

ENDOTOXINS AND β -GLUCANS AS MARKERS OF MICROBIOLOGICAL CONTAMINATION – CHARACTERISTICS, DETECTION, AND ENVIRONMENTAL EXPOSURE

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Abstract: Endotoxins and β -glucans are one of the major markers of microbiological contamination. As components of biological aerosols, they are ubiquitous in many environments. Numerous studies performed during the last three decades have proved that exposure to endotoxins and β -glucans could be associated with many diseases and/or adverse health outcomes. The aim of this review is to present the current stage of knowledge regarding endotoxins and β -glucans as biologically active components of aerosols and to characterize the quantitative methods for their detection in environmental samples. The problems of occupational exposure to both these components and their control procedures are also discussed. An overview of the available worldwide and Polish standards and threshold limit values is also given.

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INTRODUCTION

The striking increase in disease incidences related to exposure to biological agents has been globally observed during recent decades. In spite of the significant advances in medical diagnostics, some of the hazardous agents responsible for this phenomenon remain unidentified and the pathophysiological mechanisms accountable for it are not fully explained [120]. Among probable causes associated with a wide range of adverse health effects observed in both occupational and non-occupational environments, indoors and outdoors, are microbial hazards [35, 57, 146].

Microorganisms interact with the surroundings in many different ways. They can grow and colonize various natural and artificial materials and, by that, cause their biodegradation and biodeterioration. During these processes, the immu-

nologically reactive structures and substances can be released (often in a great number) into the environment and, as such, cause adverse health outcomes. This is especially important in the case of microbiological air contamination, when the exposure to a high concentration of bioaerosols may induce a series of immunopathogenic reactions. Among the numerous biologically active microbial agents are those which are ubiquitous in the air and for which airborne transport is a major way of dissemination in the environment. Among these, special attention is given to bacterial endotoxins and fungal β -glucans [33, 83, 140]. These highly conservative structures (called pathogen-associated molecular patterns, PAMPs), being selectively recognizable by the immune system cells, can substantially modulate the host response [52, 101, 102].

The aim of this review is to summarize the current state of knowledge about endotoxins and β -glucans as biologi-

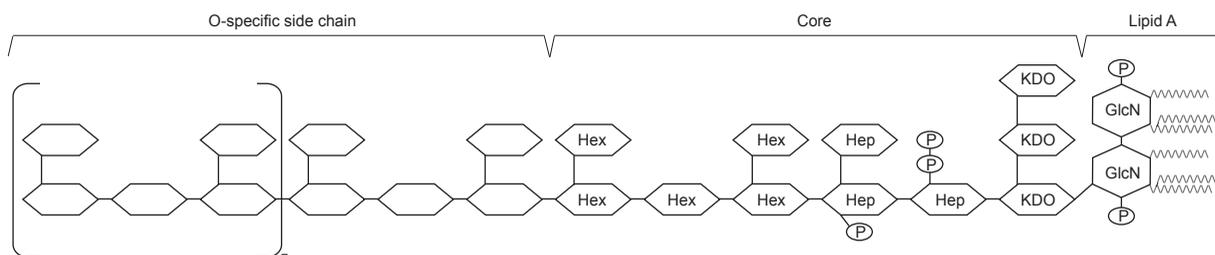


Figure 1. Schematic structure of lipopolysaccharide [84]. Hex – hexose; Hep – heptose; P – phosphoric acid residue; KDO – 3-deoxy-D-manno-octulosonic acid; GlcN – glucosamine; n – number of repeating oligosaccharide units, $\sim\sim\sim\sim\sim\sim$ – long chain fatty acid.

cally active components of aerosols, and to characterize the quantitative methods available for their detection in environmental samples. Moreover, the problems of occupational exposure to endotoxins and β -glucans, as well as the standards and threshold limits value proposals for both these microbial components existing in the scientific literature, are also discussed.

ENDOTOXINS

Endotoxin, known as lipopolysaccharide (LPS), is an integral component of the outer membrane of Gram-negative bacteria. Chemically, endotoxins are heteropolymers that include both lipid and polysaccharide moieties. In common use, the term “lipopolysaccharide” defines the chemically-purified molecule obtained via several extraction steps. On the contrary, the term “endotoxin” describes the molecule *in situ*, i.e. when the molecule is still associated with proteins and other molecules of the outer membrane. The single molecule of LPS consists of three different regions: O-specific side chain, core oligosaccharide and lipid part (called “lipid A”) (Fig. 1) [84]. These regions differ from strain to strain in both chemical composition and biological properties as well as being distinguished by their structural variability. The O-specific side chain is composed of repeating oligosaccharide units containing up to eight carbohydrate residues. The characteristic structure of this region determines the serological specificity of the LPS and plays a role of surface antigen (called “O antigen”) for individual bacterial species. The O-specific region, protruding extracellularly from the membrane, enables bacterial adhesion to the epithelial cells and, by that, evades the host immune system. The core region that links O-specific chains with lipid A is characterized by a less diverse structure compared to the O antigen. The core regions can be divided into the outer (composed of hexoses, Hex), and inner (consisted of heptoses, Hep, mainly in the L-glycero-D-manno configuration as well as 3-deoxy-D-manno-octulosonic acids, KDO) moieties. The inner core region substitutes the lipid’s glucosamine residues by the first acid residues of KDO [59, 69, 84, 87, 135].

The most conservative part of LPS is lipid A that anchors it in the outer membrane of Gram-negative bacteria. The sugar skeleton of lipid A is formed by two (1 \rightarrow 6)- β -D-

glucosamine units substituted by saturated fatty acids with long unbranched tails. The characteristic elements of lipid A are 3-hydroxy fatty acids (3-OH FAs) and, sporadically, 2-hydroxy fatty acids. The saturated fatty acids create a barrier against penetration of hydrophobic substances (such as antibiotics or bile acids) inside the cell [59, 69, 87, 135].

Biological form of endotoxins in the environment.

Endotoxins can get into the environment in the course of bacterial cell lysis, during active growth of the cell, or as a result of sudden changes in the hydration state of cell wall when Gram-negative rods are in the aerosol phase. After release, they can occur as components of the whole cell, as cell wall fragments, or as macromolecular aggregates (called “free endotoxins”) with a mass of about 1 million Da [39, 42, 55, 116].

The first ultrastructural description of biologically active form of endotoxins in the environment was published by Dutkiewicz *et al.* [42]. Using solid agar media, they have shown an ability of *Erwinia herbicola* and *Enterobacter* spp. strains to shed the membrane vesicles containing endotoxins. The subsequent preparation of lipopolysaccharides isolated from these two strains, as well as from *Alcaligenes faecalis* and *Acinetobacter calcoaceticus*, confirmed the presence of resembling vesicles. These laboratory observations were in a good agreement with the environmental data obtained by electron microscopy examination of pulverized wood samples overgrown with bacteria (used as a model substrate in this study). The formation of membrane vesicles occurs by a convolution of the outer membrane followed by a budding-like detachment of the terminal blebs. The process may progress in two ways, as external budding or as internal budding into a pouch-like structure. The resulting supermacromolecules are released into the environment as vesicular shell-like particles measuring 30–50 nm, with a characteristic “tripled-tracked” membrane resembling the outer membrane of Gram-negative rods. The immunolabeling with colloidal gold confirmed the presence in the vesicles of both lipopolysaccharide and its constituent, i.e. lipid A [39, 42, 55].

Endotoxins as immunologically reactive agent. Endotoxins belong to the most specific bacterial structures that are selectively recognized by innate immune response cells.

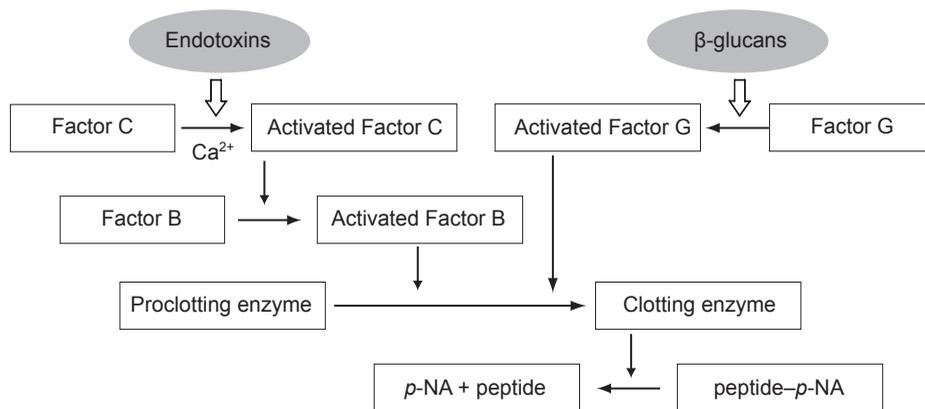
It is assumed that lipid A is responsible for most of the biological activities of endotoxin. Lipid A, when present on a surface of blood cells or incorporated into liposomes, stimulates the host organism to secrete specific antibodies [3, 180]. The toxicity of lipid A is linked to various pathophysiological reactions dependent on both infected organism and pathogen species. The inflammatory reactions caused by endotoxins are related to the activation of macrophages, neutrophils and blood platelets. Endotoxins have been reported to stimulate cells for the release of cytokines and for their production of inflammatory mediators, such as interleukins (IL-1, -6, -8 and -10), tumour necrosis factor-alpha (TNF- α), arachidonic acid metabolites (prostaglandins, leukotrienes), platelet-activating factor (PAF), free oxygen radicals, hydrogen peroxide and nitric oxide. In humans and animals, endotoxins reveal a very strong biological activity causing fever, shivering, arthralgia, influenza-like symptoms, blood leukocytosis, neutrophilic airway inflammation and asthma symptoms. The influence of LPS on human health may have both a local and a generalized character leading to a disturbance in homeostasis and septic shock [83, 135, 139, 172, 179]. It should be emphasized that the negative influence of endotoxins on host organisms is maintained despite a viability of bacteria, i.e. by both viable and dead cells. The LPS can appear in the environment as a result of bacterial cell lysis caused by antibiotic treatment or by sudden change of a hydration state of the cell wall (e.g. when bacterial particles are airborne). The release of endotoxins from cell walls of dead bacteria intensifies the toxic activity of these particles [12, 70].

Many clinical studies prove that the immune response to endotoxin exposure is a result of different interactions covering a dynamic balance between dose and duration of exposure, seasonal variations, genetic predispositions and additive or synergistic effects caused by the presence of other toxic substances in the host organisms [11, 84, 85, 129, 187]. According to literature data, an occupational exposure to endotoxins has been linked to various adverse health outcomes, from headache, cough, shortness of breath, through chest tightness and flu-like illnesses to chronic bronchitis, allergic rhinitis, asthma, or an induction of organic dust toxic syndrome (ODTS) [16, 67, 84, 107, 140]. Some researchers have reported that LPS, in synergy with other substances present in organic dust, plays an essential role in airway inflammation and bronchoconstriction [27].

The last two decades, on the other hand, have also broadened knowledge about the protective effect of environmental endotoxin exposure with regard to atopic asthma and allergy development in early childhood. According to the "hygiene hypothesis", the global increases in asthma prevalence could be due to increased susceptibility induced by a more "sterile" environment. In this context, it has been proposed that microbial infections or exposures to microbial agents such as bacterial endotoxins early in life may drive the response of the immune system – which is known

to be skewed in an atopic Th2 direction during foetal and perinatal life – into a Th1 direction and away from its tendency to develop atopic immune responses. This protective effect may be increasingly lost as the environment becomes "cleaner" [49, 50, 86, 102, 181]. If the protective effect of endotoxins is a true phenomenon, it could be helpful in development of tolerance to common antigens and in the prevention of such atopic diseases as allergic asthma, allergic rhinitis or eczema [13, 47, 106, 121, 183]. Moreover, the epidemiological evidence, as well as some animal experimental and therapeutic trial data, has demonstrated that endotoxin has antitumour properties. Nevertheless, application of endotoxin as a chemopreventive agent against cancer still requires additional toxicological and clinical studies to clarify both the biological mechanisms of anticancer effects and the complexity of dose-response relationships [4, 91, 155]. It should be remembered, however, that even a cease of excessive activations of the immune system by small amounts of LPS may lead to an overproduction of proinflammatory mediators and, by that, result in elicitation of a cascade of adverse pathophysiological reactions [39, 139, 179].

Methods for endotoxin detection in environmental samples. To confirm the presence and/or to evaluate the amount of endotoxins in environmental samples, both *in vivo* and *in vitro* assays can be applied. Among *in vivo* methods are the rabbit pyrogen test and the Schwartzman test. Among *in vitro* methods, the *Limulus* test in its several modifications (i.e. gel-clot – called also "classic *Limulus* test", turbidimetric and chromogenic assays – the two latest in both endpoint and kinetic variations), recombinant Factor C procedure and liquid or gas chromatography (alone or in combination with mass spectrometry) analyses can be distinguished. The *in vivo* tests are usually used in qualitative applications to confirm/exclude the presence of pyrogen in the sample, for endproduct testing of human and animal injectable drugs and medical devices. Chronologically, the oldest is the rabbit pyrogen test (also known as rabbit fever test) and consists in the measurements of rabbit body temperature increase induced by an intravenous injection of sterile solution of the tested product (e.g. drug) or environmental sample (e.g. dust) to be examined. It should be pointed out, however, that the rabbit assay determines a pyrogenic activity which may be caused by endotoxins as well as Gram-positive bacteria, mycobacteria, fungi and even viruses. Another *in vivo* method is based on the Schwartzman phenomenon, which occurs when a small dose of endotoxins (e.g. present in the examined sample) is subcutaneously injected, followed by an intravenous injection of a known dose of LPS to the same animal, particularly rabbit, 24 h apart. In the case of endotoxin presence in the sample, a local hemorrhagic necrosis at the site of injection is observed [29, 39, 55]. Due to the high cost, long turnaround time, animal rights issues, an analytical utilization of both these *in vivo* tests is diminished. They



p-NA—*para*-nitroaniline

Figure 2. The reaction pathways of endotoxins and β -glucans with *Limulus* amoebocyte lysate reagents used for their quantification [1, 2].

are currently rarely applied and, if at all, in combination with the *Limulus* test only.

Nowadays, the most popular method for bacterial endotoxin quantitation in raw materials, dust and air samples is the *Limulus* assay. This test is based on Bang's observation that an infection of the horseshoe crab (*Limulus polyphemus*) induced by Gram-negative bacteria results in extensive intravascular hemolymph clotting and, by that, evokes the arthropod's death [8]. The subsequent studies of Levin and Bang showed that such extracellular coagulation is caused by the reaction between endotoxin and coagulative protein in amoebocytes circulating in *Limulus* hemolymph (called *Limulus* amoebocyte lysate, LAL) [81, 82]. The biochemical principle of the *Limulus* test is based on a cascade of enzymatic reactions initiated by endotoxins in the presence of calcium ions. Starting from Factor C (a protease zymogen), this cascade pathway results in an activation of proclotting enzyme (a serine protease) in the lysate (Fig. 2) [1, 2]. The activated clotting enzyme cleaves (at the Arg-Lys and Arg-Gly linkage) soluble protein (coagulogen) from LAL in an insoluble complex (coagulin) and, finally, forms the characteristic gel matrix [114, 171, 191]. The LAL tests are very sensitive and have a broad measurement range from 1 pg/ml–10 ng/ml. In 1977, this test was accepted and recommended by the United States Food and Drug Administration (FDA) as the standard assay for bacterial endotoxin detection. According to FDA guidelines, an endotoxin concentration in the sample is expressed in endotoxin units (EUs), which describe the biological activity of endotoxins. The EU is standardized against the defined reference material, i.e. reference standard endotoxin (RSE). As stated by the FDA, both RSEs, i.e. 100 pg of the *Escherichia coli* EC-5 and 120 pg of the *E. coli* O111:B4 have activity equal to 1 EU [23, 68, 97].

In the gel-clot *Limulus* test, the serial dilutions of a tested sample (e.g. product eluate, particulate aerosol or settled dust sample extracts) are mixed with equal volumes of the *Limulus* reagent in the pyrogen-free tubes. After 60 min-

utes of incubation at 37°C in a water bath, the tubes are removed from the incubator and observed for clot formation after inverting them 180 degrees. A positive result is the formation of a solid gel-clot at the bottom of the reaction tube that withstands inversion without breaking. The concentration of endotoxin in examined sample is determined by the highest extract dilution at which coagulation is still observed. A series of RSE dilutions (usually starting from 1 EU/ml) is used to determine the sensitivity, which is the lowest endotoxin concentration forming a clot. The gel-clot test is the simplest among all *Limulus* assays and requires minimal laboratory equipment [23, 57].

The turbidimetric *Limulus* assay can be applied in two variations, i.e. as endpoint and kinetic tests. This test relies on an observation of turbidity increase in the sample, caused by protein coagulation under the influence of endotoxins. In the endpoint variation, after required incubation time (usually 1 hour), the optical densities (OD) of test-sample dilutions are measured (a single reading at $\lambda = 405$ nm for each sample) and correlated with endotoxin concentrations according to a standard curve, built based on the samples with a known amount of RSE [23, 88]. The higher the endotoxin concentration in the sample, the greater absorbance is spectrophotometrically measured. Only those concentrations that are sufficient to cause development of measurable turbidity within the given incubation time may be quantified in this assay. In contrast, in the kinetic turbidimetric method, the OD readings are taken at regular intervals throughout the whole test. The higher the endotoxin concentration in the sample, the faster the reaction and the shorter the onset time (i.e. the time which is necessary to reach a specific OD threshold). The endotoxin concentration in the studied sample is read from a standard curve, as practiced in the endpoint variation.

In 1977, Nakamura *et al.* discovered that endotoxin-activated LAL may also cleave small chromogenic peptides in the places similar to those in coagulogen [115]. In the chromogenic method, the initial part of LAL reaction with

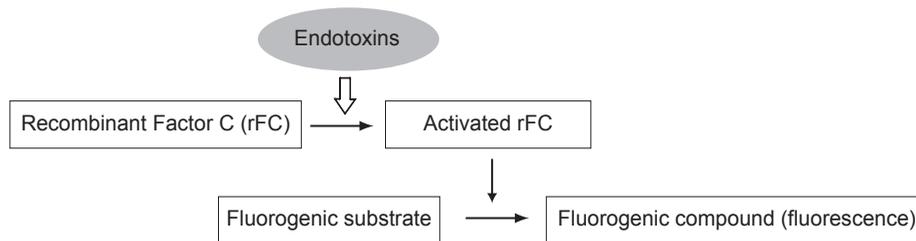
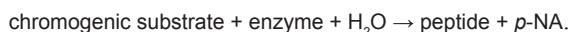


Figure 3. The recombinant Factor C mechanism of reaction [20, 68, 89, 90].

endotoxins is utilized (Fig. 2). The clotting enzyme, activated in the cascade, splits a chromophore (*para*-nitroaniline, *p*-NA) from the chromogenic substrate producing a yellow colour according to the following scheme:



After necessary incubation time (usually 1 hour), the amount of released *p*-NA, which is photometrically measured (at $\lambda = 405$ nm), is proportional to the amount of endotoxins in the samples. The greater the endotoxin concentration, the faster the reaction and the quicker the yellow colour development of the mixture [2, 66, 115]. The chromogenic LAL test appears in three variations, i.e. as two endpoint methods and a kinetic assay. In both endpoint methods, the above-described reaction is stopped after a certain period of incubation by adding acid, and the OD of the resulting mixture is photometrically measured. In the first endpoint variation, the peptide-*p*-NA complex is solely used as a source of the dye. In the second variant, called “diazo endpoint chromogenic LAL”, liberated *p*-NA is coupled to a diazo compound. When the reaction is stopped, the resulting mixture obtains a magenta colour and its OD is measured at a higher wave length $\lambda = 540$ nm. The higher endotoxin concentration in the sample, the more *p*-NA is liberated from the peptide and the deeper yellow or magenta colours of the final mixture. The endotoxin concentration in the studied sample is traditionally read from a standard curve [23].

The most sophisticated modification of the LAL assay is its kinetic chromogenic variant. The main principle of this test is very similar to those described above, i.e. the activated enzyme, catalyzing the split of *p*-NA from the colourless substrate, produces a yellow colour. The photometric measurements are carried out continuously throughout the whole incubation period (usually 50–60 min) at regular intervals (e.g. 30 s). The time required before the appearance of a yellow colour (reaction time) is inversely proportional to the amount of endotoxins present in the tested sample. In the presence of a large amount of endotoxins, the reaction occurs rapidly. When the amount of endotoxins is small, the reaction time becomes extended. The concentration of endotoxins in a sample is based on computer analysis of the speed of these changes. Irrespective of a wide range of applications and high sensitivity, the *Limulus* test

enables detection of the “active form” of endotoxins only. As opposed to the biological tests described above, the cell-bound LPS, which is treated as the absolute amount of LPS, can be assessed using other chemical (instrumental) methods [18, 57].

The LAL tests are very sensitive; however, their mechanism of reaction, reliant on enzymatic cascade initiated by activation of Factor C, can be triggered by both endotoxins and glucans. Because of this alternative glucan pathway, the LAL tests may sometimes give false positive results for endotoxin detection [68, 90]. Moreover, taking into account the “lot-to-lot” variations derived from differences in lysate pool, seasonal changes and environmental factors, the LAL reactivity can be simply altered by its biological nature [20, 68]. To eliminate all these imperfections, the recombinant Factor C (rFC), a single-step, quantitative endpoint procedure has been introduced. As mentioned earlier, Factor C has an ability to selectively recognize endotoxins and activate the protease cascade. To create an endotoxin-specific assay, Factor C has been purified and recombined. The new assay uses the rFC cloned from the horseshoe crab, *Carcinoscorpius rotundicauda*. This endotoxin-sensitive protein shares a 90.5% identical amino acid sequence with the native Factor C protein derived from *L. polyphemus* [90]. In the test (Fig. 3), the rFC is activated by endotoxin binding and such active moiety enzymatically cleaves a synthetic (peptide-coumarin) substrate, resulting in the generation of fluorogenic compound. Fluorescence is measured at time zero and after one-hour incubation with endotoxin standards at 37°C using excitation/emission wave lengths of 380/440 nm. As RSE, the *E. coli* endotoxin O55:B5 is usually used (other RSE can be used; however, their performance must be always determined). The fluorescent signal is proportional to the endotoxin concentration in the sample and is estimated based on a standard curve. A minimum detection limit of this method is 0.01 EU/ml and fluorescence-endotoxin concentration relationship is linear over the 0.01 EU/ml to 10 EU/ml [20, 68, 89, 90]. The rFC procedure has been successfully validated according to the requirements listed in the United States Pharmacopeia and, since 2009, the FDA approved the use of this procedure as the final release test [68, 90].

When a high variability in the composition of both biological and environmental samples is observed, an application

of sophisticated instrumental techniques may help to improve reproducibility and specificity for the determination of endotoxins in such complex matrices [71, 113]. Among these techniques, the most prevalent are: high-performance liquid chromatography (HPLC) [25, 92, 112, 113], gas chromatography (GC) [71, 111], gas-liquid chromatography (GLC) [26, 119], and gas chromatography-mass spectrometry (GC-MS) [98, 109, 134, 152, 164, 169]. All these analytical methods estimate the concentration of endotoxin constituents (e.g. KDO, 3-hydroxymyristic acid, 3-hydroxy-lauric acid or other 3-OH FAs), which serve as chemical markers for the quantification of LPS in the samples of different origin (e.g. cotton dust and lint [25, 98, 112, 113], grain dust [26], house dust [152, 153, 169], compost [71], sewage sludge [113], sediments [113, 119], biofuels [96], soil [192], water and mist [182], air [63, 164], cigarette, tobacco leaves and smoke particles [79] as well as vaccines [92], rabbit serum [98, 113] or Gram-negative bacteria [111]).

Nowadays, the most frequently used is GC-MS analysis, which focuses on a quantitation of 3-hydroxy fatty acids from lipid A. The typical enteric LPS contains specific 3-OH FAs with 10–18 carbon chains linked to the glucosamine disaccharide moiety of lipid A, which can be treated as chemical markers of endotoxins [109, 164, 169]. As it is not dependent on variable biological sensitivity, the GC-MS method can measure the total amount of endotoxins, i.e. both biologically active and inactive forms. Moreover, it can provide some information about the environmental sources of LPS due to the relative distributions of the individual 3-OH FAs, which differ among species of Gram-negative bacteria [109, 152, 164]. From the analytical point of view, the GC-MS method for dust samples requires the performance of several (complex and time consuming) analytical steps, including: HCl methanolysis, extraction of methyl esters with hexane and water, addition of internal standard (e.g. deuterated 3-OH-C_{14:0} methyl ester), drying (evaporation of hexane layer), redissolving in dichloromethane-hexane, application of preparations to a silica gel column, column washing, drying of the eluates (under a stream of nitrogen), and finally, chromatographic analysis of 3-OH FAs as methyl ester-trimethylsilyl derivatives (for more analytical details see: [109, 152, 164]). Despite the fact that GC-MS has limited applications for the assessment of endotoxins in environmental samples due to a low sensitivity (about 1000-fold lower than the LAL assay), this method is quite often applied to optimize both extraction and treatment procedures of environmental samples [109, 152, 164, 169].

Correlations between the methods for endotoxin quantitation. Since the relationship between endotoxin exposure and subsequent health outcomes has aroused scientific interest, there has been no agreement concerning the standard method for quantification of endotoxins as well as the laboratory elaboration and analysis of environmental

samples. For example, considering the LAL methods as the most widely used, the FDA regulates endotoxin determination for pharmaceutical and medical employments while there are no similar regulations for environmental applications [184]. Even when only one assay is exploited in the same environment, a variation between the results of quantitative analysis could be significant. The observed differences lie in: diversity of used sampling techniques and collection media, transport conditions, sample storage, treatment and extraction procedures, analytical instrumentation, and – with reference to particulate aerosol samples – instability of the biological material [36, 134, 152, 182]. Moreover, when comparing the endotoxin measurement results, a different sensitivity of the applied methods, an application of different RSE as well as “lot-to-lot” reagent variations, even when derived from the same manufacturer or supplier, should be noticed as well [29, 57, 114, 184].

As mentioned earlier, various detection assays deliver different information. Whereas the *Limulus* test detects endotoxins, which are active in this assay only, the other instrumental methods measuring the concentration of specific structural components (such as 3-OH FAs) can estimate the total LPS content in studied samples [104, 152]. The hitherto obtained data show that the concentration of LPS (i.e. total bacterial endotoxins) is measured with the same efficiency, irrespective of whether the LPS is present in a cell-wall-dissociated or -associated state or is attached to other molecules of organic origin. Despite the fact that the GC-MS gives higher endotoxin concentrations in the samples than the LAL test [125, 152, 164], the correlation between both these methods is not unequivocal, being statistically significant [58, 182] or weak [125]. The character of such quantitative relationships may depend on: the type of the sample, bacterial strains or the structure of their LPS, especially on the number and types of 3-OH FAs in the cell wall [182]. Similar observation is true when comparing results obtained by the rabbit pyrogen and LAL assays. The experiments carried out by Devleeschouwer *et al.* showed significant positive correlation between the number of organisms needed to produce LAL-positive and pyrogenic(rabbit)-positive responses [29]. For environmental samples, when various *in vitro* analytical methods are compared, e.g. gel-clot with kinetic chromogenic LAL [58], gel-clot with endpoint chromogenic and kinetic turbidimetric LAL assays [114], chromogenic LAL with GC-MS [58, 100], kinetic chromogenic LAL with GC-MS [152, 182], kinetic chromogenic and turbidimetric LAL with rFC [20, 72], GC-MS and rFC [15], the obtained endotoxin measures usually significantly correlate.

Nowadays, when availability of different analytical protocols applied to measure endotoxin content is relatively common, the studies dedicated to a comparison of obtained results are still of a great value. Due to widespread endotoxin exposure, particularly in the occupational environment, there is an urgent need to elaborate sampling strategies, standardize extraction protocols and assay procedure.

Advantages and limitations of the methods for endotoxin quantitation. The main asset of LAL assays is their high sensitivity compared to other (except the rFC – see below) endotoxin detection methods. The *Limulus* test is more economical in terms of initial cost needed, instrumentation and less time-consuming than other *in vivo* and *in vitro* analyses. For all LAL methods, a small amount of the examined sample is required. Despite the applied modification, the simplicity of the sample elaboration means that several samples might be tested daily by one trained worker. The main disadvantage of the LAL test lies in a precise implementation of the test procedure. The entire environment in which the analysis is performed, all materials and instrumentation having contact with both the sample and reagents must be pyrogen free, as a slight disturbance may influence the outcome of the assay [18, 23, 29]. The LAL reaction is enzyme mediated and, as such, has an optimal pH range, specific salt and cation requirements. The analyzed samples may occasionally contain some substances or ions which can change these optimal test conditions and, by that, decrease or increase the lysate reactivity (inhibition or enhancement phenomena) not necessarily due to deficiency or absence of endotoxins in the sample [18, 23, 122].

The rFC procedure has been found to have a comparable detection range (0.01 EU/ml–10 EU/ml) to both the chromogenic (0.005 EU/ml to 50 EU/ml) and turbidimetric (0.01 EU/ml to 100 EU/ml) kinetic LAL assays; however, as an endpoint test, can assay more samples in less time than both kinetic LAL versions [20]. The rFC enzyme solution is produced in cell culture as recombinant (cloned) protein and, as such, has greater lot-to-lot consistency, which translates into an excellent linearity of standard curves and therefore into more reproducible results [20, 68]. The rFC method does not detect (1 \rightarrow 3)- β -D-glucan activity; thus, by such an improvement in specificity (compared to the LAL assays) reduces the false positive results. Moreover, the rFC has an ability to recognize endotoxins from different source materials. The specificity, precision, accuracy, linearity, range, and quantitation limit make the rFC procedure a promising tool for endotoxin analysis in environmental samples.

The LAL and rFC assays detect an active (in these tests) form of endotoxins only. However, this seems to be more related to workers' symptoms (in terms of the LAL, at least) than the total endotoxin levels obtained by the GC-MS assay [76, 122, 164]. Moreover, the LAL methods may be most suitable when comparing exposures within similar environments, whereas GC-MS may help in optimizing sample treatment and extraction procedure as well as when comparing across different environments [134].

At present, the chemical instrumental methods (HPLC, GC-MS) have lower sensitivity compared to the LAL and rFC assays, require expensive instrumentations, are laborious and due to that are still treated rather as an experimental option than an alternative to both enzymatic procedures [122].

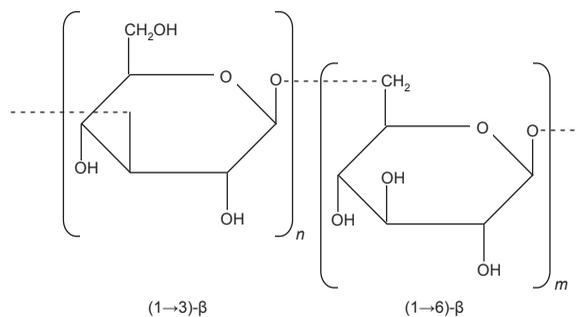


Figure 4. Schematic structure of β -glucans [185]. n – number of glucose subunits connected by intrachain glycosidic (1 \rightarrow 3)- β -linkages; m – number of glucose subunits connected by intrachain glycosidic (1 \rightarrow 6)- β -linkages.

Table 1. Characteristics of environmental β -glucans [17, 34, 78].

β -glucans	Links/Branches	Molecular mass	Source of origin
Curdlan	(1-3)- β -D	>136,000	Bacteria
Carboxymethylcurdlan	(1-3)- β -D	>95,000	Bacteria
Grifolan	(1-3)(1-6)- β -D	500,000	Fungi
Mannan	α -(1-2)(1-3)(1-6)-D	>37,000	Fungi
Laminarin	(1-3)(1-6)- β -D	16,800	Algae

β -GLUCANS

β -glucans are water-insoluble structural cell-wall components of most of the fungi and yeasts, some bacteria, most higher and many lower plants. They are also minor constituents of fungal cytosol. Glucans may account for up to 60% of dry weight of the fungal cell wall, where they are linked to proteins, lipids and carbohydrates (Tab. 1) [17, 34, 78]. Their main function is formation of a meshwork, which maintains the rigidity and integrity of the cell wall [32, 185].

From a chemical point of view, these compounds are glucose polymers, which are classified according to the type of intrachain linkage of the polymer, as α - or β -linked (Fig. 4) [185]. β -glucans may exist as a single polymer strand with helical conformation (single helix) or as a stable complex of three polymer strands forming triple helix, which is the most preferred form of glucans in nature. β -glucans derived from different sources have some differences in their structure, molecular weight, length of polysaccharide chain and degree of branching. These structural differences determine β -glucan activities [34, 48, 185].

Biological activity of β -glucans. β -glucans are non-allergenic agents, but they have immunomodulating properties and may affect respiratory health [142, 184]. It has been suggested that β -glucan has the potential to induce Th1 as well as Th2 driven immune responses [142]. Initial studies suggest a β -glucan role in the development of the adverse symptoms defined as “sick building syndrome (SBS)”

which appeared in the late 80s [32]. Since that time, numerous studies have been shown that β -glucans modulate various immunological effects by activation of macrophages to cytokine production (e.g. IL-1, -6, -8, and -12, TNF- α and IFN- γ) [127, 159], neutrophils [108, 186, 193], eosinophils [190], T-lymphocyte (Th) and natural killer cells (NK) [136]. On the other hand, their antibacterial, antiviral and anticarcinogenic activities have induced investigations on the potential biomedical applications of β -glucans [14, 117, 138]. Despite these positive effects, numerous studies have underlined the role of β -glucans in adverse health outcomes, especially in bioaerosol-induced inflammatory reactions and diseases including “building-related symptoms (BRS)”, HP and ODS [5, 32, 45, 46, 174]. The studies carried out by Rylander *et al.* revealed the relationship between the airborne level of β -glucans and appearance of negative health effects, such as: eye, nose and throat irritations, cough and itching [148]. Moreover, (1 \rightarrow 3)- β -D-glucan exposure was associated with an increase in the prevalence of atopy and myeloperoxidase (MPO) production as well as with a decrease in one-second forced expiratory volume (FEV₁) [148, 175]. It should be underlined that all the above described biological properties are not dependent on the viability of the source organism(s), and the glucans derived from, e.g. dead microorganisms may thus be responsible for observed adverse health effects to the same degree as those provoked by viable ones [32].

Another interesting phenomenon in pulmonary symptomatology is β -glucan and endotoxin interactions. Some researchers have reported that an acute exposure to endotoxins causes a massive invasion of neutrophils into the airways, while in a chronic exposure such response is almost invisible. It has been suggested that glucans themselves do not cause a neutrophil inflammation, but the endotoxin-induced neutrophil invasion into the airways is depressed by the simultaneous inhalation of β -glucans. During chronic exposure, an adaptation to endotoxins (which occurs normally) is hampered by co-exposure to β -glucans, which may lead to a greater persistence of neutrophils in the lung and organ damage [45, 46, 146, 174].

Methods for β -glucan detection in environmental samples. Nowadays, there are four assays for β -glucan detection in environmental samples: modified *Limulus* test, inhibition enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA) and monoclonal antibody-based two-site enzyme immunoassay (mAb-EIA). Among them, the most widely applied for β -glucan quantification is the modified *Limulus* test, which is based on the same principle as previously described for bacterial endotoxin measurements (Fig. 2) [1, 2]. However, glucans do not activate factor C like LPS, but factor G leading to a series of enzymatic reactions resulting in activation of the proclotting enzyme. Due to water insolubility, a different extraction procedure of β -glucans from the samples is required. Usually, the environmental sample needs to be ex-

tracted in alkaline solution (0.6 M NaOH) or by hot water (120°C–130°C) [33, 156]. The modified *Limulus* assay is very sensitive with a detection limit from 1 pg/ml–10 pg/ml, and, like its predecessor for endotoxins, occurs in the turbidimetric, chromogenic and kinetic variants [1].

Among alternative methods to the LAL assay is the inhibition enzyme immunoassay (EIA). This method (described and characterized for the first time by Douwes *et al.* in 1996 [34]) utilizes the affinity-purified anti-(1 \rightarrow 3)- β -glucan antibodies to quantify glucans in the samples. These immunospecific antibodies are obtained from the serum of rabbits immunized with bovine serum albumin-conjugated laminarin. The EIA, like modified *Limulus* assay, reacts with both linear and branched β -glucans. However, the reaction of antibodies with plant glucans shows that the EIA is not highly specific for the (1 \rightarrow 6) branched, (1 \rightarrow 3)-glucans, which are characteristic for fungi. Moreover, a low limit of detection (i.e. 40 ng/ml) narrows down the EIA applications to measurements in the environments where high exposure to organic dust is observed and to settled dust analyses [34].

Another method for β -glucan quantification is an enzyme-linked immunosorbent assay (ELISA). The main principle of this test is the use of a high-affinity receptor (galactosyl ceramide, sphingolipid) specific for (1 \rightarrow 3)- β -D-glucans as a capture reagent and a monoclonal antibody specific for complex fungal cell wall β -glucans as a detector reagent. This assay is much more sensitive than the EIA (detection level is 0.8 ng/ml); however, it has some limitations in terms of its applicability, e.g. it is not recommended for glucan measurements in metalworking fluids and for the assessment of fungal biomass [110].

The monoclonal antibody-based two-site enzyme immunoassay (mAb-EIA), which has been in common use lately, seems to be a promising tool for the environmental sample analyses. To quantify glucans with this method, the affinity-purified anti-(1 \rightarrow 3)- β -glucan antibodies produced by mouse immunization with bovine serum albumin-conjugated laminarin are used. The studies showed that the mAb-EIA is sensitive enough to detect β -glucans in inhalable dust samples, both indoors and outdoors [151]. Despite the variety of available analytical methods, it is still necessary to verify their usefulness for monitoring and epidemiological assessment in both occupational and non-occupational environments.

Correlations between the methods for β -glucan quantitation. Compared to endotoxins, the environmental measurements of β -glucan concentrations are not very common and, when performed, in the majority of cases are carried out using modified kinetic LAL or EIA assays. Hence, the data regarding either interlaboratory or field comparative studies are scarce. Sander *et al.*, studying filter extracts from poultry farms, pig stables, grain storage houses, and a laboratory animal facility, revealed that the correlation between (1 \rightarrow 3)- β -D-glucan levels obtained using the mAb-EIA and the modified (kinetic) *Limulus* test was significant. Using statistical algorithm for reproducibility

evaluation proposed by Bland and Altman, it was revealed that the ratio of assays was not influenced by the amount of (1 \rightarrow 3)- β -D-glucan on the filters in general, but by the site of sampling [151].

Environmental exposure to endotoxins and β -glucans. Endotoxins and β -glucans, as structural components of microbial cell walls, are widespread in many environ-

ments. A large number of studies performed during last 30 years have presented numerous proofs that an exposure to them can be associated with many diseases and/or adverse health outcomes. The highest exposure to endotoxins occurs in agriculture, grain processing, the waste industry and landfills, as well as in wood processing, and the metal and textile industries [37, 51, 53, 62, 73, 105, 124, 154, 161, 165, 176, 188]. On the other hand, β -glucans can play an

Table 2. Environmental levels of endotoxins and β -glucans.

Environment	Method of detection	Endotoxins (ng/m ³)	β -glucans (ng/m ³)	Data source
Poultry houses	Kinetic LAL	0.25–71995	NM	154, 161
	Gel-clot LAL	80–104220	NM	7, 124
	GC-MS	93–1669	NM	124
	Modified kinetic LAL	NM	4–5000	33, 151
	mAb-EIA	NM	2–972	151
Cattle houses	Gel-clot LAL	1.25–2810	NM	7, 41, 124
	Kinetic LAL	0.03–93.2	NM	154
	GC-MS	34–1354	NM	124
	rFC	94.3 (0.71)*	NM	15
Pig houses	Gel-clot LAL	5–75000	NM	7, 41, 124
	Kinetic LAL	0.18–14923	NM	126, 154, 161
	GC-MS	89–272	NM	124
	Modified kinetic LAL	NM	18–96	151
	mAb-EIA	NM	33–410	151
Sheep sheds	Gel-clot LAL	210–104060	NM	124
	GC-MS	2717–10029	NM	124
Horse stable	Gel-clot LAL	6–208360	NM	41, 124
	GC-MS	433–8128	NM	124
	EIA	NM	400–631000	150
Slaughterhouses	Chromogenic LAL	0.02–940	NM	76
	GC-MS	760–64000	NM	76
Meat processing	Kinetic LAL	0.26–123.5	NM	165
Dairy	rFC	112.1 (0.27)*	NM	15
Hay storage	Gel-clot LAL	60–480	NM	124
	GC-MS	12–320	NM	124
Textile plants	Kinetic LAL	0.5–840	NM	168
Hemp processing	Kinetic LAL	473.4–5980.1	NM	44
Cotton processing	Kinetic LAL	660–69360	NM	161
	Chromogenic LAL	0.66–1697	NM	21
Flax processing	Gel-clot LAL	16900–1562600	NM	40, 53
	Kinetic LAL	59.6–3583	NM	167
Grain processing	Gel-clot LAL	6.25–993900	NM	40, 53
	Kinetic LAL	1–131000	NM	61, 161, 165
	EIA	NM	120000 (4700)*	61
Horticulture:				
Tomato nursery	Kinetic LAL	0.54–342	NM	94, 165
Cucumber nursery		9–171	NM	94
Chicory nursery		3.1–67	NM	165
Mushroom cultivation		0.25–130	NM	161, 165

Table 2 (continuation). Environmental levels of endotoxins and β -glucans.

Environment	Method of detection	Endotoxins (ng/m ³)	β -glucans (ng/m ³)	Data source
Peppermint processing	Gel-clot LAL	180–208330	NM	40, 162
Nettle processing	Gel-clot LAL	800–71500	NM	40
Chamomile processing	Gel-clot LAL	5–26041290	NM	162
Potato processing	Kinetic LAL	0–90	NM	43, 167
	Gel-clot LAL	11-1893900	NM	37, 40
Fodder production	Chromogenic LAL	0.031–7000	NM	74, 165
Sugar production	Kinetic LAL	0.8–219.1	NM	165
Industrial bakery	Kinetic LAL	0.2–263.5	NM	165
Herb processing	Gel-clot LAL	41700–62500	NM	53
Corn farm	rFC	625 (4.5)*	NM	15
Wood processing industry	Chromogenic LAL	0.1-51	NM	76
	Kinetic LAL	0.5–266	NM	28, 62, 161
	GC-MS	96-7700	NM	76
	EIA	NM	3840–18940	137
Wastewater treatment plant	Chromogenic LAL	0.2–3472	NM	73, 80
	Kinetic LAL	0–416	NM	163, 166, 173
	Modified chromogenic LAL	NM	0–163	73
Waste management:				
Domestic waste collection	Kinetic LAL	0.4–718.2	NM	189
	EIA	NM	260–52500	189
Transfer and sorting	Kinetic LAL	19.5–353.6	NM	189
Landfills	Chromogenic LAL	0.4–29	NM	128
Composting plant	Chromogenic LAL	0.01–3217.4	NM	64, 165
	Kinetic LAL	0.02–3704.3	NM	178, 189
	Modified kinetic LAL	NM	0.01–14.46	64
	EIA	NM	150–206600	189
Use of biomass in power production	Kinetic LAL	<0.3–210.4	NM	189
	EIA	NM	<1–166600	189
Biofuel plants	Kinetic LAL	0.2–9917	NM	96
Tobacco industry	Chromogenic LAL	0.3–106	NM	131
Metalworking fluids	Gel-clot LAL	0.12–1.2	NM	58
	Chromogenic LAL	0.03–25000	NM	76, 77
	Kinetic LAL	0.02–2879.4	NM	58, 160, 176
	GC-MS	40–3100	NM	58, 76
Paper industry	Modified kinetic LAL	NM	4–240	149
Laboratory	Kinetic LAL	3–105	NM	161
	mAb-EIA	NM	16–38	151
Dental clinics	Gel-clot LAL	0-62.5	NM	170
Dwellings	Gel-clot LAL	0.3–0.868	NM	57
	Kinetic LAL	0.003–5.417	NM	57, 65
	Modified kinetic LAL	NM	0-19	175
Offices	Chromogenic LAL	0.05–0.3	NM	133
Schools	Modified kinetic LAL	NM	0–19	147

LAL – *Limulus* test; GC-MS – gas chromatography-mass spectrometry analysis; mAb-EIA – monoclonal antibody-based two-site enzyme immunoassay; rFC – recombinant Factor C procedure; EIA – Inhibition Enzyme Immunoassay; NM – not measured; * geometric mean (geometric standard deviation).

important role in the development of occupational diseases in farmers, especially those involved in grain and poultry farming [5, 61, 145], the paper industry [149], wood processing industry [137], waste composting facilities and among sewage treatment plant workers [64, 73, 189]. The new problem of environmental exposure to both these immunologically potent compounds observed over the last decade has been brought about by urbanization processes. Due to the dynamic development of housing construction, the human expansion on territories traditionally reserved for agricultural production, has resulted in a substantial and often uncontrolled exposure to organic dusts containing endotoxins and β -glucans [33]. Moreover, the presence of both these components is being detected more and more often in the “new” occupational environments (e.g. in the biotechnology industry) as well as in non-industrial indoor environments, including dwellings [9, 10, 58, 65, 118, 130, 132, 157, 158, 177] and schools [147, 175]. Table 2 summarizes the available data concerning endotoxin and β -glucan concentrations observed in different environments.

Endotoxin and β -glucan control procedures in working environment. Although health risk associated with biological aerosols is a known fact in many occupational environments, the widely accepted regulations for this type of airborne contaminants are scarce. Standards applied to prevent such harmful exposures should fulfill several criteria, i.e. have a strong scientific basis, invoke a specific sampling strategy and method, refer to an analytical technique and, if possible, recommend a way of interpretation of observed phenomena. For many bioaerosol agents, these rigorous requirements still need to be elaborated [54, 93, 103].

In Poland, guidelines for the assessment of workplace exposure to airborne bacterial endotoxins are given by two European Standards EN 13098:2000 and EN 14031:2003 endorsed by the Polish Committee for Standardization as Polish Standards (PN). First of them, PN-EN 13098 “Workplace atmosphere – Guidelines for measurement of airborne microorganisms and endotoxin” (adopted in 2002 and replaced in 2007) contains, among others, the basic definitions and recommendations for measurements of airborne endotoxins in the work environment. It characterizes sampling conditions, advises on sampling and analytical methods (giving their broad overview and discussing their advantages and limitations), and admits the measurements of microbial products, such as endotoxins and glucans in the assessment of microbial air contaminations [122]. The second standard, PN-EN 14031 “Workplace atmospheres - Determination of airborne endotoxin” (adopted in 2004 and replaced in 2006), provides methods for sampling, transportation, storage of samples and determination of endotoxins [123]. These both standards recommend the use of kinetic chromogenic LAL assays for detection and quantification of endotoxin levels in the workplace atmosphere; however, other methods (such as GC-MS or HPLC)

Table 3. Proposals of standard, reference or threshold limit values for bacterial endotoxins in occupational environment.

Standard/reference/threshold limit values in $\mu\text{g}/\text{m}^3$ (EU equivalent, if available)	Year of publication	Data source
0.08	1984, 1991	31, 60
0.1	1985	22
0.1–0.2	1987, 1992	99, 144
0.009	1987	19
0.03	1988	118
0.0045 (5)	1998	24
0.025	1999	75
(100)	2000	30
(200)	2003	35, 103
0.2 (2000)	2004	6
0.015	2009	95

Table 4. Health-based levels for endotoxin exposure in occupational environment [19, 143, 144, 194].

Level (EU/ m^3)	Adverse health outcomes
53	Decrease in lung function
90	Pulmonary impairment
200	Airway inflammation, mucous membrane irritation
2,000	Over-shift decline in FEV_1
3,000	Chest tightness
10,000–20,000	ODTS (Organic Dust Toxic Syndrome)

for determination of endotoxin chemical markers, although not routinely utilized, can be applied as well.

Hygienic standards and threshold limit values. In spite of the dynamic development of scientific methods enabling precise quantitative and qualitative recognition of biological contaminants in environmental samples, their proper assessment and control is still a difficult problem. On the global scale, there is a lack of commonly approved criteria for the assessment of exposure to endotoxins and glucans, as well as health-based guideline values or thresholds for acceptable levels of both these immunologically reactive biohazards. Hence, the proper interpretation of measured concentrations is hindered and microbial exposure cannot be precisely quantified. Nevertheless, taking into consideration the adverse effects and to be able to analyze the results of exposures observed particularly at many workplaces, there several attempts have been made to establish such hygienic standards. The existing proposals for threshold limit values result from the studies carried out within the past quarter-century and, unfortunately, they are solely restricted to bacterial endotoxins (Tab. 3). Many researchers have emphasized that the value of occupational exposure limit (OEL) should be based on a dose-response relationship leading to a well-defined effect(s) on human health (Tab. 4) [19, 143, 144, 194]. However, the

Table 5. Frequency of adverse health symptom occurrence in relation to airborne level of β -glucans in indoor environment [141].

Concentration of β -glucans: 0.1–5.2 ng/m ³	
Frequency of occurrence (%)	Health effects
2–30	Headache
5–35	Dry cough
11–45	Throat irritation
16–50	Nasal irritation
21–75	Fatigue

proposals, which have been built using this approach and widely applied after that are strongly limited. For example, in 1998 the Dutch Expert Committee for Occupational Standards (DECOS) proposed a value of 50 EU/m³ as the health-based recommended threshold limit for endotoxins [24]. Despite theoretical and clinical bases, the proposed value seemed to be too restricted and has been exceeded at the majority of studied workplaces, (e.g. compare with the data in Tab. 2) [83]. Hence, in 2003, taking into account the economic feasibility for some sectors of industry (especially the agricultural branches), the exposure limit was raised to 200 EU/m³ [35, 103].

In Poland in 2004, the Biological Hazard Expert Group of Interdepartmental Commission for Maximum Admissible Concentrations and Intensities for Agents Harmful to Health in the Working Environment proposed the threshold limit values for occupational exposure to bacterial endotoxins in the environments polluted with organic dust (2000 EU/m³) and in non-industrial (public buildings, dwellings) indoor environments (50 EU/m³) [6, 54]. Both these reference values are built in conformity with so-called “the environmental philosophy”. In brief: if a solid link between the concentration of investigated parameters and resulting adverse health effect cannot be effectively established, then, based on the biological agent concentration measurements, the reference values should enable evaluation of the quality of the environment, as well as determination of “what is typical and acceptable” and “what is atypical or not acceptable” for a specific type of setting [56]. These two limit values, which have been widely used in Poland, could be very helpful not only to assess an endotoxin exposure in the above-mentioned environments, but to undertake there appropriate prophylactic and preventive actions as well.

The area of hygienic standards for β -glucans is still a “grey area”. There are neither exposure standards nor proposals of threshold limit values for this biological agent; however, β -glucans have remained within the range of interest of many researches worldwide. Among these sparse data are the results obtained by Rylander, who showed that the airborne level of β -glucans in indoor environments within the range of 0.1–5.2 ng/m³ may determine the frequency of adverse health effect occurrence in the exposed population (Tab. 5) [141].

SUMMARY

The application of endotoxins and β -glucans as markers of microbiological contamination in both occupational and non-occupational environments is of a high practical value for exposure assessment. Analytical methods for their quantification are available and become relatively common in laboratory practice. Nevertheless, a lack of universally approved hygienic standards or threshold limit values still does not allow an appropriate interpretation of the environmental measurement results. On the global scale, an important problem is also the deficiency of satisfactory epidemiological data regarding dose-response relationship between airborne endotoxin and β -glucan concentrations and observed adverse health outcomes derived from such exposure. The explanation of these issues should stimulate scientists active within the areas of clinical medicine and environmental health to elucidate all these health-related complexities in the nearest future.

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