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Biological Factors Affecting Pulp Mill Effluent Induced Coagulation and Flocculation in Receiving Waters

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by

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INTRODUCTION

Recent evidence has shown that downstream from a pulp mill effluent discharge, particles form, coagulate and flocculate faster than predicted by current sediment transport models (Krishnappan, 1996b). This phenomenon has been termed pulp mill effluent induced coagulation and flocculation (PMEICF) and is the basis for this study.

Existing sediment transport models have failed to take into account the phenomenon of PMEICF. They assume all particles behave as individual particles and flocculation does not occur (Ongley et al. 1992). PMEICF can cause a build-up of organic material on the river bottom, since insufficient time has passed to allow degradation of the chemicals, if this is possible. Some of the materials may cause adverse conditions, may be toxic or induce anoxic or toxic conditions. The chemicals may still possess a significant biochemical oxygen demand, resulting in low dissolved oxygen in the river. The changed conditions near the river bottom may cause harm to benthic organisms, which could have adverse effects on the entire food chain.

The microbial involvement in biological floc formation is well-documented (Riley, 1963; Pavoni, 1972; Paerl, 1974; Biddanda, 1985; Muschenheim et al., 1989; Rao et al., 1991; Mueller, 1996). Bacteria excrete polymeric substances which may be significant in the floc formation. However, only some bacterial species are considered “floc-formers” (Friedman and Dugan, 1968). Bacteria enter into a starvation-survival phase, when in oligotrophic environments (Humphrey et al., 1983; Kjellberg and Hermansson, 1984), characterized by dwarfing, fragmentation and adsorption to each other or another surface. The physiological changes of the bacteria could influence the observed induced flocculation as well.

This study examined the microbial involvement of the induced floc formation. Through laboratory and some field work, the roles of the bacteria were studied. Attempts were made to determine individual micro-organisms involved and whether or not they alone induce flocculation of the pulp mill effluent.

BACKGROUND INFORMATION

The Nature of Pulp Mill Effluent

The composition of pulp mill effluent (PME) is extremely variable. It varies among mills, as well as over the year, depending on the type of wood or treatment process being used (Liss and Allen, 1992). Generally pulp mill effluent has an acidic pH and is high in phosphorus and nitrogen (Amblard et al., 1990). When chlorine is added during the bleaching process, chlorinated organics make up a large part of the effluent. These compounds can be referred to as chlorohumus, since the structure is usually unknown (Saski et al., 1994). However, Suntio et al. (1988) published a list of about 250 compounds, most of which are chlorinated, found in a pulp mill effluent. Each one was present at a low concentration but the number of chemicals present is

of concern. The major categories of compounds are organic acids and chlorinated organic acids, phenols and chlorinated phenols, chlorinated catechols and guaiacols, sugars, benzene and chlorinated benzene derivatives, aldehydes and chlorinated aldehydes, chlorinated acetone derivatives and chlorinated aliphatics. A switch from elemental chlorine (Cl_2) to chlorine dioxide (ClO_2) has greatly decreased the amount of chlorine by-products (Gifford, 1994). Characteristics of these by-products range from water-soluble and rapidly biodegradable substances to persistent and highly bioaccumulative substances such as dioxins and furans (Elliott et al., 1994). The fate of these compounds is largely dependent on their physical and chemical properties, resulting in various end products and final accumulation sites. PME also contains substantial concentrations of metals, such as zinc, aluminium, copper (Kukkonen, 1996) and in some cases, manganese (AEP, 1997).

After discharge into the river, the organic components of PME tend to accumulate with organic substances, such as the sediments or biological tissues, or volatilize into the air (Gifford, 1994). The hydrophilic components will likely remain in solution. The intermediates formed during the degradation process may be more biodegradable substances or more persistent compounds (Gifford, 1994). Organisms in the sediment can take up the hydrophobic compounds, initiating accumulation in the food chain (Gifford, 1994). Of concern in this study is that material removed by sedimentation. The organic halogens in particular, have been reported to accumulate downstream of pulp mills (Jokela et al., 1993). Chloroguaiacols and chlorocatechols have been reported to have high sedimentation near the mill, while chlorophenols are not as strongly affected (Kukkonen et al., 1996).

Characteristics of Flocculation

Flocculation is an essential process in natural systems because it results in the deposition of fine-grained particles. The settling velocity of flocculated particles can be up to four orders of magnitude greater than unflocculated material as measured from laboratory experiments (Krishnappan, 1996a). Stokes Law, used for cases when the Reynolds number is less than 0.3, cannot be used because of changing particle size.

The occurrence of flocculation corresponds to type 2 versus type 1 settling. Type 1 settling is discrete particle settling, without significant interaction between neighbouring particles. Type 2 settling involves coagulation and flocculation, resulting in an increased mass of the particle and increasing settling velocity (Tchobanoglous and Burton, 1991). For flocculation to occur, two conditions must be fulfilled: 1) a collision between particles and 2) adhesion between particles (van Leussen, 1988). The collision frequency is increased with increasing turbulence, but in highly turbulent waters the floc particles may shear apart. Thus a maximum floc size is obtained.

Factors affecting the collision stage of flocculation include velocity gradients within the suspending liquid (important for particles >10 μm), differential settling of particles (>10 μm) and Brownian motion (<1.0 μm) (Krishnappan, 1996a).

There are four main cohesion mechanisms (van Leussen, 1988; Krishnappan, 1996a). The first, salt flocculation, is dominant when there is a high concentration of cations. In solutions of high ionic strength, the double layer surrounding each particle is compressed, facilitating aggregation. In river systems, this process is not as important as the other three (Droppo and Ongley, 1992). The second mechanism is the formation of organic aggregates and bioflocculation. Following colonization of inorganic particles by bacteria, polysaccharides are produced by a process in which dissolved organic matter (DOM) is converted to particulate organic matter (POM). The polysaccharides adsorb onto other inorganic particles, enhancing flocculation by inter-particle bridging. Further adsorption and colonization of bacteria continue this process. The third mechanism is pelletization. Filter feeders, such as copepods, transform suspended matter into pellets, which have faster settling velocities. The final mechanism involves the chemical coatings of particles. These control the charge of the particle that will affect its cohesive abilities.

The properties of the floc particle that are affected by its environment are its size, density and strength. Growth of the floc particle has been characterized by distinct structures. At the lowest level, the inorganic particles, held together with uniform porosity are considered zero-ordered aggregates. Several of these particles together are called first-order aggregates. Several first-order aggregates clumped together form a second-order aggregate and so on. Most experimental evidence reveal a 3- or 4-level floc structure (van Leussen, 1988). As the order increases, the diameter of the floc will increase, as well as its porosity. This decreases the density as well as the floc shear strength (van Leussen, 1988; Droppo et. al., 1997). It is important to remember that floc size is a dynamic property, depending on the rate of aggregation, the rate of break-up (determined by the turbulence) and other environmental factors.

Much of the past research has focussed in natural flocculation in estuaries (Riley, 1963; van Leussen, 1988; Muschenheim et al., 1989; Eisma et al., 1991). This differs greatly from river flocculation, due to the high ionic strength of the marine waters. More recently, flocculation in rivers has gained in interest (Krishnappan, 1996a). River flocs tend to be smaller in size, due to the increased turbulence and low cation concentration (Droppo and Ongley, 1992). The lower ionic strength increases the energy barrier (i.e. the repulsive forces between the particles), inhibiting flocculation (Droppo and Ongley, 1994), as illustrated in Figure 1.

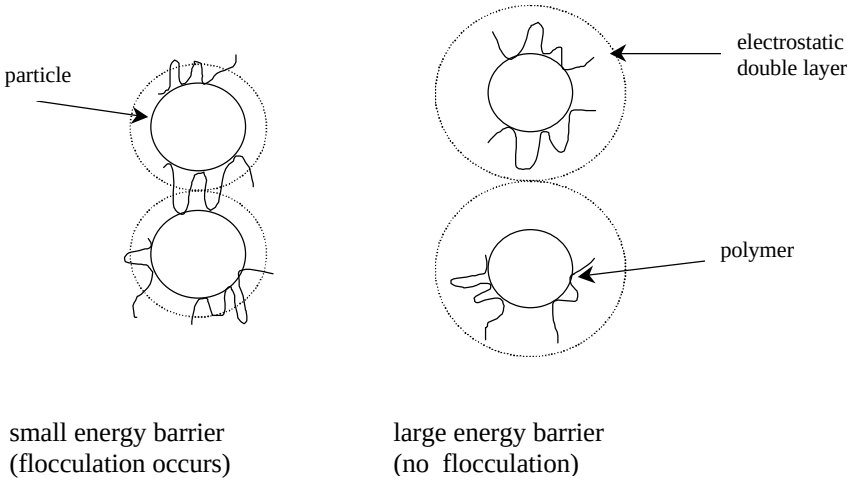


Figure 1: Bridging of the Energy Barrier by Polymers (adapted from van Leussen, 1988)

Microbial Involvement

Bacteria can be surrounded by a layer of organic material. This may be in the form of a capsule or slime layer. A capsule consists of organic matter that adheres to the cell wall, whereas a slime layer extends into the suspending medium, with finger-like appendages (Geesey, 1982). This organic layer is often referred to as extracellular polymeric substances (EPS). Most are composed of polysaccharides, but some can be proteinaceous (White, 1995) or strands of RNA or DNA (van Leussen, 1988; Pavoni et al., 1972). The exact chemical composition varies with the type of micro-organism and the environmental conditions (Geesey, 1982). They have a high length-to-width ratio, with a diameter ranging from 2 to 20 nm (Leppard, 1986).

EPS play an important role in flocculation, by aiding in the attachment of bacteria to solids (Geesey, 1982; Biddanda, 1985). Microscope work has shown that flocs viewed under high resolution resemble microbial biofilms, described in the literature (Liss et al., 1996). The colonization and growth of a biofilm at a solid-liquid interface has been described by Mueller (1996). In this case, the sediment particles can be considered the solid material. The process is broken down into six steps: 1) substratum conditioning by organic molecules, 2) transport of cells to the surface, 3) adsorption of cells to the substratum, 4) transformation of reversibly adsorbed cells to irreversibly adsorbed cells, 5) growth of the biofilm and 6) erosion of cells. The fifth step, growth of the biofilm, can lead to growth of the aggregate. This has been subsequently studied (Muschenheim et al., 1989). An immediate aggregation of particles was observed, due only to interparticle collision (i.e. a physical process). The time scale was too short to include any biological processes. A second biologically-mediated stage occurs, that results in larger aggregates. This stage involved the attachment by bacterial exudates (Biddanda, 1985; Muschenheim et al., 1989). In laboratory studies, this delayed flocculation was shown to correspond to the time when the micro-organisms enter into an endogenous growth phase (Pavoni et al., 1972).

Upon attachment to the sediment, polymers extend out into the surrounding solution, where they may attach to another particle. This mechanism of aggregation is termed interparticle bridging. For this to occur, the polymers must bridge the electrostatic double layers of both particles. The presence of some cations in the solution will compress the double layers and promote flocculation (van Leussen, 1988). Studies have shown that the presence of cations is essential for flocculation (Busch and Stumm, 1968; Levy et al., 1992; Sanin and Vesilind, 1996).

In oligotrophic environments (environments where there are few nutrients), copiotrophs (micro-organisms requiring large amounts of nutrients) enter into a starvation-survival phase (Humphrey et al., 1983). Generally this consists of two steps: 1) dwarfing and 2) fragmentation. Dwarfing is simply a reduction in size and fragmentation results in an increase in individual cell number, but without growth (Mueller, 1996). The importance of this starvation phase is that bacteria are considered “stickier” and will attach to organic matter more readily. Mueller (1996) suggested that this attachment could be used as a survival mechanism. Pavoni et al. (1972) concluded that bacterial bioflocculation would not occur until the bacteria are in an endogenous growth phase.

Some research has been done into the particular bacterial species that are involved in floc formation. In general, bacteria can be classified as floc-forming or non-floc-forming (Friedman and Dugan, 1968). Floc-forming bacteria tend to clump together when in liquid suspension. This is not always associated with the production of a capsule or slime layer (Friedman and Dugan, 1968). Most studies have attempted to isolate floc-forming bacteria from activated sludge. McKinney and Horwod (1952) identified 12 floc-forming organisms. Five were identified as *Bacillus cereus*, *Escherichia intermedium*, *Paracolobactrum aerogenoides*, *Nocardia actinomarpha* and a *Flavobacterium* spp. Friedman and Dugan (1968) added *Zoogloea ramigera* to this list. However limited work has been done to identify floc-forming bacteria from pulp mill effluent.

The other general focus of research in this area had involved identifying bacterial species in pulp mill effluent, responsible for the numerous reactions occurring. Recently, Fulthorpe et al. (1993) identified and characterized a large number of the culturable bacteria in pulp mill effluent. The isolates belonged to phenetic clusters, that were identified as *Acinetobacter* spp., *Acidovorax* spp., *Pseudomonas* spp., *Ancyclobacter aquaticus*, *Klebsiella* spp. and an unidentified cluster of pleomorphic, Gram negative methylotrophs. The first three listed were common in the river water, while the latter two were common in the mill treatment system. The majority of the isolates did not match the known fingerprints in the Biolog GN database, which contains >500 species (Fulthorpe et al., 1993). Some strains were reported to be present in both the river water and the mill treatment system. These were of the *Pseudomonas* group, including *P. stutzeri*. *A. aquaticus* was the largest of the clusters and was isolated on medium containing some clarifier effluent. The unidentified cluster, named cluster C by Fulthorpe et al. (1993), was composed of deep yellow or pale orange pigmented bacteria. They were oxidase and catalase positive, pleomorphic rods of variable thickness, or L- or C-shaped cells. *A. aquaticus* and the unidentified group were able to dechlorinate simple chlorinated aliphatics.

Bacteria with specific degradation abilities have been isolated as well. Mohn (1995) isolated bacterial species from a sequencing batch reactor in a paper mill. The bacterial species were able to grow on the resin acid dehydroabietic acid. Two of these isolates were found to be most closely related to *Sphingomonas yanoikuyae* and *Zoogloea ramigera* (by analysis of the small subunit rRNA partial sequence). Attempts have been made to identify bacteria involved in lignin degradation from a marine pulp mill enrichment culture (González et al., 1996). The majority (8 of 14) of these bacteria are in the α-subclass of *Proteobacteria* with one in the γ-subclass, three in the *Cytophagia-Flavobacterium* group and two were Gram positive.

The Role of Pulp Mill Effluent and the River System in Flocculation

Much uncertainty surrounds the ability of pulp mill effluent to induce floc formation in rivers. Initial field studies by Krishnappan (1996b) revealed that the particle size distribution downstream of the pulp mill effluent discharge at Hinton, Alberta was significantly different than the upstream particle size distribution. This was measured in-situ, using a new submersible laser particle size analyzer. Downstream of the discharge, the concentration of every size of particle had decreased, implying that the particles had settled, and the settling had been a result of flocculation.

In a subsequent laboratory study, Krishnappan (1996b) demonstrated this, using pulp mill effluent from the Weldwood of Canada Ltd. pulp mill in Hinton, Alberta, and a rotating flume. The effluent was introduced into the flume at a concentration similar to what is found in the river, during low flow conditions. The deposition rate was found to be higher than for the control, which had no effluent introduced. As well, the sediment flocs were larger in the test situation. This was the first evidence of PMEICF.

This research sparked another study with effluent from the Northwood Pulp Mill on the Fraser River in Prince George, British Columbia (Evans, 1996). These results showed that aggregation of the particles within the flume may occur, but the effect was slight and would not affect the overall transport of sediment in the Fraser River.

Additional confirmatory studies (Yang and Smith, 1999) using effluent from both the previously mentioned Weldwood site and the Weyerhaeuser pulp mill in Grande Prairie have shown that PMEICF does occur. What remains to be known is whether this is a physical-chemical process or a biological one, and whether the effect is significant in the river systems studied.

MATERIALS AND METHODS

Preliminary SEM Studies

Initial studies involved examination of the floc for the presence of bacteria. A 1:1 mixture of PME and RW from the Weyerhaeuser site was left mixing for up to 52 h. At 4.5, 28 and 52 h, 9 mL was taken from the sample, filtered and examined by SEM. Simultaneously, samples of the PME alone and the RW alone were taken and treated the same way (with no mixing time). Preparation for the SEM followed the standard procedure. More details are given in Joyce (1999). The presence and appearance of the bacteria were noted.

On the Athabasca River in Hinton, samples were taken at various locations upstream and downstream of the discharge, and examined by SEM. An upstream sample was taken, as well as samples at approximately 100 m and 2 km downstream. Again, the presence and appearance of the bacteria were noted.

Heterotrophic Cultures

Heterotrophic plates were performed on samples from the PME, the RW and a mixture of the two. Samples were collected from two sites: Weldwood of Canada Ltd. PME with Athabasca RW and Weyerhaeuser Canada PME with Wapiti RW at the end of August and beginning of December 1998. Details regarding sample acquisition and storage and provided in Joyce (1999). Using a jar test apparatus, 2 L of the final effluent was placed in one jar, 2 L of the corresponding upstream river water in a second and 1 L of effluent and 1 L of river water in a third and covered with tin foil to prevent contamination from airborne microbes. The jar test apparatus was left mixing for up to 4 d at a speed of 10 rpm. Samples were taken from both the liquid phase and the floc material that formed in the jar. Liquid samples were taken after 1 d and after 4 d by a sterile 10 mL pipet. Floc samples were only taken after 4 days to allow for attachment and establishment of any bacteria. Six serial 10-fold dilutions were used for both the floc and the liquid samples. All samples were filtered through a 0.45 μm black membrane filter (Gelman Sciences). Three types of media were used: 1) R2A (Difco), 2) a mixture of standard plate count agar (Difco) with 30% (v/v) PME (abbreviated SPAME) and 3) a medium composed of 50% by volume PME, 50% by volume deionized distilled water and 2 % (w/v) agar (abbreviated PME50). Plates were incubated for up to 5 d, to allow for growth of slow-growing organisms. The bacterial colonies present on the plate, after 48 hours of growth, were evaluated qualitatively. The colour, texture and nature of the edge of the colonies were noted. Experiments were repeated until it was evident which colonies were abundant in the sample, based on appearance only. These colonies were isolated.

Bacterial Isolation and Identification

From the culture plates obtained in section 3.2, individual colonies were isolated by streak plating onto the same medium from which they were obtained (R2A, SPAME or PME50).

Isolates were streaked onto solid medium a minimum of 5 times, to ensure purity. They were then grown in a similar liquid broth and kept frozen in a glycerol solution at -70°C until needed. To resume growth, bacteria were thawed and a small amount of freezing solution was transferred to a test tube containing the corresponding broth. The test tubes were incubated until growth was visible (turbid), from 1 to 3 d. From the test tubes bacteria were sub-cultured onto blood agar plates (BAP), MacConkey (MAC) plates and the original medium from which they were obtained. These were incubated overnight (or longer, if needed). The Gram stain and catalase and oxidase tests were performed as well as inoculation of a triple sugar iron (TSI) slant. Procedures for these tests are given in Joyce (1999). The results of all of these tests enabled classification of the bacteria into a Gram positive (GP), Gram-negative enteric (GN-ENT), Gram negative non-enteric (GN-NENT) or Gram negative fastidious (GN-FAS) category. These categories specify testing conditions necessary for use of the Biolog. They were also classified as good, poor ⊕ or poor growers, based on their growth behaviour on BAP or R2A. Poor ⊕ is a middle category, for isolates that exhibited growth behaviour between good and poor growers.

The MicroPlates were inoculated following the standard procedure outlined by Biolog Inc. They were incubated for the specified amount of time and the “fingerprint”, the pattern of purple and clear wells, was recorded. A purple well indicated a positive reaction (+) and a clear well indicated a negative reaction (-). If the well was faintly purple or only specs of purple were visible then this was indicated as borderline (/). This was entered into the computer program, which returned a top ten list of possible IDs, for the isolate.

Re-Introduction of Isolates into Sterile Effluent

Approximately 11 mL of each pure culture (prepared growth medium and bacterial growth), isolated and identified in section 3.3, was introduced into a mixture of 900 mL of sterile effluent and 900 mL of sterile RW, to determine if the bacteria could induce flocculation. Sterility of the PME and RW was obtained by successive autoclaving. A sterile, 2 L Erlenmeyer flask was used, with mixing provided by a stir bar. The mixing environment was kept as constant as possible. Three controls were used: 1) 900 mL of sterile effluent and 900 mL of sterile river water with no inoculum added, 2) 900 mL of untreated effluent (with its natural fauna) and 900 mL of river water and 3) 900 mL of sterile effluent and 900 mL of sterile river water, inoculated with 11 mL of untreated effluent. The turbidity and particle size distribution were used to measure the extent of flocculation (details given in Joyce, 1999). An initial reading (turbidity and particle size distribution) immediately following set-up was taken, as well as a final reading 4 d later.

As a validation procedure, samples from the mixtures were re-plated, to determine if only the one re-introduced species was present. The sterile control was tested for sterility. Samples were taken from only the liquid phase, diluted and plated onto the corresponding media (R2A or SPAME). If only one species was present on the plate(s), and its colonial morphology resembled that of the inoculant, it was assumed that the mixture was pure and that species was responsible

for any flocculation. If two or more species were visible (i.e. an impure solution), the experiment was repeated.

RESULTS AND DISCUSSION

Preliminary SEM Studies

For the preliminary SEM studies involving the timed experiments and the PME and RW photographs, the samples were taken from the Weyerhaeuser site in June 1998. Unfortunately, no characteristics of the RW were obtained at this time. As it was spring when the sampling was done, the river likely had a high sediment load at this time. As for the PME, the TSS had a concentration of 11 mg/L and was discharged at a rate of 559 kg/d. The pH was 7.6 and the temperature was 25°C. The biochemical oxygen demand over 5 d (BOD_5) was 16 mg/L (Final effluent monitoring results from Weyerhaeuser Canada, Ltd.).

The presence of bacteria in the 1:1 floc was unquestionable (see Figure 2). The SEM revealed numerous colonies in all samples taken at various time intervals. It was hoped to see an increase in EPS over the 52 h, however this was not evident. In all samples the bacteria did appear to be coated with a slime-like layer, determined by a deviation from their usual smooth shape. Any increase in the amount of EPS was undetectable from the SEM photographs alone.

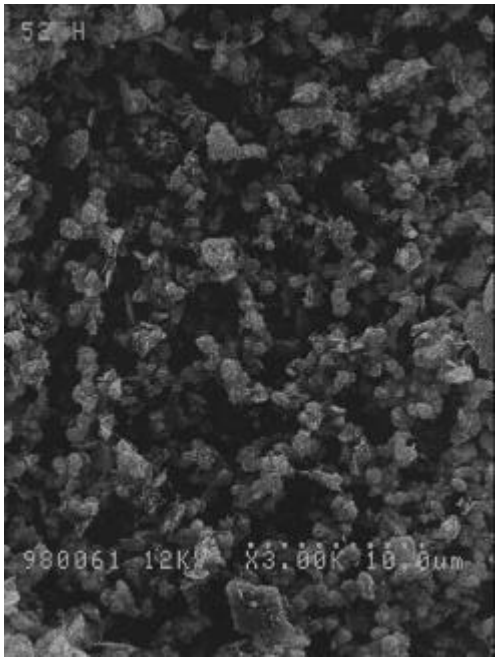
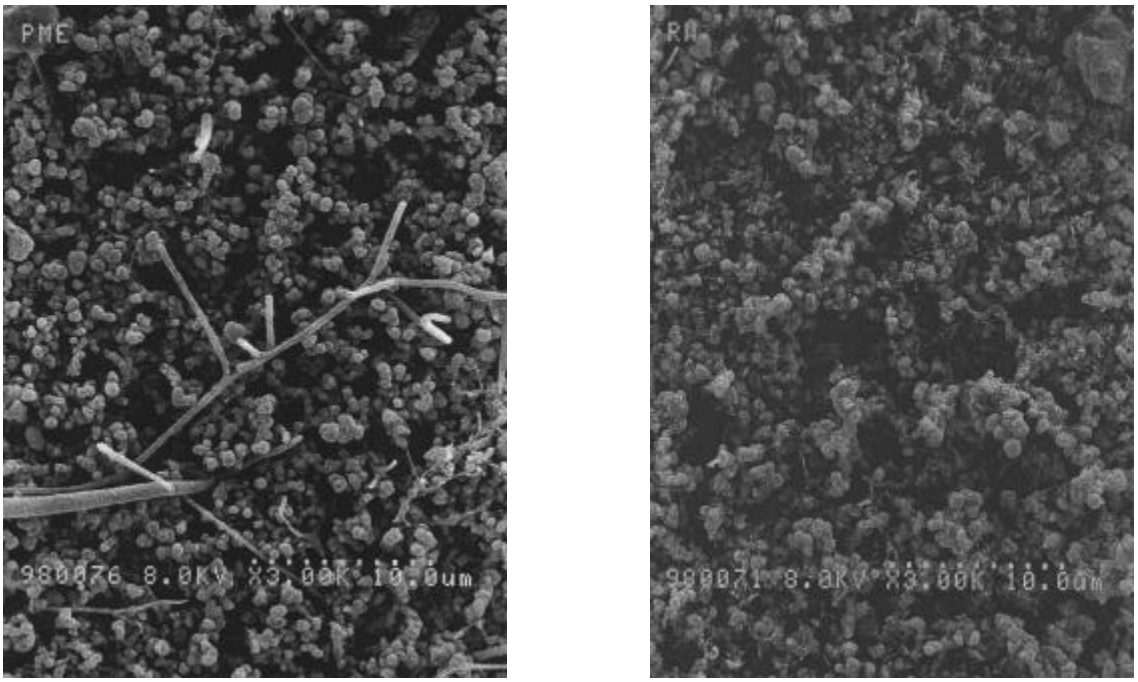


Figure 2: SEM Photograph of Filtered Sample at 52 h

The majority of the bacteria appear to be cocci in shape, although a few spirilla were present. The size ranged from approximately 0.5 μm to 1 μm. Long fibers were also present.

The samples of the PME alone and RW also revealed the presence of bacteria (see Figures 3). Although SEM is not a quantitative technique, it was evident that much higher numbers of bacteria were present in the PME sample than the RW sample. The PME sample had large numbers of cocci present, usually < 1 μm in diameter. Some spiral-shaped bacteria were present as well as some long fibers.

Much time and effort was required to locate bacterial clusters from the upstream RW sample. The majority of the bacteria were cocci and slightly smaller than the bacteria in the PME sample. The river water bacteria appear more coated than the PME bacteria, indicating that EPS production could be a survival mechanism in an oligotrophic environment (i.e. the river).



A B

Figure 3: SEM Photograph of A) PME Sample and B) RW Sample

The remaining two samples were taken at the Weldwood site in August 1998. Again, characteristics of the RW were not measured but data is available for the PME. On the day of sampling, the TSS was 20 mg/L, and discharged at a rate of 2708 kg/d. The pH was 8.0 and the temperature was 35°C (Final effluent monitoring results from Weldwood of Canada Ltd.). These are fairly typical values for the month of August.

The samples taken upstream of the pulp mill discharge at the Weldwood site (Athabasca River) were remarkably similar to the RW sample taken from the Wapiti River. The bacteria were present in clumps; another possible survival mechanism; and they appear coated again.

A common structure was observed in many of the photographs: a centre sphere with star-like appendages. An example has been enlarged in Figure 4. In both cases, it appears the appendages are directed toward bacteria. The centre sphere may actually be a bacterium, however it is difficult to tell. It is possible that the appendages are of bacterial origin, contributing to flocculation.

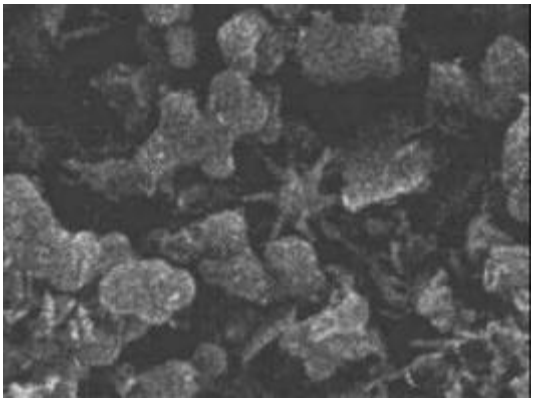


Figure 4: Star-like structure present in SEM photographs
Dimensions of photograph are 5 x 7 μm.

The samples taken at 100 m and ~2 km downstream (see Figure 5) were quite similar, and not too different from the upstream sample. Since the approximate river velocity at the time of sampling was approximately 1.5 m/s (measured in field), a distance of 2 km corresponds to a flow time of about 22 min. Since biological flocculation is a delicate process that occurs after about 1 to 2 d (Muschenheim et al., 1989), the similarity should not be a surprise. Also, the Athabasca River is a large, fast-flowing river, an environment that may not be conducive for bioflocculation.

Heterotrophic Cultures

Much growth appeared on the heterotrophic plates after 48 h. Since filtration and incubation did not occur within 24 hours of initially taking the PME and RW samples from their source, quantitative results are not accurate. Filtration and incubation did occur within 96 h, so it was assumed that the types of bacteria would not change dramatically.

In general, the RW plates had a bacterial community that was less abundant and had less variety than either the PME or 1:1 mixture plates. Each river had a characteristic fauna. On R2A medium, the Athabasca River (at Weldwood) had a group of mostly red colonies that were culturable, while the Wapiti River (at Weyerhaeuser) had few red and mostly white or clear colonies (Figure 6).

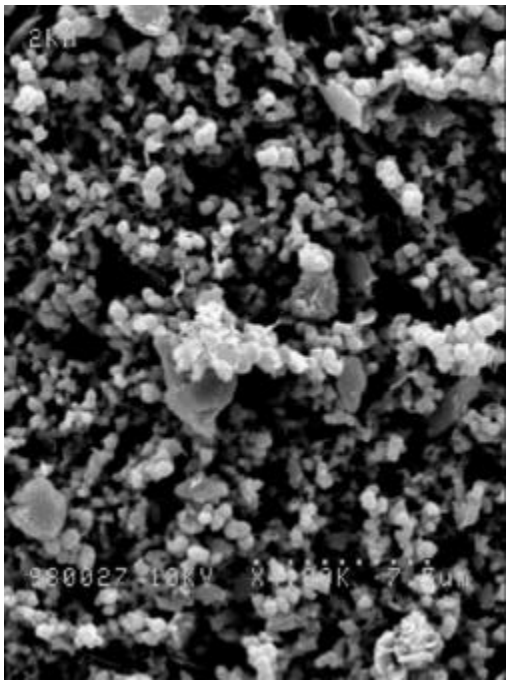
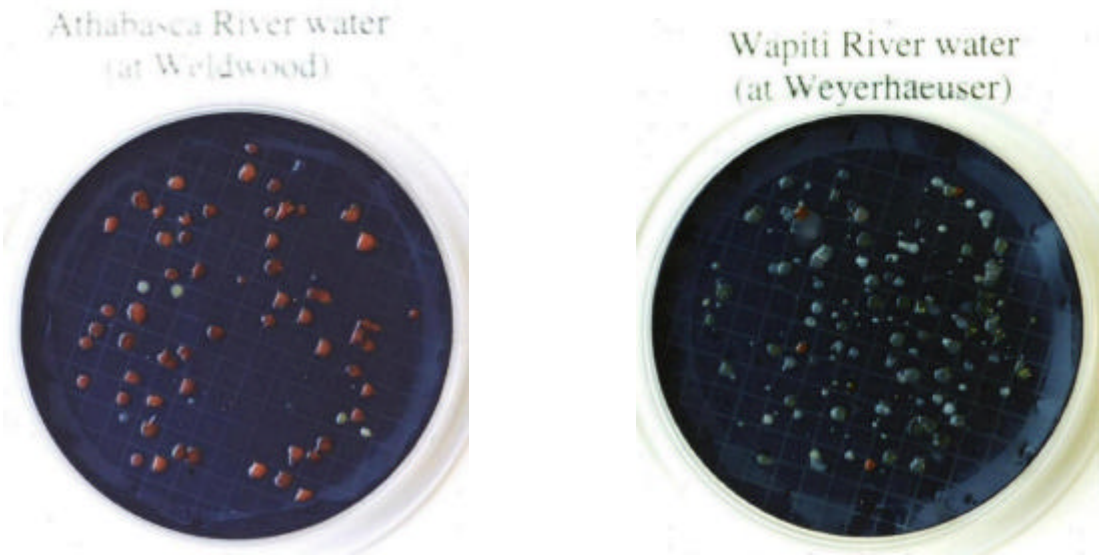


Figure 5: SEM Photograph of Bacteria ~2 km downstream

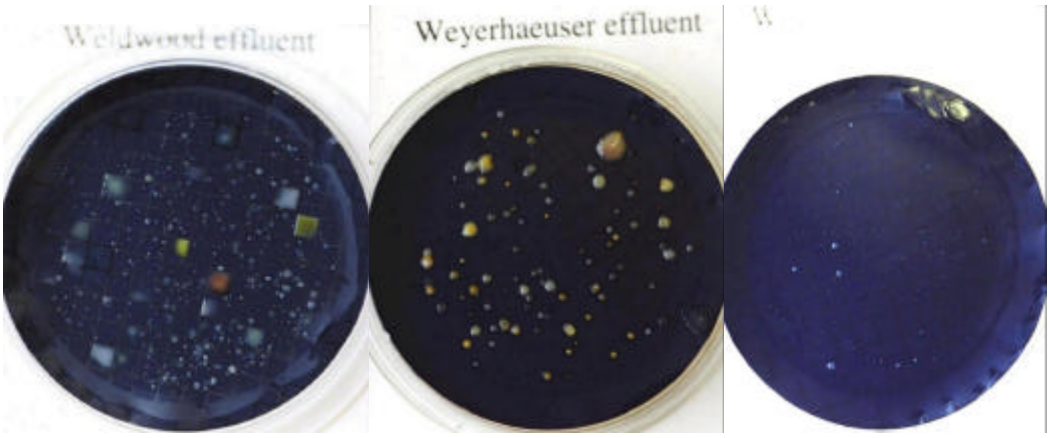


A **B**

Figure 6: Liquid phase bacteria, plated on R2A; A) Athabasca River water and B) Wapiti River water

Figure 7 illustrates the difference between the three media types and the colonies that formed. In each case it is the Weyerhaeuser effluent that is plated but onto different media.

The PME samples contained a much wider variety of bacteria. The Weldwood effluent, plated on R2A produced a fauna of mostly white bacteria, with a few coloured ones in the less diluted sample. More coloured colonies were present in the floc samples than the liquid samples. The Weyerhaeuser plates were quite similar, but generally seemed to have a wider variety of bacteria. In Figure 7 A, it appears as if there are square colonies present. These are actually a result of the membrane filter that is divided into small squares.



A **B** **C**

Figure 7: Comparison of Media when Weyerhaeuser Effluent is plated. A) R2A B) SPAME and C) PME50

The major difference with the SPAME plates was that the bacteria took a longer time to grow. After 48 hours, which was ample time for growth on R2A plates, virtually no colonies were present on the SPAME plates. After 3 days, a wide variety of colonies began to appear, but the fauna differed substantially from the R2A plates. An abundance of orange colonies were present that were not present on the R2A plates.

Slower growing still, were the colonies on the PME50 plates, which produced a much different fauna. After 5 d of growth, the bacterial colonies were tiny and appeared white (but this may be because they were so small, or a result of the medium). A reasonable explanation for the slow growth on these plates is that the nutrients are not ready to be consumed, as they are in the richer, commercial media. The bacteria must break down the molecules into smaller, usable compounds, before any growth occurs. Also, there may be fewer nutrients overall.

The plates from the 1:1 mixture of PME and RW were more similar to the PME plates than the RW plates. The Weldwood sample had mostly small, white colonies, while the Weyerhaeuser sample had a mixture of white and red colonies. This is interesting since it was the RW at Weldwood that had the abundance of red colonies. A large variety of bacteria was present from the flocculated material, from both Weldwood and Weyerhaeuser effluents.

Again, the colonies on the SPAME plates were slower growing. After 5 d, many large, orange and yellow colonies were present that were not present on the R2A plates. These colonies resembled those on the Weyerhaeuser effluent SPAME plates. The growth on the plates from the 1:1 mixture of PME and RW resembled those from the effluent on the PME50 plates, with numerous tiny white colonies present after 5 d of growth.

Bacterial Isolation and Identification

Thirty-six colonies were isolated from the heterotrophic plates (22 on R2A and 18 on SPAME) and re-plated onto the same solid medium. Isolation of bacteria from the PME50 plates was attempted but they died after the second transfer, thus none of the isolates were from the PME50 plates. A list of the isolates and their sources can be found in Appendix A, Table A 1. The cultures had to be frozen at this point, in a glycerol solution, due to time constraints. Not all species survived the freezing; there were 27 that survived. Gram staining, the oxidase test, the catalase test, a TSI test and growth on MAC plates were used to characterize these cultures. The results are summarized in Table A 2 in Appendix A. Observation under a light microscope following the Gram stain indicated that most of the bacteria were bacilli in shape, a few being C- or L- shaped. All were Gram negative, which is characteristic of bacteria in PME (Fulthorpe et al., 1993). The Gram stain was not performed on bacteria that grew well on MAC plates, a medium that is selective for Gram negative, lactose-fermenting bacteria, since it could be assumed that they were Gram negative.

From the data in Table A 2 (Appendix A), all bacteria isolates were classified as GN-NENT, except for two: SF and HC, due to their acid/acid reactions with the TSI slant. These were classified as GN-ENT and were treated separately.

The Biolog GN2-Microplates™ performed fairly well at giving a positive ID, with affirmative identification for 13 of the 27 isolates. The results are shown in Table 1. Detailed information on the identifications was presented in Joyce (1999).

Table 1: Summary of Biolog Identification

ID	Biolog ID	ID	Biolog ID
A	no ID, ressembles D in appearance and biochemical tests	HK	*Aquaspirillum metamorphum*
B	*Roseomonas* genomospecies 6	HL*	no ID, *possibly Lampropedia hylina*
C	no ID, ressembles D in appearance and biochemical tests	SA	*Brevundimonas vesicularis*
D	*Agrobacterium* like-cystic fibrosis	SB	*Flavobacterium marinotypicum*
I*	*Pasteurella pneumotropica*	SC	*Comamonas testosteroni*
HB*	*Achromobacter cholinophagum*	SD**	
HC	no ID, likely *Enterobacter* spp.	SE	no ID, likely *Pseudomonas* spp.
HD	*Acidovorax delafieldii*	SF	no ID, possibly *Aeromonas* spp.
HE	*Acidovorax delafieldii*	SG	*Bordetella bronchiseptica*
HF	*Aquaspirillum metamorphum*	SH	no ID, likely *Pseudomonas* spp.
HG*	no ID	SI*	no ID, possibly *Pasteurella trehalosi*
HH	no ID, but likely belonging to *Pseudomonas* spp.	SJ	no ID, likely *Pseudomonas aurantiaca*, resembles SN
HI*	no ID, possibly *Aquaspirillum putridiconchylium*	SN	no ID, likely *Pseudomonas aurantiaca*, resembles SJ
HJ*	*Pseudomonas echinoides*		

All isolates beginning with s (i.e. SA, SB…) were isolated from the SPAME media
* These isolates required the addition of thioglycollate.
**Even with the addition of thioglycollate, this isolate gave a false positive result

Both HF and HK were identified as *Aquaspirillum metamorphum*. *A. metamorphum* has been characterized as slow-growing, oxidase positive and it will grow on TSI but not on MAC plates. These characteristics do not seem to fit with those reported for HF and HK. HF was slow-growing, but growth did appear on the MAC plate after 48 h. HK did not grow on the MAC plates, but was oxidase negative (Table A 2). However, upon re-testing, this reaction was positive but very slow. In Bergey's Manual (Holt et al., 1994), where the above characteristics were taken from, a reaction is considered positive if 90% or more of the strains react positively. Likewise, a negative reaction refers to 90% or more of the strains reacting negatively. Thus the identification cannot be rejected on this fact alone. *A. metamorphum* has been isolated from a large variety of fresh water sources, especially stagnant areas with a high organic content (Holt, et al., 1994). So it would not be unreasonable to find *A. metamorphum* in pulp mill effluent, or river water. (Both were isolated from a 1:1 mixture, HF from the liquid portion and HK from the floc portion.)

Another *Aquaspirillum* species may have been present in the PME at Weyerhaeuser. HI had no ID, but *A. putridiconchylium* was at the top of the list with a SIM of 0.35. (A SIM of 0.5 is required for positive identification.) *A. putridiconchylium* is characterized as weakly catalase positive or negative, oxidase positive, no growth on MAC plates and growth on TSI slants. All is consistent with HI, except no growth was observed on the TSI slant (Table A 2).

Two species HD and HE were identified as *Acidovorax delafieldii*. A previous study (Fulthorpe et al., 1993) had isolated *Acidovorax* spp. from PME. The *Acidovorax* genus was created in 1990, and described as straight or slightly curved rods, 0.2 to 0.7 μm by 1.0 to 5.0 μm, occurring singly or in short chains. Three species were mentioned in Bergey's Manual (Holt et al., 1994), including *A. delafieldii*. All species are oxidase positive, consistent with HD and HE. However HD and HE were not exactly identical. Both were oxidase positive, catalase positive and did not grow on MAC plates. But HE did not grow on the blood agar plates (BAP) or the TSI, and HD did. So it is unlikely that both isolates are *A. delafieldii*.

Numerous *Pseudomonas* spp. were identified in this study, as in the previous study by Fulthorpe et al. (1993). However, the exact species was rarely able to be determined. HH, SE, SH, SJ and SN likely belong to the genus *Pseudomonas* and HJ was identified as *P. echinoides*. *Pseudomonas* spp. prefer environments where the pH is close to neutral, there is much organic matter, a mesophilic temperature and lots of dissolved oxygen (Holt, et al., 1994), an environment similar to a treatment lagoon. However *P. echinoides* in particular, has been isolated as a laboratory contaminant, so it is impossible to say whether it was actually present in the PME, or is the result of contamination of the sample. SJ and SN could not be identified by the Biolog method, but *P. aurantiaca* was top of the list, with SIMs of 0.33 and 0.41 respectively. However *P. aurantiaca* is oxidase positive and both SN and SJ were oxidase negative (Table A 2).

All *Pseudomonas* spp. are catalase positive and all of the isolates identified as *Pseudomonas* spp. were, except for HJ. Upon re-testing, HJ was found to be very weakly positive. The oxidase reactions are variable. HH had a positive ID, as *Pseudomonas* Group 2 (*Burkholderia*-like). According to Ochi (1995), the *Burkholderia* genus is very similar to the *Pseudomonas* genus, differing in their ribosomal proteins. SE had no ID, but listed only *Pseudomonas* spp. as possible IDs, and SH listed the top three possibilities as *Pseudomonas* spp.

Of all the isolates, only two were classified as enteric: HC and SF, due to their acid/acid reaction with the TSI slant. These were treated under different conditions (35°C, 4 to 6 h incubation) as specified by Biolog. *Escherichia coli* was used as a control since it was available and would give an indication of the success of the system. All three species resulted in an uncertain identification. A SIM of 0.75 is required for enterics, thus the fingerprint must be more precise. For the control, *E. coli*, the correct ID was on top of the list with a SIM of 0.67. The second species on the list was *E. coli* as well, but of a different strain. Therefore this shows that it is not unreasonable to suggest that the species on top of the list may be the correct ID.

The top four species on the list for HC were of the *Enterobacter* genus; the first with a SIM of 0.69. Thus it can be assumed that HC belongs to the *Enterobacter* genus, possibly being *E. asburiae*. *Enterobacter* spp. are straight rods that ferment glucose with the production of acid and a gas. The TSI reaction of HC indicated production of acid (by the yellow colour) but the gas was uncertain. Production of a gas is usually indicated by a gas bubble forming at the bottom of

the test tube that pushes the agar slant upwards. However, if there is a crack in the agar, the gas can escape. This was the case for HC, explaining the "questionable gas production" (?gas) noted in Table A 2. *Enterobacter* spp. are widely distributed in nature, so after analysis it seems logical that HC is an *Enterobacter* spp. with no evidence indicating otherwise. They are also found in freshwater, sewage, soil, plants and feces.

SF also had no positive ID. However all of the top ten species listed were non-enteric. There were insufficient additional GN2-MicroPlates to re-test SF under non-enteric conditions, so the ID remains inconclusive. At the top of the list was an *Aeromonas* spp. with a SIM of 0.39. If SF is actually a non-enteric bacteria, some of the reactions may have an initial lag phase and require the full 16 to 24 hours of incubation. SF fits the description of the *Aeromonas* spp. (oxidase and catalase positive) and *Aeromonas* spp. are found on fresh water, sewage and sludge. Also of interest is that SF was recorded as a late lactose fermentor, indicated by the delayed acid/acid reaction of the TSI slant. *Aeromonas* spp. are usually lactose negative, but a few strains may develop lactose fermenting abilities.

Isolate SC was identified as *Comamonas testosteroni*, with a SIM of 0.71. Second on the list was another *Comamonas* spp., *C. acidovorans*. The *Comamonas* genus was created in 1987 and included the re-classification of *Pseudomonas testosteroni* as *C. testosteroni* (Tamaoka et al., 1987). The *Comamonas* genus consists of straight or slightly curved rods, occurring singly or in pairs. It is oxidase and catalase positive, agreeing with observations of SC. No environment was given in Bergey's Manual (Holt et al., 1994) but since it is similar to *Pseudomonas* spp., it may have a similar environment. Since no observations seem to contradict the conclusion, it is likely that SC is *C. testosteroni*.

SA was identified as *Brevundimonas vesicularis*. This genus was created in 1994 (Segers et al., 1994), from a *Pseudomonas* spp: *P. vesicularis*. The reclassification was based on DNA-rRNA hybridization studies. Since these species were thought to be in the *Pseudomonas* genus for so long, basic characteristics are likely similar: straight or slightly curved rods, catalase positive and prefer neutral environments with a high organic content. SA is catalase positive, with a bacilli shape, so the observations do not contradict with the description. Therefore, SA may be *B. vesicularis*.

HB was positively identified as *Achromobacter cholinophagum*. In Bacterial Systematics (Logan, 1994), *Achromobacter* is mentioned as one of seven possible names for the *Acinetobacter* genus. According to this source, many independent isolations of the genus have led to the wide variety of names. *Acinetobacter* refers to the oxidase negative strains and *Achromobacter* refers to the oxidase positive strains (Logan, 1994). HB is oxidase positive, so at least this is consistent with the literature. *Acinetobacter* spp. has already been isolated from pulp mill effluent (Fulthorpe et al., 1993) so it is not surprising at this result. Members of the *Acinetobacter* genus are oxidase negative and catalase positive, and HB is oxidase positive and catalase negative. Both of *Acinetobacter* and *Achromobacter* spp. are glucose non-fermenting, gram negative bacteria.

SB was identified as *Flavobacterium marinotypicum*. *Flavobacterium* spp. are rods with parallel sides and rounded ends, oxidase positive, catalase positive and are usually orange-yellow pigmented. They are widely distributed in soil and water (Holt et al., 1994). SB was yellow in colour (on SPAME) but oxidase negative. The second possibility was listed as *Achromobacter cholinophagum*. The *Achromobacter* genus is considered to include the oxidase positive strains of the *Acinetobacter* genus. This is still not consistent with SB. The oxidase reaction was re-tested and may be considered weakly positive, so this particular strain may be anomalous. If this is the case, SB could be *F. marinotypicum*.

Recently six new species were proposed in the genus *Microbacterium* (Takeuchi and Hatano, 1998). Based on DNA-DNA hybridization, *F. marinotypicum* was transferred to this genus as *M. maritypicum*. comb. nov. The Biolog Gram negative database was last revised in December 1998, so it may not contain this change. According to Takeuchi and Hatano (1998), *M. maritypicum* are yellow-pigmented, Gram positive rods. *Flavobacterium* are Gram negative, but Takeuchi and Hatano (1998) performed an analysis of the cell wall components and based on this, must have reclassified it as Gram positive. SB consists of yellow-pigmented rods, so this correlates well.

The remaining isolates and their Biolog IDs were cross-referenced with appropriate sources (Holt, et al., 1994; Logan, 1994; Rihs et al., 1993; Tang et al., 1998). For the remaining isolates, the two didn't seem to match. In some cases, the characteristics of the isolate didn't match those of the species identified. In other cases, the two sets of characteristics seemed to match, but the preferred environment was very different from PME or RW. Thus, the remaining isolates remain unidentified.

From the analysis, it was concluded that some species were likely present in the effluent and/or river water. They were *Aquaspirillum* spp., *Acidovorax* sp., *Pseudomonas* spp., *Enterobacter* spp., *Comamonas* spp., *Brevundimonas* spp., *Flavobacterium* spp. and *Achromobacter* spp.

Re-Introduction of Isolates into Sterile Effluent

The particle size distributions revealed that autoclaving the effluent greatly increases the number of tiny particles. Since this seemed to be the only efficient method of obtaining sterility, the experiment had to be continued despite this difference. The sterile control would be valuable in determining if flocculation did occur, since flocculation should decrease this number of tiny particles, despite the increase in individual bacteria and possibly increase the number of larger particles. The control inoculated with a sample of pulp mill effluent should give a realistic idea of the total extent of flocculation that is achievable under these conditions. The purpose of the untreated control was to indicate how the test conditions differed from the actual conditions. The untreated control does not realistically simulate the river environment, however its physical and chemical composition would be more representative of the actual solutions.

The sterile control exhibited very little flocculation. There was a slight decrease in the particles sized 2 to 4 μm (indicated by $C/C_0 < 1$ in Figure 8). This can be attributed to any physical/chemical flocculation that might have occurred. However, it must be remembered that this is not indicative of the actual extent of physical/chemical flocculation since autoclaving the samples likely altered the physical/chemical properties of the effluent and river water. There was also a slight decrease in turbidity (see Table 2), likely due to physical/chemical flocculation as well. The larger size particles were not used for comparison, as their size would be more affected by the mixing speed, which was difficult to keep constant.

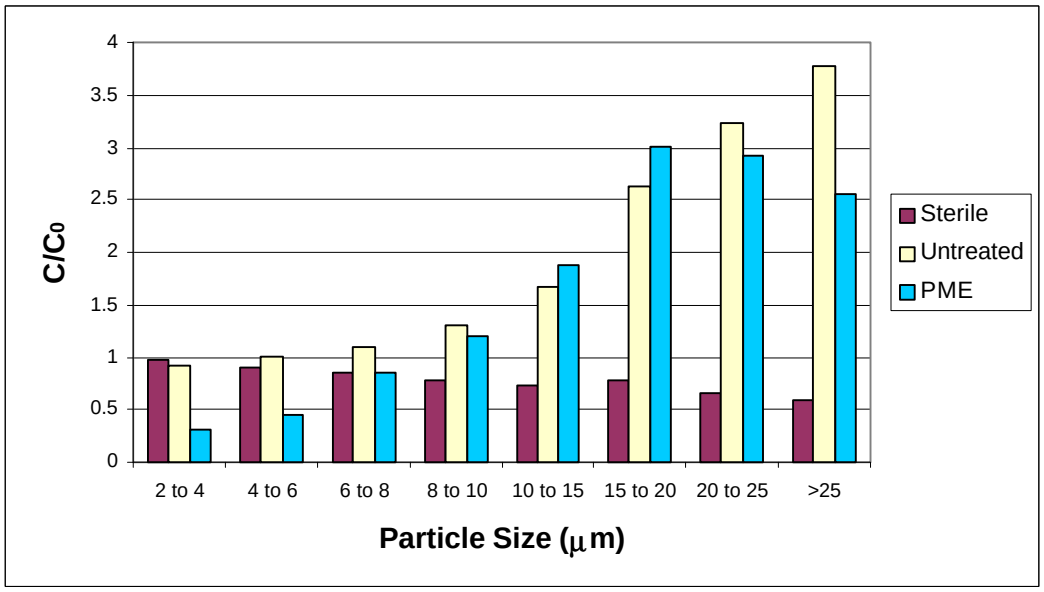


Figure 8: Comparison of the Three Controls

C_0 is the initial concentration of particles size 2 to 4 μm and C is the concentration after 4 d

The accumulation of sediment on the bottom of the flask was chosen to be ignored. It should not change the concentration of particles sized 2 to 4 μm in solution, which were used as a measure of flocculation in this experiment. The accumulation of sediment, by itself, cannot be a measure of flocculation, as some of it may have been a result of type 1 settling. A more thorough approach would be to look at a mass balance equation of the suspended and settled solids, to determine their fate.

Six PME controls were tested, three with May effluent and three with July effluent. As a first remark, after 4 d, the PME control did not produce a dramatic difference in either the turbidity or particle size distribution. After 6 d, the change was visible. A possible explanation for the delay is that the inoculated bacteria in the effluent may not have been as healthy or as abundant as those introduced by test tubes, resulting in a longer lag phase. In addition, there would have been non-flocculating bacteria present, that could have clouded the solution by increasing the number of smaller particles. Despite this observation, the 4-d analysis results will be used in statistical analysis for comparison purposes and are shown in Figure 8.

The untreated control, a 1:1 mixture of non-sterile PME and RW, was considerably different. The initial turbidity reading and particle size distribution were much lower than either of the other two controls; obviously an effect from not being autoclaved. The overall change is not as dramatic as one might expect; a difference in turbidity is barely detectable. This may be because the effluent remained in the cold room for some time before testing and some flocculation might have already occurred.

For the individual isolate runs, a combination of samples was used, from both May and July. A preliminary run for each isolate was completed with the May samples. The results were compared to the sterile control. Based on the turbidity readings, only three isolates produced a clear effluent: HC, SC and SF. The change in the turbidity for all isolates is shown in

Table **2**, with the above-mentioned isolates in bold. Since the initial turbidity varied slightly, a normalized value, T/T_0, is given for comparison purposes. (The actual turbidity readings are in Appendix B, Table B 1.) Using a decrease in the number of smallest measurable particles present (2 to 4 μm in diameter) as another indicator of flocculation, four more species demonstrated flocculation abilities as well: HH, HJ, SG and HL. Since the initial number of particles varied with each run, these were normalized as well (C/C_0, where C is the number of particles size 2 to 4 μm in 1 mL). Figure 9 compares all isolates and controls based on their C/C_0 value for particles 2 to 4 μm in diameter. From this comparison, the flocculating species are easily determined.

Table 2: Change in Turbidities for all Species and Controls

Species	T/T_0	Species	T/T_0	Species	T/T_0	Species	T/T_0
A	1.48	HD	0.78	HK	1.13	**SF**	**-0.02**
B	0.88	HE	0.78	HL	1.22	SG	0.77
C	1.11	HF	0.89	SA	1.20	SH	0.81
D	1.54	HG	0.80	SB	0.41	SI	0.86
I	0.60	HH	0.21	**SC**	**-0.03**	SJ	1.14
HB	0.21	HI	0.72	SD	0.88	SN	0.99
HC	**0.05**	HJ	0.26	SE	0.65		
Sterile (ave)	0.93	PME (ave)	0.69	Untreated (ave)	0.99		

From this analysis, species were grouped into one of three categories: 1) enhanced flocculation abilities noted, 2) mild flocculation abilities noted and 3) no flocculation abilities noted. The third category included all the species that actually increased the number of small particles. These were HD, C, D, HK and SD. Seven species were classified as capable of enhanced flocculation: HH, HC, SF, SC, HJ, SG and HL. The remaining species showed a slight decrease in the concentration of small particles, but it was not vastly different from the sterile control.

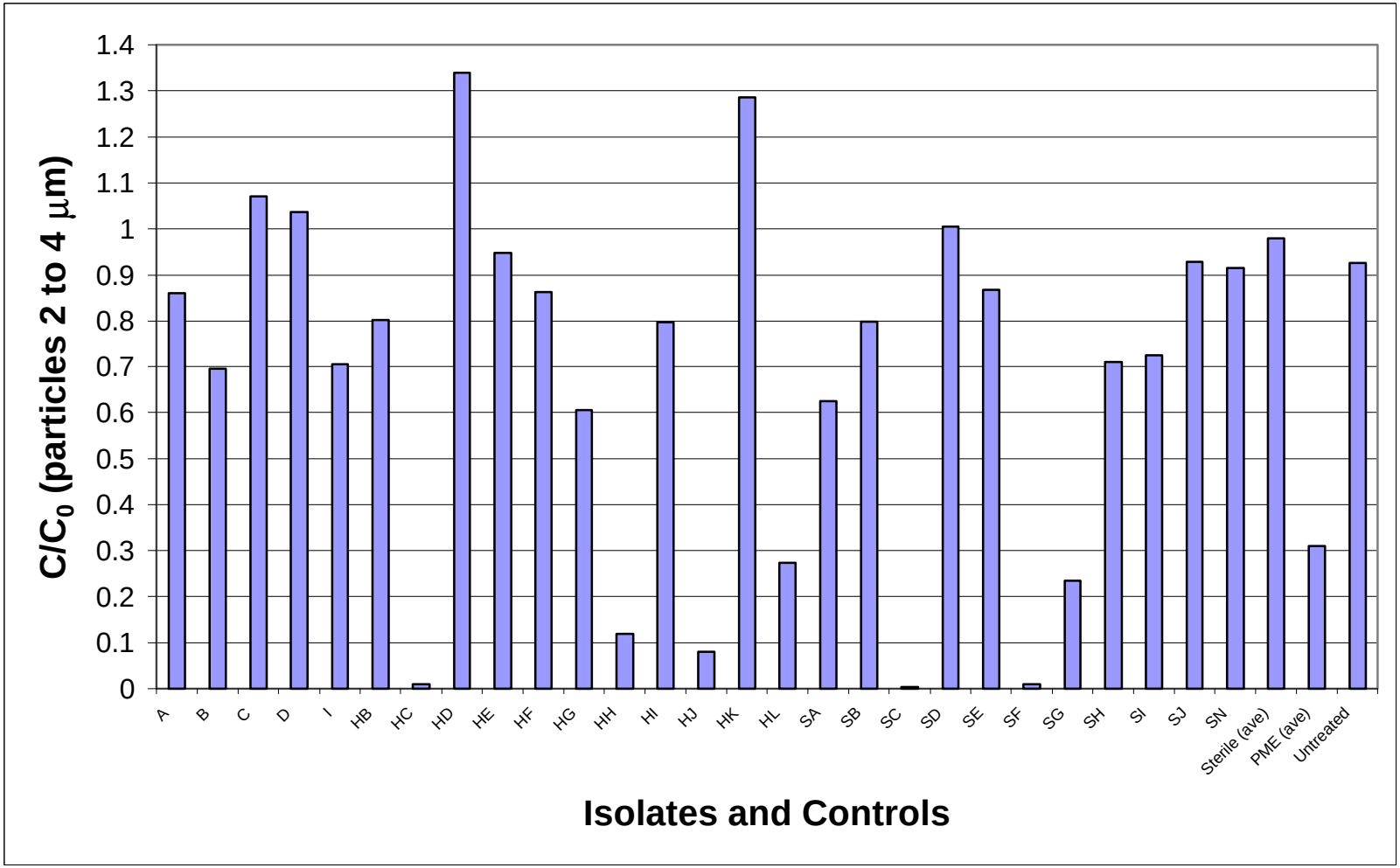


Figure 9: Comparison of all Isolates and Controls based on C/C_0 (2 to 4 μm)

C_0 is the initial concentration of particles sized 2 to 4 μm in diameter and C is the concentration after 4 d.

For statistical purposes, the experiment for the isolates demonstrating enhanced flocculation abilities were repeated three more times, with effluent and RW samples taken in different months, May and July. These results varied greatly. The initial runs had been performed with an effluent grab sample taken at the end of May 1999. This sample was all used, so a new sample had to be ordered. The new RW was much clearer and contained less sediment (a visual observation only, as no standard tests were performed for comparison). The initial readings for both turbidity and particle size distributions were much lower. However, the results were normalized for comparison purposes, and all values were averaged together. A two-mean t-test was used to determine if the results differed significantly from the mean. A summary of the statistics can be found in Appendix B, Tables B 2, B 3 and B 4.

Examining the C/C_0 parameter (2 to 4 μm), no isolates produced a significant response. This is because there was much variation among the runs and the standard deviation was very high. For all isolates classified as possessing enhanced flocculation abilities (with the exception of HL), the lowest C/C_0 value was obtained with the May effluent. Perhaps it was the high sediment load, present in the river water in May that aided in flocculation. The increased amount of sediment may have been larger in size and settled out independent of any reactions (i.e. type 1 settling). Or the increased sediment could have provided more substratum available for bacteria attachment, promoting bacterial growth, development and potentially the excretion of polymers and flocculation (i.e. type 2 settling). It appears that the flocculation abilities of the bacteria are largely dependent on their environment. It has already been demonstrated that the production of EPS by the bacteria is dependent on their environment (Flemming, 1993; White, 1995) and EPS has been shown to aid in flocculation (Frieman and Dugan, 1968). Thus it seems logical that flocculation abilities depend on the environment of the bacteria. The nature of the pulp mill effluent will change with the type of wood being processed, the process being used and the treatment of the effluent. The nature of the river water is more dependent on the seasons, with a high sediment load during the spring run-off and much lower during the rest of the year. Also, storage of the sample in the cold room will likely change physical/chemical properties, explaining the differences noted for one particular sample.

Considering the T/T_0 parameter, only HH produced a significant response. However only two runs were used for this since the other two runs were impure. (They could not be repeated due to time constraints.) It is likely that if additional runs were performed, the results would vary, as they did for the other isolates, producing a higher standard deviation and render the HH results insignificant.

A similar explanation as that used for the C/C_0 values can be used to explain these. The flocculation abilities of the bacteria seem to be largely dependent on the nature of their environment, which includes the nature of the pulp mill effluent and the river water.

From the flocculation experiments, it is obvious that some of the isolates possess flocculating abilities. However this is largely dependent on their physical, chemical and biological environment. The effect of their physical/chemical environment has been discussed.

Considering their biological environment, it is possible that some of the species may interact together. Each isolate was tested individually, however interactions between species may be significant. These may help or hinder the flocculation process. The PME control partially examined this. It was grouped with those isolates with C/C_0 (2 to 4 μm) < 0.4, however it did not produce the lowest C/C_0 value. There may not have been sufficient substratum for the increased numbers of bacteria, so more remained in solution. If this was the case, it would have increased the final concentration of particles 2 to 4 μm in diameter. Other complex interactions could also be occurring.

Those isolates that demonstrated enhanced flocculation abilities were HC, HH, HJ, HL, SC, SF and SG. Combining these results with the results of the Biolog identification, a hypothesis of the actual flocculating species can be made. HH and HJ were both positively identified as belonging to the *Pseudomonas* genus, HJ as *P. echinoides* and HH and *Pseudomonas* Group 2 (*Burkholderia*-like). If this is true, then SE, SH, SJ and SN may exhibit flocculation abilites as well, as they were also identified as belonging to the *Pseudomonas* genus. Flocculation abilities may be species specific, however the C/C_0 and T/T_0 results of the only run for these isolates (SE, SH, SJ and SN) is encompassed by the range of all runs of HH and HJ. HC and SF were both classified a enteric, HC likely belonging to the *Enterobacter* genus. SF remains unidentified, as all possible IDs were non-enteric species. It could possibly be an *Aeromonas* species. SG was positively identified as *Bordetella bronchiseptica*, however it is doubtful whether this pathogen would be abundant in pulp mill effluent or river water. SC was positively identified as *Comamonas testosteroni* and HL remains unidentified (possibly a *Pseudomonas* species).

LIMITATIONS

This study was performed in a laboratory setting and many limitations exist in its extrapolation to a field setting. However this stage is required for better understanding of phenomenon in a controlled setting. Firstly, the bacteria species were isolated on prepared media. It is of general consensus among researchers that the majority of environmental bacteria do not grow on these prepared media. So the abundant species that were isolated, may not actually be abundant in the effluent or river water.

The flocculation study used autoclaved effluent and river water, since it was the most convenient method of obtaining sterile effluent. However, this likely changed the physical and chemical properties of both. Bacteria that usually thrive in PME may not do so in the autoclaved effluent, and conversely, some species may prefer the autoclaved PME and RW combination. Also it has been shown that the production of EPS is dependent on the environment (Flemming, 1993; White, 1995). Bacteria that usually produce large amount of EPS may not do so in this autoclaved solution and the reverse may be true as well. Since the production of EPS could aid in the flocculating abilities of bacteria, this has a significant impact on the results of this study.

Finally, the 2-L Erlenmeyer flasks used in this experiment, with mixing provided by a stir bar, does not represent the turbulent mixing environment found in a river. The dilution effect present in the river is not simulated in the laboratory at all. The laboratory setting would produce more of a concentration effect, as the bacteria will increase in numbers and no dilution is present.

CONCLUSIONS

Bacteria were isolated from a sample of PME, the corresponding RW and a 1:1 mixture of the two. Identification of these isolates was conducted with the Biolog Identification System. Although there seemed to be many inconsistencies with the identification by the Biolog database, some conclusions could be made. Species that were likely present in the mixture were *Aquaspirillum* spp., *Acidovorax* spp., *Pseudomonas* spp., *Enterobacter* spp., *Comamonas* spp., *Brevundimonas* spp., *Flavobacterium* spp. and *Achromobacter* spp. *Pastuerella* spp and *Aeromonas* may have been present as well.

Through a series of flocculation experiments, it was shown that some of these isolates were capable of enhanced flocculation, in the laboratory setting used. However, this characteristic was not consistent and seemed to vary greatly from experiment to experiment. Based on the Biolog identification, the species possibly involved in enhanced flocculation were *Pseudomonas* spp., *Enterobacter* spp and *Comamonas testosteroni*. Some unidentified isolates were also involved.

With this base of knowledge, further work is recommended in this area. In the laboratory setting, a more detailed identification procedure is needed, so the relevant species can be determined with more certainty. This study focussed on mesophilic aerobes, however the river temperature may be as low as 0°C in the winter, with an ice cover, so a study of the psychrotrophs present would be worthwhile. At the same time, attention should be made to any seasonal variation. It was evident in this study that differences existed between the May and July effluents. Sampling should be conducted over an entire year to have a full picture of the annual trends. Characteristics of the effluent should be monitored, since over an entire year they are likely to change as well. Further work could also explore the production of EPS: how it changes with time, its abundance in non-flocculating solutions as compared to flocculating solutions and its role in the flocculation process.

All the previously mentioned studies can be completed at the laboratory scale. A final step would involve the determination of what actually happens in the river. Many limitations exist for this study, as were discussed, and to extrapolate the laboratory results into the river setting would greatly increase the understanding surrounding this phenomenon. The rivers involved may be oligotrophic environments, which could produce “stickier” bacteria. This may affect their flocculation abilities. The mixing environment may be too turbulent for the formation of fragile, biological flocs. Or, 4 d after discharge, the time required for biological flocculation to occur, the effluent and its components may be too dilute for any type of reaction.

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APPENDIX 1 - ISOLATES

Table A 1: Isolates, their Sources and a Description

Isolate	Source	Description (on corresponding medium)
A	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
B	WW 1:1 floc	bright, yellow colonies, very slow-growing, well-defined, ~1 mm diameter after 2 d growth
C	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
D	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
E	1:1 liquid	white
F	WW PME liquid	white
G	WW PME liquid	pale yellow
H	PME liquid	bright yellow
I	WW RW	pink colonies, ~5 mm diameter after 2 d growth, flat colonies
HA	1:1 liquid	bright yellow
HB	1:1 liquid	yellow, ~2 mm diameter after 2 d growth
HC	1:1 liquid	off-white, ~3 mm diameter after 1 d growth, variable size
HD	1:1 liquid	off-white-clear, ~1 cm after 2 d growth, flat but raised in middle,
HE	1:1 liquid	creamy white, various sizes, <1 mm-2 mm diameter after 2 d growth
HF	1:1 liquid	white, tiny colonies, <1 mm diameter after 2 d growth
HG	1:1 liquid	white, ~2 mm after 2 d growth
HH	RW	white, ~ 1mm diameter after 1 d growth
HI	PME liquid	tiny, yellow colonies, ~1 mm diameter after 2 d growth, slow-growing
HJ	1:1 floc	white/clear colonies, ~1 diameter after 2 d growth, slow-growing
HK	1:1 floc	tiny, white-clear colonies, <1 mm diameter after 2 d growth
HL	1:1 floc	clear colonies, ~1 mm diameter after 3 d growth, irregular edges, flat
HM	1:1 floc	white-clear colonies
SA	RW	pale yellow colonies, ~2 mm diameter after 2 d growth
SB	RW	yellow, 2-3 mm diameter 2 d growth, round with peak in middle
SC	RW	white-clear, oval-shaped, ~3 x ~2 mm after 1 d growth, lots of mucous
SC	RW	white-clear, oval-shaped, ~3 x ~2 mm after 1 d growth, lots of mucous
SD	RW	yellow, tiny, <1 mm diameter after 1 d growth, irregular size, shape
Isolate	Source	Description (on corresponding medium)
SE	1:1 liquid	yellowish colonies, ~1 mm diameter after 1 d growth, peak in middle
SF	1:1 floc	yellow-beige, peaked in middle, ~5 mm diameter after 1 d growth, lot of mucous
SG	1:1 floc	yellowish, ~1 mm diameter after 1 d growth, not perfectly round
SH	1:1 floc	creamy-yellow, v. spread out, jagged edges, ~ 5 mm after 1 d growth, lots of mucous
SI	1:1 liquid	pale yellow, 1-2 mm diameter after 3 d growth, smooth edges, peaked in middle
SJ	1:1 liquid	yellow-orange, very slow-growing, ~1 diameter after 3 d growth, convex
SK	1:1 liquid	white
SL	1:1 liquid	orange
SM	1:1 liquid	off-white
SN	1:1 liquid	yellow-orange, very slow-growing, ~1 diameter after 3 d growth, convex

Table A 2: Summary of Characterization Studies

ID	BAP	MAC	Gram stain and Appearance	oxidase	catalase	TSI	Comments	Growth ability
A	g (48), tiny, grey	ng (72)	g-b, curly	+	+	NC/NC		poor
B	ng (96)	ng	g-b, curly	-	-	ng (48)	grows well on R2A	poor ⊕
C	grey, opaque (fi, 48)	ng	g-b, curved, pleo, var. thickness	+	+	NC/NC		poor
D	grey, opaque (fi, 48)	ng	same as above	+	+	NC/NC (72)		poor
E	ng	ng						
F	ng	ng						
G	ng	ng						
I	small, opaque	ng (72)	g-b, in chains, mostly 2's	-	+	ng (72)	grows well on R2A	poor ⊕
HB	g (48), tiny	ng (72)	g-b, curly, chains	+	-	ng (48)	grows well on R2A	poor ⊕
HC	colif, i	pk (48)		-	+	A/A	late lactose fermenter	good
HD	small, grey	ng (72)	g-b, mostly single	+	+	Alk/NC		poor ⊕
HE	ng (72)	ng (72)	g-b, tiny, single rods	+	+	ng (48)	grows on R2A	good
HF	tiny, grey	wt (48)	g-b, single, some chains	+	+	NC/NC		poor ⊕
HG	tiny (48)	nh (48)	g-b, mostly single	+	+	Alk/NC (48)		poor
HH	tiny, grey	ng (24)	g-b, thin rods	+	+	Alk/A		good
HI	ng (48)	ng (48)	g-b, curly	+	-	ng (48)	grows on R2A	poor ⊕
HJ	small grey (48)	ng (48)	g-b, tiny, single rods	+	-	Alk/alk		poor ⊕
HK	tiny (24)	ng (24)	g-b, thin rods	-	+	Alk/NC (48)		poor
HL	tiny (24)	ng (24)	g-b, single rods	+	-	Alk/NC (48)		poor
SA	tiny, opaque (48)	wt (48)	g-b, single	+	+	Alk/NC		poor ⊕
SB	tiny, opaque (24)	ng (24)	g-b, single, short chains	-	+	NC/NC (48)		poor ⊕
SC	colif, I	wt		+	+	Alk/NC		good
SD	ng (72)	ng (72)	g-c	-	+	ng (48)	grows on R2A	good
SE	colif, h	wt		+	+	Alk/NC		good
SF	colif, h	wt		+	+	A/A	late lactose fermenter	good
SG	colif, i	wt		+	+	Alk/NC		good
SH	colif, h (48)	wt	g-b, single rods	+	+	Alk/NC		poor ⊕

Table A 3: Summary of Characterization Studies (continued)

ID	BAP	MAC	Gram stain and Appearance	oxidase	catalase	TSI	Comments	Growth ability
SI	tiny (24)	ng (24)	g-b, tiny rods	+	+	NC/NC (48)		poor
SJ	ng (48)	ng (48)	g-b, in clumps	-	+	ng (96)	grows on R2A	poor ⊕
SK	ng	ng						
SL	ng	ng						
SN	ng (48)	ng (48)	g-c, single	-	+	ng (72)	grows on R2A	poor ⊕

Abbreviations: g, growth (number of hours given in brackets); ng, no growth; g-b, Gram negative bacilli; g-c, Gram negative cocci; pleo, pleomorphic (variable length); h, hemolytic; i, indifferent; fi, fine indifferent; colif, coliform; wt, white; pk, pink, A, acid; Alk, Alkaline; NC, No change.

APPENDIX 2 – FLOCCULATION STUDIES

Table B 1: Change in Turbidities for all Species and Controls

Species	Initial Turbidity (NTU)	Final Turbidity (NTU)	T/T_0	Species	Initial Turbidity (NTU)	Final Turbidity (NTU)	T/T_0
A	160	237	1.48	HK	160	180	1.13
B	196	173	0.88	HL	222	270	1.22
C	153	170	1.11	SA	196	236	1.20
D	156	241	1.54	SB	217	89	0.41
I	281	168	0.60	**SC**	**130**	**-4**	**-0.03**
HB	282	59	0.21	SD	219	192	0.88
HC	**200**	**10**	**0.05**	SE	222	144	0.65
HD	293	230	0.78	**SF**	**165**	**-4**	**-0.02**
HE	223	175	0.78	SG	169	130	0.77
HF	200	178	0.89	SH	203	165	0.81
HG	223	179	0.80	SI	223	192	0.86
HH	190	40	0.21	SJ	204	233	1.14
HI	225	163	0.72	SN	218	215	0.99
HJ	223	57	0.26	sterile (ave)	-	-	0.93
PME (ave)	-	-	0.69	Untreated (ave)	-	-	0.99

Table B 2: Average and Standard Deviation of C/C_0 and T/T_0 for the Repeated runs of the Selected Isolates

Isolate	Number of runs	Mean (C/C_0 for 2 to 4 μm)	Std. deviation	Mean (T/T_0)	Std. deviation
HC	4	0.770	0.51	0.803	0.51
HH	2	0.453	0.47	0.465	0.36
HJ	4	0.871	0.55	0.784	0.40
HL	3	0.506	0.61	1.21	0.042
SC	4	0.931	0.69	0.668	0.48
SF	4	0.561	0.38	0.493	0.37
SG	4	0.849	0.52	0.919	0.12
Sterile	4	0.979	0.12	0.928	0.32

Note: C_0 is the initial concentration of particles sized 2 to 4 μm, C is the concentration of particles sized 2 to 4 μm after 4 d, T_0 is the initial turbidity reading and T is the turbidity reading after 4 d.

Table B 3: Statistics Summary for Comparison to Sterile Standard, based on C/C_0 (2 to 4 μm), for the Data in Table B1.

Isolate	Pooled variance estimate (s^2)	$t_{calculated}$	Degrees of freedom	$t_{critical}$ (95%)
HC	0.13725	0.80	6	2.447
HH	0.066025	2.37	4	2.776
HJ	0.15845	0.38	6	2.447
HL	0.15748	1.56	5	2.571
SC	0.24525	0.14	6	2.447
SF	0.0794	2.10	6	2.447
SG	0.1424	0.487	6	2.447

Table B 4: Statistics Summary for Comparison to Sterile Standard, based on T/T_0, for the Data in Table B1.

Isolate	Pooled variance estimate (s^2)	$t_{calculated}$	Degrees of freedom	$t_{critical}$ (95%)
HC	0.131	0.49	6	2.447
HH	0.0332	2.94	4	2.776
HJ	0.12998	0.56	6	2.447
HL	0.0621456	1.48	5	2.571
SC	0.116	1.08	6	2.447
SF	0.0690	2.34	6	2.447
SG	0.0584	0.053	6	2.447