DE GRUYTER OPEN

DOI: 10.1515/cdem-2014-0007 CHEM DIDACT ECOL METROL. 2014;19(1-2):79-85

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Escherichia coli IN SEWAGE SLUDGE - DETECTION METHOD

Escherichia coli W OSADACH ŚCIEKOWYCH - METODA WYKRYWANIA

Abstract: *Escherichia coli* is Gram-negative optionally anaerobic roads which belongs to *Enterobacteriaceae* family. Includes in a physiological bacterial flora of human and warm-blooded animals large intestine. *Escherichia coli* is being met in abiotic elements of the environment so as waters, wastewater, sewage sludge, soil and the food. This bacterium is showing the pathogenicity in named terms for the peoples, triggering diseases mainly: gastrointestinal tract and urinary.

Quality and quantitative proposed detections method of the bacteria *E. coli* contains five/six steps:

- appointment dry suspended solid,
- preparation averaged, test of sample and resuscitation of bacteria,
- making dilutions,
- enrichment and differentiation in chromogenic-selective medium,
- enumerating the amount of cfu *E. coli* in 1 g of a dry weight,
- optionally, the biochemical identification.

Keywords: *E. coli*, sewage sludge, enrichment of *Escherichia coli*, detection of *Escherichia coli*

Introduction

Sewage sludge is composed mostly of solids generated during wastewater treatment processes. Sludge treatment and disposal are probably the most costly operations in wastewater treatment plants. Neutralizing and the recycling of sludge require the knowledge, of technological and technical solutions. Wastewater treatment processes do not completely remove or inactivate pathogenic and parasitic organisms. Many of these organisms become bound to solids after wastewater treatment and are merely transferred to wastewater sludge. Further treatment is necessary to eliminate them, or at least reduce their numbers significantly. Sludge contains the huge diversity of microorganisms: viruses, bacteria, protozoa parasites and helminths eggs. The presence and concentration of pathogens is mainly determined by two factors, namely, the prevalence of pathogens among the population connected to sewage network and ability of these organisms to survive the sewage and sewage treatment processes. Sludge may become contaminated by approximately 300 species of bacteria and the number in 1 g of a dry weight reaches them from 10^9 into 10^{12} cells [1]. The survival time of bacteria in the environment is few months

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to a several months. Several pathogenic microorganisms and parasites are commonly found in domestic wastewater, sewage sludge as well as in effluents from wastewater treatment plants [2-5]. Sewage sludge bacteria belong to the following groups:

- Gram-negative facultative anaerobic bacteria: *eg*, *Aeromonas*, *Plesiomonas*, *Vibrio*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella*,
- Gram-negative aerobic bacteria: *eg*, *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Acinetobacter*,
- Gram-positive spore forming bacteria: *eg*, *Bacillus* spp.,
- Nonspore-forming Gram-positive bacteria: *eg*, *Arthrobacter*, *Corynebacterium*, *Rhodococcus.*

Escherichia coli belongs to *Enterobacteriaceae* family and along with other bacteria similar (*Enterobacter* sp., *Citrobacter* sp., *Klebsiella* sp.) are forming a group of so-called *Escherichia*. *Escherichia coli* is Gram-negative facultatively anaerobic roads. Motile by peritrichous flagella or are nonmotile [6, 7]. Chemoorganotrophic, having both a respiratory and a fermentative type of metabolism. D-glukoze and other carbohydrates are catabolized with the formation of acid and gas. Oxidase negative, catalase positive, Voges-Proskauer negative, and usually citrate negative [8]. Most *E. coli* strains are able to product indole from tryptophan and are β-glucuronidase-positive. *E. coli* strains sometimes exhibit atypical reactions in a variety of tests including H_2S, KCN, urease, adonitol, inositol and indole, making it essential to consider the overall biochemical profile rather than specific "key" reactions before eliminating *E. coli* from consideration [8].

Several strains of E. coli, many of which are harmless, are found in the gastrointestinal tract of humans and warm-blooded animals. Some kinds of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory illness and pneumonia, and other illnesses. There are several categories of *E. coli* strains, however, that bears virulence factors and cause diarrhea. There are enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC, VTEC), enteroinvasive (EIEC), and enteroaggregative (EAggEC) types of *E. coli* [9, 10].

Determinants pathogenicity *E. coli* are: enterotoxins, adhesion factors and the antiphagocitic capsle [10]. Enterotoxins are causing many gastroenteric disorders.

Enterotoxic strains (ETEC) are secreting warm-unstable toxins (LT-I and LT-II) similar structurally and functionally to *Vibrio cholerae* toxin and warm-solid toxin (ST) disturbing the transport of ions. EHEC, VTEC are producing verotoxin and hemolysins which are cytotoxic. Strains K1 of *E. coli*, has the antiphagocitic capsle built from polymer sialic acid and this strains is not inducing opsonisation [10].

Enterotoxigenic *E. coli* causes gastroenteritis with profuse watery diarrhea accompanied with nausea, abdominal cramps, and vomiting. Approximately 2-8 percent of the *E. coli* present in water were found to be enteropathogenic *E. coli*, which causes Traveler's diarrhea. Food and water are important in the transmission of this pathogen. However, the infective dose for this pathogen is relatively high, within the range of 10^6-10^9 organisms.

In the CDC (Center for Disease Ctrl and Prevention) list concerning dangerous micro-organisms and biological factors, roads *E. coli* are exchanged in the category B. Pathogenic bacteria are found in this category about the low mortality, the moderate incidence and diffusing. Bacteria *Escherichia coli* O157:H7 are mentioned near: *Salmonella*

sp., *Shigella* sp., *Staphylococcus* sp., *Vibrio cholerae*, *Brucella* sp., *Burgholderia (Pseudomonas) pseudomallei, Coxiella burnetti.*

E. coli are found in sewage sludge. The assessment of a sanitary condition of sludge is based on the indirect conclusion on the content of pathogenic bacteria and eggs of intestinal parasites and is based on determining so called sanitary indicator. Until recently, the species being a sanitary indicator among bacteria was rods *Escherichia coli*. Currently, under the Regulation of the Minister of the Environment [11], a new bacteria indicator *Salmonella* sp. is being used in sanitary assessment. However, *E. coli* presence shows to the sanitary threat of sewage sludge.

Many researchers microbiological examined sewage sludge. The presence of *E. coli* was investigated at sewage treatment plants in Austria. The sewage contained $8.5 \cdot 10^5$ cfu *E. coli* per 1 g dry weight [12]. The results show that *E. coli* were present in 61% samples of the working environment at the sewage sludge [12].

For example Nowicka and Machnicka [13], investigated surplus activated sludge using similar result: $6.4 \cdot 10^5$ cfu/g d.w. Enterohemorrhagic *E. coli* 0157:H7 was isolated by Fijałkowski et al [14, 15]. During a 1989-1990 survey of waterborne disease outbreaks in the United States, the etiologic agent in one out of 26 outbreaks was enterohemorrhagic *E. coli* O157:H7, an agent that produces shiga-like toxins (SLT) I and/or II, has a relatively low infectious dose (<100 organisms) and causes bloody diarrhea, particularly among the very young and very old members of the community [16]. Infections, if left untreated, may lead to hemolytic uremic syndrome, a leading cause of kidney damage and possible failure in children, with a 3-5 percent death rate in patients [17].

A global rise in antibiotic resistance has been reported. Antibiotic resistance represents a significant global health problem due to the use and misuse of antibiotics, which favors the emergence and spread of resistant bacteria [18]. A comparison of pre-antibiotic era strains of *E. coli* and *Salmonella enteric* to contemporary strains showed that the former were susceptible to antibiotics, whereas 20 percent of the latter displayed resistance to at least one of the antibiotics [19, 20]. For example, an investigation of the antibiotic resistance pattern of *E. coli* strains in a wastewater treatment plant in Austria showed that these strains were resistant to 16 of 24 tested antibiotics [21]. The main reason of such a situation stems from a commonly implemented antibiotic therapy in humans and its use in agriculture [18].

The risk of infecting the environment with pathogens originating from sewage sludges requires seeking more and more new methods to improve their sanitary status.

The utilized procedures of sludge processing, for example, methanogenesis or composting, do not always guarantee fulfilling requirements presented in the decree. Thus, the additional hygienization of sludge is necessary. In order to assess the sanitary state of sewage sludge, which may be utilized, the accurate, sensitive and repetitive research method, allowing the identification and diagnosis of the bacteria is required.

The article is aimed at a presentation of a new research method that may be used to determine the presence of *Escherichia coli* in sewage sludge.

Methodology of examinations

The rule of the method

The detection of *Escherichia coli* in sewage sludge is a staged procedure that consists of: determination of dry weight, preparation of a sediment sample for bacteriological analysis, preparation of its dilutions, preparation of the Chromocult agar and the supplement of *E. coli*/Coliform, and cultivation of bacteria on a selective chromogenic agar medium. What is more, it is possible to perform an additional confirmation with the help of *Enterobacteriaceae* bacteria standardized identification system. The final result constitutes of the calculation of CFU of *E. coli* in 1 gram of the sludge dry weight.

Laboratory equipment

In order to perform a microbiological analysis the following equipment is necessary:

- apparatus for sterilization: autoclave
- device for taking of sewage sludge, for example: bucket, ladle
- homogenizer
- centrifuge
- analytical balance
- vortex mixer
- thermostatic incubators regulated at (30±1) and (44±1)
- laboratory drier (105ºC),
- sterile Petri dishes, 140 mm in diameter
- sterile pipetes, glass or disposable plastic ware, capable of dispensing 1, 10 and 25 cm^3 volumes
- beakers or bottles 250 cm^3 volume
- sterile conical centrifuge tubes, 50 cm^3 volume
- standard microbiological equipment

Reagents and the growth medium

In order to carry out a bacteriological analysis one needs the following equipment:

- peptone saline solution
- Chromocult agar
- *E. coli*/Coliform supplement
- optional: nutrient agar and API 20E test.

Determination

The dry weight of sludge. The sludge dry weight is determined by a standard method described in: *Standard methods for the examination of water and wastewater* [22].

The preparation of a mean sample and research sample. The collected volume of sewage sludge should be homogenized by a thorough mixing. In order to obtain a research sample it is necessary to weigh out 10 grams of the homogenized sludge and to put it into 250 cm^3 glass bottle with a bottle top. Then, one should add the peptone saline solution in such a volume that the final mass of a research sample amounts to 100 grams. The next step is to mix the sample scrupulously for 30 minutes. The 50 cm^3 of tested sediment should be

put in a conical flask and centrifuged for 3 minutes. The rotational speed should be set at 300 rotations per min.

The preparation of dilutions. Supernatant received in the process of centrifuging is diluted to from 10^{-2} do 10^{-6} according to dilution procedure, the so-called dilution series. The dilution of 10^{-1} is obtained during the preparation of research sample.

The preparation of agar Chromocult and the *E. coli* supplement Coliform. The agar growth medium is prepared accordingly to the instruction. The supplement of *E. coli*/Coliform is added to the agar at the temperature of 45-50ºC. The mixture should be mixed rotationally.

The cultivation of bacteria on the selective chromogenic agar medium (Chromocult agar). On each Petri dish (14 mm diameter), one pours 1 cm^3 of sample (each dilution). Next, 24 cm^3 of warm Chromocult agar is introduced. The content should be mixed rotationally. The samples are left, until agar congeals in the room temperature. Then, it should be transported to an incubator and incubated in 30^{o}C for 4 hours, and then in 44ºC for 16 hours. After the process of incubation is finished, the number of claret-purple and blue colonies should be estimated.

Optionally, in order to identify bacteria from *Enterobacteriaceae* family a biochemical test (commercial name API 20E) may be utilized. Before the biochemical analysis is performed, isolated colonies of identified bacteria should be sifted on the nutritive agar and incubated in 36±2ºC for 21±3 hours.

The CFU of *E. coli* in 1 gram of the sediment dry weight. The number of *E. coli* bacteria (entities composing colonies in 1 gram of sediment dry weight is calculated according to the formula (ISO 8199:2005):

$$X = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)} \tag{1}$$

where: X - the CFU of *E. coli* in 1 gram of sediment dry weight of tested sample, N - summary figure of calculated, assumed *E. coli* colonies, n_1, n_2 - the number of repetition for dilutions F_1, F_2; V_1, V_2 - the volume of diluted sample [mm], F_1, F_2 - dilution of a sample.

Conclusions

The procedure of identification of *E. coli*, proposed in the following article, implements to routine proceedings a combination of various stages connected with: indication of sludge dry weight, preparation of sewage sludge sample for bacteriological analysis, dilution of supernatant liquor, multiplication of *E. coli* on a selective chromogenic agar medium (Chromocult agar), estimation of *Escherichia coli* colonies, which are characterized by claret-purple or dark-blue staining. Finally, the CFU in 1 gram of the sludge dry weight is calculated. The preparative stage in which the peptone saline solution is used, recovers the normal physiological activity of bacteria, it also increases the chances of identification of *E. coli.*

The microflora composing the sewage sludge is exposed to toxic substances (*eg* preliminary sedimentation tank) and stress factors for organisms, occurring during the conditioning processes and disposing of sewage sludge (*eg* aerobic and anaerobic stabilization, liming, sonification).

The accuracy of identification is influenced by a number of factors, especially: the method of sampling; transportation of samples to a laboratory; time elapsing before the start of analysis; time and method of storage (recommended time up to 24 h, temperature 4±3ºC); skills and knowledge of a lab assistant; use of aseptic equipment, growth medium and laboratory rooms. The proceeding discordant with the procedure of *E. coli* identification on each stage of diagnostic and infringements in routine bacteriological tests may cause that the obtained results will be either false positive or false negative. Moreover, the results obtained in indirect planting method unfortunately may not reflect the actual number of bacteria, which may stem from the lack of a universal growth medium, on which every *E. coli* existing in the environment of sewage sludge may grow.

The proposed method is a quick procedure (the results are obtained after 24 hours), characterized by a high level of recurrence and sensitivity of identification. The procedure implemented to test in Project Routes (2011-2014), *Novel processing routes for effective sewage sludge management. Innovative system solutions for municipal sludge treatment and management.* Grant agreement n° 265156, in which the author of the article take part.

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Escherichia coli W OSADACH ŚCIEKOWYCH - METODA WYKRYWANIA

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Abstrakt: *Escherichia coli* jest Gram-negatywną, względnie beztlenową pałeczką należącą do rodziny *Enterobacteriaceae.* Wchodzi w skład fizjologicznej flory bakteryjnej jelita grubego człowieka oraz zwierząt stałocieplnych. Spotyka się ją w elementach abiotycznych środowiska, takich jak wody, ścieki, osady ściekowe, gleba i żywność. Bakteria ta w określonych warunkach wykazuje chorobotwórczość dla człowieka, wywołując głównie schorzenia układów pokarmowego i moczowego.

Proponowana metoda jakościowa i ilościowa wykrywania bakterii *E. coli* zawiera pięć/sześć etapów:

- oznaczenie suchej masy osadu,
- przygotowanie próbki uśrednionej i badawczej oraz przywrócenie bakteriom aktywności fizjologicznej,
- wykonanie rozcieńczeń,
- namnażanie i identyfikacja na podłożu chromogennym-selektywnym,
- wyliczenie ilości jtk *E. coli* w 1 g suchej masy osadu,
- opcjonalnie identyfikacja biochemiczna.

Słowa kluczowe: *E. coli*, osady ściekowe, namnażanie *Escherichia coli*, wykrywanie *Escherichia coli*