Sewage Sludge Proteins: I. Extraction Methodology

R. N. Lerch,* K. A. Barbarick, P. Azari, L. E. Sommers, and D. G. Westfall

ABSTRACT

The extraction and quantitation of sewage sludge proteins is a prerequisite to evaluating their role as labile C and N sources in sludge-amended soil. The objective of this study was to develop extraction methodology for the routine quantitation of sewage sludge proteins. Seven sewage sludges were obtained and prepared by oven drying at 55 °C followed by extensive grinding and mixing to produce homogenous samples. Proteins were extracted using H_2O, 10% (v/v) Triton X-100 (a non-ionic detergent), and 1 M NaOH, and they were analyzed using the Lowry protein assay with bovine serum albumin standards. Protein contents of extracts ranged from (in g kg^{-1}, dry wt. basis): (i) H_2O, 1.3 to 24; (ii) 10% Triton X-100, 3.6 to 59; and (iii) 1 M NaOH, 53 to 280. Water and detergent-extractable proteins were less than, and base-extractable proteins were within, literature values reported for total sludge proteins. The coefficients of variation (CV) for each extractant and all sludges were, with one exception, below 6%. The three methodologies provided precise and reproducible data for the extraction of sewage sludge proteins. The detergent extraction procedure is recommended for routine use because of its shorter extraction time.

T HE ANALYSIS of specific sewage sludge constituents is necessary for determining their role as labile substrates for microbial growth and their potential as plant nutrient sources. Sludge proteins represent a constituent with implications to microbial growth and activity (Hattori, 1988; Boyle and Paul, 1989) as well as plant N nutrition (Barbarika et al., 1985; Hattori and Mukai, 1986). Proteins comprise 9.8 to 56.1% of sewage sludges (Hattingh et al., 1967; Siebert and Toerien, 1969; Hattori and Mukai, 1986), and they are easily degraded by soil microorganisms (Alexander, 1977; Loll and Bollag, 1983; Hattori, 1988). In addition, there are large populations of proteolytic microorganisms in soils (Alexander, 1977; Hankin and Hill, 1978; Loll and Bollag, 1983) and in anaerobically digested sludges (Siebert and Toerien, 1969; Hobson et al., 1974) suggesting that proteins are important substrates for microbial growth.

In order to study the effect of sludge proteins on microbial processes (such as C and N mineralization in sludge-amended soils), successful isolation of the proteins is required for quantitation and characterization. Macromolecular sludge or soil components are generally extracted to facilitate quantitation (Hattingh et al., 1967; Ladd, 1972; Nannipieri et al., 1980; Rudd et al., 1983). For example, protein content of sludges has been determined following KOH extraction (Hattingh et al., 1967; Kotze et al., 1968). However, the protein content of sludges (and other materials) is generally estimated by multiplying the sludge organic N content (Bremner and Mulvaney, 1982) by 6.25 (Hattori and Mukai, 1986; Helrich, 1990). This factor is based on the assumption that protein contains an average of 16% N. However, the validity of this factor has not been established for sludge proteins. Hattingh et al. (1967) provided evidence for a factor of 3.9 as the protein/organic N ratio in seven anaerobic sludges. Furthermore, sludges have been shown to contain nonproteinaceous organic N such as amino acids, amino sugars and other unidentified forms (Sommers et al., 1972; Ryan et al., 1973).

Brown and Lester (1980) evaluated six different techniques for the extraction of extracellular macromolecules from activated and synthetic-activated sludges. They reported that 2 M NaOH or 2% ethylenediaminetetraacetic acid (EDTA) were the most effective protein extracting agents. Rudd et al. (1983) also found that 2 M NaOH was an effective protein extracting agent; in addition, they reported that a cation exchange resin, Dowex 50-X8, Na^+ form (BDH Chemicals, Ltd., Poole, UK), resulted in high protein recoveries from activated sludge. The extraction with 2 M NaOH and EDTA probably lysed intact cells, which was inconsistent with the objective of obtaining extracellular proteins; therefore, these methods were not pursued further for routine use. Currently, no standard extraction procedures exist for proteins contained in aerobically or anaerobically digested sewage sludge. Therefore, the objective of this study was to develop a method for the routine extraction and quantitation of sewage sludge proteins.

MATERIALS AND METHODS

Sewage Sludges

Sewage sludges were obtained from six municipalities in the Rocky Mountain Region: (i) Aspen, CO (ASP); (ii) Cheyenne, WY (CHY); (iii) Denver, CO (DEN); (iv) Ft. Collins, CO (FTC); (v) Littleton/Englewood, CO (L/E); and (vi) Rock Springs, WY (RSP). One municipal sludge was obtained from Chicago, IL (CHI) in 1978 as a part of a national study (Sommers et al., 1991). All of the sludges were anaerobically digested except the ASP sludge (aerobically digested), and three of the seven sludges (ASP, CHY, and L/E) received polymer flocculents to aid the dewatering process. Also the CHY, CHI, and RSP sludges had been stored for 0.5, 12, and 1 yr, respectively, under conditions permitting microbial activity. The storage conditions involving stockpiling of moist sludge in fields for CHY and RSP, and the CHI sludge was stored moist in a closed 208-L container. Due to the lengthy storage time and high C/N ratio of the organic matter, the CHI sludge was assumed (on the basis that proteins are labile microbial substrates) to have low protein levels.

R.N. Lerch, Cropping Systems and Water Quality Research Unit, USDA-ARS, Columbia, MO 65211; K.A. Barbarick, Dep. of Agronomy, P. Azari, Dep. of Biochemistry, L.E. Sommers and D.G. Westfall, Dep. of Agronomy, Colorado State Univ., Ft. Collins, CO 80523. Received 5 Sept. 1991. *Corresponding author.

Abbreviations: CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; ASP, Aspen, CO; CHY, Cheyenne, WY; DEN, Denver, CO; FTC, Fort Collins, CO; L/E, Littleton/Englewood, CO; RSP, Rock Spring, WY; CHI, Chicago, IL; ANOVA, analysis of variance; LSD, least significant differences; BSA, bovine serum albumin; EC, electrical conductivity.

Effect of Oven Drying on Sewage Sludge Proteins

The effect of varying oven-drying temperature on the H_2O-extractable proteins of the L/E sludge was evaluated at temperatures of 35, 55, and 75 °C. The L/E sludge was dried for 4 d at 35 °C, 3 d at 55 °C, and 2 d at 75 °C to produce samples of less than 5% H_2O on a dry-weight basis. Moisture content of the dried sludge was determined after additional drying for 48 h at 110 °C. Further sample preparation was as described below. For each drying temperature, four representative 20- to 40-g samples were chosen. Each sample was divided into 48 subsamples (16 observations × 3 temperature treatments) for subsequent H_2O extraction. The H_2O-extraction procedure was the same as that described in the Protein Extraction Procedure section, except the shaking time was 20 h rather than 16 h. The longer shaking time represented an arbitrary choice because the optimum shaking time had not yet been determined.

One-way analysis of variance (ANOVA) was used to compare within and between temperature treatment means. Least significant differences (LSD) were calculated for cases in which the F statistic was significant at $P \leq 0.01$.

Sewage Sludge Preparation

All sludges were oven dried at 55 °C for 2 to 3 d. The moisture content of the sludges, after oven drying, were generally less than 5% on a dry weight basis (as described above). Oven-dried sludge was ground (with a stainless steel electric grinder) to pass a 2-mm sieve, followed by grinding with a mortar and pestle for 3 to 5 min. The sludge was then ground further in a ball mill for 20 to 24 h. Such rigorous grinding techniques were required for maximum cell disintegration and protein release as well as to ensure sample homogeneity. Such conditions are generally recommended for the isolation of proteins from plant, animal, and microbial cells (Scopes, 1981). The prepared sludge was divided into 20- to 40-g samples using a sample splitter, placed in plastic freezer bags, and stored at −20 °C. Storage at low temperature was required to prevent microbial degradation of the proteins (Lerch, 1991).

Characterization of Sludge Chemical Composition

For all analyses, the mass of sludge used is expressed on a dry-weight basis. The organic C content of the sludges was determined using 0.25-g samples by the modified Mebius procedure (Nelson and Sommers, 1982). Sludge total N content was determined with the Kjeldahl method (Bremner and Mulvaney, 1982) using 0.25 g sludge, about 2 g of K_2SO_4–catalyst mixture, and 5 mL concentrated H_2SO_4. The digests were heated at 225 °C for 1 h, and then at 350 °C for 6 to 8 h. The digested solutions were diluted to a volume of 75 mL with distilled–deionized water and then analyzed for NH_4^+ using a Technicon Autoanalyzer II (Tarrytown, NY). Sludge NH_4–N was determined by extracting 0.25 to 5.0 g of sludge with 50 mL of 2 M KCl (Bremner and Mulvaney, 1982) followed by NH_4^+ analysis using the Technicon Autoanalyzer II. Organic N was calculated by subtracting NH_4–N from total N. The NO_3–N concentration of the sludges was determined by extracting 0.30 to 5.0 g of sludge with 50 mL of a 0.01 M $CuSO_4$/0.001 M Ag_2SO_4 solution followed by colorimetric analysis using the NAS Szechrome procedure (Polysciences, Warrington, PA; based on a similar azo dry procedure by Szekely, 1968). The limit of sensitivity was 0.1 mg NO_3–N L^{-1}. The P, Cd, Cu, Ni, Pb, and Zn concentrations of the sludges were determined by digesting 0.25-g samples in 10 mL of concentrated HNO_3 followed by analysis using inductively coupled plasma–atomic emission spectroscopy (Havlin and Soltanpour, 1980). The pH was determined in 1:3 (sludge/water) pastes, and the EC was determined in the paste extracts. All analyses for sludge composition were performed in triplicate, and the CV for all analyses ranged from 0.4 to 19% (Table 1).

Protein Extraction Procedure

Either 1.00 g (dry wt.) sludge for H_2O and detergent extractions or 0.25 g (dry wt.) sludge for the NaOH extractions was added to a 50-mL polypropylene centrifuge tube. The sludges were treated with 25 mL of extractant and 0.125 mL $CHCl_3$ (except detergent extracts because of precipitation of the detergent upon $CHCl_3$ addition). The $CHCl_3$ was added to facilitate cell lysis, releasing intracellular proteins into solution, and to prevent microbial growth during extraction. The tubes were sealed and shaken horizontally on an Eberbach (Ann Arbor, MI) shaker with a stroke length of 4 cm and a shaking speed of 180 hz. The shaking times for the three extractants were 16 h for H_2O, 6 h for 10% (v/v) Triton X-100 polyoxyethylene(9.5)p-t-octyl-phenol (Sigma Chemical Co., St. Louis, MO), and 12 h for 1 M NaOH. The optimal shaking times, speeds, and extractant concentrations were determined by preliminary experiments (Lerch, 1991). Following extraction, the samples were centrifuged at about 10 000 × g for 30 min, and the supernatant was analyzed for protein. Samples not immediately analyzed were stored at 2 to 4 °C for 12 to 16 h.

Protein Assay

The Lowry assay (Lowry et al., 1951) was used for all protein measurements. This assay was chosen because of sensitivity, common use, and because known interferences were not expected to be present at significant levels in sludges (Ji, 1973; Bensadoun and Weinstein, 1976). Compared with the Coomassie Blue G-250 dye-binding assay (Bradford, 1976), the Lowry assay resulted in 4.3 times higher H_2O-extractable protein concentrations with one-third the variability (Lerch, 1991). The dye-binding assays are currently not considered as acceptable replacements to the Lowry assay due to the lower stability of the final color, greater variation in extinction with different proteins, and nonlinearity of standard curves with some proteins (Peterson, 1979). The protein reference standard was bovine serum albumin (BSA), fraction V (U.S. Biochemical Co. Cleveland, OH). The choice of BSA was arbitrary; however, it has been chosen commonly as a standard for protein measurements (Lowry et al., 1951; Ji, 1973; Bradford, 1976; Bensadoun and Weinstein, 1976) due to its molecular weight of 66 000, which is considered to be an average-sized protein. Standards were prepared (in the appropriate extracting agents) over a concentration range of 100 to 1500 mg L^{-1}. To prevent interferences from Triton X-100, the Lowry assay was modified. One milliliter of 10% sodium dodecyl sulfate was added to the reaction mixture to displace Triton X-100 from the proteins (Ji, 1973). Expression of protein concentration was, for all cases, in grams protein per kilogram dry sludge. The data were analyzed by two-way ANOVA as a seven (sludges) by three (extractants) factorial experiment. Comparison of extractable proteins within each sludge and each extractant were analyzed by one-way ANOVA. The LSD for mean comparisons were calculated at $P \leq 0.01$.

RESULTS AND DISCUSSION

Sewage Sludge Composition

The compositions of the sludges generally were within ranges reported for other aerobically and anaerobically digested sludges (Sommers, 1977) (Table 1). The only exception was the organic C levels of the sludges, which tended to be higher than the median (268 g kg^{-1}) levels reported by Sommers (1977). The metal levels of the sludges were generally below median levels reported by Sommers (1977) except for the CHI sludge. The pH and electrical conductivity (EC) values of the sludges ranged from 5.7 to 6.8 and 3.7 to 8.8 dS m^{-1}, respectively.

Table 1. Composition of sewage sludges.

Sludge	Organic C	Organic N	C/N	NH$_4$–N	NO$_3$–N	P	Cd	Cu	Ni	Pb	Zn
	g kg^{-1}†				g kg^{-1}				mg kg^{-1}		
ASP	420	73	5.8	3.2	<0.001	16	4.4	1000	16	60	870
	(0.8)‡	(3.1)		(3.9)		(4.5)	(3.7)	(6.1)	(2.3)	(1.1)	(9.7)
CHY	420	32	13	3.7	<0.001	19	13	1100	34	260	1500
	(2.8)	(7.8)		(3.8)		(2.0)	(1.2)	(2.4)	(1.2)	(1.8)	(2.0)
CHI	430	16	27	0.03	1.0	14	250	1700	670	1200	4900
	(3.6)	(3.2)		(12.1)	(5.2)	(5.0)	(0.9)	(0.9)	(0.9)	(0.9)	(1.0)
DEN	380	60	6.4	3.7	<0.001	16	18	610	70	200	1400
	(16.3)	(1.6)		(18.7)		(13.8)	(1.6)	(6.8)	(5.2)	(0.5)	(13.4)
FTC	430	67	6.4	5.5	<0.001	20	7.4	940	32	210	870
	(4.8)	(1.5)		(2.6)		(5.8)	(3.8)	(4.3)	(2.4)	(3.6)	(2.5)
L/E	220	54	4.1	3.4	<0.001	72	10	640	97	300	1000
	(0.4)	(4.5)		(6.4)		(2.4)	(4.2)	(2.2)	(5.2)	(2.6)	(2.7)
RSP	410	26	16	3.6	0.24	16	8.8	570	47	220	920
	(8.4)	(2.9)		(3.3)	(4.2)	(4.2)	(5.4)	(3.0)	(1.3)	(1.8)	(1.5)

† Concentrations expressed on a dry wt. basis.
‡ Numbers in parentheses are CV (%).

Sludge Preparation—Effect of Oven-Drying Temperature

Water extraction was chosen to evaluate the effect of oven-drying temperature on extractable sludge proteins because it was the mildest extractant and would not induce protein denaturation. Therefore, in addition to assessing reproducibility, any loss of protein solubility due to temperature denaturation could be evaluated. One sludge (L/E) was assessed, and the effect of drying temperature on sludge proteins was assumed to be similar for all sludges. Extractable proteins showed a decrease with increasing temperature (Table 2). The mean H$_2$O extractable proteins of the 35 °C treatment (10.6 g kg^{-1}) was significantly higher ($P < 0.001$) than the 55 °C (8.9 g kg^{-1}) and 75 °C (8.7 g kg^{-1}) treatments. Within each temperature, there were no significant differences in the water-extractable proteins at 55 °C, but the 35 and 75 °C temperature treatments both showed differences ($P < 0.001$ for each treatment). The range of the means was lowest for the 55 °C treatment (8.7–9.0 g kg^{-1}) and greatest for the 35 °C treatment (10.0–11.3 g kg^{-1}).

There are two possible explanations for the observed decrease in H$_2$O-extractable proteins with increasing temperature. One possibility was protein denaturation resulting from the 55 and 75 °C drying temperatures. While the H$_2$O solubility of the proteins was apparently af-

fected, denaturation at these temperatures would not be sufficient to cleave peptide bonds. Cleavage requires boiling in 6 M HCl for an extended period of time (Zubay, 1983). The other possibility was that microbial growth occurred during the 4-d time period required for drying at 35 °C and produced greater amounts of protein. Since 35 °C is at or near the temperatures used for the aerobic or anaerobic sludge digestion, it was likely that some microbial growth, and protein synthesis, occurred in this treatment. This possibility, combined with the irreproducible protein measurements associated with the 35 and 75 °C treatments, led to the conclusion that 55 °C was the best drying temperature for sludge preparation. Some loss of protein solubility due to denaturation at 55 °C was considered an acceptable alternative to irreproducible data.

Protein Extractants

While H$_2$O has not been used previously for protein extraction, it was considered desirable to use a mild extractant to provide a contrast to the more rigorous detergent and base extractants. Water was also expected to dissolve the most soluble and, perhaps, most labile proteins with respect to microbial degradation. The nonionic detergent Triton X-100 has been employed commonly for the isolation and purification of proteins from a variety of animal, plant, and microbial sources (Scopes, 1981, p. 1–71). Thus, its application to sludges seemed logical due to the assortment of plant, animal, and bacterial proteins in digested sludges (Hobson et al., 1974). Furthermore, the amphoteric nature of detergents provided the capability of dissolving hydrophobic membrane proteins, water-soluble proteins, and denatured proteins. Lastly, NaOH was used because of its ability to dissolve large quantities of organic compounds, including proteins, in soils (Schnitzer, 1982) and to provide an estimate of total sludge proteins.

Table 2. The effect of oven-drying temperature as a sludge pretreatment on the reproducibility of H$_2$O-extractable protein in the L/E sludge.

Sample no.†	Drying temperature			Across temperature LSD
	35 °C	55 °C	75 °C	
	g kg^{-1}‡			
1	10.0 (3.9)§	8.8 (4.7)	8.9 (2.2)	
2	10.4 (2.7)	8.7 (6.1)	8.8 (2.2)	
3	11.3 (4.9)	9.0 (4.2)	8.5 (2.2)	
4	10.8 (3.6)	9.0 (5.8)	8.6 (2.2)	
Mean	10.6 (6.0)	8.9 (5.3)	8.7 (2.9)	0.2
Within temperature LSD	0.4	NS¶	0.2	

† Each sample was divided into 48 subsamples (i.e., $n = 16$ for each treatment mean).
‡ Concentration is expressed on a dry wt. basis.
§ Numbers in parentheses are CV (%).
¶ NS = nonsignificant ($P = 0.28$).

Extractable Sludge Proteins

The amounts of protein extracted, averaged over the sludges, varied significantly ($P < 0.001$) according to the extractant used, decreasing in the order base > detergent > H$_2$O (Table 3). Within each sludge, only the H$_2$O and detergent-extractable proteins of the CHI sludge were not different at the 1% significance level. Within

Table 3. Concentrations of sewage sludge proteins determined by water extraction for 16 h, 10% Triton X-100 for 6 h, and 1 M NaOH for 12 h.

Sludges	Extractants			Mean	Within sludge LSD
	Water	Triton X-100	1 M NaOH		
	g kg^{-1}†				
ASP	24 (3.8)‡	47 (0.6)	280 (5.5)	117	17
CHY	15 (1.4)	38 (1.9)	160 (4.3)	71	7.9
CHI	1.3 (5.7)	3.6 (20.2)	53 (3.9)	19	2.4
DEN	24 (2.1)	59 (1.0)	240 (1.0)	108	2.7
FTC	19 (1.9)	50 (0.4)	270 (2.3)	113	7.0
L/E	10 (5.2)	30 (1.7)	110 (2.5)	50	3.2
RSP	4.9 (4.9)	21 (3.4)	96 (0.6)	41	1.0
Mean	14	36	173	74	
Within extractant LSD	1.4	1.7	21		

† Concentrations expressed on a dry wt. basis.
‡ $n = 3$ for means within each sludge and extractant; numbers in parentheses represent CV (%).

each extractant, extractable proteins were generally significantly different between all sludges. Exceptions were the water-extractable proteins of the ASP and DEN sludges, the base-extractable proteins of ASP and FTC sludges, and the base-extractable proteins of the L/E and RSP sludges. The ASP, FTC, and DEN sludges exhibited the highest average extractable proteins (117, 113, and 108 g kg^{-1}, respectively) while the CHI sludge exhibited the lowest (19 g kg^{-1}). A sludge by extractant interaction occurred ($P < 0.001$) due, primarily, to the ASP, DEN, and FTC sludges which changed rank within each extractant, but the large increase in base-extractable proteins of the CHI sludge, relative to its H$_2$O- and detergent-extractable levels, also contributed to the interaction. The absence of a standard method for protein determination of sludges prohibits any statements concerning the accuracy of these methods. However, the CV were below 6% for all sludges and extractions, except for the detergent-extractable proteins of the CHI sludge (Table 3), indicating that the methods employed for protein extraction were precise.

There were no comparable literature values for the H$_2$O and detergent extractable proteins in sludges. However, they were much lower than estimates of total sludge protein content (Hattingh et al., 1967; Siebert and Toerien, 1969; Hobson et al., 1974; Hattori and Mukai, 1986). Base-extractable sludge proteins (Table 3) were generally within the range of values reported by other researchers. Hattingh et al. (1967) estimated protein concentrations of seven anaerobic sludges by acid hydrolysis followed by ninhydrin reaction of liberated amino acids at 183 to 337 g kg^{-1} on a dry weight (dry wt.) basis. Kotze et al. (1968), using this same procedure, estimated the protein content of anaerobically digested sewage sludge to be 130 g kg^{-1} dry wt. Siebert and Toerien (1969) cited several studies in which total protein estimates of digested sludge ranged from 125 to 197 g kg^{-1} dry wt.; and, more recently, Hattori and Mukai (1986) estimated the protein content of six sludges to be from 98 to 561 g kg^{-1} dry wt. Therefore, the base extractable protein data presented in this study were generally within the range of reported literature estimates for total sludge protein.

The low extractable protein levels of the CHI sludge

were consistent with the assumption that the long storage time (about 12 yr prior to acquisition) and high C/N ratio would be indicative of low protein concentration and a lack of labile C and N. The low labile C and N concentrations of this sludge were evident from 12-wk soil incubations that resulted in only 2% of organic C mineralized and net immobilization of organic N (Lerch et al., 1992). The other sludges which had been stored (CHY and RSP) also exhibited lower extractable protein levels than three of the four sludges that were not stored.

Recommended Protein Extraction Method

Choice of a recommended method for protein extraction from sludge was based on the following four criteria: (i) relationship of the results to microbial processes (specifically, C and/or N mineralization in sludge-amended soil), (ii) the precision of the results, (iii) practicality as a routine method, and (iv) ease of protein characterization.

The first two criteria were satisfied by all three methods. The extractable sludge proteins were correlated to cumulative, sludge C mineralized in 12-wk soil incubations—r^2 of protein concentration vs. C mineralized was: base, 0.94**; detergent, 0.96**; and H$_2$O, 0.96** (** = statistical significance at $P < 0.01$ level of probability) (Lerch et al., 1992). Based on the high correlation to sludge C mineralization, all three extractants evidently recovered a significant portion of the labile proteins contained in the sludges. As previously mentioned, all three methods were precise for all sludges and extractions, except the detergent extraction of the CHI sludge. Thus, there was no basis for differentiating between the procedures using the first two criteria.

The H$_2$O extraction procedure required the longest extraction time (16 h), and the low levels of extractable proteins may preclude use of this procedure with some sludges. While characterization of these proteins is possible, a large volume of extract is required because of low concentrations. The base extraction procedure requires a lengthy extraction (12 h), but the high protein recovery more easily facilitated characterization studies than the H$_2$O extracts. However, base extraction apparently resulted in some peptide bond, amino acid–N, and amide–N hydrolysis based on the appearance of hydrolyzed N in the extracts and the disappearance of high molecular weight proteins in comparison to the H$_2$O extracts (Lerch, 1991; Lerch et al., 1993). The detergent extraction procedure required the least amount of time (6-h extraction), and it was the only procedure that allowed for extraction and analysis in the same day. Protein characterization was, however, prohibited by Triton X-100 because available chromatography stationary phases for detergent removal from proteins do not quantitatively recover low molecular weight proteins. Although none of the methods completely satisfied the last two criteria, detergent extraction was the most practical as a routine method. Based on the stated objective of the paper, the practicality of the method was given the most weight. Therefore, detergent extraction is recommended as a routine sewage sludge protein extraction method.

REFERENCES

Alexander, M. 1977. Introduction to soil microbiology. 2nd ed. p. 246–249. John Wiley & Sons, New York.

Barbarika, A., L.J. Sikora, and D. Colacicco. 1985. Factors affecting the mineralization of nitrogen in sewage sludge applied to soils. Soil Sci. Soc. Am. J. 49:1403–1406.

Boyle, M., and E.A. Paul. 1989. Carbon and nitrogen mineralization kinetics in soil previously amended with sewage sludge. Soil Sci. Soc. Am. J. 53:99–103.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Bremner, J.M., and C.S. Mulvaney. 1982. Nitrogen—total. p. 610–616. In A.L. Page et al. (ed.) Methods of soil analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Brown, M.J., and J.N. Lester. 1980. Comparison of bacterial extracellular polymer extraction methods. Appl. Environ. Microbiol. 40:179–185.

Hankin, L., and D.E. Hill. 1978. Proportion of bacteria in agricultural soils able to produce degradative enzymes. Soil Sci. 126:40–43.

Hattingh, W.H.J., P.G. Thiel, and M.L. Siebert. 1967. Determination of protein content of anaerobic digesting sludge. Water Res. 1:185–189.

Hattori, H. 1988. Microbial activities in soil amended with sewage sludge. Soil Sci. Plant Nutr. 34:221–232.

Hattori, H., and S. Mukai. 1986. Decomposition of sewage sludges in soil as affected by their organic matter composition. Soil Sci. Plant Nutr. 32:421–432.

Havlin, J.L., and P.N. Soltanpour. 1980. A nitric acid plant tissue digest method for use with inductively coupled plasma spectrometry. Commun. Soil Sci. and Plant Anal. 11:969–980.

Helrich, K. (ed.) 1990. Official methods of analysis. 15th ed. Assoc. Official Analytical Chem., Arlington, VA.

Hobson, P.N., S. Bousfield, and R. Summers. 1974. Anaerobic digestion of organic matter. CRC Crit. Rev. Environ. Control 4:131–191.

Ji, T.H. 1973. Interference by detergents, chelating agents, and buffers with the Lowry protein determination. Anal. Biochem. 52:517–521.

Kotze, J.P., P.G. Thiel, D.F. Toerien, W.H.J. Hattingh, and M.L. Siebert. 1968. A biological and chemical study of several anaerobic digesters. Water Res. 2:195–213.

Ladd, J.N. 1972. Properties of proteolytic enzymes extracted from soil. Soil Biol. Biochem. 4:227–237.

Lerch, R.N. 1991. Characterization of sewage sludge proteins. Ph.D. diss. Colorado State Univ., Fort Collins (Diss. Abstr. 92–07333).

Lerch, R.N., K.A. Barbarick, L.E. Sommers, and D.G. Westfall. 1992. Sewage sludge proteins as labile carbon and nitrogen sources. Soil Sci. Soc. Am. J. 56:1470–1476.

Lerch, R.N., P. Azari, K.A. Barbarick, L.E. Sommers, and D.G. Westfall. 1993. Sewage sludge proteins: II. Extract characterization. J. Environ. Qual. 22:625–629 (this issue).

Loll, M.J., and J. Bollag. 1983. Protein transformations in soil. Adv. Agron. 36:351–382.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

Nannipieri, P., B. Ceccanti, S. Cervelli, and E. Matarese. 1980. Extraction of phosphatase, urease, proteases, organic carbon, and nitrogen from soil. Soil Sci. Soc. Am. J. 44:1011–1016.

Nelson, D.W. and L.E. Sommers. 1982. Total carbon, organic carbon, and organic matter. p. 571–572. In A.L. Page et al. (ed.) Methods of soil analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Peterson, G.L. 1979. Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr, and Randall. Anal. Biochem. 100:201–220.

Rudd, T., R.M. Sterritt, and J.N. Lester. 1983. Extraction of extracellular polymers from activated sludge. Biotechnol. Lett. 5:327–332.

Ryan, J.A., D.R. Keeney, and L.M. Walsh. 1973. Nitrogen transformations and availability of an anaerobically digested sewage sludge in soil. J. Environ. Qual. 4:489–492.

Schnitzer, M. 1982. Organic matter characterization. p. 581–594. In A.L. Page et al. (ed.) Methods of soil analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Scopes, R.K. 1981. Protein purification: Principles and practices. 2nd ed. p. 1–71. Springer-Verlag, New York.

Siebert, M.L., and D.F. Toerien. 1969. The proteolytic bacteria present in the anaerobic digestion of raw sewage sludge. Water Res. 3:241–250.

Sommers, L.E. 1977. Chemical composition of sewage sludges and analysis of their potential use as fertilizers. J. Environ. Qual. 6:225–232.

Sommers, L.E., D.W. Nelson, J.E. Yahner, and J.V. Mannering. 1972. Chemical composition of sewage sludges from selected Indiana cities. Proc. Indiana Acad. Sci. 82:424–432.

Sommers, L.E., A.L. Page, T.J. Logan, and J.A. Ryan. 1991. Optimum use of sewage sludge on agricultural land. Western Regional Res. Publ. W-124. Colorado State Univ. Agric. Exp. Stn.

Szekely, E. 1968. Colorimetric determination of nitrites with p-diaminodiphenylsulphone-diphenylamine as reagent. Talanta 15:795–801.

Zubay, G. 1983. Biochemistry. p. 40. The Benjamin/Cummings Publ. Co., Menlo Park, CA.

Sewage Sludge Proteins: II. Extract Characterization

R. N. Lerch,* P. Azari, K. A. Barbarick, L. E. Sommers, and D. G. Westfall

ABSTRACT

The characterization of sewage sludge proteins is necessary to better understand their degradation by microbial populations when sludges are utilized for land application. The objective of this study was to characterize the molecular weights of extractable sewage sludge proteins by dialysis partitioning and polyacrylamide gel electrophoretic (PAGE) separation. Sewage sludges obtained from seven municipalities were extracted using H_2O and 1 M NaOH followed by purification and concentration to facilitate molecular weight determinations. Dialysis partitioning of proteins showed that the majority of these compounds had molecular weights <14 000 (i.e., they were not retained by dialysis membrane). The separation of proteins by PAGE also showed that the bulk of H_2O and 1 M NaOH soluble proteins were of low molecular weight (<17 000). This indicated considerable proteolysis during waste treatment processing. Thus, the extractable sludge proteins appeared to be primarily poly- and oligopeptides rather than intact proteins. Based on these findings, sludge proteinaceous materials would be expected to degrade rapidly in soil due to the many soil microorganisms capable of utilizing protein degradation products.

T HE CHARACTERIZATION of specific sewage sludge organic constituents is a necessary prerequisite to better understanding their transformations by microbial populations when sludges are added to soil. The importance of sludge proteins and their degradation products to C and N mineralization in sludge-amended soil has been previously demonstrated (Lerch, 1991; Lerch et al., 1992). Sewage sludge proteins extracted by H_2O, 10% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO), and 1 M NaOH were all significantly correlated (r^2 = 0.94–0.96) to C mineralization in sludge-amended soil. Low molecular weight, H_2O-extractable primary amines (presumed to be predominantly protein degradation products), combined with sludge C/N ratio, were highly correlated (r^2 = 0.91) to N mineralization in sludge-amended soil. Extraction and quantification of organic components (particularly extracellular polymers) contained in activated sludges have been extensively research because of their importance to the formation of sludge flocs and to metal complexation (Brown and Lester, 1980; Rudd et al., 1983, 1984; Kakii et al., 1986; Figueroa and Silverstein, 1989; Goodwin and Forster, 1989). The characterization of organic components extracted from digested sludge (Karapanagiotis et al., 1989) and sludge-amended soil also has been investigated (Dudley et al., 1987).

The characterization of polymeric sludge components relative to their molecular weights (MW) and chemical composition also has been pursued and indicates they are a complex chemical mixture representing a continuum of MW's (Dudley et al., 1987; Goodwin and Forster, 1989; Karapanagiotis et al., 1989). Karapanagiotis et al. (1989) reported that proteins and polysaccharides separated by gel chromatography were all greater than 5 000 MW regardless of the extraction method employed. In contrast, separation of extracted sewage sludge proteins, carbohydrates, and total organic C by membrane filtration showed that about 30 to 70% of these soluble organics were less than 500 MW (Goodwin and Forster, 1989). Dudley et al. (1987) used gel chromatography and infrared spectroscopy to examine changes in water-soluble organics from sludge-amended soils incubated over a 30-wk period. They identified a high MW fraction composed of polysaccharides, carboxylic acids, and polypeptides, and a low MW fraction composed primarily of small polypeptides and amino acids (i.e., amide-containing moieties).

A notable weakness of separating components by gel chromatography is that analysis of eluted components is based on measuring ultraviolet absorbance (typically 260 or 280 nm). Virtually any sludge extraction method will result in dissolution of humified materials, which will absorb ultraviolet light due to their aromatic nature. Therefore, MW determination of sludge components needs to be performed by more precise methods. Hence, the primary objective of this study was to characterize the MW range of extractable sewage sludge proteins by dialysis partitioning and PAGE.

MATERIALS AND METHODS

Sewage Sludges—Chemical Analyses

Sludges were collected from seven municipalities: Aspen, CO (ASP), Cheyenne, WY (CHY), Chicago, IL (CHI), Denver, CO (DEN), Fort Collins, CO (FTC), Littleton/Englewood, CO (L/E), and Rock Springs, WY (RSP). Sludge preparation was described in Lerch et al. (1993). While the 55 °C drying temperature utilized for sludge preparation likely resulted in some protein denaturation, the conditions were insufficient to cause peptide bond cleavage.

The NH_4–N in the sludges was extracted with 2 M KCl (Table 1) (Bremner and Mulvaney, 1982) and NH_4–N analyzed with a Technicon Autoanalyzer II (Tarrytown, NY). Total N (not including NO_3–N and NO_2–N) was determined by Kjeldahl analysis (Bremner and Mulvaney, 1982), and organic N was calculated as the difference between the total N and NH_4–N. Organic C of the sludges was determined by the modified Mebius procedure (Nelson and Sommers, 1982).

Sludge proteins were extracted with distilled–deionized H_2O for 16 h and 1.0 M NaOH for 12 h and were analyzed by the Lowry assay (Lowry et al., 1951) with bovine serum albumin standards. Detailed information concerning the extractions is presented in Lerch et al. (1993) and Lerch (1991). Water-soluble primary amine concentrations of the sludges (measured in the H_2O extracts discussed above for protein analysis) were measured by the trinitrobenzenesulfonic acid method of Habeeb (1966) at 345 nm (Table 1). All extractions were performed in 50-mL polypropylene centrifuge tubes using a horizontal shaker at 180 hz.

R.N. Lerch, Cropping Systems and Water Quality Research Unit, USDA-ARS, Columbia, MO 65211; P. Azari, Dep. of Biochemistry; K.A. Barbarick, L.E. Sommers, and D.G. Westfall, Dep. of Agronomy, Colorado State Univ., Ft. Collins, CO 805223. Received 5 Sept. 1991. *Corresponding author.

Table 1. Selected properties of the sewage sludges.†

Sludge	Organic N	NH_4–N‡	C/N	Primary amines§	Protein H₂O soluble	Protein 1 M NaOH soluble
	—— g kg⁻¹ ——			mg kg⁻¹	—— g kg⁻¹ ——	
ASP	73	3.2	5.8	56	24	280
	(3.1)¶	(3.9)		(0.7)	(3.8)	(5.5)
CHY	32	3.7	13	52	15	160
	(7.8)	(3.8)		(1.1)	(1.4)	(4.3)
CHI	16	0.03	27	2.4	1.3	53
	(3.2)	(12.1)		(4.7)	(5.7)	(3.9)
DEN	60	3.7	6.4	55	24	240
	(1.6)	(18.7)		(0.7)	(2.1)	(1.0)
FTC	67	5.5	6.4	54	19	270
	(1.5)	(2.6)		(0.7)	(1.9)	(2.3)
L/E	54	3.4	4.1	54	10	110
	(4.5)	(6.4)		(1.1)	(5.2)	(2.5)
RSP	26	3.6	16	50	4.9	96
	(2.9)	(3.3)		(1.1)	(4.9)	(0.6)

† All concentrations expressed on a dry wt. basis.
‡ NH_4–N determined in 2 M KCl extracts.
§ Primary amines determined in H₂O extracts.
¶ Numbers in parentheses are coefficients of variation (%).

Analytical Methods for Molecular Weight Determinations of Proteins

Protein Extraction

Sewage sludges were extracted with distilled–deionized H₂O and 1 M NaOH as described in Lerch et al. (1993) and Lerch (1991) with the following exceptions: (i) the extracts contained 2.0 g L⁻¹ NaN_3 to prevent microbial activity, instead of $CHCl_3$, because the NaN_3 could be removed by dialysis; and (ii) following centrifugation, the 1 M NaOH supernatant solutions were neutralized to pH 7.0 (± 0.1) to prevent hydrolysis of the cellulose membrane during dialysis.

Protein Partitioning into High and Low Molecular Weight Fractions

To facilitate PAGE analysis and MW partitioning, purification and concentration of the proteins in the extracts was performed by dialysis (cellulose membrane with exclusion limit of 14 000 MW) against 12 to 16 L of deionized H₂O at 2 to 4 °C for 3 d (Fig. 1). The high MW fraction (HMW_f) (i.e., the material retained in the dialysis membrane) was then freeze dried, yielding 0.275 to 2.78 mg freeze-dried extract per milliliter dialysate for the 1 M NaOH extracts and 0.075 to 1.53 mg freeze-dried extract per milliliter dialysate for the H₂O extracts (Lerch, 1991). Protein in the extracts was measured before and after dialysis, providing the total extractable (Table 1) and HMW_f proteins, respectively. The low molecular weight fraction (LMW_f) proteins were calculated as the difference in concentration between the total and HMW_f.

Polyacrylamide Gel Electrophoresis

The specific procedure used was discontinuous sodium dodecyl sulfate (SDS)-PAGE (Laemmli, 1970; Brewer et al., 1974, p. 128–160; Andrews, 1986), which employs two different acrylamide gels (stacking and separating gels). The stacking gel was composed of 50 g L⁻¹ total acrylamide (T) of which 4.5 g L⁻¹ was bisacrylamide [referred to as cross-linking concentration (C)] and a pH of 6.8. For the separating gel, the acrylamide composition was 150 g L⁻¹ T and 13.5 g L⁻¹ C with a pH of 8.8 to 8.9. Ammonium persulfate and N,N,N',N' tetramethylethylenediamine were used to catalyze gel polymerization.

All electrophoresis work was conducted using the dual minivertical electrophoresis system (Fisher Scientific, Springfield, NJ) with gel dimensions of 8 by 7 by 0.075 cm. All PAGE
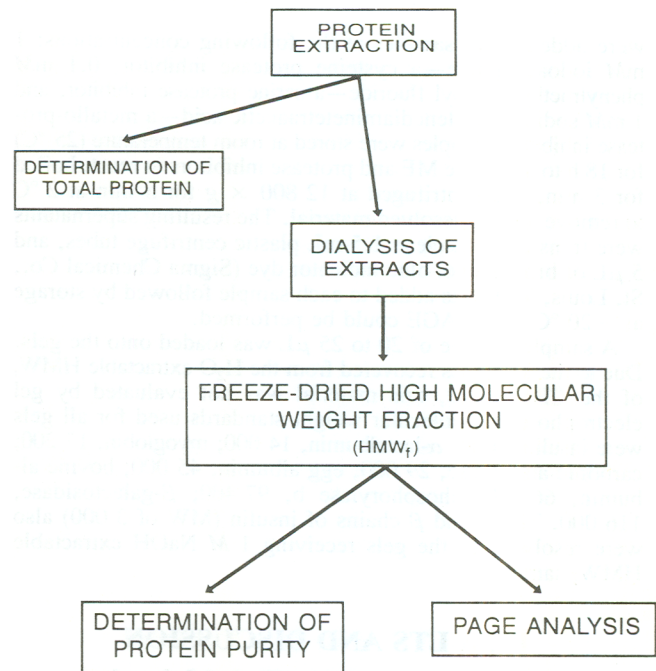

Fig. 1. Sequence of procedures and analyses for characterization of sewage sludge proteins extracted by H₂O or 1 M NaOH.
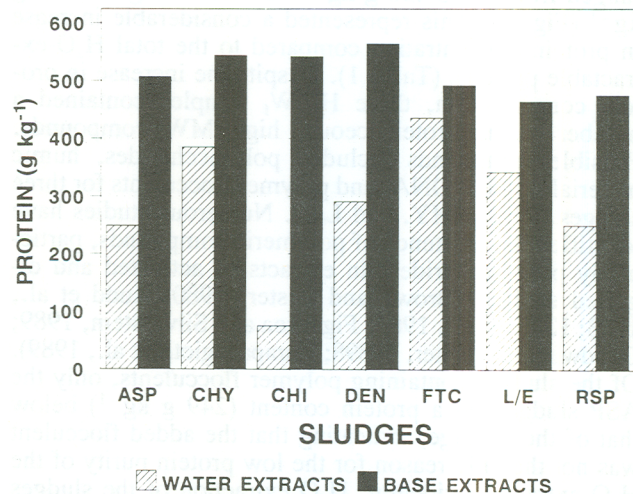

Fig. 2. Protein purity of the HMW_f samples from H₂O or 1 M NaOH sewage sludge extractions (data expressed as grams of protein per kilogram freeze-dried material).

analyses were conducted with an electrical potential of 120 V. The proteins were stained using a solution containing 2.0 g L⁻¹ Coomassie blue dye (Sigma Chemical Co., St. Louis, MO), in 500 g L⁻¹ CH_3OH and 100 g L⁻¹ CH_3COOH.

All sludge samples analyzed by PAGE were the HMW_f dissolved in buffer. Using the known HMW_f protein content (Fig. 2), an appropriate mass of HMW_f material was measured so that the samples contained equal amounts of protein. The 1 M NaOH extractable HMW_f samples contained 10 mg protein per milliliter of buffer, and the H₂O-extractable HMW_f samples contained 5 mg protein per milliliter of buffer. The buffer composition was: 0.125 M tris(hydroxymethyl)aminomethane, pH 6.8; 20 g L⁻¹ SDS; 100 g L⁻¹ sucrose; 50 g L⁻¹ 2-mercaptoethanol (ME).

To prevent endogenous protease activity, protease inhibitors

were added to all samples at the following concentrations: 1 mM iodoacetamide—a cysteine protease inhibitor, 0.1 mM phenylmethylsulfonyl fluoride—a serine protease inhibitor, and 1 mM sodium ethylenediaminetetraacetic acid—a metallo-protease inhibitor. Samples were stored at room temperature (25 °C) for 18 h to allow the ME and protease inhibitors to react, boiled for 3 min, and centrifuged at 12 800 × g for 5 min at 2 °C to remove any undissolved material. The resulting supernatants were transferred to clean 1.5-mL plastic centrifuge tubes, and 5 μL of bromphenol blue indicator dye (Sigma Chemical Co., St. Louis, MO) was added to each sample followed by storage at −20 °C until PAGE could be performed.

A sample volume of 20 to 25 μL was loaded onto the gels. Due to the low mass recovered from the H_2O extractable HMW$_f$ of the CHI sludge, this material was not evaluated by gel electrophoresis. Molecular weight standards used for all gels were insulin, 8000; α-lactalbumin, 14 000; myoglobin, 17 200; carbonic anhydrase, 29 000; egg albumin, 45 000; bovine albumin, 66 000; phosphorylase b, 97 400; β-galactosidase, 116 000. The α and β chains of insulin (MW of 3 000) also were resolved for the gels receiving 1 M NaOH extractable HMW$_f$ samples.

RESULTS AND DISCUSSION

Protein Purity of the High Molecular Weight Fraction

The protein content of the H_2O-extractable HMW$_f$ ranged from 75 to 436 g kg^{-1} with an average of 290 g kg^{-1} (Fig. 2). This represented a considerable increase in protein concentration compared to the total H_2O-extractable proteins (Table 1). Despite the increase in protein concentration, these HMW$_f$ samples contained a number of nonproteinaceous, high MW compounds. Possible compounds included polysaccharides, humic materials, RNA, DNA, and polymer flocculents for three sludges (ASP, CHY, and L/E). Numerous studies have identified the existence of polymeric compounds, particularly polysaccharides, in extracts of activated and digested sludges (Brown and Lester, 1980; Rudd et al., 1983; Kakii et al., 1986; Figueroa and Silverstein, 1989; Goodwin and Forster, 19898; Karapanagiotis et al., 1989). Of the sludges containing polymer flocculents, only the ASP sludge had a protein content (249 g kg^{-1}) below that of the average, indicating that the added flocculent was not the only reason for the low protein purity of the H_2O extracts. Evidently, H_2O extraction of the sludges dissolved other nonproteinaceous polymers.

The protein content of the 1 M NaOH-extractable HMW$_f$ ranged from 464 to 564 g kg^{-1} with an average of 513 g kg^{-1} (Fig. 2). The higher protein purity of these samples, compared to the H_2O-extractable HMW$_f$, reflected the greater quantity of proteins dissolved by NaOH. However, there was still a large amount of nonproteinaceous material contained in these extracts. The presence of humified materials in these extracts would be expected because of their solubility in NaOH (Schnitzer, 1982) and the observed brown color of the extracts. Furthermore, two of the three sludges treated with polymer flocculents contained lower than average protein contents, suggesting that the flocculents may have comprised a significant proportion of the dissolved components in these samples. Despite the impurities contained in the HMW$_f$, sample preparation by dialysis and freeze drying offered the distinctive advantages of isolation and concentration of a specific protein fraction essentially devoid of any low MW compounds and ions. This was requisite for successful PAGE separation of extractable sludge proteins.

Dialysis Partitioning of Extractable Proteins

The average proportion of H_2O-extractable proteins in the LMW$_f$ was 67% with a range of 54 to 90% (Fig. 3). The CHI and ASP sludges had the greatest proportion of H_2O-soluble LMW$_f$ proteins (greater than 80%) while the LMW$_f$ of the CHY, DEN, FTC, and RSP sludges were all between 54 and 60%. A large proportion of the proteins dissolved by H_2O extraction, therefore, were generally not retained by the dialysis membrane. Since the vast majority of proteins are of high MW (>14 000), this would indicate that the bulk of H_2O-extractable proteins were poly/oligopeptide fragments rather than intact proteins. Thus, the majority of the H_2O-soluble proteins were apparently degradation products resulting from proteolysis during the sludge treatment process. This is consistent with the large populations of proteolytic bacteria reported to exist in anaerobic digesters (Siebert and Toerien, 1969; Hobson et al., 1974).

Dialysis partitioning of the 1 M NaOH-extractable proteins showed only a slightly smaller proportion of LMW$_f$ proteins than the H_2O extracts (Fig. 4). However, NaOH extracted much greater quantities of these proteins than H_2O (Table 1). The proportion of base-soluble LMW$_f$ proteins averaged 60% of the total extracted protein with a range of 49 to 72%. Values of LMW$_f$ proteins were greatest for the CHI, CHY, and L/E sludges; only the FTC sludge had less than 50% in the LMW$_f$. For the 1 M NaOH extracts, the LMW$_f$ likely represented polypeptide fragments from the combination of degraded proteins during waste treatment and from alkaline hydrolysis of intact proteins during extraction (discussed further below). Despite the potential for hydrolysis of peptide bonds, five of the seven sludges did show a greater proportion of base-soluble HMW$_f$ proteins compared to the corresponding H_2O extracts.

PAGE Separation of Extractable Sludge Proteins

Analysis of the H_2O-extractable HMW$_f$ proteins by PAGE separation showed that the DEN, FTC, L/E, and RSP sludges contained proteins in two distinct MW ranges (Fig. 5). For all four sludges, the higher MW proteins were generally concentrated around 29 000. The DEN and FTC sludges also had some higher MW proteins as large as 66 000. The other band of proteins in these four sludges had quite low MW that covered the range from <8 000 to a maximum of about 17 000. The CHY sludge showed a distinct band of proteins in the low MW range, but the ASP sludge displayed only a very faint band at approximately 8 000 (not discernible in the photo). Since all the samples contained equal quantities of protein, lower Coomassie blue staining (particularly of the CHY and ASP samples) was interpreted as predominance of very low MW proteins (<8 000), which are poorly stained by Coomassie blue. This was substantiated by the inconsistent staining of the standards containing insulin subunits (MW of 3 000).

The 1 M NaOH-extractable HMW$_f$ proteins of all sludges were primarily concentrated in the range of 3 000
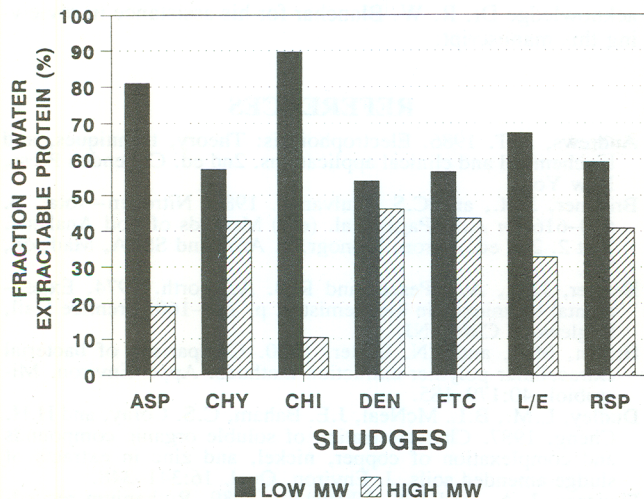
Fig. 3. Molecular weight partitioning of H_2O-extractable sewage sludge proteins by dialysis separation.
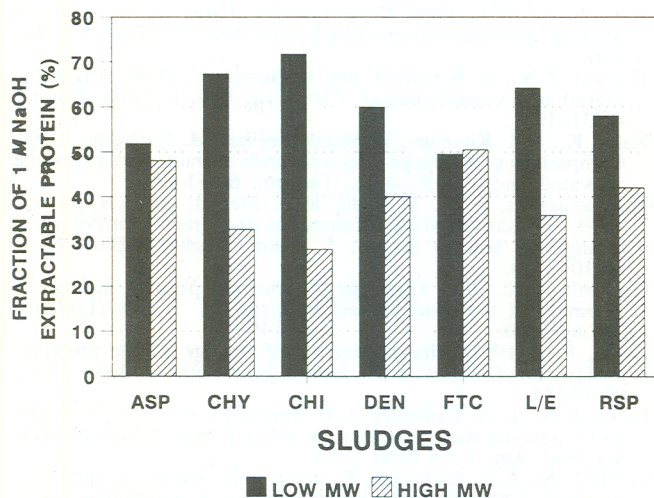

Fig. 4. Molecular weight partitioning of 1 M NaOH extractable sewage sludge proteins by dialysis separation.

Fig. 5. Separation of HMW_f H_2O-extractable proteins by PAGE: A, ASP; B, CHY; C DEN; D, FTC; E, L/E; F, RSP sludges.

Fig. 6. Separation of HMW_f 1 M NaOH-extractable proteins by PAGE: A, ASP; B, CHY; C, CHI; D, DEN; E, FTC; F, L/E; G, RSP sludges.

to 17 000 (Fig. 6). The CHY and CHI sludges showed highly resolved bands at about 3 000. While the CHY sludge had some weakly stained proteins up to 17 000, the CHI sludge had no proteins of greater than 8 000. The RSP sludge showed a pattern similar to the CHY sludge with proteins from 3 000 to 17 000, but it lacked the distinctive band at 3 000. The ASP, DEN, FTC, and L/E sludges showed very similar patterns of intense staining over the entire 3 000 to 17 000 range. These sludges also contained some proteins in the range of 17 000 to 29 000, with the FTC and L/E sludges showing proteins up to 45 000, but none of these sludges showed the distinctive band at 29 000 shown by the H_2O-extractable HMW_f. The presence of predominantly low MW proteins (< 10 000) in an activated sludge was also reported by Goodwin and Forster (1989) using membrane filtration. However, Karapanagiotis et al. (1989) reported that the major fraction of components (protein and nonprotein) from a digested sludge separated by gel chromatography were greater than 5 000 MW.

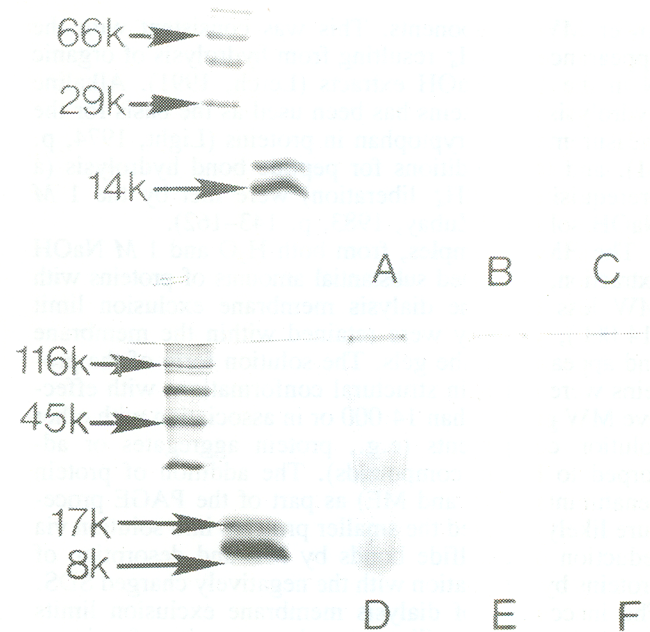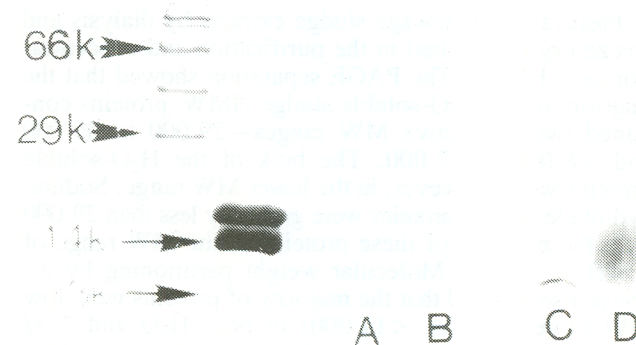Differences between the 1 M NaOH and H_2O-extract-

able HMW_f proteins were displayed by all sludges. Considerable alkaline hydrolysis of peptide bonds likely occurred during the 1 M NaOH extraction resulting in

lower MW components. This was consistent with the appearance of NH_4^+ resulting from hydrolysis of organic N in the 1 M NaOH extracts (Lerch, 1991). Alkaline hydrolysis of proteins has been used as the basis for the measurement of tryptophan in proteins (Light, 1974, p. 61), and the conditions for peptide bond hydrolysis (a prerequisite to NH_4^+ liberation) were met by the 1 M NaOH solution (Zubay, 1983, p. 143–162).

The HMW_f samples, from both H_2O and 1 M NaOH extraction, contained substantial amounts of proteins with MW less than the dialysis membrane exclusion limit (14 000), yet they were retained within the membrane and appeared on the gels. The solution form of the proteins were either in structural conformations with effective MW greater than 14 000 or in association with other solution components (e.g., protein aggregates or adsorbed to humic compounds). The addition of protein denaturants (SDS and ME) as part of the PAGE procedure likely released the smaller proteins into solution via reduction of disulfide bonds by ME and desorption of proteins by association with the negatively charged SDS. The inaccuracy of dialysis membrane exclusion limits for sludge proteins illustrates the necessity of using a more accurate and definitive procedure, such as PAGE, for protein MW determinations.

SUMMARY AND CONCLUSIONS

Preparation of sewage sludge extracts by dialysis and freeze drying resulted in the purification and concentration of a HMW_f. The PAGE separation showed that the majority of the H_2O-soluble sludge HMW_f proteins contained two distinctive MW ranges—29 000 to 66 000 and <8 000 to 17 000. The bulk of the H_2O-soluble proteins were, however, in the lower MW range. Sodium hydroxide soluble proteins were generally less than 29 000 with the majority of these proteins in the MW range of 3 000 to 17 000. Molecular weight partitioning by dialysis also showed that the majority of proteins were low MW moieties (i.e., <14 000) in both H_2O and 1 M NaOH extracts. However, the presence of proteins <14 000 on PAGE indicated the inaccuracy of dialysis membrane exclusion limits for sludge protein MW separations.

The predominance of low MW proteins in either H_2O of 1 M NaOH extracts (<17 000) suggests that sludge digesters contain microbial populations with high proteolytic activity. Apparently, the proteins separated by PAGE or dialysis partitioning were predominantly polypeptide and oligopeptide fragments rather than intact proteins. These data lead to the conclusion that sludge proteinaceous materials in amended soil would degrade rapidly as a result of the numerous soil microorganisms capable of utilizing protein degradation products.

ACKNOWLEDGMENTS

REFERENCES

Andrews, A.T. 1986. Electrophoresis: Theory, techniques, and biochemical and clinical applications. 2nd ed. Clarenden Press, New York.

Bremner, J.M., and C.S. Mulvaney. 1982. Nitrogen—total. p. 610–616. *In* A.L. Page et al. (ed.) Methods of Soil Analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Brewer, J.M., A.J. Pesce, and R.B. Ashworth. 1974. Experimental techniques in biochemistry. p. 128–160. Prentice Hall, Englewood Cliffs, NJ.

Brown, M.J., and J.N. Lester. 1980. Comparison of bacterial extracellular polymer extraction methods. Appl. Environ. Microbiol. 40:179–185.

Dudley, L.M., B.L. McNeal, J.E. Baham, C.S. Coray, and H.H. Cheng. 1987. Characterization of soluble organic compounds and complexation of copper, nickel, and zinc in extracts of sludge-amended soils. J. Environ. Qual. 16:341–348.

Figueroa, L.A., and J.A. Silverstein. 1989. Ruthenium red adsorption method for measurement of extracellular polysaccharides in sludge flocs. Biotechnol. Bioeng. 33:941–947.

Goodwin, J.A.S., and C.F. Forster. 1989. An examination of the extracellular polymers produced by activated sludge. Microbios 57:179–185.

Habeeb, A.F.S.A. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14:328–336.

Hobson, P.N., S. Bousfield, and R. Summers. 1974. Anaerobic digestion of organic matter. CRC Crit. Rev. Environ. Control 4:131–191.

Kakii, K., S.K. Kitamura, T. Shirakashi, and M. Kuriyama. 1986. Comparison of mucilage polysaccharide extracted from sewage activated sludge. J. Ferment. Technol. 64:51–56.

Karapanagiotis, N.K., T. Rudd, R.M. Sterritt, and J.N. Lester. 1989. Extraction and characterization of extracellular polymers in digested sewage sludge. J. Chem. Technol. Biotechnol. 44:107–120.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

Lerch, R.N. 1991. Characterization of sewage sludge proteins. Ph.D. diss. Colorado State Univ., Fort Collins (Diss. Abstr. 92–07333).

Lerch, R.N., K.A. Barbarick, L.E. Sommers, and D.G. Westfall. 1992. Sewage sludge proteins as labile C and N sources. Soil Sci. Soc. Am. J. 56:1470–1476.

Lerch, R.N., K.A. Barbarick, P. Azari, L.E. Sommers, and D.G. Westfall. 1993. Sewage sludge proteins: I. Extraction methodology. J. Environ. Qual. 22:620–624 (this issue).

Light, A. 1974. Proteins: Structure and function. Prentice-Hall, Englewood Cliffs, NJ.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein determination with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

Nelson, D.W., and L.E. Sommers. 1982. Total carbon, organic carbon, and organic matter. p. 571–572. *In* A.L. Page et al. (ed.) Methods of soil analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Rudd, T., R.M. Sterritt, and J.N. Lester. 1983. Extraction of extracellular polymers from activated sludge. Biotechnol. Lett. 5;327–332.

Rudd, T., R.M. Sterritt, and J.N. Lester. 1984. Complexation of heavy metals by extracellular polymers in the activated sludge process. J. Water Pollut. Control Fed. 56:1260–1268.

Schnitzer, M. 1982. Organic matter characterization. p. 581–594. *In* A.L. Page et al. (ed.) Methods of soil analysis. Part 2. 2nd ed. Agron. Monogr. 9. ASA and SSSA, Madison, WI.

Siebert, M.L., and D.F. Toerien. 1969. The proteolytic bacteria present in the anaerobic digestion of raw sewage sludge. Water Res. 3:241–250.

Zubay, G. 1983. Biochemistry. p. 143–162. The Benjamin/Cummings Publ. Co., Menlo Park, CA.