.

The optimized enzymatic protocol was trialled on whole-trawl samples collected from the western English Channel, and revealed 0.30 items of anthropogenic debris m$^{-3}$. FT-IR analysis revealed that...
while the majority of suspected plastics consisted of a synthetic polymer, ~5% of these items were made of leather. In extrapolating this data, and by defining a microplastic as <5 mm diameter, we identified an average of 0.27 items of microplastics debris m⁻² in the western English Channel trawls. This value is consistent with the broad range of concentrations (0.014–12.51 items m⁻²) observed in similar studies conducted across the globe. By using finer, 200 μm nets in combination with enzymatic digestion, we successfully identified 27 fibres, fragments and beads 25–250 μm in size across our four sub-surface trawls. Data relating to microplastics within this size range is scant. Only two of 33 studies investigating pelagic microplastics used nets finer than 300 μm aperture. Yet it is microplastics of this size that can be ingested by zooplankton and benthic invertebrates, resulting in reduced feeding rates and declining energetic reserves. Lusher et al. recently identified that 36.5% of 544 pelagic fish sampled at study site L4 in the English channel (described above) contained microplastics (comparable in size to those found within our samples) within their intestinal tracts. Within our samples, polyamides (e.g. Nylon) and polyethylene were some of the most commonly noted polymers; these pH-sensitive polymers may have been destroyed if acid, alkaline or hydrogen peroxide were used on the samples, whereas they survived enzymatic digestion. It would be presumptuous to draw further conclusions from these trial samples alone. Nevertheless, based on our findings we can recommend enzymatic protocols to detect microplastics in plankton samples in future studies.

The smallest microplastics identified in our sample were brightly coloured fragments 25 μm in diameter. Microplastics of this size may be retained on wider aperture mesh-filters and nets when they become clogged, although it is also possible that plastic litter of this size might have been internalized by zooplankton present within the sample. However, we cannot rule out the possibility that desiccating and grinding samples using a mortar and pestle may result in thermal and abrasive degradation of larger microplastics within the sample. This concern could be mitigated by using alternate preservation techniques (e.g. formalin, ethanol) and avoiding physical grinding, however, the efficacy of enzymatic digestion may be diminished where preservatives have been applied and the available surface area of the biological material has not been optimised. The range of colours identified suggest bleaching of plastics was not an issue, and stands as further proof that enzymatic digestion has minimal effects on plastics. Despite the commonality of white or clear plastics used in packaging, fishing line, clothing, personal care products and other plastic products, we did not isolate any uncoloured microplastics <250 μm in our samples. Furthermore, in considering shape, the majority of plastic litter identified in our sample (and in sediment and water-column samples from around the world) were fibrous. While such trends may be representative of the microplastics present in the marine environment, it is also important to consider that there may be a strong operator selection bias towards fibrous and brightly coloured microplastics owing to the relative ease of their identification.

The enzymatic digestion protocol was also successfully used to reveal fluorescent polystyrene beads ingested by a marine copepod. Previously, Claesens et al. trialled using nitric acid digestion to detect microplastics ingested by mussels, but found that both Nylon and polyethylene could not survive this harsh, oxidizing treatment. As enzymatic digestion is biologically specific, it is able to digest tissue without damaging plastics, and therefore proved highly applicable in this study. Fluorescent beads retained on the filter were enumerated with ease. While we anticipate that this protocol would be ideal for digesting field-collected zooplankton (and perhaps larger marine organisms) to look for evidence of microplastic ingestion, better techniques for visualizing and/or identifying very small non-fluorescent microplastics are still required.

Conclusion
We have developed a rapid method in which a proteolytic enzyme treatment can be applied to marine samples to digest away biological material without damaging any microplastics present. As research shifts from oligotrophic gyres to biologically rich coastal systems, it is hoped that this enzymatic protocol can be widely applied to better identify microplastics within these vulnerable ecosystems. By using this method in conjunction with finer sampling nets, data gaps relating to the concentration of microplastics <300 μm can be addressed. The enzymatic protocol has also been shown as an apt technique for identifying microplastics internalized by marine zooplankton. Further work is now required to trial the technique on field-collected specimens and to develop means of better visualizing very small, ingested microplastics.

Methods
Zooplankton sampling. Development of the digestion technique was performed on zooplankton collected as part of the long-term time series at a research station located in the English Channel (station L4), located 12 km south of Plymouth, UK (50°19’N, 04°13’W; Figure 2; www.westernchannelobservatory.org.uk). Horizontal, sub-surface tows using 200 μm and 500 μm plankton nets were used to collect zooplankton throughout August and September 2013. These biota-rich samples were transferred to clean, insulated containers, and transported to Plymouth Marine Laboratory (PML) within two hours of trawling.

Sample preparation. Zooplankton samples were passed through 200 μm meshes, and retained material was flushed with purified water (Milli-Q) to remove salt. Any macrozooplankton or large items of debris were rinsed thoroughly and then removed from the sample. Meshes were folded and sealed, and then oven-dried at 60 °C for 24 hours to both preserve samples and enhance the efficacy of grinding up the biological material in later steps. Desiccated samples were carefully groups with a mortar and pestle to increase the surface area of the biological material present. For method development and optimization, desiccated samples were pooled and 0.20 g DW sub-samples weighed out and then carefully transferred into digestion vessels.

Mitigating contamination. As microplastics may be airborne, present in clothing or adhered to laboratory equipment, we undertook a number of steps to prevent sample contamination. All apparatus were acid-washed and/or rinsed thoroughly with Milli-Q prior to use, while consumables were used directly from packaging. Sample preparation was conducted within a sterile algal-culturing unit. Samples and equipment were covered wherever possible to minimize periods of exposure. Personal protective equipment was worn at all times. Procedural blanks (absent of biological material or microplastics) were run in parallel with samples containing desiccated plankton, microplastics or trawled material. To demonstrate the efficacy of our preventive measures, procedural blanks were poured through mesh filters (per the methods below) and retained material analysed under a digital light microscope (Olympus SZX16) to check for contamination.

Acid and alkaline digestion. To compare the efficacy of acid or alkaline hydrolysis in digesting marine zooplankton, we used hydrochloric acid (HCl) or sodium hydroxide (NaOH) treatments. In brief: 20 mL of 1 M HCl, 2 M HCl, 1 M NaOH or 2 M NaOH (n = 3 per treatment) were added to glass flasks containing the sub-samples and maintained at room temperature for 24 h. We further trialled 5 M and 10 M NaOH at room temperature for 48 h, and 1 M and 10 M NaOH at 60 °C for 24 h (n = 3 per treatment). The optimized alkaline protocol required 40 mL of 10 M NaOH per 0.2 g dry weight (DW) sample, maintained at 60 °C for 24 h. Prior to analysis, samples were neutralized using HCl or NaOH as required.

Enzymatic digestion. The enzymatic digestion protocol developed by Lindeque and Smirnoff, was used to adapt 500 μg mL⁻¹ of Proteinase-K per 0.2 g DW sample, within universal tubes (n = 3). To maximize digestion efficacy and avoid contamination, desiccated samples were transferred into 50 mL acid-washed, screw-top glass containers with 15 mL homogenizing solution (400 mM Tris-HCl buffer, 60 mM EDTA, 105 mM NaCl, 1% SDS). Samples were physically homogenized by drawing and expelling the mixture through a 19G needle attached to a 10 mL syringe, making sure to thoroughly rinse the insides of the needle and syringe with homogenizing solution to avoid loss of material. Samples were incubated at 50 °C for 15 minutes, followed by then 500 μg mL⁻¹ of Proteinase-K added before incubating the samples at 50 °C for a further 2 hours. Next, 5 M sodium perchlorate (NaClO₄) was added and samples shaken at room temperature for >20 minutes. Solutions were physically homogenized a second time using a finer 21G needle, and then incubated at 60 °C for 20 minutes.

Ultrasonication. We employed two ultrasonication techniques to assist in the breakdown of digested samples: (a) the 5 M and 10 M NaOH digestions were placed in an ultrasonication bath for 10 minutes; (b) a sonication probe was used to directly sonicate samples that had undergone alkaline (1 M NaOH, 60 °C and 10 M NaOH, www.nature.com/scientificreports
60°C) and enzymatic (Proteinase-K, 50°C, 2 h) digestion. With both methods, samples were ultrasonicated on ice to prevent excess heat.

**Digestion efficiencies.** Post-digestion (and ultrasonication, where applicable) samples were vacuum-filtered onto pre-weighted 50 μm mesh-filters. Retained biological material was flushed copiously with Milli-Q, and then filters removed, covered and oven-dried at 60°C. Following desiccation, filters were weighed and digestion efficiencies calculated by comparing the relative removal of organic mass during the digestion. In a follow-up experiment, we compared the use of 50 μm, 20 μm and 0.02 μm filters (n = 3 per filter size) following a series of optimized enzymatic digestions.

**Microplastics.** Five types of microplastic, varying by polymer, shape, size and colour and representative of the array of microscopic plastic debris present in marine ecosystems were sourced for method validation: (a) white expanded polystyrene spheres (1.5–3.0 mm in diameter) were accrued from packaging material; (b) yellow-green Nylon monofilament fishing line (400 μm diameter, length 25 m); (c) black polyethylene fibres (10–30 μm diameter, length 0.75–1.5 mm) were individually plucked from a 100% polyester garment; (d) 100 mL of a domestic personal care product was mixed with 1 L of Milli-Q, and then filtered through a 50 μm mesh to retain a combination of black and white polyethylene fragments (60–500 μm diameter); (e) firstly, we purchased white unpigmented polyvinylchloride (uPVC) granules (60–120 μm diameter; Goodfellows). Microplastics were measured using a microscope (Olympus SZX16; x16–62 magnification) fitted with an eye-piece graticule or cellSens software (Olympus). The constituent polymers of the microplastics were confirmed using Fourier transform infrared spectroscopy (FT-IR; Bruker Hyperion FT-IR microscope) in conjunction with a reference database.

**Impact of optimized digestion protocols on microplastics.** Optimized alkaline and enzymatic digestion protocols were applied directly to each of the five selected microplastics. Following the prescribed method, microplastics were retained on 50 μm mesh-filters, rinsed with Milli-Q and then oven-dried. Microplastics were weighed (in mg) as granules, polyethylene fragments, or counted (polystyrene flakes, Nylon fibres, polyester fibres), and subsequently checked for damage under a microscope.

**Validation of the enzymatic digestion with in-situ microplastic debris.** We applied the optimized enzymatic digestion method to replicate travel samples from two sites in the western English Channel (Figure 2). Sampling was conducted in mid-October 2013 using two 200 μm aperture WP2 nets towed in parallel for approximately 500 m (SI Table 1). The volume of water filtered (V) was calculated by considering the radius of the net aperture (r) and length of tow (L) as confirmed via GPS, and applying the 95% filtering efficiency specific to 200 μm net-aperture (r) and length of tow (L) as confirmed via GPS, and applying

**Results from the ICES/GLOBEC sea-going workshop.**

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Author contributions
M.C. conceived and designed the experiments. M.C. and H.W. conducted the experimental work and analysed the data. M.C. wrote the paper. P.L., E.F., C.H. and T.G. provided their expertise and reviewed the manuscript.

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