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Confirmation of *E. coli* among other thermotolerant coliform bacteria in paper mill effluents, wood chips screening rejects and paper sludges

Chantal J. Beauchamp[a,*], *Anne-Marie Simao-Beaunoir*[b], *Carole Beaulieu*[b], *François-Philippe Chalifour*[a]

[a]*Université Laval, Département de phytologie, Sainte-Foy, Que., Canada, G1K 7P4*
[b]*Université Sherbrooke, Département de biologie, Université de Sherbrooke, Que., Canada*

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ABSTRACT

Paper sludges are solid wastes material generated from the paper production, which have been characterized for their chemical contents. Some are rich in wood fiber and are a good carbon source, for example the primary and de-inking paper sludges. Others are made rich in nitrogen and phosphorus by pressing the activated sludge, resulting from the biological water treatments, with the primary sludge, yielding the combined paper sludge. Still, in the absence of sanitary effluents very few studies have addressed the characterization of their coliform microflora. Therefore, this study investigated the thermotolerant coliform population of one paper mill effluent and two paper mill sludges and wood chips screening rejects using chromogenic media. For the first series of analyses, the medium used was Colilert broth and positive tubes were selected to isolate bacteria in pure culture on MacConkey agar. In a second series of analyses, double selective media, based on ß-galactosidase and ß-glucuronidase activities, were used to isolate bacteria. First, the presence of thermotolerant coliforms was detected in low numbers in most water effluents, but showed that the entrance of the thermotolerant coliforms was early in the industrial process. Also, large numbers of thermotolerant coliforms, i.e., 7 000 000 MPN/g sludge (dry weight; d.w.), were found in combined sludges. From this first series of isolations, bacteria were purified on MacConkey medium and identified as *Citrobacter freundii, Enterobacter sp, E. sakazakii, E. cloacae, Escherichia coli, K. pneumoniae, K. pneumoniae subsp. rhinoscleromatis, K. pneumoniae subsp. ozaenae, K. pneumoniae subsp. pneumoniae, Pantoea sp, Raoultella terrigena, R. planticola*. Second, the presence of thermotolerant coliforms was measured at more than 3700–6000 MPN/g (d.w) sludge, whereas *E. coli* was detected from 730 to more than 3300 MPN/g (d.w.) sludge. The presence of thermotolerant coliform bacteria and *E. coli* was sometimes detected from wood chips screening rejects in large quantities. Also, indigenous *E. coli* were able to multiply into the combined sludge, and inoculated *E. coli* isolates were often able to multiply in wood chips and combined sludge media. In this second series of isolations, API20E and Biolog identified most isolates as *E. coli*, but others remained unidentified. The sequences of the 16S rDNA confirmed that most isolates were likely *E. coli*, few *Burkholderia spp*, but 10% of the isolates remained

*Corresponding author.
E-mail address: chantal.beauchamp@plg.ulaval.ca (C.J. Beauchamp).

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unidentified. This study points out that the coliform bacteria are introduced by the wood chips in the water effluents, where they can survive throught the primary clarifier and regrow in combined sludges.

1. Introduction

In Canada, paper mills use water to produce pulp and paper from wood and carry them in the effluents. Eventually, the pulp is used to produce paper, but the short fibers are not retained within the paper production and are returned to the effluents. These residual sludges fibers and other materials detrimental to paper production (e.g. filler, ink, etc.) are separated from the water by decantation in the clarifier. Then, the sedimentation material is directed to the press where it becomes the sludge. The sludge is called primary sludge when it originates from the production of virgin wood fiber; or de-inked paper (DIP) sludge when it is produced by removing inks from post-consumer fiber. These two types of sludge possess high carbon (C) content, but low nitrogen (N) content (Beauchamp et al., 1998). On the other hand, the clarified water is reused, but its over flow directed to the biological reactor where nitrogen, phosphorus, air, etc. are added, before it is directed to the secondary clarifier. There, the second clarification process allows the return of the treated water to the river whereas the sedimentation material, the secondary activated sludge, is combined with the primary sludge at the press to obtain the combined sludges that are rich in C and N. The potentially toxic compounds in paper sludges are low (Beauchamp et al., 1998).

In Quebec, paper sludges are mainly used in agriculture and in restoration of sites to favor plant growth, soil C, soil water retention and soil cationic exchange capacity (CEC) (Chantigny et al., 2000; Fierro et al., 1997, 1999, 2000; Simard et al., 1998; Trépanier et al., 1996). Also, the high C : N ratio of DIP sludge enhances symbiotic dinitrogen of forage legumes (Allahdadi et al., 2004).

Up to now, very few studies have addressed the characterization of the microflora of paper sludges. Tardif (1996) reported an increase in total microbial activity and populations following DIP sludge amendment to soil. The total population of bacteria, actinomycetes and fungi increased, in general, but followed the environmental conditions. No attempt was made to identify the microorganisms for this field experiment. The microbial evolution of DIP sludge under composting conditions was followed for 24 weeks (Charest et al., 2004), where *Klebsiella pneumoniae* was isolated. This coliform bacterium (Caplenas et al., 1981, Duncan and Razzell, 1972, Huntley et al., 1976, Knittel et al., 1977, Niemelä and Väätänen, 1982, Niemi et al., 2003), as well as *E. coli*, have been reported several times from paper mill effluents (Duncan and Razzell, 1972; Huntley et al., 1976; Niemi et al., 1987; Mentu et al., 1988). However, the presence of coliform bacteria remained unclear due to the possible cross contamination with sanitary effluents in the previous studies.

Over the years, several selective media have been developed to isolate the thermotolerant coliforms, and discriminate *E. coli*, a bacterium indicative of fecal pollution, against other bacteria (Brenner et al., 1993; Francy and Darner, 2000; Niemi et al., 2003; Packer et al., 1995). New selective media have been developed to differentiate *E. coli* from *K. pneumoniae* and allow the development of knowledge on thermotolerant coliforms in paper sludges. This is particularly important since some paper mills had fecal coliform populations above the 1000 MPN/g (d.w.) limit that allows the use of paper sludges without restriction in agriculture (Gauthier and Archibald, 2001; Menv, 2004). Therefore, the present study: (i) evaluates the use of the new chromogenic and fluorogenic media to determine the presence of coliform bacteria from paper mill effluents in the absence of sanitary effluents, wood chips screening rejects and paper sludges; (ii) confirms the identity of the isolated bacteria using some biochemical characteristics or DNA sequencing; and (iii) discusses their probable source of introduction into the paper mill environments and the health impact following sludge application to agricultural soils.

2. Methodology

2.1. Sampling

For the first experiment, one paper mill in the province of Quebec (Canada) was selected to investigate the overall distribution of thermotolerant coliform bacteria in their water effluents and sludges. A total of 15 sampling points was selected in the first trial and 10 points for the second trial. For the second experiment, two paper mills in the province of Quebec (Canada) were selected to investigate the distribution of thermotolerant coliforms and *E. coli* in their wood chips screening rejects and their sludge. The samplings were repeated on three different days. The separation of their sanitary effluents has been confirmed by an independent and qualified Engineering company before undertaking these experiments.

Liquid samples were collected in sterile plastic bottles and composite samples of 2 l were made at each sampling site. Temperatures were measured at sampling. The sludges were collected at the presses and consisted of grab samples of about 2 l. Similarly, the wood chips screening rejects were collected under a covered plat-form for convenience, but still they mimic the chips entering into the paper production. All samples were placed on ice and analyzed within 48 h. Duplicate samples were taken and sent to an independent laboratory; those results are presented only if differences between laboratories were reported.

2.2. Bacterial isolation and incubation

For the first experiment, the method used was MA700tm (Menv, 1999), a method based on the chromogenic broth, Colilert (Idexx Laboratories, Westbrook, ME.) and the most probable number (MPN) counts for the bacterial estimations.