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Assessment of viability of the nematode eggs (*Ascaris*, *Toxocara*, *Trichuris*) in sewage sludge with the use of LIVE/DEAD Bacterial Viability Kit

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Abstract

Sewage sludges from wastewater treatment plants may contain live parasite eggs, which can be a source of humans and animals infection. According to the current rules, parasitological examination includes detection of the *Ascaris* spp., *Trichuris* spp. and *Toxocara* spp. eggs and estimation of their viability. The viability assessment based only on the incubation and observation of isolated egg is long and imprecise. The aim of this study was to develop sensitive and less labour-intensive methods for assessing viability of *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp. eggs in sewage sludge. For this purpose, LIVE/DEAD Kit was used. Firstly, the possibility of distinguishing between live and dead eggs in water was assessed. Secondly, an appropriate amount of dyeing mixture needed to distinguish the live and dead eggs in the sewage sludge was determined using experimentally enriched samples and naturally contaminated samples of sludge. Eggs were isolated from the samples by own method which was a combination of flotation and sedimentation, preceded by a long mixing. After the last stage of the procedure, sediment containing the eggs of parasites was stained by LIVE/DEAD kit according to the manufacturer instructions, but with the use of different variants of dyes mixture concentration. The investigation showed that live and dead eggs of these three parasites could be differed by this method with the use of proper concentration of dyes. Live eggs were stained in green (*Ascaris* and *Trichuris)* and green-blue (*Toxocara*). However, all types of dead eggs were red coloured. The study demonstrated that after some modifications (resulted from the nature of the samples) the LIVE/DEAD kit is useful for assessing the viability of *Toxocara*, *Ascaris* and *Trichuris* eggs occurring in the sludge.

Keywords

Parasite eggs, sewage sludge, wastewater treatment, fluorescence

INTRODUCTION

Sewage sludge and treated sewage from wastewater treatment plants can be reused as fertilizers on the fields [1]. Despite new ways of decontamination and sanitation, the sewage sludge may contain live parasite eggs which can be a source of infection for humans and animals. For this reason, there is a duty to monitor the safety of these substances, including the obligation for parasitological examination. According to current rules, parasitological examination includes detection of the indicator parasites, such as: *Ascaris* spp., *Trichuris* spp. and *Toxocara* spp. eggs and assesses their viability. These species were chosen because they are often found in sewage sludge. Also, they have the ability to survive in the long-term and can cause serious problems for human and animal health.

Toxocara larva migrans can cause clinical signs of visceral, ocular or cerebral toxocariasis [2, 3]. Ascariasis can affect symptoms of hepatobiliary, pancreatic or appendiceal ascariasis, intestinal obstruction and Loeffler's syndrome [4, 5, 6]. Invasion of *Trichuris* spp. can cause: colitis, dysenteric syndrome, rectal prolapse [7, 8, 9].

Production and treatment processes of sewage sludge and hygienisation of eggs can inactivate parasites. This kind of eggs, however, often do not undergo rapid degradation and over a long period of time may be able to remain almost unchanged in the sediment. This makes it difficult to assess real parasitological safety. Therefore, it is important to determine the viability of this type parasite eggs which occur in sewage sludge.

Usually, the evaluation of eggs viability is based on determining the accuracy of the morphological structure during observation under a microscope [10] or observation of developing embryos in eggs during incubation [11]. However, these methods are insufficient because they are the time- and labour-consuming. An important element in the evaluation of viability is also the subjectivity of the analyst's assessment. Moreover, eggs isolated from sewage sludge usually develop slowly. Furthermore, the glycerol covering microscopic preparations (used to obtain good humidity and transparency) causes partial cut-off of oxygen which can also hinder the development of eggs.

Therefore, there is a need for an effective alternative method of evaluation of viability, which will be simple, fast and useful for all three species of indicator nematodes.

In studies to assess the viability of bacterial cells, a large number of tests was developed based on the difference in dye absorption capacity of the live and dead cells. An example of this kind of test is LIVE/DEAD BacLight Bacterial Viability Kit, type 7007 (Molecular Probes, Invitrogen, Eugene, USA). This kit utilizes mixtures of two fluorescent stains: SYTO 9 green-fluorescent nucleic acid stain and red-fluorescent nucleic acid stain, propidium iodide. According to the

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producer, after using this mixture, live bacterial cells (with intact cell membranes) stain fluorescent green, whereas dead cells (with damaged membranes) stain fluorescent red).

The aim of this study was to determine the possibility of using LIVE/DEAD kit to assess the viability of parasites eggs isolated from sewage sludge.

MATERIALS AND METHODS

Sewage sludge

The experiments were conducted on sewage sludge from five selected municipal wastewater treatment plants from Poland.

Wastewater treatment differed by a population equivalent (pe) factor (which ranged from 14,000 – 900,000) and kind of technological processes. The main features of the treatment are presented in Table 1.

Table 1. Characteristics of municipal wastewater treatment plants tested in the experiment

Treatment plant	Factor (PE)	Fermentation	Liming	Dry matter (d.m)	No. of detected eggs / kg d.m
1	90,000	absence	liming	19.10%	<1,000
2	27,000	methane	liming	24.25%	1,000–5,000
3	60,000	methane	liming	23.20%	1,000–5,000
4	80,000	absence	absence	28.64%	1,000–5,000
5	14,000	methane	absence	15.70%	5,000- 5,0000

Parasites eggs

Parasites eggs used in the experiments were isolated from female, mature nematodes. Eggs of *Ascaris suum* were obtained from female nematode taken from the intestines of slaughtered pigs. *Toxocara canis* eggs was isolated from female nematode defecated by naturally infected dogs. *Trichuris ovis* eggs were received from females nematodes isolated from moose caecum.

Isolated eggs were stored in a 1% solution of formalin at 4 °C. Some of the eggs were inactivated by heating at 60 °C for half-an-hour. Other eggs that were not inactivated were considered as viable. Before use in experiments, the viability of the eggs was tested by incubation at 27 °C for a period of three weeks, and by microscopic observation.

Methods of detection and staining of eggs in sewage sludge

The own method, which is a combination of flotation and sedimentation, was used for the detection of eggs in sewage sludge. This was preceded by a long mixing in order to loosen the structure and breakdown of organic matter present in the sewage sludge [12]. After the last stage of the procedure (sedimentation) sediment (organic particles and eggs of parasites suspended in water) was stained by LIVE/DEAD kit according to the manufacturer's instruction, but with changing the amounts of dyes mixture. However, in the experiments, varying amounts of mixture of dyes were used. Dyes A and B were mixed immediately before use in a ratio of 1:1 and were then added to the suspensions containing the eggs (volume specified in the various experiments). After mixing the mixture of dyes with sediment, samples were incubated at room temperature (approx. 22 °C) for 15 minutes in the dark. The stained sediment was then filtered through polycarbonate filters with a diameter of 45 mm and a diameter of pore 12 μm. The filters were placed on microscopic slides.

Preparations were observed under a fluorescence microscope, using a filter for wave length 470 nm excitation and 490 nm emission. Colour and intensity of live and inactivated (dead) eggs staining and the percentage of stained eggs were visually assessed.

Course of experiments

3 experiments were conducted:

In the first one, the possibility was tested of distinguishing between live and dead eggs suspended in distilled water.

In the second experiment, an appropriate amount of dyeing mixture needed to distinguish the live and dead nematode eggs enriching the sludge was determined.

In the third part of the study, the usefulness of a LIVE/DEAD kit for staining parasite nematode eggs in various types of sewage sludge was assessed.

Experiment I

Staining of live and dead *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp. eggs suspended in distilled water.

Suspensions of live and dead eggs (each of three parasite species) contained 10,000 eggs per 1 ml distilled water were prepared. Eggs were stained by LIVE/DEAD kit: dye A and dye B were added to a suspension of distilled water containing eggs (4 μl of mixture of dyes/1 ml eggs suspended in distilled water). Then eggs were isolated from water by filtration and their colour evaluated under a microscope, similar to the case of suspension of the sediment obtained from the sewage sludge.

Experiment II

Estimation of the optimal amount of dyes for assessing viability of the eggs enriched into the sewage sludge.

The experiment was conducted on the sludge with low level of parasite eggs contamination (less than 5 eggs of each parasites species in 100 g sample detected by own method). Using own method, 600 ml of sediment was obtained. This fluid was dispensed into 30 conical tubes (Falcon type, volume 50 ml), about 20 ml to each tube. Then, 1 ml suspension of parasites eggs (10,000 each species of eggs) was added into the tubes. In this way, 5 sets of tubes were prepared. Each set of tubes contained 6 separate tubes with live and 6 tubes with dead *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp. eggs. Eggs were suspended in sediment of sewage sludge.

The samples were stained by a mixture of dyes in an amount 4, 8, 12, 16 or 20 μl per sample.

After filtration of sediment, preparations were viewed under a fluorescence microscope and colour and intensity staining of live and dead eggs were visually assessed.

Experiment III

Assessment of the viability of parasite eggs naturally occurring in sewage sludge by LIVE/DEAD kit.

Dehydrated sewage sludges from municipal mechanical-biological treatment plants were used for this experiment. Technological processes in treatment plants were different (Tab. 1): in treatment plants 1 and 4, oxygen stabilization was used, whereas in 2, 3 and 5, fermentation was used. Sewage sludge from treatment plant Nos. 1, 2 and 3 were also subjected to liming in the last part of the technological process. Samples

4 and 5 were not subjected to liming. Before the experiment, each sewage sludge was tested by own method.

A fixed amount of the mixture of dyes (20 µl dyes/20 ml sludge) were added after sedimentation.

Due to the relatively low number of eggs in each sample, the first preparations were viewed under a light microscope. Upon detection of parasite eggs, the method of observation was changed to fluorescence to evaluate the viability of eggs. Then, all detected parasite eggs were counted and on the basis of their colour (observed in fluorescence technique) their viability were determined.

RESULTS

The viability of eggs isolated from adult female nematode and the effectiveness of inactivation was assessed during the incubation at 27 °C. After 3 weeks of incubation, embryonic development in most of non-inactivated eggs of nematodes was observed. There was no development of the embryo in any of the inactivated eggs.

Experiment I

Staining of live and dead *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp. eggs suspended in distilled water.

After staining by LIVE/DEAD kit of live and dead nematode eggs suspended in distilled water, the differences in the ability to absorb the fluorescent dyes by eggs were observed.

After the staining of live eggs, 93% of *Ascaris suum* eggs were bright green-coloured, 84% of *Toxocara* spp. eggs were green-blue-coloured and 85% eggs of *Trichuris* spp. were green-coloured.

After staining of inactivated eggs, 95% of *Ascaris* spp., 95% of *Toxocara* spp., and 100% of *Trichuris* spp. eggs were red-coloured. The remaining eggs were not stained.

Staining of live and dead eggs suspended in water is presented in Figure 1.

Experiment II

Estimation of optimal amount of dyes for assessing viability of the eggs enriched into the sewage sludge.

Live and dead eggs which were enriched to sewage sludge were stained with varying intensity according to volume of

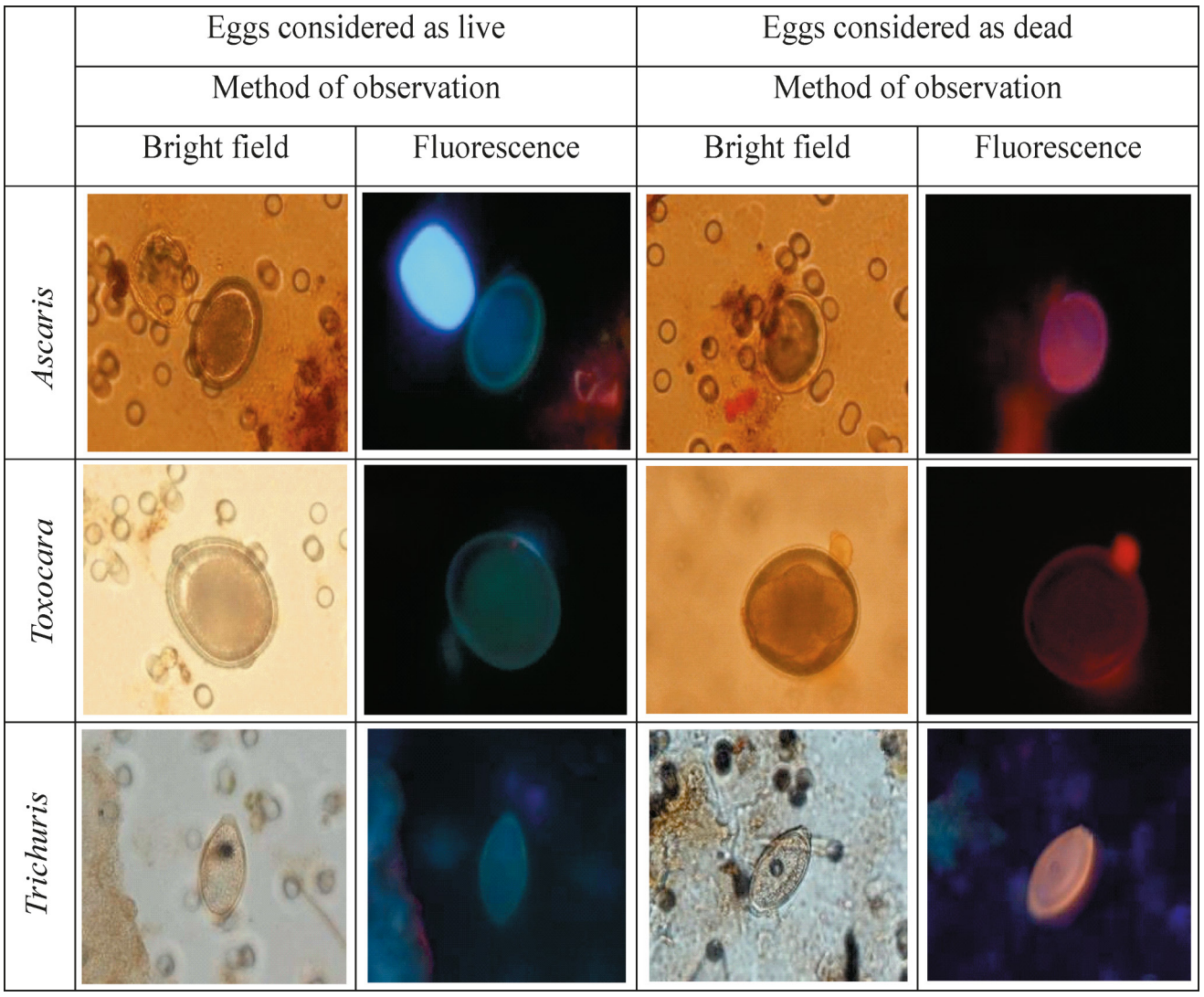

Figure 1. Eggs of *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp. in distilled water, stained by LIVE/DEAD kit

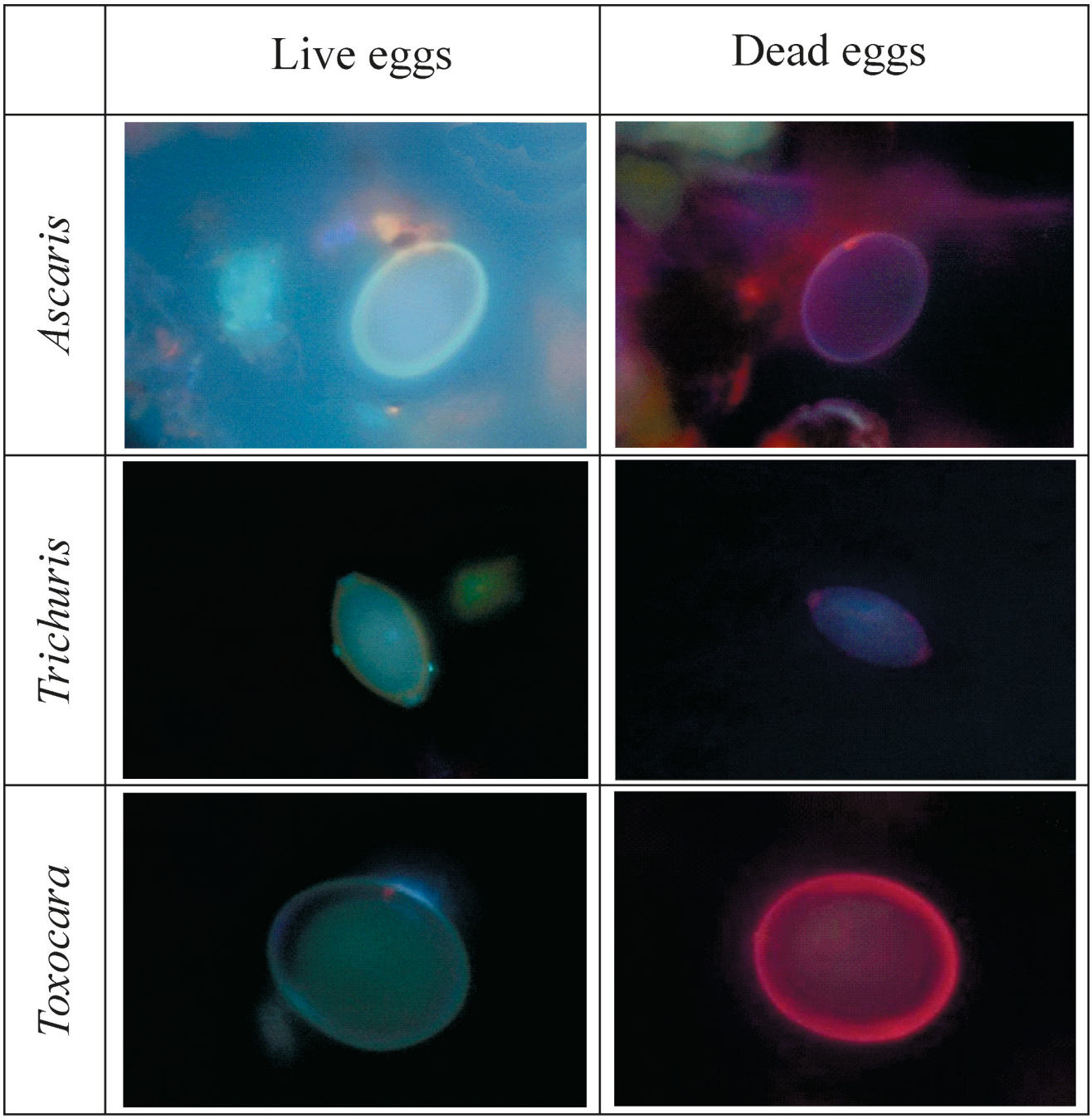

Figure 2. Eggs of *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp., isolated from experimentally enriched sewage sludge, stained by LIVE/DEAD kit

dyes mixture used. Eggs of each of three tested species were not well colored when lower concentrations of dyes 4, 8 or 12 µl/20 ml sewage sludge were used.

The use of 16 µl or 20 µl of dyes mixture per 20 ml of sediment resulted in an intensive colour of eggs: live eggs were green and dead eggs were red or orange-red.

After using 20 µl of dyes mixture per 20 ml of live *Ascaris spp.* eggs suspension, 88% of them were green stained, and in suspension of dead eggs 95% were red-coloured.

Similarly, 80% of live *Toxocara* spp. eggs were coloured green and 90% of dead eggs – red. In the case of *Trichuris* spp., the percentage of non-inactivated eggs coloured green was 80%, and inactivated eggs colored red were 98%. The remaining eggs were not stained. An example of stained eggs is shown in Figure 2.

Experiment III

Assessment of the viability of parasite eggs naturally occurring in sewage sludge by LIVE/DEAD kit.

For staining eggs naturally occurring in sewage sludge, a 20 µl mixture of dyes per 20 ml sediment was used. Significant differences in the colour of eggs were found: some eggs were stained blue-green and others red. Based on experience from the previous experiments (I and II) concerning the way of staining of non-inactivated (live) and inactivated (dead) eggs, the first ones (blue-green) were counted as live eggs

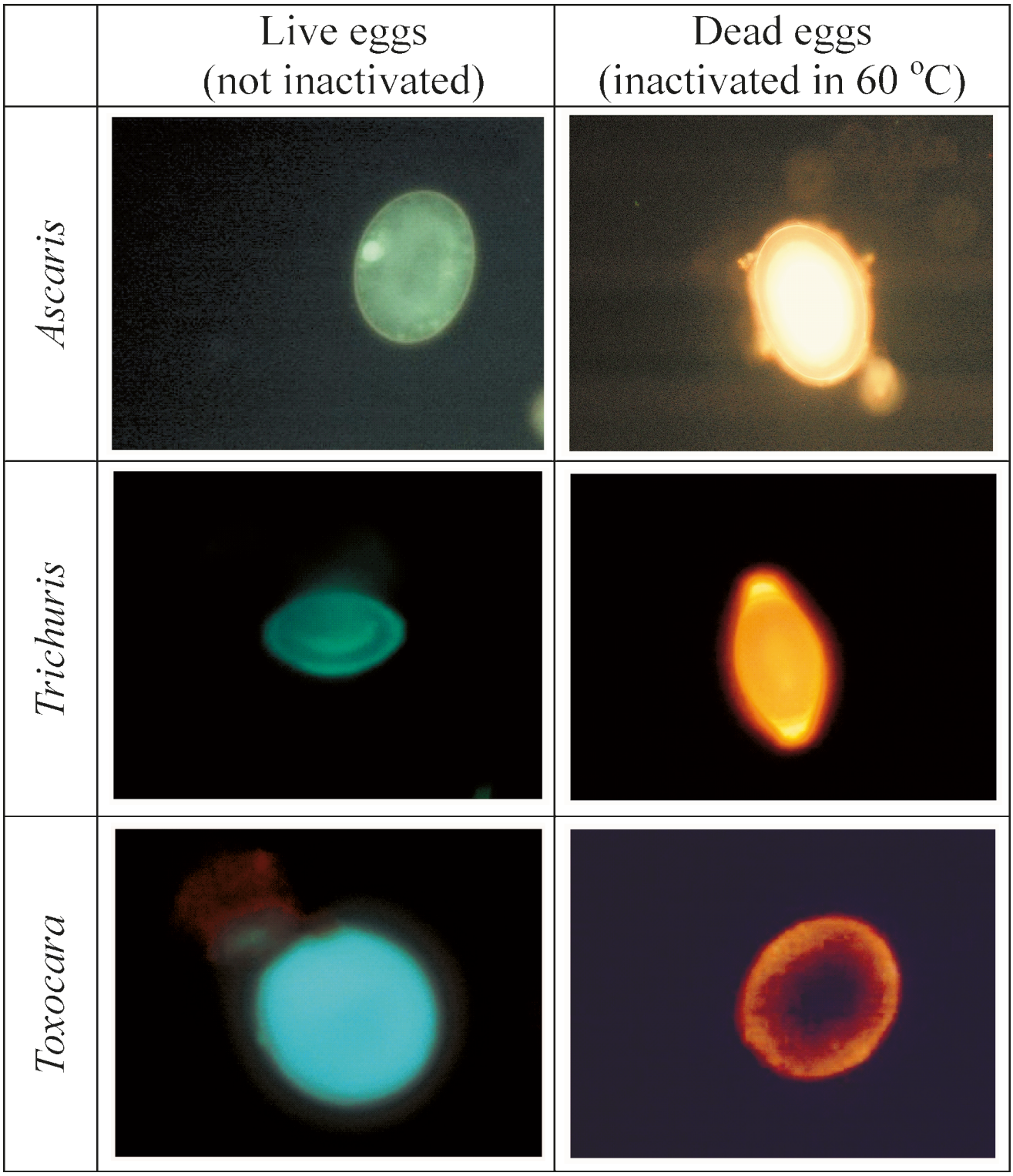

Figure 3. Eggs of *Ascaris* spp., *Toxocara* spp. and *Trichuris* spp., isolated from naturally contaminated sewage sludge, stained by LIVE/DEAD Kit

and the second ones (red) as dead. Only a slight percentage of the eggs was not stained, and were assessed as questionable results. An example of eggs staining is presented in the Figure 3.

As Tab. 2 shows, totally, 241 of parasite eggs (147 of *Ascaris* spp., 75 of *Toxocara* spp. and 23 eggs of *Trichuris* spp.) were detected in the samples. 58% of eggs (140 eggs) were assessed as live and 38% (92 eggs) as dead. Nine eggs (3.7% of the total number of eggs found) were stained insufficiently and estimation of their viability was not possible.

In each treatment plant, from 5 – 174 eggs were detected. The percentage of questionable results was 0%-22% with the highest in treatment plants 1 and 4 in which fermentation had not been applied.

The numbers of eggs classified as live/dead/questionable for individual species of parasites were, respectively: *Ascaris* spp.

Table 2. Number of eggs classified as live (L), dead (D) and questionable (Q) in sewage sludge from treatment plants

Treatment plant	No. of eggs								
	Ascaris			*Toxocara*			*Trichuris*		
	L	D	Q	L	D	Q	L	D	Q
1	1	1	1	0	1	0	1	0	0
2	1	2	1	12	12	2	2	3	0
3	1	1	0	3	1	0	3	0	0
4	7	1	0	4	5	5	0	0	0
5	88	42	0	14	16	0	6	8	0

66.7%/ 32%/ 1.3%; *Toxocara* spp. 44.6%/ 45.9%/9.5% and *Trichuris* spp. 45%/ 55%/ 0%.

The best results of staining (the lowest percentage of unstained eggs) were obtained in case of *Trichuris* spp. Eggs, and the worst – 10% of questionable results, in the case of *Toxocara* spp. eggs.

It should be noted that organic matters occurring naturally in the sediment were also partially coloured.

DISCUSSION

Determination of parasite eggs viability originating from sewage sludge meets the requirements of sanitary regulations. However, such legislation does not specify in detail how to conduct such examinations. For example, in Poland the development of the embryo in the parasite eggs during incubation is the criterion of viability. In France, the correctness of eggs morphology is estimated under a microscope, without further incubation [13]. Johnson *et al*. [14] assessed the viability on the basis of internal structural changes of parasite egg during incubation. The authors assumed that if the interior of the egg is degenerated and there is no development – the parasite egg should be counted as dead, and if there is a division of cells – as live.

Observation of development is relatively easy in the case of enriched eggs obtained by isolation from uterine of female nematodes – containing single fertilized cells. In the case of examination of sludge samples naturally contaminated with parasitic nematode eggs, the eggs are usually in the stage of cleavage and morula or blastula stage. Under a light microscope, the developing embryo takes the form of papules and the subsequent observation of cell division is practically impossible. Following our experience and literature [14], poor availability of oxygen during incubation usually causes lack of further embryo development, and viability assessment based on morphological characteristics becomes very difficult.

Therefore, attempts to distinguish live from dead eggs were made using dyes. It must be stressed that the presence of thick shells of eggs, specific matrix and organic residues found in sediments could be indicated as the most important.

Chapalamadugu *et al*. [15] studied the effect of acridine orange, propidium iodide and trypan blue on *Taenia* eggs. For better dye penetration, the shell was removed using a high temperature and chemical reagents. Then, oncospherae were released from the eggs which were easily stained. The results obtained in this experiment shows that the eggs with a damaged shell are easily stained. Dyes may then freely penetrate into the parasite egg. These studies were conducted *in vitro*.

However, staining methods were not tested as to whether they were suitable on the parasite eggs which had been subjected to technological processes, such as wastewater treatment plants. In addition, isolating the embryo from eggs is very time-consuming and is not suitable for routine examinations of parasite eggs in sewage sludge. Sewage sludge is a very difficult matrix, not only because of the content of organic substances, but also because parasite eggs occurring in the sludge are stained by bile pigments. This makes it difficult for fluorescent dyes to penetrate into the eggs. It is much easier to stain eggs suspended in water. The results obtained by de Victorica *et al*. [17], in which *Ascaris* eggs suspended in the water and sludge were stained, support this view.

The presented study shows the usefulness of LIVE/DEAD kit to assess the viability of *Toxocara*, *Ascaris* and *Trichuris* eggs occurring in sludge. The results of staining by LIVE/DEAD kit of eggs suspended in water was the most effective. It was established that inactivated *Ascaris suum*, *Toxocara canis* and *Trichuris ovis* eggs stained by LIVE/DEAD kit under fluorescence microscopy glow red, and not inactivated eggs (live) green. In the literature, there are no other studies on the use of this kit in determining the viability of nematode eggs. However, an investigation using this kit in determining the viability of protozoa *Giardia muris* cysts has been conducted [18]. In this investigation, different methods of staining of live and dead *Giardia muris* cysts were compared. The best results were obtained by these authors using LIVE/DEAD kit. However, cysts of protozoa are more easily stained because of the more permeable cell wall (which consists of a fibrous layer and membranous layer) [19] than the thick shells of nematode eggs. LIVE/DEAD kit was used to determine the viability of bacteria, for example, *E. coli* [20] and archaebacteria, which were also more easily stained than nematode eggs. Furthermore, other investigations indicated that LIVE/DEAD kit was three times more effective than DAPI in the staining of marine bacteria [21]. However, this kit has some limitations, as confirmed by investigation conducted by Ivanova *et al*. [22]. In this study, bacterial cells *Pseudomonas putida* stained in red (counted as dead) showed movement, and developed in base with zinc additive. In the presented study, there were also a single parasite eggs that were not stained. As described previously, the cause is most likely the thick shell of nematode eggs, saturation by bile pigments and strongly contaminated matrix (sewage sludge).

The study has demonstrated the high usefulness of LIVE/DEAD kit to assess the viability of *Toxocara*, *Ascaris* and *Trichuris* eggs occurring in sewage sludge. This kit could be considered as an alternative to time-consuming and subjective observation of eggs during incubation.

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