3 Material and Methods

3.1 Characterization of sulphide toxicity to *Juncus effusus*

The experiments presented here were conducted to investigate the levels of sulphide concentrations that cause toxicity effects to *J. effusus*.

3.1.1 Plant material

Plants of *Juncus effusus* from the greenhouse were taken for this study. After separating and choosing the plants, presenting homogenous and morphological traits, they were placed in the phytotechnical laboratory operating under defined environmental condition with a temperature day/night 22/16 °C; light day > 10 klux; day/night 16/8 hours.

3.1.2 Experimental set-up

Plants were placed into 7 flasks (300 ml-Erlenmeyer) with about 5 plants each (Figure 9) for 50 days. At first the plants were acclimatised for 25 days to the new environment (phytotechnical laboratory) and placed with their roots in a nutrient solution (tap water with a commercial fertilizer, Hakaphos at a concentration of 1 g L^{-1}). During this acclimatization period (25 days) plants were well adapted and no visual indication of plant stress was found.

After this acclimation period the toxicity test to sulphide started. By this the plant roots were exposed in a solution composed of tap water with 10 mM Tris/HCl buffer and 100 mg L^{-1} NaEDTA (to realize a constant pH of 7.2 ± 0.5 and keep trace metals of the tap water in the solution) to which different amounts of sodium sulphide ($Na_2S \cdot 9H_2O$) were added (0, 5, 10, 25 and 50 mg S^{2-} L^{-1}). Because of the high reactivity of sulphide with oxygen and to keep stable sulphide concentrations of these solutions, every day the old

solutions were replaced by freshly new prepared solutions.

Figure 9 Experimental set up of the sulphide toxicity test of *J. effusus* after 50 days.

3.1.3 Measurements of plant related parameters

3.1.3.1 Water uptake

The evapotranspiration of the flasks with the plants and control (without plants) was estimated by weighting each flask every 24 hours. The difference between evapotranspiration and the evaporation (control without plants) allowed the calculation of the water uptake.

Fresh plant biomass was estimated by weighting each single plant before and after 24 hours. As indicator of plants growth the ratio between the water loss and the fresh biomass was used.

3.1.3.2 Relative growth rate

The length of all shoots in each flask was measured, providing total shoot length per plant as an indicator of above-ground biomass. These data allowed for calculation of relative growth rates (RGR) using the equation:

$$RGR = (\ln W2 - \ln W1) / t2 - t1 \qquad (3.1)$$

Where W_1 and W_2 are non destructive estimates of biomass shoot length for times t_1 (beginning of period) and t_2 (end of period), respectively (Beadle, 1982).

3.1.3.3 Chlorophyll *a* fluorescence

The measurements were made using a portable chlorophyll-fluorescence meter (PAM-2000, Walz). Three mature healthy shoots per flask were randomly chosen. A small leaf-clip adapter was placed on the central part of each shoot for 15 min to achieve dark pre-adaptation before determining *F*o, the fluorescence of photosystem II in the fully oxidized state, and *Fm*, the fluorescence following a pulse of saturation light. *Fv* (*Fm-Fo)* and *Fv/Fm* data are calculated on-line and recorded in a data file. *Fv/Fm* values are used to assess the photochemical efficiency of photosystem II (Krause and Weis, 1991).

3.1.3.4 Visual observation

Each plant was daily checked. Stress symptoms of *J. effusus* were noticed by a general yellowing (chlorosis) of older shoots. Frequently this yellow shoots turned to brown (necrosis). A "die-back" response was defined by visual loss of green colour (chlorosis) and necrotic symptoms as indicator of plants death.

3.2 Treatment of sulphide containing model wastewater in the Planted Fixed Bed Reactor

3.2.1 Synthetic wastewater

In this work the alternative for the post-treatment of anaerobic effluents was investigated under conditions of subsurface horizontal flow constructed wetlands; operation, temperatures of tropical countries were taken into account. Moreover, the characteristics of the model wastewater (see Table 6) were very close to that of a typical anaerobic digester effluent of domestic wastewater. It contained sulphate and sulphide simultaneously. Figure 10 shows the mean values of the anaerobic digester effluent of Bucaramanga-Colombia.

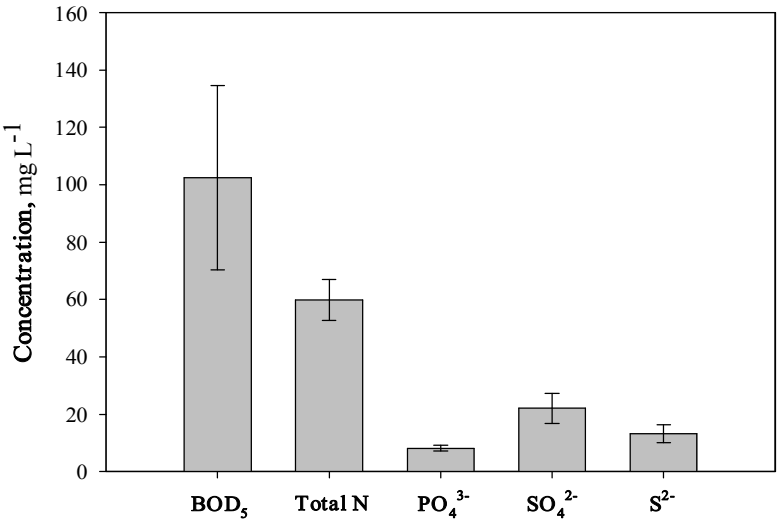

Figure 10 Effluent characteristics of the UASB-reactor of the Bucaramanga treatment plant (Colombia). (Bars are shown with standard error of the mean (n = 25, Feb-Nov-2004).

The artificial wastewater simulated an effluent of an anaerobic wastewater treatment plant with a calculated BOD_5 of about 100 mg L^{-1} derived from a carbon source

(acetate) of good bioavailability (see Table 6).

Table 6 Chemical composition of the synthetic wastewater

Compound	Concentration (mg L^{-1})
CH_3COONa	128
NH_4Cl	148
NaH_2PO_4	12
NaCl	7
$MgCl_2x6H_2O$	1.64
$CaCl_2x2H_2O$	4
$Na_2S{\cdot}9H_2O$	5
Trace mineral solution (see Table 7)	1 ml L^{-1}

The composition of the trace mineral solution is shown in the Table 7.

Table 7 Chemical composition of the trace mineral solution

Compound	Concentration (mg L^{-1})
EDTA-Na	100
$FeSO_4{\cdot}7H_2O$	100
$MnCl_2{\cdot}2H_2O$	80
$CoCl_2{\cdot}6H_2O$	170
$CaCl_2{\cdot}2H_2O$	70
$ZnCl_2$	100
$CuCl_2{\cdot}2H_2O$	150
$NiCl_2{\cdot}6H_2O$	30
H_3BO_3	10
$Na_2MoO_4{\cdot}2H_2O$	10
$Na_2SeO_3{\cdot}5H_2O$	2
HCl	3 ml L^{-1}

These compounds were dissolved in tap water with a sulphate concentration of about 150 mg S L^{-1} during the experimental phase A and in deionised water with a sulphate concentration of about 1.4 mg S L^{-1} during the experimental phases B and C. The resulting BOD_5, nitrogen and phosphorous ratio was of about 10:5:1.

To minimise abiotic sulphide oxidation in the storage tank the artificial wastewater was initially purged with nitrogen gas for at least 30 minutes to strip out dissolved oxygen. This reduced the dissolved oxygen concentration to approximately 0.1 mg L^{-1} (Yongsiri et al., 2003). Sodium sulphide ($Na_2S \cdot 9H_2O$) was then added to the water phase. The headspace of the storage tank was kept oxygen-free by continuously purging of nitrogen gas.

Despite of the nitrogen atmosphere in the feeding tank the sulphide concentration was not stable resulting in a sulphide concentration in a range of 0.92 – 1.4 mg S L^{-1}. Some thiosulphate existed already as an impurity of the sodium sulphide respectively was formed from sulphide by autoxidation during storage. That is why the artificial wastewater was prepared every 3 days anew.

Figure 11 shows mean sulphurs species (sulphate-S, sulphide-S, sulphite-S and thiosulphate-S) in the feeding tank.

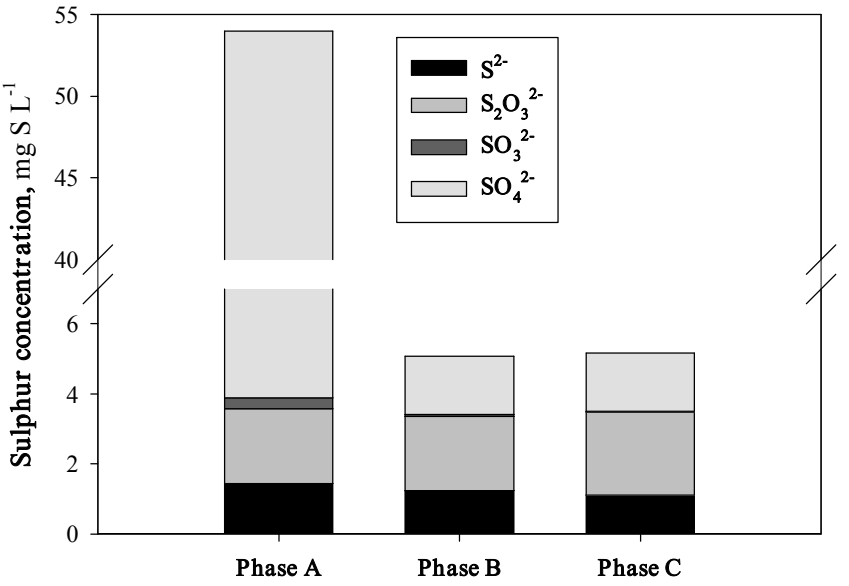

Figure 11 Sulphur species in the feeding tank of the Planted Fixed Bed Reactors.

3.2.2 Laboratory-scale reactor

Two laboratory-scale model wetlands were performed under condition of complete mixing of the filter bed by permanent circulation of the pore water. Since the internal flow conditions are comparable to an ideal mixed vessel, macro-scale gradients of concentration, Eh, pH, etc. were equalized and the effects of micro-gradient changes could be determined. The design and the principles of operation of the reactors were previously described in detail (Kappelmeyer et al., 2002; Wiessner et al., 2005a).

Figure 12 shows the scheme of the Planted Fixed Bed Reactor -PFBR. The reactor consisted of a PVC vessel with 30 cm in diameter and 30 cm tall. A basket of perforated stainless steel 28 cm in diameter and 30 cm tall was placed centrally inside the vessel. A pipe of the perforated stainless steel with 4.5 cm in diameter and 30 cm tall was placed centrally inside the basket. The basket was completely filled with gravel around the pipe. The reactors were closed with a lid containing eight holes through which the plants (18 shoots per hole) in the gravel bed grew. The gravel beds were 28 cm and the water levels were adjusted to 2.5 cm below the surface of the gravel beds.

The circulation flow was adjusted to 10 times the inflow. This permanent mixing of the process fluid made for hydrodynamic condition similar to an ideal mixed vessel inside the rhizosphere (Kappelmeyer et al., 2002).

The water level in the reactor was controlled by a sensor, which is controlled for the internal recycling system. The recirculation system is connected to a microprocessor Standard (WTW, pH-Ionen-to Put pMX 3000/pH) that permits on line measurement of the pH and the redox potential (Eh) recorded every 20 minutes.

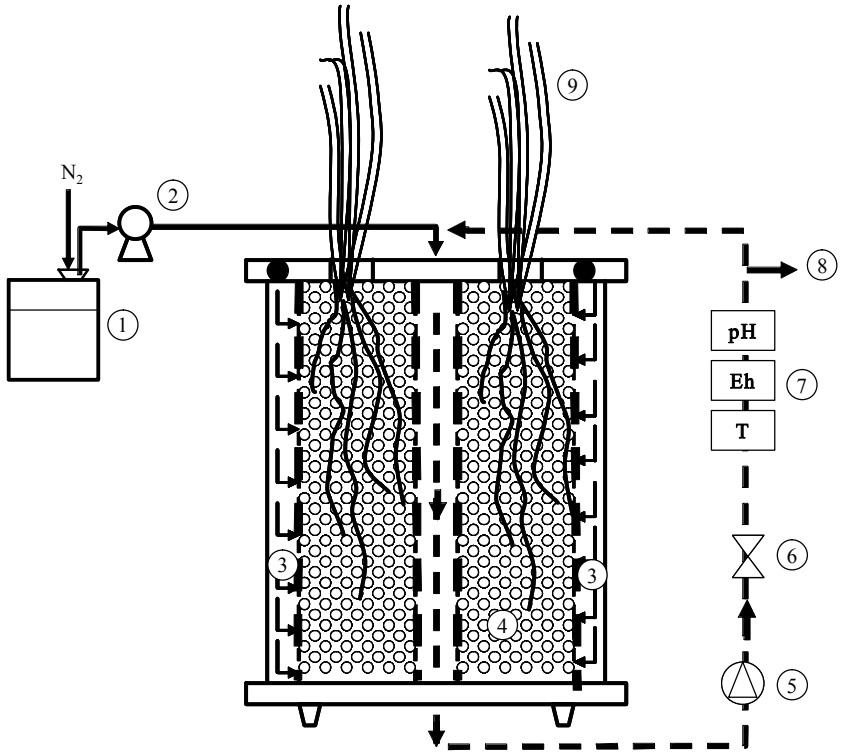

Figure 12 Diagram of the Planted Fixed Bed Reactor- PFBR (1 Feeding storage tank, 2 Pump, 3 Distribution chamber, 4 Gravel bed, 5 Recirculation pump, 6 Magnetic valve, 7 On line measurement , 8 Outflow , 9 Plants) (adapted from Kappelmeyer et al., 2002).

The physical and operational characteristics of the PFBR are shown in Table 8.

Table 8 Physical and operation characteristics of the PFBR

Characteristics	
Soil material	Gravel
Gravel diameter ,mm	2 – 8
Uniformity coefficient of the gravel, D_{60}/D_{10}	1.1
Porosity, %	0.42
Total reactor volume, L	20.1
Effective reactor volume, L	13.2
Height of the reactor, mm	280
Flow rate, ml min^{-1}	0.9 - 1.8
HRT, d	5 - 10
Circulation factor ,$V_{circulation}/V_{inflow}$	10

3.2.3 Plants biomass

Juncus effusus plants were pre-grown in hydroponic culture under greenhouse condition at a temperature of 25 °C. After choosing plants, presenting homogenous morphological traits, they were transported to the phyto-technical laboratory (temperature day/night 22/16 °C; light day > 10 klux; day/night 16/8 hours). During the acclimatization period of 1 week, the plants were fed with tap water and fertilized (NPK, Hakaphos) at a concentration of 1 g L^{-1}.

Both PFBR were planted with macrophytes (*J. effusus*). A density of 6978 and 9973 shoots m^{-2} for PFBR1 and PFBR 2 respectively were achieved.

3.2.4 Experimental conditions

The Planted Fixed Bed Reactors were run under three different conditions (phases A, B and C) realised by different sulphate concentrations in the feeding tank and different hydraulic loading rates (see Table 9).

Table 9 Operation conditions (phases A, B and C) of the Planted Fixed Bed Reactors realised by different sulphate concentrations and different hydraulic retention times of the artificial wastewater.

Parameter	Phase		
	A	B	C
Hydraulic retention time, d	5	5	10
Sulphide concentration, mg S L^{-1}	5	5	5
Sulphate concentration, mg S L^{-1}	50.1	1.4	1.4
Hydraulic loading rate, m d^{-1}	0.08	0.08	0.04
Organic loading rate, g BOD_5 m^{-2} d^{-1}	5.2	5.1	2.6

The experimental wetlands (see Figure 13) were placed in a greenhouse operating under defined environmental conditions with a temperature of 16-22°C simulating an average

summer day in moderate climates (Wiessner et al., 2005a). The experiments were run from middle of September 2004 to April 2005.

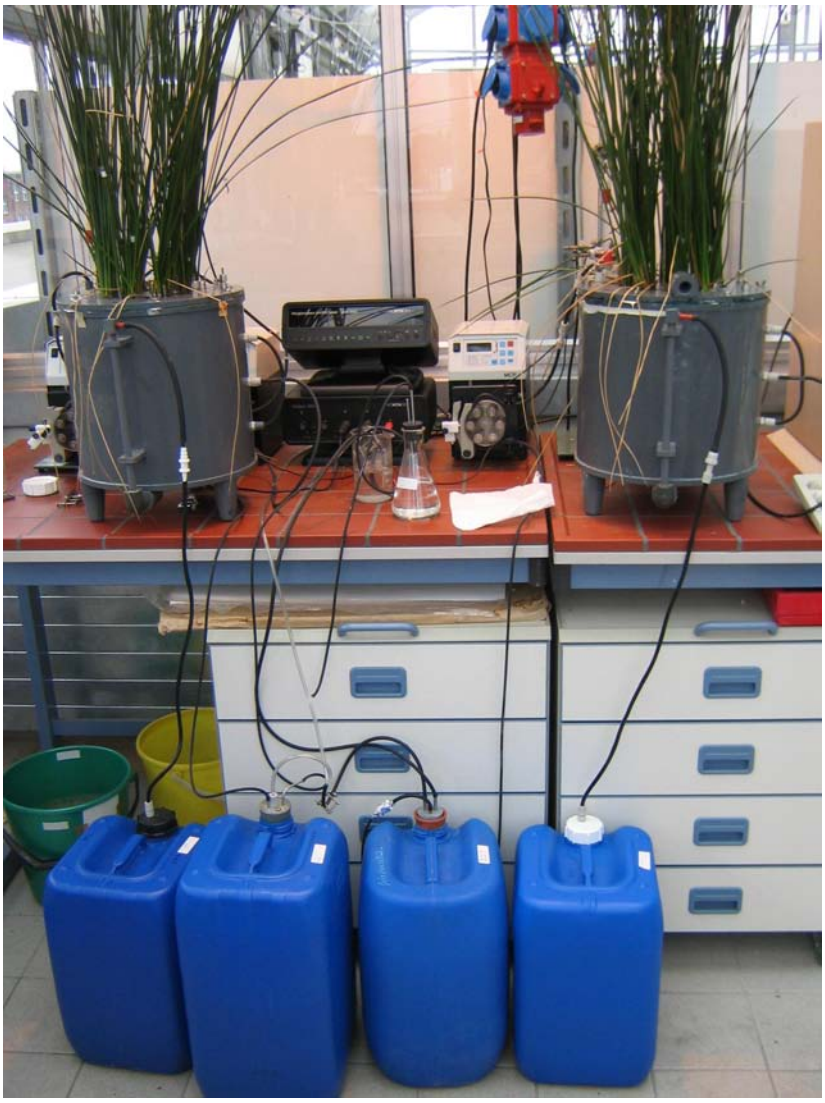

Figure 13 Experimental set up of the Planted Fixed Bed Reactor -PFBR

3.2.5 Sampling

Water samples were taken from the inlet and outlet zones of each bed (PFBR 1 and PFBR 2) with a syringe and a long needle which was rinsed in advance with N_2 gas to minimise autoxidation of sample ingredients.

3.3 Treatment of a sulphide containing model wastewater in the Laboratory-scale Horizontal Subsurface Flow Wetland

3.3.1 Synthetic wastewater

At the beginning of the experiment the wetland-scale reactors were fed with tap water to which was added trace mineral solution(1ml L^{-1}, see Table 7) and nutrient salts (Hakaphos, 0.1 g L^{-1}).

Artificial wastewater (see 3.2.1) simulating an effluent of an anaerobic wastewater treatment plant with acetate as carbon source was prepared in deionised water. Sodium sulphide ($Na_2S \cdot 9H_2O$) to reach 5 and 15 mg S L^{-1} was added into the feeding tank resulting in the mean sulphur species, which are shown in.Figure 14.

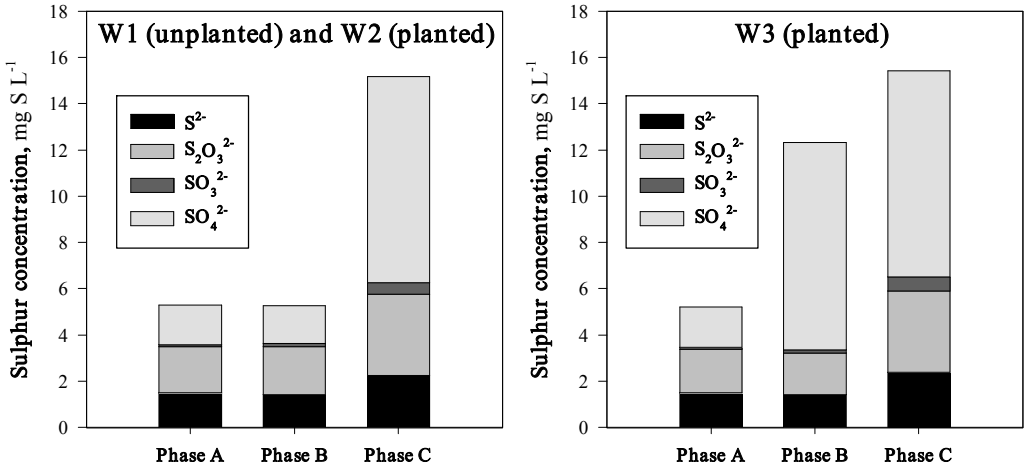

Figure 14 Sulphur species in the feeding tank of the subsurface horizontal laboratory-scale constructed wetland.

3.3.2 Laboratory-scale reactor

The diagrammatic sketch of the used laboratory-scale subsurface horizontal flow wetland is provided in Figure 15.

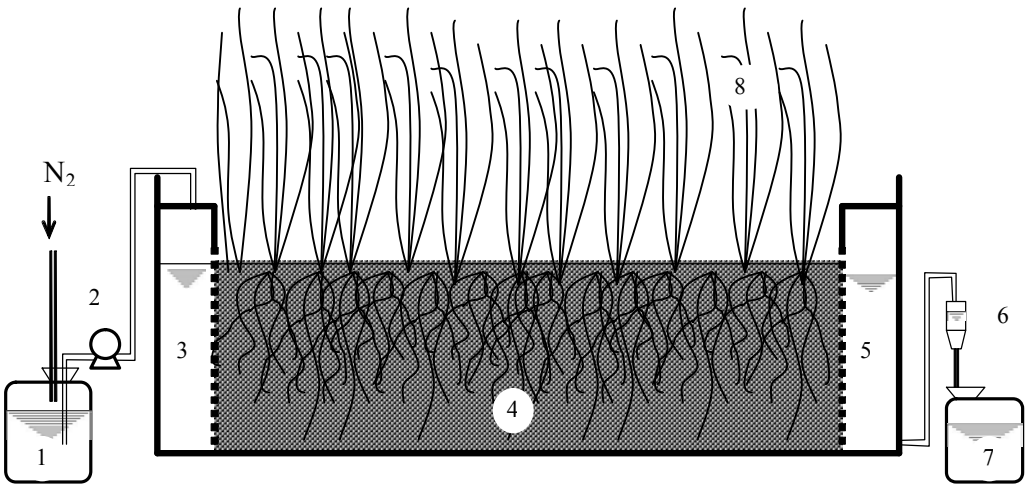

Figure 15 Horizontal flow laboratory-scale constructed wetland. (1 Feeding storage tank, 2 Pump, 3 Distribution chamber, 4 Gravel bed, 5 Outflow, 6 Flow meter, 7 Outflow storage tank, 8 Plants).

The wetlands consisted of a plastic container of 100 cm in length, 15 cm in width and 35 cm in height. The container was filled with 65.7 kg gravel (2-8 mm) up to a height of 30 cm and had a free pore water volume of 15.2 L; the water level was adjusted to 5 cm below the surface of the gravel bed. Sieves of perforated stainless steel were placed 3 cm in front of the inflow and outflow of the gravel bed. This free liquid volume should ensure an equal distribution of the inflow and a laminar liquid flow through the gravel bed (see Figure 15).

The soil material in the wetlands was washed gravel in a range between 2-8 mm. Wetland 2 (W2) and 3 (W3) were planted with macrophytes (*Juncus effusus*) with a density of 1573 and 1433 shoots m^{-2}, respectively. As a control no plants were grown on Wetland 1 (W1). Table 10 gives the main constructive details of the treatment units.

Table 10 Physical and operational characteristics of the laboratory-scale experimental wetlands

Characteristics of the wetlands systems	W_1 , W_2 and W_3
Constructed height, m	0.35
Height of the gravel bed, m	0.30
Height of the water level, m	0.25
Length, m	1.00
Width, m	0.15
Hydraulic retention time, d	5 – 2.5
Hydraulic loading rate, m d^{-1}	0.02 – 0.04
Flow rate, L d^{-1}	3.1 – 6.2

Each laboratory-scale subsurface horizontal wetland was fed separately from the same storage tank (30 L capacity). The storage tank was used for storing the synthetic wastewater (see 3.2.1) to be treated in the reactor. The tank had to be refilled before it became empty to ensure uninterrupted flow of wastewater. The storage tank with the synthetic wastewater (see 3.2.1) was kept anaerobic by bubbling nitrogen gas continuously through the head space of the storage tank.

3.3.3 Experimental conditions

The wetlands W1, W2 and W3 were run under three different experimental conditions (phases A, B and C) realised by different sulphide and sulphate concentrations in the feeding tank respectively by different hydraulic loading rates (see Table 11).

Despite the nitrogen atmosphere in the feeding tank the sulphide concentration was not stable; some thiosulphate existed already as an impurity of the sodium sulphide respectively was formed from sulphide by autoxidation during storage. That is why the artificial wastewater was prepared every 3 days anew.

Table 11 Operation conditions (phases A, B and C) of the experimental wetlands W1, W2 and W3 realised by different sulphide and sulphate concentrations respectively by different hydraulic retention times of the artificial wastewater.

Parameter	Phase		
	A	B	C
Hydraulic retention time, d	5	2.5	2.5
Sulphide concentration, mg S L^{-1}	5	5	15
Sulphate concentration, mg S L^{-1}	1.7	1.7[1)] / 8.2 [2)]	8.2
Hydraulic loading rate, m d^{-1}	0.02	0.04	0.04
Organic loading rate, g BOD_5 m^{-2} d^{-1}	1.6	2.9	3.0

[1)] W1 (unplanted) and W2 (planted), [2)] W3 (planted)

The experimental wetlands were placed in the phytotechnical laboratory (see Figure 16) operating under defined environmental conditions with a temperature of 16-22°C simulating an average summer day in moderate climates (Wiessner et al., 2005a). The operation periods run from middle Octobre 2005 to September 2006.

Figure 16 Experiment set up of the laboratory scale subsurface horizontal wetlands: W1 (unplanted), W2 and W3 (planted).

3.3.4 Sampling

Water samples were taken weekly from the middle of the inlet, middle and outlet of wetland unit with a syringe (60 ml) and a long needle which was rinsed in advance with N_2 gas to minimise autoxidation of sample ingredients.

3.4 Analytical methods

3.4.1 Dissolved sulphide

The concentration of free sulphide was determined with an ion-specific Ag^+/S^{2-} electrode (Silver/Sulphide-Electrode Ag 500, WTW, Germany) in a 6 ml sub-sample fixed immediately after collection with sulphide antioxidant buffer containing sodium hydroxide, sodium EDTA, and ascorbic acid according the WTW's instruction. The detection limit of sulphide was 0.003 mg S^{2-} L^{-1}.

3.4.2 Sulphite and thiosulphate

The inorganic highly reactive sulphur compounds sulphite and thiosulphate in the water samples were analyzed by high performance liquid chromatography (HPLC, modified method according to Rethmeier et al., 1997). The sulphur components were derivatized by monobromobimane to yield fluorescent derivatives. The derivatized sulphur compounds were detected by fluorescence emission at 480 nm. The HPLC (Beckman, USA) was equipped with a 250 mm*4 mm column filled with LiChrosphere® 60 RP select B (5 µm, Merck, Germany) and a fluorescence detector (Shimadzu, Japan). The eluents were 0.25 % acetic acid, pH 4 (solvent A) and 100 % methanol (solvent B). The flow rate of the eluent was 1 ml min^{-1} and the gradient was programmed as follows:

0-5 min 88 % A, 12 % B isocratic

5-13 min 12-30 % B linear gradient

13-16 min 30 % B isocratic

16-34 min 30-60 % B linear gradient

34-36 min 60-100 % B linear gradient

36-39 min 100 % B isocratic

39-39.1 min 100-12 % B linear gradient

39.1-42 min 88 % A, 12 % B isocratic

The lowest detectable concentration was 0.08 mg L^{-1} for sulphite and 0.112 mg L^{-1} for thiosulphate.

3.4.3 Elemental sulphur

Elemental sulphur was also determined according Rethmeier et al., 1997 by extracting samples with chloroform and the subsequent detection by HPLC (Beckman, USA) using a Li Chrospher 100, RP 18 column (5 μm, Merck, Germany) and equipped with a UV-detector at 263 nm. The detection limit for elemental sulphur was about 0.064 mg L^{-1}.

3.4.4 Total carbon and total organic carbon

The total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) of the inflow and outflow of the reactors were analyzed using TOC analyzers (Shimadzu, TOC 600, Duisburg, Germany).

3.4.5 Ion chromatography analysis (IC)

Concentration of ammonia, nitrite, nitrate and sulphate were analyzed by ion chromatography (DIONEX 100, columns AG4A-SC/AS4A-SC (for anions) and

CG12A/CS12A (for cations); Idstein, Germany) using a UV detector for nitrite and nitrate at a wave length of 215 nm and a conductivity detector for the other ions. The self generating suppressor ASRS-Ultra 4mm (for anions) and CBES-I 4 mm (for cations) were used (Wießner et al., 2005a).

3.5 Other parameters

3.5.1 Redox potential (Eh) and pH measurement

The redox potential in the Planted Fixed Bed Reactor was measured by the Pt4805-S7/120 combination Redox, METTLER TOLEDO, and the pH by the pH-electrode Sentix 41. Both parameter were measured on-line and recorded by a microprocessor Standard (pH-ION-Meter pMX 3000/pH, WTW) which allows the measurements on-line every 20 minutes.

In the laboratory-scale horizontal subsurface wetland redox potential was measured in situ every 5 minutes during 10 minutes.

The proper functioning of the electrodes are tested with WTW solution for redox potential (Pt/Ag/AgCl in 1 M KCl, +220 mV/25 °C) and for the pH, two different pH buffers (pH 4.01 and pH 7.00) solutions were used. Redox potential values were converted to the potential relative to the normal hydrogen reference electrode (Eh) taking the sample temperature into account.

3.5.2 Evapotranspiration

Initial and final conditions (weight and volume) feeding (3 to 4 days) were measured in order to determine the evapotranspiration of the system. The reactor design ensures that evaporation can be neglected. The transpiration by the plants was controlled by balancing the inflow and outflow amounts of water. The total amount of water loss was

divided by the time and the area to calculate the specific water loss (transpiration rate, L $m^{-2}d^{-1}$).

3.5.3 Shoot density

The number of the plants was estimated periodically, at approximately 30 days intervals during all experiments by counting the number of total (green and yellow) shoots and divided by the area to calculate the density of the plants.

3.5.4 Gravel analysis

The gravel bed used in this experiment was previously washed with tap water to remove unattached small particles before processing and then heated in a drying oven set at 105 °C for 2 hours. For two gravel samples were determined the gravel size, the density, porosity and uniformity coefficient.

The granulometric distribution of sizes of the gravel bed was made with the Vibratory Sieve Shaker Analysette 3 (FRITSCH). The density of the gravel was measured based on the water replacement method proposed by Balck (1986) and ASTM (1994). The porosity was calculated, dividing the volume of water that could be poured, in each graduate glass, by the total volume of the material. Uniformity coefficient (Cu) is defined like the ratio between material accumulated between the 60 and 10 percent in the granulometric curve. Table 12 shows a summary of the results.

Table 12 Characteristic of the size, density (ρ), porosity (P) and uniformity coefficient (Cu) used gravel in the Planted Fixed Bed Reactors-PFBR.

Sample	Size (mm)	ρ (kg m^{-3})	P (%)	Cu = D_{60}/D_{10}
1	2-8	1482	42.8	1.07
2	2-8	1543	40.6	1.12
Average	2-8	1512	41.7	1.10

3.5.5 Total sulphur

Total sulphur was defined as the sum of sulphide, sulphate, thiosulphate, sulphite and elemental sulphur.

3.5.6 Total nitrogen

Total nitrogen was defined as the sum of ammonia, nitrite and nitrate. Organic nitrogen was assumed to be negligible.

3.5.7 Specific removal rate

The specific removal rates of the wetland systems were calculated as the difference between the specific inflow and outflow loads (mg m^{-2} d^{-1}).

Specific (inflow/outflow*) rate = [concentration (mg L^{-1}) x flow rate (L d^{-1})]/area (m^2)

* The outflow rate include the loss water of evapotranspiration

3.5.8 Data analysis

The range and variation in physicochemical parameter in PFBR's and in planted and unplanted laboratory scale wetlands has been summarized by Principal Components Analysis (PCA) where the multivariate data has been reduced to two dimensions and displayed as "bi-plots" (Statistical Sciences Inc. 1993). PCA allows studying the relationship among descriptors and object in order to summarize important data sets and facilitate the interpretation of the data. PCA characterizes the main trends of variation of the objects (e.g. sampling sites) with respect to all descriptors (e.g. physicochemical parameters). A scatter of the objects is represented in a multidimensional diagram, with as many axes as there are descriptors in the study. The analysis summarizes the range of variation of a multivariate data set by reducing it to two dimension and display as bi-plots.