Characterisation of microplastics and unicellular algae in seawater by targeting carbon via single particle and single cell ICP-MS.

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Abstract:

The discharge of plastic waste and subsequent formation and global distribution of microplastics (MPs) has caused great concern and highlighted the need for dedicated methods to characterise MPs in complex environmental matrices like seawater. Recently, single particle inductively coupled plasma – mass spectrometry (SP ICP-MS) was demonstrated to fulfill the requirements for rapid nano- and microparticle analysis, including number and size characterisation. However, the analysis of carbon (C)-based microstructures such as MPs by SP ICP-MS is at an early stage. This paper investigates various strategies to improve figures of merit to detect and characterise MPs in complex matrices, such as seawater. Ten methods operating distinct acquisition modes including kinetic energy discrimination (KED) and ICP with tandem MS (ICP-MS/MS) and targeting ^{12}C or ^{13}C were developed and compared for polystyrene-based MPs standards in pure water and seawater. The robust analysis of MPs in seawater was accomplished by on-line aerosol dilution enabling repeatable size calibration while minimising drift effects. However, the direct analysis of seawater decreased ion transmission and required matrix-matching for accurate size calibration. Analysis of the ^{12}C isotope instead of ^{13}C improved the size detection limits (sDL) to 0.6 µm in pure water and to 1.0 µm in seawater when operating methods without cell gases or H_2 for KED. ICP-MS/MS methods reduced ion transmission but improved background signal and increased selectivity, particularly in the presence of spectral interferences. In the second part of this study, it was demonstrated that the methods were applicable for the analysis of C in unicellular organisms concurrently with polystyrene-based MPs, providing instant mass and size calibration. It was demonstrated that MPs standards were applicable for C mass calibration and cell size modelling of five different Symbiodiniaceae algae strains with diverse life-histories, including free-living and symbiotic states (e.g., as essential symbionts for scleractinian coral). The level of C in algae and the physical dimensions play important roles in the investigation of phenotypical traits associated, for example, with climate resilience as well as oceanic carbon storage. Results obtained by SP and SC ICP-MS were in line with data obtained by Coulter-counting and microscopic analysis as complementary techniques.

Keywords: SP ICP-MS, SC ICP-MS, Carbon analysis, microplastic, algae, ICP-MS/MS

Introduction

Carbon (C) associated micro-scaled structures have diverse significance across various scientific disciplines. While C is an essential building block for life, it was also the motor for the industrial revolution and the backbone of modern society which has relied on the exploitation of fossil fuels since the mid-18th century. Fossil fuels are used as feedstock for a large amount of C-based chemicals such as plastics, whose convenience and ease of manufacture have led to a widespread use [1]. In the 1970s, it was recognised that increasing plastic production and questionable waste-disposal strategies would lead to a significant discharge of plastic into the environment [2–5]. Nowadays, it is clear that the manufacture of millions of tonnes of plastics during the last century poses a significant and persistent environmental burden that threatens pristine marine ecosystems world-wide [6]. Within the marine environment, plastics are worn down by friction into powders. Exposure to radiation then causes photo-oxidation which is followed by thermal and chemical degradation producing micro-scaled plastic debris with various forms and shapes [7]. Micro-plastics (MPs) easily migrate throughout the environment where they are ingested and are accumulated by biota [8–11]. Furthermore, MPs may accumulate chemical substances and metals on their surface and therefore act as a toxic beacon [12–14]. These findings motivated the development of dedicated methods for the sampling and analysis of MPs [15–17]. Common strategies for the characterisation of MPs involve microscopy, spectroscopy, and thermal analyses [18,19]. However, recent advances in the field of elemental mass spectrometry can characterise MPs by directly targeting C or other incorporated elements associated with MPs [20].

Inductively coupled plasma – mass spectrometry (ICP-MS) has become the method of choice for the analysis of metals and metalloids in complex matrices [21]. As hyphenated techniques, gas [22] or liquid chromatography [23,24] and laser ablation coupled to ICP-MS enable speciation analysis and elemental bioimaging [25]. One promising approach to characterise individual nano- or microstructures and to differentiate between ionic and particulate elemental species is single particle (SP) ICP-MS, where particles are introduced into the

hot argon-based plasma individually. After the atomisation and ionisation of elements within individual particles, secluded ion clouds can be extracted from the plasma. These are detected as short pulses which can be resolved with several data points when operating a fast mass analyser with dwell times of around 100 µs or below. The frequency of these pulses is indicative of the number of particles and their intensity is proportional to the number of atoms enclosed in each particle [26]. Consequently, SP ICP-MS allows the sampling of hundreds to thousands of particles per minute to accurately describe parameters such as particle number concentrations and provides valuable statistical models of particle size distributions [27]. It has been shown that this strategy is applicable to the characterisation of nano- and microplastics by following the signal of metals or nanoparticles conjugated or incorporated to MPs [14,28–33]. Recently however, Bolea Fernandez et al. [20] demonstrated that SP ICP-MS may be employed to characterise MPs more directly by targeting the C content by monitoring the isotope ^{13}C. Laborda et al. [34] subsequently showed that this approach can be used to characterise MPs in consumer products. This approach has also tremendous potential for the analysis of MPs in the environment [35]. However, complex matrices call for dedicated preparation and standardisation strategies to maintain accuracy.

Biological cells can also be regarded, similar to MPs, as micro-scaled C structures in which the C correlates to mass and size. Cells have important functions regarding the fixation and storage of C through photosynthesis impacting the global climate and ecosystem. It is estimated that the oceans have absorbed approximately 50% of the C emissions since the Industrial Revolution and can be regarded as a major sink of the anthropogenic CO_2 emission [36]. A large part of this CO_2 is stored as organic and inorganic C in microbes and coral reefs [37]. While the amount of C fixed in cells is of interest for C storage strategies, it is also important for the characterisation of cell function. For example, quantifying C content in algae has led to understanding Symbiodiniaceae symbiosis with coral [38,39]. It is routine to determine the C content and the cell number (sometimes referred to as density) to normalise concentrations of other elements and organic compounds as well as to determine for example the photoactivity [40]. The cell numbers, volumes and total C concentration are often determined in separate experiments using combustion techniques coupled with infrared spectroscopy, Coulter counters, flow cytometry or microscopy. It hs been shown that SP ICP-MS can be modified to characterise individual cells in single cell (SC) ICP-MS [41]. The recent progress and the analysis of C in individual cells may allow the characterisation of the fixation of C, C storage, C metabolism, photoactivity and would also provide new models regarding the C mass distribution across cell lines or algae species. Furthermore, cell number concentrations and sizes may be calculated if additional parameters are known.

An example of a unicellular algae where the simultaneous measure of C, cell size and cell number would be valuable is the *Dinoflagellate* Symbiodiniaceae that commonly exists in a symbiotic state with invertebrates (e.g., coral) but can also attach to substrate or exist in a free-living form [42]. When in symbiosis with coral, their capacity to fix inorganic C and translocate C compounds to their host is central to healthy reef functioning [43,44]. In their free-living state, the metabolic exchange between Symbiodiniaceae and associated bacteria can result in the formation of calcified “symbiolites” [45]. The diverse ecological importance of Symbiodiniaceae have made them central across decades of research to understand how their phylogenetic, phenotypic, and environmental histories shape their functional diversity, with a focus on resolving factors that shift their relationship with coral from eubiosis to dysbiosis [39]. The latter has become increasingly important as coral reefs worldwide are under threat from climate change, predominantly due to warmer waters that trigger corals to expel their symbiotic algae. The generation of reactive oxygen species in algae within harsh environments appears to be a major factor for coral bleaching and mechanisms to mediate oxidative stress are currently being investigated [46]. Various elements are associated with the occurrence and mediation of reactive oxygen species and SP ICP-MS may be a viable technique to study metabolically active trace metals like Zn, Fe or Mn. The analysis of C may be useful as technique to normalise data sets by C mass, cell mass or size.

SC ICP-MS analysis is a viable method for the characterisation of various elements in individual cells [41,47]. For example, the marking of antigens on cell membranes with metal-coded antibodies stimulated the field of mass cytometry [48]. Von der Au et al. recently demonstrated that SC ICP-MS may be employed for the analysis of endogenous elements in individual diatoms [49]. However, it must be considered that cell suspensions are a highly dynamic living system, which sensitively respond to changes in the environment. For example, removal or exchange of cell media can cause osmotic pressures causing cellular damage, and sheer forces during the aerosol generation may rupture cell membranes. Therefore, analyses are best performed in the native matrix or quickly after matrix separation. In another study by von der Au et al., the authors report an elegant method to remove the interfering seawater matrix by employing an automated on-line high-performance liquid chromatography (HPLC) system, which trapped, washed, and eluted diatoms for online SC ICP-MS [50].

In this study, the application of ICP-MS and ICP-MS/MS was investigated for the characterisation of MPs in pure water and seawater. Different acquisition modes (no gas, KED, mass shifting) were evaluated using different cell gases. SP ICP-MS for the analysis of MPs and cells in seawater was assessed using a high matrix introduc-

tion (HMI) system. The concept was subsequently applied for the analysis of C in individual algae in seawater and MPs were used for instant mass and size calibration. In a proof of principle, five different unicellular Symbiodiniaceae species were analysed for C content and results were validated using complementary methods.

Materials and Methods

Chemicals and consumables

MPs (polystyrene) particle standards were purchased from Sigma Aldrich with diameters of 4 ± 0.1 µm and 3 ± 0.1 µm. Particles had a density of 1.05 g cm^{-3}, were 2% cross linked and were stored at 4°C as aqueous suspension at a concentration between 1 and 10% (solids). Ultra-pure water (18.2 MΩ) was obtained from an Arium® pro system (Sartorius Stedim Plastics GmbH, Germany) and used for dilution steps and to obtain blank values. Dilutions were carried out in containers made of polypropylene. Artificial seawater with IMK medium (further details below) was chosen for culturing cells and was also used for matrix-matching of MP-based standards. The determination of the transport efficiency required the estimation of the elemental responses of C in pure and seawater and was determined by analysing methanol (LC-MS grade (LiChrosolv®), Sigma Aldrich) diluted to a concentration of 54 µg C mL^{-1}. To determine the background/noise level, ultrapure water and seawater were analysed individually as blanks.

Cells and culturing conditions

Five isolates of Symbiodiniaceae (*Symbiodinium microadriaticum*, *Breviolum* sp., *Cladocopium* sp., *Durusdinium trenchii*, *Effrenium voratum*) were subcultured from a long-term laboratory stock at the University of Technology Sydney. Strains were from five genera (*Symbiodinium, Breviolum, Cladocopium, Durusdinium, Effrenium*) originating from a range of geographic locations and host organisms. Further species-specific details are given in Table 1. Isolates were grown in sterile polystyrene cell culture flasks (Falcon, Corning, New York, USA) in semicontinuous batch mode and were diluted every 4-5 days to maintain exponential growth and optically thin conditions (c. 40 000-150 000 cells mL^{-1}). All strains were cultured in sterile 0.2 µm filtered artificial seawater with Daigo's IMK medium (Nihon Pharmaceutical) and grown at 26.0 ± 0.5°C under an irradiance of *ca.* 65 µmol photons m^{-2} s^{-1} (4π LI-190SA Quantum Sensor, Licor) set to a 12 h: 12 h light: dark cycle. The abundance of cells was determined by subsampling 1 mL every four days for quantification using a haemocytometer (Neubauer Haemocytometer, Fisher Scientific, Loughborough, UK).

ICP-MS instrumentation and conditions

An 8900 series ICP-MS/MS system was operated with MassHunter software (Agilent Technologies) and equipped with Pt cones. The inner diameter of the torch was 2.5 mm and the aerosol was generated using a concentric nebuliser and a Scott double pass spray chamber cooled to 2°C for the analysis of ultrapure water and to -5°C for the analysis of seawater. The instrument was tuned daily by analysing a solution containing 1 ng mL^{-1} Li, Y, Th, Ce and Ba. To mitigate matrix effects and signal drift, an HMI system (Agilent Technologies) was used for on-line aerosol dilution. The nebuliser gas flow was 0.40 mL min^{-1} and the dilution gas flow was 0.60 mL min^{-1}. The integration time was 0.1 ms for SP ICP-MS and SC ICP-MS. Tune parameters for SP ICP-MS were obtained by visually adjusting signal to noise ratios whilst analysing a 4 µm MPs standard.

Different ICP-MS acquisition modes were compared to identify the optimal method for the detection of C in MPs and individual cells. Methods using kinetic energy discrimination (KED) or no cell gasses used a SQ mode, which means that the first quadrupole was operated with a large mass bandpass. Mass shifting methods employed H_2, He, O_2 and NH_3 as cell gases and operated in MS/MS mode. He and H_2 were obtained from Air Liquide Australia (NSW). O_2 had a purity of 99.995% (grade 4.5) and NH_3 was obtained at 10% in He from BOC (North Ryde, NSW). The extraction lenses were operated at -55.9 V (Extract 1) and -250.0 V (Extract 2),

Table 1. Information on the investigated unicellular algae species.

Genus	Species	ITS2 type	Culture isolate identity	Isolate identity used in this study	Geographic origin	Host taxa
Symbiodinium	*S. microadriaticum*	A1	CCMP2464, rt61	rt61	Florida (Caribbean Sea)	*Cassiopeia xamachana* (jellyfish)
Breviolum	*Breviolum sp.*	B1	CCMP2460, CCMP3345, rt2	rt2	Florida (Caribbean Sea)	*Aiptasia pallida* (sea anemone)
Cladocopium	*Cladocopium* sp.	C2	rt203	rt203	Palau (Pacific)	*Hippopus hippopus* (giant clam)
Durusdinium	*D. trenchii*	D1a	CCMP3408, A001, JS1313	CCMP 3408	Okinawa (Japan)	*Acropora* sp. (coral)
Effrenium	*E. voratum*	E	CCMP3420	CCMP3420	Santa Barbara (California)	Free-living

the omega lenses at -120 V (Omega Bias) and 6.0 V (Omega Lens) and the deflect potential was 7.6 V. The OctP Bias was between -1.0 and -4.0 V while operating between -20 and -11.0 V Energy Discrimination. The Axial Acceleration was between 0.0 and 2.0 V. Methods monitoring ammonia associated transitions employed cell gas flows of 1.0 mL min^{-1} He and 5% (tune parameter) NH_3. Oxygen associated transitions were monitored while employing 26% (tune parameter) O_2 and hydrogen associated transitions were monitored while employing 5.0 mL min^{-1} H_2. The KED methods employed 2.0 mL min^{-1} H_2 as collision gas.

Microscopy and Coulter Counter:

Three methods were used for cell counting and cell volume determination. First, manual cell counts were conducted using a haemocytometer with a total of eight replicate quadrats (16 squares) counted for each sample and the mean cell size for each isolate was determined according to Hennige et al. [51]. Second, 16 images (small haemocytometer quadrants) at 10x magnification were taken using a Nikon Eclipse Ci-L compound microscope (Nikon Instruments) and processed using the ImageJ2 software [52] with Fiji package [53] using a high-throughput image processing script [54] to determine cell count and cell volume. Third, 200 µL of each culture were fixed with glutaraldehyde (25%, Grade II, Sigma-Aldrich) for analysis on the coulter counter (Multisizer 4 Coulter Counter®, Beckman Coulter) using a 100 µm aperture tube. For each sample, three replicate runs were conducted, and cell volume and cell concentrations were averaged across runs.

Total organic carbon analysis

To quantify the total organic carbon (TOC) content of wet and freeze-dried cell pellets, 300 mL of each culture was subcultured into six 50 mL replicate falcon tubes (Fisher Scientific, Hampton, New Hampshire, United States), which were then divided into three replicate tubes for wet TOC (150 mL total) and three replicate tubes for TOC of freeze-dried cells (150 mL total). All tubes were centrifuged at 3,000 g for 10 min to pellet the algae cells, resuspended in 10 mL MilliQ and then centrifuged again at 1,000 g for 5 min. The supernatant was removed, and the washing step was repeated 3 times for each replicate tube to remove all media from the sample. For the freeze-dried samples, algal pellets were dried for 48 h prior to analysis (Alpha 2-4 LDplus Christ). Wet and dried (pre- and post-freeze drying) algae pellets were then weighed to four decimal places by removing as much cell pellet as possible and transferring into a new, accurately weighed falcon tube.

The carbon content of both the wet and dried cell pellets was measured using a TOC Analyser (TOC-Lesh model, Shimadzu, Kyoto, Japan) using the non-purgeable organic carbon (NPOC) protocol to detect organic carbon only. For the freeze-dried fraction, the cell pellet was resuspended in 50 mL of MilliQ water and sonicated for ~10 min to homogenise the samples. The cell pellets for both fractions were diluted into 50 mL MilliQ water in a 50 mL volumetric flask before being transferred into TOC sample vials (30 mL). Quality control suites were incorporated into each analysis run where a minimum of three analysis replicates were run for each sample and were found to be within 5% deviation of the original analysis values. Standards were run to monitor instrument drift where values were constant and within ± 0.59-0.16 standard deviation throughout the run. A spike recovery was conducted to monitor for matrix effects where the recovery value was ± 103.0 % recovery of the theoretical value. Additional procedural controls (8 replicates) of only MilliQ water were also analysed to determine the quantity of carbon introduced during the wash steps and dilution process. Carbon concentrations for each sample were then corrected (C concentration – the mean C in controls). The TOC concentration was normalised to the cell density to obtain the C mass per cell.

SP/SC ICP-MS Data processing

All ICP-MS data was acquired with MassHunter software (Agilent Technologies) and then further processed using an in-house developed Python-based software. To distinguish pulses detected in SP or SC ICP-MS from background and noise, three different thresholds were applied from which two considered a Poisson-like and one a Gaussian-like background signal distribution. At low count rates, a Poisson-filter was used as first described by Currie [55] for radioactive decay and discussed for SP ICP-MS by Tanner [56] and Mozhayeva and Engelhard [57] according to equations (6) and (7) using the mean of the background signal (b). For low backgrounds ($b < 5$) a correction factor (ε) of 0.5 was used. At higher background counts, a Gaussian filter using the 3σ criterion was used. The software subsequently counted and summed signals and determined various parameters including the transport efficiency, ionic background, noise, size detection limits, mean values and standard deviations. Microsoft Excel and OriginPro (OriginLab) were further used to model and illustrate data. The transport efficiency η was in this case calculated according to equation 1, where s is the response for an ionic standard, ρ the particle's density (1.05 g cm^{-3}), D the diameter (4 µm) of a reference particle, I the accumulated intensity for a detected reference particle, t the dwell time (0.1 ms), V the uptake rate (0.68 mL min^{-1}) and f is the mass fraction of the reference particle (0.923). The mass related response R was calculated according to equation 2, were m_{mean} is the mean mass of a reference particle and I_{mean} is the mean summed intensity per pulse. This allowed the translation of the C signal of an unknown particle into a C mass m according to equation (3). The diameter (D) of the unknown particle or cell was subsequently calculated according to equation (4) using the known density and

mass fraction of the unknown particle or cell. The size detection limit was calculated based on a study by Lee et al. [58]. When using a Gaussian filter, the noise threshold (T_{noise}) was three times the standard deviation σ of b. For comparisons, the ion transmission τ was calculated by calculating the ratio of C atoms (n) in a MPs reference and the average number of counts per MPs signal pulse (I) (equation 8).

$$\eta = \frac{s\pi\rho_p {D_p}^3}{6I\,t\,V} \quad (1)$$

$$R = \frac{m_{mean}}{I_{mean}} \quad (2)$$

$$m = RI \quad (3)$$

$$D = \sqrt[3]{\frac{6m}{\pi\rho\, f}} \quad (4)$$

$$sDL = \sqrt[3]{\frac{6T_{noise}}{\pi\rho\, fR}} \quad (5)$$

$$L_C = 2.33\sqrt{b+\epsilon} \quad (6)$$

$$L_D = 2.71 + 4.67\sqrt{b+\epsilon} \quad (7)$$

$$\tau = \frac{I}{n} \quad (8)$$

Results and Discussion

Micro-plastic analysis

The determination of MPs in environmental systems is becoming increasingly relevant, and robust methods that are capable of detecting particles at high counting rates, while also providing information regarding mass and size, are required to characterise MPs in complex matrices. SP ICP-MS is emerging as a potential solution to this problem however, the direct analysis of MP pollutants by ICP-MS requires the monitoring of C isotopes, which can be challenging, particularly in complex matrices. The first ionisation potential of C (11.26 eV) is higher than for most other elements, reducing the degree of ionisation within the plasma and lowering sensitivities. Furthermore, ambient CO_2, C contamination in the argon and high levels in investigated matrices produce elevated background signals which may limit the application of SP ICP-MS. It was previously shown that ^{13}C in MPs may be analysed via SP ICP-MS in rather simple matrices [20,34]. However, as most plastic waste is found in oceans this raises the question as to whether SP ICP-MS is also applicable to trace MPs in seawater matrices. Therefore, the feasibility and the figures of merit for the analysis of MPs in pure (MQ water) water and seawater were investigated and compared.

Figure 1A shows the detection of individual MPs in pure (blue) and seawater (red) using a method monitoring

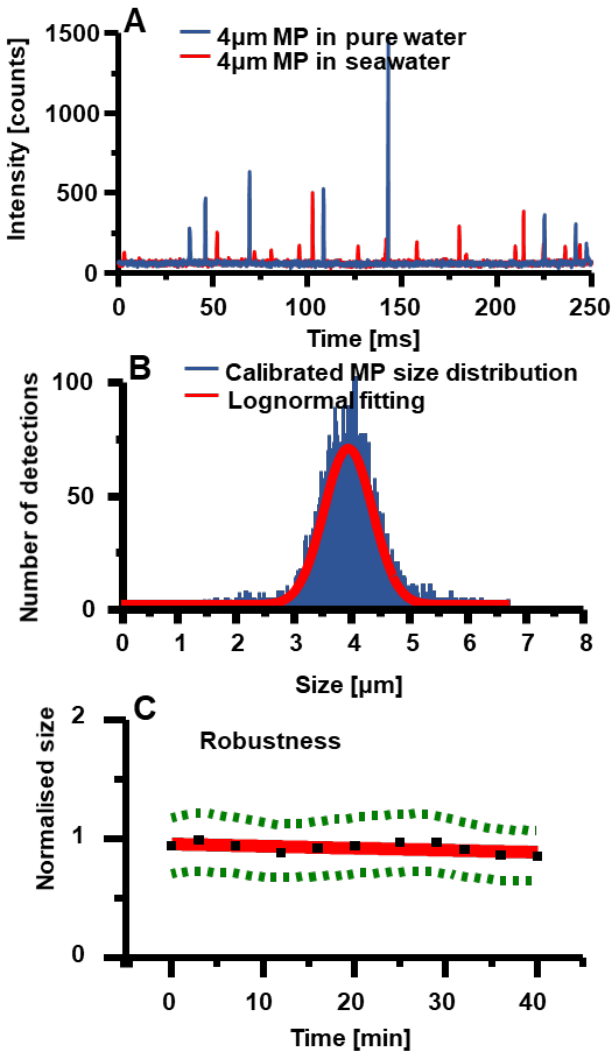

Figure 1. A: Analysis of 4 µm MPs in pure and seawater. The high ion strength of seawater reduced ion transmission leading to reduced sensitivities. **B:** Signals were calibrated according to equation 2-4 using a 3 µm MPs standard. **C:** Repeated size calibration of a 4 µm MPs spiked in seawater to investigate signal stability and drift.

the ^{13}C signal. While in both cases discrete signal pulses from MPs were detected, sensitivities decreased when analysing MPs in seawater. This was likely a result from space charge effects that influence the dynamics of extracted ions. The high abundance of ions from seawater defocuses the ion beam, resulting in deteriorated ion transmission specifically for light isotopes. This matrix-based deterioration must be considered when making accurate size calibrations and requires matrix-matching. It was found that neglecting matrix effects caused an underestimation of MPs sizes in seawater by 28%. However, the dilution of reference materials in seawater maintained accurate calibration as shown in Figure 1B, where the size distribution of a 4 µm MP standard in seawater was calibrated with a 3 µm MP standard in seawater. The analysis of matrices with

high ionic strengths induces drift effects due to the deposition of salts on the vacuum interface and ion lenses. This drift makes the analysis of seawater matrices challenging and requires additional considerations. As the developed methods were later applied to the analysis of individual cells, off-line dilution in ultra-pure water was avoided to mitigate osmotic effects and consequently, cell damage. Instead, an HMI system was employed which allowed on-line gas dilution of the sample aerosol with argon. This reduced the matrix load in the plasma but also reduced the transport efficiency from initially between 5-6% to 2-3%. To investigate method drift effects, a 4 µm MPs standard in seawater was repeatedly analysed and calibrated. Figure 1C shows the determined mean MPs sizes and sizes determined at FWHM (2.3548σ) in black and green respectively. A linear fit (red) was used to interpolate the drift rate, which was -0.1% per min. In SP ICP-MS, typical acquisition times per sample are between 30 and 90 seconds, and therefore, a 40-min signal stability allows the operator to perform between 30 and 80 separate analyses without the need for recalibration or cleaning.

Optimisation and figures of merit

The analysis of C in MPs is its infancy and so far, two studies analysing the ^{13}C isotope were reported [20,34]. In ICP-MS, the less abundant isotope ^{13}C (1.107%) is often preferred over ^{12}C (98.93%) to avoid high background intensities and spectral interferences. However, this can also be mitigated by employing tandem mass spectrometry and collision/reaction cells. In this study, we compared the figures of merit for the analysis of both ^{12}C and ^{13}C in MPs and further investigated the applicability of kinetic energy discrimination (KED) and "mass shifting" via ICP-MS/MS. In KED, the collision/reaction cell is filled with an inert gas to induce collisions reducing the kinetic energy of ions. Polyatomic interferences with large collisional cross-sections are subjected to an increased number of collisions compared to the smaller analyte ions, reducing their kinetic energy and allowing

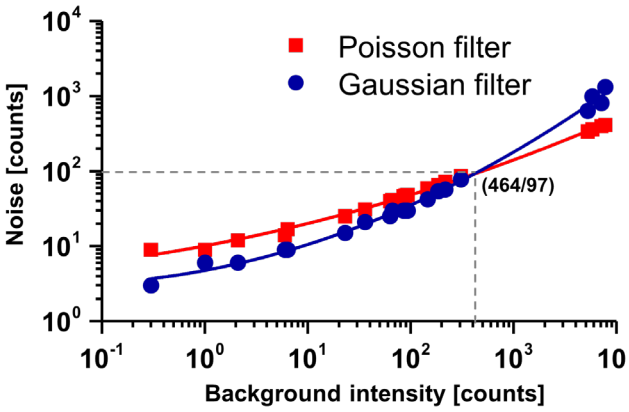

Figure 2. A Poisson filter and a Gaussian filter were compared to the set the threshold, over which a signal is recognised as MP. For count rates below 464, a Poisson filter according to equation 6 and 7 was used. Above, a Gaussian filter using the 3σ criterion was used.

them to be separated with an electrostatic barrier at the end of the collision cell [59]. In ICP-MS/MS, mass shifting is used to exploit analyte-specific chemistries by forming an adduct ion with an increased mass in the collision/reaction cell, which is then filtered in the subsequent quadrupole [60]. The *a priori* prediction of these chemistries is usually difficult as reactions occur under non-standard conditions. It became therefore common practice to investigate the performance of different cell gases, adducts and settings empirically.

In this case, ten different methods were developed and compared regarding ion transmission, noise and size detection limits (sDL). First, ^{12}C and ^{13}C were directly analysed without the use of cell gases (no gas), in which the second multipole (collision/reaction cell) was operated as a vented ion guide online. The two isotopes were analysed in "SQ" (single quadrupole) mode, in which the first quadrupole is operated as a mass filter with a large mass bandwidth [61]. In a second step, H_2 was used as a gas for KED for both ^{12}C and ^{13}C to reduce the impact of spectral interferences and noise. For mass-shifting via ICP-MS/MS, the cell gases H_2 (m/z → m/z +1), O_2 (m/z→m/z +16) and NH_3 (m/z→m/z + 15,16,17,18) were compared. ICP-MS/MS methods usually generated a low background, while KED and no-gas methods produced increasing background signals and noise. This had implications for the MPs signal recognition algorithm required for the differentiation of MPs signals from background and noise. Low background signals can be modelled via Poisson distributions as discussed by Currie [55], and proposed for SP ICP-MS by Tanner [56] and Engelhardt and Mozhayeva [57]. Limits of criticality and detection were calculated using equations 6 and 7. Contiguous signal regions above the limit of criticality that contained at least one point above the limit of detection were summed to produce a MP detection. At count rates below 5, a correction factor of 0.5 was used. However, at high counting rates, background signals approached a normal distribution and can best be described with a Gaussian distribution. To avoid the false identification of signals, a worse-case scenario approach was employed in which the method with the highest threshold was selected for peak recognition as well as to determine figures of merit. The threshold determined by the different filters were compared across background signals found for all tunes to determine the transition from Poisson-like to Gaussian-like noise as shown in Figure 2. For count rates below 464 counts a Poisson filter was used to distinguish between noise and MPs signals and a Gaussian filter was used for higher count rates.

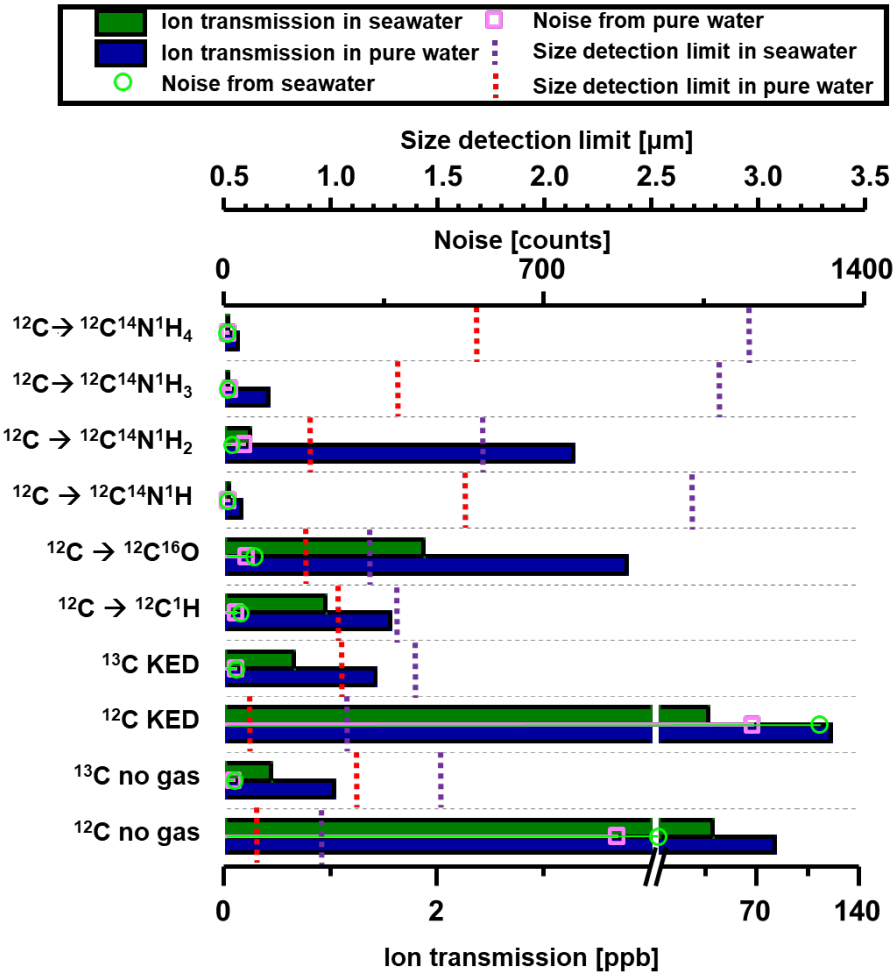

Figure 3. Figures of merit of all developed methods.

The ion transmission, noise/background levels and sDL were determined in pure and seawater for all ten methods and are shown in Figure 3. Exact values can be found in Table 2. Ion transmission was generally lower (37-90%) for C isotopes MPs analysed in seawater, which is likely caused by space charge effects, where a high number of matrix ions caused a defocussing of the ion beam and affected specifically the transmission of light isotopes such as ^{12}C and ^{13}C. Ion transmission was lowest for MS/MS methods with values between 0.1-3.77 ppb in pure water and 0.01-1.86 ppb in seawater. However, the no-gas and KED methods had ion transmissions between 1.01-121.5 ppb in pure water and 0.64-37.4 ppb in seawater. Interestingly, the KED method achieved the highest ion transmission, which may result from mitigation of space charge effects via collisional focussing in the collision/reaction cell. The noise increased with ion transmission and generally correlated with the background signal, (Figure 2), and required different models to estimate the noise levels. Background levels and noise were higher in seawater due to the abundance of organic compounds.

The sDL of each method was estimated using equation 5 combining the mass-related response and noise. Due to the higher ion transmission and decreased noise in pure water, sDL were generally 21-54% lower than in seawater. The sDL determined for ^{13}C were in line with the previous two studies [20,34]. However, analysing ^{13}C instead of ^{12}C led to 71-74% and 28-59% increased sDL in pure water and seawater, respectively. Lowest sDL were achieved with a ^{12}C no-gas method (pure water: 0.65 µm, seawater: 0.96 µm) and a ^{12}C KED method (pure water: 0.62 µm, seawater: 1.09 µm). Using ICP-MS/MS, best sDL were achieved with a method using oxygen as reaction gas and monitoring the transition $^{12}C \rightarrow {}^{12}C^{16}O$ (pure water: 0.88 µm, seawater: 1.19 µm) and similar sDL were obtained for pure water using a method employing ammonia as reaction gas monitoring the transition $^{12}C \rightarrow {}^{12}C^{14}N^{1}H_2$ (pure water: 0.90 µm, seawater: 1.72 µm). The monitoring of other ammonia reaction products deteriorated sDL substantially.

These results indicate that the robust analysis of MPs via SP ICP-MS in complex matrices like seawater is feasible by using on-line gas dilution of the sample aerosol and enables accurate size calibration when performing matrix matching. Analysing the ^{12}C isotope instead ^{13}C improved the figures of merit. Best sDL were achieved by using a method employing H_2 for KED and a method not employing any cell gases. For ICP-MS/MS, oxygen and a $+^{16}O$ mass shift achieved the highest ion transmission and best sDL in ICP-MS/MS which is relevant if

higher selectivity is required as discussed in the next section.

Table 2. Comparison of figures of merit. The Ion transmission, noise and sDL were determined for MPs in pure and seawater for ten methods using no gas methods, KED or tandem MS.

Acquisition mode	Ion transmission [ppb]		Noise [counts]		Size detection limit [µm]	
	Pure water	Seawater	Pure water	Seawater	Pure water	Seawater
$^{12}C \rightarrow {}^{12}C^{14}N^{1}H_4$	0.10	0.010	14	9	1.70	3.00
$^{12}C \rightarrow {}^{12}C^{14}N^{1}H_3$	0.39	0.014	25	9	1.32	2.86
$^{12}C \rightarrow {}^{12}C^{14}N^{1}H_2$	3.27	0.22	66	31	0.90	1.72
$^{12}C \rightarrow {}^{12}C^{14}N^{1}H$	0.14	0.02	17	12	1.64	2.74
$^{12}C \rightarrow {}^{12}C^{16}O$	3.77	1.86	72	86	0.88	1.19
$^{12}C \rightarrow {}^{12}C^{1}H$	1.54	0.93	47	59	1.03	1.32
^{13}C KED	1.40	0.64	46	48	1.06	1.40
^{12}C KED	121.5	37.4	810	1317	0.62	1.09
^{13}C no gas	1.01	0.42	40	41	1.13	1.53
^{12}C no gas	83.1	40.2	630	990	0.65	0.96

Single cell/algae analysis

SP ICP-MS is not able to distinguish between different C species and C bearing particles. While this may complicate the analysis of MPs in real seawater it also offers an elegant approach for the characterisation of unicellular organisms like algae via SC ICP-MS. C is an essential building block of life and its content varies across different cells. This is particularly relevant for algae that fix CO_2 from the atmosphere during photosynthesis. This fixation has important biological implications but also has a significant impact on the global climate. Use of algae as storage units for CO_2 is attracting increasing interest and is regarded as a future strategy to combat climate change due to anthropogenic CO_2 emissions [62]. Algae also have diverse ecological function, and as previously mentioned, Symbiodiniaceae play a major role in supporting coral health. SC ICP-MS offers an interesting and simple alternative to current methods for determining cell numbers and sizes as well as the mass of C and other biologically relevant elements.

In this study, the C content of five algae cultures (Table 1) was investigated via SC ICP-MS. Specifically, the parallel analysis of MPs reference materials allowed facilitated cell characterisation by providing immediate mass and size calibrations. Adverse effects due to the seawater matrix were overcome by using an HMI system for on-line aerosol dilution as demonstrated in Figure 1C for MPs analysis. Bright-field microscopy was employed for visual confirmation of the integrity of cells following nebulisation and the reported nebulisation parameters caused no visible damage to individual algae. As demonstrated for the analysis of MPs, different methods and acquisition modes offer different levels of sensitivity and selectivity. While SP ICP-MS/MS methods were found to be less sensitive, the exploitation of C specific reactions like the formation of CH^+, CO^+ or CNH_2^+ reduced both background and noise improving selectivity. This is useful when analysing single cells that contain a high abundance of ubiquitous and essential elements which may interfere with the analysis of C isotopes (e.g., Mg or Al). Employing oxygen as reaction gas to monitor the transition $^{12}C^+ \rightarrow {}^{12}C^{16}O^+$ showed best figures of merit of the investigated ICP-MS/MS methods in seawater and was chosen for selective analysis of algae via SC ICP-MS. A 4 µm MP reference material in seawater was analysed before and after algae analysis for calibration and no drift effects were observed. Figure 4 shows the transient analysis of *Breviolum* sp. (RT2, compare Table 1) as a representative algae species (Figure 4B) and 4 µm MPs particles in seawater (Figure 4A). Each signal pulse was recognised using a Poisson or Gaussian filter and integrated. The accumulated intensity was then converted into a histogram and fitted to

a lognormal distribution for comparison, as shown in Figure C and D. The mean intensity of the MPs signal distribution was used to determine a mass-intensity response factor which was then used to translate C signals from individual algae into a C mass per cell according to equation (3) and is shown in Figure 4E. The application of a 4 µm MPs particle suspension in seawater appeared to be optimal as they contained a similar amount of C atoms compared to detected cells and therefore caused a similar response, allowing operation within the linear dynamic range. Knowledge about the C mass fraction per cell allows the calculation of cell sizes or vice versa. In this case the C mass fraction was determined for each algae species by TOC analysis and the mass of individual cells obtained via bulk weighing and cell counting using a Coulter-counter. Application of equation 4 consequently enabled the determination of cell sizes as shown in Figure 4F. These sizes were compared to values obtained via Coulter-counting and visual size determination by microscopy with the results for all five algae species as shown in Table 3. The C mass per cell determined via TOC were within the standard deviation of results obtained via SC ICP-MS and calibrated by SP ICP-MS of MPs in seawater. It should be emphasised that the standard deviation of data obtained in SC ICP-MS, microscopy and Coulter counting was derived from inter-cellular variations whereas standard deviation for TOC analysis related to repeated (triplicate) bulk analysis. The size calibration of cells was possible by determining the C mass fraction per cell and assuming a cell density of 1 g/cm^3. The determined values were in line with results obtained via Coulter counting and microscopy and confirmed that C correlates linear with cell mass which portraits SC ICP-MS as

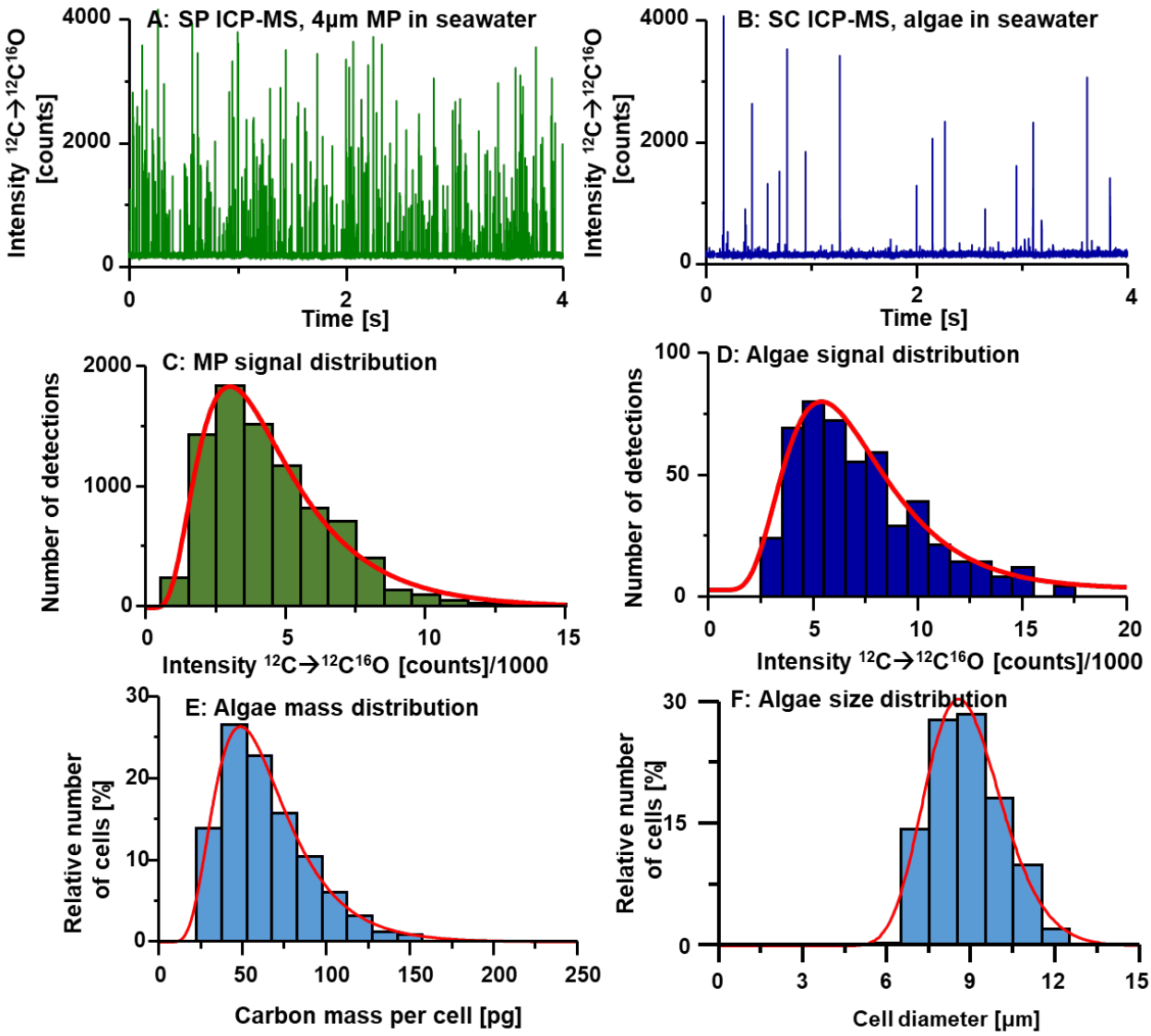

Figure 4. Combined SP ICP-MS and SC-ICP-MS for the determination of cell masses, mass distributions and size distributions. ICP-MS/MS monitoring the transition $^{12}C^+\rightarrow{}^{12}C^{16}O^+$ was used to detect individual MPs particles **(A)** and individual unicellular algae **(B)**. The construction of a signal histogram **(C)** allowed to determine the mass related response factor, which was used to translate the signal distribution per algae **(D)** into a mass-based histogram **(E)**. The off-line determined mass fraction of C per cell enabled to estimate the size distribution of algae **(F)**.

a viable method for the analysis of micro-scaled C structures. As such, SP/SC ICP-MS unifies several methods including cell counting, elemental analysis and size modelling in one platform technology.

Table 3. The C mass and size for individual cells were calculated using SC ICP-MS/MS using a 4 µm MPs standard for instant mass and size calibration. The results are compared against data obtained by microscopy, Coulter-counting and TOC. The standard deviations were calculated based on inter-algae variations. *The mass of C per cell was calculated by first weighing cell pellets followed by counting cell numbers using a Coulter counter and by TOC analysis. Here, standard deviations do not reflect the inter-cell variations but were calculated by repeated bulk analysis.

Sample ID	m(C) / cell [pg] by SP ICP-MS	m(C) / cell [pg] by Coulter counting and TOC*	Mean size [µm] by SP ICP-MS	Visual mean size [µm]	Mean size [µm] by Coulter counting	C mass fraction [m(C)/m(cell)]
RT61	56.5 ± 26.8	66.5 ± 1.1	8.6 ± 1.3	9.2 ± 6.5	9.4 ± 4.3	0.16
RT2	56.1 ± 25.2	56.0 ± 1.3	7.6 ± 1.2	8.7 ± 5.8	9.4 ± 4.3	0.17
RT203	51.9 ± 24.9	70.1 ± 1.6	8.5 ± 1.4	9.5 ± 6.9	9.7 ± 6.0	0.15
CCMP3408	57.3 ± 30.1	41.1 ± 1.3	10.1 ± 1.9	10.0 ± 7.3	7.3 ± 3.5	0.08
CCMP3420	82.7 ± 45.6	100.8 ± 1.4	9.4 ± 1.9	10.3 ± 7.8	8.5 ± 3.0	0.17
4 µm MP	32.5 ± 18.6	-	3.66 ± 0.7	-	-	-

Conclusion

The analysis of MPs by targeting C in individual MP particles was investigated by SP ICP-MS. Ten methods employing standard no-gas methods, KED and ICP-MS/MS for ^{12}C and ^{13}C were developed and compared regarding their figures of merit in pure water and seawater. Previously, ^{13}C was the favoured isotope, but a direct comparison showed that the analysis of ^{12}C improved ion transmission and consequently lowered the sDL to 0.62 µm in pure water. The robust detection and calibration of MPs in seawater was found to be feasible when using on-line aerosol dilution via an HMI system and when performing matrix matching. However, the ion transmission in seawater was significantly reduced due to space charge effects and the noise was increased as result of organic compounds in the seawater. A method using no cell gases and a method using H_2 for KED achieved comparable sDL, whereas methods performing a mass shifting of ^{12}C via ICP-MS/MS increased sDL. The ICP-MS/MS methods showed superior selectivity as well as decreased background levels and noise and were preferable when considerations of spectral interferences became relevant such as in single cell analysis. The developed methods were used for the analysis of C in individual algae of a functionally important family, Symbiodiniaceae, and the iterative analysis of MPs standards diluted in seawater and algae enabled immediate mass calibration. Additional knowledge regarding the C mass fraction in cells enabled the construction of an algae size distribution model. Five different algae species were characterised by SC ICP-MS regarding C mass and size while using SP ICP-MS for standardisation with MP standards in parallel. Results were in line with values obtained with established complementary methods (Coulter counting, microscopy, TOC).

These ICP-MS/MS methods advance the analysis of MPs by improving figures of merit and supporting the robust analysis even in complex matrices like seawater. This will gain significance given the increasing pollution of oceans by plastics and specifically MPs. Unicellular organisms may be regarded as a biological form of C-based particles and the analysis by SC ICP-MS and calibration by SP ICP-MS allow simple and rapid mass and size calibrations, which is interesting in the context of C fixation, metabolism, and size distributions of algae. As such, SC ICP-MS is a versatile technique for the analysis of C and combines various strengths and capabilities of other methods including microscopy, cell counting and elemental analyses in one platform technology.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of interest

There are no conflicts to declare.

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