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Environmental Hazard Assessment of Cheese Manufacturing Effluent Treated for Hydrogen Production

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Abstract Toxicity of effluents after treatment in an anaerobic fermentation system for hydrogen production is evaluated with three biotests: The zebrafish *Danio rerio* embryo test, the Thamnotoxkit F and the Daphtoxkit F^{TM} magna. Samples were classified from "very" to "extremely toxic". Average toxicity values for zebrafish were 1.55% (24 h) and 0.75% (48 h), for *Thamnocephalus* 0.69% (24 h) and for *Daphnia* 2.51% (24 h) and 1.82% (48 h). Statistical analysis between physicochemical parameters and LC_{50} values revealed that PO_4^{-3}, SO_4^{-2}, NH_3N and NO_3^- have the major contribution to toxicity. Based on results, this treatment is considered an environmentally ineffective way of managing the specific wastes.

Keywords Toxicity · Cheese whey · Thamnotoxkit F · Daphtoxkit F^{TM} magna · Zebrafish · Anaerobic digestion

Despite the fact that the production of milk and dairy products is limited in Greece, direct disposal of dairy effluents into soil and water recipients without any treatment consists a major environmental threat. Dairy effluents are a mixture of organic matter, nitrogen, phosphorus and bacteria (Garrido et al. 2001). They are characterized by high biological oxygen demand (BOD) and chemical oxygen demand (COD) concentrations, nutrients, carbohydrates, proteins and fats (Demirel et al. 2005).

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The treatment of these effluents has always been a concern of industrialists, for the protection of the environment. Biochemical processes through which biomass is transformed to energy, are a way to treat wastewaters of high organic content such as cheese whey and anaerobic treatment has been accepted as an effective mean of treatment for high strength wastewaters (Handajani 2004). In current study, cheese whey processed through an anaerobic fermentation system for hydrogen production in the laboratory of Biochemical Engineering and Environmental Technology in the Department of Chemical Engineering of University of Patras, was tested for toxicity. That treatment results in the reduction of BOD and COD concentrations and the production of biogases (H_2 and CO_2). The toxicity of the treated effluents was estimated, by using zebrafish embryos (*Danio rerio*) and macroinvertebrates (*Daphnia magna* and *Thamnocephalus platyurus*) in the form of microbiotests, Thamnotoxkit F and Daphtoxkit F^{TM} *magna*. Microbiotests are test-kits which contain the preserved bioindicator, experimental vessels, and reagents (Janssen et al. 2000).

The goals of this study were: to evaluate the acute toxicity of treated dairy effluents after anaerobic fermentation, using zebrafish embryos and two microbiotests, to relate the physicochemical characteristics of the effluents with their toxicity effects on the test organisms and to investigate the effectiveness of this specific treatment in the reduction of the effluent's toxicity.

Materials and Methods

For evaluating the toxicity of treated dairy effluents, a continuous stirred-tank reactor for hydrogen production (H_2-CSTR) was operated for a period of 20 months, in the cooperative laboratory of Biochemical Engineering and

Environmental Technology (Department of Chemical Engineering, University of Patras, Greece). The hydraulic retention time (HRT) was 36 h in the first 10 months of the reactor's operation, where 20 samples were collected. The HRT was reduced to 24 h for the optimum operation of the system and the remaining 20 samples were collected during that period. Each sample was divided, and a portion was stored at −20°C in order to be used for the bioassays, while the rest was stored at 4°C to be used for the physicochemical analyses.

All samples were analyzed for specific physichochemical parameters in duplicates and the results expressed as means. Standard deviation was calculated for each value. Samples were analyzed for nitrate, nitrite, ammonium, phosphate, sulphate, and total chloride ions. All analyses were conducted using Hach spectophotemeter DR2800, which is based on Standard Methods for the examination of water and wastewater (APHA 1989), while total suspended solids (TSS) and pH were measured with a portable Multiparameter Hach device (Series Ion 156).

Three toxicity tests were utilized for the evaluation of the effluent samples toxicity, two microbiotest kits and a zebrafish embryo toxicity test. All three species used in the tests are freshwater species and were selected because of the disposal of dairy effluents in surface waters.

Dormant eggs of the cladoceran crustacean *Daphnia magna* and larvae hatched from cysts of the anostracan crustacean *Thamnocephalus platyurus*, in the form of microbiotest kits, Daphtoxkit F^{TM} magna (1996) and Thamnotoxkit F (1995) respectively, were used. The tests were carried out according to the process described in the protocol of each toxkit. Incubation took place at 25°C for 24 h for the *Thamnocephalus platyurus* test and at 20°C for 48 h for the *Daphnia magna* test. Both toxicity tests consider the immobility/death of the larvae as the final endpoint and represent the effluent wastewater concentration in which mortality reaches the 50% of the initial population (LC_{50}).

The zebrafish embryo test was conducted according to the DIN (DIN 38 415-T6 2001), which is described in Nagel (2002) and Kammann et al. (2004). The embryo toxicity test with the zebrafish *Danio rerio* has been used as an alternative method for acute toxicity tests with juvenile and adult fish (OECD 2004). The main benefits of using zebrafish as a toxicological model are their size, the optimum breeding and maintenance in laboratory conditions and their optical clarity which allows observation in different developmental stages (Hill et al. 2005).

Zebrafish eggs were obtained from a group of adult fish bred under standardized conditions. The genitors were purchased from a local pet shop and kept in aquaria containing 20 L of semi-static continuously aerated filtered tap water for 1 month. The photoperiod was 12–12 h light and dark, pH was set to 7.5 and temperature was 26°C.

Embryos were obtained from spawning of 2 males and 1 female. The genitors were placed separately in a specific spawning aquarium, equipped with a mesh bottom to protect the eggs from being eaten. Spawning was induced in the morning when the lights were turned on and after 30 min, eggs from each aquarium were collected and rinsed with deionized water.

For each sample, five concentrations were tested, ranging from 10% to 0.625% or 1% to 0.0625%, depending to the range-finding test, described in the test protocol. All dilutions were made using artificial freshwater, which was prepared with specific reagents described in OECD (2004). Each test included a control that contained only artificial water. After the embryo collection and rinsing, about 40 eggs were randomly distributed into petri dishes containing 25 mL of each exposure solution in order to be exposed as soon as possible.

Only fertilized eggs were then placed in a multi-well plate. 20 eggs were used in each concentration, transferred to 10 wells that contained 2 eggs each and 4 mL of diluted sample. Exposure took place at 25°C for 48 h.

Observations of embryos were made at distinct stages, which represent important steps of zebrafish development (Table 1). Table 1 presents all the selected lethal endpoints. The observations were performed directly in the well using a stereoscope and lethal endpoints were reported after 24 and 48 h.

If the percentage of the control group mortality exceeded 10% the repetition of the tests was necessary.

Toxicity test results are expressed in LC_{50}-24 h values for the Thamnotoxkit F test, in LC_{50}-24 h and 48 h values for the Daphtoxkit F^{TM} magna microbiotest and for the zebrafish bioassay. Lethal concentrations were calculated from concentration-lethal effect curves for each sample.

SPSS 16.0 statistical package was used for data analysis and for validity control of the tests. To compare means, paired *t*-test was conducted. One sample Kolmogorov–Smirnoff test was utilized in order to test data for normality. Since all parameters failed to meet the test's criteria, Spearman's rank was used in order to check for correlations between data.

Table 1 Selected lethal endpoints for the zebrafish embryo test

Lethal endpoints	24 h	48 h
Coagulation	X	X
Missing somites	X	X
Missing tail detachment from yolk sac	X	X
Missing spontaneous movement	X	X
Missing eye formation		X
Missing heartbeat		X

Results and Discussion

All 40 samples of the treated dairy effluents were analyzed in duplicate and mean values are shown in Table 2. The physicochemical parameters of all samples are expressed as mean values of each parameter.

It must be noted that there was a great fluctuation of the values among samples as it is revealed by the standard deviation values. Concentrations of sulphate, phosphate and nitrate ions, as well as the total suspended solids concentration were high. TDS and pH values though, did not show correlation with the toxicity values.

The LC_{50} values, accompanied with the 95% confidence limits, for treated dairy samples are shown in Table 3. Chi squared values were also calculated for all tests. As probability values were higher than the 0.05 boundary the validity of the tests was testified and revealed that there is no statistical difference between predicted and observed LC_{50} values. Lethality is considered the final endpoint for both toxkits, according to their protocols. The zebrafish embryo test was applied to all 40 samples and the selected endpoints are expressed as LC_{50} values, based on the results of five different concentrations for each sample. Toxicity results of Thamnocephalus platyurus (24 h test) and zebrafish (48 h test) are similar, with a mean value of LC_{50} 0.75% and 0.69% respectively, opposed to Daphnia magna with a mean LC_{50} value of 1.82% for the 48 h toxicity tests.

In order to categorize the samples, toxicity values of all samples were transformed to toxic units based on the formula $TU = (1/LC_{50})*100$, (Isidori et al. 2000).

The 25% of the treated dairy effluent samples have been characterized as "very toxic" ($TU = 11–100$) by the Thamnotoxkit and the zebrafish embryo test while the remaining 75% are characterized as "extremely toxic" ($TU \geq 100$). On the other hand, 87.5% of the samples are characterized as "very toxic" by the Daphtoxkit test and only the 12.5% of them are characterized as "extremely toxic" (Fig. 1).

The great fluctuation in the toxicity of the samples s1–s20 (HRT = 36 h) and the normalization of the values when the HRT changed to 24 h (s21–s40), led to the grouped observation of the samples. Table 4 shows the average toxicity values for the first 10 months (G1) of reactor's function, as well as the average values for the last 10 months (G2), together with other basic statistical calculations. For all toxicity tests the LC_{50} values were lower among the samples of G2, with the differences being statistically significant for zebrafish and Thamnochephalus platyrus. Moreover, differences were also observed between different toxicity tests among the same group. The distribution of LC_{50} between the zebrafish tests and Thamnotoxkit was not statistically significant for G1 and G2, while statistically significant differences were observed in all other cases.

Agro-industrial effluents are of great concern in countries with high primary production rates, such as Greece, as they pose significant contribution to the pollution of aquatic ecosystems. Dairy product units are common and dispersed in rural areas, discharging high loaded effluents. In previous studies (Karadima and Iliopoulou-Georgudaki 2006) dairy effluents have been characterized as "toxic" to "very toxic". That fact has raised research concern on the treatment of these wastes before they have been discharged in the environment. Carbon and nitrogen removal with anaerobic filter-sequencing batch reactors (Garrido et. al. 2001), bioremediation with rohu *Labeo rohita* (Mishra et al. 2000), coagulation and decantation using aluminium sulphate, ferric chloride and calcium hydroxide (Hamdani et al. 2005) are some of the proposed treatments.

An acceptable method for treating such effluents is the anaerobic treatment, which is recognized as a more efficient approach for reducing organic load with better results than other treatment methods for such agro-industrial wastewaters (Demirel et al. 2005). In this study, a laboratory-scale anaerobic fermentation system for hydrogen production was used as an effective tool for the production of clean energy sources, from high-strength dairy industry wastes.

Treated samples were collected from a reactor for hydrogen production which was in function for about 20 months. Even after the treatment, all samples were categorized as "very toxic" and "extremely toxic" to the specific organisms. Specifically, as shown in Table 4, the toxicity impact of all samples against all organisms was greater for the first 10 months of the reactor's operation. As the conditions in the reactor were normalized after the reduction of HRT from 36 h to 24 h, toxicity seems to be

Table 2 Mean values of physicochemical parameters of treated dairy effluents samples

Samples	SO_4 (mg/L)	PO_4 (mg/L)	NO_3 (mg/L)	NO_2 (mg/L)	NH_3–N (mg/L)	Cl (mg/L)	TDS (g/L)	pH
Range	11–7932	188–2853.6	89.4–875.1	1.2–28.5	7.1–358.2	1.4–36.2	2.8–29.2	3.9–6.13
Average	825	968.5	291.9	7.6	110.8	12.1	11.4	5.05

Table 3 LC_{50} values and 95% confidence limits for treated dairy effluents for all acute toxicity tests

Sample	ZEBRAFISH	95% confidence limits		ZEBRAFISH	95% confidence limits		THAMNOTOXKIT	95% confidence limits		DAPHTOXKIT	95% confidence limits		DAPHTOXKIT	95% confidence limits	
	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 48 h (%)	Upper limit	Lower limit	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 48 h (%)	Upper limit	Lower limit
s1	1.09	1.91	0.62	*	–	–	0.22	0.30	0.16	1.87	2.76	1.27	1.21	1.78	0.82
s2	1.14	2.04	0.64	*	–	–	0.29	0.41	0.19	1.97	2.82	1.38	1.77	2.56	1.22
s3	0.82	1.82	0.37	*	–	–	0.23	0.32	0.17	1.89	2.75	1.30	1.43	2.22	0.92
s4	1.10	2.13	0.57	0.67	1.43	0.31	0.03	0.06	0.02	1.56	2.24	1.09	1.38	1.85	1.03
s5	0.11	0.20	0.06	0.08	0.16	0.04	0.07	0.10	0.04	1.62	2.38	1.10	1.25	1.67	0.93
s6	0.14	0.36	0.06	0.07	0.23	0.02	0.40	0.57	0.28	4.20	5.97	2.95	2.95	4.13	2.11
s7	0.27	0.52	0.14	0.03	0.13	0.01	0.12	0.19	0.08	2.08	2.93	1.48	1.82	2.61	1.27
s8	0.46	0.70	0.30	0.08	0.17	0.04	0.44	0.64	0.30	3.10	4.51	2.13	1.62	2.43	1.08
s9	0.61	0.86	0.43	0.42	0.61	0.29	0.27	0.38	0.19	2.09	2.86	1.53	1.55	2.18	1.10
s10	0.17	0.29	0.10	*	–	–	0.15	0.21	0.11	2.44	3.33	1.79	2.10	3.03	1.45
s11	0.35	0.51	0.24	0.20	0.33	0.12	0.20	0.27	0.15	2.37	3.32	1.69	1.73	2.64	1.13
s12	0.39	0.61	0.25	0.23	0.37	0.14	0.37	0.49	0.28	5.00	7.47	3.35	3.59	5.18	2.49
s13	0.37	0.71	0.19	0.17	0.42	0.07	0.07	0.11	0.05	1.48	2.12	1.03	1.25	1.68	0.93
s14	1.12	1.80	0.70	0.44	0.58	0.33	0.21	0.29	0.15	1.78	2.71	1.17	1.07	1.61	0.71
s15	0.21	0.35	0.12	0.18	0.31	0.11	0.27	0.36	0.20	1.98	2.92	1.34	1.53	2.18	1.08
s16	0.15	0.27	0.08	0.11	0.20	0.06	0.06	0.09	0.04	1.98	2.82	1.39	1.25	1.72	0.91
s17	1.34	2.31	0.78	0.40	0.56	0.29	0.24	0.32	0.18	1.10	1.50	0.81	0.52	0.81	0.33
s18	0.46	0.73	0.29	0.19	0.33	0.11	0.45	0.65	0.31	1.82	2.80	1.18	1.21	1.73	0.85
s19	0.62	0.88	0.44	0.58	0.83	0.41	0.51	0.72	0.36	1.34	2.01	0.90	0.88	1.23	0.63
s20	0.21	0.53	0.08	0.02	0.05	0.01	1.50	2.06	1.09	2.02	2.79	1.46	1.61	2.36	1.10
s21	0.48	0.77	0.30	0.3	0.61	0.24	2.02	2.69	1.52	3.08	4.78	1.98	2.01	2.95	1.37
s22	0.71	1.20	0.42	0.25	0.45	0.14	1.48	1.93	1.13	2.04	2.89	1.44	1.89	2.85	1.25
s23	0.62	0.91	0.42	0.29	0.54	0.16	0.76	1.05	0.55	2.04	3.07	1.35	0.79	1.41	0.44
s24	3.45	5.61	2.12	0.70	1.28	0.38	2.04	2.91	1.43	2.80	4.16	1.89	2.14	2.94	1.56
s25	3.00	4.32	2.08	2.08	2.86	1.51	1.83	2.56	1.31	1.03	1.43	0.74	0.94	1.33	0.67
s26	8.57	15.99	4.59	1.39	1.99	0.97	0.67	1.00	0.45	5.33	7.82	3.63	4.38	6.54	2.93
s27	6.82	12.20	3.81	0.82	1.44	0.47	2.04	2.91	1.43	4.01	6.05	2.66	3.36	4.75	2.38
s28	1.10	1.74	0.70	0.74	1.11	0.50	0.60	0.80	0.45	1.63	2.69	0.99	1.25	2.03	0.77
s29	4.39	7.32	2.63	2.45	3.69	1.63	0.35	0.51	0.24	2.28	3.41	1.53	1.27	1.83	0.88
s30	1.21	1.70	0.86	0.68	1.00	0.46	0.53	0.79	0.36	2.23	3.12	1.59	0.91	1.36	0.61
s31	0.76	1.14	0.51	0.66	1.02	0.43	1.28	1.85	0.88	2.31	3.20	1.67	1.98	2.92	1.34
s32	0.67	1.02	0.44	0.60	0.94	0.38	0.62	0.87	0.44	1.57	2.57	0.96	1.14	1.76	0.74
s33	1.50	2.21	1.02	1.13	1.69	0.76	0.93	1.26	0.69	2.26	3.41	1.50	1.23	1.94	0.78

Table 3 continued

Sample	ZEBRAFISH	95% confidence limits		ZEBRAFISH	95% confidence limits		THAMNOTOXKIT	95% confidence limits		DAPHTOXKIT	95% confidence limits		DAPHTOXKIT	95% confidence limits	
	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 48 h (%)	Upper limit	Lower limit	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 24 h (%)	Upper limit	Lower limit	LC_{50} 48 h (%)	Upper limit	Lower limit
s34	2.20	3.00	1.61	1.90	2.75	1.31	1.79	2.56	1.25	2.05	2.89	1.45	1.44	2.20	0.94
s35	4.32	6.39	2.92	2.02	2.89	1.41	0.93	1.26	0.69	2.16	3.09	1.51	1.35	2.03	0.90
s36	1.62	2.36	1.11	0.99	1.58	0.62	0.14	0.23	0.09	Non toxic	–	–	5.69	10.84	2.99
s37	2.22	3.17	1.55	1.75	2.72	1.13	0.31	0.45	0.21	9.09	16.25	5.08	3.76	5.78	2.45
s38	2.63	3.62	1.91	2.07	2.86	1.50	1.70	2.47	1.19	2.74	3.95	1.90	1.93	2.83	1.32
s39	2.64	4.16	1.67	1.36	1.92	0.96	1.35	1.87	0.97	3.41	5.25	2.21	2.06	2.93	1.45
s40	1.86	2.51	1.38	1.03	1.54	0.69	0.18	0.32	0.10	2.19	3.18	1.51	1.37	1.91	0.98
Average	1.55			0.75			0.69			5.51			1.82		
SD	1.82			0.69			0.64			1.44			1.04		

* Indicates the 100% mortality of all organisms

reduced significantly only for the zebrafish test, the samples of which changed category from the "extremely toxic" to the "very toxic". The toxicity values according to *Daphnia magna* do not seem to be influenced by HRT change, while *Thamnocephalus platyurus* tests presented an intermediate, but statistically significant sensitivity.

The extreme toxicity of the zebrafish (Fig. 1, sample 20) could be attributed to the change in the hydraulic retention time (HRT) of the reactor from 36 h to 24 h, at this point.

As it is shown in Fig. 1, the change of the HRT has no effect on the *D. magna*, considering that values do not show great fluctuations. This fact is also determined statistically via Spearman's correlation, as the results of the *D. magna* tests showed no correlation with any of the physicochemical parameters. The observed toxicity on *D. magna* is most likely induced by other factors, not analyzed in the present study. It must be also noted that *D. magna* tend to be less sensitive to toxic substances than other cladocerans, and this may be due to size differences (Koivisto 1995).

For all the samples, Spearman's correlation coefficient ($p = 0.05$) showed that the 24 h and 48 h zebrafish test is greatly influenced by NH_3–N ($R = 0.669$) and PO_4^{-3} ($R = 0.487$), respectively. The Thamnotoxkit test showed significant relationship with NO_3^- ($R = 0.422$) (Table 5).

In this study, the toxic potency of treated dairy effluents was tested against three freshwater organisms; *D. magna, T. platyurus* and *D. rerio*. The anaerobic treatment of dairy effluents seems to be a method that has not been effective at reducing the toxicity. Effluents after this treatment are still considered as "very toxic" to "extremely toxic".

Based on the correlation values of this study, it seems that dairy effluents' toxicity is mainly caused by ammonium, nitrites and phosphates for both zebrafish and *T. platyurus*. This indicates that further treatment is necessary in order to reduce the nutrients' concentrations and thus achieve an effluent that could be discharged to the environment with less repercussion.

Daphnia magna was proved to be the less sensitive organism for the monitoring of these effluents, while zebrafish *Danio rerio*, as a vertebrate, is considered a more indicative organism, and, is suggested as a reliable tool for the initial screening of the toxicity.

Toxicity data derived from this study are useful for risk assessment and protection of the aquatic environments, because such information is not available in the existing toxicological databases.

Finally, anaerobic treatment of dairy effluents for hydrogen production is a process that cannot be considered as an environmentally effective way of waste treatment. A combination with other suitable treatment methods should be further investigated in order to lead to an effluent that can be safely discharged into the environment.

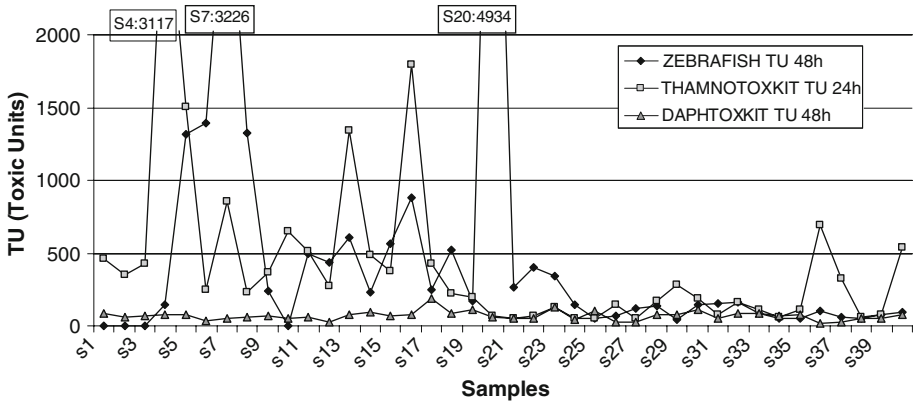

Fig. 1 Toxic unit (TU) values for treated dairy effluents for the three acute toxicity tests

Table 4 Statistical analysis of the three bioassays during the different HRT of the reactor's operation

		Mean	Std. Deviation	Paired differences				
				Mean	SD	95% confidence interval of the difference		Sig. (2-tailed)
						Lower	Upper	
Pair 1	G1DAPHTOXKIT LC50 48 h	1.586	0.682	1.51	0.61	1.18	1.83	0.00
	G1THAMNOTOXKIT LC50 24 h	0.304	0.314					
Pair 2	G1ZEBRAFISH LC50 48 h	0.240	0.200	−1.33	0.84	−1.78	−0.89	0.00
	G1DAPHTOXKIT LC50 48 h	1.586	0.682					
Pair 3	G1ZEBRAFISH LC50 48 h	0.240	0.200	0.03	0.21	−0.08	0.14	0.58
	G1THAMNOTOXKIT LC50 24 h	0.304	0.314					
Pair 4	G2ZEBRAFISH LC50 48 h	1.067	0.695	−0.88	1.45	−1.56	−0.20	0.01
	G2DAPHTOXKIT LC50 48 h	2.045	1.290					
Pair 5	G2ZEBRAFISH LC50 48 h	1.067	0.695	−0.06	1.00	−0.59	0.48	0.82
	G2THAMNOTOXKIT LC50 24 h	1.078	0.662					
Pair 6	G2DAPHTOXKIT LC50 48 h	2.045	1.290	0.86	1.59	0.01	1.71	0.05
	G2THAMNOTOXKIT LC50 24 h	1.078	0.662					
Pair 7	G1THAMNOTOXKIT LC50 24 h	0.304	0.314	−0.77	0.83	−1.16	−0.39	0.00
	G2THAMNOTOXKIT LC50 24 h	1.078	0.662					
Pair 8	G1ZEBRAFISH LC50 48 h	0.241	0.200	−0.83	0.71	−1.20	−0.45	0.00
	G2ZEBRAFISH LC50 48 h	1.067	0.695					
Pair 9	G1DAPHTOXKIT LC50 48 h	1.586	0.682	−0.46	1.49	−1.15	0.24	0.18
	G2DAPHTOXKIT LC50 48 h	2.045	1.290					

Table 5 Spearman correlation coefficient between physicochemical parameters of treated dairy effluents and the toxicity test results

	Zebrafish test LC_{50} 24 h	Zebrafish test LC_{50} 48 h	Thamnotoxkit LC_{50} 24 h
SO^{2}_{-4}	−0.371*	−0.73*	
PO_4^{-3}	−0.479**	−0.87**	−0.363*
NH_3N	−0.650**	−0.669**	−0.344*
NO_2^-	−0.312*	−0.409*	−0.351*
NO_3^-			0.422**
Cl^-	−0.367*		
TDS	−0.362*	−0.419*	

* $p < 0.05$

** $p < 0.01$

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