Université de Montréal

Étude des mécanismes d'inactivation des microorganismes suite à un traitement à l'ozone

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Thèse présentée à la Faculté des Études Supérieures
en vue de l'obtention du grade de Philosophiae Doctor (Ph.D)
En physique

Novembre 2009

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Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée :

Étude des mécanismes d'inactivation des microorganismes suite à un traitement à l'ozone

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Résumé

Notre thèse s'inscrit dans le cadre de la compréhension des mécanismes d'inactivation de microorganismes, principalement des spores bactériennes, suite à un traitement à l'ozone. Les objectifs généraux, au départ, étaient de mettre en place une stratégie expérimentale dans le but de déterminer les espèces et les phénomènes impliqués dans les mécanismes d'inactivation des spores en combinant diverses techniques pluridisciplinaires. Rappelons que les récentes avancées en stérilisation par plasma sont principalement dues aux études menées sur les mécanismes d'inactivation des microorganismes.

Dans ce contexte, en nous appuyant sur une synthèse des études publiées dans la littérature scientifique, nous avons élaboré une étude expérimentale en deux étapes : l'étude de la stérilisation à l'ozone en milieu sec puis en milieu humide. Au cours de ces études, nous avons principalement étudié la cinétique d'inactivation des microorganismes, les dommages occasionnés à ces derniers par de tels traitements et nous avons identifié les espèces responsables de l'inactivation pour, enfin, proposer un mécanisme d'inactivation en spécifiant le rôle et l'importance des différents phénomènes en jeu. L'originalité de notre approche s'appuie sur la corrélation faite entre ces deux types de traitement (ozone sec et humidifié) ainsi que la diversité et la complémentarité des moyens de caractérisation utilisés.

Dans un second volet, nous avons mis en évidence pour la première fois la possibilité de conférer à une surface de Pétri de polystyrène (PS) une importante activité biocide suite à un traitement à l'ozone. Nous avons mené une étude de caractérisation des propriétés de ces surfaces démontrant, principalement, leur grande efficacité biocide sur une large gamme de microorganismes, l'importance de l'interaction microorganismes/surface traitée, l'existence de la rémanence biocide, les modifications physico-chimiques induites en surface… L'étude des modifications physico-chimiques et microbiologiques nous a permis d'approcher les mécanismes d'inactivation en identifiant, de manière non exhaustive, les phénomènes impliqués. Enfin, nous avons examiné l'applicabilité de cette méthode à divers types de

polymères montrant que le degré d'activité biocide induite dépend de la nature du substrat.

Mots-clés : stérilisation, ozone sec et humide, spectroscopie optique d'absorption, spore bactérienne, polymères thermosensibles, caractérisation de surface.

Abstract

The thesis deals with the inactivation mechanisms of microorganisms (mainly endospores) after an ozone treatment. The general objective of this work was to provide an experimental strategy to identify the phenomena and chemical species involved in microorganism inactivation mechanism using multidisciplinary techniques. Recall that the recent advances in plasma sterilization are mainly due to comprehensive studies dealing with the inactivation mechanisms of microorganisms.

In this context, based on a review of published studies, we proposed a two-step experimental study of ozone sterilization in both dry and humid media. In the course of this work, we have examined the inactivation kinetics of some microorganisms, their damage after treatments; we have also identified the chemical species responsible for inactivation, and proposed an inactivation mechanism (for each type of ozone treatment) by describing the importance and role of the different implicated phenomena. The originality of our approach rests on the correlation achieved between the dry and humid ozone treatment, and by the diversity and complementarity of the characterization techniques used.

In a second part, we show for the first time the possibility to confer biocide activity to surfaces of polystyrene (PS) Petri dishes after their exposure to ozone. A characterization study of these treated surfaces mainly shows a high inactivation efficacy on various microorganisms, the importance of the microorganisms/treated-surface interaction, the biocide persistence of the treated surfaces and physico-chemical modifications. The study on physico-chemical and microbiological changes gives us elements for identifying the phenomena involved in the inactivation mechanisms. Finally, we have shown the possibility to confer biocide properties to polymeric surfaces in general with the same experimental process observing that the biocide efficacy depends on the nature of the polymer.

Keywords : sterilization, dry and humid ozone, absorption spectroscopy, bacterial spores, thermo-sensitive polymers, surface characterization

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Liste des notations et symboles

Abréviations

ADN	Acide désoxyribonucléique
AFM	Microscope à force atomique (Atomic Force Microscope)
B. atrophaeus	Bacillus *atrophaeus*
B. subtilis	Bacillus *subtilis*
B. pumilus	Bacillus *pumilus*
DAPI	4', 6'-diamino-2-phenylindole dilactate
DBD	Décharge à barrière diélectrique
DNase	Désoxyribonucléase
FTIR	Spectroscopie infrarouge à transformée de Fourier (Fourier transformed infrared spectroscopy)
G. stearothermophilus	Geobacillus *stéarothermophilus*
IR	Infrarouge
M	Molécule (organique ou inorganique)
MEB	Microscopie électronique à balayage
M_{ox}	Molécule oxydée
NAS	Niveau d'assurance de stérilité
NTP	Conditions normales de température et de pression
OEt	Oxyde d'éthylène
PA	Pression atmosphérique
PE	Polyéthylène
pH	Potentiel Hydrogène
PMMA	Polyméthylméthacrylate
PP	Polypropylène
PS	Polystyrène
PTFE	Polytétrafluoroéthylène (Téflon)
PU	Polyuréthane

SASP	Small acid-soluble proteins
UV	Ultraviolet
XPS	Spectrométrie photoélectronique à rayon X (X-ray Photoelectron spectroscopy)
^{o}C	Degré Celsius
%	Pourcent

Symboles latins

D	Temps nécessaire pour réduire d'une décade une population de microorganismes
h	Constante de Planck
k	Constante cinétique de décroissance
N	Nombre de survivants
No	Nombre initial de microorganismes
Qc	Énergie sous forme de chaleur
t	temps
T	Température

Symboles grecs

δ	Charge partielle portée par un atome
ε	Permittivité relative
λ	Longueur d'onde
ν	Fréquence

Bismilleh

Remerciements

J'aimerais remercier tout d'abord mon directeur de recherche, le professeur Michel Moisan, pour m'avoir permis de mener ce sujet de recherche au sein de son groupe et pour m'avoir continuellement offert, dans la mesure du possible, les moyens nécessaires pour conduire à bien mes travaux. Je tiens à le remercier pour ses qualités scientifiques, pour ses efforts dans la rédaction de la demande de brevet, et pour m'avoir initié à l'écriture d'articles scientifiques.

J'exprime également mes sincères remerciements aux membres du jury d'avoir accepté d'évaluer mon ma thèse. Je vous suis reconnaissante, à l'avance, pour avoir donné de votre temps précieux et pour vos pertinents commentaires apportés sur cette thèse.

J'aimerais également remercier le professeur Jean Barbeau et les membres de son équipe de microbiologie pour leur constant soutien. Je tiens particulièrement à le remercier pour sa disponibilité, ses qualités scientifiques et humaines. Au sein de cette belle équipe, je tiens aussi à remercier Jacynthe Séguin et Annie Leduc pour leur sympathie, leur énergie et, surtout, pour l'apport scientifique fondamental qu'elles ont apporté tout au long de cette étude. Je n'oublie pas de remercier Daniel et les stagiaires Rosalie Plantefève et Paul Grava pour leur travail en microbiologie et leur sympathie.

J'aimerais exprimer ma reconnaissance à Yassine Kabouzi à travers qui j'ai découvert la physique des plasmas et qui m'a assisté pendant environ une année sur ce sujet (à titre de post-doctorant), notamment dans la conception du montage qui allait servir à cette étude. Je remercie également Jean-Sébastien Mayer, le chef de l'atelier mécanique, pour sa disponibilité et sa contribution au montage du système expérimental sur lequel se sont effectués les travaux de cette recherche. J'exprime aussi ma gratitude envers Danielle Kéroack, assistante de recherche, pour son soutien en informatique mais aussi pour sa précieuse aide en laboratoire sans oublier sa disponibilité envers chacun des membres de ce groupe. Je remercie aussi Bachir

Saoudi, ancien assistant de recherche, pour le sérieux de son travail lors des corrections menées sur mon mémoire pré-doctoral.

J'exprime également et tout particulièrement mes plus sincères remerciements à la professeure Joëlle Margot pour ses qualités humaines, sa disponibilité, et le précieux soutien qu'elle a su m'apporter lors des moments difficiles rencontrés durant cette thèse.

Je remercie également Eduardo Castanos-Martinez pour son aide et ses précieux conseils concernant la spectroscopie optique d'absorption.

Je tiens à remercier les professeurs Benoît Cousineau, Michel Lafleur, Édouard Sacher, Alexandra Furtos et Julian Zhu pour leurs conseils scientifiques. Je tiens à exprimer mes plus vifs remerciements à Fabienne Poncin-Épaillard (Directrice de recherche au Centre National de Recherche Scientifique au Mans, France) qui nous a fait partager son expertise scientifique, et pour son intérêt et son apport à l'avancée de cette étude. J'aimerais aussi remercier les techniciens et assistants de recherche pour leur travail et leur précieux conseils, à savoir Christophe Chabanier (INRS), Mohamed Soltani (INRS), Patricia Moraille (UdM) et Suzie Poulin (École Polytechnique), Caroline Monat (UdM/Mc Gill), sans oublier le stagiaire d'été David Chiasson.

Je tiens à remercier tous mes collègues et anciens membres du Groupe de physique des plasmas pour leur grande sympathie. Merci à Nicolas Philip, Olivier Barthélemy, Crina Popovici, Thomas Fleisch, Luc Stafford, Karl Giroux, Stefano Mattei, Eduardo Castanos-Martinez, Jérôme Saussac, Jérôme Pollack, Pierre Levif, Denis Carignan, Jean-Sébastien Poirier, Pierre-Marc Bérubé, …

Je remercie également Louis Lemay, adjoint administratif, pour sa confiance en m'ayant donné la chance d'enseigner à l'Université de Montréal durant mon doctorat. Une belle expérience que j'ai agréablement appréciée. Je remercie d'ailleurs tous les étudiants que j'ai eus pour leur apport dans cette belle expérience.

Je remercie toutes les personnes formidables que j'ai pu rencontrer sur ce campus et qui ont contribué à mon épanouissement… Un très grand merci à eux tous.

Merci infiniment à mes parents pour leur soutien constant, leur patience et leurs encouragements durant cette thèse et tout au long de mes études… merci d'avoir toujours été là pour me pousser à aller de l'avant et garder confiance en moi! Merci aussi à mes beaux-parents pour leur agréable et permanent support, notamment, à la fin de la thèse. Un merci aussi à ma meilleure amie qui, par ses précieux conseils et sa gentillesse, m'a toujours été d'une grande aide et d'un grand réconfort, notamment pendant les périodes difficiles rencontrées durant cette thèse.

Enfin, j'exprime ma profonde gratitude à mon très cher mari pour ses encouragements, ses précieux conseils, et surtout pour tous ses efforts à m'aider à réaliser mes objectifs. Aussi, je le remercie sincèrement de m'avoir soutenu et ″boosté″ lors des moments difficiles traversés durant cette thèse.

Introduction

Depuis toujours, la problématique de la stérilisation des instruments médicaux ou dentaires ne cesse de préoccuper les pouvoirs publics, ainsi que les hôpitaux soucieux d'assurer une qualité de soins, pour ainsi dire, irréprochable. En effet, un nombre assez élevé d'incidents techniques ayant entraîné la mort de patients en milieu hospitalier, de par le monde mais aussi au Québec, est clairement imputable à des contaminations par des instruments médicaux mal stérilisés. Ces tragiques imprévus ne sont pas tant liés à une mauvaise utilisation des techniques de stérilisation conventionnelles qu'à leur relative inefficacité, par exemple, pour ce qui est d'éliminer les agents pathogènes nouvellement découverts tel que les prions (protéines responsables de la maladie de la vache folle). En outre, cette nouvelle difficulté repose le problème de la stérilisation des instruments à base de polymères thermosensibles (devant être effectuée à des températures ne dépassant guère 50-60 ^{o}C et recourant, à l'heure actuelle, à des méthodes chimiques relativement nocives) pour essayer de mettre au point une technique qui permette de répondre favorablement à l'ensemble des exigences énumérées ci-dessus.

À l'heure actuelle, parmi les techniques émergentes et qui présentent un réel potentiel, on retrouve la stérilisation par plasma et la stérilisation par ozone. La stérilisation par ozone est reconnue pour avoir un "champ d'inactivation" de microorganismes bien plus étendu que la stérilisation par plasma, et pourrait donc être une alternative de choix dans l'avenir; cependant elle présente l'inconvénient d'être relativement plus agressive, pouvant endommager fortement les matériaux traités, en particulier les polymères, réduisant ainsi la durée de vie des instruments.

En raison de fortes similitudes existant entre la stérilisation par ozone et celle par plasma (milieu gazeux, exposition dans une enceinte de post-décharge) et d'une évidente complémentarité dans ses deux thèmes de recherche, l'équipe du Pr. Moisan, engagé depuis plusieurs années déjà dans l'étude et le développement d'une nouvelle méthode de stérilisation à base de plasma, a intégré tout récemment le projet Ozone à ces activités de recherche.

L'ozone est un excellent agent désinfectant, connu depuis la fin du 19^{ième} siècle et employé, depuis des décennies à l'échelle mondiale, pour l'assainissement des eaux, mais aussi comme agent désodorisant et de blanchiment. La possibilité d'utiliser ses propriétés biocide et oxydante pour aller au-delà de la simple désinfection et arriver à la stérilité est assez bien connue, pas très bien comprise et peu exploitée sauf tout récemment par TSO_3 ® (Québec) qui a mis sur le marché un stérilisateur utilisant l'ozone en milieu humide.

Cependant, aussi surprenant que cela puisse paraître et compte tenu des applications industrielles et hospitalières en désinfection par l'ozone, les recherches effectuées sur ce sujet, aussi bien en milieu liquide que gazeux, n'ont toujours pas permis une identification rigoureuse des espèces et des mécanismes menant à l'inactivation des microorganismes. Selon nous, cette identification précise des espèces impliquées dans ce processus est une étape essentielle à la détermination et à la connaissance des phénomènes physico-chimiques (et donc des mécanismes) intervenant dans l'inactivation par l'ozone. Par exemple, il est bien connu qu'il faut impérativement que les microorganismes soient humidifiés pour que leur inactivation ait lieu de façon efficace, de sorte qu'il devient hasardeux d'affirmer que seule la

molécule d'ozone agit directement sur eux. Une étude de la littérature scientifique montre bien que cette question n'est toujours pas entièrement résolue.

Notre travail constitue un apport fondamental à la compréhension des mécanismes en jeu dans la stérilisation à l'ozone. Pour aborder et défricher la problématique de la stérilisation par l'ozone gazeux et tenter d'en élucider les mécanismes, nous l'avons étudié tout d'abord en milieu sec afin d'établir une bonne base pour mieux comprendre les phénomènes survenant en milieu humide (milieu chimiquement plus complexe). Cette étude nous a conduit à tisser des liens entre différentes disciplines telles que la physique, la microbiologie et les interactions physico-chimiques avec le milieu (sec et humide).

Au cours de notre étude, nous avons en plus découvert la possibilité de donner à une surface polymérique une activité biocide en l'exposant simplement à l'ozone gazeux en milieu sec. Cette découverte nous a incité à effectuer une demande (provisoire) de brevet et a donné lieu à la rédaction de plusieurs articles (récemment soumis). Ce nouvel axe d'étude s'est naturellement greffé au sujet originel et sera donc présenté comme le second volet de cette thèse s'intégrant, ainsi, à l'ensemble des travaux effectués dans le cadre du projet Ozone.

Nous abordons, tout d'abord, au chapitre 1, les principes généraux et enjeux de la stérilisation montrant, entre autres, l'intérêt de recourir au traitement à l'ozone. Nous examinons dans le chapitre 2 les propriétés de l'ozone lui conférant, notamment, ses propriétés biocides afin de bâtir une base théorique nous permettant d'étudier l'inactivation des spores bactériennes par l'ozone. Nous présentons également dans ce chapitre les outils physiques et microbiologiques ayant contribué à l'étude approfondie des mécanismes d'inactivation en milieu sec et en milieu humide. La corrélation entre ces deux milieux est un des aspects originaux de notre étude, soulevant ainsi la complexité de cette dernière et offrant alors une plus grande solidité quant aux mécanismes d'inactivation proposés. Au chapitre 3, nous abordons la question des Pétris de polystyrène (PS) traités à l'ozone et de leur activité biocide

acquise suite à ce traitement, après avoir présenté quelques outils théoriques sur les modifications de surface dues à l'action d'espèces oxydantes. Nous examinons dans cette étude : (1) les caractéristiques physiques, voire même chimiques, de ces surfaces traitées à l'ozone afin de mettre en évidence les modifications qu'elles ont subies, et, (2) microbiologiques afin d'étudier (de manière non exhaustive) l'étendue de ses propriétés biocides. Finalement, nous explorons diverses surfaces de polymères utilisés dans le domaine biomédical afin de déterminer si, suite à un traitement à l'ozone, elles pourraient également acquérir une activité antimicrobienne. Enfin, nous concluons et présentons quelques perspectives d'avenir concernant les travaux menés dans cette étude.

CHAPITRE 1
Principes généraux et enjeux de la stérilisation

1.1 Introduction

La problématique de la stérilisation des instruments médicaux préoccupe les pouvoirs publics ainsi que les hôpitaux soucieux d'assurer une qualité de soins optimale. Les professionnels ont généralement recours à des techniques de stérilisation diversifiées utilisant la chaleur (sèche et humide), des produits chimiques (oxyde d'éthylène…), le rayonnement électromagnétique (les ultraviolets, le rayonnement Gamma…) ou encore divers autres procédés physiques…[1] La relative efficacité des techniques de stérilisation conventionnelles face à certains agents pathogènes ainsi que l'avènement, à grande échelle, d'instruments médicaux composés de polymères pour la plupart thermosensibles (leur traitement ne peut

s'effectuer dans un milieu où la température dépasserait ~50-60^{o}C) sont autant d'arguments qui motivent l'émergence de la technologie plasma appliquée à la stérilisation des microorganismes. Après avoir présenté quelques notions microbiologiques de base nécessaires au physicien, nous passerons brièvement en revue les diverses méthodes conventionnelles utilisées pour traiter les instruments médicaux. Dans ce chapitre, nous montrons aussi l'intérêt d'utiliser l'action des espèces chimiques réactives issues du plasma pour stériliser et, dans le cadre de notre étude, nous nous intéresserons plus particulièrement à l'ozone.

1.2 Concept de base en stérilisation

La stérilisation est, par définition, un processus physique ou chimique qui détruit ou élimine toute forme de vie, plus spécialement les microorganismes. Par ailleurs, la désinfection est un traitement (chimique ou physique) qui vise les microorganismes pathogènes, mais ne peut garantir leur élimination totale : on dit qu'on a réduit la charge pathogène

1.2.1 Spore bactérienne

Placées dans des conditions inadaptées à leur croissance, certaines bactéries ont la capacité de sporuler, atteignant ainsi un état de dormance caractérisé par une activité métabolique extrêmement ralentie ; la phase inverse dite de germination sera provoquée lorsque les conditions environnantes seront propices à l'activité biologique de ces bactéries pour un retour à leur "vie normale".

En s'intéressant de plus près à la morphologie d'une spore, on constate qu'elle a formé, au moment de la sporulation, une "coquille" (cortex) autour de son bagage génétique enserrée par un nombre précis de couches de compositions variables selon le type de spore (Figure 1.1).

Les étapes de l'évolution de la bactérie de l'état végétatif vers la forme sporulée sont caractérisées par une déshydratation progressive du cytoplasme, l'apparition de nouveaux composés (dipicolinate de calcium…), une densification des structures

nucléaires, et enfin la synthèse d'une paroi cellulaire imperméable et hautement résistante à la chaleur.

La figure 1.1 représente les différentes tuniques ″protégeant″ le matériel génétique de la spore *Bacillus atrophaeus* (autrefois appelée *Bacillus subtilis*). On trouve essentiellement trois tuniques : (**i**) le cortex, (**ii**) la tunique interne de ~75 nm d'épaisseur composée de deux à cinq couches et (**iii**) la tunique externe d'épaisseur variant de 70 à 200 nm entourée, parfois, d'un exosporium. Les tuniques externes et internes sont formées de protéines plus ou moins réticulées. L'ADN (acide désoxyribonucléique) situé dans le ″coeur″ est protégé non seulement par l'ensemble des différentes tuniques, mais aussi par des protéines spécifiques (les SASP: small acid-soluble proteins) qui lui confèrent une grande résistance à la chaleur et aux rayonnements UV [2].

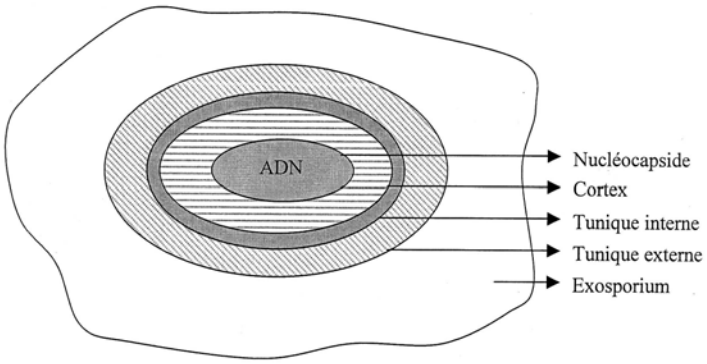

Figure 1.1 : Représentation schématique de la spore *B. subtilis* avec ses différentes couches et son matériel génétique (ADN=acide désoxyribonucléique) situé en son centre (d'après [3]).

Du fait de leur très grande résistance face aux agressions extérieures, les spores sont utilisées pour tester et valider les procédés de stérilisation : à chaque technique correspond un bio-indicateur spécifique [4] (Tableau 1.1). De manière générale, le critère utilisé pour effectuer la validation des techniques de stérilisation est le niveau d'assurance de stérilité (NAS) : un NAS de 10^{-6} correspond à la probabilité de trouver au plus un microorganisme survivant sur une population initiale de 10^{6} spores.

Technique de stérilisation conventionnelle	**Oxyde d'éthylène**	**Autoclave**	**Chaleur sèche**	**Irradiation**
Bio-indicateur correspondant	*B. atrophaeus*	*G. stearothermophilus*	*B. atrophaeus*	*B. pumilus*[*]

[*]*B.pumilus* est résistant aux radiations et plus spécifiquement aux rayonnements Gamma.

Tableau 1.1: Spores de référence (bio-indicateurs) pour la technique de stérilisation correspondante.

1.2.2 Courbe de survie

Suite à l'exposition d'un échantillon de spores à une technique donnée de stérilisation, on récupère et compte le nombre de survivants à l'aide d'un protocole microbiologique permettant, pour des conditions opératoires données, le tracé d'une courbe de survie typique. La courbe de survie [5], obéissant généralement à une loi de décroissance exponentielle (éq.2), représente le nombre de spores survivantes en fonction du temps d'exposition au procédé (éq.3).

La décroissance du nombre de survivants, dans ce cas, s'écrit : $\frac{dN}{dt} = -kN(t)$ (1)

où $N(t)$ représente le nombre de survivants au temps t et k la constante cinétique de décroissance.

L'intégration de cette équation donne : $N(t) = N_o \exp(-kt)$ (2)

où N_o est le nombre initial de microorganismes.

Sur une échelle logarithmique, on obtient : $\log_{10}\left(\frac{N(t)}{N_0}\right) = -kt = -\frac{t}{D}$ (3)

où D représente le temps nécessaire pour obtenir une réduction d'une décade de la population des microorganismes ($D=k^{-1}$).

Selon le procédé de stérilisation employé, la construction d'une courbe de survie permet de mettre en évidence, en première approximation, un ou plusieurs segments linéaires (communément appelés phases) reflétant les différentes cinétiques et pouvant permettre de dégager les mécanismes menant à l'inactivation des microorganismes [6],[7],[8]. L'un des paramètres clé déterminé à partir des courbes

de survie est le temps décimal D (éq.3) représentant le temps nécessaire pour réduire la population de spores d'un ordre de grandeur, soit une diminution de 90% de celle-ci [9]. Parmi les courbes de survie que l'on peut observer expérimentalement [5], les plus fréquemment rencontrées sont illustrées ci-dessous (Figure 1.2) : ces courbes, représentées dans l'échelle logarithmique, sont éventuellement caractérisées par a) une phase initiale de latence (cas A), b) une seule phase linéaire (cas B) et c) deux phases dont la seconde est toujours d'une cinétique moins rapide.

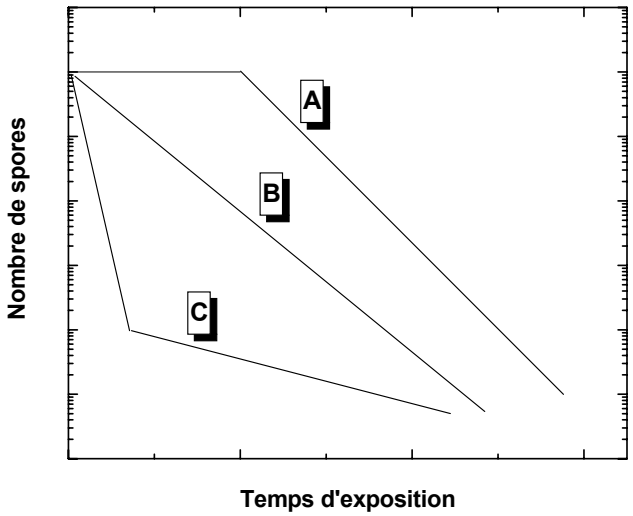

Figure 1.2 : Cas typiques de courbes de survie : A possède une phase initiale de latence ou ″épaulement″, B est dite logarithmique ou linéaire, et C est une courbe à 2 phases avec une ″traînée″ ou ″queue″ de cinétique plus faible que la première.

1.3 "Limites" des techniques conventionnelles de stérilisation

De façon habituelle, les microorganismes peuvent être inactivés par la chaleur, par un traitement chimique ou par irradiation. Nous présentons dans cette section quelques techniques de stérilisation en soulignant les difficultés actuellement rencontrées de par leur caractéristiques intrinsèques et, rappelons-le, l'apparition de ″nouveaux″ agents infectieux.

Techniques de stérilisation utilisant la chaleur humide

Généralement, la stérilisation utilisant la ″vapeur d'eau″ à haute pression (≈quelques bars) n'est applicable que si les dommages dus à la chaleur et à l'humidité ne représentent pas un problème (corrosion de certains métaux due à leur oxydation suite au traitement). Bien qu'il s'agisse d'un procédé universellement recommandé par les normes pour sa fiabilité, son faible coût et sa non-toxicité, le traitement de composés à base de polymères pose actuellement problème.

Le traitement à la chaleur humide peut induire plusieurs types de changements dans la cellule: déshydratation, destruction des protéines bactériennes… Ce procédé favorise un processus de nature chimique au cours duquel les macromolécules composant le microorganisme (chaînes peptidiques) interagissent avec les espèces réactives (telles que les espèces radicalaires) [10].

À titre d'illustration, l'autoclave fonctionne généralement à une pression de l'ordre de 1-2 bars, à une température de 121^{o}C et pour des temps d'exposition d'environ 20 min correspondant à cette température ″plateau″ (le temps de stérilisation dépendant de la température utilisée : plus elle est élevée, plus il est court).

Techniques de stérilisation utilisant la chaleur sèche

La méthode consiste à stériliser des instruments à la pression atmosphérique (PA) en ayant recours à de hautes températures (~180 ^{o}C) pour de longs temps d'exposition (au moins 90 min). Ce procédé conjugue l'action de l'oxygène et de l'azote (composants de l'air) avec celle de la chaleur.

Les capacités de réparation de la spore constituent un élément essentiel de résistance à la stérilisation par cette technique. Il existe de nombreuses contre-indications liées à l'utilisation de cette technique : détérioration des équipements, dépense d'énergie excessive, temps d'exposition trop long etc. Cette méthode n'est, généralement, pas recommandée car elle est considérée comme étant moins fiable et plus difficile à contrôler que la stérilisation par chaleur humide (autoclave).

Technique de stérilisation utilisant un composé chimique: l'oxyde d'éthylène (*OEt)*

Depuis une cinquantaine d'années, les outils biomédicaux thermosensibles sont traités chimiquement ; on peut citer, par exemple, l'utilisation de gaz tels que l'oxyde d'éthylène (OEt) pur ou mélangé à des chlorofluorocarbures [11]. L'oxyde d'éthylène présente une forte réactivité chimique : son activité biocide est principalement due à la réaction d'alkylation de certains groupements chimiques présents dans les microorganismes (ces substitutions sont létales [7]). Généralement combiné au CO_2, il est très souvent employé dans un environnement où la température et le taux d'humidité varient, respectivement, de 38°C à 54°C et de 40% à 50%.

Le principal inconvénient du traitement chimique utilisant des agents toxiques tels que OEt est qu'ils peuvent être non seulement fortement adsorbés sur une multitude de matériaux comme les plastiques mais leur éventuelle désorption peut présenter de sérieux risques pour le patient et l'utilisateur. Notons que le procédé d'aération après traitement peut durer jusqu'à plusieurs jours! [12]. Tous les inconvénients de la stérilisation par OEt sont autant d'arguments qui soutiennent sa suppression progressive du milieu hospitalier.

Technique de stérilisation utilisant le rayonnement.

Le recours à des procédés de stérilisation utilisant le rayonnement électromagnétique répond à l'existence d'objets médicaux sensibles [13] à la chaleur ou à l'humidité, rendant l'utilisation des méthodes précédentes problématique.

Chaque type de rayonnement électromagnétique (ultra-violets, rayons Gamma, rayons X…) affecte de manière différente les cellules vivantes. Utilisé depuis plus d'un siècle et reconnu comme un excellent agent biocide, le rayonnement UV est extrêmement efficace dans la gamme [230-260] nm [14-16, 11]. Ce traitement affecte efficacement la couche supérieure d'un agrégat de bactéries; par contre, son efficacité chuterait rapidement pour les couches plus profondes (effet d'écran). De ce fait, il n'est pas considéré comme pouvant mener à la stérilisation d'objets médicaux. L'action létale du rayonnement UV se traduit sur le matériel génétique, entre autres, par la formation de photoproduits altérant la structure de l'ADN. Dans le cas des spores, la formation de dimères entre les bases adjacentes de l'ADN, principalement les thymines (une des 4 bases de l'ADN), a pour effet d'inhiber les capacités de l'ADN

à se répliquer. Néanmoins, si le nombre de ces lésions n'est pas trop élevé, la réparation est possible grâce à une activité enzymatique.

Comme les UV, le rayonnement Gamma est très efficace [17]. Toutefois, ce dernier est beaucoup plus pénétrant et plus puissant que les ultraviolets puisqu'il peut traverser tous les plastiques et métaux pour ensuite tuer les microorganismes. Il sert donc à stériliser les fils chirurgicaux, les scalpels, certains implants et, il est aussi utilisé dans certains domaines de l'industrie, notamment alimentaire. L'exposition des microorganismes pendant une courte durée (quelques minutes) au rayonnement Gamma (rayonnement émis par des capsules de cobalt radioactif de synthèse ^{60}Co) a pour effet de déstructurer le génome interne de la spore bactérienne (cassure double brins de l'ADN).

1.4 Procédé plasma et procédé utilisant l'ozone : intérêt en stérilisation

Le terme « plasma » a été introduit par Langmuir en 1928 pour décrire la région d'une décharge électrique produite dans un gaz et dans laquelle la quasi-neutralité est vérifiée.

Le principal avantage d'avoir recours au plasma, dans le cadre de la stérilisation, est qu'il nous procure un milieu riche en espèces chimiquement réactives résultant de processus d'excitation, de dissociation et d'ionisation à partir de vapeurs ou de gaz [12, 18-20]. À titre d'exemple, parmi les espèces actives attendues dans une décharge contenant principalement O_2, on retrouve des radicaux tels que $O_2^{\bullet}$, $OH^{\bullet}$, $NO^{\bullet}$, mais aussi des espèces neutres comme H_2O_2, O_3 [7].

1.4.1 Le plasma : principe général

Exposition directe/indirecte

Dans le domaine de la stérilisation, il existe deux possibilités quant à l'utilisation d'une décharge plasma: les objets à stériliser peuvent être exposés

directement à la décharge (stérilisation en décharge) ou bien, ils peuvent recevoir les effluents du plasma (stérilisation en post-décharge) [9]. La principale limitation de la stérilisation par plasma dans ces deux cas est sans doute le faible pouvoir de pénétration des espèces actives [15, 21].
En ce qui concerne les nouveaux biomatériaux composés de polymères qui, pour la plupart, sont thermosensibles [22], on privilégiera la stérilisation en post-décharge [23, 24]. Dans ce cas, les espèces transportées sont majoritairement électriquement neutres [25].

L'avantage d'avoir recours au plasma, dans le cadre de la stérilisation, est qu'il peut nous procurer un milieu riche, non seulement en espèces chimiquement réactives, mais aussi en photons notamment ceux reconnus pour leur action biocide.

Agent d'inactivation : le rayonnement UV

D'un point de vue photobiologique, l'effet biocide optimal du rayonnement UV correspondrait aux radiations de la gamme des UV-C (200-290 nm) [8, 26, 27] pouvant induire, d'après la littérature scientifique, une dimérisation des bases de thymine au niveau des hélices de l'ADN (Sec.1.4).

Selon la nature du plasma, l'impact des UV sur les microorganismes différera : en effet, contrairement aux plasmas basse pression dans lesquels les UV jouent un rôle essentiel dans le processus d'inactivation, la plupart des mélanges gazeux utilisés dans les plasmas froids à la pression atmosphérique (PA) [28-30] n'émettent pas ou peu, aux longueurs d'ondes germicides, un flux de photons UV suffisant. Ceci explique que le rôle et l'importance du rayonnement UV dans le processus de stérilisation par plasma à la PA [31] restent assez controversés. En fait, l'attaque chimique des microorganismes, comme principal mode d'action du plasma, semble faire l'unanimité [32, 33]. Néanmoins, dans certaines conditions opératoires (notamment le mélange de gaz utilisé), la possibilité d'atteindre la stérilité a été démontrée [34].

Agent d'inactivation : les espèces chimiquement réactives

Dans le cadre de la stérilisation, l'intérêt des décharges plasma haute pression repose, le plus souvent, sur la nature et la densité des espèces actives créées par les processus collisionnels impliqués. Depuis une décennie, un grand nombre d'auteurs [10, 16, 32] a pu montrer que les décharges utilisant un mélange gazeux contenant O_2 ont un effet germicide, qui s'expliquerait par la présence de composés oxygénés comme $O^{\bullet}$, O_3, O_2 métastable... Cette constatation a accru l'intérêt porté à ce type de décharge et a motivé un grand nombre de travaux scientifiques [35, 36].

Dans une publication portant sur la stérilisation par plasma à la PA, Kuzmichev conclut que *"les meilleurs effets bactéricides du plasma sont atteints dans l'air et l'oxygène humidifiés"* [31]. L'humidité, comme nous le verrons par la suite, favorise la présence de radicaux tel que $HO^{\bullet}$ supposés avoir un rôle actif et oxydant sur les composants externes des cellules bactériennes. Dans le cas d'une décharge créée dans l'air, les produits de réaction tels que les oxydes d'azote (NO et NO_x) peuvent avoir une action létale sur les microorganismes [12, 31]. Quant à la présence de O_2 dans une décharge, elle mène naturellement à la génération de O_3. L'efficacité de stérilisation des décharges contenant une concentration élevée de O_3 laisse supposer que l'ozone jouerait un rôle essentiel dans les mécanismes d'inactivation [10].

Conscient de l'importance de disposer d'une atmosphère contenant des espèces oxydantes pour optimiser l'action létale sur les microorganismes, nous allons nous intéresser plus particulièrement à l'ozone et nous présentons, dans la section suivante, la génération d'ozone par une méthode plasma.

1.4.2 Génération de O_3 par plasma : exemple de la Décharge à Barrière Diélectrique (DBD)

L'utilisation de ce type de décharge a été suggérée en 1857 pour la désinfection de l'eau: ce fut sa première application dans le cadre de l'inactivation de microorganismes. Parmi les différentes applications industrielles de la DBD, nous pouvons évoquer évidemment la génération de O_3, la destruction de polluants, le traitement de surfaces, les lampes à excimère et fluorescentes, les écrans plats à

plasma, etc. Toutefois, l'ozone peut aussi être généré par irradiation UV (de 175 à 210 nm) [37]. Actuellement, la forte production d'ozone nécessaire aux applications industrielles est généralement assurée par des DBD entretenues dans l'oxygène pur ou dans l'air. La génération de O_3 dans l'air est chimiquement plus complexe, de par la présence d'azote et de vapeur d'eau, donnant lieu à la formation de nouveaux sous-produits tels que les oxydes d'azote (NO_x).

Principe général

Une décharge à barrière diélectrique s'obtient en appliquant une haute tension alternative entre deux électrodes dont, au moins, l'une est recouverte d'un matériau diélectrique permettant ainsi d'éviter le passage à l'arc. Le claquage du gaz, entre les électrodes, est caractérisé par la propagation locale de fronts d'ionisation, couramment appelés "streamers" dans la littérature scientifique. Il s'ensuit l'établissement de filaments de plasma de diamètre de l'ordre de 100 µm et dont la durée de vie est très courte, typiquement une centaine de nanosecondes.

L'écart entre les deux électrodes et l'épaisseur du diélectrique est typiquement de quelques millimètres. Selon les conditions d'excitation électrique, le plasma d'une décharge DBD peut être très inhomogène dans l'espace inter-électrodes.

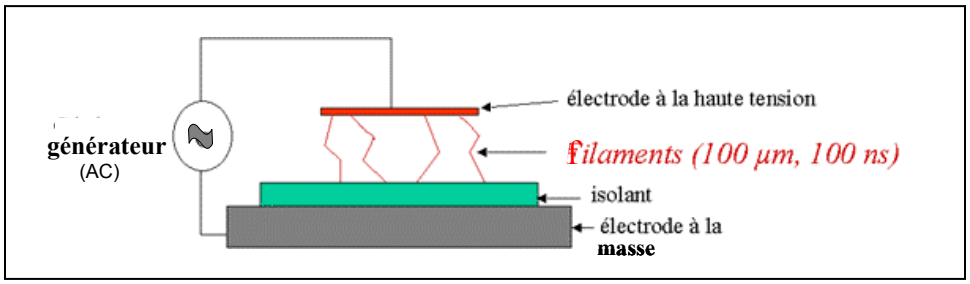

Figure 1.3: Schématisation des éléments qui composent une DBD.

Lorsque le champ électrique dans l'espace inter-électrodes est assez intense pour causer un claquage à la PA, on peut alors observer la formation d'un grand nombre de microdécharges (streamers) [38] : dans ce mode filamentaire, la conductivité électrique est physiquement restreinte aux microdécharges entre

lesquelles se trouve un gaz non ionisé servant à absorber l'énergie thermique dissipée par ces filaments de plasma, tel un réservoir, et permettant aussi la collecte et le transport de certaines espèces issues de la décharge [39]. La physico-chimie de la décharge a donc lieu dans ces microdécharges qui occupent une petite fraction du volume de la DBD et dont le nombre par unité de temps et de surface croît avec la densité de puissance.

Rôle du diélectrique. Le rôle du diélectrique est double [38]: il limite la quantité de charges et d'énergie transmise à la microdécharge et, en même temps, permet la distribution des microdécharges sur toute la surface de l'électrode. Ainsi, un diélectrique en bon état évite assurément tout claquage dans la région inter-électrodes. La fréquence de fonctionnement des décharges DBD varie habituellement entre *~1 kHz* et *10 MHz*; typiquement, la gamme *0.5-5 kHz* est utilisée afin d'éliminer tout risque d'endommagement du diélectrique. Préférentiellement, on choisira comme diélectrique le verre (avec une permittivité relative $\varepsilon_{verre}^{(borosilicate)} = 4.97$) ou encore la silice fondue ($\varepsilon_{silice\ f.} = 3.78$).

Distance inter-électrodes. La distance inter-électrodes, selon les conditions opératoires et l'application visée, peut varier de 1 mm à quelques cm. Un espace inter-électrodes étroit est souvent nécessaire pour deux raisons [39, 40] : il permet de restreindre l'augmentation de la température du gaz et, d'autre part, dans le cadre de la génération d'ozone, d'atteindre de hautes concentrations en O_3.

Physico-chimie de la décharge

D'une manière générale, dans la plupart des plasmas, les chemins réactionnels sont dominés par les réactions impliquant des particules chargées. Au contraire, dans la majorité des applications de la décharge DBD, la plupart des espèces chargées ont disparu avant même que l'on puisse observer un changement au niveau des surfaces interagissant avec le plasma. Dans ce cas, il est plus approprié de parler de *chimie des radicaux libres*, mettant en jeu principalement les espèces neutres, les fragments moléculaires, ainsi que les molécules excitées, et non les électrons ou les ions.

Chaque microdécharge plasma peut donc être considérée comme un réacteur chimique élémentaire.

Principe. Les premiers temps de la formation de la décharge sont caractérisés par la multiplication des électrons et par les phénomènes d'excitation, de dissociation et d'ionisation dus aux électrons de forte énergie. Les espèces réactives et oxydantes sont principalement générées par impacts électroniques [41]. En l'occurrence, à la PA, la densité électronique peut varier de 10^{14} à 10^{15} cm^{-3} , la densité de courant pouvant atteindre 100 à 1000 A/cm^2 : ces valeurs ont été confirmées aussi bien expérimentalement que par les modèles théoriques [38].
La génération de ces espèces pose les *conditions initiales* pour l'ensemble des réactions chimiques qui s'ensuivent : en effet, les espèces atomiques/moléculaires ioniques ou excitées enclenchent des réactions chimiques qui sont à l'origine de la synthèse d'espèces nouvelles comme O_3, ou de la destruction de polluants comme les composés organiques volatils, l'ammoniac, les sulfures...

L'échelle de temps des différents processus. À la PA, le nombre de collisions par seconde devient important: les électrons atteignent les conditions de stationnarité dans une échelle de temps de l'ordre de la ps [38]. Les microdécharges se développent dans une échelle de temps de l'ordre de la ns [42] : typiquement, on trouve ~10^6 microdécharges/cm^2 s. Les réactions faisant intervenir les radicaux libres peuvent durer de quelques µs à quelques ms [42]. Les réactions chimiques impliquant l'état fondamental prennent, quant à elles, plus de temps. La formation de O_3 dans l'air est plus longue que dans O_2 seul, soit respectivement 100 µs et 10 µs [38].

La dissociation, une étape importante. Typiquement, la première étape est la dissociation par collisions électroniques des espèces moléculaires initialement présentes. En l'occurrence, dans le cadre de la génération d'ozone, la dissociation par impacts électroniques des molécules N_2 et O_2 a été très étudiée. Par exemple, la dissociation de l'oxygène peut devenir très efficace pourvu que les énergies

électroniques moyennes soient de 4 à 8 eV: de cette façon, plus de 85 % de l'énergie des électrons est utilisée pour la dissociation.

Suivant l'application visée, il existe une concentration (ou flux) optimale de O_2 qui permet de maximiser la production de O_3 , la raison étant que O_2 est à la fois source d'oxygène atomique et un réactif de ce dernier [41].

Cinétique de formation de O_3 à partir de l'oxygène dans une décharge à la pression atmosphérique. Il est très important de pouvoir contrôler les paramètres physiques, directement reliés aux propriétés intrinsèques d'une microdécharge, afin d'optimiser la cinétique de formation de O_3 [42-44]. Initialement, la majeure partie de l'énergie gagnée par les électrons permet d'obtenir des espèces dissociées dans un état excité. La distribution des électrons de haute énergie détermine la quantité d'ozone produite [41]. Si l'on considère une collision électronique avec O_2, deux chemins réactionnels menant à sa dissociation sont possibles passant, respectivement, par les états de O_2 excités $A^3\Sigma_u^+$ et $B^3\Sigma_u^-$ ayant une énergie-seuil respective de 6 eV et 8.4 eV.

$$e + O_2 \longrightarrow e + O_2\,(A^3\Sigma_u^+) \longrightarrow e + O(^3P) + O(^3P) \qquad (4)$$

$$e + O_2 \longrightarrow e + O_2\,(B^3\Sigma_u^-) \longrightarrow e + O(^1D) + O(^3P) \qquad (5)$$

L'ozone est ensuite formé en deux étapes à partir d'une réaction à trois corps :

$$O + O_2 + M \longrightarrow O_3^* + M \longrightarrow O_3 + M^* \qquad (6)$$

M étant un troisième corps pouvant être O, O_2, O_3 ou N_2 dans le cas de l'air.

L'échelle de temps de formation de l'ozone dans l'oxygène à la PA est de quelques µs.

Les réactions consommant l'oxygène atomique et, par conséquent, en compétition avec celles conduisant à la formation d'ozone, sont les suivantes :

$$O + O + M \longrightarrow O_2 + M \qquad (7)$$

$$O + O_3 + M \longrightarrow 2O_2 + M \qquad (8)$$

$$O + O_3^* + M \longrightarrow 2O_2 + M \qquad (9)$$

Notons que les réactions de formation (équations 4, 5, 6) et de destruction (équations 7, 8, 9) de O_3 ont lieu de manière concomitante dans l'espace inter-électrodes.

1.5 Conclusion

En somme, l'étude de la stérilisation à l'ozone nécessite une approche multidisciplinaire faisant appel à la fois à la connaissance des limites et enjeux actuels dans le domaine de la stérilisation-désinfection, à des notions de physique et chimie des plasmas, à des notions de microbiologie, ainsi qu'aux interactions multiples entre ces différents domaines. Dans le chapitre 2 qui suit, nous examinons plus particulièrement quelques propriétés intrinsèques de l'ozone pour mieux appréhender son action sur les microorganismes ; ainsi, le second volet du prochain chapitre se concentrera sur l'étude de l'inactivation de microorganismes par l'ozone.

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CHAPITRE 2

Ozone : propriétés et action stérilisante

2.1 Introduction

Tel que rapporté au chapitre 1, l'efficacité de stérilisation des décharges contenant une concentration élevée de O_3 laisse supposer que l'ozone jouerait un rôle essentiel dans l'inactivation des microorganismes [1]. L'ozone serait, par exemple, transporté vers la surface du microorganisme où auraient lieu des réactions hétérogènes d'oxydation [2, 3]. Pour bien appréhender les mécanismes possibles, un relevé exhaustif des principales propriétés physico-chimiques de l'ozone s'impose. Une telle étude permettra de caractériser cet agent stérilisant et servira de base pour examiner et mieux comprendre son action sur les microorganismes.

Pour tenter de connaître les mécanismes d'inactivation mis en jeu, il est important, dans un premier temps, d'identifier les agents actifs et d'envisager leur degré d'interaction avec le substrat (c.-à-d. le microorganisme). Pour ce faire, un travail de caractérisation recourant à diverses méthodes de diagnostic a été effectué dans l'enceinte de traitement afin de déterminer les espèces présentes en phase gazeuse et sur le microorganisme afin d'évaluer les dommages occasionnés suite à son exposition à l'ozone. À titre d'exemple de diagnostics, citons la spectroscopie optique d'absorption et la spectroscopie infra-rouge (FTIR) utilisées pour la caractérisation des espèces en milieu gazeux. D'autres diagnostics physiques tels que l'imagerie (microscopie électronique à balayage (MEB)) et des évaluations microbiologiques telles que la coloration des spores ou encore la mesure d'activité enzymatique, sans oublier la détermination de la viabilité des microorganismes, ont permis d'évaluer l'effet du traitement à l'ozone sur ces derniers.

De nombreux travaux rapportés dans la littérature font état d'expériences réalisées sur divers microorganismes traités à l'ozone. Toutefois, les études portant sur la stérilisation à l'ozone en milieu sec se retrouvent en très petit nombre comparativement à celles menées en milieu humide et liquide. En effet, le traitement en milieu sec semble avoir démontré une très faible efficacité d'inactivation de sorte que la communauté scientifique a globalement délaissé cette voie au profit des études en milieu humide (voire liquide), qui représentent un grand intérêt puisque les taux d'inactivation atteints sont, de loin, bien supérieurs. Cependant, aussi surprenant que cela puisse paraître et compte tenu des applications industrielles et hospitalières de l'ozone, les recherches effectuées sur la stérilisation par O_3, aussi bien en milieu liquide que gazeux humidifié, n'ont toujours pas permis une identification rigoureuse des espèces et des mécanismes menant à l'inactivation des micro-organismes. À l'heure actuelle, on trouve dans la littérature scientifique des hypothèses relativement pertinentes mais non encore rigoureusement validées. Dans ce chapitre, nous étudierons l'inactivation de microorganismes par l'ozone gazeux (sec/humide) après avoir rappelé ses principales propriétés physico-chimiques intrinsèques.

Rappelons qu'à faible dose, l'ozone est utilisé comme désinfectant dans des atmosphères confinées et qu'à grande échelle, il est essentiellement utilisé pour désinfecter les eaux potables tendant à y supplanter le chlore. Ainsi, quelle que soit l'application visée où il y a présence humaine, la *concentration d'ozone ne doit pas dépasser 0.2 mg/m^3*. À cet effet, il existe des normes d'utilisation imposées par la législation. À titre d'exemple, aux États-Unis, la réglementation de 1997 stipule qu'un individu ne doit pas être exposé à une concentration d'ozone supérieure à :

- 0.1 ppm par volume (0.2mg/m^3 NTP (conditions normales de température et de pression)) sur une base de 8h/jour
- 0.2 ppm par volume (0.4mg/m^3) pour un temps d'exposition ne dépassant pas 10 min.

Ces valeurs de teneurs seuils d'ozone tolérables sont aussi valables au Royaume-Uni, en Europe et au Canada. Il est à noter que l'odeur spécifique attribuée à l'ozone peut être détectée à partir d'une concentration aussi faible que 0.01-0.05 ppm.

Ainsi les précautions nécessaires, telles que l'utilisation d'une hotte, de gants résistants à O_3, de masque [4], doivent être prises pour se protéger d'éventuels accidents (inhalation, fuites, explosions…) liés aux activités nécessitant la manipulation d'ozone [5].

2.2 Propriétés physico-chimiques de l'ozone

La connaissance des propriétés physico-chimiques de la molécule d'ozone est fondamentale pour comprendre son interaction avec le milieu gazeux et les microorganismes. À titre d'exemple, la forte réactivité et la relative instabilité chimique de O_3 permettent d'en faire un agent stérilisant efficace si l'on considère que les problèmes généralement liés à la présence résiduelle d'agents chimiques inactivant en fin de procédé de stérilisation sont, dans le cas de l'ozone, écartés : en effet, non seulement sa durée de vie est limitée mais une décomposition plus rapide de la molécule peut être tout à fait envisageable [6].

2.2.1 Généralités

L'ozone fut découvert en 1839 par C.F Schonbein; il faudra attendre J.L Soret pour démontrer, en 1865, l'existence de l'ozone moléculaire de masse molaire 47.998 $gmol^{-1}$ [7]. La géométrie de la molécule O_3 est représentée par une structure coudée (Figure 2.1): la distance inter-oxygène (0.127 nm) est plus courte que celle de la simple liaison O-O (0.143 nm) mais supérieure à celle de la molécule d'oxygène O=O (0.121 nm). L'énergie de liaison associée à O_3 vaut 6.12 eV. Les fréquences fondamentales de vibration de la molécule sont: ν_1= 1110 cm^{-1}, ν_2= 705 cm^{-1}, ν_3= 1043 cm^{-1}. Ces fréquences sont utilisées pour détecter et titrer O_3 par spectroscopie IR.

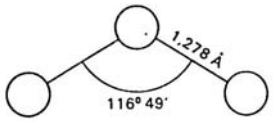

Figure 2.1 : Représentation géométrique de la molécule d'ozone (d'après [7]).

La réaction de formation est endothermique:

$$3\ O_2\ (+\ 2Q_c\ ) \longleftrightarrows 2\ O_3 \qquad (10)$$

Qc étant l'énergie (sous forme de chaleur) nécessaire à cette réaction chimique.

Son enthalpie standard de formation vaut environ 147 kJ/mol. Extrêmement instable (durée de vie de 100 à 200 s dans la stratosphère), l'ozone tend à se décomposer pour donner O_2 (avec lequel il se trouve pratiquement toujours en équilibre) et $O^{\bullet}$ (chimiquement très réactif : t<<1s): l'obtention de O_3 est globalement conditionnée par l'équilibre (10).

Sa présence est indispensable dans l'atmosphère puisque la couche d'ozone filtre la quasi-totalité du rayonnement UV d'origine solaire (97-99%) qui, dans le cas contraire, aurait éliminé toute trace de vie sur terre : son pic d'absorption est situé à la longueur d'onde λ~254nm.

2.2.2 Stabilité et décomposition

En laboratoire, il fut démontré que l'ozone est relativement instable en solution aqueuse : sa demi-vie dans l'eau distillée à 20°C est estimée à 20-30 min; toutefois, certains auteurs rapportent que, dans ces mêmes conditions, sa durée de vie serait de 20 min, d'autres de 165 min, voire même de 2 à 4 min ! Ces différences se

justifieraient par les conditions expérimentales utilisées par les opérateurs (pH, température…). En réalité, les espèces organiques et inorganiques facilement oxydables qui contaminent le milieu de traitement [8, 5] réagissent avec O_3, ce qui a pour effet de réduire sa demi-vie [9,10]. À titre de comparaison, la demi-vie de l'ozone gazeux varie de quelques heures à 3 jours (en supposant, dans ce dernier cas, que la décomposition soit purement thermique) [9].

Les chemins cinétiques menant à la décomposition de l'ozone sont les suivants:

Décomposition thermique:

L'ozone peut être converti en O_2 s'il y a un apport extérieur d'énergie sous forme de *chaleur* : l'ozone devra, pour ce faire, être porté à une température T >300 ^{o}C pour une durée de 3 s environ [11].

Sa décomposition suit le schéma suivant : $2\ O_3 \xrightarrow{T} 3\ O_2$ (11)

Décomposition radiative

La décomposition de O_3 peut être accélérée en présence d'un rayonnement ″approprié″ [12] : $O_3 + h\nu_{UV} \longrightarrow O_2 + O^*$ $\lambda \approx$[242-320] nm (12)

Décomposition catalytique

La présence d'un catalyseur (charbon, métal) accélère la décomposition de O_3 selon la réaction : $O_3 + Cat. \longrightarrow O_2 + O_{catalysé}$ (13)

La décomposition catalytique au moyen de l'hopcalite [11] est de plus en plus utilisée dans le milieu industriel, notamment en fin de procédé.

Décomposition chimique dans l'eau pure

Dans l'eau pure, O_3 se décompose, avec une cinétique dépendant de la température par une série de réactions radicalaires en chaîne, complexes, produisant des espèces intermédiaires comme le radical hydroxyle ($OH^{\bullet}$).

De manière générale, la présence d'un troisième corps M dans le milieu a pour effet d'accélérer le mécanisme de décomposition selon les réactions :

$$M + O_3 \longrightarrow M + O_2 + O \quad (14)$$

$$O + O_3 \longrightarrow 2\,O_2 \quad (15)$$

M pouvant être CCl, Br, N_2O_5, CO_2…

2.2.3 Réactivité chimique

Généralement, les composés organiques et inorganiques réagissent avec O_3 de deux façons [10] :

(a) réaction directe d'un composé organique (M) avec O_3 entraînant son oxydation

$$(M_{ox}) : O_3 + M \longrightarrow M_{ox} \quad (16)$$

(b) décomposition de O_3 dans l'eau en radical (exemple $OH^{\bullet}$), qui réagit ensuite avec

$$M : O_3 \xrightarrow[OH^-]{} OH^{\bullet} \xrightarrow[M]{} M_{ox} \quad (17)$$

Les réactions organiques avec O_3 sont sélectives [13-15]: en effet, la chimie de O_3 reste essentiellement gouvernée par sa nature électrophile. Toutefois, la molécule d'ozone peut théoriquement être représentée comme un dipôle (Figure 2.2), ce qui explique qu'elle puisse agir en mettant à profit aussi bien ses propriétés nucléophiles[1] (possible lorsqu'il existe dans une molécule un site déficitaire en électrons comme c'est le cas d'un carbone porteur d'un groupement électronégatif) qu'électrophiles[2].

---

[1] Nucléophile : C'est un composé qui ″aime″ les sites chargés positivement : il participe, dans une réaction chimique, en *cédant* des électrons à une espèce donnée (électrophile) dans le but de former une liaison chimique. Les anions et toutes les molécules ou ions ayant une paire d'électrons libres peuvent agir comme des nucléophiles.

[2] Électrophile : C'est un composé qui, littéralement, ″aime″ les électrons : il participe, dans une réaction chimique, en *acceptant* une paire d'électrons d'une espèce donnée (nucléophile) dans le but de former une liaison chimique avec cette dernière. Comme ces électrophiles sont des accepteurs d'électrons, ils sont souvent chargés positivement.

Figure 2.2 : Structure de résonance de l'ozone ; δ représentant la charge partielle portée par l'atome.

L'ozone est un puissant agent oxydant que ne surpasse que le fluor (2.87 V) mais, moins oxydant que les espèces radicalaires $O^{\bullet}$ et $OH^{\bullet}$ que l'on peut trouver en solution et dont le potentiel redox vaut, respectivement, 2.43 V et 2.80 V (Tableau 2.1). En effet, l'ozone oxyde le fer, dissous le manganèse et les composés organiques [12], attaque de plus tous les métaux sauf l'or et le platine, et recouvre l'argent d'une couche d'oxyde. La réaction d'oxydation de l'iodure de potassium est utilisée pour le dosage de l'ozone en milieu liquide.

Espèces oxydantes	Potentiel Redox (V)
Radical hydroxyle	**2.80**
Oxygène radicalaire	**2.43**
Ozone	**2.07**
Peroxyde d'hydrogène	**1.78**
Permanganate	**1.69**
Dioxyde de chlore	**1.56** (**1.27** d'après Ozonia)
Dichlore	**1.36**
Oxygène moléculaire	**1.23**

Tableau 2.1 : Potentiel d'oxydo-réduction de différents composés reconnus pour leur propriété oxydante (d'après [16]).

2.3 Étude de l'inactivation de O_3 sur les microorganismes

L'ozone ayant un fort pouvoir oxydant, nous présentons au début de cette section quelques processus d'oxydation pouvant avoir lieu lorsque les microorganismes se trouvent sous atmosphère oxydante. Cette base étant établie, le second volet de cette section aura pour but d'identifier les mécanismes d'inactivation mis en jeu : pour ce faire, il est important dans un premier temps d'identifier les

agents actifs puis d'envisager leur degré d'interaction avec le substrat (dans ce cas, les microorganismes).

2.3.1 Processus d'oxydation

Les travaux rapportés dans la littérature ont montré que l'exposition de microorganismes à une atmosphère oxydante est très souvent létale pour ces derniers [17,18] ; d'ailleurs, rappelons qu'une partie des techniques conventionnelles de stérilisation utilisait déjà des espèces oxygénés aussi bien radicalaires que neutres. À titre d'exemple: dans un plasma, l'ajout d'une concentration de 10-30 % de peroxyde d'hydrogène, connu pour ses propriétés désinfectantes, suffit pour induire un important effet biocide sur les microorganismes: ce qui pose la question du rôle des radicaux et, plus particulièrement des radicaux hydroxyles, dans le procédé de stérilisation. Toutefois, selon certains auteurs, l'efficacité de stérilisation des décharges contenant une concentration élevée d'ozone laisse supposer que la molécule O_3 jouerait un rôle essentiel dans les mécanismes d'inactivation [1].

Principe

L'étude théorique de l'oxydation d'une surface de polymère (illustrée dans la figure 2.3) a permis, d'une part, de modéliser, par analogie, les processus pouvant avoir lieu à la surface de microorganismes tels que les spores et, d'autre part, de considérer quantitativement et qualitativement l'effet d'endommagement des espèces actives sur les objets à stériliser [19].

Nous allons brièvement définir deux types d'oxydation qui, dans une décharge plasma, sont principalement dus à la présence de radicaux : l'oxydation en surface et l'oxydation en volume.

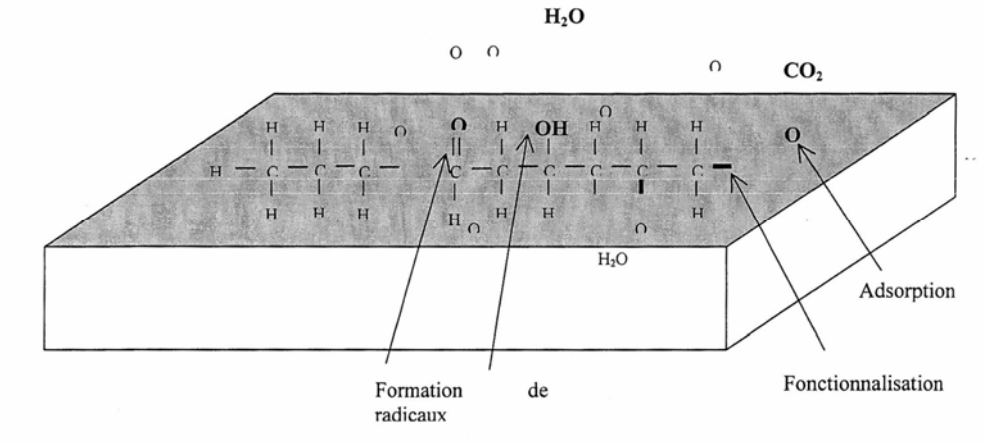

Figure 2.3 : Représentation schématique de l'oxydation et de l'érosion d'une surface de polyéthylène par un plasma d'oxygène (d'après [19]).

L'ozone est transporté vers la surface du microorganisme où ont lieu des réactions hétérogènes d'oxydation [2,3]. D'un point de vue théorique, les oxydations en surface se traduisent, entre autres, par la formation de groupements carbonyles (C=O) et de groupements hydroxyles (OH), donnant lieu à des réticulations[3] :

Oxydation $RH + O^{\bullet} \longrightarrow R^{\bullet} + HO^{\bullet}$

Réticulation $R^{\bullet} + R^{\bullet} \longrightarrow RR$

R étant une molécule organique dont la chaîne principale est essentiellement une chaîne carbonée; $R^{\bullet}$ est une molécule organique radicalaire (possédant un électron non apparié).

L'oxydation en volume dépendrait principalement de la structure du microorganisme. D'après Nicholson *et al.*, il semble que la diffusion des espèces actives (radicaux…) vers le ″coeur″ de la spore *B. atrophaeus* soit, compte tenu de considérations structurales, limitée par la tunique externe qui jouerait alors le rôle de barrière, limitant ainsi ce processus [20].

[3] Réticulation : Branchement de chaînes de polymères entre elles par des ponts ou liaisons chimiques, sous l'action par exemple de radiations, afin de constituer un réseau de masse moléculaire plus élevée et présentant des propriétés physico-chimiques différentes du polymère initial; la réticulation est un processus irréversible.

2.3.2 Inactivation de spores et bactérie par l'ozone gazeux en milieu sec

Comme nous l'avons dit auparavant, les recherches effectuées sur la stérilisation par O_3, aussi bien en milieu liquide que gazeux, n'ont toujours pas permis une identification rigoureuse des espèces et des mécanismes menant à l'inactivation des micro-organismes. De plus, les recherches sur la stérilisation par l'ozone gazeux en milieu sec sont rares : cela est regrettable si l'on considère que la mise en évidence des mécanismes d'inactivation en milieu sec est plus simple et permettrait d'abord, de façon différentielle, la compréhension des phénomènes en milieu humide où les mécanismes sont plus complexes.

En effet, les espèces chimiques, issues de la décomposition de O_3 dans l'eau, telles que le radical hydroxyle, peuvent devenir les agents oxydants les plus importants présents en solution. Contrairement à l'ozone, la réactivité du radical $OH^{\bullet}$ est non sélective et ce dernier réagit pratiquement avec tous les composés organiques (en captant un atome H) : ainsi, à peine formé, le radical $OH^{\bullet}$, d'une durée de vie de l'ordre de la µs, réagit rapidement avec les composés organiques avec un coefficient de réaction de l'ordre de 10^{13} s^{-1}. À l'heure actuelle, comme nous l'avons déjà indiqué, il est possible de trouver dans la littérature scientifique des hypothèses relativement pertinentes sur les types d'interaction avec les micro-organismes mais pas encore rigoureusement validées.

Afin de mieux appréhender la complexité de l'étude de l'inactivation par l'ozone en milieu humide, notre approche, rappelons-le, consiste, en premier lieu, à étudier et à analyser de manière exhaustive les phénomènes mis en jeu dans un milieu sec (ce qui ne semble pas avoir été fait dans la littérature scientifique, comme nous l'avons déjà souligné). Ce dernier milieu étant chimiquement moins complexe, il permettrait donc de dégager les mécanismes impliqués dans l'inactivation des spores par O_3 plus aisément, ce qui contribuerait à bâtir un raisonnement et une analyse plus rigoureuse sur les phénomènes observés en milieu humide. L'article qui suit présente une solide revue bibliographique sur le sujet ainsi que les résultats de l'étude effectuée dans nos laboratoires. Ma contribution à ce travail ainsi qu'à tous les autres articles présentés dans la thèse est décrite dans l'annexe 1 de cette thèse.

Inactivation of vegetative and sporulated bacteria by gaseous dry ozone

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Abstract. Inactivation by gaseous ozone of different types of microorganisms is successfully achieved provided, as is well known, the gaseous phase is strongly humidified. The inactivation mechanisms and species involved in this process are, however, not yet clearly identified. To gain insight, we considered exposure of bacterial spores to dry rather than humidified ozone, a less complex chemical environment. In contrast to most of the published literature, it is shown that, under strict dry ozone conditions, bacterial spores can be inactivated, but to a degree that is largely dependent on the spore type and substrate material. In this case, the O_3 molecule is determined to be responsible for the inactivation process through its diffusion into and oxidative action within the spore, as no outer erosion of the spore is detected. With humidified ozone, a higher inactivation efficiency is observed which is most probably related, in part, to the swelling of the spore, which facilitates the diffusion of oxidative species within it and up to the core; besides O_3, these oxidative agents stem from the interaction of O_3 with H_2O, which in the end leads to a heavily damaged spore structure, in contrast to dry-ozone exposure where the spore integrity is maintained.

Keywords: Ozone, dry and humidified gaseous ozone, microorganism inactivation mechanisms, vegetative bacteria, sporulated bacteria, spore structural damage

1. Introduction

1.1. Context

Ozone is a strong oxidative compound both in its gaseous form and when dissolved in water and, because of this, is known to be an efficient disinfectant for inactivating even chemically resistant microorganisms (1). Ozone can be an adequate solution where other disinfectants fail (2). Its oxidative potential is higher, for example, than that of hydrogen peroxide (3-6) and hypochlorite (7-8). Ozone was recommended as an alternative to chlorine for water treatment. On practical ground, ozone is easily generated on-site from dry air or O_2 through high-voltage corona discharges or filamentary dielectric barrier discharges at near ambient pressure and temperature (9-11). Even though ozone has been utilized in the disinfection of water for a long time, it is only recently that a humidified gaseous ozone technology has been authorized for sterilization purposes (TSO_3^{TM} sterilizer).

The growing use of heat-sensitive polymer instruments in hospitals has created new challenges in the area of sterilization. Conventional dry- and moist-heat methods, such as the Poupinel (Pasteur) oven and the autoclave respectively, can heavily damage thermosensitive materials. This is not the case with chemical sterilants such as, for example, ethylene oxide (EtO), but EtO requires long exposure and vent times (more than 10 h total) in addition to being toxic and, on the long run, carcinogenic and detrimental to the environment. As a result, EtO is already banned in many countries. Such considerations have led to look for alternate efficient sterilization processes that hopefully would operate at low temperature and inactivate rapidly (≅ 1 h) all kinds of microorganisms with low damage to medical devices (MDs) while being harmless for man and his environment. Under such requirements, both gaseous-plasma and ozone sterilization seem to offer substantial promises for sterilizing thermosensitive MDs.

1.2. A review of previous work

From its inception and for a rather long period of time thereafter, the use of O_3 as a gaseous biocide in ambient air was limited to concentration levels close to 1 ppm (as a matter of fact, 0.1 ppm was determined to be the occupational (40h/week) maximum safety-value as far as toxicity for man is concerned). In a study performed

on aerosolized bacterial suspensions in a room at known temperature and relative humidity (RH) values, Elford and Van de Eude (12) determined that concentrations in O_3 in excess of 1 ppm in the room with 60-80% RH were better conditions relatively to a dry atmosphere. Kowalski *et al* (13) studied the influence of higher concentrations of airborne ozone against *E. coli* and *S. aureus* vegetative bacteria. The microorganisms were exposed to O_3 in air, at concentrations ranging from 300 to 1500 ppm for 10 to 480 seconds with 18-20% RH: death rates in excess of 99.99% (> 4 log) were reached for both species at 1500 ppm and within 8 min.

Relying on dry gaseous ozone but at much higher concentrations, Held studied the decontamination of hospital waste (14-15) comprised of Gram-positive and Gram-negative vegetative bacteria, fungi, mycobacteria, and sporulated bacteria such as *Bacillus atrophaeus*, *Geobacillus stearothermophilus* and *Clostridium perfringens*. Ozone concentrations of 10000-12000 ppm obtained from a dry-air corona discharge were tested. This system allowed inactivation of more than 10^7 vegetative bacteria/mL (*S. aureus, B. atrophaeus, E. coli*…) within an hour of exposure time and of more than 10^7 spores/mL after two hours of treatment.

Ishizaki *et al* (16) examined the sporicidal activity of gaseous ozone on different Bacillus spores with ozone concentrations ranging from 250 to 1500 ppm (0.5 to 3 mg/L), additionally focusing on the influence of the RH level. At RH degrees of 50% or below, no appreciable decrease in the number of survivors was obtained after 6 h of exposure. However, at higher RH values, a 5 log reduction in less than 2 h was reached, which was confirmed later on by Currier *et al* (17). Aydogan *et al* (18) results also showed that increasing the O_3 concentration from 1 to 3 mg/L with a 70-95% RH level increases the inactivation rate of spores, but that beyond 3 mg/L (1500 ppm), only a weak additional increase was observed.

Three main points emerge from the above previous works: i) the higher the ozone concentration and the higher the RH (>50%), the more efficient is the inactivation process. As a matter of fact, in some cases, humidity is absolutely required to achieve sterility, as in the case of the TSO_3^{TM} sterilization system, approved by both Health Canada and FDA (US Food and Drug Administration); ii) the added value of humidified gaseous ozone with respect to dry gaseous ozone as a biocide agent is

certainly partially responsible for the fact that little work has been done under dry ozone conditions (16); iii) the inactivation mechanisms and species involved in ozone processes are, however, not yet rigorously identified according to the literature.

1.3 A review of possible inactivation mechanisms under ozone exposure

Strong oxidants are generally capable of chemical attacks on constituents of microorganisms, namely proteins, unsaturated lipids, the lipopolysaccharide layer of Gram negative bacteria, intracellular enzymes (e.g. respiratory enzymes) and nucleic acids (genetic material), as well as proteins and peptidoglycan in spore coats and virus capsids (51).

Vegetative bacteria. The inactivation of vegetative bacteria by O_3 is a complex process because ozone attacks a large number of their components; however, O_3 is believed to cause mainly protein and lipid oxidation on the bacterium cell wall and cytoplasmic membrane. The progressive degradation of these structures involves changes in permeability and cell integrity, and is often followed by cell lysis (4, 52, 53, 54). Along that line, Kim and Youssef (53) observed that damage was more pronounced for Gram-negative bacteria (*E. coli*…) probably because of their lipopolysaccharide layer than for Gram-positive ones (*S. aureus*, …) (42, 55). Hunt and Marinas (34) are proposing a different explanation for bacterium inactivation based on transmission electron microscopy (TEM) micrographs: they observed a contraction of the nucleoid of E. coli following ozone treatment. These authors concluded that O_3 was able to penetrate cells and reacts with their proteins or numerous enzymes involved in the control of DNA conformation resulting in its misfolding.

Bacterial spores. The main interest in working with bacterial spores is that they are the most resistant microorganisms and, because of that, are officially required to validate sterilization processes. Bacterial spores can withstand severe treatments including heat, irradiation, chemicals, and desiccation. The bacterial spores of Bacillus species have been shown to be particularly resistant to ozone (13) and are

therefore used in the current study. One expects a priori that bacterial spores would be less affected by O_3 than vegetative bacteria because of their multilayer protection and stress resistance. Various O_3 targets have been proposed in the literature for inducing spore lethality, which we detail:

Enzymes. Several authors referred to enzyme damage as an important inactivation mechanism by which O_3 would kill cells (56, 57). Young and Setlow (41) showed that *B. atrophaeus* mutant spores lacking a certain enzyme (second cortex lytic enzyme (SleB)) were inactivated more rapidly by O_3 than the wild-type spores (4, 41). Moreover, Takamoto *et al* (57) observed that ozone decreased enzyme activity in *E. coli* at different degrees depending on the specific nature of the enzyme considered.

DNA. It could also be a target since O_3 reacts rapidly with nucleo-bases, especially thymine, guanine and uracil (16, 39). In contrast, authors like Young and Setlow (41) claim that O_3, in water, does not kill spores by DNA damage. The case of O_3 in water is herein assimilated to ozone in a 100% RH environment.

Spore coats. Spore coat designates, starting from the spore outermost, the surface layer, then the outer coat and the inner coat. Going further inside, there are the cortex, the inner membrane and the core containing the DNA. Spore coats represent approximately 50% of the spore volume and comprise about 80% of the spore proteins, therefore constituting barriers to metabolic function damage (e.g. enzymes).

The major factor in spore resistance to biocide agents appears to be the spore coat (41, 55). This is supported by the TEM micrographs of Kim *et al* (49) in the case of aqueous O_3 treatment that reveal damage to the surface layer as well as to the outer and inner coats. Such damage opens the way to the action of O_3 on the cortex, finally causing spore inactivation through intracellular damage (41, 49). Moreover, Foegeding *et al* (58) found that *B. cereus* spores, with their coat proteins removed, were more rapidly inactivated by aqueous O_3 than intact spores: they concluded that the spore coat is a primary protective barrier against O_3 molecules. More broadly,

chemically decoated spores and spores with a defective coat (resulting from mutation in cotE, a major coat morphogenic protein) were killed much more rapidly by aqueous O_3 than spores with intact coats (41). This led Young and Setlow (41) to conclude that spore coats (especially in *B. atrophaeus* spores) are essential in spore resistance to O_3.

Inner membrane. Key proteins of the inner membrane can also be damaged by oxidizing agents, including O_3 (37). More recent findings show that the spore inner membrane could be the site of lethal injury by O_3 since a damaged inner membrane: i) prevents spores from maintaining integrity upon a normally moderate heat treatment or when their germinated form is faced with an osmotic stress and ii) because it becomes more permeable. Cortezzo *et al* (37) further demonstrated that damage to the spore inner membrane by oxidizing agents is also consistent with the observed more rapid penetration of methylamine into the core of treated spores: the inner membrane is likely the crucial permeability barrier to methylamine entry into the spore core. Rupture of this permeability barrier can possibly lead to the release of the spore core contents (4, 41).

1.4. Outline of the paper

To gain insight into the inactivation mechanisms of microorganisms following ozone exposure, we operate as a first step with dry gaseous ozone, a less complex chemical environment than humidified gaseous ozone : the addition of water vapour to gaseous ozone brings in additional oxidative agents, making it more difficult to determine their relative contribution and to identify the inactivation mechanisms. The paper focuses on the inactivation kinetics, through survival curves, of bacterial spores under strict dry ozone exposure and examines the corresponding damage using scanning electron microscopy (SEM). Such characteristic data for spores exposed to humidified ozone are also presented. A close "differential" analysis of both these sets of experimental results (dry and humidified ozone cases), making use of the literature, enables us to come up with a new and more detailed picture of the inactivation mechanisms of bacterial spores subjected to ozone.

The paper is organized as follows. Section II consists of materials and methods together with a characterization of the sterilization chamber in terms of ozone concentration and uniformity in both the dry and humidified cases. Section III concentrates on three species of bacterial spores and a vegetative bacterium to determine their survival curves and look upon their structure integrity once they have been subjected to dry gaseous ozone. Section IV considers, for comparison purposes, exposure of *B. atrophaeus* spores to humidified gaseous ozone, focusing on their inactivation kinetics, showing the extensive damage inflicted to the spores and identifying the possible chemical species involved. Section V discusses the main results on dry and humidified ozone exposure of microorganisms and correlates them to gain insight into the specific inactivation mechanisms. Section VI contains the summary and conclusion.

2. Materials and methods

Due to its high oxidative power, ozone can damage various kinds of materials more or less severely. To minimize such possible effects, which could interfere with our experiments, the sterilization chamber is made from 316 stainless steel (as required to withstand humidified ozone) and the windows used for spectroscopic observations are from fused silica. The microorganisms to be investigated are deposited on Petri dishes that are either made from polystyrene (dry ozone exposure) or Pyrex (humidified ozone exposure).

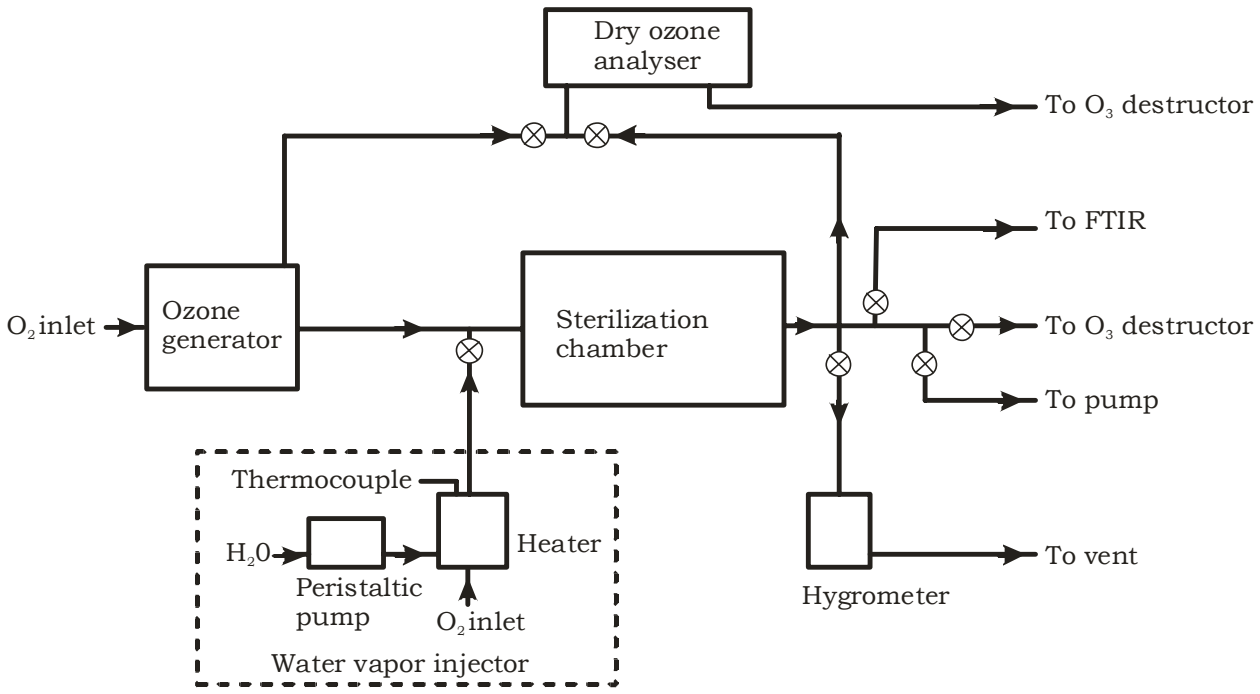

Figure 1. Schematic view of the experimental arrangement utilized to generate ozone and determine its concentration as it enters and exits the sterilization chamber. Water vapour can be added to the ozone flow and the relative humidity in the chamber measured with a hygrometer that comes with a thermometer. The nature of the effluents exiting the chamber can be analyzed through FTIR spectroscopy. An O_3-destructor (based on a chemical catalyser) is provided to abate ozone.

2.1. Ozonation system

Figure 1 shows the various elements of the system used to generate ozone and determine its concentration as it enters and exits the chamber. The chamber, made from 316 stainless steel, is a 400 mm long, 100 mm high and 220 mm wide parallelepiped (6 L volume). Ozone concentration can be monitored with an analyzer based on UV absorption. The generated effluents can also be analyzed through FTIR spectroscopy: Avatar 370 spectrometer from Thermo Nicolet using a DTGS (7800-375 cm^{-1}) detector with the number of scans and resolution set at 80 and 1 cm^{-1}, respectively. The ozone generator provides a mixture of molecular and atomic oxygen in the gas phase; it is operated within the the electrical current domain over which an increase upregulates the ozone concentration. The ozone flow is dry since the

generator is supplied from (high-purity) O_2 dry-gas bottles. The expression "dry ozone" as used herein refers to gaseous ozone having a relative humidity (RH) of less than about 2 % (determined with a hygrometer). Total gas flow is set at 5.64 standard liter/min (slm) to achieve an ozone concentration of 4 000 ppm under dry conditions.

An O_3-destructor, localised at the end of the process line, is provided to abate ozone and release it as O_2 to comply with safety (toxicity) regulations. For safety reasons also, the chamber is located within a fume hood and a vacuum dry pump is connected to make sure that the chamber effluents are fully evacuated at the end of the process.

Water vapour can be added in the process for the purpose of using humidified ozone. Water is sent through a peristaltic pump to an "oven" (heater) and the vapour produced is driven into the O_3 line by an incoming O_2 gas flow. The amount of water vapour injected, at a given temperature of the heater and given O_2 flow, depends on the H_2O flow set by the peristaltic pump. The corresponding RH level in the chamber is determined with a hygrometer (Kahn) in the sole presence of O_2 (RH up to at least 95 % can be measured accurately (±0.3%)). Total gas flow is set at 2.6 slm to achieve an ozone concentration of approximately 4 000 ppm under humidified conditions. The gas temperature in the chamber remains close to ambient (≈ 22 ^oC).

2.2 Polymer surface preparation and treatment conditions

Different types of polymer sheets (some of them used in the biomedical area) have been tested: polystyrene (PS) from Goodfellow® (ST313200), polyurethane (PU) from Johnston Industrial Plastics® (12348500), high density polyethylene (PE) from Goodfellow® (ET323100), polypropylene (PP) from Goodfellow® (PP303100), polymethylmetacrylate (PMMA) from Goodfellow® (ME303010), polymethydisiloxane (silicone) from Goodfellow® (SI303100) and polytetrafluoroethylene (Teflon®) from Goodfellow® (FP303050). Polymer sheets were cut up with appropriate techniques to provide substrates of approximately 2.5 × 2.5 cm^2. Prior to their exposure, the coupons were immersed in a (10% diluted) cleaning solution (KOH and isopropyl alcohol: Patterson) and sonicated 5 min at

ambient. They were afterwards rinsed 5 times with ultrapure and sterile water, and left to dry. Each polymer sample was further laid out on a glass Petri dish, introduced in the processing chamber (Fig. 1) and then exposed to ozone for 60 min (glass Petri dishes were used since the interaction of ozone with glass, unlike that with polymers, is expected to be minimal). The PS Petri dishes (60 x 15 mm, ref. 83.1801) came from Sarstedt.

2.3. Microorganisms: choice of vegetative and sporulated bacteria for the study, their preparation for sample deposition and number of their survivors after exposure

To characterize the biocide action of dry ozone, we have examined its effect on dried deposits of three species of bacterial spores, namely *Bacillus atrophaeus* ATCC® 9372 (formely *Bacillus subtilis*), *Geobacillus stearothermophilus* ATCC® 7953 (formerly *Bacillus stearothermophilus*), *Bacillus pumilus* ATCC® 27142, and of *Deinococcus radiodurans* vegetative bacteria ATCC® 13939. *B. atrophaeus, G. stearothermophilus* and *B. pumilus* endospores are routinely used in the validation of sterilization processes with dry heat, wet heat and gamma radiation, respectively. Moreover, *B. atrophaeus* spores are known (19-21) to be particularly resistant to ozone. *D. radiodurans* bacteria are used because of their resistance to radiation and dessication (needed feature to achieve viable dried deposits).

Preparation of microorganisms. B. atrophaeus, *G. stearothermophilus* and *B. pumilus*, endospores of Gram-positive bacilli, and *D. radiodurans* bacteria, non-spore-forming Gram-positive cocci, were prepared in the Laboratoire de contrôle des infections (Faculty of Dentistry) at Université de Montréal. The bacilli were inoculated on a sporulation medium (22) and incubated for 10 days at 37 ^{o}C for *B. atrophaeus* and *B. pumilus*, and 3 days at 56-60 ^{o}C for *G. stearothermophilus*. Spores were then collected, washed and stored at 4 ^{o}C. Viability of the spores was determined by plating on Trypticase Soy Agar (TSA) (23). *D. radiodurans* bacteria were collected after amplification in a nutritive medium at 30 ^{o}C for two days (24).

Sample preparation. Deposits of 10^6 spores in 100 µL of water are made in the center of 60 mm-diameter sterile polystyrene (Starstedt®) or Pyrex Petri dishes. *D. radiodurans* bacteria are gathered in the same fixed volume of 100 µL, but their number was sometimes much less than the intended 10^6 (sec. 3.1.4). These deposits are then dried out under similar ambient conditions and protected from light before being subjected to ozone treatment.

Pyrex Petri dishes were used as the carrier material when working with humidified ozone as is customary in such conditions (16). This is because O_3 interaction with Pyrex, as opposed to polystyrene, is expected to be minimal,

Determination of the number of survivors after exposure. Figure 2 shows the various steps involved in microorganism recovery. After exposure, a 5 ml volume of solution containing 0.5% Tween 80 ™ in saline (150mM NaCl) is added to the Petri dish and microorganisms are released from it by mechanical scrubbing using a sterile swab. The harvested bacteria are vortexed, serially diluted and various volumes (50 to 200µL) of the different dilutions are spread out onto Trypticase Soy Agar plates. When viability is expected to be very low (less than 100 microorganisms), survivors are all collected through membrane filtration. The number of colony forming units (CFUs) is determined after various periods of incubation at specific temperatures: for spores, namely *B. atrophaeus* (37 ^{o}C for 24h), *G. stearothermophilus* (56 ^{o}C for 48h) and *B. pumilus* (37 ^{o}C for 24h), and for *D. radiodurans* (ambient temperature for 4 days). Non exposed controls are recovered at the same time as the exposed microorganisms. Specific germination inducers (aniline, dipicholinic acid/calcium, lysozyme) were used to ensure that the inactivation of exposed spores was permanent.

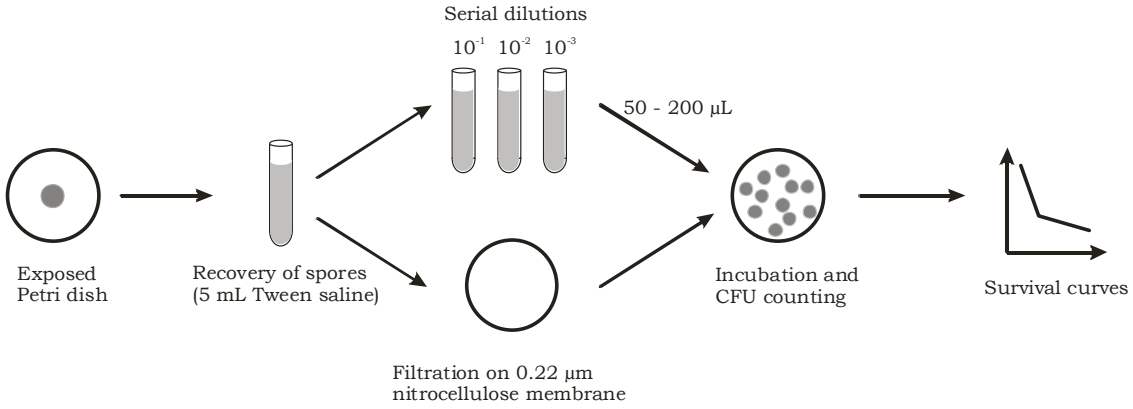

Figure 2. Diagram showing the different steps of microorganism recovery after their exposure to ozone.

2.4. Spatial distribution of gaseous ozone concentration in the chamber through optical absorption spectroscopy

A fused silica window extends axially on both sides of the chamber (figure 3) allowing visual observations and spectroscopy measurements. Axial distribution of the ozone concentration, at two different heights (y axis), is obtained through an optical absorption spectroscopy system that is movable axially (x axis) and can probe the chamber at approximately ½ and ¾ of the window height, as schematized in figure 3. A deuterium lamp is used as a continuum light source, located at the focal length of lens L_1 that provides a parallel beam directed transversally to the chamber (z axis). On the other side of the chamber, at the same x and y positions, a diaphragm (figure 3) admits only part of the light beam transmitted across the chamber, which is then focused with lens L_2 on the input side of an optical fibre linked on its other extremity to the entrance slit of a 320 mm focal length spectrophotometer.

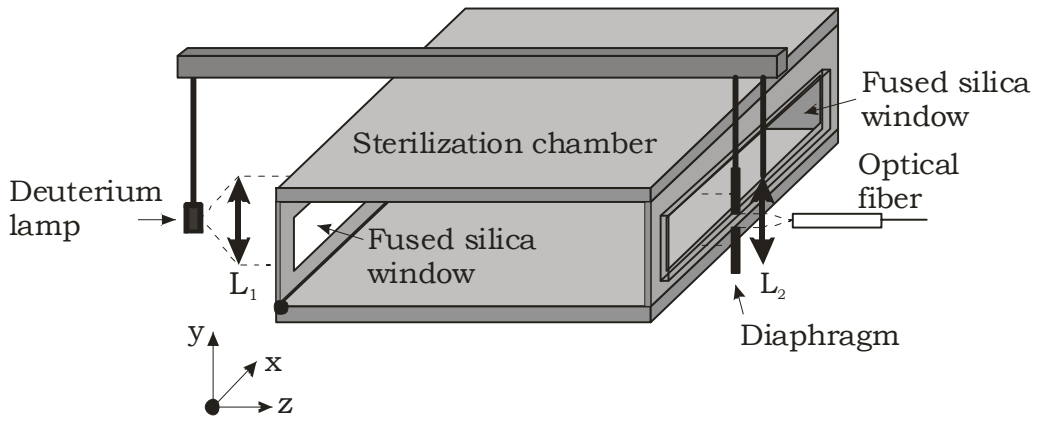

Figure 3. Schematic view of the optical-absorption measurement system probing the z-axis; it is movable along the x-axis and can be positioned at different heights y. The spectral source is a deuterium lamp that provides significant continuum emission intensity in the 240-400 nm range.

2.4.1. Spectral characteristics of ozone absorption. Figure 4(a) shows the remaining intensity of a UV beam from a deuterium lamp after it has crossed the chamber transversally (along the z axis, figure 3) as a function of wavelength, at mid-axial and mid-height positions (x = 200 mm, y = 44.5 mm, respectively). The gas flowing in the chamber is either pure O_2 (ozonator off) or O_2 partially converted into ozone (4000 ppm). Ozone molecules absorb UV radiation in a wide range of wavelengths (240 - 330 nm) [1], as shown in figure 4 (b) that displays the absorbance curve for dry and also humidified gaseous ozone. Full UV absorption in the dry case is close to 285 nm but extends on a larger wavelength range in the humidified case because of radical species such as $HO^{\bullet}$ and $HO^{\bullet}_2$, which absorb radiation in the 230-320 nm and 200-250 nm regions, respectively (25-28).

[1] It corresponds to the Hartley molecular band (29-33).

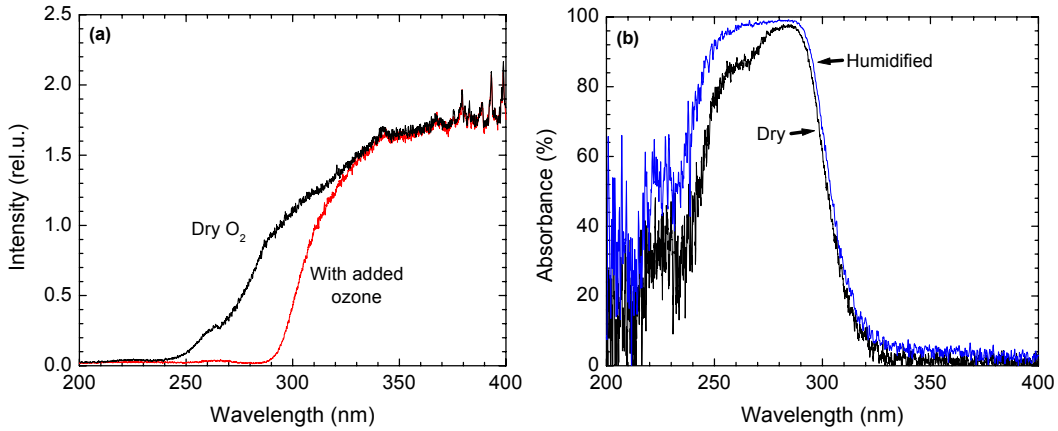

Figure 4. (a) Remaining UV intensity of the deuterium-lamp beam after crossing the chamber transversally (along the z axis, figure 3) as a function of wavelength, at mid-axial and mid-height positions (x = 200 mm, y = 44.5 mm, respectively), with pure O_2 (ozonator off) and with O_2 partially converted into ozone (4000 ppm); (b) absorbance curve for dry and humidified (70-80 % RH) ozone (4000 ppm in both cases).

2.4.2. Spatial uniformity of dry and humidified ozone concentrations. To assess the spatial uniformity of the ozone concentration in the chamber, we made absorption measurements at $\lambda = 285 \pm 0.5$ nm (maximum absorption value for O_3 in dry ozone) as functions of axial position and at two heights y in the chamber. The results indicate an almost uniform distribution for both the dry and humidified ozone concentrations reaching absorbance of 98% ± 1% and 99% ± 0.6%, respectively. Such a high degree of uniformity agrees with the fact that the same spore inactivation rate is obtained for Petri dishes exposed at different axial positions in the chamber (not shown). Therefore, each of the Petri dishes was separately placed at the geometrical center of the chamber and exposed one at a time, in both cases of dry and humidified gaseous ozone.

The relatively low volume of the chamber, 6 L, most probably facilitates reaching homogeneity in the chamber. The humidified ozone concentration needs a few minutes to stabilize while, with dry ozone, concentration stability is reached more rapidly.

3. Exposure of microorganisms to dry ozone: experimental results and inactivation analysis

3.1. Gaseous dry ozone effect on microorganism viability: inactivation kinetics study

The inactivation kinetics of the various microorganisms tested can be characterized by completing survival curves.

3.1.1. Endospores.

Influence of the endospore nature

Figure 5 shows the number of viable *B. atrophaeus*, *G. stearothermophilus* and *B. pumilus* spores (on a log scale) as a function of exposure time to dry ozone. Their inactivation rate is much different one from the other, with *B. atrophaeus* being barely inactivated in contrast to the highly inactivated *G. stearothermophilus*. The inactivation kinetics of these three kinds of spores is characterized by an initial exponential decrease (straight segment here on a log plot) followed by a slower exponential decrease, yielding what is called a two-slope (or two-phase) survival curve.

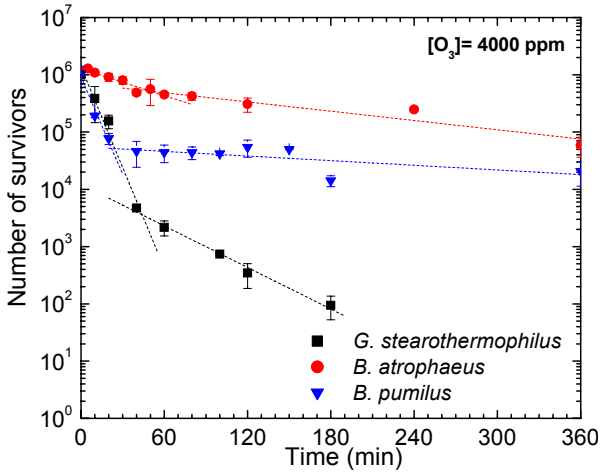

Figure 5. Survival curves for *B. atrophaeus*, *B.pumilus* and *G. stearothermophilus* endospores deposited from a 10^6 /100µL suspension (water) on polystyrene Petri dishes, left to dry for 24 h and

subjected to dry gaseous ozone at 4000 ppm. Each data point represents the average number of survivors on four different Petri dishes, and the error bar corresponds to standard deviation. Extrapolation of the *G. stearothermophilus* curve to 6 h yields less than one viable spore.

Table 1 provides the decimal (D) time, i.e. the time required to reduce the number of viable microorganisms by one log (90% decrease), allowing a more quantitative characterization of our observations.

	D_1 (min)	**D_2 (min)**
B. atrophaeus	130 ± 20	370 ± 80
B. pumilus	18 ± 3	640 ± 2
G. stearothermophilus	17 ± 2	83 ± 3

Table 1. Decimal times corresponding to the first and second phases (D_1 and D_2) of the survival curves in Fig. 5.

Although the observed lethality of *B. atrophaeus* spores is very low, it seems to be the first case of actual inactivation reported to date under similar exposure conditions[2] (RH<2%). Indeed, Ishizaki *et al* (16) reported no significant inactivation over a 6 h exposure time of different *B. atrophaeus* strains under O_3 concentrations in the 250 to 1500 ppm range, even though ozone was somehow humidified with RH values below 50%.[3] A possible explanation to this observation would be the existence of an O_3 threshold concentration for inactivation situated above 1500 ppm, as proposed by Kowalski *et al* (13). The fact that below a certain concentration threshold, ozone has no inactivation effect on this type of spores has been confirmed by other authors such as Hunt *et al* (34). At half the ozone concentration, namely 2000 ppm, for *B. atrophaeus* inactivation is weaker with a D_1 time of 170 min (not

[2] In contrast, dry gaseous ozone is known to be very effective to inactivate Gram positive and Gram negative bacteria (35), viruses (36) and some other microorganisms.
[3] Some authors consider that humidified ozone with RH values below 50% is "dry ozone"!

shown) compared to 130 min at 4000 ppm, indicating than an eventual threshold would be lower than 2000 ppm.

In an attempt to increase the inactivation rate, the Petri dishes, initially lying flat on the bottom of the chamber with the ozone gas flowing parallel to it, were put vertically, i.e. perpendicularly to the gas flow, assuming that more O_3 molecules would then reach, in fact hit, the spore deposit and thus raise the inactivation rate. Results show no significant increase in mortality, the decimal (D) value in the vertical position being 146 ± 15 min compared to 147 ± 15 min in the horizontal position: there is clearly no hydrodynamic effect of the ozone flow on inactivation.

To check to what extent spores are only very slowly inactivated by dry ozone, as is the case with *B. atrophaeus,* we first turned to *G. stearothermophilus*. This spore is a thermophile microorganism known to be resistant to humidified heat while *B. atrophaeus* withstands particularly well dry heat. The inactivation rate of dry gaseous ozone on *G. stearothermophilus* spores compared to that of *B. atrophaeus* is clearly much stronger, most probably as a result of the specific molecular composition and/or organization of their coats. We could not retrieve indications in the literature as to the inactivation of *G. stearothermophilus* spores under dry ozone exposure. Plotting the number of survivors in figure 5 as a function of dose (concentration times exposure time) instead of exposure time demonstrate, within error bars, that the inactivation rate of *G. stearothermophilus*, like that of *B. atrophaeus*, depends in the end on the applied O_3 dose (not shown).

B. pumilus spores, recognized as bioindicators resistant to gamma (ionizing) radiation, were also subjected to the same operating conditions. Their survival curve is displayed in figure 5. The decimal time of the first phase of *B. pumilus* is, within error bars, close to that of *G. stearothermophilus* (D_1 = 18 ± 3 and 17 ± 2 min, respectively), but its D_2 value of 640 min, compared to 83 min for *G. stearothermophilus*, is extremely long and rather akin to that of *B. atrophaeus*. Moreover, the number of spores inactivated in its first phase is less than two log while, with *G. stearothermophilus* for the same exposure time, it is 4 log.

In summary, in contrast to what is either found or simply not mentioned in the literature, dry gaseous ozone can affect the viability of microorganisms as resistant as

bacterial spores. It is noteworthy that the inactivation rate by dry ozone depends strongly on the spore type: *B. pumilus* inactivation rate is intermediate between *B. atrophaeus*, with the slowest inactivation rate, and *G. stearothermophilus*, with the fastest one.

Influence of the suspension “quality”

Two levels of suspension quality were tested to work out survival curves for *G. stearothermophilus* spores subjected to dry ozone. In the first suspension sample (figure 6(a)), the spores are embedded in what could be bioproducts and/or eventually chemical residues from the preparation of the spore suspension. In the second sample (figure 6(b)), the spores are cleaner, as a result of a more stringent washing procedure when preparing the suspension. One of the reasons for employing a "bad suspension" is that a priori an "external coating" of the spore should delay the access of ozone to its inner parts, and therefore results in a slower inactivation rate, a factor of practical worry as far as the inactivation efficiency of the process is concerned.

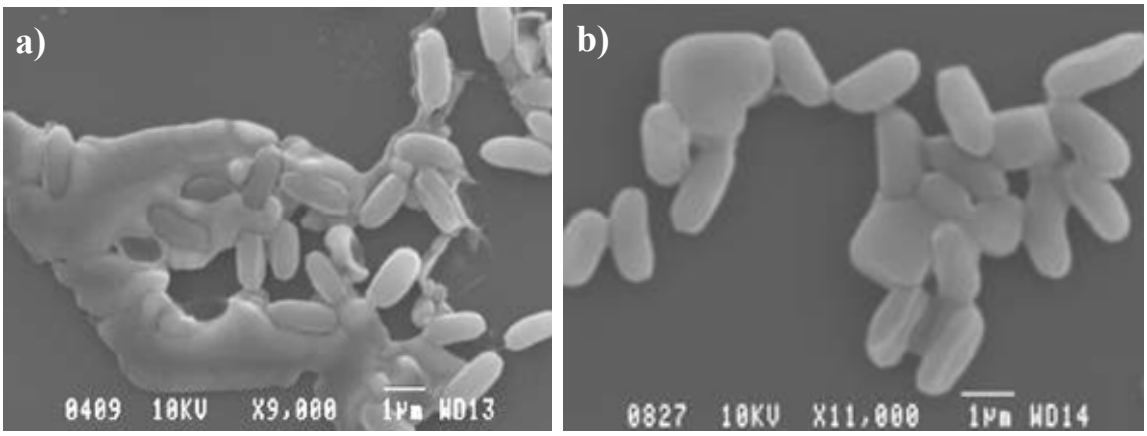

Figure 6. SEM micrographs of "embedded" and "clean" unexposed *G. stearothermophilus* spores (deposited on polystyrene Petri dishes).

The corresponding survival curves shown in figure 7 are characterized by the usual two-phase kinetics. Unexpectedly, the inactivation rate is higher for embedded spores

relatively to clean spores. However, the dispersion of the data points for "embedded" spores (figure 6(a)) is significantly larger than for clean spores (figure 6(b)) when coming to long exposure times. Figure 7(a) also shows that the higher the ozone concentration, the higher the inactivation rate. Moreover, when plotting the number of survivors as a function of ozone dose (concentration times exposure time), no significant differences (within error bars) are recorded in viability for embedded spores subjected to 2000 and 4000 ppm concentrations of dry ozone: the notion of dose thus comes out as a more general parameter than exposure time to characterize lethality.

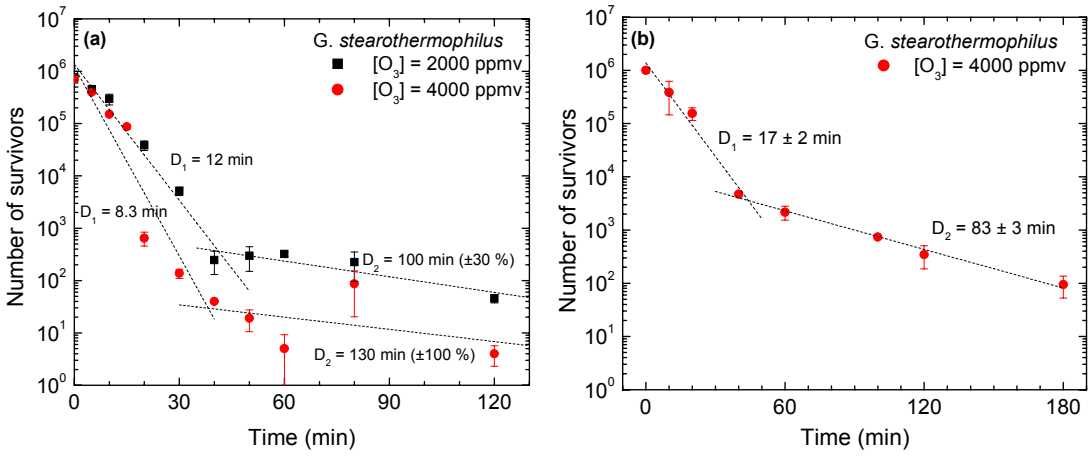

Figure 7. Survival curves for 10^6 *G. stearothermophilus* spores deposited on polystyrene Petri dishes when subjected to dry gaseous ozone: (a) at 2000 ppm and 4000 ppm concentrations in the case of "embedded" spores; (b) at a 4000 ppm concentration with "clean" spores.

To explain tentatively the fact that embedded spores are inactivated more rapidly than clean ones, even though they are thicker, we consider the following possible negative and positive contributions of the bioproducts and/or chemical residues to spore inactivation: i) the presence of extraneous material, besides shielding the spores from the biocide agent, could use up part of the ozone flow since strong oxidants highly react with organic chemical species: these two factors would imply a lowering of the ozone flow toward vital components of the spore, hence a slower inactivation rate; ii) in contrast, the chemical reaction of O_3 with bioproducts and/or chemical

residues could create additional oxidant species that would increase the inactivation efficiency. Owing to the absence of apparent damage on the outer part of "embedded" spores after exposure, these oxidant species are believed to diffuse through the spore coats and create therein lethal lesions to some metabolic mechanisms or, even, to the spore genomic material. We therefore conclude to a positive contribution to the inactivation mechanism from the material extraneous to the spore, stressing the fact that the quality of the spore suspension can strongly influence the observed inactivation rate following exposure to dry gaseous ozone.

3.1.2. Vegetative bacteria: Deinococcus radiodurans bacteria.

Because vegetative bacteria are less resistant to any given biocide agent than sporulated bacteria, one expects them to lead to a comparatively much higher inactivation rate. Since our intent is to characterize the inactivation of other microorganisms also as dry deposits (to avoid bringing water vapour in the process), we needed a bacterium that could withstand drying. Besides supporting desiccation, *Deinococcus radiodurans* is recognized as gamma (ionizing) radiation resistant and can also survive cold, vacuum, and acid.

Figure 8 shows a two-phase survival curve for *D. radiodurans* resulting from its exposure to 4000 ppm of dry O_3. The number of *D. radiodurans* bacteria initially deposited and their eventual degree of stacking can vary considerably depending on culture and drying conditions. While for an initial deposit of 3.3×10^4 bacteria (figure 8), an average of 10 CFUs was counted after a 10 min exposure, in another independent experiment with an initial deposit of 5.9×10^5 bacteria, only one CFU was encountered after the same exposure time (not shown). Clearly, dry ozone acting on this bacterium is much more efficient than with sporulated bacteria (figure 5).

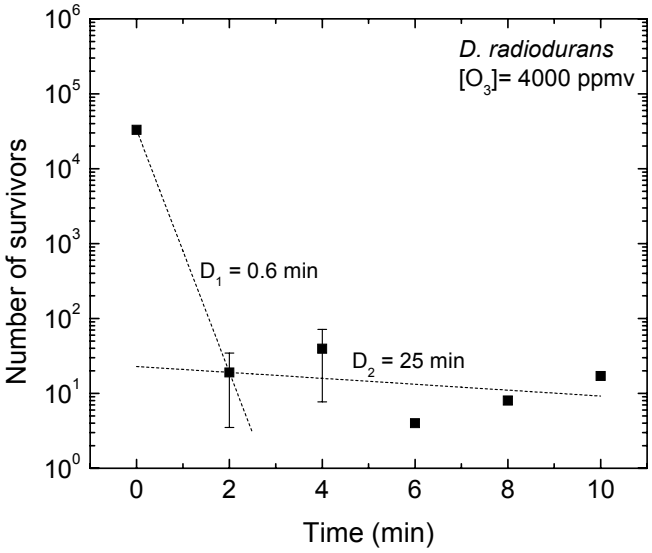

Figure 8. Survival curve for *D. radiodurans* bacteria deposited on polystyrene Petri dishes, left to dry and then subjected to 4000 ppm of dry gaseous ozone.

It must be underlined that there is no mention in the literature of the possibility of inactivating dried vegetative bacteria with dry ozone: the ozonation process is reported to be efficient on vegetative bacteria only in humid media, water, agar or with airborne bacteria (12, 13).

3.2. Dry gaseous ozone effects on microorganism integrity

Morphology and outer dimensions

Spore integrity following ozone exposure was first analysed from SEM micrographs, looking for eventual morphological changes that could give indications as to the biocidal mechanism(s). Spore integrity was further assessed by performing statistical analysis of their outer dimensions before and after ozone exposure, using the Autocad data-processing software on a minimum population of 100 spores. Both these studies are conducted for the three types of spores considered above.

Figure 9 displays SEM micrographs for the unexposed spores (control) in the left column and, horizontally in the right column, the corresponding spores once exposed to 4000 ppm of dry gaseous ozone. Both embedded and clean *G. stearothermophilus* spores are presented. Exposure time is 3 h, except for the *G.*

stearothermophilus clean spores for which it is 80 min. The SEM micrographs clearly show that there are no apparent changes in the spore morphology. The same conclusion holds even after a 6 h exposure (not shown), even though the great majority of spores have then been inactivated: for instance, with clean *G. stearothermophilus,* there should be less than one spore left out of the 1.3×10^6 spores initially deposited (figure 5). Note in passing that decreasing the number of microorganisms by 6 log allows to claim that the process leads to sterility (Safety Assurance Level).

The second test used to check for damage to the spores consisted in looking for outer dimension modifications, following different exposure times. Examining the spore length was sufficient to draw clear conclusions. The average length L of *B. atrophaeus* spores before and after exposure is the same: $L_{unexposed} \approx (1.3 \pm 0.1)$ µm, $L_{exposed\ 3h} \approx (1.25 \pm 0.05)$ µm and $L_{exposed\ 6h} \approx (1.29 \pm 0.13)$ µm where the uncertainty range is the standard deviation. As for *B.pumilus* spores, their length also remains the same as before after ozone exposure: $L_{unexposed} \approx (1.1 \pm 0.1)$ µm, $L_{treated\ 3h} \approx (1.1 \pm 0.1)$ µm. For "embedded" *G. stearothermophilus* spores, the average length does not change with $L_{unexposed} \approx (1.7 \pm 0.2)$ µm and $L_{exposed\ 80min} \approx (1.5 \pm 0.3)$ µm while for "clean" ones, $L_{unexposed} \approx (1.3 \pm 0.2)$ µm and $L_{exposed\ 60min} \approx (1.4 \pm 0.2)$ µm, indicating that "clean" spores have the same length on the average as "embedded" ones[4]. In all the analysed cases, no significant difference between unexposed and exposed spore length was found.

[4] Within the group of "embedded" spores, only those with a morphologically well defined length were considered for the histogram, ignoring those where the extraneous material embedding the spore is clearly distinct from its body.

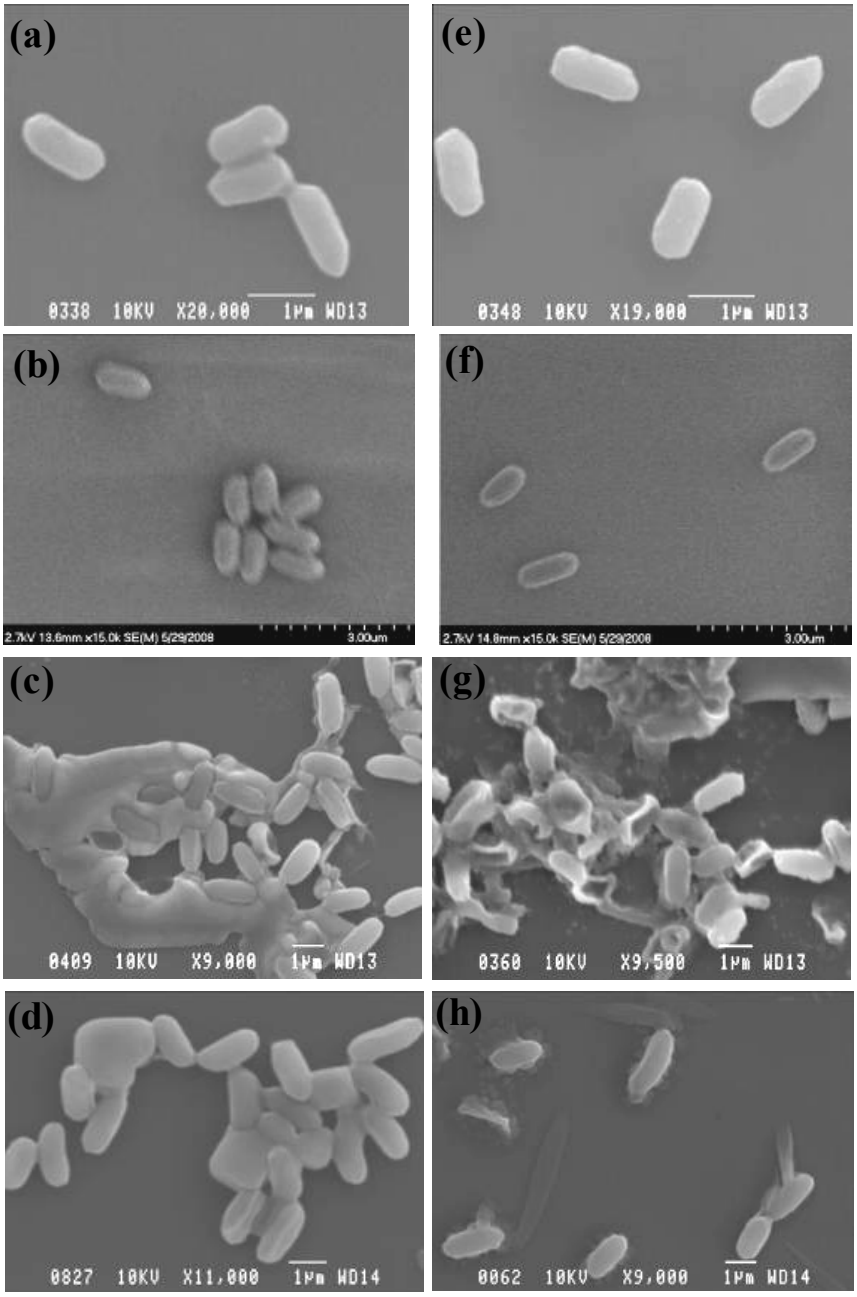

Figure 9. SEM micrographs showing vertically: first line *B. atrophaeus*, second line *B. pumilus*, third line embedded *G. stearothermophilus*, fourth line clean *G. stearothermophilus* spores deposited on Petri dishes. The left column is for unexposed (control) spores and the right one for spores exposed to 4000 ppm of dry gaseous O_3 for 3 h, except for clean spores where it is 80 min.

From both the micrographs and the average length of the spore, one can claim that there is no apparent erosion of the spore coat after ozone exposure. Having found that the length of the spores was not modified by ozone exposure, we did not care to examine their width and height as we assumed that erosion would not affect specifically their length only.

Weakening of B. atrophaeus and G. stearothermophilus spore internal structure

As we have just seen, dry ozone exposure does not affect the external morphology and dimensions of spores. This suggests that the inactivation mechanism involves penetration of the biocide agent within the spore coats, which could then somehow weaken the spore internal structure. We have observed such an effect quite unexpectedly following the use of Malachite green preparation to stain spores[5]. This dye is currently employed to assess integrity of dormant spores. Intact dormant spores stain green whereas spores in the process of germination or that have been affected by exposure to different agents turn red when adding the Safranin O counter-stain.

B. atrophaeus spores. Figure 10 (a) displays unexposed (control) spores. One can observe their regularity, smoothness and integrity. Figure 10 (b) shows unexposed spores further stained with the Malachite preparation. Under optical microscopy, the spores stain green and appear morphologically intact, although presenting some markings on their surface with "debris" in their surrounding, possibly from malachite particle deposition and/or water stains. Figure 10 (c) shows dried spores, simply subjected to 4000 ppm of dry O_3 for 60 min: the spores are still intact with very little differences with the unexposed spores in figure 10(a). Figure 10(d) shows spores exposed to dry ozone under the same conditions as in figure 10(c) and thereafter subjected to malachite green dye: some of these spores come out damaged. This procedure reveals that the spore external structure is strongly affected and it can be

[5] The Malachite green staining protocol involves heating the green dye to facilitate its eventual penetration into the spores. When the spore is not dormant, the green dye penetrates into the spores and is afterwards displaced by rinsing with water; an added red counter-stain can thus gain entry into the spore, which then becomes red in color.

observed that some ozone-exposed spores release their inner material from their surrounding coats (figure 10(d)).

We conclude that: (i) dry ozone exposure truly weakens the spore internal structure, but that an applied stress (heat and rehydration), as is the case with the malachite staining procedure, is required to reveal this structural damage; (ii) structure weakening is most probably resulting from the diffusion of O_3 into the spore and its subsequent action.

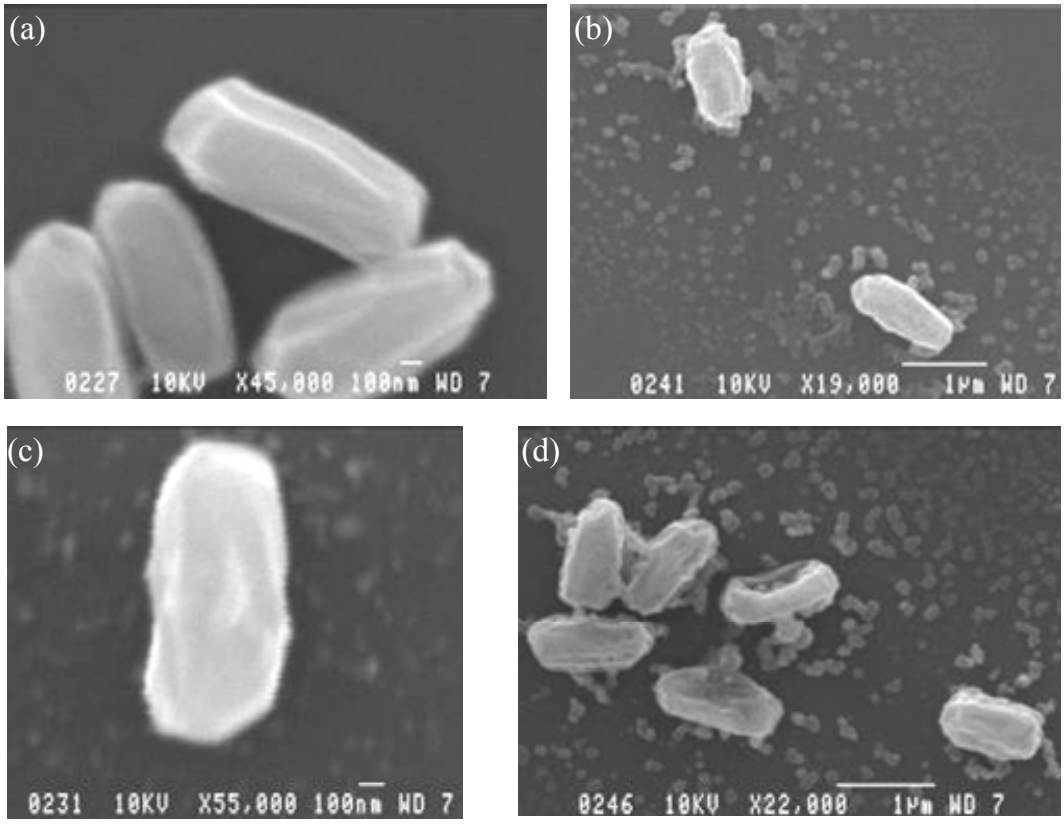

Figure 10. SEM micrographs of *B. atrophaeus* spores deposited on microscope slides showing: (a) unexposed dried spores; (b) unexposed spores after their staining with Malachite green-dye; (c) dried spores subjected for 60 min to 4000 ppm of dry O_3; (d) spores exposed to O_3 under the same conditions as in figure (c) and subjected thereafter to Malachite green stain.

G. stearothermophilus spores. A peculiarity worth noting when harvesting *G. stearothermophilus* spores to complete survival curves concerns the longer incubation

time needed for full growth of their colony forming units (CFUs) on nutrient medium in contrast to what is required, for example, for *B. atrophaeus*. Culture conditions of *G stearothermophilus* spores usually state a 48 h incubation time (at 56 ^{o}C). However, we noted that after 72 h the number of CFUs was more important than at 48 h (275 and 106 CFUs, respectively); nonetheless, it was not much greater after a week (300 CFUs), which led us to set the incubation time at 72 h. Such a growth delay suggests that still viable spores were severely damaged[6]. A similar phenomenon has been reported with spores exposed to various oxidizing agents (6, 37). In contrast, such a delay in germination is not observed, under the same operating conditions, with *B. atrophaeus* spores for which the recommended incubation time of 24 h (at 37 ^{o}C) was found suitable.

3.3. Influence of the substrate nature on the kinetics of inactivation

Figure 11 shows that the inactivation kinetics of dried spores exposed to dry gaseous ozone varies strongly with the nature of the Petri dish on which they are deposited. The inactivation rate is much higher on polystyrene substrates than on Pyrex ones for the three types of spores considered. On both types of substrates, *G. stearothermophilus* (clean) spores have the highest inactivation rate. In contrast, *B. pumilus* spores are, apparently, not inactivated at all when laid on Pyrex while they have an inactivation rate intermediate between that of *B. atrophaeus* and *G. stearothermophilus* on polystyrene. On both types of substrates, whenever the inactivation rate is significant, the survival curves are biphasic.

[6] Such an incubation delay is not observed with *G. stearothermophilus* when exposed, for instance, to the N_2-O_2 plasma afterglow in our lab.

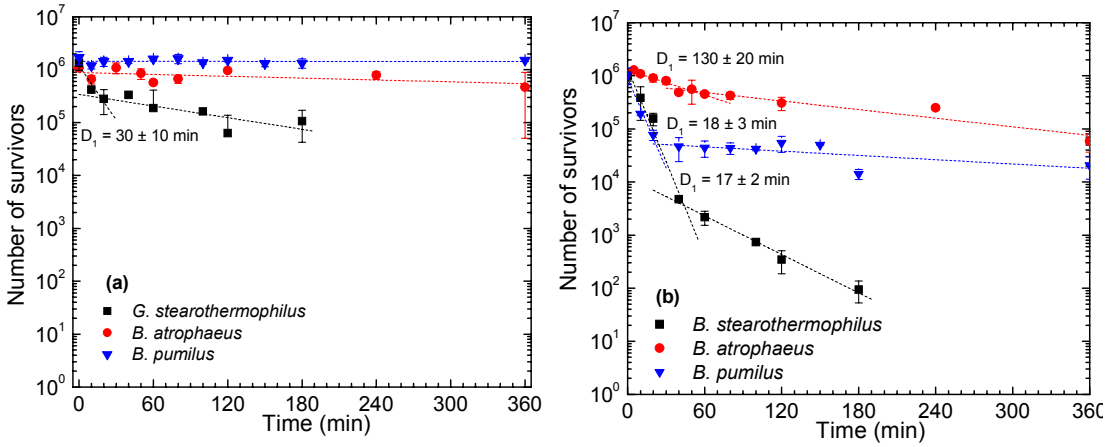

Figure 11. Survival curves for the three types of spores considered in the present study when exposed to dry gaseous ozone at a 4000 ppm concentration after having been deposited : (a) on Pyrex Petri dishes; (b) on polystyrene Petri dishes.

No dependence of the inactivation rate on the substrate nature has been reported in the literature for the case of dry gaseous ozone (i.e. RH<2%) whereas such a dependence on various other biocide agents is known. For instance: i) using an air plasma at atmospheric pressure, Kelly-Wintenberg *et al* (44) showed that *E. coli* bacteria, placed on polypropylene Petri dishes, were more readily inactivated (D_1=6s, D_2=2s) than those deposited on glass (D_1=33s, D_2=10s) or agar substrates (D_1= 70s, D_2=17s). The fact that the spores deposited on the polymer surface exhibits a greater sensitivity to plasma than those deposited on glass substrates agrees with our observations; ii) turning to ethylene oxide, Gilbert *et al* (45) determined that the nature of the surface upon which *B. atrophaeus* spores were dried had an effect on their inactivation rate. They showed that, for low RH values (33%), the inactivation efficacy was the lowest for glass, then higher with paper and, finally, among the materials tested, the greatest with cotton. They concluded that it is more difficult to kill microorganisms on impervious surfaces; iii) turning to humidified ozone (10mg/L O_3, RH =90%), Aydogan *et al* (18) determined that the inactivation rates of *B. atrophaeus* spores on glass, vinyl floor-tiles and paper were not significantly different from each other: vinyl and paper surfaces do not enhance (or delay) the inactivation of spores as compared to glass substrates. Nonetheless, this is not a general rule with humidified ozone since the same authors obtained inactivation rates for other

substrates that follow a different trend: the rate is stronger on carpet material than on glass surfaces and, in turn, less on hardwood. The outcome of their experiments suggests that spores deposited on different substrate surfaces are, generally, inactivated at different rates by gaseous disinfectants like O_3. All these observations suggest that such interactions are quite complex, strongly depending not only on the substrate nature, but also on actual experimental conditions.

The experimental data presented in table 2 compare the inactivation efficacy of *B. atrophaeus* spores deposited on various polymeric substrates after a 60 min exposure to dry gaseous ozone at a 4000 ppm concentration.

Polymers	Inactivation efficacy in % (*B. atrophaeus* spores)
Silicone	99.9985
Polyurethane	99.1
Polystyrene	79.7
High density polyethylene	36
Teflon (PTFE)	32.7
Polymethylmetacrylate	23.5
Polypropylene	15.2

Table 2. Inactivation efficacy of *B. atrophaeus* spores deposited on different polymeric substrates, dried and thereafter exposed 60 min to 4000 ppm dry gaseous ozone. Initial number of spores is 10^6 in 100 µl of water.

This table shows the spore inactivation rate observed on various polymers after exposure to dry gaseous ozone (RH < 2%): the inactivation efficacy highly depends on their nature. For comparison purposes, the corresponding inactivation efficacy on glass and PS Petri dishes is 47% and 61 %, respectively.

4. Exposure of *B. atrophaeus* spores to humidified gaseous ozone: inactivation characteristics and chemical species involved

B. atrophaeus spores were chosen in this study because of their strong resistance to inactivation by dry gaseous ozone (figure 11).

4.1 Inactivation kinetics study

The outcome of a humidified gaseous ozone flow on *B. atrophaeus* spores is illustrated in figure 12, showing a high inactivation rate in contrast to dry ozone exposure (inset of the figure). We note that the higher the relative humidity (RH) rate, the higher the inactivation rate. The corresponding inactivation kinetics, for both RH values, is again characterized by a two-phase survival curve, as also reported by several authors (13, 17, 18). Noteworthy is the fact that both *B. stearothermophilus* (not shown) and *B. atrophaeus* spores are all rapidly inactivated within a similar time frame (≈ 5 log in 40-60 min) in contrast to the dry ozone case where the inactivation rate varies considerably with the spore-coat composition.

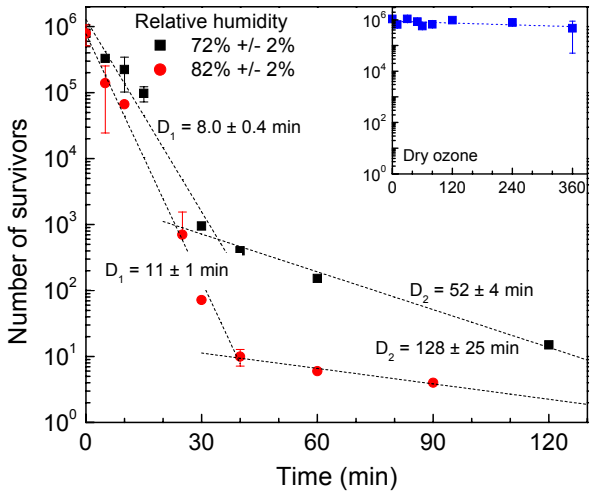

Figure 12. Survival curves of *B. atrophaeus* spores deposited on Pyrex Petri dishes subjected to humidified gaseous ozone at a concentration of approximately 4000 ppm for two values of RH. For comparison, the inset (from fig. 13 (a)) refers to exposure to dry gaseous ozone at a concentration of 4000 ppm.

A few studies have determined that for RH above 70%, the increase in inactivation rate is not significant (17, 18) since almost similar corresponding survival curves are obtained thereon. However, our survival curves in figure 12 are clearly different for RH values varying by only 10% above 70%; a similar trend has also been reported by Ishizaki *et al* (16) for whom the highest inactivation rate is actually attained at RH values above 90-95%. The same authors, however, claim that ozone looses its biocidal capacity at RH values below 50%, which is contradicted by our observations: using dry gaseous ozone, we succeeded in inactivating, although to various degrees, different species of bacterial spores (figure 11).

Some diverging results need to be cited. Ishizaki *et al* (16), operating with *B. atrophaeus* spores, obtained a 5 log reduction in less than 2 hours at 95% RH and 1500 ppm ozone concentration while, under the same conditions, Aydogan *et al* (18) reported less than a 2 log decrease. This difference in inactivation efficiency could be related to the way spores are processed after deposition and drying: Aydogan's deposits are conditioned in a desiccator (RH < 1%) whereas Ishizaki's deposits are stored in humidified air (minimum 54% RH) before ozone exposure. Our own results for samples dried in ambient humidity are close to those of Ishizaki *et al* (16), but with a still higher inactivation rate (for a slightly lower RH values, 82% vs. 95%), most probably due to a higher ozone concentration (4000 ppm vs. 1500 ppm). Increasing the drying temperature of spore suspensions lowers their resistance to a subsequent sterilization or disinfection treatment, as discussed by Cortezzo *et al* (37) and validated by us (data not presented).

4.2 Spore morphological characteristics after exposure

Figures 13 and 14 display a set of SEM micrographs of *B. atrophaeus* spores subjected to humidified gaseous ozone (≈ 75% RH) at a 4200± 200 ppm concentration, for 120 and 30 min exposure times, respectively.

Figure 13(a) shows unexposed (control) spores while figures (b) and (c) correspond to spores exposed for 120 min. The structure of all spores has been severely damaged: they have lost their original shape due to the fact that their central material has been extruded (b); some of them are even disintegrated, showing fragments of their initial structure (c). Large gathering of disintegrated material could

correspond to more than one initial spore, a situation that can be seen with unexposed spores in figure 13 (a) where 2 and 3 spores are close neighbours.

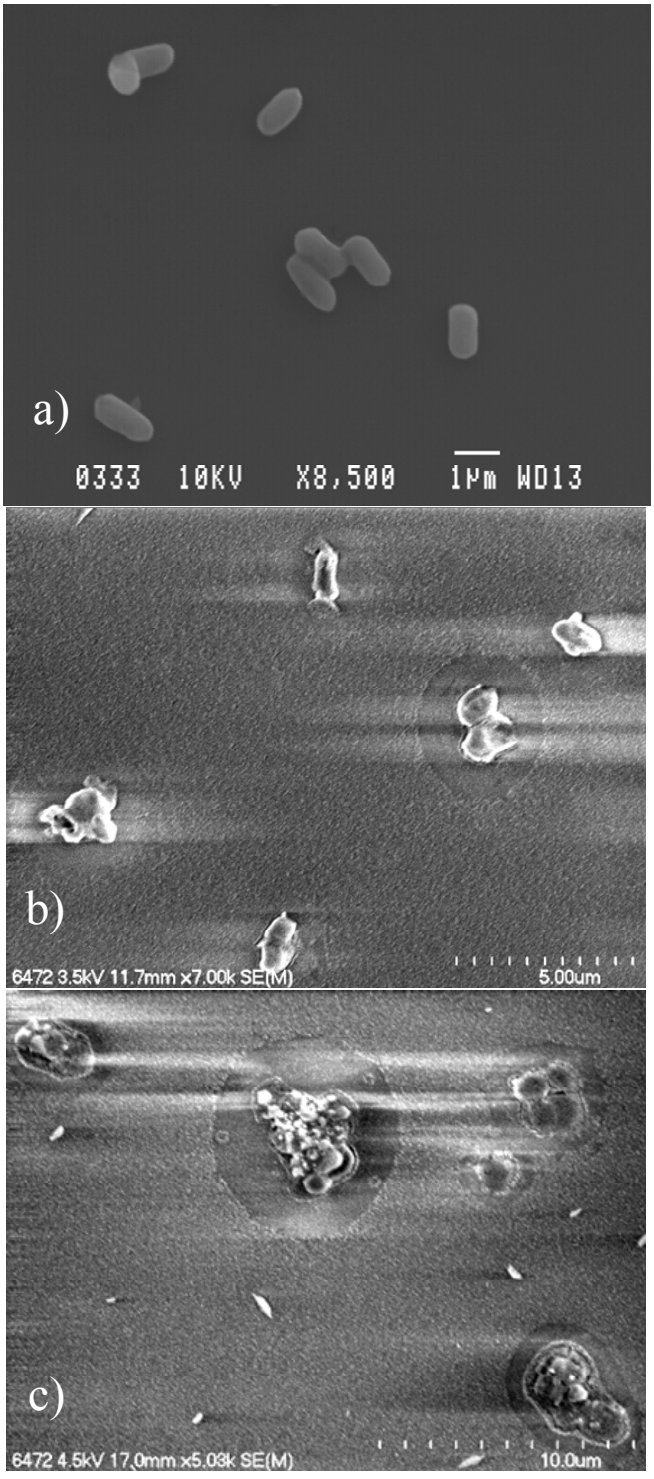

Figure 13. SEM micrographs of *B. atrophaeus* spores deposited on glass slides showing: (a) unexposed spores; (b and c) spores exposed for 120 min to humidified gaseous ozone (RH ≈ 75 %) at 4200± 200 ppm.

Figure 14 shows that the integrity and the structure of *B. atrophaeus* spores are altered even after a much shorter exposure time (30 min). However, at this time, the spores are not showing the same level of damage: some of them appear to have kept their integrity (a) while others have clearly lost part of their inner material (a and b).

These results fully contrast with dry ozone exposure where, for all spores, no apparent morphological damage is observed after 60 min and even 6 h exposure times (sec. 3.2).

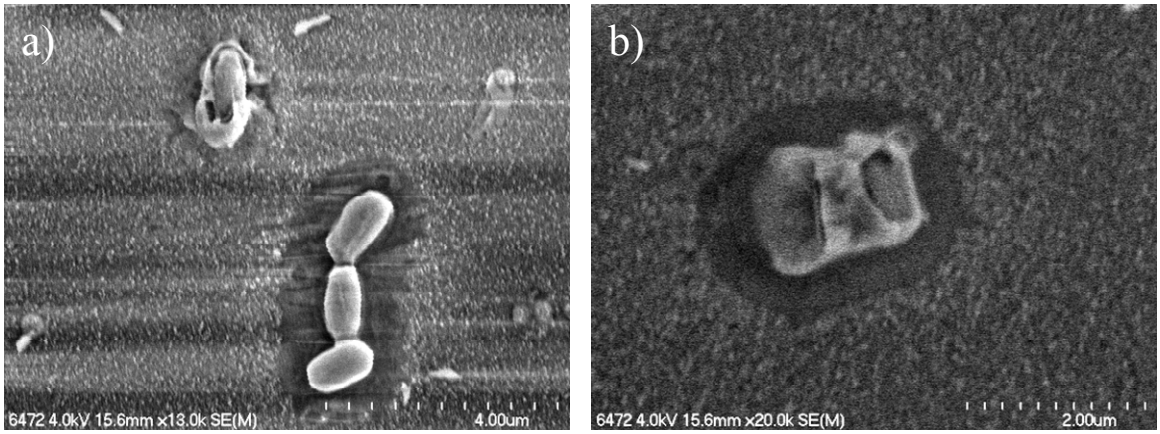

Figure 14. SEM micrographs of *B. atrophaeus* spores deposited on glass slides showing: (a) unexposed spores; (b and c) spores exposed for 30 min to humidified gaseous ozone (RH ≈ 75 %) at 4200± 200 ppm.

4.3 Identification of chemical species in the gaseous phase

Figure 15 compares infrared absorption spectra taken in dry and humidified gaseous ozone. In dry media, only the presence of O_3 is observed over the 400-6000 cm^{-1} range while, in humidified media, other species are created such as $HO^{\bullet}_2$ (hydroperoxy), as shown in figure 17 close to 1400 cm^{-1}.

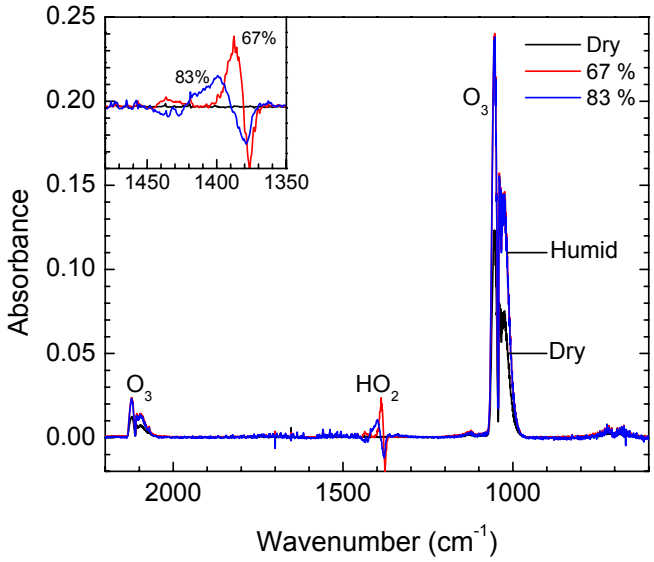

Figure 15. Infrared spectra taken at the sterilization-chamber exit under ozone concentrations of 4000 ppm: dry gaseous ozone; humidified gaseous ozone for RH ≈ 67% and RH ≈ 82.5%. The absorption band centered at 1392 cm^{-1} (see inset) corresponds to the hydroperoxy radical $HO^{\bullet}_2$, no such band being present with dry ozone.

Theory teaches that ozone decomposition in water is a radical chain-process (5, 20, 47, 48), beginning with reaction (1) where the presence of initiators (here H_2O) is required. This chain reaction finally provides highly oxidative species that include hydroxyl radicals ($OH^{\bullet}$), hydroperoxy radicals ($HO^{\bullet}_2$), superoxide radicals ($O^{\bullet}_2{}^{-}$) and H_2O_2 molecules:

$$O_3 \xrightarrow{\text{initiators } (OH^-,\ UV,\ H_2O_2 \ldots)} HO_2^{\bullet} + O_2^{\bullet -}. \qquad (1)$$

In the case of a humidified gaseous medium, the decomposition of O_3 increases with increasing RH values (49), again yielding $HO^{\bullet}_2$, $OH^{\bullet}$, H_2O_2…Therefore, maintaining a constant concentration of O_3 implies increasing its production by the ozonator.

5. Dicussion

5.1. Dry ozone treatment

5.1.1. Variability of the inactivation rate with respect to the nature of the microorganism

The changes resulting from sporulation of vegetative bacteria involve generation of new structures, namely core, cortex, plasma membrane, inner coat, outer coat and outer membrane. These structures confer resistance against several and different stresses. Besides such common structural changes following sporulation, there exist many differences among the Bacillus species considered here, which include their size, shape, resistance factors, water content and the composition and organization of their chemical constituents. For instance, the chemical composition of their coats and cortex varies considerably from one species to another (the coats of *B. atrophaeus* are essentially made up of more than 25 different proteins forming an intricate lattice while *B. pumilus* spores are characterized by the presence of an exosporium, essentially sugar). Our results clearly show that there are, indeed, strong differences in the degree and rate of inactivation among the three spores examined.

In the present case, the biocide agent, assumed to be the O_3 molecule itself, can have severals targets such as proteins, enzymes and even DNA (39). Knowing that O_3 must reach, remain onto and eventually penetrate and diffuse throughout the microorganism to damage it, the microorganism structural features can easily explain the much faster inactivation rate observed with the vegetative bacterium *D. radiodurans* (D_1 = 0.6 min) as compared with the *G. stearothermophilus* spore (D_1 =17 min). Compared to endospore multicoats, which protect a cell devoid of any activity, the cell wall of vegetative bacteria is essential for growth and metabolism, shape determination and resistance to environment fluctuation and aggression. Therefore damaging it disrupts the cell's physiology and exchanges with the microenvironment and may lead to its death.

5.1.2 Inactivation mechanisms and target identification

Our experimental observations and analyses suggest that the ozone molecules diffuse through the spore and, then, react (oxidation reactions) with targets that are essential to the spore survival. In addition, the presence of water in the core of the spore (37), although very low, could initiate chemical reactions with ozone and/or with their reaction by-products to provide further oxidant species such as radicals, oxidant molecules, which would also participate in the spore inactivation process.

The literature reports contradictory viewpoints as to the actual diffusion of O_3 through microorganisms. In the case of vegetative bacteria, some authors suggest that ozone rather attacks their cell wall, altering its permeability and cytoplasmic membrane, resulting in the leakage of the cell contents (34). For spores, ozone is assumed by some to diffuse through the surface and then permeate into the membrane (4, 40). However, some other authors claim that diffusion of O_3 throughout the spores is not possible, based, on the one hand, on the observed inability of small size reactive species (such as methylamine molecules) to diffuse through the spore coat (34, 37) and, on the other hand, because the spore inner membrane is known to have an extremely low permeability to small hydrophilic molecules (37).

As far as oxidation by O_3 is concerned, some authors suggest that the major mechanism of spore killing by chemicals is an oxidizing damage to the spore inner membrane (41). This implies the further possibility for O_3 to reach and damage the genetic material molecules in both spores and bacteria (16, 34). In the specific case of bacteria, Komanapalli *et al* (42) claim that their inactivation by ozone proceeds from inner membrane damage and not from DNA lesions, since protein and nucleic acid leakage can be observed as a result of membrane disruption.

In general, the literature mentions that, for spores, the main lethal targets of ozone are enzymes (related to their metabolic functions) and/or DNA. To check to what extent dry ozone can induce metabolic damage to spores through its attack on enzymes (proteins), we turned to lysozyme, determining its enzymatic activity after it

has been subjected to dry gaseous ozone (60 min at 4000 ppm) (data not shown). Lyzozyme was chosen because it is a strongly resistant enzyme and because its activity is easily assayed and well documented (43). Ozone exposure was shown to have no detectable effect on the enzymatic activity of lysozyme, indicating that the active sites of the lysozyme protein are not a target for O_3 under the conditions used. Nonetheless, it could happen that less resistant enzymes of spores be damaged by O_3, which may or may not lead to spore inactivation depending on their specific function.

5.2 Humidified ozone treatment

5.2.1 Species involved in microorganism inactivation.

The O_3 molecule alone is chemically selective since it mainly reacts with organic compounds M having high electronic density sites (e.g. double bonds). In contrast, radical reactions are non-selective and will react with almost all organic compounds. Considering these facts, some authors claim that molecular O_3, thanks to its high oxidizing power, is the main inactivating agent of microorganisms (reaction (2)), while others rather emphasize the high reactivity of the free radicals formed from humidified gaseous O_3 , such as $OH^{\bullet}$, $HO^{\bullet}_2$, … (reaction (3)) (34, 49) as responsible for inactivation:

O_3 direct action

$$O_3 + M \longrightarrow M_{ox} , \qquad (2)$$

O_3 indirect action (example)

$$O_3 \xrightarrow{H_2O} OH^{\square} \xrightarrow{M} M_{ox} . \qquad (3)$$

For several authors including Setlow *et al*, O_3 is not the main inactivation species but H_2O_2 and possibly free hydroxyl radicals resulting from its degradation, some of

them accessing the spore core and, at least in part, the DNA (34, 41). They suggested that the thick coating of the spore acts as a reactive barrier that uses up O_3 molecules, preventing them from reacting with critical targets located further within the spore (21). In contrast, some other studies showed, under water medium conditions, that molecular O_3, and not its derived radicals, is attacking the cell surface, altering the permeability of the inner membrane and ultimately resulting in the leakage of cell contents (34). Along the same line, Von Gunten (50) claims, based on kinetic considerations, that $OH^{\bullet}$ only plays a minor role; he specifies that the traveling distance of $OH^{\bullet}$ in a cell is estimated to be approximately 6 nm, not enough to reach the core. Furthermore, Khadre *et al* (4) demonstrated that H_2O_2, even at ≈10,000 fold higher concentration than O_3, was less effective than ozone against Bacillus spores in water.

To determine the species involved in microorganism inactivation, we turn to the "sequential approach" adopted in our study, which consists in examining first the effects of dry ozone and only afterwards those of humidified ozone. In the dry ozone case, we concluded that the O_3 molecule is directly involved in the spore inactivation since inactivation is achieved without the various radicals created in the humidified ozone case. Subsequently, this leads us to claim that, under humidified media, ozone molecules by themselves should also play an important role in spore inactivation simultaneously, in this case, with radical species that heavily damage the spore and improve markedly their inactivation kinetics.

5.2.2 Possible inactivation mechanisms under ozone exposure following our experimental study and relying on the literature.

Based on our experimental results in both the dry and humidified ozone cases, we are able to sort out specific elements from the published literature that support our findings. These can be gathered along three main lines:

(1) Inactivation of microorganisms by dry gaseous ozone: under this specific operating condition (RH < 2%), our results are markedly novel, implying that the

literature in that respect, besides being scarce, must be reconsidered: dry gaseous ozone is able to inactivate not only vegetative bacteria, but also microorganisms as resistant as bacterial spores, observing that their inactivation rate greatly depends on their nature (figure 11). This variation in the inactivation rate can be correlated with differences in coat or, eventually, cortex constituents, leading to changes in their ozone permeability as hypothesized earlier by Wickramanayake *et al* (8). Even though spore inactivation was observed to depend on their composition, they undergo no apparent structural damage (based on SEM micrographs). Nonetheless, there is strong evidence that the spore inner structure was considerably weakened, as revealed for *B. atrophaeus* and *G. stearothermophilus* (sec. 3.2). The fact that, as mentioned, there is no apparent damage to spores but weakening of their structure leads us to claim that diffusion/oxydation of O_3 is the main inactivation mechanism of spores under dry gaseous ozone (RH<2%).

(2) Importance of spore swelling under humidified media: two groups of authors have experimentally demonstrated the swelling of spores in a humidified air atmosphere, in the absence of any other gas or chemical agent (59, 60). According to Rubel *et al* (59), the collapse of the spore structure results from hydrogen-bonding sites (weak-energy bonds) formed on the macromolecular constituents of the spore. They showed that residual water loading increases with increasing humidification. Following this, Westphal *et al* (60) observed that spores consistently swell in response to increased relative humidity and shrink back to near their original size on reexposure to dry air. They highlighted two distinct time scales for swelling which they assumed to correspond to two sequential mechanisms: i) rapid diffusion of water (> 50 s) into the spore 'coat + cortex', followed by ii) a slower diffusion of water (≈ 8 min) into the spore core. They further concluded that swelling of a spore increases the diameter of its channels through which inactivating gases could pass (60).

This swelling effect therefore allows molecules and oxidative radical species to penetrate more easily within swollen or hydrated spores, diffuse therein through the so-created channels and react with internal biomolecules. As highlighted by Westphal

et al., the increased permeability of the spore structure in humidified media suggests the possibility for species to diffuse not only into the cortex but finally up to the core.

As a result, we can say that the ozone inactivation efficiency is due, in a first step, to the high RH (swelling of the spore) and, in a second step, to the specific biocide gas used (O_3 molecules and oxidative agents created in the media). In the literature, authors have always claimed that inactivation in humidified ozone process is better only because of the oxidative agents chemically created in the media without ever relating the RH effect to swelling of the spores, which is in fact essential for enhancing the inactivation efficacy. Our two-step explanation can also be applied to other biocide agents since inactivation by EtO, formaldehyde, chlorine dioxide or heat was shown to be more efficient under high RH conditions (45, 61, 62, 63). Then, the type of biocide gas used should be determinant: for instance, inactivation with O_3 is actually found to be more efficient than with H_2O_2 for a fixed RH value (4, 37).

(3) Spore collapse mechanism under humidified ozone exposure. Under humidified gaseous ozone, we observed the collapse of the spore (figures 14 (a) and 14 (b)) followed, after a longer exposure time, by its desintegration (figure 13 (c)). Since Westphal *et al* (60) used only water, not biocide agents, they only observed the swelling of the spore, not its collapse.

Our SEM micrographs show for short time exposure (30 min) that the spore collapse generally occurs at the center of the microorganism (figures 14 (a) and (b))); at longer exposure times, simple collapse can no longer be identified due to the spore desintegration (figure 13(b)). Spore collapse could result from an overpressure due to the accumulation of H_2O, oxidative species and the volatile by-products chemically created therein (64, 65). This overpressure would occur at the end point of diffusion, i.e. at the center of the spore where the pressure becomes maximum, as suggested by Westphal *et al* (60).

6. Summary and conclusion

Understanding the microorganism inactivation mechanisms resulting from exposure to humidified gaseous ozone is a complex and hard task. Our approach to that

situation was to examine, under strict dry gaseous ozone exposure (RH < 2%), the inactivation kinetics and morphological damage to three bio-indicator spores and a bacterium and, afterwards, consider the case of humidified gaseous ozone exposure: in the end, correlating the results from these two studies contributes to bringing out the various inactivation mechanisms.

Under dry gaseous ozone exposure, we have shown that O_3 molecules could efficiently inactivate certain types of spores (*G. stearothermophilus)* and much less others (*B. atrophaeus*), the difference in the inactivation rate lying presumably in the nature or arrangement of their constituents, essentially the chemical composition of their coats (and inner membrane). Morphology of the spores is not affected by dry ozone treatment, implying that diffusion/oxidation, and not erosion, is related to the action of O_3 molecules, these travelling within the spore. In addition, we showed that the ozone inactivation efficacy of spores deposited on various polymeric substrates depends on the nature of the polymer.

Under humidified gaseous ozone exposure, we consider that an important initial mechanism (prior to biocide action) is water swelling of the spores, which opens "channels" that facilitate the inner diffusion of the biocidal agents. These are the O_3 molecules and the by-products of their interaction with H_2O that yield highly oxidative species such as $HO^{\bullet}_2$, $OH^{\bullet}$, H_2O_2. The final result of these oxidation processes is the inactivation of spores that resisted to some extent the action of dry ozone (*B. atrophaeus*), all the three types of bio-indicators used here being severely damaged, and not to say pulverized after a long enough exposure time. The relative contribution of O_3 molecules and of the oxidative radicals to the humidified ozone inactivation process could be established in future work. A possible next step would be to call on molecular biology to assess the kind and level of damage suffered by the spore nucleic acids.

Acknowledgments

The authors are grateful to Mr. J S Mayer for skilful technical assistance, A Leduc for valuable assistance on enzymatic activity measurements and analysis, and Professor R Hausler at École de Technologie Supérieure (ETS) for lending us the ozonator.

Thanks are also due to Professor B Held from université de Pau and P Renault from Air Liquide (France) for pertinent comments. Financial support was provided by the Fonds Québécois pour la Recherche sur la Nature et la Technologie (FQRNT), programme Équipe.

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2.4 Conclusion

Dans ce chapitre, nous avons examiné les différents mécanismes intervenant dans l'inactivation des spores bactériennes par l'ozone sec et l'ozone humide. En milieu sec, nous avons démontré pour la première fois que ce type de traitement pouvait s'avérer efficace sur certaines spores : le principal mécanisme impliqué dans leur inactivation est la diffusion/oxydation de l'ozone. L'efficacité d'inactivation en milieu sec dépend principalement du type de microorganismes, de la concentration d'ozone et aussi du substrat sur lequel ils sont déposés. D'après la littérature, les principales cibles possibles de l'ozone seraient les enzymes et le matériel génétique : or nous avons montré que le traitement "direct" de la protéine lysozyme (enzyme très résistante) à l'ozone gazeux sec ne semblait pas altérer son activité enzymatique. Quant à l'hypothèse que l'ADN puisse être la cible de l'ozone gazeux en milieu sec, des tests préliminaires montrent que l'effet sur le matériel génétique serait de très faible intensité.

La corrélation entre l'étude en milieu sec et milieu humide nous a permis d'affirmer le rôle clé que joue la molécule d'ozone dans les mécanismes d'inactivation en milieu humide ; à partir de nos observations et de la littérature, nous avons proposé un mécanisme en montrant, distinctement, l'impact des différents facteurs participant au processus d'inactivation : l'humidité (gonflement de la spore, ouverture de canaux), O_3 et les radicaux (qui endommagent la spore par diffusion/oxydation), l'humidité et les radicaux augmentant ainsi la cinétique d'inactivation relativement au traitement en milieu sec (Annexe 2).

Au cours de l'étude de l'inactivation des spores à l'ozone gazeux sec, nous avons voulu vérifier que le faible taux d'inactivation des spores *B. atrophaeus* mesuré n'était pas un artefact dû à l'effet de l'ozone sur le substrat (Pétri de polystyrène (PS)). Pour ce faire, nous avons testé l'effet d'un dépôt de *B. atrophaeus* (n'ayant pas eu de contact avec O_3) sur des Pétris pré-traités à l'ozone sec dans les mêmes

conditions expérimentales. Par ce test, nous avons découvert que le pré-traitement à l'ozone sec de ces Pétris de PS leur conférait une activité sporicide puisque une grande proportion des spores initialement déposées semblait avoir été inactivée. Cette découverte a ouvert un nouveau volet d'étude dans le "Projet Ozone".

Au chapitre suivant, nous tenterons de caractériser le caractère biocide conféré aux Pétris de PS suite, précisément, à leur traitement à l'ozone sec : pour cela, nous examinerons, d'une part, les modifications physico-chimiques de la surface du polymère ainsi que l'étendue des dommages subis par les microorganismes. Par la suite, cette étude sera élargie en testant d'autres substrats polymériques.

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CHAPITRE 3
Traitement à l'ozone de surfaces polymériques : caractérisation de l'activité biocide induite

3.1 Introduction

Afin de valider le caractère biocide conféré à la surface d'un Pétri de polystyrène (PS) préalablement traité à l'ozone sec, il est bien évidemment nécessaire de caractériser cette nouvelle propriété en recourant à des méthodes microbiologiques afin d'en étudier l'étendue, mais aussi physiques afin d'identifier les modifications des propriétés de surface dues au traitement et ayant mené à cette activité biocide. Dans ce chapitre, nous effectuons donc l'étude de l'activité biocide des Pétris de PS

traités à l'ozone d'un point de vue microbiologique et physique, que nous élargissons par la suite à des surfaces en polymère de différentes natures (principalement des polymères utilisés dans le domaine biomédical), traitées dans les mêmes conditions. La littérature actuelle comporte un bon nombre d'articles sur la caractérisation physique de matériaux suite à un traitement oxydant mais aucun ne considère un effet biocide acquis directement après une simple exposition à l'ozone. L'originalité de cette découverte a conduit Univalor à déposer une demande de brevet (avril 2008) [1]. Le présent chapitre débute par un exposé sur les principes fondamentaux gouvernant l'interaction entre un milieu oxydant et un matériau en polymère.

3.2 Surfaces polymériques traitées par un milieu oxydant

Dans le cadre de notre étude, l'exposé des principes fondamentaux gouvernant l'interaction entre un polymère et un milieu oxydant se limitera aux surfaces polymériques traitées par un plasma d'oxygène (ou contenant de l'oxygène) menant à la formation d'espèces réactives oxygénées telles que $O^{\bullet}$, O_3… Étant donné que les polymères sont peu ou pas affectés en volume, du fait du faible pouvoir de pénétration des espèces actives issues du plasma (au plus jusqu'à quelques dizaines de nm de la surface) [2], nous traiterons donc uniquement des effets en surface.

Les travaux de Crevier concernant l'effet des espèces réactives issues de la post-décharge d'un plasma (post-décharge de N_2-O_2) sur des microsphères de PS ont mis en évidence la présence d'érosion : celle-ci s'est avérée proportionnelle (entre 0.2% et 12% O_2) au pourcentage d'O_2 injecté dans la décharge [2]. Le caractère plus "agressif" d'une exposition en décharge des microsphères de PS donne lieu à un degré d'érosion beaucoup plus important [3].

Plus généralement, les décharges d'oxygène peuvent produire deux effets distincts à la surface des polymères : la gravure par des espèces réactives telles que des radicaux ($O^{\bullet}$…) et la formation en surface de groupements fonctionnels contenant de l'oxygène [2, 4]. Rappelons que la gravure physique (bombardements d'ions

menant à la pulvérisation) a lieu uniquement en décharge alors que la gravure purement chimique (adsorption, réaction et désorption du produit de la réaction) peut se réaliser à partir des espèces neutres dans le plasma ou dans sa post-décharge [5].

Pour illustrer le premier effet, nous pouvons citer les radicaux d'oxygène, très réactifs mais aussi très électronégatifs, qui sont facilement adsorbés à la surface et se lient, par exemple, avec des carbones, des hydrogènes pour former H_2O, CO_2…[2].

La réaction dominante lors d'un traitement par plasma d'oxygène d'un polymère est l'oxydation de surface : cette oxydation mène généralement à la formation en surface de groupements carbonyles (-C=O) accompagnés de groupements hydroxyles (-OH). C'est également ce que nous observons à la surface de polymères tel que le PS, suite à un traitement à l'ozone [6]. Évidemment, un tel mécanisme modifie la composition chimique de la surface en plus de l'endommager [7- 9].

D'un point de vue théorique, lorsque des espèces actives sont adsorbées sur une surface plusieurs évènements sont envisageables : celles-ci peuvent diffuser sur la surface ou simplement se désorber sans se recombiner, ou encore, se recombiner pour, par la suite, être désorbées. Les recombinaisons sont de deux types : recombinaison de type Eley-Rideal (E-R) ou recombinaison de type Langmuir-Hinshelwood (L-H) [2].

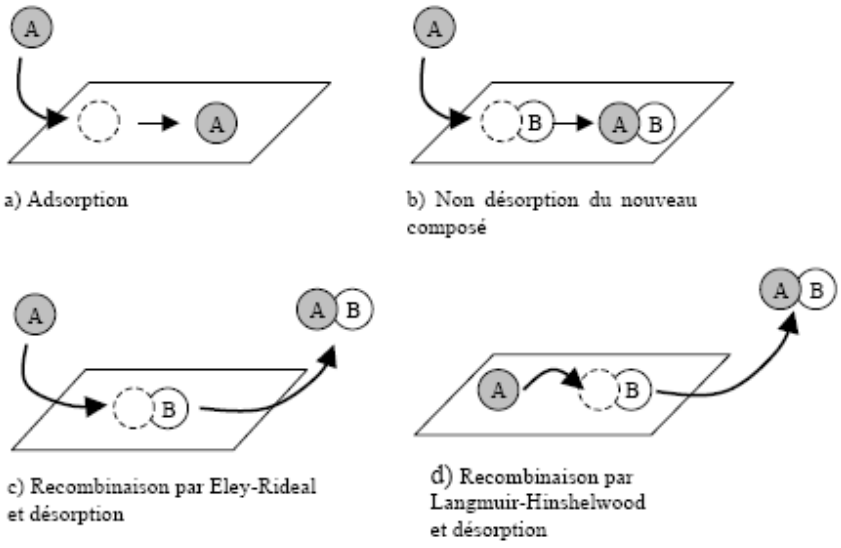

Figure 3.1. : Modèles de recombinaison d'un atome A en phase gazeuse avec un atome B de la surface (d'après [2]).

Lors de la recombinaison de type E-R, un atome incident A, non adsorbé, se recombine avec un atome B de la surface du solide. Alternativement, lors de la recombinaison de type L-H, un atome adsorbé A diffuse sur la surface et, par la suite, se recombine avec un atome B de la surface du solide. Le nouveau composé AB peut se désorber s'il forme un produit volatil (érosion) ou, simplement, rester à la surface dont il modifie ainsi la composition chimique.

Une conséquence de ces modifications chimiques est l'augmentation possible de la mouillabilité de la surface des polymères [4, 6, 7, 10, 11]. Ce résultat est dû à la formation de groupements fonctionnels tels que -C=O, -COOH et -OH qui ont une très grande affinité avec l'eau et qui, par conséquent, augmente le caractère hydrophile de la surface. La présence de ces groupements peut être mis en évidence par diverses méthodes de diagnostics physiques telles que par FTIR ou encore par XPS [12, 13].

Toutefois, il s'avère que la concentration des groupements fonctionnels établis à la surface des polymères par les espèces actives issues de la décharge peut se modifier avec le temps : on parle alors d'un phénomène de "vieillissement". Les chaînes de polymères situées à la surface ont une grande mobilité comparativement à celles situées dans le volume [14] : ainsi, la surface des polymères se réorganise constamment pour tenter d'atteindre l'état énergétique le plus stable possible. Néanmoins, le taux de réticulation des chaînes peut diminuer l'effet de vieillissement puisque la mobilité des chaînes de polymères se trouve réduite par un haut taux de réticulation (définie au chap.2, Sec 2.3.1). Les travaux menés par Crevier ont montré que, quelques heures après le traitement au plasma, la surface de polymères revient pratiquement à son état antérieur : les chaînes de polymères modifiées par le plasma semblent migrer vers l'intérieur de l'échantillon pour acquérir une configuration plus stable [2, 15]. Cette reconfiguration de la surface est particulièrement prononcée pour le silicone. Cette propriété des polymères peut, toutefois, poser problème lorsque l'on

souhaite modifier une surface de manière permanente. Le vieillissement des surfaces de polymères traitées par plasma est un phénomène très complexe qui dépend fortement de la nature du polymère, des conditions de traitement et, éventuellement, de leur stockage suite à l'exposition.

Application. La modification de surfaces polymériques par plasma (oxydation…) fut longtemps étudiée puis utilisée pour améliorer l'adhésion et la croissance des microorganismes sur ces substrats [8, 9, 16-18]. Plus récemment, ces méthodes sont employées comme étapes préliminaires pour modifier la surface afin de faciliter l'incorporation d'espèces moléculaires ayant des propriétés antibiotiques, antibactériennes ou, plus généralement antimicrobiennes, afin de prévenir une éventuelle contamination de la surface, soit en inactivant les microorganismes, en inhibant leur croissance ou, encore, en empêchant leur adhésion [11, 19, 21, 22].

3.3 Traitement à l'ozone de surfaces polymériques leur conférant un caractère biocide

Les surfaces ayant un caractère antimicrobien, notamment antibactérien, ont été largement étudiées et continuent de faire couler beaucoup d'encre compte tenu des enjeux en cause, notamment pour les surfaces polymériques [21-23]. Ces surfaces "actives" sont généralement obtenues suite à un revêtement en surface ou au greffage (sur une profondeur limitée) d'espèces actives connues pour leur activité antimicrobienne : ces surfaces nécessitent généralement une, voire plusieurs étapes de préparation pour permettre une efficacité de greffage optimale. Cette préparation peut prendre plusieurs formes selon le type de substrat et de molécules à greffer, incluant les procédés chimiques (chimie en solution) [24, 25], les procédés plasma (tel que Ar/O_2 ou O_3) [26, 27, 20, 28] ou encore des procédés se combinant à l'effet des photons (tel que O_2/VUV ou UV/O_3) [17, 29]. Leur mode d'action consiste à tuer les microorganismes, à les inhiber ou à empêcher leur adhérence à la surface. Ces surfaces sont généralement efficaces sur un large spectre de bactéries (Gram positif et Gram négatif) et, dans certains cas, sur les virus et les champignons (moisissures)

[30-32]. Par contre, rares sont les surfaces qui, après traitement (revêtement), acquièrent une activité sporicide [33].

3.3.1 Surface de Pétris de PS traitée à l'ozone sec gazeux : effet biocide et caractérisation de la surface

Comme nous l'avons annoncé en début de chapitre, nous avons examiné des Pétris de polystyrène traités à l'ozone pour déterminer l'activité biocide acquise suite à ce traitement. Une première étude a consisté à mettre en évidence, par des méthodes microbiologiques, le caractère biocide de la surface traitée et à caractériser ensuite les modifications induites en surface en ayant recours à divers diagnostics tels que le FTIR, l'AFM ou encore le XPS. Nous avons tenté, en corrélant ses résultats, de comprendre le lien entre l'effet biocide observé et les modifications de surface induites. L'article suivant (soumis à Journal of Applied Surface Science et accepté avec corrections mineures) présente les résultats de cette première étude.

Biocidal action of ozone-treated polystyrene surfaces on vegetative and sporulated bacteria

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ABSTRACT

Surfaces of materials can be modified to ensure specific interaction features with microorganisms. The current work discloses biocidal properties of polystyrene (PS) Petri-dish surfaces that have been exposed to a dry gaseous-ozone flow. Such treated PS surfaces are able to inactivate various species of vegetative and sporulated bacteria on a relatively short contact time. Denaturation of proteins seems likely based on a significant loss of enzymatic activity of the lyzozyme protein. Characterization of these surfaces by atomic-force microscopy (AFM), Fourier-transform infra-red (FTIR) spectroscopy and X-ray photoelectron spectroscopy (XPS) reveals specific structural and chemical modifications as compared to untreated PS. Persistance of the biocidal properties of these treated surfaces is observed. This ozone-induced process is technically simple to achieve and does not require active precursors as in grafting.

1. Introduction

The various possible interactions between microorganisms and surfaces of different materials have been receiving considerable attention in biomedical areas over the last decades. Two main research directions are emerging from these studies: i) the adhesion of cells on surfaces (including their growth and proliferation) and ii) the biocidal action of surfaces.

Various surface treatments/modifications have been extensively examined to control the adhesion and growth characteristics of cells on surfaces [1-4], a matter of prime importance, for instance, for tissue engineering [5]. Understanding the relationship between cells and the physicochemical properties of surfaces with which they interact (such as wettability, functional groups, topography) is, in fact, essential for the optimisation of cell adhesion, their spreading, and growth thereon [1]. Improved cellular attachment [3] is promoted by certain functional groups, which at the same time increase hydrophilicity, favouring the adsorption or adhesion of proteins [6]. For example, surface modifications with UV/O_3 [1] or with plasmas such as corona discharges [2] can increase proliferation and protein expression of cells or enhance their culture process. Similar action can be obtained with coatings with well-defined surface chemistries as they can also amplify, or prevent, bioadhesion of molecules, cells and, in some cases, bacteria [7-10].

Antimicrobials properties resulting from surface modifications and specific coatings have been the object of a large number of studies. As a matter of fact, it is known since antiquity that some heavy metals such as silver and copper possess anti-infective activity. These metals can be impregnated on surfaces such as venous, vascular or urinary catheters [11,12] or they can be immobilized in textiles and ceramics, providing antimicrobial properties [13]. Such treated materials are routinely used even nowadays in healthcare, for instance in burned skin treatment, to prevent infections [11,13,14].

More recently, surface coatings incorporating antimicrobial, antibiotic or antiadhesive molecules for preventing surface contamination have been proposed [12]. Conventional surface modification techniques for achieving coatings are usually

based on the incorporation into a polymeric surface of a leachable antiseptic. Plasmas can be used in such processes for surface modification, deposition, or as a preliminary step before grafting reactions, as needed in the fabrication of specific medical devices and biomaterials [7,15]. To illustrate the use of plasma in such applications, consider the work of Zhang et al. [16] reporting the covalent immobilization of antimicrobial bronopol and triclosan molecules by means of plasma-immersion ion implantation (PIII). This method gives excellent antimicrobial properties to polyethylene and PVC (medical grade) surfaces against Gram-positive and Gram-negative bacteria [16,17]. Initially, the surface is activated by an O_2 plasma to provide more hydrophilic groups in order for triclosan and bronopol molecules to be integrated more efficiently on the surface. Other authors showed that surfaces could also be modified chemically (e.g. thiocyanation) by turning to various gas plasmas (Ar, O_2) [17,18], with O_3 [19], with vacuum ultra-violet (VUV) irradiation in the presence of O_2 and with UV/O_3 exposure [2, 20] before grafting the antiseptic or other active species [21]. These modified surfaces exhibit antimicrobial effectiveness on a broad spectrum of Gram-positive and Gram-negative bacteria and in some case viruses and fungi [21,13,17].

The novel method that we present allows polystyrene (PS) Petri dishes to acquire biocidal features ensuring microorganism inactivation. These properties were demonstrated for both vegetative and sporulated bacteria. As a rule, surface modification studies conferring sporicidal properties are scarcer than those providing antibacterial properties [15,22]. Special attention should nonetheless be paid to endospores because they can withstand severe treatments including heat, irradiation, chemicals and desiccation, and as such are generally used to validate sterilization processes. Our method requires no grafting reactions with chemical precursors, but simply exposure to an ozone flow. Recall that because of its optical, mechanical and chemical properties, PS has commonly been used for the cost-effective production and commercialization of culture vessels replacing glass, in the mid 1960's, for cell culture and biological assays [23].

The paper is organized as follows. Section II describes the materials used and the experimental methods. Section III characterizes the biocidal activity of treated PS surfaces and its effect on the structural integrity of various vegetative bacteria, bacterial spores and lyzozyme (protein). In section IV, surface diagnostic techniques such as Fourier-transform infra-red (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and atomic-force microscopy (AFM) are used to characterize physical and chemical modifications of the PS surface after treatment. Summary and conclusion are presented in section V.

2. Materials and methods

2.1. Ozonation system and PS treatment

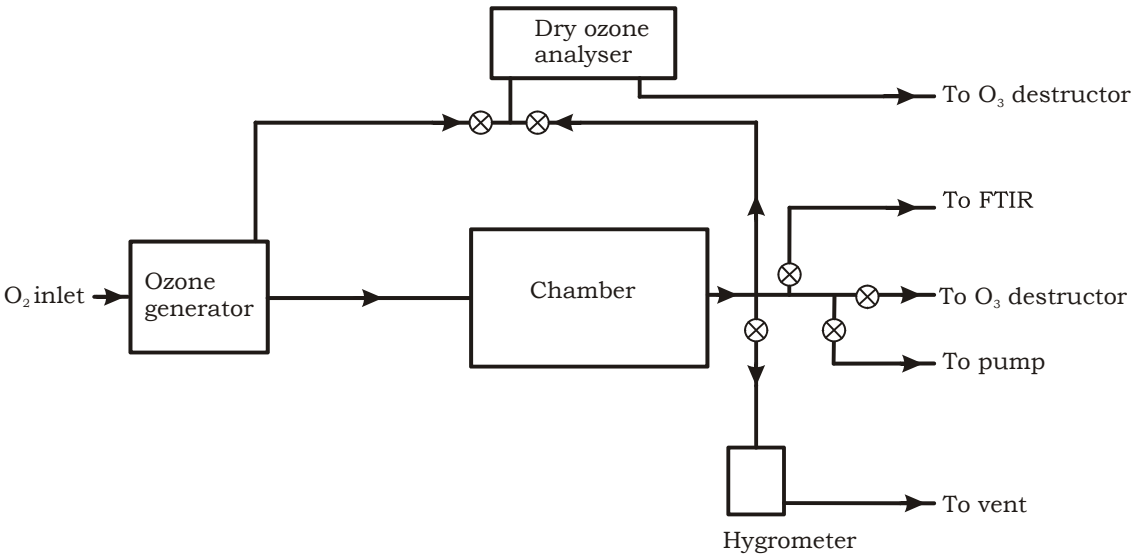


Figure 1. Schematic view of the experimental arrangement utilized to generate ozone and determine its concentration as it enters and exits the chamber. The nature of the effluents exiting the chamber can be analyzed through FTIR spectroscopy. An O_3-destructor (based on a chemical catalyser) is provided to abate ozone.

Fig. 1 shows the various elements needed to generate ozone and determine its concentration as it enters and exits the experimental chamber. This chamber, made from 316 stainless steel, is a 400 mm long, 100 mm high and 220 mm wide

parallelepiped. Ozone concentration can be monitored with a dry-ozone analyzer (based on UV absorption). In addition, the generated effluents can be analyzed through FTIR spectroscopy (Avatar 370 spectrometer from Thermo Nicolet using a DTGS (7800-375 cm^{-1}) detector; the number of scans and resolution were set at 80 and 1 cm^{-1}, respectively)

The ozone generator provides a mixture of molecular and atomic oxygen in the gas phase; it is operated within the electrical current domain over which a current increase upregulates the ozone concentration. The ozone flow is dry since the generator is supplied from (high-purity) O_2 dry-gas bottles. The gas temperature in the chamber is close to ambient (≈ 22 ^oC). The expression "dry ozone" as used herein refers to gaseous ozone with a relative humidity of less than approximately 2 %.

An O_3 destructor based on a chemical catalyser is provided to abate ozone and to release it as O_2 to comply with safety (toxicity) regulations. For safety reasons, the chamber is located within a hood and a vacuum dry pump is used to make sure that the chamber effluents are fully evacuated toward a centralized extractor system.

Sterile polystyrene Petri dishes (Starstedt®) of 60 mm diameter are introduced into the chamber (Fig. 1) for their surface treatment. The physical parameters of this ozone process are set as follows: 5.64 standard liter/min (slm) O_2 flow rate for a 4000 ppm ozone concentration, and 60 min exposure time. Aged treated samples were kept in air atmosphere and protected from light.

For comparison with the specific ozone treatment, some PS Petri dishes were exposed during 7 hours to UVC irradiation by means of a mercury lamp, generating the 254 nm germicide wavelength in air atmosphere, located at a distance of 64 cm.

2.2. Surface diagnostic techniques:physico-chemical characterization

FTIR

FTIR (microATR) spectra were recorded on a FTS 7000 Series (Digilab) spectrometer using a mercury-cadmium-telluride (MCT) detector and germanium

crystal. Spectra were obtained in attenuated total reflectance (ATR) mode with 256 scans and a resolution of 4 cm^{-1}. Samples were analyzed in air atmosphere.

AFM

AFM images were acquired in air at room temperature using a Digital Instruments (Dimension 3100, Santa Barbara, CA). Intermittent contact imaging, so-called "tapping mode", was achieved at a scan rate of 1 Hz using etched silicon cantilevers with a resonance frequency around 300 kHz, a spring constant of ≈ 42 N/m, and a tip radius of <10 nm. All images were acquired with a medium tip oscillation damping (20-30%).

Contact angle measurements

Surface wettability of PS and glass Petri dishes was characterized before and after ozone treatment, using the water-drop-shape method (VCA Optima® goniometer). Distilled water (2μL) was dropped onto 4 different sites on each sample to provide a statistical average for each sample. Experiments were conducted under ambient humidity and temperature conditions.

XPS

The surface chemical state was determined using an Escalab 220i XL, employing a polychromatic Mg radiation and operated at 15 kV and 300 W in a residual vacuum of < 1×10^{-9} mbar. Elemental surface composition (atomic %) was calculated from survey spectra measured at a pass energy of 100 eV and detailed surface chemical information (high resolution) was obtained by analysis of the carbon 1s peak envelopes at the higher resolution pass energy of 20 eV. Chemical shift data are referenced to the center of the C-C peak at 284.6 eV. The studied area (1.5mm × 1.5mm) was analyzed at 90° (normal incidence) relatively to the surface. The decomposition method used condiders a Lorentzian contribution of 30% and a Gaussian contribution of 70%.

2.3. Vegetative and sporulated bacteria used for the study: their methods of preparation and deposition on surfaces

Inoculi of 10^6 spores of *Bacillus atrophaeus* ATCC® 9372 (formely *Bacillus subtilis*), *Geobacillus stearothermophilus* ATCC® 7953 (formerly *Bacillus stearothermophilus*), and *Bacillus pumilus* ATCC® 27142 were tested. These spores are routinely used in the validation of sterilization processes. A selection of Gram-positive *Bacillus atrophaeus (vegetative cells), Staphylococcus aureus, Staphylococcus epidermis, Micrococcus luteus,* and Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa* (10^4 bacteria) were also tested. The Petri dishes containing the vegetative bacteria suspensions were hermetically sealed, preventing them from drying (to maintain them alive), in contrast to those for spore suspensions. These various microorganisms were prepared in the Laboratoire de contrôle des infections (Faculty of Dentistry) at Université de Montréal. The microbiological method of preparation was previously described in [24].

2.4. Preparation of endospore inoculi and their recovery after contact with PS surfaces

Test endospores prepared in 100 µL of distilled water were deposited at the center of the PS Petri dish either untreated (control) or pretreated with ozone under the conditions described in section 2.1. These deposits were then covered, dried in ambient air and protected from light. Some of these, deposited on control surfaces, were recovered after drying in order to determine the actual number of colony forming units (CFUs) in these deposits. The microbiological method of microorganism recovery was previously described in [24]. In some cases, a nutritive solid medium (Trypticase Soy Agar) was directly poured into the treated Petri dish with its bacterial deposit. All samples were incubated for at least 24 h for *B. pumilus* and *B. atrophaeus* spores and at least 48 h for *G. stearothermophilus* spores before recording the results: the efficacy of the treatment was determined by counting the number of CFUs. Twenty four-well PS multiplates (Costar®-Corning Incorporated)

were also used as follows. Tryticase Soy Broth (TSB) nutritive medium with a pH indicator was added in each well as soon as the *B.atrophaeus* (100µL) deposits have dried: bacterial growth was recorded whenever the pH changed and the medium became turbid. Each reported experimental value represents the average over at least 3 measurements.

Germination inducers such as aniline, dipicholinic acid/calcium and lysozyme were used to verify the incapacity of spores to germinate after having been deposited on treated PS surfaces.

Since it is known that generation of peroxides can result from an O_3 treatment, catalase (Sigma®), chemically known for reacting with H_2O_2 molecules (with production of H_2O and O_2), was used in a concentration of 10 units/ml to determine whether H_2O_2 molecules were formed and adsorbed on the PS surface after treatment.

2. 5. Structural integrity of spores

2.5.1. Scanning Electron Microscopy

A Hitachi S-4700 Scanning Electron Microscope (SEM) equipped with a « cold FEG » transmitter enables acquiring micrographs. The spatial resolution range of the apparatus is 1.7 nm and 1.2 nm nm when operated at 1 kV and 15 kV, respectively. Probe current is varied from 1 pA to ≈ 2 nA. The micrographs were taken at a voltage value of ≈ 4 kV and a probe current of 10µA. Magnification ranged from 20× to 500 000×.

2.5.2. Bacterial spores staining procedure for DNA marking: DAPI

The DNA intercalating dye 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical. The stock solution of DAPI was prepared according to manufacturer's instructions in water at 20mg/ml and conserved at 4°C until use. At time of use, the stock solution is dissolved in McIlvain's buffer pH 4.0 at a final concentration of 50 µg/ml. Spores were stained directly into the PS Petri dishes. Incubation was achieved at 21°C in the dark during 15 minutes to 1 hour. Staining solution surplus was then drained out and spores left to dry in the dark. Observations

were made with epifluorescence microscopy (Labolux 12, Leitz) with 100× immersion objective and captured with an Infinity 2 Camera with Lumenera software (Olympus, On., Canada).

2.6. Lyzozyme: enzymatic activity measurements

The effect of O_3-treated PS surfaces on proteins was tested on dried deposits of lysozyme. Since lysozyme is an enzyme, its activity can be easily measured, since any loss of activity after exposition to treated PS indicates alteration of the protein. Fourteen μg of lyzozymes in 100 μL water were deposited on treated and untreated PS and glass Petri dishes. Glass Petri dishes, for microbiological experiments, were used as negative controls since the interaction of ozone with glass, as opposed to polystyrene, is expected to be minimal.

The enzymatic assay is conducted as follows: the protein is mixed with a suspension of *Micrococcus luteus*, a Gram-positive bacterium. Active lyzozyme will lyse this bacterium. The lytic activity is monitored, at various periods of time, through changes in turbidity levels of the bacterial suspensions recorded by optical density (OD) readings at 540 nm. Changes due to the presence of the protein are calculated against blank values containing *M. luteus* bacteria only. When lysozyme has lost its enzymatic activity, there is no lysis of *M. luteus*, and therefore no changes in OD compared to its blank value. Readings recorded with lysozyme not exposed to ozone served as positive control. The assay was repeated 4 times in triplicate samples.

3. Results and Discussion

3.1. Effects of treated PS surfaces on microorganisms and biomolecules

3.1.1. Vegetative bacteria

Bacterial suspensions were deposited on PS Petri-dish surfaces (previously treated by ozone) and kept in contact with these during a given time (30 min and 3 h), after Trypticase Soy Agar (TSA), a solid nutritive medium, was added.

Bacteria	***S. aureus***	***E. coli***	***S. epidermis***	***M. luteus***	**P. aeruginosa**	***B. atrophaeus***
Characteristics	Gram + cocci	Gram - bacilli	Gram + cocci	Gram + cocci	Gram - bacilli	Gram + bacilli
Contact time : 30 min	+	+	+	+	+	+
Contact time : 3h	-	-	-	-	-	-

« + » : Positive growth : whatever the number of CFUs.
« - » : Negative growth : no bacteria were able to grow.

Table 1:Biocidal influence of the contact time on various types of vegetative bacteria when deposited on PS Petri-dish surfaces previously treated during 60 min with dry gaseous ozone at a 4000 ppm concentration. Bacteria viability was determined following addition of a nutritive medium (TSA) on samples

A significant reduction in the number of CFUs was observed after 30 min of contact with the treated surfaces and this for all species of vegetative bacteria considered (Table 1). After 3 hours of contact, no CFU was observed on TSA medium for all the tested bacteria. Since, as mentioned above, the vegetative bacteria suspensions were not allowed to dry, one could consider that their interaction with the treated surface is less direct than with dried spores.

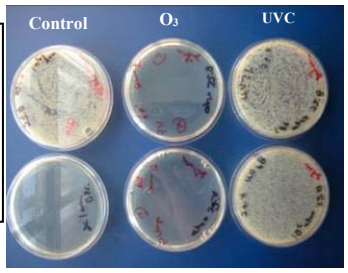
Surfaces with antibacterial characteristics resulting either from coating or grafting are known to lead to bacteria inactivation either by their killing, by inhibiting their growth or by preventing their adsorption on such surfaces [13]. For example, triclosan or bronopol coatings on polyethylene kill or inhibit bacteria by affecting the membrane structure of bacteria (these molecules cannot kill all the adhered bacteria immediately) [14,16,17]. However, certain polymeric coatings are found to definitely kill bacteria on contact by rupturing their cell membranes [21].

3.1.2. Bacterial spores

Inactivation efficacy

	Control Samples	Recently pre-treated samples	Aged pre-treated samples
B. pumilus	+	- *(n=4)*	- *(n=1)* + *(n=3:* CFU=20 ± 18.3*)*
B. atrophaeus	+	- *(n=4)*	+ *(n=4 :* CFU <100*)*
G. stearo	+	- *(n=4)*	- *(n=4)*

+ : microorganism growth (forming colonies)
- : no microorganism growth
n : number of samples examined



⇑ No survivors

Table 2 :Viability of three types of bacterial spores after their contact with ozone pre-treated PS dishes. The picture shows PS Petri dishes covered with a solid nutritive medium (TSA) after 24 h incubation time at 37°C: dishes previously pre-treated with O_3, UV light and untreated ones (control) are presented.

Pre-treatment of PS Petri dish surfaces during 10 min induced a 99-to-99.9% decrease in the initial number of CFUs (from 10^6 to 10^4-10^3). In cases where the Petri dishes were pre-treated for 60 min, all species of bacterial spores were completely inactivated (table 2). The effect of a pre-treated PS 24-well multiplate on the viability of 10^6 *B. atrophaeus* spores in 100µL of water was also tested to consolidate the experimental results obtained with PS dish surfaces: no growth was observed in 21/22 wells after addition of Trypticase Soy Broth (TSB) nutritive medium. The observed inactivation was not the result of the incapacity of spores to sense germination signals since germination inducers had no effect on the spores.

The UVC-treated PS surfaces did not provide any biocide effect, as with untreated surfaces. This shows that the surface modifications leading to the observed biocidal properties cannot be achieved through photon-induced reactions (photons/surface interaction) but probably result from direct chemical reactions.

Literature reports that peroxides, known for their antibacterial activity, can be adsorbed and eventually formed on polymeric surface after treatment with H_2O_2 vapors, ozone or other processes implying highly oxidative species [25-27]. To rule out this eventuality in the present case, catalase was added on treated Petri dishes to determine whether the antibacterial effect could be suppressed: the pre-treated PS surfaces kept their full antimicrobial activity. The chemical formation of peroxyde is thus not implicated in the inactivation of the microorganisms.

Furthermore, washing the treated surfaces with water, alcool or buffer solutions did not affect the biocide properties since no survivor was detected after adding TSA nutritive medium on dried spore deposits.

Surprisingly, aged treated surfaces still exhibited sporicide properties, which suggest persistence of the key chemical modifications. In fact, almost two months after ozone treatment, no growth was observed when *G. stearothermophilus* spores was deposited on samples while an average of 20 ± 18 CFUs was counted for *B. pumilus* spores and less than 100 for *B. atrophaeus*.

Evaluation of spore damage using SEM

SEM micrographs were taken to visualize eventual damage to the spore external structure. Results for *B. subtilis* and *B. pumilus* spores deposited on treated and untreated PS surface of Petri dish are presented in figure 2.

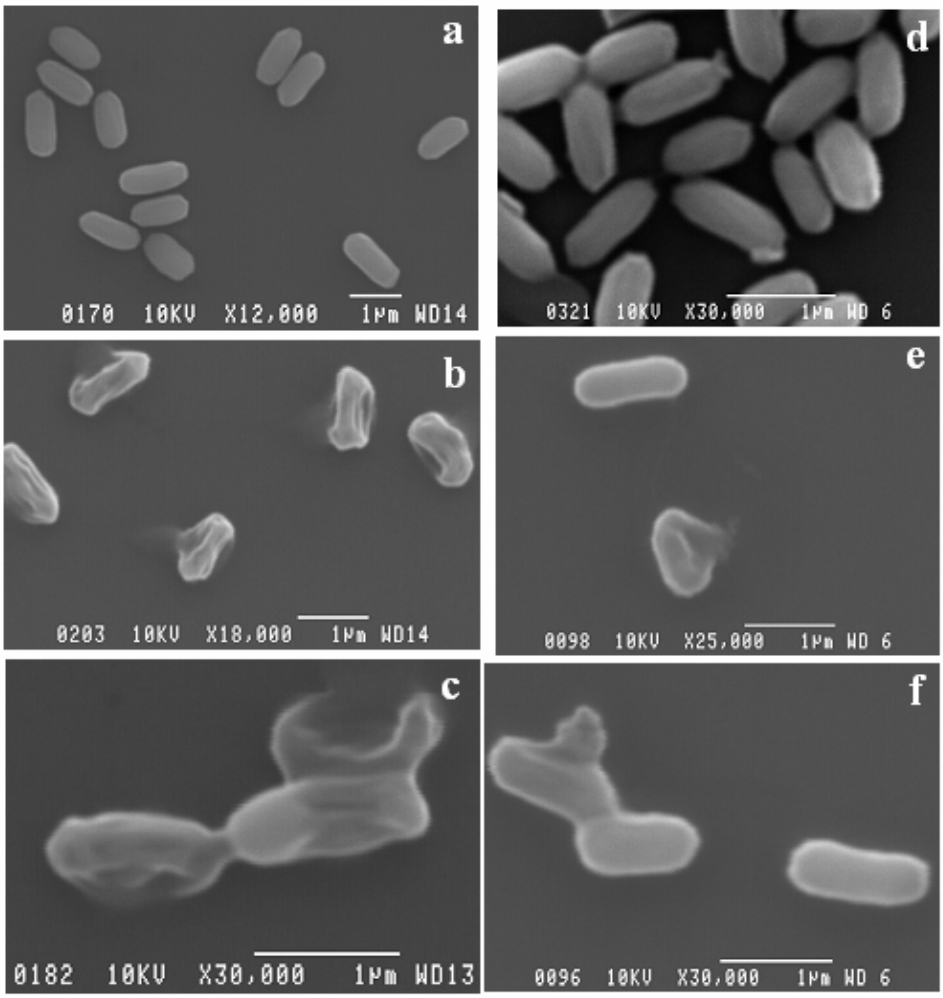


Figure 2. SEM micrographs of *B. atrophaeus* (a, b, c) and *B. pumilus* (d, e, f) spores after having been in contact with PS Petri dish surfaces previously treated 60 min with 4000 ppmv of dry gaseous O_3 (b, c, e, f) and with untreated ones (a and d).

These micrographs clearly show that the structure of *B. atrophaeus* and *B. pumilus* spores is affected. Some of them are seriously deformed and extrusion of their inner material is observed (b,c,e,f). Even though severe obvious alterations to the spore integrity are observed, the morphology of a minority of them is apparently unaffected (e,f).

There are only a few descriptions in the literature of changes in the morphology of vegetative bacteria deposited on antibacterial surfaces. It was then observed that the bacterium membrane structure (and consequently its function) can be affected leading to its inhibition or its death [16, 21]. Moreover, contact of bacteria with certain polymeric coatings can even lead to rupture of their cell membranes which definitely kill them [13]. In contrast, as far as we know, no information about spore structural modifications has been reported in the literature.

2.3. DAPI staining

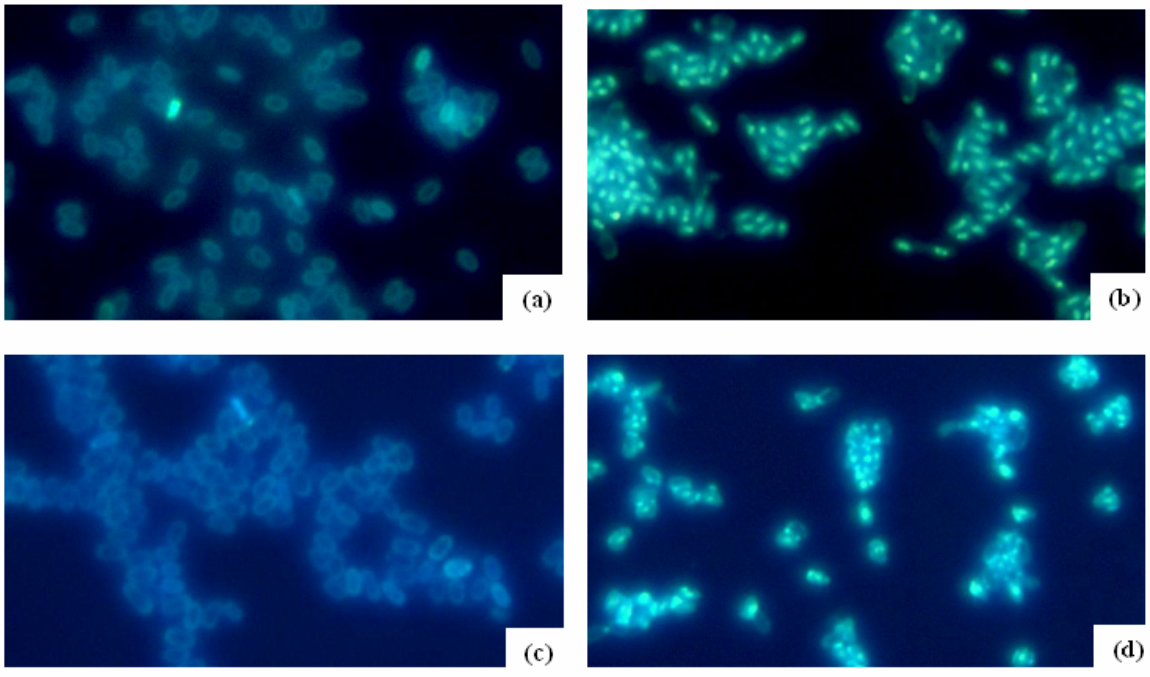


Figure 3. Epifluorescence microscopy of *B. atrophaeus* spores stained with DAPI: (a) when untreated, (b) after their treatment in autoclave for 15 min (liquid cycle used), (c) after their exposure to a UVC lamp (λ= 254 nm) and (d) after their deposition on pre-treated PS Petri dish. The stained nuclear material appears strongly brighter relatively to other spore components.

The DAPI dye is primarily nucleic acid-specific. The cortex and the coat have been shown to be permeable to small molecules and intact spores will present a peripheral staining with dyes such as DAPI. The inner membrane of intact spores is not permeable to the dye [28]. Permeabilization of spores is needed in order for the dye to reach and stain the spore DNA. Treatments such as SDS (Sodium Dodecyl Sulfate), urea, DTT (Dithiothreitol) or protease K can, for example, achieve this goal [29].

Using DAPI staining, we could readily discriminate spores which present differences in permeability after different treatments. Although DAPI staining of the spore core (DNA) does not necessarily indicate spore inactivation, it provides direct information on loss of permeability. UV light exposure (λ= 254 nm) alone is known to be sporicidal but as shown by the ring shape DAPI staining (Fig.3c) it does not alter the spore permeability. However, treatment with autoclave (steam sterilization) leads to bright staining of the core (Fig. 3b). The main killing mechanism of moist heat is protein denaturation [30], which can explain the gain of permeability that allows the DAPI stain to diffuse up to the DNA. The contact with treated PS dishes induces a clear permeabilization of the spores (Fig. 3d). The staining is even brighter than with steam treatment. Our results indicate that the active principle of ozone-treated PS reached the normally impermeable inner membrane. We can hypothesize that treated PS is more potent than steam to destabilize protective coats and inner membrane. The pattern of DAPI staining induced by treated PS should be noted: compared to what is observed with steam, stained material of spores in treated-PS seems to be fragmented and irregular. This observation in parallel with SEM micrographs is strongly in favor of core content destabilization and extrusion of DNA.

3. Lyzozyme: enzymatic activity measurements

The effect of ozone-treated surfaces on proteins was tested with dried deposits of lysozyme. Figure 4 represents the percentage of the remaining enzymatic activity of lyzozymes deposited on PS and glass Petri dish surfaces previously exposed for specific times to dry gaseous ozone. It can be seen that the enzymatic activity of the lysozyme proteins is significantly reduced upon contact with treated PS surfaces. It actually decreases with ozone exposure time, ending up with approximately a 16 % residual activity only after 6 h of pre-treatment. In contrast, more time is needed under direct O_3 flow to be able to detect a decrease in the lyzozyme enzymatic activity: the remaining activity after 3 h is still high, namely 93.5% ± 0.2%, while after 6 h, it is down to 30% ± 5%.

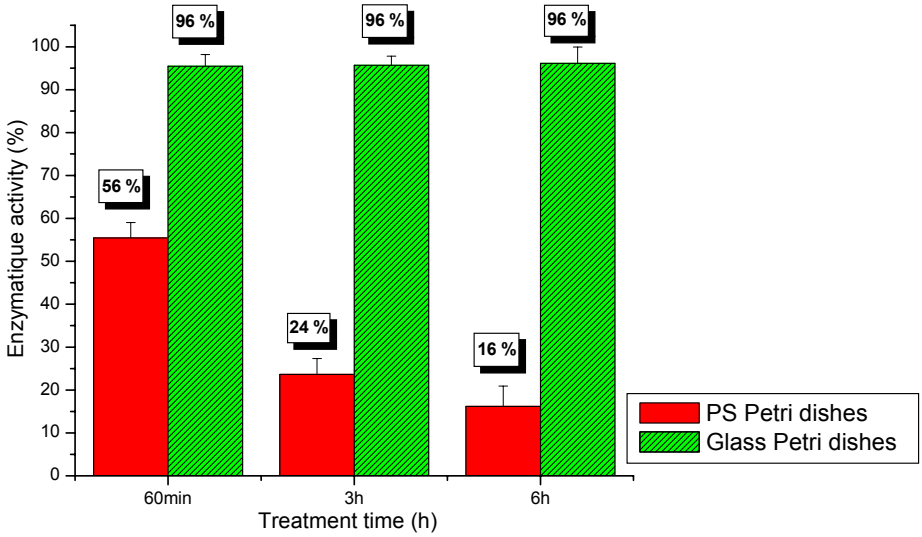


Figure 4. Enzymatic activity (in percentage), determined from optical density measurements, upon exposure of PS and glass Petri dishes to dry gaseous ozone at a 4000 ppm concentration for 1 h, 3 h and 6 h.

Whereas PS surfaces treated with O_3 degrade the enzyme expression as we just showed, PS surfaces modified by UV-O_3 treatments are reported to enhance proliferation and protein expression [2,6]. Moreover, some functional coatings used to improve the spreading and growth of certain cells and to immobilize biomolecules

(enzymes, peptides…) leave their biological activity unaltered: this contrasts with our results, which clearly showed a loss of enzymatic activity [31].

4. Physical and chemical characterization of PS surfaces after the ozone treatment

Surface modification treatments such as plasma or corona oxidation have been investigated and showed positive influence on the cell growth process, even though these surface treatments are known to be aggressive, causing damage to delicate polymer surfaces [1]. To characterize surface modifications after dry gaseous ozone exposure, techniques such as AFM (Atomic Force Microscopy), FTIR (Fourier Transformed Infra Red) and XPS (X-ray Photoelectrons Spectroscopy) were used.

4.1. AFM

Contact angle values measured before and after ozone treatment were respectively 57.7 ± 1.5 and 30.2 ± 1.0. These macroscopical measurements indicate that the surface has been modified either chemically (polarity) or/and topographically (roughness). Eventual topographic changes resulting from O_3 treatment of the PS Petri dish surface have been investigated with AFM under intermittent contact imaging mode (i.e., “tapping mode”).

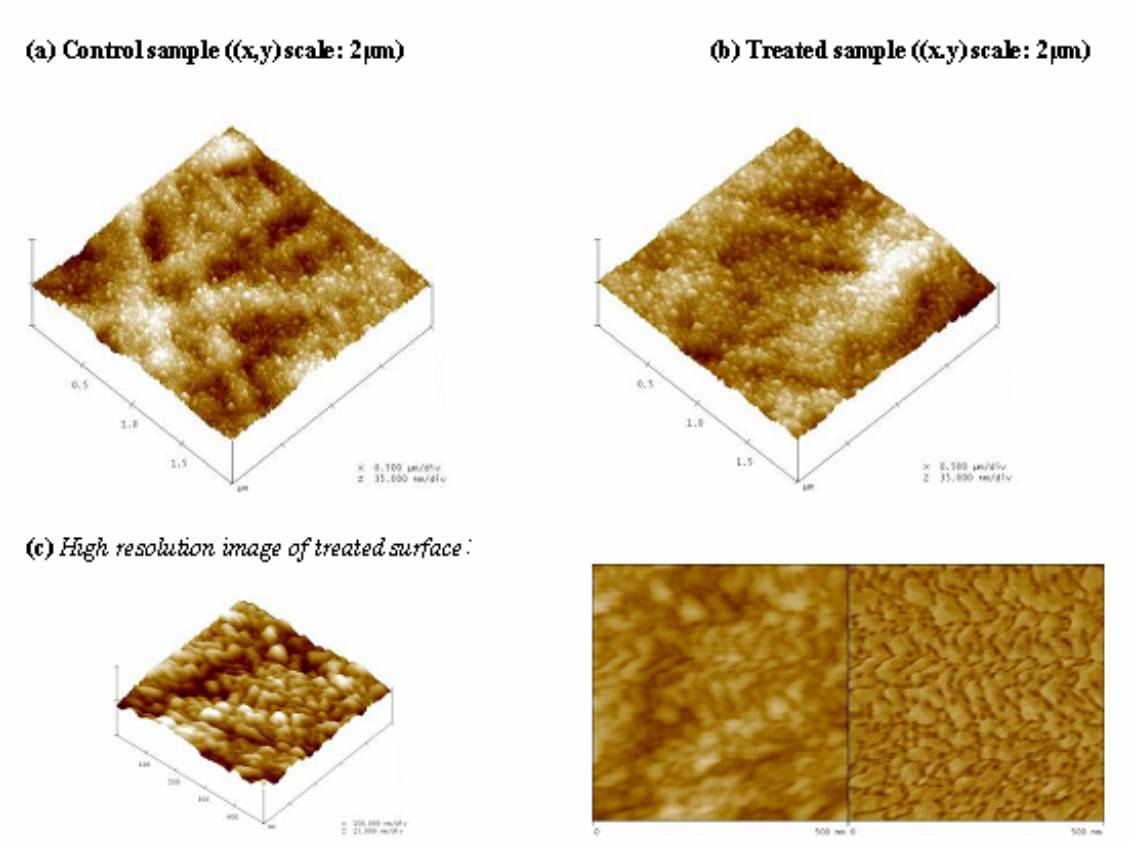


Figure 5. Tri-dimensional (3D) AFM images of Petri dishes: (a) untreated and (b) exposed for 60 min to ozone at 4000 ppm (2 x 2 μm (x, y) scale); (c) given topographical image, 3D (left) and 2D (right) in high resolution mode and its corresponding phase.

Fig. 5a shows that the untreated surface is apparently striated (along the x-y direction) and has a great number of reliefs. After treatment (Fig. 5b), the global striated aspect still remains identifiable but is less pronounced. As for the gradient in height, it is less important after treatment as we can infer from the AFM images and as confirmed by surface roughness measurements. The rms roughness of the surfaces after treatment decreases slightly from 7 nm to 5.7 nm (scale 50 μm), as shown in Table 3. For comparison, the rms surface roughness of UV/O_3 PS treated surfaces is reported to increase from 3.5 nm to 6.5 nm (scale 20μm) [4]. It was also reported that the roughness of PS surfaces treated with a CO_2 microwave plasma discharge increases, indicating that degradation of the polymer occurred during the treatment [32].

Scale (μm)	*Rugosity Rms (nm) Control sample*	*Rugosity Rms (nm) Treated sample*
50	7	5.70
10	3.75 ± 0.55	3.41 ± 0.01
2	2.09	1.70 ± 0.10
0.5		0.91 ± 0.02

Table 3. Rms surface roughness values of the ozone-treated PS Petri dish surfaces and its control under different observation scales. The 0.5 μm scale corresponds to the highest resolution image (Fig. 4c). Samples were exposed for 60 min to ozone at 4000 ppm.

AFM measurements on treated samples resulted in a very rapid wear out of the silica tip, which required to change it very often, otherwise the image resolution would then highly degrade. As a matter of fact, we noticed a great affinity between the silicon tip and the treated surface, characterized by a strong spatial drift of the tip probably related to a change in its resonant frequency (observed on the topographic and phase images) as well as a decreased resolution on topograpic images. The tip-treated polymer affinity mentioned could be related to findings by Teare et al. [1] who observed the formation of low-molecular weight oxidized materials, characterized by a highly mobile fluid-like layer called Weak Bond Layer (WBL) on the polymer surface.

As for the phase observed in Fig. 4c, it could be due to (1) a visco-elasticity contrast or (2) a chemical contrast (provided chemical modifications have occurred on the surface). The analysis of all the AFM images of the treated sample obtained under different scales, including in high resolution (Fig. 5c), enable us to claim that the observed frequency shift is due to a chemical contrast.

4. 2. FTIR

FTIR spectra were taken before and after ozone treatment of the PS dish surfaces to evaluate possible changes in the molecular structure. Two different exposure times were investigated, namely 10 min and 60 min, to eventually highlight the temporal evolution of the treated surface chemistry. The IR spectra presented in

Fig. 6 supports the occurrence of chemical modifications for PS Petri dishes treated by ozone, as already inferred from AFM.

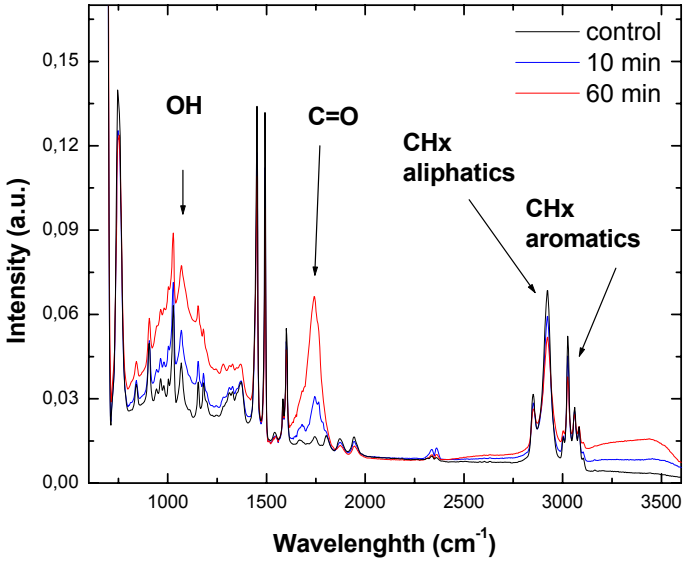


Fig. 6. FTIR spectra of untreated and treated samples in the 600-3600 cm^{-1} range. Treated samples correspond to PS Petri dish surfaces exposed for 10 min and 60 min to dry gaseous ozone (4000 ppm). New peaks and a broad continuum appear respectively at around 1720-1740 cm^{-1} and 900-1300 cm^{-1} on 60 min treated surfaces.

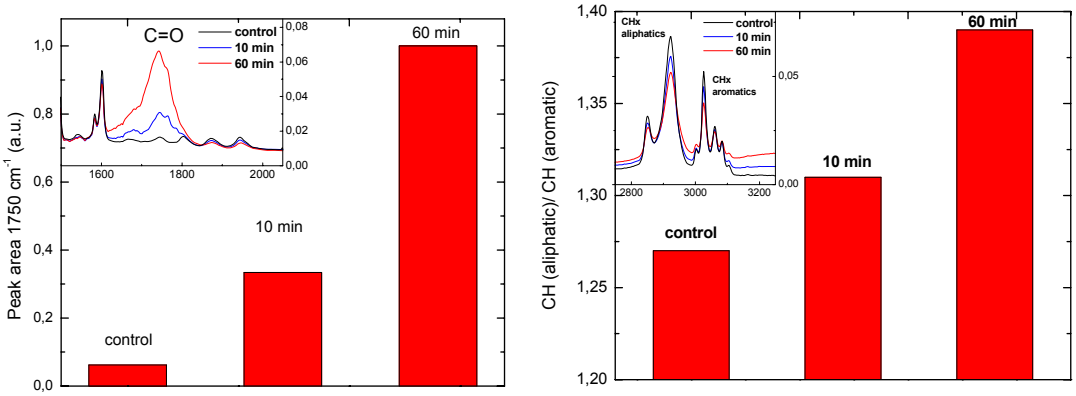


Figure 7. Relative evolution of the concentration of the **(a)** C=O and **(b)** CHx (aliphatic and aromatic) bonds with treatment time.

Much information can be taken out from these spectra. Starting with the most important one is the occurrence of a marked peak at approximately 1720 cm^{-1}. This

peak, which is barely present on untreated PS surfaces, is assigned to carbonyl bonds (C=O stretching). This peak strongly increases with exposure time (a factor of 4 on average), being much stronger after 60 min of ozone exposure than after 10 min (Fig . 7a); the control sample shows a slight oxidation level. There is thus a time dependence of the chemical reactions between substrate and ozone flow, at least for times up to 60 min. These C=O bonds could correspond to the formation of aldehyde (1725 cm^{-1}), ketone (1715 cm^{-1}) or carboxylic acid (1700 to 1730 cm^{-1}). The presence of C=O bonds (around 1740 cm^{-1}) and of OH (continuum around 1000 cm^{-1} and at 3300 cm^{-1}) [34] lead us to think that carboxilic acids (COOH) could have been attached on surface. In addition, the strong intensity of the FTIR signal could be due to the formation of a weak boundary layer (WBL) which is probably the result of surface damage.

The same peak corresponding to the C=O bonds was previously observed when PS was treated with VUV irradiation, when poly-vinyl-chloride (PVC) surfaces were processed by plasma, and when acrylate-based polymers were exposed to a combination of hydrogen peroxide vapor and gas plasma [33,20, 17, 34], respectively.

As for the other, less intense, variations observed on the FTIR spectra, let us mainly mention: (a) a peak at ≈2970 cm^{-1}, which is assigned to -CH_2 bonds (stretching) belonging to the phenyl group, (b) a peak at ≈3020 cm^{-1}, which corresponds to C-H bonds belonging to the aromatic group; the intensity of both these peaks decrease with exposure time, showing that C-H bonds (aromatic) are degraded in favour of C=O bond formation (Fig. 6a) as supported by the XPS results. Also, the intensity of the peak corresponding to the C-H bonds of aliphatic groups (≈2920 cm^{-1}) decreases, as it can be seen in figure 6b.

4.3. XPS

A few experimental facts lead us to believe that the treatment of Petri dish surfaces implies both physical and chemical modifications of the surfaces. In particular, we found that the modified surface is more hydrophilic than the untreated

one, as supported by contact angle measurements. In addition, AFM spectroscopy demonstrated, besides topographical modifications of the treated surface, a "chemical contrast". At this stage, there is thus a great interest in using XPS diagnostic (classical mode) in order to: (1) identify the chemical groups existing on the treated surfaces relatively to untreated ones, (2) compare the chemical change evolution of aged, recently treated and untreated samples, and (3) try to look for the weak boundary layer (WBL) presence on treated surfaces, as we hypothesized when using AFM diagnostic.

Fig. 8 considers quantitatively the chemical modifications (in percentage) that occurred between untreated surface, recently treated samples and a 2-month-old treated sample.

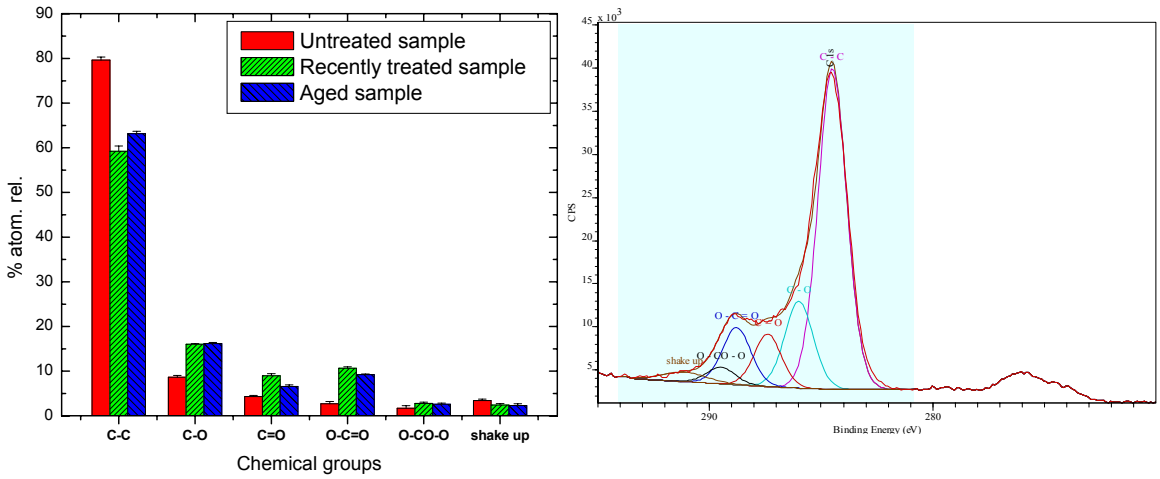


Figure 8. (a) Percentage of the main chemical groups detected on PS Petri dishes surfaces as obtained from XPS spectra of untreated, aged and recently treated samples (shake up signal corresponds to the aromatic group). The data for the recently treated sample is an average over two samples. There is approximately a two-month difference between the recent and aged samples. The treated PS Petri dish surfaces were exposed 60 min to dry gaseous ozone at 4000 ppm. **(b)** Decomposition of the C (1s) peak of the XPS spectra corresponding to a recently treated sample.

Samples	% C	% O	O/C
Untreated	85 ± 2.3	14.7 ± 1.3	0.17
Recently treated	71.8 ± 1.9	28.1 ± 0.5	0.39
Aged sample	73.7 ± 1.5	25.6 ± 1.7	0.34

Table 4. Atomic percentages of oxygen and carbon on surfaces as determined from XPS spectra.

Fig. 8 mainly shows that the number of C-C, C-H bonds on the surface, after treatment, has strongly decreased from about 80% to less than 60%. On the other hand, a general increase of oxygen bonds can be seen on the surface, since atomic oxygen represents 28.1% after treatment relatively to 14.7% before it: subsequently, an increase of simple and double oxygen bonds on surface is observed. As reported, enrichment of double bonds (unsaturated chemical bonds) at the surface could be produced by chain scission [35, 32]. Note that atomic oxygen represents 14.7% on the untreated sample surface as a result of the manufacturer surface treatment. Also, we notice that C-O and O-C=O groups give rise to the most important increase, among the different carbon/oxygen bonds presented, reaching respectively 16% from 8.6% and 10.7% from 2.7%. Zhang et al. [17] determined that the main oxygen-containing functionnal groups after plasma treatment of PVC polymer surfaces is C-O, as we have observed after ozone treatment on PS Petri dish surfaces [35]. Such changes could be explained by an oxidative fragmentation of the polymer backbone (C-C bond lysis) and eventually of aromatic groups (which was confirmed by means of FTIR diagnostic) [36]. In fact, as is well known, ozone is able to oxidize aromatic functions, which explains the decrease of the shake up signal (from 3.4% to 2.4%). As a rule, all the oxidative processes involve an increase of oxygen groups on treated polymer surfaces [17-19].

Callen et al. [23] used a remote-Ar plasma and O_3 in the presence of UV light from the plasma in order to render PS surfaces hydrophilic: as we have observed with ozone, the UV/O_3 treatment produced modifications consisting of oxygen-containing groups that were derived from, and bound to, the PS surfaces [1,23]. However, they claim, after masking PS surfaces with UV opaque materials, that O_3 exposure (for

time up to 30 min) in absence of UV radiation does not produce measurable changes on the surfaces as suggested by XPS. This observation is contradictory with ours since we have highlighted, by means of FTIR spectra, that after only 10 min of ozone treatment chemical modifications have occurred. In addition, after 30 min of ozone exposure, the inactivation efficacy of the surface on bacteria is very significant, which implies that sufficient surface chemical modifications have occurred.

An aged sample was also characterized by XPS to look for the time evolution of the surface chemical composition after treatment (Fig.8a). The comparison of the three samples, for each chemical group, might yield information about those involved in the inactivation process. The concentration of several groups was observed to slightly decrease relatively to the recently treated sample, tending toward values of the untreated sample (for instance: C-C: 79.6%>63.1%>59.2% and C=O: 9%>6.6%>4.3%). Such results suggest a rearrangement of the surface chemical structure of the sample with time since the chemical composition of the treated surface converges toward that of the untreated surface, but through different kinetics according to the specific group considered (Fig.8a): for instance, temporal evolution of the O-C=O bond percentages (2.6%<2.8%<3.4%) seems to be slower than those of C=O. In addition, the comparison of XPS experimental spectra of aged samples show that oxygen is less in depth than with recently treated samples. All these observations lead us to believe that motion occurred on the surfaces and that the eventual desorption of low molecular weight molecules as with the WBL formation could be considered.

These results do not allow identifying clearly the nature of the WBL on treated surfaces as revealed with AFM. Nevertheless, the strong affinity between the silicon tip and the treated surface could be explained by the fact that the partial charge of silicon is positive ($Si(\delta^{+})$) and that of the treated surface is broadly negative due to the strong presence of oxygen ($O(\delta^{-})$), as revealed by XPS (Fig. 8 and Table 4).

5. Conclusions

This work discloses and describes the biocidal effects of polystyrene (PS) Petri dish surfaces that have been previously subjected to a dry gaseous ozone flow [37]. To bring out such properties, we have examined their action on various species of vegetative and sporulated bacteria (known for their greater resistance to biocide agents) and on lyzozyme proteins. Our experimental investigations showed that treating PS surfaces with dry gaseous ozone can denature proteins (since the enzymatic activity of lyzozyme is strongly decreased) and inactivate various bacteria and endospores when these are deposited on this surface and kept in contact with it.

The treated surfaces were found to be chemically modified. Topographical modifications were detected with AFM images, showing rugosity variations and a chemical contrast. New chemical bonds created on PS surfaces were revealed with FTIR and XPS spectra analysis. The evolution of the chemical modifications were shown to be dependent on exposure time (as shown with FTIR spectra) and with the period of time elapsed after surface treatment (as shown with XPS diagnostic). Other analyses are needed to gain insight into the link between the chemical modifications of the treated PS surface and the strong structural damage of spores deposited on it, as observed with SEM micrographs. It would be interesting to test other kinds of polymeric surfaces to determine whether they exhibit the same properties after a similar ozone treatment.

The treatment process is simple and presents attractive characteristics since it does not require expensive pumps, or “implantation” system, and moreover, it works at atmospheric pressure and ambient temperature. In addition, no environmentally dangerous byproducts are created as checked through FTIR spectrum recording. Interestingly, these new sporicidal and antibacterial properties were observed to persist for a relatively long period of time after surface treatment. This process appears more advantageous than biocide surface coating by active molecules because of its simplicity and its great efficacy. The effect on the protein lyzozyme opens a new field of application for prions.

Acknowledgments

The authors are grateful to J. S. Mayer for skilful technical assistance and to Prof. R. Hausler at École de Technologie Supérieure (ETS) of Montréal for lending us the ozonator. The authors wish to thank M. Soltani for priceless FTIR technical assistance and C. Chabanier for support and pertinent comments on XPS measurements. The authors are particularly indebted to CNRS Research Director F. Poncin-Épaillard for valuable comments on XPS measurements and on the paper. Thanks are also due to Prof. M. Lavoie for comments and suggestions and to Dr. D. Keroack for her kind assistance.

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Plusieurs considérations importantes peuvent être déduites de l'article précédent. En particulier, on remarque, d'un point de vue microbiologique, que la surface traitée affecte la viabilité d'un large spectre de bactéries végétatives (dont certaines sont potentiellement pathogènes), mais aussi de spores bactériennes connues pour leur résistance aux conditions extrêmes. Nos résultats indiquent également que les dommages morphologiques causés aux spores sont très importants, allant jusqu'à leur perte d'intégrité (perméabilisation de la spore et extrusion du matériel interne). De plus, la perte d'activité enzymatique de la lysozyme, après avoir été en contact avec la surface traitée, laisse fortement penser qu'une dénaturation de cette protéine en est la cause ; une perspective envisageable serait de tester l'effet de la surface traitée sur des protéines pathogènes telles que la protéine prion.

D'un point de vue physique, nous retiendrons que les principales modifications de surfaces sont physico-chimiques : changement de la morphologie de surface (perte de rugosité…), apparition de nouveaux groupements fonctionnels (principalement polaires) à la surface du Pétri traité dont la concentration augmente avec le temps de traitement, et formation en surface d'une "couche moléculaire faiblement liée" ("*Weak Bond Layer*"). De plus, on retiendra le phénomène de rémanence de l'effet biocide, qui pourrait être dû à une lente réorganisation de la surface vers son état d'origine, ce qui expliquerait que l'effet biocide perdure mais de façon atténuée lorsque la surface traitée vieillit. Nous verrons, ultérieurement, que cette rémanence est très probablement liée à des modifications chimiques sur une certaine profondeur de la surface.

Bien que ce premier article nous informe sur plusieurs aspects caractéristiques de la surface traitée, des questions demeurent toutefois soulevées ; à titre d'exemple, l'étude microbiologique a été effectuée selon un examen non quantitatif. Il apparaît donc nécessaire d'approfondir notre étude pour pouvoir estimer l'étendue de l'activité biocide de la surface traitée (propriété et efficacité) et, ce, en corrélation avec les modifications physico-chimiques induites à la surface dans le but de proposer un mécanisme d'action.

Pour ce faire, nous avons donc procédé à l'examen de l'influence des paramètres physiques tels que la concentration et le temps de traitement à l'ozone, le type d'exposition (sec/humide) sur l'efficacité biocide de la surface traitée, ainsi que l'effet de paramètres microbiologiques tels que le volume de la suspension de spores, la densité de celles-ci en surface, la méthode de quantification du nombre de survivants. Afin d'interpréter ces résultats, nous avons également eu recours à une méthode microbiologique d'identification de la présence, ou non, d'ADN dans le matériel extrudé par la spore suite à son contact avec la surface traitée, ainsi qu'à la caractérisation des modifications chimiques en profondeur de cette même surface au moyen du XPS (mode angulaire). Les résultats de cette étude sont présentés dans l'article suivant (soumis à The Open Microbiology Journal).

Biocidal properties of ozone-treated polystyrene surfaces on sporulated bacteria

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ABSTRACT

Modification of surfaces by physical or chemical means is a promising approach for controlling microbial contamination. Ozone treatment of polystyrene (PS) surface can inactivate various species of vegetative and bacterial endospores on contact. The current paper reports characteristics of the biocidal action of polystyrene surfaces resulting from their exposure to a dry gaseous-ozone flow as functions of ozone exposure time, ozone concentration, level of humidity of the gaseous phase (dry or humidified) and spore load. A kinetic study reveals that the biocidal action of the treated surface is rapid: spores left to dry on Petri dishes are inactivated by 83.5% within 3 hours of contact. However, inactivation rate is reduced when surface contact is with wet spores. The persistence of the biocidal properties was correlated with an in-depth chemical modification of the surface, using X-ray photoelectron spectroscopy (XPS). Sporocidal activity can last more than one month depending on the microorganism/surface interaction. Such treated surfaces are potentially attractive in healthcare environment.

INTRODUCTION

Surface modification and production of coatings with well-defined surface chemistries [1] to prevent surface contamination have received considerable attention in recent years. Surface impregnation of venous, vascular or urinary catheters with heavy metals such as silver and copper have been used with some success [2,3,4]. Heavy metals have also been immobilized in textiles and ceramics, providing antimicrobial properties [5]. Donelli *et al.* (2001) studied the efficacy of antiadhesive, antibiotic and antiseptic coatings in preventing catheter-related infections [6,4].

Active chemical species implantations (grafting) known for their desinfectant properties like bronopol, triclosan, TiO_2 have also been tested [7,8]. In addition, polymeric surfaces (such as polyurethane) coated with chemical agents to provide biocide properties against Gram-positive and Gram-negative bacteria are also being highly investigated [9,10]. Haldar *et al.* (2006) demonstrated that certain surfaces painted with hydrophobic polycations are not only highly bactericidal but also extremely virucidal [11]. In most cases, these surfaces need some prior preparation to allow for the grafting step. This can be achieved through chemical processes such as thiocyanation [7,9,10], with gaseous plasma treatments (Ar, O_2) [12,13,14,15], O_3 [16], with Vacuum Ultra-Violet (VUV) irradiation in the presence of O_2 or with UV/O_3 exposure [17,18]. For example, wool fibers can be treated with argon plasma irradiation to activate their surface, to be then followed by grafting Ag-loaded SiO_2 nano-antibacterial agent. *E. coli* and *S. aureus* bacteria added to these treated wool fibers were reduced in number respectively by 85% and 95% after an 18h contact. [14]. Most studies have looked at the activity of these surfaces on vegetative bacteria and viruses [11,7], but scarcely on bacterial spores probably because of the recognized higher resistance of endospores to harsh treatments [20].

As a rule, the biocidal efficacy of treated surfaces is found to depend on the chemical nature of the implanted molecule(s), on their homogeneity of implantation (spatial distribution), on the type and load of the microorganisms, and on contact time.

Ozone is well known for its high oxidative power and is used for water and wastewater treatments, air purification and control of food microbial safety [21, 22, 23]. Ozone can also induce chemical modifications on some materials: as a rule, oxidative processes involve an increase of oxygen groups on treated polymer surfaces [7,24, 25]. Using X-Photoelectron Spectroscopy (XPS) diagnostic, Mahfoudh *et al*. recently showed a two-fold increase of oxygen (–O and =O) bonds on PS surface treated by ozone [26]. Previously, Kumagai *et al.* had shown the appearance of C=O bonds on three ozone-treated polymers [27, 26]. Enrichment of unsaturated chemical

bonds at the surface, such as =O and C=O, could result from chain scission [28]. Such chemical modifications can lead to important changes in surface properties [30,11].

Ozone effect on microorganisms can be strongly dependent on the nature of the media. Contrary to dry media where O_3 is the sole oxidative species, humidified media provide several oxidative elements stemming from reaction of water with ozone molecules [29, 30]. In the case of vegetative bacteria, some authors suggest that ozone attacks the cell surface, altering the permeability of the cell wall and the cytoplasmic membrane resulting in the leakage of the cell contents [31]. Komanapalli *et al* [32] claim that inactivation of bacteria by ozone proceeds from membrane damage and not from DNA lesions, since protein and nucleic acid leakage can be observed as a result of membrane disruption. For endospores, ozone is assumed to diffuse through their surface and then permeate the membrane [33, 34, 30].

Using O_3 to confer biocide properties to PS Petri dish surfaces was recently disclosed by Mahfoudh *et al.* [26, 35]. The treated surfaces showed great efficacy for inactivation of various vegetative bacteria and endospores, and for the denaturation of enzymes (proteins) such as lyzozyme. Characterization of these surfaces by atomic-force microscopy (AFM), Fourier-transform infra-red (FTIR) spectroscopy, XPS and contact angle measurements clearly indicated chemical modifications of the treated surface resulting, among other, in an increase in wettability and a decrease in roughness.

The main goal of this paper is to gain a deeper insight into the characteristic changes of ozone- treated PS Petri dishes by varying physical (mainly ozone concentration and exposure time) and microbiological parameters

MATERIALS AND METHODOLOGY

Ozonation system

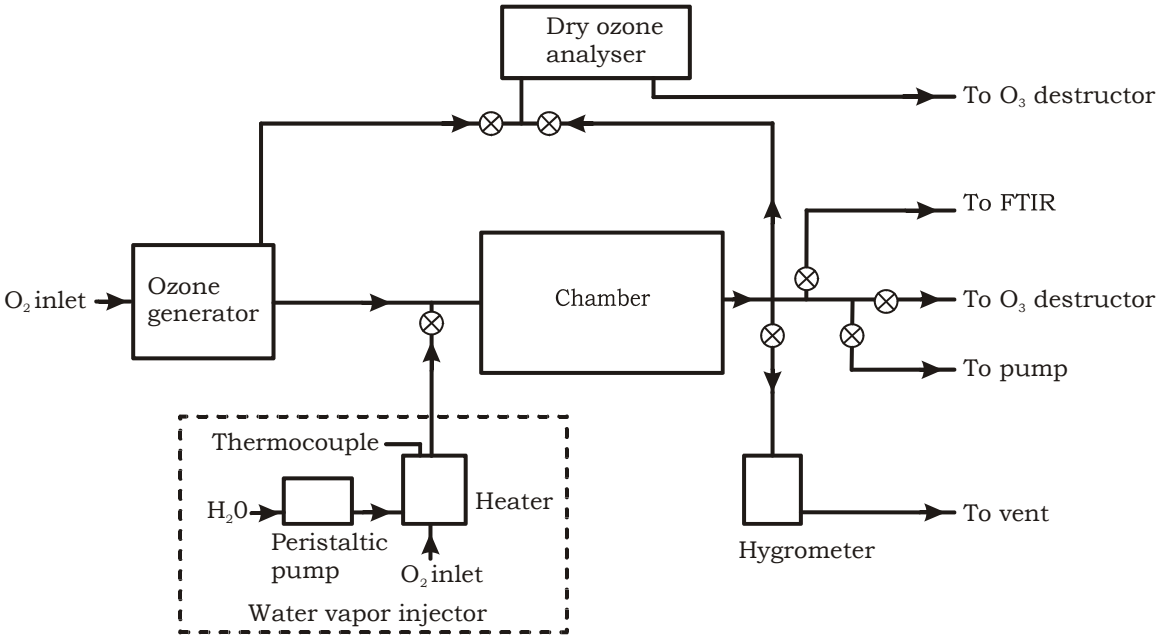

Fig. (1). Schematic view of the experimental arrangement utilized to generate ozone and determine its concentration as it enters and exits the sterilization chamber. Water vapour can be added to the ozone flow and relative humidity in the chamber measured with a hygrometer that comes with a thermometer. An ozone destructor is provided to abate ozone under different modes of operation.

Fig.(**1**) shows the various elements of the system used to generate ozone and determine its concentration as it enters and exits the chamber. The chamber, made from 316 stainless steel, is a 400 mm long, 100 mm high and 220 mm wide parallelepiped. Ozone concentration can be monitored with an analyzer based on UV absorption. The generated effluents can also be analyzed through FTIR spectroscopy. The ozone generator provides a mixture of molecular and atomic oxygen in the gas phase; it is operated within the the electrical current domain over which an increase upregulates the ozone concentration. The ozone flow is dry since the generator is supplied from (high-purity) O_2 dry-gas bottles. The expression "dry ozone" as used herein refers to gaseous ozone having a relative humidity (RH) of less than about 2 % (determined with a hygrometer). Total gas flow is set at 5.64 standard liter/min (slm) to achieve an ozone concentration of 4000 ppm under our operating conditions.

An O_3-destructor, localised at the end of the process line, is provided to abate ozone and to release it as O_2 to comply with safety (toxicity) regulations. For safety reasons also, the chamber is located within a fume hood and a vacuum dry pump is

connected to make sure that the chamber effluents are fully evacuated at the end of the process.

Water vapour can be added in the process for the purpose of using humidified ozone. Water is sent through a peristaltic pump to an "oven" (heater) and the vapour produced is driven into the O_3 line by an incoming O_2 gas flow. The amount of water vapour injected, at a given temperature of the heater and given O_2 flow, depends on the H_2O flow set by the peristaltic pump. The corresponding RH level in the chamber is determined with a hygrometer (Kahn) in the sole presence of O_2 (RH up to at least 95 % can be measured accurately (±0.3%)). The gas temperature in the chamber remains close to ambient (≈ 22 ^oC).

Polystyrene Petri dishes (Starstedt® catalog no 83-1801) of 60 mm diameter are introduced into the chamber (Fig.(**1**)) for their surface treatment. The physical parameters of the dry and humified ozone (RH ≈ 78%) processes are set as follows: 4000 ppm and 1000 ppm ozone concentrations, 60 min and 10 min exposure times. Each experiment represents the average over 3 measurements.

Endospore preparation

To characterize the biocide action of the treated PS surface, we have examined its effect on spores from *Bacillus atrophaeus* ATCC® 9372 deposited on it and left to dry. This microorganism is routinely used as a biological indicator in the validation of sterilization processes with dry heat and is known to be resistant to ozone [36, 37, 38]. An overnight culture in TSB (Trypticase Soy Broth, BD Company) of this strain was inoculated on a sporulation medium consisting by liter: 1g beef extract (Difco Laboratories), 2g yeast extract (BBL™), 5g peptone (Difco Laboratories), 5g NaCl (Fisher Scientific), 0.04 g $MnSO_4$-H_2O (Fisher Scientific) and 20g of agar technical (Difco Laboratories) adjusted to pH 7.4 in a culture flask of 150 cm^2 (Corning), and then incubated for approximately 10 days at 37^oC. Spores were harvested, by centrifugation at 6 000 x g and the pellet was washed several times with sterile distilled water. Spores are maintained in water at 4 ^oC until use. Spore quality is

monitored by Malachite green staining and viability by plating. Spore counts were determined by plating on Trypticase Soy Agar (TSA, PML Microbiology, On., Canada) and incubating at 37°C for 24h. Inoculi of 10^6 and 10^8 spores of *Bacillus atrophaeus* were used in the experiments.

Preparation of endospore inoculi and their recovery after contact with PS surfaces

The number of desired spores in 100 µL of distilled water were deposited at the center of a PS Petri dish (Starstedt ®), either untreated (as control) or pretreated with ozone under the conditions described in section 2.1. Dishes were covered after deposition and left to dry in ambient atmosphere protected from light. After drying (≈ 24h), spores were harvested with 5 ml of a solution containing 0.5%(V/V) Tween 80 ™ in 150mM NaCl added to the Petri dish by mechanical scrubbing using a sterile swab. The harvested spores were vortexed, serially diluted and various volumes (50 to 200µL) of the different dilutions were spread onto TSA plates after 24h incubation at 37°C, number of colony forming units (CFUs) were recorded. When viability was expected to be very low (less than 100 microorganisms), survivors were collected by filtration on a 0.22 µm cellulose 47mm filter, (Millipore Co). For kinetic study, spores were harvested at different times of the drying process. The effect of treated PS Petri dishes on spores maintained in suspension was assessed by putting 10^6 spores in 100 µL and 1 mL of water without the drying step: these suspensions were sealed with parafilm to avoid water evaporation during 24h before recovery.

Control spores (deposits on untreated Petri dishes) were recovered at the same time as those deposited on treated surfaces. Germination inducers such as aniline, dipicolinic acid/calcium and lysozyme were used to confirm the incapacity of germination after having been exposed to treated PS surfaces [39].

Structural damage characterization of spores

To evidence spore DNA damage, we have utilized a staining procedure for DNA marking through DAPI (4', 6'-diamino-2-phenylindole dilactate):

DAPI was purchased from Invitrogen (Canada Inc. Burlington Ontario). Stock solution was prepared according to manufacturer in water at 20 mg/ml and kept at 4°C until use. At time of use, the stock solution was dissolved in McIlvain's buffer pH 4.0 at a final concentration of 50 µg/ml and 0.1 to 0.2 ml volume was added to the dry deposit in the Petri dish. Incubation was done at 21°C in the dark during 15 minutes to 1 hour. The staining solution was then drained and spores left to dry in the dark. Observations were made with epifluorescence microscopy (Laborlux 12, Leitz) with 100× immersion objective and captured with an Infinity 2 Camera with Lumenera software (Olympus, On., Canada).

Acid treatment of spores is known to release DNA [40]. For comparison purposes with DAPI, acid effect was tested on 10^6 spores of *B. atrophaeus* in 10µl in water. These were previously deposited on a microscopic slide and then dried. Spores were then flooded with nitric acid (HNO_3) 1N during 20 minutes [41]. Acid was then drained and spores washed two times with distilled water, before the DAPI staining procedure.

DNA digestion procedure:

In order to test whether DNA was released during spore exposure to ozone-treated PS, we proceeded to DNA digestion directly on spores left 10 days in contact with PS Petri pre-exposed to ozone (60 minutes) and on spores treated with HNO_3 on a microsope slide before DAPI staining. A reactive mix of DNase I (amplification grade Rnase free) of Invitrogen was used according to manufacturer and the digestion was allowed to proceed at 21ºC during 20 minutes.

Angle resolved XPS: determination of the chemical in-depth modifications

The surface chemical state was determined by XPS (Escalab 220i XL), employing a polychromatic Mg radiation operated at 15 kV and 300 W in a residual vacuum of $< 1\times10^{-9}$ mbar. Elemental surface compositions (atomic %) were calculated from survey spectra measured at a pass energy of 100 eV and detailed surface chemical information (high resolution) was obtained by analysis of the carbon 1s peak envelopes at the higher resolution pass energy of 20 eV. Chemical shift data are referenced to the center of the C-C peak at 284.6 eV. The studied area (1.5mm × 1.5mm) were analyzed under various angles: 0°, 15°, 30°, 45°, 60° and 75° relatively to the surface normal direction. The smaller is the electron photo-emission angle, the deeper is the distance from the surface. The decomposition method used to interpretate the spectra assumed a Lorentzian contribution of 30% and a Gaussian one of 70%.

RESULTS

Effects of parameter variations on inactivation efficacy.

Physical parameters of the dry-ozone treatment: exposure time and O_3 concentration.

The inactivation efficacy of *B. atrophaeus* spores deposited on Petri dishes pre-treated with dry gaseous ozone under different exposure times and concentrations is presented in Table **1**. PS Petri dish surfaces were also exposed to humidified ozone before spore deposition.

Table **1** shows a 1.5 log reduction in the endospore population with PS Petri dishes that had been pre-treated for 10 minutes with dry ozone at 1000 ppm O_3 concentration. Spore inactivation is nearly complete after 60 min for a 4000 ppm dry-ozone concentration pre-treatment. A similar result is obtained after a 60 min pre-exposure to humidified ozone (RH = 79%) at 4000 ppm.

O_3 concentration / *Exposure time*	*1000 ppm*	*4000 ppm*	*4000 ppm*
Type of treatment	*Dry gaseous ozone*		*Humidified gaseous ozone*
Control (untreated)	$(1.2 \pm 0.2) \times 10^6$	$(1.1 \pm 0.2) \times 10^6$	$(1.2 \pm 0.1) \times 10^6$
10 min	40 604 ± 17783	188 ± 56	ND
60 min	73 ± 64	19.6 ± 20.1	18 ± 5

Table **1.** Survivor number determined on PS Petri dishes exposed to dry gaseous ozone (RH<2%) at concentrations of 4000 ppm and 1000 ppm during 10 min and 60 min before deposition of *B. atrophaeus* spores (10^6/100µl). Spores were deposited on the day of treatment and contact time is ≈24 h, which corresponds to the sample drying time.

Spore density.

Table **2** shows that the sporocidal activity of PS-treated Petri dishes is not influenced by spore density (10^6 and 10^8 spores in 100µL) in spite of the fact that the diameter of the dried spore deposit was similar in both cases, suggesting a higher number of stacked spores at the higher spore concentration.

Spore number	*Control*	*Treated sample*
10^6	$1.10^6 \pm 8.10^4$	48 ± 45
10^8	$1.10^8 \pm 1.10^7$	69 ± 49

Table **2.** Number of survivors of *B. atrophaeus* spores, deposited on dry-ozone treated Ps Petri dishes (60 min, 4000 ppm), after a contact time ≈24 h, which corresponds to the sample drying time. The initial number of spores in the suspension has no significant effect on their inactivation efficacy.

Interaction of PS treated-surfaces with microorganisms

Inactivation kinetics on drying spores and spores kept in suspension

Contact time effect

The biocidal action of PS treated surfaces on drying spore suspensions (initially 10^6 spores in 100 µL of water) as a function of contact time is illustrated in Fig. (**2a**), at times ranging from 0h to 24h. Spores' drying is completed within approximately 24 h. The corresponding inactivation-rate percentage is presented in Fig. (**2b**). Typically, a reduction of more than 80 % of the initial bacterial load is observed after a 3 hour contact.

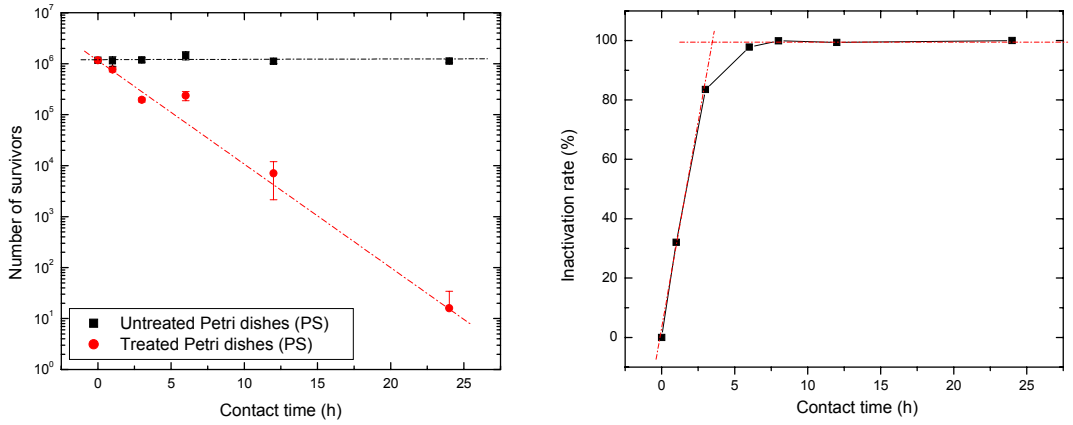

Fig. (2). Survivor number as a function of contact time of a drying spore suspension (initially 10^6 spores in 100 µL of water) with pre-treated PS Petri dish surfaces : **(a)** survivor number and **(b)** corresponding inactivation rate (in %) as functions of contact time. Drying is achieved within approximately 24 h.

The number of survivors decreases exponentially, from the beginning, with contact time (figure 2a), indicating that the biocidal activity starts immediately upon deposition. Nonetheless, as shown in figure 2a, maximum inactivation is attained after 24 h, when the spore suspension has had time to dry.

Persistence of biocidal effect over time

Spores were deposited at different days after of ozone treatment of the PS Petri dishes. A gradual loss of sporicidal activity with elapsed days can be seen in Table **3**. Strong biocide activity is maintained over a period of at least eight days after ozone treatment and is observed to decrease after a week.

Day of spore deposition after ozone treatment	*Same day*	*Day 1*	*Day 4*	*Day 7*	*Day 8*	*Day 33*
Survivor number	2.6 ± 2.1	23 ± 10	10 ± 3	404 ± 27	676 ± 200	$(2\pm 1.5)\times10^5$

Table **3.** Number of *B. atrophaeus* spore survivors, from an initial suspension of 10^6 spores in 100µL of water, collected after a 24 h contact with PS pre-treated Petri dishes, as a function of the day the suspension was deposited with respect to the day the Petri dish was subjected to the ozone treatment.

Action of the PS treated surface on spores kept in water suspension

Inactivation efficacy of the treated surface on 10^6 spores in 100 µL and 1 mL water suspensions is compared in Table **4**. Treated PS Petri dishes maintain a strong sporicidal activity even when spores are kept wet (in suspension), although this activity decreases as the suspension volume increases.

	Dry case	*100 µL*	*1 mL*
Untreated sample	$1.3\times10^6 \pm 1.5\times10^5$	$1.4\times10^6 \pm 7\times10^4$	$0.9\times10^6 \pm 4\times10^5$
Treated sample	0.8 ± 1.1	1 ± 1	287 ± 22

Table **4.** Number of *B. atrophaeus* spore survivors after exposure to PS treated Petri dishes for 24 h while maintaining the spore water suspension volume at 100 µL and 1 mL. Dried spore case exposure and untreated samples are shown for comparison and control purposes, respectively. Deposition was effected on PS Petri dishes immediately after their ozone treatment.

Regeneration of the PS-surface biocidal properties.

Two months after ozone treatment, the sporicidal activity of the treated PS surface is totally lost: $(1 \pm 0.3)\times10^6$ CFU were then recovered after a 24 h contact time. However, subjecting this same Petri dish again to ozone treatment (60 min at a 4000 ppm flow of dry ozone) yielded, after a 24 h contact time, 23.3 ± 15.3 spores. It shows that the initial biocidal properties of a given pre-treated Petri dish can be substantially regenerated by repeating the same ozone treatment.

Structural spore damage.

Staining with DAPI spores exposed to ozone-treated PS revealed loss of impermeability of their internal membrane. Unexposed spores adopted a fluorescent ring pattern [40] as the dye was excluded from the core (Fig. (**3a**)) [40]. In contrast, after exposure, the spore core took up DAPI and the staining pattern changed either to a centered or an off-centered small rod-like structure (Fig. (**3b**): see upper left arrow for off-centered case). Our results indicate that the active principle of ozone-treated PS is able to cross the normally impermeable inner membrane of some spores, nonetheless leading to 72 % ± 8 % of impermeable spores (ring pattern) relatively to the control sample (97.6% ± 0.1%). In some other spores that have lost impermeability, the DAPI-positive particulate material was seen outside but still attached to the spore, suggesting that extrusion of different molecules, among which probably DNA, occurred (Fig. (**3b**): arrow at the center of picture). Spore treatment with HNO_3 clearly showed the extrusion phenomenon (Fig. (**3c**)), which is similar to that obtained after spore contact with treated surface. On acid–treated spores and spores deposited on ozone-treated PS surfaces, a DNA digestion was done to determine whether the nucleic acid was a consituent of the extruded material: after DAPI staining, Fig. (**3d**) highlights a loss of fluorescence of the extruded material, indicating that DNA was clearly digested.

Glass Petri dishes were used as negative controls since the interaction of ozone with glass, as opposed to polystyrene, is expected to be minimal. Untreated and treated glass dishes show no structural changes with stained spores, since DAPI staining revealed that the number of impermeable spores remains the same before and after ozone-treatment (94% ± 2.5% and 95.5% ± 3.4%, respectively).

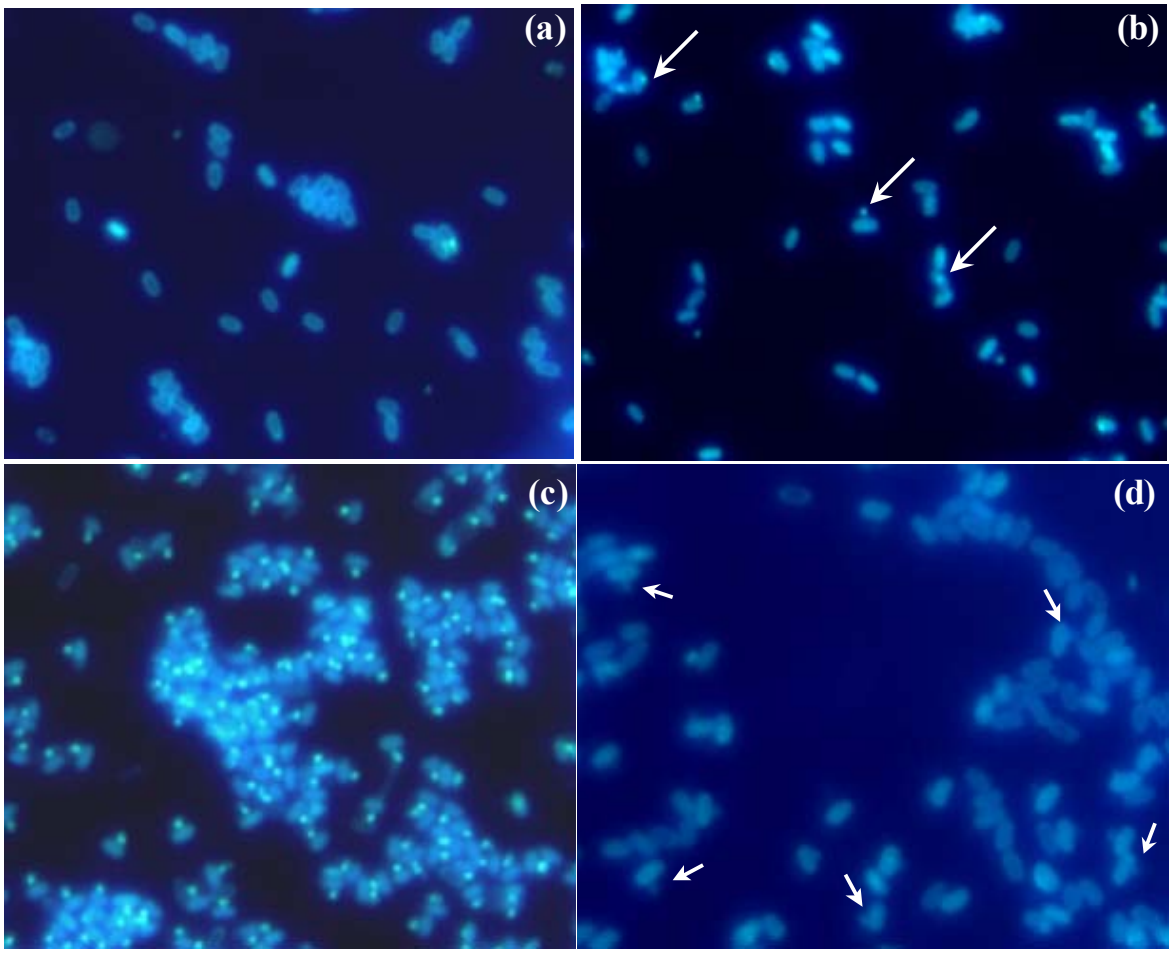

Fig. (3). Epifluorescence microscopy of *B. atrophaeus* spores deposits stained with DAPI where the nuclear material appears to be strongly brighter relatively to other spore components: **(a)** on untreated PS Petri dishes, **(b)** on ozone pre-treated PS Petri dish (60 min, 4000 ppm), **(c)** on spores (on microscope slides) treated with HNO_3, and **(d)** after their deposition on pre-treated PS Petri dish followed by DNase digestion.

Characterization of surface modifications: angle resolved XPS.

The observed biocidal persistence effect in previous sections motivated an estimation of the in-depth extent of the chemical modifications resulting from the

ozone treatment: this can be provided by angle resolved XPS technique. It allows depth probing, typically ≈ 10 nm, of each chemical group, in contrast to the classical mode that gives information limited to the immediate surface (depth < 5 nm) [26].

The tested samples were an untreated PS Petri dish (control), an aged treated sample (treated 45 days before and kept in dark under atmospheric pressure and room temperature) and recently treated samples.

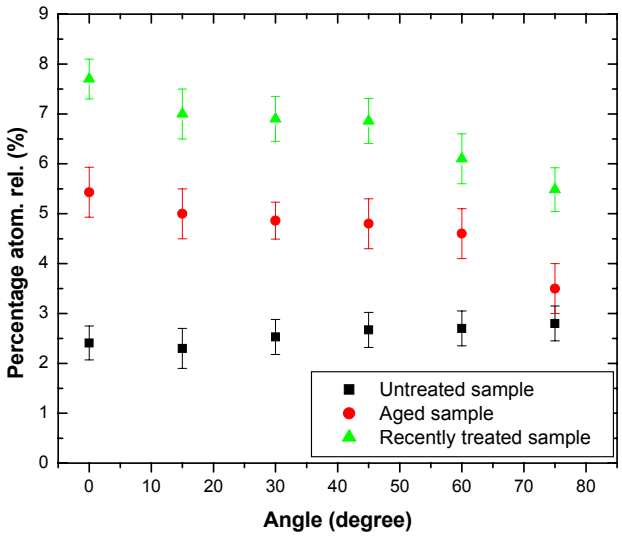

Fig. (4). Percentage of carboxylic groups [O-C=O] detected on PS Petri dish surfaces as a function of the XPS spectra of untreated, aged and recently ozone-treated samples. The time interval between the recent and aged samples is more than 45 days. On the abscissa, the reported electron photo-emission angles are directly linked to the depth (0 to ≈10 nm): the smaller the angle, the deeper the distance from the surface. Treated PS Petri dish surfaces were exposed 60 min to dry gaseous ozone at 4000 ppm. Uncertainties due to the experimental method are represented as error bars.

The XPS results show that all atomic and molecular bond profiles in-depth are different relatively to the untreated sample (with different degrees according to the kind of the bond). To illustrate this point, the depth profiles corresponding to [O-C=O] groups are presented in Fig. (**4**). The angle resolved XPS analysis shows that the relative percentage of [O-C=O] groups increases from ≈2.4% to ≈ 7.7% in depth

on the freshly-treated surface and from ≈2.8% to ≈5.5% on the aged treated surface comparatively to untreated samples. These angle-resolved XPS results confirm previous observations by means of XPS in the incidence mode: ozone treatment of polymers lead to a chemical oxidation of surfaces since a strong increase of oxygen bonds is detected to the detriment of the surface carbon bonds [26].

Two important informations can be unveiled from Fig. (**4**). First, it clearly appears that the chemical modifications resulting from the treatment spread in depth; this fact is observed for all chemical species (not shown). Such in-depth modifications could explain, at least partially, the observed remanence effect. Secondly, the curve in Fig. (**4**) corresponding to the aged treated sample is always in an intermediate position between recently treated and untreated samples, at different levels depending on the chemical groups (or bonds) considered. For example, the aged treated sample relative position for [=O] bonds (not shown) is closer, whatever the depth, to the recently treated sample (relatively to the untreated sample) than the one corresponding to the [O-C=O] groups. These observations are related with time modification of the surface chemistry: the fact that the aged treated sample profile is not superimposed with the recently treated one shows that a chemical rearrangement (such as chemical groups turn over but also macromolecular motion) occurred in surface but also in-depth, which could explain why a decrease of the inactivation efficacy was observed as time passed.

DISCUSSION

Dry gaseous ozone vs. humidified gaseous ozone

A humidified gaseous ozone medium is chemically more complex than a dry ozone environment. Dry gaseous ozone is composed of O_3, O_2 and $O^{\bullet}$ species whereas humidified gaseous ozone contains additional species, including molecules such as H_2O_2, and radicals such as $OH^{\bullet}$, $HO_2^{\bullet}$, which result from chemical reactions between O_3 and water [29, 42, 30]. Nevertheless, both ozone treatments (dry or humidified) on PS Petri dishes showed a similar high inactivation effect on *B.*

atrophaeus ATCC spores. Thus, it is the ozone molecule that plays a key role in the formation of the biocidal species responsible for the microorganism inactivation.

Effect of ozone exposure time of PS surfaces on their biocidal activity

The inactivation efficacy was shown to depend on microorganism concentration, in particular for short exposure times of PS to ozone, while this dependence is less accentuated for longer exposure times: this could be explained, in the latter case, by an excess of active chemical species with respect to the number of spores tested in the study. Nonetheless, considering that the decomposition of the ozonide (created from the reaction between O_3 and the phenyl ring of PS) is the reason for the C=O formation on the PS surface, as suggested by Kumagai *et al.* [27], this chemical reaction is, in the end, limited by the number of phenyl rings available on the samples: therefore a threshold should be reached for this reaction and, subsequently, there should be saturation of the biocidal potential of the treated surface.

Possible influence of the PS surface hydration

A possible interaction of water with the surface, before the suspension dries out, should be kept in mind. As a matter of fact, the suspension water content could be responsible for inducing the biocidal activity of the said surface that then goes on as the inoculum dries out. Therefore, we cannot exclude the possibility that hydration of the surface is needed to activate the ozone-treated surface. Moreover, this gradual drying could also change the spore resistance over the 24 hour drying period [43, 44].

Influence of the nature of the microorganism interaction with the treated surface

Our results show that even though spores can be inactivated when in suspension, optimal sporicidal activity a priori requires direct contact of the spores with the ozone-activated surface. Nonetheless, spores in suspension could reach the surface by passive sedimentation since some of the active chemical species could be leached from the surface and diffuse into the liquid overlay. This hypothesis is backed by the fact that the inactivation efficacy is higher when the suspension volume is

smaller, probably stemming from a higher biocide compound concentration and optimization of spore/surface contact.

The persistence phenomenon seems to depend strongly on the spore/treated surface interaction: a previous study showed that a longer time of persistence is observed when solid TSA nutritive medium is added on dry samples relatively to rather adding liquid TSB [26]. It can thus be concluded that the persistence of the biocidal effect is optimum when microorganism contact with ozone-activated surface is maximised.

In all the above cases, the biocide properties of the ozone-treated surfaces are observed to decrease with time. Antibacterial surfaces also have a temporal limited action [7,45,14].

Spore concentration/density

Spore stacking normally occurs when inoculi dry on a surface. Inactivation efficacy tested on a high density (10^8/100µL) inoculum revealed that spore stacking is not a hindrance to the present sporicidal activity. This indicates that the active chemical species exert their influence beyond direct spore-surface contact. This is not the case with PE samples containing triclosan or bronopol that exhibit excellent bacterial effects against *E. coli* and *S. aureus* (10^6/ml); however, when the bacterial concentrations exceed 10^8 CFU/mL, the materials lose most of their antimicrobial properties [17]. The similarity of biocidal efficacy for the different spore concentrations tested indicates that the destruction of microorganisms is a first-order process as suggested by Nurdin *et al.* [10].

Germination receptor tests for inactivated spores

Concerning the possibility that germination/growth inhibition results from contact with these treated surfaces, we have demonstrated that addition of germination inducers had no effect on the spores. In addition, structural damage of spores was highlighted using DAPI staining and SEM micrographs [26].

Remanence and regeneration

In-depth chemical modifications could be related to the persistence phenomena of the biocidal activity. Indeed, the temporal biocidal efficacy decrease of treated surfaces (aged samples) was charaterized by a depth profile (for each chemical groups or bonds) located at an intermediate position between untreated and recently treated samples. In contrast, Teare *et al*. showed that angle resolved XPS does not reveal significant differences on PS Petri dishes after their treatment with UV/O_3 [11]. Since the percentage of all chemical groups or bonds determined with XPS varies upon ozone treatment, this diagnostic alone does not enable us to identify those involved in the biocide activity.

Regeneration of the biocide properties of an aged pre-treated surface was interestingly shown to be possible: it suggests that as long as there remains raw polymer material, it can be ozone-treated and its biocide effect renewed.

Inactivation mechanisms

Setlow demonstrated that ozone could induce disruption of the spore permeability barrier of the inner membrane [40, 46, 47], the integrity of which is essential for the spore viability, without necessarily damaging the DNA. As our kinetic study of the biocide action of treated surfaces allowed a first approach to understand putative mechanisms of inactivation, the DAPI stain provided informations about spore structural damage leading to their inactivation. The inner membrane is known to be the main impermeability barrier in bacterial endospores for molecules superior to 200 Da [48]. Setlow *et al.* has shown that small fluorescent molecules are able to reach the spore core after their treatment with autoclave (steam sterilizer), strong acid and ethanol [40]. It is safe to claim that ozone-treated surfaces induce structural damage to the spore inner membrane. A first step in that direction was the observation of structural damage with SEM micrographs [26]. In the current study, inner membrane permeability modification is confirmed by DAPI penetration in the core and the presence of off-centered and extruded positive material, possibly DNA. This phenomenon was only observed with treated-PS surfaces, not with treated glass samples, meaning that the biocidal properties are specific to treated-PS samples.

HNO_3 treatment of spores normally results in a flow of material from the interior to the cortex (off-centered), sometimes leading to a rupture of the outer spore coat (extrusion) [41]. In addition, Setlow et al [40] observed that treatment of spores with acid caused a significant breakdown of the spore permeability barriers. Our results with DNA digestion of spores treated with HNO_3 and of spores deposited on ozone-treated surfaces, demonstrated in both cases, relatively to non-digested DNA, a lighter staining of extruded material, clearly indicating the presence of DNA material. However, small "ghosts" trapping DAPI remain after treatment, probably due to the presence of something else than DNA like SASP proteins (Small Acid Soluble Proteins) [48], RNA (Ribonucleic acid) [49], both located in the spore core, and/or dipicolinic acid (DPA) [49], which can bind DAPI, or also other molecules contained into the microorganism.

The effect of the ozone-treated surface on spores could involve acid species, as suggested by the similarity of our treated spores stained with DAPI with the acid-treated spores stained with DAPI reported by Setlow and Robinow. The possible action of acid species is backed by the fact that =O bonds (C=O, O-C=O…) on PS surfaces strongly increased after their ozone-treatment, as shown with XPS and FTIR [26], suggesting the presence of acid species on and below the surface. The acid species are probably not the only ones involved in this complex biocidal effect due to ozone-treated PS surfaces. We showed by means of FTIR analysis that aldehyde groups could have been created on the PS surfaces after their ozone treatment. Subsequently, as soon as the permeability barrier is lost, chemical species (such as acids or aldehydes) could be implied in the inactivation process of the spore. Nevertheless, the breakdown of the inner membrane is sufficient to induce the loss of spore viability.

Future investigations could be oriented specifically on the spore genetic material, looking for eventual modifications or bond breaking on the nucleic acid chain of the DNA, especially having in mind that certain acid (as HNO_3) and aldehyde (as formaldehyde) species are genotoxic chemical compounds [40]. Nevertheless, the spore material extrusion observed after acid treatment by Setlow *et al*. [40, 41] was not seen after an aldehyde treatment [50]. However, this does not

imply that aldehyde or other molecules could not operate as soon as spore membrane impermeability is lost.

CONCLUSION

This study revealed important biocidal properties of PS Petri dishes previously exposed to ozone, such as efficacy and remanence of such surfaces, and also allowed an approach to the inactivation mechanisms. The sporicidal activity of the surface was highlighted through spore inactivation and structural damage leading to disruption of the permeability of their inner membrane, which can lead to the extrusion of genetic material as demonstrated in this study.

The ozone treatment process presented in the current paper appears more advantageous than biocide surface coating with active molecules because of its simplicity and its great efficacy. Also, its effect on the lyzozyme protein opens the possibility of reducing the pathogenicity of prion proteins, as was previously suggested [26]. However, the human toxicity of these ozone treated surfaces remains to be characterized before determining the extent of their possible applications.

ACKNOWLEDGMENTS

The authors are grateful to JS Mayer for skilful technical assistance, to Prof. R Hausler at École de Technologie Supérieure (ETS) of Montréal for lending us the ozonator. Authors are indebted to C Chabanier and to CNRS Research Director F Poncin-Épaillard for support and pertinent comments on XPS measurements. Thanks are also due to Dr. D Keroack for valuable assistance.

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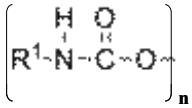
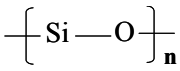
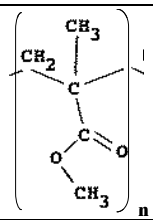
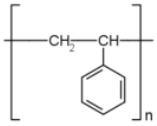
Cet article nous permet d'apprécier de manière plus précise l'étendue de l'action biocide de la surface traitée. Nous avons montré, pour un temps d'exposition ou pour une concentration plus faible en ozone lors du traitement du polymère, que le caractère biocide est bien notable même si les taux d'inactivation atteints sont relativement plus faibles. Ceci est conforme avec nos observations FTIR qui montrent que des modifications chimiques de surfaces (apparition de groupements fonctionnels) ont lieu rapidement au cours de l'exposition de la surface à l'ozone. Par ailleurs, nous avons constaté que la cinétique d'action est relativement rapide puisque la surface agit dès lors que la suspension de spores y est déposée (même si le dépôt n'est pas encore sec). La surface traitée démontre également une très grande efficacité, même pour de fortes densités de spores.

On retiendra de cette étude que l'efficacité de l'activité biocide de la surface traitée dépend fortement de la nature de l'interaction des spores avec la surface : plus celle-ci est optimale (tel que décrit dans l'article), meilleur sera le taux d'inactivation atteint sur une surface récemment traitée mais aussi sur un Pétri anciennement traité (rémanence). Nous avons montré que les modifications avaient lieu non seulement en surface mais aussi sur une certaine profondeur (≈ 10 nm) pouvant justifier la rémanence de l'activité de biocide déterminée expérimentalement. L'on retiendra aussi que les espèces chimiques responsables du caractère biocide de la surface ont pour effet de perméabiliser la spore bactérienne pouvant mener à l'extrusion de son matériel interne contenant, entre autres, le bagage génétique de cette dernière, confirmant ainsi de manière irrévocable le caractère létal d'un tel traitement. De plus, une comparaison avec la littérature nous mène à penser que les espèces acides pourraient intervenir de façon importante dans le mécanisme d'inactivation. Toutefois, nous ne pouvons exclure que d'autres espèces (aldéhydes, esters…) puissent être impliquées dans ce processus : c'est pourquoi d'autres analyses chimiques plus fines seraient probablement nécessaires pour nous éclairer sur la question. À toute fin pratique, nous avons observé la possibilité de régénérer, par un flux d'ozone, l'activité biocide de la surface traitée lorsque celle-ci a disparu avec le temps.

3.3.2 Traitement de différents polymères à l'ozone sec gazeux : effet sporicide

La grande variété des polymères (propriétés physiques diversifiées) est un atout en soi qui a donné lieu à un grand nombre d'applications dans différents domaines. Comme nous l'avons rappelé en introduction, les polymères sont utilisés, depuis longtemps, dans diverses spécialités reliées au domaine biomédical telles que la dentisterie, la chirurgie d'implants, l'orthopédie. Toutefois, les polymères utilisés dans les dispositifs médicaux doivent répondre à un certain nombre de critères tels que la biocompatibilité et la résistance à un procédé de stérilisation [22]. Les défis et enjeux actuels en matière de stérilisation et de désinfection (de haut niveau) dans le domaine biomédical nous ont, tout naturellement, conduits à examiner la possibilité de conférer un caractère biocide à des surfaces de polymères autres que les Pétris de PS, traitées dans les mêmes conditions.

Nom du polymère	**Formule**	**Applications**
Polyuréthane (PU)		Gants chirurgicaux, préservatifs, roues des fauteuils roulant..
Polytétrafluoroéthylène (PTFE- téflon)		Prothèse (vasculaire…), matériau d'implantation de choix en hémodialyse…
Polyéthylène haute densité (PE)	$-(CH_2-CH_2)_n-$	Emballage (alimentaire…), flacon (alimentaire, médicament, cosmétique…)
Silicone (polysiloxane)		Implant mammaire (liquide); prothèse, substance pharmaceutique, textile, cosmétique, industrie alimentaire, médecine.
Polyméthylméthacrylate (PMMA)		Prothèse dentaire, membranes pour hémodialyseur
Polystyrène (PS)		Emballage alimentaire, Polystyrène de sodium utilisé en médecine.

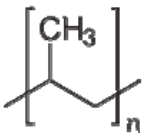
Polypropylène (PP)		Emballages alimentaires, tissus d'ameublement

Tableau 3.1. Formule chimique de polymères utilisés dans le domaine biomédical ainsi que quelques exemples de leurs domaines d'application

Les polymères utilisés lors de cette étude sont répertoriés dans le tableau ci-dessus : mis à part le silicone, ces substances sont toutes organiques. Le travail de caractérisation physique et microbiologique effectué lors de l'étude de l'effet biocide conféré aux Pétris de PS, suite au traitement à l'ozone, a été mis à profit dans l'article suivant pour étudier d'une part l'existence (ou non) d'une activité biocide de la surface du polymère traitée et, d'autre part, déterminer certaines modifications induites en surface. Cet article a été soumis à Journal of Applied Surface Science.

Biocidal action of ozone-treated polymer surfaces on sporulated bacteria

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Abstract

The current work describes a novel technique by which certain types of polymers subjected to dry gaseous ozone are acquiring the ability to inactivate microorganisms, including those as resistant as bacterial spores. The originality and advantages of this ozone-treatment method of polymers rest on its simplicity (achieved at ambient temperature and pressure, a one step process …) and its efficiency. The inactivation efficacy is found to be specific to the nature of the treated polymer: 24-hour after depositing on it 10^6 *B. atrophaeus* spores in a 100 µL suspension, high inactivation rates were observed with polymethyldisiloxane (99.997 %, almost 5 log) and polystyrene (99.7%, more than 2 log), a lower rate with polyurethane (99.1%, 2 log) and a very weak one with polytetrafluoroethylene (only 4.5 %). Changes in hydrophilicity of these surfaces are monitored by means of contact angle measurements while topographic modifications are characterized through Atomic Force Microscopy (AFM). Ozone exposure brings about important topographic changes and chemical modifications on some polymers, which can be correlated with oxidation processes, increased wettability and surface energy. Variations of the dispersive and non-dispersive components of the surface energy are partially correlated with the polymer biocide response. Furthermore, the basic component of the treated polymer (in contrast to its acid component) is shown to be linked to the biocidal activity of the treated surfaces. Chemical species bearing the ester group,

probably partially-oxidized styrene oligomers as revealed by chemical analysis, which could be involved in the biocide activity were identified. On practical ground, since some of these treated polymers can strongly reduce microorganism loads, they could be particularly useful in hospital environment.

1. Introduction

Because of their advantageous optical, chemical and mechanical properties, polymers are largely used in biomedical applications. Lately, thermosensitive polymers have emerged as composing, in part or totally, a great deal of medical devices. Methods to disinfect or sterilize such devices are restricted because of possible damaging effects whenever temperatures in excess of approximately 70^{o}C are used, which clearly excludes autoclaving. This has promoted researches on new desinfection and sterilization processes. Among them, plasma technology and ozone treatment seem to be particularly promising. Humidified gaseous ozone is already being used on hospital sites (Ref. TSO3®) for low-temperature (55^{o}C) sterilization while research on plasma sterilization is attracting more and more scientists in academia and industries in view of the possible commercialization of such a technique.

Some materials, like copper and silver, have naturally strong antibacterial properties. Copper shows inhibitory effects on various microorganisms (as opposed to silver which can inactivate them [1]) contrary to stainless steel and plastics [2]. Powders such as MgO and CaO are also bactericidal, while ZnO is bacteriostatic and eventually bactericidal for certain Gram + bacteria [3]. The primary mechanism of action of the MgO and CaO species is reported to be an alkaline effect, which leads to superoxide anions formation; as for ZnO, it affects microorganisms through the generation of H_2O_2, which is its main active agent [3].

To preserve polymers from external contaminations, various methods were explored including developing new polymers or modifying polymer surfaces to provide antibacterial properties to prevent or reduce microbial contamination. Surface modifications by coating or grafting processes have gained interest [4]: polymer coating is usually achieved following conventional surface modification techniques based, for instance, on discharge plasmas or UV radiation. Zhang et al. have studied the antibacterial efficiency of triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) and bronopol (2-bromo-2nitropropane-1,3-diol), coated PVC and polyethylene (PE) surfaces [5,6]. The surface was first activated by an O_2 plasma to produce more

hydrophilic groups so that triclosan and bronopol could be coated more efficiently on it; the next step is the covalent immobilization of antimicrobial bronopol or triclosan by means of argon plasma-immersion-ion-implantation (PIII) to ensure that the reagent bonded well on the PE surface. This technique can yield excellent microbicidal effects on the polymer surfaces against Gram + (*S. aureus*) and Gram – (*E. coli*) bacteria. Triclosan coated PVC samples exhibit 82.2% and 79.5% antibacterial effect against *S. aureus and E. coli*, respectively, whereas bronopol coated PVC samples affect respectively 98% and 77.3% of *S. aureus* and *E. coli* bacteria [6]. In these experiments, the vegetative bacteria were incubated 24h before harvesting from the surface. Triclosan or bronopol coated on polyethylene affects the membrane structure and metabolic functions of the bacteria. Haldar et al. demonstrated that certain hydrophobic polycations can be painted onto surfaces to render them not only highly bactericidal (leading to rupturing of the bacterial cell membranes) but also extremely virucidal against at least two distinct strains of influenza virus [7]. A reduction of at least three log was reached after 30 min of contact of the virus. Biocidal polyurethane chemical modifiers showed great efficacy against Gram + and Gram – bacteria, and also against certain yeasts and moulds [8,9]: inactivation rate ranged from 1 to 6 log depending on the coating nature and microorganisms.

The paper is organized as follows. Section II describes the materials used and the experimental methods. Section III examines the inactivation efficiency of treated polymer surfaces on bacterial spores (*B.atrophaeus*). Section IV presents two surface diagnostic techniques used to characterize physical modifications of the surfaces after ozone treatment: (i) topographic changes, by means of Atomic Force Microscopy (AFM), and (ii) hydrophylicity and surface energy (and its components) variations of the samples, determined from contact angle measurements. A chromatographic analysis is presented to attempt identification of low-mass molecular chemical species. Section V contains a summary and the conclusion.

2. Material and methods

2.1 Ozonation system

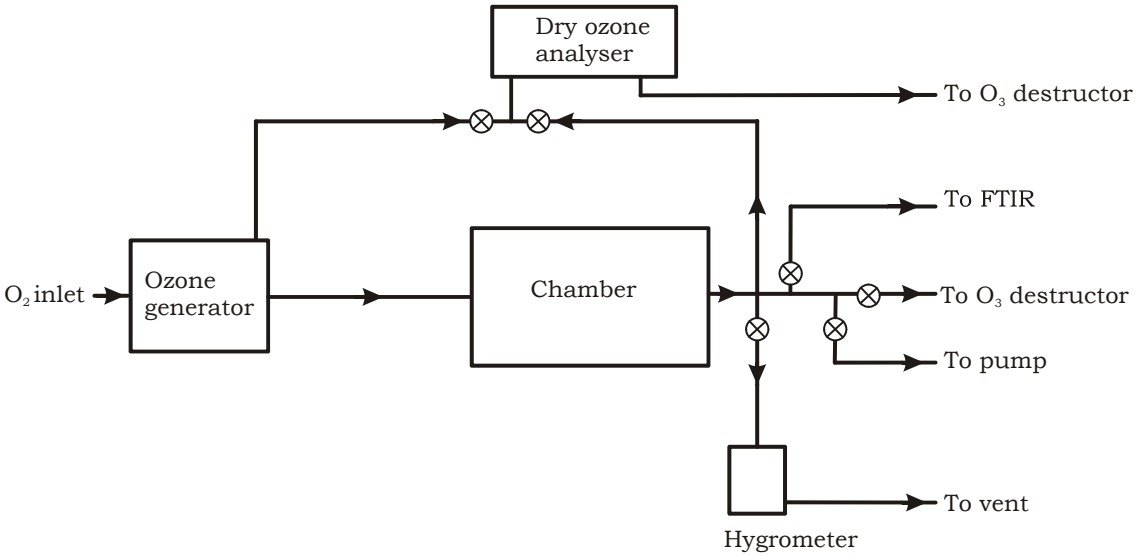

Figure 1. Schematic view of the experimental arrangement utilized to generate ozone and determine its concentration at the chamber entrance and exit. The nature of the effluents exiting the chamber can be analyzed through Fourier Transform Infra Red (FTIR) spectroscopy. An O_3-destructor (based on chemical catalyser) is provided to abate ozone.

Figure 1 shows the various elements utilized to generate ozone and determine its concentration as it enters and exits the chamber. The chamber, made from 316 stainless steel, is a 400 mm long, 100 mm high and 220 mm wide parallelepiped. Ozone concentration can be monitored with an analyzer based on UV absorption. The generated effluents can also be analyzed through FTIR spectroscopy. The ozone generator provides a mixture of molecular and atomic oxygen in the gas phase; it is operated in the electrical current domain over which increasing it increases the ozone concentration. The ozone flow is dry since the generator is supplied from (high-purity) O_2 dry-gas bottles. The expression "dry ozone" as used herein refers to gaseous ozone having a relative humidity (RH) of less than about 2 % (determined with a hygrometer). Total gas flow is set at 5.64 standard liter/min (slm) in order to

achieve an ozone concentration of 4 000 ppm under our operating conditions. The gas temperature in the chamber is close to ambient (≈ 22 ^{o}C).

An O_3-destructor, localised at the end of the process line, is provided to abate ozone and to release it as O_2 to comply with safety (toxicity) regulations. For safety reasons also, the chamber is located within a hood and a vacuum dry pump is used to make sure that the chamber effluents are fully evacuated at the end of the process.

2.2 Polymer surface preparation and treatment conditions.

Different types of polymers (used in the biomedical area) have been tested: polystyrene (PS) from Goodfellow® (LS251269), polyurethane (PU) from Johnston Industrial Plastics® (12348500), high density polyethylene (PE) from Goodfellow® (LS328449), polypropylene (PP) from Goodfellow® (LS328449), polymethylmetacrylate (PMMA) from Goodfellow® (LS251269), polymethydisiloxane (silicone) from Goodfellow® (LS251269) and polytetrafluoroethylene (Teflon®) from Goodfellow® (LS251269). Polymer sheets were cut up with appropriate techniques to provide samples approximately 2.5 × 2.5 cm^2. These were cleaned and rinsed 5 times with ultrapure water. After drying, each polymer sample was laid out on a glass Petri dish, introduced in the processing chamber (Fig. 1) and, then, exposed to ozone for 60 min (glass Petri dishes were used since the interaction of ozone with glass, as opposed to that with polymers, is expected to be minimal).

2.3 Microorganisms: sporulated bacterium choice, its preparation, deposition and recovery.

The endospores used to test the biocidal properties of the ozone-treated polymeric surfaces were *Bacillus atrophaeus* ATCC® 9372 (formely known as *B. subtilis*). These are routinely used in the validation of sterilization processes with dry heat, and are known to be very resistant to dry ozone treatment. They were prepared

in the Laboratoire de contrôle des infections (Faculty of Dentistry) of Université de Montréal.

Immediately after ozone treatment of the polymers, 10^6 spores in 100 µL of distilled water were deposited at the center of the sample. These deposits were then left to dry, protected from light, for approximately 24 hours under ambient conditions (temperature and pressure) and then harvested. The whole microbiological protocol (preparation, deposition and determination of survivor number (recovery)) is the same as that described earlier [10].

2.4 Surface characterization techniques

Contact angle measurements

The surface wettability of the polymers listed in section II.2 was characterized before and after ozone treatment, using the water-drop-shape method (VCA Optima® goniometer). Distilled water, glycerol and formamide were the three liquids used for contact angle measurements. Two µL of each liquid was dropped onto 4 different sites on each sample to provide a statistical average for each sample. Experiments were conducted under ambient humidity and temperature conditions.

Surface energy calculations

The surface energies are calculated from Fowkes (1) and Good (2) theories, based on hydrophilic-hydrophobic interactions and on Lewis acid-base interactions, respectively,

$$W_{sl} = \gamma_l (1 + \cos\Theta) = 2(\gamma_l^d \gamma_s^d)^{1/2} + 2(\gamma_l^{nd} \gamma_s^{nd})^{1/2} \quad (1)$$

$$W_{sl} = \gamma_l (1 + \cos\Theta) = 2(\gamma_l^{lw} \gamma_s^{lw})^{1/2} + 2(\gamma_l^+ \gamma_s^-)^{1/2} + 2(\gamma_l^- \gamma_s^+)^{1/2} \quad (2)$$

where W is the work of adhesion between solid and liquid, and its components (dispersive γ^d, undispersive γ^{nd}, Lischiftz-van der Walls γ^{lw} and acid-base γ^+,γ^-); γ are the associated surface energies.

Atomic Force Microscopy (AFM)

AFM images were acquired in air at room temperature using a Digital Instruments (Dimension 3100, Santa Barbara, CA). Intermittent contact imaging, so-called "tapping mode", was achieved at a scan rate of 1 Hz using etched silicon cantilevers with a resonance frequency around 300 kHz, a spring constant of ≈ 42 N/m, and a tip radius of <10 nm. All images were captured with a medium tip oscillation damping (20-30%).

Steric exclusion chromatography (SEC)

The ozone-treated Petri dishes (10, 60 and 120 min) were dissolved in tetrahydrofuran (THF) solutions, then injected into a set of columns. The eluded fractions are detected either with a refractometer or UV detectors. The calibration of elution volumes is run with different PS standards corresponding to molecular weight comprised between 3200 and 867 000 $g.mol^{-1}$.

The purpose here is to look for any degraded and oxidized oligostyrene after tetrahydrofuran (THF) extraction) from the ozone-treated Petri dish surface. For this purpose, the Petri dishes were rinsed with THF, then analysed by SEC calibrated for low molecular weight. The calibration curve is determined from different n-alcanes elutions. Therefore, the mass of eluted fractions of oxidized oligostyrene is given as n-alcane components.

3. Results and Discussion.

3.1 Biocidal activity of tested polymers: Inactivation efficacy

Figure 2 shows the number of survivors for both treated and untreated polymeric samples.

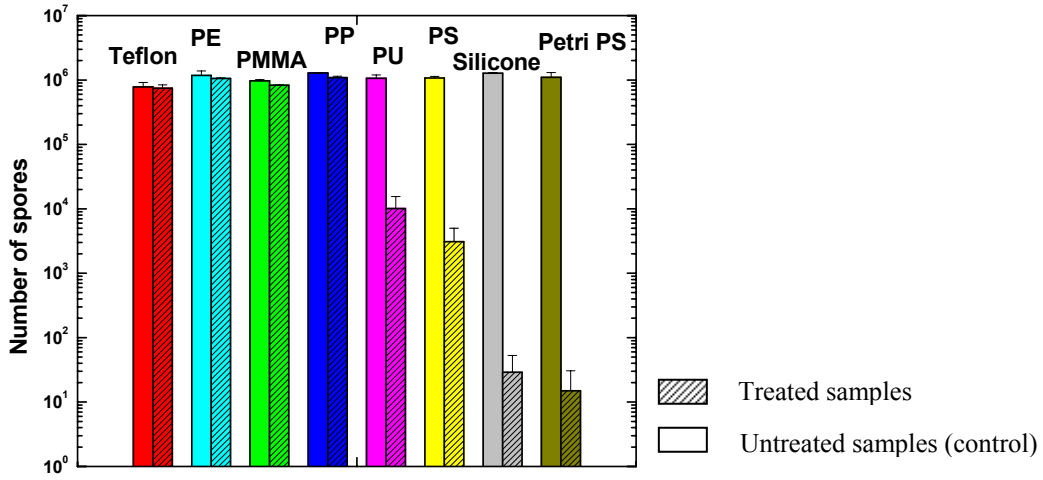

Figure 2. Representation on a log scale of the number of *B. atrophaeus* spores (initial deposit approximately 10^6/100µl) harvested after 24 h on ozone-treated and untreated polymeric samples.

A decrease in the number of survivors is observed for all samples, showing the ability to confere biocidal activity to these polymers. Nevertheless, biocidal efficacy varies strongly depending on the nature of the polymer: inactivation efficacy attained 99.1% of the spores initially deposited for PU whereas only 4.5% is reached on Teflon samples, an extremely weak value (Figure 2, Table 1). Teflon is known for its high chemical inertia (mainly because of C-F bonds), consequently modifications resulting from ozone exposure are expected to be minimal relatively to other polymers, which explains the very weak inactivation rate achieved after treatment.

Turning to the literature, surface modifications leading to antimicrobial properties generaly imply grafting or coating of active species [3, 6, 12]. The tested microorganisms in such cases are mainly vegetative bacteria and viruses while bacterial spores are rarely investigated [13]. For instance, Haldar *et al.* demonstrated that certain hydrophobic polycations painted onto glass surfaces rendered them not only highly bactericidal but also extremely virucidal against at least two distinct strains of influenza virus [7]. Makkal *et al.* tested the biocidal efficacy of PU coated with chemical pendant groups on several Gram + and Gram – bacteria: inactivation of approximately 2.6 log, 3.3 log and 3.4 log of *E.coli*, *P. aeruginosa* and *S.aureus* were

respectively obtained after a 30 min contact time (note that the bacterium suspension was sandwiched between two identically coated slides) [8].

Table 1 presents the inactivation efficacy (in percentage) reached 24-hour after depositing *B. atrophaeus* spore suspension on ozone just-treated polymer samples.

Polymers	Biocidal efficacy (%)	Deposit diameter (mm)	Contact angle reduction (%)
PS Petri dish	99.999*	11	47.7
Silicone	99.997	6-7	8.9
PS	99.7	8.5	33.6
PU	99.1	9	13.7
PP	15.2	8	11.5
PMMA	14.1	6	14.1
PE	10.5	7	22.9
Teflon	4.5	6	0.1

*Reference : [11]

Table 1. Percent inactivation efficacy observed 24-hours after depositing (10^6/100µl) *B. atrophaeus* spore suspension on different polymer samples previously exposed 60 min to a 4000 ppm dry gaseous ozone flow. The deposit diameter D measured on each type of treated polymers is indicated. Relative reduction (in %) in contact angle of the tested polymer samples after ozone exposure are also presented (distilled water was used for these measurements).

The diameter D of the deposits (Table 1) is observed to vary from 6 up to 9 mm, implying that spore density can vary significantly according to the nature of the treated polymer. This affects the polymer surface interaction with spores. A possible consideration is that the larger D, the less stacked are the spores, hence more direct is their contact with the treated surface and thus higher should be the inactivation efficacy [11]. This observation is important, but not enough to fully account for the different inactivation rates recorded between polymers: treated silicone exhibits an inactivation rate close to 99.9% with D in the range 6-7 mm whereas for PMMA and PE with D being 6 mm and 7 mm, respectively, corresponding inactivation rates as low as 14.1% and 10.5% are observed.

3.2 Contact angle modifications and biocidal actvity of tested polymers

The observed variation of the D values is expected to be related to the contact angle of the samples after their treatment. Figure 3 shows the contact-angle values determined before and after ozone treatment for each of the polymers considered while Table 1 displays their relative variation.

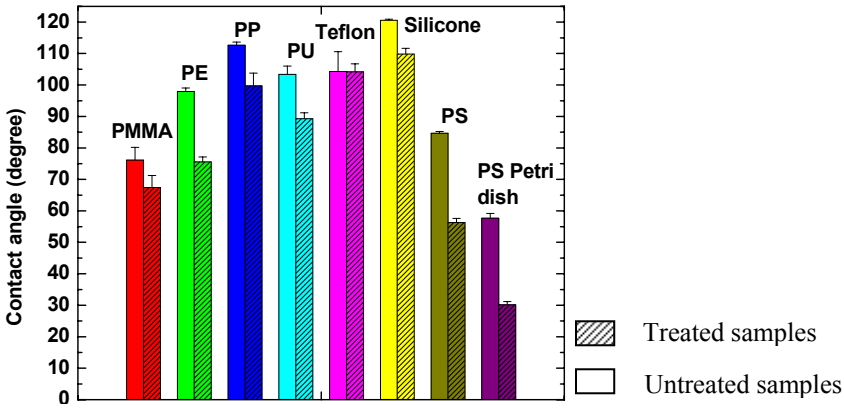

Figure 3. Contact angle measurements determined with distilled water for a set of polymers before and after ozone treatment (4000 ppm, 60 min). Error bars are also shown.

Each O_3 treated samples show an increase of hydrophilicity (or wettability), which could be related to an increase of oxygen species on their surface, leading naturally to an increase of their surface energy [11, 14, 15]. The hydrophilicity increase is more or less important depending on the polymer type.

Comparison of the biocidal efficacy with the contact angle variation (Table 1) leads us to state that inactivation rate and contact angle variations are not directly correlated. For instance, the contact angle variation for treated PS and PE samples is 33.6% and 22.9%, respectively, but the corresponding inactivation efficacy is 99.7% and 10.5%. Moreover, the contact angle determined for treated silicone and PS samples is $109.8^{o} \pm 1.8^{o}$ and $56.3^{o} \pm 1.4^{o}$, respectively, but both of them reached an inactivation rate of more than 99%.

However, note that the highest inactivation rate was obtained with PS Petri dish samples [11] that, among the treated polymers, yielded the lowest initial contact angle value and then the highest contact angle variation.

Polymers exposed to O_3 [11, 16], O_3-UV [14, 17,18] and plasma discharges containing oxygen [6, 14, 19, 20, 21] are known to yield a decreased contact angle and subsequently an increased hydrophilicity of their surface. The reported contact angle values can slightly vary from an author to another. Chemical characterization of these modified surfaces, for example through X-Photon Spectroscopy (XPS), did confirm the improved hydrophilicity with the increased oxygen content on the surface [5, 11, 14, 20].

3.3 Physical characterization of the tested polymer surfaces

The hydrophilicity increase, as just shown, is more or less important depending on the substrate nature: its specific chemical interaction with the gaseous flow could lead to different levels of physico-chemical modifications. Surface characterization of the polymers is thus needed to scrutinize the physico-chemical modifications induced by the ozone exposure.

3.3.1 Surface topography and roughness

Four polymeric samples were analyzed with the AFM technique: silicone, PS and PU, which have a very high inactivation efficacy, and PMMA, a very low one. AFM has been chosen to visualize the topologic morphology (degradation…) and to gather information on the surface roughness. The topographic modifications of these ozone-treated polymeric samples are presented in figure 4.

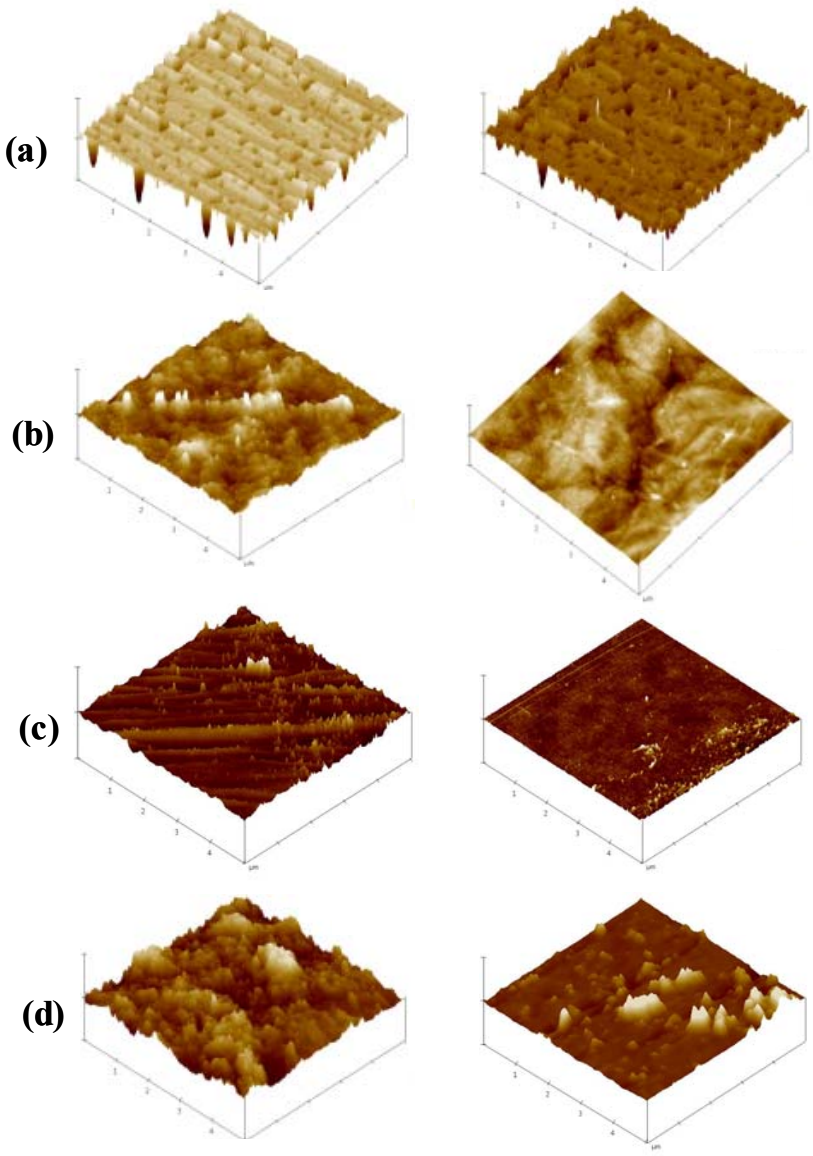

Figure 4. 3D AFM topographic images of untreated (left) and ozone-treated surfaces (60 min exposure to dry O_3 at 4000 ppm) (right) of: (a) PMMA, (b) PU, (c) PS and (d) silicone samples where full scale in (x,y) plane is 5 µm.

For all untreated samples, the strong color contrast indicates that the gradient between surface and the bottom of holes or top of peaks (reliefs) is very important. The corresponding phase image for each untreated samples (not shown) indicates that there is only one chemical entity on the whole surface; however, for untreated silicone and PU samples, a phase contrast is observed due to a great number of reliefs.

The ozone treatment effect on all these samples can be easily assimilated to a global abrasion of their surface, as seen on the 3D topographic images and in table 2. It explains that the root mean square (Rms) values of the topographic variations (the roughness) generally decrease after treatment, although reaching different levels of roughness depending on the nature of the substrate. For instance, ozone treatment of PU samples leads to a great number of "canyons" of different depths, represented by more contrasted colors although the sample roughness as a whole is very slightly affected. Hasirci *et al*. reported that after exposure to an oxygen plasma, the roughness values of PU samples slightly increased [19] whereas in our conditions a very small decrease is observed. As for the treatment of PMMA samples, it leads to a widening of the already existing holes and to additional small holes making that the hole-free part of the surface covers only 76.2 % of the total surface (25µm scale) relatively to 84.7% before ozone exposure. Concerning the textured sample of silicone, the relief is clearly smoothed after ozone treatment, representing a roughness reduction of 50% relatively to the control sample; this trend is also observed with PS samples (an 80% reduction of the roughness after treatment). Diameter and depth of the treated-PS holes were estimated to vary in the range of [1.2-3.4] µm and [12.6-46.1] nm, respectively. AFM analysis by Gejo *et al.* of PS treated with VUV radiation in presence of oxygen revealed the presence of 20 nm deep cavities [23], in agreement with our measurements.

Rms values (nm)	***PMMA***	***PU***	***PS***	***Silicone***
Untreated	6.5	9.9	4.8	> 60
Treated	4.7	9.4	0.9	32.7

Table 2. Rms values of polymeric samples before and after their ozone treatment observed on a 5 µm scale.

White dots appeared on the PMMA-treated 2D topographic images under 25 µm and also 5 µm scales, as can be seen in figure 5. The white dots observed on the 5

µm scale (see arrow, fig. 5) are in reality peaks on the topographic image (fig.4) characterized by black dots on the phase image, which indicate a chemical constrast. Such peaks have been reported on certain polymeric surfaces after an oxidative treatment [18, 22]. It appears that black dots are more numerous on the phase image than on the 2D topographic image, meaning that some peaks could be located in depth. For the other samples, it is difficult to say whether or not new peaks are created after the oxidative treatment because of their small number density or because of the initial peaks which could prevent us from observating these newly created characteristic peaks.

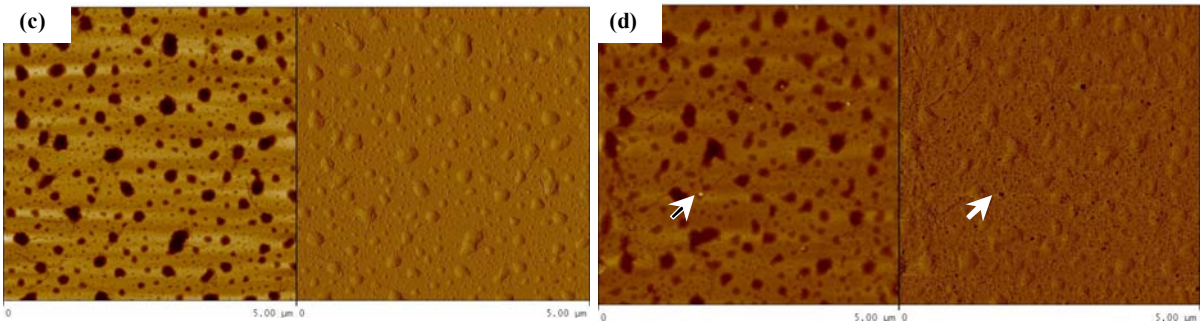

Figure 5. Topographic 2D images (left) and corresponding phase images (right) of the same untreated (c) and treated (d) samples, where full scale in (x,y) plane is 5 µm.

3.3.2 Surface energy measurements

When using equation (1), the surface energy is inferred from Fowkes theory where the total surface energy of the surface is made up of dispersive and non-dispersive (or polar) components. It comes out that the total surface energy of all samples increases but more or less significantly, depending on the polymer nature. The increase of the surface energy implies that the surface has been saturated from hydrophobic to hydrophilic groups [24].

$\gamma_{s\,(mJ.m^{-1})}$		γ_d	γ_{nd}	γ_{tot}
PP	(C)	0.02	21.04	21.06
PP	(Tr)	12.39	8.87	21.26
PE	(C)	4.30	15.34	19.64
PE	(Tr)	9.73	22.37	32.10
PS	(C)	16.60	8.01	24.61
PS	(Tr)	16.43	34.24	50.67
Teflon	(C)	5.62	5.26	10.88
Teflon	(Tr)	6.07	4.95	11.02
PMMA	(C)	6.22	16.25	22.47
PMMA	(Tr)	1.95	35.47	37.42
Silicone	(C)	0.82	8.53	9.35
Silicone	(Tr)	0.02	16.19	16.21
PU	(C)	0.33	20.75	21.08
PU	(Tr)	2.00	17.68	19.68
PS dish	(C)	9.28	34.62	43.90
PS dish	(Tr)	6.43	64.48	70.91

Table 3. Values of dispersive and non-dispersive components of the surface energy of various polymers, as calculated from equation (1), before (C) and after (Tr) ozone treatment (60 min, 4000 ppm).

At first sight, there is no direct correlation between the efficiency of the biocide activity and the variations of the dispersive and non-dispersive components (table 3). However, there is a strong increase (at least 50%) of the undispersive component of the surface energy of the PS, silicone and PS Petri dish samples, which all exhibit a high biocidal activity. PS Petri dish recorded the highest increase of the non-dispersive component together with also the highest biocidal activity.

The increase of the hydrophilicity of the PS Petri dish sample (Sec. III.2) could be explained by the increase of the polar component, indicating that polar groups (known to increase wettability) could have been created on the surface after ozone treatment. Indeed, in previous work, FTIR and XPS analysis demonstrated that, after treatment, a great number of new chemical compounds containing polar bonds such as C-O, C=O were created on surface to the detriment of C-C bonds that highly decreased [11, 25]. As a result, surface energy calculations support these previously obtained experimental results.

When using equation 2, the surface energy is based on acid-base theory where the total surface energy of the surface is composed of two components: γ^{lw} representing the Lischiftz-van der Walls interaction and $\gamma^{AB} = 2(\gamma^+ \gamma^-)^{1/2}$ representing the acid-base interaction between the solid and the liquid surfaces, with γ^+ and γ^- being the corresponding acid and basic components.

γ_s (mJ.m^{-1})	γ_+	γ_-
PP (C)	12.70	6.85
PP (Tr)	1.58	7.51
PE (C)	0.13	9.07
PE (Tr)	1.34	16.82
PS (C)	3.62	7.88
PS (Tr)	1.39	24.88
Teflon (C)	0.05	3.58
Teflon (Tr)	0.04	3.35
PMMA (C)	0.05	10.73
PMMA (Tr)	0.12	23.40
Silicone (C)	2.31	3.55
Silicone (Tr)	9.43	5.35
PU (C)	1.71	10.538
PU (Tr)	1.27	9.12
Petri PS (C)	0.75	19.66
Petri PS (Tr)	0.35	43.06

Table 4. Values of acid-base components of the surface energies of various polymers, calculated from equation (2), (C) before and (Tr) after ozone treatment (60 min, 4000 ppm).

The variations of the basic component of the surface energy can be correlated with the biocide activity of these polymeric samples (Table 4, Figure 2). In that respect, three groups of polymers could be distinguished according to the level of their biocide action: (i) highly biocidal such as PS Petri dish, silicone, PS and PU, (ii) moderately biocidal such as PP, PMMA and PE, and (iii) very slightly biocidal such as Teflon®. The results presented in Table 5 allow almost the same classification when considering the basic component (γ^-): (i) strong increase of γ^- for PS Petri dish, silicone, PS and PE, (ii) a quasi-invariability of γ^- for PU, Teflon and PP. So, except

for PU, these two classifications are well correlated, meaning that the incorporated basic and acid functional groups and theirs electron donor or acceptor properties could be related with the biocide activity of these polymers.

The higher increase of γ^- is recorded with the PS samples and PS Petri dishes: it could be associated to ester groups formation as already analyzed by FTIR and XPS spectroscopies . The correlation between the present results and previous results [11] obtained with FTIR and XPS analyses (on PS Petri dishes) seems to indicate the presence of ester groups on the treated surface.

3.3.4 Steric exclusion chromatographic analysis

To deepen the ozone-treated PS Petri dish analysis, a SEC was conducted to identify and to quantify the presence of new chemical species characterized with molecules of different weights.

Analysis of the high molecular weight fractions

The analysis of the high molecular weight fractions indicates that a surface degradation occurred on the surface after the ozone treatement. Indeed, Table 5 shows that the weight-average molecular weight (Mw) and number-average molecular weight (Mn) slightly decrease as time of treatement is increased. The polymolecularity index *I* is slightly increasing with exposure time, leading to PS chains being shortened by O_3 treatment.

PS Petri dish samples	*Mn (g.mol^{-1})*	*Mw (g.mol^{-1})*	*I*
Untreated (control)	86 460	223 600	2.586
Treated 10 min	84 520	221 660	2.623
Treated 60 min	84 930	221 770	2.611
Treated 120 min	84 810	221 800	2.615

Table 5. Weight-average molecular weight (Mw) and number-average molecular weight (Mn) and polymolecularity index (I) of the high mass fraction of the PS Petri dishes before and after ozone treatment (4000 ppm) at different exposure times.

Analysis of the low molecular weight fractions

The spectra obtained by SEC chromatography of untreated and ozone-treated (120 min) samples are presented in Figure 6. This SEC analysis confirms our previous experimental results that suggested the possible existence of a weak-bond-layer (WBL) [11], since the presence of a low molecular fraction on the sample surface (ranging from 12 to 20 cm^3) has now been clearly identified. Relatively to the untreated sample, new peaks appear corresponding to new products for which elution volumes are 13.88 and 14.86 cm^3, respectively. The other peaks were identified as being associated to additive compounds like stearate or naphtalate derivatives.

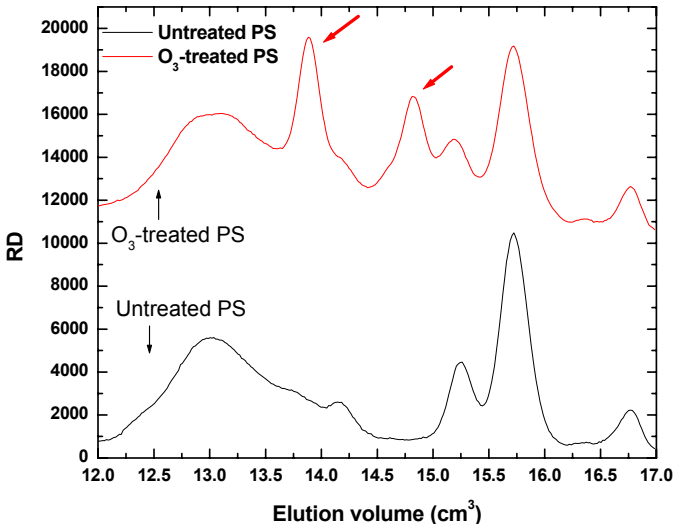

Figure 6. SEC chromatography (low molecular weight) of PS Petri dishes treated with 4000 ppm of ozone for 120 min and untreated ones (control). RD means refraction differencial.

From Figure 6, we estimate the new product proportions to be 7% for the first fraction (13.88 cm^3) and 5 % for the second one (14.86 cm^3). The peaks located at 13.88 cm^3 and 14.86 cm^3 were determined to correspond to molecules having 22 and 15 carbon atoms, respectively. These products could be identified as dimers and trimers of the partially oxidized styrene (by considering the equivalence between molecule structure and n-alcane calibration).

For elution volume of less than 12 cm^3, the PS eluated fraction (not shown in Fig.6) of the treated sample highly decreases (by a factor of 1.8), confirming that enrichment of low-weight products on surface occurred.

4. Summary and conclusions

In previous articles and a patent application [26], a method has been described, based on dry gaseous ozone treatment that confers to PS Petri dishes and polymer surfaces new properties, particularly a strong biocidal activity. The current paper completes these previous studies by examining the biocidal efficacy of such ozone-treated surfaces of different polymers (PS, PMMA,…) on *B. atrophaeus* spores, trying to relate spore inactivation to physical and chemical properties of their surface such as wettability and topography. It clearly appears that the inactivation efficacy level depends strongly on the type of polymer considered: for instance, polymers such as silicone, polyurethane and polystyrene have high inactivation rates while polypropylene, polymethylmetacrylate and polyethylene are particularly inefficient.

The fact that certain polymers have a high chemical stability implies a weak sensitivity to any oxidation process such as that involving O_3 and, therefore, one expects that their biocide properties will also be weak, as observed with Teflon sample. The biocidal efficacy is found not to be directly related with wettability modifications (obtained through contact angle measurements) and with the important topologic variations of these polymer surfaces (observed with AFM) as resulting from the O_3 oxidative action.

Analysis of the surface energy of the polymeric samples indicates: (i) a correlation of the non-dispersive components with the highest biocidal samples and, particularly, (ii) a link between acid-base components with the biocide degree of these polymeric samples : the existence of a biocide activity is correlated with the increase in the basic component of the surface energy after ozone treatment. Concerning the PS Petri dishes, chemical groups such as esters were identified as probably involved in the biocide activity. Chemical analysis (SEC) of the PS Petri

dish surfaces demonstrated the presence of a slight degradation of the surface and enrichment by low molecular weight products of the treated surface.

On practical ground, some of these treated polymers can strongly reduce microorganism loads, eventually providing a high level of disinfection. Since polymers can be manufactured in many different forms (such as powders, films, plates,…), a large number of applications is conceivable, in particular in hospital environments. However, there remains to characterize human toxicity of these treated surfaces before determining the extent of their possible applications.

Acknowledgments

The authors are grateful to JS Mayer for skilful technical assistance, to Professor R Hausler at École de Technologie Supérieure (ETS) for lending us the ozonator and to J Seguin for valuable microbiological work. Authors are indebted to P Moraille for priceless AFM technical assistance and pertinent comments and to M Amara for technical support on contact angle measurements. Thanks are also due to Dr. D Keroack and to MS Belkhous (Eng. degree) for pertinent assistance.

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Dans cet article, nous avons montré, suite à un traitement à l'ozone, la possibilité de conférer une activité biocide à la surface de différents polymères. Toutefois, il en ressort que le degré de cette activité biocide dépend très fortement de la nature du polymère puisque l'efficacité d'inactivation déterminée expérimentalement varie énormément. Afin d'examiner les modifications en surface, nous avons utilisé l'AFM pour en caractériser les changements topographiques. Nous avons également effectué la mesure d'angle de contact pour comparer les variations de mouillabilité (hydrophobe/hydrophile) de la surface avant et après traitement. La mesure de ces angles de contact nous a permis aussi de calculer l'énergie de surface et ses composantes qui, en variant, ont pu nous éclairer sur la nature des modifications donnant naissance à cette activité biocide.

Nous avons montré que les changements morphologiques sont très importants pour les différents polymères considérés, quelle que soit leur efficacité biocide, caractérisés, entre autres, par une diminution de la rugosité de la surface pour chacun d'entre eux. Concernant la mouillabilité, le traitement à l'ozone a pour effet de rendre chacune des surfaces testées relativement plus hydrophiles, ce qui va bien dans le sens de notre compréhension puisque nous avons vu précédemment (Sec.3.2.1) que ce type de traitement avait pour effet de créer des espèces oxygénées en surface, entraînant ainsi une augmentation de la mouillabilité. À partir du calcul de l'énergie de surface et de ses composantes, nous avons montré qu'il existerait un lien entre l'effet biocide de la surface et ses composantes acido-basiques. Compte tenu des études précédentes effectuées sur Pétris de PS pour lesquels nous avons indiqué que des espèces acides pourraient être impliquées dans le processus d'inactivation, on montre ici que des groupements tels que les esters pourraient aussi y participer. Par une méthode d'analyse chimique, on constate un enrichissement de la surface traitée en produits de faibles masses moléculaires. Cette étude nous a permis d'avancer dans la compréhension des mécanismes intervenant dans l'inactivation des spores bactériennes.

D'autres tests et méthodes de caractérisation seraient requis pour approfondir cette étude; toutefois, cette dernière a clairement démontré la possibilité de conférer à ces polymères le caractère biocide suite à un traitement à l'ozone, ce qui fut inclus dans la demande de brevet (avril 2008) [33].

3.4 Conclusion

Dans ce chapitre, nous avons présenté une nouvelle méthode permettant à la surface d'un Pétri de PS d'acquérir une activité biocide; puis, par la suite, nous avons élargi cette possibilité à différents types de polymères montrant, tout de même, que le degré d'efficacité dépend de la nature du polymère. Nous avons démontré que cette nouvelle propriété est due à des modifications physico-chimiques de la surface : on citera principalement l'apparition de nouveaux groupements chimiques en surface, révélée par différentes méthodes d'analyse de surface. Nous avons réussi à mettre en évidence une contribution possible des espèces acides et esters au processus d'inactivation, sans pour autant en exclure l'influence d'autres molécules (encore non identifiées de manière rigoureuse). L'ampleur des dommages causés aux spores bactériennes a été caractérisée et l'effet létal de la surface traitée sur ces microorganismes a été clairement démontré et quantifié. Nous avons aussi souligné l'importance du type d'interaction entre les microorganismes et la surface quant à l'efficacité de son action biocide (à court et à long terme). L'utilisation éventuelle de telles surfaces, par exemple en milieu hospitalier, nous a amené à en préciser quelques propriétés notamment la durée de la rémanence de l'effet biocide, la possibilité de régénérer cette activité biocide après que celle-ci ait disparu avec le temps, le recours également à un traitement à l'ozone humide…

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Conclusions et perspectives

L'optimisation des procédés de stérilisation des outils biomédicaux et l'innovation dans ce domaine sont devenues indispensables pour répondre aux besoins actuels de secteurs tels que les hôpitaux (médecine générale, chirurgie, médecine dentaire…). Un nombre assez élevé d'incidents techniques en milieu hospitalier, tel que l'augmentation des infections nosocomiales très souvent dues à des instruments médicaux mal stérilisés, a entraîné la mort de patients. Les enjeux sont de taille et les défis à relever sont donc importants. Parmi les techniques émergentes présentant un réel potentiel, on retrouve la stérilisation par plasma et, notamment, la stérilisation par l'ozone. Afin de rendre ces techniques le plus sécuritaire et efficace possible, une excellente compréhension des mécanismes physico-chimiques du procédé ainsi que des mécanismes d'inactivation des microorganismes sont nécessaires. C'est dans ce contexte général que s'est situé notre travail.

Bien qu'un grand nombre d'études ait été mené dans le cadre de la désinfection/stérilisation à l'ozone humide, les recherches effectuées sur ce sujet, aussi bien en milieu liquide que gazeux, n'ont toujours pas permis une identification rigoureuse des espèces menant à l'inactivation des microorganismes ainsi que des mécanismes mis en jeu. En comparaison, les travaux concernant le traitement à l'ozone dans un milieu sec se font rares, principalement en raison d'un certain manque d'intérêt dû à ce que ce traitement est reconnu comme étant peu efficace contrairement au procédé en milieu humide. Notre thèse a précisément consisté à étudier les mécanismes d'inactivation en jeu lors d'un traitement à l'ozone sec et à l'ozone humide. L'originalité de ce travail repose, entre autres, sur (i) la corrélation faite entre ces deux aspects du traitement à l'ozone et, sur (ii) le caractère pluridisciplinaire (physique, microbiologie, physico-chimie des polymères) de la thèse permettant une vision plus juste et plus globale de la problématique. C'est pourquoi, nous croyons, que cette étude constitue un apport essentiel à la compréhension des phénomènes intervenant lors d'un traitement à l'ozone.

Dans un premier temps, après avoir caractérisé l'enceinte de traitement, nous avons effectué une étude quantitative de l'effet d'un traitement à l'ozone sec sur différents types de spores bactériennes en ce qui a trait à leur cinétique d'inactivation et, aussi, aux dommages qui leur sont infligés. Cette étude comparative nous a permis de voir, entre autres, que l'efficacité du traitement à l'ozone sec pouvait varier de manière significative selon la nature du microorganisme. À partir de ces observations physiques et microbiologiques, le mécanisme d'inactivation proposé fut la diffusion/oxydation des espèces actives sur et à travers les microorganismes; parmi ces espèces, la molécule d'ozone a été identifiée comme jouant un rôle clé dans ce processus (alors que la communauté scientifique demeure partagée sur cette question).

Nous avons, par la suite, mis à profit le travail effectué en milieu sec pour nous orienter vers un traitement à l'ozone dans un environnement humide, milieu chimiquement plus complexe. De ce fait, la caractérisation des espèces présentes dans

la phase gazeuse humidifiée, l'étude cinétique de l'inactivation des microorganismes ainsi que la détermination des dommages qui leur sont infligés, le tout, corrélé avec certains résultats expérimentaux pertinents tirés de la littérature scientifique, nous ont permis de proposer un mécanisme d'inactivation pour le traitement à l'ozone humide.

Tout d'abord, l'humidité présente a pour effet de faire "gonfler" la spore, ouvrant ainsi des passages ou canaux dans lesquels les espèces actives vont pouvoir diffuser vers le cœur de la spore. Au cours de la diffusion, des réactions d'oxydation peuvent avoir lieu, endommageant ainsi la spore (en surface et en profondeur). Le caractère létal de ce traitement est entièrement dû à la présence de ces espèces oxydantes (O_3, espèces radicalaires…) alors que l'amélioration de la cinétique d'inactivation (relativement au cas sec) est due à la présence de l'humidité et de ces espèces chimiques en phase gazeuse. Il s'agit, croyons-nous, d'un apport de tout premier plan quant à la compréhension fondamentale des phénomènes mis en jeu dans le processus d'inactivation, incluant leur identification, leur rôle, leur importance...

Dans un second temps, notre étude s'est dirigée vers la possibilité (que nous avons découverte au cours de notre travail) de conférer une activité biocide à une surface polymérique suite à un simple traitement à l'ozone gazeux en milieu sec. Ce traitement permet à des surfaces de polymères de réaliser de très hauts degrés de désinfection (voire même, dans certains cas, la stérilité). Nous avons donc exploré ce tout nouveau volet de recherche.

Dans une première phase, nous avons déterminé l'efficacité biocide d'une surface de Pétri de PS traitée à l'ozone sur une grande variété de microorganismes (bactéries, spores bactériennes…) de manière semi-quantitative ainsi que relevé les dommages induits sur ces derniers. De plus, nous avons caractérisé les modifications de surfaces (topographiques et chimiques) survenues sur les surfaces après traitement à l'ozone. Ces études nous ont permis de mettre en évidence (i) l'existence d'une activité biocide de la surface traitée pouvant agir sur une grande gamme de bactéries végétatives, sur des spores bactériennes mais aussi sur l'activité enzymatique de la

lysozyme (protéine), (ii) une perte d'intégrité des spores, (iii) d'importantes modifications topographiques (variation de la rugosité de surface, de la mouillabilité…) et chimiques de la surface traitée (oxydation de la surface, apparition de nouveaux groupements fonctionnels en surface…), et enfin, (iv) la rémanence de l'activité biocide de la surface traitée pouvant atteindre un mois environ, selon le type de microorganisme.

Les résultats originaux obtenus lors de cette première phase de travail, nous ont encouragés à approfondir certains aspects afin de mieux connaître les propriétés caractéristiques d'une telle surface biocide (et donc l'étendue de son activité) et d'essayer de comprendre les mécanismes mis en jeu dans l'inactivation des spores bactériennes suite à leur mise en contact avec cette surface traitée. Cette étude nous a principalement permis de démontrer (i) l'étendue de l'efficacité biocide d'une surface de Pétri de PS traitée à l'ozone (efficace sur des densités de spores pouvant atteindre 10^8/100µL d'eau, existence de cette activité même si les spores se trouvent en suspension liquide…), (ii) l'influence et l'importance de la nature de l'interaction spores/surface sur l'efficacité biocide mais, aussi, sur le phénomène de rémanence, (iii) un lien possible entre la rémanence et les modifications chimiques de la surface en profondeur, (iv) une perte de l'imperméabilité de la spore pouvant mener jusqu'à l'extrusion du matériel interne contenant, entre autres, le matériel génétique (ADN). Enfin, nos résultats suggerent que l'inactivation serait due à la création d'espèces acides en surface (mais aussi d'autres molécules telles que les aldéhydes ou les esters).

Enfin, pour élargir cette étude, nous avons décidé d'explorer l'effet d'un traitement à l'ozone sur différents substrats en polymère (principalement ceux utilisés dans le domaine biomédical), notamment en ce qui concerne la possibilité de leur conférer un caractère biocide. Nous avons ainsi montré que les différents polymères testés ont tous acquis une activité biocide mais à des degrés divers selon leur nature. De plus, nous avons caractérisé les modifications physico-chimiques de leur surface : pour tous les polymères testés, d'importants changements topographiques ont été

observés. Quant aux changements de mouillabilité et donc d'énergie de surface, les résultats semblent indiquer que la variation de la composante basique serait très probablement reliée au caractère biocide de la surface, laissant penser que des molécules telles que les esters pourraient aussi (en plus des espèces acides) intervenir dans le processus d'inactivation.

Plusieurs aspects auraient mérité d'être davantage approfondis : citons quelques exemples qui nous semblent particulièrement pertinents.

(i) Essais sur objets réels

Il est évident que l'étude de l'inactivation des microorganismes par l'ozone sur des Pétris (non traités) demeure incomplète tant que des tests expérimentaux n'ont pas été menés sur des objets réellement utilisés dans le milieu hospitalier. En effet, la géométrie complexe de certains instruments médicaux pourrait réduire l'accessibilité des espèces chimiques réactives vers des sites contaminés. De plus, on évoquera la possibilité d'avoir une densité moins importante d'espèces oxydantes disponibles pour inactiver les microorganismes en raison de possibles recombinaisons en surface.

(ii) Dommages aux matériaux

Certaines modifications de surfaces causées par un traitement à l'ozone et induisant une activité biocide ont été étudiées dans le chapitre 3. Toutefois, même si nous avons montré l'existence de modifications topographiques (rugosité…) et chimiques (XPS, FTIR, mouillabilité…), nous ne savons pas si ces changements sont associés à des modifications des propriétés macroscopiques, telles que mécaniques et optiques, du matériau traité. Cet aspect mériterait d'être vérifié car de telles modifications pourraient limiter l'utilisation d'un tel procédé de désinfection/stérilisation.

(iii) Adjuvant gazeux

Dans notre travail, nous nous sommes concentrés sur l'étude d'un traitement à l'ozone gazeux (sec et humide) et son efficacité, sans toutefois examiner la possibilité

de rajouter un adjuvant gazeux (soigneusement choisi) en petite quantité (typiquement quelques %) pour tenter d'améliorer les performances du procédé de stérilisation à l'ozone sec (voire humide). Une telle étude mériterait également d'être explorée dans le cadre de l'activité biocide acquise par une surface polymérique suite à ce même traitement.

(iv) Étude sur les matériaux polymériques

Dans le cadre de l'étude de l'activité biocide acquise par un Pétri de PS suite à son traitement à l'ozone, nous avons été amenés à tester différents types de polymères pour, entre autres, vérifier si cette propriété pourrait etre étendue à d'autres substrats polymériques. Une étude approfondie visant à étudier les raisons pour lesquelles certains polymères sont plus susceptibles que d'autres d'acquérir une activité biocide serait d'un grand intérêt afin de mieux appréhender les phénomènes fondamentaux mis en jeu et ainsi améliorer les performances biocides de ces surfaces après traitement à l'ozone.

(v) Limites du traitement à l'ozone dans la formation de surfaces biocides

Tel que décrit dans le chapitre 3, nous avons mené un travail d'analyse et de caractérisation physique, microbiologique et chimique des surfaces de Pétris de PS traités à l'ozone sans, toutefois, explorer de manière exhaustive les limites d'un tel traitement. À titre d'exemple de ces limites, il serait intéressant de déterminer plus en détail les limites d'un tel procédé (en termes d'efficacité, rémanence…) en étudiant une plus grande gamme de paramètres physiques (concentration d'ozone, temps d'exposition…) et microbiologiques (nature des microorganismes, effet du volume et de la nature de la suspension liquide…) pour une meilleure compréhension des phénomènes mis en jeu.

ANNEXE 1 : Contributions de l'auteur

Cette annexe a pour but de clarifier ma contribution scientifique à chacun des articles présentés dans ce mémoire.

Article 1

A. Mahfoudh, M. Moisan, J. Séguin, J. Barbeau, Y. Kabouzi and D. Kéroack, ″Inactivation of vegetative and sporulated bacteria by gaseous dry ozone″**,** soumis au journal Ozone Science and Engineering (en révision majeure).

Ma contribution à cet article se situe au niveau de la prise de mesures telles que l'établissement des courbes de survie (exposition), les spectres d'absorption en spectroscopie optique, l'imagerie par MEB, les mesures d'activité enzymatique… Le montage de l'expérience s'est fait en collaboration avec Y. Kabouzi (à l'époque post-doctorant) et D. Kéroack (professionnelle de recherche). La récupération des microorganismes ainsi que la coloration au vert de Malachite ont été assurées par J. Séguin (technicienne en microbiologie). J'ai également réalisé, au préalable, une revue complète de la littérature. Finalement, j'ai effectué la majeure partie de l'analyse et rédigé le premier jet de l'article.

Article 2

A. Mahfoudh, J. Barbeau, M. Moisan, A. Leduc, J. Séguin, ″Biocidal action of ozone-treated polystyrene surfaces on vegetative and sporulated bacteria″, soumis au journal Applied Surface Science (accepté en decembre 2009).

Ma contribution à cet article se situe au niveau de la prise de mesures expérimentales telles que les expositions des Pétris de PS, certaines mesures de caractérisation de surface, les prises d'images par MEB, les mesures d'activité enzymatique… La viabilité, le dépôt et le comptage des microorganismes ont été traités et évalués par J. Séguin (technicienne en microbiologie). La coloration au DAPI a été assurée par A.

Leduc (assistante de recherche en microbiologie). Finalement, j'ai effectué la majeure partie de l'analyse et rédigé le premier jet de l'article.

Article 3

A Mahfoudh, A Leduc, M Moisan, J Séguin, J Barbeau, ″Biocidal properties of ozone-treated polystyrene surfaces on sporulated bacteria″, soumis au journal The Open Journal of Microbiology.

Ma contribution à cet article se situe au niveau des diverses prises de mesures visant la caractérisation des propriétés biocides acquises par les Pétris de PS suite à leur exposition. La viabilité et le comptage des microorganismes ont été évalués par J. Séguin (technicienne en microbiologie). La coloration au DAPI ainsi que les traitements à la DNase et à l'acide ont été assurés par A. Leduc (assistante de recherche en microbiologie). Finalement, l'analyse s'est effectuée en collaboration avec les spécialistes de la microbiologie (J. Barbeau, A. Leduc et J. Séguin). J'ai rédigé le premier jet de l'article.

Article 4

A. Mahfoudh, F. Poncin-Epaillard, M. Moisan, J. Barbeau, ″Biocidal action of ozone-treated polymer surfaces on sporulated bacteria″, soumis au journal Surface Science.

Ma contribution à cet article se situe au niveau de la prise de mesures expérimentales telles que la préparation et les expositions à l'ozone des échantillons de polymères, certaines mesures de caractérisation de surface et leur interprétation… La viabilité, le dépôt et le comptage des microorganismes ont été traités et évalués par J. Séguin (technicienne en microbiologie). La caractérisation chimique de la surface (SEC) a été réalisée et analysée par F. Poncin-Épaillard (Mans, France). De plus, l'analyse concernant les variations de l'énergie de surface a été effectuée en collaboration avec F. Poncin-Épaillard. Finalement, j'ai rédigé le premier jet de l'article.

ANNEXE 2: Inactivation of vegetative and sporulated bacteria by gaseous dry ozone *(complete version)*

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Abstract. Inactivation of different types of microorganisms by gaseous ozone is successfully achieved provided, as is well known, the gaseous phase is strongly humidified. The inactivation mechanisms and species involved in this process are, however, not yet clearly identified. Our approach to this situation is to consider dry ozone exposure of bacterial spores, a less complex chemical environment, in order to gain insight into the chemical reactions then taking place and their action on the biological targets. The current work is essentially experimental and calls on the literature for the possible inactivation mechanisms. As far as we know, our results demonstrate for the first time, and in opposition to most of the published literature, that it is possible to inactivate bacterial spores under strict dry ozone conditions: i) The observed inactivation rate is largely dependent on the spore type and substrate material; ii) The O_3 molecule is determined to be responsible for the inactivation process in dry media; iii) The inactivation mechanism is the diffusion and oxidative action of O_3 molecules within the spore whereas no outer erosion of the spore is detected. Comparison with exposure to humidified gaseous ozone for *B. atrophaeus* spores provides contrasting experimental results: these spores are barely inactivated under dry ozone conditions (one log in 2 hours) where O_3 is assumed to be the main biocide agent, whereas in the humidified case, the same spores are more rapidly inactivated (5 log in 40 min). The observed increased efficiency appears to be related, in part, to swelling of the spore that facilitates the diffusion of oxidative species

within it and up to the core; it is also due to the added contribution to the O_3 molecule action of the oxidative agents stemming from the interaction of O_3 with H_2O, which in the end leads to a heavily damaged spore structure in contrast to dry ozone exposure where the spore integrity is maintained.

Keywords : Ozone, gaseous, inactivation, dry ozone, humidify ozone, bacteria, spores, inactivation mechanisms, enzyme.

1. Introduction

1.1. Context

Ozone is a strong oxidative compound both in its gaseous form and when dissolved in water and, because of this, is known to be an efficient disinfectant for inactivating even chemically resistant microorganisms (1). Ozone can be an adequate solution where other disinfectants fail (2). Its oxidative potential is higher, for example, than that of hydrogen peroxide (3-6) and hypochlorite (7-8). Ozone was recommended as an alternative to chlorine for water treatment. On practical ground, ozone is easily generated on-site from dry air or O_2 through high-voltage corona discharges or filamentary dielectric barrier discharges at near ambient pressure and temperature (9-11). Even though ozone has been utilized in the disinfection of water for a long time, there has been limited interest for its use in the gaseous form for disinfection and, until only very recently, for sterilization purposes (TSO_3^{TM} sterilizer).

The growing use of heat-sensitive polymer instruments in hospitals has created new challenges in the area of sterilization. Conventional dry- and moist-heat methods, such as the Poupinel (Pasteur) oven and the autoclave respectively, can heavily damage thermosensitive materials. This is not the case with chemical sterilants such as, for example, ethylene oxide (EtO), but EtO requires long exposure and vent times (more than 10 h total) in addition to being toxic and, on the long run, carcinogenic and detrimental to the environment. As a result, EtO is already banned in many countries. Such considerations have led researchers to look for alternate efficient sterilization processes that hopefully would operate at low temperature and inactivate rapidly (≅ 1 h) all kinds of microorganisms with low damage to medical devices (MDs) and be harmless for man and his environment. Under such requirements, both gaseous-plasma and ozone sterilization seem to offer substantial promises for sterilizing thermosensitive MDs.

1.2. A review of previous work

From its inception and for a rather long period of time thereafter, the use of O_3 as a gaseous biocide in ambient air was limited to concentration levels close to 1 ppm (as

a matter of fact, 0.1 ppm was determined to be the occupational (40h/week) maximum safety-value as far as toxicity for man is concerned). In a study performed on aerosolized bacterial suspensions in a room at known temperature and relative humidity (RH) values, Elford and Van de Eude (12)**,** determined that concentrations in O_3 in excess of 1 ppm in the room with 60-80% RH were better conditions relatively to a dry atmosphere. Kowalski *et al* (13) studied the influence of higher concentrations of airborne ozone against *E. coli* and *S. aureus* vegetative bacteria. The microorganisms were exposed to O_3 in air, at concentrations ranging from 300 to 1500 ppm for 10 to 480 seconds with 18-20% RH: death rates in excess of 99.99% (> 4 log) were reached for both species at 1500 ppm and within 8 min.

Relying on much higher gaseous ozone concentrations, Held studied the efficacy of decontamination of hospital waste (14-15) on Gram-positive and Gram-negative vegetative bacteria, fungi, mycobacteria, and sporulated bacteria such as *Bacillus atrophaeus*, *Geobacillus stearothermophilus* and *Clostridium perfringens*. The decontamination vessel was supplied with dry gaseous ozone in a "cumulative mode", i.e. the vessel is closed, allowing achieving a higher ozone concentration in this chamber, approximately 10000-12000 ppm, than is possible in a continuous flow with the ozonator. Each time waste is introduced, the chamber is replenished with fresh ozone, obtained from a dry-air corona discharge and, at the end of the cycle, the effluents released from the chamber are abated. Under these conditions, the system allowed inactivation of more than 10^7 bacteria/mL (*S. aureus, B. atrophaeus, E. coli*...) as dried deposits within an hour of exposure and more than 10^7 spores/mL after two hours of treatment.

Ishizaki *et al* (16) examined the sporicidal activity of gaseous ozone on different Bacillus spores with ozone concentrations ranging from 250 to 1500 ppm (0.5 to 3 mg/L), additionally focusing on the influence of the RH level. At RH degrees of 50% or below, no appreciable decrease in the number of survivors was obtained after 6 h of exposure. However, at higher RH values, a 5 log reduction in less than 2 h was reached, which was confirmed later on by Currier *et al* (17). Aydogan *et al* (18) results also showed that increasing the O_3 concentration from 1 to 3 mg/L with a 70-

95% RH level increases the rate of inactivation of spores, but that beyond 3 mg/L (1500 ppm), only a weak additional increase is observed.

Two main points emerge from the above previous works: the higher the ozone concentration and the higher the RH (>50%), the more efficient is the inactivation process. As a matter of fact, in some cases, humidity is absolutely required to achieve sterility, as in the case of the TSO_3^{TM} sterilization system, approved by both Health Canada and FDA (US Food and Drug Administration). Furthermore, a few studies have pointed out that the inactivation rates are far from being linear with increasing RH and O_3 concentration, sometimes suggesting threshold levels for both these parameters and at times, as they are increased, a tendency for saturation of the inactivation rate (13, 18). The added value of humidified ozone with respect to dry ozone as a biocide agent is certainly partially responsible for the fact that little work has been done under dry ozone conditions (16). More experimental data are needed to reach a better insight into the ozone action mechanisms on microorganisms.

1.3. Outline of the paper

Our approach is to consider that the addition of water vapour to gaseous ozone makes it more difficult to unveil some of the basic inactivation mechanisms because it requires considering a larger number of chemical reactions. Therefore, the paper concentrates on the inactivation of bacterial spores under strict dry ozone conditions, as characterized by inactivation rates (survival curves) and spores damages (scanning electron microscopy (SEM)). Then, for comparison purposes and a complementary insight into the inactivation mechanisms, some data from exposure of spores to humidified ozone are presented.

The main interest in working with bacterial spores is that they are the most resistant microorganisms and, because of that, are officially required to validate sterilization processes. Bacterial spores can withstand severe treatments including heat, irradiation, chemicals, and desiccation: in fact, spores are at least 15 times more resistant to such stresses than vegetative bacteria. The bacterial spores of Bacillus species have been shown to be particularly resistant to ozone (13) and are therefore used in the current study.

The paper is organized as follows. Section II describes the materials used and the experimental methods. Section III examines various aspects of the exposure to gaseous dry ozone of three species of bacterial spores and of a vegetative bacterium, which include microorganism viability, structure integrity and an approach to the inactivation mechanisms. Section IV considers, for comparison purposes, a survival curve and SEM micrographs resulting from exposure of *B. atrophaeus* to humidified ozone. Finally, section V summarizes the main results on dry and humidified ozone exposure of microorganisms and correlates them to gain insight into the specific inactivation mechanisms and targets, and concludes the paper.

2. Materials and methods

Due to its high oxidative power, ozone can damage various kinds of materials more or less severely. To minimize such possible effects, which could interfere with our experiments, the sterilization chamber is made from stainless steel (as required for humidified ozone) and the windows used for spectroscopic observations are from fused silica. The microorganisms to be investigated are deposited on Petri dishes that are either made from polystyrene or Pyrex.

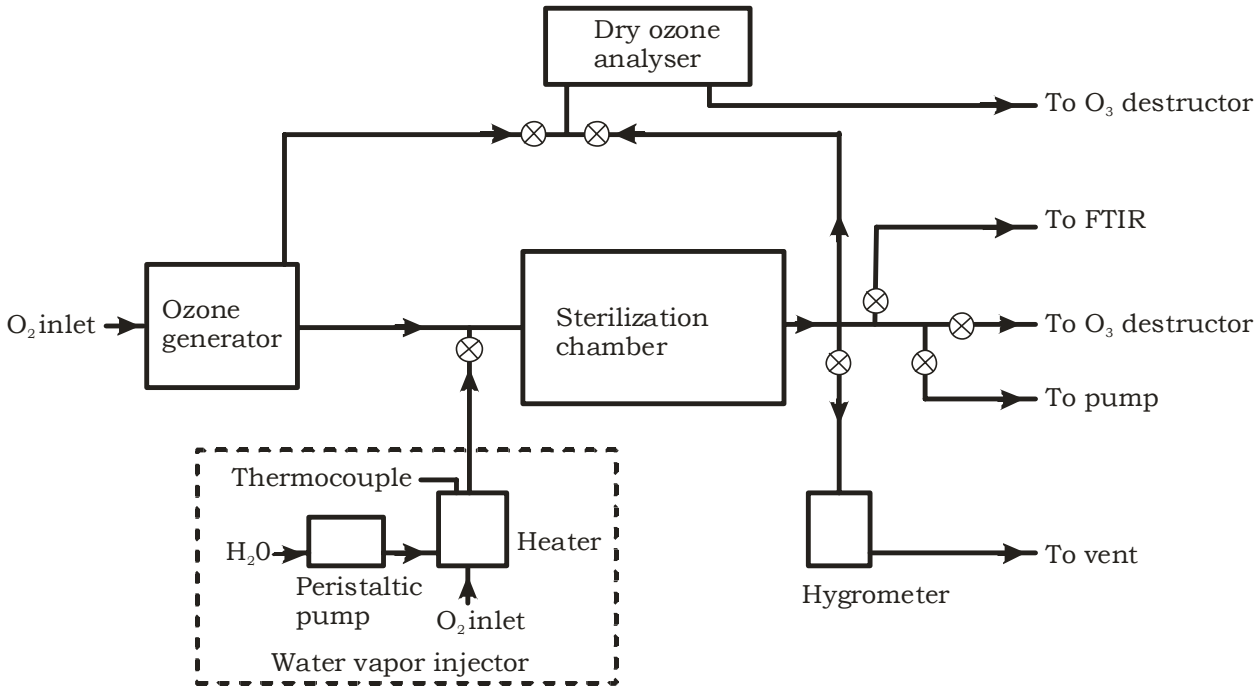


Figure 1. Schematic view of the experimental arrangement utilized to generate ozone and determine its concentration as it enters and exits the sterilization chamber. Water vapour can be added to the ozone flow and the relative humidity in the chamber measured with a hygrometer that comes with a thermometer. The nature of the effluents exiting the chamber can be analyzed through Fourier Transform Infra Red (FTIR) spectroscopy. An ozone destructor is provided to abate ozone under different modes of utilization.

2.1. Ozonation system

Figure 1 shows schematically the various elements needed to generate ozone and determine its concentration as it enters and exits the sterilization chamber. The chamber is a 400 mm long, 100 mm high and 220 mm wide parallelepiped. Since the ozone concentration cannot be monitored in the case of humidified ozone with the analyzer (based on UV absorption), it is performed through Fourier Transform Infra Red (FTIR) spectroscopy by recording an O_3 signal close to 1000 cm^{-1}. The generated effluents can also be analyzed through FTIR spectroscopy.

The corona discharge in O_2 of our ozone generator provides a mixture of gaseous molecular and atomic oxygen: decreasing the electrical current supplying the O_3 generator decreases the ozone concentration. The ozone flow is dry[7] but water vapour can be added to it, as shown in the figure. In such a case, water is sent through a peristaltic pump to an "oven" (heater). The water vapour then produced is driven in the O_3 line by an incoming O_2 gas flow. The amount of water vapour injected, at a given temperature of the heater and given O_2 flow, depends on the H_2O flow set by the peristaltic pump. The corresponding relative humidity (RH) level in the chamber is determined with a hygrometer (Kahn) in the sole presence of O_2 (RH up to at least 95 % can be measured accurately (±0.3%)). The gas temperature in the chamber is close to ambient (≈ 22 ^oC). Total gas flow is 5.64 and 2.6 standard liter/min (slm) under dry and humidified ozone conditions, respectively, to ensure a constant 4 000 ppm ozone concentration in both cases.

[7] O_2 is supplied from (high-purity) dry-gas bottles. When the sterilization chamber (figure 1) is closed to begin a process, humid air eventually present in it is flushed out by dry O_2 gas.

During ozone exposure, the gas coming out from the sterilization chamber is made to exit through an O_3-destructor. This device contains chemical catalysers destined to abate ozone and to release it as O_2 to comply with safety (toxicity) regulations*;* Note that the quantity of ambient humid air flowing back into the sterilizer through the catalyser and against the main flow (at least 2.6 slm) should then be very small. A vacuum dry pump is used at the end of process to make sure that effluents from the sterilization chamber are fully evacuated before removing exposed samples from it. Under humidified ozone operating conditions, the chamber is located within a hood.

2.2 Polymer surface preparation and treatment conditions.

Different types of polymers (used in the biomedical area) have been tested: polystyrene (PS) from Goodfellow® (LS251269), polyurethane (PU) from Johnston Industrial Plastics® (12348500), high density polyethylene (PE) from Goodfellow® (LS328449), polypropylene (PP) from Goodfellow® (LS328449), polymethylmetacrylate (PMMA) from Goodfellow® (LS251269), polymethydisiloxane (silicone) from Goodfellow® (LS251269) and polytetrafluoroethylene (Teflon®) from Goodfellow® (LS251269). Polymer sheets were cut up with appropriate techniques to provide samples approximately 2.5×2.5 cm^2. These were cleaned and rinsed 5 times with ultrapure water. After drying, each polymer sample was laid out on a glass Petri dish, introduced in the processing chamber (Fig. 1) and, then, exposed to ozone for 60 min (glass Petri dishes were used since the interaction of ozone with glass, as opposed to that with polymers, is expected to be minimal).

2.3. Microorganisms: choice of vegetative and sporulated bacteria for the study, their preparation for sample deposition and the number of their survivors after exposure

To characterize the biocide action of dry ozone, we have examined its effect on dried deposits of three species of bacterial spores, namely *Bacillus atrophaeus* ATCC® 9372 (formely *Bacillus subtilis*), *Geobacillus stearothermophilus* ATCC® 7953

(formerly *Bacillus stearothermophilus*), *Bacillus pumilus* ATCC® 27142, and on *Deinococcus radiodurans* vegetative bacteria ATCC® 13939. *B. atrophaeus, G. stearothermophilus* and *B. pumilus* endospores are routinely used in the validation of sterilization processes with dry heat, wet heat and gamma radiation, respectively. Moreover, *B. atrophaeus* spores are known (19-21) to be particularly resistant to ozone. *D. radiodurans* bacteria are used because of their resistance to radiation and dessication (needed feature to work with dried deposits).

Preparation of microorganisms. B. atrophaeus, *G. stearothermophilus* and *B. pumilus*, endospores of Gram-positive bacilli, and *D. radiodurans* bacteria, non-spore-forming Gram-positive cocci, were prepared in the Laboratoire de contrôle des infections (Faculty of Dentistry) of Université de Montréal. The bacilli were inoculated on a sporulation medium (22) and incubated for 10 days at 37 ^{o}C for *B. atrophaeus* and *B. pumilus*, and 3 days at 56-60 ^{o}C for *G. stearothermophilus*. Spores were then collected, washed and stored at 4 ^{o}C. Viability of the spores was determined by plating on Trypticase Soy Agar (TSA) (23). *D. radiodurans* bacteria were collected after amplification in a nutritive medium at 30 ^{o}C for two days (24).

Sample preparation. Deposits of 10^6 spores in 100 µL of water are made in the center of 60 mm-diameter sterile polystyrene or Pyrex Petri dishes. *D. radiodurans* bacteria are gathered in the same fixed volume of 100 µL, but their number was sometimes much less than the intended 10^6 (sec. 3.1.4). These deposits are then dried out under similar ambient conditions and protected from light before being subjected to ozone treatment.

Pyrex Petri dishes were used as the carrier material when working with humidified ozone as is customary in such conditions (16). This is because O_3 interaction with Pyrex, as opposed to polystyrene, is expected to be minimal,

Determination of the number of survivors after exposure. Figure 2 shows the various steps involved in microorganism recovery. After exposure, a 5 ml volume of a solution containing 0.5%Tween 80 ™ in saline (150mM NaCl) is added to the Petri

dish and microorganisms are released from it by mechanical scrubbing using a sterile swab. The harvested bacteria are vortexed, serially diluted and various volumes (50 to 200µL) of the different dilutions are spread out onto Trypticase Soy Agar plates. When viability is expected to be very low (less than 100 microorganisms), survivors are all collected through membrane filtration. The number of colony forming units (CFU) is determined after various periods of incubation at specific temperatures: for spores, namely *B. atrophaeus* (37 ^{o}C for 24h), *G. stearothermophilus* (56 ^{o}C for 48h) and *B. pumilus* (37 ^{o}C for 24h), and for *D. radiodurans* (ambient temperature for 4 days). Non exposed controls are recovered at the same time as the exposed microorganisms. Specific germination inducers (aniline, dipicholinic acid/calcium, lysozyme) were used to ensure that the inactivation of exposed spores is permanent.

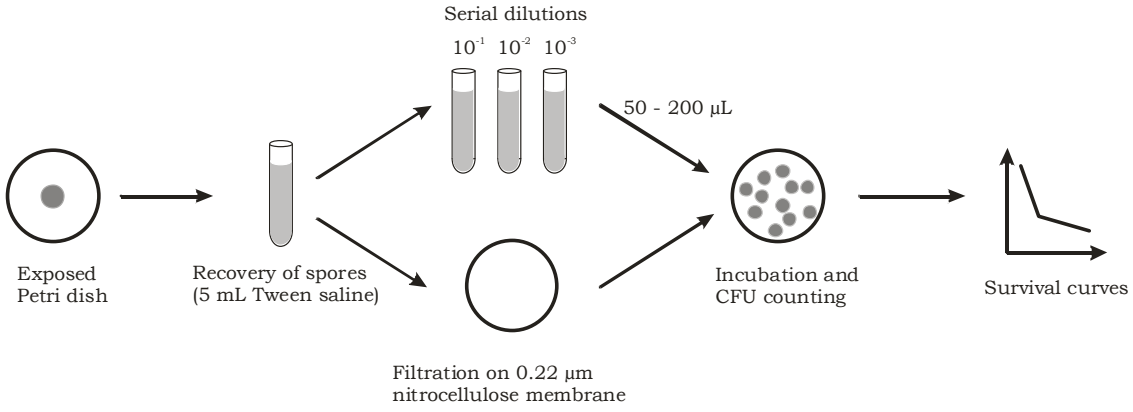


Figure 2. Diagram showing the different steps of microorganism recovery after their exposure to ozone.

2.4. Spatial distribution of the gaseous ozone concentration in the chamber through optical absorption spectroscopy

A fused silica window extends axially on both sides of the chamber (figure 3) allowing visual observations and spectroscopy measurements. Axial distribution of the ozone concentration, at two different heights (y axis), is obtained through an optical absorption spectroscopy system that is movable axially (x axis) and can probe the chamber at approximately ½ and ¾ of the window height, as schematized in figure 3. A deuterium lamp is used as a continuum light source, located at the focal

length of a lens L_1 that transforms it into a parallel beam directed transversally to the chamber (z axis). On the other side of the chamber, at the same x and y positions, a diaphragm (figure 3) admits only part of the light beam transmitted across the chamber, which is then focused with lens L_2 on the input side of an optical fibre linked on its other extremity to the entrance slit of a 320 mm focal length spectrophotometer.

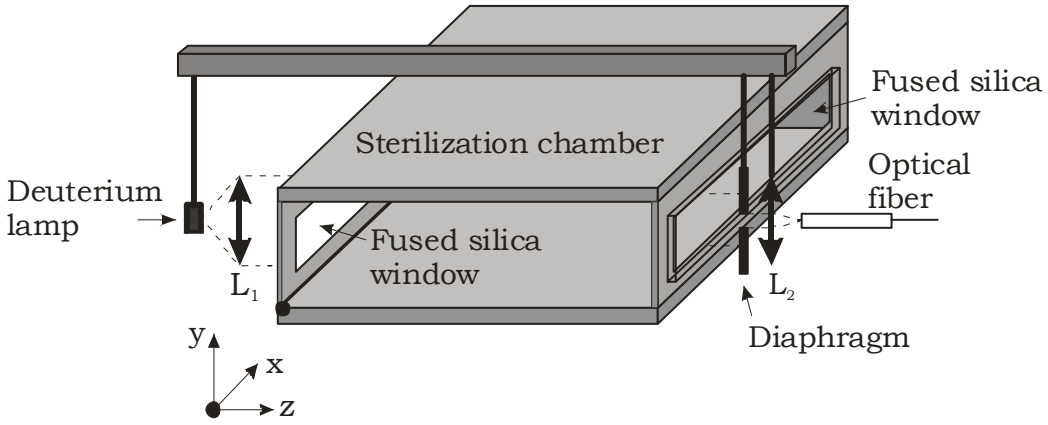


Figure 3. Schematic view of the optical-absorption measurement system probing the z-axis; it is movable along the x-axis and can be positioned at different heights y. The spectral source is a deuterium lamp that provides significant continuum emission intensity in the 240-400 nm range. The chamber is a 400 mm long, 100 mm high and 220 mm wide parallelepiped generating a 6 L volume.

2.4.1. Ozone optical-absorption spectral characteristics.

Dry ozone. Figure 4(a) shows the intensity of a UV beam from a deuterium lamp after it has crossed the chamber transversally (along the z axis, figure 3) as a function of wavelength, at mid-axial and mid-height positions (x = 200 mm, y = 44.5 mm, respectively), with and without dry ozone circulating in the chamber. The ozone molecules is known to absorb the UV beam in a wide range of wavelengths (240 - 330 nm) [8], as can be seen from figure 4 (c) that displays the corresponding absorbance curve. Full UV absorption is observed close to 285 nm.

[8] It corresponds to a Hartley molecular band (29-33).

Humidified ozone. Figure 4(b) again shows the intensity of the UV beam from the deuterium lamp after it has crossed the chamber transversally (along the z axis, figure 3) as a function of wavelength, at mid-axial and mid-height positions (x = 200 mm, y = 44.5 mm, respectively), with and without humidified ozone circulating in the chamber. Figure 4(d) shows that the spectral region of full absorption is slightly larger in humidified ozone than in dry ozone: it is mainly due to the presence of radical species such as $HO^{\bullet}$ and $HO^{\bullet}_2$, which absorb radiation in the 230-320 nm and 200-250 nm UV regions, respectively (25-28).

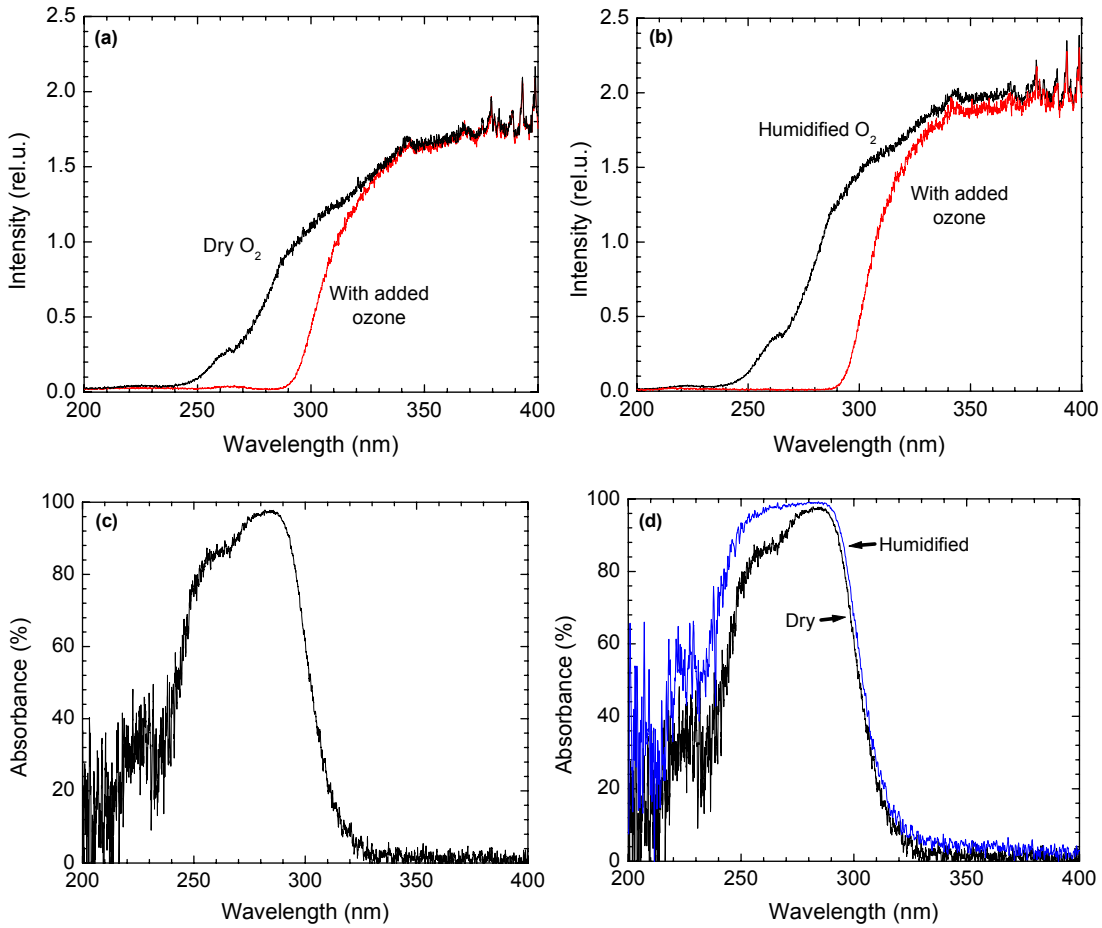


Figure 4. UV intensity of the deuterium-lamp beam after crossing the chamber transversally (along the z axis, figure 3) as a function of wavelength, at mid-axial and mid-height positions (x = 200 mm, y = 44.5 mm, respectively): (a) with and without the presence of dry ozone in the chamber, (b) with and without ozone added to humidified O_2 in the chamber; (c) absorbance plot for dry ozone corresponding to figure (a); (d) comparison of the absorbance plots in dry and humidified ozone.

2.4.2. Spatial uniformity of dry and humidified ozone concentrations. To determine the spatial uniformity of the ozone concentration in the chamber, we made absorption measurements at λ= 285 ± 0.5 nm (wavelength corresponding to the measured maximum absorption value for O_3 in dry ozone) as functions of axial position and at two heights y in the chamber. The results are shown in figure 5, for both dry ozone (a) and humidified ozone (b), indicating an almost uniform distribution (within error bars) of the dry and humidified ozone concentrations. This is supported by the fact that we obtain the same inactivation rate of spores with Petri exposed at different axial positions in the chamber (not shown). The relatively low volume of the chamber, 6 L, most probably facilitates reaching homogeneity in the chamber. The humidified ozone concentration needs a few minutes to stabilize while, with dry ozone, concentration stability is reached more rapidly.

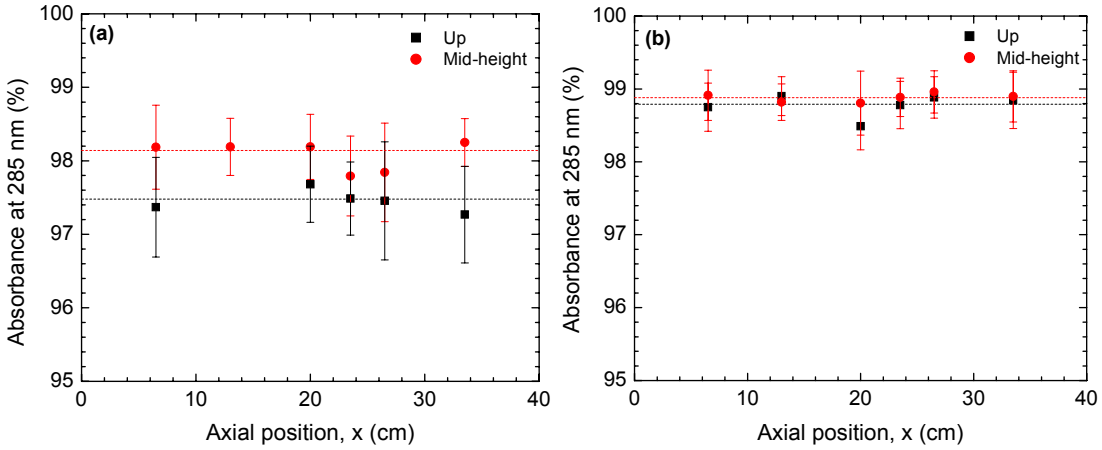


Figure 5. Measured optical absorbance at 285 nm transversely to the chamber (z axis) at two different heights (y = 44.5 mm (mid-height), y = 52 mm (up)) as a function of axial distance from the chamber entrance (gas inlet location): (a) in dry ozone; (b) in humidified ozone.

3. Exposure of microorganisms to dry ozone: experimental results and inactivation analysis

3.1. Gaseous dry ozone effect on microorganism viability and integrity of their structure

To gain insight into the inactivation mechanisms of the microorganisms subjected to gaseous ozone, we will be relying on the specific characteristics of their survival curves and be also looking for eventual morphological changes using scanning electron microscopy (SEM) micrographs.

3.1.1. Bacillus atrophaeus spore.

Survival curves

Figure 6 shows the number of viable *B. atrophaeus* spores (on a log scale) when subjected to dry ozone as a function of time (figure 6(a)) and as a function of dose (ozone concentration times exposure time, figure 6(b)). Each data point represents the average number of survivors on four different Petri dishes, and the error bar corresponds to the standard deviation. Several positions of the Petri dish in the chamber were tested and no significant variations recorded (data not shown), in agreement with the observed spatial homogeneity of gaseous ozone reported in sec. 2.4.2.

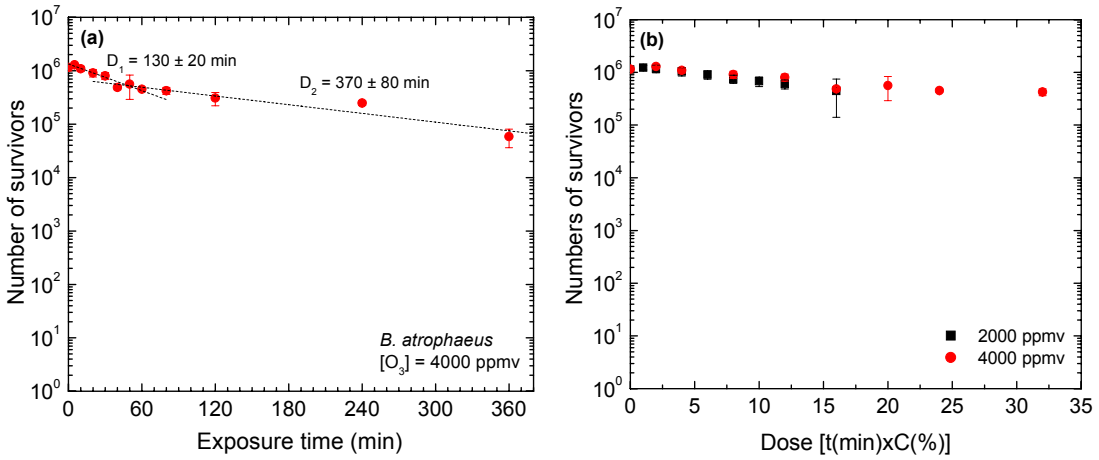


Figure 6. Results from exposure to dry ozone of 10^6 *B. atrophaeus* spores deposited on polystyrene Petri dishes: (a) survival curve under a 4000 ppm O_3 concentration; (b) number of survivors as function of the O_3 dose (exposure time times ozone concentration) when subjected to 2000 ppm and 4000 ppm concentrations of gaseous ozone.

Figure 6(a) shows a decrease, although slow, of the surviving spore number as a function of exposure time, in fact approximately 1 log in 2 h. Figure 6(b) indicates that there are no significant differences (within error bars) in viability between exposure to 2000 and 4000 ppm of dry ozone when plotted as a function of dose, the notion of dose thus coming out as a more general parameter to characterize lethality. Although lethality is low in figure 6(a), it appears to be the first case of *B. atrophaeus* spore inactivation published to date under such conditions[9]. Indeed, Ishizaki *et al* (16) reported no significant inactivation over a 6 h exposure of different *B. atrophaeus* strains under O_3 concentrations in the 250 to 1500 ppm range, even though ozone was somehow humidified (RH values tested starting from 50 %).[10] A possible explanation to this observation would be the existence of an O_3 threshold concentration for inactivation above 1500 ppm, as proposed by Kowalski *et al* (13). The fact that below a certain concentration threshold, ozone had no effect on the survival of bacteria was confirmed by other authors such as Hunt *et al* (34).

To attempt increasing the inactivation rate reported in figure 6 where the Petri dishes are lying flat, horizontally, on the bottom of the chamber with the ozone gas flowing parallel to it, we put them vertically, i.e. perpendicularly to the gas flow, assuming that more O_3 molecules would then reach, in fact hit, the spore deposit and thus raise the inactivation rate. Figure 7 shows no significant increase in mortality, the decimal (D) value in the vertical position being 146 ± 15 min compared to 147 ± 15 min in the horizontal position: there is clearly no hydrodynamic effect of the ozone flow on inactivation.

[9] In contrast, dry gaseous ozone is known to be very effective to inactivate Gram positive and Gram negative bacteria (35), viruses (36) and some other microorganisms.

[10] Because of this, some authors consider that humidified ozone with RH values below 50% is "dry ozone"!

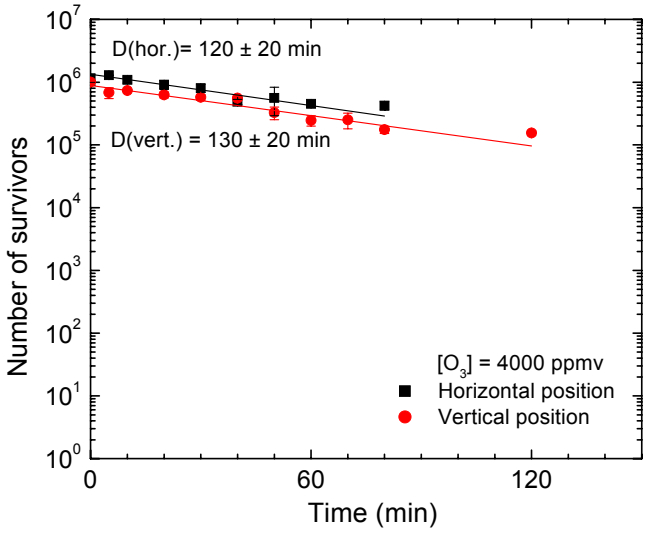
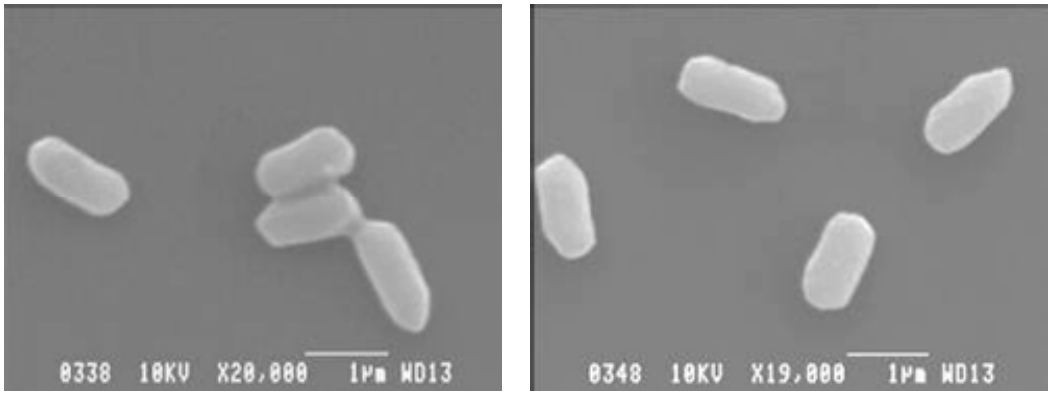


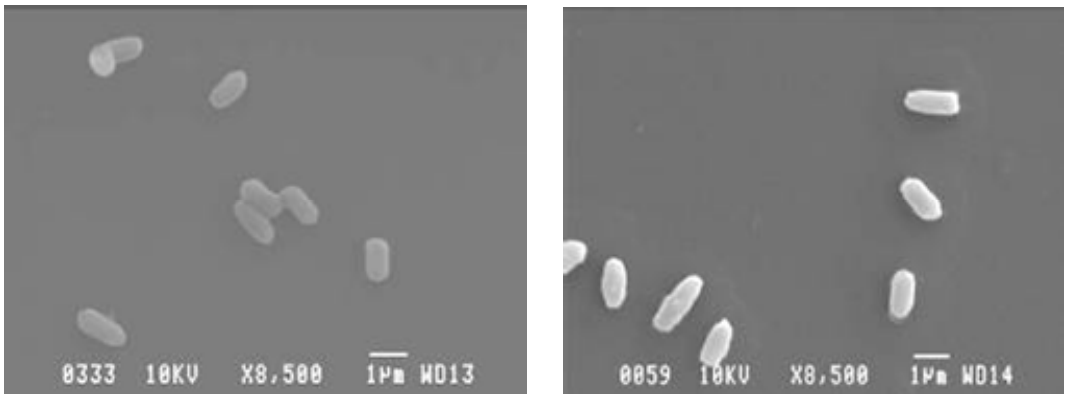
Figure 7. Survival curves corresponding to approximately 10^6 *B. atrophaeus* spores deposited on polystyrene Petri dishes and exposed to 4000 ppm of dry gaseous ozone, comparing Petri dishes in vertical (against the gas flow) and horizontal (parallel to gas flow) positions.

Morphological and dimensional integrity

To characterize the biocide action of O_3 on *B. atrophaeus* dried spores, we first examined SEM micrographs of these spores, looking for eventual morphological changes. Figures 8(a) and 8(b) display, in the left column, unexposed spores (control) and, in the right column (with the same magnification), spores subjected for 3 and 6 h to dry ozone, respectively: there are no apparent changes in their morphology, even after a 6 h exposure (approximately 5×10^4 surviving spores out of 1.3×10^6 initially deposited).



(a) Unexposed spores Exposed spores (3 h)



(b) Unexposed spores Exposed spores (6 h)

Figure 8. SEM micrographs of *B. atrophaeus* spores deposited on Petri dishes showing: (a) unexposed spores (left) and spores subjected for 3 hours to 4000 ppm of dry O_3; (b) unexposed spores (left) and spores subjected for 6 hours to 4000 ppm of dry O_3.

To make sure that no dimensional modifications actually resulted from subjecting the spores to dry ozone as suggested by the SEM micrographs, we performed a statistical analysis of the spore length using the Autocad data-processing software on a minimum population of 100 spores. The histograms in figure 9 and the corresponding average and standard deviation values indicate that no erosion has occurred: the average length L of the spores, when accounting for standard deviation, before and after exposure is the same: $L_{unexposed} \approx (1.3 \pm 0.1)$ µm, $L_{exposed\ 3h} \approx (1.25 \pm 0.05)$ µm and $L_{exposed\ 6h} \approx (1.29 \pm 0.13)$ µm (not shown). Since we found that the length of the spores was not modified, we did not examine their width and height as we assumed that erosion would not affect specifically the length only. Since erosion

is not involved in the inactivation process, we believe that the main inactivation mechanism is related to O_3 diffusion into the spore.

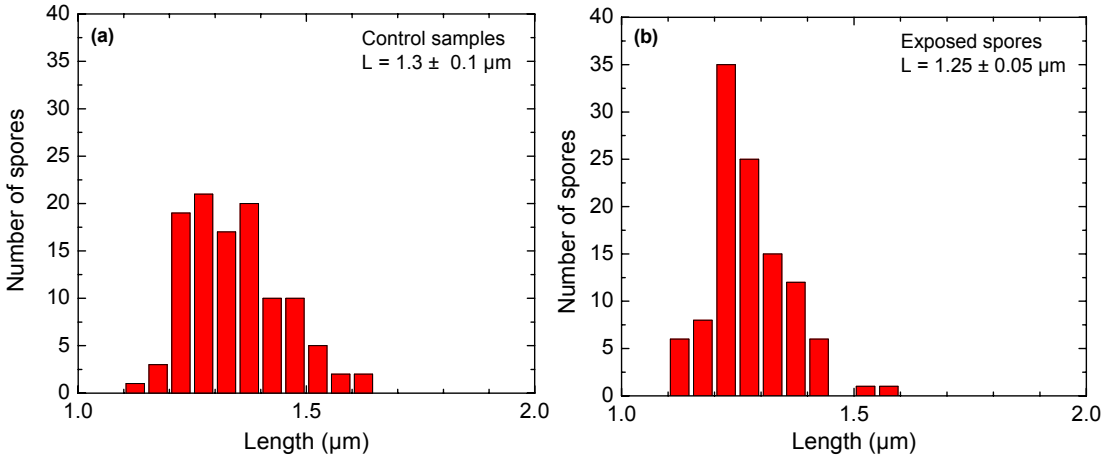


Figure 9. Statistical histograms of the length of *B. atrophaeus* dried spores deposited on Petri dishes: (a) unexposed spores; (b) spores subjected for 3 h to dry ozone.

Weakening of B. atrophaeus spore internal structure

As can be seen in figures 8 and 9, dry ozone exposure does not affect the external morphology and dimensions of *B. atrophaeus* spores that have been exposed. This suggests that the inactivation mechanism involves penetration of the biocide agent within the spore coats, which could somehow weaken the spore internal structure. We have observed such an effect quite unexpectedly following the use of Malachite green preparation to stain spores[11]. This dye is currently employed to assess integrity of dormant spores. Intact dormant spores stain green whereas spores in the process of germination or that have been affected by exposure to different agents turn red when adding the red counter-stain. This procedure actually made some ozone-exposed spores release their inner material from their surrounding coats, as can be concluded from the SEM micrograph of *B. atrophaeus* spores shown in figure 10d.

Figure 10(a) displays unexposed (control) spores. One can observe their regularity, smoothness and integrity. Figure 10(b) shows unexposed spores stained with the Malachite preparation. The spores stain green (under optical microscopy)

[11] The Malachite green staining protocol involves heating the green dye to facilitate its eventual penetration into the spores. When the spore is not dormant, the green dye penetrates into the spores and is afterwards displaced by rinsing with water; an added red counter-stain can thus gain entry into the spore, which then becomes red in color.

and appear morphologically intact, although with some markings on their surface and "debris" in their surrounding, possibly from malachite particle deposition and/or water stains. Figure 10(c) shows dried spores, simply subjected to 4000 ppm of dry O_3 for 60 min: the spores are still intact with very little difference with the unexposed spores in figure 10(a). Figure 10(d) shows spores exposed to dry ozone under the same conditions as in figure 10(c) and thereafter subjected to malachite green dye: some of these spores come out damaged. The spore external structure after exposure to dry ozone and Malachite staining (figure 10(d)) is strongly affected compared to unexposed Malachite stained spores (figure 10(b)).

In summary, we conclude that: (1) dry ozone exposure weakens the spore internal structure and that an applied stress (heat and rehydration), as is the case with the malachite staining procedure, suffices to reveal this structural damage; (2) the structure weakening mechanism is most probably due to the diffusion of O_3 into the spore.

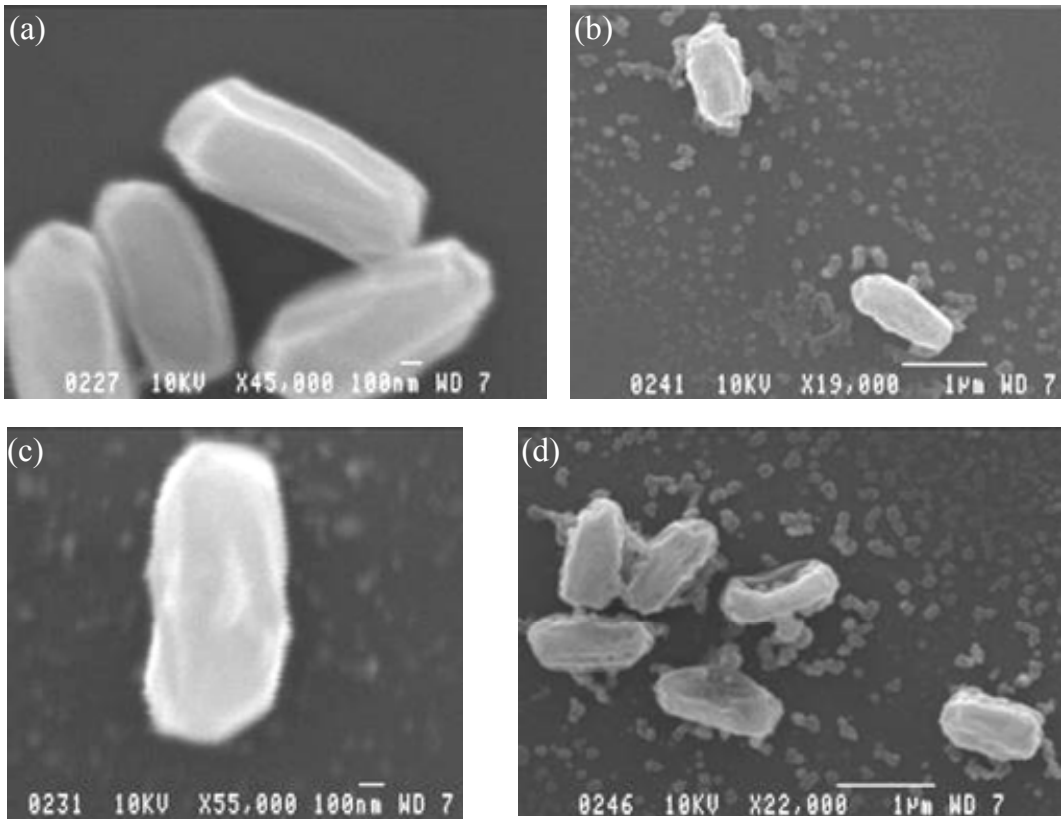


Figure 10. SEM micrographs of *B. atrophaeus* spores deposited on microscope slides showing: (a) dried unexposed spores; (b) unexposed spores after staining with Malachite green dye; (c) dried spores subjected for 60 min to 4000 ppm of dry O_3; (d) spores exposed to O_3 under the same conditions as in figure (c) and subjected thereafter to Malachite green stain.

3.1.2. Geobacillus stearothermophilus spores.

Survival curves

Generally speaking, the (scarce) literature discussing the exposure of bacterial spores to dry ozone mentions that these microorganisms are not, or very little, affected by such a treatment, whereas subjecting them to humidified ozone (RH ≥ 50-60%) eventually leads to their complete inactivation (17). As we have just seen (figure 6), *B. atrophaeus* is only very slowly inactivated by dry ozone. To check to what extent this trend holds true with other species of spores, we first tested *G. stearothermophilus*. This spore is a thermophile microorganism known to be resistant to humidified heat while *B. atrophaeus* withstands particularly well dry heat.

Experimental survival curves obtained for *G. stearothermophilus* spores are presented in figures 11(a) and (b). The essential difference between these two sets of data is that the spores are cleaner in figure 11(b) (see figure 13 below), as a result of a more stringent washing procedure when preparing their suspension. In the case of figure 11(a), the spores are embedded in what could be bioproducts and/or eventually chemical residues from the preparation of the spore suspension; it is interesting to compare these two cases since, at first, a thicker "coating" of the spore should delay the access of ozone to its inner parts, hence a slower inactivation rate. Nonetheless, in both cases, the inactivation efficiency of dry gaseous ozone on *G. stearothermophilus* spores compared to that of *B. atrophaeus* is clearly much stronger, most probably as a result of the specific molecular composition and/or organization of their coats (sec. 3.2.2). We could not find indications in the literature concerning the inactivation of *G. stearothermophilus* spores when subjected to the present conditions.

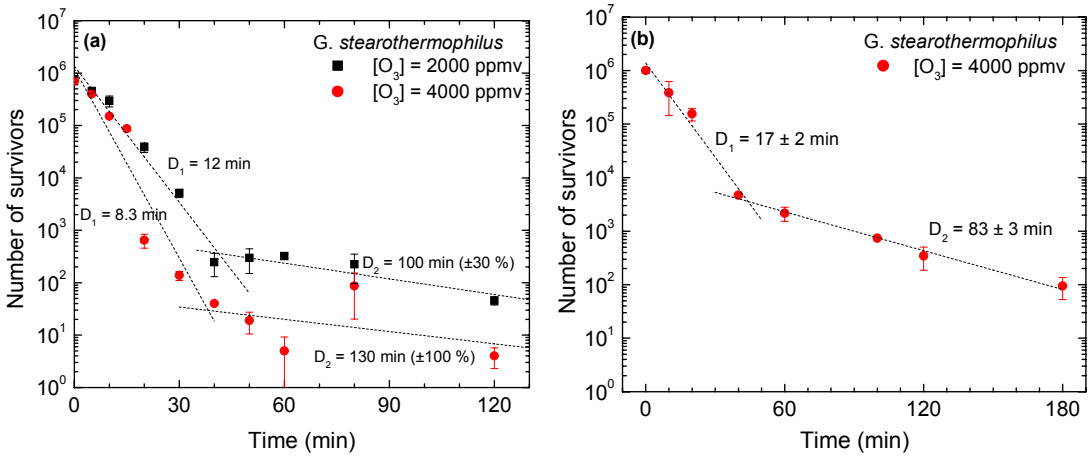


Figure 11. Survival curves for 10^6 *G. stearothermophilus* spores deposited on polystyrene Petri dishes after exposure to dry gaseous ozone: (a) at 2000 ppm and 4000 ppm concentrations on "embedded" spores; (b) at a 4000 ppm concentration on "clean" spores (see figure 13 for SEM micrographs of "embedded" and "clean" spores).

The survival curves in figure 11 are characterized by a two-phase kinetics, the first phase having a much shorter D time. The dispersion of the data points on the survival curves with "embedded" spores (figure 11(a)) is significantly larger than with clean spores (figure 11(b)) for long time exposure. Figure 11(a) also underlines that the higher the concentration of the biocide agent, the higher the inactivation rate. However, plotting the number of survivors in figure 11(a) as a function of dose instead of exposure time reveals, within error bars, that the inactivation rate of *G. stearothermophilus*, like that of *B. atrophaeus*, depends directly on the applied dose of O_3 (not shown).

A peculiarity encountered when recovering *G. stearothermophilus* spores to work out survival curves concerns the longer incubation time needed for full growth of their colony forming units (CFU) on nutrient medium compared, for example, to that for *B. atrophaeus*. Culture conditions of *G stearothermophilus* spores usually specify incubation for 48 h (at 56 ^{o}C). However, as shown in figure 12, we observed that after 72 h the number of CFU was more important than at 48 h; nonetheless, it was not much greater after a week, which we set as the incubation time (72 h). Such a growth

delay suggests that still viable spores were severely damaged[12]. Such a phenomenon has been reported with spores exposed to various oxidizing agents (6, 37). In contrast, such a delay in germination is not observed, under the same operating conditions, with *B. atrophaeus* spores for which the recommended incubation time of 24 h (at 37 ^{o}C) was found suitable.

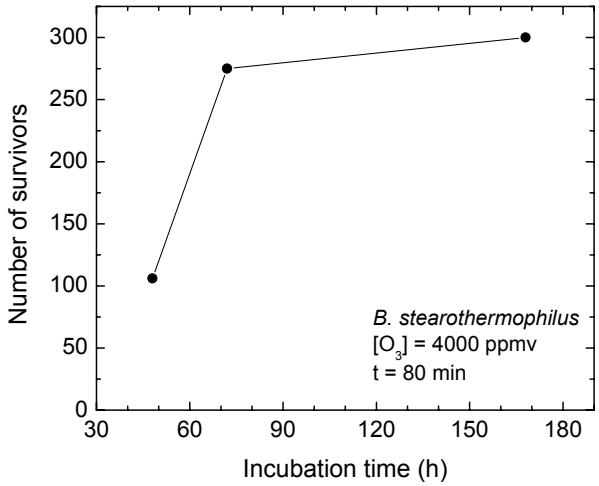


Figure 12. Effect of the incubation time on the number of CFU recovered from 10^6 "embedded" *G. stearothermophilus* spores deposited on polystyrene Petri dishes and subjected to dry gaseous ozone for 80 min at 4000 ppm.

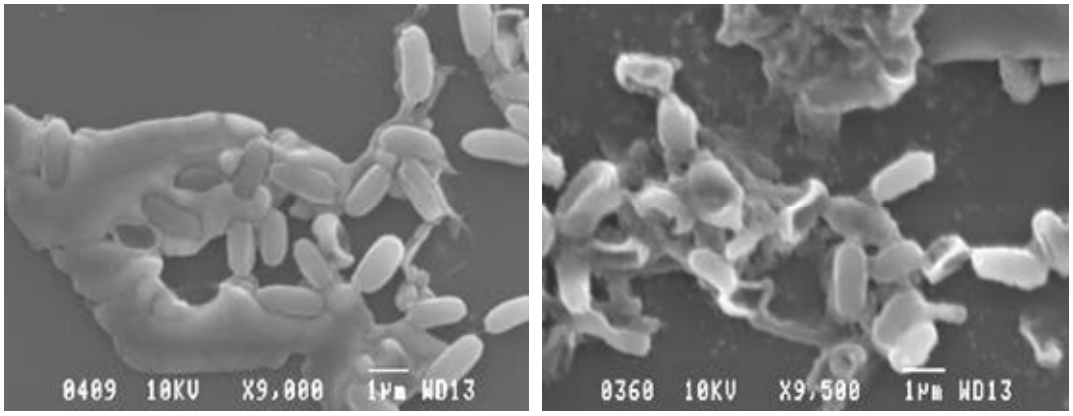
Morphological and dimensional integrity

Figure 13 displays two sets of SEM micrographs of *G. stearothermophilus* deposited spores, identified as "embedded" (figures 13(a) and (b)) and "clean" (figures 13(c) and (d)). The external appearance, i.e. morphology and texture, of both the "clean" and "embedded" spores is not significantly modified after exposure to dry ozone, even after 6 h (figure 13(d)).

Integrity of the exposed spores is demonstrated by performing statistical analysis of their length, as in the previous section. For "embedded" spores (figure 13(a) and figure 13(b)), we get the histograms in figure 14. No significant erosion has occurred since the average length L of the spores including its standard deviation value, is the

[12] Such an incubation delay is not observed with *G. stearothermophilus* when exposed to the N_2-O_2 plasma afterglow.

same before and after exposure: $L_{unexposed} \approx (1.7 \pm 0.2)$ µm, $L_{exposed\ 80min} \approx (1.5 \pm 0.3)$ µm. The histograms for "clean" unexposed and exposed spores appear in figures 14(c) and 14(d), respectively. We find $L_{unexposed} \approx (1.3 \pm 0.2)$ µm, $L_{exposed\ 60min} \approx (1.4 \pm 0.2)$ µm, i.e. again no significant difference between unexposed and exposed spores, but the "clean" spores seem indeed slightly shorter on the average than the "embedded" ones[13].



(a) Unexposed "embedded" spores (b) Dry O_3 exposed "embedded" spores (80 min)

(c) Unexposed "clean" spores (d) Dry O_3 exposed "clean" spores (6 h)

Figure 13. SEM micrographs of "embedded" and "clean" *G. stearothermophilus* spores (deposited on polystyrene Petri dishes): (a) and (c) are unexposed while (b) and (d) have been exposed to 4000 ppm of dry O_3, for 80 min and 6 h, respectively.

[13] Within the group of "embedded" spores, only those with a morphologically well defined length were considered for the histogram, ignoring those where the extraneous material embedding the spore is clearly distinct from its body.

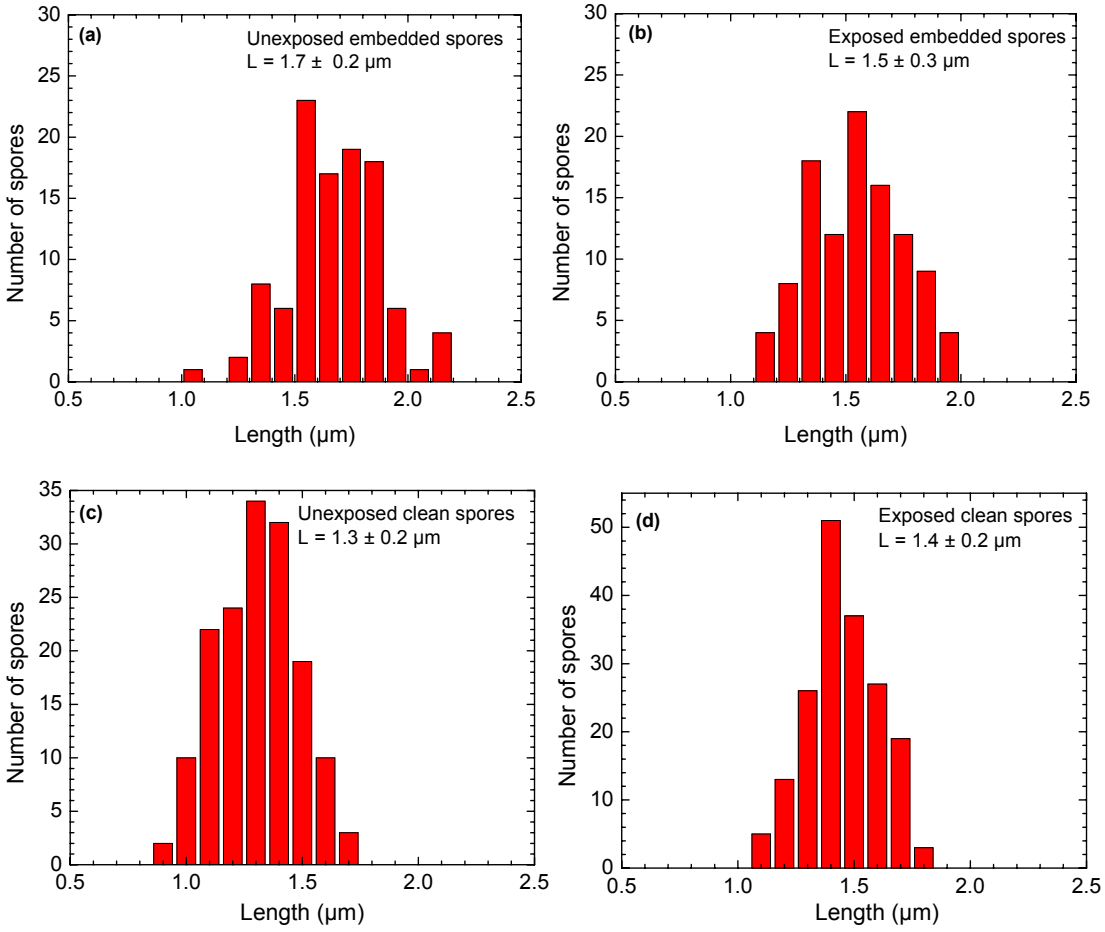


Figure 14. Statistical histograms of the length of *G. stearothermophilus* spores deposited on Petri dishes: (a) unexposed "embedded" spores; (b) "embedded" spores subjected for 80 min to 4000 ppm of dry ozone; (c) unexposed "clean" spores; (d) "clean" spores subjected for 60 min to 4000 ppm of dry ozone.

Unexpectedly, "embedded" spores are inactivated more rapidly than clean ones, suggesting a positive chemical assistance of the spore extraneous material to the inactivation mechanism. The following considerations can be made as to the possible negative and positive contributions of the bioproducts and/or chemical residues to spore inactivation: i) the presence of extraneous material, besides shielding the spores from the biocide agent, could use up part of the ozone flow since strong oxidants highly react with organic chemical species: these two factors imply a lowering of the ozone flow toward vital components of the spore, hence a slower inactivation rate; ii)

in contrast, the chemical reaction of O_3 with bioproducts and/or chemical residues could create additional oxidant species that increase the inactivation efficiency. Owing to the absence of apparent damage on the outer part of "embedded" spores after exposure, these oxidant species certainly diffuse through the spore coats and create therein lethal lesions to some metabolic mechanisms or, even, to the spore genomic material. Since the first-phase inactivation rate is lower with clean spores (D_1 = 8.3 min for embedded spores and D_1 = 17 min for clean spores), we conclude to a positive contribution to the inactivation mechanism from the material extraneous to the spore.

3.1.3. B. pumilus spores.

Survival curves

B. pumilus spores, recognized as bioindicators resistant to gamma (ionizing) radiation, were also subjected to dry gaseous ozone to gain further insight into the action of O_3 on spores. A typical survival curve obtained after exposure to 4000 ppm of dry O_3 is presented in figure 15. It exhibits a two-phase inactivation kinetics, as already noted for *G. stearothermophilus* spores. The decimal time of the first phase of *B. pumilus* is, within error bars, the same as that of *G. stearothermophilus* (D_1 = 18 ± 3 and 17 ± 2 min, respectively), but its second phase, D_2 = 640 min compared to 83 min for *G. stearothermophilus* (figure 11(b)), is extremely long and rather akin to that of *B. atrophaeus* (figure 6(a)). Moreover, the number of spores inactivated in its first phase is less than two log while, with *G. stearothermophilus* for the same exposure time, it is 4 log.

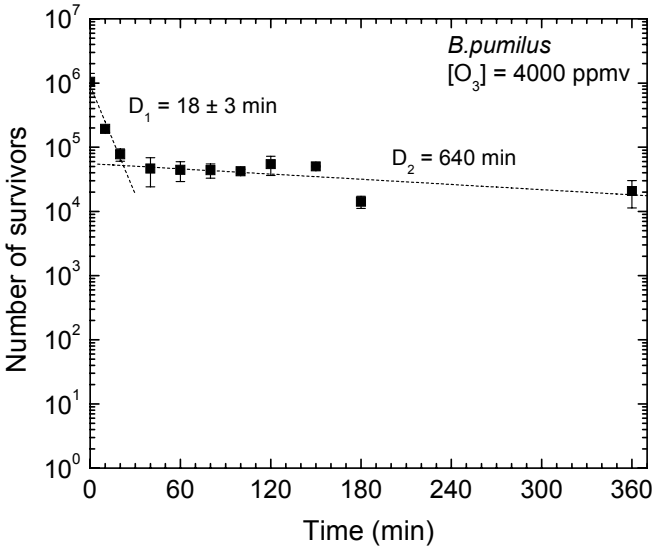
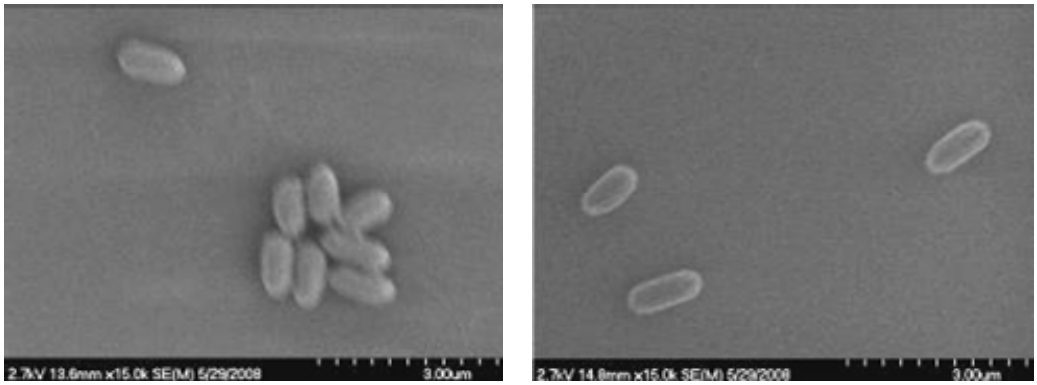


Figure 15. Survival curve for 10^6 *B. pumilus* spores deposited on polystyrene Petri dishes following exposure to 4000 ppm of dry gaseous ozone.

Morphological and dimensional integrity

Figure 16 shows SEM micrographs of *B. pumilus* spores unexposed (left) and exposed (right) to 4000 ppm of dry gaseous ozone for three hours. According to their survival curve (figure 15), there remains approximately 2 10^4 living spores after a 3 hour exposure.



Unexposed spores Exposed spores (3 h)

Figure 16. SEM micrographs of unexposed *B. pumilus* spores and after a 3 hour exposure to 4000 ppm of dry gaseous O_3.

These micrographs clearly show that the spore morphology is not affected by exposure to dry ozone. Statistical analysis of the spore length, performed as in section 3.1.1, leads to the histograms in figure 17. The corresponding average and standard deviation values indicate that no dimensional change has occurred since the average length L of the spores, when accounting for standard deviation, is the same before and after exposure: $L_{untreated} \approx (1.1 \pm 0.1)$ µm, $L_{treated\ 3h} \approx (1.1 \pm 0.1)$ µm.

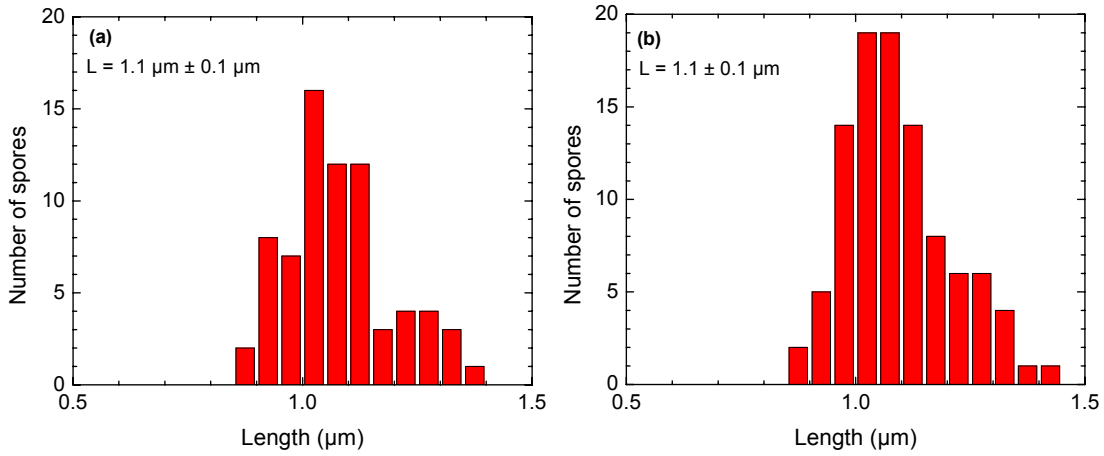


Figure 17. Statistical histograms of the length of *B. pumilus* spores deposited on Petri dishes: (a) unexposed spores; (b) spores subjected for 3 h to 4000 ppm of dry ozone.

Figure 18 gathers the survival curves of the three types of spores that we have just considered. Clearly, in contrast to what is found in the literature, dry gaseous ozone can affect the viability of microorganisms as resistant as bacterial spores. It is noteworthy that the inactivation rate of this ozonation process depends strongly on the spore type (more on this in sec. 3.2.2): *B. pumilus* inactivation rate is intermediate between *B. atrophaeus*, which shows the slowest inactivation rate, and *G. stearothermophilus*, the fastest total rate. Interestingly, in all cases, the morphology and dimensions of the spores following exposure to dry O_3 are unaffected provided they are not subjected to some external treatment (hydration, heat…) thereafter.

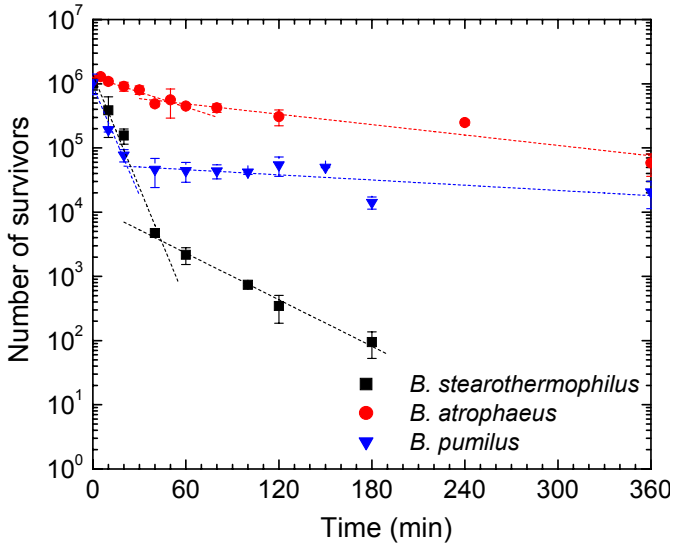


Figure 18. Comparison of the survival curves of the three types of spores considered in the preceding sections, all deposited on polystyrene Petri dishes and subjected to dry gaseous ozone at 4000 ppm.

3.1.4. Deinococcus radiodurans bacteria. Because vegetative bacteria are less resistant to any given biocide agent than sporulated bacteria, one expects them to have a comparatively much higher inactivation rate. Since our intent is to characterize the inactivation of microorganisms under the same conditions as that of our dry spore deposits, we needed a bacterium that could withstand drying. Besides supporting desiccation, *Deinococcus radiodurans* is recognized as gamma (ionizing) radiation resistant and can also survive cold, vacuum, and acid. It is, in fact, known as a polyextremophile bacterium and has been listed as "the world's toughest bacterium".

Survival curves

Figure 19 shows a two-phase survival curve for *D. radiodurans* resulting from their exposure to 4000 ppm of dry O_3. Clearly, dry ozone acting on this bacterium is much more efficient than with sporulated bacteria (figure 18). Nonetheless, there is no mention in the literature of the possibility of inactivating dried vegetative bacteria with dry ozone: the ozonation process is reported to be efficient on vegetative bacteria in humid media, water, agar or with airborne bacteria (12, 13).

The number of *D. radiodurans* bacteria initially deposited and their eventual degree of stacking can vary considerably depending on culture and drying conditions. While for an initial deposit of 3.3×10^4 bacteria (figure 19), an average of 10 CFU was counted after a 10 min exposure, in another independent experiment with an initial deposit of 5.9×10^5 bacteria, only one CFU was encountered after the same exposure time. No SEM micrographs of these bacteria were taken.

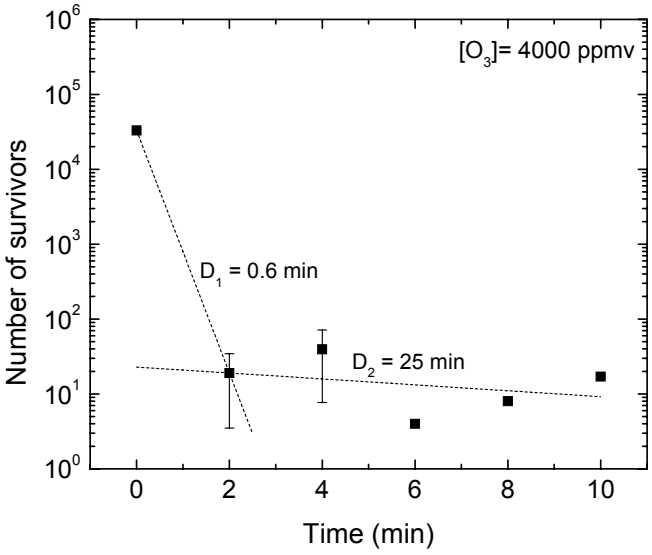


Figure 19. Survival curve for *D. radiodurans* bacteria deposited on polystyrene Petri dishes dried and then subjected to 4000 ppm of dry gaseous ozone.

3.2. Inactivation rate by gaseous dry ozone with respect to the nature of the microorganism: vegetative and sporulated bacteria

Spore forming Gram-positive bacilli, such as the Bacillus species, respond to stimuli as nutrient deprivation by entering a state of dormancy, becoming endospores. Such a dormant state implies that the metabolic activity of the spore is greatly reduced, but that the spore retains the capacity to germinate back to the vegetative state. This is because the changes resulting from sporulation involve compartementation with new structures, namely core, cortex, plasma membrane, inner coat, outer coat and outer membrane. These structures confer resistance to several and different stresses. Besides common structural changes resulting from sporulation, there exist many differences among Bacillus species, which include their

size, shape, resistance factors, water content and the composition and organization of their chemical constituents. For instance, the chemical composition of their coats and cortex is quite different: the coats of *B. atrophaeus* are essentially made up of more than 25 different proteins forming an intricate lattice, that of *B. pumilus* is characterized by the presence of an exosporium (essentially sugar) while *G. stearothermophilus* coat comprises specific peptidoglycans that provide a strong physical and chemical barrier.

Knowing that O_3 must reach, remain onto and eventually penetrate and diffuse throughout the microorganism to damage it, its structural features can easily explain the much faster inactivation rate observed with the vegetative bacterium *D. radiodurans* (D_1 = 0.6 min) as compared with the *G. stearothermophilus* spore (D_1 =17 min). Compared to endospore multicoats which protect a cell devoid of any activity, the cell wall of vegetative bacteria is essential for growth and metabolism, shape determination and resistance to environment fluctuation and aggression. Therefore damaging it disrupts the cell's physiology and exchanges with the microenvironment and may lead to cell death.

As shown in figure 18, there are clear and strong differences in the degree and rate of inactivation among the three spores examined. The "large" O_3 molecule cannot penetrate the spore material as easily as a photon and its diffusion, a priori, depend significantly on the spore composition and on the specific chemical properties of the spore metabolic enzymes. Indeed, in the case of UV irradiation, the essential target for achieving inactivation is the genomic DNA (38) while, in the present case, the biocide agent, assumed to be the O_3 molecule itself, can have severals targets such as proteins, enzymes and even DNA (39).

3.3 Inactivation by dry ozone: mechanisms and target identification

3.3.1. Inactivation mechanisms and possible targets. Our experimental observations and analyses are driving us to assume diffusion of the O_3 molecules followed by the reaction of these oxidative species with vital components as being the main inactivation mechanism of microorganisms subjected to dry ozone.

As far as diffusion of O_3 through microorganisms is concerned, there are contradictory reports. Some authors suggest that ozone attacks the cell surface, altering the permeability of the cell wall and cytoplasmic membrane, resulting in the case of vegetative bacteria in the leakage of the cell contents (34). For spores, ozone is assumed to diffuse through their surface and then permeate into the membrane (4, 40). However, some other authors claim that such a diffusion of O_3 throughout spores is not possible, based, on the one hand, on the observed inability of small reactive species (such as methylamine molecules) to diffuse through the spore coat (34, 37) and, on the other hand, because the spore inner membrane has an extremely low permeability to small hydrophilic molecules (37).

As far as oxidation by O_3 is concerned, some authors suggest that the major mechanism of spore killing by chemicals is an oxidizing damage to the spore inner membrane (41). This implies the further possibility for O_3 to reach and damage the genetic material molecules in both spores and bacteria (16, 34). In the specific case of bacteria, Komanapalli *et al* (42) claim that their inactivation by ozone proceeds from membrane damage and not from DNA lesions, since protein and nucleic acid leakage can be observed as a result of membrane disruption.

At this point, our results strongly suggest that the ozone molecules diffuse through the spore and then react with targets that are essential to the spore survival. In that process, even though the water content in the core of the spore is very low (37), it would be enough to react chemically with ozone and/or with their reaction by-products to provide other oxidant species such as radicals, oxidant molecules, which could in addition participate efficiently in the spore inactivation process. Nonetheless, in general, the literature mentions that, for spores, the main lethal targets of ozone are enzymes (related to their metabolic functions) and/or DNA.

To check whether dry ozone can induce metabolic damage to spores as a result of its attack on enzymes (proteins), we turn to lysozyme determining its enzymatic activity after it has been subjected to dry gaseous ozone. Lyzozyme was chosen because it is a strongly resistant enzyme as well as a mucolytic enzyme (antibiotic properties). Its activity is easily assayed and well documented (43).

3.3.2. Enzymatic activity test. Lysozyme is a small globular protein found in a great number of tissues and secretions of animals (such as tears, saliva, blood serum etc) and also in plants, molds and egg white among other sources. The commercial protein used for the assay is isolated from chicken egg white. It has 129 amino acids in the primary sequence and 4 intrachain disulfide bridges between sulfhydryl containing amino-acid residues. The enzymatic activity of lysozyme consists in the lysis of bacterial cell wall mucopolysaccharides by hydrolysis of specific linkages.

Enzyme assay

Dried lysozyme-protein deposition samples were subjected for 60 min to dry gaseous ozone under a 4000 ppm concentration, as we did previously with spores. Then, the enzymatic activity of unexposed and exposed lysozyme proteins was evaluated and compared.

The enzyme assay is conducted as follows: the protein is mixed with a suspension of *Micrococcus luteus*, a Gram-positive bacterium. An active lysozyme will lyse this bacterium. The lytic activity is monitored, at various periods of time, through changes in turbidity levels of the bacterial suspensions recorded by optical density (O.D.) readings at 540 nm. Changes due to the presence of the protein are calculated against blank values containing *M. luteus* bacteria only. If lysozyme had lost its enzymatic activity, there would be no lysis of *M. luteus*, and therefore no changes in O.D compared to its blank value. Readings recorded with lysozyme not exposed to ozone serve as positive control. The assay was repeated 4 times in triplicate samples. To make sure that under our conditions, the enzyme activity causing cell lysis is not an artefact, we used another protein, namely Bovine Serum Albumin (BSA), which has no enzymatic activity and no effect on *M. luteus*, as a negative control.

Results

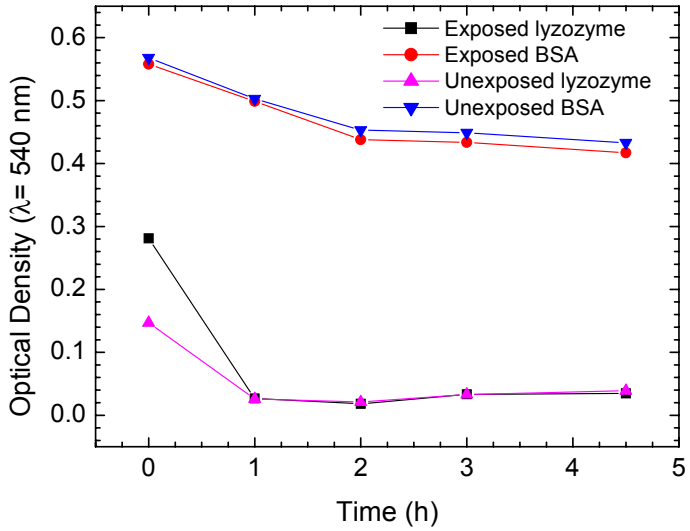


Figure 20. Time evolution of the lytic activity of lysozyme and Bovine Serum Albumin (BSA) proteins in contact with *M. luteus* bacteria, monitored through the average optical density (OD) of samples, before and after a 60 min exposure to 4000 ppm of dry O_3. BSA is used as a negative control. The reduction in OD_{540nm} readings towards unexposed lysozyme implies that it has retained its enzymatic activity.

In conclusion, exposure for 60 minutes to gaseous dry ozone under a 4000 ppm concentration had no detectable effect on the enzymatic activity of lysozyme, indicating that the active sites of the lysozyme enzyme are not a target for O_3 under the conditions used. Nonetheless, it could happen that less resistant enzymes of spores could be damaged by O_3, while not necessarily leading to spore inactivation. Moreover, in contrast to the case of a pure and isolated enzyme or protein, O_3 oxidative by-products could be induced in the spore that could lead to important metabolic damage.

3.4. Influence of the substrate nature on the kinetics of inactivation

Figure 21 shows that the inactivation kinetics of dried spores exposed to dry gaseous ozone varies strongly with the nature of the Petri dish on which the spores were deposited. The inactivation rate is much higher on polystyrene substrates than on Pyrex ones for the three types of spores considered. On both types of substrates, *G. stearothermophilus* spores have the highest inactivation rate. In contrast, *B. pumilus*

spores are, apparently, not inactivated at all when deposited on Pyrex while they have an inactivation rate intermediate between that of *B. atrophaeus* and *G. stearothermophilus* on polystyrene. On both types of substrates, whenever the inactivation rate is significant, the survival curves are biphasic.

The dependence of the inactivation rate on the nature of the substrate has been reported in the literature for at least three gaseous biocide agents, but not for dry ozone: 1) Making use of an air plasma at atmospheric pressure, Kelly-Wintenberg *et al* (44) showed that *E. coli* bacteria, placed on polypropylene Petri dishes, were more readily inactivated (D_1=6s, D_2=2s) than those deposited on glass (D_1=33s, D_2=10s) or agar substrates (D_1= 70s, D_2=17s). The fact that the spores deposited on the polymer surface exhibits a greater sensitivity to plasma than those deposited on glass substrates agrees with our observations. 2) Turning to ethylene oxide, Gilbert *et al* (45) determined that the nature of the surface upon which *B. atrophaeus* spores were dried had an effect on their inactivation rate. He showed that, for low RH values (33%), the inactivation efficacy was the lowest for glass, then higher with paper and, finally, among the materials tested, the greatest with cotton. He concluded that it is more difficult to kill microorganisms on impervious surfaces. 3) Calling on humidified ozone (10mg/L O_3, RH =90%), Aydogan *et al* (18) determined that the inactivation rates of *B. atrophaeus* spores on glass, vinyl floor tiles and paper were not significantly different from each other: vinyl and paper surfaces do not enhance (or delay) the inactivation of spores as compared to glass substrates. Nonetheless, this is not a general rule with humidified ozone since the same authors obtained inactivation rates for other substrates that follow a different trend: the rate is stronger on carpet material than on glass surfaces and, in turn, less on hardwood. The outcome of their experiments suggests that spores deposited on different substrate surfaces are, generally, inactivated at different rates by gaseous disinfectants like O_3. Aydogan *et al* (18) interpreted these results by assuming that these substrates were using up the O_3 molecules before they reach the spores, eventually leading to the formation of volatile organic carbons compounds that could be toxic to the spores (as supported by the action of the fumigative emission of the carpet material tested). However, the generation of volatile organic compounds does not always imply a higher inactivation

rate than on chemically inert substrates (46, 16). As a matter of fact, these observations suggest that such interactions are much more complex, strongly depending not only on the substrate nature, but also on actual experimental conditions.

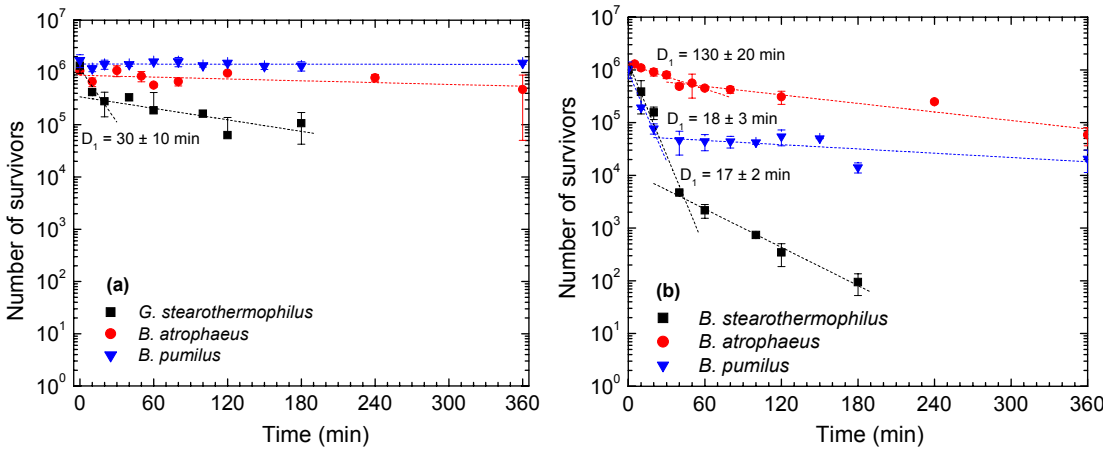


Figure 21. Comparison of the survival curves of the three types of spores considered in the present study when exposed to dry gaseous ozone at a 4000 ppm concentration after being deposited : (a) on Pyrex Petri dishes; (b) on polystyrene Petri dishes.

The experimental data presented in table 1 compare the inactivation efficacy of *B. atrophaeus* spores deposited on various polymeric substrates, after a 60 min exposure to dry gaseous ozone. The substrates were initially cleaned and rinsed 5 times with ultrapure water before spore deposition. The deposits were exposed to ozone as soon as they had dried. The polymers tested are commonly used in the biomedical field.

Polymers	Inactivation efficacy in % (*B. atrophaeus* spores)
Polystyrene	79.7%
Silicone	99.9985%
Polyurethane	99.1%
Polymethylmetacrylate	23.5%
High density polyethylene	36%
Polypropylene	15.2%
Teflon (PTFE)	32.7%

Table 1. Inactivation efficacy of *B. atrophaeus* spores deposited on different polymeric substrates and exposed 60 min to 4000 ppm dry gaseous ozone. Initial number of spores is 10^6 in 100µl of water.

This table shows, for the first time to our knowledge, that spore inactivation after exposure to dry gaseous ozone (RH < 2%) is possible not only on polystyrene Petri dishes but also on various other polymers. The inactivation efficacy level highly depends on the nature of the substrate. Such a result could be of interest for hospitals and biomedical applications in general, especially for polymers such as silicone, polyurethane (PU)...

4. Humidified gaseous ozone effect on *B. atrophaeus* spores

According to the optical absorption measurements presented in Material and Methods (sec. 2), the system is operated under conditions such that the spatial distribution of humidified gaseous O_3 in the chamber is homogeneous. Therefore, a single Petri dish, placed at the geometrical center of the chamber, was exposed at a time. *B. atrophaeus* spores were chosen in this study because of their strong resistance to inactivation by dry gaseous ozone (figure 22 (b)).

4.1 Survival curves

The effect on the inactivation rate of a humidified gaseous ozone flow on *B. atrophaeus* spores is illustrated in figure 22 (a), showing a high inactivation rate in contrast to dry ozone exposure in figure 22 (b). We note that the higher the relative humidity (RH) rate, the higher the inactivation rate. The corresponding inactivation kinetics, for both RH values, is characterized by two phases on the survival curve as reported by several authors (13, 17, 18).

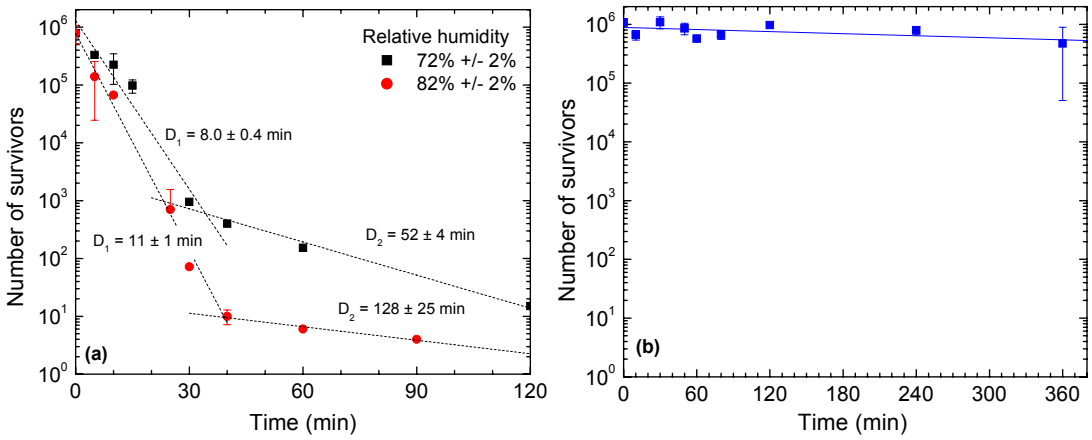


Figure 22. Survival curves of *B. atrophaeus* spores deposited on Pyrex Petri dishes: (a) subjected to humidified gaseous ozone at a concentration of approximately 4000 ppm for 2 values of RH; (b) subjected to dry gaseous ozone at a concentration of 4000 ppm.

According to literature, the highest microbial inactivation rate by humidified gaseous O_3 is attained at relative humidity (RH) rates of 90-95% while it would lose its inactivation property at RH ≤ 50% (47). However, the second part of this statement does not fit with our observations since, with dry gaseous ozone, we succeeded in inactivating, although to various degrees, different species of bacterial spores (figure 18).

Ishizaki *et al* (16), in studying the sporicidal activity of O_3 on *B. atrophaeus* spores, obtained a 5 log reduction in less than 2 hours for a 95% RH and a 1500 ppm ozone concentration; in contrast, Aydogan *et al* (18) results showed that less than a 2 log reduction was reached for identical ozone concentration and RH conditions. The difference in sporocidal activities between these two studies could be explained in the way spores are processed after deposition: Aydogan's samples were dried at room temperature in a safety cabinet for 3 h and further conditioned in a desiccator (RH < 1%) for > 3 days compared to Ishizaki's study (their deposits were dried in safety cabinet for 2.5 h; spore strips were afterward conditioned at constant RH values (minimum 54% RH) before ozone exposure). It has been reported that increasing the drying temperature of spore suspension lowers their resistance to a sterilization or disinfection treatment, as discussed by Cortezzo *et al* (37), and verified by us (data not presented). Our own results are close to those of Ishizaki *et al* (16), but with a

higher inactivation rate, most probably because of a higher ozone concentration (4000 ppm vs. 1500 ppm).

A few studies have determined that for RH above 70%, the increase in inactivation rate is not significant (17, 18), based on almost similar corresponding survival curves. However, our survival curves in figure 22 (a) are clearly different for RH values varying by only 10% above 70%. Therefore, it tends to prove that the RH degree is an important factor throughout its range since a little increase in its value can clearly yield an increased inactivation rate, as also reported by Ishizaki *et al* (16).

4.2 Spore morphological characteristics after exposure

Figure 23 displays a set of SEM micrographs of *B. atrophaeus* dried spores. Figure 23(a) shows unexposed (control) spores while figures (b) to (f) correspond to spores subjected to humidified gaseous ozone (≈ 75% RH) at a 4200± 200 ppm concentration. Humidified ozone, in contrast to dry ozone (figure 10(c)), alters the integrity and the structure of *B*. *atrophaeus* spores even after short exposure times (30 min for figures 24(b) to (d)). Note the clear zone surrounding the spores: they could be attributed to leaching of material resulting from the lysis of cell membranes. Longer exposures (2 hours in figures 24(b) to (d)) cause still more damage. This situation fully contrasts with dry ozone exposure where no apparent morphological damage is observed after 60 minute and even 6 h exposures (sec. 3.1.1).

In figures 24(b) to (d) (30 min exposure), two main trends can be observed: some spores have preserved their structure while others have lost their original shape, part of their central material having been lost. In figures 24(e) and (f) (2 hour exposure), all spores have been damaged and some of them have been disintegrated. Under humidified conditions, O_3 molecules appear definitely more efficient in attacking the spores.

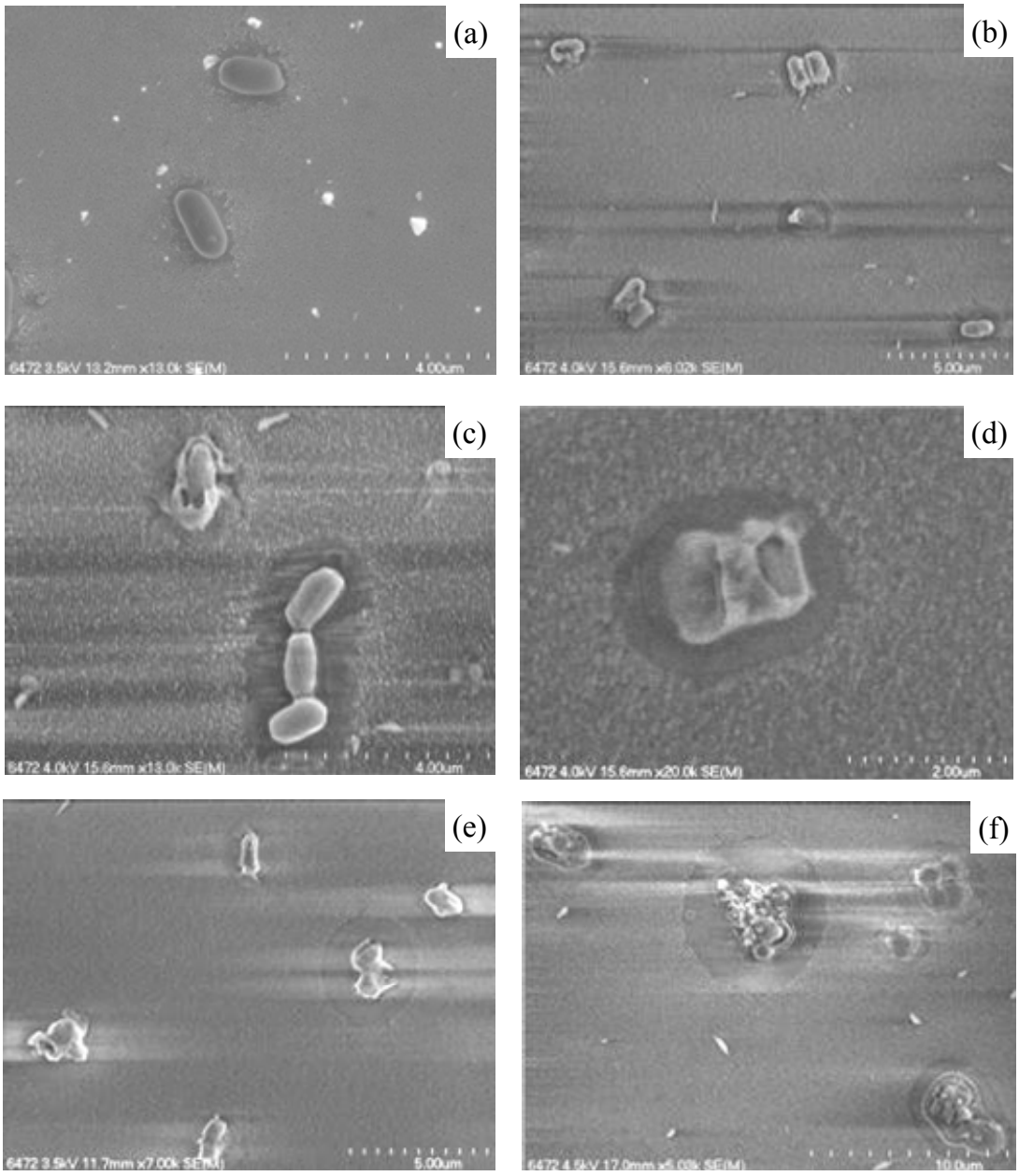


Figure 23. SEM micrographs of *B. atrophaeus* spores deposited on glass slides showing: (a) unexposed spores; (b-c-d) spores exposed for 30 min to humidified gaseous ozone at 4200± 200 ppm; (e-f) spores exposed for 2 h to humidified gaseous ozone at 4200± 200 ppm. For both exposure times, RH was set at approximately 75%.

4.3 Identification of chemical species in the gaseous phase

Figure 24 compares infrared spectra taken in dry and humidified gaseous ozone. In dry media, only the presence of O_3 is observed over the 400-6000 cm^{-1} range, while in humidified media, other species are created such as $HO^{\bullet}_2$ (hydroperoxy), as shown in figure 24.

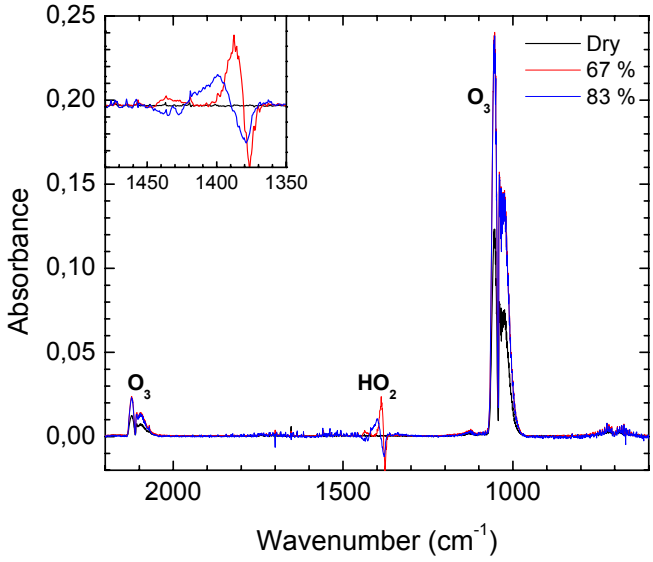


Figure 24. Infrared spectra taken at the sterilization-chamber exit under ozone concentrations of 4000 ppm: dry gaseous ozone (black curve); humidified gaseous ozone for RH ≈ 67% (red curve) and RH ≈ 82.5% (blue curve). The 1392 cm^{-1} signal corresponds to the hydroperoxy radical $HO^{\bullet}_2$, no signal being present with dry ozone.

Theory teaches that ozone decomposition in water is a radical chain-process (5, 20, 47, 48), beginning with reaction (1) where the presence of initiators (here H_2O) is required. This chain process finally provides highly oxidative species that include hydroxyl radicals ($OH^{\bullet}$), hydroperoxy radicals ($HO^{\bullet}_2$), superoxide radicals ($O^{\bullet}_2{}^{-}$) and H_2O_2 molecules:

$$O_3 \xrightarrow{\text{initiators } (OH^-,\ UV,\ H_2O_2 \ldots)} HO_2^{\bullet} + O_2^{\bullet -} . \qquad (1)$$

In the case of a humidified gaseous medium, the decomposition of O_3 increases with increasing RH values (49), again yielding $HO^{\bullet}_2$, $OH^{\bullet}$, H_2O_2…Therefore, maintaining a constant concentration of O_3 implies increasing its production by the ozonator.

The O_3 molecule by itself is chemically selective since it can directly react with organic compounds M having high electronic density sites (e.g. double bonds). In contrast, $OH^{\bullet}$ reactions are non selective and will react with almost all organic compounds. Taking these facts into account, some authors claim that molecular O_3, thanks to its high oxidizing power, is the main inactivator of microorganisms (reaction (2)), while others rather emphasize the high reactivity of the free radicals formed from humidified gaseous O_3 , such as $OH^{\bullet}$, $HO^{\bullet}_2$, … (reaction (3)) (34, 49):

O_3 direct action

$$O_3 + M \longrightarrow M_{ox} , \qquad (2)$$

O_3 indirect action

$$O_3 \xrightarrow{H_2O} OH^{\bullet} \xrightarrow{M} M_{ox} . \qquad (3)$$

4.4 Species involved in microorganism inactivation

For several authors like Setlow *et al*, it is not O_3 that is the main inactivation species but H_2O_2 and possibly free hydroxyl radicals resulting from its degradation, some of them accessing the spore core and, at least in part, the DNA (34, 41). In fact, these authors consider that the O_3 molecules would first react with a large amount of proteins and other constituents in the coats before reaching the core (21). The thick coating of the spore would then act as a reactive barrier that uses up much O_3, preventing it from reacting with critical targets located further within the spore.

In contrast, some other studies showed, under water medium conditions, that molecular O_3 and not the radicals attacks the cell surface, altering the permeability of the membrane and ultimately resulting in the leakage of cell contents (34). Based on kinetic considerations, von Gunten (50) claims that $OH^{\bullet}$ only plays a minor role: the

traveling distance of $OH^{\bullet}$ in a cell is estimated to be approximately 6 nm, not enough to reach the core. Furthermore, Khadre *et al* (4) demonstrated that H_2O_2, even at ≈10,000 fold higher concentration than O_3, was less effective than ozone against Bacillus spores in water.

In humidified media, as Aydogan *et al* (18), Kim *et al* (49) and others, we observed that the inactivation rate of ozone molecules on spore viability increases with RH under constant ozone concentration (figure 22(a)), although O_3 decomposes more rapidly at high than at low RH values. In the dry ozone case, inactivation is achieved without the various radicals created in the humidified ozone case, allowing us to state that the O_3 molecule is directly involved in the spore inactivation (sec. 3.1). This leads us to claim that, under humidified media, ozone molecules should also play an important role in spore inactivation (based on dry case) but that radical species are also involved in the process since spores are heavily damaged and the inactivation kinetics markedly improved.

5. Discussion and conclusion

5.1 Possible inactivation mechanisms under ozone exposure: critical review and analysis

Strong oxidants are generally capable of chemical attacks on constituents of microorganisms, namely proteins, unsaturated lipids, the lipopolysaccharide layer of Gram negative bacteria, intracellular enzymes (respiratory enzymes) and nucleic acids of the genetic material, as well as proteins and peptidoglycan in spore coats and virus capsids (51).

Vegetative bacteria

The inactivation of vegetative bacteria by O_3 is a complex process because ozone attacks a large number of their components; however, O_3 is believed to cause mainly protein and lipid oxidation on the bacterium cell wall. The progressive degradation of the cell wall involves changes in membrane permeability and cell integrity, and it is often followed by a lysis reaction (4, 52, 53, 54). In that respect, Kim and Youssef (53) observed that damage was more pronounced for Gram-negative bacteria (*E.*

coli…) probably because of their lipopolysaccharide layer than for Gram-positive ones (*S. aureus*, …) (42, 55). Hunt and Marinas (34) are proposing a different explanation for bacterium inactivation based on transmission electron microscopy (TEM) micrographs: they observed that the *E. coli* nucleoid seemed to have contracted. They concluded that O_3 was able to penetrate the cells and reacts with proteins or numerous enzymes involved in the control of DNA conformation resulting in its precipitation.

Bacterial spores

One expects a priori that bacterial spores would be less affected by O_3 than vegetative bacteria because of their multilayer protection and stress resistance. Nonetheless, SEM micrographs of *B. atrophaeus* spores show that, in contrast to dry ozone exposure, heavy damages result from humidified gaseous ozone exposure (figures 23(b) to 23(f) compared to figure 10(c)). Various O_3 targets have been proposed in the literature for inducing spore lethality, which we detail:

Enzymes

Several authors referred to enzyme damage as an important inactivation mechanism by which O_3 kills cells (56, 57). Young and Setlow (41) showed that *B. atrophaeus* mutant spores lacking a certain enzyme (second cortex lytic enzyme (SleB)) were inactivated more rapidly by O_3 than were wild-type spores (4, 41). Moreover, Takamoto *et al* (57) observed that ozone decreased the enzyme activity in *E. coli* at different degrees depending on the nature of the enzyme.

DNA

DNA could also be a target since O_3 reacts rapidly with nucleo-bases, especially thymine, guanine and uracil, as reported, for instance, by Isihizaki *et al* (16), Swadeshi *et al* (39). In contrast, authors like Young and Setlow (41) claim that O_3, in water, does not kill spores by DNA damage.

Spore coat

Spore coats designate, starting from the spore outermost, the surface layer, the outer coat and the inner coat. Going further inside, there are the cortex, the inner membrane and the core containing the DNA. Spore coats represent approximately 50% of the spore volume and comprise about 80% of the spore proteins, therefore constituting barriers to metabolic function damage (e.g. enzymes).

The major factor in spore resistance to biocide agents appears to be the spore coat (41, 55). This is further supported by the TEM micrographs of Kim *et al* (49) in the case of aqueous O_3 treatment, revealing damage to the surface layer as well as to the outer and inner coats. Such damage opens the way to the action of O_3 to the cortex, finally causing spore inactivation through intracellular damage (41, 49). Moreover, Foegeding *et al* (58) found that *B. cereus* spores, with their coat proteins removed, were more rapidly inactivated by aqueous O_3 than intact spores: he concluded that the spore coat is a primary protective barrier against O_3 molecules. More broadly, chemically decoated spores and spores with a defective coat (resulting from mutation in cotE, a major coat morphogenic protein) were killed much more rapidly by aqueous O_3 than spores with intact coats (41). This led Young and Setlow (41) to conclude that spore coats (especially in *B. atrophaeus* spores) are essential in spore resistance to O_3.

Inner membrane

Key proteins of the inner membrane can also be damaged by oxidizing agents, including O_3 (37). More recent findings show that the spore inner membrane could be the site of lethal damage by O_3 since a damaged inner membrane: i) prevents spores from maintaining integrity upon a normally moderate heat treatment or when their germinated form is faced with an osmotic stress and ii) because it becomes more permeable. Cortezzo *et al* (37) further demonstrated that damage to the spore inner membrane by oxidizing agents is also consistent with the observed more rapid penetration of methylamine into the core of treated spores: the inner membrane is likely the crucial permeability barrier to methylamine entry into the spore core. Rupture of this permeability barrier can possibly lead to the release of the spore core contents (4, 41).

Relation between humidification and spore collapse.

Under humidified gaseous ozone, we have observed the collapse of the spore (figure 23(d)) followed, after a longer exposure time, by its pulverization (figure 23(f)). According to Rubel *et al* (59), the collapse of the spore structure results from hydrogen-bonding sites formed on the macromolecular constituents of the spore. They show that the residual water loading increases with increasing humidification. Following this, Westphal *et al* (60) observed that spores consistently swell in response to increased relative humidity and shrink to near their original size on reexposure to dry air. They highlighted two distinct time scales for swelling which they assumed to correspond to two sequential mechanisms: i) rapid diffusion of water into the spore 'coat + cortex', followed by ii) a slower diffusion of water into the spore core. This is consistent with the fact that the coat+cortex are commonly thought to be readily permeable to water and to contain free water, whereas the core is thought to contain structured or bound water. They further concluded that swelling of a spore increases the diameter of channels through which inactivating gases could pass.

Proposed inactivation mechanisms following our experimental study and based on the literature

Based on our experimental results in both the dry and humidified ozone cases, we are able to sort out specific elements from the published literature that support our findings. These can be gathered along three main lines:

(1) Inactivation of microorganisms by gaseous ozone in dry case: under this specific operating condition (RH < 2%), our results are markedly novel, implying that the literature in that respect, besides being scarce, must be reconsidered and updated: dry gaseous ozone is able, in short exposure times, to inactivate vegetative bacteria and also, but on a longer time scale, microorganisms as resistant as bacterial spores, observing that their inactivation rate greatly varies from one type of spore to another (figure 18). This variation in the inactivation rate can be correlated with differences in coat or, eventually, cortex constituents, leading to variations in its ozone permeability

as hypothesized earlier by Wickramanayake *et al* (8). Another point worth mentioning that stems from our observations is that SEM micrographs after dry ozone exposure do not show structural damage of the spores. Nonetheless, among these spores even those with the lowest inactivation rate (*B. atrophaeus)*, there is strong evidence that their inner structure is considerably weakened, as revealed by malachite green staining (figure 10(d)). These two last observations lead us to claim that diffusion/oxydation is the main inactivation mechanism of spores under dry gaseous ozone (RH<2%).

(2) Importance of spore swelling under humidified media: two groups of authors have experimentally demonstrated the swelling of spores in a humidified air atmosphere, in the absence of any other gas or chemical agent (59, 60). Taking this phenomenon into account enables us to bring further insight into the inactivation mechanism when O_3 molecules are added: molecules and oxidative radical species can then penetrate more easily within swollen or hydrated spores, diffuse therein through the so-created channels and react with internal biomolecules. As a result, we can say that the inactivation efficiency is due, as a first step, to the high RH (swelling of the spore) and, as a second step, to the specific biocide gas used. This point is very important because authors have always claimed that inactivation in humidified ozone process is better only because of the oxidative agents chemically created in the media ; no studies (in the sterilization or desinfection context) have related the RH effect to the swelling of the spores, which is in fact essential for enhancing the inactivation efficacy. This reasoning can also be applied to the known fact that inactivation by EtO, formaldehyde, chlorine dioxide or heat is more efficient under high RH conditions (45, 61, 62, 63). Then, the type of biocide gas used should be determinant: for a given humidified media, inactivation with O_3 is actually found to be more efficient than with H_2O_2 (4, 37).

(3) Westphal *et al* (60) speculate that swelling occurs on two time scales: i) rapid diffusion of water (> 50 s) into the spore coat and cortex; ii) a slower diffusion of water (≈ 8 min) into the spore core. The increased permeability of the spore structure in humidified media suggests the possibility for O_3 to diffuse not only into the cortex but finally up to the core.

Our SEM micrographs show, for short time exposure (30 min) that the spore collapse generally occurs at the center of the microorganism (figure 23 (b-c-d)); at longer exposure times, simple collapse can no longer be identified due to the spore pulverization. Spore collapse can be thought of as resulting from an overpressure due to the accumulation of H_2O, oxidative species and the volatile by-products chemically created therein (64, 65). As suggested by Westphal *et al* (60), this overpressure occurs at the end point of diffusion, i.e. at the center of the spore where the pressure becomes maximum. Since Westphal *et al* (60) used only water, no biocide agents, they only observe the swelling of the spore, not its collapse.

5.2 Conclusion

Understanding the microorganism inactivation mechanisms following humidified gaseous ozone exposure is a complex and hard task. Our approach to that situation was to examine, under strict dry gaseous ozone exposure (RH < 2%), the inactivation kinetics and morphological damage of three bio-indicator spores and a bacterium and, afterwards, consider the case of humidified gaseous ozone exposure, assuming that specific mechanisms would then add up to those evidenced under dry ozone conditions, all of them concurring to the inactivation process. In the course of experiments, we have also taken great care in ensuring that a single parameter was varied at a time (ozone concentration, RH (measured with great accuracy), O_2 gas flow…).

Under dry gaseous ozone exposure, we have shown that O_3 molecules could efficiently inactivate certain types of spores (*G. stearothermophilus)* and much less others (*B. atrophaeus*), the difference in the inactivation rate lying presumably in the nature or arrangement of their constituents, essentially the chemical composition of their coats (and inner membrane). Morphology of the spores is not affected by dry ozone treatment, implying that diffusion/oxidation, and not erosion, is related to the action of O_3 molecules, these travelling within the spore. Having shown, through a resistant enzyme, that metabolic functions were probably not affected by ozone exposure, it seems logical to consider that a possible target for O_3 is the genetic material, as supported by molecular biology tests of collaborators (Monat and

Cousineau, MS in preparation). In addition, we showed that the ozone inactivation efficacy of spores deposited on polymeric substrates depends on the very nature of the polymer.

Under humidified gaseous ozone exposure, we consider that an important initial mechanism (prior to biocide action) is water swelling of the spores, which opens "channels" that facilitate the inner diffusion of the biocidal agents. These are O_3 molecules and by-products of their interaction with H_2O that yield highly oxidative species such as $HO^{\bullet}_2$, $OH^{\bullet}$, H_2O_2. The final result of these oxidation processes is the inactivation of spores that resisted to some extent the action of dry ozone (*B. atrophaeus*), all the three types of bio-indicators used here being severely damaged, and not to say pulverized after a long enough exposure time. The relative contribution of O_3 molecules and of the oxidative radicals in the humidified ozone inactivation process could not be established in the present work. Nevertheless, recall that, in the dry ozone case, the action of O_3 molecules within the spore itself was found essential to the inactivation process. A next step would be to determine, through molecular biology tests, the damage suffered by the spore nucleic acids. A priori, in humidified media, the genetic material would be more readily and rapidly damaged than in the dry ozone case due to spore swelling and to the added contributions of the various oxidative radicals and O_3 molecules.

Acknowledgments

The authors are grateful to Mr. J S Mayer for skilful technical assistance, A Leduc for valuable assistance on enzymatic activity measurements and analysis, and Professor R Hausler at École de Technologie Supérieure (ETS) for lending us the ozonator. Thanks are also due to Professor B Held from université de Pau and P Renault from Air Liquide (France) for pertinent comments. Financial support was provided by the Fonds Québécois pour la Recherche sur la Nature et la Technologie (FQRNT), programme Équipe.

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