DIRECTION ENVIRONNEMENT
(ENVIRONMENT DIVISION)

DETERMINATION OF THE BACTERIOSTATIC AND BACTERICIDAL EFFICIENCY OF A FLOOR COVERING

CRIQ File N^{o} 640-PE35964

Technical Report

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QUÉBEC, MARCH 15, 2006

Centre de recherche industrielle Québec

Le présent rapport a été traduit en anglais à la demande du client. En cas d'incompatibilité entre la version française et la version anglaise, la version française prévaudra.

This report was translated in English at the client's request. In case of incompatibility between the French and the English version, the French one will prevail.

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1. INTRODUCTION

FORBO LINOLEUM INC. manufactures and distributes a linseed oil-based floor covering marketed under the name Marmoleum. This covering has already been tested in the past to prove its bactericidal and bacteriostatic efficiency against different microorganisms.

2. PROJET OBJECTIVE

The objective of this project was to determine the bactericidal and bacteriostatic efficiency of the Marmoleum covering against the *Clostridium difficile* bacterium according to three different test methods to which the Marmoleum covering had already been subjected, namely :

- a qualitative evaluation of bactericidal or bacteriostatic efficiency (TNO seed layer method);
- a quantitative evaluation of bactericidal efficiency according to the AATCC 100 standard;
- an evaluation of the contact time necessary for the Marmoleum covering to be in contact with the bacterium to determine bactericidal or bacteriostatic activity (in vivo simulations method).

3. DESCRIPTION OF THE WORK

A Marmoleum covering sample was delivered to the CRIQ on February 2, 2006. The tests were performed between February 20 and March 10, 2006. Each test was performed on a covering sample as received and on a sample which had been subjected to one hour of leaching in tap water and then dried in ambient air.

In all cases, the tests were performed in bouillon or on a gelose containing the Reinforced Clostridium Medium and the strain used was *Clostridium difficile* ATCC 9689.

3.1 PREPARATION OF THE SAMPLES

Before proceeding with each test, the samples were cut to the required shape and dimensions. They were then disinfected with a 70 % ethyl alcohol solution.

Half of the samples were then subjected to one hour of leaching in tap water. For this purpose, they were placed in a shallow container and a quantity of water sufficient to cover them was added. The containers were placed on an agitating plate adjusted to a high enough speed to allow weak displacement of the water to the surface of the covering parts. After one hour, they were removed from the solution, drained and left to dry on a stainless steel grille for one hour at room temperature.

3.2 QUALITATIVE EVALUATION OF THE BACTERICIDAL OR BACTERIOSTATIC EFFICENCY ACCORDING TO THE TNO SEED LAYER METHOD

The principle of this evaluation is to put a bacterial suspension of known concentration in a liquefied gelose into contact and transfer a small quantity of this suspension to the surface of the coating. During the incubation period, the samples are inspected periodically to determine whether or not there has been development of the bacterium.

A *Clostridium difficile* culture was produced by inoculating 10 ml of Reinforced Clostridium Medium bouillon and incubating it under anaerobic conditions at 35 ℃ for 48 hours. This culture then was diluted in a liquefied gelose containing the same culture medium to obtain a final concentration of about 10^{5} CFU (colony forming units) per millilitre.

Two teflon disks 1 cm in diameter and 0.5 cm thick then were placed on a 5 cm by 5 cm coating sample and each was filled with 0.5 ml of inoculated gelose. After solidification of the gelose, the samples were deposited in 100 x 15 mm Petri dishes and incubated for 4 days at 35 ℃ under anaerobic conditions. A control sample was also prepared by transferring the same quantity of inoculated gelose to a disk placed at the bottom of a sterile Petri dish and incubated under the same conditions, as well as controlling the sterility of the gelose before its inoculation.

A count was also performed on the freshly inoculated gelose by subjecting it to successive dilutions and filtering a known volume of the dilution on a hydrophobic grid-membrane filter. The membrane then was deposited on the surface of a Reinforced Clostridium Medium gelose and incubated for 72 hours at 35 ℃ under anaerobic conditions. At the end of the incubation period, the count was performed.

The bacteriostatic activity was evaluated daily by examining the presence or absence of growth on the samples. The absence of growth confirms bacteriostatic or bactericidal activity and, conversely, growth shows that this activity is not recorded.

3.3 QUANTITATIVE EVALUATION OF BACTERICIDAL EFFICIENCY ACCORDING TO AATCC 100

The American Association of Textile Chemists and Colorists offers a method for quantitative evaluation of the bactericidal activity of a surface on which a known quantity of bacteria is present. This technique was developed to test fabrics but is totally applicable to a floor covering.

A *Clostridium difficile* suspension at a concentration of about 10^5 CFU per 0.1 ml was prepared in Reinforced Clostridium Medium bouillon. Coating samples 48 mm in diameter were cut out and placed individually at the bottom of sterile 500 ml plastic bottles. The samples were inoculated with 0.1 of the suspension and a count was performed on half of them by adding 100 ml of a neutralizing solution containing 0.1 % peptone and 0.1 % polysorbate 80 and by filtering 1 ml of this solution on a membrane filter as described in the preceding test. Control samples moistened with 0.1 ml of sterile water were also prepared.

The other half of the samples was placed in the incubator at 35 ℃, after hermetically sealing the containers, for 24 hours. A bottle containing only 0.1 ml of inoculum without a sample was also inserted to validate the inoculum's viability. After the incubation period, a new count was performed by adding 100 ml of neutralizing solution to each container and filtering 1 ml of the resulting solution.

3.4 EVALUATION OF THE TIME NECESSARY FOR THE MARMOLEUM COATING TO BE IN CONTACT WITH THE BACTERIUM TO DETERMINE BACTERICIDAL OR BACTERIOSTATIC ACTIVITY (IN VIVO SIMULATIONS METHOD)

This evaluation consists of determining the contact time required between a piece of Marmoleum and the bacterium, either to prevent growth and multiplication, or to eliminate it.

The *Clostridium difficile* suspension at a concentration of about 10^5 CFU per 0.1 ml was prepared in Reinforced Clostridium Medium bouillon. A 0.1 ml volume of this suspension was spread with a curved glass rod on the surface of each gelose contained in a 100 x 15 mm Petri dish and left to dry for 15 minutes under a laminar flow hood.

The 1 cm by 1 cm coating samples were deposited on a gelose facing the bacteria and incubated at 35 ℃ under anaerobic conditions. Sample pieces were removed after 1, 2, 4, 6 and 24 hours. After each removal, the geloses, now deprived of the coating sample, were put back in the incubator under the same conditions and examined every 24 hours up to 72 hours. Totally inhibited growth at the end of 72 hours indicates a bactericidal or bacteriostatic effect, while partial inhibition of growth in the first 48 hours followed by growth after 72 hours instead shows bacteriostatic activity. The method is validated by the growth of bacteria on the surface of an inoculated gelose which was not placed in contact with the samples and by insertion of an uninoculated gelose on which no growth must be observed.

4. RESULTS OBTAINED

4.1 RESULT OF THE QUALITATIVE EVALUATION OF EFFICIENCY MEASURED ACCORDING TO THE TNO SEED LAYER METHOD

The count performed on the inoculum used to perform this test was 1.2 x 10^5 CFU/ml. Table I presents the results obtained after 72 hours of incubation. The plus (+) symbol indicates that there was growth while the minus (–) symbol represents the absence of growth of the *Clostridium difficile* bacterium.

TABLE I: GROWTH OBSERVED AFTER 72 HOURS OF INCUBATION (*TNO SEED LAYER METHOD*)

SAMPLE	GROWTH OBSERVED
Marmoleum as is	-
Marmoleum soaked for one hour	-
Positive control (inoculated gelose)	+
Sterility control	-

The result obtained shows that the Marmoleum coating possesses a definite bacteriostatic activity and potentially bactericidal activity against *Clostridium difficile.*

4.2 RESULT OF THE QUANTITATIVE EVALUATION OF BACTERICIDAL EFFICIENCY ACCORDING TO AATCC 100

The inoculum used in this evaluation contained 1.3 x 10^5 CFU/0.1 ml. Table II presents the results obtained in the counts performed immediately after inoculation and after 24 hours of contact between the bacterium and the coating at 35 ℃.

Efficiency is calculated as follows:

$R = 100 (B - A)/B$

where R = % reduction after 24 hours of contact
A = count of the inoculated sample after 24 hours of contact
B = count of the inoculated sample after inoculation

TABLE II: BACTERICIDAL EFFICIENCY

SAMPLE	COUNT (CFU/SURFACE)		% EFFICIENCY
	UPON INOCULATION	AFTER 24 HOURS OF CONTACT	
Marmoleum as is (inoculated)	1.2×10^5	< 100	> 99.9
Marmoleum as is (uninoculated)	< 100	< 100	---
Marmoleum soaked for one hour (inoculated)	1.2×10^5	< 100	> 99.9
Marmoleum soaked for one hour (uninoculated)	< 100	< 100	---
Inoculum	1.3×10^5	$> 10^8$	---

The increase in the number of bacteria in the inoculum after 24 hours of incubation proves its viability over time. Moreover, the fact that the count of the uninoculated samples is less than 100 proves that they were not contaminated during handling.

4.3 RESULTS OF THE CONTACT TIME EVALUATION (IN VIVO SIMULATIONS METHOD)

The following table presents the results obtained after 72 hours of incubation following different contact times between the sample and the inoculum. The plus (+) symbol indicates that there was growth while the minus (–) symbol represents the absence of growth of the *Clostridium difficile* bacterium. The inoculum used for this test contained 5×10^5 live cells of *Clostridium difficile* at the beginning of the test.

TABLE III: GROWTH OBSERVED ACCORDING TO THE CONTACT TIME

SAMPLE	GROWTH OBSERVED				
	T = 1 HOUR	T = 2 HOURS	T = 4 HOURS	T = 6 HOURS	T = 24 HOURS
Marmoleum as is	-	-	-	-	-
Marmoleum soaked for one hour	-	-	-	-	-
Positive control (inoculated gelose)	+				
Sterility control	-				

Since no growth was recorded after reincubation of the samples once the coating was removed, Marmoleum thus seems to be bactericidal against *Clostridium difficile.*

5. CONCLUSION

Marmoleum floor covering has bacteriostatic and bactericidal properties against the *Clostridium difficile* ATCC 9689 strain. Moreover, soaking the sample in water for one hour does not seem to alter its antibacterial properties. Thus, it can be concluded that:

- Marmoleum proves its bacteriostatic efficiency against *Clostridium difficile* when tested according to the TNO Seed layer Method*;*
- Marmoleum possesses bactericidal activity exceeding 99.9 % against *Clostridium difficile* when tested according to the AATCC 100 method;
- After one hour of contact with the *Clostridium difficile* bacterium, Marmoleum proves its bactericidal efficiency when tested according to the in vivo simulations method*.*