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# Studies on the Survival of Enterohemorrhagic and Environmental *Escherichia coli* Strains in Wastewater and in Activated Sludges from Dairy Sewage Treatment Plants

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Abstract

Survival of *Escherichia coli* O157:H7 strain isolated from milk in Poland and an environmental *E. coli* strain in wastewater from Garwolin and Łowicz dairies and in activated sludges from dairy sewage treatment plants as well as in dairy wastewater with activated sludges was examined. Environmental materials were contaminated with about $10^8$ of target bacteria/ml of sample. The experiments were performed under temperature conditions typical of autumn-winter (6°) and spring-summer (24°C) seasons. It was found that the non-pathogenic *E. coli* strain survived longer in all media than the enterohemorrhagic serotype. *E. coli* O157:H7 bacteria were not detected (in direct plating method) in activated sludges after 21–28 days; in dairy wastewater as well as in wastewater with activated sludges after 21–25 days. These periods for environmental *E. coli* strain were 35–42 days (activated sludges), 25–28 days (wastewater with activated sludges). At higher temperature environmental *E. coli* were not detected in wastewater from Łowicz dairy sewage treatment plant after 25 days, but the bacteria were still present in wastewater from Garwolin dairy sewage tratment plant after 34 days.

The obtained results show that the lack of environmental *E. coli* bacteria (as a indicator bacteria of fecal contamination) in dairy wastewater and in dairy wastewater with activated sludges could indicate the absence of pathogenic *E. coli* bacteria. Prolonged existence of the enterohemorrhagic serotype in activated sludges shows the need to treat activated sludges prior to the utilization of these materials as fertilizer.

Key words: *E. coli* O157:H7, environmental *E. coli* strains, dairy wastewater, activated sludge

## Introduction

*Escherichia coli* strains are present in the group of microorganisms that grow in the intestine of people and warm-blooded animals and aid in digestion. Approximately 0.1% of total bacterial count within an adult's intestine is represented by *E. coli* strains. These bacteria are released into the environment in feces; their presence in samples indicates fecal contamination, perhaps accompanied by pathogens. Although most *E. coli* strains are harmless, some serotypes can cause severe illness. *E. coli* O157:H7 is one of the pathogenic, verotoxin-producing serotypes that is more significant than other well-recognized foodborne pathogens for reasons that include the severe consequences of infection, low infection dose, acid tolerance, high survival rate in cooling and freezing conditions and relatively low sensitivity to some preservatives used in the food industry (Benjamin and Data, 1995; Buchanan and Doyle, 1997; Kwiatek, 2000; Leyer *et al*., 1995). Undercooked ground beef, fermented sausages, raw milk and dairy products have been identified as the principal vehicle in most outbreaks (Buchanan and Doyle, 1997).

The main reservoir of *E. coli* O157:H7 bacteria is the intestinal tract of ruminants. In calves naturally infected with *E. coli* O157:H7 no clinical signs but only shedding of the bacteria in their feces was noted (Brown *et al*., 1997; Cray *et al.*, 1995). Published research in many countries on cattle colonized with *E. coli* O157/*E. coli* O157:H7 have reported prevalence rates of 1.8–10.8% (Faith *et al.*, 1996; Hancock

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*et al*., 1997; Rice *et al*., 1997; Van Donkersgoed *et al*., 1999; Orr, 2000; Zhao *et al*., 1995). Monitoring studies conducted in the nothern region of Poland, indicated that 4 (0.73%) of 551 cattle were positive for serotype *E. coli* O157 (Uradziński, 2001). *E. coli* O157:H7 from dung can get to a cow's udders, the hide of animals or milking equipment, this being followed by milk contamination.

In Poland the *E. coli* O57:H7 serotype has been isolated from food samples. Różańska and Kwiatek (1996) found that 3 (5.5%) of 54 samples of raw milk as well as 8 (10.7%) of 75 samples of ground beef and minced pork-beef meat were contaminated by pathogenic bacteria. Szteyn *et al.* (1999) have reported the isolation of four *E. coli* O157:H7 strains from 141 samples of raw milk originating from cows of the Olsztyn region.

Contamination of environmental materials by fecal pathogens is currently monitored by testing them for the presence of indicator bacteria such as *E. coli*. It is very important to know the period of *E. coli* O157:H7 survival in the above mentioned environmental materials because if this pathogen can survive longer than other *E. coli* of fecal origin, the present testing procedure might be inadequate for detecting contamination.

The aim of this study was to determine the survival of the pathogenic *E. coli* serotype O157:H7 compared to non-pathogenic environmental *E. coli* strain in dairy wastewater and in activated sludges from dairy sewage treatment plants in the aspect of their possiblility of potential environmental contamination as well as public health hazard.

## Experimental

### Materials and Methods

**Target bacteria.** In model experiments, the pathogenic *E. coli* O157:H7 strain isolated from milk by employees of the Veterinary Institute in Puławy and *E. coli* strain isolated from Czerniakowskie Lake water by employees of the Institute of Agricultural and Food Biotechnology were used. The pathogenicity of *E. coli* O157:H7 strain (carrying *stx 1*, *stx 2*, *eae* and $hly_{933}$ genes) was confirmed by the studies of Łękowska-Kochaniak *et al.* (2002).

**Inoculum preparation for model experiments.** Suspensions of *E. coli* O157:H7 serotype as well as the environmental *E. coli* strain necessary for environmental materials inoculation were prepared according to Czajkowska *et al*. (2005). Two-stage culture processes were used. In the first stage, one loop of biological material was introduced into 10 ml of bioMerieux Trypcase Soy Broth and incubated for 6 h at 37°C. The entire content of the test tube was then transferred to 250 ml of above-mentioned broth and incubated at the same temperature for 24 h. Subsequently, the bacterial suspensions were centrifuged (6350 × g, 5 min), washed two times with saline solution (0.85% NaCl) and resuspended in 25 ml of the same solution. Plate counts of bacteria were estimated on Trypcase Soy Agar after incubation at 37°C for 24 h. An inoculum contained about $10^{10}$ bacterial cells per 1 ml.

**Model experiments.** Samples of wastewater were taken from Garwolin (A) and Łowicz (B) dairies and samples of activated sludge – from Garwolin and Łowicz dairies sewage treatment plants (A and B, respectively) in September/October, 2003. These materials were characterized with respect to their physicochemical and biological properties and level of natural microflora. Experiments on the survival of pathogenic and non-pathogenic *E. coli* strains in environmental materials were performed in several variants: (a) *E. coli* 157:H7 serotype or environmental *E. coli* strain in activated sludge after 1 hr sedimentation process (150 ml of activated sludge in 300 ml Erlenmayer flasks); incubation in stationary conditions, (b) *E. coli* 157:H7 serotype or environmental *E. coli* strain in dairy wastewater (150 ml of wastewater in 300 ml Erlenmayer flasks); incubation in orbital shaker (80 rpm), (c) *E. coli* 157:H7 serotype or environmental *E. coli* strain in dairy wastewater with activated sludge after 1hr sedimentation process (100 ml and 50 ml, respectively in 300 ml Erlenmayer flasks), incubation in orbital shaker (80 rpm).

In the experiment on the survival of *E. coli* strains in the variant wastewater with activated sludge, wastewater from Garwolin dairy was water diluted (1:10 vol/vol) to obtain the COD (chemical oxygen demand) and BOD (biochemical oxygen demand) values compared with the COD and BOD (biochemical oxygen demand) values of Łowicz dairy wastewater. In the variant with wastewater from Garrwolin dairy alone, initial wastewater was used.

Each Erlenmayer flask was inoculated with 3.0 mL of *E. coli* O157:H7 or *E. coli* bacterial suspension. The flasks were incubated at 6°C or at 24°C. The frequency of analyses on the *E. coli* strains survival depended on the death rate of bacteria in the specific variant of the experiments. Environmental samples (1 ml) were serially diluted (1/10) in saline solution and assayed for *E. coli* O157:H7 count or environmental *E. coli* count by direct plating (0.1 ml or 0.5 ml of initial sample and each dilution) in duplicate on bioMerieux Coli O157:H7 ID Agar with additional selective substances (0.5 g of sodium tellurite and 0.01 g of cefixime per 1l of medium) or Oxoid TBX medium, respectively. Petri dishes were incubated for 24 hours at temperature 37°C or 44°C, respectively. The experiment was terminated when pathogenic bacteria were not detected in three consecutive daily analyses.

Confirmation of the presence of *E. coli* O157:H7 bacteria in the environmental materials during incubation was performed according to Czajkowska *et al*. (2004). Material from suspect colonies was transferred to Petri dishes with bioMerieux Endo Agar. After 24 h incubation at 37°C, the presence of metallic colonies was checked. These colonies were subject to testing with the use of Merck Singlepath *E. coli* O157 test (immunochromatographic rapid test based on gold-labeled antibodies specific to *E. coli* O157).

**Microbiological analyses of environmental materials.** Total bacterial count was determined on Oxoid Plate Count Agar. Petri dishes were incubated at 20°C for 72–96 hours. Coliform count was determined on Oxoid *E. coli*/Coliform Agar and β-glucuronidase-positive *E. coli* count on Oxoid TBX Agar. Petri dishes were incubated for 24 hours at 37°C or 44°C, respectively.

**Physicochemical and biological analyses of environmental materials.** The following physicochemical and biological methods were used to characterize the environmental materials:

– dry matter of activated sludges was determined by drying 10 ml samples (after centrifugation and washing) to a constant weight at temperature 105°C (Hermanowicz *et al*., 1967).

– organic substances content in activated sludges was determined as the difference between dry mass content and inorganic substances content determined by burning a dry sample in muffle furnace at 500°C for 1 hour (Hermanowicz *et al*., 1967).

– chemical oxygen demand (COD) of the wastewater was determined by the dichromate method (Polish Standard 74/C-04578/03),

– biochemical oxygen demand (BOD) of the wastewater was determined by the dilution method (Hermanowicz *et al*., 1967),

– salinity of wastewater was determined using inoLabCond Level 1 Tetracon 325 conductometer,

– settling of activated sludge was estimated by the determination of volume of the settleable solids from the 100 ml of activated sludge after 30 minut (Hermanowicz *et al*., 1967).

## Results and Discussion

Wastewater used in these experiments originated from dairies with different production profile. Garwolin dairy (A) manufactures mainly cottage cheese; Łowicz dairy (B) – mainly soft and hard cheeses. This was the reason for differences in wastewater pH (6.67 and 5.93, respectively) (Table I). Moreover, wastewater from Garwolin dairy (A) was characterized by very high COD and BOD levels – over 10-fold higher than wastewater from Łowicz dairy (Table I).

The characteristics of activated sludges from both sewage treatment plants was almost the same. pH values were close to neutral (7.35 – plant A and 6.87 – plant B), settling was 300 and 250 ml/l, dry matter content – 4,6 and 4.1 g/l; organic matter content – 64.2% and 68.3% of dry weight, respectively.

Microbiological analyses of the environmental materials showed that the total bacterial count in dairy wastewater as well as in the activated sludges was almost the same and averaged $10^7$ CFU/ml; coliform count changed in the range between $7.0\times10^4$ and $5.4\times10^5$ CFU/ml. The number of β-glucuronidase *E. coli* bacteria was higher in activated sludges (about $10^3$ CFU/ml) than in wastewater (about $10^2$ CFU/ml).

In experiments on the survival of enterohemorrhagic and environmental *E. coli* in materials from dairies and from sewage treatment plants it was found that environmental *E. coli* strain survived longer than the

Table I
Physicochemical and biological characteristics of dairy wastewater and activated sludges from dairy sewage treatment plants

| Tested parameters | Dairy wastewater A | Activated sludge from treatment plant A | Dairy wastewater B | Activated sludge from treatment plant B |
|---|---|---|---|---|
| COD mg $O_2$/l | 12960 | – | 966 | – |
| BOD mg $O_2$/l | 8750 | – | 824 | – |
| pH | 5.93 | 7.35 | 6.67 | 6.87 |
| Salinity g/l | 1.2 | – | 1.0 | – |
| Settling ml/l | – | 300 | – | 250 |
| Dry matter content g/l | – | 4.6* | – | 4.1* |
| Organic matter content (% of d.m.) | – | 64.2 | | 68.3 |
| Total bacterial count, CFU/ml | $1.5\times10^7$ | $9.7\times10^6$ | $2.2\times10^7$ | $1.6\times10^7$ |
| Coliform count, CFU/ml | $5.4\times10^5$ | $7.0\times10^4$ | $2.5\times10^5$ | $1.4\times10^5$ |
| *Escherichia coli* count, CFU/ml | $3.6\times10^2$ | $4.2\times10^3$ | $5.0\times10^2$ | $1.6\times10^3$ |

* Dry matter content of activated sludge after 1 h sedimentation

enterohemorrhagic strain (Fig. 1, 2 and 3). Moreover, both strains survived better at 6°C than at 24°C. At 6°C, an undetectable level (<1 CFU/ml) of pathogenic bacteria in activated sludges (as determined by agar plate) was noted within 65 and 78 incubation days, whereas at 24°C after 21–28 days of incubation (Fig. 1). In the above mentioned incubation time, log CFU/ml of environmental *E. coli* bacteria ranged from about 3 to 5 at 6°C and from about 2 to 5 at 24°C. Both *E. coli* strains died faster in the variants: dairy wastewater with activated sludges and dairy wastewater alone than in the variant with activated sludges (Fig. 1, 2 and 3). An undetectable level of *E. coli* O157:H7 serotype in the wastewater with activated sludge was noticed at 6°C after 34–44 days; at this time log CFU/ml of environmental *E. coli* bacteria ranged from 1.0 to 3.0 units; at 24°C, *E. coli* O157:H7 bacteria were not detected after 21–25 days

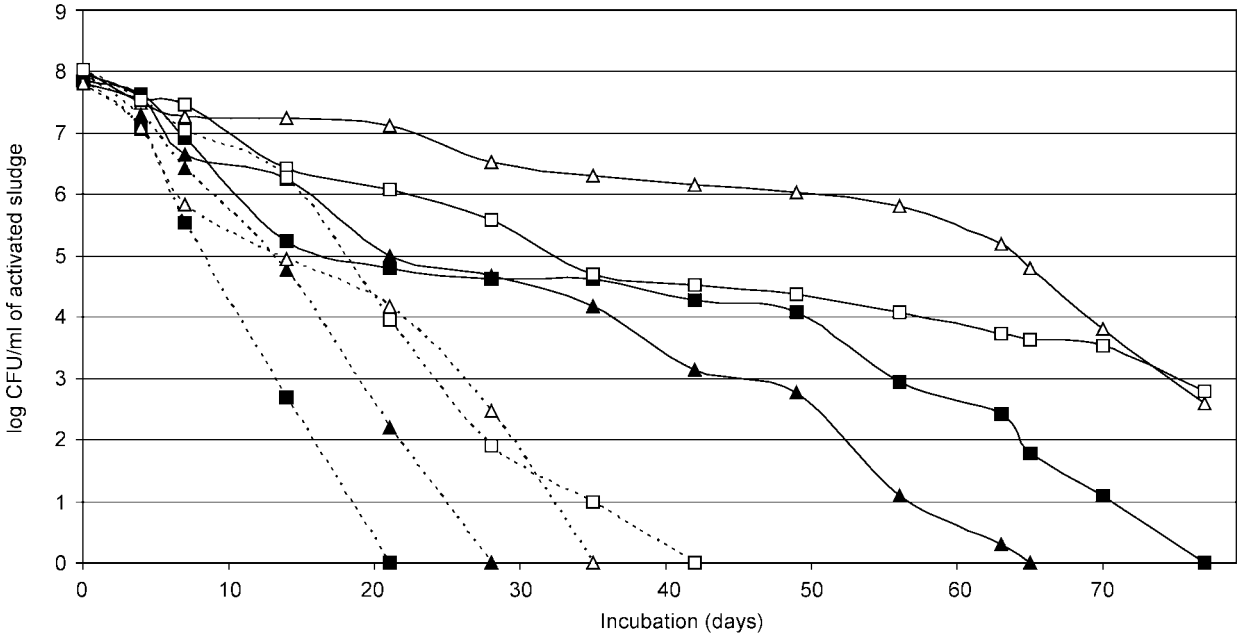


Fig. 1. Survival of enterohemorrhagic and environmental *E. coli* bacteria in activated sludges from dairy sewage treatment plants at 6°C and 24°C.

Activated sludge plant. – ■ – A, *E. coli* 0157:H7, 6°C; – ▲ – B, *E. coli* 0157:H7, 6°C; – □ – A, *E. coli*, 6°C; – Δ – B, *E. coli*, 6°C; – ■ – A, *E. coli* 0157:H7, 24°C; - - ▲ - - B, *E. coli* 0157:H7, 24°C; - - □ - - A, *E. coli*, 24°C; - - Δ - - B, *E. coli*, 24°C.

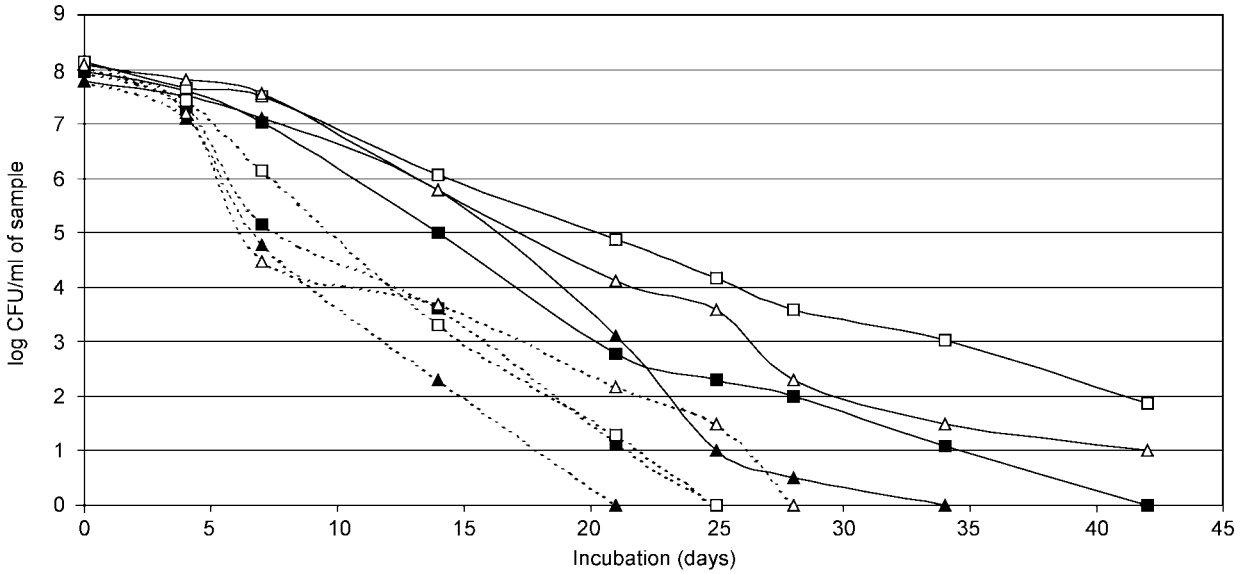


Fig.2. Survival of enterohemorrhagic and environmental *E. coli* bacteria in dairy wastewater contaning activated sludges at 6°C and 24°C.

Activated sludge with wastewater. – ■ – A, *E. coli* 0157:H7, 6°C; – ▲ – B, *E. coli* 0157:H7, 6°C; – □ – A, *E. coli*, 6°C; – Δ – B, *E. coli*, 6°C; - - ■ - - A, *E. coli* 0157:H7, 24°C; - - ▲ - - B, *E. coli* 0157:H7, 24°C; - - □ - - A, *E. coli*, 24°C; - - Δ - - B, *E. coli*, 24°C.

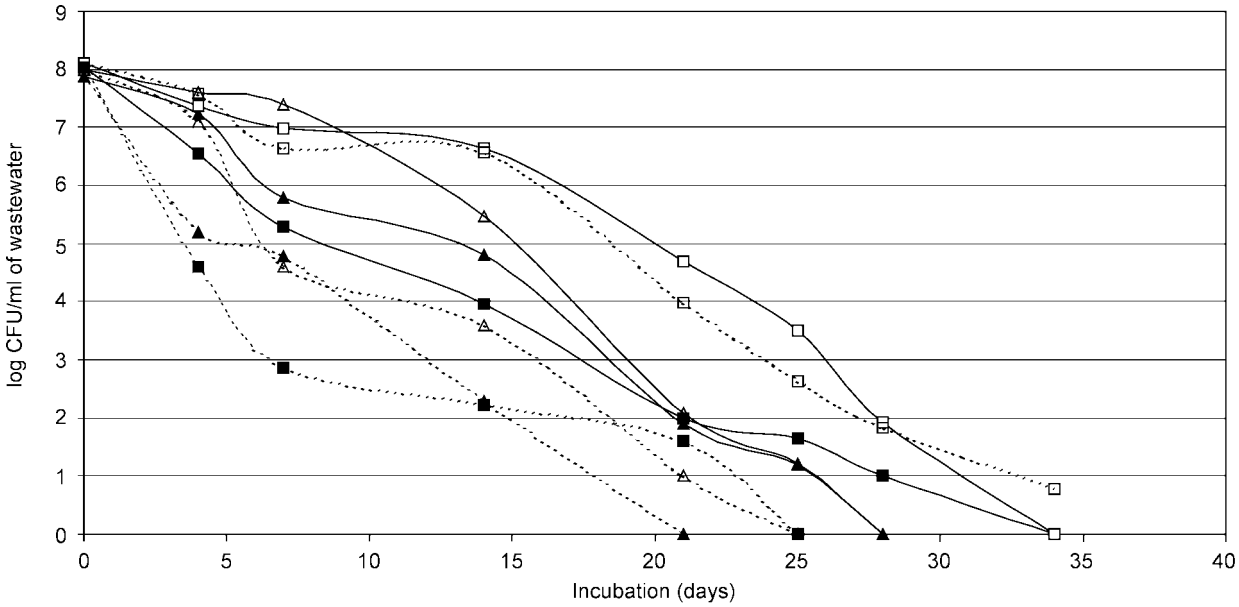


Fig. 3. Survival of enterohemorrhagic and environmental *E. coli* bacteria in dairy wastewater at 6°C and 24°C.
Dairy wastewater. – ■ – A, *E. coli* 0157:H7, 6°C; – ▲ – B, *E. coli* 0157:H7, 6°C; – □ – A, *E. coli*, 6°C; – Δ – B, *E. coli*, 6°C; - - ■ - - A, *E. coli* 0157:H7, 24°C; - - ▲ - - B, *E. coli* 0157:H7, 24°C; - - □ - - A, *E. coli*, 24°C; - - Δ - - B, *E. coli*, 24°C.

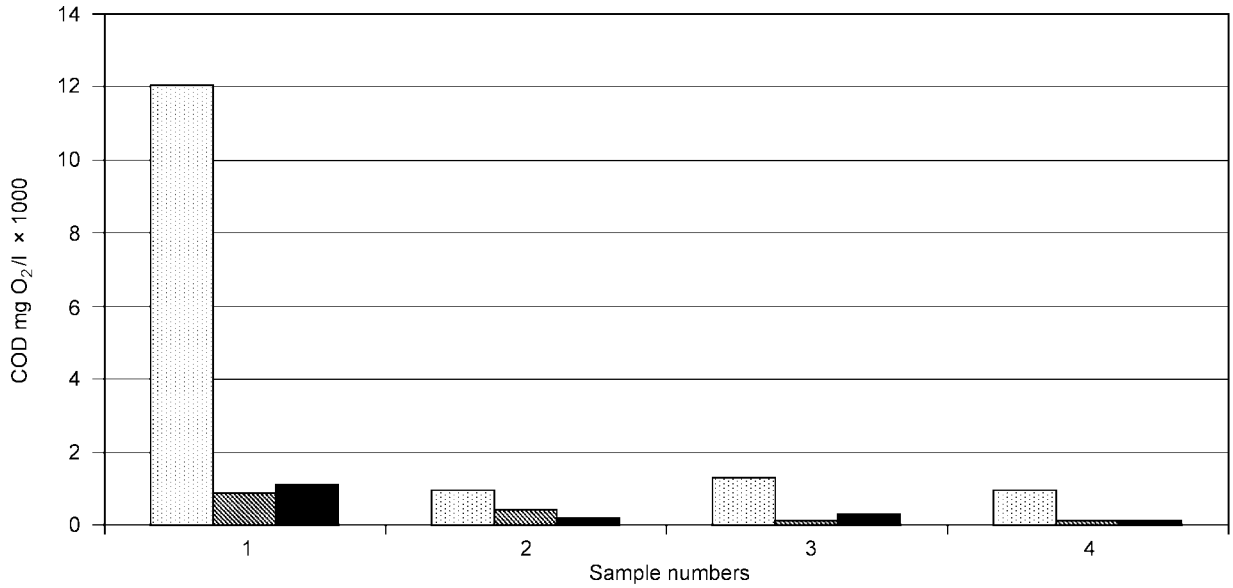


Fig. 4. Changes in COD values after incubation of *E. coli* O57:H7 in wastewater (1 – dairy A; 2 – dairy B) and wastewater with activated sludges (3 – dairy and sewage treatment plant A; 4 – dairy and sewage treatment plant B) at 6°C and 24°C.
COD on 0 day of incubation; COD on the last day of incubation at 6°C; COD on the last day of incubation at 24°C.

of incubation and environmental *E. coli* bacteria after 25–28 days of incubation (Fig. 2).

The differences between the survival of both *E. coli* strains in dairy wastewater at 6°C and 24°C were less distinct (Fig. 3). At 6°C, neither *E. coli* strain was detected after 28–34 days of incubation; at temperature 24°C, pathogenic bacteria were not detected after 21–25 days but environmental *E. coli* bacteria – after 25 days (wastewater from dairy B) or their count was about 10 CFU/ml (wastewater from dairy A) after 34 days of incubation. It should be emphasized that high values of BOD and COD of wastewater from dairy A did not effect the survival of pathogenic and environmental *E. coli* bacteria (Fig. 3).

The effect of temperature on the survival of pathogenic and indicator bacteria in the environment has been reported by numerous authors. Czajkowska *et al.* (2004 and 2005) have found that *E. coli* O157:H7 serotype survived in cultivable and meadow soils for 60 and 158 incubation days (5°C) and for 48 and 90 days

(20°C); in water for 32 and 51 days (6°C) and for 21 and 32 days (24°C); in water sediments for 73 and 100 days (6°C) and for 30 and 60 days (24°C). Wang and Doyle (1998) found that populations of *E. coli* O157:H7 bacteria decreased in water by 1 to 2 log unit by 91 days at 8°, whereas the bacteria were not detected (≥3 log unit decrease) after 49 to 84 days at 25°C. According to Paluszak *et al.* (2003), fecal streptococci survived at 4°C in peat soil with addition of cattle slurry 18.2 weeks longer and *E. coli* 3.2 weeks longer than at 20°C. This phenomenon has also been observed by Rhodes and Kator (1988). In frames of around 12 days long experiment the number of bacteria *E. coli* and *Salmonella* decreased at high temperature from the level of 6 log CFU/ ml respectively to the level <1 log CFU and around 2 log CFU/ml, whereas at low temperature to the level of around 3.5 and 4.5 log CFU/ ml.

Incubation of dairy wastewater and dairy wastewater with activated sludge samples at both temperatures caused an increase of pH values to the level of 8.0–8.5. In the variant – wastewater from dairy B and temperature 24°C, pH increased to even 9.2–9.3 at the end of incubation (data not presented). These pH values were beyond the maximum pH value for *E. coli* strains (Roberts *et al*., 1996). This could cause the faster death of *E. coli* strains in wastewater from dairy B than in wastewater from dairy A. No regularities were observed with respect to changes in total count of bacteria in the tested media at the final periods of sample incubation. An increase or decrease in the quantity of bacterial population, irrespective of the incubation temperature used, rarely exceeded one logarithmic unit (data not presented). There was a distinct tendency for the number of coliform bacteria to decline This phenomenon has also been observed by Czajkowska *et al*. (2005) in experiments on the survival *E. coli* O157:H7 serotype in water and water sediments.

After incubation of wastewater and wastewater with activated sludges at both temperatures, a reduction of COD values was noted. In the samples with wastewater and pathogenic bacteria, the COD value dropped at the final stages of incubation from the beginning values of about 12000 $O_2$/l (dairy A) to about 880 $O_2$/l (6°C) and 1100 $O_2$/l (24°C) and from about 970 $O_2$/l (dairy B) to about 400 $O_2$/l and 200 $O_2$/l (Fig. 4). In experimental variants with dairy wastewater containing activated sludge, the reduction of COD values in the range of several-fold was noted. With regard to the COD results presented above, the wastewater treatment process should still be continued after the death of pathogenic bacteria. It can be assumed that the likelihood of the presence of *E. coli* O157:H7 bacteria in wastewater from dairy after processing is very low. Moreover, the lack of indicative *E. coli* bacteria in wastewater can be judged as the absence of dangerous pathogens. On the other hand, a very significant risk exists resulting from the relatively long survival of pathogenic bacteria in activated sludges. The principle should be the application of the composting, pasteurization, alkaline treatment processes before utilization of activated sludge on lands.

In this studies only the effect of physical and chemical factors of wastewater and activated sludges on the survival of pathogenic and environmental *E. coli* strains has been tested. Experiments should be continued to evaluate how selected biocenotic factors affect this process, because Wcisło and Chróst (2000) found that the major factor that responsible for mortality of *E. coli* in water was autochtonic microflora, especially microflagellate.

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