

COMPARATIVE LIPOQUINONE ANALYSIS OF  
INFLUENT SEWAGE AND ACTIVATED  
SLUDGE BY HIGH-PERFORMANCE  
LIQUID CHROMATOGRAPHY  
AND PHOTODIODE ARRAY  
DETECTION

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(Received June 7, 1996; Accepted October 7, 1996)

Reverse-phase high-performance liquid chromatography and photodiode array detection were used to analyze the microbial quinones of influent sewage and activated sludge in a sewage treatment plant. Significant differences in quinone patterns were noted between the sewage and activated sludge. Unlike the activated sludge, the sewage had low ratios of menaquinones to ubiquinones, and contained menaquinone-6 as the most abundant menaquinone and negligible amounts of partially hydrogenated menaquinones. A photodiode array analysis revealed that the sewage also contained considerable amounts of plastoquinones and vitamin K<sub>1</sub>, both of which are specific to photosynthetic electron transport in cyanobacteria and chloroplasts. These results suggest that the microbial population structure of sewage is markedly different from that of activated sludge. Relationships between changes in the community structure of the sewage and activated sludge were also discussed on the basis of the results of a numerical analysis of lipoquinone patterns.

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Microbial cellular constituents which are directly extractable from the environment provide in situ measures of microbial populations in terms of quantity, quality, and activity (17). The membrane lipid molecules, isoprenoid quinones, are particularly useful biomarkers in this respect, because they are essential components of the respiratory or photosynthetic electron transport systems of microorganisms, have great structural diversity, and have been fully realized for their significance in microbial chemotaxonomy (2,3).

Recent advances in high-performance liquid chromatography (HPLC) and personal computer software have made it possible to detect lipoquinones from environmental samples rapidly and accurately. The HPLC method has been used for the quinone analysis of mixed microbial populations in sediments (5), microbial mats (7), and biological wastewater treatment systems (6-11,19) as well as of microbial cultures for chemotaxonomic characterization (2,3,14-16,18,20). Although HPLC is a powerful technique for quinone analysis, technical difficulty is sometimes encountered with peak identification, especially when analyzing quinone mixtures from complex microbial communities. An approach to overcome this difficulty is the on-line spectral analysis of HPLC peak components with the aid of a photodiode array detector. This technique has been used to determine lipoquinone contents in the classification of some bacterial taxa (14).

We have applied the HPLC separation technique in conjunction with photodiode array detection (PAD) to conduct a comparative analysis of microbial quinones in raw sewage and activated sludge. The distribution of isoprenoid quinones as biomarkers of bacterial populations in activated sludge has, in recent years, received intensive study (6-9,11,19). This biomarker approach, as well as molecular methods with rRNA-targeted oligonucleotide probes (22-24) and PCR-involved 16S rDNA libraries (1), have proven to be useful for in situ characterization of microbial communities of activated sludge without isolating and cultivating. Despite this, there has been no available information in literature on chemotaxonomic and molecular approaches to the characterization of microbial populations in raw sewage. The chemical and microbial compositions of influent sewage may affect the community structure of activated sludge and purification efficiency in sewage treatment processes. However, little is known about the relationship between influent sewage and activated sludge in terms of microbial population structures. In this study, we demonstrate characteristic features of the quinone composition of raw sewage using the HPLC-PAD system. Marked differences in microbial population structures between sewage and activated sludge are discussed on the basis of their quinone profiles. To our knowledge, this is the first report to determine the quinone composition of microorganisms in raw sewage.

#### MATERIALS AND METHODS

*Sewage and sludge samples.* Sewage and mixed liquor of activated sludge were collected from the Shinjiko-tobu sewage treatment plant in Matsue, Shimane

prefecture, Japan, from September 1991 to March 1992. This plant consists of two major processing lines, the north line and south line. The process of the north line consists of main activated sludge stages in an anaerobic-aerobic system and a nitrification stage, whereas the south line includes anaerobic-aerobic activated sludge systems only. In this study, activated sludge was collected from a main aeration tank in the north line with concomitant sampling of raw sewage from the sewage inflow line. All samples were taken in sterile polyethylene bottles, transported to the laboratory with freezing at  $-20^{\circ}\text{C}$ , and stored at  $-80^{\circ}\text{C}$  until analysis.

*Extraction and purification of quinones.* Suspended solids and/or sludge in thawed samples were harvested by centrifugation ( $12,600\times g$ , 10 min), washed with 50 mM phosphate buffer (pH 6.8) containing 1 mM ferricyanide, and resuspended in the same buffer at a total volume of 10 ml. Quinones were extracted three times with 2.5 volumes of a chloroform-methanol mixture (2:1, v/v), evaporated under vacuum conditions, and reextracted three times with *n*-hexane-water (1:1, v/v). Then, the crude quinone extract in *n*-hexane was concentrated and purified by aluminum oxide column chromatography and silica gel thin-layer chromatography (TLC). Detailed information on the procedures used for the extraction and purification has been described previously (11). Two quinone fractions (i.e., ubiquinone and menaquinone fractions) were obtained by TLC and dissolved in 100  $\mu\text{l}$  of acetone for HPLC analysis. In this study, we also used silica column chromatography with Sep-Pak Plus Silica (Nihon Millipore Ltd., Tokyo, Japan) as an alternative method for partial purification of the quinone extract. The crude extract in *n*-hexane was applied to Sep-Pak and eluted once with 20 ml of *n*-hexane-diethyl ether (98:2, v/v) and the second time with 20 ml of *n*-hexane-diethyl ether (90:10, v/v) at a flow rate of 20 ml/min. The elutes with 2 and 10% diethyl ether contained quinone components corresponding, respectively, to those of the menaquinone and ubiquinone fractions obtained by the TLC noted above. The resultant menaquinone fraction contained a menaquinone analog, vitamin K<sub>1</sub> (phyloquinone), and plastoquinones in some cases as described later. It has been shown that the mobilities of phyloquinone and plastoquinones are more similar to each other than to that of ubiquinones upon quantitative column chromatography (21).

*HPLC analysis.* Quinone components were separated and identified by the HPLC-PAD method. The HPLC system consisted of a Beckman 110B solvent delivery pump (Beckman Instruments (Japan), Ltd., Tokyo, Japan), a Zorbax ODS column (4.6 i.d.  $\times$  250 mm) (Du Pont Co., Wilmington, DE, U.S.A.) in a column oven at  $30^{\circ}\text{C}$ , a Beckman 168 diode array detector, and an IBM 55/SX personal computer for data analysis. A mixture of methanol-isopropyl ether (9:2, v/v) was used as the eluent at a flow rate of 1 ml/min. The injection volume of the sample was 20  $\mu\text{l}$ . The detector signal was monitored at 275 and 283 nm for ubiquinones and at 255 and 270 nm for components of the menaquinone fraction, with on-line scanning at a wavelength range of 220 to 350 nm. A post-run analysis of the data was performed using the chromatography system program Beckman

System Gold. For the identification and measurement of quinone homologues, parameters for peak identification and calibration of detector response factors for each component were set in the program on the basis of HPLC data on known concentrations of standard quinones, which had been measured spectrophotometrically using respective extinction coefficients (13). The response factors to give a concentration (nmol/100  $\mu$ l acetone) were 0.710 to 0.692 for ubiquinones (Q-6 through Q-11) at 275 nm, 0.530 for plastoquinone (PQ-9) at 255 nm, and 0.645 to 0.630 for the naphthoquinones (K<sub>1</sub> through MK-12) at 270 nm. Thus, the molar ratios (%) of quinone components were calculated automatically on the personal computer.

*Two-dimensional and Ag<sup>+</sup>-loaded TLC.* Two-dimensional silver-ion-modified TLC with Whatman SC5 Multi-K pre-coated plates (Whatman K.K., Tokyo, Japan) was used as a supplementary method to identify partially hydrogenated menaquinones as described previously (11, 12).

*Mass spectrometry.* Mass spectra were recorded on a Shimadzu Model QP-2000 mass spectrometer, using a direct insertion probe, an ionization voltage of 70 eV and a temperature range of 180 to 200°C.

*Standard quinones and abbreviations for quinones.* Standard ubiquinones and phylloquinone were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The menaquinone standards were prepared from known species of bacteria (11). The plastoquinones were prepared from a cyanobacterium, *Synechococcus leopoliensis* strain M-6, obtained from the culture collection center of the Institute of Molecular and Cellular Biosciences, The University of Tokyo. In this report, ubiquinones, menaquinones, and plastoquinones with *n* isoprene units in each side chain were abbreviated as Q-*n*, MK-*n*, and PQ-*n*, respectively. Partially hydrogenated menaquinones were expressed as MK-*n*(H<sub>*x*</sub>), where *x* indicated the number of hydrogen atoms saturating the side chain. Phylloquinone was abbreviated as K<sub>1</sub> as needed.

*Numerical analysis.* A numerical analysis of quinone profiles was performed with the "overlap" method. The dissimilarity index *D* (%) was used for classifying and clustering samples. For example, *D*(*t*<sub>1</sub>, *t*<sub>2</sub>) and *D*<sub>sewage</sub>(*t*<sub>1</sub>, *t*<sub>2</sub>) indicated the dissimilarity (%) between samples *t*<sub>1</sub> and *t*<sub>2</sub> and between sewage samples *t*<sub>1</sub> and *t*<sub>2</sub> themselves, respectively. Detailed information on these procedures has been described previously (11).

## RESULTS

### *Preliminary studies for quinone analysis*

The HPLC-PAD system has the advantage of providing information on spectral patterns of separated peak components as well as on their elution times. This may mean that it is not always necessary to separate the quinone fractions from the crude extracts prior to HPLC analysis. Therefore, early in the development of the protocol for quinone analysis in this study, we attempted to analyze the

crude extract directly by the HPLC-PAD system. However, this attempt gave unsatisfactory results, because the background of the HPLC profiles was so high and the peak components found were too abundant to isolate each quinone homologue clearly. This was, in particular, the case for peak components with short retention times below 10 min.

As the next step, we attempted to save time for the fractionation of ubiquinones and menaquinones by using Sep-Pak Plus Silica columns as an alternative to the alumina column chromatography-TLC system commonly used. The concentrated crude extracts in hexane were applied to Sep-Pak and eluted with 2% diethyl ether-hexane and then with 10% diethyl ether-hexane. HPLC analysis of the fraction with 2 and 10% diethyl ether provided clear separation of the peaks of menaquinone and ubiquinone homologues, respectively, allowing them to be identified on the basis of their HPLC retention times and spectral patterns. This simplified method also had the advantage over the conventional method in that the former recovered 1.6 to 2.5 times higher amounts of quinones from the samples than did the latter. For example, the average quinone contents of the sewage recorded with the Sep-Pak and alumina column-TLC systems were, respectively, 0.56 and 0.28  $\mu\text{mol}$  for ubiquinones and 0.21 and 0.11  $\mu\text{mol}$  for menaquinones, per gram of dry weight of suspended solids.

Therefore, in this paper, we report only the results obtained with samples prepared with Sep-Pak column chromatography.

#### *HPLC profiles of sewage and sludge quinones*

As noted above, the HPLC system used provided good separation of Sep-Pak-fractionated quinone components from the influent sewage and activated sludge samples tested. Figure 1 shows examples of HPLC elution profiles of the quinone mixtures from these samples. In all cases, the ubiquinone fraction gave three major peaks upon HPLC (Fig. 1A and B). One of these components corresponded to Q-8, which eluted as the most prominent peak with a retention time of about 10 min. The other two components were Q-9 and Q-10, which appeared with longer retention times according to the lengths of their side chains. Other ubiquinone components were detected only as minor peaks. A spectral analysis showed that all these components had an absorption maximum at 274 to 275 nm (data not shown), thereby ascertaining them to be ubiquinones.

While the menaquinone fractions of the sludges gave complicated HPLC profiles (Fig. 1D), those of the sewage samples showed relatively simple elution patterns (Fig. 1C). In the latter case, MK-6 was detected as the major peak with an elution time of 9.3 min. It is noteworthy that the sewage samples also produced a component eluting with a retention time of about 19 min and showing the absorption spectrum characteristic of plastoquinones ( $E_{\text{max}} = 255 \text{ nm}$ ) (Fig. 2, solid line). This component was identified as PQ-9 by comparing the HPLC elution time with that for the major plastoquinone from the cyanobacterium *Synechococcus leopoliensis*. A mass spectrometry analysis confirmed this assignment, as the 19-min

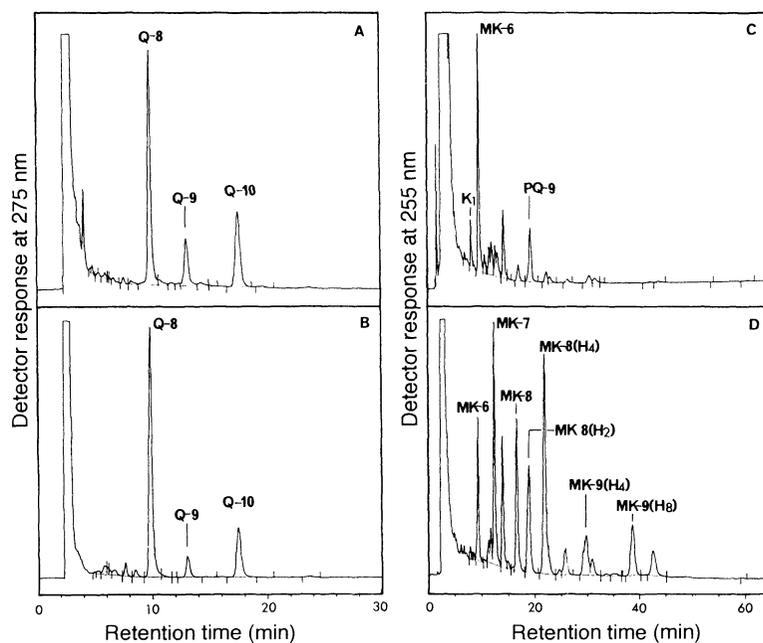


Fig. 1. HPLC elution profiles of microbial quinones from influent sewage and activated sludge.

A, ubiquinone fraction of sewage; B, ubiquinone fraction of activated sludge; C, menaquinone fraction of sewage; D, menaquinone fraction of activated sludge.

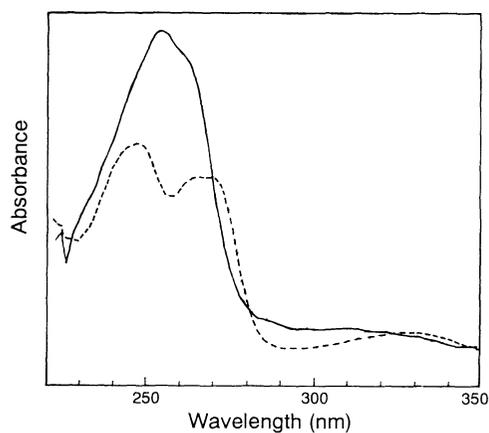


Fig. 2. Ultraviolet absorption spectra of the HPLC components of the menaquinone fraction with an elution time of about 19 min.

Solid line, component from sewage showing a spectrum typical of plastoquinones; broken line, component from sludge showing a spectrum typical of menaquinones.

component purified gave a molecular ion peak of  $m/z$  749 ( $M + 1$ ) and a base peak of 189 corresponding the quinone ring (4). The menaquinone fraction of the sludge produced a peak showing the same retention time as PQ-9 as well. However, it showed an absorption spectrum typical of menaquinones (Fig. 2, broken line), and was thus identified as dehydrogenated menaquinone-8 (MK-8( $H_2$ )). The occurrence of MK-8( $H_2$ ) and other partially hydrogenated menaquinones in the sludge was supported by the results of two-dimensional TLC and silver-ion TLC.

In addition to the above-noted menaquinones, significant amounts of some unknown components were detected frequently in the menaquinone fractions (e.g., those with a retention time of 15 and 14 min from sewage and sludge, respectively). These unknown components could be easily distinguished from the menaquinones and plastoquinones by measuring the absorption spectrum (data not shown).

Therefore, unless the HPLC-PAD system is used, it is difficult to identify various quinone components, such as those having similar HPLC retention times.

#### *Quinone composition of sewage and sludge*

Table 1 summarizes the quinone compositions of all the sewage and sludge samples tested as determined by the HPLC-PAD system (cf. Fig. 1). As reported elsewhere (7), the proportion of ubiquinone homologues of the sludge usually decreased in the following order: Q-8 > Q-10 > Q-9 > others. This was also the case in the sewage samples, but the relative content of ubiquinones other than Q-8 was higher as compared to that of sludge. Menaquinone patterns in the sewage were quite simple compared to those in the sludge, as all sewage samples contained MK-6 exclusively as the major menaquinone with much smaller amounts of other menaquinone homologues present. The predominant menaquinones detected in the sludge differed from sample to sample, being either MK-7, MK-8, or MK-8( $H_4$ ). Also, MK-6 or MK-8( $H_2$ ) occurred in significant amounts in some sludge samples. The sewage was also characterized by the occurrence of significant proportions of  $K_1$  and PQ-9 and by low molar ratios of menaquinones to ubiquinones, varying between 0.293 and 0.417. In the activated sludge, no or trace amounts of phyloquinone and PQ-9 were detected, and the MK/Q ratios ranged from 0.726 to 0.990.

#### *Numerical analysis of quinone profiles*

On the basis of the results presented in Table 1, a numerical analysis of the quinone profiles was conducted to provide quantitative information on differences in the microbial community structures among the samples (Table 2). The percentage dissimilarities ( $D$  values) among the sewage samples were less than 18.4%, whereas those among the sludge samples were less than 19.4%. These results suggest that similar seasonal variations in the community structure occurred in the influent sewage and sludge at a dissimilarity level up to about 20% as estimated by quinone profiling. The percentage dissimilarities between the sewage and sludge

Table 1. Quinone composition of influent sewage and activated-sludge samples collected from September 1991 to March 1992.<sup>a</sup>

Sample	Raw sewage												Activated sludge											
	Sep. 19 1991	Oct. 16 1991	Nov. 13 1991	Dec. 12 1991	Jan. 16 1992	Feb. 12 1992	Mar. 12 1992	Sep. 19 1991	Oct. 16 1991	Nov. 13 1991	Dec. 12 1991	Jan. 16 1992	Feb. 12 1992	Mar. 12 1992	Sep. 19 1991	Oct. 16 1991	Nov. 13 1991	Dec. 12 1991	Jan. 16 1992	Feb. 12 1992	Mar. 12 1992			
<b>Benzoquinones</b>																								
Q-6	0.09	0.68	1.00	0.56	0.64	0.68	0.45	0.10	0.15	0.20	0.43	0.47	0.31	0.60	0.10	0.15	0.20	0.43	0.47	0.31	0.60			
Q-7	0.42	0.74	1.07	1.16	0.82	0.67	1.10	0.54	0.91	0.99	1.00	1.07	0.71	1.40	0.54	0.91	0.99	1.00	1.07	0.71	1.40			
Q-8	39.70	36.86	32.89	32.25	34.63	32.81	32.56	31.39	34.15	36.55	39.74	42.27	35.63	36.62	31.39	34.15	36.55	39.74	42.27	35.63	36.62			
Q-9	7.51	10.04	10.50	13.52	14.03	15.23	11.30	3.75	3.69	3.41	3.40	3.40	3.44	3.59	3.75	3.69	3.41	3.40	3.40	3.44	3.59			
Q-10	19.14	18.40	20.47	21.33	21.63	18.03	20.18	14.18	12.45	12.79	11.09	10.46	10.77	10.34	14.18	12.45	12.79	11.09	10.46	10.77	10.34			
Q-11	0.31	—	—	—	—	—	—	0.28	0.23	0.22	0.21	0.19	0.27	0.27	0.28	0.23	0.22	0.21	0.19	0.27	0.27			
PQ-9	3.28	4.03	6.12	4.46	4.24	3.76	4.74	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
<b>Naphthoquinones</b>																								
K <sub>1</sub>	1.51	2.61	2.77	1.66	2.14	1.44	2.37	0.03	0.01	0.22	0.27	0.14	0.12	0.25	0.03	0.01	0.22	0.27	0.14	0.12	0.25			
MK-6	12.69	15.34	14.58	17.77	15.44	21.57	19.99	0.43	2.71	3.39	4.04	3.98	4.62	3.87	0.43	2.71	3.39	4.04	3.98	4.62	3.87			
MK-7	5.52	2.72	2.25	1.70	1.96	1.12	2.27	3.87	5.29	6.08	7.04	7.67	8.23	7.79	3.87	5.29	6.08	7.04	7.67	8.23	7.79			
MK-8	2.64	2.20	1.99	1.50	1.44	1.25	1.77	9.42	9.89	8.41	8.02	6.74	7.38	5.97	9.42	9.89	8.41	8.02	6.74	7.38	5.97			
MK-9	0.66	0.91	0.69	0.59	—	0.49	—	2.22	2.02	1.18	1.17	0.84	0.74	0.60	2.22	2.02	1.18	1.17	0.84	0.74	0.60			
MK-10	1.11	1.11	0.96	0.93	0.07	0.8	—	2.05	2.08	1.79	1.32	1.15	1.12	1.06	2.05	2.08	1.79	1.32	1.15	1.12	1.06			
MK-11	0.43	0.35	0.28	0.30	0.25	0.20	0.31	1.75	2.14	1.77	1.56	1.91	2.19	2.06	1.75	2.14	1.77	1.56	1.91	2.19	2.06			
MK-12	0.57	0.27	0.07	0.15	0.19	—	—	—	—	—	0.10	—	0.01	0.03	—	—	—	0.10	—	0.01	0.03			
MK-8(H <sub>2</sub> )	—	—	—	—	—	—	—	5.79	8.43	7.06	5.95	5.69	6.91	6.04	5.79	8.43	7.06	5.95	5.69	6.91	6.04			
MK-8(H <sub>4</sub> )	1.46	1.36	1.67	0.84	0.64	0.70	0.77	10.72	8.59	9.38	7.18	8.53	8.75	10.82	10.72	8.59	9.38	7.18	8.53	8.75	10.82			
MK-8(H <sub>6</sub> )	0.05	—	—	—	—	—	—	0.22	0.21	—	0.22	0.24	0.19	0.26	0.22	0.21	—	0.22	0.24	0.19	0.26			
MK-9(H <sub>2</sub> )	0.77	0.55	0.75	0.38	0.37	0.29	0.60	4.17	3.85	2.16	1.84	1.80	2.01	1.66	4.17	3.85	2.16	1.84	1.80	2.01	1.66			
MK-9(H <sub>4</sub> )	1.42	1.59	1.57	0.73	0.69	0.47	1.09	3.99	2.26	3.64	1.77	2.92	2.56	2.45	3.99	2.26	3.64	1.77	2.92	2.56	2.45			
MK-9(H <sub>6</sub> )	0.03	0.02	0.05	—	—	—	—	0.23	0.21	0.22	0.16	0.19	0.13	0.17	0.23	0.21	0.22	0.16	0.19	0.13	0.17			
MK-9(H <sub>8</sub> )	0.53	—	0.19	0.04	—	0.03	—	4.06	—	—	3.02	0.01	3.68	3.70	4.06	—	—	3.02	0.01	3.68	3.70			
MK-10(H <sub>2</sub> )	—	—	—	—	—	—	—	0.33	0.44	0.34	0.32	0.31	0.19	0.28	0.33	0.44	0.34	0.32	0.31	0.19	0.28			
MK-10(H <sub>4</sub> )	0.16	0.22	0.14	0.11	0.19	—	0.12	0.48	0.28	0.19	—	—	0.04	0.17	0.48	0.28	0.19	—	—	—	0.04			
Unidentified	—	—	—	0.02	0.58	0.46	0.38	—	0.01	0.01	0.15	0.02	—	0.17	—	0.01	0.01	0.15	0.02	—	0.17			
MK/Q molar ratio	0.417	0.399	0.382	0.364	0.293	0.396	0.408	0.990	0.938	0.842	0.780	0.726	0.953	0.883	0.990	0.938	0.842	0.780	0.726	0.953	0.883			

<sup>a</sup> Data are expressed as mol%.

Table 2. Percentage dissimilarities ( $D(t_1, t_2)$  values) among sewage and sludge samples.

Sample	Raw sewage							Activated sludge						
	Sep. 19 1991	Oct. 16 1991	Nov. 13 1991	Dec. 12 1991	Jan. 16 1992	Feb. 12 1992	Mar. 12 1992	Sep. 19 1991	Oct. 16 1991	Nov. 13 1991	Dec. 12 1991	Jan. 16 1992	Feb. 12 1992	
<b>Raw sewage</b>														
Oct. 16/1991	8.4													
Nov. 13/1991	12.3	6.2												
Dec. 12/1991	15.8	9.8	7.3											
Jan. 16/1992	15.0	8.2	8.1	4.8										
Feb. 12/1992	18.4	11.9	12.2	6.7	8.6									
Mar. 12/1992	15.9	9.2	6.7	5.3	7.0	7.4								
<b>Activated sludge</b>														
Sep. 19/1991	36.3	38.6	39.1	42.5	44.0	44.2	42.7							
Oct. 16/1991	32.2	35.1	36.8	40.7	40.4	42.2	40.6	10.5						
Nov. 13/1991	28.7	31.7	35.8	39.7	38.9	41.2	39.5	12.4	6.7					
Dec. 12/1991	26.1	32.3	36.4	40.4	39.7	41.8	40.3	16.6	12.6	8.6				
Jan. 16/1992	27.5	33.2	37.4	41.2	40.7	42.7	41.1	19.4	13.1	8.7	6.2			
Feb. 12/1992	30.0	33.7	36.7	40.7	39.9	41.9	40.6	14.8	10.8	7.9	6.7	7.9		
Mar. 12/1992	30.0	33.5	37.1	40.9	40.3	42.4	40.9	15.1	13.7	9.2	7.2	7.6	4.7	

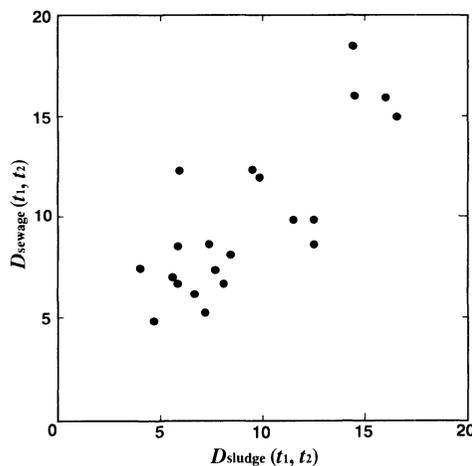


Fig. 3. Relationship between changes in sewage ( $D_{\text{sewage}}(t_1, t_2)$ ) and sludge ( $D_{\text{sludge}}(t_1, t_2)$ ) quinone profiles as a function of time.

The correlation coefficient ( $r$ ) is 0.798 ( $n=21$ ), significant at a 99.9% level.

samples ranged from 26.1 to 44.2%, being much larger than those for each above-noted case.

The foregoing results suggest that the community structures of the sewage and sludge, as estimated by quinone profiling, differed significantly from each other but changed in parallel. This relationship became clearer by plotting data on the dissimilarities in the respective quinone profiles of the sewage and sludge as a function of time (i.e.,  $D_{\text{sewage}}(t_1, t_2)$  vs.  $D_{\text{sludge}}(t_1, t_2)$ ). As shown in Fig. 3, there was a positive relationship between  $D_{\text{sewage}}(t_1, t_2)$  and  $D_{\text{sludge}}(t_1, t_2)$ , with a correlation coefficient of 0.798 ( $n=21$ ).

#### DISCUSSION

During the past decade, microbial quinones from mixed populations in activated sludge and some other wastewater environments have been analyzed successfully using HPLC techniques (6-11, 19). Previous research has shown that standard activated-sludge systems in sewage treatment plants contain both menaquinones and ubiquinones with MK/Q molar ratios of 0.6 to 1.0 (6, 8, 11). In general, the ubiquinone profiles of the sludge are characterized by the occurrence of Q-8 as the predominant homologue, Q-10 as the second most common type and Q-9 and other ubiquinones as minor components, whereas the menaquinone compositions of the sludge are more complicated, as shown by the fact that either MK-7, MK-8, or MK-8(H<sub>4</sub>), and in some cases one of other homologues, is predominant depending upon the time and space.

In this study, we used the HPLC-PAD system to enhance the accuracy and reliability of quinone analysis of sewage and activated sludge. Our approaches to

this study expanded previous results and also provided new information on the quinone patterns of influent sewage and their relationships with the sludge quinone profiles in the same plant. Our new observations on the sewage quinones are as follows: (a) the MK/Q ratios are much lower ( $<0.42$ ) compared to those for sludge; (b) MK-6 is predominant in the menaquinone fraction; (c) partially hydrogenated menaquinones occur only in trace amounts; and (d) K<sub>1</sub> (phylloquinone) and PQ-9 are present in significant proportions.

The low MK/Q ratios found in the sewage suggest that bacterial members with ubiquinones (i.e., those belonging to the class *Proteobacteria*) occur in much greater proportions of the total population in sewage than in activated sludge. Since Q-8, a major ubiquinone detected in the sewage, is known to be the predominant quinone in all recognized species of the beta subclass and many members of the gamma subclass of the *Proteobacteria*, these bacteria are suggested to constitute a major part of the bacterial flora of raw sewage, as well as of activated sludge. A recent molecular analysis by PCR cloning of 16S rDNA showed that bacteria belonging to the beta subclass of *Proteobacteria* most frequently occurred in activated sludge (1). The contribution of eucaryotic microorganisms such as yeast, fungi, and protozoa to the occurrence of ubiquinones in wastewater environments may be negligible because these environments yielded a much lower biomass of eucaryotes than procaryotes as estimated by microscopy (A. Hiraishi, unpublished observations).

The low MK/Q ratios, together with the occurrence of trace amounts of partially hydrogenated menaquinones, also indicate that the population of such menaquinone producers as Gram-positive bacteria, at least those of the high guanine + cytosine DNA group, is low or negligible in influent sewage, unlike activated sludge. However, the finding that MK-6 was the predominant menaquinone in sewage indicates that the occurrence of MK-6-containing bacteria is a characteristic feature of raw sewage. Menaquinone-6 is present as a major quinone not only in some species of Gram-positive bacteria (3) but also in limited members of other phylogenetic groups, including those of the *Cytophaga-Flavobacterium* group (15, 16) and *Planctomyces* (18).

An additional important observation in this study is that considerable amounts of phylloquinone and plastoquinones were detected in the influent sewage. These quinones are essential mediators of oxygenic photosynthetic electron transport in cyanobacteria and chloroplasts. Our data suggests that oxygenic phototrophs occur in much higher proportions of the microbial populations in raw sewage than in activated sludge. However, at this point in time, we do not know which kinds of oxygenic phototrophs contribute to the occurrence of photosynthetic quinones in these environments. What is known is that the photosynthetic quinones, in addition to MK-6, may be used as positive indicators of sewage microbial populations in general.

The data presented here covered variations in quinone profiles over a half-year period in a sewage treatment plant. A numerical analysis of the quinone profiles showed that microbial communities in sewage or in activated sludge were relatively

constant regardless of time, as revealed by low  $D$  values of less than 20%. In other words, the upper limit of seasonal variations in microbial communities in a sewage treatment plant, as indicated by  $D$  values, is about 20% if the plant is operated under normal conditions. This supported the view previously reported with respect to the interpretation of  $D$  values for wastewater treatment processes (8). Numerical studies also show that the community structure of raw sewage and activated sludge differed significantly from each other but may change in parallel in response to environmental stress. Namely, changes in the chemical composition of influent sewage and some other physico-chemical factors affect the microbial community structure thereof directly, and these changes in chemical and microbial compositions subsequently have major effects on the taxonomic composition and activity of the microbial populations in activated sludge.

In summary, this study demonstrates that the spectrochromatographic method is a powerful tool for lipoquinone analysis of complex microbial populations in wastewater environments. This method is essential for a comparative quinone analysis of raw sewage and activated sludge because of the occurrence of different quinone types showing the same HPLC retention time.

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