Interaction of storage carbohydrates and other cyclic fluxes with central metabolism: A quantitative approach by non-stationary ^{13}C metabolic flux analysis, **Supplementary Material #2**

1.Concentrations of intracellular metabolites

Table S2-1. Concentrations of intracellular metabolites at different growth rates

Metabolite (measurement method)	Averaged concentrations (µmol g_{DW}^{-1})			
	Dilution rate (h^{-1})			
	0.054	**0.101**	**0.207**	**0.307**
Metabolites of the Glycolysis				
Glc_int (GC-MS)	9.25 +/- 0.28	5.57 +/- 0.43	1.35 +/- 0.23	1.67 +/- 0.47
Glc6P (GC-MS)	3.88 +/- 0.06	5.42 +/- 0.15	9.20 +/- 0.12	9.02 +/- 0.29
Fru6P (GC-MS)	1.02 +/- 0.02	1.50 +/- 0.04	2.50 +/- 0.03	1.88 +/- 0.05
M6P (GC-MS)	1.32 +/- 0.02	1.76 +/- 0.05	2.80 +/- 0.03	2.33 +/- 0.07
FBP (GC-MS)	0.20 +/- 0.03	0.23 +/- 0.03	0.56 +/- 0.06	1.66 +/- 0.10
DHAP (GC-MS)	0.25 +/- 0.01	0.54 +/- 0.02	0.78 +/- 0.01	0.75 +/- 0.01
GA3P (GC-MS)	0.019 +/- 0.002	0.032 +/- 0.001	0.032 +/- 0.001	0.044 +/- 0.001
G3P (GC-MS)	0.146 +/- 0.032	0.207 +/- 0.029	0.147 +/- 0.050	0.039 +/- 0.006
3PG (GC-MS)	6.95 +/- 0.06	4.99 +/- 0.17	5.76 +/- 0.07	3.30 +/- 0.08
2PG (GC-MS)	0.864 +/- 0.004	0.587 +/- 0.018	0.632 +/- 0.015	0.314 +/- 0.005
PEP (GC-MS)	3.04 +/- 0.04	2.41 +/- 0.04	2.29 +/- 0.02	1.04 +/- 0.01
Pyr (GC-MS)	2.39 +/- 0.04	2.20 +/- 0.02	3.40 +/- 0.16	3.39 +/- 0.20
Metabolites of the Storage Carbohydrate pools				
T6P (GC-MS)	0.336 +/- 0.010	0.374 +/- 0.009	0.039 +/- 0.002	0.016 +/- 0.001
Treh (GC-MS)	145.93 +/- 3.22	162.16 +/- 2.86	1.74 +/- 0.01	0.29 +/- 0.02
Glycogen (glucose equivalent) (Enzymatic assay)	439 +/- 18	593 +/- 17	121 +/- 2	98 +/- 1
Glc1P (LC-MS/MS)	0.22 +/- 0.01	0.36 +/- 0.03	0.46 +/- 0.03	0.42 +/- 0.03
UDPG (LC-MS/MS)	3.40 +/- 0.07	3.85 +/- 0.03	3.82 +/- 0.09	3.10 +/- 0.03
Metabolites of the Pentose Phosphate Pathway				
6PG (GC-MS)	0.6 +/- 0.01	0.9 +/- 0.05	1.42 +/- 0.04	1.46 +/- 0.1

Rul5P (GC-MS)	0.269 +/- 0.004	0.278 +/- 0.010	0.496 +/- 0.003	0.281 +/- 0.005
Rib5P (GC-MS)	0.53 +/- 0.01	0.58 +/- 0.01	0.78 +/- 0.01	0.59 +/- 0.02
Xyl5P (GC-MS)	0.57 +/- 0.01	0.61 +/- 0.02	1.08 +/- 0.01	0.64 +/- 0.01
E4P (GC-MS)	0.0138 +/- 0.0003	0.0101 +/- 0.0007	0.0116 +/- 0.0002	0.0085 +/- 0.0003
Sed7P (GC-MS)	5.50 +/- 0.05	3.23 +/- 0.07	5.99 +/- 0.03	2.32 +/- 0.07
Metabolites of the TCA cycle				
CIT (GC-MS)	11.67 +/- 0.31	6.96 +/- 0.15	6.49 +/- 0.05	5.76 +/- 0.10
isoCIT (GC-MS)	0.31 +/- 0.01	0.40 +/- 0.02	0.43 +/- 0.01	0.17 +/- 0.01
Glyoxylate	*Not available*	*Not available*	*Not available*	*Not available*
AKG (GC-MS)	2.61 +/- 0.02	1.82 +/- 0.04	3.35 +/- 0.03	2.31 +/- 0.06
SUCC (GC-MS)	1.27 +/- 0.14	0.96 +/- 0.01	3.17 +/- 0.12	6.55 +/- 0.07
FUM (GC-MS)	0.57 +/- 0.03	0.67 +/- 0.01	1.21 +/- 0.01	0.70 +/- 0.02
MAL (GC-MS)	2.31 +/- 0.18	3.01 +/- 0.05	4.89 +/- 0.06	2.63 +/- 0.05
Amino Acids				
Ala (GC-MS)	39.89 +/- 0.85	36.67 +/- 0.96	64.79 +/- 0.41	21.31 +/- 0.68
Gly (GC-MS)	4.60 +/- 0.09	1.29 +/- 0.03	2.31 +/- 0.03	4.71 +/- 0.94
Val (GC-MS)	11.50 +/- 0.33	15.53 +/- 0.12	28.46 +/- 0.26	13.72 +/- 0.22
Leu (GC-MS)	1.35 +/- 0.05	0.52 +/- 0.01	0.59 +/- 0.01	0.64 +/- 0.08
Ile (GC-MS)	2.44 +/- 0.07	1.77 +/- 0.02	2.74+/- 0.01	1.74 +/- 0.13
Pro (GC-MS)	5.42 +/- 0.10	1.75 +/- 0.03	2.58 +/- 0.05	1.96 +/- 0.10
Ser (GC-MS)	3.73 +/- 0.09	1.37 +/- 0.01	2.25 +/- 0.04	4.28 +/- 0.81
Thr (GC-MS)	4.27 +/- 0.08	4.48 +/- 0.03	9.27 +/- 0.03	13.25 +/- 0.34
Meth (GC-MS)	0.262 +/- 0.006	0.160 +/- 0.001	0.256 +/- 0.004	0.240 +/- 0.011
Asp (GC-MS)	13.82 +/- 0.16	20.12 +/- 0.36	31.74 +/- 0.38	36.82 +/- 0.53
Orn (GC-MS)	5.69 +/- 0.22	1.63 +/- 0.05	1.09 +/- 0.01	1.94 +/- 0.10
Phe (GC-MS)	0.90 +/- 0.02	0.62 +/- 0.01	0.67 +/- 0.01	0.59 +/- 0.05
Cys (GC-MS)	0.23 +/- 0.01	0.36 +/- 0.01	0.26 +/- 0.01	0.29 +/- 0.01

Glu (GC-MS)	154.85 +/- 3.35	104.24 +/- 1.51	117.55 +/- 0.61	65.96 +/- 0.73
Lys (GC-MS)	10.40 +/- 0.24	4.90 +/- 0.08	8.43+/- 0.07	9.43 +/- 0.10
Asn (GC-MS)	7.18 +/- 0.14	3.47 +/- 0.06	3.83 +/- 0.03	2.75 +/- 0.02
Gln (GC-MS)	58.73 +/- 0.85	22.19 +/- 0.34	23.21 +/- 0.26	14.91 +/- 0.29
Tyr (GC-MS)	0.96 +/- 0.02	0.67 +/- 0.01	0.56 +/- 0.01	0.47 +/- 0.04
His (GC-MS)	3.53 +/- 0.08	4.49 +/- 0.07	2.61 +/- 0.01	3.33 +/- 0.05
Trp (GC-MS)	0.278 +/- 0.006	0.233 +/- 0.004	0.176 +/- 0.002	0.142 +/- 0.021
Others				
Acetyl-CoA (LC-MS/MS)	0.24 +/- 0.03	0.25 +/- 0.05	0.60 +/- 0.10	0.43 +/- 0.07
CoA (LC-MS/MS)	0.14 +/- 0.03	0.12 +/- 0.02	0.16 +/- 0.04	0.11 +/- 0.02
Cofactors				
NADH (LC-MS/MS)	0.33 +/- 0.11	0.47 +/- 0.05	0.46 +/- 0.03	0.59 +/- 0.06
NAD (LC-MS/MS)	2.10 +/- 0.25	1.83 +/- 0.20	1.72 +/- 0.23	1.62 +/- 0.30
NADPH (LC-MS/MS)	1.00 +/- 0.09	1.88 +/- 0.16	1.66 +/- 0.14	1.65 +/- 0.24
NADP (LC-MS/MS)	0.18 +/- 0.13	0.41 +/- 0.11	0.52 +/- 0.15	0.43 +/- 0.21
FAD (LC-MS/MS)	0.11 +/- 0.01	0.13 +/- 0.01	0.18 +/- 0.04	0.19 +/- 0.01
Nucleotides				
AMP (LC-MS/MS)	0.46 +/- 0.05	0.55 +/- 0.04	0.64 +/- 0.05	0.96 +/- 0.12
ADP (LC-MS/MS)	2.03 +/- 0.07	2.15 +/- 0.06	2.62 +/- 0.05	3.75 +/- 0.60
ATP (LC-MS/MS)	8.61 +/- 0.36	9.70 +/- 0.22	12.71 +/- 0.48	14.62 +/- 1.99
Sum (ATP+ADP+AMP)	11.10 +/- 0.48	12.40 +/- 0.32	15.97 +/- 0.58	19.33 +/- 2.71
UMP (LC-MS/MS)	0.18 +/- 0.01	0.11 +/- 0.02	0.13 +/- 0.03	0.35 +/- 0.05
UDP (LC-MS/MS)	0.63 +/- 0.01	0.74 +/- 0.03	1.07 +/- 0.06	1.53 +/- 0.36
UTP (LC-MS/MS)	2.19 +/- 0.13	1.84 +/- 0.06	3.18 +/- 0.08	4.30 +/- 0.83
Sum (UTP+UDP+UMP)	3.00 +/- 0.30	2.70 +/- 0.52	4.38 +/- 0.95	6.18 +/- 2.09

Steady-state concentrations of the intracellular metabolites at the different dilution rates were determined from three independent samples taken at different times during each steady state but after five residence times had been elapsed. Although the measured concentrations of the intracellular metabolites varied with the growth rate, some of them shared a common pattern. While metabolite concentrations of the upper glycolysis showed an increasing trend with the growth rate (e.g., G6P in Figure S2-1A), the concentration of metabolites in the lower glycolysis decreased (e.g. 3PG in Figure S2-1B). Two remarkable exceptions were observed, intracellular glucose in the upper glycolysis and pyruvate in the lower glycolysis. In the first case, the intracellular glucose decreased from about 9 μmol g_{DW}^{-1} at low growth rates to about 1.5 μmol g_{DW}^{-1} at high growth rates. On the other hand, pyruvate increased with the growth rate from about 2.2 μmol g_{DW}^{-1} at low growth rates to about 3.4 μmol g_{DW}^{-1} at high growth rates. Though concentrations alone may not be enough to draw a definite conclusion, they do suggest that these two pools may have been subject to an extra phenomena that make these metabolites to behave differently from other metabolites belonging to the same pathways.

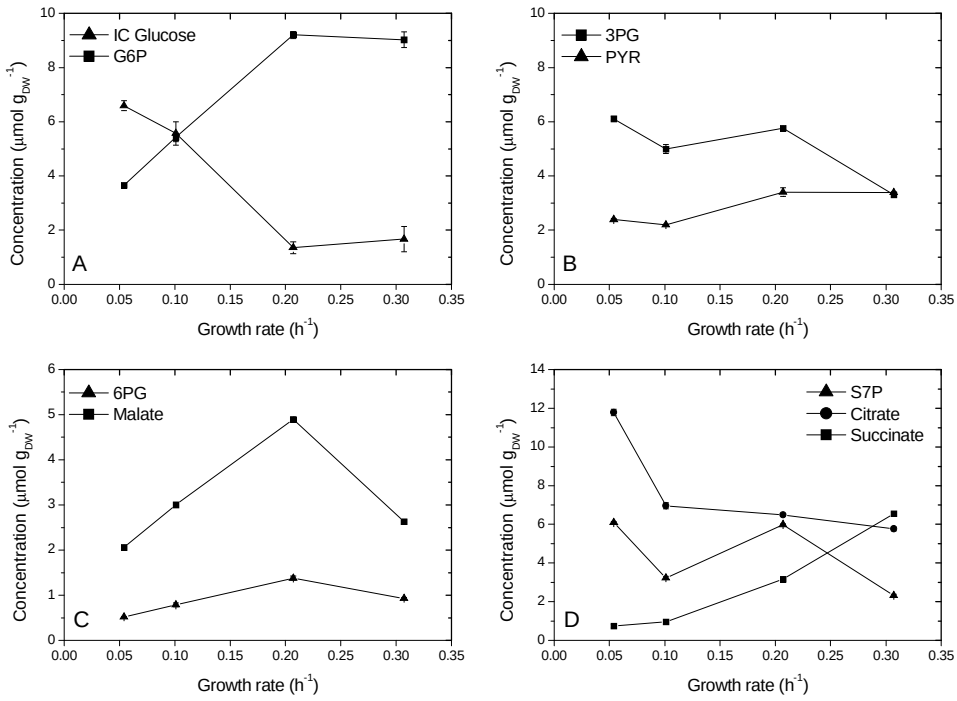

Figure S2-1. Concentration of selected metabolites vs Growth rate. A) and B) Metabolites of the upper (IC-glucose, G6P) and lower (3PG, PYR) glycolysis respectively; C) Metabolites of the PPP (6PG) and TCA (Malate); D) Metabolites whose behavior deviates from other metabolites of the same pathway. Data points are the average of three different samples at each particular growth rate and error bars indicate the standard error.

Metabolites of the PPP and TCA cycle shared a common behavior with the growth rate. In general, concentrations increased with the growth rate exhibiting a maximum at D = 0.207 h^{-1}, followed by a lower concentration at D = 0.307 h^{-1} (e.g., 6PG and malate in Figure S2-1C, respectively). S7P in PPP, and citrate and succinate in TCA, were the exceptions to the regular pattern (Figure S2-1D). In particular, succinate seemed to be a growth-related metabolite since its concentration steadily increased with the growth rate.

Quality check of the generated metabolite data

The reliability of the data can be assessed by: a) direct inspection of the standard deviation of the measurements; b) confronting the results with previously published data (e.g., Canelas et al 2008 for D = 0.1 h^{-1}); and c) evaluating the mass action ratios of some sound reactions. Although most of the results showed a relative deviation from the mean of less than 5% (Figure S2-2) some exceptions were observed. For instance, FBP and G1P exhibit relative deviations from the mean in the order of 10% while G3P up to 50%. A usual behavior that we have observed is that whenever the concentration of a particular pool (e.g., FBP) drops close to zero, the relative deviation becomes more significant with respect to the corresponding pool size.

Although the absolute values for the concentration of intracellular metabolites may differ from those obtained during similar experiments due to small differences in medium composition or dilution rate, it is still valid to check whether the data is consistent by comparing different datasets. We have confronted our results at D = 0.101 and 0.054 h^{-1} with those reported by other authors (Canelas et al., 2008; Mashego et al., 2007; Wiebe et al., 2008), having similar results for most of the metabolites for a similar D. As mentioned above, some of the small differences may be explained due to differences in medium composition (e.g., addition of ethanol in Canelas and Mashego experiments) and sample processing (mainly the method used for quenching and washing samples). The high reproducibility of the present measurements indicated by their standard errors as well as the mass action ratios between different metabolites, which we have found to be in agreement with the K_{eq}-values reported in the literature (Table S2-2), suggest that this set of data is consistent for all the dilution rates tested. Moreover, it can be assumed that these values represent the *in-vivo* K_{eq} considering that the Q-values were nearly constant, while the fluxes changed at least six-fold (due to a six-fold change in the growth rate). Note that RPI at D=0.207 h^{-1} was an exception.

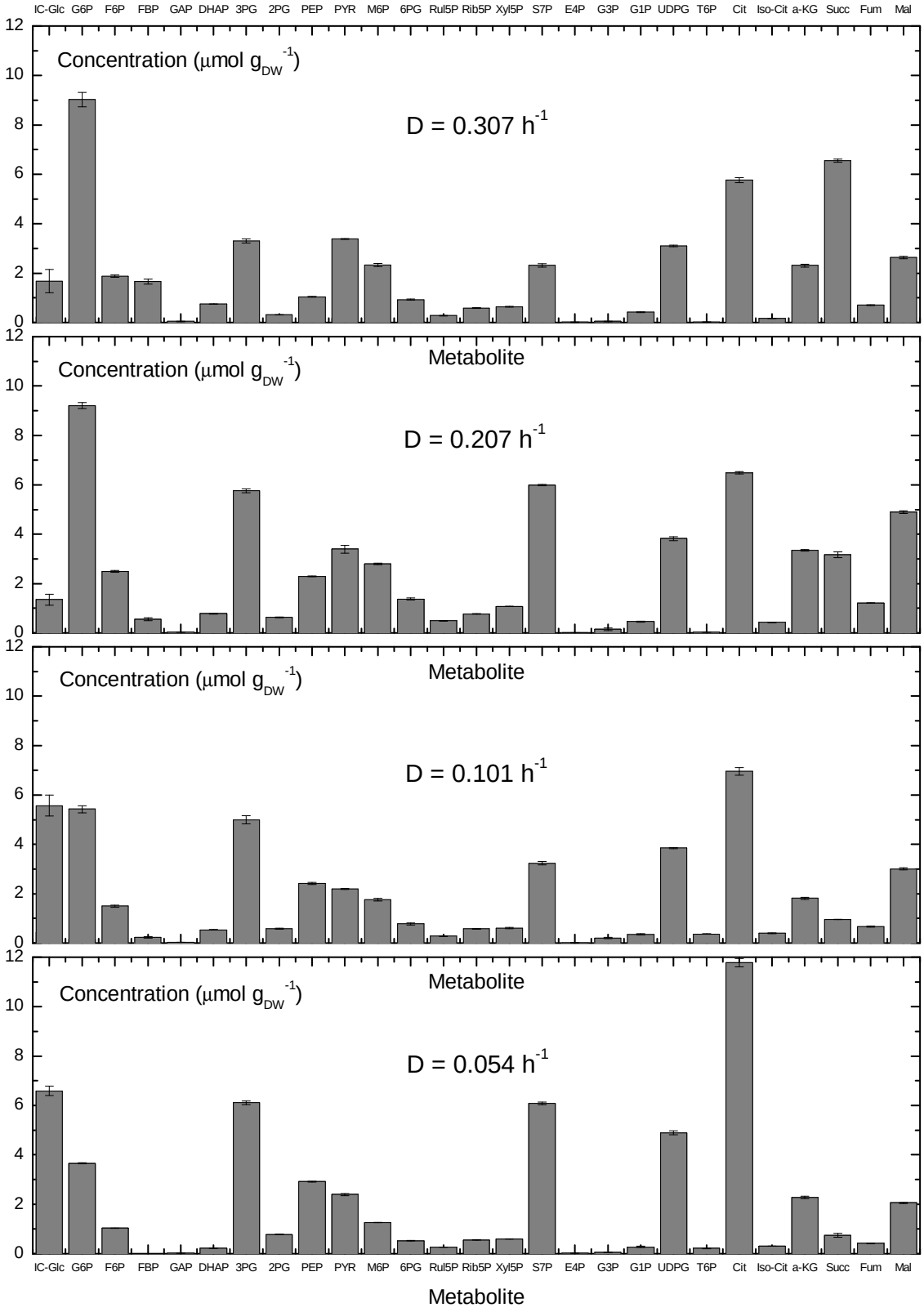

Figure S2-2. Comparison of concentrations of selected metabolites for the four dilution rates tested. Error bars indicate the standard error of measurements from 3 different samples

The suitability of the setup used in this study for generating realistic *in vivo* metabolite data is also supported by the energy status of the microorganisms at the different dilution rates. Thus, we found that the obtained values for the adenylate pools (ATP, ADP and AMP), energy charge and the adenylate kinase mass action ratio (Table S2-3) were in agreement with those previously reported (Atkinson, 1968; Canelas et al., 2008; Mashego et al., 2007). This also indicates that our approach (several dilution rates in one experiment) was comparable to single dilution rate chemostats and therefore suitable for generating consistent data in order to perform flux estimations.

Table S2-2. Estimated mass action ratios of selected reactions

Reaction	**μ= 0.054 h^{-1}**	**μ= 0.101 h^{-1}**	**μ= 0.207 h^{-1}**	**μ= 0.307 h^{-1}**	**K_{eq} Literature**
PGI: F6P/G6P	0.262 +/- 0.006	0.277 +/- 0.011	0.272 +/- 0.005	0.209 +/- 0.009	0.259 +/- 0.003[a]
GPM: 2PG/3PG	0.124 +/- 0.002	0.118 +/- 0.005	0.110 +/- 0.003	0.095 +/- 0.003	0.1158 +/- 0.0008[a]
ENO: PEP/2PG	3.522 +/- 0.046	4.115 +/- 0.014	3.631 +/- 0.090	3.304 +/- 0.069	4.01 +/- 0.09[a]
PGM: G1P/G6P	0.057 +/- 0.002	0.066 +/- 0.006	0.050 +/- 0.003	0.046 +/- 0.003	0.063 +/- 0.003[a]
PMI: M6P/F6P	1.307 +/- 0.028	1.174 +/- 0.050	1.120 +/- 0.020	1.236 +/- 0.048	1.18 +/- 0.01[a]
FMH: Mal/Fum	4.080 +/- 0.384	4.466 +/- 0.108	4.028 +/- 0.057	3.750 +/- 0.126	4.3 +/- 0.7[b]
RPI: Rib5P/Rul5P	1.968 +/- 0.054	2.080 +/- 0.084	1.570 +/- 0.025	2.108 +/- 0.072	2.1 +/- 0.1[a]

a) Canelas et al. (2011); b) Goldberg et al. (2004)

Table S2-3. Adenylate system in *S. cerevisiae* at different dilution rates

Dilution rate (h^{-1})	**0.054**	**0.101**	**0.207**	**0.307**
ATP (μmol g_{DW}^{-1})	8.61 +/- 0.36	9.70 +/- 0.22	12.71 +/- 0.48	14.62 +/- 1.99
ADP (μmol g_{DW}^{-1})	2.03 +/- 0.07	2.15 +/- 0.06	2.62 +/- 0.05	3.75 +/- 0.60
AMP (μmol g_{DW}^{-1})	0.46 +/- 0.05	0.55 +/- 0.04	0.64 +/- 0.05	0.96 +/- 0.12
Total AXPs (μmol g_{DW}^{-1})	11.1 +/- 0.48	12.40 +/- 0.32	15.97 +/- 0.58	19.33 +/- 2.71
Energy Charge	0.87	0.87	0.88	0.85
Adenylate kinase	1.04	0.87	0.84	1.00

References

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