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Equivalence of Microbial Biomass Measures Based on Membrane Lipid and Cell Wall Components, Adenosine Triphosphate, and Direct Counts in Subsurface Aquifer Sediments

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Abstract. An uncontaminated subsurface aquifer sediment contains a sparse microbial community consisting primarily of coccobacillary bacteria of relatively uniform size which can be counted directly with appropriate staining. The morphological simplicity and the relatively decreased cell numbers, when compared with surface soils and sediments, make the subsurface an ideal natural community with which to compare the utility of chemical measures of microbial biomass to direct microscopic counts. The membrane phospholipids (estimated as the polar lipid fatty acids, the lipid phosphate, and phosopholipid glycerol phosphate), lipopolysaccharide lipid A (estimated as the LPS hydroxy fatty acids), cell walls (estimated as the muramic acid), and adenosine triphosphate all give essentially identical estimates of cell numbers and dry weight as the direct counts, using conversion factors determined on subsurface microorganism monocultures. Assays of microbial cell components are thus validated by comparison with the classical direct count in at least one soil/sediment.

Introduction

The determination of the microbial biomass in complex environmental samples, such as soils and sediments, has presented problems as the classical techniques of viable counting most often recover a small and variable proportion of the cells detectable by direct counting [1]. In the water column, direct counts of pelagic bacteria not attached to surfaces are significantly higher than viable counts on the same samples [20]. With sediments and biofilms, the problems with classical methods are more severe. In addition to the problems of providing a universal growth medium in the Petri plate for a diverse microbial community, the organisms must be quantitatively removed from the surfaces and from each other. Direct counting of microorganisms in soils presents "formidable technical difficulties" [21]. Direct microscopic methods that require quan-

titative release of the bacteria from the biofilm can have the problem of inconsistent removal from some surfaces. High speed blending of sediments to remove the microbiota prior to staining and direct counting in epifluorescent illumination was neither quantiative nor reproducible when compared with chemical assay of the muramic acid of the prokaryotic cell wall in one study of marine sediments [26]. In another study, a marine sandy sediment from the North Sea showed no detectable muramic acid after blending for direct counting (D. C. White, unpublished data). Direct microscopy can be performed on the sediment particles or thin biofilms if vital staining of the microbes can be used to distinguish the microbes from abiotic particles. In surface soils with high microbial densities and great morphologic diversity, estimations must be made for the organisms rendered invisible by sediment granules or overlapping organisms in biofilms [8]. The problems in calculation of cell volumes with the complex morphologies of the diverse communities of surface soils can be overcome by the application of computer-based image enhancing [6]. This methodology works best when the density of organisms in the sediments or biofilms is low and overlapping is minimal. Ghiorse and Balkwill [14, 15] developed a method of directly counting acridine orange (AODC) stained subsurface microbes after detachment and concentration in the presence of sodium pyrophosphate. In sediments from unpolluted subsurface sites the direct counts are reproducible [3] and when compared with a carefully controlled extraction and assay of adenosine triphosphate (ATP) gave similar estimates of microbial biomass [39]. Viable counts from these same subsurface sediments, however, remain considerably more variable despite the use of dilute media [4, 32]. Recent evidence shows that the viability of soil organisms increases with increases in their size [2].

In their review of methods for measuring microbial biomass in soils, Jenkinson and Ladd [21] state that no measurement of components of the microbiota fulfills the criteria of (1) presence in all parts of the soil in the same concentration per cell at all times, (2) presence in living but not dead cells or nonliving parts of cells, (3) availability of methods for quantitative extraction, and (4) availability of methods for determination in soil extracts. In 1984, Nannipieri [27] stated "Measurement of microbial biomass with methods based on specific biomass constituents, such as muramic acid, hexosamines, nucleic acid, etc., have provided, with the exception of the ATP technique, erroneous estimates,"; he relied on responses to fumigation for microbial biomass estimations.

The development of assays of cellular components for microbial consortia as biomass measures primarily in sediments had been documented prior to 1984 [40, 43–47] and has continued despite the pessimism cited above in the soil literature. Components and bacterial cells added to soil and sediment samples have been recovered quantitatively. Because the total community is examined in these procedures without the necessity of removing the microbes from surfaces, the microstructure of multi-species consortia is preserved. The method involves the measurement of biochemical properties of the cells. Chemical methods can also be utilized for extracellular products (uronic acids) not determined by the classical techniques of culture or direct counting. Those components generally distributed in cells are utilized as measures of biomass.

Components restricted to subsets of the microbial communities can be utilized to define the community structure (the lipopolysaccharide lipid A-hydroxy fatty acid methyl esters [LPS-OHFAME] in gram-negative bacteria, for example). The concept of "signatures" for subsets of the community based on the limited distribution of specific components has been validated by using antibiotics and cultural conditions to manipulate the community structure. The resulting changes agreed both morphologically and biochemically with the expected results [45]. Other validation experiments involved isolation of specific organisms, biomarker identification, and their detection in appropriate artificial mixtures or after specific manipulations. The utilization of specific inhibitors of stimuli for classes of microbes or the effects of specific predation and disturbance have been reviewed [42].

Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities, the bacteria contain a relatively constant proportion of their biomass as phospholipids [43, 44]. Phospholipids are not found in storage lipids and have a relatively rapid turnover in some sediments so the assay of these lipids gives a measure of the "viable" cellular biomass [46]. The total polar lipid fatty acids (PLFA), the phosphate of the phospholipids, and the glycerol-phosphate and acid-labile glycerol from phosphatidyl glycerol-like lipids that are indicators of bacterial lipids can be assayed to increase the specificity and sensitivity of the phospholipid assay [13, 43, 44, 46].

From the residue of the lipid-extracted biofilm, muramic acid, a unique component of the bacterial cell wall, can be recovered [11]. Muramic acid in the bacterial cell wall exists in a 1:1 molar ratio with glucosamine. As the analysis gives both glucosamine and muramic acid, and the chitin walls of many microeukaryotes yield glucosamine, the glucosamine to muramic acid ratio gives insight into the prokaryote to eukaryote ratio. This complements the information developed from the ester-linked PLFA.

Gram-negative bacteria contain distinctive patterns of amide or ester-linked aliphatic and hydroxy fatty acids in the lipid A of their lipopolysaccharide wall polymers [33]. This has proven to be an extremely valuable assay in the definition of gram-negative bacteria. With this assay it is possible to detect bacteria in mammalian tissue or secretions [31].

The experiments reported herein show measurements of the same subsurface soil sample give essentially identical estimates of the cellular biomass and the direct count, and fulfill the requirements of Jenkinson and Ladd [21]. Estimates of microbial biomass were based on the membrane polar lipids estimated as the total fatty acids, the lipid phosphate, the lipid glycerol phosphate, the content of LPS-OHFAME, the content of muramic acid, and the content of ATP.

Materials and Methods

Unisil (100–200 mesh) silicic acid was obtained from Clarkson Chemical Co., Inc. Williamsport, Pennsylvania. Glass-distilled solvents (Burdick and Jackson, Muskegon, Wisconsin) or freshly redistilled analytical grade chloroform (Mallinckrodt, St. Louis, Missouri) were used. Derivatizing

reagents and authentic standards were purchased from Pierce Chemical Co., Rockford, Illinois; Aldrich Chemical Co., Milwaukee, Wisconsin; U.S. Industrial Chemicals Co., Tuscola, Illinois; and Sigma Chemical Co., St. Louis, Missouri. Fused silica capillary GLC Durabond columns (DB-1) were supplied by J&W Laboratories Inc., Rancho Cordova, California.

Sampling Site

Uncontaminated sandy clay sediments were collected from subsurface sediments from the unconsolidated sediment in the the flood plain margin of a small river at Lula, Oklahoma. The sample collection methods [48] prevented contamination with surface soil microorganisms. This was established by comparison of the phospholipid ester-linked fatty acid patterns from the subsurface sediment and the surface soils [36]. The soil profile and characteristics have been described [3, 48]. The sediments were sampled for ATP in the field within minutes of recovery. Reconstruction experiments have shown that recovery is quantitative. The sediments for AODC and lipid membrane and cell wall component analyses were collected in sterile wide-mouth jars, sealed, packed in ice, and shipped to the laboratories by air express. Analyses with processing within a week of sampling have been shown to yield values identical to those processed immediately after sampling.

Total Cell Counts

The bacterial cells were enumerated with AODC modified from that reported previously [37, 48]. A 2.5 g subsurface soil sample was suspended in 22.5 ml of filter-sterilized 0.1% sodium pyrophosphate ($Na_2P_2O_7 \cdot 10\ H_2O$; pH 7), stirred with a magnetic stirrer at room temperature for 30 min and allowed to settle for 2 min. A 9 ml portion of the suspension was then transferred to a sterile centrifuge tube, 0.1 ml of 50% glutaraldehyde was added, the tube was warmed to 50°C, and sterile molten Noble-Agar to a final concentration of 0.1%. After vigorous mixing on a vortex mixer, the sample was stored at 4°C until AODC could be performed. To count, 5 μl of the suspension was spread uniformly on a 1.04 cm^2 circular area on a clean glass slide, air dried for 2 min, stained with 0.01% acridine orange for 2 min, rinsed with distilled water, and blotted dry. A drop of distilled water was added, and a 22 × 22 mm coverslip was applied. Excess water was drawn from beneath the coverslip with bibulous paper, the edges were sealed, and the slide was examined with a Nikon Optiphot epifluorescence light microscope at a final magnification of 1,000×. Counting fields were selected randomly, but only within areas of the sample smear located close to the periphery that represented regions of average smear thickness (see [37]). The numbers of bacteria in 50 fields were counted, and the number per g dry weight was calculated with appropriate dilution factors. Microbial cell volumes were calculated from photographs like those illustrated in Fig. 1A.

Electron Microscopy

Release and concentration of microbial cells from subsurface solids for subsequent thin sectioning and transmission electron microscopy (TEM) utilized centrifugal washings of the suspension used for counting [4]. The supernatant suspension was centrifuged at 650 × *g* for 5 min for five washings with the 0.1% pyrophosphate. The supernatants of the five washings were combined and the cells recovered after centrifugation for 20 min at 23,000 × *g*. The fixation, dehydration, embedding, thin sectioning, and TEM have been described [3, 14, 48].

Membrane Lipid Analysis

Subsurface sediments were lyophilized, the lipids were extracted with the one-phase chloroform: methanol extraction, and the extract was filtered and fractionated into neutral-, glyco-, and polar-lipid fractions as described [36]. The glycolipid fraction was utilized for the analysis of poly beta-hydroxy alkanoate (PHB) by purification, ethanolysis, and analysis by gas chromatography (GC) as described [9, 10]. The polar lipids were recovered and the phosphate and glycerol phosphate determined [13, 44, 46]. A portion of the lipid was subjected to mild alkaline methanolysis, the ester-linked phospholipid fatty acids (PLFA) were recovered, purified by thin layer chromatography, and analyzed by GC as described [17, 36]. Recovery of added standards and bacteria to the sediment has been shown to be quantitative [17, 36, 45].

The hydroxy fatty acids of the LPS were recovered from the lipid-extracted residue after hydrolysis, purified, derivatized, and assayed by GC as described [28, 33]. Recovery from sediments has been shown to be quantitative [28, 33].

Cell Wall Assay

Muramic acid was recovered after hydrolysis of the lipid-extracted sediment, and the carbohydrate was derivatized and assayed by GC [11]. Recovery of added bacteria or cell walls from sediments has been shown to be quantitative [11, 24–26].

ATP Assay

ATP was recovered from sediments using the specific conditions established to be most productive for subsurface sediments, and assayed enzymatically with purified preparations [38]. The method has been established to correlate with AODC cell counts [39] and with activity towards biodegradation of pollutants [49]. The recovery of adenine nucleotides from these subsurface sediments has been shown to be quantitative [38].

Recovery

Each of the assays based on analysis of cell constituents described above has quantitative recovery of authentic standards, and bacteria containing assayed amounts of the constituent have been shown in reconstruction experiments with sediments.

Results

Morphology and Distribution of Subsurface Microbes

Figure 1A shows a typical microscope field of the AODC-stained microorganisms. The cell volume determined from photographs like this was 1.07 ± 0.71 μm^3, $n = 43$. The electron micrographs (Fig. 1B and C) show that the microbes detected by AODC have a typical morphology. Gram-positive coccoid bacteria are illustrated.

The community nutritional status of the microorganisms can be determined by the ratio of poly-beta-hydroxy alkonates (PHA) to PLFA [12]. The organisms

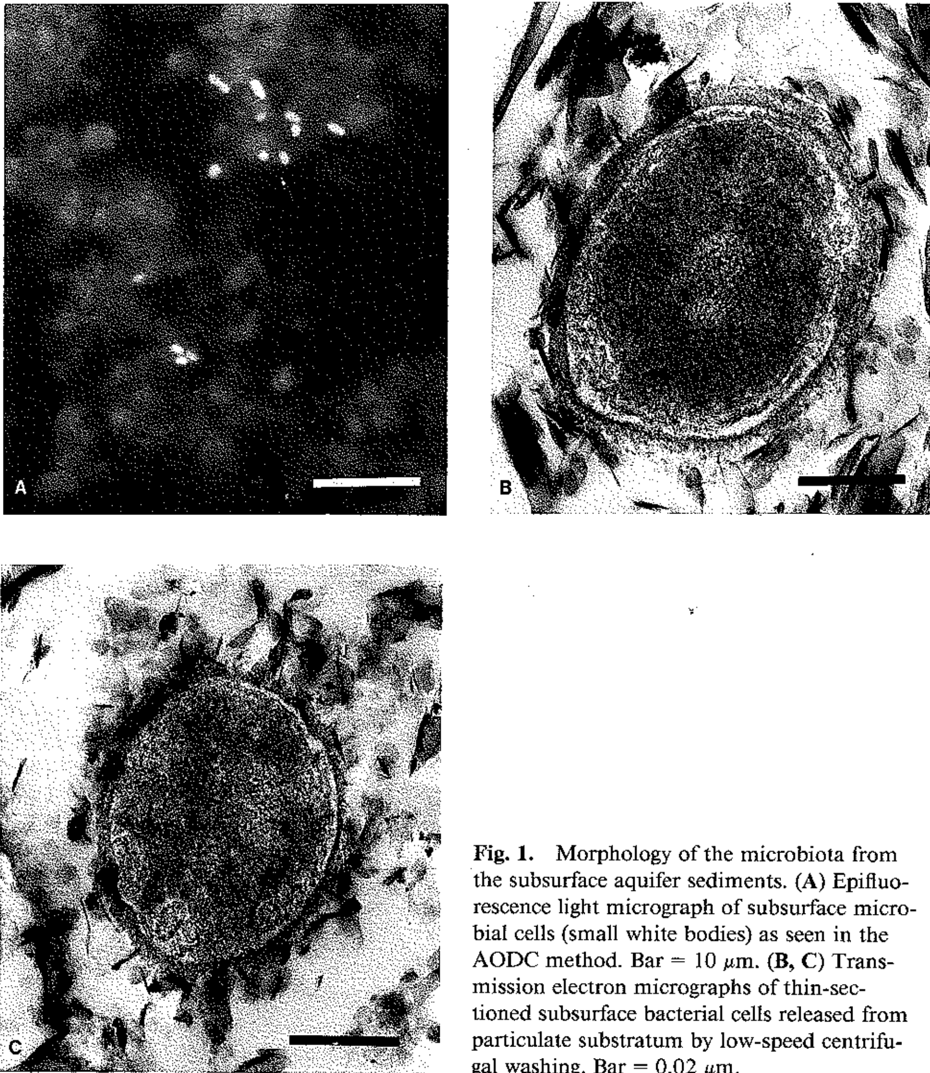

Fig. 1. Morphology of the microbiota from the subsurface aquifer sediments. (**A**) Epifluorescence light micrograph of subsurface microbial cells (small white bodies) as seen in the AODC method. Bar = 10 μm. (**B, C**) Transmission electron micrographs of thin-sectioned subsurface bacterial cells released from particulate substratum by low-speed centrifugal washing. Bar = 0.02 μm.

from the subsurface samples used in this study contained 2.74 ± 0.58, n = 3, nmol 3-OH butyric acid in the PHA per g dry weight compared with 2.54 ± 0.58 nmol per g dry weight in the surface soil at that site. The ratio of PHA 3-OH butyrate to phospholipid phosphate was 9.09 ± 1 for the subsurface soil compared with 0.61 ± 0.09 for the soil at the surface. This indicates that the organisms containing the high levels of PHA had been subjected to conditions of unbalanced growth [29].

Table 1. Equivalence of biomass determinations by membrane lipid and cell wall components, and ATP to direct counts in subsurface sediments

Component	Concentration nmol/g dry wt sediment	Conversion factor μmol/g dry wt cells	Direct count equivalence[a] 10^6 cells/g dry wt sediment	Dry weight cells μg cells/g dry wt sediment
Direct count			7.4 ± 3.5	1.3 ± 1.1[b]
Membrane				
Phospholipid fatty acids	0.35 ± 0.08	100	7.0 ± 0.2	3.5 ± 0.1
Lipid phosphate	0.11 ± 0.03	50	4.4 ± 0.1	2.2 ± 0.05
Glycerol phosphate	0.22 ± 0.09	50	8.8 ± 0.3	4.4 ± 0.15
LPS-OHFAME	0.09 ± 0.04	15	12.0 ± 3.0	6.0 ± 1.5
Cell Wall				
Muramic acid	0.22 ± 0.1	58.5	7.5 ± 5.0	3.7 ± 2.5
ATP	1.39[c] ± 0.42	1.7[d]/10^7	8.2 ± 1.1	1.4 ± 0.8[e]

[a] Calculated with 2×10^{13} cells/g dry wt
[b] Calculated with a cell volume of 1.07 μm^3 and 1.72×10^{-13} g/cell
[c] ATP given in ng/g dry wt
[d] ATP given in ng/10^7 cells
[e] Calculated assuming 10^7 cells weigh 1.72 μg

Equivalence Between Direct Count and Cell Constituent Analysis of Microbial Biomass

The data in Table 1 indicate that estimates of cell numbers by direct counts and by analysis of cell membrane, LPS-OHFAME, muramic acid in the cell wall, and extractible ATP give essentially equivalent numbers for both cell numbers and dry weight biomass.

The primary difficulties in this analysis come in the application of "conversion" factors.

Discussion

Conversion Factors

The number of cells corresponding to the actual amount of membrane, to LPS OH fatty acid, or to cell wall component determined per g dry weight of cells and the number of cells per g dry weight must be determined. The dry weight of bacteria isolated from litter and soil has ranged between 1.66 and 67.5 × 10^{-13} g/cell, 15.5 × 10^{-13} average, n = 17 [16]; this gives 6.4 × 10^{11} cells/g dry weight. Determinations based on chemostat-grown *Escherichia coli* gave 2.3 ± 0.9 × 10^{-13} g/cell, n = 73, or 4.3 ± 1.2 × 10^{12} cells/g dry weight [19]. Based on respiration rate, cell number, and growth rates, plated bacteria gave a biomass of 4.0 × 10^{-13} g/cell (2.5 × 10^{12} cells/g dry weight) [23]. From measurements of the relationship between biomass and biovolume, Bratbak

and Dundas [5], found an average biovolume of 0.48 ± 0.2 μm^3 of *Pseudomonas putida* grown on three different media and of bacteria in a mixed bacterial population isolated from brackish water in an estuary in Norway. They also found 4.8 ± 2.3 × 10^{-13} g/cell or 2.08 ± 1.7 × 10^{12} cells/g dry weight. In the calculations comparing the number of cells determined by AODC count with the various measures of bacterial components in Table 1, a conversion factor of 2 × 10^{12} cells/g dry weight was used [5]. Webster et al. [39] determined the conversion factor directly as 1.7 ng ATP per 10^7 cell-forming units. They used cultures of two strains of *Arthrobacter* sp. and one of *Pseudomonas* sp. which were isolated from this subsurface sediment, grown in the dilute peptone-yeast extract medium of Ghiorse and Balkwill [14], and allowed to starve for between 4 and 9 weeks. The high PHA/phospholipid ratio in the subsurface bacteria, as compared with the bacteria from the surface soil, is strong evidence of starvation in the Lula subsurface microbiota. Using the conversion factor based on the measurement with starving bacteria isolated from the sediment gives essentially the same number of cells as observed in the AODC count and as calculated from the membrane lipid, LPS, OHFAME, and cell wall component measurements.

To calculate the dry weight of cells per g dry weight sediment, the biovolume of 42 cells from the Lula subsurface sediment was determined to be 1.07 ± 0.79 μm^3. Norland et al. [30] developed a relationship between cellular dry weight and volume based on measurements of 337 individual bacterial cells with a range in volume between 0.01 and 7 μm^3. The allometric relationship showing that smaller bacteria have a higher dry weight to volume ratio than larger bacteria was developed. Applying this relationship to the average biovolume estimated for the organisms used in the AODC count in this study gave a value of 1.72 ± 0.11 × 10^{-13} g per cell. This value and the AODC count gave dry weight values essentially equivalent to the biomass estimates from the three membrane lipid components, the LPS OH-fatty acids, and the muramic acid of the cell wall. The conversion factors used for the component measures were derived experimentally as previously reported using the appropriate bacterial monocultures (polar lipid fatty acid and phospholipid phosphate [43, 44, 46], polar lipid glycerol phosphate [13], LPS-OHFAME [33], and muramic acid [10]). The weight of 10^7 bacteria, which was used to calculate the bacterial dry weight from the ATP measurement, was calculated using the measured biovolume (1.07 μm^3) and the allometric expression of Norland et al. [30].

Suitability of Biomass Measures Based on Microbial Cellular Components

Of the four criteria required by Jenkinson and Ladd [21] mentioned in the introduction, the universal distribution of polar lipids, gram-negative bacteria containing lipid A OHFAME, and bacteria containing muramic acid in subsurface sediments has been demonstrated. Subsurface sediments recovered even 410 m below the surface have been shown to contain them [47]. The constancy of phospholipid content among eubacterial species grown under conditions in the subsurface has been established [43]. Based on cultural conditions, the

component of the LPS least subject to variability is the lipid A OHFAME composition [34]. The muramic acid content of many eubacterial strains, both gram-positive and gram-negative, has been established [11, 24, 25]. ATP is clearly a universal component of living cells subject to rapid changes with changes in the physiological status of the cells [7]. The second criterion of Jenkinson and Ladd [21] requires that the biomarkers be sufficiently labile on cell death to indicate viable or potentially viable microbiota. Phospholipids, phospholipid fatty acids, adenosine nucleotides, muramic acid, isolated bacterial cell walls, and the lipopolysaccharide of dead bacteria are rapidly lost from marine sediments [7, 22, 25, 34, 43, 44, 46]. This indicates that these chemical markers provide good estimates for the standing viable or potentially viable microbiota. The third and fourth criteria [21] of quantitative extraction and sensitive assay methods have been demonstrated with reconstruction experiments utilizing authentic standards and nongrowing bacterial monocultures. Thus, the admonition of Nannipieri [27] has been turned around. The cell biomass assays based on constituents of the cells are more accurate (in terms of the reproducibility) and less subject to errors in interpretation (biovolume determination) than the AODC counts. With the biochemical component methods there is no necessity for quantitative recovery of attached cells from the substratum. Preliminary evidence showed the method of removal by flotation using pyrophosphate and other polymers, although useful, is selective as the PLFA patterns of the total sediment and the bacteria released from the sediment are not identical (D. L. Balkwill and D. C. White, unpublished experiments). In these experiments a small proportion of the cells were left attached to the substratum. Viable count methods are the least accurate methods of determining biomass [3, 32].

Determination of the Microbial Community Structure and Nutritional Status

Use of biochemical component analysis to determine microbial biomass in soils and sediments provides further advantages over classical measures. The development of specific "signature" biomarker patterns of PLFA, LPS OHFAME, and to some extent ratios of glucuronic to muramic acids in cell wall preparations provides insight into the community structure. The utility of subsurface sediments as a test system for the equivalence of various biomass methods is due to the morphologic simplicity of the microbiota. Microeukaryotes, if present, are essentially insignificant in the biomass of subsurface sediments. This is easily demonstrated by the absence of polyenoic PLFA of chain length >20 carbons in these sediments [36]. The PLFA patterns can also be utilized to provide indications of the conditions in the microniche [41]. Some bacteria shift the PLFA patterns with changes in nutrients conditions (some do not) and accumulate both cyclopropane and *trans* monoenoic PLFA with starvation stress [18].

The presence of high proportions of PHA in lipid phosphate found in this subsurface sediment indicates starvation with unbalanced growth. Certain bacteria form the endogenous lipid storage polymer PHA under conditions where the organisms can accumulate carbon but have insufficient total nutrients to

allow growth with cell division [29]. The GC assay of PHA increased the sensitivity and specificity of the assay and provided a useful means of defining the nutritional status of microbes in various environmental habitats [12]. Uncontaminated subsurface aquifer sediments show a microbiota with high levels of PHA relative to the phospholipids [47], as well as a specific PLFA pattern that is similar in clays, sands, and limestones from various locations and distinctly different from surface soils [36]. Contamination of the subsurface sediments with aromatic phenols induces bacterial growth in the vadose zone with a decrease in the rate of PHA biosynthesis [35].

Conclusions

Measures of microbial biomass in soils and sediments based on components such as the membrane polar lipids, the cell wall, LPS OHFAME, and ATP provide estimates of biomass and cell numbers that agree with AODC counts (Table 1). Biomass determinations based on assay biochemical components do not require recovery of the organisms from the soil substratum and can provide insight into the community structure and nutritional status of the microbial consortia.

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