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Single cell measurements of microbial stoichiometry and phylogeny

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Abbreviations

Br	Bromine
Bulk	*here:* many microbial cells measured together with elemental analysis
C	Carbon
CARD-FISH	Catalyzed Reporter Deposition Fluorescent *in situ* Hybridization
CoA	Coenzyme A
CoV	Coefficient of variation
DAPI	4',6'-diamidino-2-phenylindole
EDX	Energy Dispersive X-ray Spectroscopy
EDX_S	standardized EDX result
EELS	Electron Energy Loss Spectroscopy
F	Fluorine
FA	formamide
FISH	Fluorescent *in situ* hybridization
HRP	Horseradish peroxidase
min.	minute
N	Nitrogen
P	Phosphorus
PBS	phosphate buffered saline
RT	room temperature
sec.	second
XRMA	X-ray microanalysis

Key words

EDX; EELS; halogen labeled FISH; single cell stoichiometry; ecological stoichiometry

Chapter 1:

Comparison of different methods to measure microbial stoichiometry

An experimental investigation with two bacterial species at different growth phases and contrasting resource supplies

Introduction

In this study, I investigate how bulk and single cell measurements of microbial stoichiometry compare. As studies of microbial stoichiometry measure either single cells or bulk cell material, it is important to determine if both methods yield similar results and how the mean values and variance of elemental content differs between species, growth phases, and nutrient supply.

Why stoichiometry?

Ecological stoichiometry is the study of balance between several chemical substances in ecological processes and interactions [1]. Carbon (C), nitrogen (N), and phosphorus (P) are the main building blocks of life on earth [1], and most research in ecological stoichiometry focuses on their ratios [2]. In this thesis I will focus on the relative proportion of these three elements, but the methods and ideas presented here can be extended to include a range of other elements.

There has been a long history of stoichiometric measurements in biology [1], but most of the studies have focused on multicellular organisms (e.g. zooplankton, algae) [3,4,5,6,7], rather than on microorganisms. As an example, several studies have been conducted to investigate the stoichiometry of zooplankton, and it was found that their stoichiometry was strikingly stable even over seasonal changes in their food supply [6]. Furthermore, it was discovered that even though species may be very similar in their phenotype and genotype, the stoichiometry between e.g. zooplankton species, and even of those inhabiting the same habitat, can be surprisingly different [8]. The stoichiometry of individual species in a food web is the link to food-web dynamics and determines the overall capacities of an ecosystem for biogeochemical cycles [9]. While measuring the stoichiometry of

single zooplankton specimen is relatively straight forward, it becomes more difficult when one wants to measure single bacteria, which have a size of only several μm.

What determines the stoichiometry of an organism?

Looking at stoichiometric ratios is in a way a very simplifying view on organisms and one has to keep in mind where these differences in stoichiometry are arising from. Molecular compounds are enriched or depleted in certain elements. Proteins for example, do not contain any P (average C:N = 2.7, C = 53 %, N = 17 % (% by weight)). Carbohydrates contain only C, O, and H and no N or P (C = 37 %, N = 0 %, P = 0 %). Phospholipids, on the other hand, have a low N content, but contain a relatively high amount of P (average C:N:P = 39:0.8:1 C = 65 %, N = 1.6 %, P = 4.2 %). Nucleic acids, which as DNA make up a rather small proportion of cell content, but as RNA can make up to 40 % of microbial biomass, are rich in P and have similar N values as proteins (C:N:P = 9.5:3:7.1; C = 32.7 %, N = 14.5 %, P = 8.7 %)[1]. These compounds are examples of major biochemical classes in all cells, and suggest that the relative proportion of these molecules ultimately determines biomass stoichiometry. For example, differences in the proportions of proteins and nucleic acids will not result in large changes of biomass C:N ratios, but will lead to large disparities of P content [1]. This has been demonstrated empirically in microorganisms using Raman microspectroscopy [10], but research in this field has only started recently.

Nutrient recycling

Bacteria play a major role in recycling nutrients through degradation of organic matter, though mostly not by direct excretion of nutrients, but rather because they accumulate high amounts of nutrients in their biomass and act as a source of energy for grazers [11]. It is therefore important to note that the biomass stoichiometry of microorganisms affects how nutrients are recycled and in which proportions they are released back into the environment [12]. While the direct relationship of excretion and ingestion of elements being dependent on consumer stoichiometry has been successfully demonstrated for herbivores [4], an in-depth investigation of this relationship has yet to be elucidated for microorganisms. Differential recycling of certain elements by microbes has been shown to have effects on other members of food webs such as herbivores, zooplankton, and algae

[13] while changes in their communities also induce changes in bacterial community composition and activity [13,14]. The direct implications of stoichiometric differences in different bacterial groups on higher trophic levels of the food web could most likely be investigated by comparing rates of bacterivory in zooplankton species that differ in P demand [3], but this relationship will not be part of my study.

The range of C:N:P in bacteria

In 1958, Redfield published his well known work about ratios of C, N, and P in algae, which in nowadays is commonly referred to as the 'Redfield ratio' (C:N:P = 106:16:1 (molar ratio) [15]). Bacteria tend to have relatively higher amounts of N, and especially P, compared to the Redfield ratio [16]. One of the first estimates of the bacterial C:N:P ratio was published in 1987 by Goldman [17], who discovered a ratio of 45:9:1 in bacteria. In 2007, Cleveland & Liptzin [18] estimated a ratio of 60:7:1 for soil bacteria. While C:N ratios were found to be relatively constant over bacterial species and growth conditions (~ 3.5 – 12) [3,19], large variations in C:P (~ 8 – 1000 [20,21]) and N:P (~10 – 200 [21]) have been reported.

A number of studies have addressed the factors that might be responsible for differences in microbial stoichiometry. Some of the factors, which have been identified to play a role for organism stoichiometry are temperature [22], growth rate [20], growth phase [19], autotrophy/ heterotrophy [23], species [16], and the habitat [16,24].

Different ways to measure bacterial stoichiometry

There are two different methods, which are commonly used to measure microbial stoichiometry these days. The first one is X-ray microanalysis (XRMA), which has been used since more than a quarter of a century to measure the elemental content of single bacteria of various habitats [16,24], of individual isolates [25,26], under different growth conditions [19,27] or nutrient limitation, and in interaction with other organisms [28]. On the other hand, similar questions about microbial stoichiometry have been investigated by measuring bulk cell material of many cells with wet chemical analyses [3,20,22,29,30].

Energy Dispersive X-ray Spectroscopy (EDX), also know as **X-ray microanalysis** (XRMA), combined with Transmission Electron Microscopy (TEM),

was first used to quantify the stoichiometry of single bacteria in 1985 by Heldal et al. [31], and an improved protocol was published in 1995 by Norland et al. [32]. EDX measures the high-energy photons (X-rays), which are emitted when accelerated electrons interact with matter [32]. Every element emits characteristic X-rays when the accelerated electrons interact with electrons from the element's shells. The energy of the emitted X-rays matches the differences in energy levels between the different shells of the atoms. As every element has its characteristic energy differences (X-ray emission lines of C, N, and P.: [33]), the X-rays can be used to qualify and quantify the elements in a sample [32].

Measurements of **bulk cell stoichiometry** are usually performed by elemental analysis with a CHN analyzer (C, N), and different ways of P digestion (e.g. [22]). For C and N analysis, cells are either filtered directly onto combustible GF/F filters, or they are harvested through centrifugation from the medium and pelleted into tin capsules. Either of them is then analyzed in an elemental CHN analyzer and an average value for all cells in the sample is achieved.

A third method to measure elemental composition, which has been used in biology [34], but not applied yet for measurements of microbial stoichiometry, is **Electron Energy Loss Spectroscopy** (EELS) combined with TEM. EELS measures the changes of kinetic energy, which occur when the electron beam interact with a sample [35]. These changes are specific for every element and resulting energy edges can be quantified and qualified. Several studies have shown that for elements with an atomic number of $Z < 17$ [36] (C = 6, N = 7, P = 15), the analytical sensitivity of EELS is higher compared to EDX.

In this thesis I will evaluate the advantages and disadvantages of the different methods to measure bacterial stoichiometry using two bacterial species harvested at logarithmic and stationary growth phase and grown at a range of different resource stoichiometries.

Methods

Cultivation

Two bacterial species, *Pectobacterium carotovorum* (formerly *Erwinia carotovora* [37] (Fig. 1A & 1E), and *Verrucomicrobium spinosum* (Fig. 1B & 1F), were grown in twelve different resource treatments on minimal media containing different ratios of C, N, and P (Table 1), and harvested each in logarithmic and stationary phases of the growth (see Keiblinger et al. 2010 for detailed description [38]).

Bulk cell material of all treatments was analyzed previously (Table 2; method described below). Three treatments of each species were chosen for single cell elemental analysis based on the results from the bulk analysis in order to cover a broad range of biomass stoichiometry for each species.

Table 1: Initial resource concentrations used to culture *Pectobacterium carotovorum* (here: E) and *Verrucomicrobium spinosum* (V). NH_4Cl was used for culturing *P. carotovorum* and $NaNO_4$ for *V. spinosum*.

Treatment	Concentration (mM)			Molar ratios			*P.*	*V.*
#	Glucose	$NH_4Cl/NaNO_4$	NaH_2PO_4	C:N	C:P	N:P	*carotovorum*	*spinosum*
1	25	12.5	0.25	12	600	50		X
4	25	1.25	0.025	120	6000	50	X	X
7	5	5	0.1	6	300	50	X	
8	5	5	0.01	6	3000	500		X
9	5	0.5	0.1	60	300	5	X	

Bulk cell analysis

C, N, and P of bulk cell material from each culture was measured with wet chemical analyses on freeze-dried, pelleted biomass. Briefly, C and N were measured from biomass weight into tin capsules (~2 mg/ sample) using an elemental analyzer (EA1110 CE Instruments Milan Italy). Total phosphorus was determined through acid extraction of dried samples in a nitric acid-perchloric acid mixture (HNO_3:$HClO_3$, 4:1) and subsequent photometric measurements [38,39].

TEM grid preparation

After cultivation, cells were harvested by centrifugation, washed three times with NaCl, centrifuged again, and finally resuspended in 130 mM NaCl. Aliquots were fixed with paraformaldehyde to a final concentration of 4 % for two hours at 4°C, washed in PBS, and then stored in a 1:1 mixture of 96% Ethanol and 1x phosphate buffered saline (PBS). Until further processing, samples were stored at -20°C.

Before TEM grid preparation, cell density was estimated by DAPI staining on black polycarbonate filters and counting with epifluorescent microscopy [40]. Cultures were washed to remove salts by diluting the cell cultures with Milli-Q water before centrifugation (10 min., 10000 rpm), and subsequent removal of the supernatant. Cells were re-dissolved Milli-Q water depending on the estimated cell densities. Between 6 – 7 µl of washed cell culture from each was treatment pipetted directly onto the coated (= shinny) side of a carbon coated copper grid (300 mesh; Agar Scientific catalog #S160-3), while holding the grids with forceps. The grids were air dried on filter paper or dried under pressurized air. To see if there were cells on the grids they were checked with a TEM Philips 208 and photographs of prepared cells were recorded.

Table 2: Average biomass stoichiometry as determined by chemical analysis of bulk cells for the logarithmic growth phase cells (L) as well as of the stationary phase cells (S). This table was used to select three treatments from each species in order to cover a wide range of stoichiometric variation (selected treatments: **bold**)

Treatment	**C:N**	**C:P**	**N:P**		**C:N**	**C:P**	**N:P**
	Logarithmic growth phase (L)				*Stationary growth phase (S)*		
E1L	4.6	55.1	12.0	E1S	6.1	131.9	21.6
E2L	5.1	111.3	21.7	E2S	6.0	194.6	32.5
E3L	4.3	36.0	8.4	E3S	6.8	45.1	6.6
E4L	**5.3**	**142.2**	**26.8**	**E4S**	**6.8**	**223.2**	**32.9**
E5L	5.3	36.6	7.0	E5S	6.9	41.1	5.9
E6L	4.6	34.7	7.5	E6S	7.1	68.9	9.7
E7L	**4.9**	**95.6**	**19.6**	**E7S**	**5.1**	**122.3**	**23.9**
E8L	4.8	92.1	19.0	E8S	5.8	173.4	29.7
E9L	**4.6**	**31.6**	**6.9**	**E9S**	**6.5**	**32.7**	**5.0**
E10L	5.4	113.8	21.1	E10S	6.4	174.8	275
E11L	5.2	35.3	6.8	E11S	7.0	37.4	5.3
E12L	6.0	66.7	112	E12S	7.3	76.6	10.6
V1L	**4.5**	**58.3**	**12.9**	**V1S**	**6.3**	**177.3**	**27.3**
V2L	6.1	191.9	29.7	V2S	6.6	261.6	37.0
V3L	6.0	78.0	13.0	V3S	4.8	63.5	13.1
V4L	**5.0**	**98.6**	**19.8**	**V4S**	**5.3**	**95.7**	**18.2**
V5L	6.0	11.1	1.8	V5S	8.0	95.1	12.0
V6L	5.8	51.2	8.7	V6S	7.8	111.7	15.5
V7L	5.1	76.7	15.1	V7S	6.4	120.7	19.1
V8L	**7.5**	**314.2**	**41.7**	**V8S**	**8.2**	**388.1**	**47.4**
V9L	7.2	84.4	11.8	V9S	8.3	122.7	14.8
V10L	6.8	223.2	32.7	V10S	7.0	27.0	39.1
V11L	6.5	33.0	5.1	V11S	8.9	103.7	12.0
V12L	7.3	88.9	12.3	V12S	7.3	97.9	13.7

Elemental analysis

Elemental content of C, N, and P of single cells from each species of the selected treatments was measured with Energy Dispersive X-ray Spectroscopy (EDX) and Electron Energy Loss Spectroscopy (EELS). All of the selected treatments described above were measured with EDX and a subset of the treatments was measured with EELS. EDX and EELS were performed on a FEI TECNAI G20 *(Vienna University of Technology, University Service center for Transmission Electron Microscopy (USTEM))*. The acceleration voltage was set to 200kV, and the microscope was operated in transmission electron mode (TEM). A tilt angle of around 15° was used for optimal X-ray detection by the EDX detector. Grids were inserted with the carbon-coated side facing the electron source.

Electron Energy Loss Spectroscopy (EELS)

In terms of accuracy of light element detection, EELS would be the method of choice, since it is much more sensitive in detecting light elements, such as C, N, and P (atomic numbers: C = 6, N = 7, P = 15), compared to EDX [36]. EELS spectra were measured of exact the cells, which had before been measured with EDX, for having a good basis of comparison of the two methods. EELS measurements were performed in EFTEM mode (energy filtered TEM mode) with a 2 mm spectrometer entrance aperture arranged around one cell at a time. At magnifications between 7400 – 18500 X, spectra of single cells as well as particle free areas next to each cell were recorded using the software DigitalMicrograph [41]. Pictures of some individual cells were taken with the same software. For each measurement point (i.e. cell or background), two spectra were collected, a zero loss spectrum and one spectrum without the zero loss peak (ZLP), shifted +160 eV, in order to increase resolution in the core loss area, where the C, N, and P edges are located. The optimal recording time was assigned separately for each spectrum before the spectrum was acquired.

For evaluation, the two spectra were calibrated (the ZLP was set to 0 eV and the steepest area of carbon edge was set to 284 eV), spliced and the absolute amounts of C, N, and P per area were computed using the EELS Quantification tool of DigitalMicrograph. The convergence angle used for the evaluation was 0.1 mrad and the collection angle 80 mrad. The cell spectra were background corrected by subtracting the elemental analysis results from a spectrum recorded in a cell-free

area of the carbon film, which had been recorded, calibrated, and quantified analogous to the cell spectra.

Energy Dispersive X-ray Spectroscopy (EDX)

From each treatment between 10 and 36 cells (Table 3) were measured with EDX. For every cell, a background spectrum was measured in a cell-free area close to the cell with the exact same parameters as the cell spectrum.

For EDX the microscope was operated at a dispersion of 5ev/ch 102.400 µs, a live time (= accumulation time) of 60 s, the spot size set to 1, and a beam current between 2.64 and 8.5 µA (= Emission step: 1 – 5). The beam current was adjusted from cell to cell in order to achieve an optimal dead time of around 40%. Spectra of single cells were collected with the software TEM Image & Analysis (FEI, [42]) at magnifications between 8300 – 26000 X (depending on cell size) after positioning the electron beam around a single cell by changing the beam intensity. For each cell, a spectrum of an adjacent particle free area was collected with the exact same settings as for the cell.

After data collection, the spectra were evaluated with the TIA software. First, the background spectrum was subtracted from the cell spectrum. The 'peak identification tool' was used in order to position the peaks correctly and the 'calibration shift tool' was used, if necessary, to match the assigned peak lines to the maximum peak positions. Afterwards a background area in the spectrum was selected in order to remove it before performing the elemental analysis. The background area was selected similarly for all cells ranging from 4 to 7.5 keV and from 9.3 keV to the end of the spectrum (around 20 keV). Spectra were analyzed for C, N, and P only. All of these were calculated together by using the 'EDX quantification tool', which includes background correction (based on the background set before), peak fitting, and quantification. Quantification results were reported in ratios of the selected elements in weight % as well as atom % (= molar ratio). All subsequent analyses were performed with the molar ratios.

Cells were first analyzed for content of C, N, and P without a standard and later corrected against Coenzyme A (CoA: $Na_3C_{21}H_{36}N_7O_{16}P_3S$; Fig. 1E), which was used as a standard with known elemental composition [32]. CoA was added onto slightly damp TEM grids by gently rubbing the carbon-coated side of a TEM grid

against the inner side of the storage vial. This way, crystals of different sizes got stuck to the carbon film. Particles of similar sizes as microbial cells were measured the same way as bacterial cells. The spectra were then used as a reference for evaluation in TIA by evaluation all cell spectra against one of the standard spectra.

C:P ratios above 1000, resulting from % P values close to the detection limit of EDX, were excluded from the results.

On one of the grids, spectra of several cells were recorded on two different days to evaluate the accuracy of the method between different measuring dates and with slightly different settings.

Statistical evaluation

To evaluate if bulk cell measurements and single cell measurements yield similar results, a one-way ANOVA was performed with the elemental ratios of the EDX results, the standardized EDX results (EDX_S) and the bulk results for each species, treatment, and growth phase. If significant differences ($p < 0.05$) were detected, a post hoc Least square means Tukey HSD was performed to find out which results where responsible for the significant differences. All statistical analyses were performed using the statistical software JMP [43].

Table 3: Number of cells/ samples measured with each method (EDX, EDX_S and bulk analysis). a: EDX measurements (not standard corrected) b: Standard corrected EDX measurements c: measurements of bulk cell material with wet chemical analyses.

	n EDX[a]	n EDX_S[b]	n Bulk[c]	n EELS
E4L	34	34	3	-
E7L	30	30	3	-
E9L	37	36	3	-
V1L	27	14	3	-
V4L	32	27	4	-
V8L	29	21	3	-
E4S	20	19	3	6
E7S	19	16	3	7
E9S	10	20	3	10
V1S	33	30	4	6
V4S	14	14	4	10
V8S	16	14	4	9

Results

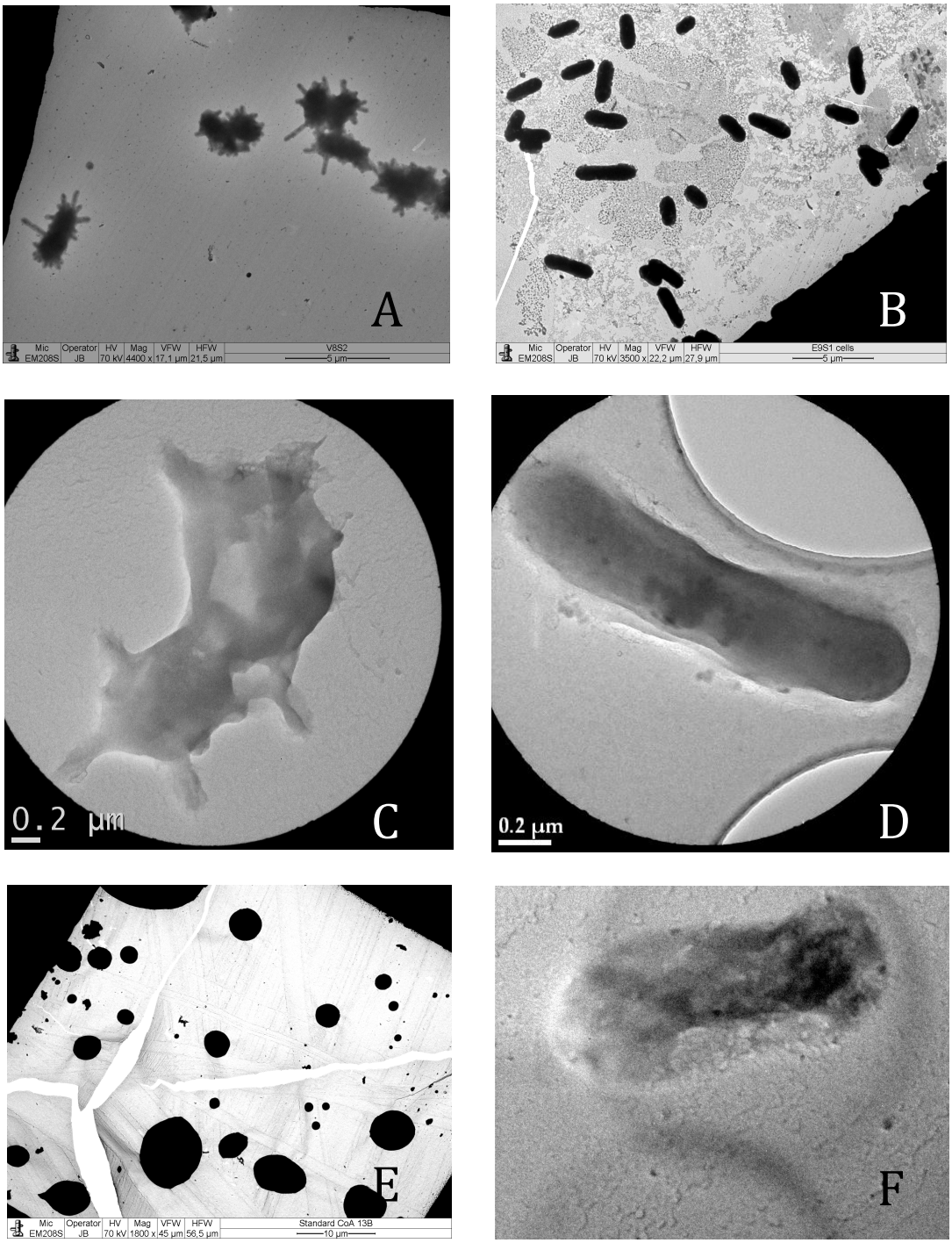

Figure 1: TEM pictures of *Verrucomicrobium spinosum* (A, C), *Pectobacterium carotovorum* (B, D), Coenzyme A particles (E), and a *P. carotovorum* cell with two rings around it (probably a damage caused by the electron beam) (F) on carbon-coated TEM grids. Pictures A, B, and E were taken with a TEM Philips 208, pictures C, D, and F with a FEI TECNAI G20.

Electron Energy Loss Spectroscopy

I first evaluated the cells with EELS to see if that had additional analytical power for the light elements (i.e. C and N). Evaluation of the EELS spectra (typical EELS spectrum: Figure 2) resulted in negative amounts of P, and very low amounts of N calculated for all cells. Several adjustments in the measuring technique were tried out (e.g. changing the shift of the coreloss spectrum and longer measurement times), but brought no improvements. Therefore, I conducted no further analysis with EELS.

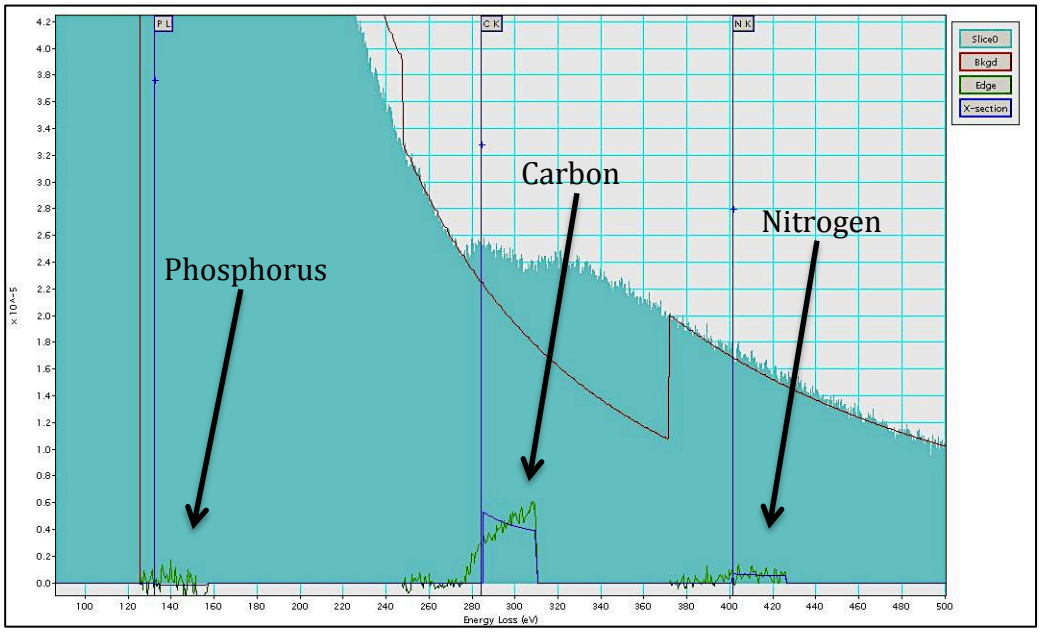

Figure 2: Example of an EELS spectrum with the position of the edges for C, N, and P. X-axis: Energy loss (keV), y-axis: relative amount. The left part of the spectrum, where the zero loss peak is located, is cut off to show only the Plasmon area in which C, N, and P are situated. P - K-edge: 132 keV, C –K-edge: 384 keV, N – k-edge: 401 keV.

Energy dispersive X-ray Spectroscopy

Methodological remarks

Initially it was difficult to separate the peaks of C and N in the EDX spectra (Fig. 3a: EDX spectrum where the N peak is situated right on the shoulder of the C peak). This was improved by changing the dispersion settings to 5 eV/ ch, 102.400 µs, which lead to separation of C and N peaks (Fig. 3b).

Several CoA particles of slightly different sizes were measured, and all measurements resulted in spectra showing all the elements expected from the chemical structure of CoA (Fig. 4), indicating that the given settings were able to

successfully evaluate multiple elements from a single sample. In addition, polystyrene latex beads (C_8H_8) with a diameter of 0.855 were used as an internal standard to estimate the amount of error in the evaluation of N and P, as polystyrene beads consist only of carbon and hydrogen. While EDX analysis correctly detected 0.00 atom % for N, for P an average of 1.19 atom % was calculated.

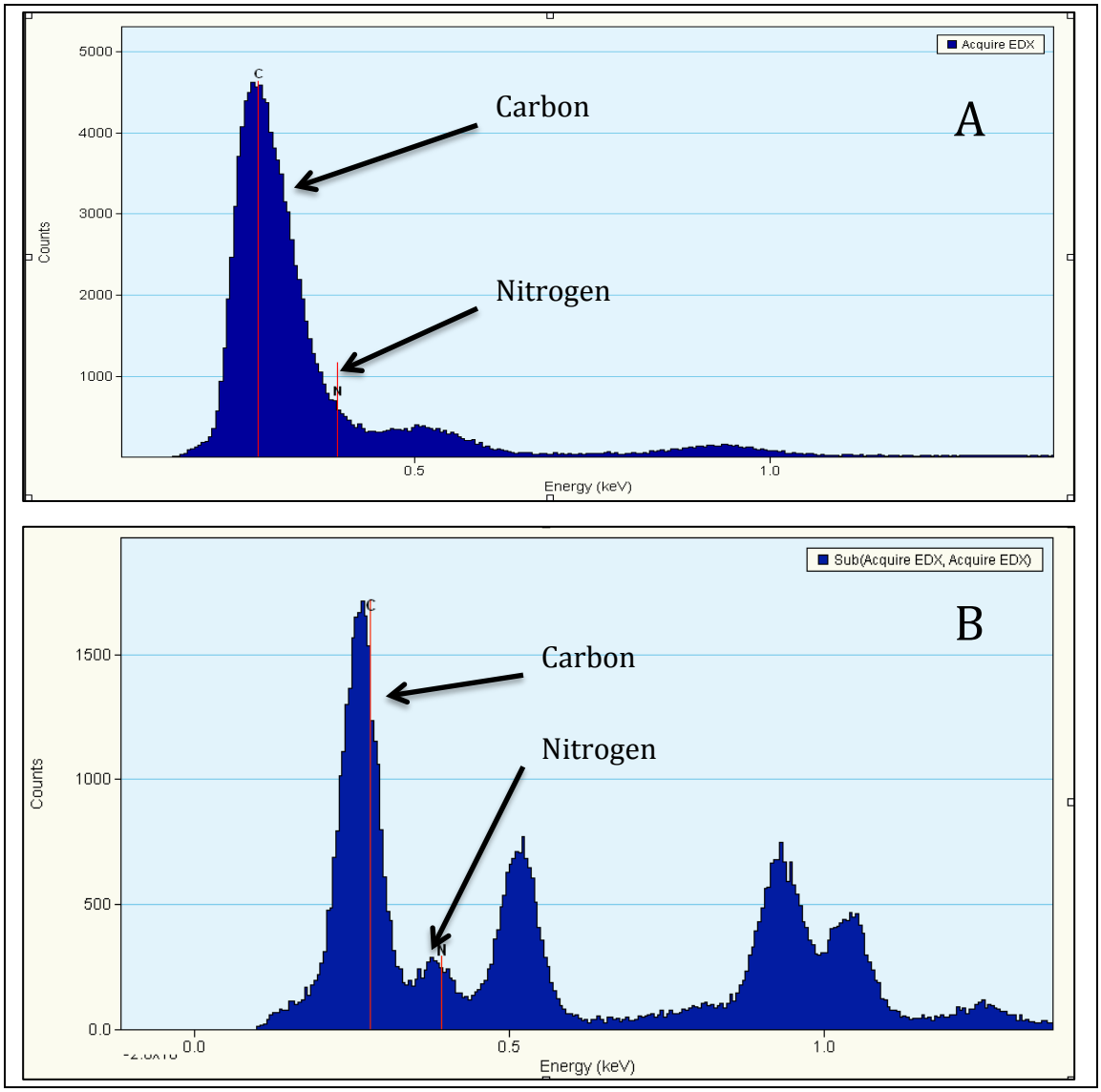

Figure 3: First part on an EDX spectrum showing the position of the C and N peak. A: Spectrum where the N peak is not clearly separated from the C peak. B: Clearly differentiable C and N peaks. X-axis: X-ray energy value (keV), y-axis counts per time. The area below a Gauss-curve fitted for the peak of each element (marked with a vertical line), correlates to the amount of the given element in the sample.

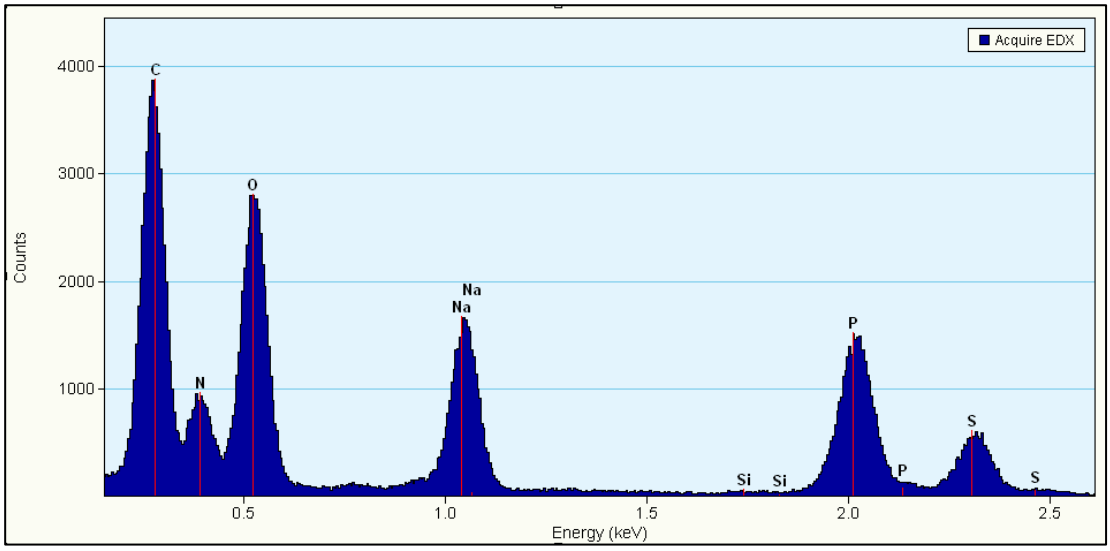

Figure 4: EDX spectrum of Coenzyme A (CoA: $Na_3C_{21}H_{36}N_7O_{16}P_3S$). The spectrum contains peaks for all elements present in this molecule (except for hydrogen as H is too light to be detected with EDX). Axis labels: same as in Fig. 3.

Stoichiometric ratios of single cells vs. bulk cell measurements

Average cell stoichiometry of single cells not evaluated against CoA (*from here called:* EDX) ranged for C:N from 5 – 13, for C:P from 85 – 806, and for N:P from 17 – 149 by atom. Standard corrected cell ratios (EDX_S) ranged from 6 – 11 for C:N, from 51 – 608 for C:P, and from 6 – 99 for N:P (Table 4). The overall average of C:N:P of EDX results was 365:67:1, for EDX_S it was 228:33:1, and for bulk measurements 148:24:1. Treatments that received the lowest amount of P in the media (cf. Table 1) had C:P ratios up to eight fold higher (EDX), respectively six fold higher (EDX_S), and four fold higher (bulk) than the C:P of the Redfield ratio, while treatments that received high amounts of P with the media where either close to the Redfield ratio C:P or even below it.

I furthermore evaluated if stoichiometry was different between species and between growth phases (Table 5). Significant differences ($p < 0.05$, t-test with Welch correction) were detected for nearly all stoichiometric ratios measured with EDX and EDX_S when evaluated for effect of growth phase (combined for both species), when growth phases were divided by species, when ratios were divided by species (regardless of growth phase), and when species and growth phase effects were combined. Highly significant differences were evaluated especially for elemental ratios grouped by species ($p < 0.001$ in all cases). Bulk

cell results did not show this high degree of significant differences, but averages followed the same trend.

Table 4: Mean elemental ratios in atom % for C, N, and P of the not-standard-evaluated EDX spectra (EDX), EDX spectra evaluated against CoA (EDX_S), and bulk measurements (Bulk) for all treatments, species, and growth phases.

	EDX				EDX_S				Bulk			
	n	**C:N**	**C:P**	**N:P**	***n***	**C:N**	**C:P**	**N:P**	***n***	**C:N**	**C:P**	**N:P**
E4L	*34*	7,50	289,46	38,87	*34*	6,25	153,87	24,80	*3*	5,31	142,36	26,85
E7L	*30*	8,30	245,49	29,67	*30*	7,30	131,51	18,14	*3*	4,86	95,69	19,67
E9L	*37*	7,53	126,51	16,87	*36*	6,53	65,11	10,05	*3*	4,60	31,59	6,86
V1L	*27*	7,99	202,17	24,67	*14*	6,55	97,44	14,91	*3*	4,51	58,31	12,91
V4L	*32*	8,70	274,34	31,55	*27*	7,64	151,63	19,72	*4*	4,98	98,73	19,80
V8L	*29*	13,23	805,66	149,41	*21*	11,11	607,78	98,67	*3*	7,54	314,55	41,71
E4S	*20*	7,64	675,26	105,83	*19*	6,50	298,61	46,00	*3*	6,78	223,25	32,94
E7S	*19*	8,01	161,51	28,02	*16*	7,37	100,78	14,50	*3*	5,12	122,29	23,90
E9S	*10*	4,70	85,34	18,06	*20*	7,98	50,90	6,44	*3*	6,52	32,69	5,01
V1S	*33*	7,78	559,55	134,83	*30*	6,68	293,00	43,34	*4*	6,29	177,28	27,34
V4S	*14*	8,02	234,91	29,20	*14*	7,02	137,85	19,49	*4*	5,34	95,69	18,18
V8S	*16*	11,47	717,99	197,28	*14*	8,97	646,93	85,28	*4*	8,19	388,10	47,44
Average		**8,41**	**364,85**	**67,02**		**7,49**	**227,95**	**33,44**		**5,84**	**148,38**	**23,55**

Table 5: Mean C:N, C:P, and N:P evaluated for overall differences between growth phases (L, S) when species were combined and independently, as well as differences between the two species (E, V) when growth phases were combined. A two-sided t-test was performed with Welch-correction for unequal variances (significance level: $p < 0.05$), because the bulk analysis contained many fewer measurements than the single cell work and variances between EDX and EDX_S were significantly different (see below). Bold values: no significant difference.

L: logarithmic growth phase S: stationary growth phase E: *P. carotovorum* V: *V. spinosum*

	EDX			EDX_S			Bulk		
	C:N	**C:P**	**N:P**	**C:N**	**C:P**	**N:P**	**C:N**	**C:P**	**N:P**
Overall Growth phase effect									
L	8.79	275.48	46.80	7.40	155.69	27.28	5.28	122.23	21.22
S	8.09	389.75	94.67	7.30	241.33	35.41	6.41	179.95	26.54
t-test	*0.013*	*0.001*	*0.001*	***0.651***	*0.000*	*0.041*	*0.002*	***0.102***	***0.193***
P. carotovorum growth phase effect									
E L	7.75	216.71	28.08	6.67	115.21	17.49	4.92	89.88	17.79
E S	7.18	313.23	57.99	7.29	150.98	22.45	6.14	126.08	20.62
t-test	*0.036*	***0.070***	*0.000*	*0.016*	*0.037*	***0.069***	*0.001*	***0.286***	***0.595***
V. spinosum growth phase effect									
V L	9.97	355.70	68.28	8.57	233.53	43.60	5.61	151.35	24.31
V S	8.77	479.67	124.76	7.32	330.06	47.70	6.61	220.36	30.99
t-test	*0.009*	*0.036*	*0.034*	*0.000*	*0.025*	***0.620***	***0.102***	***0.211***	***0.249***
Species effect									
E	7.56	243.60	37.44	6.89	128.36	19.32	5.53	107.98	19.20
V	9.47	402.58	91.17	7.97	283.58	45.62	6.15	188.99	27.95
t-test	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	***0.096***	*0.016*	*0.028*
Combined effect of species and growth phase: E L vs. V L									
t-test	*0.000*	*0.001*	*0.002*	*0.000*	*0.000*	*0.001*	***0.153***	***0.149***	***0.214***
Combined effect of species and growth phase: E S vs. V S									
t-test	*0.000*	*0.016*	*0.008*	***0.930***	*0.000*	*0.000*	***0.334***	***0.065***	***0.089***

Comparing EDX results with bulk results showed that 28 % of the stationary growth phase cells and 44 % of the logarithmic growth phase cells were not significantly different from the bulk results ($p < 0.05$, evaluated with a one-way ANOVA and post-hoc Tukey HSD; stationary growth phase: Fig. 5, logarithmic growth phase: Fig. 6). When evaluated against CoA, 67 % of the stationary phase cells, and 89 % of the ratios from the logarithmic phase cells were significantly not different from the bulk measurements. Overall, this means that 36 % of the elemental ratios calculated with EDX and 78 % of the ratios corrected with CoA could statistically not be differentiated from the bulk cell results. Furthermore, only 28 % of the EDX results from the stationary growth phase and 17 % from the logarithmic growth phase (average: 22 %) showed no significant difference to the EDX_S results.

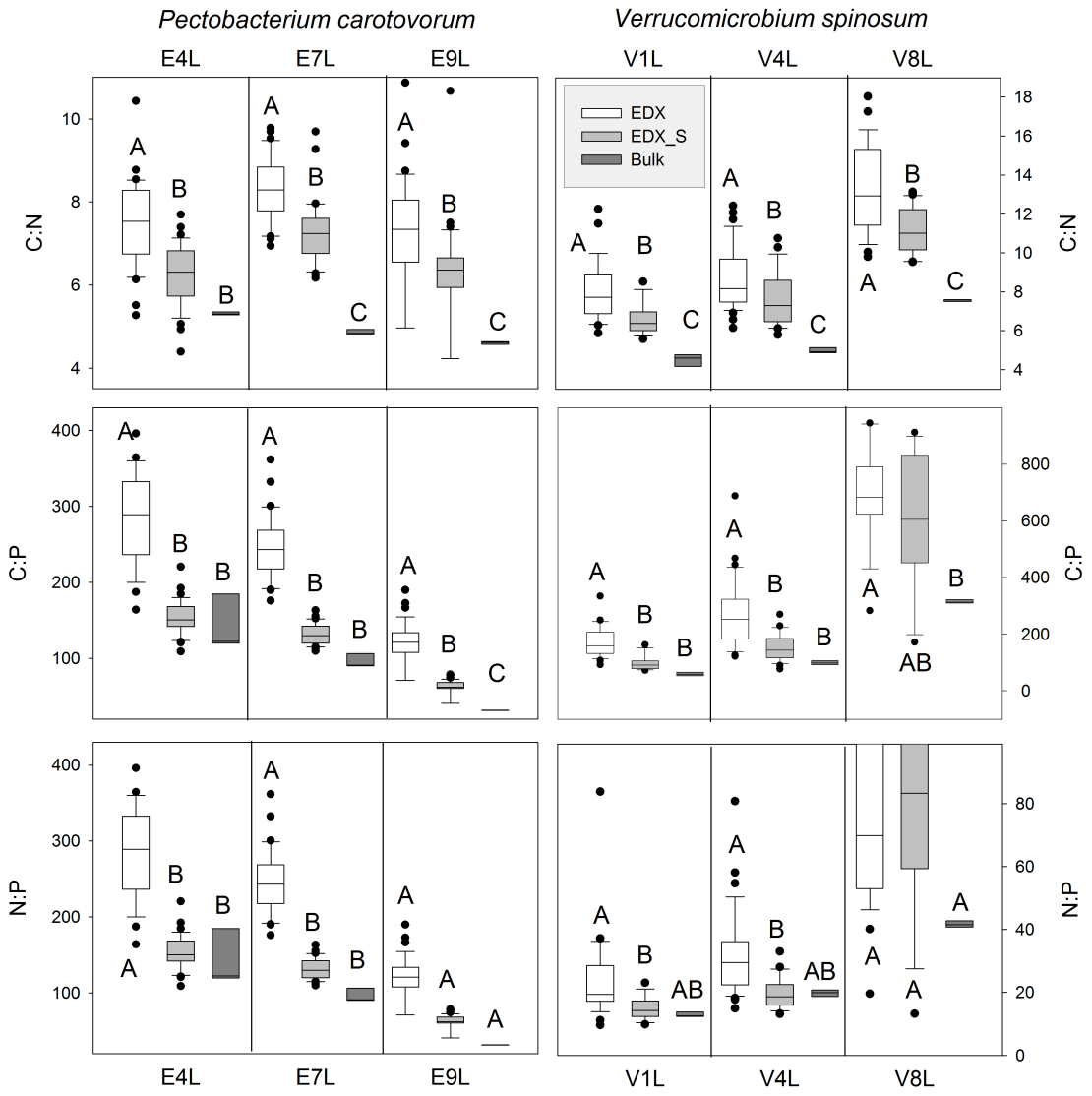

Figure 5: Elemental ratios (C:N, C:P, & N:P) of logarithmic phase treatments of *Pectobacterium carotovora* (E4L, E7L & E9L) and *Verrucomicrobium spinosum* (V1L, V4L, & V8L). White boxes show EDX results not corrected with a standard (n = 14 - 34), light grey boxes show EDX results corrected with Coenzyme A spectra (EDX_S; n = 11 – 34), and dark grey boxes bulk cell results (n = 3 – 4, measured with wet chemical analyses). The plots show the median, 25% and 75% quartiles (boxes), 10% and 90% percentiles (bars), as well as outliers (points). The letters indicate significance differences (i.e. same letters indicate that the difference between the plots is not significant), calculated by a Least Square Means Tukey HSD with the software JMP [43].

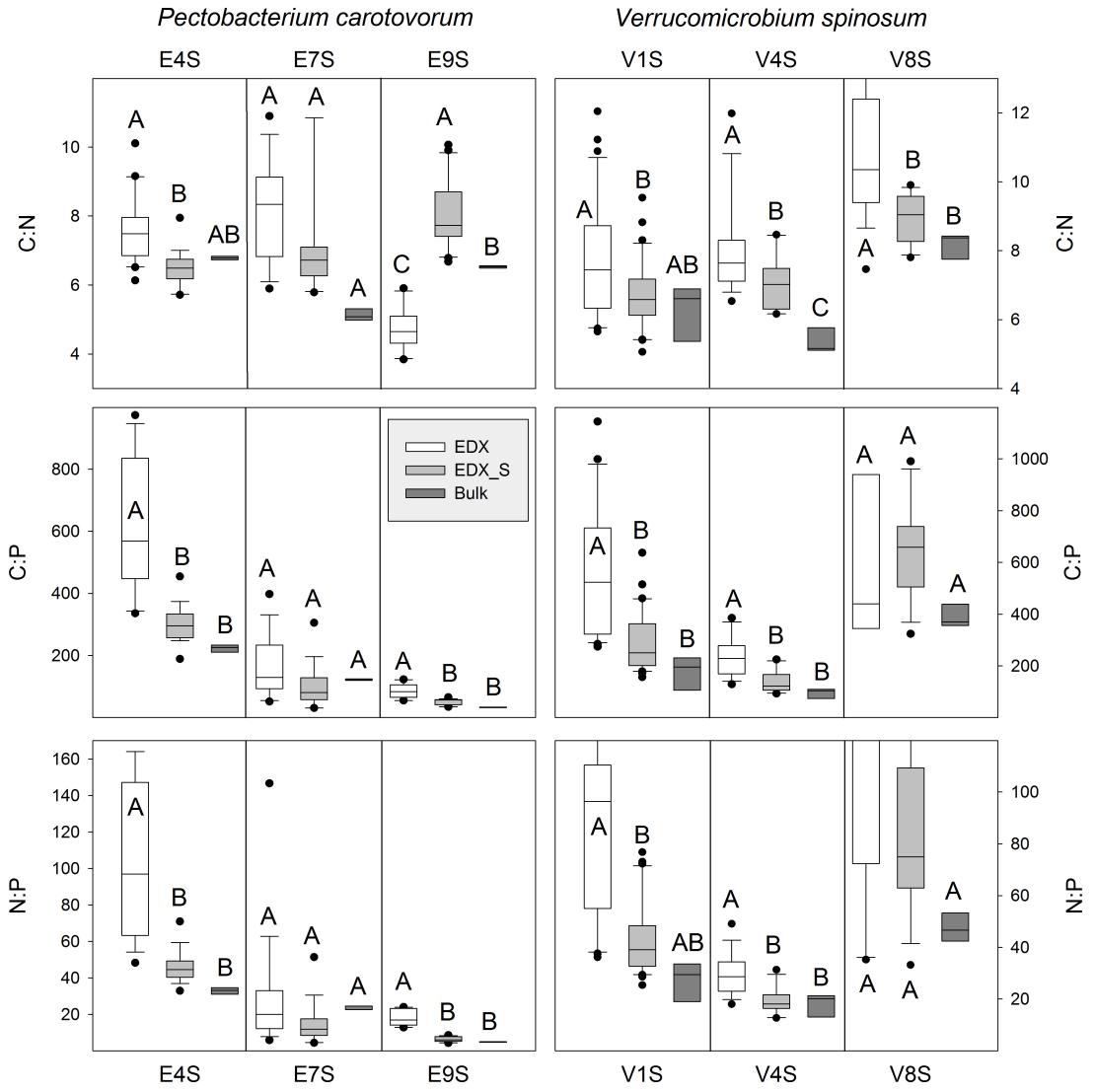

Figure 6: Elemental ratios (C:N, C:P, & N:P) of stationary phase treatments of *Pectobacterium carotovorum* (E4L, E7L, & E9L) and *Verrucomicrobium spinosum* (V1L, V4L, & V8L). White boxes show EDX results not corrected with a standard (n = 14 - 34), light grey boxes show EDX results corrected with Coenzyme A spectra (n = 11 – 34), and dark grey boxes bulk cell results (n = 3 - 4, measured with wet chemical analyses). The plots show the median, 25% and 75% quartiles (boxes), 10% and 90% percentiles (bars), as well as outliers (points). The letters indicate significant differences (i.e. same letters indicate that the difference between the plots is not significant), calculated by a Least Square Means Tukey HSD with the software JMP [43].

Differences in variances

Comparison of variances between EDX and EDX_S

In 83% of all treatments, the EDX_S results had a smaller variance compared to the EDX results, calculated by the dividing the coefficient of variation (CoV) of the EDX_S treatments by the CoV of the EDX treatments (Table 6). The difference in variance of C:N ratios between EDX and EDX_S for all treatment was not significant. However, for the C:P and N:P ratios, the variance differed significantly ($p < 0.05$ & $p < 0.001$, respectively). On average, the variance of all elemental ratios was higher in the EDX results compared to the EDX_S results. When looking at the differences within each treatment, only one treatment (E7S) had on average higher variances in the EDX_S results whereas all other treatments had higher variances in the EDX results.

Table 6: Comparison of variance within EDX results (EDX) and standardized EDX results (EDX_S) for every treatment and growth phase. Numbers show the result of dividing the CoV of EDX_S from the CoV of EDX. Numbers > 1 indicate a bigger variance in the not standardized results, whereas numbers < 1 indicate larger variance in the standardized results (bold numbers).

	C:N (n.s.)	C:P (*)	N:P (***)	Average
E4L	1.19	1.70	1.64	1.51
E7L	**0.85**	1.62	1.42	1.30
E9L	1.01	2.07	1.81	1.63
V1L	1.58	1.18	2.40	1.72
V4L	1.04	1.37	1.67	1.36
V8L	1.64	**0.60**	1.62	1.29
E4S	1.65	1.73	2.24	1.87
E7S	**0.48**	**0.93**	1.52	**0.97**
E9S	1.04	1.49	1.09	1.20
V1S	1.45	1.16	4.31	2.31
V4S	1.53	**0.95**	**0.99**	1.16
V8S	3.43	1.99	2.52	2.65
Average	1.41	1.40	1.93	1.58

Comparison of variances between stationary and logarithmic growth phase

I also compared the variances between the stationary and the logarithmic growth phase, using only the standardized EDX results (= EDX_S), by dividing the CoV of the logarithmic growth phase cells by the CoV of the stationary growth phase cells (Table 7). On average, 67 % of the stationary growth phase treatments had a higher variance compared to the logarithmic growth phase treatments. When separated by elemental ratios, the variance of the logarithmic growth phase was

higher for the C:N ratios (67 %), but lower for C:P (16 %) and N:P (16 %). The average variance over all ratios was 1.91 fold higher.

Table 7: Difference in the variance of single cell measurements between stationary and logarithmic growth phase, calculated by dividing the CoV of the logarithmic growth phase cells by the CoV of the stationary growth phase cells. Treatments with number > 1 have a higher variance in the stationary growth phase compared to the logarithmic growth phase (67 % of all treatments), while treatments with a number < 1 have a higher variance in the logarithmic growth phase treatments (bold letters).

Treatment	C:N	C:P	N:P	Average
E4	**0.68**	1.40	1.34	1.14
E7	3.72	6.33	6.48	5.51
E9	**0.89**	2.18	2.52	1.86
V1	1.22	1.44	1.31	1.32
V4	**0.64**	1.04	1.08	**0.92**
V8	**0.77**	**0.72**	**0.61**	**0.70**
Average	1.32	2.18	2.22	1.91

Comparison of variances between EDX_S and bulk measurements

Except for two ratios for treatment E4L (C:P and N:P), the variance of the individual elemental ratios of the EDX_S results was higher compared to the variance of the bulk material (Table 8). Averaged over all treatments and ratios, the variance of the bulk material was 43 % of the variance of the EDX measurements. For C:N, the variance of the bulk results was 29 % of the EDX results, for C:P 50 % and for N:P 48 %.

Table 8: Comparison CoV of bulk measurements and the standardized EDX measurements. The CoV of the bulk measurement is expressed as proportion of the CoV of the EDX_S measurements. Bold numbers: > 1, indicating a larger variance in the bulk measurements as compared to the EDX_S measurements.

Treatment	C:N	C:P	N:P	Average
E4L	0.06	**1.81**	**1.82**	**1.23**
E7L	0.12	0.91	0.69	0.57
E9L	0.05	0.05	0.11	0.07
V1L	0.57	0.44	0.25	0.42
V4L	0.17	0.25	0.21	0.21
V8L	0.05	0.05	0.03	0.05
E4S	0.09	0.27	0.29	0.22
E7S	0.09	0.03	0.06	0.06
E9S	0.04	0.13	0.11	0.09
V1S	0.94	1.00	0.93	0.96
V4S	0.69	0.65	0.99	0.78
V8S	0.64	0.45	0.27	0.45
Average	0.29	0.50	0.48	0.43

Over- and underestimation of elements with EDX

A comparison of atom % C, % N, and % P of the EDX (respectively EDX_S) results plotted against the corresponding bulk results combined for all species, treatments, and growth phases revealed an overestimation of % C in the EDX and EDX_S results and an underestimation of % N and % P, compared to the bulk (Fig. 7). The slopes of the calculated regression lines for EDX_S (1.104 for % C, 0.935 for % N, and 1.399 for % P) are all fairly close to 1, indicating strong similarities. The coefficient of determination (r^2) of the regression line is 0.514 for % C, 0.466 for % N, and 0.783 for % P, which means that 51 % of variation within the EDX_S results of % C can be explained by variation of the bulk results (respectively 46 % for %N, and 78 % of % P). Atom % of not-standardized EDX results were also plotted against bulk results. On average, the fit of the not-standardized results to the 1-to-1-line was weaker (slope: 0.409 for % C, 0.164 for % N, and 2.360 for % P) and the coefficient of determination explains less of the variation between the points (r^2: % C = 0.155, % N = 0.029, and % P = 0.737).

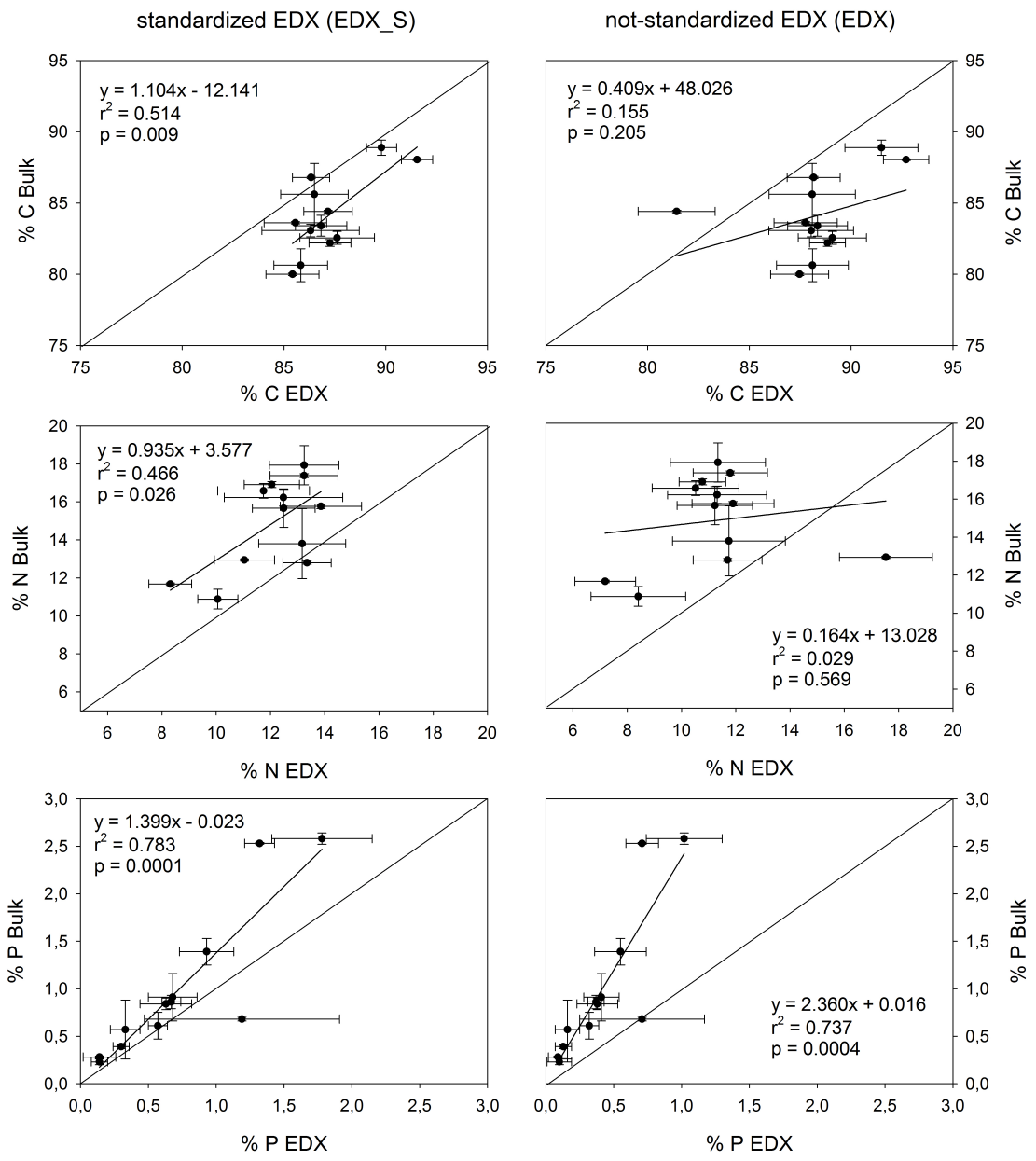

Figure 7: Averages (dots) and standard deviation (error bars) of atomic % C, N, and P of EDX results (x-axis) plotted against atomic % C, N, and P of the bulk measurements combined for species, treatments, and growth phases. The long solid line shows the 1-to-1-line, while the small line shows a regression through all measured averages. The left side of the plot shows the comparison for the standardized EDX results (EDX_S), while the right side shows the same for the not-standardized EDX results (EDX). The equation (y = ax + b) gives the slope (a) and the axis intercept (b) for each regression line. R^2, the coefficient of determination, shows how well the regression fits the points (1 = perfect fit).

Comparison of data collected on different dates from the same grid

Finally, to compare how well cells maintain their composition after being placed on a TEM grid over time, I measured the same grid on two different dates and compared results between dates. The data showed no significant differences in the molar ratios as well as the elemental ratios (t-test, $p < 0.05$; Table 9). The variance between the two sampling dates was also compared (by dividing the CoV of the first measurement time (V1S) by the CoV of the second measurement time (V1S_2)) and values close to 1 have been calculated, indicating very similar variances. The largest deviation from 1 was recorded for N:P ratios, which showed 71 % similarity, compared to 97 % similarity in the C:N ratios.

Table 9: Averages of atom % as well as elemental ratios of data from the same grid collected on different dates (V1S & V1S_2). Data has been tested with a t-test ($p < 0.05$) and differences in variance have been evaluated by dividing the CoV of V1S by the CoV of V1S_1.

	C Atom %	N Atom %	P Atom %	C:N	C:P	N:P	*n*
Average V1S	86.490	13.170	0.332	6.682	293.002	43.335	*30*
Average V1S_2	86.437	13.174	0.373	6.678	275.120	40.818	*13*
t-test	*0.927*	*0.995*	*0.376*	*0.990*	*0.668*	*0.657*	
CoV V1S/V1S_2	0.9549	0.9671	0.8150	0.9742	0.8390	0.7131	

Discussion

One of the main goals of this study was to compare single cell stoichiometric measurements performed with Transmission Electron Microscopy to measurements of bulk cell material performed with classical chemical methods.

EELS

Analysis with EELS would have an advantage over EDX as EELS is more sensitive to light elements [44] and in theory should provide more accurate values for C, N, and possibly P. However, in this study I was unable to detect P above background levels and results for N were very low. One reason why EELS did not work is probably the thickness of the cells (~400 nm or more), which leads to plural inelastic scattering and a dramatic increase in background levels [45], and thus makes accurate quantifications challenging. This increase in background levels was observed for cells spectra compared to background spectra. Further analysis may be able to circumvent these effects and to still use EELS for single-cell measurements if cells are first embedded in resin and then sliced into sub-micron slices. Using uniform slices would control the thickness effects and even cells of various sizes and diameters would become comparable.

Comparison of EDX and bulk measurements

Standardization

The use of Coenzyme A as a standard decreased the difference between the outcome of single cell EDX measurements and bulk cell measurements. Almost 80 % of all standardized resource ratios were significantly indifferent from the bulk measurements, whereas only 36 % of the not standardized resource ratios showed no significant difference to the bulk results. The remaining 20 % difference between standardized EDX results and bulk results might be due to the low bulk sample numbers in this study (either 3 or 4 samples per treatment), or the use of only one and not several different standards to fine-tune the method, as Norland et al. [32] have done it for their measurements. Furthermore, it cannot be clearly evaluated, if either EDX measurements or bulk analyses yield better results. It is possible, that EDX results are more accurate regarding elemental ratios, because all elements are measured with the same method and on the same cells with, whereas bulk cell measurements are using a different method for

measuring P than for C and N. Even small differences in absolute weight of the sample for C/N and P bulk analyses can have strong affects on the elemental ratios.

How do results compare to previous studies

All the elemental ratios measured in this study range within what has previously been measured for bacterial C:N, C:P, and N:P (e.g. [19,20,21]). On average, the measured ratios were larger than the Redfield ratio [15] (Redfied: C:N:P = 106:16:1; here: EDX_S: 228:33:1, bulk: 148:24:1). This deviation from the Redfield ratio is mainly driven by low amounts of P in several of the treatments, which drove the overall C:P ratio higher. My results fit the expectation that low levels of P in the medium the bacteria are growing in lead to very high C:P ratios in the cells, as it has been reported in a previous study by Cotner et al. for freshwater ecosystems, as well as experimentally enriched bacteria [21].

Over- and underestimation

When atom % values were combined for all species and growth phases, and compared for differences between the EDX and the bulk measurements, a pronounced equalization of the EDX method towards the bulk results was observed when evaluated against CoA (Fig. 8). This evaluation has, on the one hand, demonstrated the importance of a standard when EDX is used to measure single cell stoichiometry and, on the other hand, it gives an idea about which elements are over- or underestimated by each method in comparison to the other method. From my results it seems that EDX, when evaluated against CoA, slightly overestimates C and underestimates N and P, as compared to bulk measurements. The underestimation of P might be due to the relatively low amount of P in the cells, which sometimes falls out of the detection limit of P with EDX, as compared to bulk analyses, where the analysis of many cells at a time conceals the low amounts of P. The overestimation of C is possibly due to deposition of C on the grids while in the TEM. To further investigate if this is the case I propose to measure the exact same cells on a grid several times and evaluate if the % C increases over time. To sum up these findings, I have to state that it is impossible for me at this stage to say if either EDX measurement or bulk measurements yield better results. The 20 % difference between EDX and bulk results might be

aroused by either (or both) methods and further studies will have to evaluate the accuracy and clarify this finding.

Comparison of variances

The decrease in variance when EDX spectra were evaluated against CoA is most likely due to an overall improvement of evaluation when CoA is used as a standard. The variation between the EDX_S results (Table 8) was, except for C:P and N:P of one single treatment, always higher than the variation between the bulk cell analysis, which suggests that we miss a lot of variation when performing bulk analyses (i.e. measuring the average of many cells) with chemical methods as compared to EDX measurements. In addition, it may be that the sample number of the bulk measurements is too small to be compared to EDX measurements, or both. Measuring more replicates with bulk cell material of the same treatments might enlighten if the sample number is crucial in this case. If these differences are not caused by differences in sample size, the results suggests that with respect to variance, bulk cell measurements and EDX measurements yield very different results and are not directly comparable. One reason for this might be the sample preparation for bulk measurements, through which unquantifiable amounts of every other compound in the medium (e.g. salts, nutrients, and other small organisms) will be included in the overall result as the preparation technique does not select solely for bacteria. Improvements of the protocol of bulk cell measurements may reduce the impact of the stoichiometry of the medium on the bulk cell measurements.

Influence of growth phase and species on cell stoichiometry

Previous studies have shown that bacteria tend to have higher ratios of C:N:P in stationary growth phase compared to logarithmic growth phase [19]. This is most likely due to depletion of nutrients, especially P and N in the medium over time in a batch culture, which decreases the absolute amounts of P and N in the cells and thus increases the ratios of C:N and C:P. In this study, I have also been able to compare mean biomass stoichiometry between growth phases (cf. Table 7). For most ratios, I found significantly higher amounts of P in the logarithmic growth phase (i.e. lower C:P and N:P) compared to the stationary growth phase. Higher C:P and N:P ratios in the stationary phase were observed both in the EDX analysis and the bulk results, but the differences between ratios measured with the bulk

analysis were not significant. One reason for this is possibly again the smaller sample size of bulk samples, which measures an average over all cells in the sample as compared to the single cell measurements, where ratios are calculated for each cell separately (bulk: 3-4 samples per treatment, EDX: 10-34 cells per treatment). C:N ratios were on average higher in the logarithmic growth phase cells when measured with EDX and higher in the stationary growth phase when measured as bulk cell material (Table 5). The higher C:N ratios in the logarithmic growth phase measured with EDX contradict a previous study by Vrede et al. [19], who, using EDX found on average higher C:N, C:P, and N:P ratios in stationary phase cells. The difference between these two studies might possibly be explained by the fact that C:N ratios are generally less variable [3], which might lead to an over-interpretation of differences in C:N. The lesser variability in C:N might also explain why bulk samples did in fact follow the expected difference pattern (higher C:N in the stationary growth phase), as their sample size was a lot smaller. To evaluate if either bulk measurements or EDX measurements yield more accurate results for C:N, a higher sample size of bulk samples would be necessary.

The above-described trends of higher elemental ratios in the stationary growth phase were observed both when dividing the growth phases by species, and when growth phases were evaluated including both species (Table 5). This suggests that it is a general trend, which, at least for the two species I investigated, was not species dependent.

However, when I compared differences between the two species, I also found highly significant differences ($p < 0.001$) in C:N, C:P, and N:P between *P. carotovorum* and *V. spinosum* (the only not significant difference being C:N measured by bulk analysis, which again is probably a result of low sample size of bulk material). This result indicates that large stoichiometric variation between species exists [20] and highlights the importance of also investigating the species affiliation when measuring microbial stoichiometry.

Additional issues

EDX analysis, as I have performed it, is not perfect yet and some aspects still have to be regarded to improve the method. One of the problems I was facing regards the preparation of TEM grids. The preparation technique in some cases led to

large amounts of contamination on the grids. As large amounts of these particles can probably be accredited to salts in the medium, improvements to this were possible by washing the cells with Milli-Q water. However, an additional washing step did not always improve the results and thus it might be promising to harvest the cells by centrifugation on TEM grids [32] as with this technique, salts and other dissolved substances will remain in the medium.

Another problem is the damage caused by the electron beam when TEM is applied [46] (Fig. 1D). I have performed measurements of single cells from the same grid on two different dates and did not measure any significant difference between the stoichiometry of the single cells, but it is unlikely that the same cells were measured on the two different dates. It has not been investigated yet for biological samples, if the electron beam can cause changes in cell stoichiometry by damaging cell structures and molecular bindings. Further investigations of this finding would be very important in order to determine to optimal electron dose for exact stoichiometric measurements without changes in stoichiometry during the time the electron beam is applied to one cell.

Conclusion

My study has demonstrated that EDX measurements and bulk cell measurements are comparable if EDX spectra are evaluated against a standard, as both methods yield on average similar results. However, differences in variance were observed and should be further investigated to evaluate if they are artifacts of small sample numbers of the bulk analyses, or if they in fact demonstrate that a lot of variance is lost when only bulk measurements are conducted. Further fine-tuning of EDX measurements might improve the technique and might make it possible to use the modern TEM equipment available in Vienna for fast stoichiometric analysis of single cells. For samples with very low amounts of P, however, bulk analyses might still be the better choice, as very low amounts of P cannot be measured accurately by EDX. The way to solve this might be to perform EELS on thin slices (~50 µm thick) of embedded cells.

This study also pointed out significant differences between growth phases and species on the cell stoichiometry. Especially the stoichiometric differences in

species have not been regarded enough in present studies and might be one of the reasons for stoichiometric difference between habitats.

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Chapter 2:

Combining single cell stoichiometry & phylogenetic identification in microorganisms

Introduction

Microbial biomass stoichiometry is an important factor in the environment [1]. The amount of C, N, and P required to build up a biomass and to sustain growth will determine the proportions of these elements incorporated into biomass [2] and released back into the environment by any organism [3]. Several studies have evaluated the relationship between biomass stoichiometry, nutrient incorporation, and nutrient recycling for natural and cultured organisms. However, most studies focused on large decomposers such as zooplankton, phytoplankton, and fish [2,3,4,5,6,7]. Microbes (bacterial and archaeal heterotrophs), however, are the most abundant organisms in any ecosystem and are responsible for a lot of the nutrient recycling on our planet [2]. The importance of aquatic microbes for the recycling and thus returning of carbon was defined as the 'microbial loop' [8]. Since then, a lot of research has focused on the importance of microbes for biogeochemical processes in various habitats and the factors determining nutrient recycling [9,10,11].

Studies of macroorganisms have demonstrated, that different species, and even those that are closely related, can differ greatly in biomass stoichiometry [2,12,13,14]. This has been mainly shown for larger organism (e.g. zooplankton, invertebrates, and fish), but a set of studies has also investigated the range of stoichiometric differences between bacterial species at different environmental conditions and growth phases [13,14,15,16]. Recently, Hall et al. [1] put together a mechanistic framework that links species specific stoichiometry to biogeochemistry. Direct implications of the variances in bacterial stoichiometry for the recycling of nutrients have not been regarded systematically so far. Considering the range of stoichiometric differences, and keeping studies about macroorganisms is mind though, it would be unlikely if microbial stoichiometry

would not account for differences in decomposition. Even though the differences between the stoichiometry of microbial groups and species might be limited, the effect of these differences can be large due to the high abundance of microbes and their high turnover rate of organic material.

In the first chapter of this thesis, two bacterial species that were grown in very similar nutrient regimes had significantly different biomass stoichiometries. While this result is consistent with previous work, suggesting a link between phylogeny and biomass stoichiometry, it is currently not possible to link phylogeny with stoichiometry in environmental microbial communities.

In this study, I attempt to simultaneously measure bacterial biomass stoichiometry and phylogeny. Knowing more about how biomass stoichiometry is linked to community composition will lead to a better understanding of nutrient recycling and decomposition. In addition, it will broaden our understanding of how microorganisms affect nutrient recycling between different growth conditions (e.g. habitats and seasons). The technique might also be used to identify capabilities of certain species for services in our daily life, such as waste water treatment and decontamination.

I applied fluorescent *in situ* hybridization (FISH) and catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH) labeling with simultaneous stoichiometric measurements of single cells with EDX and EELS (EELS & EDX: see chapter 1). Specifically, I used probes (FISH) and tyramides (CARD-FISH) containing either bromine (Br) or fluorine (F). These halogens were selected as they do not occur naturally in bacteria and should therefore only be present in the cells and detectable with EDX/ EELS if cells had been labeled. My goals were a) to investigate if EDX and/or EELS can detect the applied halogen labels, and b) to study the effect FISH/ CARD-FISH labeling on biomass stoichiometry of bacterial cells by comparing their elemental ratios to unlabeled cells measured in the first chapter. In theory, Br is a good candidate for EDX detection, as it is a relatively heavy element (z = 35), and success of detection is better for heavier elements as compared to light elements. F, on the other hand, should be easier to be detected with EELS, as it is a light element (z = 9) and it has a pronounced K edge in the core loss region.

Methods

I selected the logarithmic growth phase treatments of *P. carotovorum,* analyzed for their stoichiometry in the first chapter of this thesis, since they were less heterogeneous in their biomass stoichiometry (cf. table 5, chapter 1) and more likely to have higher ribosome content than stationary phase cells. FISH was conducted with a Br containing probe and CARD-FISH with tyramides containing either F or Br. I evaluated the ability of EDX and EELS to detect halogen labels on *P. carotovorum* cells that differed in their biomass stoichiometry based on the results of the first chapter and the effects of FISH/ CARD-FISH on biomass stoichiometry.

FISH

The general FISH protocol by Fuchs and Pernthaler [17] was modified to work in liquid media, instead of the usual procedure on filters or glass slides. This facilitated applying cells on TEM grids after FISH labeling.

Fixed cells (fixation: chapter 1) were washed by adding 1 mL of 1x PBS to 150 µl of fixed culture and subsequently centrifuged (10,000 rpm, 10 min, 20°C, in 1.5 mL Eppendorf). Afterwards, the supernatant was decanted and 1 mL of 1:1 96 % EtOH : 1x PBS was added. The supernatant was removed again before an ethanol series was conducted with 50, 80, and 96 % ethanol to dehydrate and permeabilize the cells. 200 mL of ethanol was added and centrifuged after 1 min. of incubation (10,000 rpm, 10 min, 20°C). The ethanol was then removed and the step was repeated with the subsequent concentrations. After the complete ethanol series had been conducted, the tube was left open to dry (5 min.), before the probe (Table 1) was added in a 1:10 ratio with the hybridization buffer (containing 20% formamide). The tube was kept in the hybridization oven (46°C) for 1.5 hours and subsequently centrifuged (10,000 rpm, 10 min., 40°C). The supernatant was then removed and the tube was filled with 1.5 mL preheated washing buffer, incubated a 48°C (10 min.) and centrifuged again (10,000 rpm, 10 min., 40°C). The washing buffer was removed and cells were re-dissolved in 1x PBS. Outcome of the FISH labeling was checked by epifluorescent microscopy. Cell were washed and resuspended in Milli-Q water prior to putting them on the TEM grids in order to decrease the amount of salts in the solution.

Table 1: FISH probes used in this study

Probe	Sequence	Halogen	Reference
Br_6-EUB338-Cy3	5′-Cy3-GcTGccTcccGTAGGAGT-3′ (c = 5-bromo-2′deoxycytidine)	Br	Li et al., 2008 [18]
Non-EUB-Cy3	5′-Cy-3-ACTCCTACGGGAGGCACG-3′	-	Amann et al, 1990 [19]

CARD-FISH

To increase the amount of halogen label in the cells and the likelihood to get positive halogen detection with EDX and EELS, I used CARD-FISH, adapted from Pernthaler et al. 2002 [20,21]. I was unable to see a fluorescent signal using liquid CARD-FISH, and so all CARD-FISH essays were performed on slides.

Fixation and washing of the cultures was performed analogous to FISH. 10 µl of fixed culture was added onto 10-well slides and slides were dried (pressurized air). After dehydration in an ethanol series (50, 80, 95 % ethanol), the slides were dipped in low gelling point agarose (0.2 %) to prevent cell loss, and air-dried. Proteinase K was added onto each well (15 µl/well, 10 min. incubation at room temperature (RT)) for cell permeabilization. Slides were then washed in Milli-Q water (1 min.) and afterwards incubated in 0.01 M HCl to bleach endogenous peroxidase and inactivate proteinase K (20 min., RT). After dipping the slides in Milli-Q water and 96 % EtOH, horseradish peroxidase probe (HRP-EUB338-I, resp. HRP-NonEUB) and hybridization buffer were added in a 1:10 ratio onto the dried slides. The slides were incubated in a humid chamber at 46°C (3 hours) and then put into pre-warmed washing buffer (48°C, 15 min.). Directly afterwards, the slides were dipped in ice cold Milli-Q water and then incubated in 1x PBS (10 min., RT). Slides were dapped on blotting paper without letting them run dry and 15 µl of tyramide containing substrate mix was put into each well (tyramides: Table 2; synthesized according to Pernthaler et al. [21]). After incubation in a humid chamber with Milli-Q water (45 min., dark), the slides were washed in 1x PBS (10 min., RT, dark), then in Milli-Q water (1 min., RT, dark), and finally in 96 % EtOH (15 sec., RT, dark). Successfully labeled cells were removed from the slides with 1x PBS (pipetting PBS up and down and gently scratching the glass surface with the pipet tip). To remove salts, the solution was centrifuged (10,000 rpm, 10 min.) and the cells were resuspended in Milli-Q water.

Table 2: Succinimidyl esters used for tyramide labeling (protocol: [21])

Succinimidyl ester	Emission wavelength	Halogen	Reference
5-carboxy-2',4',5',7'-tetrabromosulfonefluorescein	544	Br	Behrens et al., 2008 [22]
Oregon Green 488-X	517	F	Behrens et al., 2008 [22]

Grid preparation and analytical measurements

TEM grid preparation and EDX measurements were performed the same way as described the first chapter. 28 of the FISH labeled cells (treatment E4L), as well as 26 bromine-labeled CARD-FISH cells and 20 fluorine-labeled CARD-FISH cells (treatment E7L) were measured with EDX. After data collection, the spectra were analyzed as described in chapter 1. Data was evaluated with a standard (CoA) for C, N, and P and without a standard for the amount of halogens (F, Br). Several CARD-FISH cells were also measured with EELS for their content of F and Br as described in chapter 1.

Statistical evaluation

The amount of halogen in the labeled cells (FISH, CARD-FISH Br & CARD-FISH F) was compared with a one-way ANOVA and post-hoc Tukey HSD to evaluate if a significant difference in halogen content ($p < 0.05$) could be detected. Differences in elemental ratios between the FISH treated cells and the control cells from the same treatment (results chapter 1) were analyzed with a two-sided t-test with Welch's correction for unequal variances. Stoichiometry of the two CARD-FISH treatments and control cells was compared with a one-way ANOVA and post-hoc Tukey HSD to evaluate the influence of FISH and CARD-FISH on single cell stoichiometry. All analyses were performed with JMP [23].

Results

FISH

Cells of the E4L and the E9L treatments had a positive fluorescent signal with the Br_6-Eub338-Cy3 probe when checked against non-EUB-Cy3 hybridized cells of the same treatments. Since only cells from the E4L treatment were successfully collected on TEM grids, solely cells from this treatment were analyzed for their elemental composition and for halogen label detection. Unfortunately, Br was below detection of EDX for all 28 cells analyzed from treatment E4L and thus this method was not further pursued (peak position: Fig. 1).

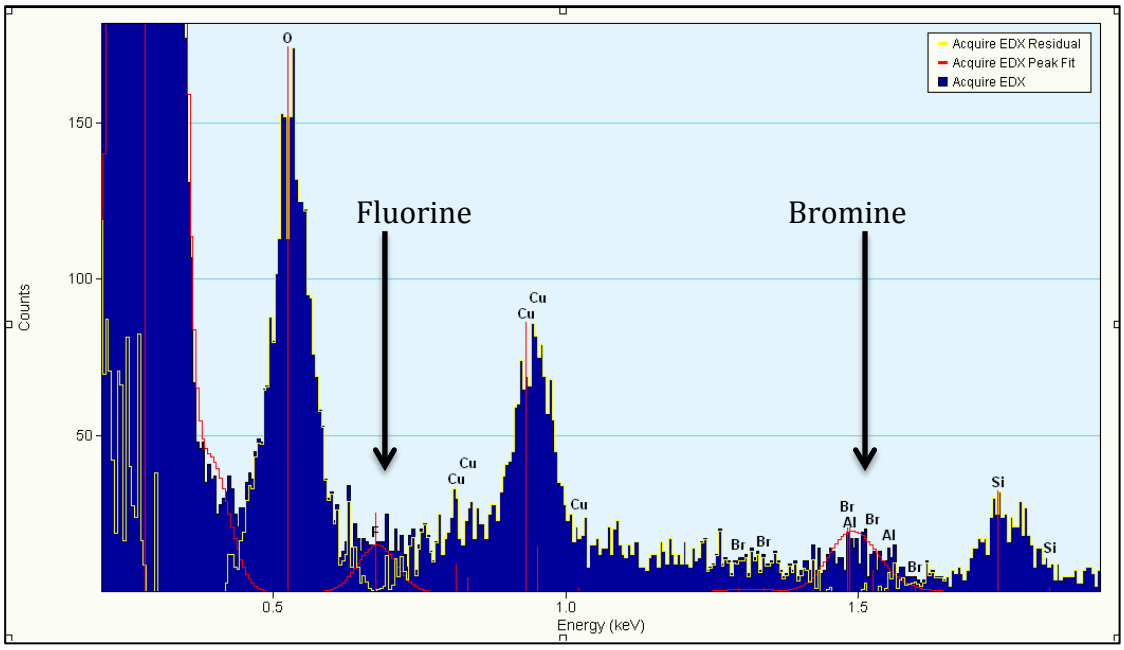

Figure 1: Position of K edges of F and Br in an EDX spectrum. X-axis: counts, y-axis: energy (keV).

CARD-FISH

All treatments of *P. carotovorum* investigated in the first chapter (E4L, E7L & E9L), hybridized successfully with the halogen containing tyramides, as positive fluorescent signals were observed. Brightest signals were observed in treatment E9L (second brightest: E7L). These treatments also had the lowest C:P values (i.e. the highest amount of P; chapter 1, Table 1). As cells from treatment E9L were not successfully brought onto TEM grids, the second brightest treatment was evaluated for C, N, P, F, and Br.

Unfortunately, none of the brominated cells (CARD-FISH Br), had a positive bromine value when evaluated with EDX. A positive fluorine value, however, was calculated for all cells labeled with either of the tyramides. The calculated percentage of fluorine was significantly higher in the CARD-FISH Br cells (Tukey HSD; $p < 0.001$) as compared to the CARD-FISH F cells (Fig. 2). The percentage of fluorine was also evaluated for the FISH labeled cells (Br probe) and compared to the CARD-FISH labeled cells. No significant difference in fluorine content was measured between the FISH and the CARD-FISH F cells (Tukey HSD; $p > 0.05$), but the FISH cells differed significantly from the CARD-FISH Br cells.

P. carotovorum cells labeled with CARD-FISH Br and CARD-FISH F were also measured with EELS to investigate if halogen label detection is possible. Unfortunately again, EELS evaluations for Br and F were below detection (Fig. 3).

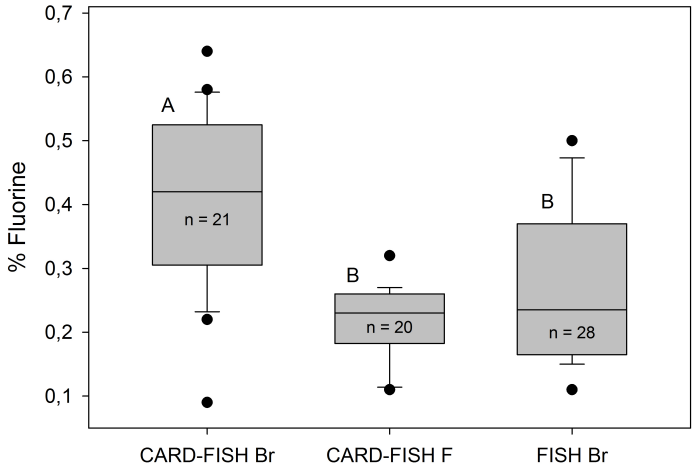

Figure 2: Fluorine content (atom % of fluorine as a percentage of C, N, P, F, and Br) of *P. carotovorum* treatments after CARD-FISH (E7L) and FISH (E4L). Boxes represent the median, 25% and 75% quartiles, bars show 10% and 90% percentiles and points outliers. Statistical analysis: one way ANOVA with post-hoc Tukey HSD ($p < 0.05$). Different letters show significant difference.

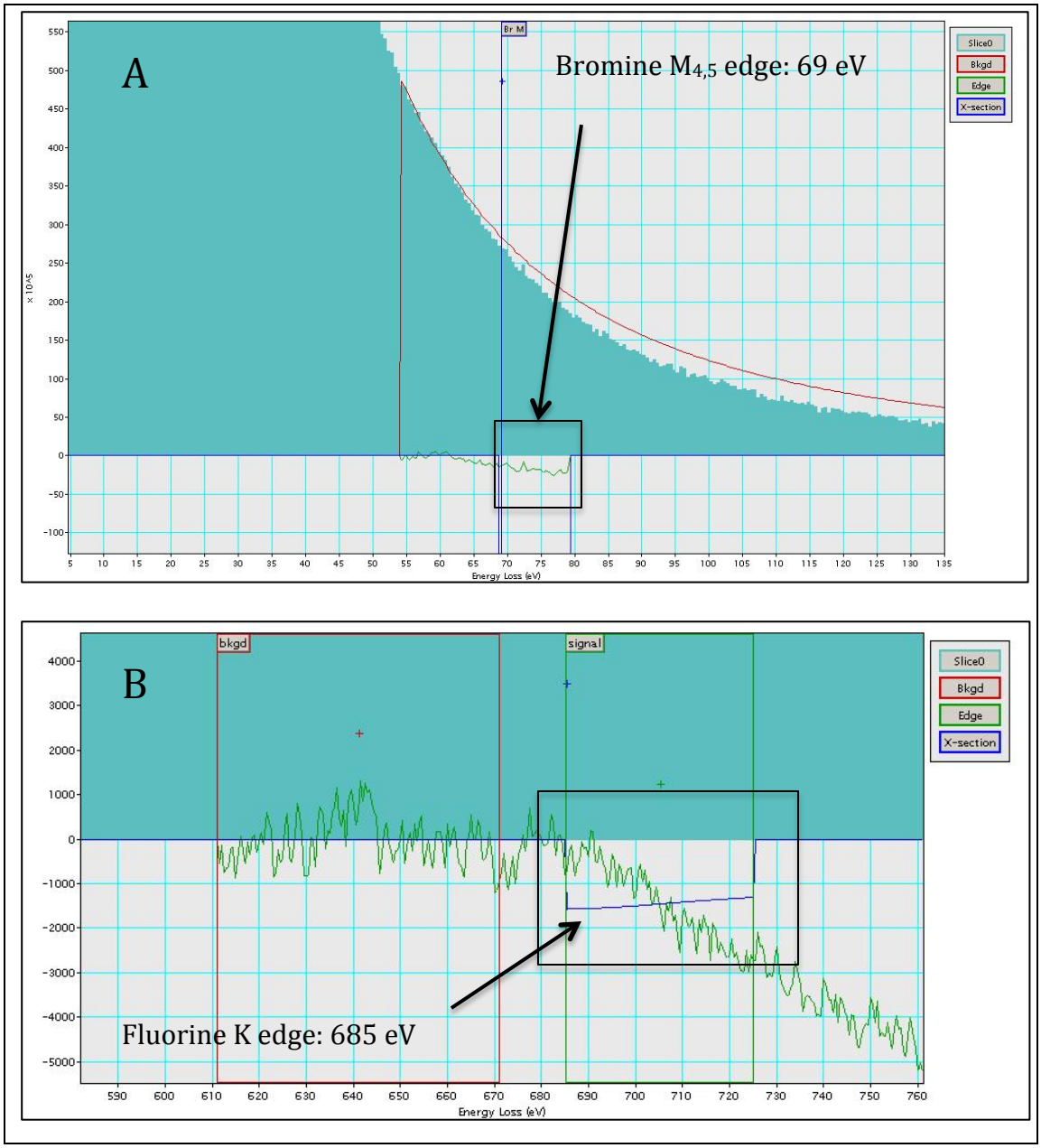

Figure 3: Examples of Br (A) and F (B) edge evaluation. The left part of the horizontal line marks the area used for background subtraction and the right part marks which area is used for calculation of elemental content. X-axis: energy loss (eV), y-axis: relative scale of counts per time.

Effect of FISH/ CARD-FISH labeling on the cell stoichiometry

While the ability to detect a halogen label with FISH or CARD-FISH was not possible, I was still able to test how FISH and CARD-FISH influenced the biomass stoichiometry of *P. carotovorum.* Standardized cell stoichiometry of the FISH labeled cells (Fig. 4) differed significantly from the control spectra of unlabeled cells from the same treatment (control cells: data from chapter 1) in C:N ($p < 0.01$, $t = 4.062$, $df = 53$; Welch-corrected t-test), C:P ($p < 0.01$, $t = 4.890$, $df = 34$), and N:P ($p < 0.01$, $t = 3.682$, $df = 37$). In addition, FISH labeled cells had significantly

higher variance in C:P (F-test; $p < 0.001$, Table 3) and N:P ($p < 0.001$) compared to the control cells, while the variance in C:N was not significantly different from the control cells ($p = 0.053$). Notably, variance of C:P was 13 x higher and variance of N:P 7 x higher between the FISH cells compared to the control cell (Fig. 4).

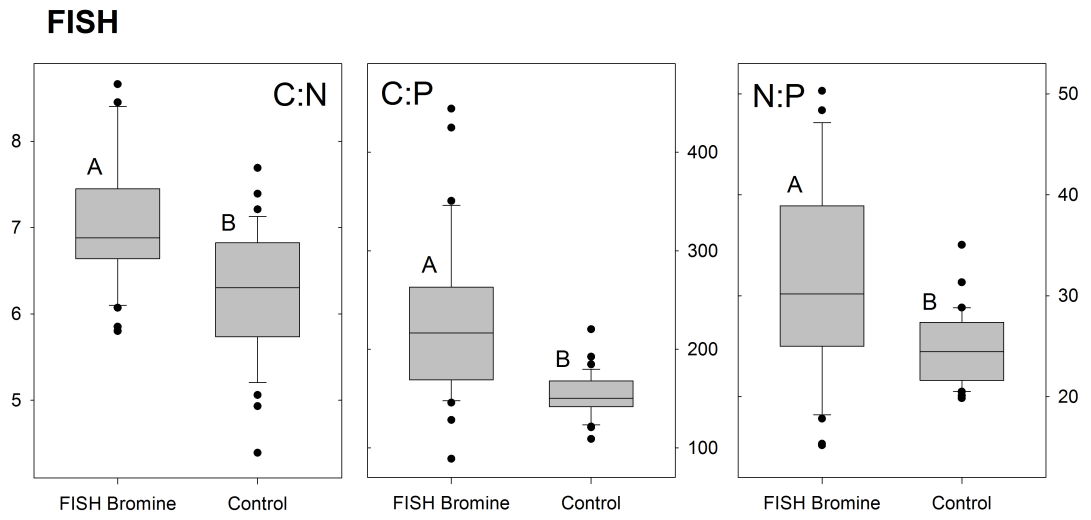

Figure 4: Standard-corrected elemental ratios of *P. carotovorum* labeled with the Br_6-Eub338-Cy3 probe and unlabeled control cells of treatment E4L. Y-axis: molar ratio. Statistical analysis: two-sided t-test with Welch's correction for unequal variances. Description of boxes and letters: see above (Fig. 2).

Table 3: Comparison of variance (σ^2) of the molar ratios (atom %) as well ratios of C:N, C:P, and N:P of the FISH cells & unlabeled control cells. Variance of the control cells is given as a proportion of FISH variance, statistical comparison was conducted with the F-test (**bold** values: significant difference)

	% C	**% N**	**% P**	**C:N**	**C:P**	**N:P**
σ^2 **Control/** σ^2 **FISH**	1,273	1,207	0,251	0,498	0,075	0,136
F-test	*0,507*	*0,606*	***0,000***	*0,053*	***0,000***	***0,000***

Biomass stoichiometry of CARD-FISH labeled cells was also compared to unlabeled cell stoichiometry of the same treatment (Fig. 5, treatment: E7L; control cell data: chapter 1). While no significant difference in C:N was found between the CARD-FISH F cells and the control cells, CARD-FISH Br cells had significantly low C:N than CARD-FISH F cells (one-way ANOVA with post-hoc TUKEY HSD comparison). For C:P, the two CARD-FISH labeled treatments did not differ from each other, but they both differed significantly from the control cells ($p < 0.05$). N:P ratios were significantly different between all of the three ($p < 0.05$). Interestingly, significant differences in variance between control cells and

CARD-FISH F, respectively CARD-FISH Br labeled cells were found for C:P and N:P (Table 2; $p < 0.001$; F-test). Variance in C:N did not differ significantly. Variance between CARD-FISH Br and CARD-FISH F cells also did not differ significantly for C:N, C:P, or N:P, but significant difference in % P was detected.

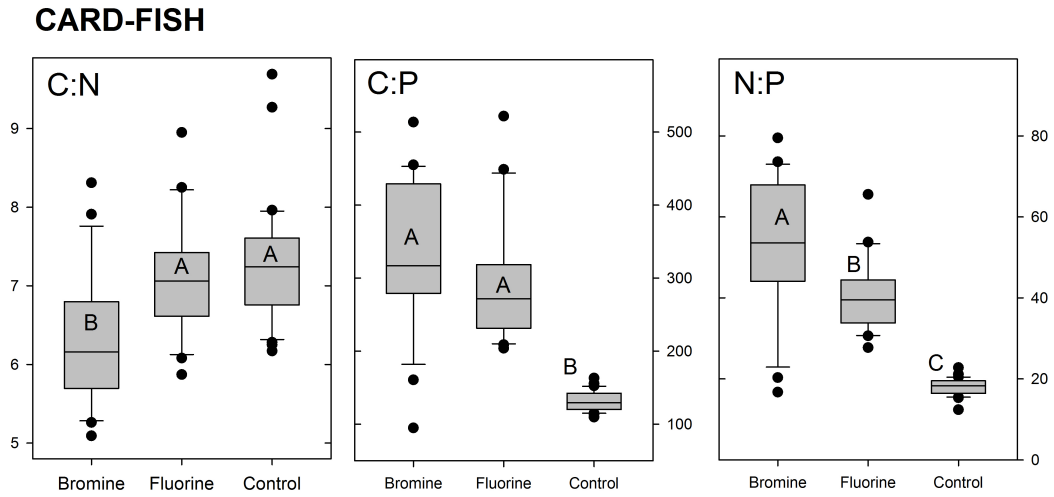

Figure 5: Standard-corrected elemental ratios of *P. carotovorum* cells labeled with CARD-FISH probes for EUB338-I and tyramides containing either Br or F and unlabeled control cells of treatment E7L. Description of boxes and statistical analysis: see above (Fig. 2).

Table 2: Comparison o variance (σ^2) of molar ratios (atom %) as well ass C:N, C:P, and N:P ofcontrol cells and CARD-FISH labeled cells. Variance of the control cells is given as a proportion of CARD-FISH variance, statistical comparison was conducted with the F-test (**bold** values: significant difference)

	% C	% N	% P	C:N	C:P	N:P
σ^2 Control/ σ^2 CARD-FISH Br	0,491	0,491	0,174	0,877	0,018	0,017
F-test	*0,079*	*0,079*	***0,000***	*0,732*	***0,000***	***0,000***
σ^2 Control/ σ^2 CARD-FISH F	0,883	0,928	0,876	1,131	0,027	0,054
F-test	*0,746*	*0,837*	*0,730*	*0,794*	***0,000***	***0,000***
σ^2 CARD-FISH Br/ σ^2 CARD-FISH F	1,799	1,892	5,048	1,290	1,506	3,176
F-test	*0,206*	*0,170*	***0,001***	*0,583*	*0,377*	*0,015*

To summarize the results, I have to state that halogen detection was not successful with either FISH or CARD-FISH. Furthermore, the FISH as well as CARD-FISH labeling procedure does have a pronounced effect on the stoichiometry of the single cells.

Discussion

Halogen labeling

Using both FISH and CARD-FISH, halogen detection with EDX or EELS was not successful. So far it has not been possible to directly link phylogeny and stoichiometry for *P. carotovorum* with the methods I used in this study.

There are several possible explanations why the methods were unsuccessful. For halogen detection with EDX, the amount of halogen in the cells probably lies below the detection limit of this method for F and Br. This can be due to the relatively low amounts of halogen brought into the cells (FISH: 5 Br atoms per probe; number of probes per cell depends on the # of ribosomes in a cell; CARD-FISH: 5 F atoms/ 4 Br atoms per tyramides) and the amounts of RNA in the cells. Furthermore, the position of the peaks of Br, and especially of F, are situated in an area of the spectrum, which can be highly influenced by background scattering (Fig. 1) and differentiation between background and halogen levels are therefore very difficult. For halogen detection with EELS, the thickness of the cells is probably the main problems (cf. chapter 1).

The effect of *in situ* hybridization on cellular stoichiometry

FISH and CARD-FISH labeling of *P. carotovorum* had strong effects on the biomass stoichiometry, affecting both the ratios of C:N, C:P, and N:P as well as the variance between the cells. This effect may be due to how the probes are introduced into the cells and hybridized to the rRNA. Both with FISH and with CARD-FISH, the C:P and N:P ratios increased compared to the control cells, meaning that either the absolute amount of P in the cells decreased or the amount of C and/or N increased. This might be due to loss of phospholipids and ribosomes during the permeabilization of the cell wall. Both phospholipids and RNA can be major contributors to the total P budget in the cell. When observed with the TEM, several cells looked deflated, indicating that they might have lost parts of their cell content. Furthermore, the increased variance in C:P and N:P between the FISH/ CARD-FISH treated cells might be due to individual differences between the cells in the response to the permeabilization and the loss of certain cell compounds.

Future directions

Even though I did not succeed in combining stoichiometry and phylogeny, there are several possibilities to improve the method. One possibility is to perform FISH/ CARD-FISH directly on TEM grids as this might decrease the damage caused to the cells through aggressive treatments, such as numerous steps of centrifugation (here used for FISH), or rough removal of the cells from glass slides (CARD-FISH). Decreasing the forces on the cells during FISH/ CARD-FISH treatment might positively affect the amount of label retained in the cells.

A different possibility is to use immunogold labeling [24,25] instead of halogen containing probes. For this method, antibodies containing submicron scale gold particles are brought into the cells after a specific FISH treatment. Gold particles are only deposited in cells that have been labeled with specific FISH probes which are recognized by antibodies carrying the gold particles. After enhancement of the size of the metal particles through silver enhancement [24], the immunogold particles can be used for visual identification of the labeled bacterial species with the TEM [24]. In theory, labeled cells should be visually differentiable from the other cells, because they contain little black dots, as the density of gold prevents electrons from passing through. Since neither gold nor silver is a common element in bacterial cells, these elements should not affect the cell stoichiometry. It is of course not clear how the whole labeling procedure effects cellular stoichiometry. However, it is reasonable to improve the immunogold protocol to reduce cell disruption and still get a visible signal of deposited particles, as very high magnifications are possible with the TEM to visualize the cells.

Finally, one of the problems with FISH labeling, if this method should be applied to environmental samples, is the high phylogenetic diversity within any given sample. Labeling specific groups of the community and then performing EDX on the cells may lead to a high number of cells that need to be analyzed before the cells of interest can be found. Using cell sorting of FISH labeled cells with Flow Cytometry (FACS) may simplify the whole method, as cells can be divided by various phylogenetic levels and label detection with EDX or EELS would not be necessary.

One final possibility is to use slices of embedded cells and perform FISH/ CARD-FISH on them. The advantage of the slicing technique would be that cell slices

would most likely be thin enough for EELS measurements of halogen labels. F is hard to measure with EDX (see above), but should have an easily measurable energy edge in EELS if the cells are sliced, this might increase the sensitivity of F detection.

Conclusion

FISH and CARD-FISH labeling with halogen containing probes/ tyramides has not been successful with the methods applied in this study, but there is a large potential to improve the method. Both FISH and CARD-FISH labeling have a significant effect on single cell stoichiometry, affecting the average elemental ratios (C:N:P), as well as on the variance between the individual cells. Future research has to address these effects in order to produce comparable information about single cell stoichiometry and phylogeny. Some possibilities for improvements are: performing FISH/ CARD-FISH on TEM grids directly, immunogold labeling, cell sorting with Flow Cytometry, and performing FISH/ CARD-FISH on sliced cells to improve detection of F with EELS.

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Summary

Ecological stoichiometry is the study of the relative proportions of elements in organisms and ecosystems and the relationship of balance between elements in ecological processes and interactions. In this study, I investigated how different methods to measure the stoichiometry (C:N:P) of bacteria compare, and if it is possible to combine measurements of single cell stoichiometry to species identification with Fluorescent *in situ* hybridization (FISH). I used two bacterial species, *Pectobacterium carotovorum* and *Verrucomicrobium spinosum,* grown in several treatments at different resource ratios and harvested each in stationary and logarithmic growth phase. Single cell measurements were conducted with Energy Dispersive X-ray spectroscopy (EDX) and bulk cell analyses were performed with a CHN analyzer and phosphorus digestion. In addition, I tried to measure single cell stoichiometry with Electron Energy Loss Spectroscopy, but due to the thickness of the cells, these measurements were not successful. The average C:N:P was 365:67:1 for unstandardized EDX results, 228:33:1 for standardized EDX (EDX_S), and 148:24:1 for bulk measurements. EDX results were compared to bulk results and only 22 % of the EDX results, when standardized with Coenzyme A, were significantly different from the bulk cell analyses ($p < 0.05$). When unstandardized EDX results were compared to bulk results, 64 % of the elemental ratios differed significantly between the two methods. These results indicate that, if EDX results are standardized, both methods may be used for microbial elemental analysis and it is currently unclear which of the two methods drives the 22 % of significant differences. Significant differences were also found between the elemental ratios of cells harvested at different growth phases and between two species, pointing out the importance of these variables when microbial stoichiometry is studied. Standardized EDX results furthermore had significantly lower variance in C:P and N:P as compared to unstandardized EDX results. In addition, the variance of the bulk results differed significantly from the EDX results, which might be an effect of low sample number for the bulk analyses (3 – 4) compared to single cell measurements (10 – 37). In the second part of the study I aimed to combine measurements of single cell stoichiometry to phylogenetic identification and used FISH with bromine (Br) labeled probes and CARD-FISH with Br or fluorine (F)

containing tyramides in order to identify these rare elements in labeled cells with EDX. Unfortunately, the Br label was not detectable with EDX, and no significantly higher amount of F could be measured for F labeled cells. However, there are various possibilities to improve the method, such as immunogold labeling, cell sorting by Flow Cytometry, performing FISH labeling directly on TEM grids, or the slices of resin-embedded cells for EELS measurements of F. Future research also has to address the significant change of single cell stoichiometry found after cell labeling when the elemental ratios of FISH and CARD-FISH labeled cells were compared to ratios of unlabeled cells.

Zusammenfassung

Die ökologische Stöchiometrie beschäftigt sich mit den Verhältnissen von Elementen in einzelnen Organismen und Ökosystemen sowie dem Gleichgewicht zwischen den Elementen in ökologischen Prozessen und Interaktionen. In der vorliegenden Arbeit habe ich Unterschiede zwischen verschiedenen Methoden untersucht, die häufig für die Messung der Stöchiometrie (C:N:P) von Bakterien verwendet werden (1. Kapitel). Des Weiteren habe ich versucht, stöchiometrische Messungen einzelner Bakterien mit phylogenetischen Bestimmungen mittels Fluoreszenz *in situ* Hybridisierung (FISH) zu verbinden (2. Kapitel). Für die Untersuchungen wurden die beiden Bakterienarten *Pectobacterium carotovorum* und *Verrucomicrobium spinosum* verwendet. Diese wurden vorab in mehreren Ansätzen bei unterschiedlichen Nährstoffverhältnissen angezogen und sowohl in der stationären als auch in der logarithmischen Wachstumsphase für weitere Analysen fixiert wurden. Mittels energiedispersiver Röntgenmikroanalyse (EDX) wurden die C:N:P Verhältnisse von einzelnen Bakterien gemessen. Die Stöchiometrie vieler Bakterien zusammen („Bulk“) wurde mit Hilfe eines CHN Analysators (C, N) und Phosphoraufschluss (P) bestimmt. Ich habe ferner versucht mit Elektronenenergieverlustsspektroskopie (EELS) die Stöchiometrie einzelner Bakterien zu messen, jedoch waren diese Messungen aufgrund der Dicke der Bakterien nicht erfolgreich. Im Mittel wurde ein C:N:P Verhältnis von 365:67:1 für EDX Ergebnisse ohne Standardkorrektur durch Koenzym A, 228:33:1 für standardkorrigierte EDX Ergebnisse (EDX_S) und 148:24:1 für Bulk bestimmt. Fast 80 % der EDX_S Ergebnisse, sowie 36 % der nicht standardkorrigierten EDX Ergebnisse, waren statistisch nicht signifikant unterschiedlich von den Bulk Ergebnissen ($p < 0.05$). Diese Resultate haben gezeigt, dass sowohl EDX als auch Bulk Analysen ähnliche Ergebnisse erzielen, sofern für die EDX Auswertung ein Standard verwendet wird. Welche Methode für die verbleibenden Unterschiede verantwortlich ist, ist dabei noch ungeklärt. Signifikante Unterschiede wurden darüber hinaus zwischen den Elementverhältnissen der verschiedenen Wachstumsphasen sowie den beiden untersuchten Arten gefunden, was darauf hindeutet, wie wichtig diese beiden Faktoren bei stöchiometrischen Analysen sind. Die Varianz zwischen EDX_S Ergebnissen einzelner Zellen war signifikant höher für C:P und N:P als bei nicht

standardisierten EDX Ergebnissen. EDX_S Ergebnisse haben, verglichen mit den Bulk Ergebnissen, eine signifikant höhere Varianz gezeigt. Dies könnte durch die deutlich kleinere Probenanzahl bei Bulk Messungen (3 – 4 Proben pro Ansatz) im Vergleich zu den EDX Messungen (10 – 37 Zellen pro Ansatz) bedingt sein. Im zweiten Teil der Arbeit habe ich versucht Messungen der Stöchiometrie einzelner Bakterien mit deren phylogenetischer Bestimmung zu verbinden und habe dafür brommarkierte FISH Sonden sowie CARD-FISH mit brom- oder fluormarkierten Tyramiden verwendet. Brom ließ sich mit EDX nicht in den Zellen nachweisen und die gemessene Menge an Fluor war in den fluormarkierten Zellen nicht signifikant höher als in den brommarkierten Zellen. Etliche Möglichkeiten sind denkbar um diese Methode zu verbessern, so zum Beispiel eine Markierung der Zellen mit Immunogold, Zellsortierung mittels Durchflusszytometrie (FACS), ein verändertes FISH-Protokoll, bei dem die Zellen direkt auf dem TEM Grid markiert werden, sowie Schnitte von eingebetteten Bakterien um dann mittels EELS eine genau Messung von F durchzuführen. Weitere Analysen müssen ferner durchgeführt werden um die Veränderung der Stöchiometrie, welche bei FISH-markierten Zellen gemessen wurde, zu minimieren.

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I want to thank Ed Hall and Tom Battin for supervising me with this thesis and giving me the possibility to learn several new techniques and ecological concepts Furthermore, I want to thank Michael Stöger-Pollach from the University of Technology in Vienna, who taught me how to operate the TEM and Roland Hatzenpichler and Markus Schmid for advising me in microbial ecology, and the whole Battin group for comments and ideas.

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Curriculum Vitae

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Date and Place of birth:

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University Education:

Okt. 2005 – Sep. 2008:	Bachelor of Environmental Sciences, University o Bielefeld (Germany)
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Sep. 2007 – Aug. 2008:	Student Exchange to Uppsala, Sweden (ERASMUS)
Okt. 2008 – Jan. 2009:	Studies of Environmental Sciences at the University of Applied Life Sciences Vienna (Austria)
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School Education:

1991 – 1995:	St. Dionysius Grundschule Havixbeck (Primary School)
1995 – 2004:	Gymnasium Marienschule Münster (Secondary School), completed with "Abitur" (diploma from German secondary school qualifying for university admission or matriculation) in 2004

School Exchanges:

Sep. 2001 – Dec. 2001:	School exchange to Ireland (Limerick)
Aug. 2002 – Sep. 2002:	School exchange to Australia (Sydney)

Social volunteer year:

Sep. 2004 – Aug. 2005:	Social volunteer year in Romania (Brasov), working with elder people and children.

Language skills:

German (mother tongue), English, Romanian, Swedish

Computer literacy:

Microsoft Office, iWork, SigmaPlot, ArcGIS, JMP, AnyLogic

Appendix I: Row data chapter 1

Treatment	Method	% C	% N	% P	C:N	C:P	N:P
E4L	EDX_S	80.87	18.44	0.67	4.39	120.70	27.52
E4L	EDX_S	84.70	14.75	0.54	5.74	156.85	27.31
E4L	EDX_S	85.94	13.66	0.39	6.29	220.36	35.03
E4L	EDX_S	85.76	13.74	0.49	6.24	175.02	28.04
E4L	EDX_S	83.82	15.66	0.50	5.35	167.64	31.32
E4L	EDX_S	85.39	14.11	0.49	6.05	174.27	28.80
E4L	EDX_S	86.61	12.93	0.45	6.70	192.47	28.73
E4L	EDX_S	82.57	16.76	0.66	4.93	125.11	25.39
E4L	EDX_S	84.66	14.81	0.52	5.72	162.81	28.48
E4L	EDX_S	82.85	16.37	0.76	5.06	109.01	21.54
E4L	EDX_S	85.49	13.86	0.64	6.17	133.58	21.66
E4L	EDX_S	84.78	14.58	0.62	5.81	136.74	23.52
E4L	EDX_S	85.92	13.51	0.55	6.36	156.22	24.56
E4L	EDX_S	84.26	15.16	0.56	5.56	150.46	27.07
E4L	EDX_S	85.86	13.58	0.54	6.32	159.00	25.15
E4L	EDX_S	84.19	15.23	0.56	5.53	150.34	27.20
E4L	EDX_S	84.25	15.15	0.59	5.56	142.80	25.68
E4L	EDX_S	86.90	12.53	0.55	6.94	158.00	22.78
E4L	EDX_S	85.37	14.12	0.49	6.05	174.22	28.82
E4L	EDX_S	87.04	12.43	0.51	7.00	170.67	24.37
E4L	EDX_S	86.96	12.45	0.58	6.98	149.93	21.47
E4L	EDX_S	86.27	13.20	0.52	6.54	165.90	25.38
E4L	EDX_S	88.01	11.45	0.52	7.69	169.25	22.02
E4L	EDX_S	87.54	11.85	0.59	7.39	148.37	20.08
E4L	EDX_S	86.24	13.19	0.55	6.54	156.80	23.98
E4L	EDX_S	87.28	12.10	0.61	7.21	143.08	19.84
E4L	EDX_S	86.74	12.78	0.47	6.79	184.55	27.19
E4L	EDX_S	86.84	12.54	0.61	6.93	142.36	20.56
E4L	EDX_S	86.25	13.15	0.59	6.56	146.19	22.29
E4L	EDX_S	86.54	12.85	0.60	6.73	144.23	21.42
E4L	EDX_S	85.82	13.56	0.61	6.33	140.69	22.23
E4L	EDX_S	87.04	12.35	0.60	7.05	145.07	20.58
E4L	EDX_S	85.27	14.10	0.62	6.05	137.53	22.74
E4L	EDX_S	84.95	14.34	0.70	5.92	121.36	20.49
E4L	EDX	84.23	15.30	0.45	5.51	187.18	34.00
E4L	EDX	86.85	12.85	0.29	6.76	299.48	44.31
E4L	EDX	88.67	11.14	0.17	7.96	521.59	65.53
E4L	EDX	89.22	10.48	0.28	8.51	318.64	37.43
E4L	EDX	86.42	13.31	0.26	6.49	332.38	51.19
E4L	EDX	87.09	12.67	0.22	6.87	395.86	57.59
E4L	EDX	87.10	12.63	0.25	6.90	348.40	50.52
E4L	EDX	85.70	13.99	0.30	6.13	285.67	46.63
E4L	EDX	87.40	12.20	0.38	7.16	230.00	32.11
E4L	EDX	83.61	15.86	0.51	5.27	163.94	31.10
E4L	EDX	87.57	12.07	0.35	7.26	250.20	34.49
E4L	EDX	87.73	11.96	0.30	7.34	292.43	39.87
E4L	EDX	89.03	10.68	0.27	8.34	329.74	39.56
E4L	EDX	86.08	13.47	0.43	6.39	200.19	31.33
E4L	EDX	86.08	13.47	0.43	6.39	200.19	31.33
E4L	EDX	85.87	13.77	0.35	6.24	245.34	39.34
E4L	EDX	86.77	12.96	0.26	6.70	333.73	49.85

E4L	EDX	88.49	11.13	0.36	7.95	245.81	30.92
E4L	EDX	87.45	12.29	0.24	7.12	364.38	51.21
E4L	EDX	88.89	10.73	0.36	8.28	246.92	29.81
E4L	EDX	89.46	10.20	0.32	8.77	279.56	31.88
E4L	EDX	88.14	11.60	0.25	7.60	352.56	46.40
E4L	EDX	90.99	8.72	0.28	10.43	324.96	31.14
E4L	EDX	88.90	10.73	0.36	8.29	246.94	29.81
E4L	EDX	88.56	11.18	0.25	7.92	354.24	44.72
E4L	EDX	88.76	10.94	0.28	8.11	317.00	39.07
E4L	EDX	89.00	10.66	0.33	8.35	269.70	32.30
E4L	EDX	89.14	10.47	0.38	8.51	234.58	27.55
E4L	EDX	88.95	10.79	0.25	8.24	355.80	43.16
E4L	EDX	88.72	10.88	0.38	8.15	233.47	28.63
E4L	EDX	88.10	11.50	0.38	7.66	231.84	30.26
E4L	EDX	89.25	10.45	0.28	8.54	318.75	37.32
E4L	EDX	87.92	11.77	0.30	7.47	293.07	39.23
E4L	EDX	87.75	11.87	0.37	7.39	237.16	32.08
E4L	Bulk	83.65	15.65	0.68	5.35	122.45	22.91
E4L	Bulk	83.51	15.77	0.70	5.30	119.71	22.60
E4L	Bulk	83.68	15.86	0.45	5.28	184.90	35.04
E7L	EDX_S	86.50	12.79	0.69	6.76	125.36	18.54
E7L	EDX_S	85.44	13.85	0.70	6.17	122.06	19.79
E7L	EDX_S	89.54	9.66	0.78	9.27	114.79	12.38
E7L	EDX_S	85.72	13.66	0.60	6.28	142.87	22.77
E7L	EDX_S	86.51	12.81	0.66	6.75	131.08	19.41
E7L	EDX_S	86.56	12.64	0.79	6.85	109.57	16.00
E7L	EDX_S	87.38	11.86	0.75	7.37	116.51	15.81
E7L	EDX_S	86.37	12.97	0.65	6.66	132.88	19.95
E7L	EDX_S	88.04	11.41	0.54	7.72	163.04	21.13
E7L	EDX_S	85.58	13.69	0.71	6.25	120.54	19.28
E7L	EDX_S	86.88	12.45	0.66	6.98	131.64	18.86
E7L	EDX_S	87.50	11.80	0.68	7.42	128.68	17.35
E7L	EDX_S	86.72	12.56	0.70	6.90	123.89	17.94
E7L	EDX_S	86.37	12.93	0.69	6.68	125.17	18.74
E7L	EDX_S	87.67	11.62	0.69	7.54	127.06	16.84
E7L	EDX_S	87.56	11.76	0.67	7.45	130.69	17.55
E7L	EDX_S	88.03	11.33	0.63	7.77	139.73	17.98
E7L	EDX_S	87.70	11.59	0.70	7.57	125.29	16.56
E7L	EDX_S	88.31	11.10	0.58	7.96	152.26	19.14
E7L	EDX_S	87.69	11.68	0.61	7.51	143.75	19.15
E7L	EDX_S	87.33	12.06	0.59	7.24	148.02	20.44
E7L	EDX_S	87.15	12.24	0.60	7.12	145.25	20.40
E7L	EDX_S	86.91	12.45	0.62	6.98	140.18	20.08
E7L	EDX_S	88.15	11.21	0.62	7.86	142.18	18.08
E7L	EDX_S	86.44	12.84	0.73	6.73	118.41	17.59
E7L	EDX_S	90.10	9.30	0.58	9.69	155.34	16.03
E7L	EDX_S	87.19	12.05	0.75	7.24	116.25	16.07
E7L	EDX_S	87.30	11.92	0.77	7.32	113.38	15.48
E7L	EDX_S	87.29	12.07	0.62	7.23	140.79	19.47
E7L	EDX_S	87.93	11.32	0.74	7.77	118.82	15.30
E7L	EDX	88.29	11.27	0.42	7.83	210.21	26.83
E7L	EDX	87.13	12.56	0.29	6.94	300.45	43.31
E7L	EDX	89.46	10.19	0.33	8.78	271.09	30.88
E7L	EDX	88.52	11.13	0.33	7.95	268.24	33.73

E7L	EDX	87.46	12.20	0.33	7.17	265.03	36.97
E7L	EDX	88.79	10.84	0.36	8.19	246.64	30.11
E7L	EDX	87.96	11.52	0.50	7.64	175.92	23.04
E7L	EDX	88.23	11.41	0.35	7.73	252.09	32.60
E7L	EDX	89.14	10.52	0.33	8.47	270.12	31.88
E7L	EDX	87.96	11.64	0.39	7.56	225.54	29.85
E7L	EDX	87.48	12.04	0.46	7.27	190.17	26.17
E7L	EDX	89.04	10.64	0.31	8.37	287.23	34.32
E7L	EDX	89.10	10.49	0.40	8.49	222.75	26.23
E7L	EDX	88.43	11.21	0.34	7.89	260.09	32.97
E7L	EDX	90.35	9.24	0.40	9.78	225.88	23.10
E7L	EDX	89.55	10.07	0.36	8.89	248.75	27.97
E7L	EDX	88.76	10.82	0.41	8.20	216.49	26.39
E7L	EDX	89.54	10.03	0.41	8.93	218.39	24.46
E7L	EDX	90.41	9.33	0.25	9.69	361.64	37.32
E7L	EDX	89.73	9.98	0.27	8.99	332.33	36.96
E7L	EDX	88.27	11.32	0.40	7.80	220.68	28.30
E7L	EDX	89.05	10.59	0.35	8.41	254.43	30.26
E7L	EDX	88.78	10.84	0.37	8.19	239.95	29.30
E7L	EDX	88.85	10.82	0.32	8.21	277.66	33.81
E7L	EDX	87.25	12.27	0.46	7.11	189.67	26.67
E7L	EDX	90.18	9.46	0.34	9.53	265.24	27.82
E7L	EDX	89.32	10.25	0.41	8.71	217.85	25.00
E7L	EDX	89.59	10.01	0.39	8.95	229.72	25.67
E7L	EDX	89.43	10.14	0.42	8.82	212.93	24.14
E7L	EDX	89.23	10.33	0.43	8.64	207.51	24.02
E7L	Bulk	82.05	17.02	0.90	4.82	90.97	18.87
E7L	Bulk	82.08	16.97	0.91	4.84	90.06	18.62
E7L	Bulk	82.47	16.72	0.78	4.93	106.03	21.50
E9L	EDX_S	90.37	8.47	1.15	10.67	78.58	7.37
E9L	EDX_S	85.07	13.66	1.25	6.23	68.06	10.93
E9L	EDX_S	85.02	13.59	1.37	6.26	62.06	9.92
E9L	EDX_S	85.53	13.08	1.38	6.54	61.98	9.48
E9L	EDX_S	86.02	12.69	1.27	6.78	67.73	9.99
E9L	EDX_S	86.71	11.88	1.40	7.30	61.94	8.49
E9L	EDX_S	84.04	14.65	1.30	5.74	64.65	11.27
E9L	EDX_S	85.28	13.37	1.34	6.38	63.64	9.98
E9L	EDX_S	87.04	11.77	1.17	7.40	74.39	10.06
E9L	EDX_S	85.31	13.42	1.25	6.36	68.25	10.74
E9L	EDX_S	83.28	15.21	1.49	5.48	55.89	10.21
E9L	EDX_S	85.97	12.60	1.41	6.82	60.97	8.94
E9L	EDX_S	85.09	13.51	1.38	6.30	61.66	9.79
E9L	EDX_S	85.16	13.44	1.39	6.34	61.27	9.67
E9L	EDX_S	85.06	13.56	1.37	6.27	62.09	9.90
E9L	EDX_S	85.69	13.06	1.24	6.56	69.10	10.53
E9L	EDX_S	85.84	12.88	1.26	6.66	68.13	10.22
E9L	EDX_S	85.77	12.91	1.30	6.64	65.98	9.93
E9L	EDX_S	87.14	11.62	1.22	7.50	71.43	9.52
E9L	EDX_S	86.30	12.28	1.41	7.03	61.21	8.71
E9L	EDX_S	85.19	13.39	1.40	6.36	60.85	9.56
E9L	EDX_S	86.57	12.30	1.12	7.04	77.29	10.98
E9L	EDX_S	84.53	14.09	1.37	6.00	61.70	10.28
E9L	EDX_S	85.72	13.02	1.24	6.58	69.13	10.50
E9L	EDX_S	85.16	13.62	1.21	6.25	70.38	11.26

E9L	EDX_S	85.49	13.16	1.34	6.50	63.80	9.82
E9L	EDX_S	83.30	15.19	1.49	5.48	55.91	10.19
E9L	EDX_S	85.44	13.00	1.55	6.57	55.12	8.39
E9L	EDX_S	85.25	13.31	1.43	6.40	59.62	9.31
E9L	EDX_S	84.32	14.26	1.40	5.91	60.23	10.19
E9L	EDX_S	84.35	14.24	1.40	5.92	60.25	10.17
E9L	EDX_S	85.19	13.51	1.29	6.31	66.04	10.47
E9L	EDX_S	84.21	14.43	1.34	5.84	62.84	10.77
E9L	EDX_S	no data	no data	no data	no data	no data	no data
E9L	EDX_S	85.08	13.66	1.24	6.23	68.61	11.02
E9L	EDX_S	86.69	12.21	1.09	7.10	79.53	11.20
E9L	EDX_S	82.96	15.73	1.30	5.27	63.82	12.10
E9L	EDX	91.13	8.38	0.48	10.87	189.85	17.46
E9L	EDX	87.07	12.33	0.58	7.06	150.12	21.26
E9L	EDX	89.90	9.55	0.54	9.41	166.48	17.69
E9L	EDX	87.19	12.10	0.70	7.21	124.56	17.29
E9L	EDX	87.30	11.92	0.76	7.32	114.87	15.68
E9L	EDX	89.11	10.18	0.69	8.75	129.14	14.75
E9L	EDX	86.29	12.97	0.73	6.65	118.21	17.77
E9L	EDX	88.29	10.82	0.88	8.16	100.33	12.30
E9L	EDX	88.06	11.42	0.51	7.71	172.67	22.39
E9L	EDX	88.81	10.53	0.64	8.43	138.77	16.45
E9L	EDX	85.08	14.13	0.77	6.02	110.49	18.35
E9L	EDX	88.17	11.12	0.69	7.93	127.78	16.12
E9L	EDX	88.41	10.76	0.81	8.22	109.15	13.28
E9L	EDX	87.43	11.89	0.66	7.35	132.47	18.02
E9L	EDX	86.10	13.19	0.70	6.53	123.00	18.84
E9L	EDX	88.15	11.25	0.59	7.84	149.41	19.07
E9L	EDX	87.88	11.25	0.85	7.81	103.39	13.24
E9L	EDX	87.37	11.91	0.71	7.34	123.06	16.77
E9L	EDX	88.12	11.16	0.71	7.90	124.11	15.72
E9L	EDX	87.18	12.09	0.72	7.21	121.08	16.79
E9L	EDX	87.21	11.87	0.91	7.35	95.84	13.04
E9L	EDX	88.84	10.52	0.62	8.44	143.29	16.97
E9L	EDX	85.35	13.84	0.79	6.17	108.04	17.52
E9L	EDX	86.28	13.13	0.57	6.57	151.37	23.04
E9L	EDX	85.85	13.33	0.80	6.44	107.31	16.66
E9L	EDX	89.00	10.29	0.70	8.65	127.14	14.70
E9L	EDX	85.59	13.62	0.78	6.28	109.73	17.46
E9L	EDX	88.55	10.51	0.93	8.43	95.22	11.30
E9L	EDX	87.71	11.57	0.71	7.58	123.54	16.30
E9L	EDX	86.38	12.85	0.75	6.72	115.17	17.13
E9L	EDX	85.60	13.40	0.99	6.39	86.46	13.54
E9L	EDX	86.52	12.70	0.76	6.81	113.84	16.71
E9L	EDX	87.63	11.71	0.65	7.48	134.82	18.02
E9L	EDX	86.38	12.82	0.78	6.74	110.74	16.44
E9L	EDX	88.17	11.11	0.71	7.94	124.18	15.65
E9L	EDX	89.63	9.89	0.47	9.06	190.70	21.04
E9L	EDX	84.83	14.42	0.74	5.88	114.64	19.49
E9L	Bulk	80.08	17.29	2.54	4.63	31.47	6.79
E9L	Bulk	80.03	17.37	2.52	4.61	31.72	6.88
E9L	Bulk	79.90	17.49	2.53	4.57	31.58	6.91
V1L	EDX_S	88.97	10.46	0.55	8.51	161.76	19.02
V1L	EDX_S	no data	no data	no data	no data	no data	no data

V1L	EDX_S	84.88	14.10	1.00	6.02	84.88	14.10
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	85.61	13.44	0.93	6.37	92.05	14.45
V1L	EDX_S	83.82	15.06	1.11	5.57	75.51	13.57
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	86.53	12.54	0.92	6.90	94.05	13.63
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	85.32	14.05	0.61	6.07	139.87	23.03
V1L	EDX_S	84.78	14.36	0.85	5.90	99.74	16.89
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	86.28	12.99	0.71	6.64	121.52	18.30
V1L	EDX_S	86.83	12.05	1.10	7.21	78.94	10.95
V1L	EDX_S	85.49	13.29	1.20	6.43	71.24	11.08
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	87.53	11.30	1.15	7.75	76.11	9.83
V1L	EDX_S	85.53	13.41	1.05	6.38	81.46	12.77
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	85.02	14.02	0.95	6.06	89.49	14.76
V1L	EDX_S	no data	no data	no data	no data	no data	no data
V1L	EDX_S	84.84	14.27	0.87	5.95	97.52	16.40
V1L	EDX	90.05	9.67	0.27	9.31	333.52	35.81
V1L	EDX	88.63	10.62	0.73	8.35	121.41	14.55
V1L	EDX	85.11	14.49	0.39	5.87	218.23	37.15
V1L	EDX	87.53	11.92	0.53	7.34	165.15	22.49
V1L	EDX	87.84	11.52	0.63	7.63	139.43	18.29
V1L	EDX	86.10	13.35	0.53	6.45	162.45	25.19
V1L	EDX	86.70	12.54	0.74	6.91	117.16	16.95
V1L	EDX	85.78	13.66	0.55	6.28	155.96	24.84
V1L	EDX	87.76	11.58	0.64	7.58	137.13	18.09
V1L	EDX	87.05	12.59	0.35	6.91	248.71	35.97
V1L	EDX	86.99	12.64	0.36	6.88	241.64	35.11
V1L	EDX	89.79	9.36	0.84	9.59	106.89	11.14
V1L	EDX	88.15	11.21	0.63	7.86	139.92	17.79
V1L	EDX	88.24	11.03	0.72	8.00	122.56	15.32
V1L	EDX	88.57	10.82	0.59	8.19	150.12	18.34
V1L	EDX	89.60	9.41	0.98	9.52	91.43	9.60
V1L	EDX	91.55	7.96	0.47	11.50	194.79	16.94
V1L	EDX	88.16	11.42	0.40	7.72	220.40	28.55
V1L	EDX	89.30	10.25	0.44	8.71	202.95	23.30
V1L	EDX	86.90	12.37	0.72	7.03	120.69	17.18
V1L	EDX	92.35	7.54	0.09	12.25	no data	83.78
V1L	EDX	89.97	9.50	0.52	9.47	173.02	18.27
V1L	EDX	88.30	11.11	0.58	7.95	152.24	19.16
V1L	EDX	89.38	10.09	0.52	8.86	171.88	19.40
V1L	EDX	86.10	13.24	0.64	6.50	134.53	20.69
V1L	EDX	86.86	12.75	0.37	6.81	234.76	34.46
V1L	EDX	85.95	13.54	0.49	6.35	175.41	27.63
V1L	Bulk	80.94	17.59	1.42	4.60	56.85	12.35
V1L	Bulk	79.35	19.08	1.52	4.16	52.29	12.57

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V1L	Bulk	81.61	17.11	1.24	4.77	65.78	13.79
V4L	EDX_S	85.22	13.89	0.87	6.14	97.95	15.97
V4L	EDX_S	86.30	12.72	0.97	6.78	88.97	13.11
V4L	EDX_S	85.96	13.27	0.76	6.48	113.11	17.46
V4L	EDX_S	85.58	13.54	0.87	6.32	98.37	15.56
V4L	EDX_S	no data	no data	no data	no data	no data	no data
V4L	EDX_S	87.26	12.11	0.61	7.21	143.05	19.85
V4L	EDX_S	88.67	10.92	0.40	8.12	221.68	27.30
V4L	EDX_S	no data	no data	no data	no data	no data	no data
V4L	EDX_S	no data	no data	no data	no data	no data	no data
V4L	EDX_S	88.98	10.44	0.56	8.52	158.89	18.64
V4L	EDX_S	88.77	10.89	0.33	8.15	269.00	33.00
V4L	EDX_S	no data	no data	no data	no data	no data	no data
V4L	EDX_S	89.20	10.19	0.59	8.75	151.19	17.27
V4L	EDX_S	89.61	9.96	0.42	9.00	213.36	23.71
V4L	EDX_S	91.06	8.47	0.45	10.75	202.36	18.82
V4L	EDX_S	90.33	9.17	0.49	9.85	184.35	18.71
V4L	EDX_S	90.59	8.81	0.59	10.28	153.54	14.93
V4L	EDX_S	89.06	10.22	0.71	8.71	125.44	14.39
V4L	EDX_S	87.58	11.73	0.68	7.47	128.79	17.25
V4L	EDX_S	no data	no data	no data	no data	no data	no data
V4L	EDX_S	88.71	10.80	0.48	8.21	184.81	22.50
V4L	EDX_S	87.27	11.96	0.75	7.30	116.36	15.95
V4L	EDX_S	88.25	11.30	0.43	7.81	205.23	26.28
V4L	EDX_S	87.33	12.10	0.55	7.22	158.78	22.00
V4L	EDX_S	85.48	14.00	0.50	6.11	170.96	28.00
V4L	EDX_S	86.62	12.66	0.71	6.84	122.00	17.83
V4L	EDX_S	86.31	12.97	0.70	6.65	123.30	18.53
V4L	EDX_S	85.92	13.30	0.77	6.46	111.58	17.27
V4L	EDX_S	89.21	10.38	0.39	8.59	228.74	26.62
V4L	EDX_S	85.90	13.38	0.71	6.42	120.99	18.85
V4L	EDX_S	85.88	13.41	0.69	6.40	124.46	19.43
V4L	EDX_S	84.33	14.56	1.10	5.79	76.66	13.24
V4L	EDX	88.96	10.71	0.31	8.31	286.97	34.55
V4L	EDX	87.70	11.79	0.49	7.44	178.98	24.06
V4L	EDX	87.61	11.86	0.51	7.39	171.78	23.25
V4L	EDX	87.76	11.77	0.45	7.46	195.02	26.16
V4L	EDX	85.38	13.91	0.69	6.14	123.74	20.16
V4L	EDX	87.71	11.74	0.53	7.47	165.49	22.15
V4L	EDX	90.31	9.43	0.24	9.58	376.29	39.29
V4L	EDX	89.72	9.71	0.55	9.24	163.13	17.65
V4L	EDX	88.76	11.03	0.19	8.05	467.16	58.05
V4L	EDX	90.36	9.31	0.31	9.71	291.48	30.03
V4L	EDX	89.36	10.50	0.13	8.51	687.38	80.77
V4L	EDX	90.86	8.72	0.40	10.42	227.15	21.80
V4L	EDX	90.96	8.78	0.24	10.36	379.00	36.58
V4L	EDX	91.86	7.84	0.29	11.72	316.76	27.03
V4L	EDX	92.13	7.64	0.22	12.06	418.77	34.73
V4L	EDX	91.07	8.62	0.29	10.56	314.03	29.72
V4L	EDX	92.30	7.44	0.25	12.41	369.20	29.76
V4L	EDX	89.68	9.83	0.48	9.12	186.83	20.48
V4L	EDX	88.47	11.13	0.38	7.95	232.82	29.29
V4L	EDX	88.38	10.87	0.73	8.13	121.07	14.89
V4L	EDX	90.90	8.80	0.28	10.33	324.64	31.43

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V4L	EDX	87.99	11.64	0.35	7.56	251.40	33.26
V4L	EDX	89.44	10.19	0.35	8.78	255.54	29.11
V4L	EDX	87.92	11.69	0.38	7.52	231.37	30.76
V4L	EDX	86.49	13.16	0.34	6.57	254.38	38.71
V4L	EDX	88.41	11.30	0.28	7.82	315.75	40.36
V4L	EDX	87.88	11.68	0.42	7.52	209.24	27.81
V4L	EDX	88.69	10.81	0.49	8.20	181.00	22.06
V4L	EDX	88.85	10.93	0.20	8.13	444.25	54.65
V4L	EDX	90.12	9.46	0.41	9.53	219.80	23.07
V4L	EDX	87.79	11.90	0.30	7.38	292.63	39.67
V4L	EDX	86.73	12.56	0.69	6.91	125.70	18.20
V4L	Bulk	82.34	16.81	0.83	4.90	99.77	20.37
V4L	Bulk	83.19	16.01	0.77	5.20	108.50	20.88
V4L	Bulk	82.18	16.88	0.91	4.87	89.83	18.45
V4L	Bulk	82.49	16.63	0.85	4.96	96.81	19.51
V8L	EDX_S	92.80	7.07	0.11	13.13	843.64	64.27
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	91.16	8.79	0.03	10.37	no data	293.00
V8L	EDX_S	91.40	8.47	0.11	10.79	830.91	77.00
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	91.86	8.07	0.05	11.38	no data	161.40
V8L	EDX_S	90.34	9.49	0.15	9.52	602.27	63.27
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	90.37	9.42	0.20	9.59	451.85	47.10
V8L	EDX_S	90.69	9.19	0.11	9.87	824.45	83.55
V8L	EDX_S	90.80	9.04	0.15	10.04	605.33	60.27
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	90.42	9.48	0.09	9.54	no data	105.33
V8L	EDX_S	91.40	8.25	0.30	11.08	304.67	27.50
V8L	EDX_S	91.40	8.49	0.09	10.77	no data	94.33
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	92.33	7.11	0.54	12.99	170.98	13.17
V8L	EDX_S	92.31	7.61	0.07	12.13	no data	108.71
V8L	EDX_S	91.53	8.31	0.14	11.01	653.79	59.36
V8L	EDX_S	92.35	7.44	0.19	12.41	486.05	39.16
V8L	EDX_S	91.16	8.72	0.10	10.45	911.60	87.20
V8L	EDX_S	92.39	7.50	0.09	12.32	no data	83.33
V8L	EDX_S	92.11	7.84	0.03	11.75	no data	261.33
V8L	EDX_S	91.84	8.15	no data	11.27	no data	no data
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX_S	92.67	7.27	0.05	12.75	no data	145.40
V8L	EDX_S	91.10	8.89	no data	10.25	no data	no data
V8L	EDX_S	no data	no data	no data	no data	no data	no data
V8L	EDX	93.86	6.03	0.10	15.57	938.60	60.30
V8L	EDX	93.96	5.91	0.02	15.90	no data	295.50
V8L	EDX	91.83	8.10	0.06	11.34	no data	135.00
V8L	EDX	92.93	6.91	0.14	13.45	663.79	49.36
V8L	EDX	93.49	6.48	0.01	14.43	no data	648.00
V8L	EDX	94.17	5.77	0.05	16.32	no data	115.40
V8L	EDX	91.38	8.55	0.05	10.69	no data	171.00
V8L	EDX	91.91	7.94	0.13	11.58	707.00	61.08
V8L	EDX	91.12	8.74	0.13	10.43	700.92	67.23
V8L	EDX	90.89	9.05	0.04	10.04	no data	226.25

V8L	EDX	91.47	8.39	0.12	10.90	762.25	69.92
V8L	EDX	94.42	5.47	0.10	17.26	944.20	54.70
V8L	EDX	92.02	7.92	0.04	11.62	no data	198.00
V8L	EDX	92.13	7.70	0.15	11.96	614.20	51.33
V8L	EDX	92.02	7.88	0.09	11.68	no data	87.56
V8L	EDX	93.15	6.70	0.14	13.90	665.36	47.86
V8L	EDX	91.90	7.99	0.09	11.50	no data	88.78
V8L	EDX	93.21	6.45	0.33	14.45	282.45	19.55
V8L	EDX	94.05	5.92	0.02	15.89	no data	296.00
V8L	EDX	92.42	7.40	0.16	12.49	577.63	46.25
V8L	EDX	92.99	6.89	0.11	13.50	845.36	62.64
V8L	EDX	92.94	7.00	0.04	13.28	no data	175.00
V8L	EDX	92.68	7.17	0.14	12.93	662.00	51.21
V8L	EDX	92.65	7.21	0.12	12.85	772.08	60.08
V8L	EDX	90.67	9.26	0.05	9.79	no data	185.20
V8L	EDX	93.68	6.23	0.07	15.04	no data	89.00
V8L	EDX	94.66	5.25	0.08	18.03	no data	65.63
V8L	EDX	91.83	8.15	0.01	11.27	no data	815.00
V8L	EDX	93.83	6.01	0.15	15.61	625.53	40.07
V8L	Bulk	88.03	11.69	0.27	7.53	322.18	42.78
V8L	Bulk	87.98	11.72	0.28	7.50	312.09	41.59
V8L	Bulk	88.10	11.61	0.28	7.59	309.37	40.77
E4S	EDX_S	no data	no data	no data	no data	no data	no data
E4S	EDX_S	86.34	13.46	0.19	6.41	454.42	70.84
E4S	EDX_S	86.11	13.64	0.23	6.31	374.39	59.30
E4S	EDX_S	87.25	12.46	0.27	7.00	323.15	46.15
E4S	EDX_S	84.94	14.70	0.34	5.78	249.82	43.24
E4S	EDX_S	86.68	13.01	0.29	6.66	298.90	44.86
E4S	EDX_S	84.81	14.85	0.33	5.71	257.00	45.00
E4S	EDX_S	86.57	13.17	0.24	6.57	360.71	54.88
E4S	EDX_S	86.90	12.83	0.26	6.77	334.23	49.35
E4S	EDX_S	88.53	11.15	0.30	7.94	295.10	37.17
E4S	EDX_S	85.80	13.88	0.31	6.18	276.77	44.77
E4S	EDX_S	86.29	13.45	0.24	6.42	359.54	56.04
E4S	EDX_S	86.35	13.31	0.33	6.49	261.67	40.33
E4S	EDX_S	86.78	12.92	0.29	6.72	299.24	44.55
E4S	EDX_S	86.72	12.92	0.35	6.71	247.77	36.91
E4S	EDX_S	85.90	13.74	0.34	6.25	252.65	40.41
E4S	EDX_S	87.05	12.64	0.29	6.89	300.17	43.59
E4S	EDX_S	85.73	13.93	0.33	6.15	259.79	42.21
E4S	EDX_S	84.73	14.80	0.45	5.73	188.29	32.89
E4S	EDX_S	86.81	12.87	0.31	6.75	280.03	41.52
E4S	EDX	87.14	12.78	0.06	6.82	no data	213.00
E4S	EDX	86.54	13.29	0.15	6.51	576.93	88.60
E4S	EDX	87.69	12.20	0.09	7.19	974.33	135.56
E4S	EDX	90.02	9.96	no data	9.04	no data	no data
E4S	EDX	85.82	14.01	0.16	6.13	536.38	87.56
E4S	EDX	88.23	11.63	0.12	7.59	735.25	96.92
E4S	EDX	87.60	12.23	0.15	7.16	584.00	81.53
E4S	EDX	86.96	12.93	0.10	6.73	869.60	129.30
E4S	EDX	90.08	9.84	0.06	9.15	no data	164.00
E4S	EDX	90.90	9.00	0.09	10.10	no data	100.00
E4S	EDX	88.31	11.50	0.18	7.68	no data	63.89
E4S	EDX	88.13	11.77	0.08	7.49	no data	147.13

Row data

E4S	EDX	86.75	12.99	0.24	6.68	361.46	54.13
E4S	EDX	88.11	11.80	0.08	7.47	no data	147.50
E4S	EDX	88.02	11.77	0.19	7.48	463.26	61.95
E4S	EDX	89.69	10.13	0.16	8.85	560.56	63.31
E4S	EDX	88.64	11.14	0.20	7.96	443.20	55.70
E4S	EDX	88.57	11.31	0.10	7.83	885.70	113.10
E4S	EDX	87.18	12.54	0.26	6.95	335.31	48.23
E4S	EDX	88.76	11.15	0.07	7.96	no data	159.29
E4S	Bulk	86.73	12.89	0.37	6.73	233.54	34.71
E4S	Bulk	86.77	12.80	0.41	6.78	209.94	30.97
E4S	Bulk	86.88	12.72	0.38	6.83	226.26	33.14
E7S	EDX_S	86.53	12.64	0.81	6.85	106.83	15.60
E7S	EDX_S	no data	no data	no data	no data	no data	no data
E7S	EDX_S	86.97	12.32	0.69	7.06	126.04	17.86
E7S	EDX_S	no data	no data	no data	no data	no data	no data
E7S	EDX_S	85.95	13.41	0.62	6.41	138.63	21.63
E7S	EDX_S	85.33	14.37	0.28	5.94	304.75	51.32
E7S	EDX_S	87.62	11.26	1.11	7.78	78.94	10.14
E7S	EDX_S	86.09	13.07	0.73	6.59	117.93	17.90
E7S	EDX_S	no data	no data	no data	no data	no data	no data
E7S	EDX_S	86.53	12.17	1.29	7.11	67.08	9.43
E7S	EDX_S	85.20	13.23	1.56	6.44	54.62	8.48
E7S	EDX_S	85.09	12.13	2.77	7.01	30.72	4.38
E7S	EDX_S	94.08	5.27	0.63	17.85	149.33	8.37
E7S	EDX_S	83.80	13.38	2.80	6.26	29.93	4.78
E7S	EDX_S	84.32	14.60	1.07	5.78	78.80	13.64
E7S	EDX_S	88.08	11.22	0.68	7.85	129.53	16.50
E7S	EDX_S	84.46	14.49	1.03	5.83	82.00	14.07
E7S	EDX_S	86.07	12.58	1.34	6.84	64.23	9.39
E7S	EDX_S	84.88	13.51	1.60	6.28	53.05	8.44
E7S	EDX	89.19	10.30	0.50	8.66	178.38	20.60
E7S	EDX	90.26	9.73	no data	9.28	no data	no data
E7S	EDX	88.95	10.67	0.36	8.34	247.08	29.64
E7S	EDX	91.14	8.79	0.06	10.37	no data	146.50
E7S	EDX	87.53	12.16	0.29	7.20	301.83	41.93
E7S	EDX	85.89	13.42	0.67	6.40	128.19	20.03
E7S	EDX	89.04	10.48	0.46	8.50	193.57	22.78
E7S	EDX	87.23	12.43	0.33	7.02	264.33	37.67
E7S	EDX	91.37	8.39	0.23	10.89	397.26	36.48
E7S	EDX	89.89	9.30	0.80	9.67	112.36	11.63
E7S	EDX	86.62	12.51	0.85	6.92	101.91	14.72
E7S	EDX	88.07	10.18	1.74	8.65	50.61	5.85
E7S	EDX	90.12	9.87	no data	9.13	no data	no data
E7S	EDX	85.88	12.59	1.51	6.82	56.87	8.34
E7S	EDX	85.08	13.98	0.93	6.09	91.48	15.03
E7S	EDX	89.06	10.38	0.54	8.58	164.93	19.22
E7S	EDX	84.92	14.41	0.65	5.89	130.65	22.17
E7S	EDX	87.58	11.51	0.90	7.61	97.31	12.79
E7S	EDX	85.00	13.72	1.26	6.20	67.46	10.89
E7S	Bulk	82.97	16.34	0.67	5.08	124.59	24.54
E7S	Bulk	83.56	15.73	0.70	5.31	119.95	22.58
E7S	Bulk	82.69	16.61	0.68	4.98	122.35	24.58
E9S	EDX_S	87.36	11.12	1.51	7.86	57.85	7.36
E9S	EDX_S	85.64	12.14	2.20	7.05	38.93	5.52

E9S	EDX_S	86.75	11.73	1.50	7.40	57.83	7.82
E9S	EDX_S	86.67	11.17	2.14	7.76	40.50	5.22
E9S	EDX_S	88.04	10.07	1.87	8.74	47.08	5.39
E9S	EDX_S	86.19	12.28	1.52	7.02	56.70	8.08
E9S	EDX_S	88.47	9.93	1.58	8.91	55.99	6.28
E9S	EDX_S	86.14	11.29	2.55	7.63	33.78	4.43
E9S	EDX_S	87.69	10.22	2.08	8.58	42.16	4.91
E9S	EDX_S	84.82	12.72	2.45	6.67	34.62	5.19
E9S	EDX_S	87.22	11.32	1.45	7.70	60.15	7.81
E9S	EDX_S	88.19	9.55	2.25	9.23	39.20	4.24
E9S	EDX_S	89.54	8.90	1.55	10.06	57.77	5.74
E9S	EDX_S	86.84	11.67	1.47	7.44	59.07	7.94
E9S	EDX_S	87.41	11.22	1.35	7.79	64.75	8.31
E9S	EDX_S	86.75	11.31	1.93	7.67	44.95	5.86
E9S	EDX_S	89.38	9.03	1.58	9.90	56.57	5.72
E9S	EDX_S	87.52	11.03	1.44	7.93	60.78	7.66
E9S	EDX_S	86.79	11.52	1.68	7.53	51.66	6.86
E9S	EDX_S	85.84	12.66	1.49	6.78	57.61	8.50
E9S	EDX	82.90	16.16	0.92	5.13	90.11	17.57
E9S	EDX	81.64	17.13	1.21	4.77	67.47	14.16
E9S	EDX	80.82	18.38	0.79	4.40	102.30	23.27
E9S	EDX	83.00	16.31	0.68	5.09	122.06	23.99
E9S	EDX	81.94	16.97	1.08	4.83	75.87	15.71
E9S	EDX	80.72	17.87	1.40	4.52	57.66	12.76
E9S	EDX	79.50	19.30	1.19	4.12	66.81	16.22
E9S	EDX	80.72	18.48	0.79	4.37	102.18	23.39
E9S	EDX	78.20	20.34	1.44	3.84	54.31	14.13
E9S	EDX	84.86	14.39	0.74	5.90	114.68	19.45
E9S	Bulk	84.34	12.92	2.65	6.53	31.82	4.87
E9S	Bulk	84.50	12.89	2.53	6.56	33.36	5.09
E9S	Bulk	84.35	13.00	2.56	6.49	32.89	5.07
V1S	EDX_S	86.60	13.11	0.27	6.61	320.74	48.56
V1S	EDX_S	84.00	15.49	0.49	5.42	171.43	31.61
V1S	EDX_S	85.77	13.93	0.29	6.16	295.76	48.03
V1S	EDX_S	83.21	16.44	0.34	5.06	244.74	48.35
V1S	EDX_S	85.73	13.81	0.45	6.21	190.51	30.69
V1S	EDX_S	no data	no data	no data	no data	no data	no data
V1S	EDX_S	87.91	11.85	0.23	7.42	382.22	51.52
V1S	EDX_S	85.52	13.92	0.55	6.14	155.49	25.31
V1S	EDX_S	no data	no data	no data	no data	no data	no data
V1S	EDX_S	85.70	13.84	0.45	6.19	190.44	30.76
V1S	EDX_S	no data	no data	no data	no data	no data	no data
V1S	EDX_S	84.60	15.17	0.21	5.58	402.86	72.24
V1S	EDX_S	87.50	12.29	0.19	7.12	460.53	64.68
V1S	EDX_S	85.09	14.46	0.44	5.88	193.39	32.86
V1S	EDX_S	86.78	12.93	0.28	6.71	309.93	46.18
V1S	EDX_S	86.71	12.99	0.29	6.68	299.00	44.79
V1S	EDX_S	84.00	15.55	0.44	5.40	190.91	35.34
V1S	EDX_S	84.11	15.48	0.40	5.43	210.28	38.70
V1S	EDX_S	89.11	10.74	0.14	8.30	636.50	76.71
V1S	EDX_S	85.90	13.70	0.36	6.27	238.61	38.06
V1S	EDX_S	86.90	12.74	0.35	6.82	248.29	36.40
V1S	EDX_S	87.76	11.96	0.27	7.34	325.04	44.30
V1S	EDX_S	87.41	12.41	0.17	7.04	514.18	73.00

V1S	EDX_S	87.04	12.71	0.23	6.85	378.43	55.26
V1S	EDX_S	86.56	13.20	0.40	6.56	216.40	33.00
V1S	EDX_S	89.58	10.16	0.25	8.82	358.32	40.64
V1S	EDX_S	87.74	11.88	0.37	7.39	237.14	32.11
V1S	EDX_S	90.31	9.48	0.20	9.53	451.55	47.40
V1S	EDX_S	87.95	11.74	0.30	7.49	293.17	39.13
V1S	EDX_S	87.26	12.30	0.43	7.09	202.93	28.60
V1S	EDX_S	86.18	13.45	0.36	6.41	239.39	37.36
V1S	EDX_S	85.42	14.08	0.48	6.07	177.96	29.33
V1S	EDX_S	86.34	13.30	0.34	6.49	253.94	39.12
V1S	EDX	89.07	10.75	0.17	8.29	523.94	63.24
V1S	EDX	85.74	14.06	0.19	6.10	451.26	74.00
V1S	EDX	89.46	10.51	0.01	8.51	no data	1051.00
V1S	EDX	84.82	15.02	0.14	5.65	605.86	107.29
V1S	EDX	88.51	11.37	0.11	7.78	804.64	103.36
V1S	EDX	91.50	8.41	0.08	10.88	1143.75	105.13
V1S	EDX	89.95	10.00	0.03	9.00	no data	333.33
V1S	EDX	88.12	11.56	0.31	7.62	284.26	37.29
V1S	EDX	90.57	9.38	0.04	9.66	no data	234.50
V1S	EDX	87.92	11.94	0.12	7.36	732.67	99.50
V1S	EDX	87.90	12.03	0.05	7.31	no data	240.60
V1S	EDX	86.99	12.91	0.09	6.74	966.56	143.44
V1S	EDX	86.83	12.93	0.22	6.72	394.68	58.77
V1S	EDX	85.06	14.65	0.28	5.81	303.79	52.32
V1S	EDX	87.64	12.03	0.32	7.29	273.88	37.59
V1S	EDX	88.86	10.82	0.30	8.21	296.20	36.07
V1S	EDX	84.74	15.01	0.24	5.65	353.08	62.54
V1S	EDX	85.03	14.82	0.14	5.74	607.36	105.86
V1S	EDX	89.04	10.75	0.20	8.28	445.20	53.75
V1S	EDX	85.47	14.36	0.15	5.95	569.80	95.73
V1S	EDX	88.10	11.60	0.28	7.59	314.64	41.43
V1S	EDX	86.23	13.55	0.20	6.36	431.15	67.75
V1S	EDX	91.76	8.18	0.05	11.22	no data	163.60
V1S	EDX	92.32	7.67	no data	12.04	no data	no data
V1S	EDX	88.02	11.85	0.12	7.43	733.50	98.75
V1S	EDX	89.84	10.05	0.10	8.94	898.40	100.50
V1S	EDX	89.26	10.56	0.16	8.45	557.88	66.00
V1S	EDX	91.16	8.74	0.09	10.43	no data	97.11
V1S	EDX	89.85	10.04	0.09	8.95	998.33	111.56
V1S	EDX	87.88	11.81	0.30	7.44	292.93	39.37
V1S	EDX	87.61	12.24	0.13	7.16	673.92	94.15
V1S	EDX	86.06	13.66	0.26	6.30	331.00	52.54
V1S	EDX	85.60	14.33	0.05	5.97	no data	286.60
V1S	Bulk	86.24	13.27	0.48	6.50	181.00	27.84
V1S	Bulk	87.10	12.53	0.36	6.95	238.51	34.30
V1S	Bulk	86.68	12.90	0.41	6.72	209.45	31.16
V1S	Bulk	82.42	16.52	1.03	4.99	80.17	16.07
V4S	EDX_S	88.18	11.40	0.41	7.74	215.07	27.80
V4S	EDX_S	88.66	10.50	0.83	8.44	106.82	12.65
V4S	EDX_S	85.81	13.49	0.68	6.36	126.19	19.84
V4S	EDX_S	86.30	12.95	0.73	6.66	118.22	17.74
V4S	EDX_S	87.41	12.19	0.39	7.17	224.13	31.26
V4S	EDX_S	87.18	12.20	0.60	7.15	145.30	20.33
V4S	EDX_S	86.92	12.13	0.94	7.17	92.47	12.90

V4S	EDX_S	85.36	13.80	0.83	6.19	102.84	16.63
V4S	EDX_S	86.71	12.59	0.68	6.89	127.51	18.51
V4S	EDX_S	89.03	10.52	0.43	8.46	207.05	24.47
V4S	EDX_S	85.35	13.83	0.80	6.17	106.69	17.29
V4S	EDX_S	85.27	13.84	0.88	6.16	96.90	15.73
V4S	EDX_S	85.67	13.51	0.80	6.34	107.09	16.89
V4S	EDX_S	87.57	11.84	0.57	7.40	153.63	20.77
V4S	EDX	88.74	10.89	0.36	8.15	246.50	30.25
V4S	EDX	90.20	9.35	0.43	9.65	209.77	21.74
V4S	EDX	87.98	11.69	0.32	7.53	274.94	36.53
V4S	EDX	88.19	11.39	0.41	7.74	215.10	27.78
V4S	EDX	88.49	11.27	0.23	7.85	384.74	49.00
V4S	EDX	88.88	10.80	0.31	8.23	286.71	34.84
V4S	EDX	87.09	12.21	0.68	7.13	128.07	17.96
V4S	EDX	89.17	10.46	0.35	8.52	254.77	29.89
V4S	EDX	87.91	11.64	0.43	7.55	204.44	27.07
V4S	EDX	92.04	7.68	0.26	11.98	354.00	29.54
V4S	EDX	87.13	12.35	0.51	7.06	170.84	24.22
V4S	EDX	86.22	13.20	0.57	6.53	151.26	23.16
V4S	EDX	87.51	11.94	0.53	7.33	165.11	22.53
V4S	EDX	87.29	12.33	0.36	7.08	242.47	34.25
V4S	Bulk	82.94	16.27	0.77	5.10	107.20	21.03
V4S	Bulk	83.16	16.07	0.75	5.18	110.65	21.38
V4S	Bulk	82.99	16.15	0.83	5.14	99.43	19.34
V4S	Bulk	84.49	14.17	1.29	5.96	65.46	10.98
V8S	EDX_S	no data	no data	no data	no data	no data	no data
V8S	EDX_S	89.30	10.58	0.10	8.44	893.00	105.80
V8S	EDX_S	90.77	9.17	0.05	9.90	no data	183.40
V8S	EDX_S	89.79	10.12	0.08	8.87	no data	126.50
V8S	EDX_S	90.56	9.30	0.13	9.74	696.62	71.54
V8S	EDX_S	89.82	10.02	0.15	8.96	598.80	66.80
V8S	EDX_S	90.37	9.49	0.13	9.52	695.15	73.00
V8S	EDX_S	88.66	11.16	0.17	7.94	521.53	65.65
V8S	EDX_S	88.50	11.35	0.13	7.80	680.77	87.31
V8S	EDX_S	90.35	9.52	0.12	9.49	752.92	79.33
V8S	EDX_S	90.31	9.49	0.19	9.52	475.32	49.95
V8S	EDX_S	89.12	10.78	0.09	8.27	990.22	119.78
V8S	EDX_S	no data	no data	no data	no data	no data	no data
V8S	EDX_S	89.06	10.79	0.14	8.25	636.14	77.07
V8S	EDX_S	89.96	9.85	0.18	9.13	499.78	54.72
V8S	EDX_S	90.43	9.27	0.28	9.76	322.96	33.11
V8S	EDX	91.25	8.73	0.01	10.45	no data	873.00
V8S	EDX	92.38	7.39	0.21	12.50	439.90	35.19
V8S	EDX	94.78	5.21	no data	18.19	no data	no data
V8S	EDX	92.35	7.64	no data	12.09	no data	no data
V8S	EDX	93.31	6.62	0.06	14.10	no data	110.33
V8S	EDX	90.17	9.77	0.04	9.23	no data	244.25
V8S	EDX	90.35	9.55	0.08	9.46	1129.38	119.38
V8S	EDX	92.00	7.93	0.06	11.60	no data	132.17
V8S	EDX	90.05	9.82	0.12	9.17	750.42	81.83
V8S	EDX	87.93	11.79	0.27	7.46	325.67	43.67
V8S	EDX	91.04	8.88	0.07	10.25	no data	126.86
V8S	EDX	90.99	8.97	0.02	10.14	no data	448.50
V8S	EDX	94.86	5.07	0.05	18.71	no data	101.40

V8S	EDX	90.31	9.64	0.04	9.37	no data	241.00
V8S	EDX	91.57	8.37	0.05	10.94	no data	167.40
V8S	EDX	90.50	9.24	0.25	9.79	362.00	36.96
V8S	Bulk	89.06	10.69	0.24	8.33	367.23	44.06
V8S	Bulk	89.15	10.59	0.25	8.42	352.84	41.90
V8S	Bulk	89.22	10.58	0.19	8.43	460.81	54.66
V8S	Bulk	88.10	11.66	0.24	7.56	371.52	49.15

Appendix II: Row data chapter 2

Treatment	Label	Method	%C	%N	%F	%P	%Br	%I	C:N	C:P	N:P
E7L	CARD-FISH BR	EDX	85.35	13.95	0.51	0.17	0.00	0.00	6.12	502.06	82.06
E7L	CARD-FISH BR	EDX	86.50	13.01	0.29	0.18	0.00	0.00	6.65	480.56	72.28
E7L	CARD-FISH BR	EDX	87.63	12.01	0.22	0.13	0.00	0.00	7.30	674.08	92.38
E7L	CARD-FISH BR	EDX	89.22	10.05	0.54	0.17	0.00	0.00	8.88	524.82	59.12
E7L	CARD-FISH BR	EDX	83.85	15.42	0.56	0.16	0.00	0.00	5.44	524.06	96.38
E7L	CARD-FISH BR	EDX	82.55	14.72	0.55	0.16	0.00	0.00	5.61	515.94	92.00
E7L	CARD-FISH BR	EDX	87.17	12.35	0.32	0.13	0.00	0.00	7.06	670.54	95.00
E7L	CARD-FISH BR	EDX	86.42	13.14	0.28	0.14	0.00	0.00	6.58	617.29	93.86
E7L	CARD-FISH BR	EDX	82.12	13.26	0.42	0.17	0.00	0.00	6.19	483.06	78.00
E7L	CARD-FISH BR	EDX	87.51	11.74	0.64	0.09	0.00	0.00	7.45	972.33	130.44
E7L	CARD-FISH BR	EDX	87.29	12.07	0.46	0.16	0.00	0.00	7.23	545.56	75.44
E7L	CARD-FISH BR	EDX	86.28	13.20	0.38	0.11	0.00	0.00	6.54	784.36	120.00
E7L	CARD-FISH BR	EDX	88.80	10.52	0.36	0.30	0.00	0.00	8.44	296.00	35.07
E7L	CARD-FISH BR	EDX	84.38	15.02	0.42	0.16	0.00	0.00	5.62	527.38	93.88
E7L	CARD-FISH BR	EDX	86.46	13.02	0.39	0.11	0.00	0.00	6.64	786.00	118.36
E7L	CARD-FISH BR	EDX	86.86	12.56	0.45	0.10	0.00	0.00	6.92	868.60	125.60
E7L	CARD-FISH BR	EDX	85.09	14.21	0.58	0.10	0.00	0.00	5.99	850.90	142.10
E7L	CARD-FISH BR	EDX	88.07	11.52	0.28	0.11	0.00	0.00	7.64	800.64	104.73
E7L	CARD-FISH BR	EDX	85.00	14.06	0.43	0.49	0.00	0.00	6.05	173.47	28.69
E7L	CARD-FISH BR	EDX	86.42	13.35	0.09	0.11	0.00	0.00	6.47	785.64	121.36
E7L	CARD-FISH BR	EDX	86.18	13.34	0.32	0.15	0.00	0.00	6.46	574.53	88.93
E7L	CARD-FISH BR	EDX_S	84.87	14.82	-	0.30	-	-	5.73	282.90	49.40
E7L	CARD-FISH BR	EDX_S	85.86	13.80	-	0.32	-	-	6.22	268.31	43.13
E7L	CARD-FISH BR	EDX_S	87.01	12.74	-	0.24	-	-	6.83	362.54	53.08
E7L	CARD-FISH BR	EDX_S	88.97	10.70	-	0.32	-	-	8.31	278.03	33.44
E7L	CARD-FISH BR	EDX_S	83.34	16.36	-	0.29	-	-	5.09	287.38	56.41
E7L	CARD-FISH BR	EDX_S	84.06	15.62	-	0.30	-	-	5.38	280.20	52.07
E7L	CARD-FISH BR	EDX_S	86.63	13.11	-	0.24	-	-	6.61	360.96	54.63
E7L	CARD-FISH BR	EDX_S	85.80	13.93	-	0.26	-	-	6.16	330.00	53.58
E7L	CARD-FISH BR	EDX_S	85.60	14.07	-	0.32	-	-	6.08	267.50	43.97
E7L	CARD-FISH BR	EDX_S	87.30	12.51	-	0.17	-	-	6.98	513.53	73.59
E7L	CARD-FISH BR	EDX_S	86.86	12.83	-	0.29	-	-	6.77	299.52	44.24
E7L	CARD-FISH BR	EDX_S	85.76	14.01	-	0.21	-	-	6.12	408.38	66.71
E7L	CARD-FISH BR	EDX_S	88.27	11.16	-	0.55	-	-	7.91	160.49	20.29
E7L	CARD-FISH BR	EDX_S	83.77	15.93	-	0.29	-	-	5.26	288.86	54.93
E7L	CARD-FISH BR	EDX_S	85.96	13.82	-	0.20	-	-	6.22	429.80	69.10
E7L	CARD-FISH BR	EDX_S	86.45	13.35	-	0.19	-	-	6.48	455.00	70.26
E7L	CARD-FISH BR	EDX_S	84.69	15.10	-	0.19	-	-	5.61	445.74	79.47
E7L	CARD-FISH BR	EDX_S	87.55	12.23	-	0.20	-	-	7.16	437.75	61.15
E7L	CARD-FISH BR	EDX_S	84.22	14.87	-	0.89	-	-	5.66	94.63	16.71
E7L	CARD-FISH BR	EDX_S	85.65	14.13	-	0.20	-	-	6.06	428.25	70.65
E7L	CARD-FISH BR	EDX_S	85.57	14.14	-	0.27	-	-	6.05	316.93	52.37

E7L	CARD-FISH F	EDX	88.33	11.24	0.18	0.23	0.00	0.00	7.86	384.04	48.87
E7L	CARD-FISH F	EDX	87.49	12.07	0.22	0.21	0.00	0.00	7.25	416.62	57.48
E7L	CARD-FISH F	EDX	86.21	13.27	0.27	0.22	0.00	0.00	6.50	391.86	60.32
E7L	CARD-FISH F	EDX	85.83	13.70	0.26	0.19	0.00	0.00	6.26	451.74	72.11
E7L	CARD-FISH F	EDX	87.10	12.48	0.26	0.14	0.00	0.00	6.98	622.14	89.14
E7L	CARD-FISH F	EDX	88.60	11.15	0.11	0.12	0.00	0.00	7.95	738.33	92.92
E7L	CARD-FISH F	EDX	89.21	10.49	0.19	0.09	0.00	0.00	8.50	991.22	116.56
E7L	CARD-FISH F	EDX	87.85	11.68	0.25	0.20	0.00	0.00	7.52	439.25	58.40
E7L	CARD-FISH F	EDX	87.19	12.42	0.18	0.20	0.00	0.00	7.02	435.95	62.10
E7L	CARD-FISH F	EDX	87.93	11.63	0.25	0.17	0.00	0.00	7.56	517.24	68.41
E7L	CARD-FISH F	EDX	88.22	11.33	0.27	0.15	0.00	0.00	7.79	588.13	75.53
E7L	CARD-FISH F	EDX	87.46	12.08	0.24	0.19	0.00	0.00	7.24	460.32	63.58
E7L	CARD-FISH F	EDX	87.83	11.70	0.23	0.21	0.00	0.00	7.51	418.24	55.71
E7L	CARD-FISH F	EDX	88.35	11.22	0.27	0.14	0.00	0.00	7.87	631.07	80.14
E7L	CARD-FISH F	EDX	88.30	11.26	0.20	0.21	0.00	0.00	7.84	420.48	53.62
E7L	CARD-FISH F	EDX	87.40	12.19	0.23	0.16	0.00	0.00	7.17	546.25	76.19
E7L	CARD-FISH F	EDX	90.23	9.44	0.21	0.11	0.00	0.00	9.56	820.27	85.82
E7L	CARD-FISH F	EDX	87.04	12.46	0.32	0.16	0.00	0.00	6.99	544.00	77.88
E7L	CARD-FISH F	EDX	89.49	10.15	0.15	0.18	0.00	0.00	8.82	497.17	56.39
E7L	CARD-FISH F	EDX	88.60	11.11	0.11	0.17	0.00	0.00	7.97	521.18	65.35
E7L	CARD-FISH F	EDX_S	87.65	11.91	-	0.43	-	-	7.36	203.84	27.70
E7L	CARD-FISH F	EDX_S	86.81	12.79	-	0.38	-	-	6.79	228.45	33.66
E7L	CARD-FISH F	EDX_S	85.52	14.06	-	0.41	-	-	6.08	208.59	34.29
E7L	CARD-FISH F	EDX_S	85.12	14.51	-	0.35	-	-	5.87	243.20	41.46
E7L	CARD-FISH F	EDX_S	86.49	13.23	-	0.27	-	-	6.54	320.33	49.00
E7L	CARD-FISH F	EDX_S	87.95	11.82	-	0.22	-	-	7.44	399.77	53.73
E7L	CARD-FISH F	EDX_S	88.68	11.14	-	0.17	-	-	7.96	521.65	65.53
E7L	CARD-FISH F	EDX_S	87.24	12.39	-	0.36	-	-	7.04	242.33	34.42
E7L	CARD-FISH F	EDX_S	86.47	13.15	-	0.36	-	-	6.58	240.19	36.53
E7L	CARD-FISH F	EDX_S	87.34	12.34	-	0.31	-	-	7.08	281.74	39.81
E7L	CARD-FISH F	EDX_S	87.68	12.03	-	0.28	-	-	7.29	313.14	42.96
E7L	CARD-FISH F	EDX_S	86.83	12.81	-	0.35	-	-	6.78	248.09	36.60
E7L	CARD-FISH F	EDX_S	87.19	12.40	-	0.39	-	-	7.03	223.56	31.79
E7L	CARD-FISH F	EDX_S	87.81	11.91	-	0.27	-	-	7.37	325.22	44.11
E7L	CARD-FISH F	EDX_S	87.66	11.94	-	0.39	-	-	7.34	224.77	30.62
E7L	CARD-FISH F	EDX_S	86.77	12.93	-	0.29	-	-	6.71	299.21	44.59
E7L	CARD-FISH F	EDX_S	89.76	10.03	-	0.20	-	-	8.95	448.80	50.15
E7L	CARD-FISH F	EDX_S	86.47	13.22	-	0.30	-	-	6.54	288.23	44.07
E7L	CARD-FISH F	EDX_S	88.88	10.77	-	0.34	-	-	8.25	261.41	31.68
E7L	CARD-FISH F	EDX_S	87.91	11.77	-	0.30	-	-	7.47	293.03	39.23
E4L	FISH Br	EDX	90.10	9.50	0.15	0.23	0.00	0.00	9.48	391.74	41.30
E4L	FISH Br	EDX	87.63	11.85	0.26	0.24	0.00	0.00	7.39	365.13	49.38
E4L	FISH Br	EDX	88.92	10.67	0.20	0.20	0.00	0.00	8.33	444.60	53.35
E4L	FISH Br	EDX	88.20	11.34	0.16	0.27	0.00	0.00	7.78	326.67	42.00
E4L	FISH Br	EDX	90.83	8.23	0.88	0.02	0.01	0.00	11.04	_	_
E4L	FISH Br	EDX	88.65	10.95	0.11	0.25	0.02	0.00	8.10	354.60	43.80
E4L	FISH Br	EDX	89.61	9.99	0.16	0.21	0.00	0.00	8.97	426.71	47.57
E4L	FISH Br	EDX	88.52	10.90	0.39	0.16	0.00	0.00	8.12	553.25	68.13
E4L	FISH Br	EDX	88.15	11.24	0.34	0.22	0.02	0.00	7.84	400.68	51.09
E4L	FISH Br	EDX	90.06	9.50	0.24	0.18	0.00	0.00	9.48	500.33	52.78
E4L	FISH Br	EDX	89.11	10.28	0.23	0.35	0.00	0.00	8.67	254.60	29.37
E4L	FISH Br	EDX	90.74	8.93	0.18	0.11	0.02	0.00	10.16	824.91	81.18
E4L	FISH Br	EDX	89.09	10.41	0.19	0.29	0.00	0.00	8.56	307.21	35.90
E4L	FISH Br	EDX	89.69	9.96	0.16	0.16	0.00	0.00	9.01	560.56	62.25

Row data

E4L	FISH Br	EDX	87.62	11.78	0.32	0.24	0.02	0.00	7.44	365.08	49.08
E4L	FISH Br	EDX	87.59	11.78	0.38	0.24	0.00	0.00	7.44	364.96	49.08
E4L	FISH Br	EDX	88.70	10.89	0.21	0.18	0.00	0.00	8.15	492.78	60.50
E4L	FISH Br	EDX	87.61	12.01	0.16	0.17	0.01	0.00	7.29	515.35	70.65
E4L	FISH Br	EDX	87.65	11.67	0.39	0.27	0.00	0.00	7.51	324.63	43.22
E4L	FISH Br	EDX	87.80	11.47	0.44	0.26	0.00	0.00	7.65	337.69	44.12
E4L	FISH Br	EDX	86.76	12.51	0.47	0.23	0.00	0.00	6.94	377.22	54.39
E4L	FISH Br	EDX	85.35	13.66	0.50	0.48	0.00	0.00	6.25	177.81	28.46
E4L	FISH Br	EDX	88.97	10.46	0.27	0.28	0.00	0.00	8.51	317.75	37.36
E4L	FISH Br	EDX	86.52	12.81	0.31	0.34	0.00	0.00	6.75	254.47	37.68
E4L	FISH Br	EDX	85.81	13.62	0.23	0.32	0.00	0.00	6.30	268.16	42.56
E4L	FISH Br	EDX	88.34	11.30	0.15	0.19	0.00	0.00	7.82	464.95	59.47
E4L	FISH Br	EDX	89.40	10.07	0.31	0.21	0.00	0.00	8.88	425.71	47.95
E4L	FISH Br	EDX	89.15	10.46	0.18	0.18	0.00	0.00	8.52	495.28	58.11
E4L	FISH Br	EDX_S	88.27	11.36	-	0.36	-	-	7.77	245.19	31.56
E4L	FISH Br	EDX_S	85.65	13.78	-	0.55	-	-	6.22	155.73	25.05
E4L	FISH Br	EDX_S	87.79	11.79	-	0.40	-	-	7.45	219.48	29.48
E4L	FISH Br	EDX_S	87.12	12.56	-	0.30	-	-	6.94	290.40	41.87
E4L	FISH Br	EDX_S	89.21	10.56	-	0.21	-	-	8.45	424.81	50.29
E4L	FISH Br	EDX_S	86.89	12.63	-	0.46	-	-	6.88	188.89	27.46
E4L	FISH Br	EDX_S	87.52	12.10	-	0.36	-	-	7.23	243.11	33.61
E4L	FISH Br	EDX_S	87.86	11.84	-	0.28	-	-	7.42	313.79	42.29
E4L	FISH Br	EDX_S	86.77	12.67	-	0.54	-	-	6.85	160.69	23.46
E4L	FISH Br	EDX_S	91.26	8.19	-	0.54	-	-	11.14	169.00	15.17
E4L	FISH Br	EDX_S	87.19	12.11	-	0.68	-	-	7.20	128.22	17.81
E4L	FISH Br	EDX_S	88.82	10.97	-	0.20	-	-	8.10	444.10	54.85
E4L	FISH Br	EDX_S	88.74	10.80	-	0.45	-	-	8.22	197.20	24.00
E4L	FISH Br	EDX_S	88.73	10.98	-	0.27	-	-	8.08	328.63	40.67
E4L	FISH Br	EDX_S	86.53	13.01	-	0.44	-	-	6.65	196.66	29.57
E4L	FISH Br	EDX_S	86.50	12.95	-	0.53	-	-	6.68	163.21	24.43
E4L	FISH Br	EDX_S	86.48	13.03	-	0.48	-	-	6.64	180.17	27.15
E4L	FISH Br	EDX_S	86.81	12.84	-	0.33	-	-	6.76	263.06	38.91
E4L	FISH Br	EDX_S	86.61	13.05	-	0.32	-	-	6.64	270.66	40.78
E4L	FISH Br	EDX_S	86.59	10.00	-	0.40	-	-	8.66	216.48	25.00
E4L	FISH Br	EDX_S	86.21	13.27	-	0.51	-	-	6.50	169.04	26.02
E4L	FISH Br	EDX_S	85.44	14.08	-	0.47	-	-	6.07	181.79	29.96
E4L	FISH Br	EDX_S	86.88	12.68	-	0.42	-	-	6.85	206.86	30.19
E4L	FISH Br	EDX_S	84.47	14.56	-	0.95	-	-	5.80	88.92	15.33
E4L	FISH Br	EDX_S	85.93	13.67	-	0.39	-	-	6.29	220.33	35.05
E4L	FISH Br	EDX_S	87.48	11.90	-	0.60	-	-	7.35	145.80	19.83
E4L	FISH Br	EDX_S	85.78	13.82	-	0.38	-	-	6.21	225.74	36.37
E4L	FISH Br	EDX_S	84.99	14.52	-	0.47	-	-	5.85	180.83	30.89
E4L	FISH Br	EDX_S	86.90	12.71	-	0.38	-	-	6.84	228.68	33.45
E4L	FISH Br	EDX_S	87.39	12.22	-	0.38	-	-	7.15	229.97	32.16
E4L	FISH Br	EDX_S	87.64	12.09	-	0.25	-	-	7.25	350.56	48.36