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Extended Abstract (4 X A4 pages)

ISOLATION AND IDENTIFICATION OF ANTHROPOGENIC PARTICLES IN FISH STOMACHS BY RAMAN SPECTROSCOPY: A NEW METHOD

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Introduction

In 2013, 299 megatons of plastic were produced worldwide[1]. It is estimated that 10% of this production ends up in the seas[2]. Among these marine debris, two size classes are commonly defined: macroplastics (> 5 mm) and microplastics (MP). Impacts caused by marine macroplastics have been described since the late sixties while MPs have been studied more recently, likely due to the difficulty to isolate and identify them precisely[3]. Nevertheless, MP are widespread in the marine environment[4,5,6] and there are recent concerns about their toxicity on wildlife[7,8]. They affect organisms at the first levels of marine food chains. Several studies used the color, size and shape as criteria for MP identification[9,10] while visual examination is subjective. Consequently, the use of these criteria is very hazardous to discriminate accurately the exact nature of the particles. In fish, anthropogenic particles (AP) were found in different species and identified as MP[11,12] but other products can have similar colors, shapes or sizes. Consequently analytical methods are required to identify the chemical composition of the particles[13]. Methods to monitor the abundance of anthropogenic debris (including plastics) in marine environment vary considerably between countries and organizations. As a consequence, the OSPAR Commission is currently taking steps to introduce standardized protocols[14,15]. The main objective of this study is to propose a new method to isolate AP (MP and textile fibers of natural origin) from stomach contents of fish. The method is adapted to Raman spectroscopy in order to identify the chemical composition accurately. The Raman spectroscopy analyses suffer often from fluorescence, particularly when applied to biological samples. The present method avoids as much as possible this type of problems. It has been tested on stomach content of three planktivorous clupeiform species: the Atlantic herring *Clupea harengus*, the European pilchard *Sardina pilchardus* and the European anchovy *Engraulis encrasicolus*.

Materials and methods

Reference samples

Several plastic families and one piece of cotton were used to set up the method. These families were chosen due to their high production and worldwide consumption. Five replicates of one piece of cotton and seven polymers families [polycarbonate (PC), polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and Nylon] were treated in order to ensure their resistance to the different solutions used in this method. Before and after the treatments, particles were analyzed by Raman spectroscopy. It allowed us to attest that plastics and cotton are not chemically affected.

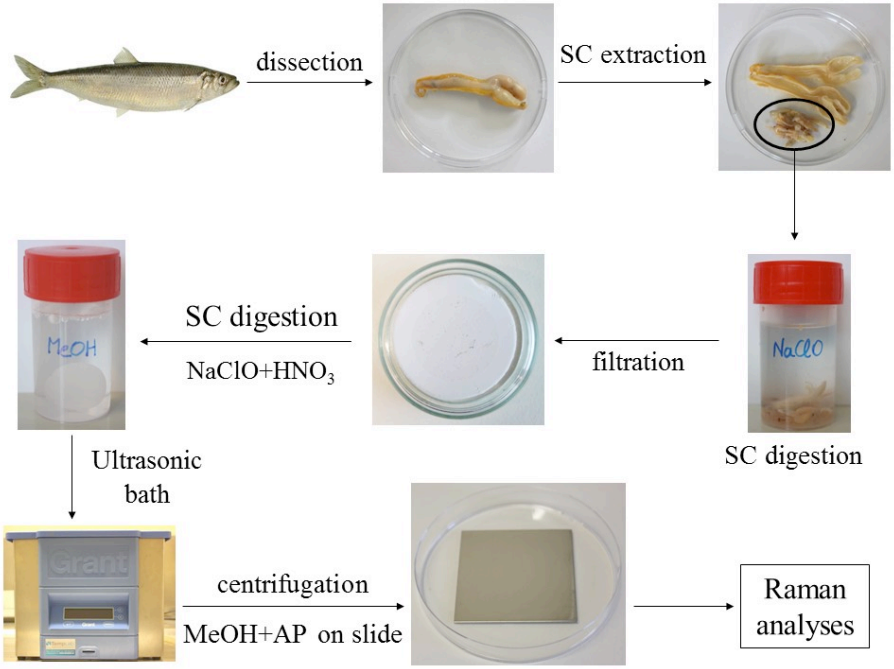
Fish sampling

Stomach contents of one individual of three species were sampled: an Atlantic herring *Clupea harengus*, a sardine *Sardina pilchardus* and an European anchovy *Engraulis encrasicolus*. The herring and the sardine were sampled during the International Bottom Trawl Survey (IBTS) and the anchovy was sampled during the Pélagiques Méditerranée (PELMED) survey, both organized by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). The herring and the sardine were sampled in January 2013, in the North Sea and in the English Channel respectively. The anchovy was sampled in July 2013 in the Gulf of Lions.

Sample preparation

In order to digest organic matter, stomach contents were stored in a 9 % sodium hypochlorite solution during one night. This solution was then filtered with a cellulose acetate filter membrane. It was rinsed with a nitric acid solution (65 %) diluted with the same NaClO solution (1:10 v/v ratio). The $NaClO/HNO_3$ volume was then brought up to 30 ml. After 5 minutes the $NaClO/HNO_3$ solution was filtered. The membrane was then put into a 15 ml methanol solution. The solution was then exposed to ultrasounds during 5 minutes. Afterwards, the membrane was manually removed. The methanol solution, containing AP, was then centrifuged at 5000 rpm during 10 minutes. After centrifugation, the bottom was collected and put on a 26 cm² polished stainless-steel plate. The small volume of methanol dried in approximately 2 hours. The isolation protocol is summarized in Figure 1.

Figure 1 Summary diagram of the isolation method. SC = stomach content, AP = anthropogenic particles.



Raman spectroscopy
Forty control samples (35 MP and 5 pieces of cotton) and 26 particles found in fish stomach were analyzed using a LabRam 300 spectrometer (Jobin-Yvon) provided with an Olympus confocal microscope and a CCD detector. Two lasers were used depending on the particle color. The laser spot was focused on the target using a CCD camera. The integration times ranged from 5 to 50 seconds per spectral portion, depending on the sample. Where necessary, a baseline correction was applied to the recorded spectra using a polynomial regression model and homemade software. Matchings between recorded spectra and references from commercially available or homemade libraries were performed using the Thermo Specta 2.0 software.

Particles images and weights
After Raman analyses, AP on stainless steel were photographed using a MOC-510 Mueller-Optronic 5 megapixel CMOS camera set on a stereo microscope. AP were weighed with an analytical balance with an accuracy of 0.01 mg. All particles from one sample were weighed together, and not separately. The software ImageJ was used to measure the maximum length of each AP.

Results and discussion:
Our isolation method was first tested twice on commercial plastics and cotton samples by following the mass variation after each step. Only one solution causes an important mass loss; methanol provoked an important loss on PVC particles. The Raman spectra were found identical before and after treatments. It proves that our

isolation method does not affect the chemical nature of the studied compounds. In addition, the fluorescence level was low despite the chemical treatment.
The isolation method revealed that the three stomachs contained 26 non-degraded particles. Interestingly, only eight of the 26 particles were made of plastic. Three plastic types were found: PE, PP and PET. The majority of "non-plastic" particles were made of vegetal material such as cellulose.
AP length in fish stomachs ranged between 0.18 mm and 9.49 mm with a mean of 1.60 mm (n=23, SE=±0.42). Plastic particles had a mean length of 2.59 mm, with a minimal value of 0.22 mm and a maximal value of 9.49 mm. The longest particle was a plastic particle (PP) found in the sardine's stomach.
The method for the isolation of anthropogenic particles developed in the present study seems reliable. Out of 26 particles found, 23 were from anthropogenic sources. This method fitted very well to a Raman spectroscopy analysis as there was no alteration of chemical composition observed after the isolation. Plastic particles are however easily identified by Raman spectroscopy because they produce strong Raman spectra whereas vegetal materials produce weak spectra. Methanol was chosen because of its high volatility and its low density compared to plastic or cellulose ones. PE, PP, PET, PS and PVC, which are the most common plastics[16] have a density superior to 0.9 g/cm³ whereas methanol weighs 0.79 g per cm³ [17]. This method could fit to stomach contents of carnivorous fish or even of other animals if concentrations of solutions are adapted.

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