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1 **Amino acids promote black carbon aggregation and microbial colonization in coastal** 2 **waters off Vietnam**

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11

12 **Abstract**

13 The combustion of fossil fuels and biomass produces pyrogenic organic matter usually
14 known as ‘black carbon’ (BC), which are transported across the atmosphere as particulate
15 aerosol, eventually deposited on land and oceans. Soil studies have investigated the potential
16 microbial colonization and remineralization of BC particles, but this process has been seldom
17 studied in marine waters. BC provides a significant input of organic carbon to the oceans, yet
18 its fate and role in its biogeochemical cycling remains unknown. Here we explored the
19 microbial colonization of BC particles in coastal seawater samples collected in Halong Bay
20 (northern Vietnam). Using high-resolution mass spectrometry and microscopy methods, we
21 observed an increasing colonization of BC particles by marine microbes in the presence of
22 amino acids. Our results suggest that natural organic matter (NOM) present in seawater may
23 promote the microbial colonization and eventual remineralization of BC particles. Future
24 experiments should explore the potential microbial remineralization of BC particles to unveil
25 the role of this massive source of carbon to coastal marine ecosystems.

26

27 **Keywords:** pyrogenic organic matter; nanoSIMS; SEM; aggregates; Halong Bay

1 **1. Introduction**

2 The ability of the oceans to sequester CO₂ depends on organic particle production via
3 photosynthesis and their microbial remineralization in the water column. If sinking organic
4 particles are remineralized by microbial activity in shallow waters the resulting CO₂ is
5 released back to the atmosphere, whereas if they reach depths deep enough to avoid release
6 of CO₂ back to the atmosphere net carbon sequestration takes place. The balance between
7 these two processes determines the effectiveness of CO₂ sequestration by the ocean, a process
8 known as the biological carbon pump.

9 Black carbon (BC) is pyrogenic (i.e. thermally altered) organic matter produced during
10 incomplete combustion of fossil fuels, biofuels and biomass, which is an unaccounted-for
11 source of carbon in marine ecosystems. BC is emitted to the atmosphere at an estimated rate
12 of 2 to 29 Tg per year (Bond et al., 2013), and is deposited onto land and ocean surfaces via
13 dry and wet deposition. Deposition rates are estimated at ~12 Tg C per year on the ocean
14 (Jurado et al., 2008) and ~5 Tg C per year on land (McBeath et al., 2015). In addition to
15 direct atmospheric input of BC to the ocean, a fraction of the BC deposited on land can reach
16 the coastal ocean via riverine runoff. Estimates of the fluvial flux of BC to the coastal ocean
17 vary widely from 8 to 37 Tg C per year (Coppola et al., 2018a), exceeding the estimated
18 deposition of BC on land. While BC impacts the global ocean as a whole, its effects are likely
19 aggravated in coastal zones which receive BC via atmospheric deposition summed to riverine
20 runoff. The coastal ocean only accounts for 10% of the global ocean surface, but supports
21 nearly 20% of marine primary production (Del Giorgio and Duarte, 2002) and contributes
22 about ~30% of the CO₂ sequestered by the oceans on a global scale (Borges et al., 2005).
23 Hence, the effect of BC on coastal ocean carbon cycling needs to be elucidated.

24 Due to their porous structure (Rockne et al., 2000; Zerda et al., 2000) and surface
25 coverage with oxygen-containing functional groups (Masiello, 2004), BC particles are highly

26 reactive (Liang et al., 2006) and have been observed to adsorb natural organic matter (NOM)
27 and nutrients from seawater (Mari et al., 2014, 2017). The interaction between BC and
28 microbes may impact the biological carbon pump in two ways: (i) the adsorption of NOM
29 and nutrients onto BC particles decreases the concentration of these solutes in seawater
30 limiting growth and activity of marine microbes, and/or (ii) NOM-enriched BC particles
31 attract microbes with particle-attached lifestyles promoting particle degradation (Weinbauer
32 et al., 2012). BC may thus impact marine carbon cycling significantly (Weinbauer et al.,
33 2017), however most of our knowledge on the interaction between BC and microbes comes
34 from soils studies, while fewer have sought to find the fate of these particles at sea (Cattaneo
35 et al., 2010a; Mari et al., 2014; Pradeep Ram et al., 2018).

36 Atmospheric BC particles deposited on land through wet or dry deposition are known to
37 alter microbial activity in soils (Liang et al., 2010), affecting the efficiency of NOM
38 degradation (Wu et al., 2017). BC particles (reference material or naturally deposited
39 particles) added to seawater are rapidly colonized by bacteria, resulting in an enhancement of
40 particle-attached bacterial production (Malits et al., 2015; Mari et al., 2014). Here we
41 investigated whether microbial colonization of BC particles in the presence of amino acids.
42 Using nanoscale secondary ion mass spectrometry (nanoSIMS) and microscopy methods, we
43 document an increasing colonization of BC particles with time over 48 h.

44 The projected increase in BC deposition in the coming years (OECD, 2016), which is
45 especially acute in Asia, calls for the urgent need of understanding how it affects marine
46 microbial communities, which are behind all major element cycles sustaining life in the
47 oceans (carbon, nitrogen, phosphorus, sulfur), and have a crucial role in determining the
48 ocean's ability to regulate global climate. While the metabolic processes behind the bacterial
49 colonization and degradation of BC, and the extent to which these contribute to the biological
50 carbon pump are currently unknown, our results provide a step forward on our understanding

51 of BC-microbial interactions in the ocean.

52

53 **2. Materials and methods**

54 *2.1. Sampling and experimental setup*

55 Samples were taken from Halong Bay in the northern coast of Vietnam on 6 November
56 2018 using a small boat. Surface seawater was collected with a hydrochloric acid-cleaned
57 bucket from the side of the boat. Surface microlayer (SML) samples were collected from the
58 bow of the boat (1 m above sea level) using a 40 x 25 cm glass plate sampler vertically
59 dipped and slowly withdrawn. The SML adhering to the glass plate was collected into
60 Nalgene bottles (Cunliffe and Wurl, 2014). Each plate dip recovers about 8 mL of SML, so
61 the plate was dipped about 75 times to obtain 600 mL.

62 The obtained 600 mL of SML were mixed with 5400 mL of surface seawater (11.11%
63 v/v). BC reference material obtained from diesel combustion (Sigma Aldrich NIST2975) was
64 added to a final concentration of 0.16 g L⁻¹, which is representative of the BC concentrations
65 found in sea surface slicks (Mari et al., 2014). We note however that BC deposited in the
66 ocean is a mix of several combustion sources, and hence the reference material used here
67 only represents ‘natural’ BC inputs partially. On top of the reference BC material added,
68 background ‘natural’ BC may have been present in our samples. While we did not measure
69 BC concentrations in our samples, previous studies have reported highly variable
70 concentrations in the SML ranging from 8 to 25610 μM C (Mari et al., 2017).

71 The seawater, SML and BC mix was distributed in fifteen 250 mL transparent
72 polycarbonate bottles. Three sets of triplicates were amended with a mix containing >20 algal
73 amino acids labelled with ¹⁵N (98 atom%, Sigma Aldrich catalogue no. 608947). This mix
74 was added to represent 10% of usual in situ dissolved organic nitrogen concentrations (i.e.,
75 between 5 and 38 μmol L⁻¹; Mari et al., 2017). Each triplicate set of isotope-amended bottles

76 were incubated for 12, 24 and 48 h in a stirring plate placed inside an MLR 351 Sanyo
77 Versatile Environmental Test Chamber programmed to mimic the in situ diel light cycle
78 (10:17 hours of light and 11:29 hours of darkness) and surface seawater temperature (28°C).
79 Two sets of unamended triplicates were used as ‘time zero’ (not incubated), and ‘control’ (48
80 h incubation without ¹⁵N-labeled amino acid amendments). Each bottle was filtered onto 0.2
81 µm polycarbonate filters, fixed with 4% paraformaldehyde (final concentration) for 1 h at
82 room temperature and stored at -20°C. The filters were used for microscopy and nanoscale
83 secondary ion mass spectrometry (nanoSIMS) analyses as outlined below.

84

85 2.2. NanoSIMS

86 Circles of 1 cm diameter were excised from the polycarbonate filters and placed onto
87 nanoSIMS sample holders. The analyses were performed using a N50 nanoSIMS (Cameca,
88 Gennevilliers, France) at the French National Ion MicroProbe Facility according to
89 previously described methods (Benavides et al., 2017). A 1.3 to 3 pA 16 keV Cesium (Cs⁺)
90 primary beam focused onto ~100 nm spot diameter was scanned on a 256 x 256 pixel raster
91 with a raster area of 20 x 20 µm, and a counting time of 1000 µs per pixel. Samples were pre-
92 sputtered with Cs⁺ for 250 sec to remove surface contaminants and increase conductivity.
93 Negative secondary ions ¹²C⁻, ¹²C¹⁴N⁻, ¹²C¹⁵N⁻ and ³¹P⁻ were detected with electron multiplier
94 detectors, and secondary electrons were simultaneously imaged. Fifteen serial quantitative
95 secondary ion mass planes were generated and accumulated to the final image. Mass
96 resolving power was ~8000 in order to resolve isobaric interferences. Data were processed
97 using the LIMAGE software (L. Nittler, Carnegie Institution of Washington,
98 <https://sites.google.com/carnegiescience.edu/limagesoftware/>). All scans were corrected for
99 any drift of the beam during acquisition. Isotope ratio images were generated by dividing the
100 ¹³C⁻ ion count by the ¹²C⁻ ion count, and the ¹²C¹⁵N⁻ ion count by the ¹²C¹⁴N⁻ ion count. ¹⁵N-

101 enriched areas were selected as regions of interest (ROI). For each ROI, the $^{15}\text{N}/^{14}\text{N}$ ratios
102 were calculated. The number of ROIs analyzed were 60 per control (non ^{15}N -amended)
103 sample and 110 for ^{15}N -labeled samples.

104

105 *2.3 Cell counts*

106 Each polycarbonate filter was stained with $1\ \mu\text{g mL}^{-1}$ 4',6-diamidino-2-phenylindole
107 solution (DAPI) to determine bacterial abundance using an epifluorescence microscope
108 (Zeiss Axioplan, Jana, Germany) fitted with a 352-402 nm excitation filter.

109

110 *2.4. Scanning electron microscopy (SEM) analyses*

111 The samples of the nanoSIMS experiments (above) were used for SEM imaging after gold
112 sputtering for 10 minutes at 15mA on an Edwards S150B sputter coater apparatus. Imaging
113 was carried out on a FEI Teneo running in high vacuum, at 20 kV and using the built-in
114 Everheart Thornley detector for secondary electrons.

115

116 **3. Results**

117 Over the course of our experiment, we observed an increasing adsorption of ^{15}N -labeled
118 amino acid and microbial colonization on BC particles. Visual examples of this are shown in
119 the nanoSIMS images of Fig. 1. The morphology of the BC particles is depicted by ^{12}C
120 counts (Figs. 1A-D). We observed an increase in ^{15}N -labeled amino acid adsorption onto BC
121 particles with incubation time, as depicted by the increasing ^{15}N -enrichment from 12 to 48 h
122 (Figs. 1E-H). The increasing ^{15}N -enrichment of BC particles was paralleled by an increased
123 presence of bacteria, as proxied by ^{31}P -enrichment (Figs. 1I-L).

124 The average isotope enrichment values for controls and samples incubated with ^{15}N -
125 labeled amino acids at 12, 24 and 48 h are shown in Fig. 2. The $^{15}\text{N}/^{14}\text{N}$ ratio of the BC

126 particles analysed increased significantly from 0.07 to 3.5 between 12 and 48 h as compared
127 to the control (one-way ANOVA $p < 0.05$; Fig. 2A). We observed a 97% increase in ^{31}P
128 enrichment at 24 h and of 99% at 48 h (Fig. 2B), coinciding with a 60 and 76% increase in
129 bacterial abundance as depicted by DAPI counts at 24 h and 48 h, respectively (Fig. 2C). ^{31}P -
130 enrichment values and bacterial DAPI counts were positively and significantly correlated (r^2
131 = 0.98, $p < 0.05$).

132 Colonizing bacteria were not spotted in SEM images of control samples (not incubated
133 with ^{15}N -labeled amino acids; Fig. 3A), while those amended with amino acids showed
134 colonizing bacterial assemblages as biofilms covering BC particles (Figs. 3B-C).

135

136 **4. Discussion**

137 BC particles provide a substantial input of organic carbon to the oceans, yet their fate
138 and its interactions with the marine biota are unknown. BC was long thought to be strictly
139 refractory and thus unavailable for microbial processing (Masiello, 2004). However, BC
140 particles have a fractal morphology which confers them a high specific surface area and
141 highly adsorptive properties. Such characteristics render BC particles useful for agricultural
142 soil treatments to enhance nutrient and water retention (Gao et al., 2019). Recent studies have
143 shown that at least a fraction of BC can be microbially degraded in soils, but the degree of
144 degradation may vary with BC origin (source material), combustion temperature, pH, as well
145 as the diversity and activity of the *in situ* microbial community (Zimmerman, 2010).

146 The microbial degradation of BC particles in soils may be enhanced or decreased
147 according to the availability of other substrates such as labile NOM or nutrients (Hamer et al.,
148 2004). The so-called ‘priming effect’ refers to the stimulation (positive priming) or
149 suppression (negative priming) of BC mineralization in the presence of organic or inorganic
150 substrates. While this effect has been documented in soil studies (Hamer et al., 2004),

151 whether it also takes place in marine waters or how it may affect the biological carbon pump
152 is unknown (Weinbauer et al., 2012). Here we observed an increase in BC-associated bacteria
153 of up to 76% over 48 h incubation in the presence of ¹⁵N-labeled amino acids (Fig. 2C). Our
154 results are in agreement with previous incubation experiments where following BC addition
155 to seawater, a decrease in bulk dissolved organic carbon concentrations accompanied by an
156 increase in BC particle-attached bacterial production and abundance was observed (Cattaneo
157 et al., 2010b; Mari et al., 2014). Similarly, other studies observed a rapid bacterial
158 colonization of BC reference material in seawater, increasing from 10-25% after ~30 min to
159 >40% after 24 h (Cattaneo et al., 2010a; Weinbauer et al., 2009). These studies identified the
160 increased particle-attached microbial abundance and activity as a response to NOM
161 adsorption onto BC surfaces.

162 The microbial colonization of BC will depend on the particle's surface reactivity and
163 porosity, which determine the interactions with bacterial cell surface components (Hill et al.,
164 2019). Mari et al. (2014) observed that BC enhances aggregation processes and the transfer
165 of dissolved organic carbon to the particulate phase. Cattaneo et al. (2010a) observed that the
166 microbial colonization of BC-induced aggregates increases linearly with particle size. In our
167 experiments we observed a clear increase in aggregation in samples amended with ¹⁵N-
168 labeled amino acids as compared to controls (Fig. 4). These results support the stimulation of
169 BC particle aggregation due to amino acid adsorption and their subsequent bacterial
170 colonization as depicted by nanoSIMS analyses (Fig. 1).

171 The structural and chemical reactivity characteristics of BC particles depend on the
172 combustion conditions that the original material experienced before becoming BC (Lehmann
173 et al., 2011), as well as on its abiotic degradation during atmospheric transportation and/or
174 subsequent deposition in seawater (Zimmerman, 2010). For example, UV radiation has been
175 observed to promote the oxidation of BC and the leakage of nutrients and organic molecules

176 in seawater enhancing bacterial activity (Malits et al., 2015). Hence, future experiments
177 should explore the variability of microbial BC colonization and remineralization according to
178 its structural and chemical characteristics. Finally, the colonizing microbial community may
179 be dynamic, with opportunistic microbial communities (*r*-strategists or rapidly reproducing
180 organisms typical of unstable environments) colonizing BC particles and degrading them
181 thanks to substrate primer availability, and specialist microbes (*k*-strategists or slowly
182 reproducing organisms typical of less variable environments) appearing later in the
183 degradation process as the BC particle becomes more refractory (Liang et al., 2010;
184 Zimmerman et al., 2011). The successional colonization of BC particles at sea remains to be
185 explored (Datta et al., 2016).

186 BC additions may induce changes in soil chemistry, affecting the composition and
187 metabolism of the microbial communities it contains, and overall carbon cycling in these
188 environments (Hill et al., 2019; Lehmann et al., 2011). Similarly, the microbial colonization
189 and remineralization of BC particles deposited at sea may contribute to carbon sequestration
190 in the ocean (Weinbauer et al., 2012), a process that remains undocumented despite the
191 important flux of BC to the oceans (Coppola et al., 2018; Jurado et al., 2008). The significant
192 proliferation of bacteria observed along the course of our experiments could imply that the
193 added amino acids acted as a priming effect, promoting the colonization of BC particles and
194 presumably their degradation. However, the bacterial colonization of BC observed here does
195 not imply that the particles were eventually remineralized, and bacteria could have been
196 merely attracted by the adsorbed amino acids. Further experiments measuring CO₂ release or
197 oxygen consumption are needed to verify if remineralization does actually happen when BC
198 particles are incubated with marine microbes and if it is facilitated by the availability of
199 organic molecules, as previously done in soil experiments (Hamer et al., 2004; Liang et al.,

200 2010). Such experiments will allow constraining the role of BC in the carbon pump and the
201 extent to which it affects CO₂ sequestration by the oceans.

202

203 **5. Conclusions**

204 Current estimates of BC introduction into the ocean ascend to ~50 Tg C per year (Coppola et
205 al., 2018; Jurado et al., 2008). This flux could impact the biological pump in unknown ways.

206 While BC has been traditionally considered a refractory material unavailable for biological
207 use, soil studies have documented BC particle bacterial remineralization in the presence of
208 priming factors such as inorganic nutrients or organic molecules. Here we document the
209 microbial colonization of BC particles in coastal seawaters off Vietnam, which occurred only
210 in the presence of amino acids. Our results provide a step forward on our understanding of
211 BC-microbial interactions in the ocean laying the ground for future studies constraining the
212 impact of BC in the marine biological carbon pump.

213

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223

224

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350 **Figure captions**

351 Figure 1: NanoSIMS images of BC particles. ^{12}C enrichment of BC particles in control (not
352 labelled, A) and ^{15}N -labeled amino acid samples after 12 h (B), 24 h (C) and 48 h (D) of
353 incubation. Algal amino acid adsorption onto BC particles as depicted by the $^{15}\text{N}/^{14}\text{N}$ isotope
354 ratio in control (E), and incubated samples (12, 24 and 48 h – F, G and H, respectively).
355 Presence of bacteria as depicted by ^{31}P enrichment in control (I), and incubated samples (12,
356 24 and 48 h – J, K and L, respectively).

357

358 Figure 2: ^{15}N and ^{31}P enrichment of BC-amended seawater samples labelled with ^{15}N -labeled
359 amino acids at 12, 24 and 48 h: (A) $^{15}\text{N}/^{14}\text{N}$ isotope ratio, (B) ^{31}P counts above an arbitrary
360 threshold measured by the nanoSIMS. (C) Bacterial DAPI counts at the same time points.
361 Dashed lines indicate the values of each variable measured in control samples at 48 h (not
362 incubated with ^{15}N -labeled amino acids).

363

364 Figure 3: (A) SEM image of a BC particle in a control sample (not amended with ^{15}N -labeled
365 amino acids). (B, C) Two examples of BC particles from samples incubated with ^{15}N -labeled
366 amino acids after 48 h of incubation, showing bacterial biofilms (marked with red arrows).

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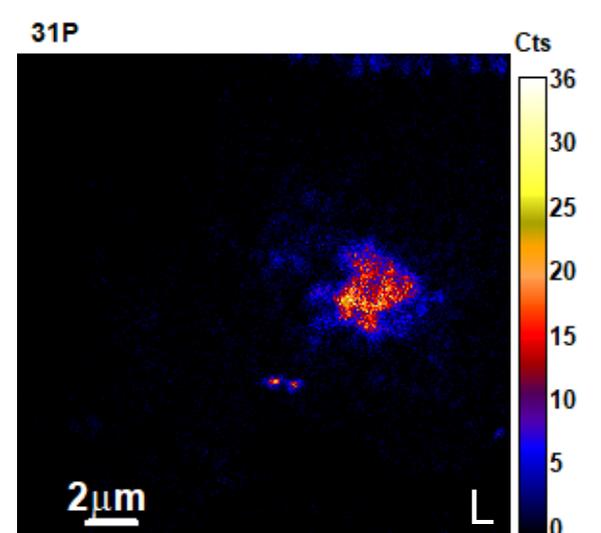
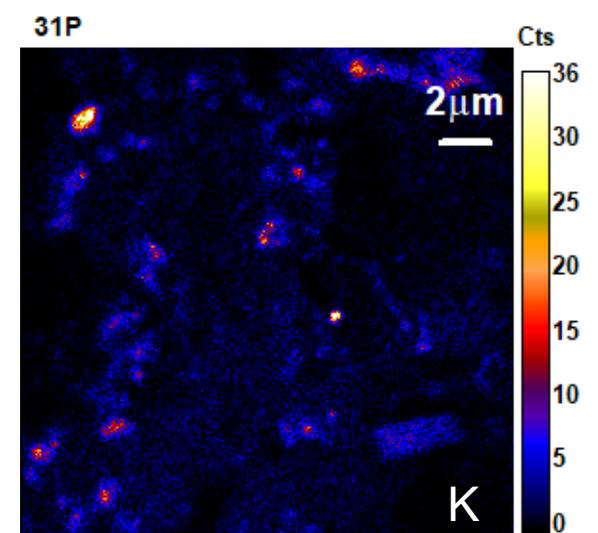
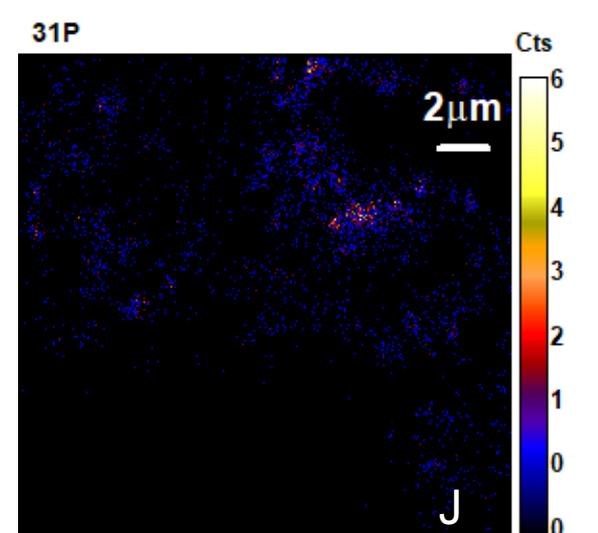
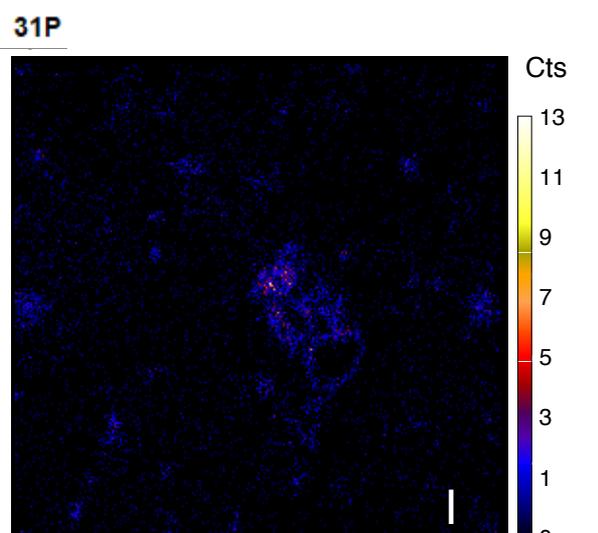
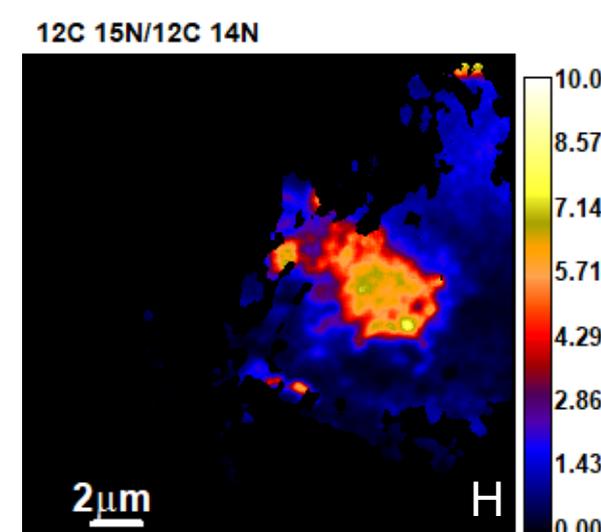
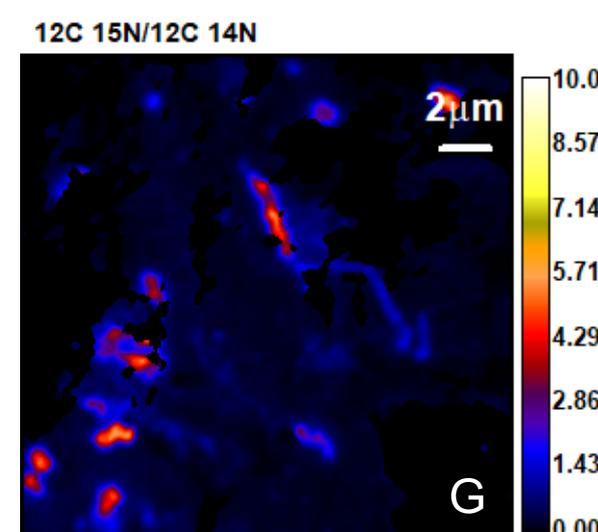
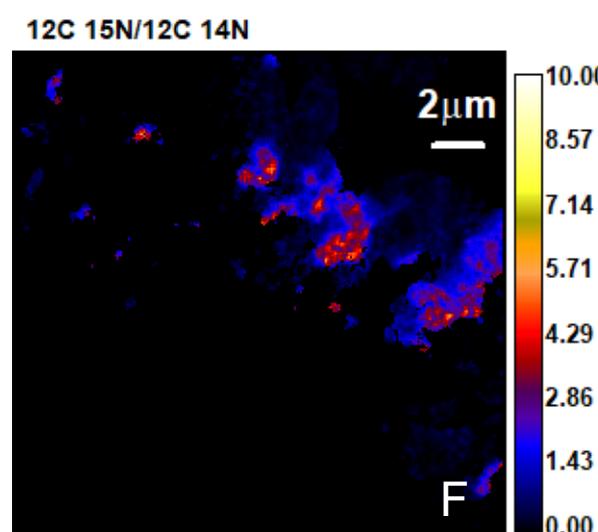
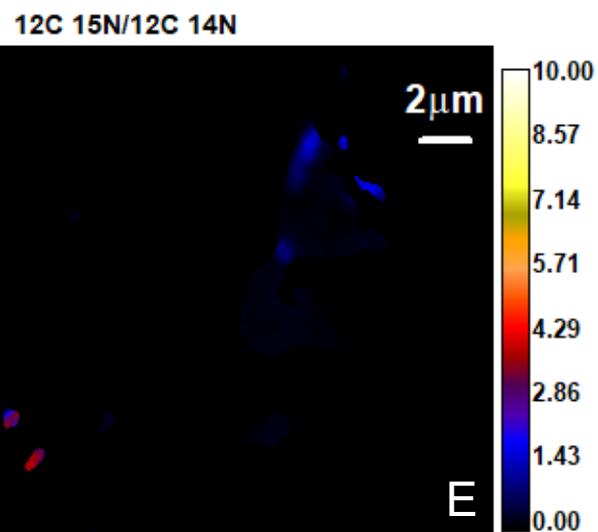
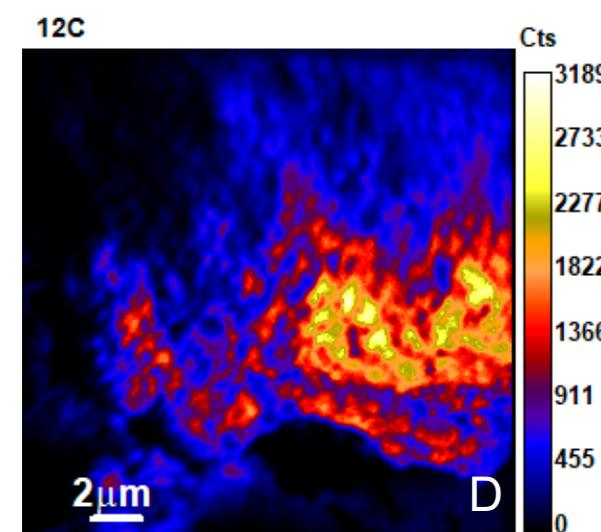
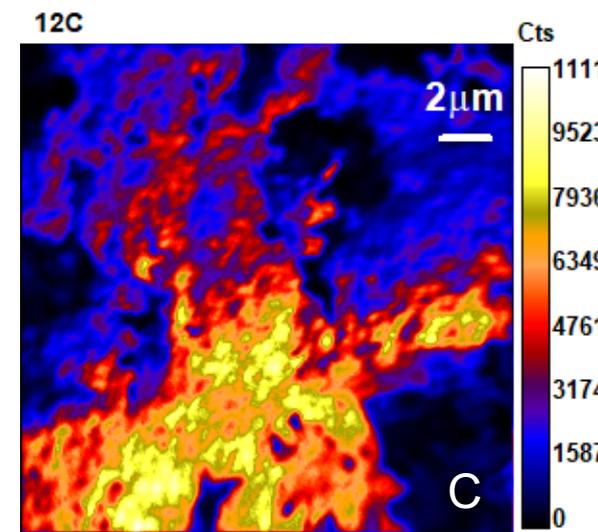
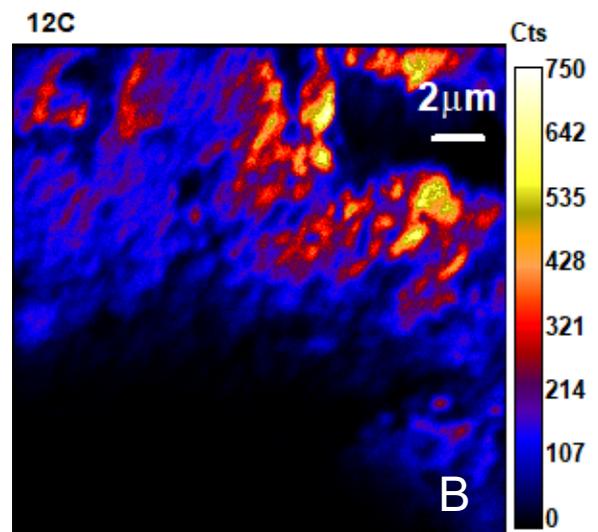
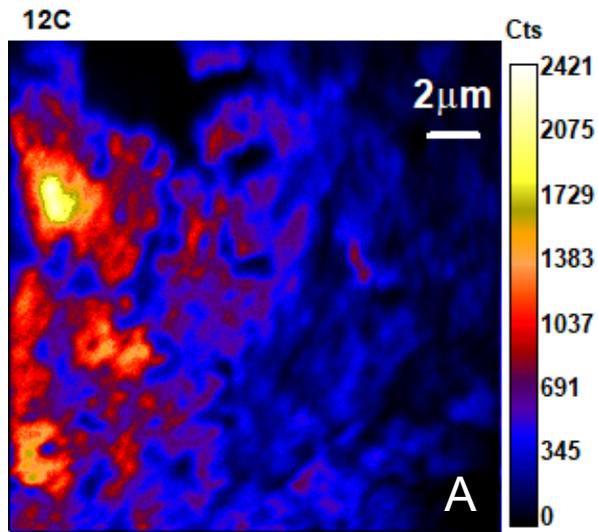
368 Figure 4: Left two bottles: two replicate incubation bottles amended with ^{15}N -labeled amino
369 acids after 48 h of incubation, right bottle: control sample (not amended with ^{15}N -labeled
370 amino acids) after the same incubation period. The picture depicts how the addition of amino
371 acids favoured BC particle aggregation (two left bottles), while in unamended samples BC
372 tended to remain in dissolved form.

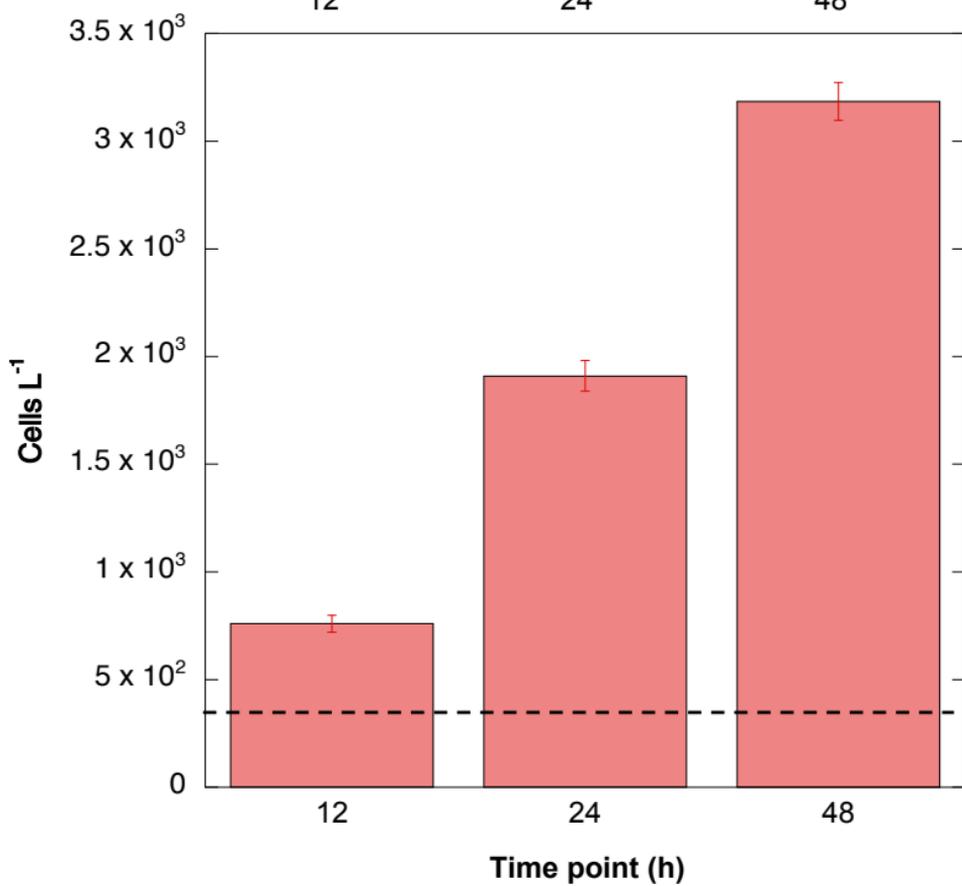
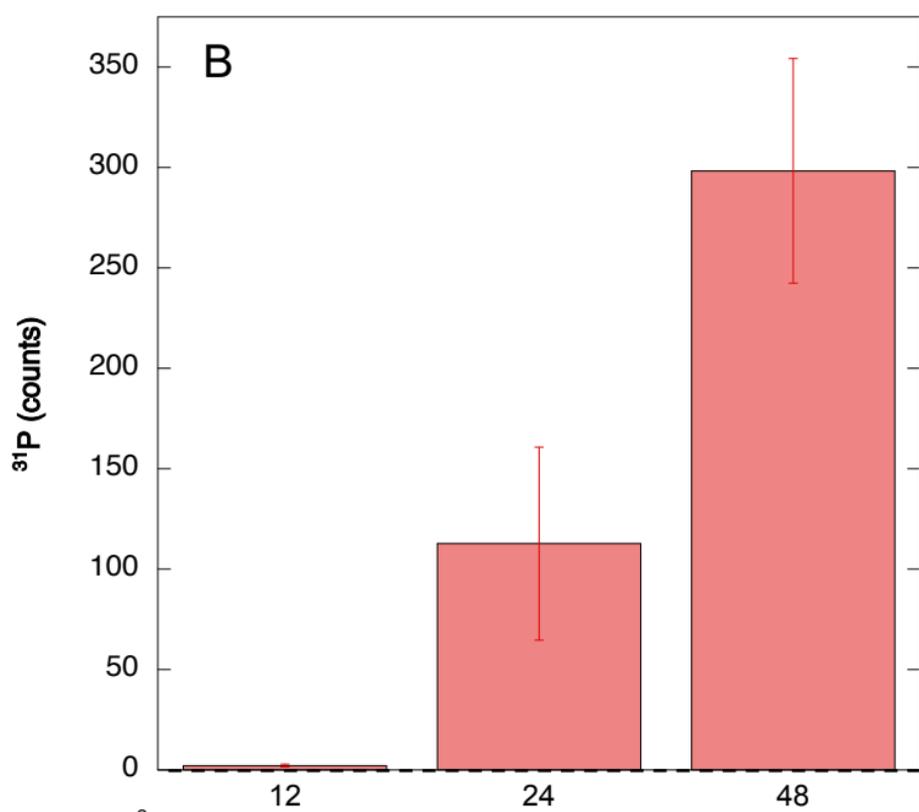
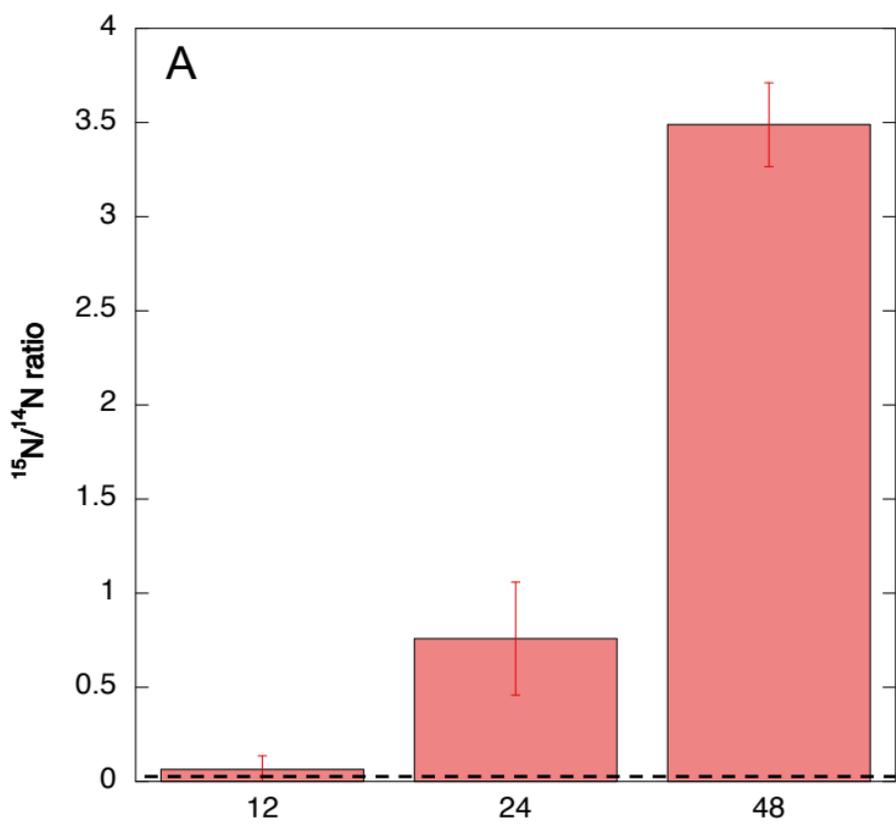
Control

12 h

24 h

48 h

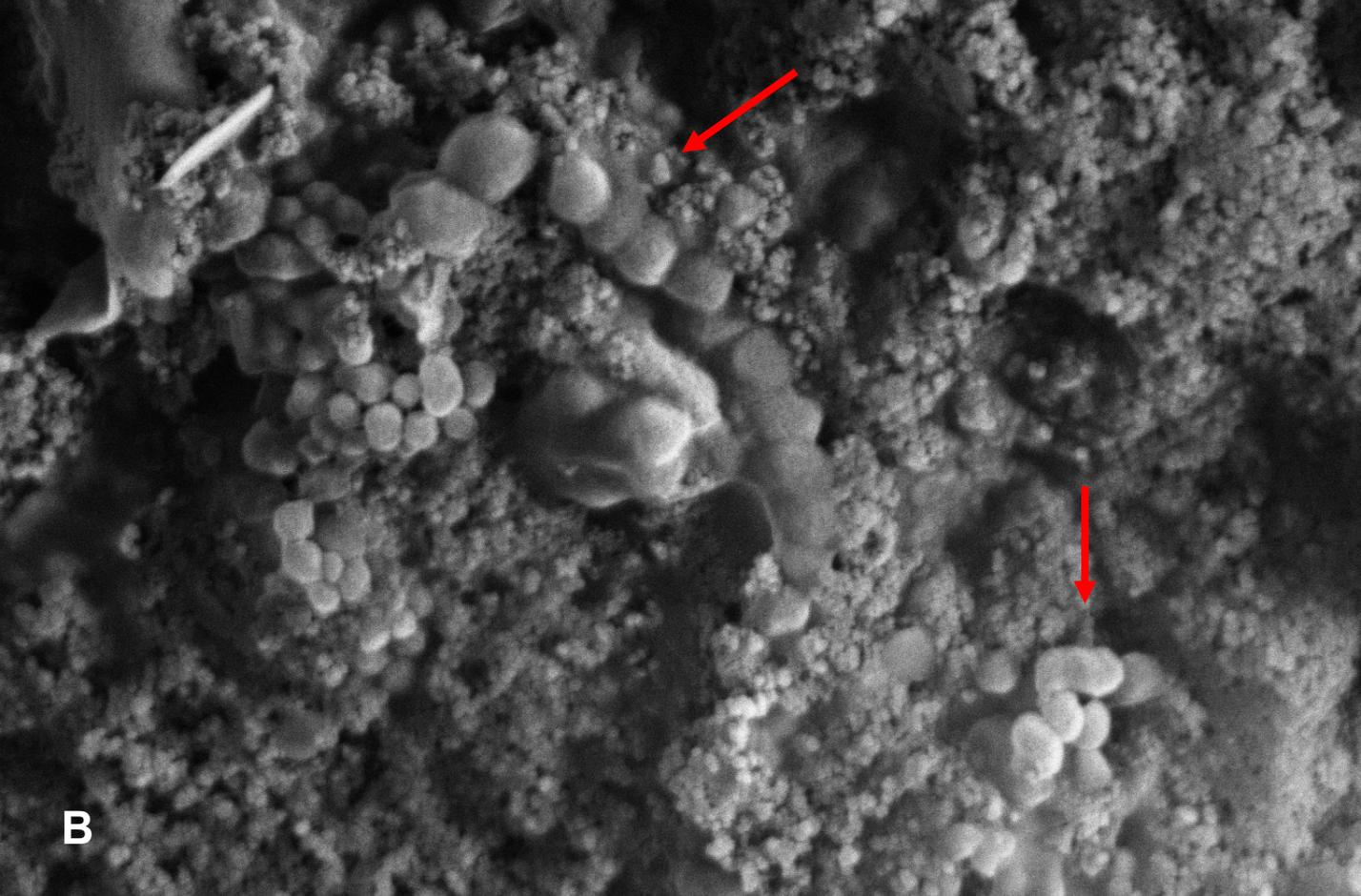






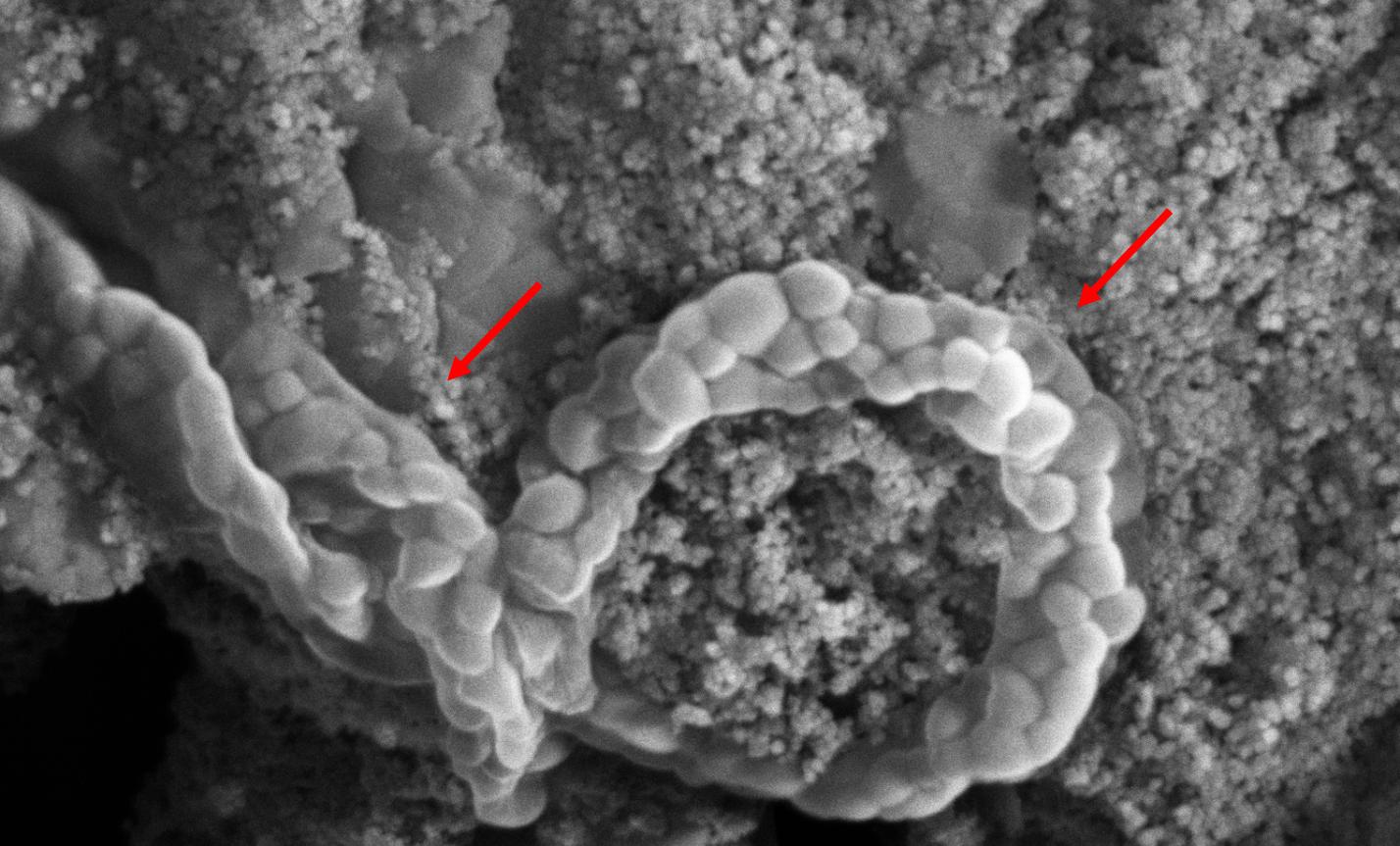
A

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|--|---------------|-------------|----------|-----|--------|---------|---------|------|-----------|
| | x: 28.2557 mm | 2/14/2019 | HV | det | WD | HFW | mag | 5 μm | FEI Teneo |
| | y: 3.1198 mm | 11:54:48 AM | 10.00 kV | ETD | 9.4 mm | 15.9 μm | 8 000 x | | |



B

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|--|----------------|-------------|----------|-----|--------|---------|----------|------|-----------|
| | x: -23.3493 mm | 3/12/2019 | HV | det | WD | HFW | mag | 2 μm | FEI Teneo |
| | y: 18.3207 mm | 11:22:51 AM | 20.00 kV | ETD | 5.4 mm | 8.47 μm | 15 000 x | | |



C

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|--|----------------|-------------|----------|-----|--------|---------|----------|------|-----------|
| | x: -23.3704 mm | 3/12/2019 | HV | det | WD | HFW | mag | 2 μm | FEI Teneo |
| | y: 18.3827 mm | 11:36:54 AM | 20.00 kV | ETD | 5.5 mm | 8.47 μm | 15 000 x | | |



Benavides

Don. A
t48

Benavides

Don. B
t48

Benavides

Don. Control
t48

