INVESTIGATION OF THE MICROBIAL POPULATIONS IN THE ACTIVATED SLUDGE OF THE HOECHST-CELANESE WASTEWATER TREATMENT PLANT

by

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ABSTRACT

The microbial populations in the Hoechst-Celanese activated sludge were examined. Heterotrophs, denitrifiers, sulfate-reducers, protozoa and filamentous bacteria were enumerated. Variations in microbial populations were compared with influent and effluent constituent concentrations, and with aeration basin characteristics, such as dissolved oxygen and F/M ratio, to determine whether any microbial type could be used by plant operators to monitor process performance. Results indicated that filamentous bacteria may be useful to plant operators for monitoring process performance because an inverse relationship between filamentous bacteria, food-to-microorganism ratio and sludge volume index was suggested by this study. Protozoa may also be useful for operators, although more data is needed. *Microthrix parvicella* and Type 0041 were the most common filament types. Filament Type 1701 was most prevalent during a period of low dissolved oxygen. A strong relationship between stalked ciliates and effluent quality was mention in the literature, but was not found in this study. Enumeration methods were evaluated.

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“We dance round in a ring and suppose,
But the Secret sits in the middle and knows.”

THE SECRET SITS
Robert Frost

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Chapter I

INTRODUCTION

1.1 GENERAL

Hoechst Celanese, Inc. (Celco) manufactures an average of five million pounds of cellulose acetate per week at its plant in Narrows, Virginia. The conversion of cellulose into cellulose acetate involves the replacement of methyl functional groups on the cellulose molecule with acetate groups. Cellulose is insoluble in water and is essentially unreactive. In order to make the reaction occur, the cellulose is immersed in concentrated sulfuric and acetic acids. The reaction is stopped, after two of three functional groups have been replaced, by the addition of magnesium oxide. The sulfuric acid cannot be recovered.

The process results in high concentrations of magnesium and sulfate ions in the treatment plant influent. Other major constituents include: acetic acid, isopropyl alcohol, isopropyl acetate, methyl cyanate, mesityl oxide, and the metals: copper, nickel, lead and chromium. The wastewater is low in nitrogen, so urea must be added to satisfy the nitrogen requirement of the activated sludge. There is a large surplus of phosphorus, so phosphorus supplementation is not necessary. The equalized influent to the treatment plant commonly exceeds 100° F.

Like many industries, Celco has had problems with bulking and poor settling of the activated sludge in its wastewater treatment plant. In many instances, the growth of filamentous organisms has been observed. Filaments may be within or outside activated sludge flocs. The latter situation may prevent flocs from compacting; this is referred to as *bulking*. Often, operational factors contribute to the growth of filaments. Such factors include low food/microorganism (F/M) ratio, low dissolved oxygen (DO), widely fluctuating organic loads, nitrogen deficiency, acidic conditions and long mean cell residence time, θ_c.

The bacteria and protozoa comprising activated sludge become acclimated to the conditions under which they live. However, wastewater constituents and amounts vary daily, necessitating adaptation by the microorganisms. Because of differing sensitivities and resilience, microorganism populations constantly shift and fluctuate. Increases in a predator population may result in a decrease in prey numbers, for example.

1.2 SCOPE OF RESEARCH

At the time of this study, no attempt had been made to identify microbial populations in the Celco activated sludge. Dr. Clifford Randall had observed some population fluctuations during 11 years of working with the company (1991, personal communication); however, no quantification had been attempted. Plant operators were interested in knowing whether population shifts could be used as indicators of process performance. The objective of this research was to identify: 1) major microbial groups, such as denitrifiers and filamentous bacteria, that could be identified and enumerated using simple procedures, and 2) any relationships between population dynamics and operating parameters or process performance. Pursuit of the objectives required evaluation of microbial identification and enumeration techniques, and this became a secondary objective.

The characterization of the microbial populations in activated sludge is too complex to be attempted in a Master's thesis. Apart from the difficulty of identifying bacteria to the generic level, the fact that populations are dynamic may make the identification and tracking of all population shifts an impossible task. Upon the recommendation of the committee, and because samples were to be taken every two weeks, the research was limited to the microbes that could be grown and tracked using simple techniques. Biweekly sampling was determined by the 14 to 18 day sludge age. The following populations were studied: denitrifiers, sulfate reducers, protozoa, filamentous bacteria and any bacteria that would grow on Standard Methods Agar.

Chapter II

LITERATURE REVIEW

2.1 BACTERIA

2.1.1 General

The activated sludge (AS) process involves the breakdown of wastewater contaminants by bacteria. The species and numbers present depend upon the constituents and physical characteristics of the influent. Most of the bacteria are aerobic heterotrophs that feed on dissolved organic matter (BOD) from the influent, waste products of other bacteria and compounds released from dead cells. Some bacteria are chemolithotrophs, which oxidize inorganic compounds, such as sulfide, to obtain energy. In zones of low dissolved oxygen, facultative anaerobes may reduce sulfate to sulfide or nitrate and nitrite to nitrogen gas. The latter may be encouraged to grow in nitrogen-removing treatment plants.

Denitrifiers

Painter (1970) conducted a review of the literature on inorganic nitrogen metabolism. He classified nitrogen metabolism into two groups: assimilative, in which nitrate is reduced to ammonia and incorporated into cell constituents, and dissimilative, in which NO_3^- is used as the terminal electron receptor, instead of O_2. If the final product of dissimilation is N_2 or N_2O, it is called *denitrification*. The following is a suggested pathway (Tiedje 1982):

$$NO_3^- \Rightarrow NO_2^- \Rightarrow [\text{Enz NO}] \Rightarrow N_2O \Rightarrow N_2$$

The most common denitrifying bacteria found in activated sludge plants are *Pseudomonas* spp. . Sometimes found are: *Achromobacterium* spp., *Denitrobacillus* spp., *Spirillum* spp., *Micrococcus* spp., and *Xanthomonas* spp. . Denitrifying bacteria are facultatively anaerobic, and prefer to use oxygen. The presence of oxygen inhibits the synthesis of denitrifying enzymes (Tiedje, 1982). Tiedje states: "...the population density may have no direct relationship to denitrifying enzyme concentration or activity."

Sulfate-reducers

Given that acetate and sulfate are abundant in the Hoechst-Celanese wastewater, the following reaction from Horan (1990) is particularly appropriate:

$$CH_3COOH + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O$$

In anaerobic ponds, this reaction is carried out by *Desulfuromonas acetoxidans* and *Desulfotomaculum*, for example. Some bacteria utilize larger fatty acid molecules and excrete acetate. In aerobic zones, sulfide can be oxidized to elemental sulfur or thiosulfate by *Thiobacillus* or the filamentous bacterium *Beggiotoa.*

Flocculation

Bacteria in activated sludge do not have a net surface charge. The secretion of extracellular polymer molecules with charged functional groups, such as carboxyl groups, may cause the bacteria to react like negatively charged colloids. Polyvalent cations in the solution neutralize the charge and the cells aggregate into particles with a high zone-settling velocity (Forster and Dallas-Newton, 1980). The extracellular polymers may also act as a slime layer which physically traps other cells or particles. Tenney and Stumm (1965) suggest that these polymers are excreted at all growth phases, but that during rapid growth "new surfaces are created faster than surfaces can be covered with such polymers ... polyacids of bacterial origin have been used successfully to flocculate bacteria."

Floc formation makes it difficult to isolate individual cells for culture and enumeration (Allen, 1944). Some of the problems associated with the identification of organisms in activated sludge include the following (Sterritt and Lester, 1988):

- dilutions for plate counts most likely result in colonies from clumps of cells, not individuals
- non-floc cells would give rise to individual colonies and would give a distorted picture of dominant species
- some organisms isolated may have been present in the influent and not a part of the activated sludge system.

Methods for dispersing flocs have ranged from shaking to ultrasonication (Sterritt and Lester, 1988). According to Sterritt and Lester, Allen (1944) forced samples through a small orifice. The difference between homogenized and unhomogenized plate counts was ten to 100-fold. Yin and Moyer (1968) tested 37 surfactants (nonionic, cationic and anionic) and found that none dispersed flocs significantly. Williams, *et al.* (1970) used a flexural sonicator to set up standing waves along a length of partially submerged wire. They found that large flocs were disrupted more rapidly than small flocs, and because large flocs are anaerobic at the center, their disruption does not appreciably increase viable cell counts. Pike, *et al.* (1972) compared methods for deflocculating and enumerating viable heterotrophic bacteria in activated sludge. They concluded that the best combination was sodium tripolyphosphate as a diluent, homogenization with the Kerry method for one minute and spread plating on casein-glycerol-yeast extract (CGY) medium, incubating at

22° C for six days. The Kerry procedure involves the use of a sonication device to break bacteria away from flocs. They found that substituting filter-sterilized activated sludge supernatant for all or part of the water did not significantly increase the counts on CGY medium.

2.1.2 Enumeration

Total counts

Estimates of the number of bacteria present in various stages of sewage treatment are provided by Pike and Carrington (1972). They estimate the number of viable bacteria in mixed liquor at a conventional loading rate to be 4.9×10^7 bacteria per mL.

The enumeration of bacteria in activated sludge, because it is based on statistical probability, is at best an approximation. As discussed in section 2.1.1, isolation of individual cells is difficult. Homogenization of activated sludge shears flocs and separates cells to some extent. However, the cells must be prevented from reflocculating, through charge restabilization or dilution. Pike, Carrington and Ashburner (1972) suggest sodium tripolyphosphate in deionized water as a diluent.

Procedures for the enumeration of bacteria include culture methods such as spread plate, pour plate or drop plate; or direct counting methods using counting chambers such as a Helber chamber (Pike and Carrington, 1972). Culture methods involve the serial dilution of a sample and the inoculation of a culture medium. The medium is incubated and the resulting colonies are counted.

Direct counting is similar to that used in blood-cell counts in medical laboratories. A cell of known volume underlain by an accurate graticule 1 mm^2 , is subdivided into 400 squares. A drop of homogenized sample is placed in the cell and the bacteria are counted.

Because bacterial cells are close in size to the optical limits of light microscopes, it may be difficult to differentiate between cells and inorganic particles. No information about cell viability is obtained. Therefore, counts may be overestimated using this technique.

Most Probable Number

Another method for enumerating bacteria is the multiple-tube most probable number (MPN) technique. Multiple tubes of each dilution (usually 10^{-3} to 10^{-8}, in tenfold dilutions) are incubated, then read for a positive biochemical reaction. The number of positive tubes at each dilution is used with MPN tables to estimate the number of organisms per mL of original sample (*Standard Methods*, 1985). This technique is useful for denitrifying and sulfate-reducing bacteria.

2.1.3 Identification

Various staining techniques have been used to differentiate bacterial genera visually. Gram's stain colors cell walls purple. Those bacteria that do not possess a cell wall (most activated sludge bacteria) stain pink. Neisser stain reveals the presence of polyphosphate. Other procedures differentiate bacteria possessing sulfur or poly-β-hydroxybutyrate granules, flagella, capsules or other structural components. Despite the number of visual differentiating techniques, most activated sludge bacteria look very similar and require biochemical tests on pure cultures for identification.

Biochemical identification methods entail the inoculation of specific nutrient media with pure cultures. The ability to grow on the nutrients provided or produce certain wastes, such as gas, are characteristic to an organism. Because a variety of such tests are required to identify each organism, some packaged test panels have been manufactured. Two of these are the Biolog and API® systems. The latter was designed for diagnostic testing in the health care field. Both systems have plastic plates with wells containing reagents and nutrients. Inoculation of the wells with the specified concentration of cells, then incubation at the correct humidity and temperature, are all that is required. After incubation the wells are compared to a control or to the descriptions in the instructions. In the case of the Biolog system, the tests were designed such that a positive result to any test is indicated by a purple well, while negative cells remain clear. The location of the positive and negative cells is compared to a data base and the most probable identification (given a limited database) is produced.

2.2 PROTOZOA

2.2.1 Types

Of the three phyla of protozoa, i.e., Ciliophora, Mastigophora and Sarcodina, the most important in activated sludge plants is Ciliophora, the ciliates. Curds and Cockburn (1970) performed the most comprehensive study of protozoa and effluent quality. They studied 56 activated sludge plants from March through December 1968 in Great Britain. The species of ciliates they most commonly observed were: *Trachelophyllum pusillum*, *Vorticella convallaria*, *V. microstoma*, *V. alba*, *Opercularia coarctata*, *Euplotes moebiusi* and *Aspidisca costata*. The subclass peritrichia, the stalked ciliates, was by far the most abundant and important. The other subclasses are the holotrichs, which are characterized by the presence of locomotory cilia distributed uniformly over the cell surface, and spirotrichs, which have locomotory cilia primarily on the lower surface. Also present were ciliate carnivores, i.e., Suctoria and "errant hunters", which prey on other protozoa.

Flagellates, the Mastigophora, were found in the most heavily loaded plants. They have one or more flagella for locomotion and feeding. While some flagellates possess chloroplasts, the important class in activated sludge systems is Zoomastigophora, which are heterotrophic. An example is *Bodo caudatus* (Horan, 1990).

The third phylum important in activated sludge systems is Sarcodina, the amoebae. Their diverse structures range from no skeleton to a shell of protein, silicate or carbonate. Horan (1990) offers *Amoeba proteus* as an example.

2.2.2 Relationships

Predator-prey

Protozoa are predators in the activated sludge system, feeding on dissolved organic matter, bacteria and other protozoa. They may be found free-swimming, attached to or crawling on floc surfaces. Curds and Fey (1969) studied the effect of ciliated protozoa on *Escherichia coli* populations. They found that the ciliates were primarily responsible for the destruction of the *E. coli* and that the ciliates reduced the total viable bacteria population and improved effluent quality. Curds and Cockburn (1970a) state that *Chilodonella cucullus* has the oral apparatus to ingest filaments, algae, fungal hyphae and certain bacteria. Curds (1971) examined the population dynamics of attached and free-swimming ciliates. He ran two simulations, one for sludge containing free-swimmers, the other containing attached ciliates. He found that when the concentration of floc-forming bacteria increased, the concentration of dispersed bacteria and substrate decreased, attached ciliate numbers increased and free-swimmers decreased. Because ciliates feed on dispersed bacteria, they may have a polishing effect on the effluent.

Populations

During startup of a plant, a succession of protozoan types has been observed. Curds (1971) states that at the time of his study, these population changes were assumed to be the result of environmental changes. He proposed that the succession may be explained by growth kinetics and settling properties. Attached and crawling ciliates settle out with the floc. Free-swimmers are washed out with the effluent. He states:

> ...at steady state the specific growth rate of an attached ciliate is low and equal to the specific wastage rate whereas the specific growth rate of a free-swimming ciliate is much higher and equals the dilution rate...the lower the growth rate the lower the concentration of dispersed bacteria present.

Therefore, a plant containing free-swimmers will produce more turbid effluent "since dispersed bacteria are competing with sludge bacteria for substrate, the effect of the high population of dispersed bacteria in a reactor containing free-swimming ciliates is to lower the concentration of sludge bacteria present."

Indicators

Because of the correlation between peritrich populations and effluent turbidity, some workers have studied the possibility of using qualitative and quantitative observations of protozoa in mixed liquor as indicators of process performance. Reynoldson (1942) studied *Vorticella* as an indicator organism. He found a degree of correlation between *Vorticella* numbers and results from a "three-minute oxygen absorption from acidified potassium permanganate" test (n = 32, r = -0.7703; n = 23, r = -0.6925) for two periods. The correlation for BOD_5 : n = 25, r = -0.7661; n = 15, r = -0.8380.

Baines, *et al.* (1953) studied domestic sewage treatment plants. They found a good inverse correlation between the number of peritrichs and the effluent BOD_5 . The relationship did not hold for free-swimmers. Peak free-swimmer (*Lionotus* and *Aspidisca)* populations coincided with peaks of peritrich populations. Except during the summer, the effluent BOD and ciliate populations coincided. They also stated that since *Vorticella* has a free-swimming form, it could explain differential counts based on locomotion. Although the number of free-swimming forms increased when an activated sludge sample was allowed to sit in a bottle unaerated, there was no correlation in an activated sludge plant.

Curds and Cockburn (1970b) developed "association ratings", whereby up to 34 protozoa were identified to the species level. They used averaged monthly effluent BOD values, divided into four basic concentration groups, and attempted to correlate them with protozoa numbers and types. They state:

> ...there was generally a tendency for a given species to occur more frequently in plants delivering effluents within a particular quality category. An arbitrary total of 10 points was awarded to each species, and these were distributed between the four effluent categories so that the greatest number of points was given to the effluent category with which that species was most frequently found associated, for example, a ciliate species occurring with frequencies of 60, 80, 40 and 20 per cent in plants delivering effluent in the four categories would be awarded the ten points in the ratios of 6:8:4:2, that is, 3,4,2 and 1.

The number of points awarded for each species in each of four categories of effluent BOD was used as a predictor. They analyzed data from 34 additional sites to see whether predicted effluent BOD values matched actual values. They claim their method was successful in 83% of the cases.

In his studies, Poole (1984) found that peritrichs were associated with high effluent BOD and ammonia-N, and low MLSS. Monogononta rotifers were associated with low effluent BOD and ammonia-N and high MLSS.

He divided the plants into three categories. The first type were nitrifying and had low sludge loading and long sludge age. Type II were non-nitrifying, had high sludge loading and short sludge age. Type III were non-nitrifying, had low sludge loading and low sludge age. Holotrichs occurred regularly in Types I and II, although species differed. *Chilodonella uncinata* and peritrichs occurred more frequently in Type II, especially *V. microstoma*, *Opercularia coarctata*, and *O. microdiscum*. Hypotrichs and flagellates occurred in all types. Shelled amoebae were found primarily in Type I. He concluded that there is a distinct community of organisms associated with nitrifying sludges.

More recently, Al-Shahwani and Horan (1991) sampled two activated sludge plants (one domestic and one industrial) biweekly for 18 months. Their method required the identification of twenty-one ciliates to the genus level. Using the association ratings of Curds and Cockburn (1971), Al-Shahwani and Horan predicted the daily effluent BOD with a success rate of only 66% for the domestic and 48% for the industrial plant. After 77 sampling results had been analyzed using multiple regression, the shortened indicator list predicted the daily effluent BOD, with a success rate of 84% for the domestic plant and 57% for the industrial one. They suggested that because there are inaccuracies in sludge wastage determinations, an advantage of their technique is the confirmation of actual operating conditions. They considered biweekly sampling more useful than monthly sampling because protozoa populations reflect the environmental conditions of the previous few days, and process performance can be monitored more closely with frequent sampling.

2.2.3 Enumeration

Baines, *et al.* (1953) and Reid (1969) used a haemocytometer to count protozoa. In their 1991 study, Al-Shahwani and Horan counted protozoa directly using phase contrast microscopy, dilution with tap water and a calibrated Lund cell. They counted cells in the order: free-swimming, other motile protozoa, flagellates, then peritrichs and other attached or slow movers. In other studies, no mention was made of how protozoa were counted.

2.3 FILAMENTOUS BACTERIA

2.3.1 General

Some species of bacteria grow in a filamentous form that may act as a backbone for floc formation. Common genera are: *Beggiotoa, Nocardia, Thiothrix, Sphaerotilus, Nostocoida, Microthrix*, and *Heliscomenobacter*. Some are identified only by a number, such as types 021 N, 0092 and 1701.

The morphology of filaments varies considerably. The trichome, or filament strand, may be as short as 10 µm, or as long as >800 µm, depending on the species. Some have a gliding motility. Individual cells may be rectangular, oval, discoid or barrel-shaped. Depending on the stain used, some have been shown to have granules within the cells.

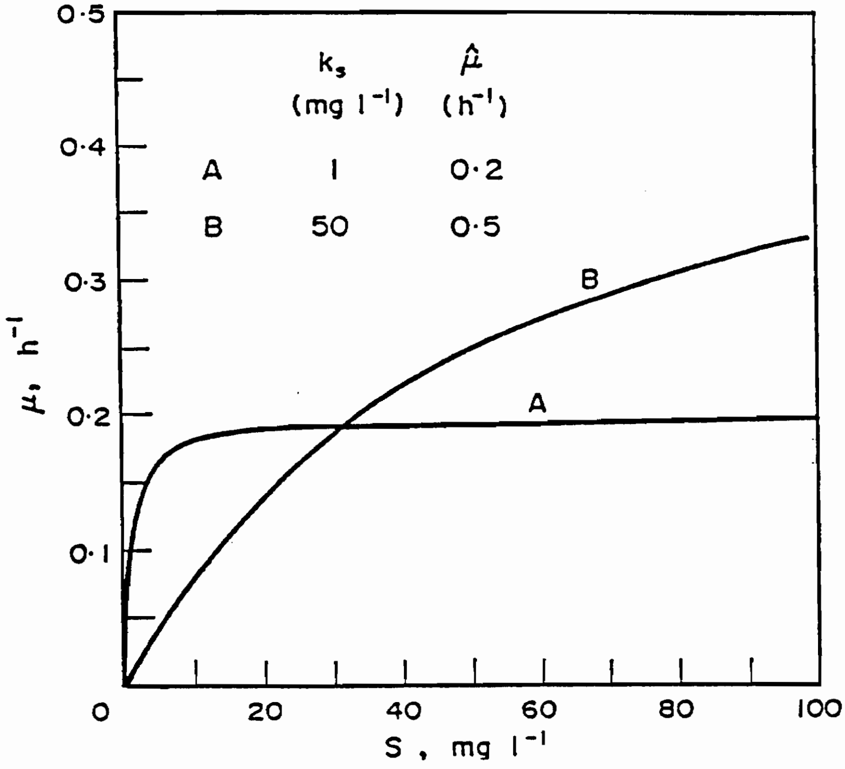

Figure 1. Graphical presentation of the principle of selection of microorganisms in mixed cultures. (From Chudoba, *et al.*, 1973).

The cause of filament growth has been the subject of considerable speculation and research. Chudoba, *et al.* (1973) studied the kinetics of growth. The plot of growth rates of two species versus substrate concentration showed that substrate concentration favors the selection of some organisms over others in a mixed culture (Figure 1). They found that filaments and floc bacterial growth tended to follow the relationship shown in the figure, and filamentous growth should be favored at certain substrate concentrations.

Similarly, Sezgin and Parker (1978) concluded that outgrowth of filaments from flocs occurs when conditions within the flocs allow for a higher growth rate for filaments. Because the dissolved oxygen in the floc center may be considerably lower than that in the bulk solution, filaments that grow in conditions of low dissolved oxygen will have a competitive advantage. They suggested that because filaments extending from the floc surface could be sheared off, trichomes outside of flocs would tend to be longer.

Pipes (1967) suggests that because filamentous bacteria have a larger surface area/volume ratio, that they may have an advantage in competing for nutrients. *Microthrix parvicella* may be hardly affected by dissolved oxygen concentration, but it is affected by sludge age (Eikelboom, 1977). Strom and Jenkins (1984) provided a table of operating conditions and associated filament types. These species may be used as indicator organisms to monitor process performance. Eckenfelder (1989) stated that filamentous growth is affected by: 1) wastewater composition. The more easily degraded the organic matter, the more likely filamentous growth is to occur. 2) The depth of O_2 penetration in the floc depends on the O_2 concentration in the aeration basin and on the oxygen utilization rate (OUR), which is proportional to the food to microorganism (F/M) ratio. Low dissolved oxygen encourages filament growth, although filaments are not anaerobes. 3) Low nitrogen and phosphorus concentrations encourage filamentous growth.

Certain species tend to be associated with particular operating conditions. Types 0092 and 0041 tend to occur in plants with low BOD loading; Types 021N and 1701 tend to occur under conditions of low DO (Strom and Jenkins, 1984). Type 0581 may only grow well in conditions of low loading and low DO.

2.3.2 Effect on Flocs/Process performance

So long as filaments are short and remain almost entirely within flocs, there appears to be a beneficial effect on sludge settlement. The extension of filaments into the solution contributes to the entrapment of small particles, resulting in a good quality effluent, given a sufficiently long settling time. Flocs formed without a filament backbone tend to be weak and easily sheared into smaller particles in the aeration tank. The resulting tiny flocs take longer to settle and may be carried out of the sedimentation tank with the effluent (Gray, 1989). Filaments provide a firmer base of support for the flocs to withstand shear forces. They become a problem when they protrude significantly from the floc surface, or entrain bubbles and float to the tank surface. These conditions are referred to as *bulking* and *foaming*, respectively. Protruding filaments sometimes resemble hair when viewed under the microscope. They extend into the solution and may bridge flocs, slowing settling and preventing compaction, although, if filaments wrap around flocs or each other, a highly filamentous sludge may have a low sludge volume index (SVI) (Pipes, 1979). Some workers have shown that filament length has as much effect as filament number (Sezgin, *et al.*, 1978; Palm, *et al.*, 1980; Lee, *et al.*, 1982; Baker and Veenstra, 1986). Sezgin, *et al.* (1978) found that "a filament length of 10^7 µm per mL appears to be a good dividing point between a filamentous bulking sludge and a non-bulking sludge." Farquhar and Boyle (1971b) studied settling characteristics, dissolved oxygen (DO), pH, temperature and filament types in activated sludge plants, some of which had problems with bulking. They found *Thiothrix*, *Sphaerotilus*, *Toxothrix*, *Vitreoscilla*, lactic acid bacteria, *Nocardia*, *Beggiotoa* and *Microscilla*. At some plants, filament growth was classified as "slight" and had a low SVI. At one, though, the SVI was 139. "Moderate" growth classification usually corresponded to an SVI of <200, although, in three cases, SVI was >200 (range: 234-300). "Excessive" growth corresponded to an SVI of >200 (range: 219-342). High SVI "moderate" plants had *Thiothrix*, as did "slight" plants with a high SVI. The lowest SVI of an "excessive" group had no *Thiothrix*. According to Jenkins, *et al.* (1984), *Thiothrix* I has a trichome length of 100-500+ µm, which is long.

Eikelboom (1977) studied 1200 sludge samples from 220 treatment plants for the presence of filaments. Approximately 40% of the plants treating mostly domestic sewage were in a bulking condition. Of those treating primarily industrial wastewater, 60% were bulking. The industries studied were dairy, meat/poultry, potato and fruit factories. The most common filamentous bacteria found were *Haliscomenobacter hydrossis*, Type 0092, Type 021 N and Type 0041. In this paper he presented an identification key for use by microbiologists and operators. Farquhar and Boyle (1972) studied a pilot plant that was bulking. The influent was septic and black. *Thiothrix* I and III were identified. Pre-aeration was chosen to remove the sulfide, and the filament numbers declined. Cessation of pre-aeration resulted in the regrowth of *Thiothrix*.

2.3.3 Identification

Because different species are more likely to occur under certain operating conditions, the identification of the filaments present are crucial to monitoring process performance. Farquhar and Boyle (1971) utilized chemical tests to differentiate filaments. To detect a sheath, they used a lysozyme-detergent test that disintegrated cells, leaving the sheath intact. It was then stained with dilute crystal violet or methyl blue in alcohol. Some organisms oxidize ferrous iron and deposit it in the sheath. Prussian blue reaction detected deposited iron. A manganese oxidation test separated *Leptothrix* from *Sphaerotilus*. It required culturing of the organisms on manganese agar. The detection of intracellular lipid and sulfur deposits was used to differentiate filaments. In the same study, Farquhar and Boyle used Burdon's Method of staining with Sudan black B. Unfortunately, this method is not useful for mixed cultures. The detection of sulfur deposits separates *Thiothrix* sp. from *Leucothrix* spp., which are attached, and *Beggiotoa* spp. from *Vitreoscilla* spp., which are gliders. They also used physiologic stains such as Gram, acid-fast, spore and India ink negative stains.

Van Veen (1973) grouped filamentous bacteria into five categories. Group I filaments were characterized by a sheath. Group II were non-motile, Gram negative and orange or yellow pigmented. Group III were Gram negative, gliding, red colony-formers. Group IV were Gram positive and nonmotile. Group V were nonmotile unidentified. Some Group V were Gram positive with some Gram negative autolyzed cells.

The species van Veen associated with Group I were *Sphaerotilus* and *Streptothrix*. These were subpolarly flagellated; some oxidized manganese and some had carotenoid pigments. Some of Group II resembled *Flavobacterium*. They contained carotenoid pigments, and consisted of chains of variable length. Cell septa were sometimes unclear. *Flexibacter* and *Microscilla* were assigned to Group III. These had shorter trichomes than Group II, but flocs were less compact. Cells were rod-shaped. Group IV included *Nostocoida limicola*. These were chains of coccoid cells (in pure culture) or rods (in activated sludge). According to van Veen, they resembled Cyanophyceae. Group V filament cells contained electron-dense granules, often observed close to cell interfaces.

No cells could be seen under phase-contrast illumination. This group included *Microthrix parvicella.*

In 1975, D.H. Eikelboom studied 1100 sludge samples, from plants with and without bulking problems, to identify the filaments present. He used Gram and Neisser stains, phase-contrast and electron microscopy, and culture techniques to aid in identification. In his 1975 and 1977 studies, he used the following morphologic features to classify filaments into seven basic categories:

- presence or absence of a sheath or slime layer
- gliding motility
- branching; true or false
- nature, length and shape of the filaments
- result of Gram stain
- diameter, length and shape of the cells
- presence of cell septa
- presence or absence and composition of cell inclusions (poly-β-hydroxybutyrate, polyphosphate or sulfur)

He noted that his preliminary experiments showed that the morphogenesis of filaments may vary with the culture conditions, although the conditions must be extremely different from those present in the original sample. Jenkins, *et al.* (1984) used these features, as well as the presence of attached growth (epiphyte), location of trichomes (whether within, protruding from or attached to the floc surface), and the formation of rosettes or gonidia.

Eikelboom (1977) commented that plant operators and even researchers often do not try to identify the filaments causing bulking in the plants under study. This necessitates trial and error control methods. The key he presented in 1977 allows for the identification of most of the filaments in activated sludge.

2.3.4 Enumeration

Methods used to enumerate and measure filaments are varied. Rensink (1974) used a "+" to "+++" scale for filament growth.

Finstein and Heukelekian (1967) measured filament lengths by diluting samples three- to ten-fold with distilled water. One-tenth milliliter portions were spread uniformly over a 10 cm. area of microscope slides. Four slides per sample were made. They projected the images onto a sheet of paper. One hundred fields per slide were selected at random, and the outlines of flocs and filaments were traced on the paper. Measurements were obtained from the tracings and converted into actual dimensions. Filament lengths were measured with a map measure.

Sezgin, *et al.* (1978) counted flocs and filaments in diluted activated sludge. They diluted 2 mL samples with one liter of distilled water and stirred with a jar test apparatus for one minute at 95 rpm ($G = 85\ s^{-1}$). Filaments were counted and sized using size ranges: 0-10 μm, 10-25 μm, 25-50 μm, 50-100 μm, 100-200 μm, 200-400 μm, 400-800 μm, and >800 μm. Filaments >800 μm were counted individually.

Forster and Dallas-Newton (1980) used a ten-point scale to give a sample a filament number (FN) to plot against the settling characteristics. Details of the assessment were not provided, although they claim the agreement between numbers from the authors was within half of a point.

Pipes (1979) counted filaments using a Neubauer haemocytometer. He diluted the samples with supernatant from settled mixed liquor, and observed them under 200x magnification.

Jenkins, *et al.* (1984) used a simplified counting technique that involved determining the area of a coverslip, and counting the number of times any filament intersects with the hairline of the microscope eyepiece. The sample is observed using 100x magnification. They used the following formula:

$$\text{Filament Count}/\mu\text{L} = \frac{\text{(filament intersections in field counted)}}{50\,\mu\text{L}} \times 12\ .$$

Walker (1982) developed an enumeration method that takes into account the number and length of filaments. He placed a small quantity into a Lund nanoplankton counting chamber and examined it under a light microscope equipped with a Whipple eyepiece graticule (calibrated using a stage micrometer), using 10X magnification. By making the assumption that the length of a filament crossing a square is on average equivalent to the width of that square, the length of the filament can be calculated. He calculated the filament length per unit weight of dry solids as follows:

$\text{Filaments (cm/mg MLSS)} = \dfrac{\text{N x F x 1000}}{\text{MLSS (mg/L)}}$, where N= count of filaments per 100 squares and $\text{F} = \dfrac{\text{Width of square (cm)}}{\text{area of 100 squares (cm}^2\text{) x cell depth (cm)}}$.

In an attempt to predict bulking, Green (1982) needed a method to count and measure filaments accurately. He used a MOP - VIDEOPLAN image analyzer, a computerized, semi-automatic device. Black and white photographs were taken, placed on a measuring tablet under an acetate sheet, and the filaments were measured with a felt-tip stylus. Species were not differentiated except for *Microthrix parvicella* and *Nocardia*, because they form "patches". *M. parvicella* was measured separately and a conversion factor was used.

2.4 SUMMARY

Activated sludge is a complex ecosystem. Floc formation exacerbates the usual difficulty in isolating bacteria, and the identification of bacteria to the genus level was beyond the scope of a Master's thesis. The literature provided simple techniques with which to culture and enumerate microorganisms such as denitrifiers, although enumeration was based on probability or rough guess.

Some organism populations have been found to be related to influent constituent concentrations or process performance. Sometimes the individual types of organisms involved influence process performance; otherwise, the number of organisms is important. Knowledge of these organisms may be useful to plant operators to monitor the activated sludge health or efficiency.

Chapter III

METHODS AND MATERIALS

3.1 PHYSICAL PARAMETERS OF AERATION BASIN

This study was designed to determine to what extent conditions in the aeration basin of the Celco activated sludge wastewater treatment plant affect bacteria and protozoa populations. Information on wastewater and mixed liquor characteristics is kept on a computer spreadsheet by operators.

3.2 SAMPLING

Aeration basin samples were taken from the unit in the treatment plant control building where lines from each process are fed. Approximately 50 mL were placed in a plastic bottle that provided at least 50 mL of airspace to keep the sample aerobic. During laboratory processing, the samples were kept in a refrigerator to minimize chemical or biological changes. All samples were processed on the day of sampling.

3.3 MOST PROBABLE NUMBER PROCEDURES

The number of organisms in the original sample cannot be counted directly. At best, the number can be estimated using Most Probable Number (MPN) procedures. These entail a serial dilution of the sample, then the inoculation of an artificial medium to grow the organisms. Confirmation of one or more specific biochemical reactions is scored as a positive response. The most probable number was estimated using the procedure given in Chapter 39 of *Methods of Soil Analysis* (1982), including Table 39-1, p. 818.

3.3.1 Denitrifiers

A culture medium of nutrient broth and potassium nitrate was prepared as described in *Methods of Soil Analysis* (1982), pp. 1018-1019. Ten milliliters of medium was pipetted into each of 40 10-mL screw-cap culture tubes and autoclaved. From a fresh sample, ten-fold dilutions were made from 10^{-2} to 10^{-8}, using an 0.85% saline solution as the diluent.

Five tubes were used per dilution. Each was inoculated with 0.1 mL of the appropriate dilution. If 8 mL tubes were used, the inoculum volume was adjusted to maintain a ten-fold dilution. The tubes were marked with the sample number and the final dilution factor, then left in a dark cupboard at room temperature for two weeks.

At the end of the two-week incubation period, the tubes were tested for the presence of nitrate and nitrite. Approximately 1 mL was poured into a small test tube and several drops of diphenylamine reagent was added. The diphenylamine reagent is oxidized by the anions to diphenylbenzidine, then to the blue quinoid imonium ion.

3.3.2 Sulfate-reducers

The first method used for enumerating sulfate-reducers was taken from *Methods of Soil Analysis* (1982). The medium contained agar and was difficult to inoculate and obtain consistent results.

The second method was taken from *Standard Methods* (1985), p. 1026. A culture medium of sulfate-reducing medium was prepared. Commercially prepared nutrient broth was supplemented with 1.0 g/L peptone powder in order to meet the requirements. L-ascorbic acid was substituted for sodium ascorbate. The medium contained no agar and was easy to inoculate. Ten milliliters of medium was pipetted into each of 40 10-mL screw-cap culture tubes and autoclaved. From a fresh sample, ten-fold dilutions were made from 10^{-2} to 10^{-8}, using an 0.2% saline solution as the diluent.

Five tubes were used per dilution. On the sampling day, the ferrous ammonium sulfate and ascorbic acid were added, and each tube was inoculated with 0.1 mL of the appropriate dilution. If 8 mL tubes were used, the inoculum volume was adjusted to maintain a ten-fold dilution. The tubes were marked with the sample number and the final dilution factor, inverted several times to stir the contents, and left in a dark cupboard at room temperature. After two weeks, any tubes that turned black were considered to be positive.

The most probable number was obtained using the procedure given in Chapter 39 of *Methods of Soil Analysis* (1982), and Table 39-1, p. 818.

3.4 HETEROTROPHIC PLATE COUNT

A heterotrophic plate count provides a method to estimate the number of organisms in the original sample that will grow on Standard Methods Agar. The procedure used was taken from *Standard Methods* (1985). The medium was prepared as outlined on the product label. Serial ten-fold dilutions from the denitrifier or sulfate-reducer procedures were used to inoculate two plates per dilution, 0.1 mL per plate. Two control plates were inoculated with sterile dilution fluid. The plates were poured and placed in an incubator at

35° C for at least 24 hours before inoculation, in order to dry the medium (inoculate beaded on fresh medium, making the counting of individual colonies difficult). Plates were placed in a dark cupboard at room temperature for 48 hours. Colonies were counted using the procedure given on p. 866 of *Standard Methods* (1985).

3.5 PROTOZOA

3.5.1 Counting

Initially, protozoa were counted individually over the entire slide. However, because this was so time consuming, the heat from the microscope lamp began to dry the slide. Therefore, the Palmer-Maloney (P-M) Method from *Standard Methods* (1985), p. 1062, was adopted. A plastic slide with a well of known volume was filled with undiluted sample. A cover slip pressed lightly onto the ring ensured the correct volume of sample. The ocular was fitted with a grid, the area of which was measured using a micrometer. The sample was viewed under 200X power and any protozoan within or touching the grid was counted. In order to obtain accurate results, the entire depth of field had to be examined by focusing up and down. The slide was randomly moved under the objective and 10-20 grids or "fields" were examined. The number of fields counted depended on the number of protozoa in the sample. If there were few protozoa, more fields had to be counted in order to make the results significant. The numbers of protozoa were totaled and the count was repeated until the counts were roughly within 10% of the average. One slide required as many as ten recounts. The organisms were categorized whenever possible and the relative numbers were noted.

3.5.2 Analysis

The estimation of the number of protozoa per mL was made using the following calculation:

Protozoa / mL = (# protozoa)(CF)[chamber area / (grid area x # grids)],

where "# protozoa" was the average number of the two or three counts, CF was a correction factor for the volume of the P-M cell, and the grid area was calibrated at 200X magnification. For the P-M cell and grid used, CF was 8.92 mL^{-1}, chamber area was 232.2344 mm^2 and the grid area was 0.122 mm^2. The correction factor is unique to each P-M cell.

3.6 FILAMENTS

3.6.1 Counts

Filaments were counted by using a combination of the procedure given in Jenkins, *et al.* (1984) and the Palmer-Maloney method from *Standard Methods* (1985). One drop (approximately 50 μL) of undiluted sample was spread onto a clean slide. Unless a slide was very clean, water would not adhere to it to give a uniform smear. After drying, the slide was stained using Neisser stain. Because the smear was irregularly shaped, a square area was drawn, which touched the sides of the smear and included any areas not covered by the smear. The number of grids (see the section on Protozoa Counts) along the length and width of the square was counted in order to calculate the area. Random fields, including those outside the smear, were counted as follows: any filament touching or crossing the centerline of the ocular grid was counted. Twenty fields were counted and totaled. The process was then repeated with the same slide, as often as necessary in order to obtain consistent results. The average number of filaments per grid was multiplied by the total number of grids. This was the estimated number of filaments per drop.

3.6.2 Identification

An attempt was made to identify the filament types, although most observations were made from Gram- and Neisser-stained preparations. Filaments were examined under oil immersion for the following characteristics: relative abundance; average length, diameter and shape of trichome; reaction to Gram and Neisser staining (presence of intracellular granules or staining of trichome); location in relation to flocs; cell dimensions; presence of septa, sheath, branching and epiphyte growth. Descriptions, photographs and drawings provided by Jenkins, *et al.* (1984), Strom and Jenkins (1984) and Eikelboom (1974) were compared to Celco samples. Filament diameter and length were measured using a micrometer that was calibrated at the various magnifications used.

Chapter IV

RESULTS

4.1 GENERAL

In this chapter, microbial population data will be presented. Comparisons of these data with Celco influent, aeration basin and effluent characteristics will also be presented. One of the original parameters of study was aeration basin temperature. The equalization basin influent is >100° F. During the hot summer months the aeration basin influent can exceed 85° F, although normal temperatures range from <60° F to >85° F. Operators supplied information on dissolved oxygen, sludge age, suspended solids and pH.

Conditions at the plant did not vary much during the course of the study. There were no real shock loads to the aeration basin, and the effluent rarely exceeded quality limits. Above average winter ambient temperatures did not provide wide aeration basin temperature fluctuations.

Because one of the objectives of this study was the investigation and evaluation of culture and enumeration techniques, there are gaps and inaccuracies in the data resulting from changes or errors in technique. A complete discussion of the problems with the techniques chosen will be given in the following chapter, as will suggestions to improve future results.

4.2 BACTERIA

The results of the heterotrophic plate counts (HPC) from March through September are shown in Figure 2. A sample taken on 17 April was largely unusable due to a dilution error. In other cases, results were inconclusive due to contamination of the Petri plates. These data were not included. Every attempt was made to inoculate the plates in a relatively clean room, on a surface that had been cleaned with Lysol®. Any plate with 30-300 colonies was counted, and plates of the same dilution were averaged to obtain the results shown.

The heterotrophic plate counts were plotted with the average dissolved oxygen concentrations in the aeration basin for the two weeks preceding sampling (Figure 3). The bacteria numbers from this counting procedure follow the dissolved oxygen concentrations somewhat, although it must be stressed that the numbers shown are only for bacteria that grow on the medium used. It is unknown how many bacteria were present in the original sample. The plate count was also plotted against the oxygen uptake rate (OUR) (Figure 4). Clearly, the results of the plate count do not correlate with changes in the OUR.

The target mean cell residence time (MCRT) was 14 days until mid-September when it was increased to 18 days. The heterotrophic plate counts appeared to fluctuate inversely with MCRT (Figure 5).

No correlation between plate counts and influent BOD was apparent (Figure 6), nor to F/M ratio (Figure 7). HPC and effluent nitrogen may be inversely related (Figure 8). On 11 June the HPC count was highest, and total nitrogen levels were low. When the effects of individual influent organic compounds were examined, there was a possible inverse relationship between HPC and methyl ethyl ketone and mesityl oxide (Figures 9 & 10, respectively). No apparent relationship existed between isopropyl alcohol, methyl cyanate, acetone or acetic acid levels and HPC (Figures 11, 12 & 13).

4.3 DENITRIFIERS

4.3.1 General

The method used to culture denitrifiers was consistent. As stated in **Methods and Materials**, this method may not necessarily enumerate denitrifying bacteria. It may, however, indicate denitrifying potential of an activated sludge. A system that is aerobic, not anoxic, will not denitrify significantly. This method reduces the oxygen concentration in the medium to <1 mg/L, stimulating the synthesis and activity of denitrifying enzymes. This method will be discussed further in the following chapter.

4.3.2 Comparison to Aeration Basin Characteristics and Process Performance

The denitrifier (DN) population varied from <0.5 x 10^5 to > 2.0 x 10^7 cell/mL during the study . Denitrifiers are aerobic and utilize NO_3^- only when O_2 is absent (Tiedje, 1982). From mid-April to the end of the study, the DN counts appeared to vary as the DO concentrations in the aeration basin (Figure 14). These DO values did not reflect the condition in the center of flocs.

In order to determine whether denitrifier populations fluctuated similarly with nitrogen loading, the total nitrogen to the systems was calculated and compared to denitrifier counts (Figure 15). Again, no relationship was apparent.

Denitrifier populations were also examined with regard to effluent nitrogen concentrations (Figure 16). During the first half of the study period the DN counts appeared to vary inversely with effluent nitrogen concentrations although NO_X levels were not affected. Only after the 27 May sample did DN counts and NO_X show an inverse

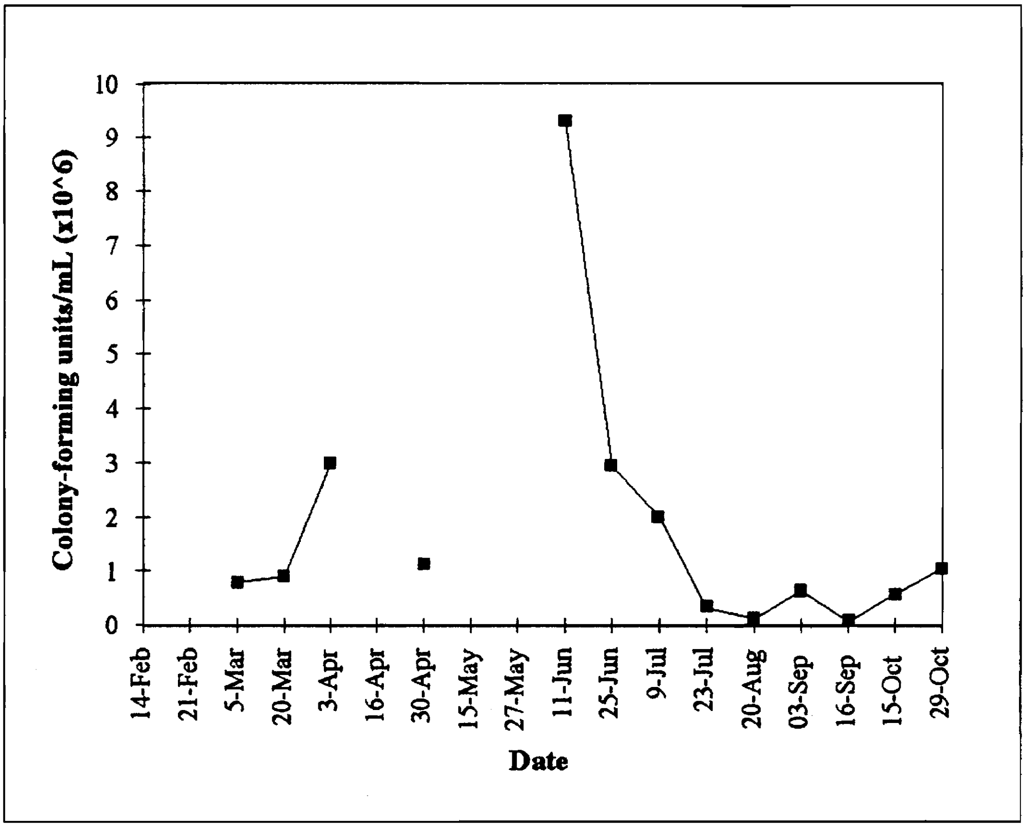

Figure 2. Heterotrophic plate counts during study. Celco activated sludge, 1992.

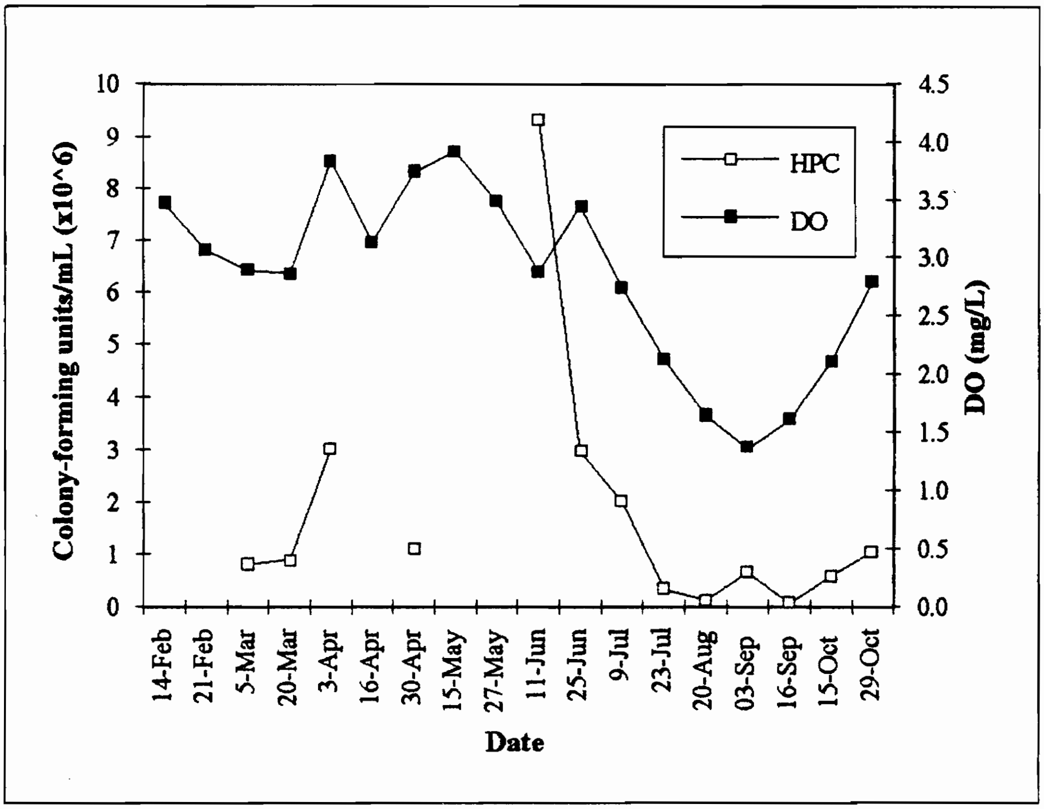

Figure 3. Comparison of dissolved oxygen (DO) in aeration basin to heterotrophic plate counts in Celco activated sludge, 1992. DO values were averaged over the period preceding sampling equal to the target sludge age.

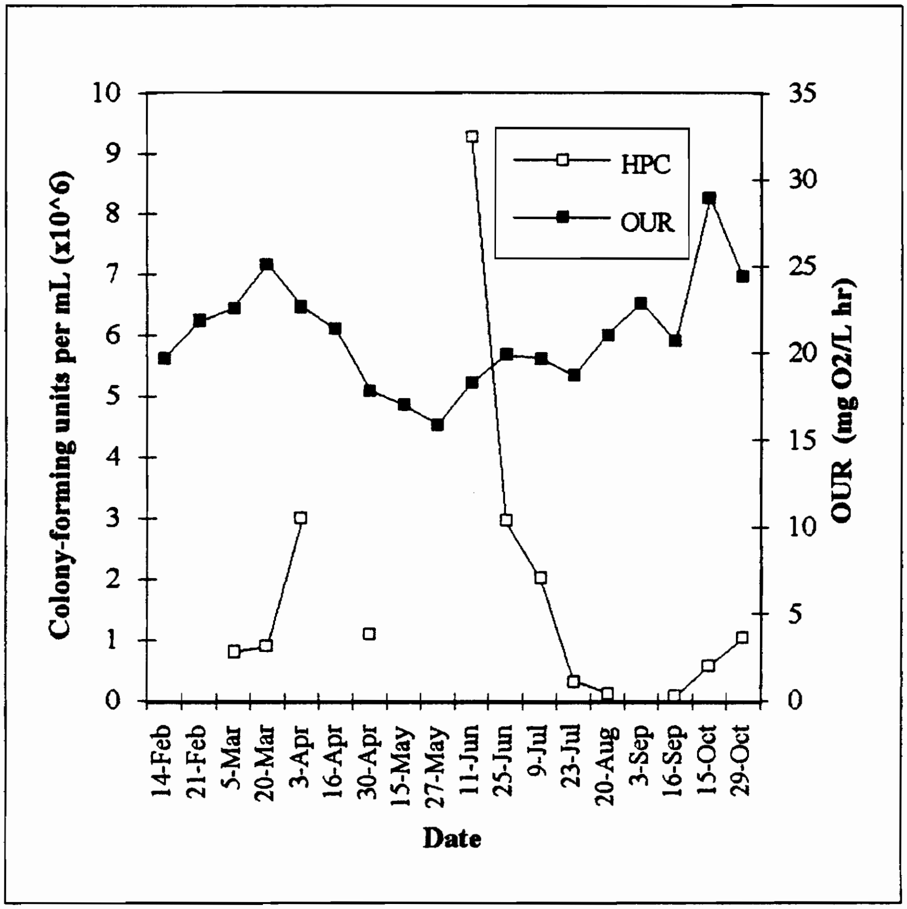

Figure 4. Comparison of mixed liquor oxygen uptake rates (OUR) to heterotrophic plate counts of Celco activated sludge, 1992. OUR values were averaged over the period preceding sampling equal to the target sludge age.

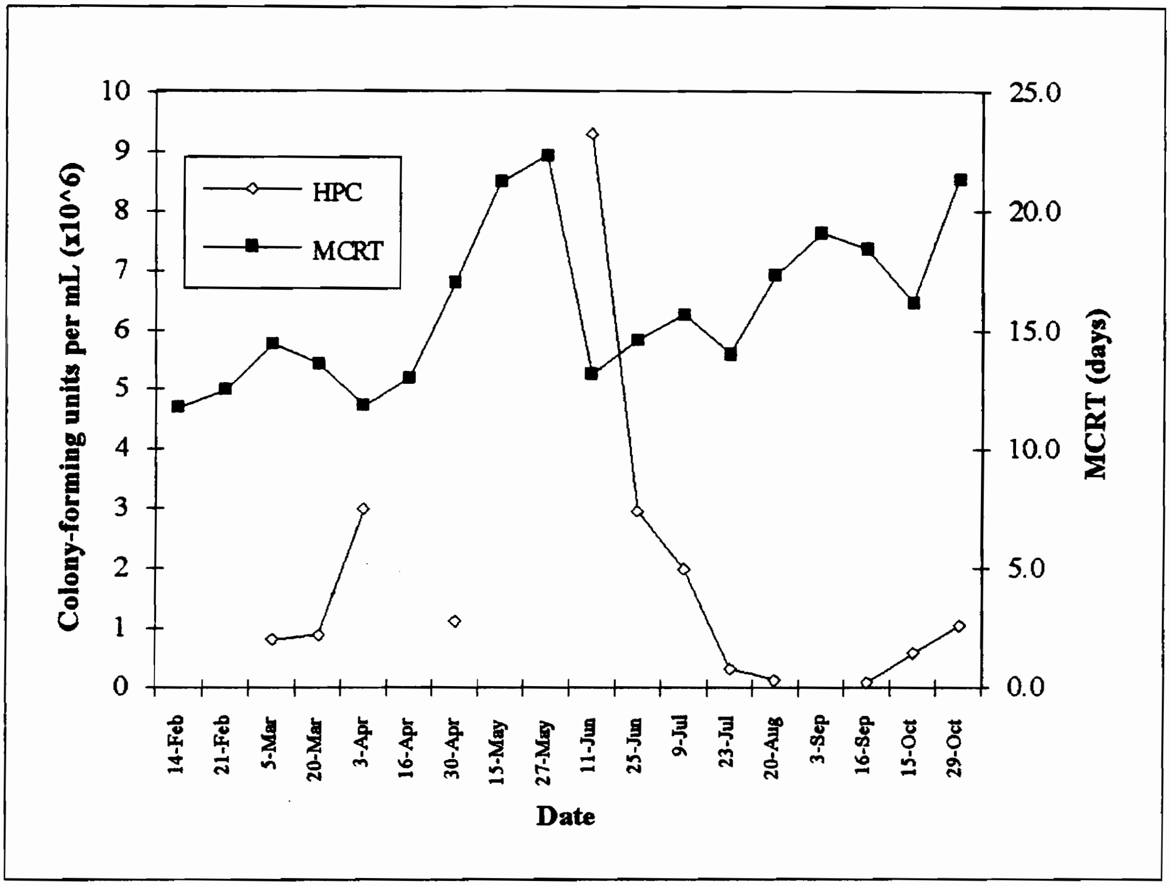

Figure 5. Comparison of changes in mean cell residence time (MCRT) to heterotrophic plate counts of Celco activated sludge, 1992. MCRT values were averaged over the period preceding sampling equal to the target sludge age.

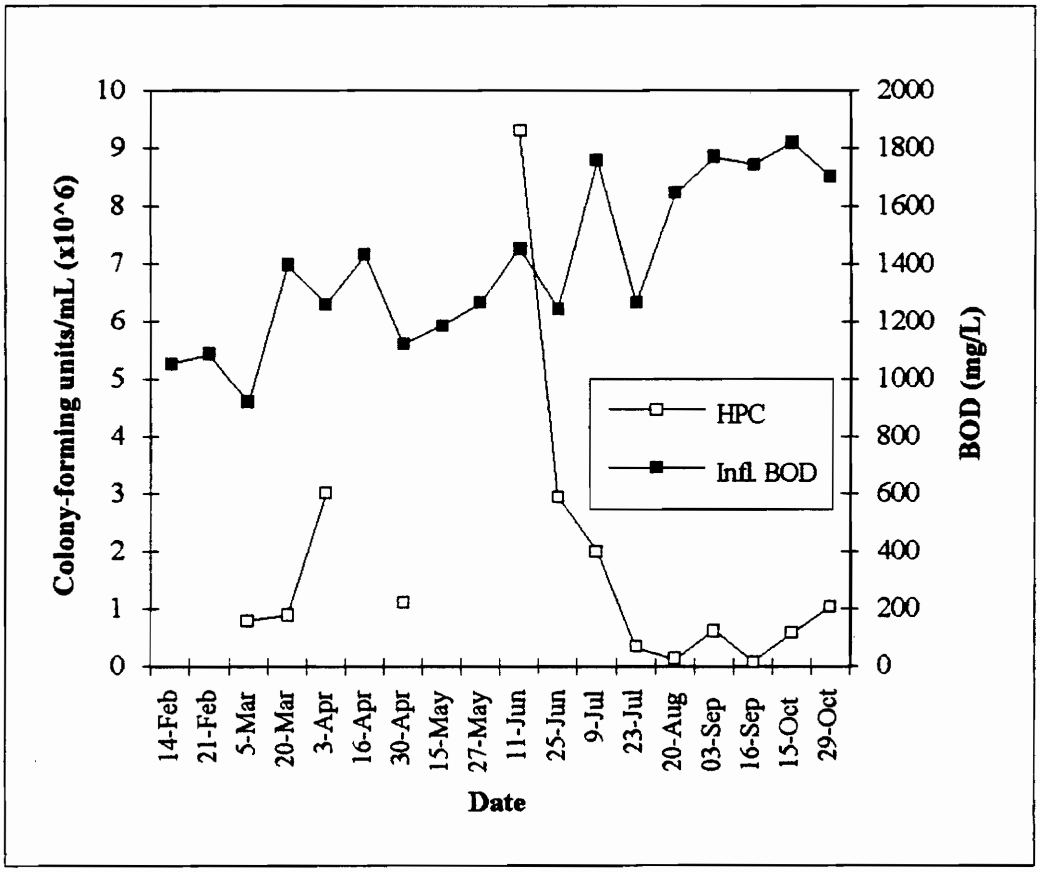

Figure 6. Comparison of influent biochemical oxygen demand (BOD) to heterotrophic plate counts of Celco activated sludge, 1992. BOD values were averaged over the period preceding sampling equal to the target sludge age.

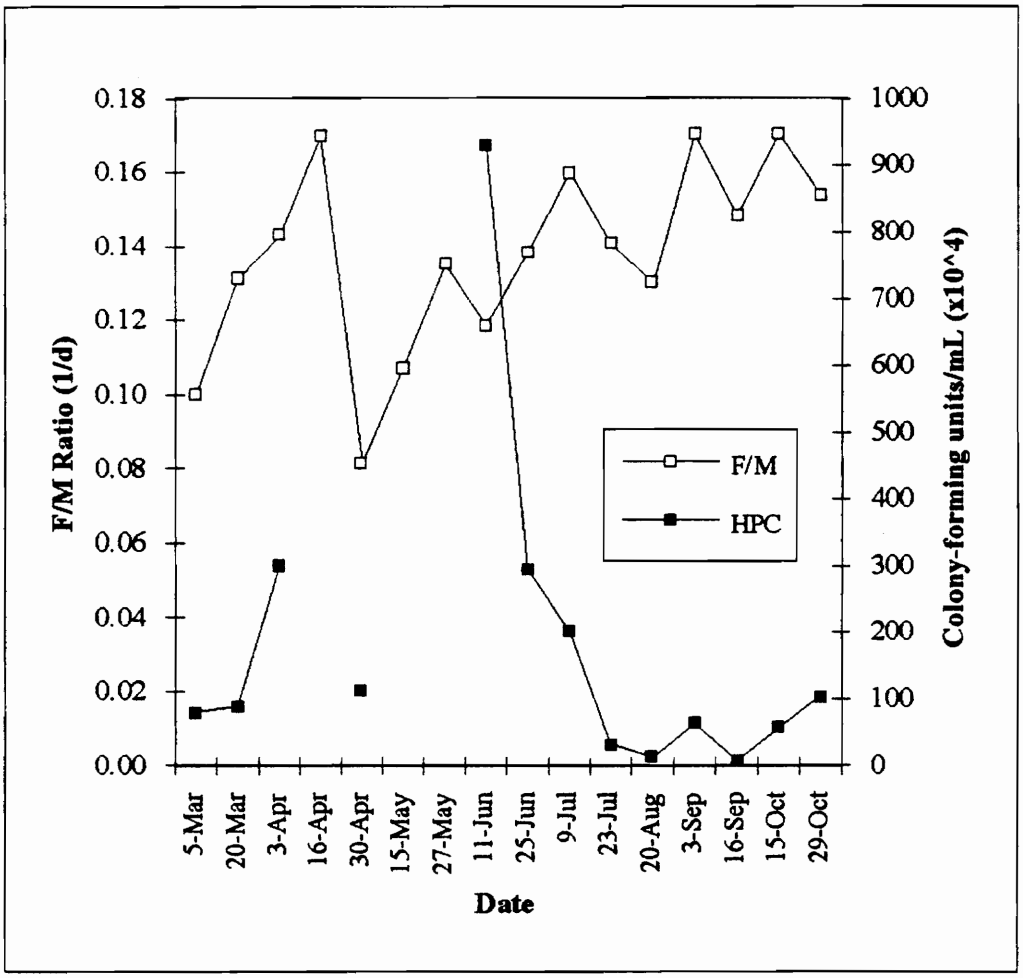

Figure 7. Comparison of food-to-microorganism (F/M) ratio to heterotrophic plate counts of Celco activated sludge, 1992. F/M ratio values were averaged over the period preceding sampling equal to the target sludge age.

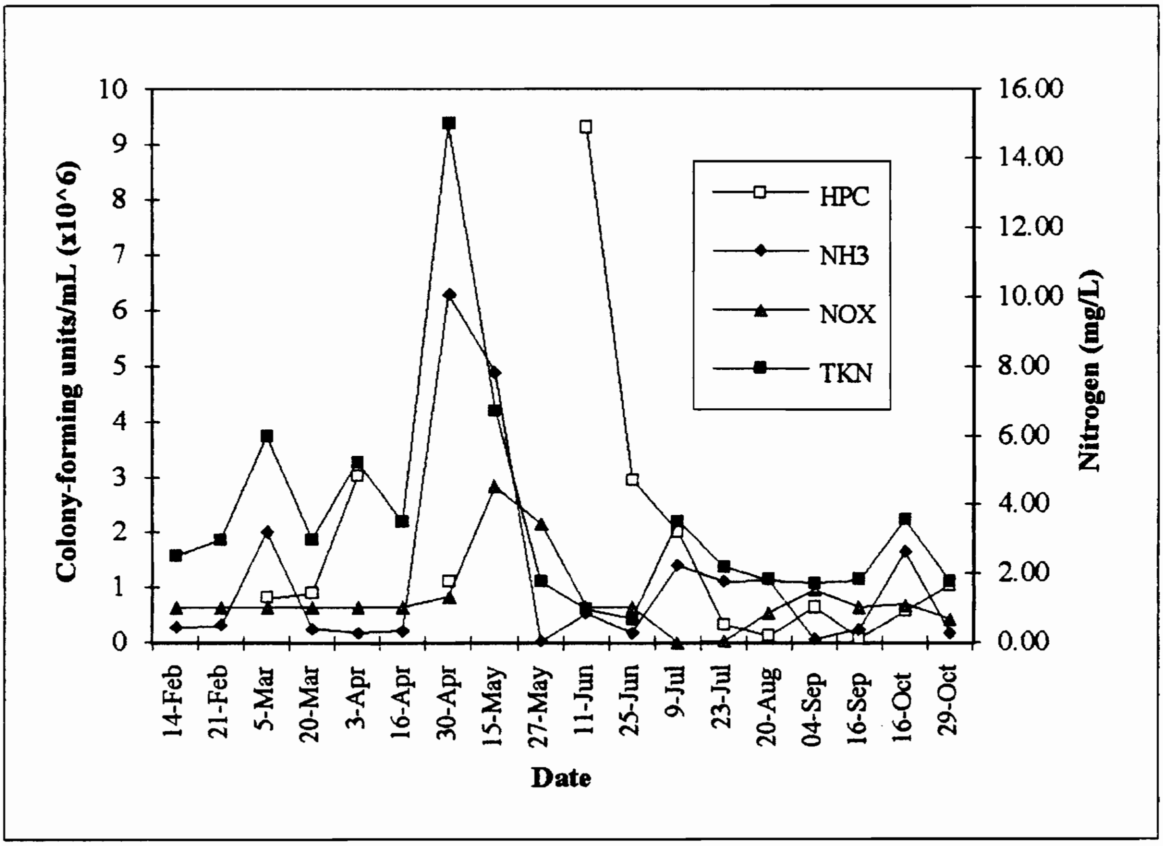

Figure 8. Comparison of heterotrophic plate counts to effluent nitrogen of Celco activated sludge, 1992. Nitrogen values were averaged over the period preceding sampling equal to the target sludge age. NH_3 = ammonia; TKN = total Kjeldahl nitrogen; NOx = nitrate + nitrite.

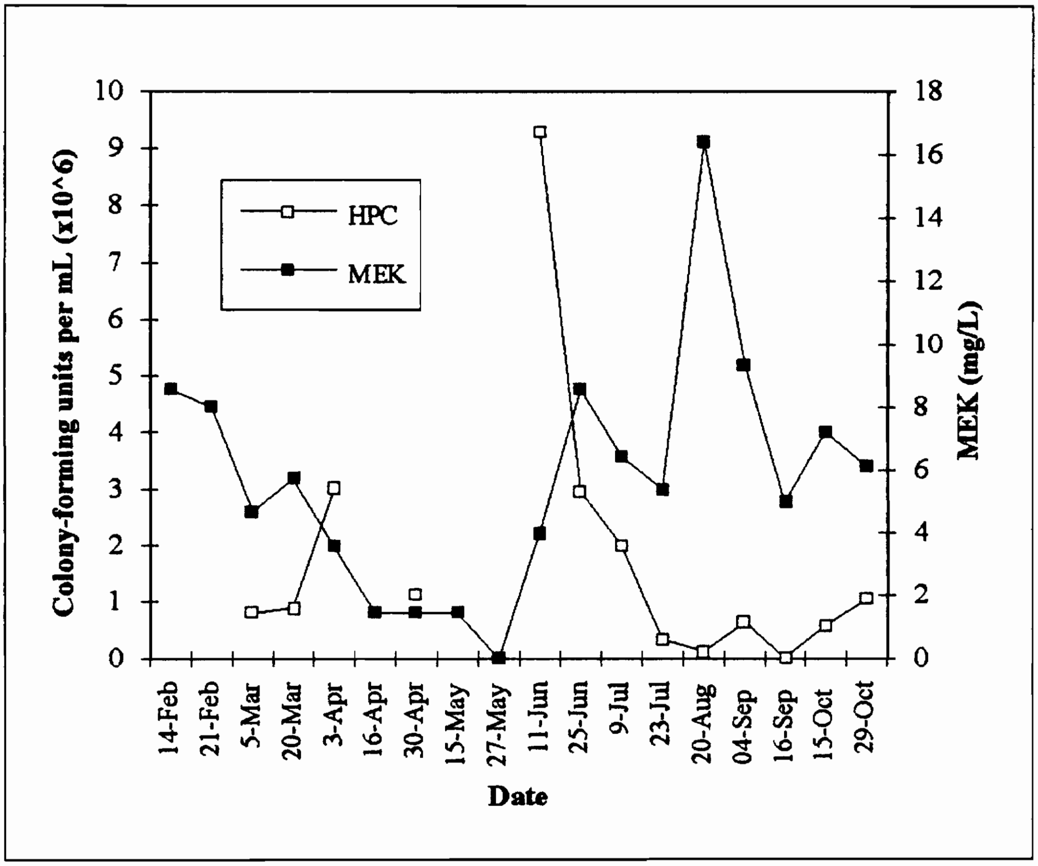

Figure 9. Comparison of influent methyl ethyl ketone (MEK) concentration to heterotrophic plate counts to Celco activated sludge, 1992. MEK values were averaged over the period preceding sampling equal to the target sludge age.

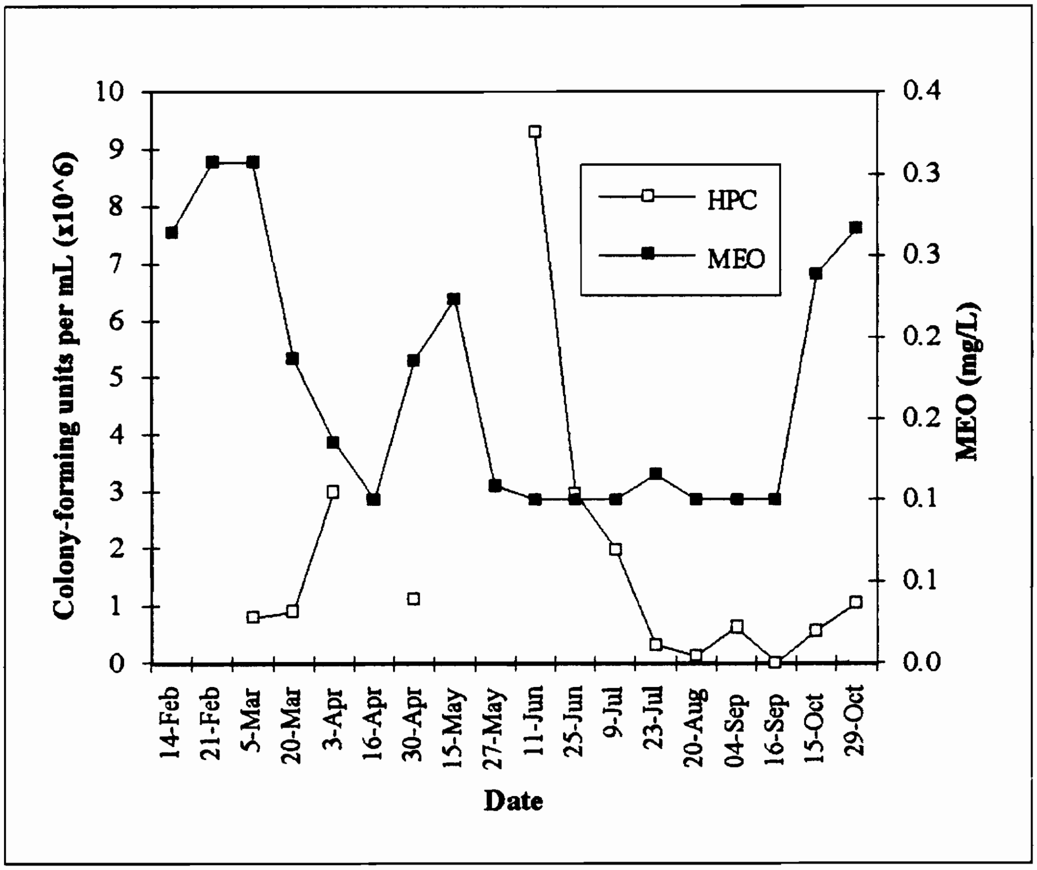

Figure 10. Comparison of influent mesityl oxide (MEO) concentration to heterotrophic plate counts of Celco activated sludge, 1992. MEO values were averaged over the period preceding sampling equal to the target sludge age.

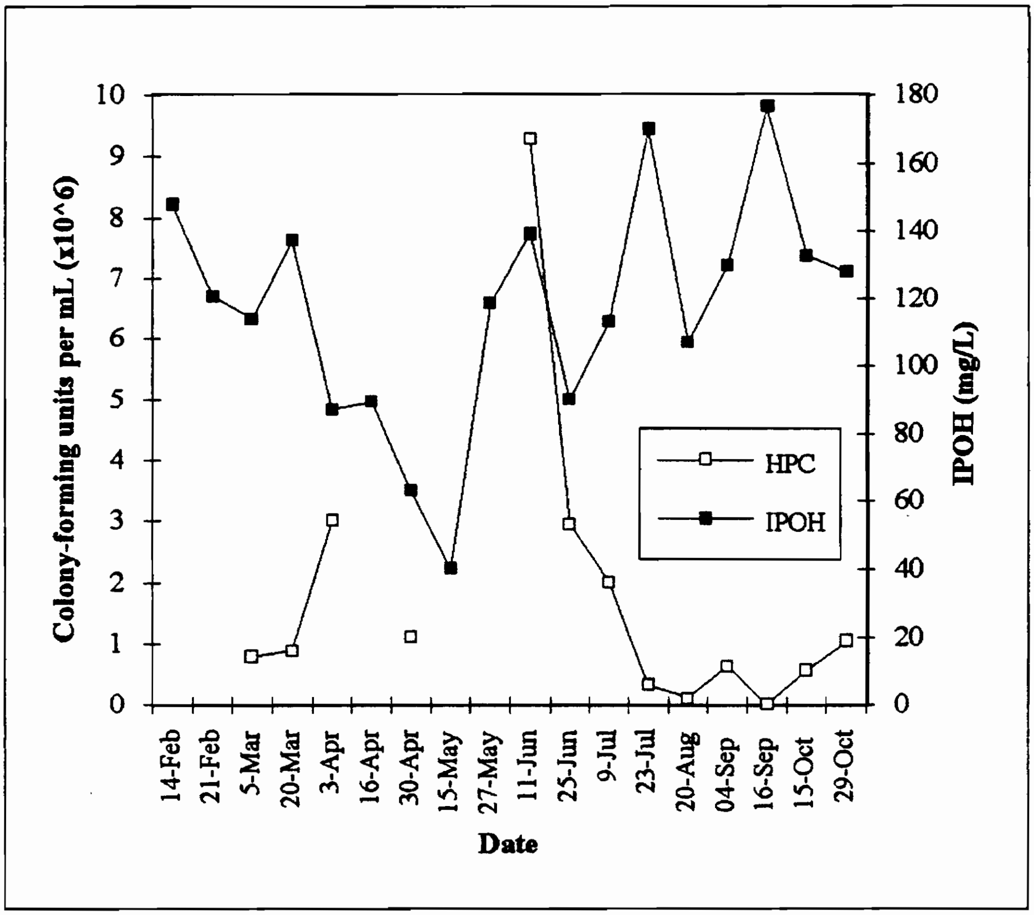

Figure 11. Comparison of influent isopropyl alcohol (IPOH) to heterotrophic plate counts of Celco activated sludge, 1992. IPOH values were averaged over the period preceding sampling equal to the target sludge age.

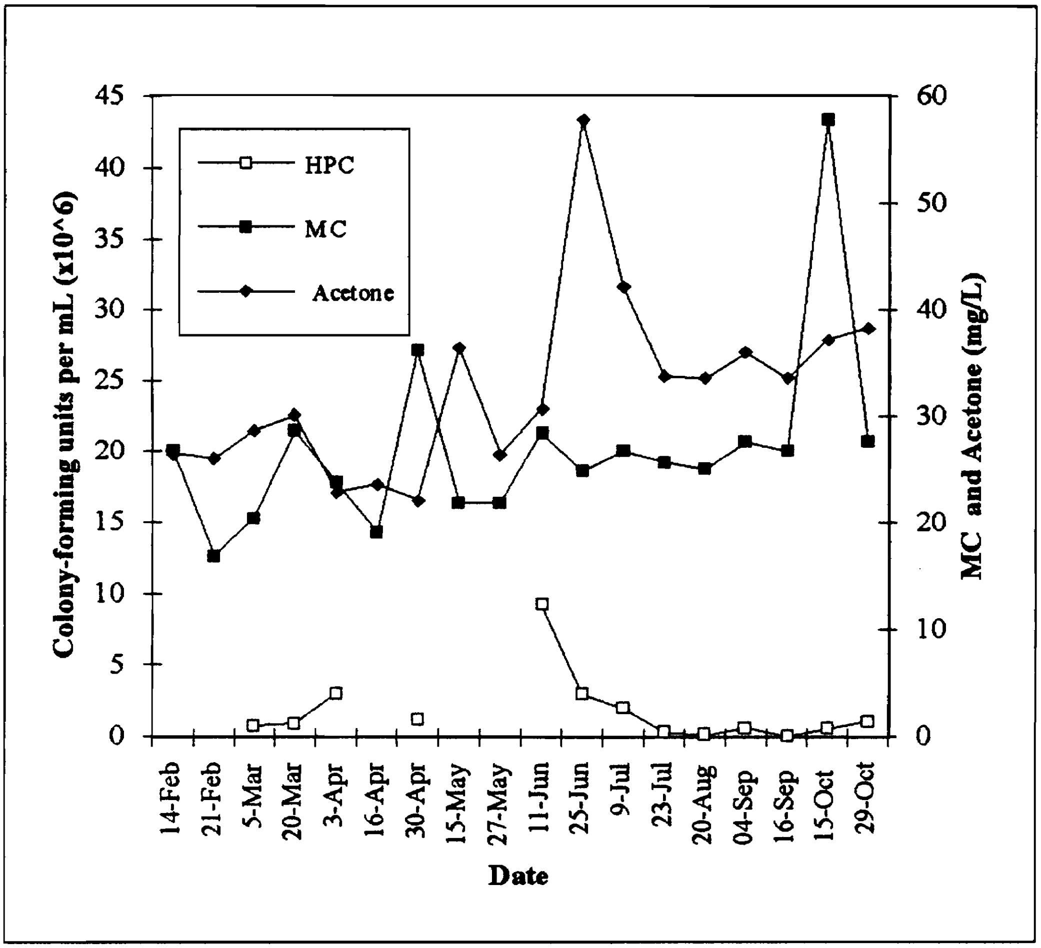

Figure 12. Comparison of influent methyl cyanate (MC) and acetone to heterotrophic plate counts of Celco activated sludge, 1992. MC and acetone values were averaged over the period preceding sampling equal to the target sludge age.

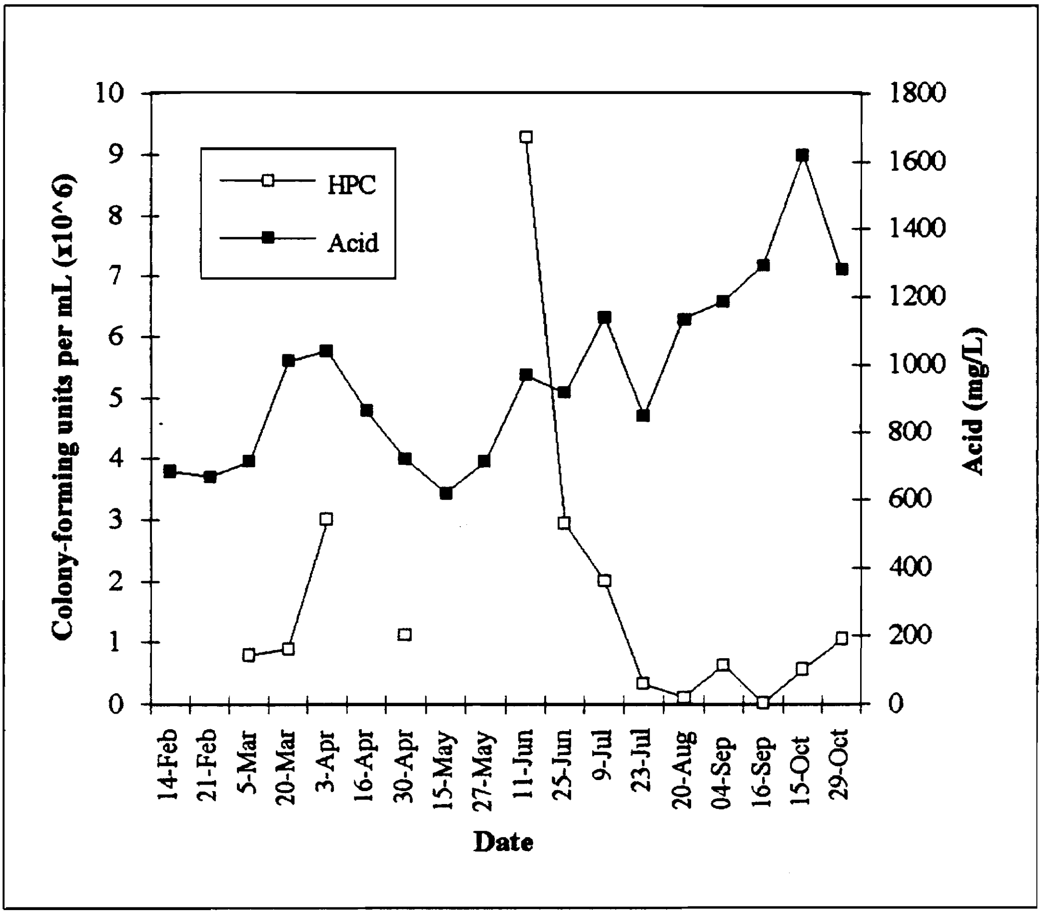

Figure 13. Comparison of influent acid to heterotrophic plate counts of Celco activated sludge, 1992. Acid values were averaged over the period preceding sampling equal to the target sludge age.

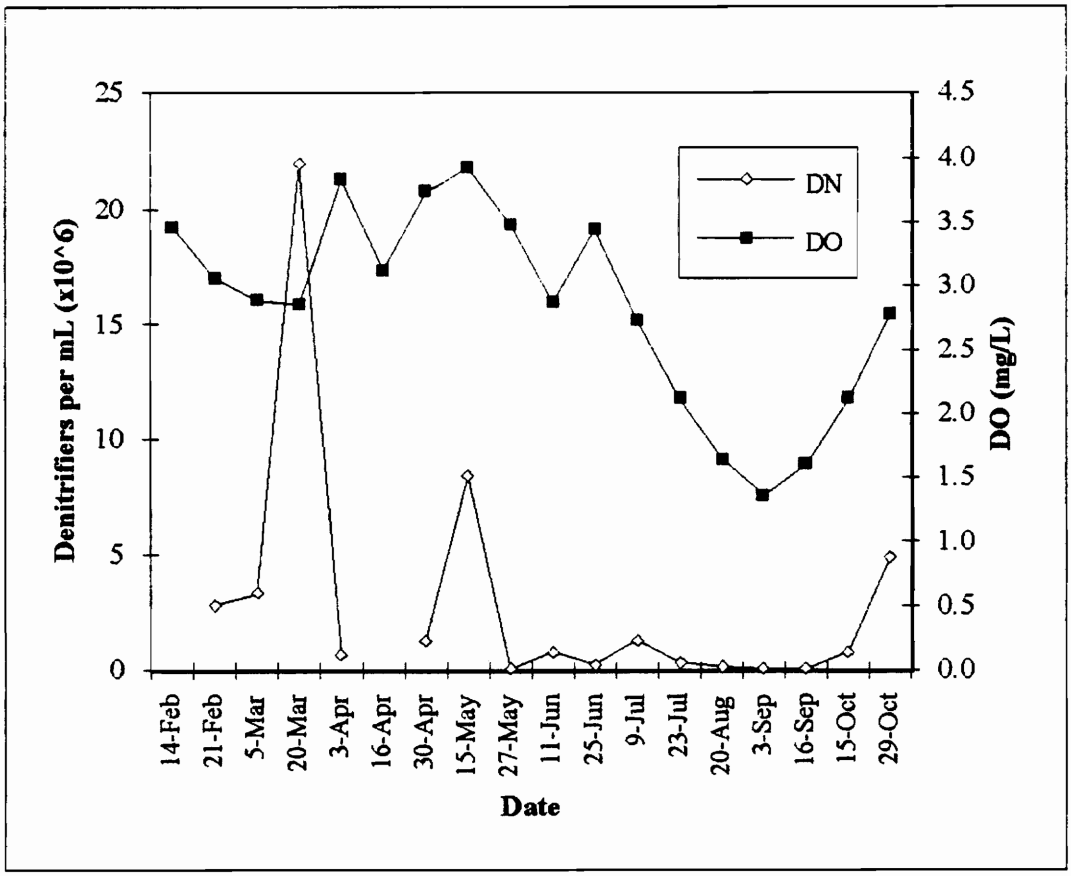

Figure 14. Comparison of dissolved oxygen (DO) concentration in aeration basin to MPN denitrifiers (DN) in the Celco activated sludge, 1992. DO values were averaged over the period preceding sampling equal to the target sludge age.

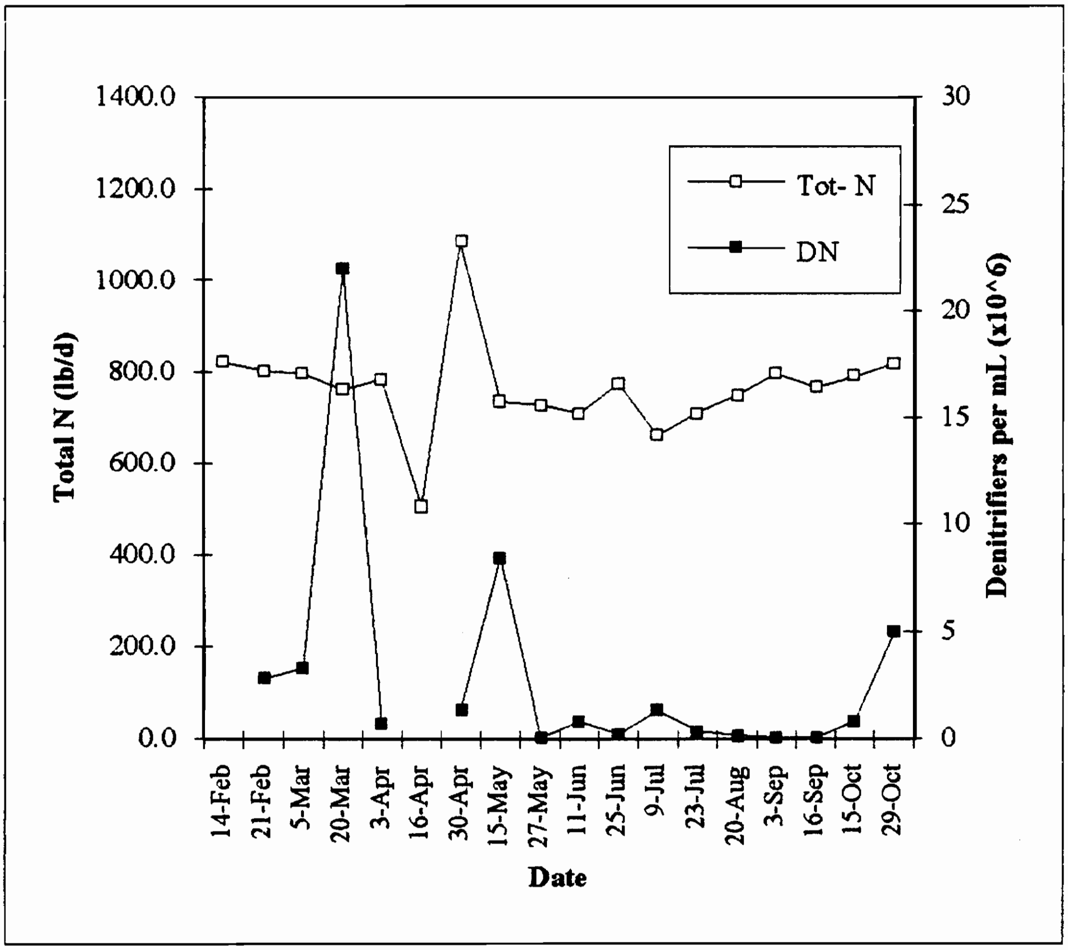

Figure 15. Comparison of MPN denitrifiers (DN) in the Celco activated sludge to the total nitrogen (Tot-N) entering the biological system. Nitrogen values were averaged over the period preceding sampling equal to the target sludge age (1992).

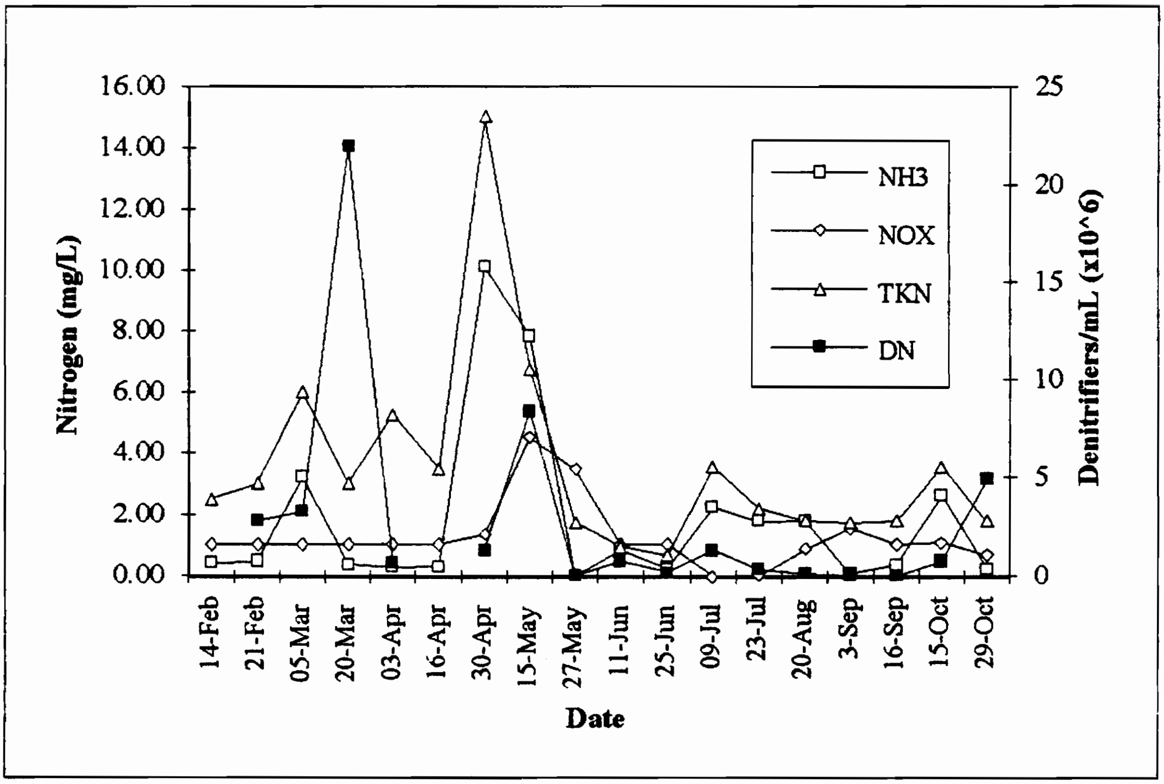

Figure 16. Comparison of the denitrifier (DN) population in the Celco activated sludge (1992) to the effluent nitrogen concentrations. Nitrogen values were averaged over the period preceding sampling equal to the target sludge age. NH_3 = ammonia; TKN = total Kjeldahl nitrogen; NOx = nitrate + nitrite.

relationship. It should be noted that from August through September the average DO concentration is <2.0 mg/L, which overlaps this period.

Causes of turbid effluent include bulking, rising sludge and deflocculation. Denitrification produces N_2 and N_2O. Because N_2O is very water-soluble, only N_2 bubbles could contribute to rising sludge. To determine whether DN counts could be related to effluent suspended solids the average SS was plotted against DN counts (Figure 17). DN counts, effluent suspended solids and sludge volume index did not appear to be related (Figure 18).

4.4 SULFATE-REDUCERS

The sulfate-reducer data is presented in Figure A-1. The first method chosen for enumerating sulfate-reducers was taken from *Methods of Soil Analysis* (1982). However, this method was neither consistent nor effective in culturing sulfate-reducing bacteria from the Celco activated sludge (see **Methods and Materials.**) A culture and enumeration method was taken from *Standard Methods* (1985) that was effective and consistent.

The Celco influent has a high concentration of sulfate due to the use of sulfuric acid in the synthesis of cellulose acetate. Sometimes black mats have been observed floating in the clarifiers. During this study, some samples in the refrigerator turned black within two days, while others never did. Because so few reliable data were obtained, no correlation between blackening samples and culture results could be investigated.

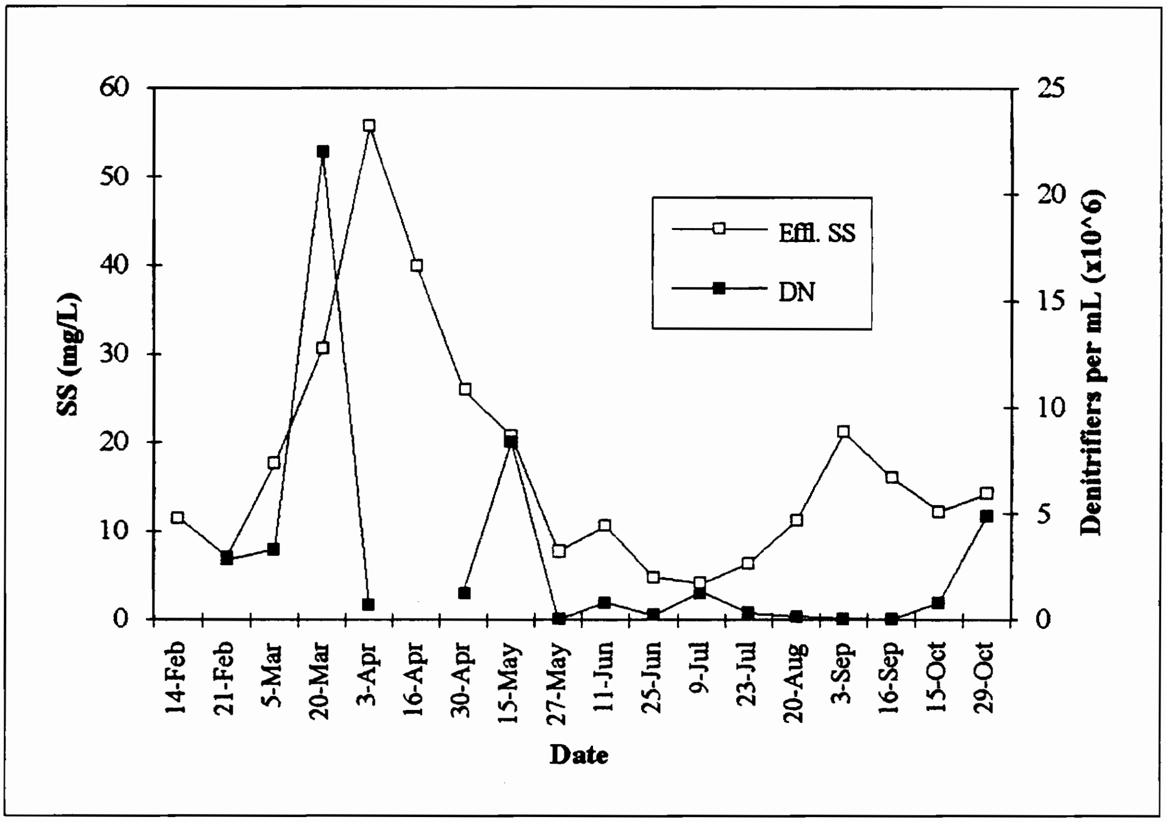

Figure 17. Comparison of the Celco activated sludge denitrifier (DN) MPN values to effluent suspended solids (SS) concentrations (1992). Suspended solids values were averaged over the period preceding sampling equal to the target sludge age.

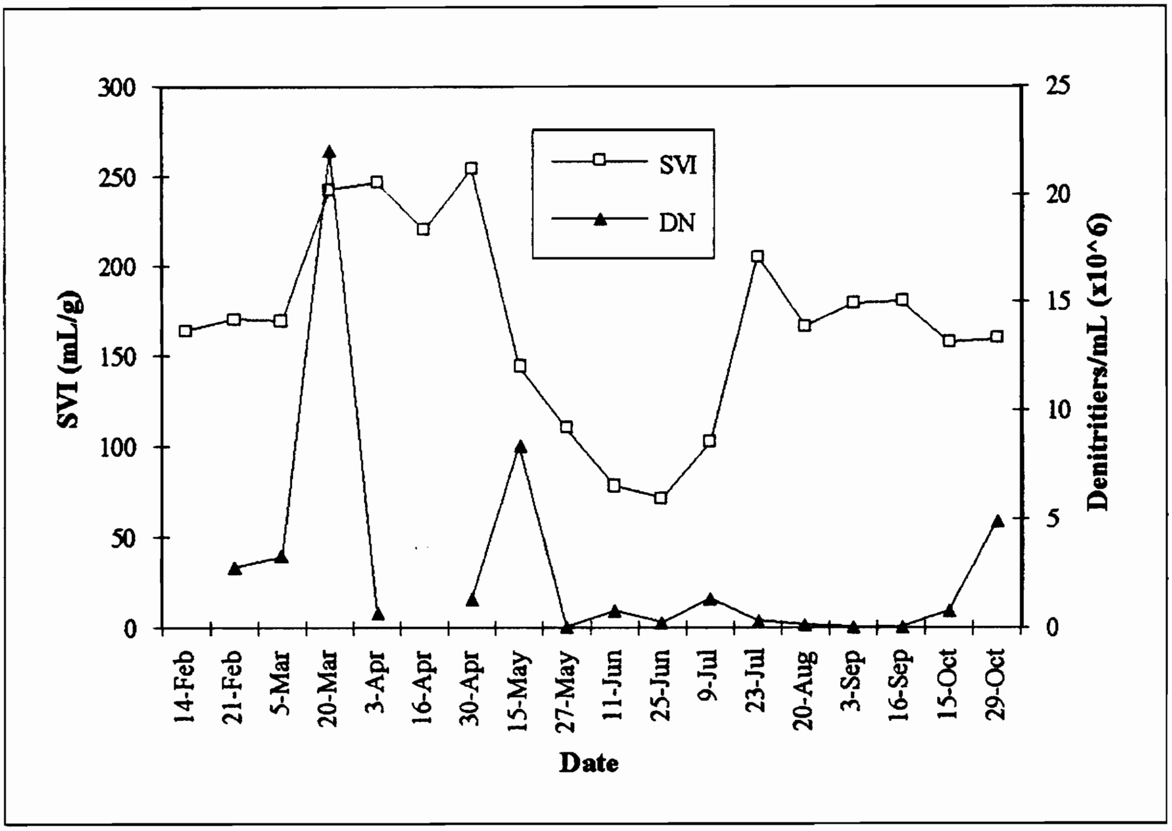

Figure 18. Comparison of the Celco activated sludge denitrifier (DN) MPN values to the sludge volume index (SVI) (1992). SVI values were averaged over the period preceding sampling equal to the target sludge age.

4.5 PROTOZOA

4.5.1 General

The results of the protozoa counts from March through October are shown in Figure 19. The Palmer-Maloney method was begun in April. Before that, an attempt was made to count the protozoa in the entire area under a cover slip. However, the slide dried too quickly and some protozoa were too motile to make it a feasible method. Counts made during that period were rough estimates of the percentage of the slide actually counted. Protozoa were differentiated into *free-swimming* (FS), *stalked*, *flagellated* (Flag) and *predators* (Pred). The relative numbers were plotted with time (Figure 20). No attempt was made to identify the species present, although sketches were made during counting. Genera observed were *Vorticella*, *Lionotus* (or *Litonotus*), *Euplotes* and possibly *Chilodonella*. In samples from 23 July to 16 September (A to B), an organism with "wormlike" movement was observed. There were a large number in samples from 4 and 16 September, although it was not possible to count them accurately. First, they were detectable almost entirely by deduction from the movement of flocs. Second, movement ceased soon after counting began. When visible, they were attached to or entwined in the flocs, and resembled nematodes, although the size of protozoa. When not moving, they were undetectable. On 16 September (C), there were very small free-swimmers that were too numerous, and too fast, to count. Had they been slowed or killed, they may have been undetectable. The spike in total protozoa number for 16 September reflects the "worms" that could be counted before they stopped moving. The actual number of total protozoa should be much higher because of the small free-swimmers, which were not counted. The dates of these unusual and transient populations are noted on the figures.

No amoebae were identified. Some types resemble oval cells and may have been overlooked. A large organism, approximately 125 µm long, with a large, muscular buccal organ and no apparent cilia, was often observed. Because one was observed attempting to ingest a stalked ciliate, it was assumed to be a predator and was recorded as such. Another crawler, approximately the same size, had a trilobed "foot". The term *predator* is used to describe these two organisms, which may have been rotifers.

4.5.2 Comparison to Heterotrophic Plate Count

Ciliates were plotted against heterotrophic plate counts (Figure 21). No clear relationship existed. Stalked ciliates were numerous at the same time that a dominant population of "tetrad" non-floc bacteria (15 May) was observed. These were bacteria that appeared to have remained attached to each other after cell division. The groups resembled cubes, and had many more than four cells per side. Protozoa counts also appeared to be inconsistent with fluctuations in OUR (Figure 22).

4.5.3 Comparison to aeration basin conditions

Mean Cell Residence Time

The mean cell residence time (MCRT), or sludge age, was compared to stalked and free-swimming ciliate populations to see whether the data agreed with the 1971 study by Curds (Figure 23) (see Chapter II), in which he concluded that free-swimming ciliate populations increased as the wash-out rate decreased. The MCRT was averaged over the period preceding sampling equal to the target MCRT. The target MCRT was 14 days prior to mid-September, when it was increased to 18 days.

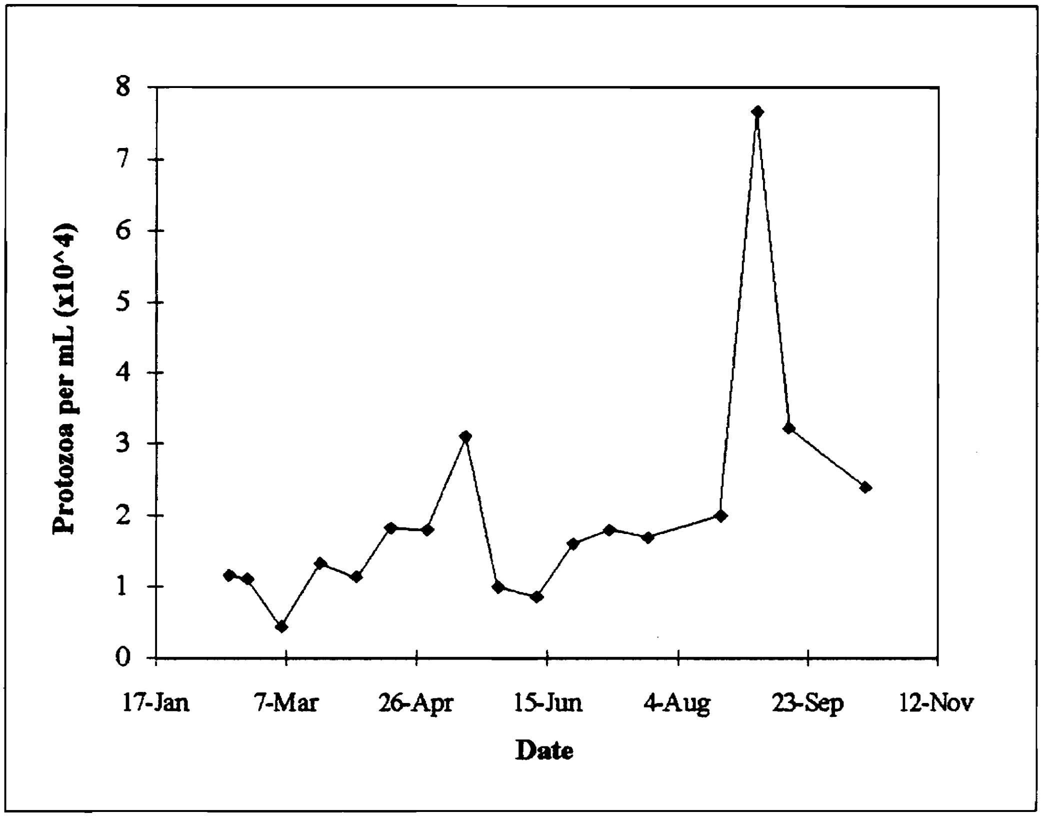

Figure 19. Estimated protozoa populations during study of Celco activated sludge, 1992.

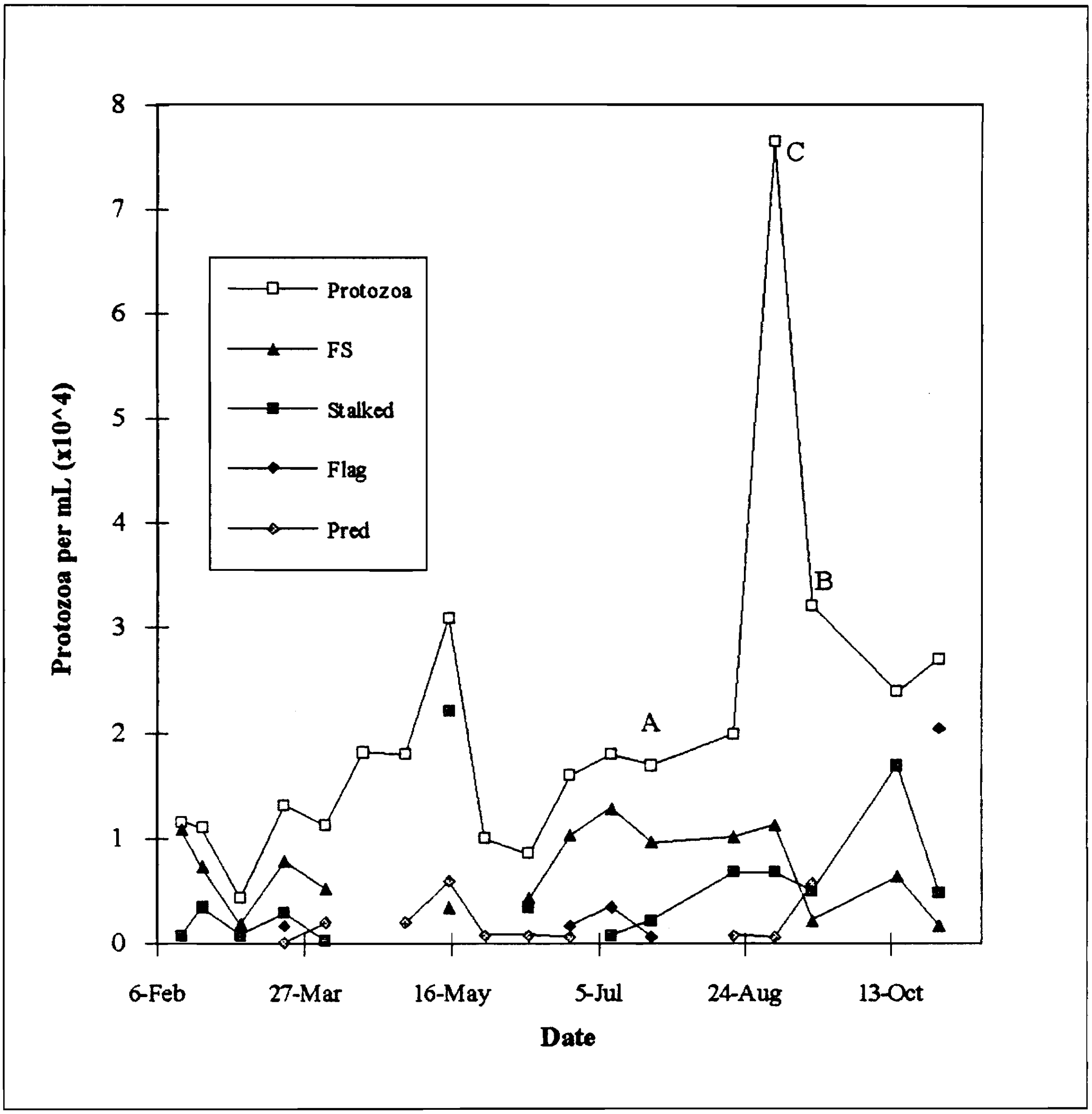

Figure 20. Differential protozoa counts during study of Celco activated sludge, 1992. FS = free-swimming ciliates; Stalked = stalked ciliates; Flag = flagellates; Pred = predators; Protozoa = total protozoa.

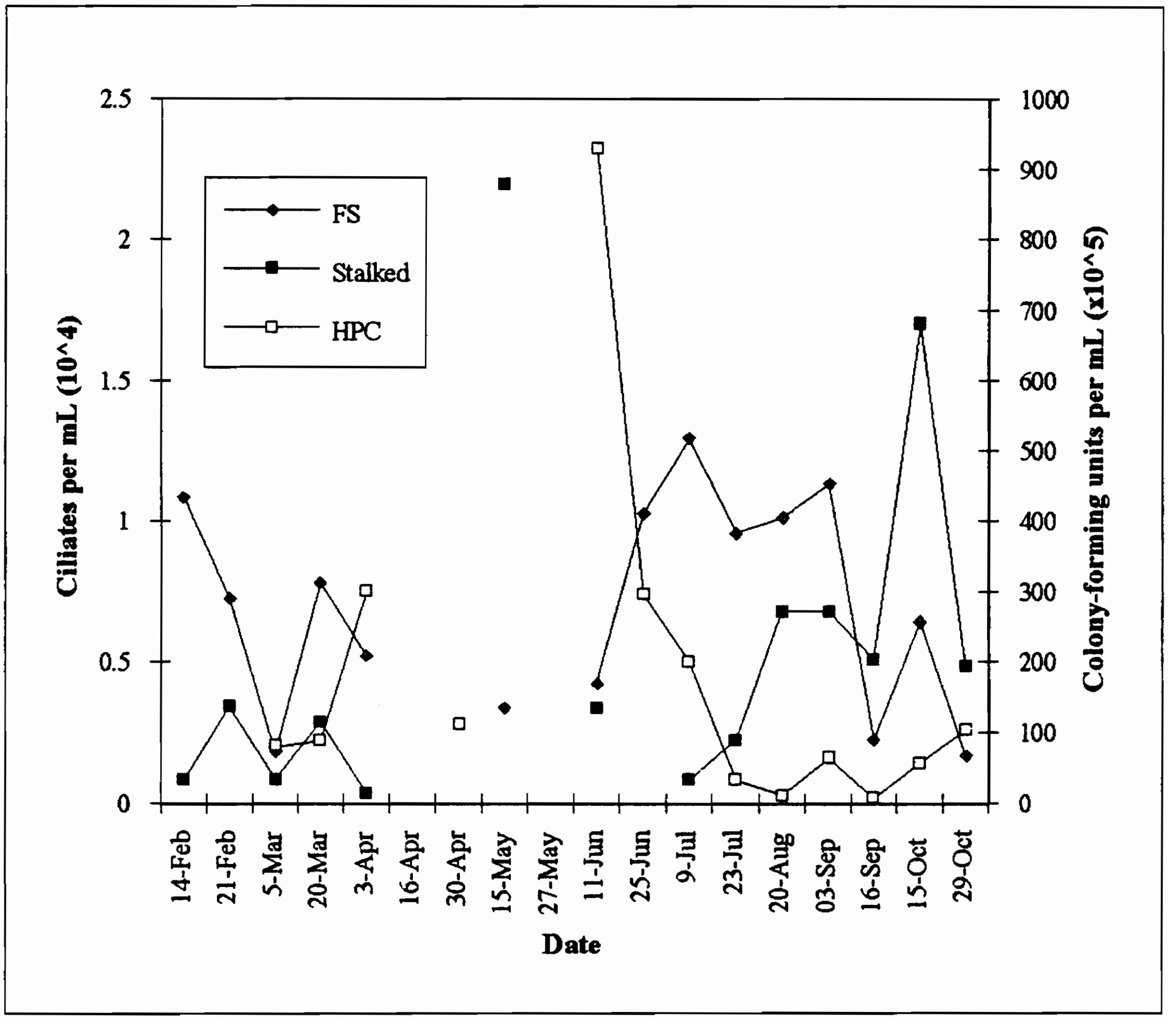

Figure 21. Comparison between free-swimming (FS) and stalked ciliate populations, and heterotrophic plate counts during study of Celco activated sludge, 1992.

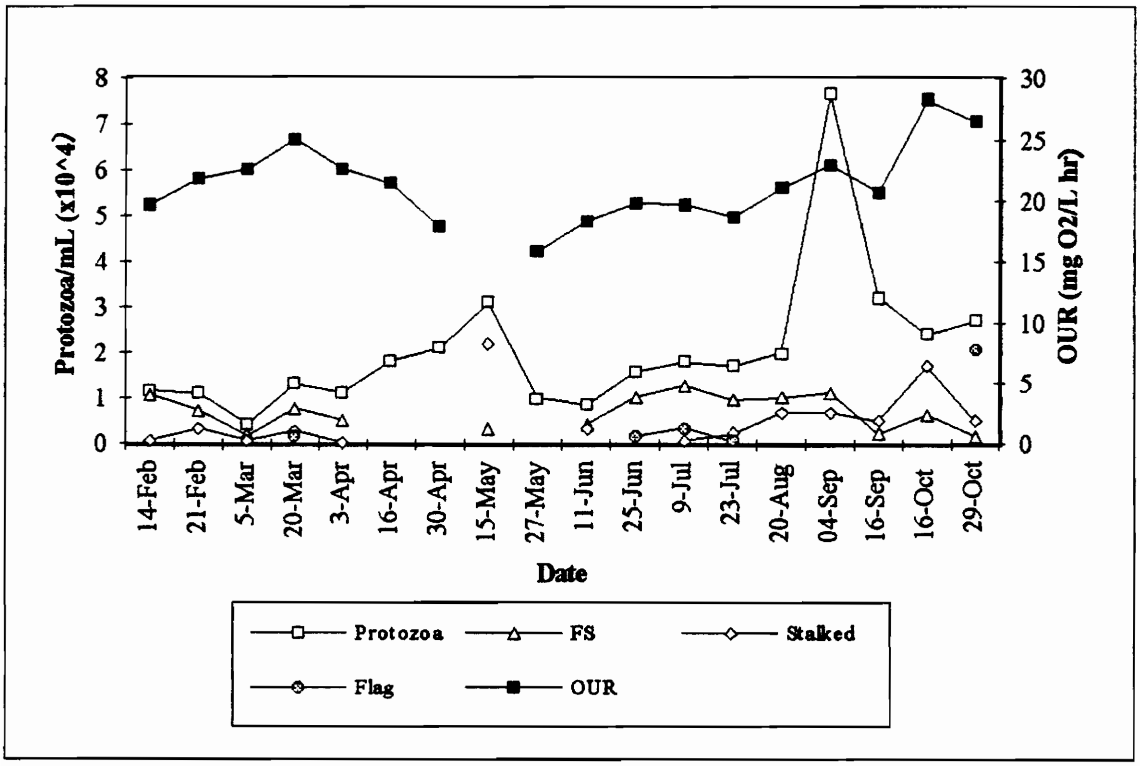

Figure 22. Comparison between protozoa populations and oxygen uptake rates (OUR) of Celco activated sludge, 1992. OUR values were averaged over the period preceding sampling equal to the target sludge age. FS = free-swimming ciliates; Flag = flagellates; Protozoa = total protozoa; Stalked = stalked ciliates.

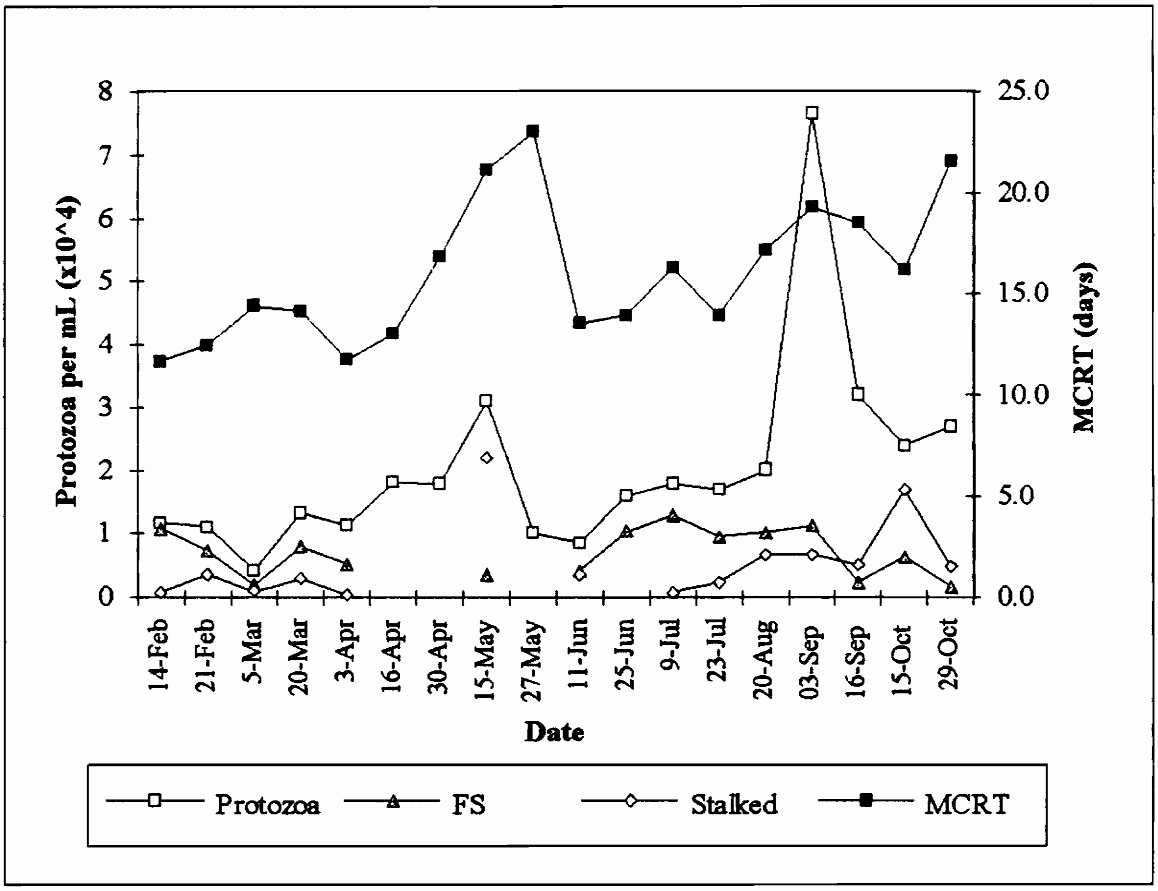

Figure 23. Comparison of changes in mean cell residence time (MCRT) to protozoa populations in Celco activated sludge, 1992. MCRT values were averaged over the period preceding sampling equal to the sludge age. FS = free-swimming ciliates; Stalked = stalked ciliates; Protozoa = total protozoa.

According to the figure, stalked ciliates tended to increase more rapidly than free-swimming (FS) ciliates when the average MCRT was >15 days. For a period from 3 April to approximately 11 June, the average MCRT was 15 days or greater and the number of stalked ciliates increased while the FS ciliate population decreased. Stalked ciliates also increased more rapidly than FS ciliates from 23 July to 20 August. However, the period from 16 September to 29 October did not follow this trend and may be the result of other conditions (see next section). When protozoa were plotted against MCRT, relationships were not evident (Figures 24 and 25), although increasing MCRT appeared to affect stalked ciliate populations more than free-swimming ciliate populations. A linear regression on protozoa vs. MCRT produced an r^2 equal to 0.179 (see Appendix).

Dissolved oxygen

The effect of DO on ciliate numbers is shown in Figure 26. The sudden increase in the protozoa count on 3 September corresponded to a low average DO concentration in the aeration basin. Specifically, the "wormlike" organisms previously described were most abundant during the period. Stalked and FS ciliate populations were not lowest at that time. When protozoa were plotted against dissolved oxygen, no relationships were evident (Figures 27 and 28), and line- and curve-fitting *Excel* spreadsheet functions performed on DO vs. total protozoa produced r^2 values equal to 0.3 and 0.23, respectively (see Appendix).

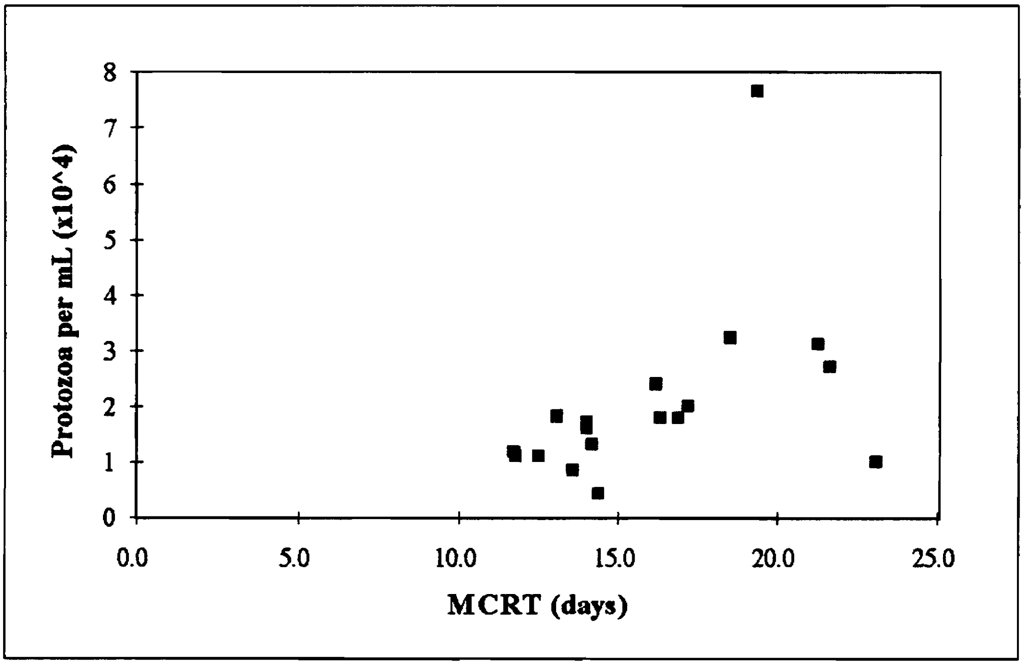

Figure 24. Protozoa vs. mean cell residence time (MCRT) for the Celco activated sludge, 1992.

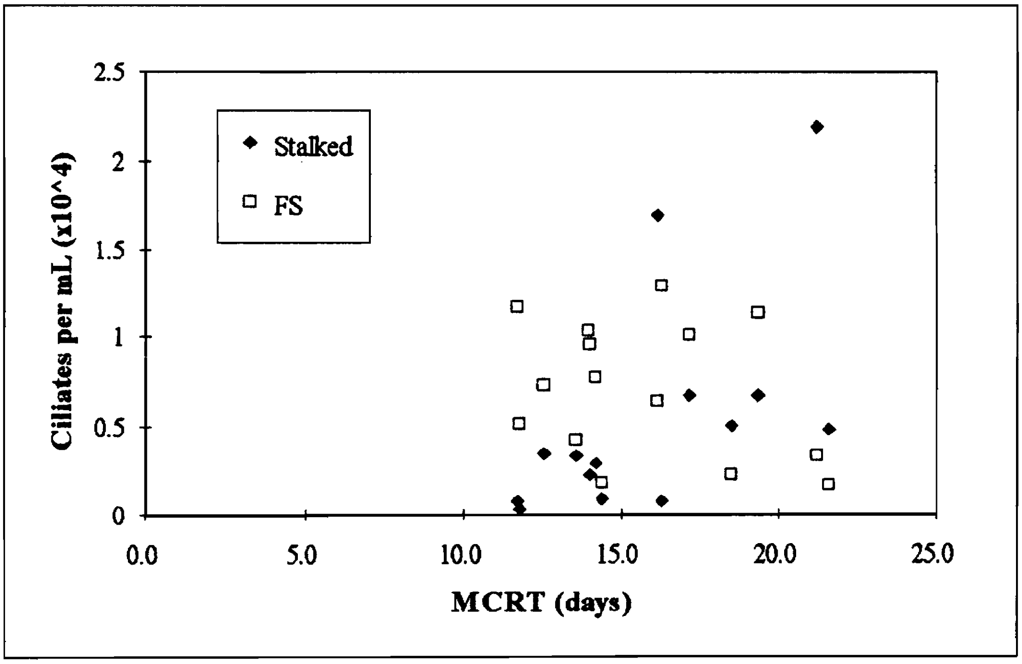

Figure 25. Ciliates vs. mean cell residence time (MCRT) for Celco activated sludge, 1992. FS = free-swimming ciliates; Stalked = stalked ciliates.

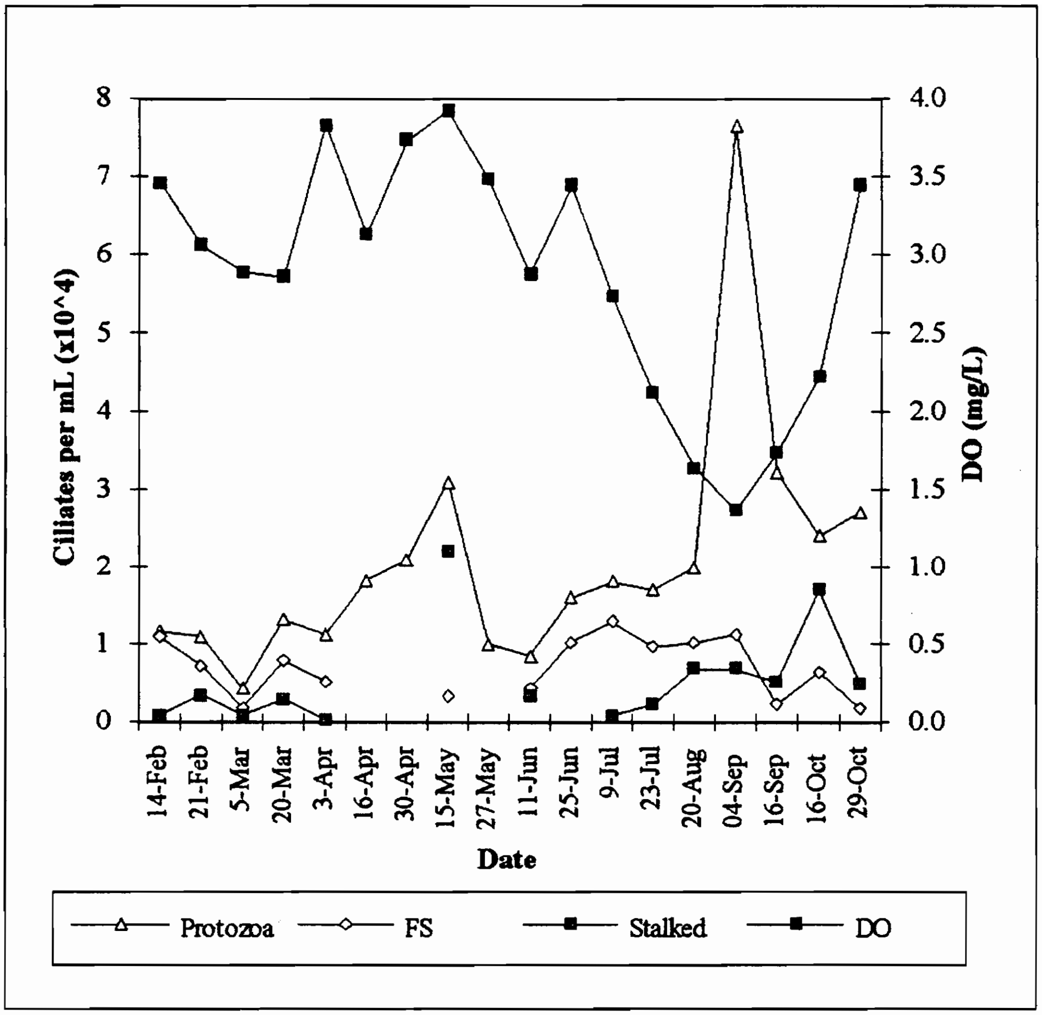

Figure 26. Comparison of dissolved oxygen (DO) to protozoa populations in Celco activated sludge, 1992. DO values were averaged over the period preceding sampling equal to the target sludge age. FS = free-swimming ciliates; Stalked = stalked ciliates; Protozoa = total protozoa.

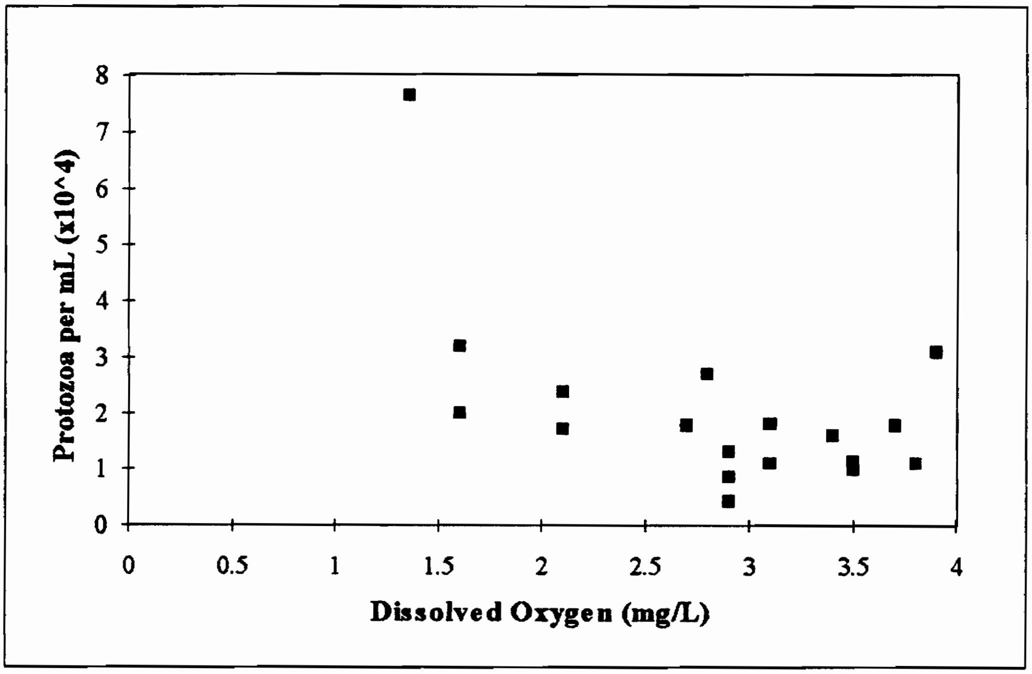

Figure 27. Protozoa vs. dissolved oxygen (DO) in Celco activated sludge, 1992.

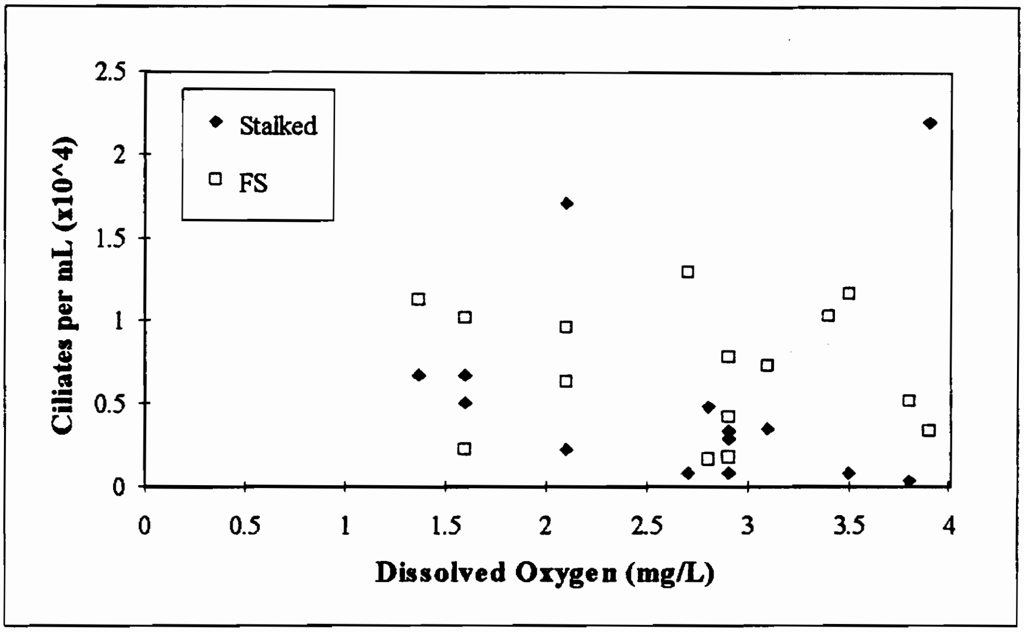

Figure 28. Ciliates vs. dissolved oxygen (DO) in Celco activated sludge, 1992. FS = free-swimming ciliates; Stalked = stalked ciliates.

Influent constituents

Influent constituent loading was analyzed for effects on ciliate populations (Figures 29-34). A significant amount of acid entered the system on 9 May and from 8-10 July. The 9 May load coincided with a sudden increase in non-floc bacteria and stalked ciliate populations. A shockload of isopropyl alcohol may have occurred on 27 May; an unusually high value for acetone was entered in plant logs on 21 June. Loading variations for other constituents were insignificant.

Effluent suspended solids and BOD

When ciliate counts were plotted against effluent BOD and SS , the relationships were not clearly discernible (Figures 35-38). Unfortunately, the BOD data record was incomplete between 27 May and 23 July. Lab notes for differential protozoa counts were also incomplete between 3 April and 9 July. The data gaps precluded the detection, if present, of a relationship between ciliates and effluent BOD and SS. Effluent BOD and SS data are summarized in Figure 39.

4.6 FILAMENTOUS BACTERIA

4.6.1 General

The variations in filamentous bacteria populations are shown in Figure 40. Specific types of filamentous bacteria were classified according to their abundance in the samples, using the following system in order of abundance: excessive, abundant, very common, common, some and few.

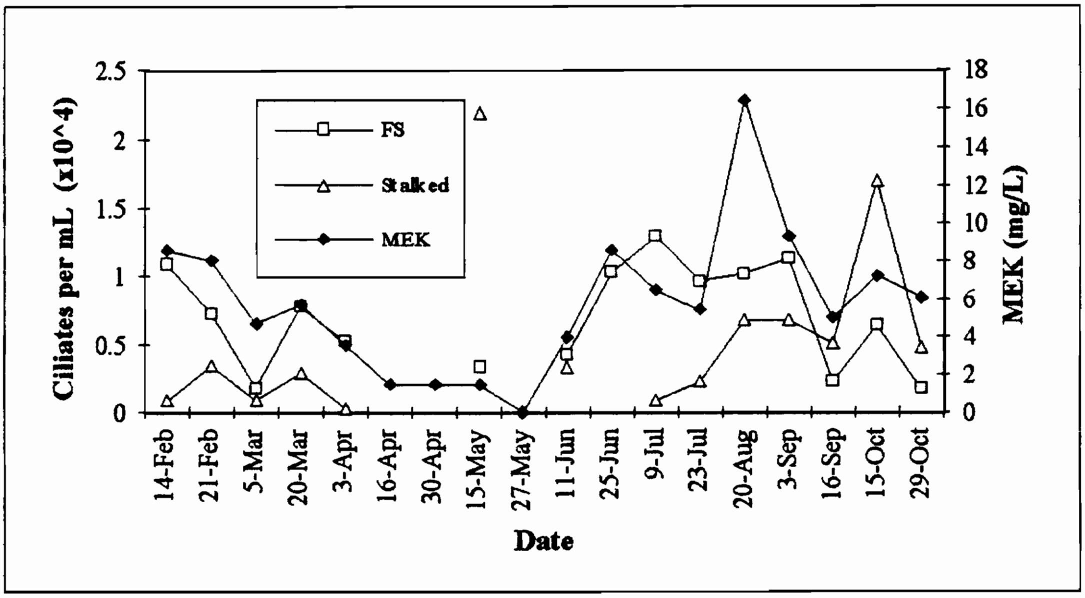

Figure 29. Comparison of influent methyl ethyl ketone (MEK) to protozoa populations in Celco activated sludge, 1992. MEK values were averaged over the period preceding sampling equal to the target sludge age. FS = free-swimming ciliates; Stalked = stalked ciliates.

Sample I was observed to have abundant *M. parvicella*. These are very thin trichomes, with some 20-50μm and others 100+μm long. There was some to common *Nocardia*, some Type 0041 and *N. limicola*, and common Type 1701. Sample J also had abundant *M. parvicella*. Type 0041 and *Nocardia* were common to very common. A filament that may have been Type 1701 was abundant in Sample J. Sample K had very common *M. parvicella*, and some Type 0041, and some *N. limicola*. Filaments were common within flocs, although they were not identified. Sample L also had abundant *M. parvicella* and common Type 0041. Sample M had abundant to excessive *M. parvicella*, common Type 0041, some *Nocardia*, and some tangled thick strands covered with epiphyte. In Sample N, *H. hydrossis* was common to very common, and some *Nostocoida limicola* II and *Microthrix* were present. Common thick (2-3μm diameter), long (±800 μm) trichomes were also present, although they were unidentified. Types 1701 and 0041 were common in Sample O, as was *H. hydrossis*. Also present was a filament within the flocs acting as a backbone. It was very difficult to see detail. Sample P was very similar to Sample O. The sample from 25 June had some to common *M. parvicella*. Type 0041 was common to very common, and some *H. hydrossis* filaments were present. A filament that may have been Type 1701 was common. Due to an oversight, only one slide of a Neisser stain was prepared for Sample R. In Sample R, Types 0041 and 1701 were common. Filaments were abundant overall. *M. parvicella* was very common in Sample T. Also common was a thick filament (present in all samples) that looked like Type 0041, except that it did not possess Gram positive inclusions consistently. Type 1851 was abundant, and some *N. limicola* was present.

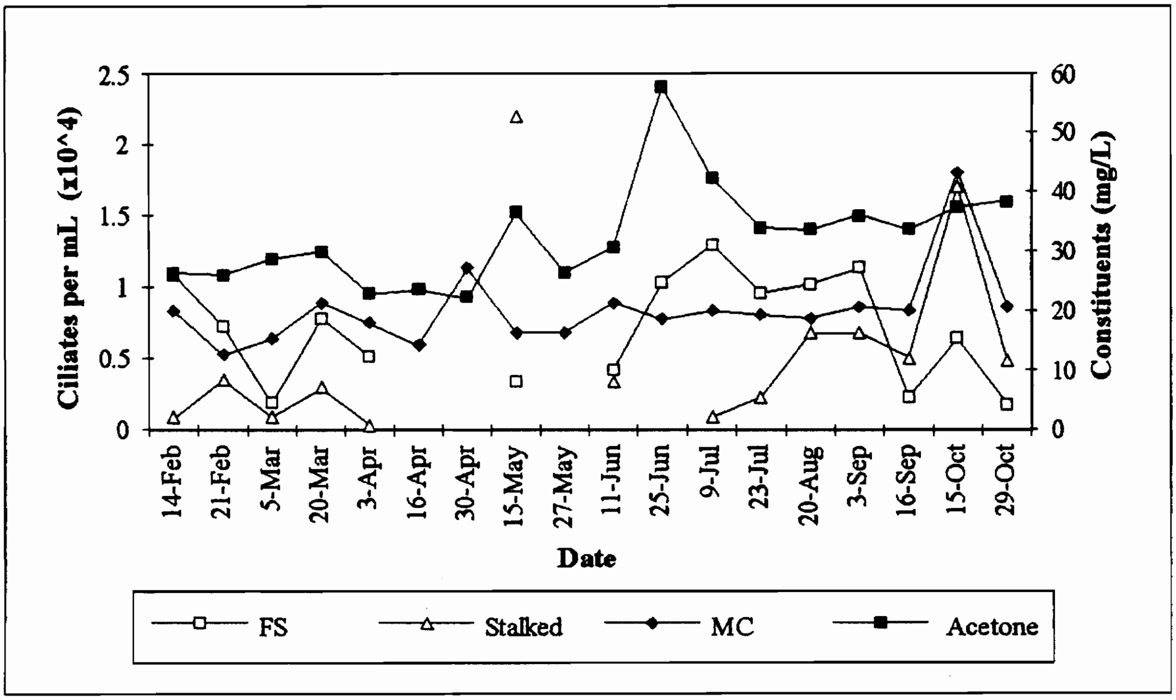

Figure 30. Comparison of influent methyl cyanate (MC) and acetone to protozoa populations in Celco activated sludge, 1992. MC and acetone values were averaged over the period preceding sampling equal to the target sludge age. FS = free-swimming ciliates; Stalked = stalked ciliates.

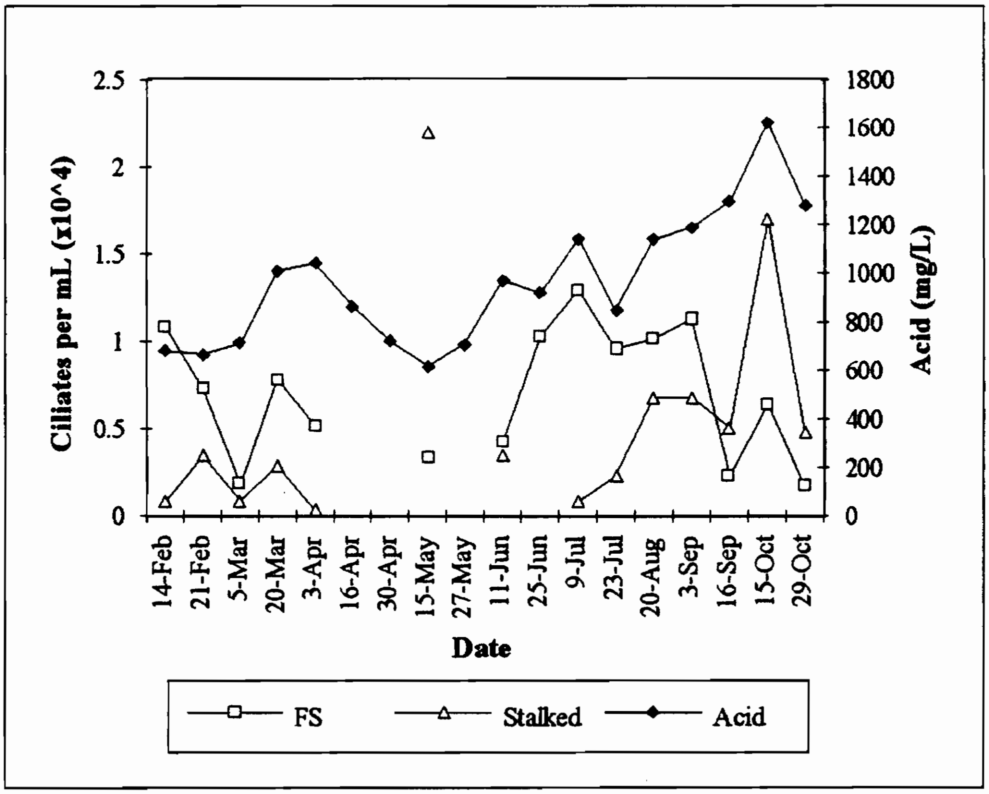

Figure 31. Comparison of influent acid to free-swimming (FS) and stalked ciliated populations in Celco activated sludge, 1992. Acid values were averaged over the period preceding sampling equal to the target sludge age.

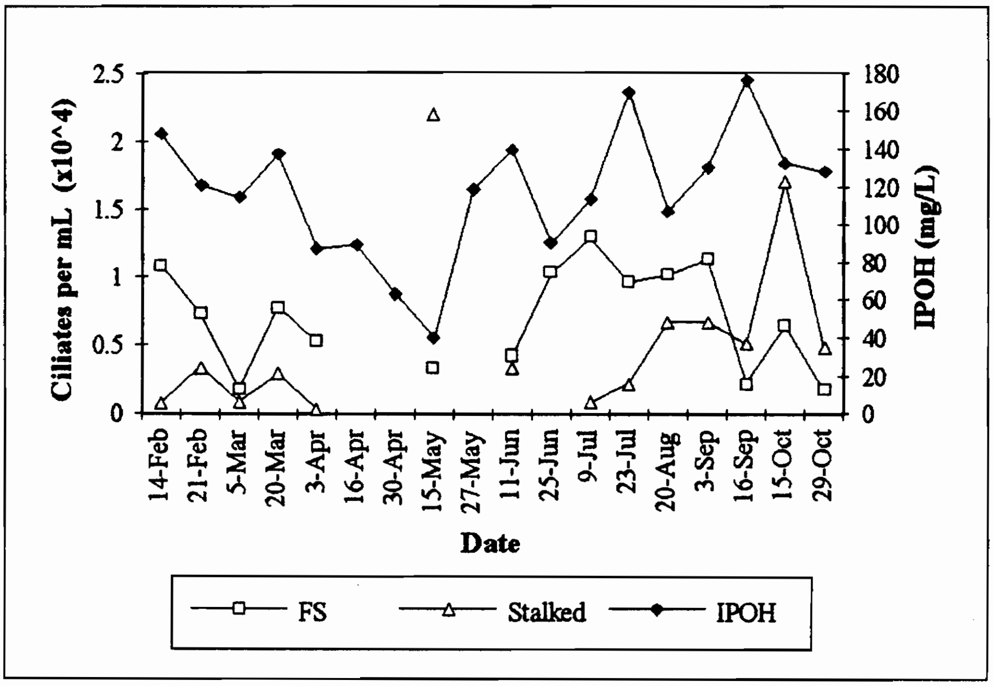

Figure 32. Comparison of influent isopropyl alcohol (IPOH) to free-swimming (FS) and stalked ciliated populations in Celco activated sludge, 1992. IPOH values were averaged over the period preceding sampling equal to the target sludge age.

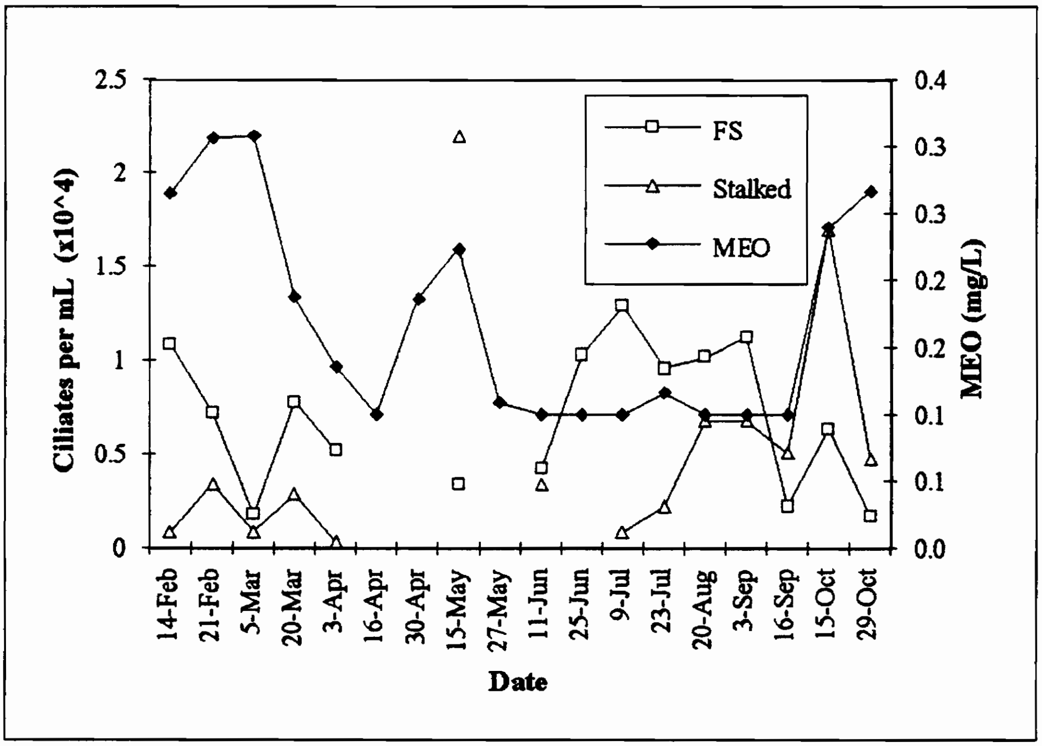

Figure 33. Comparison of influent mesityl oxide (MEO) to free-swimming (FS) and stalked ciliate populations in Celco activated sludge, 1992. MEO values were averaged over the period preceding sampling equal to the target sludge age.

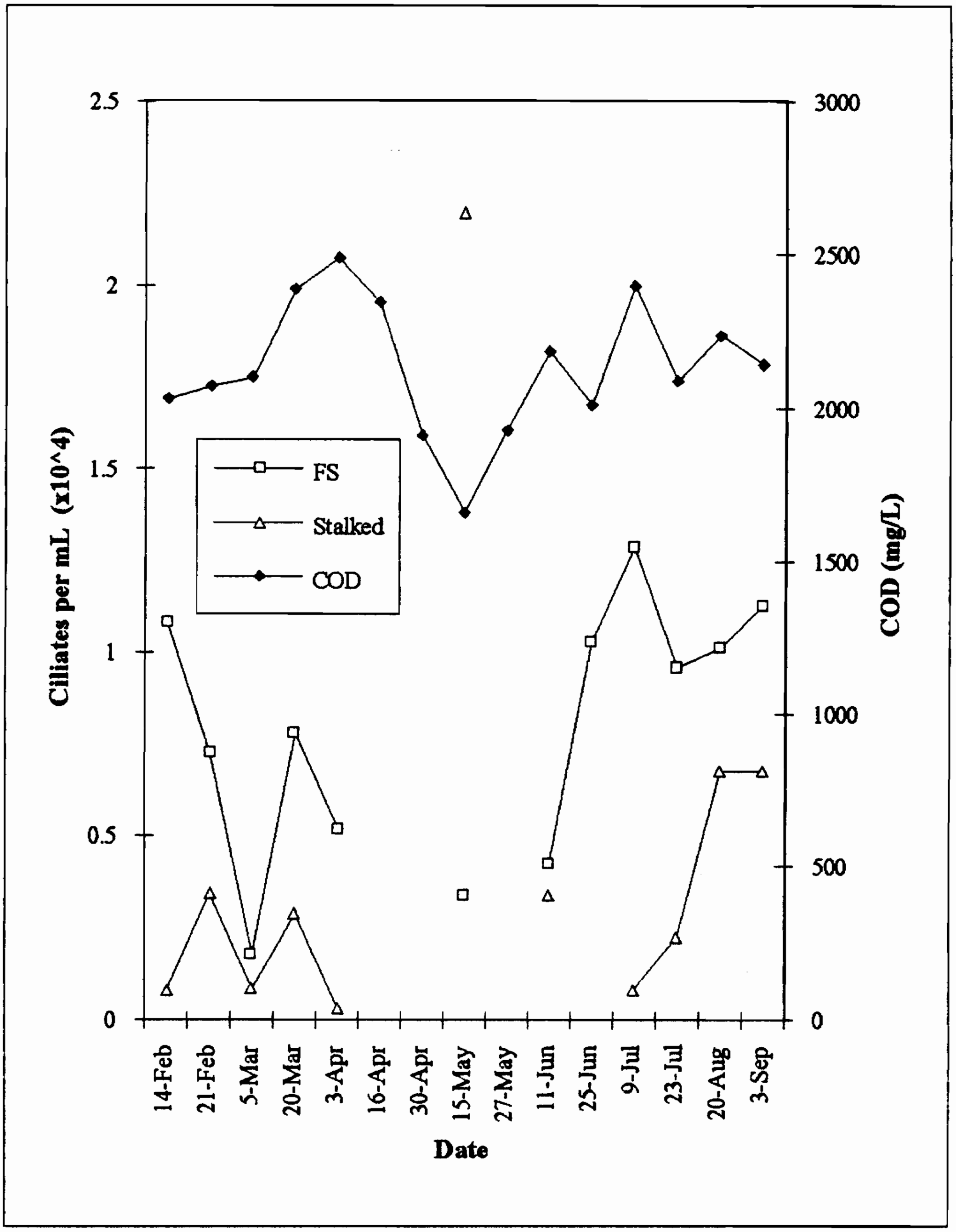

Figure 34. Comparison of influent chemical oxygen demand (COD) to stalked and free-swimming (FS) ciliates in the Celco activated sludge, 1992. COD values were averaged over the period preceding sampling equal to the target sludge age.

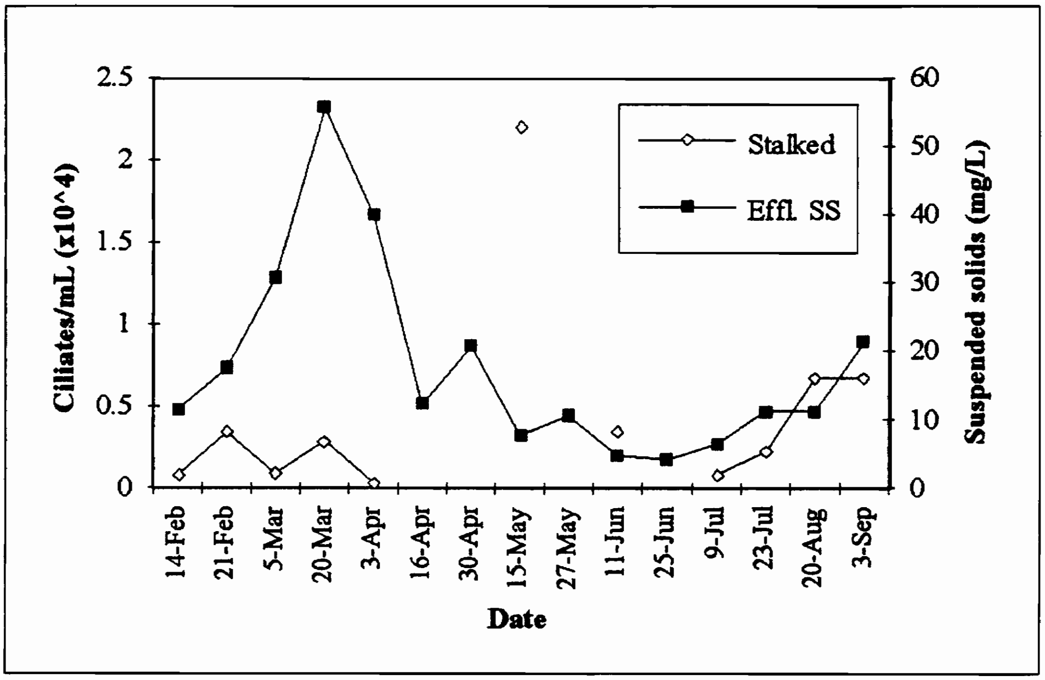

Figure 35. Comparison of stalked ciliate population to effluent suspended solids (Effl.SS). Celco activated sludge, 1992. SS values were averaged over the period preceding sampling equal to the target sludge age.

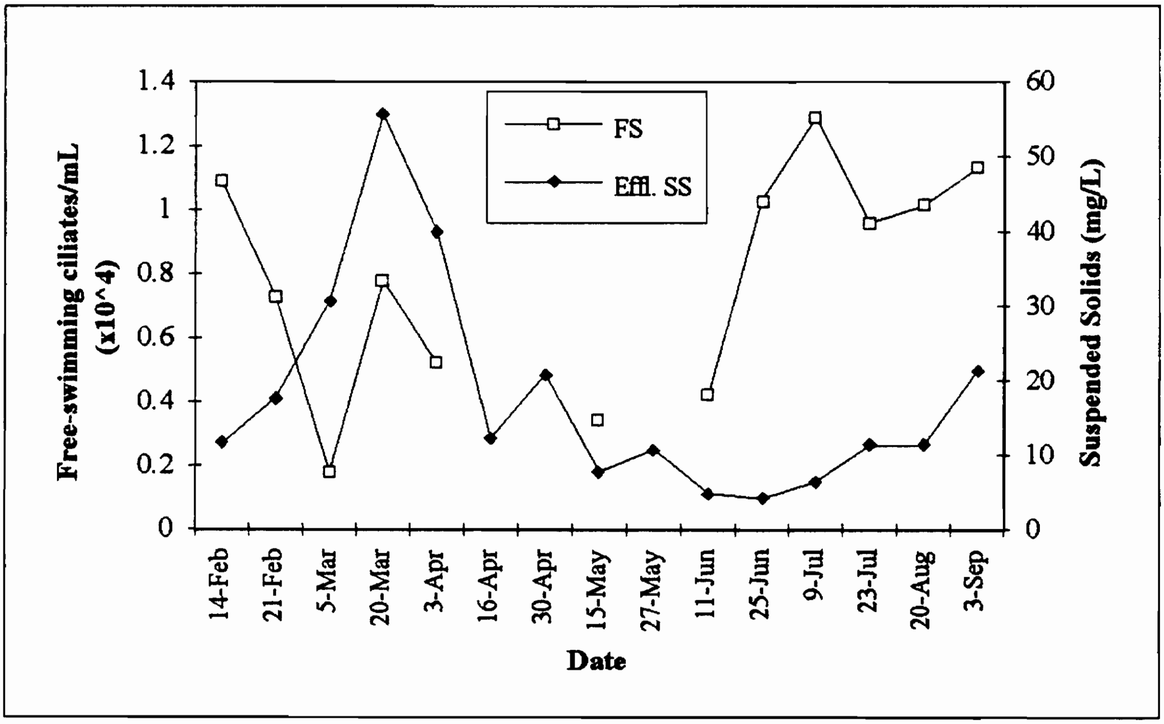

Figure 36. Comparison of free-swimming (FS) ciliates to effluent suspended solids (Effl.SS). Celco activated sludge, 1992. SS values were averaged over the period preceding sampling equal to the target sludge age.

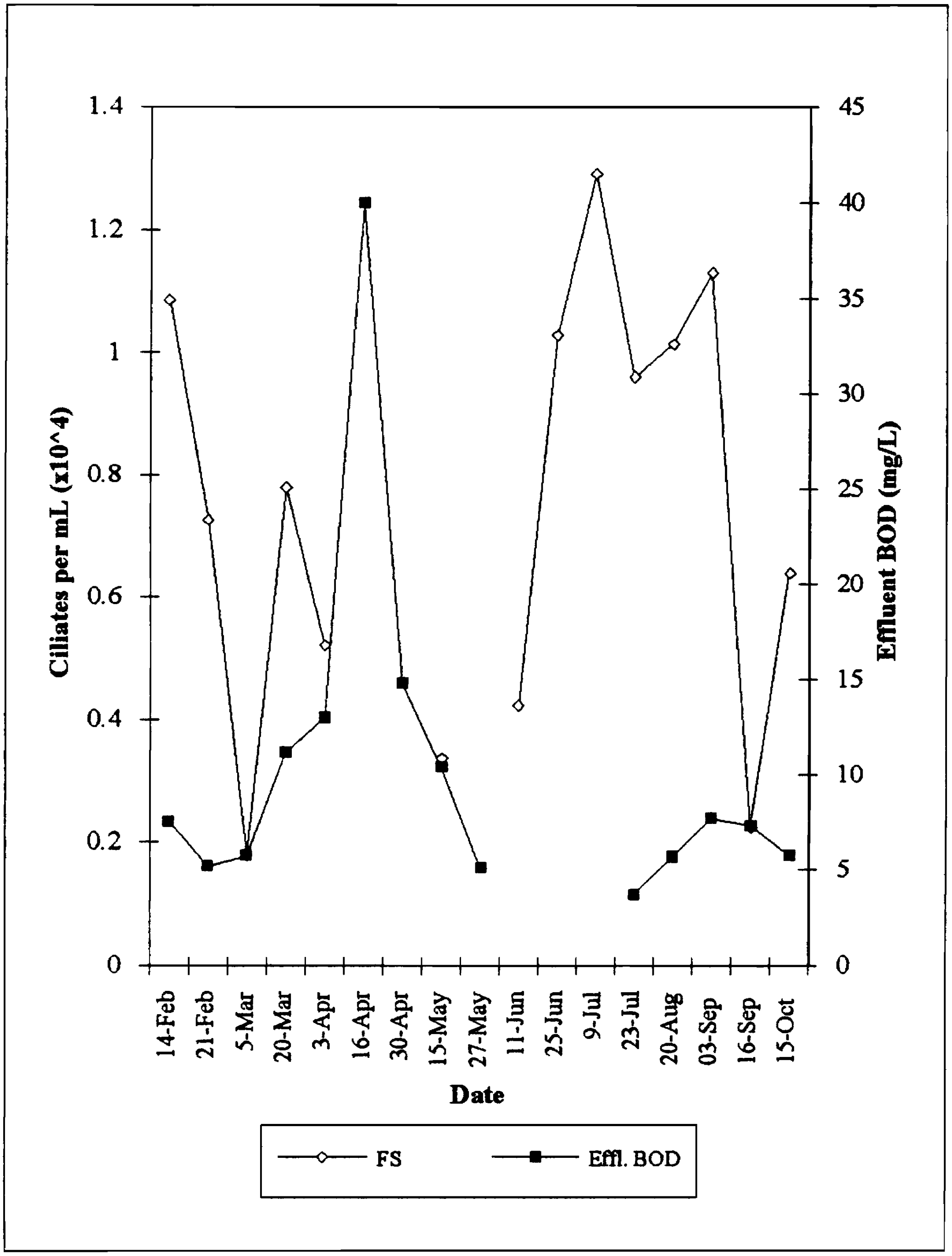

Figure 37. Comparison of free-swimming (FS) ciliates to effluent biochemical oxygen demand (BOD). Celco activated sludge, 1992. BOD values were averaged over the period preceding sampling equal to the target sludge age.

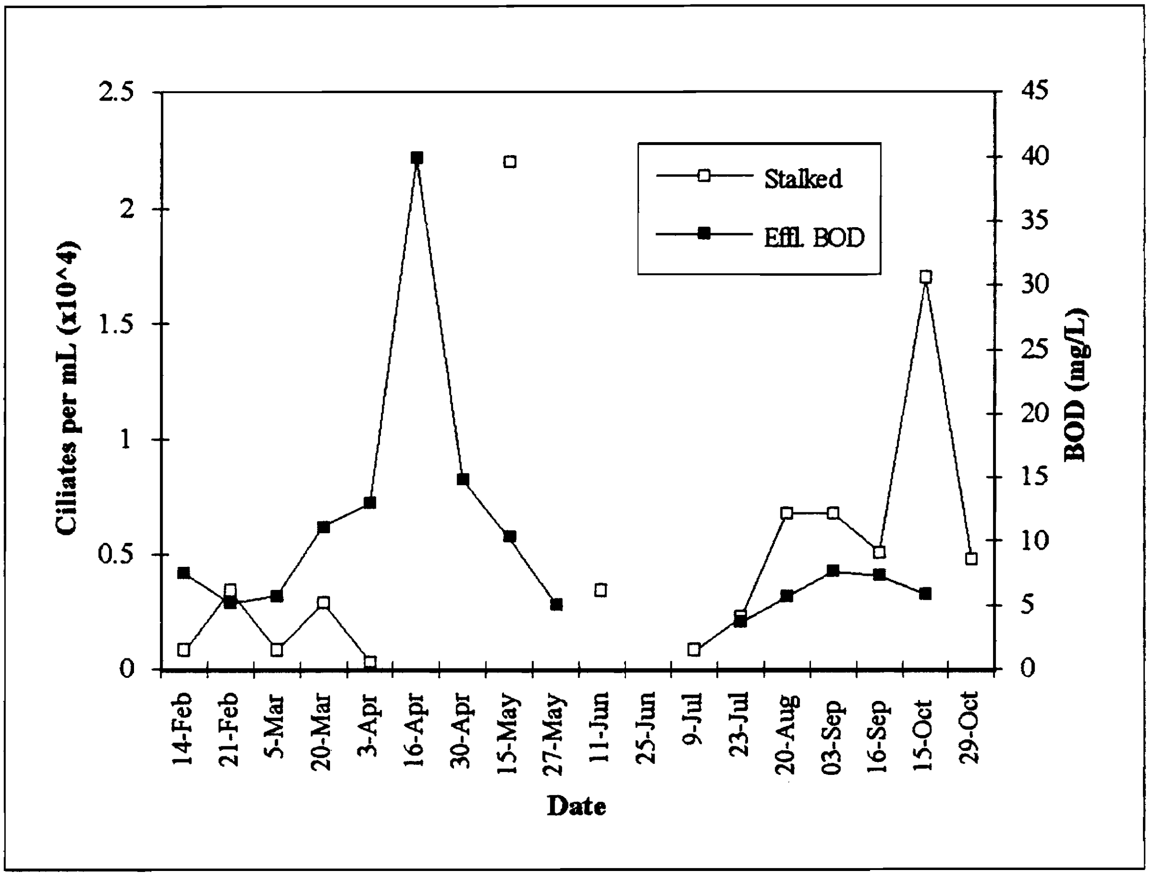

Figure 38. Comparison of stalked ciliates to effluent biochemical oxygen demand (BOD). Celco activated sludge, 1992. BOD values were averaged over the period preceding sampling equal to the target sludge age.

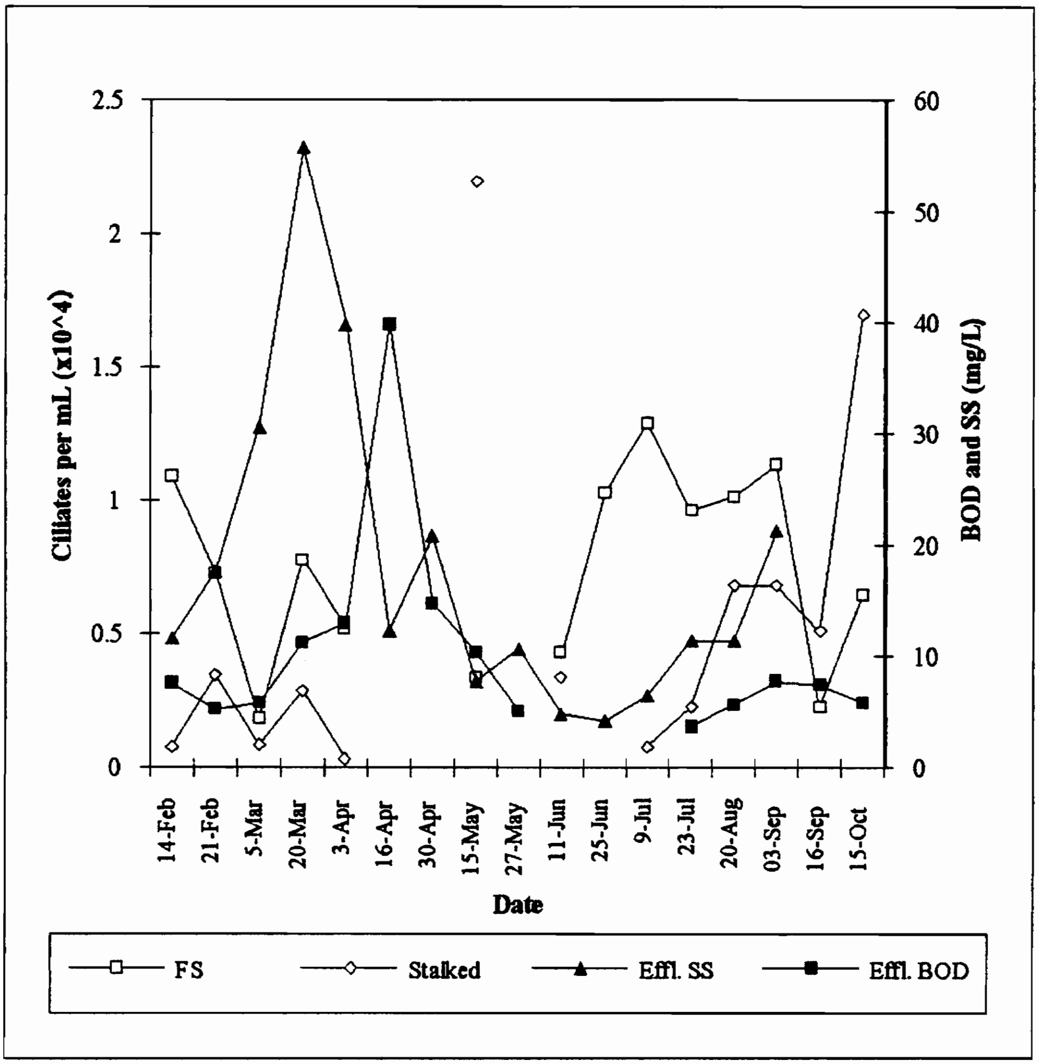

Figure 39. Summary chart of comparison of free-swimming (FS) and stalked ciliate populations to effluent biochemical oxygen demand (Effl.BOD) and suspended solids (Effl.SS). Celco activated sludge, 1992. BOD and SS values were averaged over the period preceding sampling equal to the target sludge age.

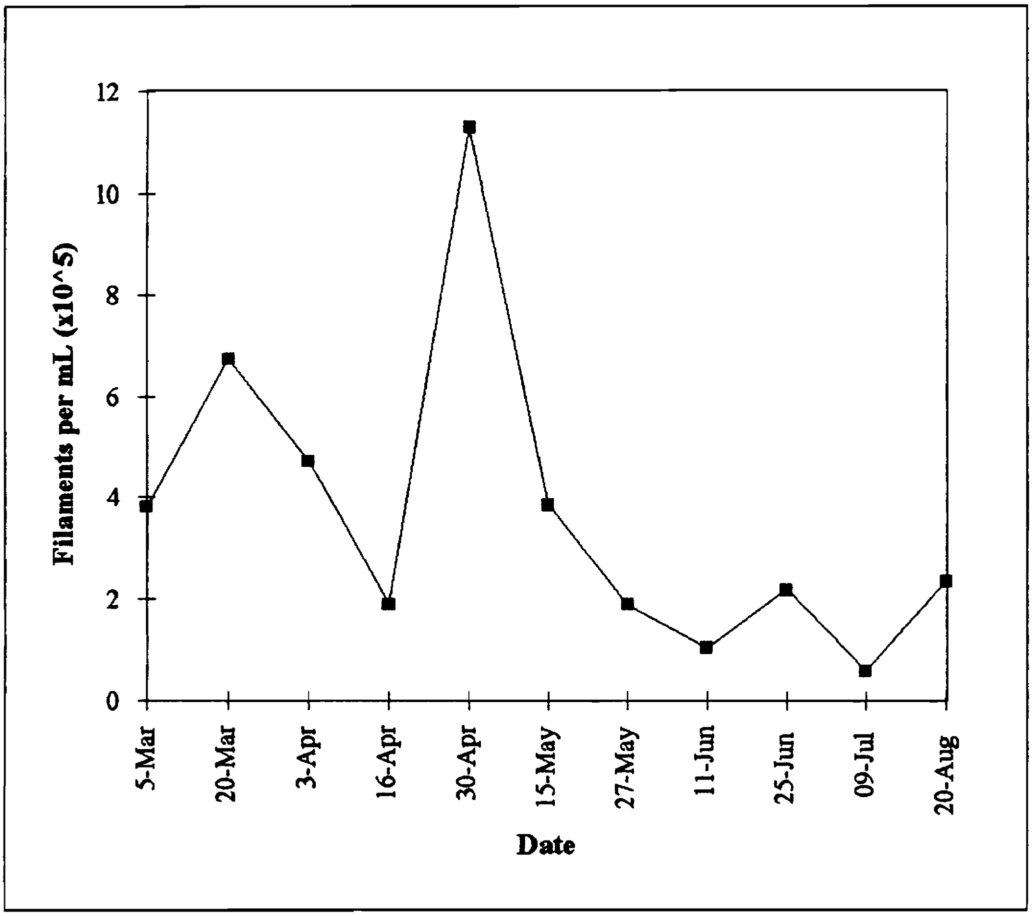

Figure 40. Variations in filamentous bacteria population in activated sludge of Celco wastewater treatment plant, 1992.

4.6.2 Comparison to Aeration Basin Conditions

Nitrogen loading

Filament populations appeared to be responsive to changes in nitrogen loading (Figure 41). The Celco system is nitrogen-poor, with the bulk of the nitrogen being added as urea. Filament types associated with nutrient deficient activated sludge are *Thiothrix*, *S. natans*, Type 021N, and possibly *H. hydrossis* and Types 0041 and 0675 (Jenkins, *et al.*, 1984). Type 0041 was found in most samples and was classified as "common" from 16 April to 20 August. The highest average nitrogen concentration corresponded to Sample M, which contained very common to excessive growth of *Microthrix*.

Dissolved oxygen

Filaments commonly associated with low dissolved oxygen are Type 1701, *S. natans* and *H. hydrossis* (Strom and Jenkins, 1984). The sample corresponding to the lowest average DO concentration was taken on 20 August (Figure 42). It contained common *M. parvicella*, Type 0041 and abundant Type 1851. The sample corresponding to the highest average DO concentration was taken on 30 April. It contained primarily Type 0041. Samples from 27 May through 9 July contained Types 0675 (from 27 May) and 1701 (11 June through 9 July).

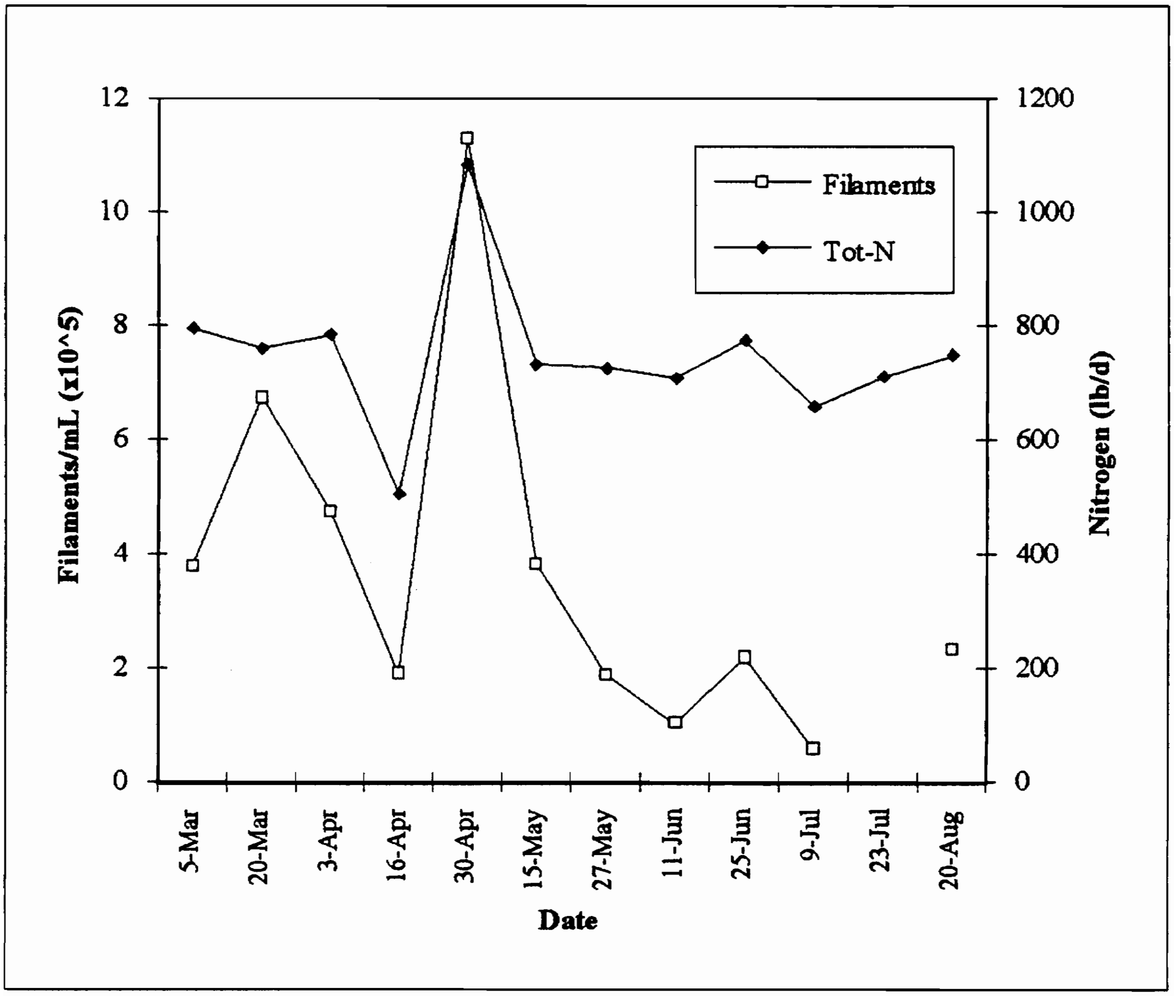

Figure 41. Comparison of total nitrogen loading (Tot-N) to filamentous bacteria population in Celco activated sludge, 1992. Nitrogen values were averaged over the period preceding sampling equal to the target sludge age

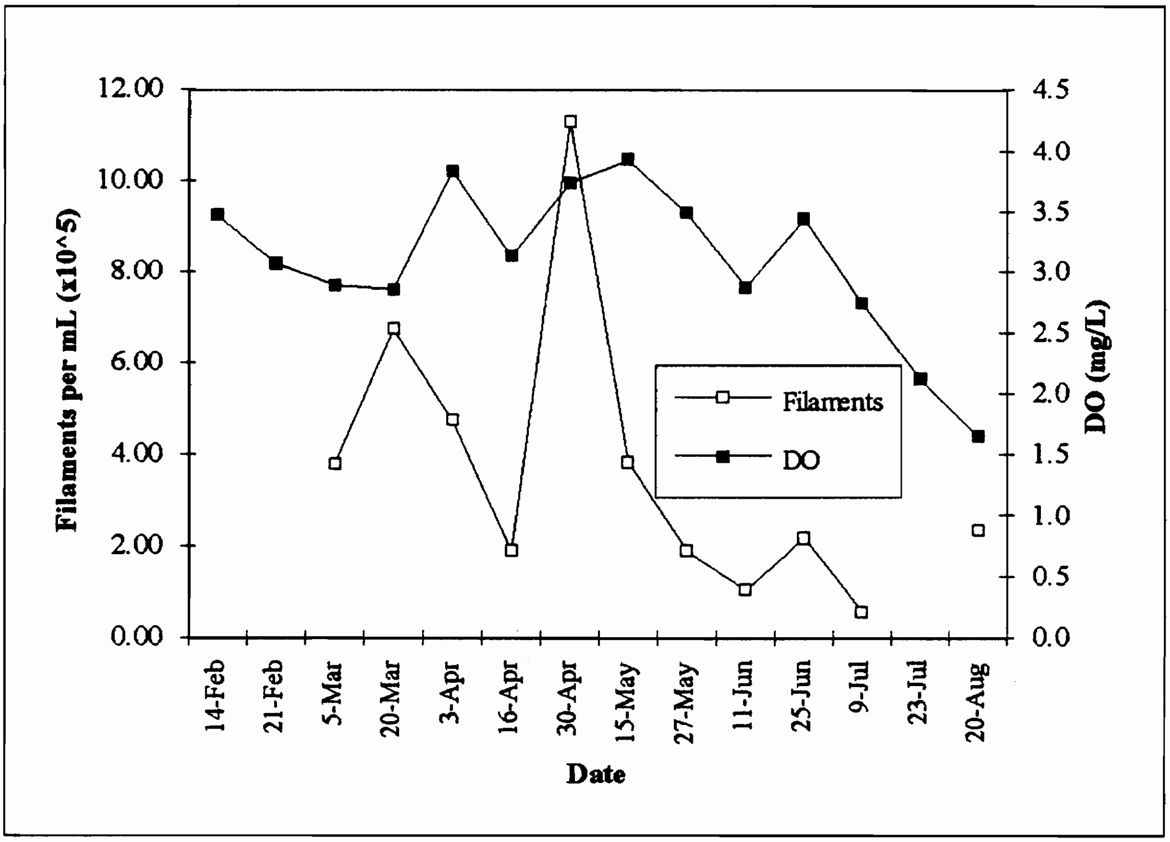

Figure 42. Comparison of dissolved oxygen (DO) to filament population in Celco activated sludge, 1992. DO values were averaged over the period preceding sampling equal to the target sludge age.

F/M ratio

An abundance of *M. parvicella*, *H. hydrossis*, *Nocardia* and Types 021N, 0041, 0675, 0092, 0581 and 0961 have been associated with a low food-to-microorganism ratio (Strom and Jenkins, 1984). On 30 April the average F/M ratio was at its lowest point during the study (Figure 43). *M. parvicella* was very common on that day. The next sample, taken on 15 May, had very common *M. parvicella*, in tangles, and very common *H. hydrossis*. Some Types 0411 and 0675 were also present. By 27 May, the amount of *M. parvicella* was greatly reduced. Types 1701 and 0041 and *H. hydrossis* were common. *Wastewater Engineering*, 3rd ed.(1991), p.550, states that the desired F/M ratio value for complete mix reactors at 0.2-0.6 d^{-1}. The average F/M ratio during the period varied from approximately 0.08 to 0.16 d^{-1}. It should be noted that when the F/M ratio was 0.1+ d^{-1} the filament counts were low.

Mean cell residence time

MCRT appeared to have more effect on individual genera than on the overall filament population (Figure 44). *M. parvicella* was usually present in samples taken during the study. The population decreased significantly when the average MCRT was ≥20 days. During that period, *H. hydrossis* became very common. *H. hydrossis* was classified as "few" when the average MCRT decreased to 15 days.

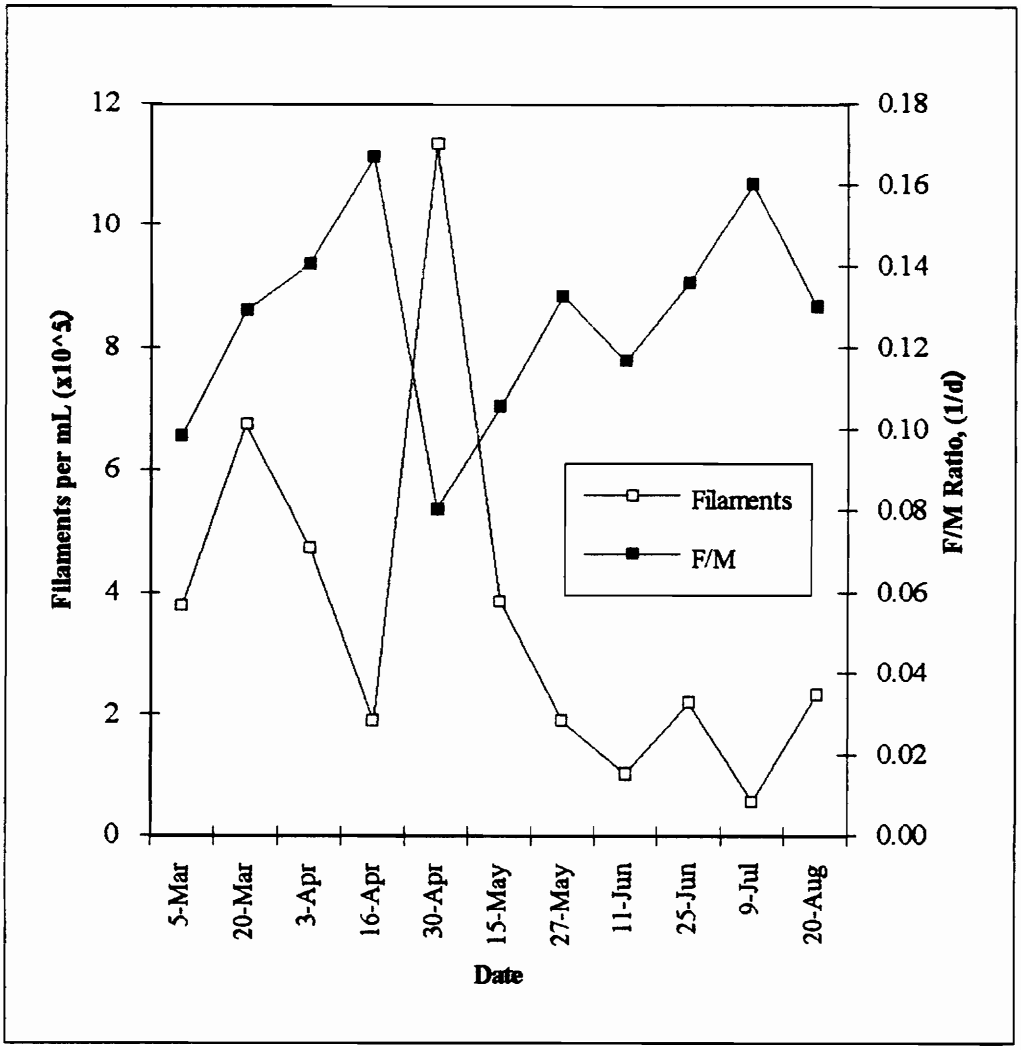

Figure 43. Comparison of food-to-microorganism (F/M) ratio to filament population of Celco activated sludge, 1992. F/M ratio values were averaged over the period preceding sampling equal to the target sludge age.

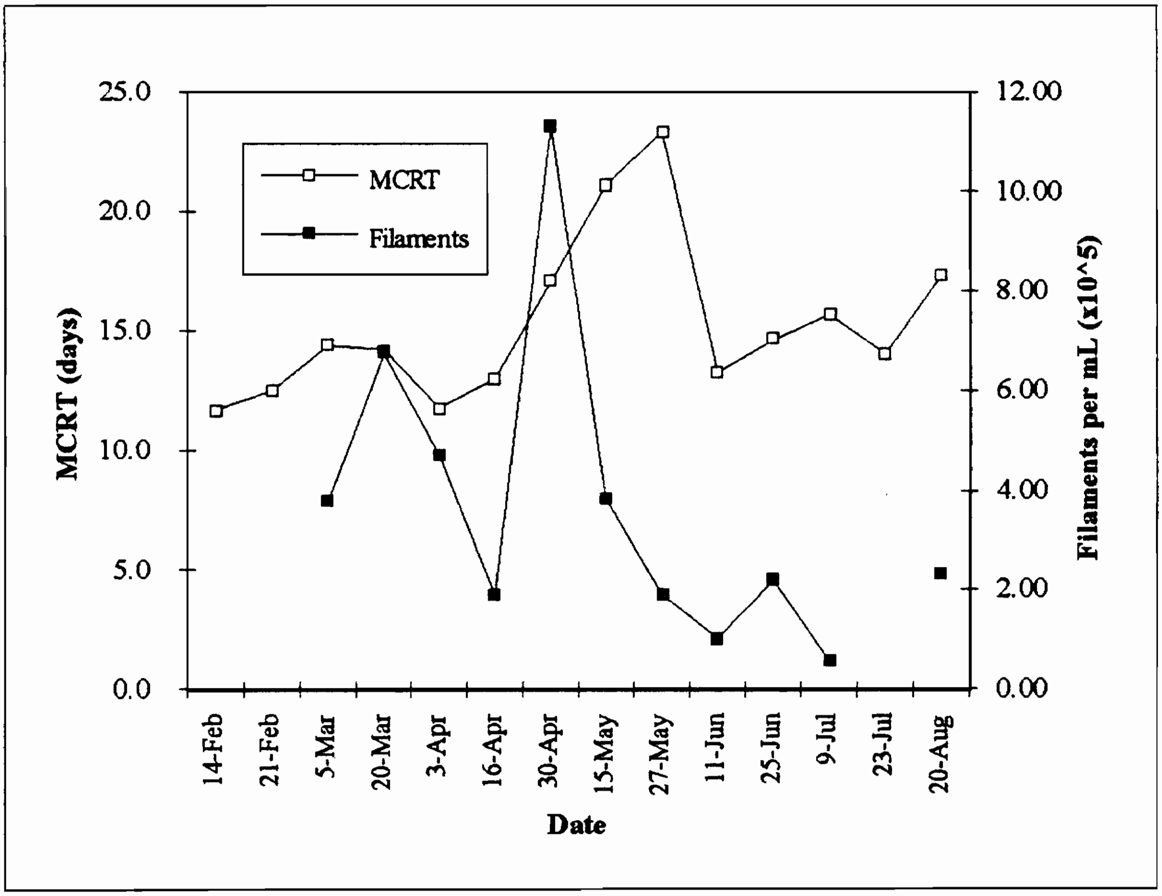

Figure 44. Comparison of changes in mean cell residence time (MCRT) to filament populations of Celco activated sludge, 1992. MCRT values were averaged over the period preceding sampling equal to the target sludge age.

4.6.3 Comparison to Influent Constituents

Filament numbers were plotted against influent constituents to determine whether there was a relationship (Figures 45-49). In every case examined there was no obvious relationship. However, filament numbers tended to be low from 27 May to 20 August. During this period, average acid, isopropanol, methyl ethyl ketone and acetone concentrations were generally elevated.

4.6.4 Comparison to Plant Performance

Suspended solids

The presence of filaments may act as a backbone for flocs or as a mesh that can entrap particles during settling. Effluent suspended solids increased from 5 March to 3 April then decreased to a low of approximately 5 mg/L on 9 July (Figure 50). *M. parvicella* was common to abundant from 5 March to 30 April. Type 0041 was also common. The filament most common during the period of low average effluent suspended solids was Type 1701.

SVI

Filaments may also prevent the compaction of flocs, a condition known as *bulking* . Filament counts fluctuated similarly with the sludge volume index (Figure 51). The highest average SVI value corresponded to the highest filament count from the sample taken 30 April. This relationship did not continue after Sample P. Unfortunately, slides of Sample S, taken on 23 July, were not made. Sample Q was not unusually filamentous, although Sample R was. In Sample R, flocs appeared diffuse, although this observation was made from a prepared slide. A wet mount examined at the plant on 21-July was

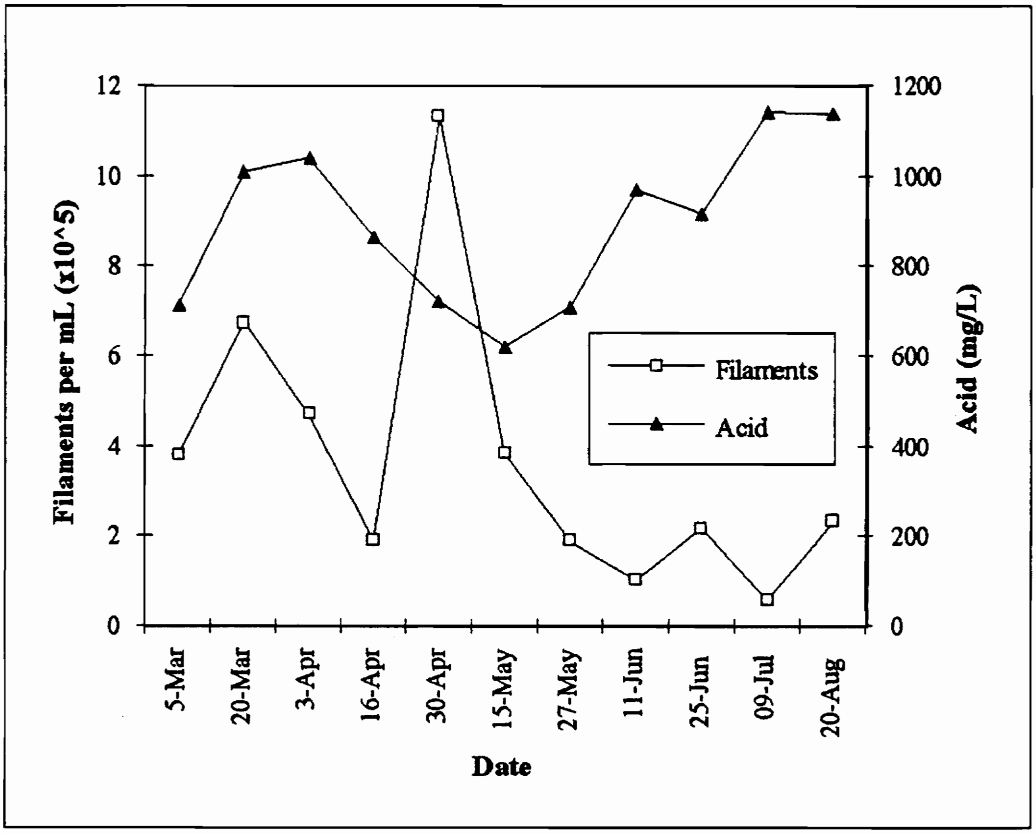

Figure 45. Comparison of influent acid concentrations to filament population of Celco activated sludge, 1992. Acid values were averaged over the period preceding sampling equal to the target sludge age.

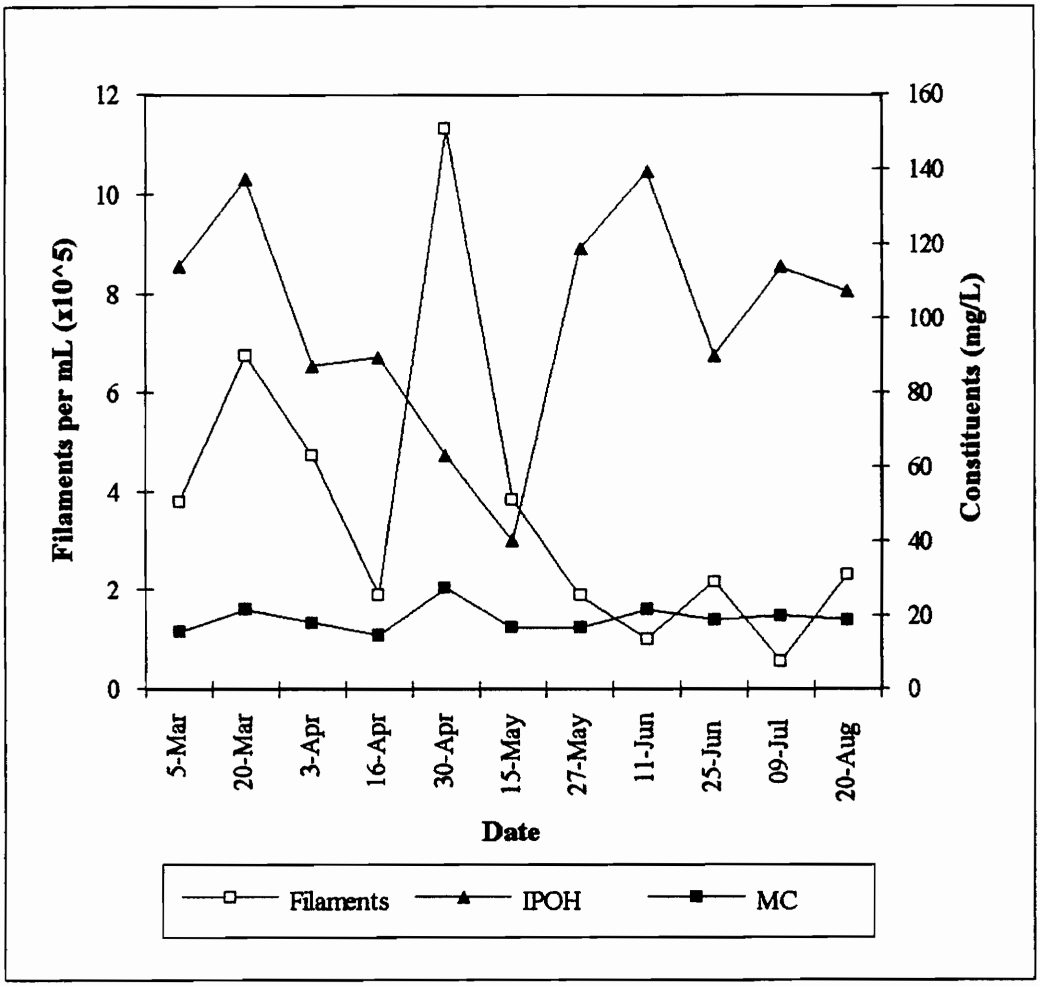

Figure 46. Comparison of influent isopropyl alcohol (IPOH) and methyl cyanate (MC) to filament population of Celco activated sludge, 1992. Constituent values were averaged over the period preceding sampling equal to the target sludge age.

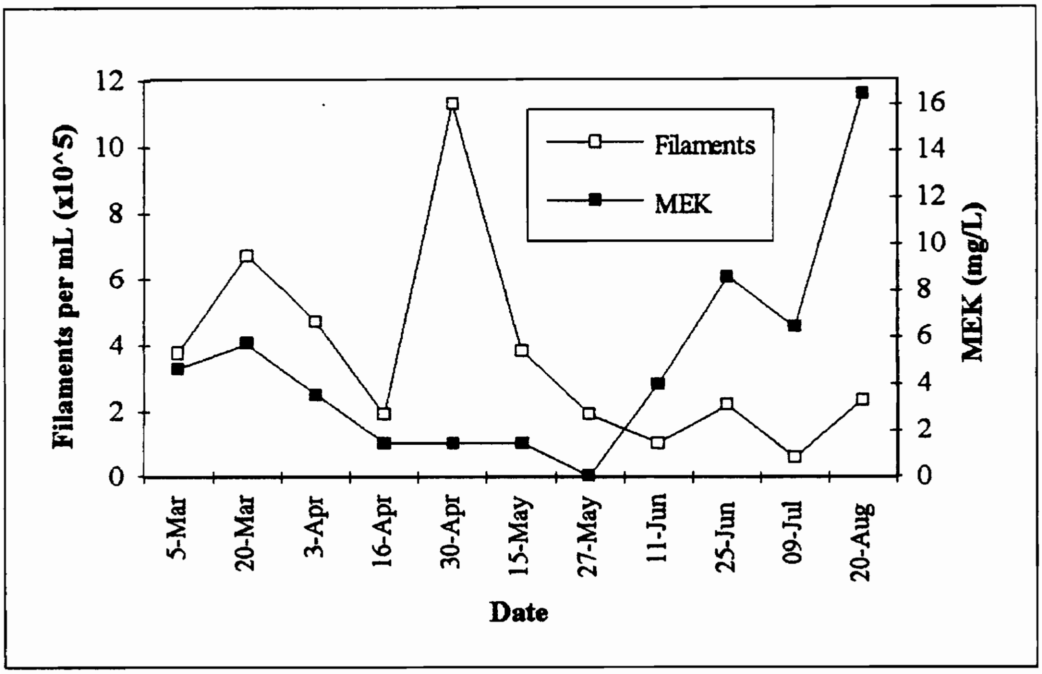

Figure 47. Comparison of influent methyl ethyl ketone (MEK) to filament population of Celco activated sludge, 1992. MEK values were averaged over the period preceding sampling equal to the target sludge age.

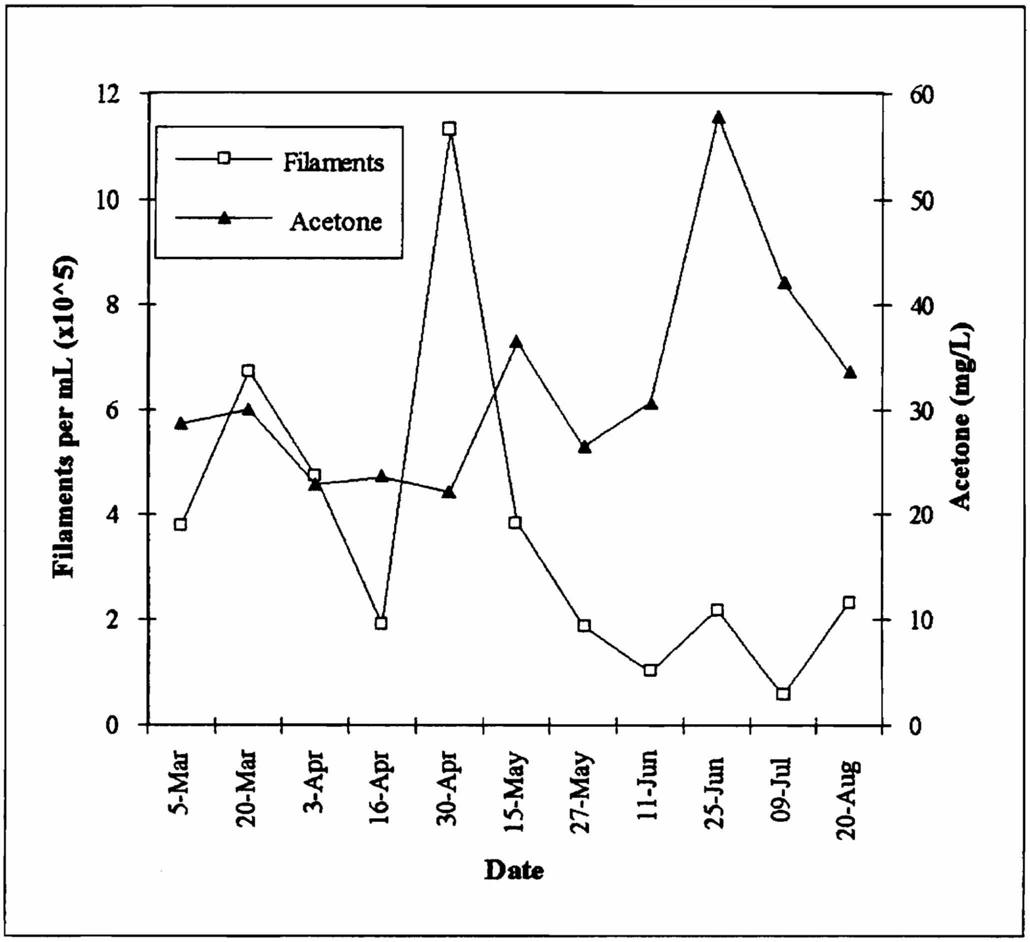

Figure 48. Comparison of influent acetone to filament population of Celco activated sludge, 1992. Acetone values were averaged over the period preceding sampling equal to the target sludge age.

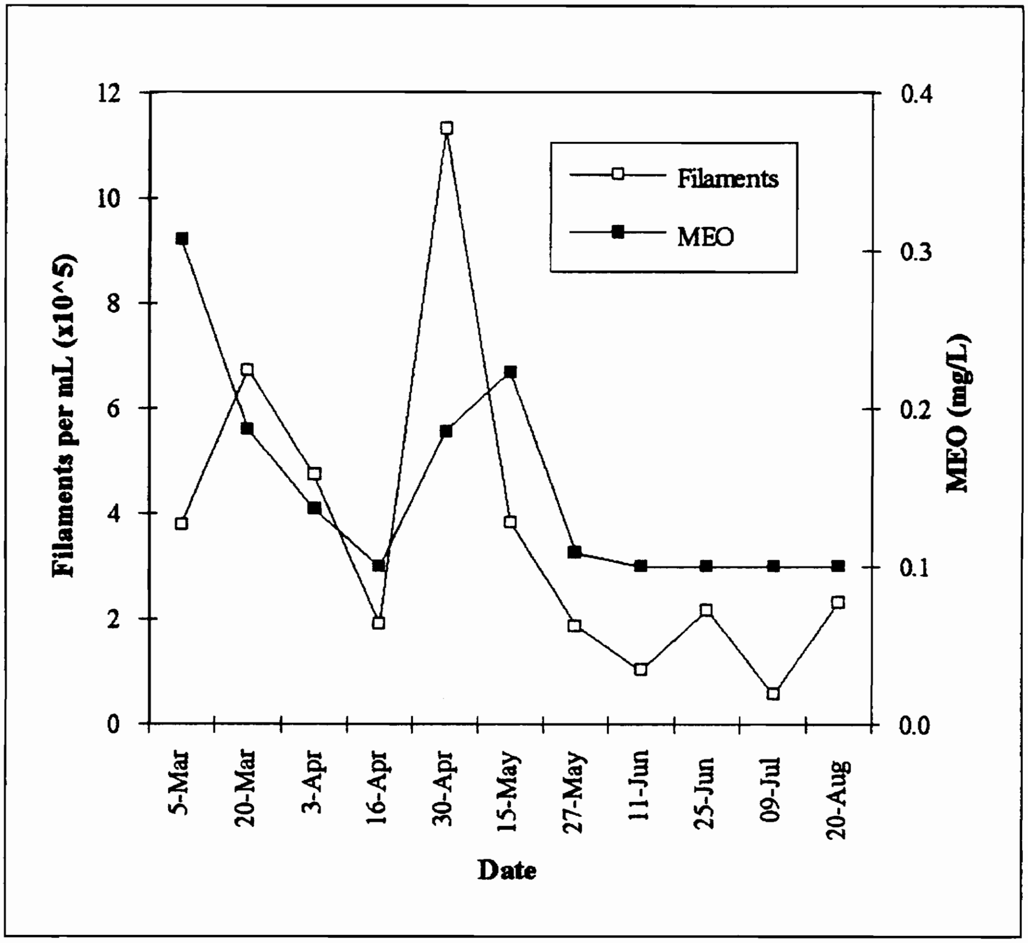

Figure 49. Comparison of influent mesityl oxide (MEO) to filament population of Celco activated sludge, 1992. MEO values were averaged over the period preceding sampling equal to the target sludge age.

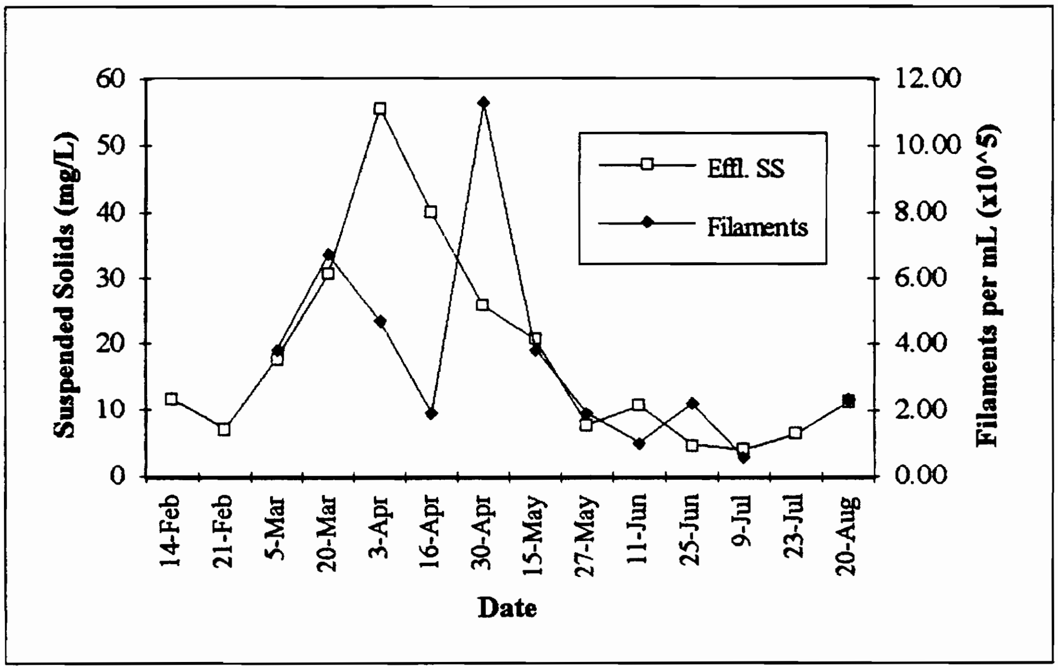

Figure 50. Comparison of filament population to effluent suspended solids (Effl.SS). Celco wastewater treatment plant, 1992. SS values were averaged over the period preceding sampling equal to the target sludge age.

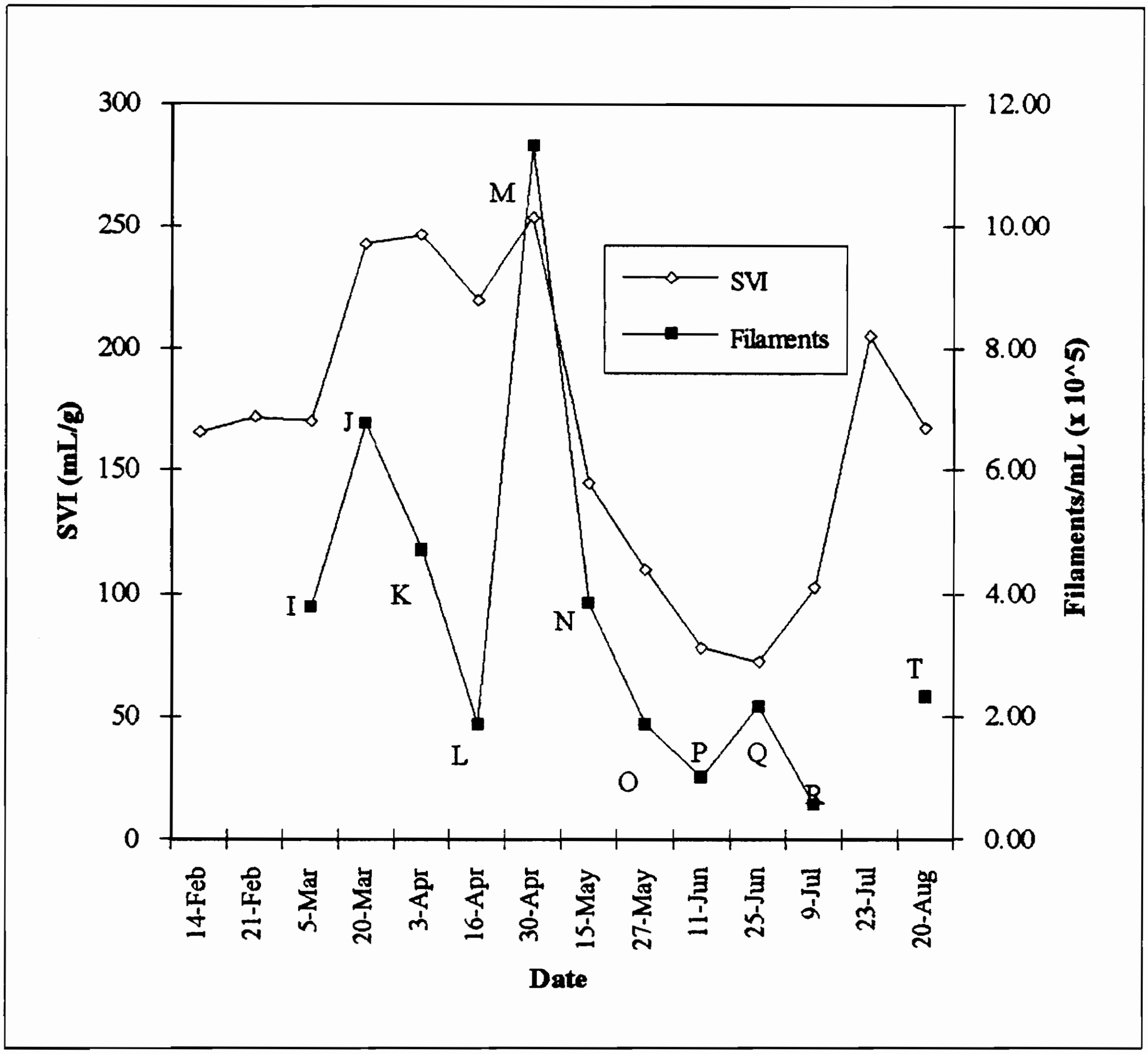

Figure 51. Comparison of filament population to sludge volume index (SVI). Celco wastewater treatment plant, 1992. SVI values were averaged over the period preceding sampling equal to the target sludge age. (See text for explanation of filament types prevalent in samples.)

reported to be filamentous, with most filaments protruding from floc surfaces. Flocs were also reported as small (C.W. Randall, 1992, personal communication).

Chapter V

DISCUSSION

5.1 HETEROTROPHIC PLATE COUNTS

5.1.1 Aeration Basin Conditions

Dissolved oxygen

As stated in the Results chapter, the dissolved oxygen values do not reflect conditions at the center of flocs. Under conditions where DO concentration is low in the bulk solution, the floc center could be anaerobic. Even when DO levels are not low, bacteria could be in a growth phase and utilizing oxygen faster than it diffuses into the floc, creating anoxic conditions. This could affect the number of viable cells present in the activated sludge. In *Wastewater Engineering* , 3rd. ed. (1991), a minimum DO concentration of 2.0 mg/L is recommended. Samples taken on the 3rd and 16th of September corresponded to periods of average DO of <2.0 mg/L (Figure 3). The fact that HPC did not vary similarly with the oxygen uptake rate (OUR) may be another indication that the HPC is not a useful procedure for operators at this plant (Figure 4).

Mean cell-residence time

When MCRT is increased there is a corresponding increase in the amount of biomass in the mixed liquor. Jenkins and Garrison (1968) commented that an increase in biomass does not necessarily mean an increase in viable cell numbers. The low HPC during the period of high average MCRT could substantiate the idea.

Influent biochemical oxygen demand

Organics in the influent were primarily simple molecules such as acetic acid, isopropyl alcohol and acetone. Acetic acid is easily degraded by activated sludge microorganisms. As influent BOD gradually increases more food would be available to the microbes and growth would be stimulated. The relationship between MCRT and F/M ratio is

$$\frac{1}{\theta} = Y\frac{F}{M}\frac{E}{100} - k_d \quad \text{and} \quad \frac{F}{M} = \frac{S_o}{\theta X}$$

where Y=cell yield coefficient, lb. cell produced per lb. organic matter removed

E=process efficiency, %

k_d=endogenous decay coefficient, time $^{-1}$

S_o=influent BOD or COD concentration, mg/L

θ=hydraulic detention time of aeration tank, d

X=concentration of volatile suspended solids in the aeration tank, mg/L

In order to maintain an equilibrium, an increase in influent BOD would necessitate a decrease in MCRT. Relationships between MCRT and F/M ratio are shown in Figures A-3 and A-4. A comparison of Figures 5 and 6 reveals that on 11 June the average influent BOD was approximately 1450 mg/L and the average MCRT was approximately 13 days. On that date the HPC was estimated at 9.0 x 10^6 colony-forming units/mL. From 23 July to 15 October, the average influent BOD was >1200 mg/L and the average MCRT was >15 days. The HPC decreased to very low levels, indicating that although biomass increased, the number of viable cells did not. The high 11 June HPC value may have been the result of very high flows around 5 June. An observer at the plant noted that the aeration basin was almost flooded. A higher than normal flowrate and lower MCRT could have stimulated bacterial growth.

5.1.2 Influent Constituents

Although the charts suggested there may have been some relationship between influent MEK and MEO concentrations it is likely that the changes in viable bacterial populations resulted from the MCRT and overall influent BOD relationships discussed in the previous section.

5.1.3 Effluent Nitrogen

The Celco wastewater is not carbon-limited like most domestic wastewaters. Because of that, the heterotrophic bacteria may use ammonia (NH_3) to satisfy their nitrogen requirement, out-competing nitrifiers for nitrogen. High NH_3 concentrations tended to coincide with low HPC counts (Figure 8).

5.2 DENITRIFIERS

5.2.1 General

The MPN procedure was simple to perform and was consistent throughout the study. Unfortunately, denitrification is more a function of enzyme activity than denitrifier numbers (Tiedje, 1982). According to Teidje, the synthesis and activity of denitrifying enzymes are inhibited by the presence of O_2. He also asserted that

> ...because the denitrifying enzymes are not constitutive, their presence (or quantity if present) in denitrifying cells is not known. These

physiological properties often make enumeration of denitrifiers of little value since the population density may have no direct relationship to denitrifying enzyme concentration or activity.

5.2.2 Dissolved Oxygen

The MPN procedure for denitrifiers was performed and analyzed to determine whether it could be used as a test of *potential* denitrification activity in the samples. The y-axis label on the figures, "Denitrifier per mL", is a misnomer; however, it is used for consistency with the procedure described by Tiedje (1982). Viewed as denitrification potential, the MPN of denitrifiers generally increased and decreased with changes in the average DO concentration (Figure 14). Because denitrifiers are aerobes, this may indicate that denitrifier population changes, rather than changes in enzyme concentration or activity, were responsible. Low average DO concentrations at the same time as elevated dentrifier counts would suggest increased synthesis or activity of denitrifying enzymes. The relationship between the denitrifier population and dissolved oxygen during the study is shown in Figure A-5.

5.2.3 Influent and Effluent Nitrogen

Most of the influent nitrogen was in the form of urea, supplied by operators. The effect of influent nitrogen concentrations on denitrifiers depends upon the number and activity of nitrifying bacteria. Urea is hydrolyzed by heterotrophs to ammonia, which is oxidized by nitrifiers to nitrate and nitrite. Nitrifiers grow slowly relative to other activated sludge bacteria, and are difficult to grow in the laboratory. A sufficiently high MCRT is required to maintain a nitrifier population. If the system was being operated with sufficient nitrogen in the influent, a higher MCRT would be expected to result in a higher amount of NO_x in the mixed liquor. Under anoxic conditions, in the clarifiers or floc centers, denitrification could occur. However, insufficient nitrogen was added for nitrification during the period of this investigation.

Nitrate and nitrite concentrations in the effluent were elevated during periods of MCRT >15 days (Figure 16). The same figure shows that a peak dentrifier count did not coincide with a change in effluent NO_x concentration. The relationship between the denitrifier population and NOx during the study is shown in Figure A-6.

5.2.4 SVI and Effluent Suspended Solids

Nitrogen gas is produced during denitrification, potentially becoming trapped in flocs if bubbles form. There was no real evidence that nitrogen bubbles contributed to increased effluent suspended solids or to poorly settling sludge (Figures 17 & 18).

5.3 PROTOZOA

5.3.1 General

At the time of counting, sketches of the protozoa seen and the relative numbers were recorded. Samples were not treated to kill protozoa to make them easier to count. Motionless objects that could have been amoebae were examined, but it was not clear whether they were amoebae or dying protozoa of other types. In some samples there were many dead or dying protozoa. There was often no consistent structure by which to group or identify the cells. Nevertheless, the types of organisms observed in this study were consistent with those reported by Baines, *et al.* (1953), Curds and Cockburn (1970a), Poole (1984) and Al-Shahwani and Horan (1991).

5.3.2 Relationships

Predator-prey

No clear relationship was found between free-swimming and stalked ciliates and heterotrophic plate counts (Figure 21), but there were data gaps. Curds and Fey (1969) did not differentiate between protozoa types when they described ciliates as being responsible for bacterial destruction. Curds (1971) described an inverse relationship between stalked ciliates and dispersed bacteria. Dispersed bacteria were more likely to form colonies on the culture media (Sterritt and Lester, 1988). Consequently, the dispersion method used in this study may be better for studying the relative peritrich and dispersed bacteria populations than would one using a more rigorous dispersion method. Peritrichs cannot ingest large flocs. The stalked ciliate population increased dramatically, coinciding with the equally dramatic increase in non-floc "tetrad" bacteria, in the 15 May sample.

Relative populations

Curds (1971) reasoned that when the sludge age is increased, the attached or crawling ciliate population should increase because a larger number would be returned to the aeration basin in the return sludge. Because the hydraulic detention time would remain essentially the same, free-swimming ciliates would have no advantage over attached ciliates. In this study, the stalked ciliate population generally increased relative to free-swimming ciliates whenever the average MCRT increased (Figure 23), although the relationship was not strong (Figure 25). The flagellate population was only significant in the 15 October sample.

Protozoa and dissolved oxygen

Protozoa numbers increased during a period of low dissolved oxygen (Figures 26 and 27). The DO was below 2.0 mg/L many times during the period. Not only was the aeration basin relatively warm because of the season, the average MCRT was increasing. The average oxygen uptake rate also increased at the same time, indicating an increased oxygen demand due to respiration, nitrification or decay. Dead and lysed cells would have provided food for ciliates. Also, the protozoa were present in the bulk solution or on floc surfaces, where oxygen was available. Very low DO may have been the cause of the sudden appearance of a large number of "wormlike" organisms and small, fast ciliates. It is possible that those organisms had an advantage in a situation of low DO. The large populations were ephemeral, disappearing once average DO values were ≥2.0 mg/L.

Ciliates and influent constituents

In general, there was no clear cause-effect relationship between influent constituents and ciliate population. An exception may be the relatively high concentration of acid on 9 May, which was followed by sudden increases in non-floc bacteria and stalked ciliate populations (Figure 31).

Ciliates and plant performance

Strong evidence of an inverse relationship between peritrichs and effluent BOD could not be developed, partly because of gaps in BOD and protozoa data (Figure 38). Baines, *et al.* (1953) found an inverse relationship between peritrichs and effluent BOD_5, as did Reynoldson (1942). Conversely, Poole (1984) found that peritrichs were associated with high effluent BOD_5. Curds and Cockburn (1970b) focused on identifying species that were associated with effluent BOD ranges. Most peritrich species were associated with an effluent $BOD_5 \leq 20$ mg/L. Al-Shahwani and Horan (1991) found a correlation between *Carchesium polypinum* (a peritrich) and effluent BOD ($r= -0.342$) for the plant treating primarily industrial wastewater.

No relationship was discernible between free-swimming ciliates and effluent BOD (Figure 37), partly because of the data gaps. The lack of relationship agreed with the conclusion of Baines, *et al.* (1953).

Workers who studied relationships between stalked and FS ciliates and effluent quality found that good effluent quality was associated with the presence of ciliated protozoa, especially the peritrichia . Previous work (Curds and Cockburn, 1970a and b; Reynoldson, 1942; Curds 1971; Al-Shahwani and Horan, 1991) indicated that when the stalked ciliate populations are high, effluent SS should be low. Stalked ciliates prey on individual bacteria and organic compounds in the bulk solution. Despite the large number of "tetrad" groups and individual non-floc bacteria in the activated sludge from 15 May to 3 September, effluent suspended solids were not present in high concentrations during that period (Figure 35). In fact, the effluent SS concentration was at or below limits throughout the study. There may have been an inverse relationship between stalked ciliates and effluent SS during the first half of the study, but the relationship was not present during the latter half. What the effluent SS consisted of was not recorded. Relationships between ciliate populations and effluent quality are shown in Figures A-7 through A-10.

5.4 FILAMENTOUS BACTERIA

5.4.1 General

Identification

Jenkins, *et al.* (1984) were the most helpful for the identification of filamentous bacteria. Wet mounts are more useful than stained preparations because the filaments can be observed for motility and the presence of sulfur granules and a sheath. Phase-contrast microscopy requires a wet mount and allows more accurate measurements of trichomes and cells. Eikelboom's (1975) descriptions were based on observations of domestic wastewater treatment plants, primarily. Often, filaments growing in industrial treatment plants have different characteristics than they do when growing in plants treating domestic wastewater. For instance, a filament growing in municipal activated sludge may have epiphyte growth, whereas the same species growing in a plant treating industrial wastewater may have none. Response to stains may also differ.

The treatment of the samples is important for maintaining the appearance of filaments. Pipettes with relatively small openings were used so that single, uniform drops could be placed on the slides. Estimation of the number of filaments per milliliter required knowing the volume of the sample placed on the slide. However, the use of a small orifice may shear filaments, breaking them or tearing them away from flocs. Several slides were made for almost every sample, although the slides were not made independently from amounts drawn at random from a well-stirred sample.

Doubtless not all of the filaments in each sample were identified. An attempt was made to identify the most common ones in each sample. Sometimes the identity of a filament was obvious, others seemed to fit no description available. While Neisser staining is straightforward, Gram staining requires practice to avoid over-decolorization, which can hamper identification. In some cases it is easy to confuse filament types such as 0041 and 021N. Strom and Jenkins (1984) discussed this and listed Types 0041 and 021N as common in activated sludge from plants treating chemical wastewaters.

5.4.2 Aeration Basin Characteristics

Nitrogen loading

As stated previously, the Celco wastewater is nitrogen-poor even though urea is added. The filament found most often during the study was Type 0041, which is associated with nutrient-poor conditions (Gray, 1989, Jenkins, *et al.*, 1984). Apparently, even though some filaments are associated with nutrient-deficient conditions, in this plant their growth is limited by nitrogen availability (Figure 41). Gray cited Strom and Jenkins (1984) as concluding that some filamentous bacteria have been associated with nutrient deficiency, but they did not make that conclusion.

Dissolved oxygen

Like all values examined, those for dissolved oxygen in the aeration basin were averages from the 14 days preceding sampling. During July and August there were 27 days in which the DO was ≤2.0 mg/L. On 12 days the DO was ≤1.0 mg/L and on two days it reached 0.0 mg/L (Figure A-2).

Although *H. hydrossis* may have been present in samples before Sample N (15 May), there is no evidence that the population increase was stimulated by low DO. Low DO may have contributed to the presence of Type 1701 in June and July. Type 1701 is usually found mostly within and extending from flocs. This would give them a competitive advantage, according to Sezgin and Parker (1978). *Microthrix parvicella* did not appear to be affected by DO concentration and this agreed with Pipes (1967). Strom and Jenkins (1984) found Type 0041 in plants suspected of having low DO.

Food-to-microorganism ratio

The inverse relationship between filament counts and F/M ratio (Figures 43 and A-11) supports previous workers (Pipes, 1967 and Eckenfelder (1989). When the F/M ratio decreases the filaments have a competitive advantage, perhaps, as Pipes suggests, because of their surface area/volume ratio. The increasing F/M ratio probably stimulated heterotroph growth because the oxygen uptake rate generally increased during the same period.

According to Jenkins, *et al.* (1984): "the absolute dissolved oxygen concentration required to prevent the growth of...'low DO' filamentous organisms is a function of the F/M and that 'low DO' bulking can occur at aeration basin DO concentrations that are not 'low' in the usually accepted sense of the term."

Mean cell residence time

Eikelboom (1977) commented that growth of *M. parvicella* depended more on sludge age than on DO concentration. The result of this study supported him (Figure 44). The bacterium was much less prevalent when the average MCRT was >20 days (15 May to 27 May).

Although no mention was found in the literature specifically linking *H. hydrossis* to sludge age, it was very common when sludge age was >20 days. Prevalence decreased to "few" after the average MCRT decreased to 15 days.

5.4.3 Influent Constituents

Filament counts did not appear to have a direct relationship to average influent constituents. However, the averaged values masked occasional high values. For example, from 2-8 May, the average acid concentration was 510 mg/L, with a range of 350 to 770 mg/L (Figure 45). On 9 May, the concentration increased to 1190 mg/L, then decreased to an average of 650 mg/L with little variation until 15 May. From 8-10 July, influent acid concentrations were 950 mg/L, 1780 mg/L and 1010 mg/L, respectively. Average concentrations were 900-1000 mg/L at the 20 March and 3 April samplings, but they were the result of more gradual fluctuations, not "shock loads."

A significant shock load of isopropyl alcohol may have occurred on 27 May. Records show an influent concentration of 900 mg/L. However, given the normally low levels, it may have been an entry error by operators.

No significant MEK, MC or MEO loading variations occurred during the study. MEK values were almost always 0.0 or 1.0 mg/L, and occasionally 2.0 mg/L. Filament counts were likely influenced by other factors.

On 21 June, the acetone concentration was reported to be 400 mg/L. It was the only entry during the study that exceeded 100 mg/L. There was no apparent effect, and it is possible it was not accurately reported.

5.4.4 Plant Performance

Effluent suspended solids

A relationship between filament counts and effluent suspended solids was not apparent. More important were the types of filaments present. The most common type found during the period of low effluent SS was Type 1701, which is characterized by epiphytic growth (Jenkins, *et al.*, 1984). The presence of epiphyte may reduce the amount of dispersed bacteria in the solution.

Sludge volume index

The sludge volume index has been studied as a function of filament number and length (Eikelboom, 1977; Sezgin, *et al.*, 1978; Pipes, 1979; Palm, *et al.*, 1980; Lee, *et al.*, 1982 and Baker and Veenstra, 1986). During this study, SVI was often >100 mL/g, almost always when *M. parvicella* was very common to abundant. *M. parvicella* has a thin trichome, 100-400 μm long, on the average. Type 0041, another common filament type found in the study, is 100-500 μm long. Type 1701 and *H. hydrossis*, numerous during periods of relatively low SVI, have average lengths of 20-80 μm and 20-100 μm, respectively. These findings support previous workers.

5.5 EVALUATION OF METHODS

5.5.1 Heterotrophic Plate Count

The heterotrophic plate count (HPC) was chosen because it is a standard method. The HPC procedure, as used in this study, was not particularly useful. First, only a subpopulation was tracked--those bacteria that grew on the medium. The specific genera cultured were unknown. Second, simple shaking probably did not develop sufficient shear force to separate cells, so the number of cells per mL in the original sample was probably underestimated.

Floc dispersion methods such as sonication or shaking are only effective if flocs can be prevented from reforming. Sodium chloride was ineffective, as were ionic and nonionic surfactants (Yin and Moyer, 1968). Sodium tripolyphosphate was used successfully as a diluent by Pike, *et al.* (1972).

5.5.2 Identification of Heterotrophs

Identification of heterotrophic bacteria is difficult. Historically, it has required a biochemical test series for each species, and a comparison of the results to dichotomous keys. This is still the best method. There are shortcuts available in the form of commercially prepared kits, such as Biolog and API®(see Section 2.1.3). These kits were designed for the health care field, and their databases do not include all environmentally important bacteria.

An attempt to use the Biolog system in this study was unsuccessful. Directions for its use specified a cell-density range for the inoculate. The turbidity of the standards provided were measured using available equipment. The cell density of the unknown bacterial sample was adjusted continuously, yet at no time was the measured turbidity as high as the lower value for the standards. This may have been a problem for the one bacterial type being tested. The Biology system requires the cultures to be grown on Tryptic Soy Agar.

5.5.2 MPN Denitrifiers

As mentioned previously, the MPN denitrifier test is worth little as an estimation of denitrifier numbers because denitrification is more a function of enzyme activity than cell numbers. However, it may be possible to view the test as an indicator of a sludge's denitrification potential, and to use it to identify trends, as it was used in this study. The test was easy to perform and was consistent, but it requires 14 days of incubation.

5.5.3 MPN Sulfate-reducers

Unfortunately, much time was spent attempting to make the method from *Methods of Soil Analysis* (1982) work. The medium was agar-based and was difficult to inoculate with the requisite amount of inoculum. The medium also appeared different each time it was prepared, and controls sometimes turned black near the bottom of the tubes. The procedure from *Standard Methods* (1985) was much easier to prepare and inoculate. It was also consistent. Like the MPN Denitrifier procedure, it requires at least 14 days of incubation.

5.5.4 Palmer-Maloney Counting Method

This was an extremely useful method with which to count protozoa. The method, as used in this study, could be improved by performing multiple counts of each of several P-M cell volumes from each sample. An additive, such as glycerin, could be employed to slow the protozoa. Dead organisms can be hard to identify.

5.5.5 Filament Counts

Counts should be made from both wet mounts and stained slides. Multiple volumes should be drawn from each gently well-mixed sample, such that the smears represent the sample.

Other methods that take into account filament length would be useful, such as that of Walker (1982). The method described by Jenkins, *et al.* (1984) recommends using 100X magnification. However, the 200X magnification used in this study was too weak to guarantee that the very thin filaments were not missed, and a method using higher magnification would be more accurate.

5.5.6 Filament Identification

Because of time constraints, filament identification was not rigorously pursued. Wet mounts are necessary for the observation of motility, inclusions, cell septa and sheaths. Gram stains require practice to avoid over-decolorization. Neisser stains are simple to perform. Careful calibration of an ocular grid is necessary for accurate measurement of filament width and length, as well as cell dimension. Phase contrast is necessary for observation of sheaths, cell walls and cell indentations in wet mounts.

Chapter VI

CONCLUSIONS

This study fulfilled its first objective as an initial investigation of the microbial populations in the Hoechst-Celanese activated sludge. Activated sludge is a complex ecosystem and as such was not easy to characterize. It was also difficult to identify cause and effect relationships because so much about the species involved was unknown.

The second objective of the study, to identify relationships between population dynamics and process performance, was hampered by the relative stability of the activated sludge. During the study, effluent standards were exceeded rarely, if at all. An unusually warm winter prevented large variations in aeration basin temperature. Influent constituents did not really constitute shock loads at any time.

Some relationships reported in the literature may have been supported by this study, but additional data is needed. The following relationships were inconclusive or suggested by this study:

1) Stalked ciliate population increases relative to free-swimming ciliates when mean cell residence time increases;
2) Effluent suspended solids concentration decreases when stalked ciliate population increases;
3) Filaments *M. parvicella* and Type 1851 predominate during periods of low dissolved oxygen;

4) Filament *H. hydrossis* population increases when average mean cell residence time (MCRT) ≥20 days;

5) Filament *M. parvicella* population decreases when average MCRT ≥20 days;

6) Filament Type 1701 common during periods of low effluent suspended solids

7) Filament *M. parvicella* associated with sludge volume index ≥150.

According to this study, the best organisms to use to monitor process performance are the filamentous bacteria and protozoa. Relative populations and overall numbers can be determined quickly because culturing is not necessary. Identification of filamentous bacteria requires training and experience, but the information gained from both groups of organisms could be worthwhile.

Various procedures for identifying and enumerating microbial organisms were evaluated as a secondary objective. Some methods that were unsatisfactory for use with this activated sludge were identified, and some were modified to suit the needs and time schedule of the experimenter. It was found that the heterotrophic plate count (HPC) and Most Probable Number procedures are not useful for close monitoring of plant performance. The HPC detects a subset of heterotrophs, and the species are unknown. Monitoring denitrifier populations would not benefit operators because the plant does not nitrify significantly, and because the MPN technique does not yield population numbers. Sulfate-reducer monitoring may be beneficial as a measure or indication of the sulfate-reducing potential of the activated sludge for design purposes, should effluent sulfate ever be regulated. The MPN techniques also require at least 14 days for incubation.

Chapter VII

RECOMMENDATIONS

7.1 HETEROTROPHIC BACTERIA

It was clear from this study that the heterotrophic plate count was not useful in monitoring heterotroph populations (see Section 5.5.1). However, Standard Methods Agar is a general medium that is a good starting point for isolating bacteria for identification. Casein-glycerol-yeast extract medium has also been used (Pike, *et al.*, 1972).

If identification of heterotrophic bacteria is desired, consultation with a microbiologist is recommended for those workers not familiar with, or current in, aseptic and isolation techniques. Commercially prepared systems are only as good as their databases, but they may be a place to start if they are available or economical. However, it is likely that biochemical test will have to be prepared manually and the results compared to standard authorities.

7.2 DENITRIFIERS

The MPN Denitrifier procedure requires 14 days of incubation, making it useless for evaluating or closely monitoring activated sludge. In this study, only the presumptive test was used. Tiedje (1982) states that the ability to reduce NO_x is not limited to denitrifiers, and that the test should be coupled with a test for the presence of N_2 to confirm denitrification. Another confirmatory procedure is to add 1.0 mL of acetylene (C_2H_2) to the headspace of each tube after autoclaving. Acetylene inhibits the reduction of

N_2O. After 14 days, 0.5 mL of headspace is removed and injected into a gas chromatograph as per instructions on page 1019 of *Methods of Soil Analysis* (1982).

7.3 SULFATE-REDUCERS

The MPN Sulfate-reducer procedure requires at least 14 days of incubation, making it of little use for evaluating and closely monitoring activated sludge. It was found in this study that of the two methods used, the one from *Standard Methods* (1985) is more appropriate for the Celco activated sludge.

7.4 PROTOZOA

The Palmer-Maloney counting method from *Standard Methods* (1985) is recommended for the Celco activated sludge (see Section 5.5.4). Differentiating free-swimming and stalked ciliates, flagellates and amoebae is important for evaluating and monitoring of activated sludge. Relative numbers of the above groups of protozoa should be collected over an extended period. The data can be analyzed and compared to plant data to determine whether certain populations can be correlated with particular operating conditions. It is doubtful that the identification of individual protozoa species would provide sufficient information to be worthwhile.

7.5 FILAMENTOUS BACTERIA

Filamentous bacteria identification is recommended to plant operators. The growth of some species is associated with certain operating conditions, so an examination of the activated sludge may help to predict or diagnose problems. The methods Eikelboom (1975, 1977 and 1982) and Jenkins, *et al.* (1984) may be most useful.

Experiments with enumeration methods were not performed in this study. A comparison of results of the methods used by previous workers would be worthwhile. For day to day plant operations, the classification scheme of Jenkins, *et al.* (1984) should be sufficient for enumeration.

Chapter VIII

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APPENDIX

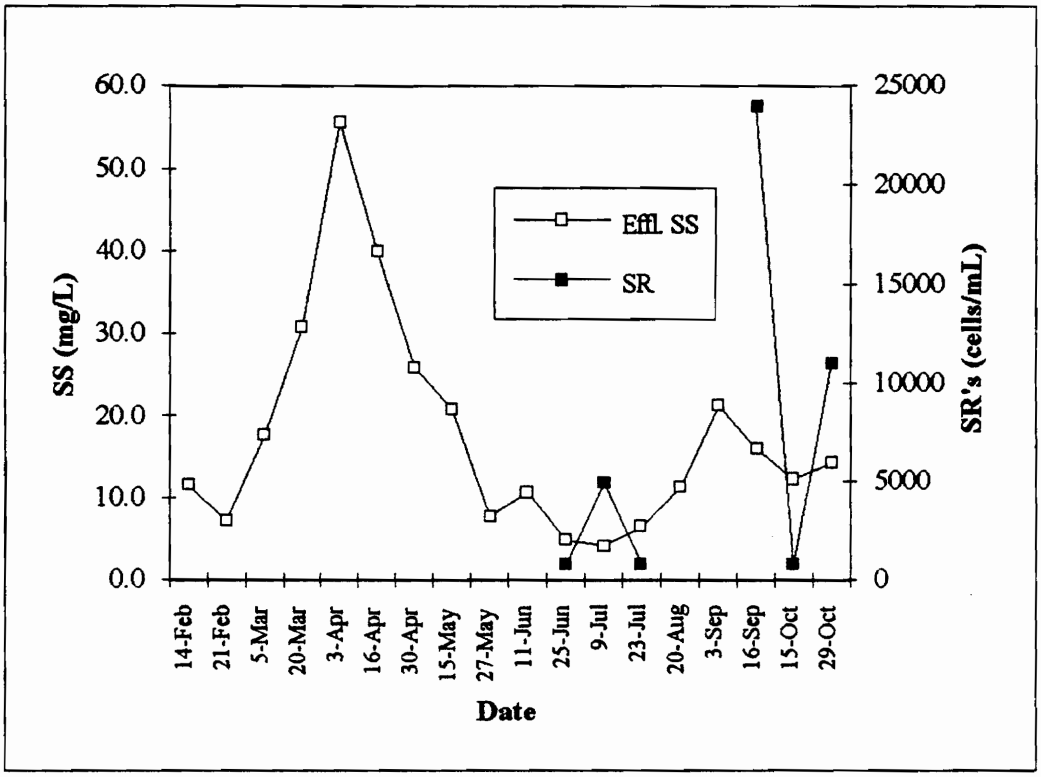

Figure A-1. Effect of sulfate-reducer population on effluent suspended solids. Celco wastewater treatment plant, 1992. SS values were averaged over the period preceding sampling equal to the target sludge age. All data were produced using the second culture method.

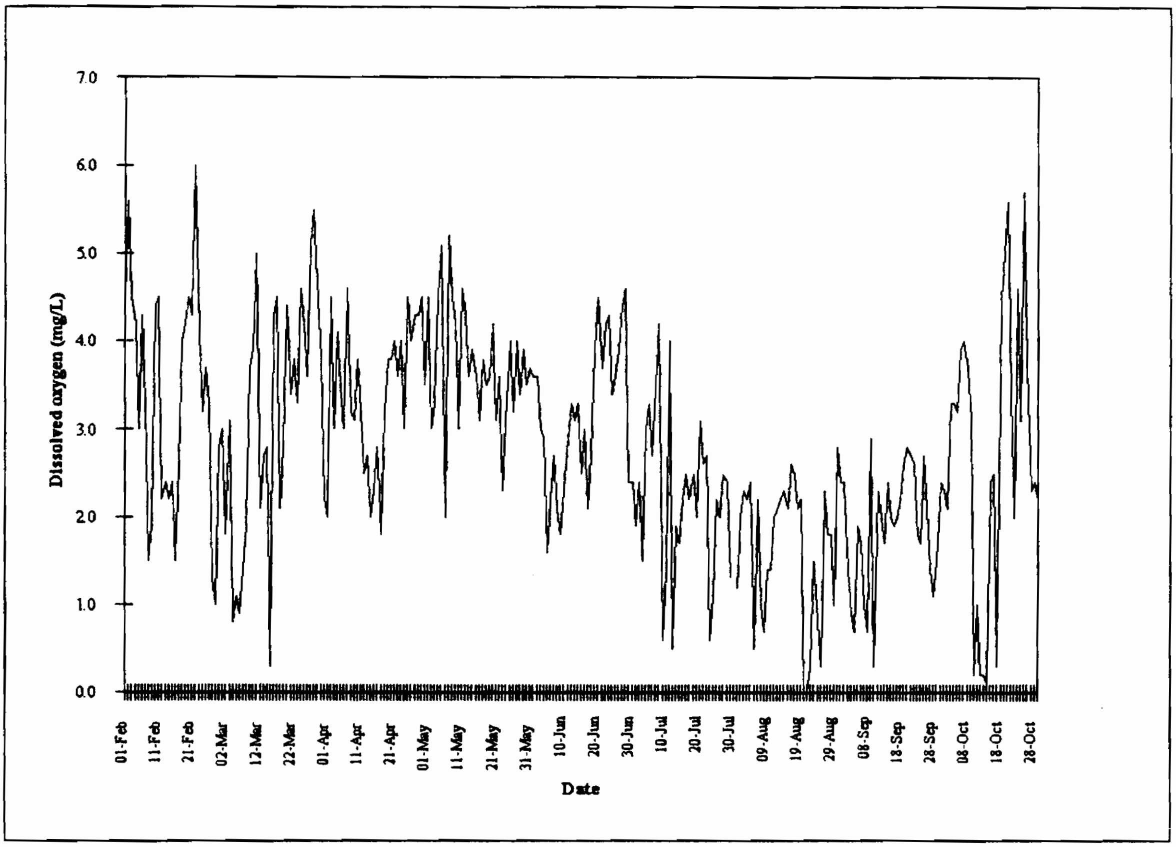

Figure A-2. Dissolved oxygen in Celco activated sludge, 1992.

Table A-1. Results of linear regression on mean cell residence time vs. protozoa of Celco activated sludge, 1992.

Regression Statistics

Multiple R	0.459
R Square	0.211
Adjusted R Square	0.161
Standard Error	1.461
Observations	18

Analysis of Variance

	df	*Sum of Squares*	*Mean Square*	*F*	*Significance F*
Regression	1	9.107	9.107	4.268	0.055
Residual	16	34.138	2.134		
Total	17	43.246			

	Coefficients	*Standard Error*	*t Statistic*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-1.336	1.671	-0.799	0.435	-4.878	2.207
x1	0.210	0.102	2.066	0.054	-0.005	0.426

Table A-2. Results of line- and curve-fitting spreadsheet functions on dissolved oxygen vs. protozoa of Celco activated sludge, 1992.

LINEST			
Slope:	**-1.116**	**Y-int:**	**5.203**
SEn:	0.4239	SEb:	1.243
r2:	**0.3023**	SEy:	1.373
F:	6.9325	df:	16
SSreg:	13.073	SSresid:	30.173
LOGEST			
Slope:	**0.6777**	**Y-int:**	**5.042**
SEn:	0.1737	SEb:	0.509
r2:	**0.2387**	SEy:	0.563
F:	5.017	df:	16
SSreg:	1.5891	SSresid:	5.068

SEn = standard error for slope coefficient
SEb = standard error for y-intercept
r2 = coefficient of determination
SEy = standard error for y estimate
F = F statistic
df = degrees of freedom
SSreg = regression sum of squares
SSresid = regression sum of residuals

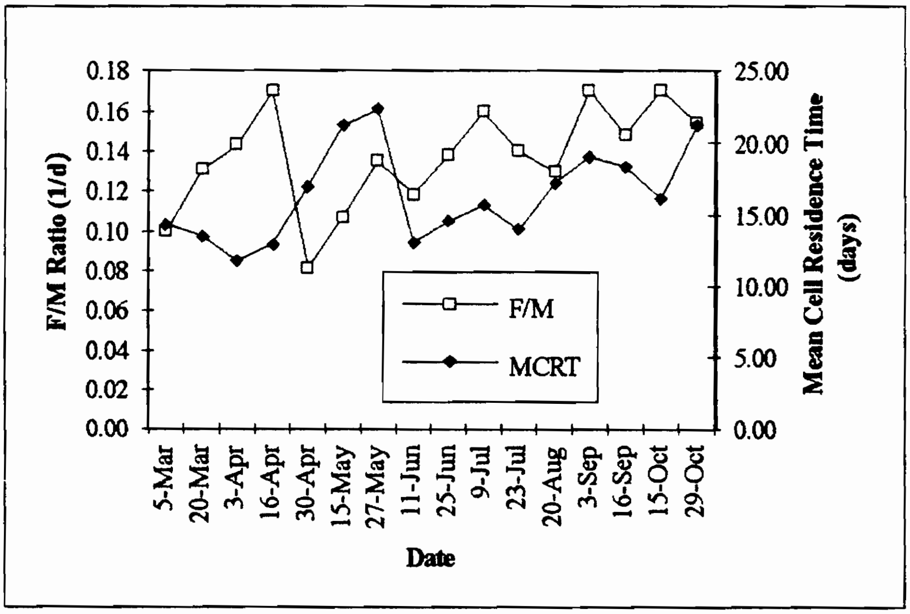

Figure A-3. Food to microorganism (F/M) ratio and mean cell residence time (MCRT) vs. Time. Celco activated sludge, 1992.

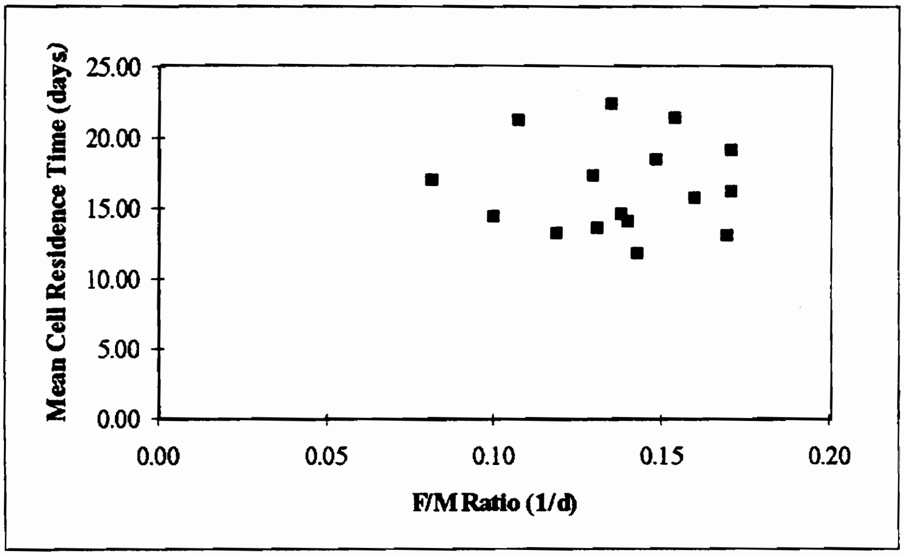

Figure A-4. Relationship between mean cell residence time (MCRT) and food to microorganism (F/M) ratio in Celco activated sludge, 1992.

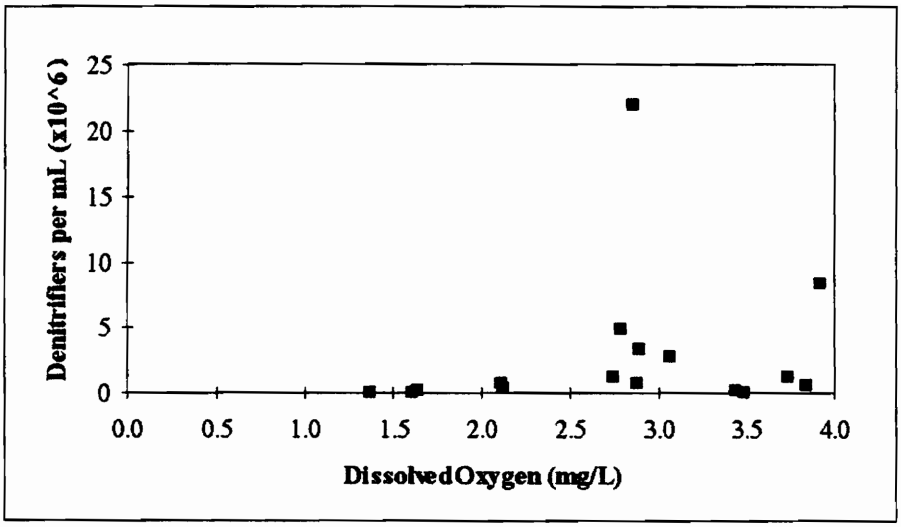

Figure A-5. Relationship between denitrifier (DN) population and dissolved oxygen (DO) in Celco activated sludge, 1992.

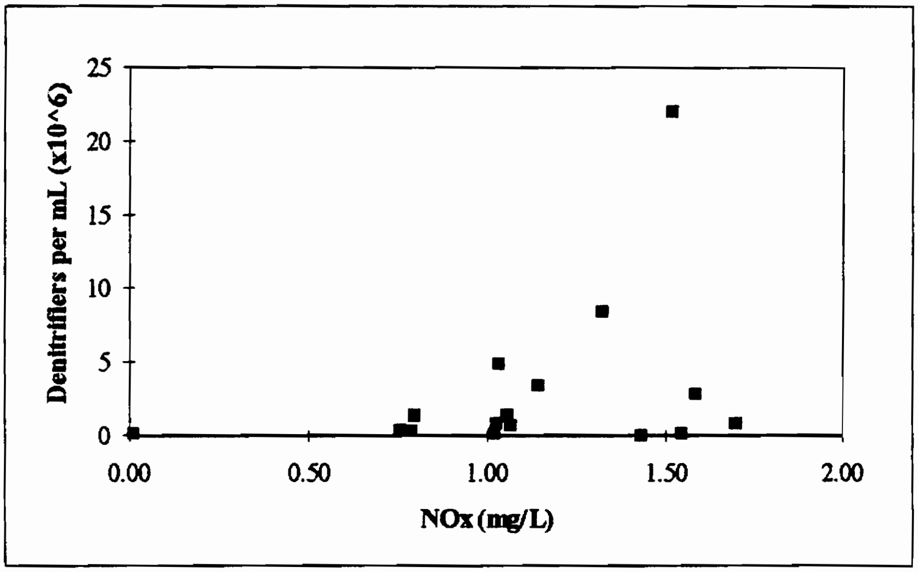

Figure A-6. Relationship between denitrifier (DN) population and effluent nitrate + nitrite (NOx) in Celco activated sludge, 1992.

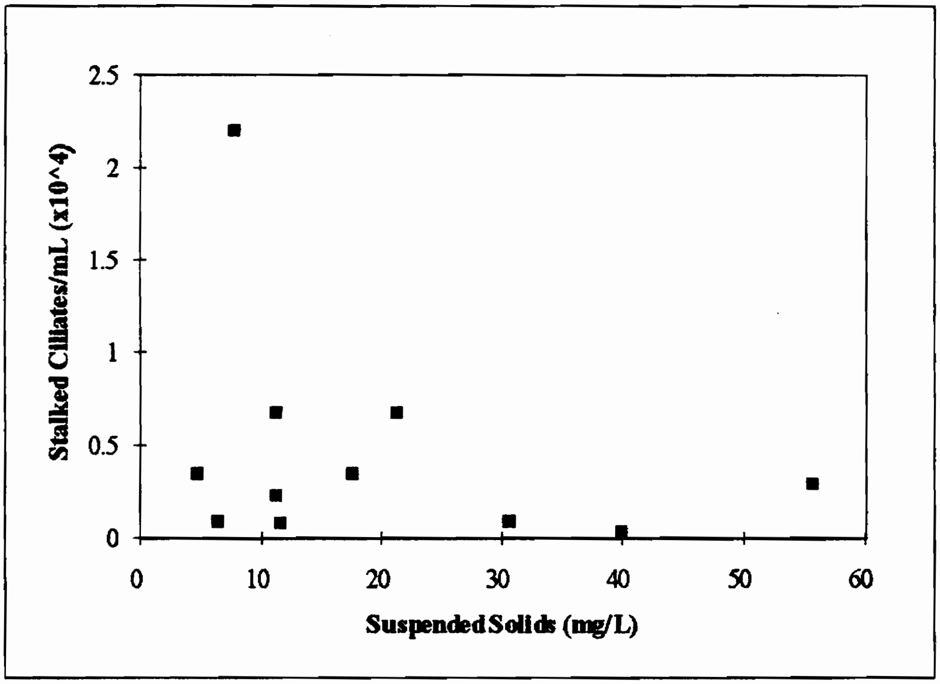

Figure A-7. Relationship between stalked ciliate population and effluent suspended solids (Effl.SS) in Celco activated sludge, 1992.

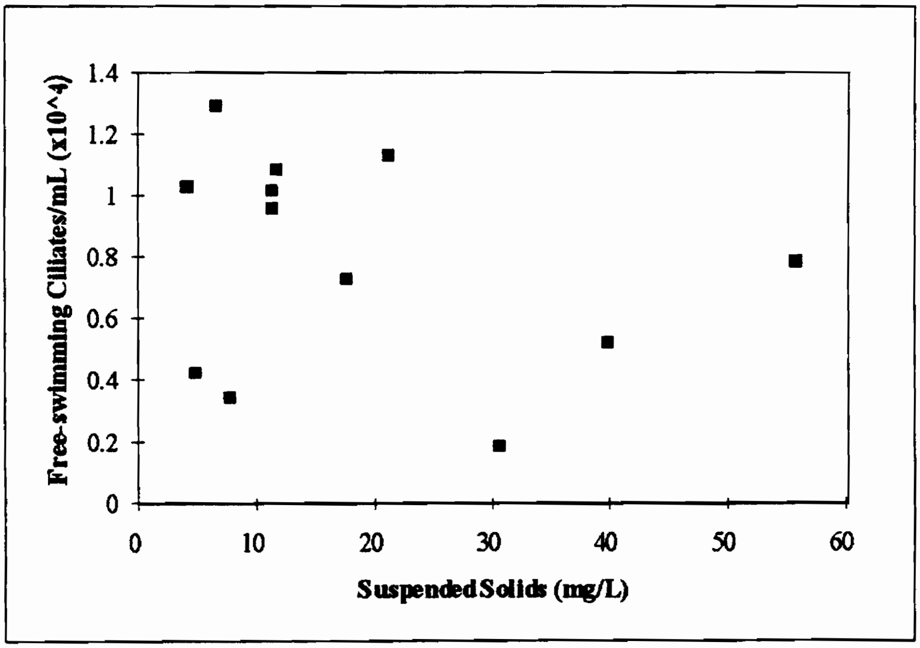

Figure A-8. Relationship between free-swimming (FS) ciliate population and effluent suspended solids (Effl.SS) in Celco activated sludge, 1992.

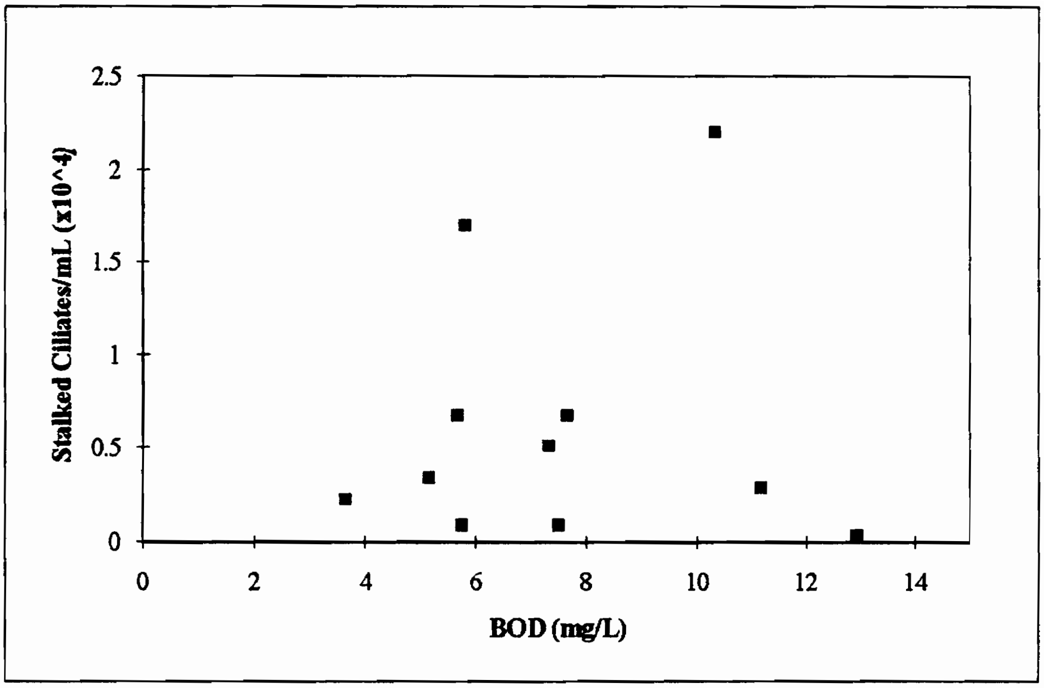

Figure A-9. Relationship between stalked ciliate population and effluent biochemical oxygen demand (BOD) in Celco activated sludge, 1992.

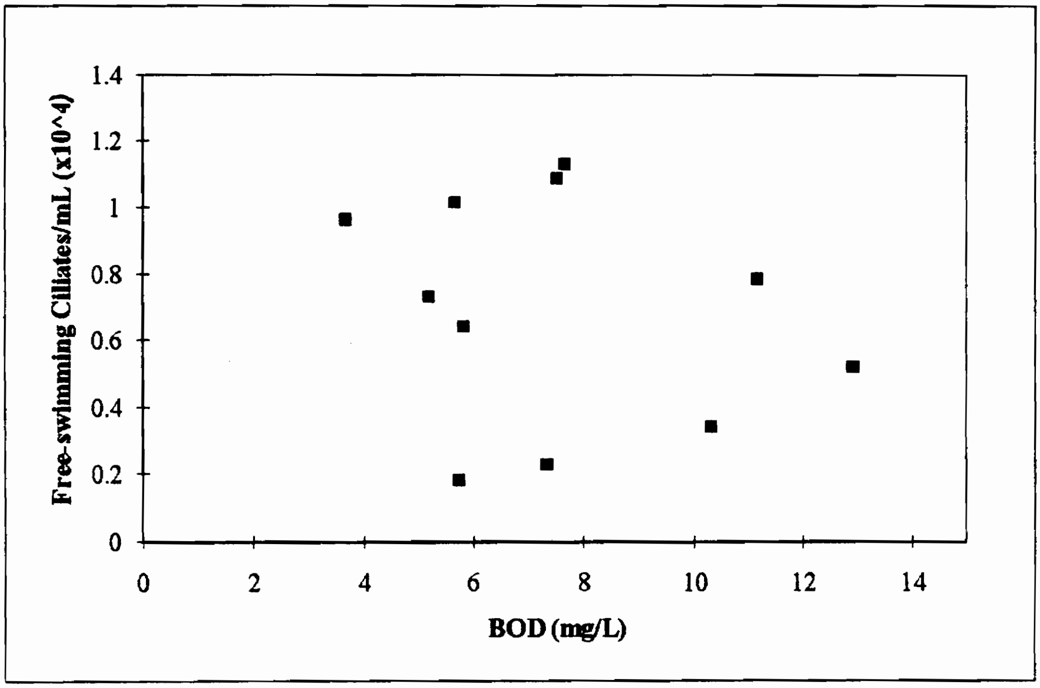

Figure A-10. Relationship between free-swimming (FS) ciliate population and effluent biochemical oxygen demand (BOD) in Celco activated sludge, 1992.

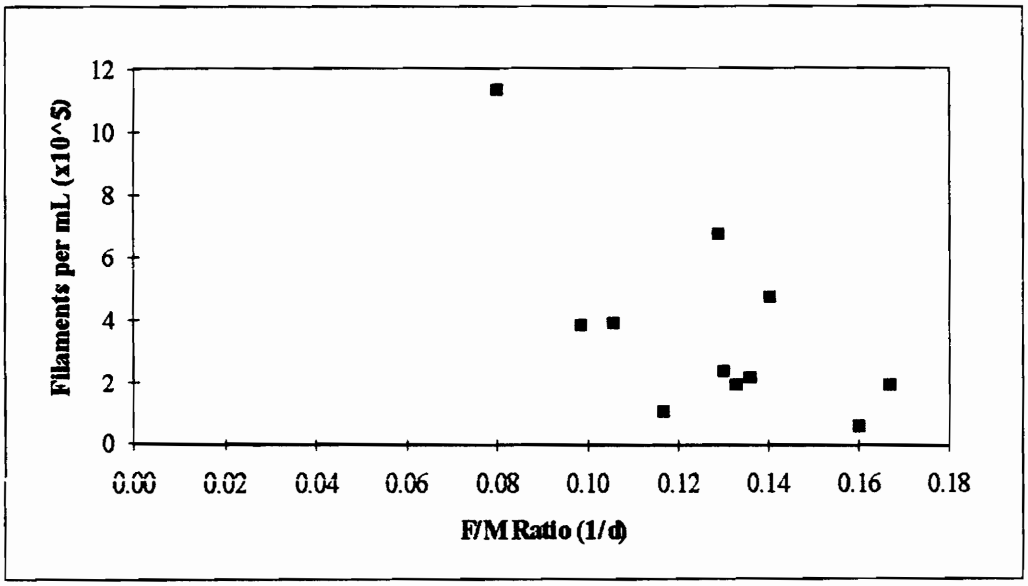

Figure A-11. Relationship between filamentous bacteria population and food to microorganism (F/M) ratio in Celco activated sludge, 1992.

VITA

Karen was born in Kingsville, Maryland in 1960. She moved with her family to an antebellum farm in Lexington, Virginia in 1976. She earned a Bachelor of Science in Biology from VPI&SU in 1985 and a Master of Science in Public Health, with an emphasis in medical parasitology, from the University of North Carolina at Chapel Hill in 1986. She worked in the environmental program of the Virginia Department of Health before returning to Va Tech to earn her Master of Science in Environmental Engineering. Her interests include reading, bonsai, fighter aircraft and miniature furniture. She hopes to work in surface water quality management.

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