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Coliform Bacteria and Nitrogen Fixation in Pulp and Paper Mill Effluent Treatment Systems

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The majority of pulp and paper mills now biotreat their combined effluents using activated sludge. On the assumption that their wood-based effluents have negligible fixed N, and that activated-sludge microorganisms will not fix significant N, these mills routinely spend large amounts adding ammonia or urea to their aeration tanks (bioreactors) to permit normal biomass growth. N_2 fixation in seven Eastern Canadian pulp and paper mill effluent treatment systems was analyzed using acetylene reduction assays, quantitative nitrogenase (*nifH*) gene probing, and bacterial isolations. In situ N_2 fixation was undetectable in all seven bioreactors but was present in six associated primary clarifiers. One primary clarifier was studied in greater detail. Approximately 50% of all culturable cells in the clarifier contained *nifH*, of which >90% were *Klebsiella* strains. All primary-clarifier coliform bacteria growing on MacConkey agar were identified as klebsiellas, and all those probed contained *nifH*. In contrast, analysis of 48 random coliform isolates from other mill water system locations showed that only 24 (50%) possessed the *nifH* gene, and only 13 (27%) showed inducible N_2-fixing activity. Thus, all the pulp and paper mill primary clarifiers tested appeared to be sites of active N_2 fixation (0.87 to 4.90 mg of N liter^{-1} day^{-1}) and a microbial community strongly biased toward this activity. This may also explain why coliform bacteria, especially klebsiellas, are indigenous in pulp and paper mill water systems.

Good performance of activated-sludge biotreatment systems depends on the concentrations of several key nutrients, including bioavailable "fixed" nitrogen. Unlike municipal sewage, pulp and paper mill wastewaters are typically rich in carbohydrates but poor in fixed nitrogen, due to the high C/N ratio typical of wood. Therefore, careful dosing of the raw effluent with fixed N and P prior to biotreatment is essential. This is a substantial expense. A ratio of 100:5:1 for bioavailable carbon (C) to nitrogen (N) to phosphorus (P) in the feed (raw effluent) is usually recommended. Excess fixed N is also undesirable, as it may result in fish-toxic free ammonia reaching the receiving waters and eutrophication (19). Successive nitrification and denitrification of excess N may also cause "rising sludge" in the secondary clarifier due to entrapment of N_2 gas bubbles, resulting in biosolid losses to the receiving waters (16).

The biological fixation of N_2 usually requires the following conditions: (i) readily available carbohydrates as an energy source (5, 12), (ii) low fixed-nitrogen concentrations (5), and (iii) absence or very low concentrations of dissolved oxygen (DO) (5, 12–14). N_2 fixation has been reported from some pulp and paper mill aerated lagoons (4, 8), which may have regions supplying these key conditions. The N_2 fixation in these lagoons was shown to be capable of supplying the entire N requirements of the system, corresponding to more than 600 kg of N day^{-1} (8). In contrast, N_2 fixation is unlikely to occur in activated-sludge aeration tanks or secondary clarifiers of pulp and paper mills because (i) the DO level typical of activated-sludge operation is too high (usually 1 to 3 mg of O_2 liter^{-1}) for nitrogenase to function (8, 21), (ii) nutrient nitrogen has already been added as NH_3 or urea, repressing N_2 fixation (18), and (iii) levels of free sugars are usually very low because of the intense competition for carbon and energy sources by the activated-sludge biomass (16).

A mill primary clarifier is an unmixed settlement tank or basin, continuously removing suspended wood fibers and particles, and fillers or coaters such as clay, starch, and calcium carbonate from combined, pH-adjusted raw mill effluents. Primary clarifiers usually meet the three criteria given above for the growth and activity of N_2-fixing microorganisms. Since their function is to mechanically settle particles and fibers, there is no aeration (low DO), and as supplemental fixed N has not been added yet in most mills, the C/N ratio is likely to be high. Previous work on the ecology of coliform bacteria in several pulp and paper mill effluent systems found permanent coliform populations, with *Klebsiella* strains predominant, in all primary clarifiers examined (11a). Indeed, the presence of N_2-fixing members of the *Enterobacteriaceae*, including *Klebsiella* sp. strains, in pulp and paper mill water systems has long been known (2, 4, 6, 8, 9, 15, 18, 20–22).

In this study we used both acetylene reduction (AR) (an indirect measurement of nitrogenase activity) and functional gene probing (for the *nifH* nitrogenase gene) to detect N_2-fixing bacteria and N_2 fixation in seven primary-clarifier and bioreactor ecosystems. To measure the abundance and composition of N_2 fixers in one particular clarifier, we enumerated the culturable bacterial community, the coliform bacteria, and we tested a subsample of the resulting colonies for the *nifH* gene by colony hybridization. We then identified a subset of those organisms that tested positive using biochemical characterization. This is the first report demonstrating N_2-fixing activity by both cultured isolates and active in situ populations in pulp and paper mill primary clarifiers. The results also indicate the predominance of the genus *Klebsiella* in these nitrogen-fixing communities.

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TABLE 1. Characteristics of the mill treatment systems studied

Mill	Process: product[a]	Furnish[b] (% recycled fibers added)	Biotreatment[c]	Vol of primary clarifier[d] (m^3)	Vol of effluent treated (m^3 day^{-1})
A	TMP: newsprint, GWSpec	SW (15)	A/S	NA[e]	68,800
B-1	GWSpec, KR papers, Bl. KR pulp	SW	SBR	3,175	5,760
B-2	GWSpec, KR papers, Bl. KR pulp	SW	SBR	11,065	43,200
C	Bl. KR.: fine papers	HW (15)	A/S	40,000	120,000
D	TMP: newsprint	SW	SBR		
E	TMP: newsprint, GWSpec	SW (15)	A/S	11,000	61,000
F	Sulfite: newsprint	SW (16)	A/S	10,000	40,000
G	Bl. KR: market pulp	HW	A/S	20,000	71,000

[a] Thermomechanical (TMP), Kraft (KR), and sulfite pulping processes were used. GWSpec, groundwood specialties; Bl. KR pulp, bleached kraft pulp.
[b] SW, softwood; HW, hardwood.
[c] A/S, aerobic activated sludge; SBR, sequencing batch reactor.
[d] Approximate volume. Mill B has two primary clarifiers; B-1 treats debarking process wastewaters. The clarified B-1 effluent and the other effluent streams from the mill are treated in clarifier B-2. Mill D has no separate primary clarifier.
[e] NA, not available.

MATERIALS AND METHODS

Sampling procedures. Seven Eastern Canadian pulp and paper mills were chosen in order to include a broad range of pulp and papermaking processes and biotreatment system designs (Table 1). Over 17 months, samples from the primary clarifiers and from the aeration tank biomass and liquor were collected. The primary-clarifier samples were collected at several depths using a 1.2-liter Kemmerer autosampler. Grab samples were transported in 500-ml polypropylene bottles on ice. AR assays and bacterial cultures were done within 4 and 24 h of sampling, respectively.

Total counts, coliform isolates, and pure culture controls. Total bacterial counts used triplicate serial dilutions and plating of samples onto 1/5-strength Trypticase soy agar (TSA) plates (Difco) supplemented with 1% (wt/vol) NaCl and 1% (wt/vol) glucose. Coliform counts used triplicate dilution plating on both MacConkey and eosin methylene blue agar (Difco). All plates were incubated at 37°C for 18 to 24 h. Total and fecal coliforms (TC and FC) were selected for an enumerated as described previously (11a) using the most-probable-number (MPN) methods recommended in *Standard Methods for the Examination of Water and Wastewater*, sections 9221B and 9221E (1). Isolations were carried out as described previously (11a) using MacConkey agar. Strains were identified using the standard API 20E biochemical test procedure (Biomérieux).

Control bacterial strains are listed in Table 2. Prior to DNA extraction, *Azoarcus tolulyticus* was grown aerobically in modified R2A broth (ATCC culture medium 2120). All other pure (control) cultures (Table 2) were grown aerobically in 10 ml of nutrient broth (Difco).

Flask cultures. Coliform colonies isolated from MacConkey agar were transferred successively onto "high-N" glucose-thioglycolate agar plates and into 10-ml tubes of the corresponding "N-free" broth containing, per liter, 10.0 g of D-glucose, 6.3 g of K_2HPO_4, 1.7 g of NaH_2PO_4, 0.1 g of $MgSO_4$, 0.008 g of Na_2MoO_4, 0.008 g of ferric citrate, 0.5 g of Na-thioglycolate, and 0.001 g of resazurin. The high-N broth also contained 0.2 g of yeast extract $liter^{-1}$ and 0.5 g of Casamino Acids $liter^{-1}$. In situ N_2 fixation activity was measured by the AR assay as described by Knowles et al. (18). Assays were initiated by the removal of 5 ml of N_2 and the addition of 5 ml of acetylene (final concentration, 10% [vol/vol]), followed by incubation at room temperature with shaking (120 rpm). To assay N_2 fixation by isolates, 1 ml of a "preenrichment" culture growing on 9 ml of fresh "N-free" medium was pipetted into 50-ml flasks, and the assay was performed as for clarifier samples (above). The AR assay was used essentially as described previously (18).

***nifH* gene probe.** From the published sequence of the Mo-Fe nitrogenase genes of *Azotobacter chroococcum* (10, 17), we designed primers to amplify a portion of the *nifH* gene from a purified plasmid (pER4) containing *nifHDK* from *A. chroococcum*. The two primers, designated NIFN3 (5′-ATCCACCAC CACTCAGAACC) and NIFC3 (5′-ATAACGCCGAACTCCATCAG) amplified a 780-bp region of *nifH* (sequence positions 285 to 1064; GenBank accession no. M20568). To obtain labeled probe, we amplified the probe sequence by PCR, incorporating digoxigenin (DIG)-labeled dUTP according to the manufacturer's protocol (PCR DIG probe synthesis kit; Roche Molecular Biochemicals). The amplified probe was purified after 1% agarose gel electrophoresis (with an agarose gel DNA extraction kit from Roche Molecular Biochemicals).

DNA extraction. DNA was extracted from pure cultures using a sodium dodecyl sulfate (SDS)-based method (3). Well-mixed primary clarifier grab samples (500 ml) were blended in a Waring blender (1 min), then filtered through cheesecloth and centrifuged (10,000 × *g*, 10 min, 4°C). The pellet was resuspended in 1 to 3 ml of lysing solution containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM EDTA (pH 8.0), and 2% (wt/vol) SDS. One milliliter of resuspended cells was placed in a 2-ml screw-cap tube containing 1 g of zirconia-silica beads (diameter, 0.1 mm) (Biospec Products, Bartlesville, Okla.) and 1 ml of phenol-chloroform (1:1, vol/vol). The cells were lysed by 5 min in a beadbeater and then centrifuged (2,000 × *g*, 15 min), and the aqueous DNA-containing supernatant was repeatedly phenol-chloroform and chloroform extracted. Phase separation was carried out at 10,000 × *g* for 15 min. The purified DNA was treated with RNase, precipitated with isopropanol, washed with 70% ethanol, dried, and resuspended in 200 to 500 μl of Tris-EDTA (TE) buffer. Purified DNA was quantified on a 1% agarose gel using an AlphaImager 1200 with AlphaEase software (Alpha Innotech).

ATP extraction. ATP was extracted from primary-clarifier and activated-sludge samples using a trichloroacetic acid-EDTA-based ATP release method and was measured using commercial luciferin-luciferase assay reagents (FL-AAM, FL-AAS, and FL-AAB; Sigma). Light production was measured using an LKB model 1250 luminometer.

Dot blot hybridization. Purified DNA (1 μg) was alkali transferred to Hybond N^+ membranes (Amersham) using a dot blot manifold. After UV cross-linking (Stratagene Stratalinker), membranes were rinsed in 0.5 M Tris-HCl (pH 7.0) to ensure removal of alkali, because the probe's DIG label is alkali labile. The hybridization and wash procedures were modified from earlier methods (25, 27). The membranes were prehybridized in roller bottles (42°C, 4 h) in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.7]–5× Denhardt's solution–0.2 mg of salmon sperm DNA ml^{-1}. Denhardt's solution is, per liter, 0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, and 0.2 g of bovine serum albumin. Hybridization was carried out for 14 to 16 h at 42°C in 10 ml of prehybridization solution with 10% (wt/vol) dextran sulfate and 10 ng of denatured *nifH* probe ml^{-1}. The membrane was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (15 min at room temperature [RT]), 0.5× SSC–0.1% SDS (15 min at RT), 0.1× SSC–0.1% SDS (15 min at RT), and 0.1× SSC–1% SDS (15 min at 42°C). Detection was carried out by using nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as a colorimetric substrate for alkaline phosphatase according to the manufacturer's protocols (Roche Molecular Biochemicals).

Colony hybridization. Random colonies from both TSA and MacConkey plates were chosen for hybridization analysis. These isolates and pure culture controls were transferred to TSA plates. After 24 h at 37°C, colonies were transferred to Hybond N^+ membranes (Amersham) and lysed (23). Hybridization with *nifH*, washes, and detection were done as for the dot blots.

TABLE 2. Pure culture DNA used as controls in this study

Strain	Source or reference
Klebsiella pneumoniae[a]	
KPN3 (primary clarifier isolate)	This study
KPG1 (activated sludge isolate)	This study
KPG2 (primary clarifier isolate)	This study
KPG3 (primary clarifier isolate)	This study
Escherichia coli K12 (MM294A)	11
Azoarcus tolulyticus	28
Pseudomonas stutzeri JM300	7
Pseudomonas stutzeri Zobell (ATCC 14405)	B. Ward
Sinorhizobium meliloti RCR 2011 (SU47)	26
Rhodobacter sphaeroides f. sp. *denitrificans*	24
Paracoccus denitrificans (ATCC 17749)	R. Ye
Corynebacterium nephridii (ATCC 11425)	R. Ye
Pseudomonas sp. strain G-179	27

[a] Based on API 20E identification.

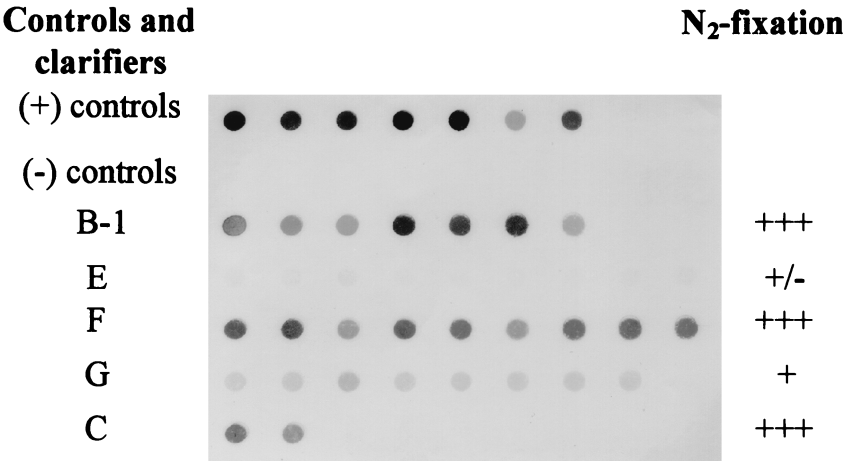

FIG. 1. Dot blot hybridization of 1-μg DNA samples to the *nifH* gene probe. Positive controls (from left to right) are primary-clarifier isolates KPN3, KPG2, and KPG3, *A. tolulyticus*, *Pseudomonas stutzeri* JM300, *Sinorhizobium meliloti*, and *Rhodobacter sphaeroides*. Negative controls (from left to right): KPG1 (activated sludge isolate), *Escherichia coli* K-12, *P. stutzeri* Zobell, *Paracoccus denitrificans*, *Corynebacterium nephridii*, *Pseudomonas* sp. strain G-179, no DNA (NaOH) control. Seven primary-clarifier samples (see Table 1 for mill treatment system characteristics) were taken from mill B-1, nine from mill E, nine from mill F, eight from mill G, and two from mill C. Samples were taken at various depths at different locations across the clarifier diameter. The actual biochemical N_2-fixing activity indicated, in milligrams of N fixed per liter per day, is an average of in situ activity measured at all sampling points (±, 0 to 0.5; +, 0.5 to 1.0; ++, 1.0 to 1.5; +++, 1.5 to 2.0).

RESULTS AND DISCUSSION

Occurrence of N_2 fixation in mill aeration tanks (bioreactors). Existing reports of N_2 fixation in pulp and paper mill treatment systems are limited to aerated lagoons (4, 8) and laboratory-scale activated-sludge systems held at abnormally low DO and fixed-N levels (11a, 20; D. J. Gapes, N. M. Frost, T. A. Clark, P. H. Dare, R. G. Hunter, and A. H. Slade, presented at the 6th IAWQ Symposium on Forest Industry Wastewaters, Tampere, Finland, 6 to 10 June, 1999). In this study, full-scale on-line activated-sludge systems and primary clarifiers were examined for N_2-fixing activity. The biomass and suspended liquor from the activated-sludge aeration tanks (bioreactors) of the seven mills described in Table 1 were sampled, and AR assays were run within 4 h. While the *nifH* gene was detected in the samples, none showed any N_2 fixation activity (data not shown).

Occurrence of N_2 fixation in mill primary clarifiers. In contrast, both the *nifH* gene and active N_2 fixation were present in all of the six mill primary clarifiers tested (Fig. 1; Table 3). Since all dots contained the same total DNA, the *nifH* probe signal amplitudes (Fig. 1) indicate the relative proportion of N_2-fixing genes in the total population, not the absolute population size. The amount of *nifH* gene probe that hybridized to these primary-clarifier DNA samples varied from very low to as much as that seen using control DNA from pure cultures of known N_2-fixing organisms. This suggests that a high proportion of the total microbial consortium in the primary clarifier carries the *nifH* gene. We also observed a general correlation between the *nifH* probe signal intensity and the average in situ nitrogen-fixing activity (Table 3) calculated from all sampling sites we tested in each clarifier (Fig. 1). The maximum N_2 fixation rates observed in the six primary clarifiers ranged between 0.87 and 4.90 mg of N liter^{-1} day^{-1} (Table 3), comparable to the maximum rates previously reported from an aerated lagoon system (5.5 mg of N liter^{-1} day^{-1}) (8).

The primary clarifiers, which are responsible for mechanical settling, had, as expected, only a fraction of the biomass density of the corresponding activated-sludge-containing aeration tanks. At a depth of 4 m, the mill C primary clarifier had 167 ng of ATP ml^{-1}, while the mill C aeration tank contained 3,400 to 5,200 ng of ATP ml^{-1}.

Monitoring the mill C primary clarifier for 6 months indicated that N_2 fixation probably occurs continually (Table 3). Because the primary clarifiers are unmixed and thus highly heterogeneous, the amount of N_2 fixation (and biomass density) varies greatly with the sampling site selected (Table 3). To see if there was net N_2 fixation in the primary clarifier, the fixed N concentrations of the mill C primary-clarifier input and output were compared (three sets of grab samples collected on three different days). The input effluent had on average a total Kjeldahl nitrogen concentration (TKN) of 3.8 ± 0.2 mg/liter, and the clarified output effluent had an average TKN of 4.8 ± 0.7 mg/liter, indicating a substantial increase in fixed N (about 120 kg, based on 120,000 m^3 of raw effluent clarified day^{-1}), despite the decrease in total C accompanying the removal of settleable solids by the primary clarifier. Because of the fluctuations in the operation of each mill and the large variations between mills, these can only be considered preliminary data

TABLE 3. Maximum observed N_2 fixation rates in mill primary clarifiers examined

Mill	Date (day-mo-yr)	Sampling depth (m)	DO level (mg liter^{-1})	N_2 fixation rate (mg of N liter^{-1} day^{-1})
B-1	19-07-99	3.0	0.0	4.26
	29-11-99	4.0	2.9	0.87
B-2	19-07-99	4.5	0.0	1.30
	29-11-99	5.0	3.8	ND[a]
C	6-07-99	4.0	0.0	4.70
	22-11-99	4.0	0.0	3.03
	7-06-99	5.0	NT[b]	4.71
	4-10-99	5.0	0.0	1.85
	24-05-99	6.0	NT	2.19
E	29-11-99	5.0	2.7	ND
	3-08-99	7.5	0.0	1.00
F	16-08-99	5.5	0.0	4.90
G	16-08-99	5.5	0.0	0.89

[a] ND, no activity detected.
[b] NT, not tested.

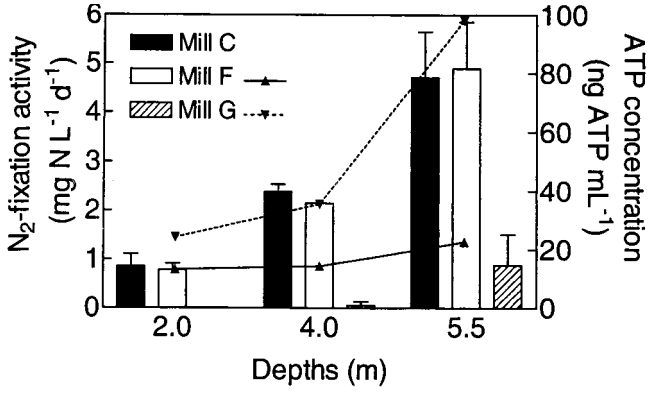

FIG. 2. Influence of depth on N_2 fixation activities (bars) and ATP concentrations (lines) of three primary clarifiers. Sampling was performed at the middle of the clarifier radius except for mill G (3 m from the center). N_2 fixation is reported as means of duplicate samples ± standard deviations. ATP concentrations were not measured for the mill C primary clarifier.

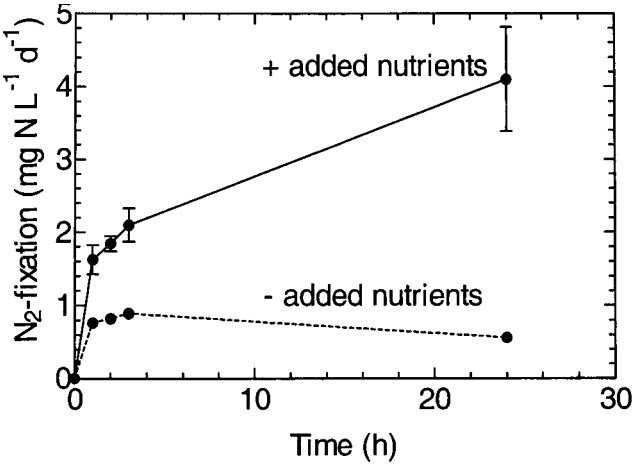

FIG. 3. Effects of added nutrients on N_2 fixation in the mill C primary clarifier. The sample was obtained at a 5-m depth. Glucose and sodium acetate (final concentrations, 0.05% [wt/vol]) were added. Values are means of duplicate samples ± standard deviations. Error bars that are not visible are smaller than the symbols.

for mill C, and applicable only to mill C. Since the mill measures the C-to-N ratio of the 107 tonnes of primary biosolids removed day^{-1} by this clarifier to be 650:1 to 800:1, it can be calculated that the settled biosolids decreased the output TKN by about 1.0 mg/liter, meaning that another 120 kg of N is produced day^{-1} in the primary clarifier of mill C. The structure and bioavailability of the N entering and exiting the primary clarifiers are unknown. After passage into the mill C aeration tank, most of this N appears to become part of the activated sludge, as the mill adds ammonia and ammonium pyrophosphate to a C/N/P ratio of 100:4.0 to 4.5:0.8. If all this deliberately added N is taken up, the activated (secondary) sludge should have a C/N ratio between 20:1 and 25:1. In fact, the measured ratio is typically 9:1, strongly suggesting that a large amount of the N arriving from the primary clarifier is captured in the activated sludge. Conversely, the fact that the primary-clarifier diazotrophs fix N in the presence of an influent TKN of 3.8 mg/liter suggests that the N originating in the mill is not readily available to them.

In most of the six primary clarifiers, higher N_2 fixation rates were observed at greater sampling depths (Table 3 and Fig. 2). There are three likely reasons. Firstly, ATP measurements showed higher biomass concentrations in deeper samples, which contain more solids (Fig. 2). Secondly, because nitrogenase is O_2 sensitive, the low oxygen tension observed in deeper samples may allow more N_2 fixation per unit of biomass (Table 3). Thirdly, the settling of wood particulates in primary clarifiers may also improve carbohydrate availability at greater depths, and readily available carbohydrates are essential for N_2 fixation, since a large amount of ATP is required.

The rate of N_2 fixation was shown to be nutrient limited in the mill C primary clarifier, at least in bottom sludge samples, where in situ fixation was highest (Fig. 3). Added glucose and acetate increased the N_2-fixing activity more than sevenfold over 24 h. Although the *Enterobacteriaceae*, particularly *Klebsiella pneumoniae*, are metabolically versatile (20), it is likely that the glucose was the major driver of increased N_2 fixation. Bruce and Clark (4) observed that the AR rate of *K. pneumoniae* isolates growing on Kraft mill effluent in an aerated stabilization basin (lagoon) was greatly stimulated by 5 g of glucose $liter^{-1}$. Thus, the nutrient limitation that exists in the aeration tank biomass (16) also occurs in the primary clarifier, at least for N_2 fixation.

Importance of *Klebsiella* in the primary-clarifier N_2-fixing community. Previously, we demonstrated that coliform bacteria, predominantly *Klebsiella* strains, were numerous in several of the primary clarifiers examined (11a). To determine the dominant taxa in the primary-clarifier N_2-fixing community, nonselective (dilute TSA) agar plate counts were done. From the same plates 188 colonies, 94 each from samples A and B (Table 4), were screened by colony hybridization. Of these isolates, 94 (50%) were *nifH* positive. Identification of 20 randomly selected *nifH* positive isolates revealed that 18 (90%) were klebsiellas. Thus, N_2-fixing coliform bacteria belonging to the genus *Klebsiella* account for 45% of the total bacterial population culturable on a nonselective medium. The API 20E system we used for colony characterization classed all the isolated klebsiellas as *K. pneumoniae*. However, previous work revealed that a high proportion of the *K. pneumoniae* isolates found in pulp and paper mill ecosystems could be more appropriately referred to as *Klebsiella terrigena* or *Klebsiella planticola* due to their inability to produce gas from lactose at 44.5°C (11a). The high numbers and proportions of *Klebsiella* strains growing on the nonselective TSA plates indicate a higher proportion of coliform bacteria in primary-clarifier samples than was estimated by the TSA/MacConkey plate count

TABLE 4. Occurrence of *nifH*-containing members among total culturable bacteria and coliform bacteria from a selected primary clarifier, and predominance of *Klebsiella* spp. among N_2-fixing bacteria isolated

Sample[a]	Total bacteria			Coliform bacteria		
	Count[b] (10^7 CFU ml^{-1} ± SEM)	No. hybridizing to *nifH*/no. tested		Count[c] (10^6 CFU ml^{-1} ± SEM)	No. hybridizing to *nifH*/no. tested	
		All bacteria	*K. pneumoniae*[d]		All bacteria	*K. pneumoniae*[d]
A	3.2 ± 0.2	51/94	8/10	3.5 ± 0.1	92/94	10/10
B	1.2 ± 0.1	43/94	10/10	1.2 ± 0.1	94/94	10/10
Avg	2.2 ± 0.2	50%	90%	2.3 ± 0.2	98%	100%

[a] Primary clarifier samples (A and B) correspond to mill C bottom sludges obtained from the center and the middle radius of the settlement basin, respectively.
[b] On triplicate TSA plates.
[c] On triplicate MacConkey plates.
[d] Identified by the API 20E test.

TABLE 5. Distribution of coliforms, N_2 fixation, and the *nifH* gene in several pulp and paper mill water systems

Species[a]	No. of isolates		
	Total tested	With the *nifH* gene[b]	With AR activity
Klebsiella pneumoniae	32	18	9
Enterobacter cloacae	8	2	2
Citrobacter freundii	4	2	2
Enterobacter agglomerans	1	0	0
Escherichia coli	3	2	0
Total	48	24	13

[a] Based on API 20E results for isolates from various treatment system sources (effluent feeds, clarifiers, and aeration tanks) from seven mills.
[b] Determined by colony hybridization.

ratios (Table 4). Thus, a combination of gene probing, API identification, and classical nonselective growth techniques shows that, at least in this primary-clarifier system, the MacConkey agar MPN counts seriously underestimate the total number of coliform bacteria present.

Colony hybridization of 188 MacConkey isolates showed that 98% of the coliform population in the primary clarifier carried *nifH*. Of the 186 *nifH*-bearing isolates, 20 were randomly chosen, and all (20 of 20) were identified as *Klebsiella* strains (Table 4). The seven mill systems surveyed here were all shown previously to support numerous coliform bacteria (11a), but this is the first report demonstrating that primary clarifiers have large populations of bacteria actively fixing nitrogen in situ, and even larger populations with the genetic potential for N_2 fixation. Unlike previous results from a lagoon treatment system (4), our findings indicate that N_2-fixing *Klebsiella* strains are the major diazotrophs in pulp and paper mill water and treatment systems.

Does the presence of the *nifH* gene correlate with measured N_2-fixing activity? A total of 48 coliform isolates from several mills were tested for the *nifH* gene and their ability to reduce acetylene (Table 5). Of the 48 isolates, 13 (27%) showed AR activity while 24 (50%) possessed the *nifH* gene. No isolate testing negative for *nifH* could reduce acetylene, and all strains reducing acetylene tested positive for *nifH*. Comparable active-to-potential N_2 fixation ratios were observed with *K. pneumoniae* isolates, with 9 of 32 (28%) and 18 of 32 (56%) displaying N_2-fixing activity and the presence of the *nifH* gene, respectively. The percentage of N_2-fixing *K. pneumoniae* isolates among isolates from the seven mill water systems (28%) is consistent with the findings of a previous report in which 32% of klebsiella from several ecosystems, including pulp mills, possessed N_2-fixing ability (18). The low proportion of *nifH*-positive *K. pneumoniae* isolates (56%) seen in Table 5 compared with the proportion in Table 4 (100%) is presumably due to the fact that the coliform bacteria for which results are shown in Table 5 were isolated from many places in the seven pulp mill water systems, not just from primary clarifiers. Other evidence for the selection of N_2 fixers by the primary-clarifier environment is the increase in the proportion of *nifH*-positive *K. pneumoniae* isolates, from 56% (18 of 32) for isolates from all sampling locations (Table 5) to 83% (15 of 18) when only *K. pneumoniae* isolates from primary-clarifier samples are considered. In the primary clarifier, N_2-fixing klebsiellas likely possess selective growth advantages over non-N_2-fixing bacteria, such as the following: (i) raw mill process effluents typically have high C/N ratios (2, 11a, 12, 18); (ii) the temperatures in primary clarifiers can rise to 40°C, also selecting for klebsiellas, since >90% of the strains from these seven paper mill water systems are thermotolerant (grow at 44.5°C) (11a); and (iii) the very low DO levels observed in primary clarifiers (Table 3) presumably select for facultative bacteria, such as coliforms (4, 21).

Conclusions. (i) The combination of in situ and isolate *nifH* gene probing and AR assays with classical microbial enumeration, isolation, and identification was very effective in elucidating the nature, magnitude, and microbiology of N_2 fixation in mill water systems.

(ii) *Klebsiella* strains actively fixing N_2 are major components of pulp mill primary-clarifier microbial communities. This probably explains why coliform bacteria are indigenous to pulp and paper mill water systems (2, 11a).

(iii) N_2 fixation by klebsiellas and related coliform bacteria is commonplace and continuous in these primary clarifiers and may contribute significantly to the fixed N required by the activated-sludge biomass. It should also increase the value of the combined dewatered sludges as fertilizers.

(iv) Every pulp and paper mill biotreatment system is unique. The relative importance of the N_2 fixed by coliforms in each primary clarifier, and whether it can be substantially increased by adjustments to the clarifier's mode of operation, remains to be shown.

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REFERENCES

1. **American Public Health Association.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
2. **Archibald, F. S.** 2000. The presence of coliform bacteria in Canadian pulp and paper mill water systems—a cause for concern? Water Qual. Res. J. Can. **35:**1–22.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
4. **Bruce, M. E., and T. A. Clark.** 1994. *Klebsiella* and nitrogen fixation in pulp and paper mill effluents and treatment systems. Appita J. **47:**231–237.
5. **Cannon, M., S. Hill, E. Kavanaugh, and F. Cannon.** 1985. A molecular genetic study of *nif* expression in *Klebsiella pneumoniae* at the level of transcription, translation and nitrogenase activity. Mol. Gen. Genet. **198:** 198–206.
6. **Caplenas, N. R., M. S. Kanarek, and A. P. Dufour.** 1981. Source and extent of *Klebsiella pneumoniae* in the paper industry. Appl. Environ. Microbiol. **42:** 779–785.
7. **Carlson, C. A., and J. L. Ingraham.** 1983. Comparison of denitrification by *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrificans*. Appl. Environ. Microbiol. **45:**1247–1253.
8. **Clark, T. A., P. H. Dare, and M. E. Bruce.** 1997. Nitrogen fixation in an aerated stabilization basin treating bleached kraft mill wastewater. Water Environ. Res. **69:**1039–1046.
9. **Duncan, D. W., and W. E. Razzell.** 1972. *Klebsiella* biotypes among coliforms isolated from forest environments and farm produce. Appl. Microbiol. **24:** 933–938.
10. **Fallik, E., Y.-K. Chan, and R. Robson.** 1991. Detection of alternative nitrogenases in aerobic gram-negative nitrogen-fixing bacteria. J. Bacteriol. **173:** 365–371.
11. **Finn, T. M., B. Kunkel, G. D. Vos, and X. E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.

11a. **Gauthier, F., and F. S. Archibald.** The ecology of "fecal indicator" bacteria commonly found in pulp and paper mill water systems. Water Res., in press.

12. **Hardy, R. W. F., R. C. Burns, and R. D. Holsten.** 1973. Applications of the

acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. **5:**47–81.
13. **Hill, S.** 1988. How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. **54:**111–130.
14. **Hill, S., G. L. Turner, and F. J. Bergensen.** 1984. Synthesis and activity of nitrogenase in *Klebsiella pneumoniae* exposed to low concentrations of oxygen. J. Gen. Microbiol. **130:**1061–1067.
15. **Huntley, B. E., A. C. Jones, and V. J. Cabelli.** 1976. *Klebsiella* densities in waters receiving wood pulp effluents. J. Water Pollut. Control Fed. **48:**1766–1771.
16. **Jenkins, D., M. G. Richard, and G. T. Daigger.** 1993. Manual on the causes and control of activated sludge bulking and foaming, 2nd ed. Lewis Publishers, Chelsea, Mich.
17. **Jones, R. L., P. Woodley, A. B. Zinoni, and R. L. Robson.** 1993. The *nifH* gene encoding the Fe protein of the molybdenum nitrogenase from *Azotobacter chroococcum*. Gene **123:**145–146.
18. **Knowles, R., R. Neufeld, and S. Simpson.** 1974. Acetylene reduction (nitrogen fixation) by pulp and paper mill effluents and by *Klebsiella* isolated from effluents and environmental situations. Appl. Microbiol. **28:**608–613.
19. **Möbius, C. H.** 1991. Nitrogen and phosphorus limits for nutrient deficient industrial wastewaters. Water Sci. Technol. **24:**259–267.
20. **Neilson, A. H., and A.-S. Allard.** 1985. Acetylene reduction (N_2-fixation) by *Enterobacteriaceae* isolated from industrial wastewaters and biological treatment systems. Appl. Microbiol. Biotechnol. **23:**67–74.
21. **Neilson, A. H., and L. Sparell.** 1976. Acetylene reduction (nitrogen fixation) by *Enterobacteriaceae* isolated from paper mill process waters. Appl. Environ. Microbiol. **32:**197–205.
22. **Niemela, S. I., and P. Vaatanen.** 1982. Survival in lake water of *Klebsiella pneumoniae* discharged by a paper mill. Appl. Environ. Microbiol. **44:**264–269.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Satoh, T., Y. Hoshino, and H. Kitamura.** 1976. *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. Arch. Microbiol. **108:**265–269.
25. **Smith, G. B., and J. M. Tiedje.** 1992. Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Environ. Microbiol. **58:**376–384.
26. **Vincent, J. M.** 1941. Serological properties of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. Proc. Linn. Soc. N. S. W. **66:**145–154.
27. **Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje.** 1993. Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. Appl. Environ. Microbiol. **59:**250–254.
28. **Zhou, J., M. R. Fries, J. C. Chee-Sandford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. **45:**500–506.