Endotoxins Detection and Control in Drinking Water Systems

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ABSTRACT

Endotoxins are a constituent of the lipopolysaccharide (LPS) complexes present in the outer layer of the cell wall of most Gram-negative bacteria and some cyanobacteria. The ingestion by a typical adult of amounts exceeding 1,000 endotoxin units (EUs) can cause fever, diarrhoea, vomiting, acute respiratory illnesses, and lung inflammation. In contrast, much smaller doses may lead to protective immunity against allergic diseases.

Endotoxins can be released in the air as well as in the water; previous studies have mainly focused on airborne endotoxins. Although many studies on endotoxins in raw and treated drinking waters have been performed, few have assessed seasonal variations and none have been conducted in Eastern Canada. Furthermore, a clear understanding of removal of endotoxins by various water treatment processes is still required.

Two methods to measure the concentrations of endotoxin were used and compared, the Limulus Amebocyte Lysate test (LAL) and the recombinant Factor C test (rFC). Raw water samples were taken from various drinking water sources around the Island of Montreal. The effects of free chlorine, UV radiation, and ozone were studied in batch experiments on filtered water samples via typical dosages and fluences used in drinking water treatment facilities. Residual concentrations for free chlorine were 0.8 and 1.6 mg/L; ozone doses were 0.5 and 1 mg/L; UV fluences were 40 and 100 mWs/cm^2. Detention times of 20 and 60 minutes were tested for chlorine and 5 and 20 minutes for ozone. Grab sampling from three drinking water treatment plants in the Montreal area was performed during the months of June and late August/September 2006 and January 2007. Processes at these plants include coagulation and flocculation, sand filtration, ozonation and disinfection by chlorine. To test the variation in endotoxin concentrations during a sand filter cycle, samples were withdrawn directly from a filter in one of the treatment plants studied. The filtration cycle, from one backwash to the next one, lasts 72 h. Samples were collected immediately before the backwash, at the beginning and at the end of the ripening period, at the beginning of the filtration cycle and 48 h later, which corresponds to a half cycle period.

Of the two endotoxin detection methods used, LAL consistently gave slightly higher values compared to rFC; rFC also required more expensive hardware, but the method was less tedious and reagent costs were lower. Results presented, unless otherwise stated, were obtained with the rFC method. Endotoxin levels decreased in raw water samples between June and September. Concentrations ranged from 20 to 30 EU/mL in June, and decreased to 10 to 14 EU/mL in August and beyond. For the disinfection processes, the UV and free chlorine doses tested had little or no effect on the endotoxin concentrations, but ozone reduced the concentrations by up to 75%. Sand filtration and flocculation showed significant endotoxin removal efficiencies (50 – 60%). Levels remained around 5 EU/mL throughout the remaining treatment processes regardless of the influent concentration. Hence, endotoxin inactivation by free chlorine and UV does not occur with typical doses used in drinking water treatment plants; in contrast, flocculation and sand filtration, as well as ozonation, are much more effective.

RÉSUMÉ

Les endotoxines sont un composant des complexes de lipopolysaccharides (LPS) présents sur la couche extérieure de la paroi cellulaire de la majorité des bactéries Gram-négatif et de certaines cyanobactéries. Leur ingestion en quantités dépassant les 1,000 unités d'endotoxine (EUs) par un adulte peut causer de la fièvre, de la diarrhée, des vomissements, des maladies respiratoires aiguës ou encore une inflammation des poumons. En revanche, des doses plus réduites peuvent amener une immunité protectrice contre certaines maladies allergiques.

Les endotoxines peuvent être libérées dans l'air ainsi que dans l'eau, la majorité des études sur ce sujet se sont centrées sur leur transport par voies aériennes. Bien que plusieurs études sur les endotoxines dans les eaux brutes et traitées pour la consommation humaine aient été réalisées, seules quelques-unes ont estimé les variations saisonnières et aucune d'entre elles n'a été effectuée dans l'est du Canada. En outre, une compréhension affinée de la neutralisation des endotoxines suivant différents procédés de traitement d'eau reste à développer.

Deux méthodes pour mesurer les concentrations des endotoxines ont été utilisées et comparées, le test du Limulus Amebocyte Lysate (LAL) et le test du facteur C recombinant (rFC). Les résultats montrés, à moins qu'indique, ont été obtenus avec le test rFC. Des échantillons d'eau brute ont été prélevés sur plusieurs sources le long de l'île de Montréal. Les effets du chlore libre, de la radiation UV ainsi que de l'ozone ont été étudiés dans des expériences en laboratoire sur des échantillons d'eau filtrée. Les doses et les fluences analysées sont celles habituellement utilisées dans les usines de production d'eau potable. Des concentrations résiduelles de chlore libre de 0,8 et 1,6 mg/L ont été choisies ainsi que des doses d'ozone de 0,5 et 1 mg/L et de fluences d'UV de 40 et 100 mWs/cm^2. Le chlore a été testé pour un temps de contact de 20 et 60 minutes et l'ozone pour un temps de 5 et 20 minutes. Des échantillons en provenance de trois usines de production d'eau potable de la région de Montréal ont été analysés pendant les mois de juin, août et septembre 2006 ainsi que janvier 2007. Les procédés suivis dans

chacune des usines incluent coagulation et floculation, filtration par sable, ozonation et désinfection par chlore. Afin de tester les variations sur les concentrations des endotoxines lors d'un cycle de filtration, des échantillons ont été prélevés à partir d'un filtre dans une des usines étudiées. La durée totale du cycle de filtration, entre un lavage et le suivant, est de 72h. Les échantillons ont respectivement été collectés : immédiatement après le lavage du filtre, au début et à la fin de sa période de maturation, au début du cycle de filtration et 48h après (moitié d'un cycle).

L'écart entre les concentrations mises à jour par la LAL et la rFC s'est révélé consistant. Ces concentrations se sont avérées légèrement plus élevées pour la LAL que pour la rFC. Par ailleurs, la rFC requiert un équipement plus onéreux, cependant cette méthode est moins contraignante et le coût des agents réactifs est moins élevé. D'autre part, le niveau des endotoxines dans les échantillons d'eau brute a diminué entre juin et août. Les taux de concentration obtenus ont varié selon un intervalle compris entre 20 et 30 EU/mL en juin et ont diminué jusqu'à un intervalle compris entre 10 et 14 EU/mL en août. Concernant les procédés de désinfection, les doses utilisées pour la radiation UV et le chlore libre ont eu un effet minimal sur les concentrations des endotoxines, voire aucun effet. Par contre, l'ozone a provoqué la réduction de ces concentrations jusqu'à 75%. La filtration par sable et la floculation se sont montrées efficaces d'une façon significative pour extraire les endotoxines (50 – 60%). Leur taux s'est en effet stabilisé autour de 5 EU/mL pendant les étapes du traitement restantes indépendamment de la concentration initiale. Par conséquence, l'inactivation des endotoxines par le chlore libre et la radiation UV ne se produit pas avec les doses typiquement utilisées dans les usines de production d'eau potable. Néanmoins, la floculation, la filtration par sable aussi bien que l'ozonation s'avèrent particulièrement plus efficaces.

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1. INTRODUCTION

The hygiene hypothesis claims that an early childhood exposure to infectious agents decreases susceptibility to allergic diseases (Strachan, 1989). Although research has focused mainly on the study of the interaction of endotoxins with humans cells by inhalation (Enterline et al., 1985; Gereda et al., 2000; Braun-Fahrlander et al., 2002; Smit et al., 2005), little effort has been concentrated on the consequences and absorption mechanisms of endotoxins through ingestion. Moreover, it is equally important to clearly identify endotoxin levels in raw and drinking water samples in addition to the effect that drinking water treatment processes may have on the removal and control of endotoxins.

The analysis and quantification of endotoxin levels in water bodies as well as in drinking water systems is a relatively recent subject of study. The first measurements, performed by Diluzio and Friedman, date back to 1973. Since then, several other studies (Jorgensen et al., 1976; Sykora et al., 1980; Burger et al., 1989; Rapala et al., 2002; Rapala et al., 2006) have been performed to assess the concentrations of endotoxins in cities across the United States, Denmark, Namibia, South Africa and Finland but the lack of information on untreated surface water and groundwater endotoxin concentrations as well as the presence of few studies of endotoxin levels in drinking water reported in the literature has been evident (Anderson et al., 2002). In addition, Diluzio and Friedman (1973) noticed the need to establish correlations between seasonal endotoxin contents in raw water samples and bacteriological levels. Although a study by Da Silva (2005), as a prelude to this project, evaluated the levels of endotoxins around the island of Montreal, including samples from three drinking water treatment plants, it did not assess seasonal variations, thus the necessity of a larger study for Montreal's area that could include the examination of these variations and the confirmation of results previously found.

A number of experimental methods to quantify endotoxins have been described in the literature (Binding et al., 2004; Li et al., 2004; Priano and Battaglini, 2005; Rybka and Gamian, 2006) however, they involve complex techniques such as gas-liquid chromatography-mass spectrometry. On the other hand, several commercial kits have

been developed for endotoxin measurement. Specifically, the chromogenic Limulus amebocyte lysate test (CAMBREX, 2006a) and the pyrogene recombinant factor C assay (CAMBREX, 2006b), both from Cambrex Laboratories (now known as the Lonza Group Ltd.) may be used to detect endotoxin in drinking water samples; however, based on the performance characteristics of these two methods, their suitability to quantify endotoxins in raw and drinking water samples is to be determined.

It was Diluzio and Friedman (1973) who observed that endotoxin levels are reduced during purification of drinking water but at the same time expressed the need to find out which stages in the purification process reduce these levels. More recently, the effect on endotoxin inactivation of different water treatment processes, namely UV light and several oxidants such as free chlorine and potassium permanganate has been studied by Anderson et al. (2003a; 2003b). However, these studies utilized doses much higher than those normally used in drinking water treatment facilities. Furthermore, the effect of other treatments such as ozonation and slow sand filtration has not been investigated. It is for this reason that testing the efficiency of UV light and free chlorine at working doses, as well as ozone and slow sand filtration, on endotoxin inactivation and removal is of importance.

Hence, the general objective of this study is to provide information on endotoxin concentrations in different raw and treated drinking water samples across Montreal, as well as to assess the effects of various drinking water treatment processes on the inactivation and removal of endotoxins.

In addition, the following specific objectives have been established:

- The first stages of the project will include a search for alternative methods of quantifying endotoxins. Following a literature review of all available methods, laboratory tests will be done using only commercially available kits and will take into account the practical feasibility of the methods as well as their overall cost in order to assess the most convenient(s) one(s).

- With the best performing method, tests will be carried out on different water samples (tap water, raw water and deionized water) to find possible correlations between several water parameters such as TOC, turbidity, total coliforms and water temperature to try to determine if these characteristics have any influence on the endotoxin levels. In addition, samples will be studied in different months to determine if seasonal variations have any impact.

- Several authors have studied the effects that conventional drinking water treatment processes have on the removal of endotoxins. These processes include coagulation, settling and filtration, disinfection by free and combined chlorine, UV and ozone. Therefore, another objective is the confirmation and extension of these results. Processes to be studied will include sand filtration, and disinfection by chlorine, UV and ozone.

2. LITERATURE REVIEW

2.1. Endotoxins description and properties

Endotoxins are a constituent of the outer layer of the cell wall of most Gram-negative bacteria and some cyanobacteria (Sykora et al., 1980; Anderson et al., 2002). They are located in the outermost film of the membrane, facing into the external environment, forming part of a greater macromolecular complex called the lipopolysaccharide (LPS) (Anderson et al., 2002). Three main components can be identified in these LPS compounds, from the exterior to the interior: a surface O-specific polysaccharide (carbohydrate polymer) chain, composed in turn of a series of similar O-specific oligosaccharide repeating subunits; a core oligosaccharide, divided into an inner and an outer section; and an acylated glycolipid, the lipid A, composed of a hydrophilic, negatively charged bisphosphorylated diglucosamine backbone and a hydrophobic domain of either six or seven acyl chains in amide and ester linkages (Priano and Battaglini, 2005) which anchors the LPS molecule in the outer membrane (Stewart et al., 2006). In addition, lipid A appears to be a complex array of lipid residues rather than a single molecular structure (Anderson et al., 2002) and is the "endotoxic" (innate immune stimulating) component of the LPS (Stewart et al., 2006). A schematic diagram of this molecule is shown in Figure 2.1.

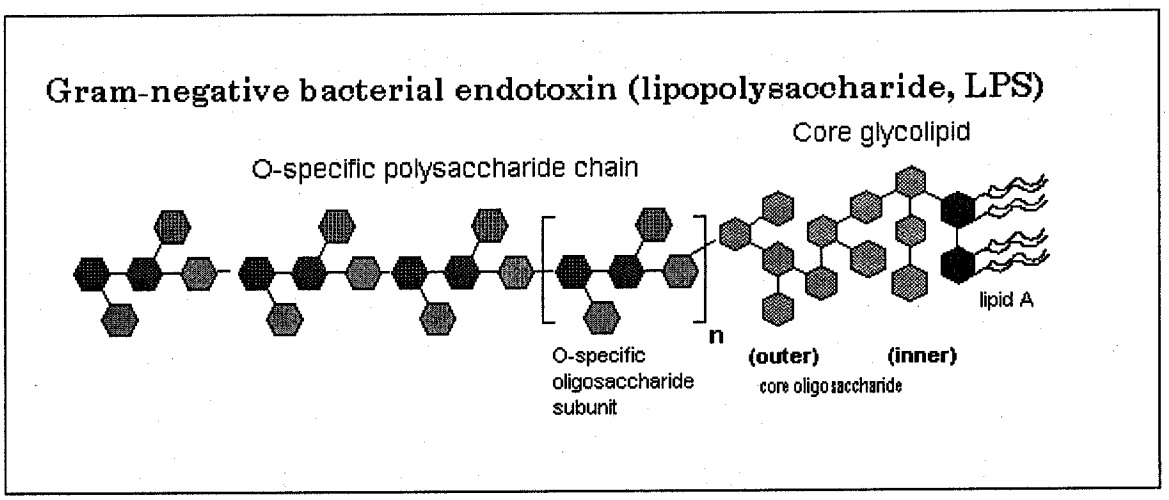

Figure 2.1. Schematic diagram of a lipopolysaccharide molecule (Barclay, 2007).

Stewart et al. (2006) noticed that the term endotoxin was used in the literature to describe several types of toxins within bacterial cells, nevertheless, they concluded that the terms endotoxin and LPS could be used interchangeably. They highlight nevertheless that LPS should refer to the purified molecule whereas endotoxin describes macromolecular complexes of LPS, protein, phospholipids and nucleic acids, which appears to contradict the abovementioned definition. On the other hand, Anderson et al. (2002) state that the terms endotoxin and LPS should not be interchangeable since it has been found that the lipid A component of the LPS is the critical element for biological reaction. Hence, there is still some contradiction when attempting to arrive at an exact definition of endotoxins, however, for the sake of simplicity, the terms endotoxin and LPS will be considered in this study as interchangeable.

Endotoxins are relatively heat stable (Anderson et al., 2003b), they are negatively charged and their size ranges between $20x10^3$ to $1x10^6$ Daltons (Bryans et al., 2004). Different factors such as bacterial origin and presence of divalent cations influence the molecular size and the state of aggregation; additionally, endotoxins are dispersible in polar solvents such as water and can be rinsed away from inert or uncharged surfaces (Bryans et al., 2004).

Several authors (Keleti and Sykora, 1982; Anderson et al., 2002; Rapala et al., 2002) have established a few differences between cyanobacterial endotoxin and gram-negative bacteria endotoxin. Rapala et al. (2002) affirm that, although being significantly thicker, the wall from cyanobacterial cells resembles that of gram-negative bacteria. However, Keleti and Sykora (1982) state that even if LPS from both cells are basically similar, they differ in both chemical and biological characteristics. These authors also found that cyanobacterial LPS contained glucose, xylose, mannose and rhamnose whereas gram-negative bacteria LPS contained 2-keto-3-deoxyoctonate, heptose, galactose and glucosamine. As for the lipid A component, the one from cyanobacteria contains long-chain saturated and unsaturated fatty acids and hydroxyl fatty acids but lacks phosphates, which are present in lipid A from gram-negative bacteria (Keleti and Sykora, 1982). In a previous study, Keleti et al. (1979) determined that the total phosphorus in cyanobacterial

LPS was as low as 3% whereas the carbohydrate content could range between 60 and 80% and the protein content between 0.13 and 20%.

Even though studies from Keleti and Sykora (1982), Anderson et al. (2002) and Rapala et al. (2002) conclude that cyanobacterial LPS is as much as 10 times less active than that from gram-negative bacteria such as *Salmonella*, Stewart et al. (2006) establish that the health implications of cyanobacterial LPS are weakly understood and the topic needs more research.

2.2. Endotoxin – cell interaction

Endotoxins are typically released upon cell lysis and during multiplication (Anderson et al., 2002). Morrison et al. (1994) concluded that, although host responses to endotoxin were believed to be a combination of cellular, pathologic, physiologic and pharmacologic activities, LPS induces the production and release of immunologically active cytokines and other mediators of the host inflammatory response. They also identified the polymorphonuclear leukocyte, endothelial cell and mononuclear phagocyte as primary targets (Morrison et al., 1994).

The mechanism by which the cell recognizes the presence of endotoxins is complex, but it has been well documented in the literature (Morrison et al., 1994; Kaiser, 2005). Once the LPS complexes have been released from the surface of gram-negative cells, they interact with several proteins and bind to specific LPS receptors on host cells, activating the production and discharge of proinflammatory mediators (Morrison et al., 1994). In order to efficiently bind to the cell, free LPS attach first to a specific LPS-binding protein (LBP). The LPS – LBP complex then binds to a receptor molecule called CD14, located on the surface of body defence cells, usually called macrophages (Kaiser, 2005) and also on any LPS responsive cell. The CD14 receptor is in charge of activating the cell responses (Morrison et al., 1994). This bonding stimulates another receptor, the toll-like receptor TLR-4, which has the ability to respond to LPS by commanding the macrophage to release several defence regulatory chemicals called cytokines (Kaiser,

2005). CD14 and the TLR-4 are physically associated and both components are needed for signals to be sent into the cell after the LPS binds. Amongst the cytokines generated by endotoxin-stimulated macrophages, interleukin 1 (IL-1) and tumour necrosis factor (TNF) affect the cell by increasing the systemic inflammatory processes and inducing vasodilatation (Morrison et al., 1994). Moreover, these and other cytokines, once attached to cytokine receptors, activate the complement and the coagulation pathways (Kaiser, 2005). An excess in the production of clotting factors may cause acute respiratory distress syndrome (ARDS) and disseminated intravascular coagulation (DIC); complement proteins on their side may cause shock and multiple organ system failure (MOSF) when damaging the vascular endothelium (Kaiser, 2005). A diagram for the endotoxin – cell interaction pathway is shown in Figure 2.2.

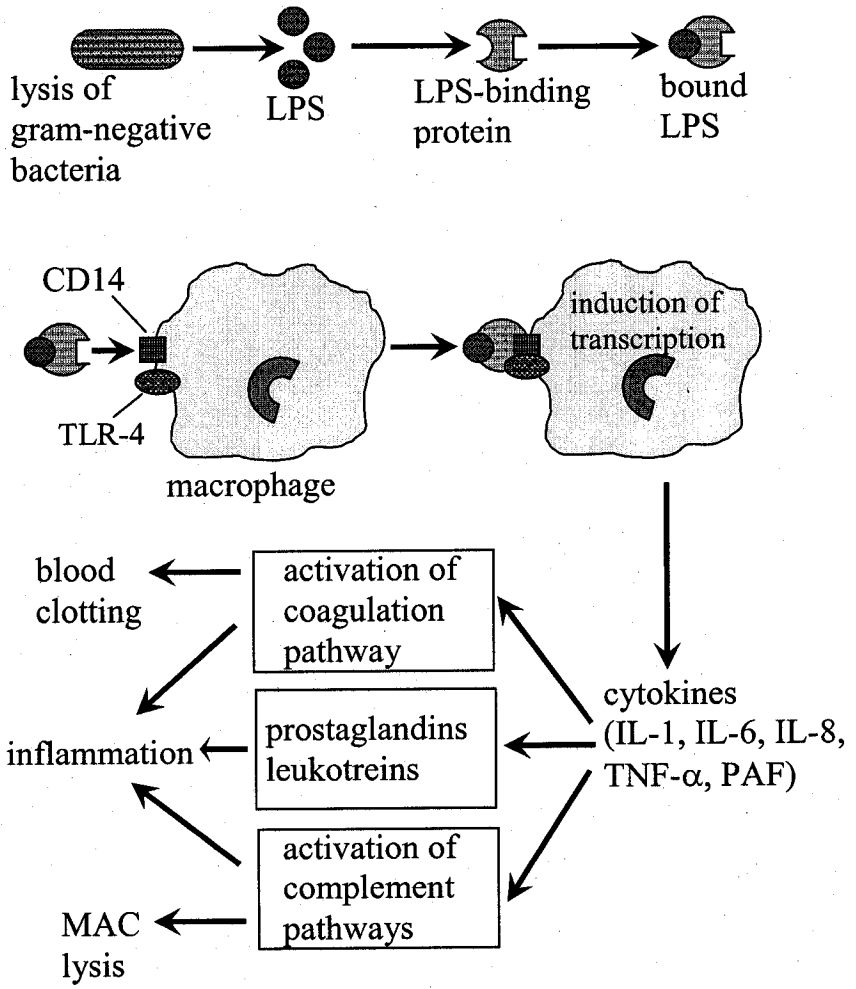

Figure 2.2. Physiologic action of lipopolysaccharide (LPS) from the gram-negative cell wall (modified from Kaiser, 2005).

2.3. Human reaction and symptoms

Human response to endotoxin is widely reported in the literature, for both gram-negative and cyanobacterial endotoxins. Symptoms depend to a large extent on the exposure route, concentration and exposure intervals. Different exposures routes have been established; Anderson et al. (2002) identify the intravenous way, air and aerosolized water inhalation and water ingestion. Regardless of the exposure route, they cite fever, diarrhoea, vomiting, hypotension, shock, intravascular coagulation and death as general symptoms where the latter only occurs at very high concentrations.

Sykora et al. (1980) name several high dose phenomena such as leucopenia, leucocytosis, Schwartzman phenomenon, induction of interferon production and dissolution of cancerous tumours among the physiological responses. However, they omit to mention that the ingestion of LPS produced by algal and bacterial blooms in treated water stored in uncovered reservoirs may be responsible for some waterborne disease outbreaks which they classify as 'of unknown etiology'. Rapala et al. (2002) affirm that endotoxins in water and water aerosols have been related to acute respiratory illness, inhalational fever, gastrointestinal disorders and inflammation at the alveolar level. In addition, Stewart et al. (2006) identify nausea and vomiting as a normal physiological response to the ingestion of LPS; other general symptoms include gastrointestinal illnesses, headaches, dizziness, cramps, blistering of mucous membranes and skin reactions. When the exposure route is inhalation of aerosolised cells other symptoms may appear such as dyspnoea, chest tightness, shivering, fatigue and malaise in normal subjects and bronchoconstriction in asthmatic subjects. These symptoms are referred to by Heederik et al. (2000) as 'organic toxic dust syndrome'. In addition, Heederik and Douwes (1997) establish a difference between acute and chronic effects after inhalation of endotoxins: dry cough and shortness of breath accompanied with a decrease in lung function, fever reactions and malaise as well as joint aches are identified as acute effects whereas chronic bronchitis and reduced lung function are related to chronic endotoxin exposure. Moreover, the authors suggest that either acute and chronic effects may be induced through inflammatory responses in the lungs having the alveolar macrophage

playing a key role (Heederik and Douwes, 1997). Priano and Battaglini (2005) add that, although LPS is chemically inert itself, its presence in blood, identified as endotoxemia, provokes an array of exaggerated host responses known as septic shock.

Several studies have focused on the analysis of endotoxin exposure through inhalation by wastewater treatment workers. Thorn et al. (2002) reported similar symptoms among wastewater workers and they emphasize that fever, shivering and headache have been identified in workers constantly in contact with sludge. In this sense, Smit et al. (2005) agree that fever and other flu-like symptoms have been related to endotoxins in plants where sludge was heat-dried into powder. In their study, they identified three sets of correlated symptoms, as opposed to analysing individual symptoms: lower respiratory and skin symptoms, flu-like and systemic symptoms and upper respiratory symptoms where the latter two groups had a higher dose-response relationship. Interestingly, they concluded from their results that employees exposed on a regular basis to sewage or sludge may develop some tolerance for microbial agents (Smit et al., 2005). A more recent study by Visser et al. (2006) lists the same symptoms as those already mentioned but, in addition, the authors identify aerosols from raw sewage and sludge as the main route of exposure for wastewater workers and, since cleaning activities are the principal producers of high levels of aerosols, they are considered as a significant factor of exposure in wastewater treatment plants.

The exposure to endotoxin via dust inhalation has also been widely studied, moreover, different types of dust, including cotton-, organic-, house- and farming-dust have been analysed. A study by Enterline et al. (1985) suggests that workers in cotton-textile mills have uncommonly low death-rates for cancer, especially respiratory cancer due to the chronic exposure to airborne endotoxins in the work environment. Furthermore, the death-rates decrease even more when the exposure periods are longer or when the exposure concentrations are higher (Enterline et al., 1985). The authors propose several mechanisms through which endotoxins may reduce the development of cancer cells in the lungs: stimulation of the activity of macrophages, release of interferon, mitogen activity or induction of tumour necrosis factor (Enterline et al., 1985). The effect

of endotoxins in organic dust has been reported by Heederik et al. (2000); other than the biological responses already cited, the authors mention the induction of specific immunoglobulin E (IgE). Additionally, they identify two mechanisms that lead to the elimination of particle-associated endotoxins: mucociliary transport when endotoxins are deposited in the upper airways and macrophage and polymorphonuclear leukocyte phagocytosis when endotoxins are deposited in the deeper airways (Heederik et al., 2000). The authors also hypothesize that endotoxins may have an adjuvant effect by increasing non-specifically the immune response to antigens in humans (Heederik et al., 2000). This idea is further analyzed by Gereda et al. (2000) and also by Braun-Fahrlander et al. (2002) who studied, respectively, the relationship between house-dust and environmental endotoxin exposure and asthma in infants and school-age children. Results show that indoor endotoxin exposure early in life may protect against allergen sensitisation since the homes of allergen-sensitised infants had lower concentrations of house-dust endotoxins than those of non-sensitised infants (Gereda et al., 2000). On the other hand, after having found that endotoxin levels measured in samples of dust from children's' mattresses were inversely related to the incidence of hay fever, atopic asthma and atopic sensitization, Braun-Fahrlander et al. (2002) concluded that environmental exposure to endotoxins may have an essential role in the development of tolerance to allergens found in natural environments. In contrast, based on the fact that the protective effects of endotoxins have only been recognized for atopy and allergic asthma, without necessarily reducing the incidence of non-allergic asthma which in fact could be prompted by higher endotoxin encounters later on, Douwes et al. (2002) state that, apparently, endotoxin exposure may prevent the primary antecedent for allergic asthma but it may be the source for non-allergic asthma, either primary or secondary.

2.4. Units of measurement and exposure limits

Irrespective of the exposure route, there is little agreement on maximum safe levels of endotoxins for human health. Even though there is more information concerning the inhalation and intravenous doses, the recommended numbers for ingestion are almost non-existent and an overall standard needs to be established. Furthermore, the use of

different measuring units throughout the literature makes it difficult to compare the results between distinct studies. In effect, while older studies on endotoxins (Diluzio and Friedman, 1973; Jorgensen et al., 1976; Sykora et al., 1980) reported their results in terms of endotoxin weight, expressed in nanograms or micrograms per cubic meter for air samples or per millilitre for liquid samples, more recent studies (Korsholm and Sogaard, 1988; Burger et al., 1989; Anderson et al., 2002; Rapala et al., 2006; Visser et al., 2006) prefer to express the endotoxin concentrations in terms of endotoxin units (EU) per cubic meter or per millilitre depending on the nature of the sample. This preference is due to the fact that endotoxin potency varies depending on the type of bacteria (Anderson et al., 2002), species and the specific batch of bacteria from which the reference endotoxin is obtained (Anderson et al., 2003b) and weight units may not necessarily reflect the actual activity of the endotoxin analyzed. The establishment of a standardised unit allows comparing endotoxin activities irrespective of their source although conversion factors between endotoxin weight and endotoxin activity must be always provided (Anderson et al., 2002). According to the United States Pharmacopeia (2000), an Endotoxin Unit (EU) is defined as the endotoxin activity of 0.2 ng of Reference Endotoxin Standard EC-2, giving a ratio of 5 EU/ng. Several authors (Heederik and Douwes, 1997; Thorn et al., 2002; Anderson et al., 2003b) agree that a fairly accurate conversion factor to shift between units of weight and units of activity lies between 5 and 10 endotoxin units per nanogram of endotoxin.

As mentioned before, numbers are highly variable with regards to the healthy endotoxin exposure levels and they change even more depending on the exposure route. For instance, in the case of inhalation, Anderson (2002) quotes the suggested guidelines proposed by the International Committee on Occupational Health in 1993 which determine that concentrations should not be higher than 200 ng/m^3 in order to avoid organic toxic dust syndrome, not higher than 100 ng/m^3 to avoid systemic effects and lower than 10 ng/m^3 to avoid airway inflammation. Heederik and Douwes (1997) refer to the Dutch Expert Committee on Occupational Standard whose recommendation for personal inhalable dust exposure in an eight hour time-weighted average should be less than 50 EU/m^3 or 4.5 ng/m^3; however, in another paper, this same limit is as high as 30

ng/m^3 (Anderson et al., 2002). Furthermore, Heederik and Douwes (1997) reveal that no occupational exposure limit has been established, either in the Netherlands nor other countries and they broaden the 'No Effect Level' interval within 170 and 9 ng/m^3 (1700 – 90 EU/m^3). Based on acute respiratory effects, the authors establish the No Observed Adverse Effect Level (NOAEL) to be 9 ng/m^3 (Heederik and Douwes, 1997).

Concerning the intravenous doses Anderson et al. (2002) report that the required endotoxin doses to increase human body temperature by 1.9° C range between 1 and 10 ng/kg body weight for different species of *Salmonella* and *Escherichia coli*. The United States Pharmacopeia (2000) defines four types of water, three for injection and one for inhalation, with a corresponding endotoxin limit. Water for injection as well as sterile water for injection have an endotoxin limit of 0.25 EU/mL whereas bacteriostatic water for injection and sterile water for inhalation are allowed to contain as much as 0.5 EU/mL. The British Pharmacopoeia (1993) also establishes a limit of 0.25 EU/mL for water for injection.

Regarding the ingestion of endotoxins, existing information is merely qualitative in part because the mechanisms through which endotoxins are assimilated in the organisms though digestion are not yet fully understood. Snella and Rylander (1977) suggest that there may be a possible natural defence system at the intestinal epithelium level, in the form of antibodies, that neutralises the endotoxins. The authors add that endotoxins should not represent any danger to human beings unless they have a deficiency in their immunologic system (Snella and Rylander, 1977). Thus, as previously mentioned, further studies to determine the absorption of endotoxins by ingestion are needed in order to establish a standard exposure limit. Rapala et al. (2002) confirm these needs by mentioning that the knowledge on the occurrence and removal of endotoxins in water samples is so limited that guidelines cannot yet be set. On their side, Diluzio and Friedman (1973) affirm that the presence of endotoxins in drinking water does not seem to be a health risk as long as absorbed levels are limited and adequate removal and inactivation mechanisms exist; what is more, they expect a certain tolerance to be developed if small quantities are ingested. Other studies on endotoxins in drinking water

samples (Jorgensen et al., 1976; Burger et al., 1989; Rapala et al., 2006) limit themselves to the analysis of endotoxin concentrations and removal efficiencies, without relating these levels to any standard exposure limit. Furthermore, Rapala et al. (2002) mention that studies need to be performed in order to find harmless endotoxin levels in drinking water as no guidelines exist.

2.5. Endotoxin quantification experimental methods

Several experimental methods have been proposed in order to identify and quantify the amounts of endotoxins in liquids. In an experiment developed by Binding et al. (2004) it was possible to quantify endotoxins from occupational and environmental samples by GC-MS determination of 3-hydroxy fatty acids (3-OH FAs) present in the lipid A region of the LPS molecule. Overall, the method follows three basic steps: the hydrolytic cleavage of the 3-OH FAs, the derivatisation of the hydroxyl and the carboxyl functionality and the chromatographic separation of the derivatives. The authors concluded that their technique allows obtaining quantitative information on the endotoxin content in both aqueous and dust samples. However, they recognize that the absolute amount of LPS cannot be determined since LPS from different bacterial species will have different 3-OH FAs (Binding et al., 2004) which also explains the poor correlations they obtained with their method and the Limulus Amebocyte Lysate (LAL) assay (see Section 2.6.1).

In a similar study, Li et al. (2004) were able to measure endotoxin levels by liquid chromatography (HPLC) using fluorescence detection to quantify the derivatized fatty acids obtained from the lipid component of various LPS molecules. Their procedure involved similar steps to those performed by Binding et al.: hydrolysis of fatty acids in order to release them from their lipid source, transfer into an organic solvent, derivatisation of the hydroxyls C12:0 and C14:0, and HPLC quantisation. These specific hydroxyl groups were analyzed because they are indicators of the lipid A of endotoxins (Li et al., 2004). In addition, the authors compared the levels of the OH fatty acids obtained by HPLC with results obtained by LAL testing and arrived at the same

conclusion as Binding et al. (2004): while LAL measures specific biological activity of endotoxin, HPLC only measures the physical incidence of OH groups in the lipid A fraction of endotoxin (Li et al., 2004).

Rybka and Gamian (2006) also used gas–liquid chromatography–mass spectrometry to detect the levels of endotoxins but, instead of measuring the OH groups, they measured another component of the LPS molecule, the 3-deoxy-D-manno-2-ocyulosonic acid (Kdo) which is located between the lipid A and the core oligosaccharide, forming an unstable acid link between the two. Their methodology included drying and methanolysis of LPS samples, dephosphorylation and acetylation of the samples and finally GLC-MS analysis. The conclusion for this study is that Kdo may be used for LPS quantification but, since Kdo is not unique to LPS and can also be found in other polysaccharide molecules, the results obtained by this method may not be regarded as absolute for endotoxin detection. The authors suggest, nevertheless, that Kdo may be considered as a good candidate for chemical detection of LPS in specific environments like human body fluids. The presence of different bacteria in drinking water samples, however, may not render the method suitable for the measurement of endotoxins in this type of sample.

Priano and Battaglini (2005) devised an electrochemical experiment to detect endotoxins with the motivation to find a simple and fast method capable of carrying out on-line measurements in different liquid samples. The principle is based on a gold electrode and a recombinant endotoxin neutralizing protein (ENP). The endotoxin detection is done by competition of LPS present in the sample and a horseradish peroxidase-labelled LPS conjugate (LPS-HRP). One advantage of this method is that it allows one to measure endotoxin concentrations below 0.25 EU/mL; however, LPS is not only absorbed by the neutralizing protein (ENP) but also by other polymeric matrices which leads to possible interferences of proteins. The authors claim, nevertheless, that the method is still efficient when applied to extremely pure aqueous solutions mostly composed of inorganic ions where protein interferences could be discarded. Being a recent method, other factors such as correlating the results obtained with a LAL assay, refining the LSP-HRP conjugate activity and reducing interferences are still to be

explored. Thus, as with the method proposed by Rybka and Gamian, its suitability for analyzing drinking water samples would also need to be assessed.

2.6. Endotoxin quantification commercial kits

2.6.1. Limulus amebocyte lysate test

The Limulus amebocyte lysate test (LAL) is currently one of the most commonly used methods to measure endotoxin concentrations in different environmental as well as pharmaceutical samples. The principle of this method was first described in the works of Levin and Bang (1968) who discovered that the blood of the horseshoe crab, the *Limulus polyphemus* (Young et al., 1972), contains a single cell, the amebocyte, which clots in the presence of bacterial endotoxin. The mechanism through which these cells coagulate involves the aggregation of amebocytes immediately after the blood is extracted; the cells then degranulate, leading to the formation of a liquid phase which in turn forms a gel when in contact with endotoxin. This coagulation system was described as a defence mechanism in the Limulus, acting as the control method towards bacterial infection since this species lives in a rich gram-negative bacteria environment.

The authors found that only the amebocytes contained the clottable protein present in the Limulus blood. In their experiments, they were able to show that the rate of gelation was independent of the concentration of coagulable protein (Levin and Bang, 1968) but directly proportional to the concentration of endotoxin present. Moreover, it was suggested that the reaction between the cellular protein and endotoxin is enzymatic. Further experiments (Young et al., 1972) confirmed this idea by demonstrating that endotoxin activates an enzyme present in one fraction of the amebocyte lysate, rather than directly reacting with the clottable protein. It was also found that the reaction was pH and temperature dependent.

Modern LAL assays are still based on these discoveries. This is the case of the chromogenic Limulus amebocyte lysate test (LAL) developed by CAMBREX laboratories (CAMBREX, 2006a). In this specific test, the initial part of the LAL endotoxin reaction is used to activate an enzyme which later on will release p-nitroaniline (pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA thus producing a yellow colour that can be photometrically measured at 405 – 410 nm. The rate of activation is dependent on the endotoxin concentration. A calibration curve must be prepared every time endotoxin concentrations in different samples are to be determined in order to correlate the absorbances of the unknown concentrations with those of the standards.

A couple of weaknesses have been identified in the LAL assay (ERDG, 2007): the limitation to distinguish between live and dead bacteria and the inability to recognize different species of bacterial endotoxin. On the other hand, Eduard et al. (2004) noticed that LAL tests may underestimate endotoxin levels when performing aqueous extraction of dust samples since only soluble endotoxins are determined by this method. However, regardless of these inconveniences LAL is, to date, one of the most widespread methods used to quantify endotoxin concentrations in a variety of samples.

2.6.2. Recombinant factor C test

Studies performed by Nakamura et al. (1986) and later by Iwanaga (1993) were able to show a more detailed description of the activation of the Limulus clotting system when induced with LPS. From this perspective, it was found that there are three sequential activations of intracellular hemolymph zymogens. A first double chain glycoprotein, called factor C, is autocatalytically converted to an activated form, factor C, in the presence of LPS (Iwanaga, 1993). This activated form reacts in turn with another single chain glycoprotein called factor B and converts it into a second active factor designated factor B, composed of two different chains. This new form of factor B activates a second single chain glycoprotein known as proclotting enzyme which is converted to clotting enzyme, a two chain active form composed of a light and a heavy chain. Finally, coagulogen present in the cell reacts with the clotting enzyme to form

coagulin gel, a fibrous component of the extracellular clot, completing thus the cascade pathway. The overall processes present in the clotting mechanism can be classified as cell adhesion, aggregation, degranulation and gel formation (Iwanaga, 1993). A diagram of the activation pathway is shown in Figure 2.3.

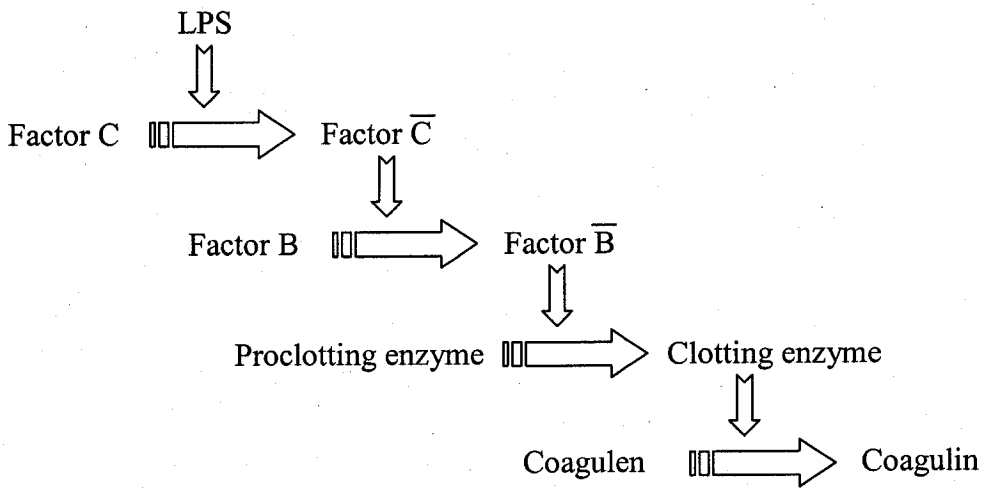

Figure 2.3. Coagulation cascade of the Limulus amebocyte (Modified from Nakamura et al., 1986)

Additional studies by Muta et al. (1993) provided more details related to the structure and physicochemical characteristics of factor C. It was found that this protein is composed of two polypeptide chains, a heavy and a light one, connected by a disulfide linkage. It is composed of five repeating units formed of 60 amino acid residues each showing a unique mosaic protein structure. The optimal activation of factor C by endotoxin occurs at pH 7.2 and it is stable at 0° C for several weeks. It was also observed that factor C is localized in large granules present in the cell.

A commercial recombinant factor C endotoxin detection system developed by CAMBREX laboratories (CAMBREX, 2006b) is based in the ability of factor C to selectively recognize endotoxin and activate the protease cascade but, instead of following the complete pathway described in Figure 2.3 ultimately leading to the conversion of coagulogen into coagulin, a purified and cloned species, the recombinant

factor C, is activated by endotoxin binding which then reacts with a fluorogenic substrate included in the assay in order to produce a fluorescent signal (Figure 2.4). This signal is proportional to the endotoxin concentration in a given sample. A calibration curve needs to be prepared together with the samples, both standards and samples are incubated for one hour at 37° C. Fluorescence is measured using excitation/emission wavelengths of 380/440 nm at the beginning as well as at the end of the incubation period and corrected with the negative controls. Endotoxin concentrations are then calculated relative to the standard curve.

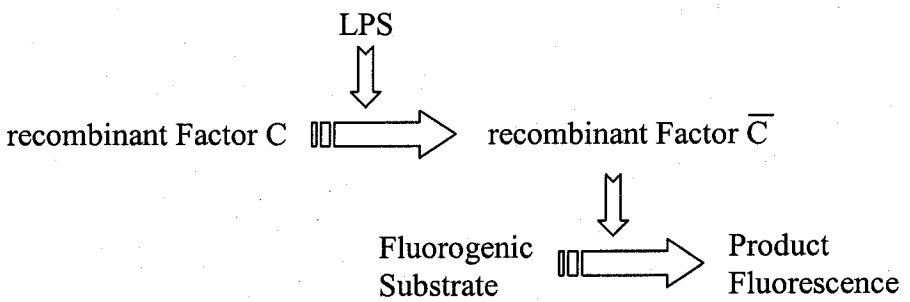

Figure 2.4. Endotoxin detection by recombinant factor C (Modified from CAMBREX, 2006b)

2.7. Quantification of endotoxins in drinking water systems

Studies that quantify the levels of endotoxin in drinking water systems date back to the early 1970s. One study performed by Diluzio and Friedman (1973) analyzed water samples from eighteen sources around the Mississippi River and the Gulf of Mexico area. Although the authors say that results were presented qualitatively, numerical values were included for some of the samples, ranging between 1 and 400 μg/mL. A sample from Mexico City's tap water was also analyzed and found to have 800 μg/mL; milk samples from New Orleans ranged between 30 and 130 μg/mL; however, the endotoxin content increased sixteen-fold when milk was maintained at room temperature for 24 h with concomitant bacterial proliferation; on the other hand, no endotoxin alteration was observed when the samples were refrigerated. Other samples studied included chemically pure water, sodium chloride solution, beer, cola drinks and wine but none of them

showed any endotoxin content; however, when analyzing laboratory deionized water, detectable amounts of endotoxin were found.

Jorgensen et al. (1976) analyzed samples from both drinking water and wastewater from different cities around the United States. Concentrations measured ranged between 0.625 and 1,250 ng/mL, significantly lower than those measured by Diluzio and Friedman. The authors give a preliminary explanation to this difference by stating that the use of a different lysate in each study may lead to different results; this should be combined with the fact that Jorgensen et al. were careful to either freeze or refrigerate all their samples while Diluzio and Friedman were not; although freezing a sample would prevent its degradation, in the case of endotoxin it also prevents bacterial growth thus keeping endotoxin levels lower. The highest treated water concentration was measured in a wastewater plant whose treatments included activated sludge and chlorination whereas the lowest occurred in a wastewater treatment plant using a trickling filter and settling. In general, drinking water treatment plants using sedimentation, coagulation and filtration showed lower endotoxin concentrations while in plants with activated carbon columns, concentrations were higher (Jorgensen et al., 1976).

Evans et al. (1978) performed a similar exercise by measuring endotoxins in different water samples coming from the East Gallatin River near the Bozeman, Montana area. Endotoxin levels varied between 1.19 ng/mL for a drinking water tap sample at Montana State University and 1049 ng/mL for a sample at a sewage outfall. It is important to mention that, while the previous two studies did not, this one discriminates between total, bound and free endotoxin. The free endotoxin was obtained by measuring the supernatant fluid obtained by centrifugation of the samples and the second by subtracting the concentration of free endotoxin from the total endotoxin concentration.

Sykora et al. (1980) studied five water systems in Pittsburgh, Pennsylvania by sampling the treatment plant effluent, water from the reservoir and from selected points in the distribution network. The geometric means for endotoxin concentrations ranged between 0.63 and 130 ng/mL, however, concentrations as high as 3,200 ng/mL were

registered in an open reservoir where the concentration of algae and SPC bacteria were also high. In addition, the authors found good correlations between total phytoplankton and LPS concentrations and explained that additional chlorination in reservoir waters may be the reason for low LPS levels. It was also found that systems whose source water was ground water normally showed lower LPS concentrations in the plant effluent. Nevertheless, based on other observations, the authors concluded that it is not the quality of the source water but the degree of treatment carried out at the plant that has a greater effect on the concentrations of endotoxins in effluent waters. The work by Diluzio and Friedman is also referred to in this paper and the occurrence of non-specific reactions produced by organic compounds other than LPS is given as an explanation for the high endotoxin values reported.

Another study by Haas et al. (1983) examined two water treatment plants whose location is not revealed. The mean values for total endotoxin concentrations were found to be between 3.46 μg/L for a sampling point following pre-chlorination and alum coagulation and 13.40 μg/L for a point after sedimentation. Free and bound endotoxin levels were calculated in a similar way to the one performed by Evans et al. (1978), however it was concluded that bound endotoxin values were not reliable since, in most of the cases, free endotoxin concentrations were higher than total endotoxin levels. It was also concluded that the presence of endotoxins may reflect the long-term quality of the water samples, as opposed to short-term variations, which implies that it is important to measure and compare endotoxin concentrations seasonally.

A Danish study by Korsholm and Sogaard (1988) had as a primary objective to correlate LPS levels in water samples with acridine orange direct counts (AODC). For that, 233 samples of drinking water were obtained from private wells, ground water sources and several water treatment plants. Although the nature of the samples with the minimum and maximum values was not identified, concentrations ranged between 0.075 and 600 EU/mL. Additionally, it was found that total LPS and AODC were highly correlated but variations in endotoxin content within samples made it difficult to set up a general conversion factor between these two parameters.

Endotoxin concentrations were also measured in two reclamation plants at Windhoek (Namibia) and the Cape Flats (South Africa) by Burger et al. (1989). Levels were as high as 1,215 EU/mL at the maturation pond effluent and as low as 4 EU/mL at a borehole sample. This high decrease in the endotoxin content demonstrates that treatments employed at the two plants studied were very efficient for endotoxin removal. Moreover, it was found that sand filtration, ozonation and chlorination contributed to a higher reduction in the concentration, whereas activated carbon columns increased the endotoxin levels, probably due to bacterial growth present in this type of column (Burger et al., 1989).

In order to quantify endotoxin concentrations in cyanobacterial water blooms, a more recent study by Rapala et al. (2002) measured 151 freshwater samples from several places in Finland with results showing values to be in the $10^2 - 10^3$ EU/mL range, with a mean of 1,400 EU/mL. Another part of the experiment included the analysis of nine drinking water treatment plants. In this case, raw water concentrations ranged between 18 and 356 EU/mL; after different treatments were applied, concentrations decreased to between 3 and 15 EU/mL where the lowest value corresponds to a plant with alum coagulation, flotation, sand filtration and chlorination. Samples measured throughout the distribution network had values in the same order of magnitude, ranging between 14 and 32 EU/mL. A few years later, the same research group measured endotoxin activities in one treatment plant serving 36,000 people. Raw water concentrations remained within the 35 – 430 EU/mL range, however, concentrations as high as 3,300 EU/mL were reported after a heavy rainfall (Rapala et al., 2006). After different treatments were applied, levels decreased to between 4 and 60 EU/mL with coagulation and sand filtration being the most efficient methods.

A summary of different endotoxin concentrations encountered in the literature can be found in Table 2.1.

Table 2.1. Endotoxin concentrations found in previous studies.

Sample	Endotoxin Range		Reference
	ng/mL	EU/mL*	
Drinking water	1,000 – 800,000	–	(Diluzio and Friedman, 1973)
Drinking water	0.625 – 500	–	(Jorgensen et al., 1976)
Wastewater	0.78 – 1,250	–	(Jorgensen et al., 1976)
Raw water	252 – 1,049	–	(Evans et al., 1978)
Drinking water	1.19 – 53.19	–	(Evans et al., 1978)
Drinking water	0.63 – 130 (3,200)	–	(Sykora et al., 1980)
Drinking water	3.46 – 13.40	–	(Haas et al., 1983)
Drinking water	–	0.075 – 600	(Korsholm and Sogaard, 1988)
Raw water	–	1,080 – 1,215	(Burger et al., 1989)
Treated water	–	4 – 72	(Burger et al., 1989)
Raw water	–	18 – 356	(Rapala et al., 2002)
Drinking water	–	3 – 15	(Rapala et al., 2002)
Raw water	–	35 – 430 (3,300)	(Rapala et al., 2006)
Drinking water	–	4 – 60	(Rapala et al., 2006)

* 10 EU ~ 1 ng

2.8. Removal of endotoxins through water treatment processes

The removal of endotoxins at laboratory scale using different methods has also been studied. Anderson et al. (2003a) tested the effect of medium-pressure UV lamps on endotoxin inactivation. Deionized water spiked with 300 and 400 EU/mL was used for the experimental set up and high UV fluences between 100 and 600 mJ/cm^2 were applied, although practical UV doses for drinking water treatment normally range between 40 and 100 mJ/cm^2 (Anderson et al., 2003a). With these parameters, a reduction of 0.55 (EU/mL)/(mJ/cm^2) was reached. The authors claim that, with this inactivation, up to a 55% removal could be reached if the initial endotoxin concentration of the sample is between 50 and 200 EU/mL.

This same research group also studied the effect of various oxidants on endotoxin inactivation. Selected disinfectants were free chlorine, monochloramine and potassium permanganate with residual doses of 2 and 100 mg/L, 3 and 100 mg/L and 3 mg/L, respectively (Anderson et al., 2003b). For all three disinfectants, high values for retention times as compared to those normally used in drinking water treatment facilities were chosen and ranged between 24 and 210 h. The reduction rates were 1.4, 1.0 and 0.7 (EU)/mL·h for free chlorine, monochloramine and potassium permanganate, respectively. These values were considered to be quite small, especially because common retention times are no greater than 48 h. Based on these results, endotoxin inactivation by physical rather than chemical processes was recommended.

A study to assess the removal of endotoxins with a biosand filter (BSF) was performed in Mozambique by Bojcevska and Jergil (2003). Three types of filters were used: one regular biosand filter, one with an extra layer of granulated activated carbon (GAC) and one more with an extra layer of charcoal. Endotoxin concentrations in raw water ranged between 2.8 and 38.9 EU/mL and the mean removal rates for the three filters were 5% for the charcoal supplemented filter, 18% for the regular filter and 37% for the GAC filter. Removal rates were found to increase with time which was thought to occur due to the formation of a biological layer. No correlation between the endotoxin concentrations and chlorophyll a were found. In addition, the authors concluded that, due to the lack of guidelines, it could not be established if the endotoxin levels in filtered waters were safe.

Endotoxin removal from dialysis water by using ceramic membranes was tested by Czermak et al. (2005). In their study, three types of commercial tubular ceramic membranes used for ultra- and nanofiltration were challenged with endotoxin concentrations varying between 0 and 2,000 EU/mL. The critical parameter was set to be 0.25 EU/mL, the maximum endotoxin concentration allowed by the United States Pharmacopeia (2000) in dialysis water. When applying an endotoxin concentration of 100 EU/mL, concentrations lower than the permitted value were obtained for all three

membranes; however, when the concentration was increased to 1,000 EU/mL none of the membranes was able to reach the target value of 0.25 EU/mL. The explanation given for this behaviour is that at low endotoxin concentrations, trapped endotoxins may obstruct the permeation of free molecules thus increasing the membrane efficiency but, when concentration is increased, endotoxin excess may produce concentration polarization provoking a reduction in the membrane efficiency (Czermak et al., 2005). Bender et al. (2000) concluded that commercial nanofiltration ceramic membranes were not efficient enough for the removal of endotoxins in dialysis fluids.

An experiment performed by Wang et al. (2005) showed that endotoxin could be removed from aqueous solutions by means of a synthetic calcium silicate hydrate adsorbent. The removal mechanism is based on the interaction between the anionic phosphate group in LPS molecules and the cationic ligands on the sorbents. The authors claim that the method is highly efficient, attaining removals of up to 99.99994%, by reducing concentrations as high as 5,000 EU/mL down to 0.003 EU/mL. Furthermore, the addition of an electrolyte may enhance the removal efficiency by increasing the negative zeta potential of the adsorbent and thus the electrostatic attraction between this and the positively charged LPS molecules. Although the method seems to be a good alternative for biotechnological and pharmaceutical processes, the high operation and maintenance costs make it impractical for large scale applications such as water treatment.

3. MATERIALS AND METHODS

3.1. Sampling points

With the purpose of assessing the seasonal variations as well as the effect of large scale drinking water treatment processes on endotoxin concentrations in water samples around the Montreal area, samples were collected from three different drinking water treatment plants, during the months of June and September 2006 and January 2007. An additional sample collection was done only for raw waters in late October 2006 in order to compare endotoxin levels to those measured a year earlier by Da Silva (2005). Figure 3.1 shows the flow diagram for the three plants studied; hollow arrows indicate the exact locations where the samples were taken from.

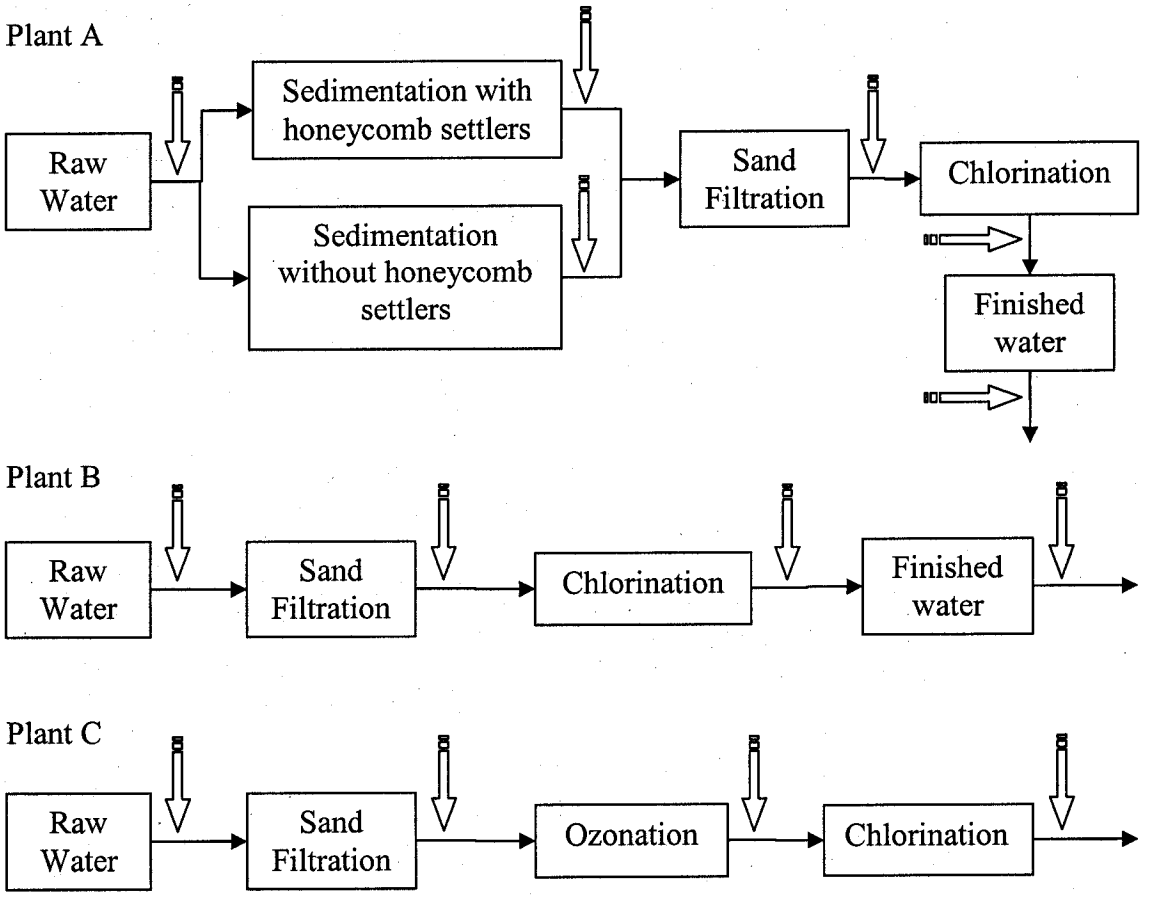

Figure 3.1. Flow diagram for the three drinking water treatment plants studied.

In order to evaluate the differences in endotoxin concentrations throughout the drinking water distribution system, three more sampling locations were selected, having as their principal characteristic to be the furthest points of the hydraulic profile of the network. These points were located at 15, 29 and 35 km, respectively, downstream from their corresponding drinking water treatment plant. The idea behind this choice was to examine if there was any effect of chlorine residuals and piping conditions on endotoxin levels.

For the experiments performed to test the effect of UV light on endotoxin inactivation, samples were collected at the raw water intake as well as at the outlet of one of the sand filters of plant B. In subsequent experiments dealing with the effect of free chlorine and ozone on endotoxin inactivation as well as the effect of large scale sand filtration on endotoxin removal, samples from the outlet of one of the filters from plant B were used.

Regardless of their source, all samples were collected in either 250 mL or 1 L brown glass bottles with pyrogen-free flat disk septa. Bottles were also rendered endotoxin-free by heating in the oven at 250° C for at least 30 minutes, as performed by other researchers (Anderson et al., 2003b). During transport, they were kept cold at 2 – 4° C. In the case of samples used to test seasonal variations, 1.8 mL aliquots were transferred upon arrival to the laboratory into pyrogen-free microcentrifuge tubes (Fisher Scientific, Whitby, ON) which were immediately frozen for later endotoxin measurement. Filtered water samples used to test the effect of the different drinking water treatment processes were kept in the brown glass bottles at one third of their capacity and frozen by keeping the bottles in a horizontal position.

3.2. Glassware and pipettes

In addition to the brown glass bottles where samples were transported, all other glassware used throughout the different experiments was made endotoxin free by washing with tap water and rinsing twice with distilled water, then baking at 250° C for

at least 30 minutes. In the case of pipette tips and microplates, they were bought pyrogene free; 1–200 μL and 100–1000 μL universal fit pipette tips (Corning Inc, Corning, NY) covered all of the pipetting needs. It was thought that autoclaving would be enough to remove all the endotoxin content in pipette tips but it was found (Martin and Dailey, 2001) that this is not the case thus new pipette tips were used at the beginning of every experiment to minimize environmental endotoxin contamination.

3.3. Limulus amebocyte lysate test

3.3.1. Experimental procedure

Endotoxin concentrations measured using this method were performed by means of the QCL-100® chromogenic LAL endpoint assay (catalogue number 50-648U, Cambrex Bio Science Walkersville, Inc, Walkersville, MD) consisting of Limulus amebocyte lysate, a lyophilized endotoxin standard and a chromogenic substrate; in addition, a stop reagent such as acetic acid or sodium dodecylsulfate is needed. Given that the number of samples analyzed was high, the microplate method was preferred over the test tube method. Even though the pH for the first samples was measured and adjusted to be within the 6.0 – 8.0 range it was later found that by diluting the samples with LAL reagent water (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) pH adjustment was automatically done.

Standards and dilutions were prepared in borosilicate glass disposable culture tubes (Borex®) made endotoxin free with the method previously described. Endotoxin concentrations were determined as described by the manufacturer. Lyophilized endotoxin from the *E. coli* O111:B4 strain with a known activity ranging between 15 – 40 EU was reconstituted by adding 1.0 mL of LAL reagent water at room temperature. The actual activity of the vial was thus determined by the value stated on the certificate of quality. Reconstituted endotoxin was vigorously vortexed for at least 15 minutes. Since the method is linear for a concentration range between 0.1 and 1.0 EU/mL, four endotoxin standards were prepared from the reconstituted endotoxin. The first standard, containing

a concentration of 1.0 EU/mL was obtained by diluting 0.1 mL of endotoxin stock into (X-1)/10 mL of LAL reagent water, where X equals the endotoxin activity of the vial. The remaining three standards were prepared by diluting 0.5 mL of the 1.0 EU/mL solution into 0.5 and 1.5 mL of LAL reagent water and 0.1 mL of the 1.0 EU/mL solution into 0.9 mL of LAL reagent water in order to get, respectively, 0.5, 0.25 and 0.1 EU/mL concentrations. Tubes containing the four standard solutions were vigorously vortexed for at least one minute. Samples previously frozen in 2 mL microcentrifuge tubes were thawed and vigorously vortexed for at least one minute. Dilutions were prepared by diluting the appropriate volume of sample into the necessary volume of LAL reagent water in order to yield the required values; dilution tubes were also vortexed for at least one minute immediately after the dilution was done. The Limulus amebocyte lysate and the chromogenic substrate were reconstituted with 3.0 and 6.5 mL of LAL reagent water, respectively. The substrate was prewarmed to 37° C ± 1.0° C and appropriate amounts of both reagents, according to the number of tests, were pipetted in reagent reservoirs (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) immediately before the assay was to start.

Standards, negative controls, samples and spiked samples were always run in duplicate. During the first trials, triplicate samples were used but after having tested the reproducibility of the test (see Section 3.3.2) it was found that duplicates were enough. A 96 well microplate (Corning Inc, Corning, NY) was pre-equilibrated at 37° C ± 1.0° C in a FL600 microplate reader (BioTek Instruments Inc, Winooski, VT) which has incubation capabilities, and 50 µL of sample or standard were carefully dispensed in the appropriate well. Spiked samples were done by adding 10 µL of a 2 EU/mL solution into the appropriate wells to yield a known spiked concentration of 0.4 EU/mL per well, as suggested by the manufacturer. Knowing the sensitivity of the test, special care was given to the pipetting rate. At t = 0, 50 µL of LAL were pipetted from the reagent reservoir into the first column of the microplate using an 8-channel pipettor; a 10 second interval pipetting rate was then used between each one of the remaining columns. Once all wells were filled with LAL the microplate was slightly tapped to facilitate mixing. The microplate was returned into the incubator for 10 minutes and at the end of this time 100

µL of the prewarmed substrate were added following the same pipetting rate as the one used to add LAL. Mixing was done again by tapping the side of the microplate and this one was returned into the incubator for another 6 minutes. At t = 16 minutes, 100 µL of 25% V/V glacial acetic acid (LabChem Inc, Pittsburgh, PA) were added as a stop reagent, keeping the same pipetting rate that was used with the previous two reagents. Once all wells were filled, mixing was performed for a third time. Absorbance was read at 405 nm for each one of the wells with an ELx808 microplate reader (BioTek Instruments Inc, Winooski, VT) using KC4 microplate reader software (BioTek Instruments Inc, Winooski, VT).

Endotoxin concentrations were then calculated by linear regression of the endotoxin standards absorbance values. The mean absorbance for each one of the four standards was corrected with the mean absorbance of the negative control by subtracting the latter from the previous ones in order to give a mean Δ absorbance value for each standard. A calibration curve was built by plotting these four mean Δ absorbance values against endotoxin concentrations (Microsoft® Office Excel 2003) and by adjusting a best-fit straight line. It was not necessary to force this line to pass through the origin since the mean Δ absorbance values had already been corrected with the mean absorbance of the negative control. Absorbance of samples measured in the same microplate as the standards was also corrected by subtracting the mean absorbance of the negative control. Unknown endotoxin concentrations could then be obtained from the linear equation of the calibration curve (see appendix A).

To ensure that other substances in the samples were not interfering with the LAL reaction giving as a result a lower final Δ absorbance, concentrations of the spike were found by subtracting the diluted unspiked sample concentration from the diluted spiked sample concentration. According to the manufacturer (CAMBREX, 2006a), this difference should equal the known concentration of the spike, 0.4 EU/mL ± 25%; if not, the sample will require higher dilutions to overcome inhibition.

3.3.2. Reproducibility

In order to guarantee high-quality data and to ensure that good experimental techniques were implemented, a reproducibility test was performed on triplicate samples during the first endotoxin concentration measurements done with the LAL assay. The success of the test is reflected in a low coefficient of variation (CV) which is defined as 100 times the standard deviation of a set of values divided by the mean and expressed as a percent (CAMBREX, 2006a). Values below 10% for the coefficient of variation of the absorbances read with the LAL assay are acceptable; moreover, coefficients of variation around 3–4% for the 1 EU standard were attained as stated by the manufacturer. Based on these results, and for budgetary reasons, it was decided to reduce the number of replicates from 3 to 2. Subsequent reproducibility tests were performed on duplicates showing similar 3–4% C.V. values.

3.4. Recombinant factor C test

3.4.1. Experimental procedure

A second method was used to measure endotoxin concentrations: the 192 test kit pyrogene® recombinant factor C endotoxin detection system (catalogue number 50-658U, Cambrex Bio Science Walkersville, Inc, Walkersville, MD). This comes with a lyophilized endotoxin standard, an rFC enzyme solution, a fluorogenic substrate and an rFC assay buffer. As with the LAL method, samples needed to be within the 6.0 – 8.0 pH range, a situation that was solved by diluting them using the same LAL reagent water as before. Standards and dilutions were prepared similarly in borosilicate glass disposable culture tubes (Borex®).

The methodology to prepare the standards and samples was done exactly as stated by the producer (CAMBREX, 2006b); however, the procedure to measure the endotoxin concentrations had to be adjusted to fit the specific microplate reader model and software used.

Lyophilized endotoxin from *E. coli* O55:B5 strain was reconstituted with LAL reagent water to yield a 20 EU/mL stock solution; depending on the activity of a specific vial more or less reagent water was added. Once reconstituted, endotoxin stock solution was vigorously mixed for at least 15 minutes at room temperature. The recombinant factor C assay is linear in the 0.01 – 10 EU/mL range hence four standards have to be prepared within this range. For this, a first standard containing 10 EU/mL was prepared by adding 0.5 mL of the 20 EU/mL stock solution into 0.5 mL of LAL reagent water. Two serial dilutions were subsequently done by pipetting 0.1 mL of the 10 EU/mL solution into 0.9 mL of LAL reagent water to give a 1.0 EU/mL solution and then 0.1 mL of this solution into 0.9 mL of LAL reagent water to give a 0.1 EU/mL solution. Although the protocol suggests that one should prepare a final 0.01 EU/mL standard, it was decided to prepare a 0.05 EU/mL solution instead in order to avoid having very close values with the negative control. This last solution was obtained by pipetting 0.5 mL of the 0.1 EU/mL solution into 0.5 mL of LAL reagent water. As with the LAL assay, all solutions were vigorously vortexed for at least one minute.

In a 96-well microplate (Corning Inc, Corning, NY), 100 µL of negative controls, standards, samples and spiked samples were pipetted in duplicate to the appropriate wells. Reproducibility tests also showed that duplicates were sufficient instead of triplicates. Spiked samples were done by adding 10 µL of the 1 EU/mL solution to the corresponding wells. Once all wells were filled, the microplate was pre-incubated for a minimum of 10 minutes at 37° C in a FL600 microplate reader (BioTek Instruments Inc, Winooski, VT). During this time, the working reagent was prepared by adding the rFC enzyme solution, assay buffer and fluorogenic substrate in a 1:4:5 ratio, respectively, to a reagent reservoir and mixed gently. Special attention was given to the order of mixing, as recommended. Carefully, 100 µL of the working reagent were added to each well using an 8-channel pipettor. Fluorescence was read at time zero with the FL600 microplate reader at emission/excitation wavelengths of 360:40 and 460:20, respectively, after having shaken the microplate for 10 seconds at level 1. The sensitivity of the reader was set at 85 so that all four standards would fall into the fluorescence range (see section 3.4.2 for the sensitivity determination), and fluorescence values were acquired using KC4

microplate reader software (BioTek Instruments Inc, Winooski, VT). The microplate with the samples was incubated for one hour and at the end of this period fluorescence was read again under the same conditions as before. The difference between the one-hour and time zero readings for the mean fluorescence values of the standards, samples and spiked samples (Δ mean fluorescence) was corrected with the negative control by subtracting the latter from the previous ones to give the net Δ mean fluorescence.

The calibration curve was obtained by plotting the log net Δ mean fluorescence of the standards against log endotoxin concentrations (Microsoft® Office Excel 2003) and adjusting the best-fit curve. Endotoxin concentrations in the samples and spiked samples were obtained according to the standard curve previously obtained (see appendix B). Similarly to the LAL method, product inhibition can occur when substances in the sample interfere with the enzyme reaction giving a lower net Δ mean fluorescence. Lack of product inhibition was determined by subtracting the endotoxin concentration of the unspiked sample from the endotoxin concentration of the spiked sample. The difference between these two values should equal the concentration of the spike, in this case 0.1 EU/mL, within the range of 50 – 200% (CAMBREX, 2006b).

3.4.2. Sensitivity determination

Relative Fluorescence Unit (RFU) is the standard unit for fluorescence signals. This unit is arbitrary since the real fluorescence signal is converted to an electronic signal by adjusting the sensitivity setting or the gain setting of the specific microplate reader used. A weak signal can be boosted by adjusting to a higher sensitivity setting and in the same manner a signal that is too strong can be tuned down by adjusting to a lower sensitivity setting.

In the case of the rFC assay, the 0.01 – 10 EU/mL endotoxin concentration range corresponds to a 3-log range which correlates linearly with a 3-log RFU range. When the sensitivity of the reader is tuned too low the 0.01 EU/mL may be difficult to detect, or its

fluorescence value may be too close to that of the negative control. To avoid this, sensitivity should be tuned higher; however, if it is tuned too high, the 10 EU/mL standard will be off-scale. The microplate reader used has a fluorescence range from 0 to 99999 thus a test was performed to adjust the reader sensitivity so that the higher fluorescence value (10 EU/mL standard) was lower than 99999 and the smallest (negative control) would not be too close to the 0.01 EU/mL standard.

To do this, a microplate with four duplicate standards was prepared and the instrument was set at three different sensitivities selected based on the recommendations on both Cambrex laboratories and BioTek instruments. The sensitivity values chosen were 50, 60 and 80. The BioTek FL600 model allows one to take simultaneous measurements at different sensitivities. With these values, the normal rFC protocol was followed and fluorescences were read at time zero and one hour later. It was found that at sensitivity 80, the uncorrected mean fluorescence for the highest standard was 46684 so in subsequent trials sensitivity was increased to 85. With this setting, the mean fluorescence increased to 98000 leaving a gap of almost 100 RFU between the lowest standard and the negative control (see appendix C). The 85 sensitivity was used as the standard in further experiments.

3.5. Endotoxin inactivation by UV

Although Anderson et al. (2003a) tested much higher doses, fluences of 40 and 100 $mW \cdot s/cm^2$ were selected in order to be in compliance with those commonly used in drinking water treatment facilities in Europe and established in North American guidelines (Hofmann et al., 2004). Water samples from plant B were labelled "raw water" and "filtered water", according to their source, and additional spiked samples were created by adding a known amount of endotoxin (E8029-1VL endotoxin standard, Sigma-Aldrich, Oakville, ON) to both raw and filtered water samples to yield an initial endotoxin concentration of about 100 EU/mL. The purpose of this was to compare the effect of inactivation of high initial endotoxin concentration samples vs. natural water sample concentrations.

UV irradiation was performed with a collimated beam apparatus. A mercury low-vapour pressure UV lamp emitting at a wavelength of 254 nm was mounted over a collimating tube. UV fluences were determined following the method of Bolton and Linden (2003) to correct the incident intensity for the reflection at the water surface as well as the absorption of UV light by water in order to calculate the average intensity of the lamp. UV transmittance at 254 nm of each one of the samples was calculated with an Ultrospec 3300 pro UV/visible spectrophotometer (Biochrom, Cambridge, UK) and the incident intensity of the UV lamp was measured with an IL1400A radiometer furnished with an SUL240 probe (International Light, Inc, Newburyport, MA).

Volumes of 10 mL of the unspiked and spiked raw and filtered water were poured into 30 mm diameter x 21 mm deep Petri dishes (Kimble glass, Inc. Vineland, NJ) in duplicate and placed one by one beneath the collimated beam for the time necessary to reach the required UV fluence. Samples were continuously stirred and covered with aluminium foil immediately after the exposure time had elapsed. Aliquots of each sample were transferred into 2 mL microcentrifuge tubes, covered and frozen for later endotoxin determination. Negative controls were performed by stirring a sample for the same time but without exposing it to UV light.

All samples and controls were measured by both the LAL and the rFC assays. A total of four sample sets was done, two for raw water and two for filtered water. Endotoxin concentrations were calculated once with the LAL test and once with the rFC test.

3.6. Endotoxin inactivation by free chlorine

3.6.1. Chlorine demand curves

Since free chlorine experiments were performed in filtered water from plant B, the chlorine demand curves for this specific type of water had to be established. This was done by adding known amounts of sodium hypochlorite to corresponding 100 mL water

samples and then measuring the free residual chlorine by amperometric titration, as described in Standard Methods (APHA et al., 1998). An amperometric titrator was used (Hach Company, Loveland, CO) equipped with a titrastir to keep the sample stirred, and a 5 mL graduated Kimax manual burette (Kimble Products, Vineland, NJ) was used to add a standard phenylarsine oxide solution (0.00564 N, Fisher Scientific, Fair Lawn, NJ). As the phenylarsine oxide was added, the changes in current were observed on the display of the titrator and the titration was stopped when the numbers did not change any further. Free residual chlorine was then calculated with the following equation:

$$mg\ Cl\ as\ {}^{Cl_2}\!/_{L} = \frac{mL\ titrant \times 200}{mL\ sample} \qquad (3.1)$$

This process was repeated with nine different samples and the free residual chlorine values were plotted against the chlorine added (Fig. 3.2). With these values it was possible to determine the amounts of chlorine to be added in order to get 0.8 and 1.6 mg/L free residual.

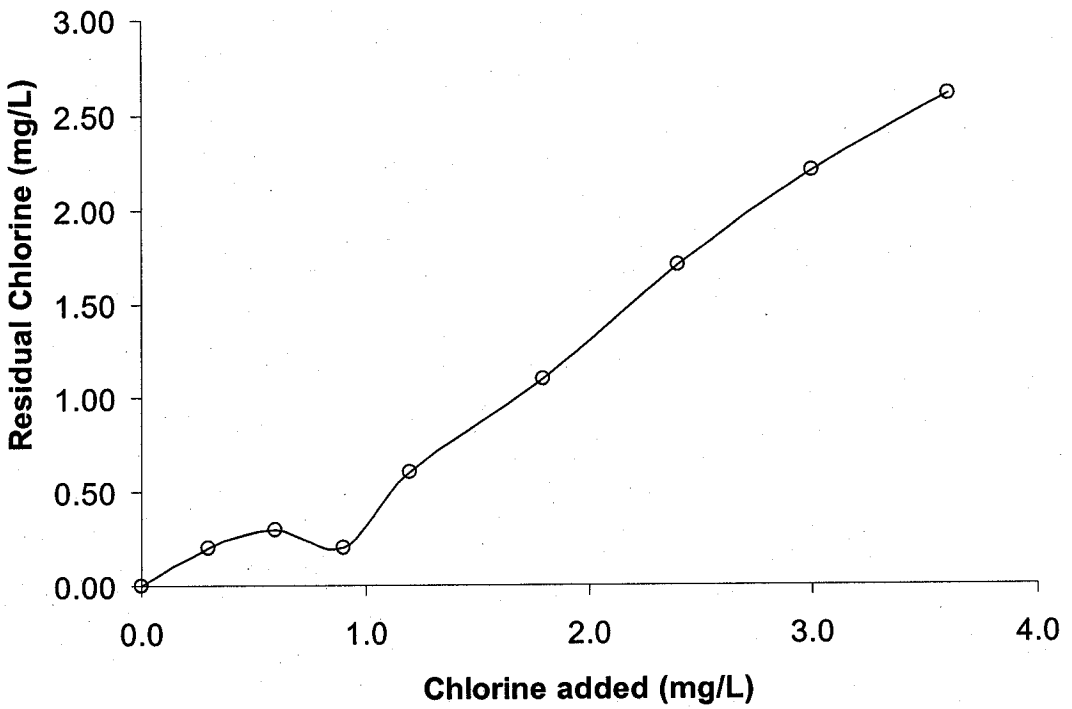

Figure 3.2. Chlorine demand curve for filtered water from Plant B.

3.6.2. Experimental procedure

Endotoxin inactivation by free chlorine tests was performed exclusively with filtered water samples from plant B as opposed to UV experiments where raw water samples were also analyzed. Furthermore, samples were not spiked. This decision was taken in order to keep realistic conditions considering that drinking water treatment facilities usually perform chlorination after filtration. Thus the objective of these experiments was to test the effect of chlorine on low endotoxin concentrations. The mean pH of the samples was 7.6 which indicates that no additional adjustments had to be done since this value was already within the required range for both LAL and rFC assays.

Sodium hypochlorite was chosen as the preferred oxidant and two different free chlorine residual doses, 0.8 mg/L and 1.6 mg/L, as well as two detention times of 20 minutes and 1 hour were selected, once again with the intention of maintaining realistic conditions used in drinking water treatment facilities. Tests were performed in 250 mL graduated Erlenmeyer flasks which were made chlorine-demand free by exposing them to water with 10 mg/L of chlorine overnight and rinsing them with chlorine-demand free water prior to be rendered endotoxin-free by following the usual method. Flasks were covered and wrapped with endotoxin-free aluminium foil to keep them in the dark in order to avoid potential photocatalytic reactions, as suggested by Anderson et al. (2003b).

A set of 14 flasks was prepared following the protocol described in Table 3.1 in order to have duplicates for all controls, chlorine residuals and retention times. Volumes of 50 mL of sample were poured into the flask and, at time zero, the appropriate amount of sodium hypochlorite (as 4%), obtained from the chlorine demand curve (see Section 3.6.1), was added to all of them but two which were used to calculate the initial concentration of endotoxin in the samples. Flasks were mixed in a Junior Orbital Shaker (LabLine Instruments, Inc., Melrose Park, Ill) at approximately 150 rpm for 20 minutes for flasks from 1 to 6 and for 1 hour for flasks from 7 to 12. At the end of each detention time, sodium thiosulfate (N/10 solution, Fisher Scientific, Nepean, ON) was added to

four out of the six flasks in the amounts calculated as twice the equimolar requirement in order to quench any remaining chlorine residual; the other two flasks where no sodium thiosulfate was added served as the oxidant-free controls. Flasks were mixed for another minute to guarantee that all chlorine was quenched and 1.8 mL aliquots were transferred into 2 mL microcentrifuge tubes that were covered and frozen for later endotoxin concentration determination.

The complete experiment was performed three times during the month of September 2006 and endotoxin concentrations in all the samples were measured using the rFC assay no more than 24 hours after the endotoxin inactivation by chlorine test was carried out.

Table 3.1. Protocol for the free chlorine experiments.

Flask Number	Sample Volume (mL)	Desired Residual Chlorine (mg/L)	Chlorine to be added (mg/L) (from Fig. 3.2)	4% Chlorine to be added (μL)	Detention time (min)	Sodium thiosulfate needed (μL)
1	50	0.8	1.4	35	20	22
2	50	0.8	1.4	35	20	22
3	50	1.6	2.4	60	20	43
4	50	1.6	2.4	60	20	43
5[1]	50	0.8	-	-	20	22
6[1]	50	1.6	-	-	20	43
7	50	0.8	1.4	35	60	22
8	50	0.8	1.4	35	60	22
9	50	1.6	2.4	60	60	43
10	50	1.6	2.4	60	60	43
11[1]	50	0.8	-	-	60	22
12[1]	50	1.6	-	-	60	43
13[2]	50	-	-	-	-	-
14[2]	50	-	-	-	-	-

[1] Oxidant-free controls.
[2] Initial endotoxin concentration controls.

3.6.3. rFC – chlorine interference test

Initial results from samples taken inside the treatment plants as well as throughout the network consistently showed higher endotoxin concentrations in samples where the presence of chlorine was identified (samples taken immediately after chlorination, chlorine booster station). This observation led to the conclusion that chlorine could be interfering with the rFC assay. To prove this hypothesis, several chlorinated sample were tested.

In the first experiment, samples following chlorination from plants A and B were measured for endotoxin concentration before and after the addition of sodium thiosulfate (Fisher Scientific, Nepean, ON). Duplicate measurements were performed following the standard rFC method.

In a second experiment, distilled water was spiked with approximately 80 EU/mL of *E. coli* O55:B5 strain endotoxin (Cambrex Bio Science Walkersville, Inc, Walkersville, MD). To test the effect of free chlorine, two samples were prepared by adding 40 µL and 80 µL of a 200 mg/L sodium hypochlorite solution to two 50 mL samples of the previously spiked distilled water to yield, respectively, chlorine doses of 0.8 and 1.6 mg/L. Two more samples were prepared to obtain a combined chlorine dose of 0.8 and 1.6 mg/L by adding 40 µL and 80 µL of a 200 mg/L sodium hypochlorite solution and 56 µL and 112 µL of a 0.001 mol ammonium hydroxide solution (LabChem Inc, Pittsburgh, PA) mixed in a 1:1 molar ratio to 50 mL volumes of spiked distilled water in order to test the effect of combined chlorine. The initially spiked sample was used as a negative control and was simultaneously measured with the remaining four samples for endotoxin concentration. In this second experiment none of the samples was quenched with sodium thiosulfate in order to determine the endotoxin concentration in samples whose chlorine concentration was known. Endotoxin detection was performed in duplicate in a standard rFC assay.

3.7. Endotoxin inactivation by ozone

3.7.1. Experimental procedure

Endotoxin inactivation by ozone followed the same protocol as that used with free chlorine in terms of the samples used. Filtered water samples from plant B were exposed to different ozone doses and retention times. In order to do this, a deionized water volume was saturated with ozone as shown in Figure 3.3. Extra dry 99.6% oxygen (MEGS Speciality Gases, Inc., Montreal, QC) was fed into an Ozo 2 VTT ozone generator (Ozomax, Ltd., Granby, QC) with a nominal production rate of 10 g/hr when fed with oxygen and equipped with a gas flow rate reader. Oxygen pressure was controlled by a regulating valve connected to the oxygen cylinder. Ozone was pumped into a gas washing bottle by means of a glass-fritted diffuser and transferred into the deionized water until the solution was supersaturated with a steady state concentration of 3.5 mg/L of ozone at room temperature. The complete setup was housed inside a fume hood to avoid possible ozone exhaust inside the laboratory. The actual ozone production rate of the generator was calculated by iodometric titration as described in Section 3.7.2 giving as a result 8.7 g/hr.

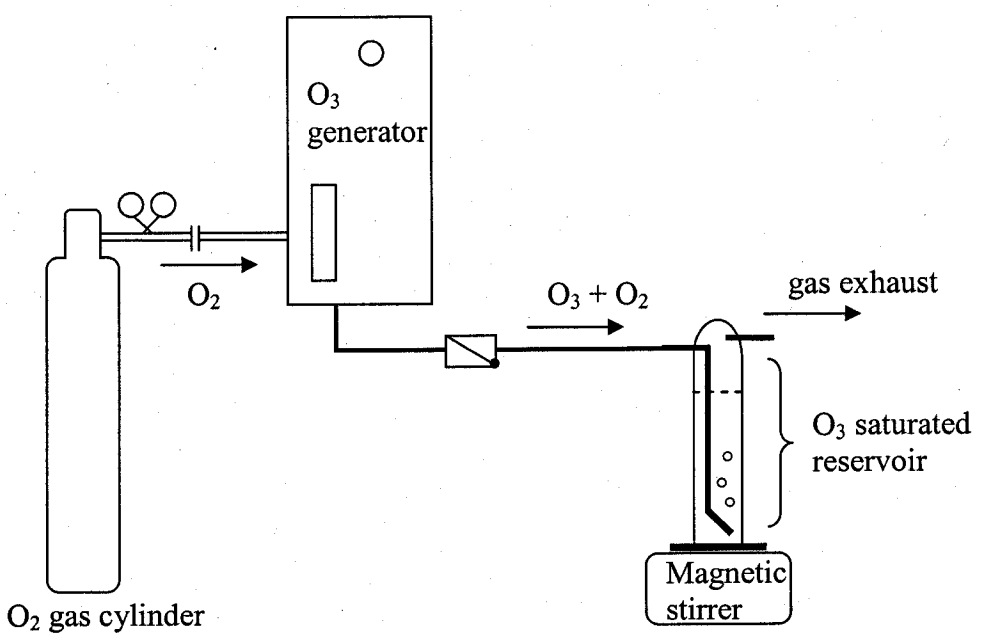

Figure 3.3. Diagram of ozone contacting apparatus.

Two ozone residual concentrations of 0.5 and 1.0 mg/L measured after two retention times of 5 and 20 minutes were chosen based on values used in typical drinking water treatment facilities in order to yield Ct values of 2.5 and 20 (mg·min)/L. Tests were performed in a similar fashion as previously done for free chlorine. The ozone-saturated solution was added in the amounts described in Table 3.2 to the corresponding volumes of filtered water samples into 150 mL Erlenmeyer flasks. These were previously treated to render them ozone-demand free by soaking them in a saturated ozone solution overnight and endotoxin-free by the usual method. Sample and ozone-saturated solution volumes were chosen so that as much as possible of the flask was filled to avoid a headspace (ozone degasification). Flasks were covered with endotoxin-free aluminium caps, wrapped in aluminium foil to avoid photocatalytical reactions and mixed in a Junior Orbital Shaker (LabLine Instruments, Inc., Melrose Park, Ill) at approximately 150 rpm for either 5 or 20 minutes. Duplicates for each Ct value as well as initial and ozone-free controls were prepared.

At the end of each retention time, a 5 mL sample aliquot was withdrawn in order to measure ozone residuals (see Section 3.7.3); immediately after sodium thiosulfate (N/10 solution, Fisher Scientific, Nepean, ON) was added to the corresponding flasks in the amounts described in Table 3.2. Flasks were mixed one more minute to ensure that ozone was completely quenched. Sample aliquots of 1.8 mL were transferred into 2 mL microcentrifuge tubes which were immediately frozen and stored for endotoxin concentration determination.

The complete experiment was performed twice during the month of November 2006 and rFC was chosen as the measuring method for endotoxin concentration in all of the samples.

Table 3.2. Protocol for the ozone experiments.

Flask Number	Ozone Dose (mg/L)	Detention time (min)	Ct value (mg min/L)	Sample Volume (mL)	Ozonated solution to be added (mL)	Sodium thiosulfate needed (μL)
1[1]	-	-	-	140	-	-
2[1]	-	-	-	140	-	-
3	0.5	5	2.5	120	20	26
4	0.5	5	2.5	120	20	26
5[2]	-	5	2.5	140	-	26
6[2]	-	5	2.5	140	-	26
7	1.0	20	20.0	100	40	59
8	1.0	20	20.0	100	40	59
9[2]	-	20	20.0	140	-	59
10[2]	-	20	20.0	140	-	59

[1] Initial endotoxin concentration controls.
[2] Ozone-free controls.

3.7.2. Determination of the ozone production rate (gas phase)

The ozone production rate of the ozone generator was determined by iodometric titration, as described by Rakness et al. (1996). A slightly buffered potassium iodide stock reagent (called $NBKI_e$) was prepared by adding 20 g of KI (Fisher Scientific, Nepean, ON) to 1 L of freshly distilled water. Immediately afterwards, 7.3 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$, Fisher Scientific, Fair Lawn, NJ) and 3.5 g of monopotassium dihydrogen phosphate (KH_2PO_4, Anachemia, Montreal, QC) were added to the solution. The stock reagent was stored in a brown bottle and refrigerated until it was used.

A 50 mL burette was filled with a 0.1 N sodium thiosulfate solution (Fisher Scientific, Nepean, ON) and kept ready for titration. Two gas washing bottles equipped with a fritted-glass diffuser were each filled with 250 mL of the 2% $NBKI_e$ solution and they were connected in series with the ozone generator. The second bottle acted as a guard detector. Experiments were performed at 24° C. Ozone was bubbled through the KI solution at a flow rate of 2.83 L/min and a pressure of 10 psi (68.94 kPa) for 1 minute. Formation of iodine was observed in both bottles since the solution in both of them

turned yellow. The KI solution from both bottles was mixed in a single bottle and then a 100 mL volume was transferred into a 250 mL Erlenmeyer flask. This sample was acidified with 2.5 mL of sulphuric acid and immediately titrated with the 0.1 N sodium thiosulfate solution kept in the burette until the KI solution turned to a pale yellow. At this point, 1.5 mL of starch indicator solution (0.5%, LabChem Inc, Pittsburgh, PA) were added, turning the solution violet, and titration continued until the violet colour had completely disappeared. The final titrant volume was recorded and the ozone concentration (C_{O3}) in the gas as well as the ozone production rate (OPR) were calculated, respectively, using the following two equations:

$$C_{O_3} = \frac{24 \times V_t \times N_t}{V_{O_3}} \qquad (3.2)$$

$$OPR = C_{O_3} \times Q_{O_3} \qquad (3.3)$$

where: V_t = volume of sodium thiosulfate titrant used, in mL.

N_t = normality of sodium thiosulfate, 0.1 N.

V_{O3} = volume of ozone gas bubbled through the KI solution, 2.83 L.

Q_{O3} = flow rate of ozone gas bubbled, 2.83 L/min.

The iodometric test was performed three times giving an average concentration of 51.30 ± 1.58 mg/L and an ozone production rate of 145.2 ± 4.48 mg/min, which is equivalent to approximately 8.7 g/h, slightly lower than the 10 g/h nominal production rate claimed by the manufacturer.

3.7.3. Determination of ozone residuals (liquid phase)

Ozone residuals were measured by the indigo colourimetric method, as described in Section # 4500–Ozone from Standard Methods (APHA et al., 1998). An indigo stock solution was prepared by adding 770 mg of potassium indigo trisulfonate ($C_{16}H_7N_2O_{11}S_3K_3$, Sigma-Aldrich, Montreal, QC) to 1 L distilled water and 1 mL concentrated phosphoric acid. The stock solution was stored in a brown glass bottle and

kept in the dark. Since the residual ozone concentrations were expected to be higher than 0.3 mg/L, an indigo reagent II solution was prepared by diluting 100 mL of the indigo stock solution, 10 g of sodium dihydrogen phosphate (NaH_2PO_4) and 7 mL of concentrated phosphoric acid into 1 L distilled water.

The gravimetric method was chosen as the preferred option since sample weights were more accurately measured than sample volumes producing thus better quality data. Sample aliquots of 5 mL were withdrawn with a syringe from either the gas washing bottles, through a septum located on the side of the bottle, or from the flasks in which the ozone inactivation experiments were carried out. A 10 mL volume of indigo reagent II was added to a 100 mL volumetric flask and diluted with distilled water; this sample was labelled as the blank. A second 100 mL volumetric flask was weighed with a Mettler PC 2000 balance (Mettler Instruments, Zurich), this value was recorded as *tare weight*. 10 mL of indigo reagent II were added as well as 4 mL of ozonated sample and the flask was weighed again, this new value was recorded as *weight before distilled water*. Finally, distilled water was added to the 100 mL mark and the flask was weighed for a third time, recording data as *total weight*. The flask containing indigo reagent II, sample and distilled water was carefully swirled until the solution turned a light blue.

The absorbance of both solutions (blank and sample) was immediately measured at 600 nm using 5 cm cells and a Hewlett Packard 8452 diode array spectrophotometer with a wavelength range 190 – 1100 nm and a 2 nm resolution. The ozone residuals were then calculated with the following equation:

$$mg\ O_3 / L = \frac{(A_B \times 100) - (A_s \times V_T)}{f \times V_s \times b} \qquad (3.4)$$

where: A_B, A_s = absorbance of blank and sample, respectively

V_s = volume of sample, mL

= [(weight before distilled water – tare weight) x 1.0 mL/g] – 10 mL

V_T = total volume of sample plus indigo, mL

= [(final weight – tare weight) x 1.0 mL/g]

b = path length of the cell, 5 cm

f = sensitivity factor based on a 20000/cm change in the absorbance per mole of added ozone per litter, 0.42 (cm mg/L)$^{-1}$

3.8. Endotoxin removal by filtration

The extent of endotoxin removal by slow sand filtration was tested during a complete filtration cycle in order to determine if concentrations changed at different points of the cycle and if backwashing of the filter had any effect on the removal rate. One full-scale filter from plant B was chosen for this test. The dimensions of the filter are 15.24 x 7.32 m (50 x 24 ft), giving a surface area of 111.5 m^2. The filter bed is a 75 cm depth sand layer with an effective diameter of 0.6 mm. The filter treats a water flow of approximately 12 m^3/min and the turbidity of the water on the days the samples were collected was approximately 0.21 NTU. The filtration cycle lasts 72 h and the filter is backwashed with air and water on a time-based schedule. The backwash cycle consists of a 2 minutes air phase followed by a 5 minutes water phase. After these two steps, a 13 minutes pre-filtration stage is carried out before the water flow is switched back into circulation. Backwash water is not recycled into the plant's influent but is sent into the sewer.

Six samples were collected at the outlet of the filter throughout the 72 h cycle. For practical reasons the first sample was taken at the end of the cycle, immediately before the backwash sequence started. The second sample was collected seven minutes later immediately after the water backwash phase was over. Thirteen minutes later, when the prefiltration phase was completed, a third sample was obtained and a fourth one a few minutes after that, when the water flow was back in circulation. The fifth sample was collected two days later to obtain the endotoxin concentration after approximately half of the filtration cycle. Finally, one sample was withdrawn at the top of the filter during the water backwash phase to test the endotoxin levels in the water used for backwashing. All samples were collected in endotoxin-free 250 mL brown glass bottles and transported to

the laboratory in a refrigerated box. Aliquots were transferred into 2 mL microcentrifuge tubes and frozen for endotoxin determination within 24 h.

All samples were measured in duplicate with the rFC assay and the test was performed twice during the months of November and December 2006.

3.9. Environmental contamination assay

Based on the first results obtained during the UV inactivation experiments, it was thought that airborne endotoxin could have been contaminating the samples since samples exposed longer to UV light, and thus to air, showed either no endotoxin decrease or higher endotoxin concentrations. In order to clarify this issue, an environmental contamination assay experiment was performed. Samples were prepared and treated under the same conditions as the rest of the samples analyzed in the UV experiments but without being exposed to UV light. UV exposure times ranged between 3 and 9 minutes therefore these time intervals were chosen plus a 1-hour interval as an extreme condition. Triplicate sample aliquots were extracted for later LAL endotoxin measurement.

4. RESULTS AND DISCUSSION

Please note that, due to the rFC – chlorine interference described in Sections 3.6.3 and 4.5, endotoxin concentrations in samples from Sections 4.1, 4.2.2 and 4.2.3 where the presence of chlorine is likely (i.e. samples were withdrawn after chlorination) may be over-estimated since chlorine was not quenched before endotoxin testing.

4.1. LAL vs. rFC comparison

During the first measurements performed in June 2006, samples from plant C were tested with both LAL and rFC methods in order to compare their respective results as well as to establish if there was any consistency between the two assays. Also, two different dilutions for each sample were performed to allow concentrations to fall within the linear intervals where the methods work. Dilutions chosen were 1/10 and 1/40 for LAL and, since the linear range for the rFC assay is broader, dilutions of 1/10 and 1/100 were chosen for this method. The comparison between the two assays is presented in Figure 4.1.

It was found that concentrations measured with the rFC method were consistently lower than those measured with the LAL method for all the samples tested. This difference can be explained mainly by the fact that endotoxin concentrations are measured in terms of activities and these activities are always related to the standard curve used. In the case of rFC the standard curve is prepared with the *E. coli* O55:B5 strain whereas LAL used the *E. coli* O111:B4 strain; reported activities for these strains vary from 5 to 20 EU/ng. Even though both strains come from the same species of bacteria, the activities and the presence of each specific strain in each one of the samples tested may be different.

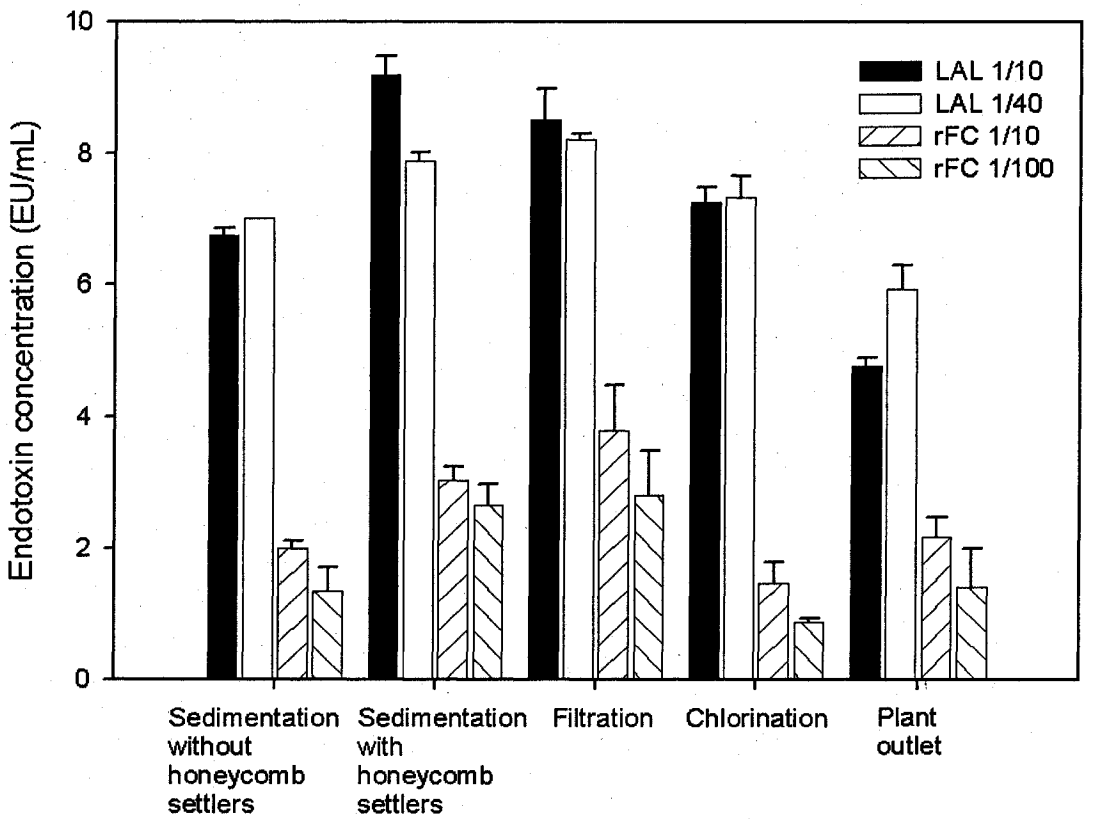

Figure 4.1. LAL/rFC comparison for spring samples from plant C

Based on these results it was then decided to use a 1/40 dilution for measurements performed with LAL and a 1/100 dilution for samples measured with rFC; both of these dilutions were small enough to make the concentration fall within the linear range but large enough to overcome any endotoxin inhibition that could happen due to other elements present in the sample. Also, after having used both LAL and rFC several times it was confirmed that the kinetics as well as the addition of fewer reagents in the rFC assay minimized systematic and human errors; it was thus decided to measure, with a few exceptions, subsequent samples exclusively with this method. Results presented hereafter are, unless specified otherwise, all measured with the rFC test.

4.2. Seasonal variations

4.2.1. Raw waters

Raw water samples at the inlet of the three plants studied were collected during the months of June, August and October 2006 and January 2007. Endotoxin concentrations for these samples are shown in Figure 4.2.

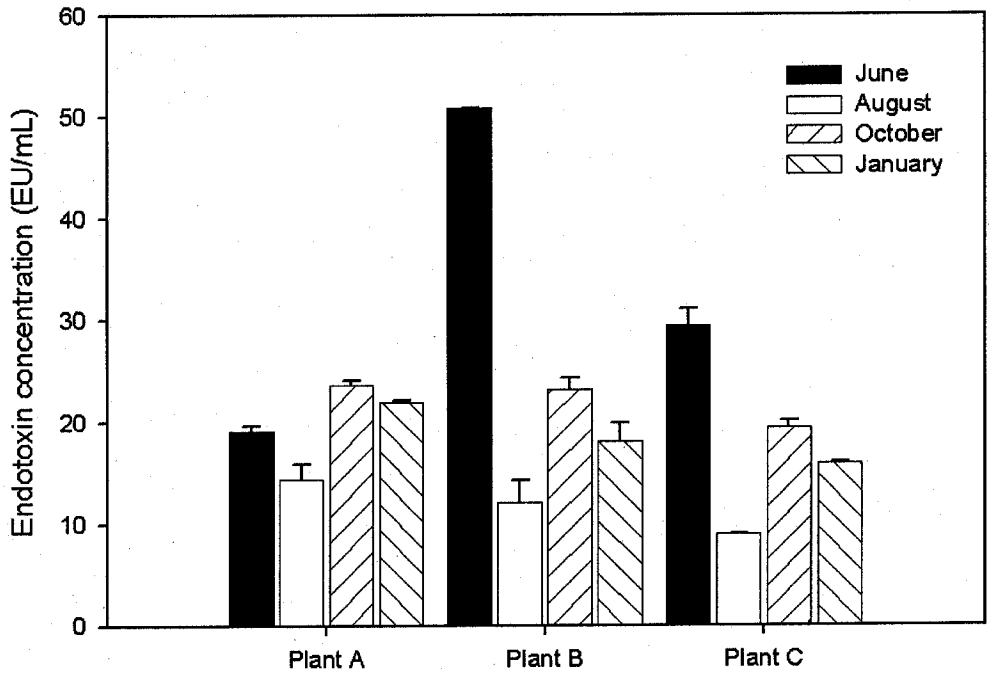

Figure 4.2. Endotoxin concentrations in raw waters

With the exception of the sample taken at plant B in June, all other concentrations ranged between 9 and 30 EU/mL. The high concentration of almost 50 EU/mL for this sample is unlikely and may have been due to contamination of the sample during transport or sample handling inside the lab, since for the remaining three months concentrations for plant B were lower and similar to those from plants A and C. Overall, there is a tendency for concentrations to decrease during August and to increase during the spring and fall (June and October). Contrary to what was expected, concentrations during the winter, when water temperatures are at their lowest levels, were not the lowest of the year; in fact, concentrations during the summer were the lowest. Thus, low temperatures do not necessarily mean low endotoxin levels. One possible explanation for

this trend is that, due to Quebec's weather, spring runoff into the raw water sources may be contributing to the increase in endotoxin concentrations. In contrast, due to the fact that endotoxin can be found free or bound to the cell, although bacteria may have already died, endotoxins may still be present in water bodies, irrespective of the low temperatures during the winter. This idea is supported by Sykora et al. (1980); in their study they found that bacterial counts did not always occur concurrently with high endotoxin levels in water samples.

The differences in concentrations between plants B and C for each season are minor; this is logical since both plants obtain their water from the same intake in the St. Lawrence River. On the other hand, plant A takes its raw water from Lake St. Louis and, except for the sample taken in June, concentrations in samples taken in August, October and January are slightly higher than those for plants B and C. In general, both sources provide water with an endotoxin level within the same range.

Levels found were, generally, lower or in the lower part of the interval than those described in the literature. The three studies that give endotoxin concentrations in raw water samples are those of Burger et al. (1989), Rapala et al. (2002) and Rapala et al. (2006) (see Table 2.1. Section 2.7). While the first study found values as high as 1,100 EU/mL, concentrations in the last two ranged between 4 and 356 EU/mL. One can observe from these studies that endotoxin levels in raw water samples are highly variable and mainly dependent on the water source; however, even though two different sources were included in this study, endotoxin levels did not vary considerably from one source or season to the other.

4.2.2. Drinking water treatment plants

Samples were collected after each treatment stage (see Figure 3.1) at the three treatment plants during spring and summer 2006 and winter 2007 in order to compare, on the one hand, the effect of each process on endotoxin removal and, on the other, the variations throughout the different seasons. Endotoxin concentrations for all collected samples are presented in Figure 4.3.

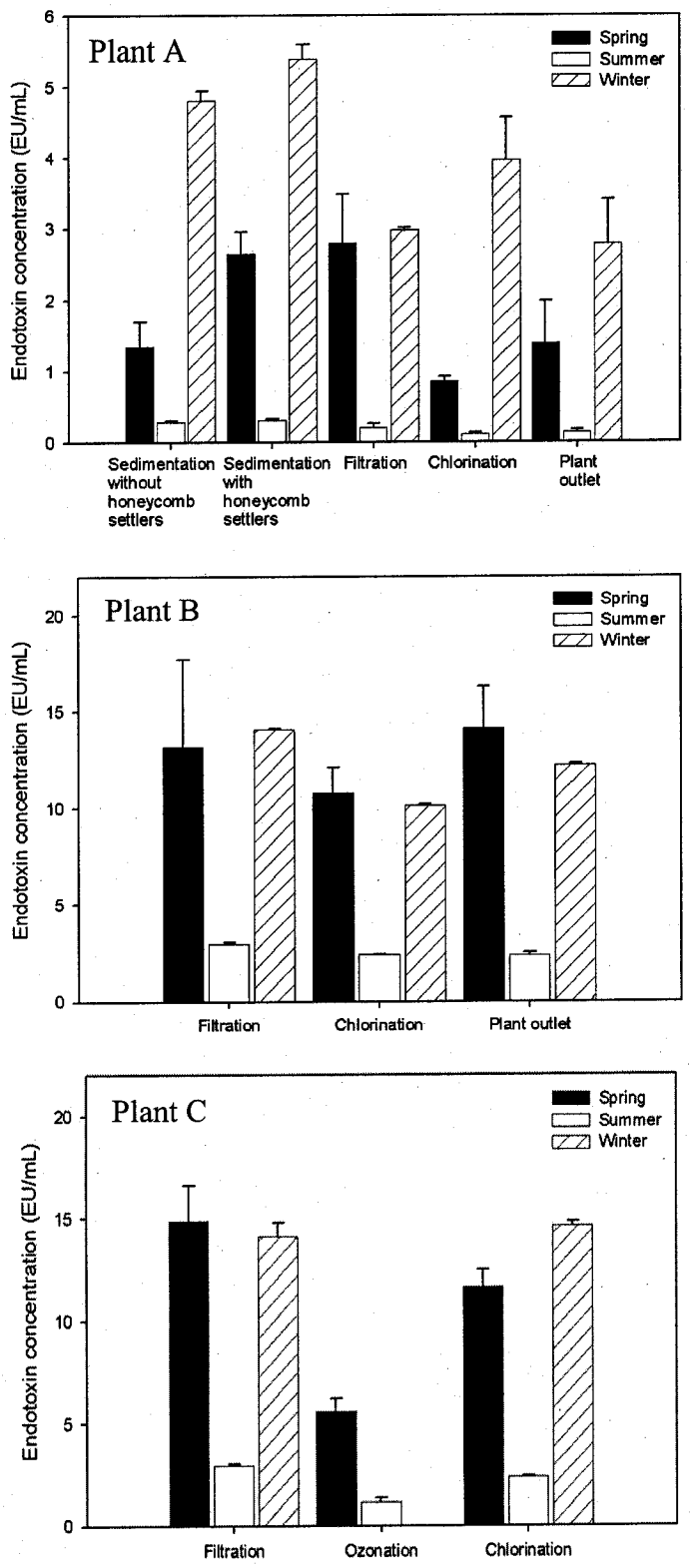

Figure 4.3. Endotoxin concentrations for samples from three water treatment plants

In general, as for the raw water samples (see Figure 4.2), there is a tendency for endotoxin concentrations to be lower during the summer. In the case of plants B and C, concentrations during spring and winter showed only small variations within each process but concentrations during the summer were clearly lower. Similarly, samples from plant A consistently showed higher levels in the winter than in the summer. This may be due to the fact that at the time when the samples were collected in the spring a temporary pre-chlorination step was being implemented at the plant and this could have helped to decrease the levels in the rest of the samples.

Table 4.1 Percentage removals for the three plants studied.

Plant	Sedimentation without settlers*	Sedimentation with settlers*	Filtration	Ozonation	Chlorination**	Overall removal
A	95%	80%	69%	-	46%	90%
B	-	-	70%	-	20%	80%
C	-	-	50%	60%	-4%	73%

* Plant A has two parallel streams, either with or without honeycomb settlers.

** This percentages may be under-estimated due to chlorine interferences.

From Table 4.1, one can see that water treatment processes help to reduce endotoxin levels at large scale facilities. While endotoxin concentrations in raw waters from plant A ranged between 15 and 23 EU/mL, they decreased to approximately 1 EU/mL to 6 EU/mL. In the case of plants B and C a similar reduction is observed from endotoxin concentrations between 9 and 30 EU/mL to between 2 and 14 EU/mL.

Further analyzing each one of the treatment stages, one can see that, concerning the two samples from plant A involving sedimentation (Figure 4.3A), there was a consistent high decrease (between 93 and 98%) in endotoxin concentration for the settling tank devoid of honeycomb settlers as opposed to a removal efficiency of between 75 to 86% for the settler provided with honeycomb settlers. This situation can be explained by the fact that although the honeycomb settlers improve particulate settling, at the same time

there is a larger accumulation of organic matter and other elements, including bacteria, on the honeycomb walls, a situation that facilitates the accumulation of endotoxin inside the tank. From Figure 4.3A, it also appears that filtration was more efficient during the summer and the winter (69% and 46% removal efficiencies, respectively) than during the spring. Chlorination on the other hand, decreased the concentrations more efficiently in the spring (69%) than in the winter (46%). Finally, endotoxin concentrations in the sample taken at the outlet of the plant increased 62% after chlorination during the spring but decreased 30% during the winter.

For samples from plant B (see Figure 4.3B) chlorination decreased endotoxin concentrations between 18 and 28% following sand filtration in all three seasons; however, these concentrations increased again by between 20 and 31% at the outlet of the plant, possibly due to endotoxin accumulation throughout the piping. Chlorination did not have the same effect on samples from plant C (see Figure 4.3C) where endotoxin concentration increased as much as 104% after chlorination following ozonation. Unfortunately the ozone generator at plant C was shut down on the day on which the winter samples were taken so it is not possible to compare the effect of this process with the remaining samples. However, from samples measured in the spring and the summer, it is evident that ozonation has a great effect on endotoxin inactivation with removal efficiencies as high as 60%. Even after filtration has reduced endotoxin levels, ozonation is able to further decrease these levels.

From these results one can see that treatments such as sedimentation, filtration and ozonation are more efficient for endotoxin inactivation and removal, while chlorination has a smaller effect. The fact that sedimentation and filtration are physical processes explains their higher removal efficiency, while it appears that endotoxins are resistant to chemical processes and killing or inactivating bacteria does not necessarily imply the destruction of endotoxins.

Overall, processes carried out at plant A were more efficient for endotoxin removal than those used in plants B and C. In addition, plant A levels were consistently lower due to the fact that the endotoxin concentrations in the raw water were also lower.

These results are in agreement with those found by Burger et al. (1989). In their study, they found that ozonation applied after sand filtration further reduced endotoxin levels in a similar manner to the results shown here. They also found that endotoxin removal was higher at the early stages of the treatment process, a situation that occurred in all three plants A, B and C. While sedimentation for plant A and sand filtration for plants B and C as well as ozonation for plant C removed endotoxin to a higher degree, chlorination decreased endotoxin concentrations but to a lower extent.

Rapala et al. (2002) reported overall reductions in different drinking water treatment plants ranging from 59 to 97%, depending on the type and combination of processes used. In the case of plant A endotoxin reductions ranged between 87 and 93%, placing it as a very efficient one. Plant B showed removal efficiencies between 32 and 80% and interestingly the fact that the ozonation in plant C was inactive during the winter decreased the removal efficiency to only 7%. With ozonation working the efficiency for plant C increased to 73%.

Another study by Rapala et al. (2006) shows high removal efficiencies for sand filtration (36 – 96%) and ozonation (33 – 35%) but lower ones for chlorination (< 0%), in agreement with the results obtained herein.

4.2.3. Distribution system

Three more samples were collected during the spring and summer 2006 and winter 2007. These samples were taken at the furthest hydraulic point in the water distribution network from plants B and C, with the objective to assess the effect of chlorine residuals as well as piping conditions and seasonal variations on endotoxin concentrations. Sampling points were located at 15 km from plant C and 29 as well as 35

km from plant B. Two samples were supplied for plant B from different directions in the network, thus giving additional information. Endotoxin concentrations for these points are presented in Figure 4.4.

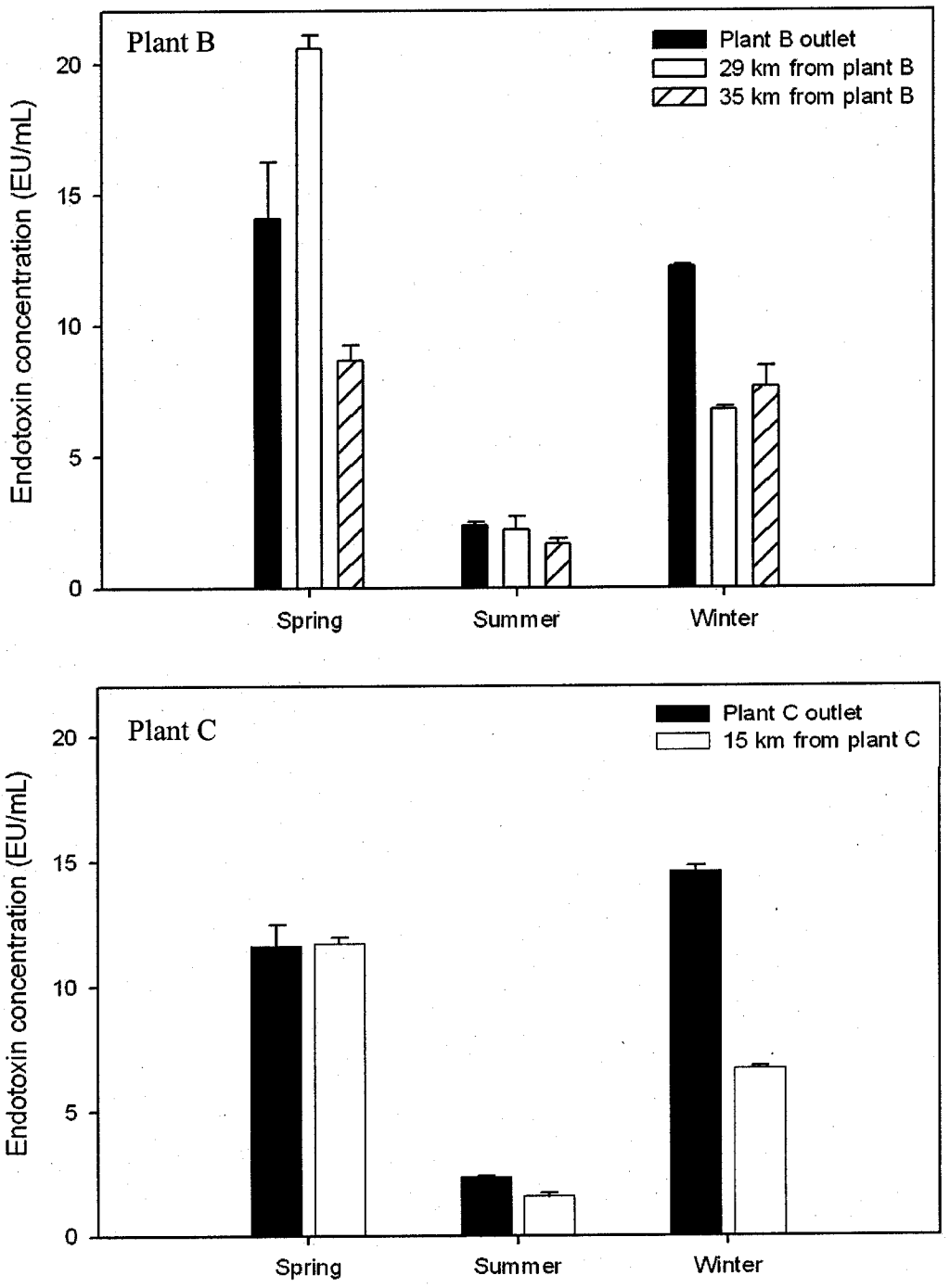

Figure 4.4. Endotoxin concentrations throughout the distribution network supplied by plants B and C

As in the previous cases, there is a general tendency for endotoxin concentrations to be lower during the summer, between 1 and 2 EU/mL, increasing to 6 – 7 EU/mL during the winter and up to 8 – 20 EU/mL during the spring. From Figure 4.4 it can be seen that there was a relatively high concentration during the spring for the point located at 29 km when compared with the remaining two points; this effect is reproduced, to a lesser extent, during the summer. It was then found that this sampling point is downstream from a chlorine booster station and the addition of chlorine may explain a higher endotoxin concentration, since as will be shown later, the presence of chlorine can yield falsely high endotoxin values.

Besides the situation occurring with the chlorine booster station previously discussed, a dependency between endotoxin concentrations and distance between a sampling point and the treatment plant cannot be established since samples come from two different plants. Although in the winter concentrations increased 13% from the point located at 29 km to the one located at 35 km, the increase from 15 km to 29 km was only 1%. During the summer, endotoxin levels increased 8% between the first and the third points but in the spring there was a decrease of 26%. Levels did not increase to concentrations higher than those found in the raw waters, which suggests that the distribution network is able to maintain the low levels reached during treatment.

Only one study by Rapala et al. (2002) measured endotoxin concentrations in a drinking water distribution system. Levels found ranged between 14 and 32 EU/mL in sampling points between 3.5 and 33 km from the plant. Results found in this study do not show a direct relationship between endotoxin concentration and distance from the treatment plant either; endotoxins decreased by as much as 24% between two points but also increased by as much as 68% for a subsequent sample.

4.3. Endotoxin inactivation by UV light

Experiments to assess endotoxin inactivation by UV light were performed in both raw and filtered water samples from plant B. Samples were measured with both rFC and LAL assays because in fact these experiments were performed early in the study, and the

final choice of methodology had not been made. The results for endotoxin inactivation by UV light in raw and filtered water samples can be found, respectively, in Figures 4.5 and 4.6 with sections (a) showing the measurements performed with the rFC method and sections (b) those found with the LAL method. The bars represent the mean of duplicate measurements with T lines delimiting half the range between the two measurements.

As found previously in Section 4.1, rFC values were consistently lower than those obtained with LAL; however, due to improvements in the experimental techniques for both methods, the differences in the concentrations were lower than those observed in Section 4.1.

In general, experiments carried out in raw water samples showed a higher removal, ranging between 8 and 22%. Nevertheless, there is not a clear tendency for endotoxins to decrease as the UV fluence increases since, in some cases, there was a higher endotoxin removal at the lower UV fluence of 40 $mW \cdot s/cm^2$ with a subsequent increase in the concentration when UV fluence was increased to 100 $mW \cdot s/cm^2$. This effect is reproduced irrespective of the sample being spiked or unspiked; a situation that suggests that endotoxin inactivation by UV light is not affected by the initial endotoxin concentration in the sample.

Results from the filtered water samples were more variable than those from the raw water samples. This is shown by the larger error bars obtained, especially when using the LAL assay but, to a lesser extent, also in the case of rFC. Unexpected results such as the endotoxin concentration for the unspiked sample at 40 $mW \cdot s/cm^2$ and the increase in concentration in the spiked samples in Figure 4.6a are probably due to human or procedural errors during the inactivation. This situation led to the idea that environmental contamination could have been affecting the results obtained (see Section 4.8); however, it was found there that increases in endotoxin concentrations by environmental exposure were not significant.

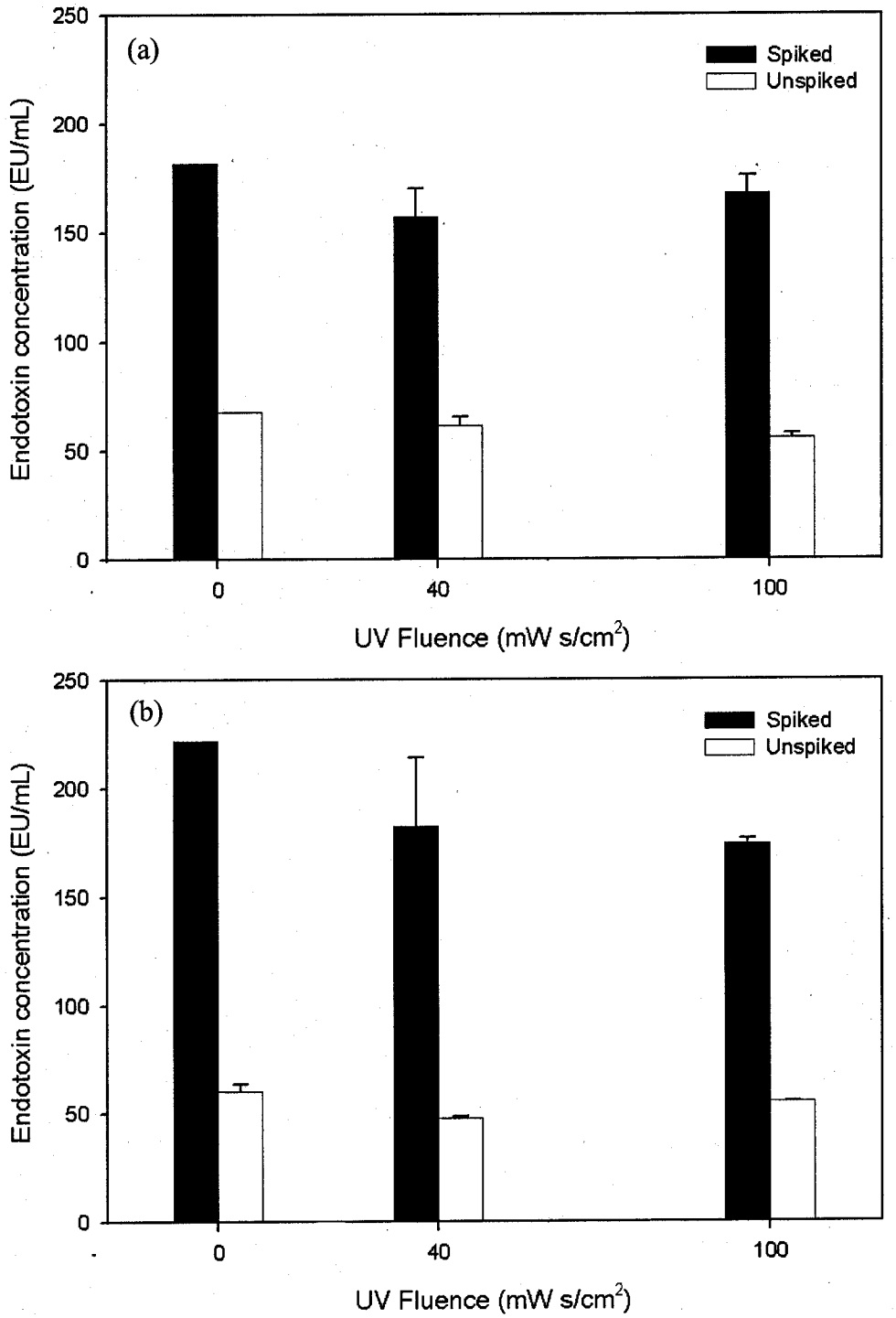

Figure 4.5. Endotoxin inactivation by UV in raw water measured with (a) rFC and (b) LAL

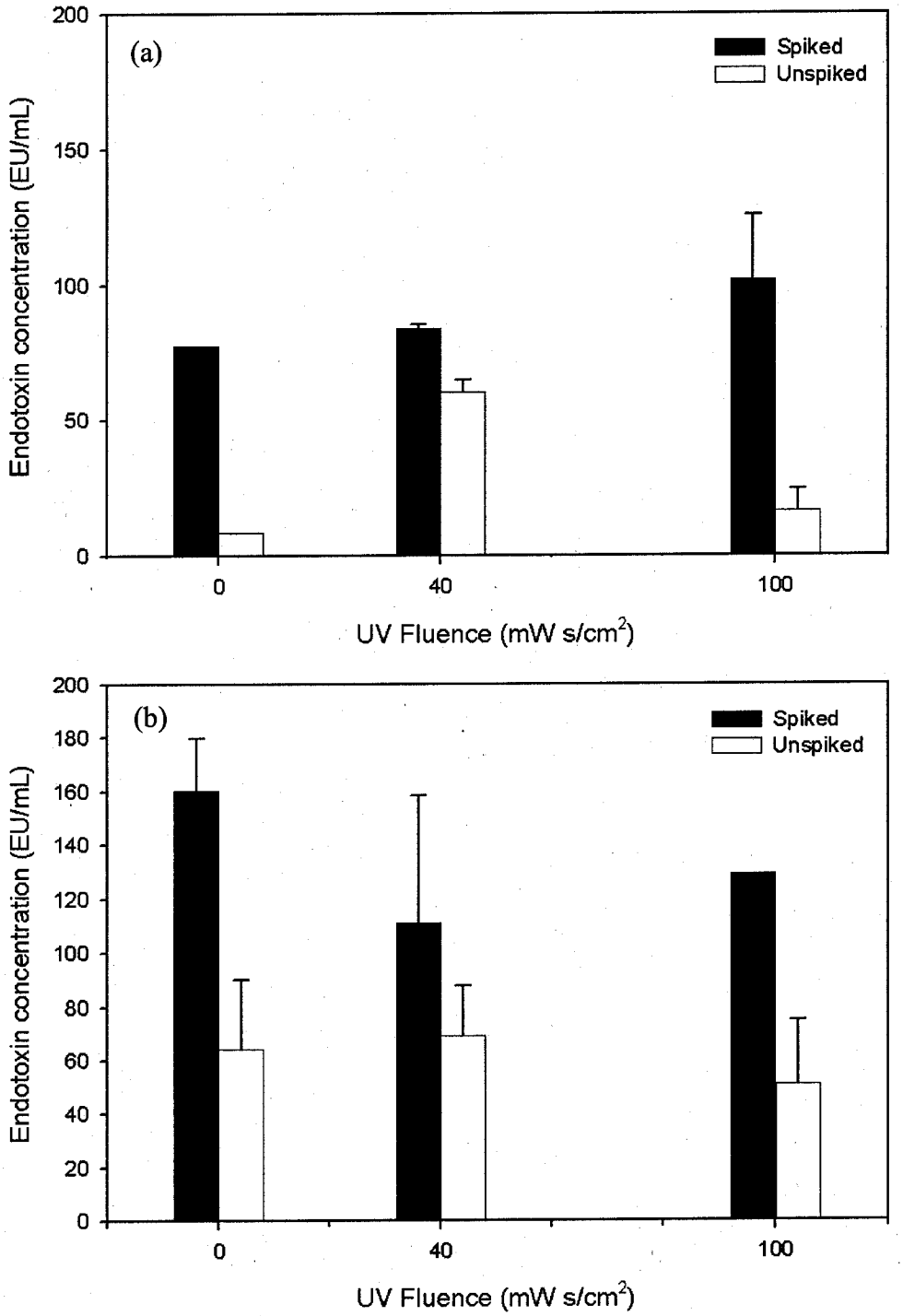

Figure 4.6. Endotoxin inactivation by UV in filtered water measured with (a) rFC and (b) LAL

Endotoxin removal efficiencies for filtered water samples ranged between 20 and 31% for the cases where there was an actual endotoxin reduction. However, endotoxin increases as high as 30% were also measured suggesting that endotoxin inactivation by UV light at 40 and 100 mW•s/cm^2 doses are, in general, erratic and insignificant overall.

A similar experiment was performed by Anderson et al. (2003a) with higher endotoxin removals achieved. However, the authors used much higher UV fluences ranging from 100 to 600 mW•s/cm^2, as well as a medium-pressure UV lamp as opposed to the low-pressure lamp used in the current experiments. Anderson et al. (2003a) developed a linear model to predict theoretical endotoxin concentrations after UV light exposure for smaller UV fluences depending on the initial endotoxin concentration of a given sample. With their model, a final concentration of 178 EU/mL and 145 EU/mL can be achieved when using, respectively, fluences of 40 mW•s/cm^2 and 100 mW•s/cm^2 in a sample with an initial concentration of 200 EU/mL. These values represent 13 and 28% in terms of removal efficiencies.

In the current experiments performed with spiked raw water samples, an initial concentration of approximately 200 EU/mL was used. When applying a fluence of 40 mW•s/cm^2, an endotoxin concentration of 182 EU/mL was achieved, representing a 13% removal; this is consistent with the model proposed by Anderson et al. However, the endotoxin concentration reached with the 100 mW•s/cm^2 dose was only 174 EU/mL, or 8% removal, compared to the 28% expected by Anderson et al.'s model. Larger discrepancies were found with this model when attempting to use it with the unspiked raw water sample. Hence, although their model behaved linearly for the 100 – 600 mW•s/cm^2 interval, it may not do so for lower UV fluences (40 – 100 mW•s/cm^2).

4.4. Endotoxin inactivation by free chlorine

Endotoxin inactivation by free chlorine was tested on filtered water samples from plant B using two chlorine doses of 1.8 and 2.4 mg/L in order to yield, respectively, residual levels of 0.8 mg/L and 1.6 mg/L. Two retention times of 20 and 60 minutes were

used to yield four Ct values of 16, 32, 48 and 96 mg•min/L. The results for endotoxin inactivation by free chlorine are presented in Figure 4.7 with each point representing the mean of duplicate measurements and error bars omitted for clarity.

The problem of chlorine causing interference with the rFC assay, discussed in Section 4.8, was not an issue for the samples analyzed in this section since all were quenched with sodium thiosulfate. Thus values shown in Figure 4.7 reflect the actual effect of chlorine on endotoxin inactivation and not the possible interference between this one and the assay.

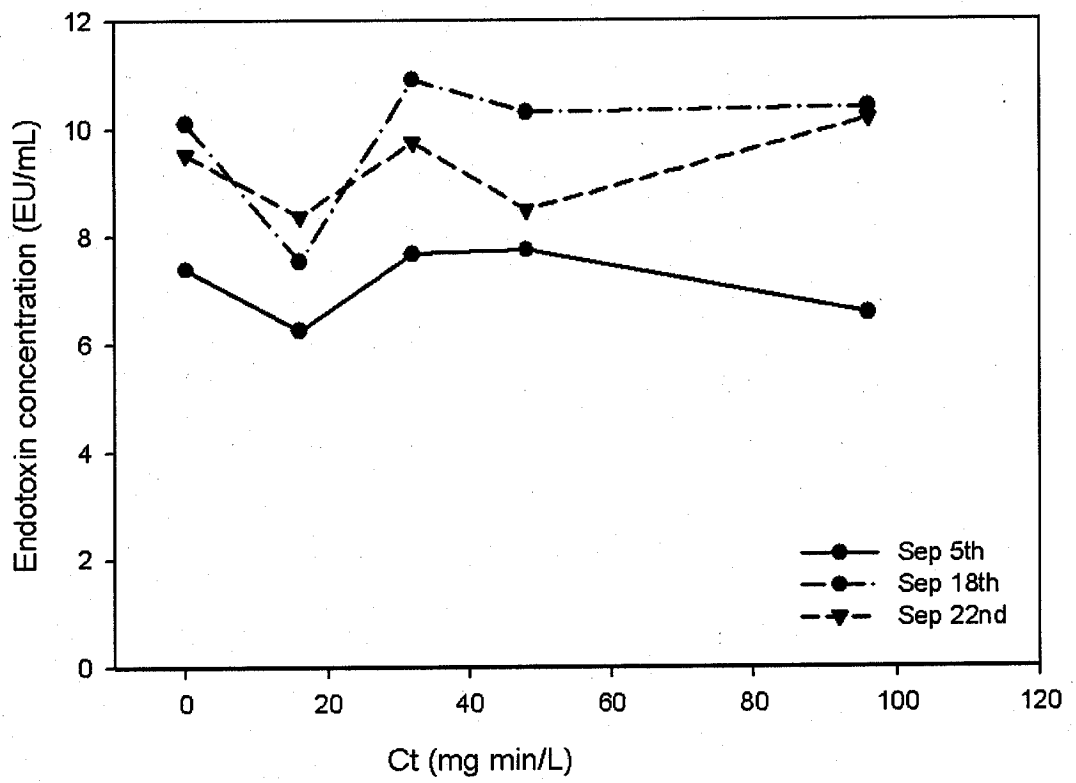

Figure 4.7. Endotoxin inactivation by free chlorine

Initial concentrations in the samples (controls) ranged between 7 and 10 EU/mL. Since the samples were not spiked, these were the actual endotoxin concentrations found in filtered water samples. Endotoxin removals were within the 11 – 25% interval. However, as occurred in the endotoxin inactivation by UV light experiments, increases as

high as 8% were also measured, which suggests that chlorine would have little or no effect on endotoxin levels.

In a study performed by Anderson et al. (2003), the effect of several oxidants, including free chlorine, on endotoxin inactivation was analyzed. In the case of free chlorine, considerably higher residual doses of 2 and 100 mg/L were applied. Also, much longer retention times of 24, 120 and 169 h were used, thus giving Ct values from 2,880 to 1.014×10^6 mg•min/L. As mentioned earlier, the smaller concentrations and retention times chosen for the current study were meant to mimic realistic conditions at drinking water treatment facilities. With their parameters, Anderson et al. were able to achieve endotoxin inactivation rates of 1.3 and 1.4 EU/mL-h when using, respectively, 2 and 100 mg/L of chlorine residuals. The close values between the two doses used suggests that inactivation rate is independent of the chlorine residual dose, and in fact is virtually zero.

With the results from Figure 4.7 it is difficult to establish similar inactivation rates since values decrease and increase without following a well-established trend.

In their study, Anderson et al. also concluded that endotoxin inactivation rates found were relatively small in terms of significance for drinking water treatment. This idea is confirmed by the results from Figure 4.8 where it can be seen that, when used at typical drinking water doses, chlorine is, indeed, only slightly effective for endotoxin inactivation. These results also support those described in Section 4.2.2 where it was shown that chlorine was not very effective at large scale facilities either, especially at plants B and C.

4.5. rFC – chlorine interference test

Figure 4.8 shows the results for the experiment performed to test the possible interference between chlorine and the rFC assay. Since all results presented so far were obtained with the rFC method, LAL – chlorine interference was not tested. Endotoxin concentrations were measured in samples following chlorination from plants A and B;

these concentrations correspond to the bars labelled *before*. The bars labelled *after* represent the endotoxin concentration in the two samples once sodium thiosulfate had been added.

There was a decrease of 60% in the endotoxin concentration in both samples after the addition of sodium thiosulfate, which suggests an interference of chlorine with the rFC assay. Since the rFC assay is based in the change in fluorescence of a sample during a 1h period, this interference may be due to chlorine emitting its own fluorescence. The total fluorescence read would be equivalent to the addition of the sample fluorescence plus the one added by the effect of chlorine.

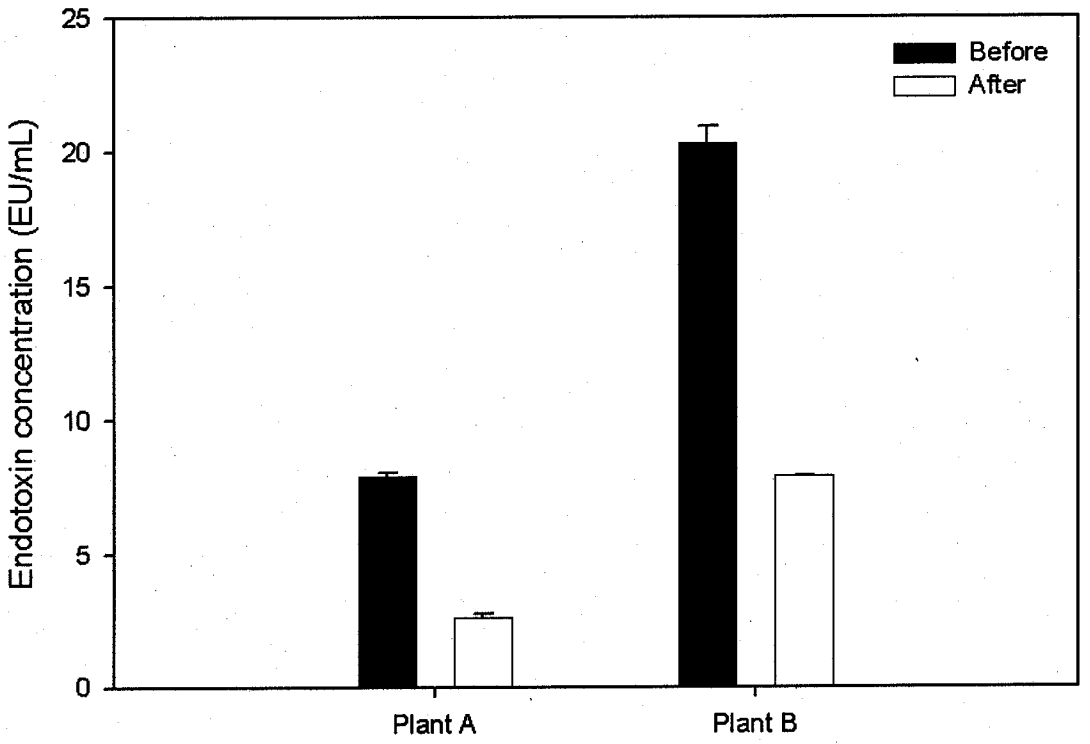

Figure 4.8. Endotoxin concentrations in chlorinated samples before and after the addition of sodium thiosulfate

To confirm this, the results from a second experiment performed with free and combined chlorine are presented in Figure 4.9. Endotoxin concentrations were measured

in a chlorine-free control sample and after the addition of 0.8 and 1.6 mg/L of free and combined chlorine. The error bars are shown for duplicates in each case.

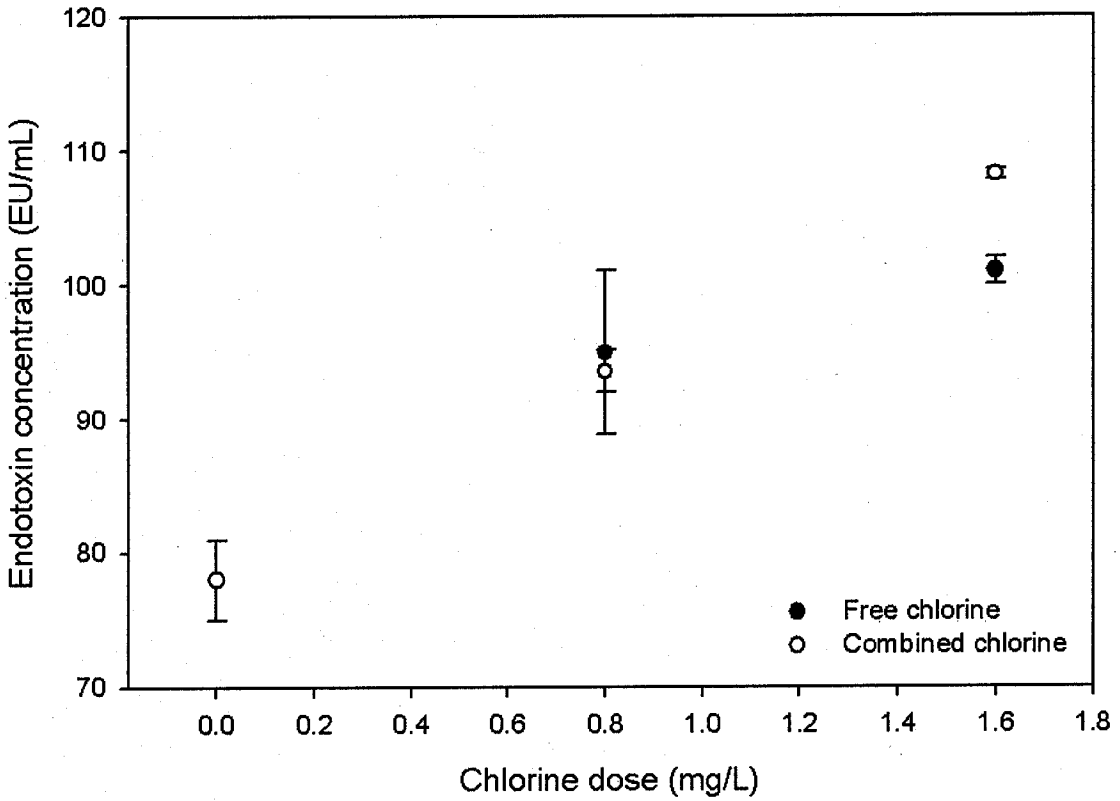

Figure 4.9. Endotoxin concentrations in chlorinated samples

The increase in apparent endotoxin concentration is evident, with 22 and 20%, respectively, for free and combined chlorine at a dose of 0.8 mg/L and 30 and 29% when a dose of 1.6 mg/L was applied. A linear regression between endotoxin concentration and chlorine dose was performed and correlation coefficients of 0.9308 for free chlorine and 0.9997 for combined chlorine were obtained. Equations 4.1 and 4.2 show, respectively, the linear relationship between endotoxin concentration and free and combined chlorine doses.

$$C_e = 14.38 \times FCl + 79.774 \qquad (4.1)$$

$$C_e = 18.88 \times CCl + 78.104 \qquad (4.2)$$

where: C_e = final endotoxin concentration (EU/mL)

FCl = free chlorine dose (mg/L)

CCl = combined chlorine dose (mg/L)

The slopes from Equations 4.1 and 4.2 represent the increase in endotoxin concentration per mg/L of chlorine added and the intercepts the initial endotoxin concentration present in the sample. In this experiment, the samples were spiked with 80 EU/mL, and the equations suggest that one could expect an increase of approximately 15 EU/mL per mg/L of free chlorine and almost 19 EU/mL per mg/L of combined chlorine.

These effects may be due to two reasons. The first, as mentioned before, is that chlorine may be emitting its own fluorescence, thus even small quantities can affect the results, independently if free or combined chlorine were added. The second is that one or both sodium hypochlorite and ammonium hydroxide solutions could have been contaminated with small amounts of endotoxin. This would explain the fact that the addition of combined chlorine increased the levels slightly more, since two solutions were mixed and added, compared to free chlorine where only one solution was added. However, given the oxidation capacities of these compounds, one would expect endotoxin levels in the solutions to be close to zero. In addition, an rFC test on a chlorine solution was performed but no enzymatic reaction occurred, thus the endotoxin concentration in the solution could not be determined. It is therefore more plausible that the differences in endotoxin concentrations found are due to an additional fluorescence emission by chlorinated compounds.

Based on these results it is concluded that endotoxin concentrations in chlorinated samples where chlorine residuals were not quenched will be overestimated. This overestimation can be quantified with the values from Equations 4.1 and 4.2. In contrast, laboratory scale results showing the limited capacity of chlorine to inactivate endotoxin (Section 4.4) remain valid since all samples analyzed there were chlorine-quenched prior to endotoxin quantification, hence eliminating the possible interference herein described.

4.6. Endotoxin inactivation by ozone

Since ozonation is normally applied after filtration in large scale facilities, laboratory scale experiments for endotoxin inactivation by ozone were performed on filtered water samples from plant B. Results for these experiments are shown in Figure 4.10 with each point representing the mean of duplicate measurements and bars delimiting the range of the highest and lowest values. An ozone residual dose of 0.5 mg/L was combined with a retention time of 2 minutes to yield a Ct value of 2.5 mg•min/L and a second ozone residual dose of 1 mg/L was applied for 20 minutes in order to achieve a Ct value of 20 mg•min/L. Initial endotoxin concentrations were measured by the controls at Ct = 0 mg•min/L. Additional ozone-free controls were performed at 2.5 and 20 mg•min/L to ensure that the reductions in endotoxin concentrations were due exclusively to the effect of ozone and also to verify that no environmental contamination had occurred during the test. Remaining ozone was quenched to stop the inactivation reaction.

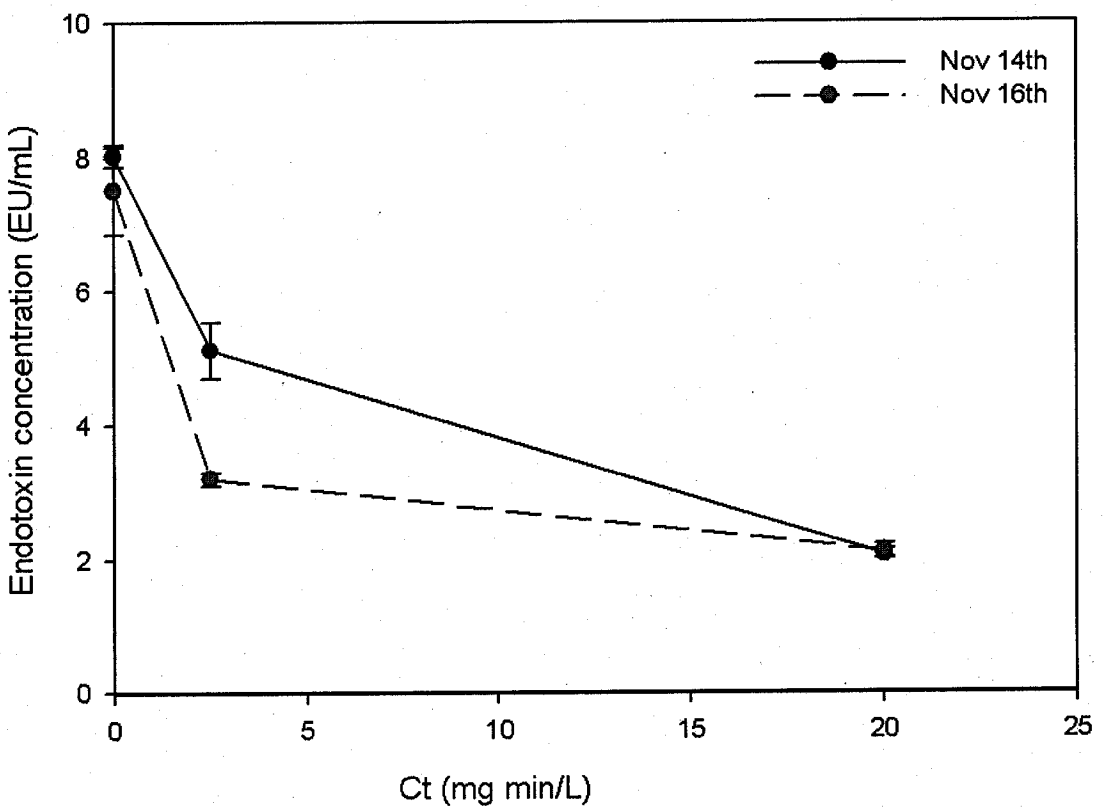

Figure 4.10. Endotoxin inactivation by ozone

Initial endotoxin concentrations were similar to the values measured in the free chlorine experiments and ranged between 7.5 and 8 EU/mL. Samples were not spiked in order to test the effect of ozone on concentrations normally found in real drinking water samples.

Due to the limited number of samples used, it is difficult to perform any statistical analyses. However, because the protocol for the rFC assay included adding a fixed amount of endotoxin to serve as an offset (hence to work in the linear range) and to ensure that no endotoxin inhibition was occurring, and negative controls were performed, one can be confident that these reductions are due to the effect of ozone only. Results from Figure 4.10 show that up to 57% endotoxin inactivation can be achieved at 2.5 mg•min/L and as much as 74% when increasing the Ct value to 20 mg•min/L. Endotoxin concentrations decreased to 3 – 5 EU/mL and to 2 EU/mL, respectively, for the two Ct doses used. When the endotoxin concentrations are converted to a log-scale and the reduction levels are calculated it is found that it is possible to achieve a 0.6 log endotoxin inactivation with the highest Ct dose applied. These results are in agreement with large scale endotoxin inactivation by ozone for plant C (see Section 4.2.2 and Figure 4.3) where it was shown that ozonation was able to achieve close to 60% inactivation.

The effect of ozonation on endotoxin inactivation at laboratory scale has not been published. However, as previously mentioned, Rapala et al. (2006) reported large scale inactivation rates between 33 and 35% for ozonation following coagulation and sand filtration. This lower rate compared to the ones attained at plant C as well as in the laboratory scale experiments may be due to the fact that endotoxin concentrations were previously decreased in the coagulation-sand filtration phase. Another factor that may explain this difference is the Ct value used at the plant studied by Rapala et al., unfortunately, this value is not mentioned, making it difficult to establish if the difference is indeed due to a lower Ct dose used.

In a previous study by Rapala et al. (2002) only one of the nine treatment plants analyzed included ozonation as one of the treatment processes. The overall endotoxin reduction for that plant was 96%, placing it as one of the most efficient ones. As in the other study, the extent of endotoxin inactivation due to the effect of ozonation was much lower, reaching merely 8%. Again, no information was available on the ozone doses and retention times used. Similarly, Burger at al. (1989) reported a reduction of 30% due to ozonation following sand filtration.

4.7. Endotoxin removal by sand filtration

Endotoxin concentrations on samples from a large scale sand filter collected over a 72h period are shown in Figure 4.11. The endotoxin concentration in a backwash water sample is included in Figure 4.11a to illustrate the large difference in the concentration in this sample and the concentrations in the others. The 72h hour cycle begins a few minutes before the backwash cycle is started; at this moment, a sample was taken and labelled *before backwash*. This sample corresponds to the end of a normal filtration cycle but, as previously mentioned, it was decided to place this sample on the first position for practical and illustrative reasons. The second sample, identified as *beginning of prefiltration* is the sample taken immediately after the backwash cycle was completed. The following two, *end of prefiltration* and *beginning of filtration* correspond, respectively, to the moment before and after the water flow is back in circulation. The sample labelled *half filtration cycle* is the one taken 48h after the first sample was collected. Finally, *backwash water* is the sample taken at the top of the filter while backwashing occurred.

Figure 4.11b presents all the samples previously described except for the backwash water in order to show the differences in endotoxin concentrations during the filtration cycle, since elevated concentrations in the backwash water, as can be seen on Figure 4.11a, make it difficult to notice changes in the other samples.

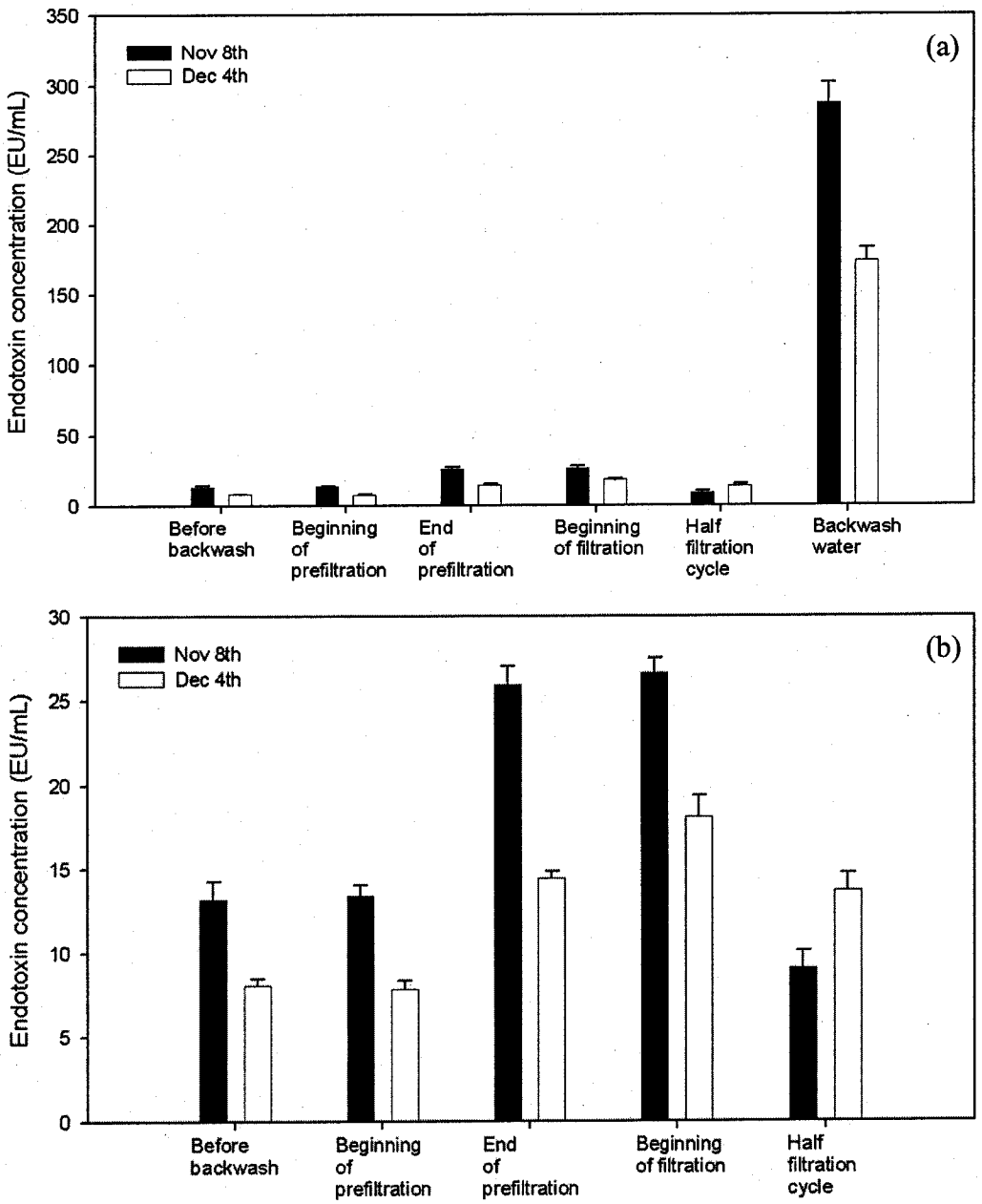

Figure 4.11. Endotoxin concentrations (a) in a complete sand filtration cycle and (b) over a cycle but excluding the backwash water.
Note the differences in endotoxin concentration scales.

From Figure 4.11a it was found that endotoxin concentrations are as much as 13 and 15 times higher in the backwash water than in the remaining samples. This is a very interesting result and it shows that most of the endotoxins are retained in the filter and then flushed away during the backwash process. This high concentration is explained by

the difference between the backwash water flow and the flow of water coming into the filter. The backwash flow is 4.5 times larger than the inflow, yielding a higher endotoxin concentration per volume unit. This situation, explains the capacity of the filter to remove endotoxins.

It is important to mention that, concerning the efficiency of a filter, the term endotoxin removal should be used rather than endotoxin inactivation since, as previously discussed in Section 4.2.2, the filtration process is physical and endotoxins are, indeed, removed from the water flow, as opposed to other methods such as chlorination or ozonation where a chemical process, which is not yet fully understood, destroys the endotoxin complexes.

From Figure 4.11b it is interesting to notice that endotoxin concentrations remain unaltered before and after backwashing. On the other hand, there is an increase of 90% in the concentrations during the prefiltration phase and a subsequent stabilization during the early part of the filtration cycle. These two results indicate that endotoxin concentrations are not affected by the backwash process by itself but by the prefiltration period. This stage is also known as filter ripening (Amburgey and Amirtharajah, 2005) who explain how the efficiency of sand filters improves from the beginning of the filtration cycle during ripening periods. This situation is clearly identified when looking at the concentrations on the samples labelled as *end of prefiltration* and *beginning of filtration*. Even when the water flow is switched back into circulation, the endotoxin concentration is at its highest level of the filtration cycle. It is not until 48h later, when the filter has recovered its efficiency, that endotoxin levels decrease as much as 66%. One possible solution to prevent such high endotoxin concentrations to be so high when the filtration cycle starts would be to extend the prefiltration period a little longer until endotoxin levels have started to decrease. However, the practical and operational consequences of this action would have to be assessed.

The interaction between endotoxins and sand filtration has not been reported in detail in the literature. Endotoxin concentrations before and after filtration have been assessed

(Burger et al., 1989; Rapala et al., 2006) but neither of the articles specify the exact moment in the cycle when the samples were collected. Therefore, it is not possible to compare the results obtained with previous work; moreover, a confirmation of these findings is necessary. Sample collection at shorter intervals during the 72h filtration period could give information on how endotoxins decrease during the first few hours after the filter is placed back in operation and a mass balance for endotoxin in the filter would help to understand how exactly endotoxins are removed during backwash.

4.8. Environmental contamination assay

Endotoxin concentrations were measured in triplicate for the four samples exposed in the environmental contamination assay. These concentrations are presented in Figure 4.12. The error bars show one standard deviation. The first bar represents the control sample which was not air exposed in order to establish the initial concentration of endotoxin already present in the sample.

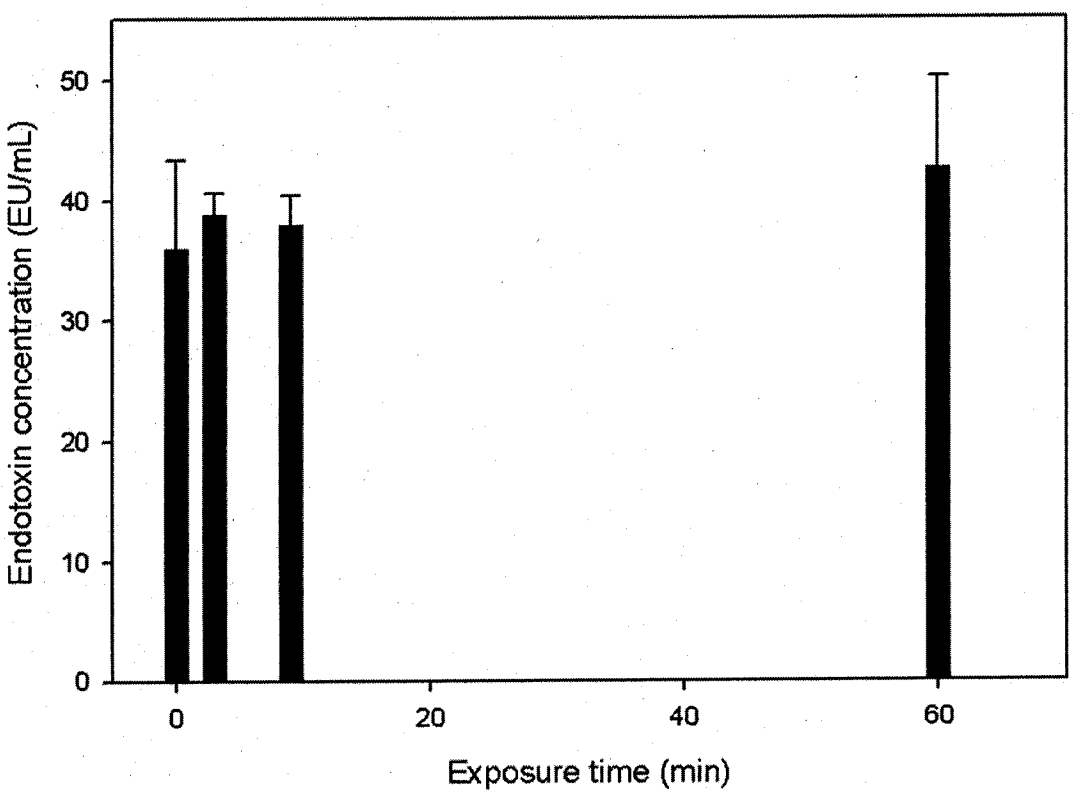

Figure 4.12. Endotoxin concentrations after environmental exposure

Endotoxin concentration increments of 8, 6 and 18% were found, respectively, after 3, 9 and 60 minutes of exposure. However, large standard deviation values of the triplicates make it difficult to establish if these increments are, indeed, due to environmental contamination or if the differences were due to human and procedural errors.

A test for statistical outliers was performed on the four samples and it was found that none of the values were outliers for a 1% level of significance ($T < 1.15$ when $n = 3$), thus differences due to environmental exposure were not significant. Based on these results, it was concluded that endotoxin inactivation in experiments performed with UV light, where samples had to be exposed to air, was occurring exclusively by the effect of UV since it was demonstrated that air contamination had no significant effect on endotoxin concentrations.

Since experimental protocol allowed doing so, samples in experiments involving free chlorine and ozone were covered with endotoxin-free aluminium foil to provide a higher level of confidence that environmental contamination was minimized.

4.9. Correlations with other drinking water parameters

Although some correlations between bacterial parameters and endotoxin concentrations have been reported in the literature (Jorgensen et al., 1976; Evans et al., 1978; Korsholm and Sogaard, 1988; Rapala et al., 2002) no study has made an attempt to correlate these concentrations with other drinking water parameters. Hence, with data acquired from the three drinking water treatment plants, a linear regression analysis was performed between endotoxin concentrations and other parameters for most of the samples studied. These parameters included water temperature, turbidity, total organic carbon, total coliforms and heterotrophic bacteria. However, given the limited data

available for total coliforms and heterotrophic bacteria, these variables showed very low correlation coefficients ($r^2 < 0.1$) therefore they were not considered for further analysis.

A stepwise multiple linear regression analysis was performed with the remaining three variables (water temperature, turbidity and total organic carbon). In the first step of the model, the three variables and all three crossed factors were considered as independent variables. Even though a high correlation coefficient ($r^2 = 0.915$) was obtained, all six factors were statistically insignificant ($p > 0.05$), hence the model was rejected. In the second step, the variable with the highest p value (TOC) was not considered for the regression but the remaining variables were still statistically insignificant. The same procedure was repeated by removing the temperature – turbidity factor in the next step which lead to a four-variable model in which all of the factors were statistically significant ($p < 0.05$) with a high correlation coefficient (r^2=0.869). However, although the model is mathematically and statistically acceptable, the physical and practical meaning of the crossed factors (Temperature)x(Turbidity), (Temperature)x(TOC) and (Turbidity)x(TOC) is hard to interpret, therefore, the multiple regression model with crossed factors had to be rejected.

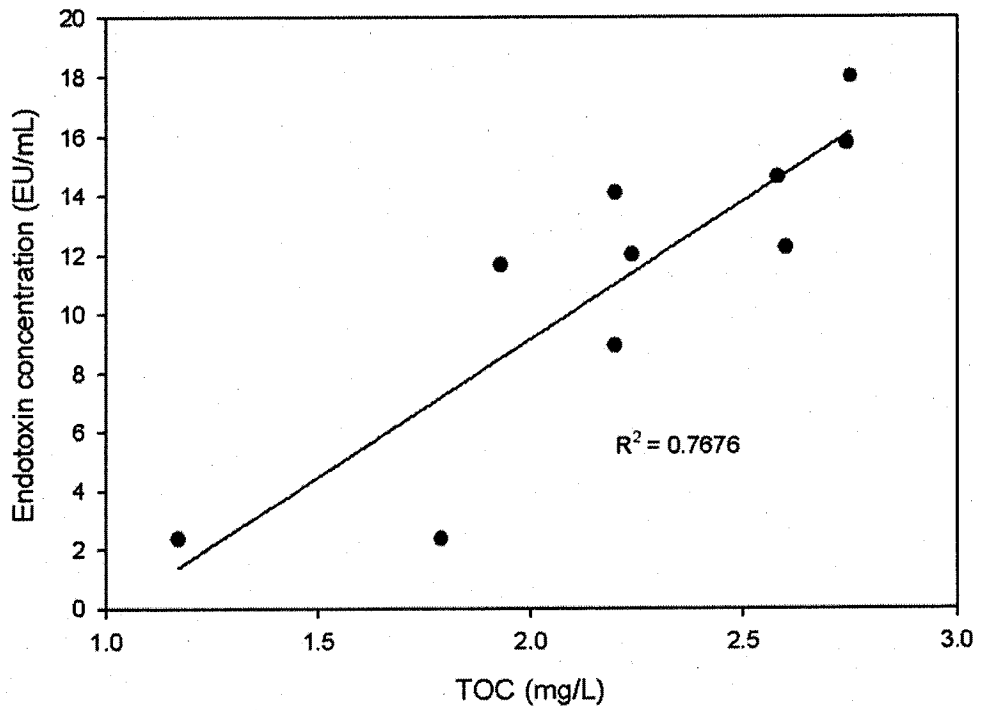

Figure 4.13. Endotoxin concentrations vs. total organic carbon

Finally, a multiple linear regression analysis was performed including only water temperature, turbidity and total organic carbon as the independent variables. The highest correlation occurred with total organic carbon as the independent variable. This model was statistically significant ($p = 0.007$) with a correlation coefficient $r^2 = 0.7676$. A graph showing the correlation between endotoxin concentrations and total organic carbon is presented in Figure 4.13.

It is important to mention that samples used for this analysis come from the same source, i.e. plants B and C which obtain their source water from the St. Lawrence River.

The fact that water temperature did not correlate with the endotoxin concentrations can be explained because of the large difference between winter and summer temperatures. This difference can be seen in Figure 4.14. While there are two separate tendencies, one for cold temperatures (between 1 and 4 °C) and one for warmer temperatures (between 16 and 20 °C), both showing a decrease in endotoxin concentrations as temperatures increase, there is no overall tendency for both cold and warm months.

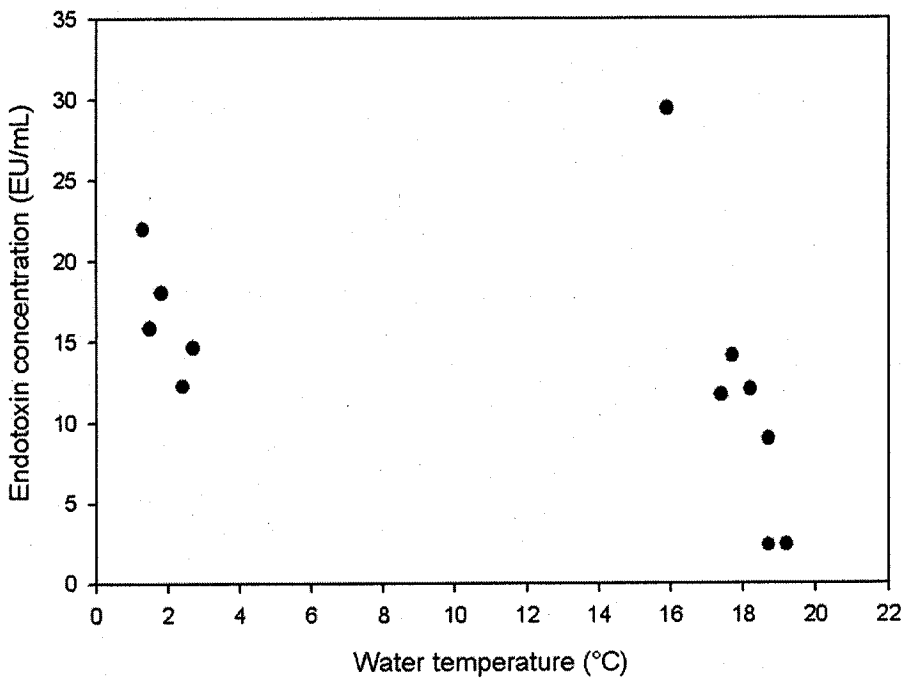

Figure 4.14. Endotoxin concentrations and water temperature

In the case of turbidity, no correlation was found mainly because the turbidity values remained pretty stable throughout all the samples. For the raw water samples the numbers were between 1.63 and 2.38 NTU; for the remaining samples values were between 0.12 and 0.43 NTU. This narrow range does not allow one to establish a reasonable correlation.

5. CONCLUSIONS, CONTRIBUTIONS AND FUTURE WORK

5.1. Conclusions

The overall objectives for this study were to provide information on endotoxin levels in different raw and drinking water samples across Montreal as well as to assess the effects of various drinking water treatment processes on the inactivation and removal of endotoxins.

The methodology used included two different assays to quantify endotoxins in aqueous samples: the Limulus amebocyte lysate (LAL) test and the recombinant factor C (rFC) test. For the same samples, concentrations measured with the rFC test were lower than those measured with the LAL test but changes in endotoxin levels were consistent within the two methods. The rFC assay was preferred over the LAL assay because of its less tedious experimental procedure, as well as a better endotoxin concentration off-set protocol which considerably reduced human and systematic errors. Moreover, although rFC requires more expensive equipment, the reagents cost per test was lower than for LAL.

Endotoxin concentrations in raw water samples were lower than those measured in previous studies (Burger et al., 1989; Rapala et al., 2002; Rapala et al., 2006) and they ranged from 9 to 30 EU/mL with a tendency to decrease during the warmest summer months. Highest concentrations were found during the months of June and October.

Some large scale water treatment processes were effective for endotoxin removal. Efficiencies for sedimentation and sand filtration reached 90 and 60%, respectively. Chlorination achieved only 28% reduction in the best of the cases. In contrast, ozonation was able to inactivate 60% of the endotoxins. The overall efficiencies for the three plants studied ranged between 30 and 90%, depending on the season.

Endotoxins measured throughout the drinking water distribution system also showed lower concentrations during the warmest months and highest concentrations during the spring.

On the one hand endotoxin concentrations found in raw and treated drinking water samples in Montreal were lower than those reported elsewhere, but no guidelines for maximum endotoxin concentrations in water samples exist, therefore it is not possible to comment on the health aspect of Montreal's water *vis-à-vis* endotoxins. On the other hand, the hygiene hypothesis still needs to be verified for this water.

Laboratory scale experiments showed that UV light and free chlorine had only a small effect on endotoxin inactivation at doses typically used at large scale facilities. Inactivation rates as high as 22% were reached with UV light but a consistent reduction could not be established. Similarly, free chlorine was able to inactivate endotoxins by as much as 25%, but inconsistently. On the other hand, ozone proved to be a superior method, reaching maximum inactivation levels of 60% with Ct values as low as 2.5 and 20 mg•min/L.

In a parallel test during the UV experiments, it was found that contamination of the samples due to environmental exposure was negligible. Moreover, proper handling and the use of pyrogen-free material helped to decrease errors in the measurements.

Measurements on large scale sand filters showed that the physical action of removing endotoxins is more efficient than the chemical inactivation effect, with an increase of the endotoxin concentration in the backwash water as much as 15 times. Furthermore, it was found that endotoxin levels increase considerably during the filter's ripening period and subsequently decrease as the filter recovers its efficiency.

From the water parameters analyzed, only total organic carbon correlated with endotoxin concentrations, with a linear correlation coefficient r^2 of 0.7676.

An interference of chlorine with the rFC assay was found, possibly due to an extra emission of fluorescence, thus erroneously yielding higher results. In order to avoid this, samples containing chlorine should be quenched before endotoxin quantification.

5.2. Contributions to knowledge

The main contribution of this study is the demonstration that filtration and ozonation were effective for endotoxin inactivation and removal, whereas chlorine and UV were not at typical doses used in large scale drinking water facilities. It was also demonstrated that concentrations were still high at the end of the ripening period of a sand filter, thus this aspect of filter operation may need further attention. Finally, endotoxin concentrations in water samples around Montreal were found to be lower than those reported elsewhere; concentrations were highest in June and January and lowest in August.

5.3. Recommendations for future work

During the literature review many studies on endotoxin inhalation were found; in contrast, very few on endotoxin ingestion have been published. The experiments and measurements done in this study can help to comprehend how endotoxins behave in drinking water samples and how they react to different treatment processes. Following this, the exact mechanism by which the human organism interacts with endotoxins when they are ingested still needs to be ascertained.

There was a reduction in the endotoxin levels when using chemical processes such as chlorine, however, the precise mechanisms of endotoxin destruction still needs to be determined. Previous studies have also detected these reductions but none of them has described the exact phenomenon through which endotoxins are decomposed into simpler molecules. Similarly, the inactivation kinetics due to ozone should be investigated further. Greater inactivation rates for ozone than for chlorine or UV were found but a more detailed description of the kinetics of the process is lacking.

Finally, in order to fully understand how endotoxins are removed in a sand filter, a detailed mass balance analysis could provide useful information. Furthermore, a closer look into the sand particles–endotoxin interaction could help to describe the mechanisms through which endotoxins are attached and removed.

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APPENDIX A: Example to calculate endotoxin concentrations with the LAL method

1. With at least three standards in duplicate within the 0.1 and 1.0 EU/mL interval, prepare a calibration curve. The absorbance of a negative control should be determined as well, and absorbances for the remaining standards and samples must be corrected with this value, as shown in Table A.1. The calibration curve resulting from the values from Table A.1. is presented in Figure A.1.

Table A.1. Absorbances for the standards and the negative control in an LAL assay

Endotoxin standard (EU/mL)	Absorbance	Mean Absorbance	Mean Δ Absorbance
0	0.103	0.103	0.000
0	0.103		
0.1	0.177	0.177	0.074
0.1	0.176		
0.25	0.230	0.232	0.129
0.25	0.233		
0.5	0.334	0.339	0.236
0.5	0.344		
1	0.536	0.554	0.451
1	0.571		

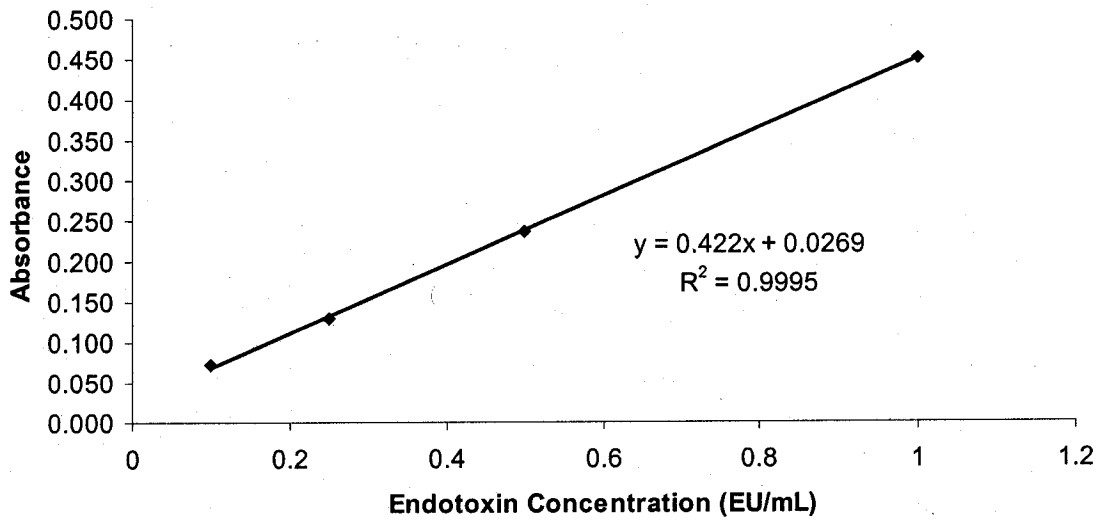

Figure A.1. Calibration curve for an LAL assay

2. With the equation obtained from the calibration, the endotoxin concentration for a given sample can be determined from the absorbance corrected values as follows:

$$C_e = \frac{ABS - 0.0269}{0.422} \qquad \text{(A.1)}$$

where: C_e = Endotoxin concentration in the sample (EU/mL)

ABS = Corrected absorbance value in the sample

3. If the sample has been diluted, the C_e value should be multiplied by the dilution factor to obtain the total endotoxin concentration in the undiluted sample.

APPENDIX B: Example to calculate endotoxin concentrations with the rFC method

1. With the fluorescence values of at least three endotoxin standards within the 0.01 and 10 EU/mL interval, prepare a calibration curve. Fluorescence values for all the standards and samples must be corrected with the one from the negative control. An example of a calibration curve for the rFC assay is shown in Table B.1 and Figure B.1.

Table B.1. Fluorescences for the standards and the negative control in an rFC assay

Sensitivity: 85

C1	C2	C3	C4	C5	C6	C7	C8	C9
	t = 0		t = 1 h					
Endotoxin standard (EU/mL)	Fluoresc.	Mean Fluoresc.	Fluoresc.	Mean Fluoresc.	Δ Mean Fluoresc.	Net Δ Mean Fluoresc.	log endotoxin standard	log Net Δ Mean Fluoresc.
0	4108	4058	4132	4096	38	0	-	-
	4007		4059					
0.05	4004	4042	4675	4683	641	603	-1.3	2.7803
	4080		4691					
0.1	4035	4009	5414	5413	1404	1366	-1	3.1353
	3983		5411					
1	4199	4177	15931	15763	11587	11549	0	4.0625
	4154		15595					
10	4208	4189	76317	76540	72352	72314	1	4.8592
	4169		76763					

where:

$$C6 = C5 - C3 \quad \text{(B.1)}$$

$$C7 = C6_i - C6_{blk} \quad \text{(B.2)}$$

$$C8 = \log(C1) \quad \text{(B.3)}$$

$$C9 = \log(C7) \quad \text{(B.4)}$$

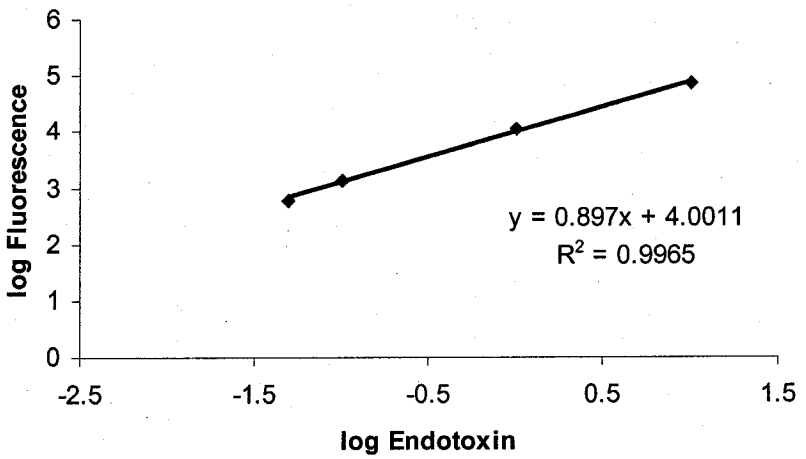

Figure B.1. Calibration curve for an rFC assay

2. From the equation obtained with the calibration curve, the endotoxin concentration in a sample, once the fluorescence value has been corrected with the negative control, can be obtained with the following equation:

$$C_e = \frac{FLR - 4.0011}{0.897} \qquad \text{(B.5)}$$

where: C_e = Endotoxin concentration in the sample (EU/mL)

FLR = Corrected fluorescence value in the sample

3. Total endotoxin concentration in the sample can be found by multiplying the value obtained by the dilution factor.

APPENDIX C: rFC Sensitivity Determination

Table C.1. Fluorescence values for the rFC sensitivity determination

Sensitivity: 50

Endotoxin standard (EU/mL)	t = 0 Fluorescence	t = 0 Mean Fluorescence	t = 1 h Fluorescence	t = 1 h Mean Fluorescence	Δ Mean Fluorescence	Net Δ Mean Fluorescence
0	101	93	113	106	13	0
	85		98			
0.01	82	86	85	98	12	-1
	89		110			
0.1	104	93	104	104	11	-2
	82		104			
1	85	95	330	303	208	196
	104		275			
10	55	93	1245	1175	1082	1070
	131		1105			

Sensitivity: 60

Endotoxin standard (EU/mL)	t = 0 Fluorescence	t = 0 Mean Fluorescence	t = 1 h Fluorescence	t = 1 h Mean Fluorescence	Δ Mean Fluorescence	Net Δ Mean Fluorescence
0	256	281	269	307	27	0
	305		345			
0.01	259	273	311	302	29	3
	287		293			
0.1	296	289	345	356	67	41
	281		366			
1	311	289	1190	1157	868	842
	266		1123			
10	266	277	5176	4914	4637	4611
	287		4651			

Sensitivity: 80

Endotoxin standard (EU/mL)	t = 0 Fluorescence	t = 0 Mean Fluorescence	t = 1 h Fluorescence	t = 1 h Mean Fluorescence	Δ Mean Fluorescence	Net Δ Mean Fluorescence
0	2426	2426	2451	2465	39	0
	2426		2478			
0.01	2325	2414	2457	2523	109	70
	2503		2588			
0.1	2405	2423	3204	3131	708	670
	2441		3058			
1	2493	2426	10764	10685	8259	8220
	2359		10605			
10	2582	2559	49305	46684	44125	44086
	2536		44062			

Note: Values have been rounded to closest integer.

APPENDIX D: Microplate sampling protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A		B	ST1	ST2	S1D1	S1D1	S2D1	S2D1	S3D1	S3D1	S4D1	S4D1
B		B	ST1	ST2	K1D1	K1D1	K2D1	K2D1	S3D1	K3D1	K4D1	K4D1
C		KB	ST3	ST4	S1D2	S1D2	S2D2	S2D2	S3D2	S3D2	S4D2	S4D2
D		KB	ST3	ST4	K1D2	K1D2	K2D2	K2D2	K3D2	K3D2	K4D2	K4D2
E	S5D1	S5D1	S6D1	S6D1	S7D1	S7D1	S8D1	S8D1	S9D1	S9D1	S10D1	S10D1
F	K5D1	K5D1	K6D1	K6D1	K7D1	K7D1	K8D1	K8D1	K9D1	K9D1	K10D1	K10D1
G	S5D2	S5D2	S6D2	S6D2	S7D2	S7D2	S8D2	S8D2	S9D2	S9D2	S10D2	S10D2
H	K2D2	K2D2	K6D2	K6D2	K7D2	K7D2	K8D2	K8D2	K9D2	K9D2	K10D2	K10D2

Figure D.1. 96-wells microplate diagram

where:

			rFC	LAL
ST1	standard 1	=	10 EU/mL	1 EU/mL
ST2	standard 2	=	1 EU/mL	0.5 EU/mL
ST3	standard 3	=	0.1 EU/mL	0.25 EU/mL
ST4	standard 4	=	0.05 EU/mL	0.1 EU/mL

B	blank
KB	spiked blank
S1D1	sample 1 dilution 1
K1D1	spiked S1D1
S1D2	sample 1 dilution 2
K1D2	spiked S1D2

APPENDIX E: UV experimental protocol

Preparation

- Oven-bake UV dishes for 30 minutes at 250^{0}C
- Soak stir bars in ethanol

Procedure

- Turn on and **zero the radiometer**
  - Place radiometer where readings will be taken
- **Turn on UV lamp** and let warm-up for at least 10 minutes
- **Measure the transmittance** T of samples at **254 nm**
  - Use deionized water as a reference
  - Fill cuvette using sterile pipette tip to avoid contaminating sample
  - Run samples three times and take average T
- **Prepare spiked samples**
  - In one container prepare enough spiked sample for three samples, plus 6 mL
  - Spike to 100 EU/mL (calculations based on endotoxin standard and volume)
  - Fill three vials with initial spiked sample and label
  - Transfer 10 mL of spiked samples into UV dishes, add stir bar that has been pulled through a flame, cover with aluminum foil and label UV40, UV100, and UVC
- **Prepare unspiked samples**
  - Fill three vials with initial unspiked sample and label
  - Using a new glass pipette, add 10 mL of unspiked sample to UV crystallization dishes, add sterilized stir bar, cover with aluminium foil and label
- **Measure the incident intensity** of the UV lamp in mW/cm^2
  - Measure three times and take average
- **Calculate the average intensity**
  - $$I^*_{avg} = (0.975) I^*_o \left[\frac{(1 - e^{-d \ln\left(\frac{1}{T}\right)})}{d \ln\left(\frac{1}{T}\right)} \right]$$

where: I^*_o = incident intensity (mW/cm^2)
d = depth of sample (2.2 cm)
T = transmittance (where cell path length = 1.0 cm)

- **Calculate the duration** (in sec) required for doses of 40 and 100 mJ/cm^2
  - $$t = \frac{Dose}{I^*_{avg}}$$

- Uncover sample to be tested and place sample on stirrer (stirrer should be placed on thin black height adjustment) beneath UV beam
- Start timer when you open the collimating beam, and use tape to hold trap open
- Immediately after irradiation, shield dish so that photoreactivation is prevented
- **Re-measure the incident intensity** of the UV lamp in mW/cm^2
    - Measure three times and take average
- Once all samples have been run, **transfer aliquots into vials**, label, wrap in foil and put in freezer until next endotoxin detection run

APPENDIX F: Free chlorine experimental protocol

1. Obtain break-point chlorination curves for samples.
2. Set the residuals doses for free chlorine to be used.
3. Set the detention times to be used for each chosen dose.
4. Measure pH and ammonia.
5. Experiment should be performed in sealed flasks (250 mL)
6. Initial concentration of chlorine to be added depends on the residual concentration wanted. This concentration can be obtained with the break-point chlorination curve.

Materials

- 250 mL graduated Erlenmeyer flasks sealed with aluminum caps
- Orbital shaker
- Sodium hypochlorite
- Amperometric titration equipment

Equipment Sterilization

All glassware coming in contact with samples must be freshly unwrapped or rendered pyrogen-free by being heated in the oven at 250°C (482° F) for 30 minutes.

Glassware must be baked at 400° C for 2 hours and rinsed with a dilute solution of HOCl (20 mM) followed by Nanopure water to ensure that the glassware will not exert a chlorine demand or,

Glassware must be exposed to water containing at least 10 mg/L chlorine for 3h or more before use and rinsed with chlorine-demand-free water.

pH Testing and Adjustment Procedure

Test pH

- Pipette 30 mL of well-mixed sample into small clean beaker
- Test pH of aliquot

Adjust pH

If pH is outside of 6.0 – 8.0 range, pH needs to be adjusted

- For pH too high, use 0.1N hydrochloric acid to lower pH of aliquot
- For pH too low, use 0.1N sodium hydroxide to raise pH of aliquot

Free Chlorine

- Set total volume of samples to be analyzed.
- Run each dose and each detention time test in duplicate.
- Spike samples with known amount of endotoxin.
- Set two oxidant-free control samples for each dose and each detention time.

- Add as much sodium hypochlorite as necessary to achieve the residual concentration.
- At proper detention time, add sodium thiosulfate at twice the equimolar requirements to quench any chlorine residual.
- Measure free chlorine residual and endotoxin activity at the end of the detention time.

Experimental values

- Sample volume: 100 mL. Smaller volumes are difficult to measure for residual chlorine since the electrode for the amperometric titration will not be fully covered.
- Chlorine residuals to be used: 0.8 mg/L and 1.6 mg/L. Based on the chlorine demand curves previously obtained for Atwater's Raw Water, the concentrations of chlorine to be added to obtain the abovementioned residuals are 1.8 mg/L and 2.4 mg/L respectively.
- Detention times to be used: 20 minutes and 1 h.
- Number of flasks to be used:
  - 0.8 mg/L residual, 20 min detention time: 2
  - 0.8 mg/L residual, 1 h detention time: 2
  - Oxidant free control: 1
  - 1.6 mg/L residual, 20 min detention time: 2
  - 1.6 mg/L residual, 1 h detention time: 2
  - Oxidant free control: 1

    Total: 10
- Sodium thiosulfate volume needed to quench residual chlorine:
  - For 0.8 mg/L: 43 µL
  - For 1.6 mg/L: 86 µL

APPENDIX G: Ozone experimental protocol

1. Obtain saturation concentration for deionised water sample (ozonated solution).
2. Set the residuals concentrations for ozone to be used.
3. Set the detention times to be used for each chosen dose.
4. Calculate Ct values with the residual doses and detention times chosen.
5. Measure pH.
6. Experiment should be performed in sealed flasks/bottles with sample filling the whole volume to avoid headspace (ozone degasification)
7. Initial concentration of ozone to be added depends on the residual concentration wanted. This concentration can be obtained by diluting the ozone saturated solution more or less with the water sample.

Materials

- 250 mL graduated Erlenmeyer flasks sealed and covered from light
- Orbital shaker
- Ozone generator and equipment
- Indigo method equipment and reagents

Equipment Sterilization

All glassware coming in contact with samples must be freshly unwrapped or rendered pyrogen-free by being heated in the oven at 250°C (482° F) for 30 minutes.

Glassware must be baked at 400° C for 2 hours and rinsed with a dilute solution of ozone followed by Nanopure water to ensure that the glassware will not exert an ozone demand or,

Glassware must be soaked in a concentrated aqueous ozone solution before use and rinsed with ozone-demand-free water.

pH Testing and Adjustment Procedure

Test pH

- Pipette 30 mL of well-mixed sample into small clean beaker
- Test pH of aliquot

Adjust pH

If pH is outside of 6.0 – 8.0 range, pH needs to be adjusted

- For pH too high, use 0.1N hydrochloric acid to lower pH of aliquot
- For pH too low, use 0.1N sodium hydroxide to raise pH of aliquot

Ozone

- Set total volume of samples to be analyzed.
- Run each dose and each detention time test in duplicate.

- Spike samples with known amount of endotoxin.
- Set two oxidant-free control samples for each dose and each detention time.
- Add as much ozone saturated solution as necessary to achieve the residual concentration.
- At proper detention time, add sodium thiosulfate or sodium formate at twice the equimolar requirements to quench any ozone residual.
- Measure ozone residual and endotoxin activity at the end of the detention time.

Experimental values

- Sample volume: That necessary to reduce headspace as much as possible.
- Ozone residuals to be used: 1.0 mg/L and 0.5 mg/L. Based on the indigo method the concentration of saturation is 3.5 mg/L. To achieve the desired residuals the saturated solution needs to be diluted 2:7 and 1:7 respectively.
- Detention times to be used: 5 min and 20 min.
- Ct values used: 2.5, 5, 10 and 20 mg·min/L.
- Number of flasks to be used:
  - 2.5 mg·min/L : 2
  - 5 mg·min/L : 2
  - 10 mg·min/L : 2
  - 20 mg·min/L : 2
  - Oxidant free control: 2

  Total: 10
- Sodium thiosulfate volume needed to quench residual ozone:
  - For 0.5 mg/L: 26 µL
  - For 1.0 mg/L: 59 µL