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Large-Scale, Continuous-Flow Production of Stressed Biomass (*Desulfovibrio vulgaris* Hildenborough)

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VIMSS Virtual Institute for Microbial Stress and Survival

http://vimss.lbl.gov/

Abstract

The Protein Complex Analysis Project (PCAP, http://pcap.lbl.gov/), focuses on high-throughput analysis of microbial protein complexes in the anaerobic, sulfate-reducing organism, *Desulfovibrio vulgaris* Hildenborough (DvH). Interest in DvH as a model organism for bioremediation of contaminated groundwater sites arises from its ability to reduce heavy metals. *D. vulgaris* has been isolated from contaminated groundwater of sites in the DOE complex. To understand the effect of environmental changes on the organism, midlog-phase cultures are exposed to nitrate and salt stresses (at the minimum inhibitory concentration, which reduces growth rates by 50%), and compared to controls of cultures at midlog and stationary phases. Large volumes of culture of consistent quality (up to 100 liters) are needed because of the relatively low cell density of DvH cultures (one order of magnitude lower than *E. coli*, for example) and PCAP's challenge to characterize low-abundance membrane proteins.

Cultures are grown in continuous flow stirred tank reactors (CFSTRs) to produce consistent cell densities. Stressor is added to the outflow from the CFSTR, and the mixture is pumped through a plug flow reactor (PFR), to provide a stress exposure time of 2 hours. Effluent is chilled and held in large carboys until it is centrifuged. A variety of analyses – including metabolites, total proteins, cell density and phospholipid fatty-acids – track culture consistency within a production run, and differences due to stress exposure and growth phase for the different conditions used. With our system we are able to produce the requisite 100 L of culture for a given condition within a week.

Biomass Production Flow

Media
- Defined 60mM lactate/50 mM sulfate medium
- Maintained anaerobic with N2 blanket

CFSTR
- **Continuous flow stirred tank reactors**, in parallel, turbidostat mode.
- Continuously sparged w/ N2
- redox potential -600 mV, pH 7.5, 30 deg C

GLS
- **Gas/Liquid Separator**
- Releases entrained gas before flow to PFR
- Stressor mixing (delivered by peristaltic pump)
- Fraction of flow wasted to ensure vessel does not drain

PFR
- **Plug Flow Reactor**, minimal dispersion
- 2 hour exposure time for stressor

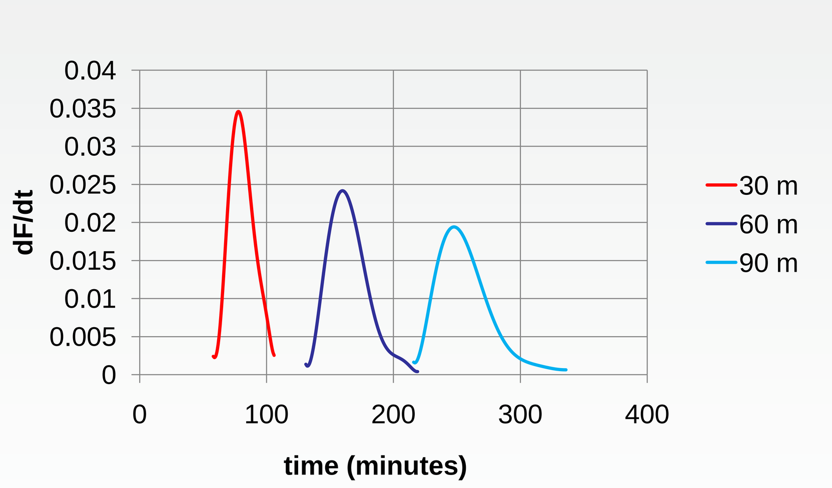
Chiller
- Broth flows through ice bath to chill to 4 deg C
- Chilled carboy holds chilled broth until batch centrifugation

At the end of the run, flow to PFR is stopped and stressor is added to the CFSTR. The CFSTR is harvested through the chiller after two hours.

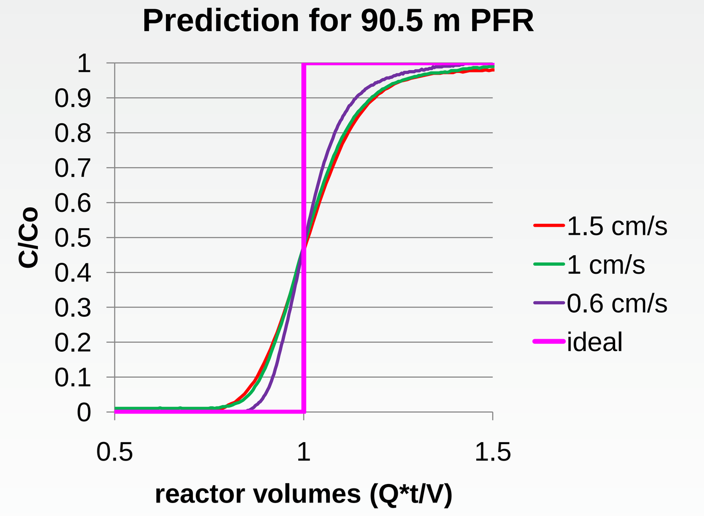
Characterization of Dispersion in Plug Flow Reactor (PFR)

- Want biomass to have constant exposure (2 hrs) to stressor during continuous flow operation.
- Need to assess dispersion, i.e., deviation from mean residence time in PFR
- Tracer tests performed with 3 mg/L methylene blue dye, step-input using ¼" ID polypropylene tubing of 3 lengths
- Analysis of the RTD was performed using methods from Dankwerts (1953) and Imhoff (2009)

Residence Time Distributions



Prediction for 90.5 m PFR



Results:
- Gaussian residence time distribution (RTD) curves
  - 68% of the mass has exposure time of 1.8-2.2 hours
  - 95% of the mass has exposure time of 1.6-2.4 hours.

Summary of Production Conditions

Condition	CFSTR Dilution rate (1/hr)	PFR material and ID	PFR length (m)	PFR Flow velocity (cm/s)	Residence time (hrs)	Notes
control (midlog phase)	0.12 ± 0.03	tygon 1/4"	76 & 87	1.3	2.4-2.5	1,3,4, 5
stationary phase	0.06 ± .007	teflon 3/16"	81 & 112	0.8-1.5	2.0-3.7	2,3,4, 5
96 mM NaNO3	0.10 ± .001	teflon 3/16"	112	0.5-1.5	2.0-5.5	5
265 mM NaCl	0.11 ± .005	teflon 3/16"	112	1.7-1.9	1.6-1.8	6
390 mM NaCl	0.10 ± 0.02	tygon 1/4"	30 & 61	0.5-1.1	1.9-2.9	3,4,5

Notes: Teflon PFA is gas impermeable, tygon has very low gas permeability. 1. Pure water used as "blank stressor"; 2. 3 reactors run in parallel; 3. Gas was entrained in PFR; 4. PFR lengths were changed during run because of one reactor going off-line; 5. Media delivery tube was submerged below liquid level in reactor – this resulted in more lactate consumption by biofilm growth on submerged portion of tube; 6. Media delivery tube dripped into reactor.

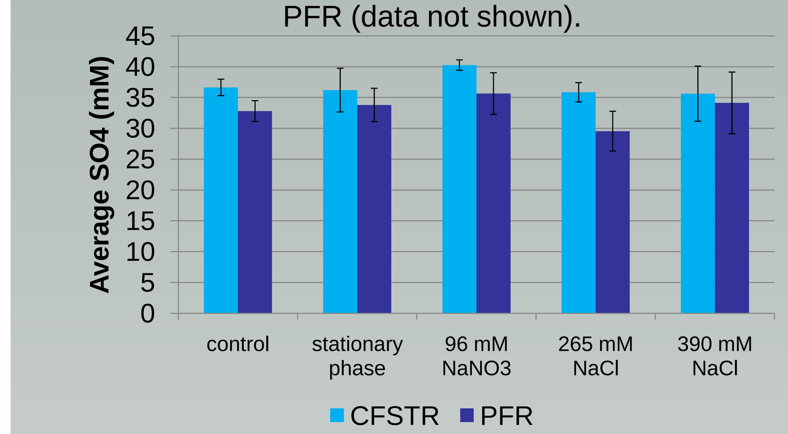
Results of Biomass Monitoring

Observations of heterogeneous biomass distribution in PFR:

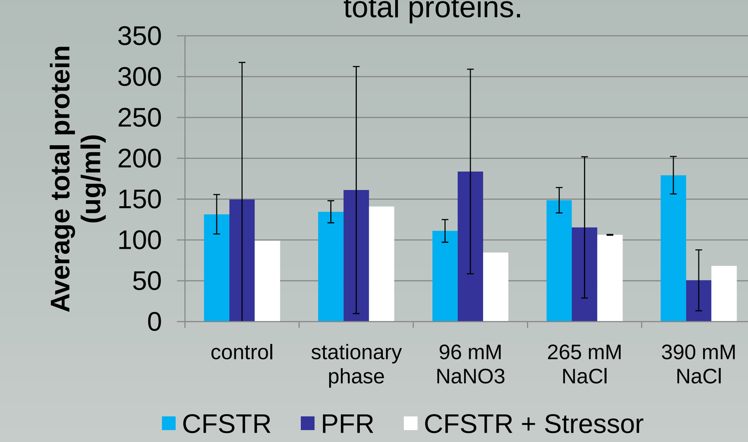
- Small (1 mm diameter) light brown biomass clumps in first section of PFR, clumps grew in size as they traveled through PFR, and were up to 5 mm in diameter, black in color when exiting the PFR.
- Some settling of biomass occurred on the bottom of a few sections of the horizontal runs of tubing, probably when flow was stopped.
- Trapped gas bubbles in the PFR resulted in some biomass settling and some enhanced mixing as biomass flowed around the bubbles.

Sulfate and Total Protein Concentrations

Some sulfate reduction occurs in PFR, with the smallest reduction occurring with 390 mM NaCl. Most of the lactate was consumed in CFSTR before flow to PFR (data not shown).

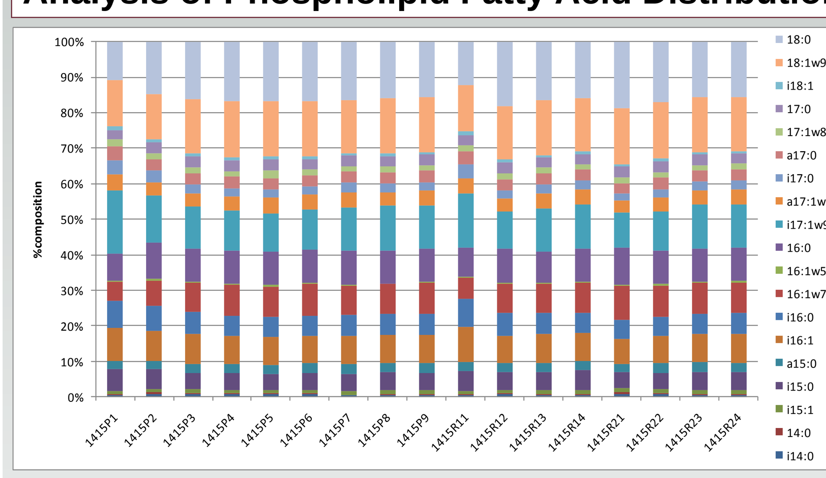


Large variability in protein concentrations due to heterogeneous biomass distribution in PFR. Averages indicate that NaCl stressor had the greatest effect on total proteins.



Sulfate concentrations are measured by ion chromatography (Dionex ICS 2000). Micro BCA protein assay is used for total proteins (Pierce Biotechnology). Values shown are the average of samples taken over the duration of the 5-day operation for each condition (CFSTR samples 1x/day, PFR samples 2x/day). Once flow to the PFR is stopped, stressor is added to the CFSTR and "CFSTR + stressor" samples are taken two hours later. "CFSTR + stressor" samples are from either one reactor, or averaged from two reactors.

Analysis of Phospholipid Fatty Acid Distributions

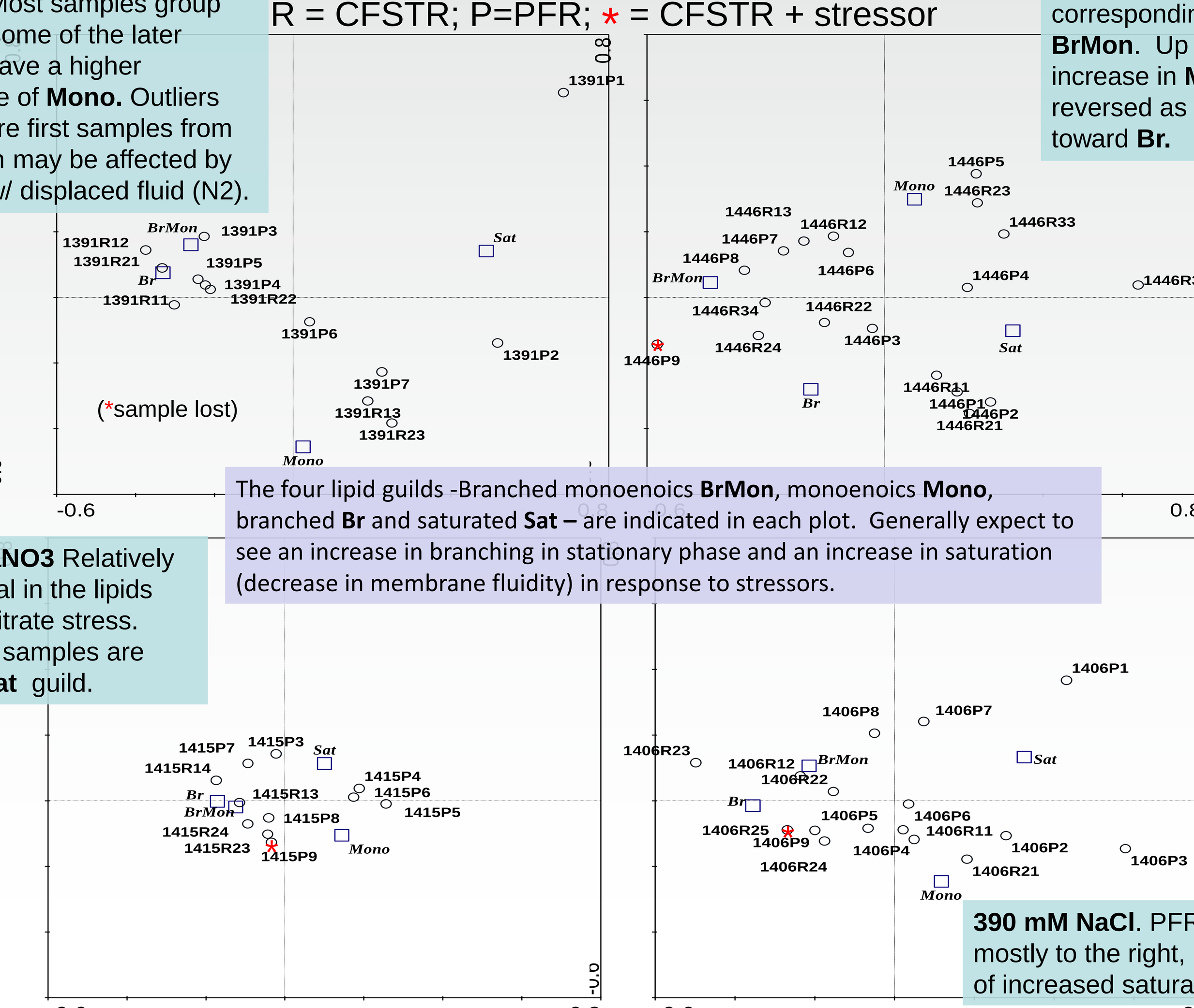


Lipid distribution, 96 mM NaNO3

Analysis: Percent composition data is either analyzed directly using Correspondance Analysis (CA) or combined with supporting environmental data to produce ordination diagrams (Canonical Correspondance Analysis, CCA). The proximity of the samples to each other on the diagrams is related to their similarity in lipid composition.

Correspondance Analysis

R = CFSTR; P=PFR; ★ = CFSTR + stressor



Control Most samples group together, some of the later samples have a higher percentage of **Mono.** Outliers P1 & P2 are first samples from PFR which may be affected by interface w/ displaced fluid (N2).

(*sample lost)

stationary phase. PFR samples generally move from right to left – corresponding to an increase in **BrMon**. Up until P5 there is an increase in **Mono** but this trend is reversed as it trends downward toward **Br.**

The four lipid guilds -Branched monoenoics **BrMon**, monoenoics **Mono**, branched **Br** and saturated **Sat** – are indicated in each plot. Generally expect to see an increase in branching in stationary phase and an increase in saturation (decrease in membrane fluidity) in response to stressors.

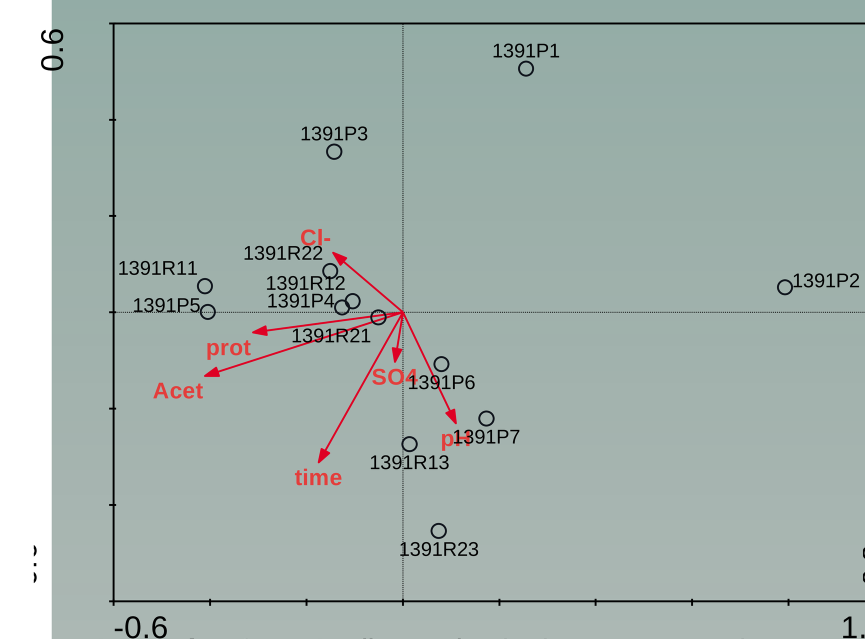
96 mM NaNO3 Relatively small signal in the lipids from the nitrate stress. Most PFR samples are close to **Sat** guild.

390 mM NaCl. PFR samples mostly to the right, in the direction of increased saturated lipids.

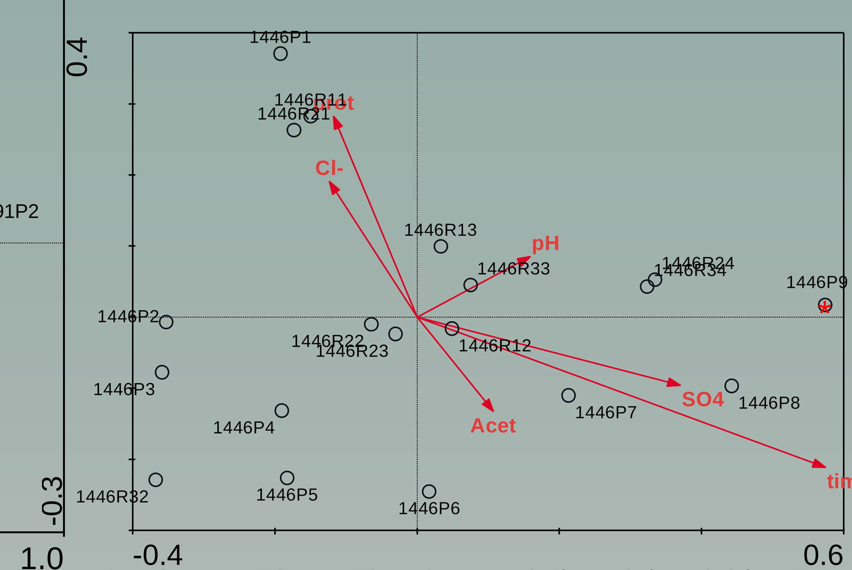
Canonical Correspondance Analysis

Percent composition data is combined with supporting environmental data to produce ordination diagrams. The proximity of the samples to each other on the diagrams is related to their similarity in lipid composition. Angles between environmental variables determine their relative correlation and variables increase in the direction of the arrows.

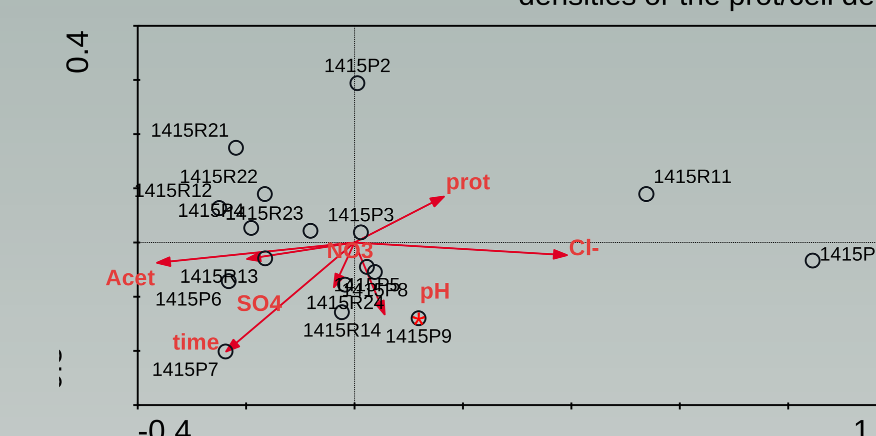
R = CFSTR; P=PFR; ★ = CFSTR + stressor



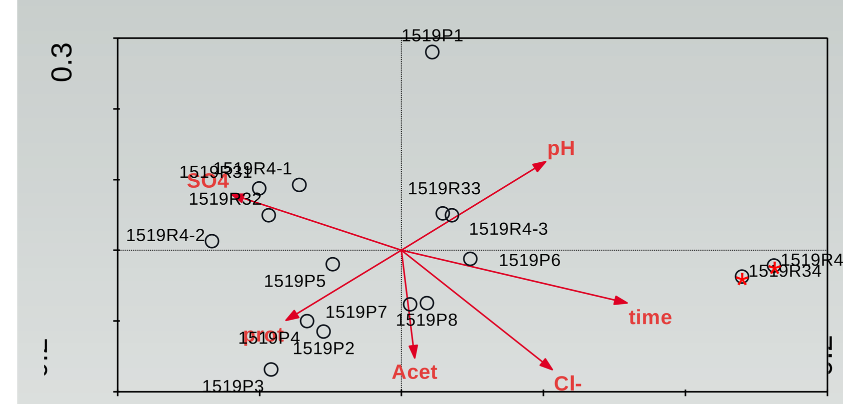
Control. After P3, all samples in the Reactor and PFR are similar in lipid composition and values of the variables. (*sample lost)



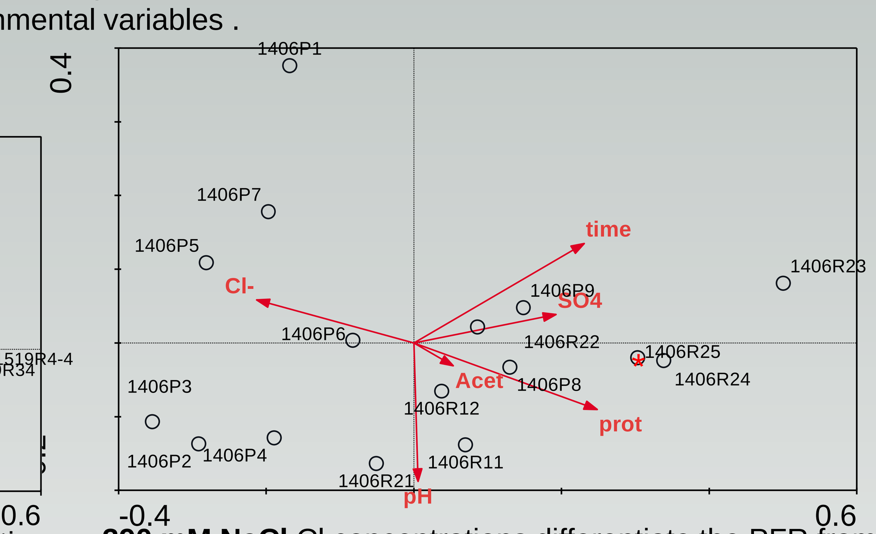
Stationary Phase Time is most influential variable. Protein varies in almost the opposite direction, implying that the cell densities or the prot/cell decreases over time.



96 mM NaNO3 The effect of NO3 on the lipids is small, and is much less influential than the effects of the other environmental variables .



265 mM NaCl Cl concentrations influences the distribution but not as strongly as 390mM NaCl (to the right). Outliers ★ experienced operational disturbance.



390 mM NaCl Cl concentrations differentiate the PFR from Reactor. The reactor samples also have increased protein relative to the PFR samples.

Generally, P1 is an outlier, perhaps affected by interface with displaced fluid (N2)

Conclusions

- We have designed a continuous flow system to provide controlled stress-exposure to DvH, consisting of one or multiple CFSTRs that produce steady-state log-phase culture, stress-addition to the effluent, and flow through a PFR to provide a defined stress exposure-time to the biomass. This system can produce up to 100 L in one week, which is a much higher production rate than possible in batch mode.
- Flow velocities in the PFR produce near-ideal plug-flow of solutes. Visual observation indicates that the small biomass flocs in the initial portion of the PFR move with the flow. These flocs grow in size and always as they move through the PFR, however they do not settle unless operational disturbances occur. Change in flow velocity (or intermittent flow cessation) due to CFSTR performance problems. Change in flow velocity also affects the residence time of the broth in the PFR, and hence stress-exposure time.
- The absence of mixing in the PFR produces a heterogeneous distribution of biomass, which is seen in highly variable total protein concentrations, as well as cell density and optical density values (data not shown here) in the PFR effluent.
- The effect of stress is not strongly evident in the biomass monitoring data, however, total protein concentrations were reduced by the addition of NaCl stressor – this reduction increased with higher NaCl concentration.
- PLFA was tested as a monitoring tool of biomass consistency in the PFR because lipid fractions are independent of biomass concentration. Correspondance Analysis of PLFA data do not indicate a significant effect of the PFR on biomass, as compared to biomass grown in the CFSTR. The lipid distributions can be very sensitive to operational disturbances.
- Incorporating environmental data (total proteins, metabolite concentrations, stressor concentrations) into the correspondance analysis of the PLFA data more clearly separates CFSTR and PFR samples. In the case of the NaCl stressor, lipid composition is correlated with NaCl and protein concentrations. NaNO3 stressor did appear to affect lipid composition.

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