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Development and Validation of a Customizable Chemically Defined Medium for Soil Microbial Community Growth in Serial Batch Culture Studies

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Abstract

A customizable chemically defined aqueous medium (CDM) paradigm was developed for the study of microbe - metal interactions. Metal speciation in the medium was predicted and controlled using MINEQL+, a chemical speciation program. The medium was optimized for the growth of a soil microbial community from an agricultural farm soil (AD) in central New Jersey. The combination of minimal amounts of basal salts, trace micronutrients, growth factors and carbon source was prepared with the following constraints: the metal (and chemical) speciation was known and controlled; the medium was buffered to a target pH of 6.2; and bioavailable metal (of interest) concentrations were maintained constant throughout growth. The medium design consisted of 17 individual components with dextrose as the carbon source. CDM was used successfully in the growth of mixed soil microbial communities under dextrose limiting conditions from an agricultural soil. Growth parameters of CDM were compared to those in a traditional soil extract medium (SES) as a control. These CDM culture growth data were comparable (if not better than) to results observed with the same inoculum grown in SES. The AD culture grown in CDM had apparent specific growth rates of up to 0.30 ± 0.07 h^{-1} with the corresponding doubling time averaging 2.3 h. Maximum specific growth rates of 0.4 ± 0.09 h^{-1} and maximum cell densities of 10^8 - 10^9 per ml were routinely obtained. Community metabolic profiles (BIOLOG) and bacterial community denaturing gradient gel electrophoresis (DGGE) profiles of populations from CDM and SES showed that the defined medium provided for development of comparably or more active and complex microbial communities as did the less selective SES. The successful growth of a competent soil microbial community demonstrating comparable biokinetic parameters is indicative of the utility of the customizable medium paradigm to the creation of a chemically defined medium for more accurate microbial assessments of metal interactions with soil microbial communities at fully defined metal concentrations.

Keywords: *Bioavailable; Defined Medium; Metal Bioavailability; MINEQL+; Respirometry; Metal Speciation; Chemical Speciation Program; Respirometry*

Abbreviations

CDM: Chemically Defined Medium; SES: Soil Extract Medium; DGGE: Denaturing Gradient Gel Electrophoresis; EDTA: Ethylenediaminetetraacetic Acid; SC: Seed Culture; SEP: Sequential Extraction Procedure; SBLC: Serial Batch Liquid Culture

Introduction

Despite the general recognition that a "total" metal concentration measurement cannot provide a meaningful reflection of the metal's influence on soil bacteria, a considerable amount of the scientific literature describes these effects with respect to total metal concentrations [1,2]. Although the total concentration of metals in soil may give some indication of the degree of contamination, alongside the fact that there are reports claiming that even bound metals may be available and thus exert an effect on bacterial activity [3], a total metal concentration gives no indication to, what Semple., *et al.* [4] terms, bio accessibility or the potential of microbial exposure to the metal (bioavailability). Total metal concentration necessarily includes all water soluble (available) and insoluble (nonavailable) metal states resulting from the interaction with the myriad of soil components and metal ligands. Added complexity results from the heterogeneous distribution of autochthonous and allochthonous compounds and components present in soil in various physical phases and all this contributes to a difficulty, as recognized by Boivin., *et al.* [5] in determining actual bacterial exposure to metals. As such, the quantity of total metal loading provides little insight into the behavior and bioavailability of metals to the soil microbial community [6] and gives no information on the chemical reactivity of the different forms of the metal [1,7]. In short, the only critical metal factor that results in a biological response is the concentration of the bioavailable metal fraction, which is generally proportional to the concentration of the free metal ion, $[M^{z+}]_{free}$ [8].

With the understanding that a determination of the soluble metal fraction is a critical factor in microbial activity, it would be, as Farrell., *et al.* [9] suggested, unrealistic to attempt to make a quantitative determination of all metal-ligand species in soil samples a part of a routine soil metal examination. Furthermore, given the heterogeneity of soil and the fact that chemical composition of the soil solution is dependent on the local (microsite) properties of the soil matrix, total soil measures of available metal likely would not be indicative of microsite impacts on microbial function [10]. Thus it is difficult to make generalizations about soil microbial community activities from one soil microsite to the next, where the amount of bioavailable metal in the soil solution may be dramatically different.

Kim., *et al.* notes that for several decades, efforts to examine the physico-chemical forms of various soil elements have been made to better understand metal bioavailability through chemical extraction methods, for example, sequential extraction procedures (SEP) [11]. In practice, such arduous and time-consuming procedures likely do not represent actual bioavailability, in part because of the dynamics and overall complexity in the biological system [1,12]. While some sequential extraction procedures have gained wide acceptance [13], there must be recognition that SEPs do not provide a direct characterization of metal speciation but rather indicates chemical reactivity [14]. Although extractable metals are not an accurate measure of free or bioavailable metal concentrations, the determination and recognition of this fraction of metals is a dramatic improvement over the use of total metal concentrations [15]. However, theoretical difficulties notwithstanding, there remains the more fundamental debate as to the utility of the procedure. For example, as reported by Kabala and Singh and others [16-18], there are formidable concerns regarding the non-selectivity of the reagents and the fact that extraction procedures themselves may redistribute elements between assay-defined fractions. Therefore, even the best attempts to link bacterial response to specific bioavailable metal concentrations using undefined media should be expected to produce questionable results.

Although defined media exist for the growth of specific organisms, generally such media have not been available for study of metal toxicity on interacting soil microbial consortia, especially where comparative kinetic determinations are important. Here we present a promising approach towards the elucidation of the relationship between expressed microbial function in soil and bioavailable metal concentration. Our approach was consistent with the methodology described by Twiss., *et al.* where the critical importance of exposure media in bioavailability and toxicity studies was recognized; that is, the composition of the media must be known "as precisely and accurately as possible". We developed a defined medium that combines a minimum of components that supported predictable growth rates and cell yields for soil microbial communities. Specifically, more traditional concepts of microbial growth media requirements are combined with current chemical speciation programs to produce a defined medium in which the chemical form and concentration of all components are known. This medium design paradigm is not unique, with the artificial algal media, Aquil [19] being a prime example.

Thus, the objective of this work was to develop a chemically defined medium that allows the maintenance of a known and constant concentration of "free" metal (throughout an assay culture run), permits the manipulation of target free metal concentration between tests, and is buffered to maintain a stable pH. Additionally, the medium should be clear and free of precipitants to allow for optical density (OD) measurements, which can be important since many researchers applying defined media for pure cultures tend to generate growth curves from OD measurements [20-22].

The availability of computer-based speciation programs eases the burdensome (iterative forward) calculations required by even the most minimally defined media, where multiple metal and ligand combinations present complicated interactions. Using one of many Windows-based chemical speciation programs, such as MINTEQ, MINEQL+, and others [23,24], this functionality renders the medium as easily customizable. A researcher with only basic computer skills can easily utilize this newly developed defined medium as a platform for examining microbial response to a number of metals at a user-defined metal concentration. To be as applicable as possible, we used the most widely used chemical equilibrium modeling software [23], MINEQL+ (Ver 4.5; Environmental Research Software, Hallowell, ME, USA).

Materials and Methods

Chemicals

All chemicals utilized were of analytical grade and the highest quality available from Fisher Scientific Company (Pittsburgh, PA) and Sigma Aldrich Chemicals Company (St. Louis, MO).

Medium composition

SES

Soil samples (from a Freehold sandy loam agricultural farm soil in Adelphia, NJ) were extracted by shaking 20 g of soil with 100 ml 0.1 M Tris buffer (pH 7.5) for 10 min. The resultant slurry was centrifuged for 10 min. at 2600 g. The supernatant liquid was removed and autoclaved to produce a sterile soil extract medium.

CDM

The defined medium composition was based on a minimal basal salt recipe plus a carbon source and pH buffer. Essential metals (Fe, Mn, Zn, Co, Se, and Cu) were added with a chelator, ethylenediaminetetraacetic acid (EDTA). The EDTA was used as a metal buffering agent to ensure the constancy of soluble metal concentrations. EDTA also served to minimize effects from unaccounted for metals (contaminants or those metals not captured by the ion exchange treatment). pH buffering was provided by Bis-Tris, instead of phosphate, to avoid metal phosphate precipitates.

To achieve the goal of minimizing trace element and nutrient components while still allowing unrestricted growth, Excel-based spreadsheet calculations were performed to ascertain the necessary medium nutrient concentrations. The spreadsheet was constructed using reference data [25] and inserting anticipated culture growth yields and expected final biomass concentrations. Nitrogen and phosphorus requirements were adjusted by applying known K_S values, 1.0 and 0.2, respectively [25] and developing a Monod relationship between specific growth rate (μ) and substrate concentration (S) to define the nutrient concentration and its effect on the growth rate (Figure 1).

To preclude the possibility of these nutrients from becoming growth limiting for the duration of substrate availability, the minimal nutrient concentrations exceeded the minimum metabolic requirements (as determined experimentally) throughout the growth curve. This ensured that any inhibition to growth resulted from toxicity or substrate depletion rather than nutrient deficiency.

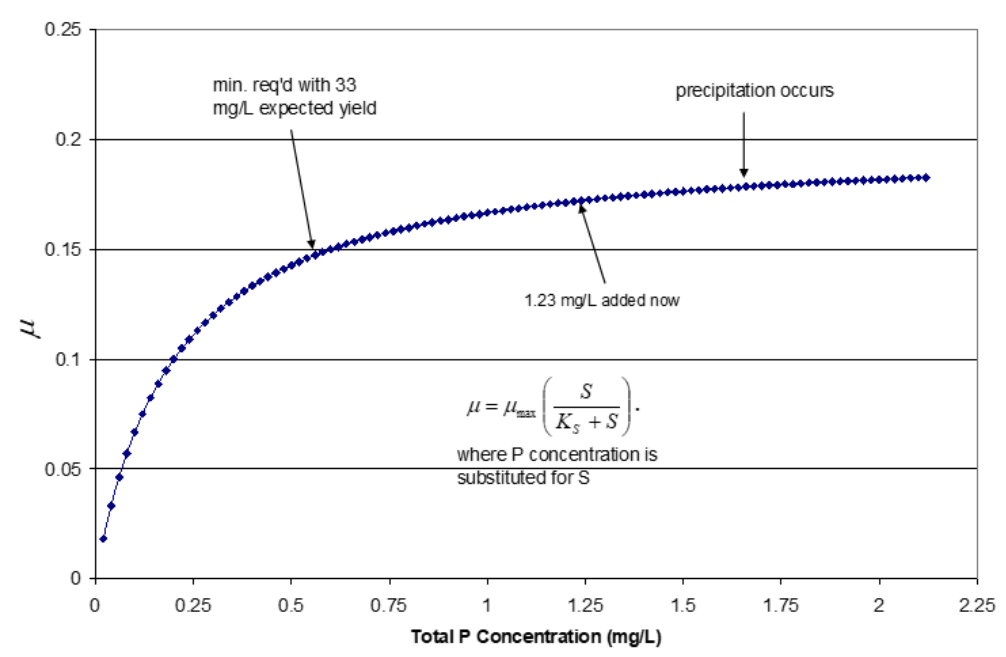

Figure 1: *The effect of N and P concentrations on specific growth rate (μ). (Assuming 0.20 h^{-1} maximum specific growth rate).*

The quantities of trace elements were based on the use of MINEQL+ Version 4.5 [26] by constraining "free" metal concentrations at a target value (e.g., 10^{-13} M copper for the base CDM) and providing total EDTA and salt concentrations. Liquid stock solutions were made from each component group as shown in Table 1.

Component	Stock Solution Concentration [g $liter^{-1}$]	Final Medium Concentration [μM] (unless otherwise specified)
Base medium		
Bis-TRIS	83.68	0.1M
$MgSO_4$	0.394	0.01
$CaCl_2 \cdot 6H_2O$	1.176	2000.0
KCl	0.596	1.0
$(NH_4)_2SO_4$	16.91	579.9
$Na_2HPO_4 \cdot H_2O$	1.340	25.0
$Na_2EDTA \cdot H_2O$	37.224	0.01
Trace elements		
$FeCl_3 \cdot 6H_2O$	4.876	18.0
$MnSO_4 \cdot H_2O$	0.443	2.24
$CuSO_4 \cdot 5H_2O$	2.721	10.9
$ZnSO_4 \cdot 7H_2O$	2.875†	0.01

SeO_4	1.0 ‡	9.05 nM
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	1.01‡	1.61 nM
$CoCl_2\cdot 6H_2O$	0.585§	2.5 nM
Vitamins		
Vitamin B_{12}	0.5¶	0.00037
Biotin	0.005¶	0.002
Thiamine-HCl	0.100¶	0.3
Carbon source		
Dextrose	6.25	0.052%

Table 1: *Composition of the chemically defined medium (CDM).*

† A $ZnSO_4$ stock solution (10^6X) was made by adding this amount to a 1liter polycarbonate volumetric and bringing to volume with 0.1N Trace Metal Clean HCl (deionized and Millipore filtered water) followed by a 1000 ml dilution.

‡ A SeO_4/ $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ stock solution (10^9X) was made by adding this amount to a 1liter polycarbonate volumetric and bringing to volume with deionized and Millipore filtered water followed by two 1/1000 ml serial dilutions.

§ $CoCl_2\cdot 6H_2O$ stock solution (10^9X) was made by adding this amount to a 1liter polycarbonate volumetric and bringing to volume with deionized and Millipore filtered water followed by two 1/1000 ml serial dilutions.

¶ A Vitamin B_{12}/Biotin (10^6X)/Thiamine HCl (10^3X) stock solution was made by adding the above amounts of Vitamin B_{12} and Biotin to a 1 liter polycarbonate volumetric and bringing to volume with deionized and Millipore filtered water. 1ml of this solution was transferred to a 1liter polycarbonate volumetric
and adding the above amount of Thiamine HCl and brought to volume with deionized and Millipore filtered water.

For components utilized in the medium, constants contained in the MINEQL+ thermodynamic database were reviewed against the National Institute of Standards and Technology (NIST) database [27] and any necessary modifications were made (after correcting to zero for ionic strength) for CuEDTA and CuHEDTA (log K = 20.54 and log K = 24.08, respectively [28]. Since some compounds were not in the MINEQL+ thermodynamic database, (e.g. Bis-Tris) using NIST database information, necessary equilibrium constants were added (all corrected for ionic strength and temperature).

Preparation of Labware

Prior to use, all plastic containers were soaked in 0.1N Trace Metal Clean grade HCl solution for at least one day and triple- rinsed with Milli-Q water system filtered (Millipore Corporation, Bedford, MA) water. The containers were air dried upside down in a laminar flow hood, to prevent contamination by air-borne particulates [6,19].

For containers used in the respirometric studies, the seals between the reaction vessels and caps were of great importance that to ensure the exclusion of atmospheric oxygen from entering and mixing with the headspace gas of respirometer vessels and custom designed caps and regular leak testing was required.

Care was taken to acid wash any materials that came in contact with the medium to prevent possible metal contamination.

Medium preparation

Medium components were prepared separately in the following manner: a) preparation of base medium buffers, basal salts**,** and nutrients, b) chelation followed by the addition of trace metal salts (TMS), c) sterilization, then vitamin and dextrose addition, and final product use and manipulation.

Preparation of base medium

Deionized and Milli-Q system filtered (Milli-Q water) water was used to prepare stock solutions based on the recipes (Table 1) in 4 l volumes as follows. Bis-Tris, $MgSO_4$ and $CaCl_2 \cdot 6H_2O$ were combined in a 1 liter polycarbonate volumetric flask and brought to volume with deionized Milli-Q water. KCl, $(NH_4)_2SO_4$, $NaNO_3$, $Na_2HPO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$ were mixed in another 1 liter polycarbonate volumetric flask and brought to volume with Milli-Q water. These two solutions were combined in a 10l carboy, 1.7l of Milli-Q water was added and the mixture placed on a magnetic stirrer. The pH was adjusted to 6.2 with 11.3N Trace Metal Clean HCl. Milli-Q water was added (post-chelexation) to bring the total volume to 3954 ml. The final mixture was brought to final volume with a 46 ml (containing the TMS, vitamin, and dextrose solutions).

Chelexation of base medium

Prior to entering the ion exchange 2.5 x 75 cm Flex-Column (Kontes, Vineland, NJ), the medium was filtered (inline 0.2 µm pore sized aqueous solution filter capsule (nylon membrane, Whatman, Inc., Clifton, NJ)) to prevent any precipitate accumulation in the column resin. The ion exchange column contained Chelex 100 cation exchange resin (200 - 400 mesh, sodium form; Bio-Rad Laboratories, Richmond, CA), designed to bind transition metals [29], was prepared as described by Price., *et al.* The resin scavenged unwanted (and unaccounted for) metals carried in the solution. The Chelex column flowrate was set at 1 drop (~50 µl) every 3s.

Sterilization

Microwave sterilization was preferred to avoid potential metal introduction and possible carbonate precipitation from autoclave steam [19]. Microwave sterilization was carried out in a Sharp Carousel microwave oven (model R-5080DK; Sharp Electronics Corp., Mahwah, NJ), which operated at a frequency of 2450 Mhz and produced a maximum output of 1200W. To achieve the appropriate power rating, following the microwave sterilization procedure as described by Keller., *et al.* [30] the microwave was operated at a 60% power level. Randomly selected CDM preparations were tested and shown to be sterile by plating CDM samples in triplicate on nutrient agar.

Final media preparation

After allowing the media to cool to room temperature, 1 ml of the vitamin stock solution was added (see Table 1) brought to volume with Milli-Q water and then filter-sterilized with a 0.2 µm pore sized Acrodisk syringe filter (Pall Corporation, East Hills, NY)). Lastly, 8 ml of the filter sterilized dextrose solution was added to deliver 54.7 mg COD l^{-1} (derived from the theoretical 1.067 mg of COD mg^{-1} of dextrose). The dextrose solution was prepared by adding dextrose (3.0g) to a volumetric flask brought to 500 ml volume with Milli-Q water.

Oxygen headspace depletion respirometry

The growth of the liquid cultures in 8 bioreactors was monitored via a computer software program, Challenge AER-ANR (CES, Inc., Fayetteville, AR), that collected oxygen utilization data reported by a headspace oxygen depletion respirometer (AER-208 respirometer, CES Inc., Fayetteville, AR). Oxygen headspace depletion was monitored by a "Flo-cell" sensor, which detected passage of a metered volume of pure oxygen (Zero Grade oxygen, Airgas East, Piscataway, NJ) to the reaction vessel. This equipment (using low oxygen permeability tubing (Tygon® FP tubing with 3/32"ID x 3/16"OD (Cole-Parmer Instrument Co., Vernon Hills, IL and inline "snubbers")) delivered oxygen to replace that utilized by the microbial community and reported continuous oxygen uptake data at a 20-minute frequency. A manifold (3rd stage) oxygen pressure regulator, released excess manifold pressure and maintained (with the help of a barometric pressure controller) a constant manifold pressure 2 mm of H_2O to the "Flo-cell" input. Waste carbon dioxide was scavenged from the headspace using granular soda lime suspended above the reaction mixture.

Growth conditions

Reaction vessel monitoring occurred in a covered water bath (Challenge Environmental Systems Inc., Fayetteville, AR), maintained at 18˚C, to prevent photochemical reactions [6] and algal growth.

Seed culture (SC)

Inocula for the experiments were from an agricultural Freehold sandy loam farm soil (Table 2). The seed culture inoculum in the liquid medium was 5.0% (w/v) soil (i.e. 5g of soil placed in each reactor). Seed cultures were grown in four reactors, two replicates containing 95 ml of CDM and two replicates containing soil extract solution (SES). One liter of SES was made using 100 ml of Adelphia agricultural farm (AD) soil extract, combined with 892 ml of 0.1 M Tris and similar to CDM, 8 ml dextrose.

Characteristic	Value
Soil source	Agricultural farm soil
pH	6.35
Texture	sandy loam
Organic matter	7.6%
CEC (meq/100 g)	9.8
Kjeldahl Nitrogen	0.22%
Inorganic Nitrogen - NO_3^- (mg kg^{-1})	64
Inorganic Nitrogen – NH_4^+ (mg kg^{-1})	8
Phosphorus	196
†Total Zn (µg g^{-1})	39.9
‡ Available Zn (µg g^{-1})	4.3
†Total Cu (µg g^{-1})	7.1
‡ Available Cu (µg g^{-1})	3.6

Table 2: *Characterization of the inoculating soil and the source of the soil extract.*

† SW846 6010B Inductively Coupled Plasma Spectrophotometer (ICP) method, analytical data provided by Shoayuan Zhang, Cook College, Rutgers University, NJ.

‡ Mehlich 3 extraction values (Soil Testing Laboratory, NJ Agricultural Exp. Station).

For the first 20h (lag phase), the reactors' mixing speed was set at 50 rev min^{-1}. For the remaining time period (post lag phase), the mixing speed was increased to 250 rev min^{-1}. The biomass growth was monitored until the oxygen uptake data indicated that all available substrate had likely been consumed. At this time, preparations began for SC sampling and the SI inoculation.

Serial inoculation (SI)

Four reactors containing 98 ml of sterile CDM and 4 reactors with 98 ml sterile SES were prepared as the seed cultures approached maximum cell density (approximately 90 - 100% substrate utilization), 2 ml of the suspended CDM seed cultures were transferred to two replicate CDM reactors (CDM/CDM) and to 2 replicate SES (CDM/SES) reactors. The same was done with the SES seed culture, yielding replicate SES/CDM and replicate SES/SES cultures (Figure 2). The inoculated reactors were placed on the respirometer with the mixing rate at constant 250 rev min^{-1}.

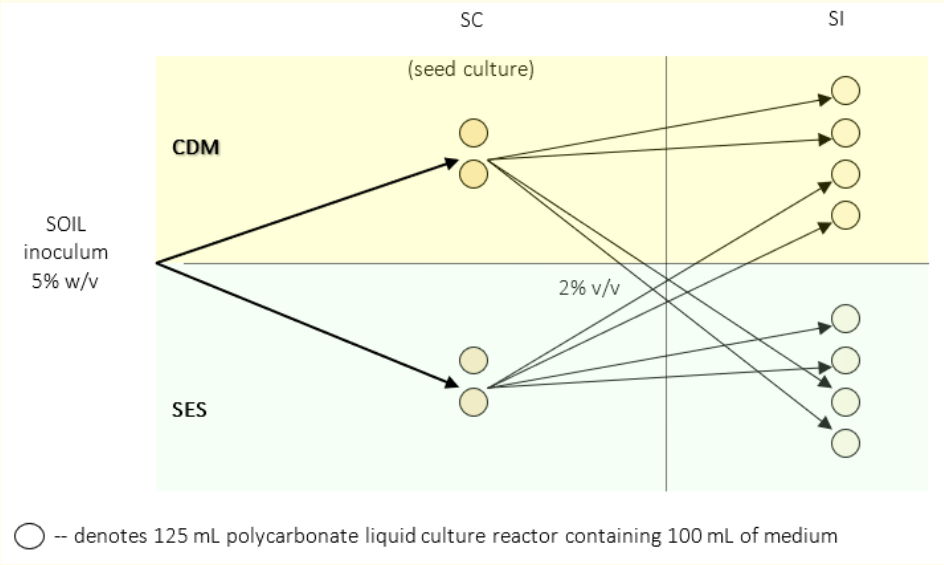

Figure 2: *Experimental design for CDM validation (comparison between CDM and SES liquid media).*

Data collection

Oxygen uptake measurements for the seed cultures and the SI reactors (collected automatically at 20-min intervals) were used for kinetic parameter estimation. The SC and SI cultures were sampled, at 90 - 100 % substrate utilization, for bacterial enumeration. Samples from the SI cultures were collected for community composition profiling (DGGE assays) and for the metabolic diversity measurements (BIOLOG™ system).

Kinetic parameter estimation

General growth kinetics estimates (apparent specific growth rate *(μ)*, half-saturation coefficient (K_S) and growth yield (Y)) were derived from the respirometer O_2 uptake measurements obtained from the serial batch liquid culture (SBLC) reactors. Efforts were undertaken to ensure growth parameters were constrained only by the substrate (S) concentration and not by nutrient or O_2 limitations, which prevent unrestricted growth and thus preclude any determination of growth rates, K_S values and yields from respirometric data [31]. As O_2 limitations may arise from insufficient culture mixing [32], a stirring rate of 250 rev min^{-1} was maintained to ensure appropriate O_2 availability.

All kinetic parameter estimation calculations began with oxygen uptake data validation to determine if there was any evidence of growth limitation (aside from that caused by decreasing substrate concentration). Non-substrate limited growth data precluded the use of curve fitting the data to a Monod expression [31] and required alternative methods of kinetic parameter estimation.

Metabolic Catabolic Profiling

Metabolic profiles for the bacterial communities present in the CDM and SES media were assayed with the BIOLOG™ system (Biolog, Inc., Hayward, CA), using a modified Kelly and Tate procedure. To prevent the blank well (control) from developing any color, dilution testing indicated that a minimum of 17 ml of sterile TRIS buffer to 1 ml inoculant was required prior to ECO plate inoculation. The

inoculated plates were incubated at 30°C, and color development was read using the microplate reader (BIOLOG, Inc., Hayward, CA). Plates were read beginning at 14h and subsequently at 2-h intervals. At each interval, average well color development (AWCD) plate values were calculated. The plate readings were continued at 2-h intervals until 0.25 AWCD was approached, at which point reading intervals were reduced to allow a more accurate measurement of the time required to reach a plate reading of 0.25 AWCD. The BIOLOG data was analyzed in two ways: determination of a color development rate chart and principal component analysis (PCA) using the Statistical Analysis System (SAS/STAT®) software [33].

DNA extraction

SBLCs required a preparation step before extracting community DNA (UltraClean Soil DNA Kit; Mo-Bio Laboratories Inc., CA). The procedure was performed as follows: frozen 5 ml SBLC samples were thawed and vortexed for 5s. Biomass from 5.0 ml of SBLC was concentrated prior to extraction and the MoBio UltraClean Soil DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) recommendations for "maximum yield" were followed. After agarose gel electrophoresis, bacterial DNA was UV visualized with ethidium bromide staining.

PCR and DGGE

A 492 bp fragment of bacterial 16S rDNA between *Escherichia coli* positions 27 to 519 was amplified using universal bacterial primers 27f (5¢-AGA GTT TGA TCC TGG CTC AG-3¢) and 519r (5¢- GWA TTA CCG CGG CKG CTG -3¢). Amplification was performed using a 2400 GeneAmp PCR system thermocycler (Perkin-Elmer, Foster City, CA) programmed to incubate reactions through one 5 minute 94ºC denaturation cycle, followed by 30 cycles of 30 s at 94ºC, 30 s at 55ºC, 1 ½ min at 72ºC, and a final 10-minute elongation at 72ºC and cooling at 4ºC. In addition, to improve PCR amplification specificity, yield and suppress primer annealing, a pseudo "Hot Start" technique was employed. Amplicons were confirmed on ethidium bromide stained agarose (1%) gels and visualized on a UV transilluminator (Fisher Scientific, Piscataway, NJ) and Polaroid Photo Documentation Camera w/Hood (Fisher Scientific, Pittsburgh, PA).

The amplification products, after purification with a QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA), were separated by Denaturing Gradient Gel Electrophoresis (DGGE) and was carried out as described by Sambrook., *et al.* [34] with a Dcode™-universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gel was prepared with a denaturant gradient ranging from 20% to 80% and electrophoresis was performed at 70 V at 60ºC for 16h. The gel was visualized with SYBR® Green I Nucleic Acid Gel Stain (Molecular Probes, Inc., Eugene, OR) and imaged with the KODAK Electrophoresis Documentation and Analysis System 290 (EDAS 290) (Eastman Kodak Company, New Haven, CT).

Results

Media formulation is presented in Table 1. Note that the N and P concentrations exceed minimum bacterial growth requirements and ensure that minimum nutrient concentrations would only be reached at the end of the growth curve and not be growth limiting (this P concentration did not produce precipitation). Addition of trace amounts of cobalt and selenium resulted in greatly improved growth rates and yields.

SC (seed culture) apparent specific growth rates *(μ)* for CDM ranged from 0.23 to 0.25 h^{-1} (derived via linear regression of OUR/OU) with a lag phase of 31.5 ± 3.1h, whereas the SES seed cultures' μ ranged from 0.13 to 0.18 h^{-1} with a lag duration of 26.1 ± 2.7 h. The SI inoculant had SES viable plate counts of 2.36 ± 0.31 x 10^8 colony forming units (CFU) ml^{-1} and were 3.45 fold greater than the viable plate count for CDM at 6.84 ± 0.67 x 10^7 CFU ml^{-1}. SI CDM doubling times averaged 2.30 h and the SI CDM lag phase averaged 13.73h, with the shortest average lag period (11.66h) exhibited by the CDM/SES culture (Table 3). SI apparent specific growth rates ranged from 0.30 h^{-1} (OUR/OU method) to 0.1 h^{-1} (Ln OU method) for the SES/SES cultures. K_S determinations maximally averaged 5.48 mg l^{-1} for CDM.

Kinetic Parameter	CDM (2 ml)	SES (2 ml)	CDM (2 ml)	SES (2 ml)
	CDM Liquid Medium		SES Liquid Medium	
Lag duration (h)	13.71 ± 1.88	13.78 ± 1.92	11.66 ± 1.66	16.45 ± 2.08
μ (h^{-1})	0.30 ± 0.07	0.30 ± 0.08	0.18 ± 0.09	NM ‡
μ_{Ln} † (h^{-1})	0.39 ± 0.06	0.37 ± 0.14	0.13 ± 0.04	0.1§
μ_{max}KM ¶ (h^{-1})	0.37§	0.36§	NM ‡	NM ‡
μ_{max} (h^{-1})	0.40 ± 0.09	0.40 ± 0.08	NM ‡	NM ‡
Y_{KM} ¶	0.35§	0.40§	NM ‡	NM ‡
K_S ¶ (mg l^{-1})	5.48§	3.82§	NM ‡	NM ‡

Table 3: *Kinetic parameter estimates derived from respirometric data.*

The values below are the best estimates of maximum observed values of specific growth rates (μ_{max}), specific growth rates (μ), half-saturation constants (K_S) and biomass yield (Y) using various derivation methods for the test soil microbial communities. Values are reported as averages and standard error (where possible) calculated from four oxygen uptake curves.

† - μ_{Ln} reported values were generated via a natural log transformation (see Appendix) and a linear regression through the relevant data points.

‡ - NM = not measurable

§ - represents calculation from only 2 oxygen uptake curves

¶ - calculation performed using an unpublished Microsoft Excel spreadsheet-based regression analysis program by James Young and modified by Robert M. Cowan.

Viable plate counts (on soil extract agar) were an order of magnitude greater with SI CDM-based cultures (CDM/CDM: 8.14 ± 1.6 x 10^8; SES/CDM: 2.4 ± 0.53 x 10^8) than the SI SES based cultures (CDM/SES: 2.07 ± 0.79 x 10^7; SES/SES: 2.5 ± 1.1 x 10^7). This relationship is similarly represented in the relative amounts of DNA extracted from each of the bioreactor samples for PCR. Viable plate counts from SC cultures were greater for the SES cultures (1.1 ± 0.66 x 10^9) than for the CDM cultures (2.6 ± 0.59 x 10^8), as was expected since the SC contained 5 g of soil compared to the SI, which did not contain soil.

Oxygen utilization curves (Figure 3) for the bioreactors reflected greater yields and growth rates for CDM-based cultures compared to the SES-based cultures. The SES/CDM cultures had the maximal estimated yield of approximately 0.4 mg volatile suspended solids (VSS)/mg COD removed, followed by CDM/CDM at 0.35 mg VSS/mg COD removed. (Note that for the SES/CDM cultures, 2 ml of SES SC was inoculated in CDM for SI.) The estimates for CDM/CDM maximum observed specific growth rates were 0.40 ± 0.09 (linear regression) and 0.37 (Kinetic Model) h^{-1} and 0.40 ± 0.08 (linear regression) and 0.36 (Kinetic Model) h^{-1} for SES/CDM. The amount of oxygen uptake in the CDM/SES and SES/SES cultures was severely limited, which precluded any yield or apparent specific growth rate measurements.

The CDM and SES microbial communities each had distinct metabolic profiles (Figure 4). This analysis produced separation mostly along PC 1, which accounted for 29% of the cumulative variance indicating differences between community metabolic profiles of the 2 media. PC 1 and PC 2 together accounted for 52% of the cumulative variation. The SES/CDM and CDM/SES cultures were separated along PC 1 between the SES/SES and CDM/CDM culture, which suggested the mixed enrichment and selection pressures differed between the two media.

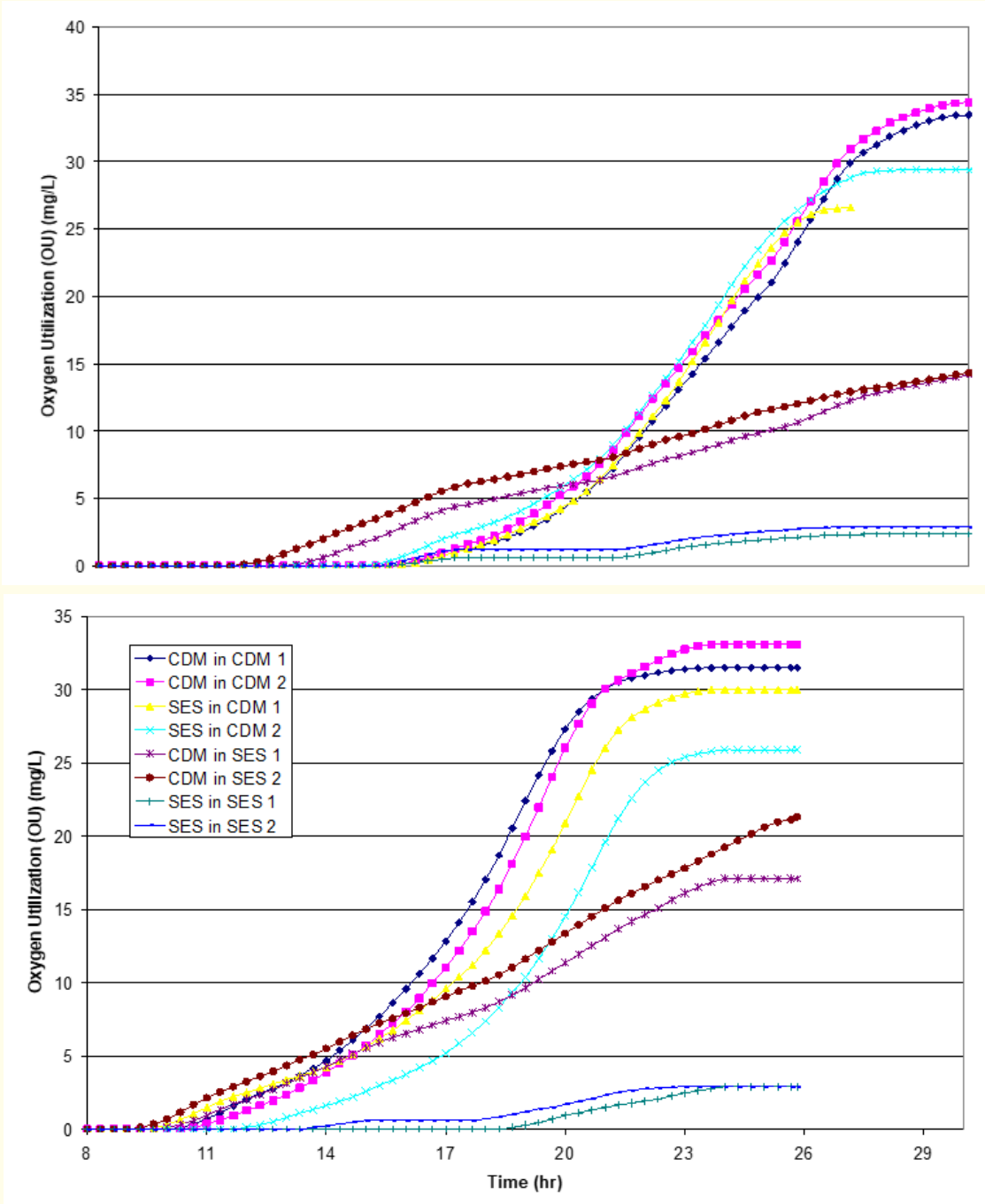

Figure 3: *Respirograms for the CDM and SES Cultures (072003 (a) and 070203 (b)) showing the success of the CDM-based cultures. Cumulative oxygen uptake data was smoothed (interpolated and a 5-point rolling average).*

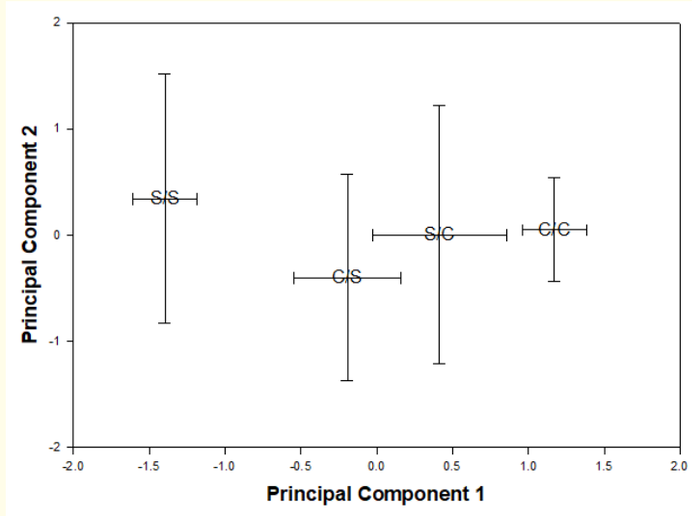

Figure 4: *Principal Component Analysis of BIOLOG (ECO plate) metabolic profile data of CDM and SES BIOLOG profile averages. Each point represents the average of 6 data points with the error bars denoting one standard deviation. This analysis compared sets of data reflecting AWCD values closest to 0.25. The two principal components (PC) describe 51.9% of the variation with PC 1 accounted for 28.9% of the variance in the data and PC 2 accounted for 22.9% of the data. C/C = CDM in CDM, S/C = SES in CDM, C/S = CDM in SES, S/S = SES in SES.*

The separation profiles of amplified 16S rDNA 492 bp sequences obtained from community DNA by DGGE resolved in most lanes, distinct banding patterns of variable intensity. The DGGE profile (Figure 5) provided evidence that CDM was causing the enhancement for a particular banding pattern in lanes 2-7, in relation to lane 1, which represented the banding pattern for the bacterial DNA extracted from the parent AD soil. This particular banding pattern was not observed in lanes 8 and 9. Lanes 8 and 9, representing the SES/SES DNA extracts, demonstrated no particular enhancement for any band and presented more of a smear than any distinct band resolution. It is worthy of note that there appears to be a common band in all lanes, indicating that one band is a common feature in all tested cultures.

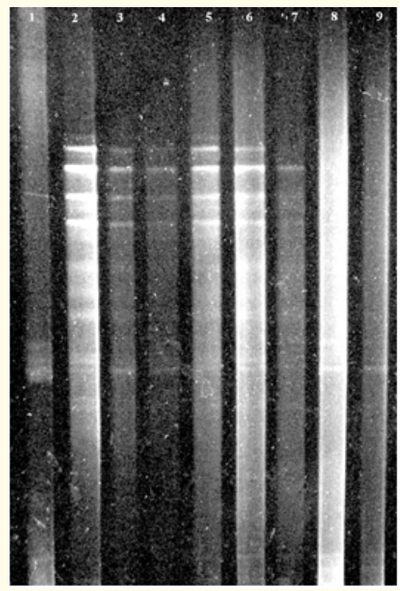

Figure 5: *DGGE analysis of test microbial communities based on 16S rDNA specific PCR. DGGE patterns did not exhibit a marked difference in band number nor did there appear to be a difference in migration positions for lanes 2 - 8. Lanes: 1, AD soil DNA extract. Lanes 2 and 3, CDM/CDM. Lanes 4 and 5, SES/CDM, Lanes 6 and 7, CDM/SES. Lanes 8 and 9, SES/SES.*

Discussion

CDM's design was to have a minimum concentration of nutrients and yet permit unrestricted community growth, with growth being limited by only a single parameter, the carbon (and energy) substrate concentration. Initial substrate (S_0) COD concentration was carefully controlled to limit excessive biomass growth and allow for a feasible mathematical calculation of K_S [31,35]. Successfully achieving that design goal would allow one to define the specific concentration and form of metal available to the microorganisms. To gauge CDM-derived kinetic measurements and to assess our success in developing a sound testing platform medium, CDM supported growth was compared to that of the control medium, SES. CDM shortcomings in preliminary comparisons against SES media became evident, as it was apparent that CDM culture growth rates became limited before the carbon and energy source was depleted. Minimum nutrient requirements were re-evaluated using estimates of biomass yield and final biomass at substrate depletion along with a μ approximation and K_S to calculate the additional amount of nutrient (above that which would be incorporated into the biomass) needed to keep these substrates from becoming growth rate limiting.

Examination of the potential for N-limitation of culture growth showed expected observations (i.e. that the form of N was nearly as important to growth as its concentration). The nitrogen added (as described in the Material and Methods) was non-limiting.

Focusing on the next most common potentially limiting nutrient, phosphorus was added (amount calculated as described in the Materials and Methods) and was present in sufficient concentrations to preclude growth limitation for the CDM cultures. However, for the SES cultures, the phosphorus concentration was found to have a dramatic effect on the SES cultures. Figure 6 shows the effect of amending the SES SI cultures with the same phosphorus concentration that was added to the CDM cultures. The SES/SES+P cultures had improved growth rates and yield with the bacterial population densities increased 10 fold. Of course, the CDM/SES+P culture improved even more dramatically, since the inoculant had already seen selection pressure for the more oligotrophic CDM medium in SC. The SES/SES+P cultures, in contrast, had to acclimate (note the longer lag periods for all the cultures with respect to CDM/SES+P) to the change in their environment from SC to SI, where the soil is no longer a substantial source of nutrients.

In terms of assay suitability, CDM was compatible with a number of commonly performed assays on the soil solution and the soil microbial community. In this study, the compatibility with metabolic diversity profiling (BIOLOG™ system) was shown with the following constraint: the inoculant had to be diluted at a 1:17 ratio, at a minimum. Dilutions with 0.1 M Tris buffer lower than 1:17 resulted in false positive results (oxidation of DOC carried over from the sample). In this case (and whenever metal amendments are anticipated), Tris is recommended (as opposed to phosphate buffer, a common solution for the soil extraction of microbial communities) as the buffer does not form any problematic metal precipitates, which could void the utility of the BIOLOG™ system assay [36].

CDM growth comparison to SES

Any intrinsic kinetic parameters (e.g. μ_{max}, Y, K_S,) estimated for characterization and comparison efforts of the cultures (and in this work, grown under dextrose substrate limiting conditions) required a prior "critical inspection" of the data to decide the appropriate derivation technique to apply. As some data (e.g. 072003 CDM-based cultures) appeared to permit the unrestricted growth of the culture (i.e. not limited by anything other than substrate) by meeting the criteria assumed by the Monod model [31], kinetic parameter estimation for the "Kinetic Model" (unpublished model by James Young and modified by Robert C. Cowan) was performed via a least squares approach using Microsoft Excel's solver program. It was observed that the media seed cultures apparent specific growth rates (μ) were surprisingly greater with the CDM medium, (averaging 0.24 h^{-1}), whereas the CDM culture had an expected longer period of lag time (31.5 ± 3.1h), taking 4.5 hours longer than the SES culture before the onset of measurable growth. The nature of expectation arises from the fact that the CDM and SES seed cultures had the same inoculum source and the SES liquid medium was most similar to that of the inoculant's native environment. However, in line with the lag expectations, the viable plate counts for the SES seed cultures were, on average, an order of magnitude greater than the CDM seed culture.

Referring to figure 3 does not necessarily require a visit to table 3 to reason that the CDM-based cultures (CDM/CDM and SES/CDM) had the largest growth yields.

The SES/CDM culture (of 070203) with a slightly lower cumulative oxygen uptake in figure 3 (compared to CDM/CDM) had the greatest yield of 0.4. These cultures did not appear to be appreciably limited by any nutrient other than substrate (S) concentration. These cultures to a small degree did show some evidence of something occurring biologically that the Kinetic Model could not explain. The complexity present in the oxygen uptake curve explains the large K_S value generated by the model. An exaggerated example of this complexity can be seen in the OUR versus OU plots, which show up as a succession of small peaks atop a plateau. These are nutrient limitations or possibly changes in dominant community members. In any event, these features preclude the use of curve fitting the data to the Monod-based Kinetic Model.

The remaining curves in figure 3 (SES-based cultures) reflect growth rates and yields that are dramatically affected by one or many limiting nutrients. Preliminary testing indicated that the SES cultures were always limited when compared to the initial culture, where the soil inoculum appeared to serve as a source of trace minerals. These conditions, too, preclude the possibility of estimating the kinetic parameters in any way other than linear regression of the OUR/OU and Ln OU plots.

However, in the SI, the lag phase duration across the cultures was roughly half of the SC lag phase. While the CDM-based cultures had approximately the same lag duration (averaging 13.73 to 13.74h), the SES-based cultures presented the extremes in duration. It appears that the CDM/SES had the shortest lag (11.66 ± 1.66 h) and SES/SES, the longest (16.45 ± 2.08h), in spite of the fact that the initial biomass (X_o) for SES was larger than CDM cultures. The SES liquid-based cultures (i.e. lacking soil) also demonstrated dramatically reduced growth rates and yields from their SC measures as well as their SI CDM counterparts. The disparity in apparent specific growth rates and yields between the two media types in SI cultured with dextrose as the carbon source apparently resulted from nutrient deficiency in the SES. As mentioned earlier, while testing indicated that the SES trace metal or nitrogen deficiencies did not play a role in the disparity; phosphorus was shown to be a limiting factor in microbial growth. Such a finding might provide a simple and easy improvement to those wishing to enhance the growth characteristics of a soil extract liquid medium.

Conclusion

Whereas a more detailed study would permit the elucidation of CDM's effects on the microbial community, this study shows that CDM was successful in supporting a competent microbial community with growth parameters at least comparable to those grown in SES. In addition, the liquid medium was found to be compatible with a number of commonly performed assays on the soil solution and the soil microbial community. The simple composition of the CDM made it a feasible medium for trace metal work with soil microbial cultures and fulfilled the most basic necessity in microbe testing where the specific metal concentrations and their forms available to microorganisms are known. Although this medium may grow only a segment of the viable soil community (as would any medium), it still provided a foundation with which to compare disparate soils and their inhabitants.

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