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Identification of the need for a comprehensive investigation of endotoxin in water sources

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ABSTRACT

With the increase of global environmental concerns, the importance of emerging pollutants is increasing daily. Endotoxin is one of the emerging pollutants whose presence in water sources causes serious risks to human health. This review article aims to investigate the amount of endotoxin in aquatic ecosystems and the potential dangers of endotoxin in these sources. In this study, the general characteristics of endotoxin have been investigated along with practical methods for removing this pollutant from water sources. At first, an overview of endotoxins and their importance in water pollution was given, and the importance of studying endotoxin contamination in water sources and the objectives of this study were determined. Then, the sources of endotoxin contamination in water, the effect of endotoxin on human health, its associated risks, and the relationship between endotoxin levels and its adverse consequences on human health were discussed. The following examined the methods used to measure and detect endotoxin in water samples and the advantages and limitations of each of these methods. Also, different strategies were proposed to control and reduce endotoxin contamination in water sources. Finally, existing regulations and guidelines for endotoxin levels in drinking water and other water sources are explained, and regulatory gaps and frameworks are discussed. The results of this study emphasize the importance of continuous monitoring and management of endotoxin contamination in water sources and the need to increase information about this emerging pollutant to provide practical strategies to maintain water quality and public health.

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1. Introduction

Assessing the current status of running water is a crucial aspect of water resources management, and it becomes even more critical in developing countries such as Iran, where water ecosystems are severely threatened by pollution. Therefore, managing and protecting these resources is essential to ensure the continued provision of water and ecological services (Sasi, 2015; Taban et al., 2018). The requirement to determine the pollution of an aquatic environment is to have the physical, chemical, and biological information of that environment. This information is complementary, and all of them are necessary for correct analyses (Khezri et al., 2019). Water resources are essential both for the environment and the economy. It is possible to measure the quality of water resources by monitoring their biological and physicochemical properties. By doing so, we can ensure that we maintain and utilize these resources effectively (Pazira et al., 2016).
Investigating the biological characteristics of water is crucial due to pollutants caused by living organisms, which have resulted in significant health issues in public health (Gorbet and Sefton, 2005).

Though these pollutants may be removed from the water, the toxins and compounds they leave behind can remain. Insufficient information regarding these factors and harmful compounds exists in water and other environments. These dangerous factors and compounds are typically classified as emerging pollutants (Yousefi et al., 2018). Emerging pollutants refer to natural, synthetic, and chemical micro-pollutants that result from the activity of microorganisms or their metabolites. These pollutants are not typically found in nature and can enter the environment, causing adverse effects on both humans and the physical environment. These effects can be either known or unknown (Calvo-Flores et al., 2018). Endotoxin is an emerging pollutant found in the cell walls of gram-negative bacteria and some cyanobacteria. It is composed of lipopolysaccharides and has a general structure that comprises three main parts: lipid A, main oligosaccharide, and antigen O (Anderson, 2002; Engler et al., 2023). The size of the O antigen polysaccharide chain determines the weight of the endotoxin molecules. The lipid A part of the endotoxin molecule is on the cell wall membrane (Stewart, 2006), while the main oligosaccharide core and O antigen are exposed to the outer environment of the cell. These parts are the primary targets of antibodies produced by gram-negative bacteria (Poxton, 1995). Lipid A is the endotoxin molecule's biologically active part and is highly harmful to humans. It acts as the active site of the antigen and maintains various endotoxin activities (Raetz and Wilfield, 2002). Even though the endotoxin is attached to the cell wall of gram-negative bacteria, it is continuously released into the surrounding environment during cell growth and division. As the cell divides, lipid A is released into the extracellular environment, which increases the biological activities and inflammatory responses to endotoxin in humans and other organisms. The inflammatory response in humans and organisms is determined by the characteristics and arrangement of acyl chains and phosphate groups in lipid A (Erridge et al., 2002). Humans are inevitably exposed to endotoxin, a pollutant produced by gram-negative bacteria such as Pseudomonas, Klebsiella, and Enterobacteriaceae. These bacteria are commonly found in natural environments and release endotoxin in water sources, soil, airborne particles, and vegetation. Exposure to endotoxin can cause several symptoms in humans, such as fever, vomiting, inflammatory reactions, diarrhea, low blood pressure, and septic shock (Yan et al., 2023). Studies have found that a concentration of 1 ng/ml of endotoxin can lead to septic shock and even death (Cheng et al., 2019). To avoid any serious complications, it is advised to limit the amount of endotoxin to 0.2 Eu/ml (where 1 endotoxin unit (1EU) equals 0.1 ng) per kilogram of body weight (Posha and Sandhyarani, 2020). Although a 0.2-0.1 ng/kg dose does not cause any apparent clinical discomfort, it can still increase circulating pro-inflammatory cytokines and white blood cells (Krogh-Madsen et al., 2008). Endotoxins can contaminate water consistently because traditional water purification methods do not remove endotoxins; some may even release more endotoxins into the water. Also, Endotoxin contamination can come from bacteria and suspended particles. After water purification, bacteria can remain on the walls of transmission pipes, adding to the endotoxin levels in purified water. Conversely, the World Health Organization (WHO) has not yet issued guidelines for safe endotoxin levels in water and wastewater. Therefore, it is crucial to monitor endotoxin levels in drinking water after treatment processes and find ways to reduce and eliminate this harmful pollutant. Unfortunately, there is limited research on endotoxin levels in water resources and no clear guidelines for safe levels of endotoxin concentration. As a result, the purpose of this study is to review the existing research on endotoxin production sources, its effects on human health, and methods to reduce and eliminate it comprehensively.

2. Material and Methods

This study utilizes a descriptive review method to investigate the contamination of water sources with endotoxin and its effects and risks on human health. The objective is to review and summarize the current body of knowledge on endotoxin contamination, recognize significant research trends, and analyze the progress made in understanding and dealing with endotoxin contamination in water sources. The search for relevant studies began in 1960, based on the value of the subject, the scientific progress made, and the available relevant studies. A systematic approach was followed to conduct the literature search. We

used electronic databases like PubMed, Scopus, Web of Science, Science Direct, and Springer to ensure comprehensive coverage of relevant articles, with keywords like endotoxin, water resources, human health, and endotoxin detection. Boolean operators like AND, OR, and NOT combined keywords and controlled vocabulary terms to construct search queries. The keywords were carefully selected to cover fundamental concepts and subtopics related to endotoxin contamination. Different permutations and combinations of related keywords, synonyms, and related terms were used to search. The search was limited to titles, abstracts, and keywords of reviewed scientific articles in English. In addition to the database search, a manual search strategy was employed, where the reference lists of the retrieved articles and relevant review articles were carefully examined to identify any possible missed studies.

3. Results and discussion

The results of the investigation on the sub-topics of sources of endotoxin contamination, health effects of exposure to endotoxin on humans, the relationship between the level of endotoxin and its adverse health consequences on humans, the methods used to measure and detect endotoxin, the advantages and limitations of the method Endotoxin detection methods, strategies to control and reduce endotoxin contamination in water sources, and existing regulations and guidelines for endotoxin levels in drinking water and other types of water sources were grouped. The results of various studies related to each section were reviewed.

3.1. Sources of endotoxin contamination in water

Water contamination caused by gram-negative bacteria and certain cyanobacteria results in the production of endotoxins. Therefore, any factors leading to increased bacterial contamination of water and algal bloom will also increase endotoxin levels. A comprehensive review of relevant literature can provide valuable information on the sources of endotoxin contamination in water. Various studies have discovered the presence of endotoxins in different water sources, leading to high pollution levels. Annadotter et al. (2005) found endotoxins in Chiuro Lake at levels ranging from 1000-7750 EU/ml, which they attributed to the activity of cyanobacteria. Similarly, in a study by Rapala et al. (2002), endotoxin activity during cyanobacteria blooms in freshwater in Finland was up to 38,000 EU/ml. Ohkouchi et al. (2007) reported endotoxin activity of 2430 EU/ml in the Yudo River basin in Japan, caused mainly by sewage effluent. Goyal and Gerba (1982) also observed high endotoxin levels in groundwater (480 ng/ml) due to sewage contamination. Zhang et al. (2013) research showed that the total endotoxin activity in the inlet and effluent of a water treatment plant in Beijing, China, ranged from 11-16 EU/ml, with sewage being the primary source of pollution. Huang et al. (2011b) detected endotoxin activity of 600-2510 EU/ml in two treatment plants in China, which was attributed to secondary effluent with an anaerobic process. De Man et al. (2014) discovered that the high endotoxin activity in city fountains was due to the overflow of mixed wastewater. Anderson et al. (2002) suggested that inhalation of water aerosol droplets is one of the routes of human exposure to endotoxin. Anderson et al. (2008) also stated that biological filters are the net endotoxin producers in water treatment plants with biological processes. In one of the studies conducted in 2022, one of the main ways of increasing endotoxin downstream of the river was the entry of sewage (de Santana et al., 2022). Finally, Guizani et al. (2009a) identified effluent from treatment plants as a source of water pollution. Studies have shown that sewage and cyanobacteria bloom are the two leading causes of endotoxin production in water resources. Thus, controlling wastewater entry into water sources and monitoring for cyanobacteria is essential to prevent their bloom. These measures can effectively reduce the amount of endotoxin present in the water.

3.2. Potential health risks associated with exposure to endotoxins

The action of endotoxins in the human body is complex, but four main pathways have been identified (Prescott et al., 1993). These pathways begin with activating the Hageman factor, a blood clotting factor. Once activated, the Hageman factor can initiate blood coagulation, leading to thrombosis and acute intravascular coagulation. This, in turn, causes the release of platelets and coagulation factors, resulting in internal bleeding and organ failure.

The complement system can also be activated, leading to inflammation. Hageman factor also converts the plasminogen activator to plasmin, which stimulates the production of plasmin. Plasmin mediates blood clot lysis, which can help to stop bleeding. It can also contribute to the inflammatory response initially caused by endotoxin by activating complement. Hageman factor can also cause a series of enzymatic reactions leading to the release of vasoactive peptides, which cause the leakage of the liquid part of the blood into the interstitial spaces, resulting in decreased blood volume and a subsequent drop in blood pressure. If hypertension is not treated, it can lead to circulatory shock and death. The human body is susceptible to bacterial endotoxin, and even a minimal amount (1 to 5 nanograms per kilogram of body weight) of endotoxin can increase body temperature, causing a fever reaction that gradually subsides after about four hours (Mehta et al., 2010). Fever is a common symptom because endotoxins stimulate host cells to release proteins called endogenous pyrogens that affect the temperature-regulating part of the brain (hypothalamus) (Braude, 1982). Several studies have been conducted to examine how the body responds to endotoxins. Thorn and Beijer (2004) found that handling dried sludge increased levels of C-reactive protein and fibrinogen degradation products, indicating inflammation of the airways. Yin et al. (2013) investigated the relationship between endotoxemia and liver diseases and emphasized the role of Toll-like receptors-4, CD14 receptor, and Kupffer cells in endotoxin-induced liver injury. Amoureux (2004) highlighted the pathophysiological role of endotoxins in various diseases, including respiratory distress syndrome, multiple organ failure, septic shock, liver diseases, and graft-versus-host disease. Moreover, Olson et al. (1988) reported that drinking certain liquids, such as alcohol or fructose drinks, increases the permeability of the gastrointestinal tract, allowing endotoxin to enter the gastrointestinal tract. Elin et al. (1981) found that lower doses of endotoxin affect the metabolic function of the human body and cause dysfunction in small blood vessels. Furthermore, Bidne et al. (2018) discovered that exposure to endotoxin can reduce women's reproductive performance and cause inflammation, low birth weight, and adverse pregnancy outcomes such as premature delivery. In addition, a dose of 0.2-0.1 ng/kg may not cause any noticeable clinical discomfort, but it can increase circulating pro-inflammatory cytokines and raise IL-6 and TNF-α levels by 4-20 times (Krogh-Madsen et al., 2008). Finally, Wichmann et al. (2004) study showed that drinking water did not affect the release of cytokines. However, all river water samples caused the release of pro-inflammatory cytokines, and free endotoxin was the main driver of pro-inflammatory cytokines in river water samples.

3.3. Effects of endotoxin on human health

Endotoxin causes factors such as fever and breathing problems in humans, which disappear after a few hours if these symptoms do not worsen. Also, in most cases, the death is suspected to be septic, which is not directly related to endotoxin. Hence, reports on the risks of endotoxin-contaminated water to human health underestimate the danger. This review discusses studies highlighting the dangers of endotoxins on human health. Residents of Tampere, Finland, experienced an epidemic of cough, dyspnea, chills, fever, headache, muscle pain, and arthralgia due to the inhalation of endotoxins in their drinking water, as reported by Aro et al. (1980). The symptoms began with chills and fever 3-6 hours after exposure to bath water and returned to normal within 24 hours (Muittari et al., 1980). Annadotter et al. (2005) also reported that toxic pneumonitis was prevalent in Zimbabwe 1.5 to 6 hours after bathing due to algal cyanobacteria blooming in drinking water tanks. Rylander et al. (1993) study showed that exposure to endotoxin-contaminated water in an air conditioner leads to fever and mild respiratory symptoms along with an increase in the number of leukocytes, the level of immunoglobulins and antibodies. Anderson et al. (2007) stated that endotoxin risks occur following exposure to drinking water through inhalation of bioaerosols from showers and humidifiers. They indicated that endotoxin risks occur when the endotoxin activity of drinking water in humidifiers is 1000 EU/ml. McGregor et al. (1993) reported that swimming in a pool at a recreation center in Colorado, USA, which had high endotoxin activity levels, caused symptoms such as cough and fever in the lifeguards of this pool. Following the algal bloom of cyanobacteria in hot spring waters in Gaza, the activity of microcystin and endotoxin increased

simultaneously and caused synergistic toxicity for hot spring consumers (Mohamed, 2008). Atterholm et al. (1977) stated that following an increase in endotoxin activity in a water source, symptoms of acute fever with dry cough and muscle pain appeared in 56 patients after a warm bath, and these symptoms persisted for 6 to 15 hours. Hindman et al. (1975) studied endotoxin contamination following a cyanobacterial bloom in source water. The study found that endotoxin contamination was the leading cause of febrile reactions in 23 hemodialysis patients, one of whom died. In 1996, an epidemic of varying severity was observed in 126 patients due to the use of polluted city water from a local reservoir containing cyanobacteria, leading to the death of several people (Pouria et al., 1998). Muittari et al. (1982) reported that symptoms of high fever were observed in people who received an inhalation dose of 0.03-0.01 micrograms of endotoxin in the body after a water source was contaminated with it. In another study, Kennedy et al. (1987) surveyed a group of cotton workers in Shanghai, China. Cotton dust usually contains measurable levels of endotoxin. The first group consisted of 439 workers employed in a silk factory who had low or no exposure to endotoxin and were considered the control group. The second group consisted of 443 workers who worked in two cotton mills containing endotoxin. The cotton mill workers were divided into four possible endotoxin exposure groups based on their mills. This study showed that these workers had a higher prevalence of chronic bronchitis, defined as cough or phlegm in the chest for at least 3 months per year for 2 or more consecutive years. Byssinosis, which is chest stiffness or shortness of breath related to constant work shifts and improvement away from work, showed a similar trend in the workers of this factory. Another study investigated the effect of endotoxin on the immune and inflammatory systems of workers for one year. The results showed that exposure to bacterial endotoxin increases serum levels of CRP-day (GM=1.4 mg/l) and SAA-day (GM=12 mg/l) among workers (Madsen et al., 2023). Shaheen et al. (2009) conducted a study to investigate the levels of environmental endotoxin in indoor air dust samples collected from two schools - one in a rural area and the other in an urban area. The researchers performed a history of clinical examination, allergen skin test, and basic pulmonary function test on 200 children. The results showed that the level of environmental endotoxin was lower in rural schools than in urban schools. Urban students had a significantly higher history of allergy symptoms than rural ones, and the mean pulmonary function parameters in urban students were significantly lower than in rural students. Skin prick test results also showed a significant reaction to all tested allergens in urban children compared to rural children ($P<0.05$). In a 2015 study, Ohkouchi et al. found that natural bacterial/cyanobacterial flora in water environments and distribution systems can trigger human inflammatory responses. Some occupational settings have reported gastrointestinal symptoms such as diarrhea and stomach pain as a result of endotoxin exposure. These symptoms were prevalent in wastewater treatment plants (Lundin and Checkoway, 2009).

3.4. Endotoxin detection methods

There are two methods for measuring endotoxin: bioassay and chemical analysis. Bioassays measure the relative reactivity of endotoxins with enzymes, and chemical analyses focus on quantifying endotoxin biomarkers.

3.4.1. Bioassay methods

3.4.1.1. Rabbit pyrogen method

The oldest and simplest endotoxin detection technique, the rabbit pyrogen test, involves injecting the desired biological sample into live rabbits and waiting for the fever to develop (Dullah and Ongkudon, 2017). This method works on the principle that rabbits and humans have similar fever patterns under the influence of endotoxins. It has been found that an increase in temperature of 0.5 degrees Celsius within 180 minutes after injection causes fever (Gimenes et al., 2015). This measurement technique has a detection limit of 0.5 EU/ml, which was considered an accurate method at the time of the development of this method in 1912 (Hoffmann et al., 2005). In this method, many rabbits were examined, which is ethically problematic. In addition to ethical concerns about animal testing, this method has many disadvantages. The rabbit pyrogen method is a time-consuming procedure since body temperature is monitored starting 2 weeks before injection to ensure a relatively stable body temperature and continuing until 3 hours

after injection. Also, measuring core body temperature alone does not provide a specific diagnostic method for endotoxin, as other endogenous or exogenous substances may cause changes in body temperature. Most importantly, this method does not provide any trace amount of endotoxin. It is criticized due to the need for many samples and its almost obsolete sensitivity and accuracy compared to other methods (Vipond et al., 2016).

3.4.1.2. Limulus Amebocyte Lysate (LAL) Test

Limulus Amebocyte Lysate (LAL) assays have primarily replaced the rabbit pyrogen test in experimental research and the pharmaceutical and food industries. The LAL assay is simpler, more accurate, and cost-effective than the rabbit pyrogen test. Moreover, it is more sensitive to lower amounts of Lipopolysaccharide (LPS) than the rabbit pyrogen method. The LAL assay is up to 300 times more sensitive than the rabbit pyrogen test for pure, undegraded LPS and correlates well with it (Su et al., 2015). Additionally, the LAL assay has inherent advantages that reduce the likelihood of false negative results compared to the rabbit pyrogen test. The LAL assay uses a blood extract from the Limulus polyphemus horseshoe crab species (Jin et al., 2018). To clot, the LAL assay uses three zymogens (or proenzymes) found in horseshoe crab hemolymph: factor C, factor B, and a pre-coagulation enzyme. Zymogens are enzymes that remain inactive until a stimulus dissociates the enzyme and changes its structure, freeing the active site. In the LAL assay, the presence of LPS stimulates the activation of factor C, which activates factor B. The activation of factor B causes the activation of the pre-coagulation enzyme, and the active coagulation enzyme then activates the coagulant protein, producing a clot. The clot formation process is efficient and quickly leads to the formation of a clotting gel, which makes the solution cloudy (Fig. 1).

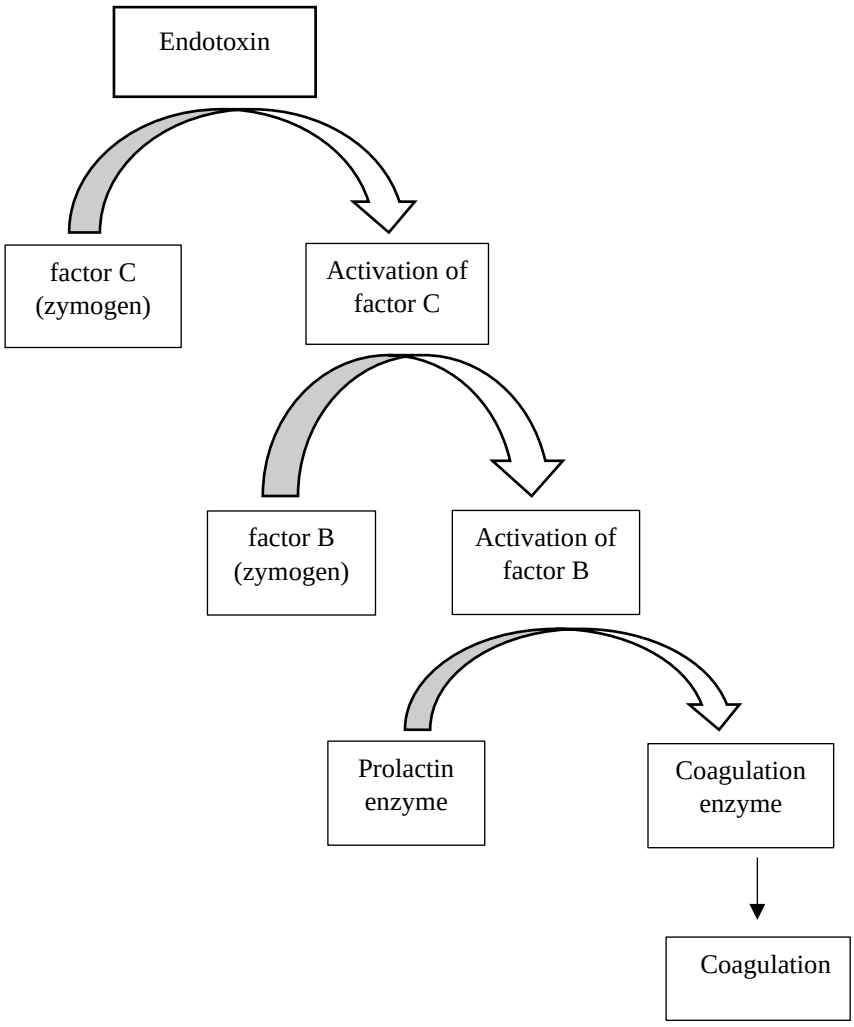

Fig. 1. Coagulation reaction in horseshoe crab in the presence of endotoxin

3.4.1.2.1. Methods of using LAL assay

Horseshoe crab blood extract is used in three ways: gel clot, chromogenic, and turbidity.

3.4.1.2.1.1. The clot gel method: This method involves mixing equal parts of the extract and the desired sample. If the gel is formed and the mixture remains intact at the end of the tube, the test is positive (Sakai et al., 2004). This means the sample has at least enough endotoxin to produce a positive reaction with a detection limit of 0.03-0.06 EU/ml. Chromogenic and turbidity methods are known as photometric tests because they require an optical reader for analysis.

3.4.1.2.1.2. Chromogenic and turbidity method: Chromogenic method is performed by replacing a natural substrate, coagulin, with a chromogenic or colored substrate. The chromogenic substrate is reacted with an endotoxin-activated coagulase enzyme, and the chromogenic molecule is released from the substrate into the suspension measured by spectrophotometry (Dolejs and Vanousova, 2015). The chromogenic method uses a set of amino acids and p-nitroaniline (pNA) as the chromogenic agent. The enzyme pNA cleaves the complex and turns the suspension yellow, directly proportional to the endotoxin concentration in the solutions (Dullah and Ongkudon, 2017). There are three types of chromogenic assays: two endpoint assays and one kinetic assay (Dawson, 1995). In end-point chromogenic assays, the release reaction of pNA is stopped by adding acid. The difference between the two endpoint chromogenic methods is incorporating a diazo compound with pNA, which reads the absorbance at 540 nm compared to 405 nm for pNA using the latter method. The change in absorbance avoids interference for samples that absorb light at 405 nm. The kinetic chromogenic assay measures pNA uptake at regular intervals throughout the experiment. The turbidity method is similar to the chromogenic method, but this method measures the turbidity of the solution. The rate of turbidity and absorbance change is proportional to endotoxin concentration. In the clot and gel turbidity assay, the coagulation enzyme converts coagulin to coagulin, which creates the gel and opacifying agent. These tests are widely accepted as official endotoxin tests in the water resources and pharmaceutical community (Chen and Mozier, 2013) and have different detection ranges (Table 1).

Table 1. Endotoxin detection methods using the LAL kit and the detection limit of each method (Schneier et al., 2020)

Method	Detection time(minute)	Detection Limit (EU/ml)
Endpoint chromogenic	14-30	0.01-0.1
Turbidity measurement	60	0.01
Chromogenic Synthetic	60	0.005
Florgenic endpoint	60	0.01-0.005
Gelchelate	30	0.25

3.4.1.2.2. Disadvantages of the LAL method

As previously mentioned, while the LAL method is more accurate than the rabbit pyrogen test, it has some disadvantages. For instance, the LAL test can produce false negative and false positive results. False-negative outcomes occur when endotoxins are combined with certain products such as buffer compounds (e.g., citrate and phosphate), cell culture medium compounds, and surfactants (e.g., polysorbate 20 and polysorbate 80) (Schwartz et al., 2017). Interfering agents create a protective layer around endotoxin molecules that makes them unavailable for reaction with LAL reagents, a phenomenon called low endotoxin recovery (LER) that leads to false adverse outcomes (Schwartz et al., 2017). Additionally, the LAL assay can produce false positive results by stimulating the factor G protease enzyme pathway with (1→3)-β-D-glucan, a primary component of the cell membrane of pyrogens, which forms the same final product as coagulin protein found in LAL reactions (Morita et al., 1981). Furthermore, the protein chains relied upon by the LAL assay can be disrupted in samples with free metal ions, proteins, peptides, and polymers that bind to the active site of endotoxins and neutralize their biological activity (Kushibiki et al., 2014). After discovering factor C as an endotoxin-activated part of the protein chain, efforts have been made to replace the conventional LAL test using recombinant factor C. As technology advances, alternative techniques have been considered to reduce pressure on the horseshoe crab.

3.4.1.3. Recombinant factor C (RFC) assay

RFC is a synthetic protein sensitive to endotoxins and derived from factor C DNA. It is used as a replacement for LAL testing (Maloney et al., 2018). This method involves endotoxin binding, activating a fluorescein substrate called amino methyl coumarin, which

activates synthetic factor C molecules. This produces a fluorogenic compound, and the fluorescence is measured at 440/380 nm in the presence and absence of endotoxins (Fig. 2). The difference in fluorescence is directly proportional to the concentration of endotoxins in the sample. This method eliminates the possibility of producing false positive results, which is a risk with the LAL method (Ding and Ho, 2001). The range of enzyme sensitivity to endotoxin in this method is 500-0.05 EU/ml (Pui et al., 1998). However, despite its low detection limit in laboratory conditions, the factor C assay is prone to contamination in laboratory environments, which can significantly compromise its analytical application (Barnett et al., 2012). Additionally, it is a fluorescence-based assay that requires a fluorometer and is less cost-effective than the LAL method (Reynolds et al., 2002).

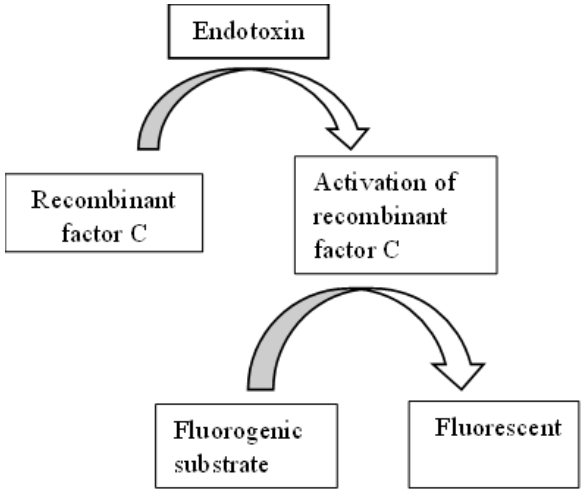

Fig. 2. Recombinant factor C assay reaction

3.4.1.4. Bovine Whole Blood Assay (bWBA)

The testing method involves drawing blood from the animal and mixing it with an endotoxin solution. When white blood cells in bovine blood are exposed to endotoxin, they produce prostaglandin E2 (PGE2), a cytokine that causes an inflammatory response similar to humans (Wunderlich et al., 2015). The production of PGE2 is directly correlated to the concentration of endotoxin. This test is highly accurate and can detect endotoxins in concentrations as low as 0.25 EU/ml, close to the concentration at which humans start showing symptoms of endotoxin exposure (Wunderlich et al., 2015). The test is relatively easy to conduct and requires minimal preparation time (Dullah and Ongkudon, 2017). However, it has some limitations. False positive results can be produced, and the required blood can only be collected from very young calves, which is difficult to obtain in large quantities. Additionally, certain countries do not allow the collection or use of cow's blood due to cultural or religious beliefs.

3.4.1.5. Monocyte Activation Test (MAT)

The Monocyte Activation Test (MAT) has been developed since 1995 (Vipond et al., 2019). This test is a laboratory alternative to the rabbit pyrogen test and detects a wide range of pyrogens, including endotoxins and non-endotoxin pyrogens. The test involves placing the sample being tested in contact with human monocyte cells, miming febrile reactions in the human body. When pyrogens are present, monocytes are activated and produce different types of cytokines, including interleukin-1β (IL-1β) and interleukin-6 (IL-6). The commercially available MAT kit uses frozen monocytes in human blood to test the reaction to endotoxins. The response to endotoxin is determined by measuring induced pro-inflammatory cytokines using enzyme-linked immunosorbent assay (ELISA) (Liebers et al., 2009). The cytokine released by monocytes in the presence of endotoxins binds to a primary antibody and to a secondary antibody with a peroxidase enzyme (Avidin-HRP) that metabolizes the substrate tetramethylbenzidine (TMB) and turns it from blue-green to yellow (Lequin, 2005). The spectrophotometer measures the color density absorption at 450 nm, similar to the LAL chromogenic test (Hoffmann et al., 2005). The MAT test has advantages, such as detecting all pyrogens and inflammatory substances that are harmful to humans (Hasiwa et al., 2013). The method has

a detection limit of 10 EU/ml. The most significant limitation of this method is the short half-life (>2 hours) of living monocytes in human blood in laboratory conditions.

3.4.2. Chemical analyses for the detection of endotoxin

Endotoxin detection tests involve gas chromatography/mass spectrometry (GC/MS) to quantify 3-OHFAs in endotoxin lipid A. These compounds act as indirect biomarkers of endotoxin levels. Unlike bioassays, the analysis of 3-OHFAs allows the determination of total cell-bound and non-cell-bound endotoxins. In some studies, gas-liquid chromatography (GLC) has been used. However, GLC has lower selectivity than GC/MS, as it identifies chemicals only by their retention time. In contrast, GC/MS detects molecular weight (mass-to-charge ratio) in addition to retention time, allowing it to identify the analytes more accurately. As a result, most studies currently use GC/MS for endotoxin detection (Shende et al., 2022). Among the various methods mentioned, the LAL kit method is the most commonly used method to detect endotoxin in water sources and detect human inflammatory activities. Despite the availability of various methods in endotoxin detection, the LAL kit method remains the most preferred one. Table 2 lists some studies that have used different methods for endotoxin detection.

Table 2. Studies conducted to detect endotoxin using different methods

	Diagnosis method	**Samples**
Bernardova et al. (2008)	LAL Kit	Laboratory and field cyanobacteria
Dobrovolskaia et al. (2014)	LAL Kit	Clinical grade nano-formulations
Gehr et al. (2008)	LAL Kit and factor C	Water resources of Montreal Island
Guizani et al. (2009a)	LAL Kit	Sewage
Dudziak, 2015	LAL Kit and factor C	Bacterial endotoxin
Zhang et al. (2016b)	LAL Kit	Wastewater
Maloney et al. (2018)	factor C	Water resources
Fung et al. (2017)	Capillary electrophoresis using laser	Wastewater
Neun and Dobrovolskaia, 2011	LAL Kit	Nanoparticles
O'Toole et al. (2008)	LAL Kit	Wastewater
Akbar John et al. (2012)	LAL Kit	Biological liquid
O'Toole et al. (2009)	LAL Kit	Water resources
Spaan et al. (2008)	LAL Kit and GC/MS	Wastewater treatment
Fujita et al. (2011)	LAL Kit	Water resources
El-Hakim et al. (2007)	LAL Kit	Water purification system
Dubczak et al. (2021)	factor C	medicinal water
Abate et al. (2007)	LAL Kit and factor C	medicinal water
Hoffmann et al. (2023)	GC/MS	Water resources
McClymont, 2017	factor C	medicinal water
Duan et al. (2007)	LAL Kit	Water river
Guizani et al. (2009b)	LAL Kit	Sludge treatment center
Guizani et al. (2016)	LAL Kit	treated wastewater
Huang et al. (2011a)	LAL Kit	Treated sewage effluent

3.4.3. Biosensors technique

As a means of enhancing and progressing endotoxin detection methods, researchers have created new techniques. These methods are novel diagnostic tools scientists aspire to use as a substitute for the conventional rabbit pyrogen test and LAL kit. The techniques can be categorized into electrochemical, optical, and mass-based sensors.

3.4.3.1. Electrochemical sensor

Electrochemical sensors operate on the principle of electrochemical impedance spectroscopy. In impedance spectroscopy, electrodes are placed in the solution to be tested, and an alternating current signal is passed through the solution. This signal typically varies between 2 and 10 mV, and changing its frequency can create an impedance spectrum (Honeychurch, 2012). To reduce electrical resistance, the electrodes are coated with metal. Specific proteins that are highly selective for endotoxin components bind to these electrodes. If the endotoxin comes into contact with the electrode-protein complex, it binds to the proteins (Syaifudin et al., 2011). The binding of endotoxin to the proteins on the electrodes increases the resistance of the electrode.

3.4.3.2. Optical sensors

Liquid crystal-based optical sensors are known for their high sensitivity in detecting endotoxins. Optical techniques can be classified into three main categories: luminescence, surface plasmon resonance (SPR), and bioelectrochemistry, all producing visual changes. Aptamer-based optical sensors, among other optical sensors, have a linear detection range for endotoxin from 0.05 to 1000 EU/ml (An and Jang, 2019).

3.4.3.3. Surface plasmon resonance (SPR) and mass-based techniques

The plasmonic biosensor works through bent optical fiber probe technology, which employs octadecyl trichlorosilane on the surface of optical fiber probes to capture endotoxin from aqueous solutions using its hydrophobic part. The endotoxin binding occurs in real time by measuring the change in the refractive index (Manoharan et al., 2019). Many attempts have been made to replace biological methods with more advanced diagnostic techniques such as biosensors to detect pollutants like endotoxin. Although some biosensors have been developed with a very low minimum detection limit for endotoxin and have proven to be accurate in detecting this pollutant, no biosensor has been commercialized or officially approved by organizations like the FDA (Food and Drug Administration) and the World Health Organization. This technology is still under development. Table 3 presents some of the biosensors that have been used to detect endotoxin and their corresponding detection ranges.

Table 3. Types of biosensors made for endotoxin detection and its detection limit

	Biosensor	**Detection limit**
Manoharan et al. (2019)	Plasmonic with optical fiber	0.04 EU/ml
Liu et al. (2017b)	Quartz crystal microbalance (QCM) with advanced signal	10- 0.005 EU/ml
Zandieh et al. (2018)	Silver nanostructures	340 pg/ml
Wang et al. (2014)	Gold nanotubes	0.0084 μM
Liu et al. (2019)	Nano molecular membrane	$1.3*10^{-5}$ μg/l
Tang et al. (2020)	Fluorescence	1-0.1 EU/ml
Xiong et al. (2012)	Piezoelectric biosensors	0.1 pg/ml
Ma et al. (2020)	Super sensitive and selective detection microfluidic	500 pg/ml
Inoue and Takano, 2010	Zymogen-based Electrochem	1000-5000 EU/ml
Yu et al. (2019)	Electrochemistry of graphene oxide and gold nanoparticles	1-0.0001 EU/ml
Priano et al. (2007)	Amperometric with neutralizing protein	0.07 EU/ml
Sakti et al. (2001)	Thickness Shear Mode (TSM)	100 mg/l
Reta et al. (2019)	Electrochemistry based on porous silicon membranes	18 EU/ml
Liu et al. (2017a)	Electrochemistry based on porous silicon membranes	0.004 EUml
Inoue et al. (2012)	Electrochemistry based on potentiometric method	1-5 EU/ml
Noda et al. (2010)	Bioluminescence	0.0005 EU/ml
Prasad et al. (2018)	Fluorescent	10 EU/ml

3.5. Control and reduce endotoxin contamination in water sources

Implementing endotoxin removal solutions requires careful consideration of several factors. These include water quality requirements, the scale of treatment required (Domestic, community, or industrial), and the associated operating and maintenance costs. Among the various methods available, coagulation and sedimentation are commonly used water purification technologies that work well against hydrophobic pollutants with considerable molecular weight, such as endotoxins. These processes, which include coagulation, sedimentation, and filtration, effectively reduce endotoxins in water sources. Several studies have investigated these processes in water, wastewater, and treated sludge to evaluate the effectiveness of different treatment methods on endotoxin levels and to improve these methods. Can et al. (2013) found that traditional purification processes in a Beijing water treatment plant reduced total endotoxin levels by 63%. In another study, Rapala et al. (2002) observed an 83-86% reduction in endotoxin activity during coagulation, sedimentation, and filtration of sand in the water treatment process in Finland. Zhang et al. (2013) discovered that coagulation and sedimentation removed 60% of total endotoxin, 52% of free endotoxin, and 72% of bound endotoxin in a Beijing water treatment plant. Dai et al. (2018) reported that polyaluminum coagulation followed by gravity sedimentation reduced the free and bound endotoxin ratio by 86.5 ± 8.5 and 60.8 ± 37.5%, respectively. Guizani et al. (2016) discovered that a coagulation-flocculation process removed up to 40.5% of endotoxin from secondary wastewater samples at a treatment plant in Japan. Duan et al. (2007) conducted studies on two treatment plants in Wuhan, China, which showed that the treatment process reduced total endotoxin activity by 7 and 12%, respectively. In another study, Rapala et al. (2006) found that

sand filtration in water treatment plants increased endotoxin removal efficiency in drinking water from 36 to 96%. Goyal and Gerba (1982) reported a 90-99.9% reduction in endotoxins during wastewater infiltration in 100-250 cm of soil. Guizani et al. (2011) reported that the removal efficiency of endotoxin activity reached 64.3% and 86.7% in very coarse sand and fine sand, respectively, for the secondary effluent from a full-scale activated sludge process in a sewage treatment plant. After 75.6% soil filtration, the removal efficiency was observed. In two wastewater treatment plants in South Africa, Berger et al. (1989) found that endotoxins were reduced by 84.2% and 97.2% after sand filtration. According to O'Toole et al. (2008) research, endotoxin concentrations in reclaimed water can be reduced to comparable levels in drinking water. However, higher endotoxin concentrations may occur for some reclaimed waters where membrane filtration is not performed. Simazaki et al. (2018) research showed that coagulation and chemical precipitation significantly reduce free and particle-bound endotoxins. It is important to note that filters sometimes pose the greatest risk of endotoxin contamination. A study by Anderson et al. (2008) revealed that the average activity of endotoxins increased from 6 EU/ml in water to 16 EU/ml in the filter effluent when using dual active environmental filters (Anthracite/sand). Furthermore, after an 18-hour filter shutdown test, the activity of endotoxin in filter effluent reached 745 EU/ml. These findings highlight the relationship between bacterial growth and the condition and performance of filters. Therefore, it is advisable to avoid long-term shutdowns to reduce the volume of filters. Additionally, waste of filter capacity should be avoided to ensure timely backwash for filtration. Purification processes can result in increased endotoxin levels in water in some cases, such as chlorination, while methods like using ultraviolet rays and ozone can effectively remove endotoxin. A study by Oh et al. (2014) found that ultraviolet radiation (480 mJ/cm^2) reduced endotoxins in river water by 2.4%. In addition, exposure to 2 mg/l ozone for 10 minutes resulted in a reduction of endotoxin activity in river water from 0.431 ng/ml to 0.384 ng/ml. Gehr et al. (2008) study showed that ozone can effectively reduce endotoxin in drinking water, with a maximum reduction of 57% at a rate of 2.5 mg/min/l, and up to 74% at a rate of 20 mg/min/l. Zhang et al. (2016a) demonstrated that dielectric barrier discharge plasma reduced total endotoxin activity in standard endotoxin solutions from 2.3 EU/ml to 0.5 EU/ml, a decrease of 78.3% within 480 seconds. This study indicates that the low-temperature plasma method resulted in a significant reduction of free endotoxins in water. However, the results of Mohamed et al. study (2022) showed that final chlorination only reduced bound endotoxins by 1 EU/ml but increased free endotoxin (112-37 EU/mL) in purified water. It is essential to remember that the efficiency of purification techniques in eliminating endotoxin is affected by specific factors such as the initial levels of endotoxin, water quality parameters, and other contaminants in the water that coexist with endotoxin. Therefore, combining multiple treatment methods may be necessary to achieve the optimum endotoxin removal.

3.6. Legal framework regulations

Establishing comprehensive regulations and standards to determine the endotoxin limit in water sources is a complicated task due to the varied structure of endotoxin in bacteria and cyanobacteria, as well as their diverse toxicity. As a result, international organizations, such as the World Health Organization, have yet to establish specialized guidelines for this contaminant (Zhang et al., 2019). Developing guidelines and harmonized approaches across different regions can ensure consistent water quality protection. However, some countries have general instructions on endotoxin without taking specific details into account. The National Health Council in the Netherlands has recommended that the occupational exposure limit for endotoxin should not exceed 4.5 ng/m^3 (Andrews et al., 1985). Similarly, Palchak et al. (1988) cited Laitinen et al. (1992) and suggested that the exposure limit to endotoxin in workplaces should be 30 ng/m^3 with an 8-hour weighted average. According to the American Thoracic Society's guidelines from 1998, the concentration of endotoxin in the workplace air should not exceed 200 ng/m^3 to prevent toxic organic dust syndrome, 100 ng/m^3 to prevent systemic effects, and 10 ng/m^3 to prevent inflammation of the respiratory tract (Kennedy et al., 1998). Moreover, the Dutch Health Council recommended that the maximum allowed endotoxin concentration in

the workplace air should be 90 EU/m^3 per year (DECOS, 2010). Although local standards have been established, there is still a lack of a specific endotoxin standard or a guidebook for sampling and measuring endotoxins by different methods. Moreover, there is no universal standard for endotoxin production from various sources and occupational exposure to endotoxin.

4. Conclusion

Endotoxin as an emerging pollutant in water sources, the amount of this pollutant, the risks caused by it on human health, the ways of diagnosis, and related laws were investigated during this study. Identification and control of endotoxin are necessary to reduce potential adverse effects on human health. According to the review, more collaborative efforts among researchers, policymakers, and stakeholders are needed to address the complex challenges associated with endotoxin contamination. By embracing innovative technologies and maintaining a proactive approach, we can protect both our ecosystems and the well-being of future generations.

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