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Ultrastructural and electron energy-loss spectroscopic analysis of an extracellular filamentous matrix of an environmental bacterial isolate

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Summary

Strain F8, a bacterial isolate from “river snow”, was found to produce extracellular fibers in the form of a filamentous network. These extracellular filaments, which were previously shown to be composed of DNA (Böckelmann *et al.,* 2006), have been studied for the first time by ultrastructural and electron energy loss spectroscopy in the present work. 'Whole mount' preparations of strain F8 indicate these polymers are ultrastructurally homogeneous and form a network of elemental filaments, which have a width of 1.8 to 2.0

31 nm. When incubated at pH 3.5 with colloidal cationic ThO_2 tracers they become intensely
32 stained (electron dense), affording direct evidence that the fibers are negatively charged
33 and thus acidic chemically. Elemental analysis of the extracellular filaments by Energy-
34 filtered Transmission Electron Microscopy revealed phosphorus to be the main element
35 present and, since pretreatment of F8 cells with DNase prevented thorium labeling, the
36 fibers must be composed of extracellular DNA (eDNA). Neither ultrathin sections nor 'whole
37 mount negative stain' caused DNA release by general cell lysis. Additionally, cells infected
38 with phages were never observed in ultrathin sections and phage particles were never
39 detected in whole mount samples, which rules out the possibility of phages being directly
40 involved in eDNA release.

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45 **Introduction**
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48 In most natural environments bacteria live in association with surfaces in structures known
49 as biofilms, which is the prevailing microbial lifestyle. Surface association is an efficient
50 means of lingering in a favorable microenvironment rather than being swept away by the
51 current (Watnick and Kolter, 2002). Extracellular polymeric substances (EPS) act as
52 multifunctional elements in these biofilms, forming the matrix and stabilizing the spatial
53 structure (Wolfaardt *et al.,* 1999). Moreover the EPS is involved both in the interaction with

54 the environment and with other biofilm organisms. Embedded in EPS, biofilm organisms
55 can establish stable arrangements and function multicellularly as synergistic
56 microconsortia. The major components of EPS are not only polysaccharides but also
57 proteins, lipids, and to a smaller extent nucleic acids (Flemming and Wingender, 2001),
58 which are the focus of the present study.

59 The model organism in biofilm studies, *Pseudomonas aeruginosa*, has long been known
60 to excrete large amounts of DNA (Hara and Ueda, 1981). Furthermore some other bacteria
61 produce substantial quantities of extracellular DNA. Olishevsky and colleagues (2004)
62 discovered amounts of unmethylated CpG in the extracellular DNA of two *Bacillus subtilis*
63 strains. It was also reported that the flocculation ability of a marine photosynthetic
64 *Rhodovulum sp.* was due to secreted nucleic acids (Nishimura *et al.,* 2003). Whitchurch et
65 al. (2002) could show that *Pseudomonas aeruginosa* produces a large amount of eDNA
66 during alginate biosynthesis, which is obviously required for the initial biofilm
67 establishment of the strain. The extracellular DNA was continuously present in
68 *Pseudomonas aeruginosa* biofilms (Steinberger *et al.,* 2002). The investigation of the
69 universality and persistence of eDNA in dual-species biofilms revealed, that significantly
70 more eDNA was produced in *P. aeruginosa* and *P. putida* biofilms than in *Rhodococcus*
71 *erythropolis* or *Variovorax paradoxus* biofilms (Steinberger and Holden, 2005). While the
72 amount of eDNA in dual-species biofilms was of the same order of magnitude as that

73 from single-species biofilms, the amounts were not predictable from single-strain
74 measurements. The authors concluded that extracellular DNA production in unsaturated
75 biofilms (from unsatured environments such as terrestrial subsurface or on plant leaves) is
76 species dependent and that the phylogenetic information contained in this DNA pool is
77 quantifiable and distinct from either total or cellular DNA. Since unsaturated biofilms are
78 not subjected to hydrodynamic shear, the structural roles for eDNA in this context are
79 questionable. In contrast to this Allesen-Holm et al. (2006) revealed that *Pseudomonas*
80 *aeruginosa* produces extracellular DNA which functions as a cell-to-cell interconnecting
81 matrix component in biofilms. The eDNA is generated via a mechanism which is dependent
82 on acyl homoserine lactone and Pseudomonas quinolone signalling, as well as on flagella
83 and type IV pili. The eDNA is obviously located primarily in the stalks of mushroom-shaped
84 multicellular structures, with a high concentration especially in the outer part of the stalks
85 forming a border between the stalk-forming bacteria and the cap forming bacteria. These
86 results are in agreement with our study on an aquatic biofilm forming bacterium, strain F8,
87 isolated from the South Saskatchewan River in Saskatoon, Canada (Böckelmann *et al.,*
88 2006). This strain produces a stable filamentous network of extracellular DNA,
89 demonstrating a new function and relevance for eDNA. Moreover immunologists observed
90 that human neutrophils were also able to produce an extracellular network of DNA and

91 proteins. These neutrophil extracellular traps (NETs) degraded virulence factors and killed

92 bacteria (Brinkmann *et al.,* 2004).

93 In all the above studies the confirmation of the nature of the DNA present in the

94 extracellular material was performed by different staining and molecular biological

95 techniques. Matsukawa and Greenberg (2004) visualized the DNA in *Pseudomonas*

96 *aeruginosa* biofilms with the specific fluorescent double-stranded DNA stain PicoGreen.

97 Allesen-Holm *et al.,* (2006) applied propidium iodide, ethidium bromide and DDAO (7-

98 hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)) to stain the eDNA. In our recent

99 study (Böckelmann *et al.,* 2006) we used different DNA specific SYTO dyes especially

100 SYTO9 to visualize the filamentous network of strain F8, whereas Brinkmann *et al.,* (2004)

101 stained the eDNA with SYTOX GREEN, DRAQ5, Bisbenzimide33342 and ToPro3. Digestion

102 with DNase (Whitchurch *et al.,* 2002; Steinberger and Holden, 2005; Allesen-Holm *et al.,*

103 2006; Böckelmann *et al.,* 2006) as well as PCR and RAPD-PCR (Steinberger and Holden,

104 2005; Allesen-Holm *et al.,* 2006; Böckelmann *et al.,* 2006), Southern blot analysis (Allesen-

105 Holm *et al.,* 2006) and restriction endonuclease treatment (Nishimura *et al.,* 2003;

106 Böckelmann *et al.,* 2006,) was applied to the extracellular material to verify its DNA nature.

107 As electron microscopical studies on the extracellular material are scarce (Hara and Ueda,

108 1981; Brinkmann *et al.,* 2004) the aim of the present study was the ultrastructural analysis

of the extracellular threadlike matrix of strain F8 by electron energy-loss spectroscopy in order to confirm its DNA composition.

Results and discussion

Ultrastructural analysis of ultrathin sectioned and whole mount preparations of cells and extracellular material of F8

In our recent work (Böckelmann *et al.,* 2006) we investigated the proliferation of extracellular DNA, in the form of a filamentous network, of strain F8 (AY077611). The bacterium was isolated from lotic aggregates of the South Saskatchewan River, Saskatoon, SK., Canada, grown on low nutrient freshwater basal medium (FBM). It is a typical rod shaped Gram-negative bacterium ~ 2 µm in length and 1 µm in width. Phylogenetic analysis revealed strain F8 as a deep branching gamma-Proteobacterium with 96 % sequence identity to *Rheinheimera baltica* (AJ002006 DSM 14885) (Brettar *et al.,* 2002). When strain F8 was cultivated on FBM or R2A medium filament formation started at day four and continued up to day seven. In younger (than four days) or older (than seven days) cultures of strain F8, or cultures of strain F8 that were provided with high concentrations of nutrients as TSA or NB medium microfilaments could not be detected. DNA-specific stains

130 and DNA degrading enzymes indicated the filamentous networks consisted of DNA, and a
131 proteinaceous or polysaccharide origin could be excluded.

132 Here we have used ultrastructural and electron-loss spectroscopic analysis by Energy-
133 filtered Transmission Electron Microscopy (EFTEM) to substantiate these findings. At the
134 base of ultrathin sections the 'filamentous state' of F8 cells was analysed with untreated
135 cells or with those after DNase treatment. As a rough indication of the presence of DNA,
136 bacteria were labeled with cationic 1.5 nm ThO_2 colloids, which bind strongly to negative
137 charged structures and can be discriminated and identified because of their high
138 particulate contrast in the electron microscope. Under the conditions applied, i.e. binding
139 of ThO_2 colloids at pH 3.0 after fixation with glutardialdehyde, a distinct labeling of
140 negatively charged biomolecules was visible (Fig. 1). Beside electron dense staining of the
141 outer membrane, traces of rudimentary EPS and murein (Fig. 1, twin arrows), was
142 observed with both DNase treated and untreated cells. The main difference was in the
143 existence of an extracellular threadlike matrix, which could not be detected with F8 cells
144 that had been DNase treated. At high magnification (Fig. 1b) these microfilaments were
145 decorated with particulate electron dense stain and form an irregular network (Fig. 1b,
146 quadruple arrows).

147 The morphological analysis of the filamentous state of strain F8 in the electron
148 microscopic preparation (Fig. 2a) revealed overall structural features, which are very

149 similar to the epifluorescence microscopic images as shown in our recently published work
150 (Böckelmann *et al.,* 2006). The characteristic fibers of the extracellular DNA net, which
151 often appear as diffusive strings in the light microscope, are easily recognized recognized
152 as a mixed network of loosely organized strands (Fig. 2a, #), associated with straight fibers
153 (Fig. 2a, arrows). At a higher magnification the threadlike network often shows groups of
154 strands oriented in parallel, running in close proximity (Fig. 2, arrows) and those in the
155 vicinity of cells are multidirectionally oriented. These clusters contain a mixed population
156 of intensely stained living bacteria and electron translucent ghost-like cells (Fig. 2b, #).
157 Interspersed within the extracellular DNA matrix distinct particulate matter can be
158 recognized (Fig. 2b, and c, triple arrow). At high resolution (Fig. 2c, d) the dark stained
159 tracks reveal the presence of one to multiple individual strands (Fig. 2d, arrowheads),
160 which are about 1.7 nm in width (mean: 1.97+/- 0.31 nm; N = 76). These structures
161 apparently appear isomorph to cellulose protofilaments (see for comparison Benziman *et*
162 *al.,* 1980). But the enzymatic treatment of the microfilamentous network of strain F8 with
163 cellulase under stringent conditions did not lead to degradation of the filaments even after
164 an incubation time of 3h up to 24h (data not shown). Treatment of the F8 sample with the
165 fluorescence brightener Calcofluor White (Maeda and Ishida, 1967; Benziman *et al.,* 1980)
166 did not result in any staining (data not shown).
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169 *Electron spectroscopic analysis of the F8 extracellular threadlike network*

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171 Phosphorous is well known to be the most prevalent element of nucleic acids, since it
172 constitutes 9.6% of the elemental composition of DNA (Mizuno *et al.,* 1976). Thus it
173 seemed reasonable to analyze strand-like patches with identical sampling size, but
174 different network densities, by parallel electron energy-loss spectroscopy (PEELS: see Fig.
175 3) and additionally to do element mapping of phosphorus within these areas by electron
176 spectroscopic imaging (ESI; see Fig. 4). It was found that 'whole mount' preparations of
177 strain F8 yielded unambiguous P-signal intensities relative to the corresponding ultrathin
178 sections either by PEELS or ESI. EELS intensities are directly proportional to the local
179 elemental concentration (Egerton, 1996) and can be recognized from peak maxima at
180 141.3eV (Fig. 3b) by comparing extracellular strands in both MP01 and MP02 areas (5,3
181 μm^2 at a diameter of 2.6 µm, Fig. 3a). Obviously area MP02 contains more filaments
182 relative to MP01, which reflects about 20 units higher intensities. For comparison,
183 especially to estimate the appearance of nucleic acid-related electron energy loss near
184 edge structures (ELNES), reference spectra were recorded of purified herring sperm DNA
185 (Fig. 3: DNA). As can be seen especially with MP02 relative to herring sperm DNA, all three
186 relevant ELNES intensity maxima are in place (arrows) and provide strong evidence of

187 significant chemical identity of both samples. It should be realized that $P_{L2,3}$- ELNES
188 features and intensities can be obscured by $U_{O4,5}$ tailing intensities, as can be seen from a
189 comparison of phosphorus and uranium spectra (Ahn *et al.,* 1983; Reimer *et al.,* 1992).
190 Therefore, as a non-interfering alternative lead acetate has been used as staining agent.
191 Lead acetate as well as lead perchlorate were successfully applied to stain pure DNA, giving
192 similar results in contrast as those of conventional uranyl acetate (Stoeckenius, 1961).
193 Thus background intensities, which were corrected according to the 'potence' underground
194 function, were mainly based on high mass-thickness of the underlying carbon-Butvar™
195 layer of approximately 75 nm thickness. Because of these experimental restrictions it was
196 not rational to attempt P-quantification, as a comparative optimum specimen thickness of
197 17.5 nm is required when operating with exciting electron energies of 80 keV (Wang *et al.,*
198 1992). Nevertheless, at the qualitative level the actual PEELS data unambiguously indicate
199 the element phosphorus and thus nucleic acids to be constituents of the threadlike matrix.
200 Additionally, because of the high density of DNA strands in suitable sample areas,
201 phosphorus elemental mapping by ESI was performed and distinct intensities could be
202 related to structural features (Fig. 4). As noted above the high mass density of the support
203 film did not allow phosphorus quantification of the extracellular DNA although optimum
204 background correction was used according to the 'multi window exponential difference'
205 method. This background correction method, based on three pre-edge images at 110,

206 121, and 134 eV and two post-edge images at 144 and 154 eV, is similar to the N-
207 windows method, described by Quintana *et al.,* (1998). In the present work it was found to
208 give optimum results for phosphorus detection. This is obvious from the comparison of the
209 P-elemental map (Fig. 4d) with the zero loss image (Fig. 4a) and it is evident that the
210 threadlike network follows the phosphorus distribution. Only those network areas,
211 composed of just a few DNA strands, could not be resolved adequately in the phosphorus
212 map (Fig. 4a,d, arrow). Similarly, because of the non-linear energy-spread of the electron
213 beam and the actual current settings there is a fall-off in intensities at the periphery of the
214 CCD detector, as is indicated by signal undersaturation of the R-map shown by red pixels
215 (see Fig. 4C, #)
216 From this ultrastructural and electron energy-loss spectroscopic analysis it is obvious, and
217 shown for the first time in the present paper, that the extracellular threadlike network of
218 strain F8 contains phosphorus as the main element. Thus the strands, in agreement with
219 our previously published data (Böckelmann *et al.,* 2006), were unambiguously identified as
220 extracellular DNA. These findings highlight the role of bacterial eDNA in the context of
221 general biofilm formation both in the environment and in artificial systems encountered in
222 medical and industrial environments. As such eDNA should become an area of future
223 fruitful studies including the importance for the bacterial producer up to precise
224 applications in nanotechnology.

Experimental procedures

Bacterial strain and growth conditions

Strain F8 (AY077611) was isolated from “river snow” of the South Saskatchewan River, in Saskatchewan, Canada as described by Böckelmann *et al.,* (2006). For eDNA production the strain was grown on freshwater basal medium (FBM) agar plates. It contained 3.0 g Na_2SO_4, 0.4 g $MgCl_2 \times 6H_2O$, 1.2 g NaCl, 0.3 g NH_4Cl, 0.15 g $CaCl_2 \times 2H_2O$ and was supplemented with yeast extract (0.01% w/v) plus glucose (0.5 mM), trace elements and vitamin solution (Böckelmann *et al.,* 2000). For all investigations a loop of bacterial material was transferred and resuspended in 1ml 20 mM HEPES, pH 7.4, fixed with 1% glutardialdehyde at ambient temperature and stored at 4 °C for several days.

Digestion with DNase, Proteinase and Cellulase

A suspension of filament forming cells of strain F8 (20 mM HEPES, pH 7,4) was used for digestion experiments. 200 µl of the unfixed suspension were treated either with 20 µl DNase I (10 mg ml^{-1}, 50 mM TRIS, pH 7, 10 mM $MgCl_2$, 150 mM NaCl) or 2 µl Proteinase K

247 (20 mg ml^{-1}) (both Sigma, Deisenhofen, Germany) according to the manufacturer's
248 protocol. Cellulase treatment was performed following the protocol of White & Brown,
249 1981 and used in two different concentrations (1mg and 5mg ml^{-1} citrate buffer pH 4.8)
250 each 20 µl. As a proof of the enzymatic activity of the cellulase, the digestion of nitrate
251 cellulose filter paper was used. In all cases the enzymatic treatment of the microfilaments
252 was checked microscopically every 30 min for up to 3 h for DNAse incubation, and up to
253 24 h for cellulase treatment.
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256 *Electron Energy Loss Spectroscopy*
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258 For negative staining a prefixed sample aliquot (0.5% glutardialdehyde - 20 mM HEPES, pH
259 7.4) was diluted 1:2 with 1% (w/v) lead acetate (freshly prepared with boiled distilled
260 water). A 60 µl droplet of the solute was layered with four freshly prepared Butvar™ coated
261 300 mesh Cu grids for 3 minutes at ambient temperature in a humid chamber. The
262 chamber's atmosphere was freed from CO_2 by placement of 2 to 3 NaOH platelets on a
263 moistened filter paper. Grids were quickly blotted with filter paper, loaded and washed with
264 freshly boiled distilled water, immediately blotted and air-dried.
265 For high resolution ultrastructure analysis unstained, prefixed F8 cells (0.5%
266 glutardialdehyde - 20 mM HEPES, pH 7.4), adsorbed within 30 seconds on to a carbon-

coated Formvar™ foil, were negatively stained with 4% (w/v) uranylacetate, pH 4.5, following the general protocol.

Electron Energy Loss Spectroscopy (EELS)

Electron Spectroscopic Imaging (ESI)

EELS analysis of strain F8 in the phase of extracellular DNA production was performed with whole mount samples, negatively stained with Pb-acetate (see above; Pb-acetate was used, since, in contrast to the U-$O_{4,5}$ near edge structures, there is no interference from P-$L_{2,3}$ edge). ESI data were recorded of motives with slightly dark-stained (electron dense) fiber strands originating from or in the vicinity of individual bacterial cells. 512X512 images of adequate energy loss were recorded for 2 seconds with a 1024x1024 CCD detector with two-fold binning. Background subtraction for calculating the phosphorus element map was performed by the 'multi window exponential difference' method, with energy settings from low to high 110 eV, 121 eV, 134 eV (= pre-edge), and144 eV, 154 eV (=post-edge) with an energy slit, set to 10 eV width. Corresponding survey views were recorded in the 'elastic bright-field' mode at 0 eV. Aperture settings were 12 mrad (60 μm) for objective aperture and 1.2 mrad (= 200 μm) of the condenser aperture. Beam current was set to 3 μA. EELS-analysis and electron microscopy was performed with an Energy-

288 Filtered Transmission Electron Microscope (EF-TEM) with an integrated filter of the
289 Castaing-Henry type (CEM 902, Zeiss Company, Oberkochen, Germany). Spectra and image
290 registration were recorded with a cooled 1024 × 1024 CCD camera (Proscan CCD HSS
291 512/1024; Proscan Electronic Systems, Scheuring, Germany).
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294 *Parallel electron energy loss spectroscopy (PEELS)*
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296 Spectra were recorded within specific regions of the ESI-motives. The area was set to 2.6
297 µm in diameter (= 5.31 μm^2) and spectra were recorded for 37 seconds by three-fold
298 integration within an energy-range from 92.7 eV to 194.3 eV with a channel width of 0.1
299 eV, spectrum energy resolution was about 1.6 eV at zero-loss (FWHM). Recorded PEELS
300 data were corrected for background, applying the 'potence' underground function of the
301 EsiVision Pro Software (EsiVision Pro, Vers. 3.2; SIS - Soft Imaging Systems, Münster,
302 Germany) and were 'medium'-filtered (settings: 1,5 eV width).
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305 *Preembedding label with cationic ThO_2 colloids, embedment and ultrathin sectioning of F8*
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307 Both DNase-treated and untreated F8 cells were fixed in 1% (v/v) glutardialdehyde - 20 mM
308 HEPES, pH 7.4. Samples were processed for ThO_2 labeling and embedding as described in
309 detail elsewhere (Lünsdorf *et al.,* 2006).

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312 *Epifluorescence microscopy*

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314 10 µl of a cell suspension were stained with 10 µl of the DNA-stain SYTO9 (Molecular
315 Probes, Eugene, Oregon, 1:1000 diluted) according to the manufacturer`s instructions
316 before microscopic examination. Microscopy was performed with a Zeiss Axioskop
317 microscope (Oberkochen, Germany) equipped with Zeiss light filter sets no. 01 and HQ
318 light filter 41007 (AF Analysentechnik, Tübingen, Germany) (Böckelmann *et al.,* 2000).

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426

427 **Figure legends**

428

429 **Fig. 1.** Ultrathin sections of strain F8 cells without (A, B) and with 3h DNase treatment (C).
430 Negative charges (arising from nucleic acids) have been traced with cationic colloidal ThO_2.
431 In addition to the outer membrane surface the majority of the filaments at the periphery (A,
432 B: quadruple arrows) appear intensely labeled. Characteristically the microfibers are
433 decorated with electron dense particles (B). The filaments are absent from the DNase
434 treated sample (C), but cells appear coccoid and larger in size, relative to untreated cells.
435 Often the outer membrane is detached from the cytoplasm or is budding off the cell
436 (arrowheads). Twin-arrows indicate residual EPS and/or murein.

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438 **Fig. 2.** Ultrastructural analysis of 'whole mount' preparations of F8 cell in the 'extracellular
439 DNA' -state. (A) Low magnification view of cells stained with uranyl acetate, which appear
440 interconnected by a threadlike matrix. (B) Detailed view of a cluster of cells and cell ghosts
441 (#). The main part of the network appears mostly parallel oriented over long distances
442 (arrows), but loosely dispersed within the cell cluster. (C) High resolution details of the
443 network show the threadlike matrix to be 'cross-linked' by aggregates of amorphous
444 cellular material (triple arrows). (D) Cutout of the framed area in (C), which clearly reveals
445 the weakly stained matrix is composed of individual strands (arrowheads).

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Fig. 3. PEELS-analysis of the threadlike matrix. (A) Measured areas with high microfilament density (blue circle, MP02) relative to an area with lower density (red circle, MP01) are indicated. The corresponding PEELS (after background-correction) are outlined in (B) (MP01, MP02). Herring sperm DNA was used as a reference for nucleic acid and phosphorus reference. The boxed area indicates the $P_{L2,3}$-ELNES and the left margin is the ionization onset. Arrows point to intensity maxima corresponding to phosphate-coordinated phosphorus.

Fig. 4. Net phosphorus mapping of the threadlike matrix of F8. (A) Elastic survey view of a cell end, linked to a threadlike matrix and negatively stained with lead acetate. Arrows point to filaments, missing in the elemental map in (D). Arrowheads indicate membranes and corresponding phospholipids with high phosphorus intensities in (D) (B) Survey view, overlaid with net phosphorus map. (C) R-map, which shows only a few red low level (#) and blue high level (arrow) signal pixels. (D) Net phosphorus map (colored green), which shows signal loss mainly in the upper and lower right corners, marked in red in the R-map.

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