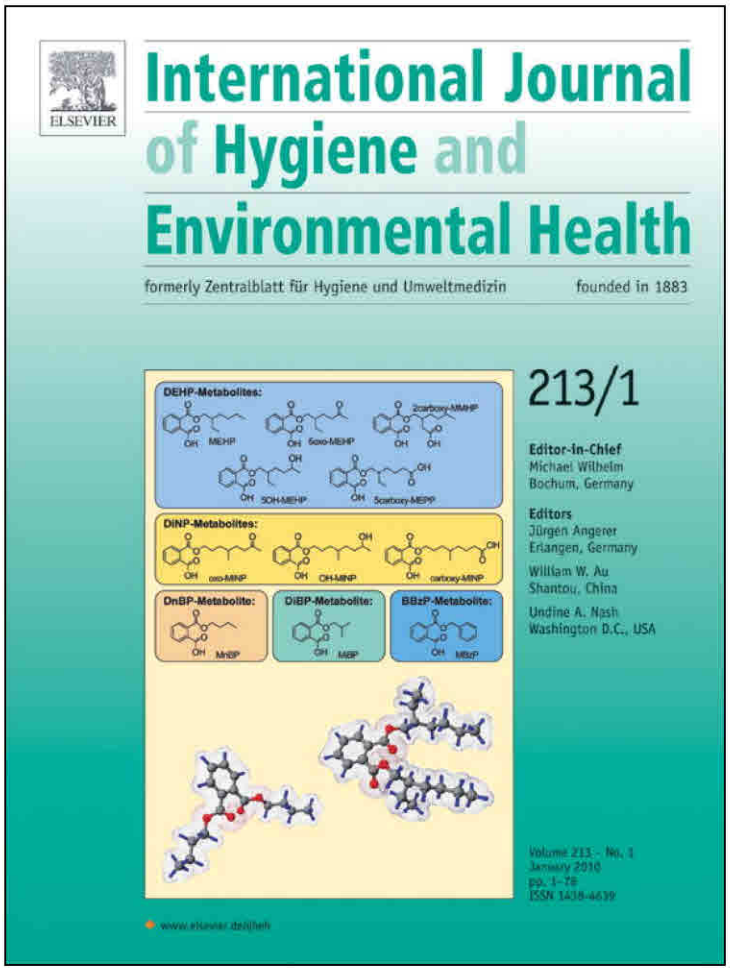

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Constructed wetlands – Are they safe in reducing protozoan parasites?

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ABSTRACT

Constructed wetlands have been promoted in recent literature for use in rural communities in developed as well as in developing countries as an appropriate technology to be handled with low operational maintenance costs. Within a joint project supported by BMBF (Project No O2WA0107 and No 02WA0108) research was done concerning the sanitation effect of constructed wetlands on wastewater effluents. This article will focus on the detection and the removal of cysts of *Cryptosporidium parvum* and *Giarda lamblia*, those being the most frequently identified pathogenic protozoan parasites worldwide with increasing medical and economical consequences.

Two plants, one installed in 2000 as a pilot plant at Langenreichenbach near Leipzig (Saxony, Germany), the other one in routine operation since 1993 in a training center at the town of Belzig (Brandenburg, Germany) were tested for three years. Detection methods from the US EPA (ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure (EPA/814-B-95-003;US EPA 1995) were employed in order to assess protozoal and bacterial reduction in the wastewater passing through different combinations of filter beds and fillings.

Removal of cysts of *Cryptosporidium* and *Giardia* spp. turned out to be a 2 log reduction in all plants. The most effective structural element was a two-stage combination of filter beds leading to the highest removal efficiency both for the protozoan and the bacterial indicator organisms. Also, washed sand (0-2 mm grain size) in the filter bed proved to be most effective filter material; the planted reed (*phragmites* spp.) or willow (*salix* spp.), however, turned out to be of minor importance for the filtering activity.

Introduction

Hygienic concerns in treatment of wastewater come to the fore when water is to be reused or discharged into sensitive receiving rivers, lakes, and ponds. Whenever there is a shortage of water, as is the case in arid subtropical areas of the world, wastewater traditionally is needed for irrigation. However, this application includes the risk of epidemics, especially in developing countries where wastewater treatment is limited for financial reasons. Crops may contain pathogenic microorganisms after irrigation with raw wastewater, and the discharge of untreated sewage may contaminate drinking water resources (Exner et al., 2001; Baeder-Bederski et al., 2004).

Infections with the protozoan parasites, *Cryptosporidium* and *Giardia* spp. are most frequently associated with diarrhea that persists for 7 to 10 days (Thielman and Guerrant, 2004). Waterborne transmission of *Cryptosporidium* oocysts and *Giardia* cysts is well documented (Exner et al., 2001; Slifko et al., 2001) and occurs after ingestion of infective (oo)cysts, which are voided in the feces of an infected person or animal. Cysts from human or animal feces can enter surface water directly or through runoff from fields where manure or sewage sludge is used as fertilizer (Bukhari et al., 1997). The most important source of surface water contamination in densely populated areas are sewage effluents from wastewater treatment plants (Hänninen et al., 2005).

Constructed wetlands (or planted soil filters) may be installed either as vertical or as horizontal flow filter constructions. Horizontal systems may be further classified, depending on the pathway of water flow, as surface and subsurface flow systems. Constructed wetlands have demonstrated effective removal of protozoan parasites mainly with horizontal subsurface flow gravel-based systems (Rivera et al., 1995). Within these configurations, the wastewater is flowing through the media and also below the surface level. The influence of vegetation in constructed

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wetlands on parasite removal is still unclear. Utilization of reeds, *Phragmites* spp. for instance, is common in most types of constructed wetlands; however the presence of marsh plant in subsurface flow gravel beds may not be a significant factor for the removal of protozoan parasites (Quiñónez-Díaz et al., 2001).

Recent documentation on wetland systems concerning wastewater quality improvement has focused on the efficiency of removing waterborne pollutants. Several studies have shown effectiveness of wetlands in treating domestic wastewater for reduction of biochemical oxygen demand, suspended solids and nitrogen (Hagendorf, 1997; Karpiscak et al., 1996). In the meantime, constructed wetlands are known as an attractive and cost-effective wastewater treatment alternative compared to conventional processes. However, information on removal of pathogenic microorganisms is limited; only a few studies have been performed to determine the fate of pathogenic microorganisms and protozoan parasites at the same time (Reed, 1995; Hagendorf et al., 2002; Stott et al., 2003). A variety of processes have been identified concerning the removal of bacterial and viral pathogens, but there is a lack of detailed studies of parasite removal and inactivation mechanisms.

In our study, which was supported by Federal Governmental Funding (BMBF-No.: 02WA0108) the microbiological water quality was evaluated by determining not only fecal indicators, such as *Escherichia* (*E.*) *coli*, Enterococci, coliform bacteria, but also other microbiological parameters like colony forming units (cfu) and protozoan parasites as well.

This paper reports the removal of pathogenic protozoan parasites (*Cryptosporidium* oocysts and *Giardia* cysts; cf. Redder, 2007) and discusses advantages and disadvantages of constructive details of the wetlands tested.

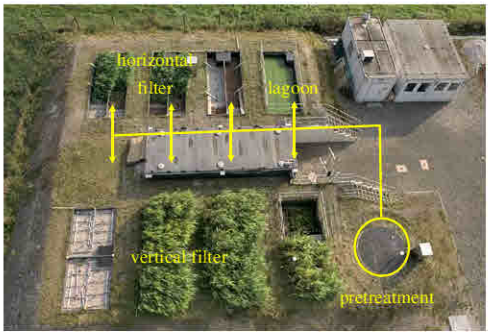

Fig. 1. Pilot plant at Langenreichenbach, Germany (photo: A. Kuenzelmann, UFZ, Halle-Leipzig).

Materials and methods

The Langenreichenbach plant

A pilot plant system was set up in 2000 by the UFZ Centre for Environmental Research Leipzig-Halle (Germany) in the village of Langenreichenbach near Leipzig, Germany (Fig. 1). The water resource was a main sewer carrying municipal raw sewage of about 10,000 population equivalent to the plant. The raw water was mechanically pre-treated in a straw filter. The plant itself consisted of 14 coated steel container elements each measuring 6.7 m^2, which were filled with identical waste water from a ring piping. Different scenarios for evaluating different constructive elements and following microbiological and chemical parameters were tested from 2002 to 2005.

To study the influence of two filter materials, 7 basins were filled with washed sand (grain size, 0-2 mm Heinrich Niemeier GmbH & Co KG, Sprotta, Germany), while the other 7 filters were filled with a mixture of expanded clay (Fibo Exclay Deutschland GmbH, Lahmstedt, Germany) and sand, grain size 2-4 mm and 0-2 mm, respectively. This quality has been developed especially for comparative tests to examine the influence of different types of filter materials. The filters were planted with reed (*Phragmites australis*) with a density of six plants per square meter. To determine the influence of the reed on the reduction performance, 4 of 14 filter beds remained unplanted.

After running through the pilot plant, the water was then returned to the municipal sewage plant. Combinations of 6 different soil filters were tested within the pilot project (Table 1). In this two-stage operation, the water – discharged from the 1st vertical or horizontal flow filter – was transferred at intervals to the 2nd horizontal flow filters using peristaltic pumps. This specific setup was operated for 12 months.

The Belzig plant

The routine plant at Belzig has been in operation since 1993 for 300 population equivalents at a residential area with a seminar center in Belzig, Germany. The two-stage system consists of two vertical flow filters with recirculation planted with *Phragmites australis* (Fig. 2). The sample sites of both plants are shown in Table 1.

Parasitological analysis

Samples were collected every two weeks at the Langenreichenbach pilot plant for a period of 9 months. Sampling spots

Table 1
Sample sites of the pilot plant at Langenreichenbach, Germany and of the routinely run plant at Belzig, Germany (excluded: influent of the system).

Plant	Filter bed combinations	flow	filter material	Plants
Langenreichenbach	combination I			
	1st stage	vertical	clay/sand	*Phragmites australis*
	2nd stage	horizontal	sand	
	combination II			
	1st stage	vertical	sand	*Phragmites australis*
	2nd stage	horizontal	clay/sand	
	combination III			
	1st stage	horizontal	clay/sand	None
	2nd stage	horizontal	sand	
	lagoon	–	none	none
		–		
Belzig	1st stage	vertical	sand	willow
	2nd stage	vertical	sand	willow

were selected within the system as follows: (1) influent, raw wastewater; (2) effluent of the 1st filter flow; (3) effluent from the 2nd filter flow; (4) effluent from a facultative pond (without filter material and without reeds). At the plant at Belzig samples were collected over a period of 3 months.

Sample collection and processing was performed according to the US EPA (United States Environmental Protection Agency) filtration method "ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure" (EPA/814-B-95-003; US EPA 1995). The test method includes detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in water by a fluorescent antibody procedure. Protozoa were concentrated (cf. Gornik and Exner, 1991; Koch, 2004) from a large volume (100 L) by retention on a yarn-wound filter, 1 μm nominal porosity (Micro-Wynd® MW D-PPPY, Cuno Europe SA, Mainz, Germany). Retained particulates were eluted from the filter using an eluting solution (Tween 80) and were concentrated by centrifugation (1500 × g for 10 minutes). *Cryptosporidium* oocysts and *Giardia* cysts were separated from other particulate debris by flotation on a Percoll-sucrose solution with a specific gravity of 1.18. A monolayer of the water layer/Percoll-sucrose interface is placed on a membrane filter, indirectly stained with fluorescent antibody

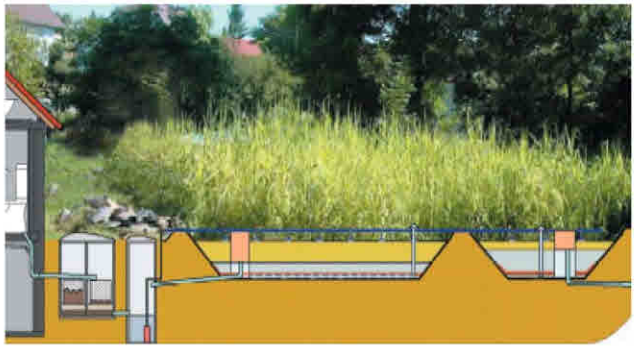

Fig. 2. Routinely run plant at Belzig, Germany (www.oecotec.de).

Hydrofluor™-Combo (Indirect Immunofluorescent Detection Procedure for *Giardia* Cysts and *Cryptosporidium* Oocysts in Environmental Samples, Strategic Diagnostics Inc., Newark, DE, USA), and examined under the fluorescence microscope.

Using epifluorescence (Axiolab®, Zeiss, Jena, Germany), slides were scanned at 400′ magnification for apple-green fluorescence of *Cryptosporidium* oocysts and *Giardia* cysts shapes. If apple-green fluorescing cyst and oocyst structures were observed, *Cryptosporidium* and *Giardia* were identified at a 1000 × magnification. Cysts and oocysts were classified according to specific criteria (immunofluorescence, size and shape). Each well was scanned systematically and the number of oo-/cysts were counted and documented as counts per actual volume per sample. Results were reported in terms of the categories per 100 L.

According to WHO guidelines (1989) enumeration of cysts and oocysts was determined as total number of protozoa per liter, the performance of removal was expressed as a $\log_{10}$ reduction factor ($\log_{10}$ (concentration of protozoa) influent – $\log_{10}$ (concentration of protozoa) effluent of filterbed).

Statistical analysis

Analysis was carried out using the program Stat graphics plus (professional version 4.0 for windows, Statistical Graphics Corp.)

For statistical evaluation the Mann-Whitney test was used. The test is constructed by combining the two samples, sorting the data from smallest to largest, since the p-value is greater or equal to 0.05, there is not a statistically significant difference between the medians at the 95.0% confidence level. All p-values are two-sided.

Results

Results obtained at the Langenreichenbach plant

A total of 93 samples were gathered at the pilot plant at Langenreichenbach. The influent contained a primary mean concentration of approx. 150 *Giardia* cysts/100 L and 14 *Cryptosporidium*

Table 2
Results for *Cryptosporidium* oocysts gained at the pilot plant in Langenreichenbach, Germany (m=mean, sd=standard deviation, RF=$\log_{10}$ Reduction Factor, p-value=significant value calculating the Mann-Whitney test).

Combination of filter beds	*Cryptosporidium* oocysts								
	m/100 L	sd	Log10/100 L	sd	corrected factor 10 (Log10/1000 L)	RF	p-value	Median/100 L	Log10/100 L
c I									
influent (n=10)	13.60	14.71	1.13	0.70	–			11.50	1.06
pretreatment (n=16)	7.69	5.93	0.89	0.46	–	0.25	0.397734	8.00	0.90
first stage (n=10)	1.80	3.82	0.26	0.37	–	0.63	0.0224439	0.00	0.00
effluent second stage (n=8)	0.00	0.00	0.00	0.00	–	0.26	not cal.	0.00	0.00
c II									
influent (n=10)	13.60	14.71	1.13	0.70	2.13			11.50	1.06
pretreatment (n=17)	6.82	6.29	0.83	0.51	1.83	0.30	0.339558	8.00	0.90
first stage (n=11)	0.18	0.60	-0.74	0.09	0.26	1.58	0.00293408	0.00	0.00
effluent second stage (n=7)	0.00	0.00	0.00	0.00	0.00	0.26	not cal.	0.00	0.00
c III									
influent (n=10)	13.60	14.71	1.13	0.70	2.13			11.50	1.06
pretreatment (n=17)	6.71	6.26	0.83	0.51	1.83	0.31	0.339558	7.00	0.85
first stage (n=9)	0.78	1.72	-0.11	0.24	0.89	0.93	0.0154541	0.00	0.00
effluent second stage (n=9)	0.44	1.33	-0.36	0.20	0.64	0.25	0.586669	0.00	0.00
lagoon									
influent (n=7)	9.43	10.23	0.97	0.65	–			11.00	1.04
pretreatment (n=10)	9.80	5.41	0.99	0.35	–	-0.02	0.921236	10.00	1.00
lagoon (n=10)	8.20	14.34	0.91	0.53	–	0.08	0.0521032	4.00	0.60

oocysts/100 L (Tables 2 and 3). *Cryptosporidium* oocysts only appeared sporadically in the influent of the pilot plant. In the effluent of the two stage systems less than 1 oocyst/100 L was identified. The lagoon had negligent efficacy. The mean concentration of *Giardia* in the influent was 150 cysts/100 L ($\pm$100) and for *Cryptosporidium* 13.6 oocysts/100 L ($\pm$14.7) respectively. The passage of the 1st stage of the combination of filter beds resulted in a comparably high removal rate of both *Cryptosporidium* and *Giardia* spp. Calculating the Mann-Whitney test a significant difference between the effluent of mechanically pretreatment in a straw filter and each of the effluent of the 1st filter was determined (Tables 2 and 3). Nevertheless, low concentrations of protozoa were still found in samples taken at the effluent of the 1st filter. In the effluent of the 2nd stages less than 1 oo-/cyst/100 L was found in most of the samples, average recovery level of oo-/cysts and cysts being 6.5% and 14.7%, respectively. Because of the low amount of protozoa in the effluent of the 2nd stages no significant difference between the primary effluent of the effluent of the 1st filter and each of the effluent of the 2nd filter was determined (Tables 2 and 3). If no protozoa were detected in the effluent of the 2nd filter a calculation of the Mann-Whitney test was not possible.

The average removal of *Giardia* ranged between 0.08 and 1.58 $\log_{10}$ with the highest reduction rates in the first stages of the treatment process in all combinations. In the effluent of the treatment process less than 1 oocyst per 100 L was detected in all combinations. The $\log_{10}$ reduction factor of *Cryptosporidium* oocysts varied between 0.25 and 1.58 with highest in the first step of combination II. The average $\log_{10}$ reduction factor (RF) for the two-stage-system (combination I) was 2.2. Similar reductions were observed for combination II (($\log_{10}$)=1.9) and for the unplanted combination system III (RF($\log_{10}$)=2.0). Calculating the Mann-Whitney test no significant difference between the pretreatment and the effluent of the lagoon (*Cryptosporidium* p=0.0521032; *Giardia* p=0.307124) could be determined. Therefore, it can be assumed that filtering of the wastewater is the main effective mechanism reducing the protozoan parasites. The removal of *Cryptosporidium* oocysts and *Giardia* cysts, however, seemed to be independent of the filter types used. The reed *Phragmites australis* generally did not have a noticeable influence on the removal of the protozoan parasites tested.

Results gained at the Belzig plant

At the Belzig plant a total of 14 water samples were gathered: A primary concentration of about 670 oo-/cysts/100 L was

Table 3
Results for Giardia cysts obtained at the pilot plant in Langenreichenbach, Germany (m=mean, sd=standard deviation, RF=log10 Reduction Factor).

Combination of filter beds	*Giardia*cysts								
	m/100 L	sd	Log10 /100 L	sd	corrected factor 10 (Log10/1000 L)	RF	p-value	Median /100 L	Log10 /100 L
c I									
influent (n=10)	157.80	89.24	2.20	0.27	3.20			157.00	2.20
pretreatment (n=16)	113.88	78.42	2.06	0.34	3.06	0.14	0.152374	84.50	1.93
first stage (n=10)	6.40	7.57	0.81	0.55	1.81	1.25	0.000132531	3.00	0.48
effluent second stage (n=8)	0.38	1.06	−0.42	0.17	0.58	1.23	0.030679	0.00	0.00
c II									
influent (n=10)	153.10	79.33	2.18	0.26	–			157.00	2.20
pretreatment (n=17)	126.12	89.97	2.10	0.36	–	0.08	0.379506	96.00	1.98
first stage (n=11)	4.36	5.84	0.64	0.48	–	1.46	0.0000142457	2.00	0.30
effluent second stage (n=7)	1.14	1.95	0.06	0.29	–	0.58	0.212788	0.00	0.00
c III									
influent (n=10)	171.60	98.75	2.23	0.29	–			163.50	2.21
pretreatment (n=17)	126.94	89.31	2.10	0.35	–	0.13	0.258449	96.00	1.98
first stage (n=9)	3.33	4.21	0.52	0.46	–	1.58	0.0000391855	0.00	0.00
effluent second stage (n=9)	1.11	2.67	0.05	0.31	–	0.48	0.246598	0.00	0.00
lagoon									
influent (n=7)	149.86	100.46	2.18	0.30	–			154.00	2.19
pretreatment (n=10)	88.60	48.24	1.95	0.22	–	0.08	0.187412	66.00	1.82
lagoon (n=10)	128.90	172.82	2.11	0.46	–	−0.24	0.307124	45.00	1.65

Table 4
Results obtained at the routinely run plant in Belzig, Germany (m=mean, sd=standard deviation, RF=log10 Reduction Factor).

Combination of filter beds		*Giardia*cysts							
	m/100 L	sd	Log10/100 L	sd	corrected factor 10 (Log10/1000 L)	RF	p-value	Median /100 L	Log10/100 L
influent (n=4)	147.00	54.50	2.20	1.74	3.17			129.50	2.11
first stage (n=5)	0.40	0.89	−0.40	−0.05	0.60	1.80	0.019964	0.00	–
effluent 2nd stage (n=5)	0.00	–	–	–	–	0.60	not cal.	0.00	–
		Cryptosporidium oocysts							
influent (n=4)	523.25	236.33	2.72	2.37	3.72			433.00	2.64
first stage (n=5)	0.80	1.10	−0.10	0.04	0.90	1.92	0.017451	0.00	–
effluent 2nd stage (n=5)	0.00	–	–	–	–	0.90	not cal.	0.00	–

detected at the influent of the routine plant system. The mean of *Cryptosporidium* spp. was 147 per 100 L and 523 per 100 L for *Giardia* cysts, respectively (Table 4). Less than 1 *Cryptosporidium* oocyst could be found after running through the first stage of the treatment process. *Giardia* cysts sporadically were determined in the effluent of the first stage. The average reduction factor for *Cryptosporidium ssp.* was 1.80 and 1.92 for *Giardia* spp. The average removal of protozoan parasites was about 3 $\log_{10}$, resulting after passage of a two-stage combination. The main reduction took place in the first stage of the filter system with a factor of 2.65 in all parameters determined. However, protozoan parasites still were present in the effluent of the first stage but not in the effluent of the second stage. Calculating the Mann-Whitney test a significant difference between the influent and the effluent of the 1st filter was determined (*Cryptosporidium* p=0.017451, *Giardia* p=0.019964). Since no protozoa were detected in the effluent of the 2nd filter calculation of the Mann-Whitney test was not possible (Table 4).

Discussion

The World Health Organization has compiled standards governing the hygienic quality of irrigation water. However, this can only be achieved if raw wastewater is adequately treated. According to the literature the removal of protozoan pathogens is an important yet often neglected issue, particularly in developing countries, where water obtained from sewage plants is used for agricultural application.

Constructed wetlands appear to be an alternative to municipal plants. Our study proved constructed wetlands in a pilot and a field scale to achieve reduction rates of ≈ 2 log for the protozoan pathogens *Cryptosporidium* oocysts and *Giardia* cysts. These results are in agreement with Caccio et al., who investigated municipal wastewater plants in Italy observing a reduction rate of 1-2 log concerning *Giardia* cysts (Caccio et al., 2003).

In our study the most important results were that several consecutive stages appeared to be the only alternative to guarantee the removal of protozoan pathogens. This two-stage system consisting of a subsurface horizontal flow filter led to an almost complete removal of parasite pathogens in the pilot plant at Langenreichenbach. Hagendorf et al. found comparable results in a two-stage wetland with a horizontal combined serial to a vertical flow filter. *Cryptosporidium* oocysts were reduced by about 1, *Giardia* cysts up to 3 log steps (Hagendorf et al., 2002):

Small particle sizes (0-2 mm sand particles) seemed to favor parasite reduction by direct mechanical removal. This relates to the results of Williams et al. (1995) and Stott et al. (1997, 2003), who found particulate matter accumulating especially within the first 10-20 m in the reed bed, suggesting that parasite eggs might be removed by mechanical filtration in subsurface flow systems.

Within the pilot plant at Langenreichenbach limiting the hydraulic load to a range of 40-60 mm/day turned out to be essential. Higher loads might have led to a breakdown of the system by overloading (Baeder-Bederski et al., 2005).

In our study the microbiological water quality was evaluated also determining fecal indicators, especially *E. coli* (DIN 19650 (1999), WHO, 1989). Generally, at the input of the pilot plant at Langenreichenbach *E. coli* was detected up to an average of 10^7 cfu/100 ml. All filterbeds exhibited reduction factors up to 5 with a range of 0.5 to 5.9. Concerning the lagoon the reduction factor was 1.4 with a range of 0.4 to 2.5. *E. coli* concentrations showed to be relatively constant. Similar results were gained at the Belzig plant. The highest reduction rates were achieved with horizontal flow filter beds. Although natural wastewater treatment systems in general are known to be more effective at removal of protozoan parasites than conventional (mechanical) systems (Stott et al., 1997, 2003), the rate of reduction usually turned out to be relatively low in comparison to bacterial removal. No direct correlation between protozoan parasites reduction and those of other indicator organisms could be shown. These results are found in other studies (Füchslin, 2005; Bischoff and Feuerpfeil, 2001).

The lack of correlation between bacterial indicator organisms and parasites underlines the necessity of adequate diagnostics for parasitic load in hygienized water. Monitoring a range of indicator organisms in reclaimed effluent is more likely to be predictive concerning presence of parasitic pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested (Harwood et al., 2005).

Additional research in improving water and sewage treatment practices is needed. Timely and efficient detection of infectious *Cryptosporidium parvum* and *Giardia lamblia* in environmental samples requires the development of rapid and sensitive techniques. A major factor complicating proper detection is the problem of efficiently concentrating cysts from environmental samples, while limiting the presence of extraneous materials. Molecular-based techniques are the most promising methods for sensitive and accurate detection in the near future., e.g. a multiplex PCR for the simultaneous detection of *Cryptosporidium parvum*, *Giardia lamblia* and further waterborne protozoan pathogens (Carey et al., 2004).

In conclusion, the tested filter systems are able to reduce the number of protozoa to some extent and thus to reduce the potential risk of infections associated with wastewater reuse. *Phragmites australis* seems to have little influence on protozoan (as well as bacterial) reduction performance. Wastewater effluents from the tested types of plants may be used as irrigation water, e.g. for open areas and parks according to the WHO guidelines; however, in the event of strong evidence for high parasitic load, these requirements might not be met. Therefore point intensive control of protozoan parasites is necessary, this being the most important issue since the literature shows no direct correlation to other indicator organisms. Thus, any use of the obtained water must be accompanied by close quality controls.

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