

CHAPTER 3

BIOLOGICAL PRETREATMENT OF *Cenchrus ciliaris* cv. Molopo (BUFFELSGRASS)

ABSTRACT

The biological treatment of acid mine drainage using sulphate reducing bacteria is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction. Biological pretreatment of complex carbon sources by white rot fungi was investigated to enhance the biodegradability of the lignocellulose substrate. The effect of substrate particle size was evaluated for natural and steam pasteurized *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) which was inoculated with *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Schizophyllum commune*. Uninoculated controls were run in parallel. *In vitro* dry matter digestibility was used to measure the effectiveness of the fungal delignification processes. In all treatments, the initial digestibility was high (64.8%), rapidly decreasing after 1 to 2 weeks. None of the treatments increased the digestibility of the natural substrate. Only *P. ostreatus* was capable of improving the initial digestibility of steam pasteurized grass by 7% over 6 weeks. Substrate particle size influenced substrate degradation in terms of fungal community structures, but did not influence the overall extent of degradation by the inoculated fungi and the naturally occurring microflora. The pH of all natural grass treatments increased rapidly to an alkaline pH in excess of 9. It is known that alkaline treatment can increase forage digestibility, however, in this study the alkaline pH decreased the substrate digestibility by leaching potentially digestible compounds. This was confirmed by the brown leachate that was produced over the experimental period in all treatments. The leachate that was produced could be a direct result of the breakdown of lignocellulose, yielding potentially readily utilisable carbon.

1. INTRODUCTION

Agricultural wastes and lignocellulose are potential inexpensive and available sources of fermentable carbon compounds. During the development of a mixed aerobic-anaerobic microbial treatment process for acid-mine drainage using straw as substrate, substrate availability became the limiting factor in terms of sulphate reduction (Béchar *et al.*, 1994). The authors assumed that the biodegradation of the straw would provide organic carbon necessary to sustain the treatment process. However, the long-term stability of their bioreactors could not be maintained and supplementation with urea and sucrose was required. Carbon was the primary limiting factor in the treatment process.

Plant biomass is made up of predominantly cellulose, hemicellulose and lignin. Up to 50% of the lignocellulose biomass in nature consists of cellulose and the potential for producing fermentable sugars from the cellulose biomass is undergoing intense investigation. However, cellulose fibers are embedded into a lignin matrix, which protects them from enzymatic hydrolysis (Leonowicz *et al.*, 1999; Tomme *et al.*, 1995). Lignin makes up about 20-30% of wood and up to 15% of grasses, and is found in cell walls in a complex with cellulose and hemicellulose polysaccharides (Jeffries, 1990; Mueller-Harvey and Hartley, 1986). In this natural composite material, the cellulose fibrils provide tensile strength, and the hemicellulose and lignin provide cross-linking, binding the structure together (Jeffries, 1990; Tomme *et al.*, 1995).

The biological process for converting the lignocellulose to fermentable carbon compounds requires delignification to liberate cellulose and hemicellulose from their complex with lignin, and depolymerization of the carbohydrate polymers to produce free sugars or short sugar chains (Lee, 1997). These compounds can then be fermented to organic acids by microbial consortia occurring in either aerobic or anaerobic environments, which in turn will provide a carbon source for sulphate reducing bacteria. Water-soluble carbohydrates and phenolics are released from grass lignocellulose during hot water treatment (Milstein *et al.*, 1981; Vered *et al.*, 1981). Such a leachate can contain a mixture of growth promoting substances, antimicrobial compounds and fermentable substrates.

If the purpose of biological delignification is to obtain a cellulose enriched substrate that can be further fermented under anaerobic conditions to low molecular weight organic acids, then the mechanism of delignification should be very specific. White rot fungi are the only microorganisms capable of degrading lignin completely (Reid, 1995). Different species of these fungi exhibit different degradative specificities towards cellulose, hemicellulose and lignin (Kuhad *et al.*, 1997). Many species with accurate lignin degradation capabilities have been described in literature (Lee, 1997; Reid, 1989). An additional requirement should be that the polysaccharide component of lignocellulose must be left untouched. *Pleurotus ostreatus* is one fungus that meets these requirements (Camarero *et al.*, 1998; Hadar *et al.*, 1992; Platt *et al.*, 1984). Therefore, white rot fungi can potentially be used for the biological pretreatment of lignocellulose biomass to make the cellulose and hemicellulose components more accessible for further use in a passive AMD treatment.

Biological pretreatment of complex carbon sources must be accomplished on site in order to meet the requirements for a passive AMD treatment system. This implies that the substrate cannot be sterilized and that the pretreatment agents should be able to compete effectively with the natural microflora present on the substrate. Survival of *P. ostreatus* in soil has been demonstrated, but not for *Phanerochaete chrysosporium* (Lang *et al.*, 1997; Radtke *et al.*, 1994). This implies that potential pretreatment agents should be screened for their ability to survive on the natural substrate without inhibition of its delignification capabilities. *Schizophyllum commune* is frequently observed on decaying trees and mechanisms for ensuring efficient colonization and degradation must exist within this fungus. *Phanerochaete chrysosporium* is the model organism used for studying lignocellulose biodegradation. Ease of handling and rapid growth makes it a favourite choice for lignocellulose biodegradation studies.

The objective of this study was to investigate the biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) with *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Schizophyllum commune*. The fungal community structure of the decaying grass was also studied.

2. MATERIALS AND METHODS

2.1 Fungal strains

Pleurotus ostreatus was obtained from the Department of Botany basidiomycete culture collection, University of Pretoria. *Phanerochaete chrysosporium* strain WR 230 was obtained from the Council for Scientific and Industrial Research (CSIR) fungi culture collection. *Schizophyllum commune* was isolated from decayed plant material found in a suburban garden. All strains were maintained on Malt Extract Agar (MEA) at 25°C.

2.2 Substrate preparation

Buffelsgrass (*Cenchrus ciliaris* cv. Molopo) was obtained from a farm near Pienaarsrivier, 60 km north of Pretoria. A hammer mill was used to prepare two particle sizes: fine cut (1-2 cm pieces) and medium cut (5-10 cm pieces). Uncut grass (longer than 15 cm) was used as the third particle size.

2.3 Preparation of fungal inocula and experimental setup

2.3.1 Preparation of pre-inoculum

Agar squares (6 mm x 6 mm) from 10 d old *Pleurotus ostreatus* and *Schizophyllum commune* pure cultures were inoculated aseptically into Malt Extract (ME) broth (supplemented with 250 mg/l chloramphenicol) and incubated on a rotary shaker (130 rpm) at 28°C. Growth was detected visually after 24 to 72 h. The broths were aseptically homogenised for 20 s with a high-speed homogeniser (Ultra-Turrax supplied by Janke & Kunkel Ika-werk).

50 g grass was placed in a 2 l Erlenmeyer flask, moistened with 150 ml of distilled water (dH₂O) and autoclaved twice at 121°C for 30 min. The autoclaved grass was inoculated separately with 5 ml of the liquid inoculum prepared for *P. ostreatus* and *S. commune* and 20 or more agar squares (6 mm x 6 mm) from 10 d old *P.*

chryso sporium pure cultures. The inoculated grass was incubated at room temperature for 7 d or until sufficient fungal colonization was visible.

2.3.2 Preparation of large-scale inoculum

Medium cut grass was soaked in tap water for 18 h and steamed for 4 h. 288 g steamed grass was aseptically inoculated with 12 g of the fungal pre-inocula and packaged into plastic tubing reactors (20 cm x 50 cm x 50 µm) (Figure 3.1). Ventilation holes were made 5 cm apart. The experimental reactors were incubated at 20°C – 25°C until sufficient fungal growth was visible.

2.4 Substrate inoculation

280 g natural grass were moistened with 500 ml of tap water and inoculated with 20 g (wet weight; 5 g dry mass) of inoculum. 740 g steamed grass was inoculated with 60 g (wet weight) of the inoculum. The inoculated substrate was placed in plastic tubing reactors (20 cm x 50 cm x 50 i m) sealed and ventilation holes were made 20 cm apart. Completed reactors were incubated at 20°C-25°C. Reactors were constructed in triplicate for all substrate treatments and particle sizes. Uninoculated controls were run in parallel.



Figure 3.1: Completed plastic tubing reactor constructed for the biological pretreatment of Buffelsgrass.

2.5 Sampling

Series containing triplicate reactor sets were harvested on a weekly basis for six weeks (Figure 3.2). One reactor per set was stored at 4°C in case additional analyses were required. The entire contents of the second reactor was dried and a composite sample milled (hereinafter referred to as “Dry sample”). The remaining reactor was used for wet chemistry analyses (hereinafter referred to as “Chem sample”). Composite samples from this reactor were used for drying, microbiological analysis, pH and digestibility experiments.

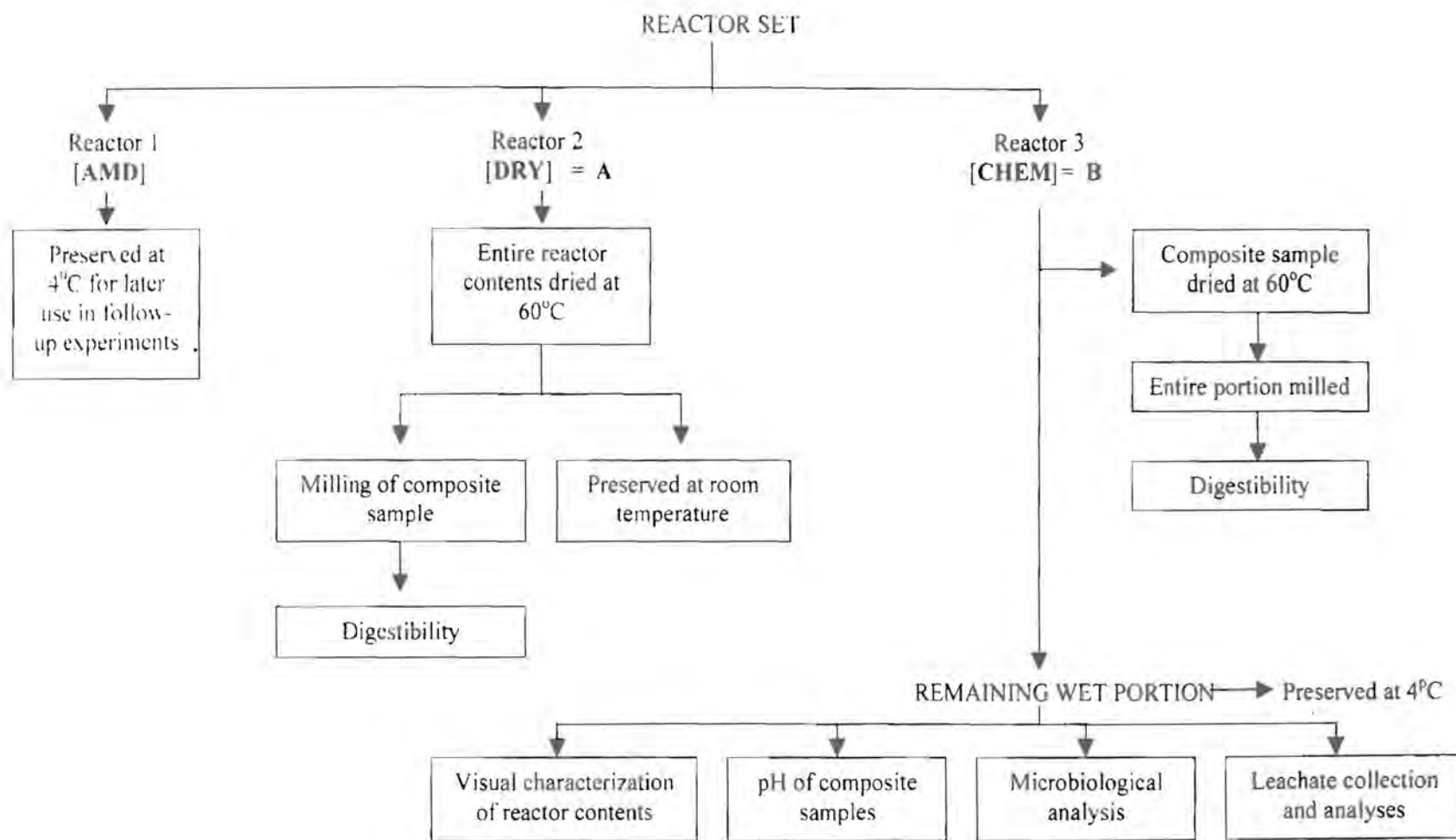


Figure 3.2: Allocation of analyses to individual reactors per reactor set.

2.6 Physical and chemical analyses

2.6.1 Dry mass loss

Dry mass loss was estimated after drying at 60°C for 48 h and expressed as the percentage of the starting material dry mass. Composite samples were milled in a Willey mill to pass a 1 mm sieve.

2.6.2 pH

5 g of wet composite sample was mixed with 200 ml of distilled water and kept overnight at 4 °C. The pH for duplicate (fine and medium grass) or triplicate (uncut grass) samples was determined the following day using a Beckman ϕ 34 pH meter.

2.6.3 *In vitro* dry matter digestibility of experimental reactor contents

A modified two-stage Tilley & Terry (1963) procedure was used for measuring the *in vitro* dry matter digestibility (IVDMD) of the dried samples. Dry matter (DM) determinations were made on separate samples at 100°C. The percentage dry matter (% DM) was used to correct the initial sample mass to dry matter.

Phase 1

Fresh artificial saliva solution was prepared using NaHCO₃ (9.80 g/l), KCl (0.57 g/l), NaCl (0.47 g/l), MgSO₄·7H₂O (0.12 g/l), anhydrous CaCl₂ (0.04 g/l), and anhydrous Na₂HPO₄ (3.71 g/l). The solution was thoroughly saturated with CO₂ at 39°C. Duplicate subsamples (0.2 g) from the Dry and Chem samples were weighed into glass centrifuge tubes. Alfalfa of known *in vivo* digestibility (75-77%) was included as a positive control. Rumen contents were obtained from a fistulated sheep maintained on lucerne. Rumen liquor was filtered through 4-layer cheesecloth and mixed with the artificial saliva in a 1:3 ratio. This mixture was continuously gassed with CO₂. To each tube 2 ml of 8.68 g/l urea solution and 20 ml of the artificial saliva/rumen solution were added. The space above the liquid in each tube was thoroughly flushed with CO₂. "Blank" tubes contained no sample and were filled with the urea and

artificial saliva/rumen mixtures only. The tubes were sealed with a rubber stopper fitted with a Bunsen gas release valve. The tubes were then incubated at 39°C for 48 h in a shaking water bath.

Phase 2

A fresh solution of 4 g pepsin in 1 l of 0.1 M HCl was prepared in a 1 l glass beaker. The rubber stoppers were removed and gently rinsed with water to remove solid particles adhered to the sides of the stopper and the tube. The tubes were centrifuged at 2500 rpm for 15 min. The supernatant was carefully decanted and discarded. 20 ml of the pepsin/HCl-solution was added to each tube. The tubes were sealed with the rubber stoppers and incubated at 39°C for 48 h in shaking water bath. The tubes were then centrifuged at 2500 rpm for 15 min. The supernatant was gently decanted and 20 ml warm tap water was added. The tubes were again centrifuged at 2500 rpm for 15 min. The supernatant was gently decanted and the tubes were dried at 100°C for 18 h, cooled in a dessicator and weighed. The IVDMD of the residue was calculated as follows:

$$\begin{aligned} \text{IVDMD} &= 100 - \text{Undigested material} \\ &= 100 - \frac{[(\text{Final sample} - \text{Blank}) - \text{Tube mass}]}{\text{Initial sample} \times \% \text{ DM}} \end{aligned}$$

Digestibility data was corrected for dry mass loss as follows:

$$\text{Digestibility (corrected)} = \frac{\text{Digestible mass} + \text{Dry mass lost}}{\text{Starting Dry mass}}$$

2.7 Microbiological analysis

1 g sample of plant material was taken from sites in the reactor representing visually dominant fungi, diluted in 9 ml of distilled water and vortexed for 15 s. Dilution series were prepared and plated on Potato Dextrose Agar (PDA) (Biolab) plates. All steamed grass samples were plated on normal PDA plates (pH ± 5.6) while the remaining samples were plated on modified PDA plates (pH adjusted to 9.3 prior to autoclaving)

to simulate the pH of the decaying plant material. Chloramphenicol (250 mg/l) was added to the microbiological media. Fungal and yeast colonies were selected and isolated for identification. Water agar (12 g agar/l distilled water) was used for identification of *Fusarium* species. Light microscopy was used to identify the fungal species and scanning electron microscopy (SEM) was used to study substrate decomposition.

3. RESULTS

3.1 Physical and chemical analyses

- **Fine natural grass**

Dry mass

The dry mass in the control experiment decreased from 100% to 71.3% over the experimental period. This decrease occurred gradually at a consistent rate over the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *S. commune* decreased from 100% to 80.3% (19.7%) over the experimental period. Dry mass loss occurred rapidly during the first week (13.0%) and then slowly for the rest of the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *P. ostreatus* decreased from 100% to 76.9% (23.1%) over the experimental period. Dry mass loss occurred rapidly during the first week (14.6%) and remained constant for the rest of the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *P. chrysosporium* decreased from 100% to 74.7% (25.3%) over the experimental period. Dry mass loss occurred rapidly within the first week (17.0%) and remained constant for the rest of the experimental period (Figure 3.3).

Digestibility

The digestibility of the control experiment grass decreased gradually at a consistent rate from 59.166% to 34.016% (25.150%) over the experimental period (Figure 3.3).

The adjusted digestibility decreased gradually from 59.2% to 45.4% over 5 weeks after which it again increased to 52.9%. The digestibility of the grass inoculated with *S. commune* decreased from 59.166% to 27.025% (32.141%) over the experimental period (Figure 3.3). A more rapid decrease was observed for the first 2 weeks (by 30.522%) and then remained constant for the rest of the experimental period. Adjusted digestibility followed the same trend, stabilizing after 2 weeks at 41.4%. The digestibility of the grass inoculated with *P. ostreatus* decreased from 59.166% to 22.068% (37.098%) over the experimental period (Figure 3.3). A more rapid digestibility decrease was observed within the first week (by 24.523%) and decreased slowly for the rest of the experimental period. The adjusted digestibility decreased rapidly within 1 week and then remained constant for the rest of the experimental period. The digestibility of the grass inoculated with *P. chrysosporium* decreased from 59.166% to 26.031% (33.135%) over the experimental period (Figure 3.3). A more rapid decrease in digestibility was observed within the first week (21.339%) and then remained constant for the rest of the experimental period.

pH

The pH in the control experiment increased from 6.00 to 10.06 over the 6 week experimental period. A rapid increase to pH 9.95 was observed within the first week. The pH of the grass inoculated with *S. commune* increased from 6.00 to 9.95 over the 6 week experimental period. A rapid increase to pH 9.88 was observed within the first week. The pH of the grass inoculated with *P. ostreatus* increased from 6.00 to 10.09 over the 6 week experimental period. The pH increased within the first 2 weeks to 8.02. The pH of the grass inoculated with *P. chrysosporium* increased from 6.00 to 10.05 over the 6 week experimental period. A rapid increase to pH 9.81 was observed within the first week.

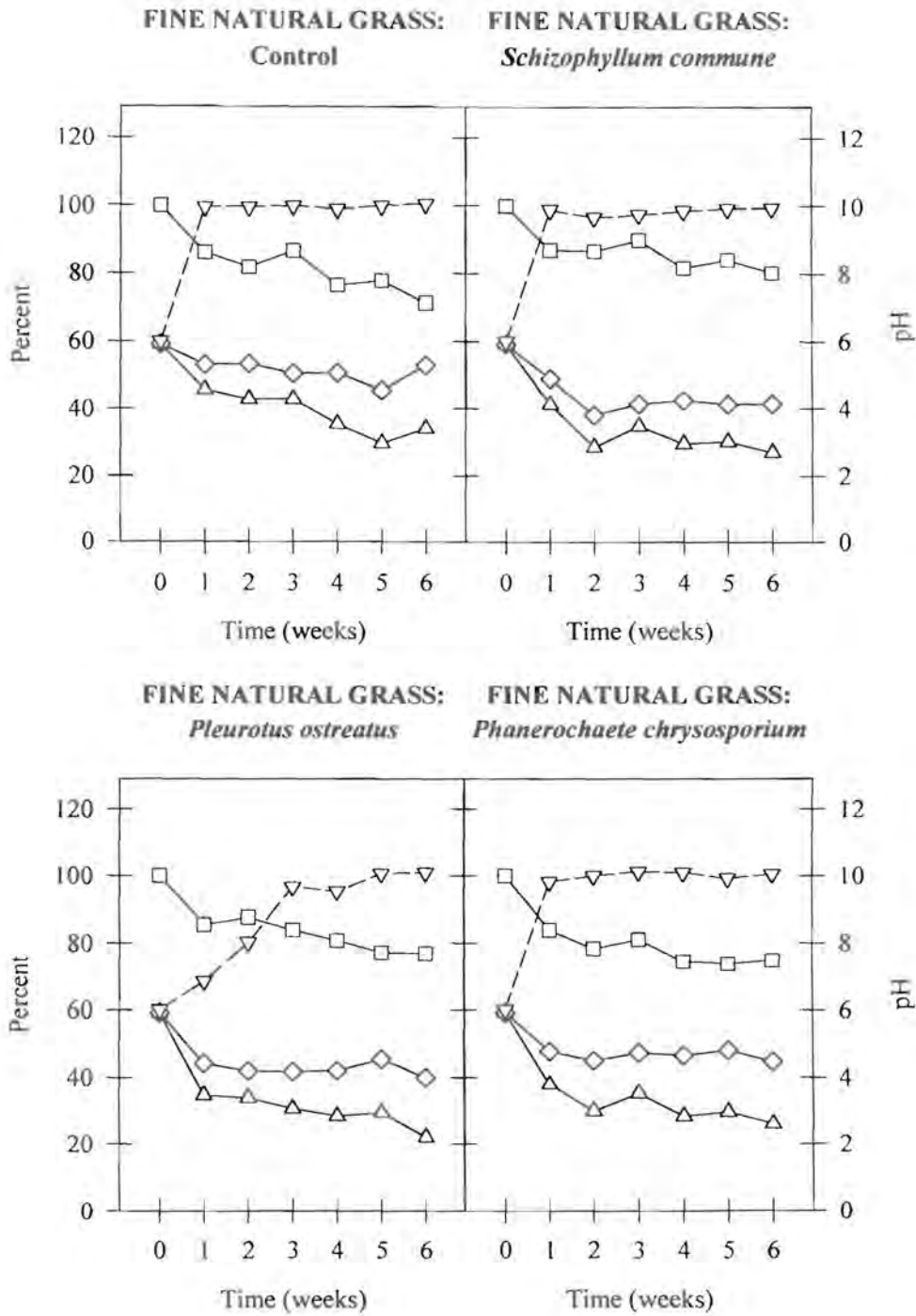


Figure 3.3: Influence of different treatments on solid-state fermentation of fine natural grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽).

- **Medium natural grass**

Dry mass

Dry mass in the control experiment decreased gradually from 100% to 71.7% (28.3%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *S. commune* decreased gradually from 100% to 69.3% (30.7%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *P. ostreatus* decreased gradually from 100% to 68.1% (31.9%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *P. chrysosporium* decreased gradually from 100% to 68.5% (31.5%) over the experimental period (Figure 3.4).

Digestibility

The digestibility of uninoculated medium-cut grass decreased gradually from 59.166% to 24.752% (34.414%) over the experimental period (Figure 3.4). The adjusted digestibility decreased very slightly to 46.1% over the experimental period. The digestibility of medium-cut grass inoculated with *S. commune* decreased from 59.166% to 16.594% (42.572%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first week (36.009%) and then remained constant. The adjusted digestibility decreased over the first three weeks but increased to 42.5% over the experimental period. The digestibility of medium-cut grass inoculated with *P. ostreatus* decreased from 59.166% to 17.383% (41.783%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first week (33.625%) and then remained constant. The adjusted digestibility also decreased within the first week but increased to 43.7% over the experimental period. The digestibility of medium-cut grass inoculated with *P. chrysosporium* decreased from 59.166% to 23.226% (35.940%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first 2 weeks (29.748%) and then remained constant. The adjusted digestibility decreased within the first 2 weeks but increased to 47.4% over the experimental period.

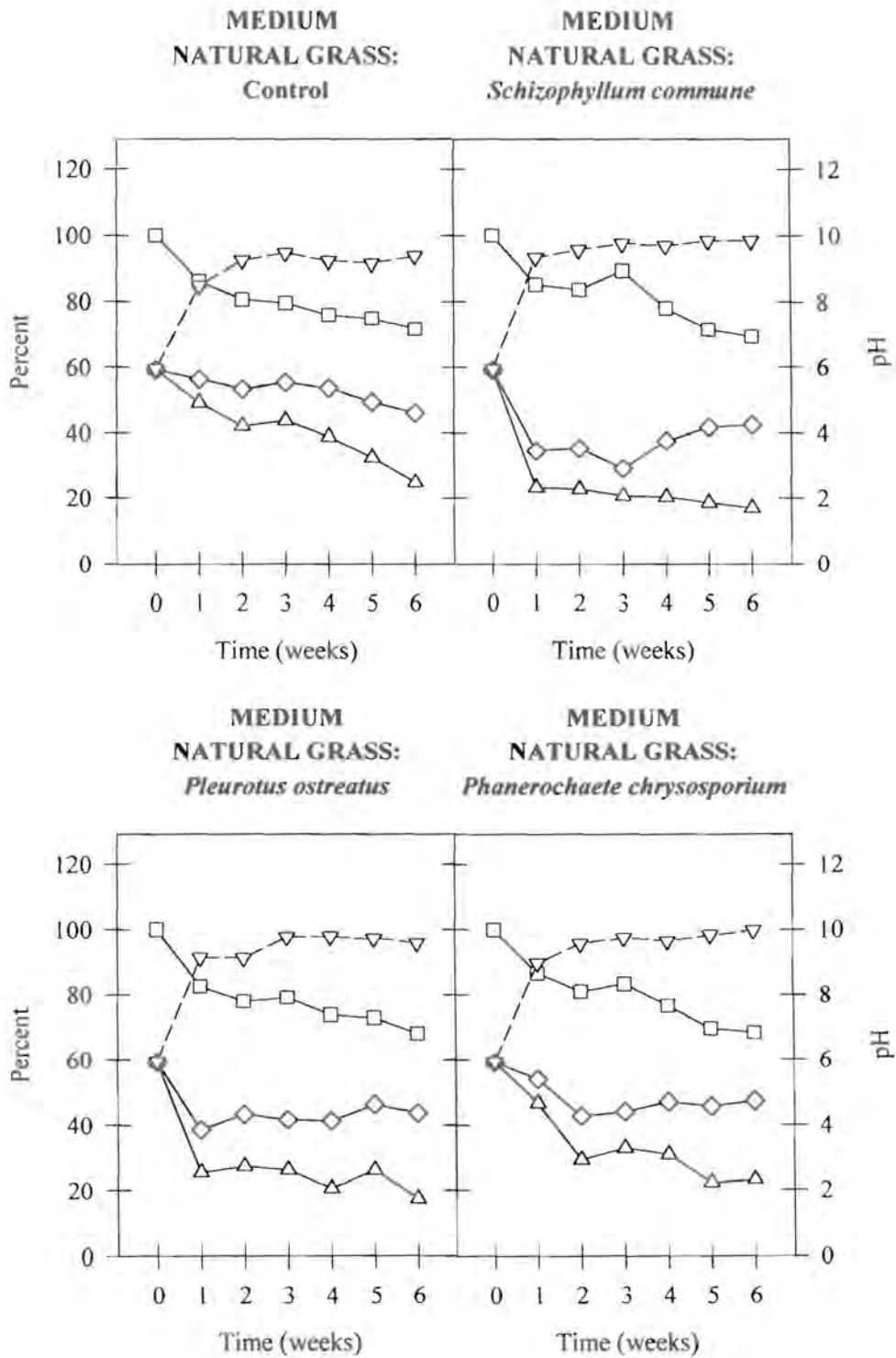


Figure 3.4: Influence of different treatments on solid-state fermentation of medium natural grass as described by dry mass loss (\square), corrected digestibility (\diamond), digestibility of the remaining residue (\triangle), and pH (∇).

pH

The pH of the uninoculated medium-cut grass increased rapidly from 5.94 to 9.37 over the experimental period. A rapid increase to pH 8.47 was observed within the first week. The pH of the medium-cut grass inoculated with *S. commune* increased from 5.94 to 9.84 over the experimental period. A rapid increase to pH 9.32 was observed within the first week. The pH of the medium-cut grass inoculated with *P. ostreatus* increased from 5.94 to 9.58 over the experimental period. A rapid increase to pH 9.13 was observed within the first week. The pH of medium-cut grass inoculated with *P. chrysosporium* increased from 5.94 to 9.98 over the experimental period. A rapid increase to pH 8.96 was observed within the first week.

- **Uncut natural grass**

Dry mass

Dry mass in the control experiment for uncut grass decreased from 100% to 97.6% (2.4%) over the experimental period (Figure 3.5). An increase in dry mass was observed after the first 2 weeks. Dry mass in uncut grass inoculated with *S. commune* remained unchanged over the six week experimental period (Figure 3.5). Periodic dry mass increases and decreases were observed. After the first 3 weeks the dry mass increased to 105.7% (5.7%) and decreased to 88.4% (11.6%) after the fifth week of the experimental period. Dry mass in uncut grass inoculated with *P. ostreatus* decreased from 100% to 97.3% (2.7%) over the experimental period (Figure 3.5). The dry mass increased after the first 2 weeks to 131.7% (31.7%) and decreased gradually to 97.3% over the experimental period. Dry mass in uncut grass inoculated with *P. chrysosporium* remained unchanged at 99.1% over the experimental period (Figure 3.5). The dry mass increased to 115.9% (15.9%) and 109.5% (9.5%) respectively after the first and fourth weeks of the experimental period.

Digestibility

The adjusted digestibility of uncut grass decreased from 64.8% to 40.7% (24.1%) over the incubation period (Figure 3.5). The residue digestibility followed the same trend. Digestibility decreased gradually over the first 4 weeks to 33.9% (30.9%) after which it again increased to 40.7% (24.1%). The adjusted digestibility of uncut grass inoculated with *S. commune* decreased from 64.8% to 38.3% (26.5%) after the experimental period (Figure 3.5). A more rapid decrease in digestibility was observed within the first 2 weeks and then remained constant. The residue digestibility followed the same trend. The adjusted digestibility of uncut grass inoculated with *P. ostreatus* decreased from 64.8% to 34.7% (30.1%) over the experimental period (Figure 3.5). A more rapid decrease in digestibility to 19.5% (45.3%) was observed within the first 2 weeks after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of uncut grass inoculated with *P. chrysosporium* decreased from 64.8% to 26.3% (38.5%) over the experimental period (Figure 3.5). The digestibility initially decreased to 34.9% (29.9%) within the first 2 weeks after which it again increased to 55.8%. The digestibility again decreased for the remainder of the experimental period.

pH

The pH of the uncut grass control experiment increased from 6.04 to 10.03 over the experimental period (Figure 3.5). The pH increased within the first 2 weeks to 9.12. The pH of uncut grass inoculated with *S. commune* increased from 6.04 to 9.87 over the experimental period (Figure 3.5). The pH increased within the first week to pH 9.40. The pH of uncut grass inoculated with *P. ostreatus* increased from 6.04 to 10.08 over the experimental period (Figure 3.5). The pH increased within the first week to 9.51. The pH of uncut grass inoculated with *P. chrysosporium* increased from 6.04 to 9.89 over the experimental period (Figure 3.5). The pH increased within the first week to 9.61.

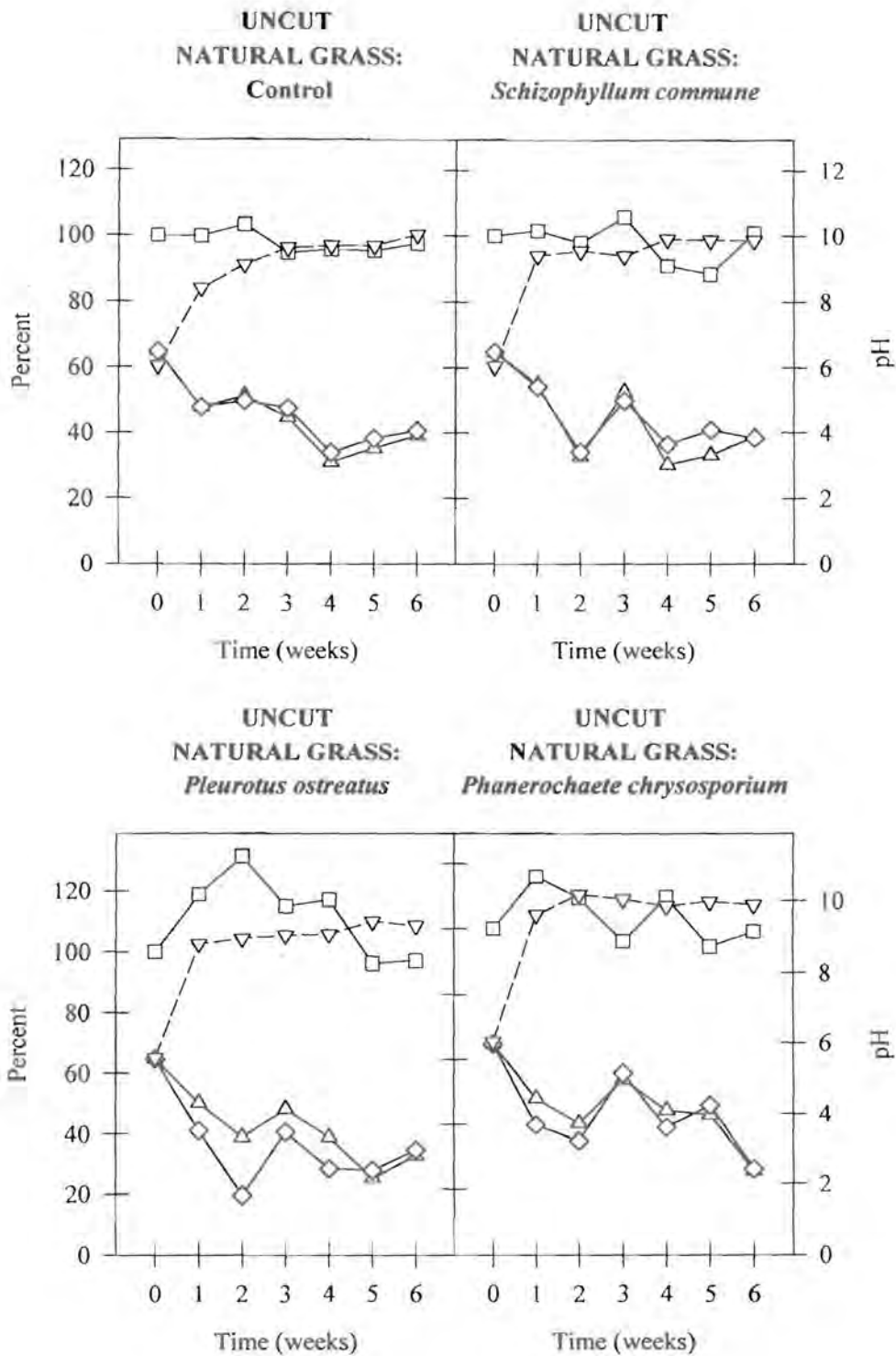


Figure 3.5: Influence of different treatments on solid-state fermentation of uncut natural grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽).

- **Fine steamed grass**

Dry mass

Dry mass in the steamed grass control experiment decreased from 100% to 54.3% (45.7%) over the experimental period (Figure 3.6). Dry mass loss was very slow during the first 5 weeks but increased rapidly during the sixth week of the experimental period. Dry mass in steamed grass inoculated with *S. commune* decreased from 100% to 97.6% (2.4%) over the experimental period (Figure 3.6). Dry mass increased 104.6% (4.6%) within the first week of the experimental period. Dry mass in steamed grass inoculated with *P. ostreatus* decreased from 100% to 87.6% (12.4%) over the experimental period (Figure 3.6). Dry mass increased after the first 2 weeks to 107.7% (7.7%) after which it again decreased. Dry mass in steamed grass inoculated with *P. chrysosporium* decreased from 100% to 71.4% (28.6%) over the experimental period (Figure 3.6). No increase in dry mass was observed.

Digestibility

The adjusted digestibility of the control experiment grass increased from 39.2% to 61.6% (22.4%) over the experimental period (Figure 3.6). The digestibility initially decreased within the first 2 weeks (11.6%) after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *S. commune* decreased from 39.2% to 36.0% (3.2%) over the experimental period (Figure 3.6). The digestibility increased to 41.7% (2.5%) within the first 2 weeks after which it again decreased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *P. ostreatus* increased from 39.2% to 46.2% (7%) over the experimental period (Figure 3.6). The digestibility decreased within the first 3 weeks to 26.8% (12.4%) after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *P. chrysosporium* increased from 39.2% to 47.8% (8.6%) over the experimental period (Figure 3.6). The adjusted digestibility decreased within the first 2 weeks to 27.7% (11.5%) after which it again increased. The residue digestibility followed the same trend up to week 4 from where it continued its downward trend.

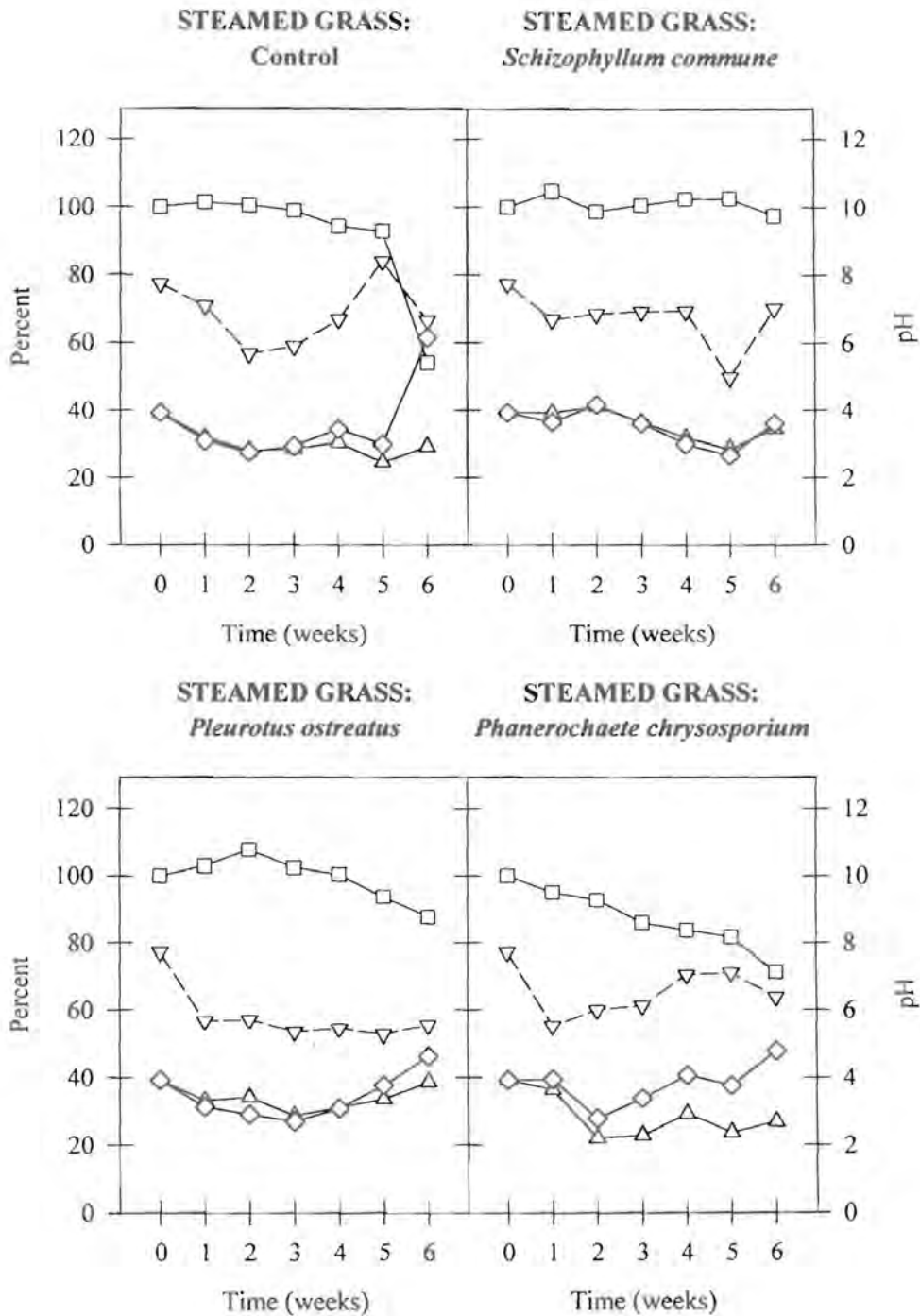


Figure 3.6: Influence of different treatments on solid-state fermentation of fine steamed grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽). Digestibility of the remaining residue was similar to the corrected digestibility, except for *P. chrysosporium*, for which a downward trend was maintained.

pH

The pH of the control experiment grass decreased to from 7.73 to 6.66 over the experimental period (Figure 3.6). The pH decreased to pH 5.68 after the first 2 weeks, after which it increased to 8.40 after the fifth week of the experimental period. The pH of steamed grass inoculated with *S. commune* decreased from 7.73 to 7.03 over the experimental period (Figure 3.6). The pH decreased to 5.00 after the fifth week of the experimental period. The pH of steamed grass inoculated with *P. ostreatus* decreased from 7.73 to 5.53 over the experimental period (Figure 3.6). The pH decreased to 5.25 within the fifth week of the experimental period. The pH of steamed grass inoculated with *P. chrysosporium* decreased from 7.73 to 6.38 over the experimental period (Figure 3.6). The pH decreased to 5.51 within the first week of the experimental period, after which it increased again.

3.2 Microbiological analysis

Complex and dynamic fungal communities exist on decaying plant material consisting mainly of common saprophytes (Figure 3.7). Dominant species in this study were selected based on their numerical superiority on agar plates and were identified using light microscopy as *Aspergillus niger*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium equiseti*, *Fusarium chlamydosporum* and *Acremonium kiliense* (Figure 3.8). *Scopulariopsis brevicaulis* was the dominant organism isolated from uncut natural grass treatments. At least four different yeast genera were isolated. *Coprinus* spp. was visible on uncut grass inoculated with *S. commune* and *P. ostreatus* after 4 weeks (Figure 3.9). Slime moulds were detected on natural fine-cut grass inoculated with *P. ostreatus*.

Succession amongst the different fungi was observed (Figure 3.7). *Aspergillus* spp., *Rhizopus/Mucor* spp. and various yeast genera dominated the early stages of fine and medium natural grass treatments. Initially, these “sugar fungi” utilized easily metabolizable sugars that was present in the decaying grass. The later stages were then dominated by *Fusarium* spp. and *Geotrichum* spp.

Key

- Fusarium* spp.
- Geotrichum* spp.
- Trichoderma* spp.
- Acremonium* spp.
- Yeast spp. (various genera)
- Penicillium* spp.
- Mucor* spp. / *Rhizopus* spp.
- Scopulariopsis brevicaulis*
- Aspergillus* spp.
- Memmoniella* spp.

WEEKS	1	2	3	4	5	6
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FINE NATURAL HAY



Figure 3.7: Schematic representation of the fungal community structure showing the dominant fungi present on decaying grass.

WEEKS	1	2	3	4	5	6
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FINE STEAMED HAY



MEDIUM NATURAL HAY



UNCUT NATURAL HAY



Figure 3.7 (continued): Schematic representation of the fungal community structure showing the dominant fungi present on decaying grass.

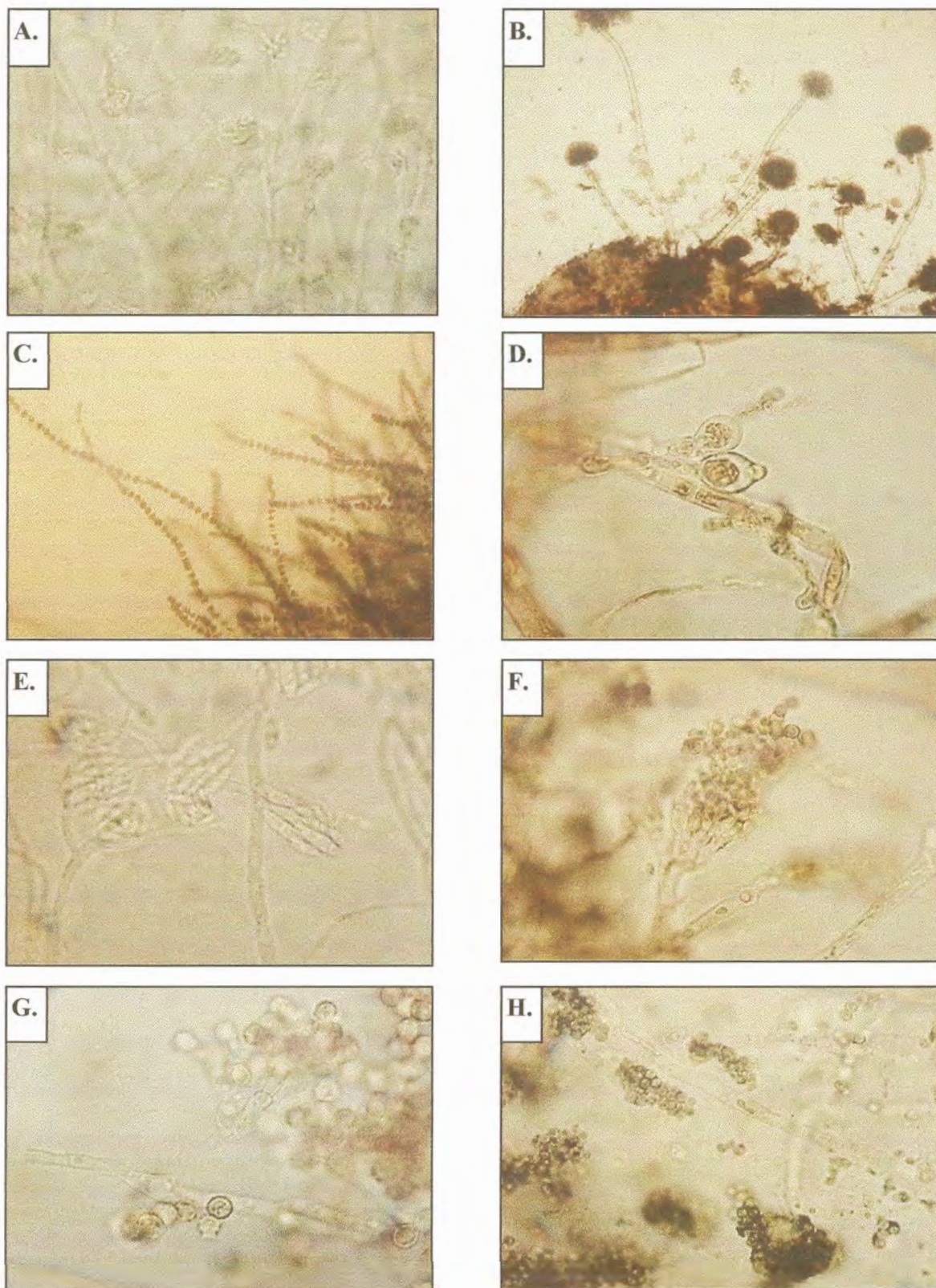


Figure 3.8: Light microscope images of some of the dominant fungi isolated from the decaying plant material. A) *Acremonium kiliense*, B) *Aspergillus niger*, C) *Candida* spp. (10X magnification of culture plate), D) *Fusarium chlamydosporum*, E) *Fusarium moniliforme*, F) *Penicillium* spp., G) *Scopulariopsis brevicaulis*, and H) *Trichoderma* spp.

These organisms are known for their ability to produce toxic compounds and that probably enabled them to survive on the decaying substrate. *S. brevicaulis* was not a dominant organism, but was present on the grass for the entire experimental period. A similar succession pattern was observed for steamed grass treatments.

A large number of genera were present on the steamed grass during the first two weeks of the experimental period. *Trichoderma* spp., *Fusarium* spp. and *Penicillium* spp. became the dominant genera on the steamed grass during the later stages of the experiment. Medium natural grass treatments maintained active populations consisting mainly of various yeast genera, *Geotrichum* spp. *Fusarium* spp. and to some extent *Aspergillus* spp. Uncut natural grass treatments were completely dominated from the onset of the experiment by *S. brevicaulis*, but this changed by week 5 and 6 when various genera were able to suppress *S. brevicaulis*.

Scanning electron microscopy revealed extensive colonization of the substrate surfaces by various fungi, yeasts and bacteria (Figure 3.10).

Within 1 week, inoculated steamed grass was completely colonised by *Pleurotus ostreatus* and *Phanerochaete chrysosporium*. After 2-3 weeks, the steamed grass inoculated with *P. chrysosporium* showed severe secondary colonisation by another fungi (*Trichoderma* spp.).



Figure 3.9: *Coprinus* spp. mushrooms on uncut natural grass inoculated with *P. ostreatus* (week 5). Broad substrate specificities and significant lignolytic enzyme activities at alkaline pH are some of the unique characteristics of this genus (Heinzkil *et al.*, 1998).

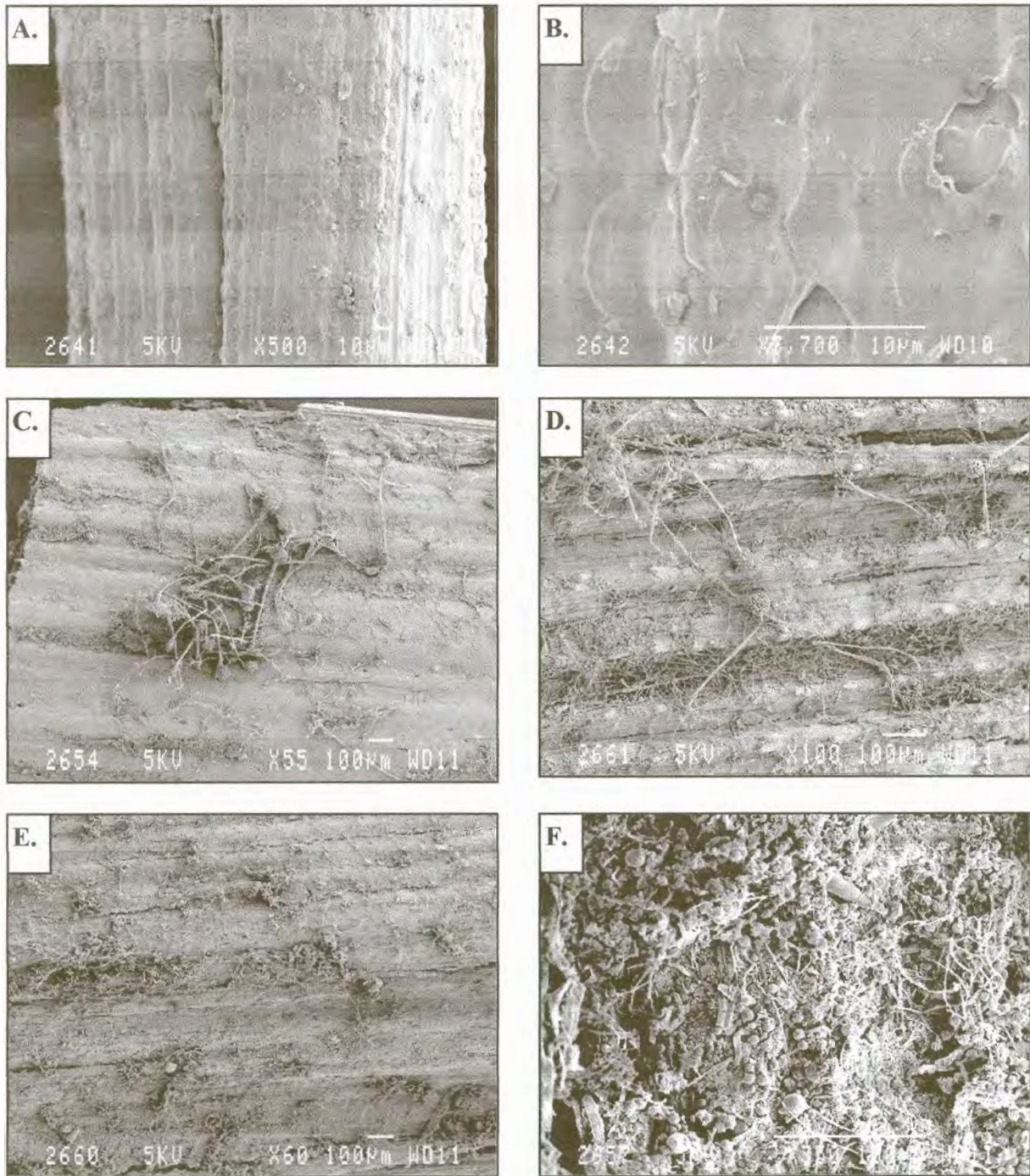


Figure 3.10: Scanning electron microscope images of control (autoclaved) and uncut natural grass treatments. The autoclaved grass seen here has an intact structure (A-B). The natural substrates were colonised to different extents. Localised (C), moderate (D-E), and severe surface colonisation (F) were noticed. Figure 3.10C represents uncut natural grass inoculated with *P. chrysosporium* after 6 weeks. The relative degradation of the substrate can be followed from week 1, control (E); week 3, *S. commune* (D) and week 5, *P. ostreatus* (F).

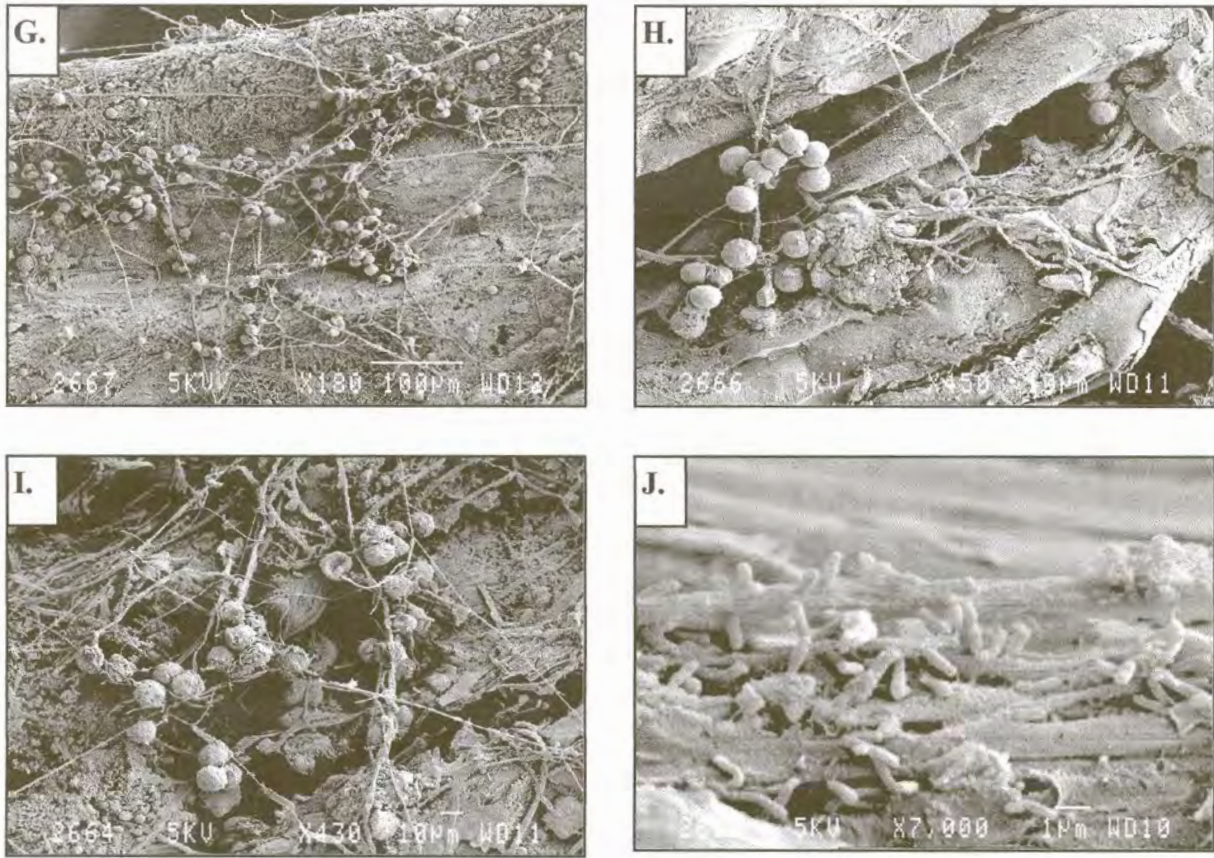


Figure 3.10 (continued): Scanning electron micrographs of uncut natural grass. Uninoculated uncut natural grass have been densely colonised by fungi, bacteria and yeasts (Figure 3.10 (G-I)). Figure 3.10J shows large numbers of bacteria attached to the substrate.

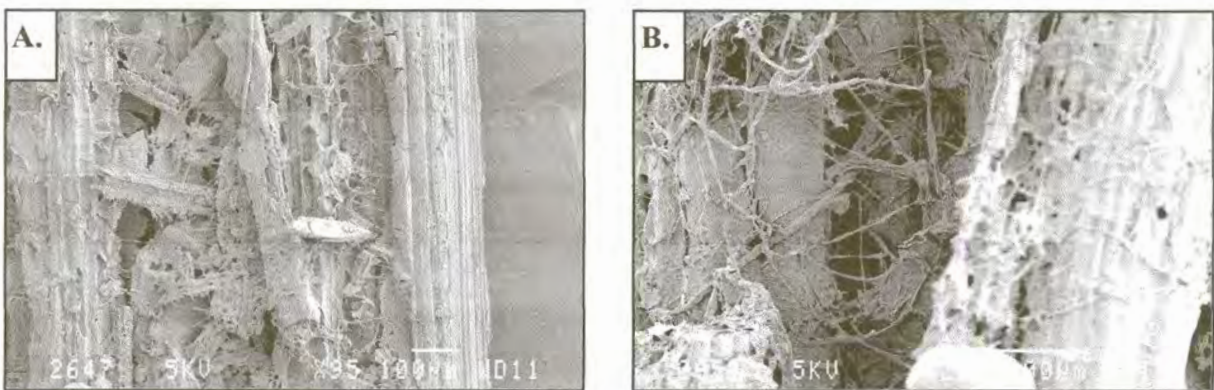


Figure 3.11: Scanning electron micrographs of fine steamed grass inoculated with *S. commune*. Damage to the fiber structure and evidence of fungal colonisation were apparent after 1 week, compared to the control (Figure 3.10A-B). Yeasts and bacteria were also observed but not to the same extent when compared to uncut natural grass treatments.

4. DISCUSSION

4.1 Influence of biological pretreatment on digestibility of Buffelsgrass

Dry mass loss for the control experiments was gradual, whereas the inoculated treatments showed a rapid dry mass loss within the first week, after which the process slowed down substantially. This was attributed to degradation of readily biodegradable compounds and the simultaneous leaching of water-soluble compounds. This was also indicated by the leachate that already formed within the first week. Further weight loss was attributed to fungal activity. This resulted in the increase of leachate volume over time. This may be exactly what is required, since degradation is taking place at a slow rate, whilst leachate is being produced.

The digestibility results confirm the conclusions drawn from the dry mass results, that readily biodegradable material was utilized initially, after which degradation slowed down as a result of a more recalcitrant substrate. This is in agreement with Valmaseda *et al.* (1991) who indicated that during the initial colonization phase of wheat straw, free sugars were available, but were rapidly metabolized by the fungi.

4.2 The effect of substrate particle size on Buffelsgrass degradation

Particle size influenced the ability of the naturally occurring microflora to degrade natural grass (Figures 3.3 - 3.6). The natural consortia, consisting of bacteria, yeasts and fungi, better degraded medium sized natural grass than fine-cut natural grass. Although dry mass increased for uncut natural grass treatments, the digestibility decreased for all treatments. This is an indication that fungal degradation occurred while biomass accumulated. The extent of degradation of uncut natural grass was not more extensive than medium-cut natural grass treatments. Also, the fungal community structure (Figure 3.7) showed that a higher degree of diversity existed on fine- and medium-cut natural grass compared to the lower degree of diversity on uncut natural grass. The spaces between the uncut natural grass particles allowed for sufficient aeration and this favoured substrate colonization by fungi. The spaces between the fine-cut particles were much smaller and were water clogged. This prevented

sufficient gas transfer, removal of volatile metabolic products and facilitated the spread of bacteria (Reid, 1989). Therefore, the fine-cut particle sizes, and to some extent the medium-cut particle size, created favourable conditions for extensive substrate colonization by fungi and bacteria. The colonization and degradation of steamed fine-cut grass by *P. ostreatus* was not impeded. This implies that the substrate particle size did not influence colonization and degradation of the natural substrate by *P. ostreatus*, but was rather influenced by competition with the microflora or the alkaline pH.

The fine- and medium-cut particle sizes facilitated the continuous degradation of the Buffelsgrass. The uncut grass was also degraded but showed dry mass increases due to biomass accumulation. Therefore, particle size influenced substrate degradation in terms of the fungal community structures but did not influence the overall extent of degradation by the inoculated fungi and the naturally occurring microflora.

4.3 The influence of pH on the Buffelsgrass digestibility

The pH of all treatments increased rapidly within one or two weeks to an alkaline pH in excess of 9.8. The origin of the alkali conditions remains unknown. Alkali treatment has been used for years to increase the digestibility of forages (Jackson, 1977). Alkali treatment produces a number of substantial changes in cell wall composition and organization i.e. the partial solubilization of hemicellulose, lignin and silica; the hydrolysis of uronic and acetic acid esters (Chesson, 1981). In grasses, a high proportion of ferulic and *p*-coumaric acids are esterified to hemicellulose and lignin (Jeffries, 1990). Ester linkages are readily cleaved by low levels of alkali that may account for the substantial solubilization of phenolic material (Chesson, 1981). Increased cellulose digestibility was attributed to the removal of phenolics and not the weakening of the cellulose structure (Chesson, 1981). Alkali resistant material showed considerable resistance to microbial action and was not released from cell walls until over 50% of the cellulose and hemicellulose was degraded (Chesson, 1981). More recent studies on this phenomenon showed that polyphenols (pyrogallol, gallic acid and 1,2,4-trihydroxybenzene) oxidize rapidly under alkaline conditions (Armstrong *et al.*, 1994), forage upgrading by NaOH and Ca(OH)₂ treatment proved viable (Haddad

et al., 1994), and that industrial alkaline pulping removed lignin from the neighboring polysaccharide moieties (Sun *et al.*, 1998).

We propose that the alkaline pH decreased the substrate residue digestibility by leaching potentially digestible compounds. The mechanisms of creating and maintaining the alkaline pH are unknown, nor whether intrinsic chemical reactions or microbial metabolic actions are responsible.

4.4 Microbiology of Buffelsgrass degradation

Microbial consortia degraded lignocellulose faster than pure cultures of white-rot fungi (Blanchette and Shaw, 1978). Significant increases in weight loss and stimulation of mycelial growth was observed in wood decay treatments combining bacteria and yeasts with white-rot fungi (Blanchette and Shaw, 1978). Our results indicated that the natural populations of microflora present of the Buffelsgrass caused continuous degradation of the substrate over time. The naturally occurring microflora increased the rate of substrate degradation in the presence of the inoculated fungi, but substrate degradation proceeded very slowly after a threshold level was reached within 1 or 2 weeks. Therefore, the naturally occurring microflora on Buffelsgrass and the inoculated fungi synergistically degraded the substrate.

The micrographs presented here (Figures 3.10 and 3.11) suggest a mutualistic association among bacteria, yeasts, and fungi during lignocellulose decay. This is in agreement with the observations by Blanchette and Shaw (1978). The fungi isolated from the decaying grass are classified as primary saprophytes that metabolize easily degradable sugars and other compounds. These fungi may not directly cause decomposition, but can synergistically increase rates of decay when they develop mutualistic relationships with, and flourishing alongside species decomposing cellulose and lignin (Dix and Webster, 1995). Microorganisms unable to degrade the ligninpolymer can obtain energy from the low molecular weight intermediates released from the degradation of lignin by true white-rot fungi. A similar situation arises where competent fungi degrade cellulose.

Trichoderma spp. is common to soil and grow very rapidly, producing a large amount of conidia and they have the ability to secrete a range of enzymes, including cellulases. They are able to withstand severe nutrient stress and are known as highly combative fungi, being able to repel attacks by other microorganisms and capture nutrient resources. *Trichoderma hirzianum* was identified as the causal agent of green mould disease that plague mushroom farms worldwide (Castle *et al.*, 1998). The colonization of steamed grass by *P. chrysosporium* was severely interrupted by *Trichoderma* spp. This is an example of a negative interaction the white rot fungus encountered on the steamed substrate surfaces. The *Trichoderma* spp. was able to displace *P. chrysosporium* from the substrate. This is also a classic example of how lignocellulose degradation by the white rot fungus was exploited by an organism unable to degrade lignin. The white rot fungus removed the lignin shield and was subsequently displaced from the substrate by the more aggressive microorganism.

Fungi are known for their ability to survive and grow at extreme pH ranges. *Coprinus* spp. comprises lignolytic enzymes characterized by broad substrate specificities and significant enzyme activities at alkaline pH (Heinzkill *et al.*, 1998). Lignin model compounds were efficiently degraded by species of this genus (Guiraud *et al.*, 1999). The microfungi isolated from the decayed grass were able to survive the alkaline conditions on the Buffelsgrass. The ability of the fungi to survive at the alkaline pH gave them an advantage over fungi that could not. The introduction of urea to forest litter induced the appearance of unusual fungi (Lehman, 1976). Urea treatment of pine needles stimulated the development of some fungi, but suppressed others (Lehman and Hudson, 1977). Lehman and Hudson (1977) described the effect of alkali on the appearance of the leaf litter: darkening, water soaking and a rise in pH. This is in agreement with our own observations of the reactor contents. The action of the urea remained unclear, but it was considered unlikely to be merely an effect of adding a nitrogenous compound. The release of ammonia was also observed in our study and the study by Lehman and Hudson (1977). The origin of the alkaline conditions remains unknown

The nutritional state of the steamed substrate, not the particle size, influenced its colonization and degradation by the white-rot fungi. *S. commune* comprises potent

cellulose degrading enzymes as well as esterases which can disrupt the lignin-hemicellulose linkages, thereby solubilizing the lignocellulose matrix into smaller lignin-carbohydrate complexes (Fang *et al.*, 1998; Haltrich *et al.*, 1995; Jeffries, 1990; Mankel and Kothe, 1999; Willick *et al.*, 1984). The nutrient deficient substrate (in terms of the lack of enzyme substrates) influenced the ability of *S. commune* to effectively colonize this substrate. *S. commune* removes lignin through solubilization and modification and does not comprise the powerful lignin oxidizing enzymes described for *P. ostreatus* and *P. chrysosporium*. The latter two organisms were able to colonize and degrade the substrate because their action of lignin degradation is based on potent lignin oxidizing enzymes.

Various interactions occurred between the fungal populations on the decaying grass. Patterns of succession were observed and mutualistic and antagonistic interactions between these populations were described. The inoculated fungi increased the degradation rate of natural grass. *P. ostreatus* was the only fungus capable of improving the starting digestibility of the steamed grass by the end of the experiment.

4.5 Leachate origin and significance

Hot water treatment of wheat and cotton straw yielded solutions containing sugars and phenolics (Milstein *et al.*, 1981; Vered *et al.*, 1981). Most of the sugars were bound to either polysaccharides or phenolics, indicating that hot water treatment released lignin-carbohydrate complexes and sugar containing polymers. The time of steam exposure affected the extent of nutrient loss. Rapid release of phenolics and carbohydrates into liquor took place within 5 h of hot water treatment and increased steadily thereafter (Vered *et al.*, 1981). Thus, although the microbiological obstacles were partially removed during the 4 h steaming of the Buffelsgrass, much of the potentially digestible nutrients were lost. The remaining fraction contained a higher proportion of recalcitrant polymers in the form of lignin-carbohydrate complexes. The low starting digestibility of the steamed grass can therefore be ascribed to the steam pasteurization of the substrate.

The production of a leachate can be viewed two ways. The negative implication of leaching is that potentially digestible compounds are released from the lignocellulose matrix and is either lost by seeping or through mineralization by the naturally occurring microflora. This leachate can also contain antimicrobial compounds that might be inhibitory when applied to a biologically driven process. However, literature indicated that microbial consortia in various environments were capable of detoxifying potentially toxic lignocellulose degradation intermediates (Cornu *et al.*, 1994; Eglund *et al.*, 1997; Wilson and Mertens, 1995). Sulphate reducing bacteria are also able to degrade lignin degradation products either in pure culture or synergistically (Boopathy, 1997; Lovley *et al.*, 1995). The advantages of leaching are that it can provide microbial consortia with easily fermentable substrates that can be fermented to low molecular weight organic acids. These fermentation products can be utilized by sulphate reducing bacteria as a carbon and nutrient source.

5. CONCLUSIONS

- In all treatments, the initial digestibility was high, rapidly decreasing after 1 to 2 weeks. This was attributed to readily biodegradable substrates being utilised during the first 2 weeks, leaving a more recalcitrant substrate which was slowly digested. None of the treatments directly increased the digestibility of the substrate. However, the leachate which was produced, could be a direct result of the breakdown of the substrate yielding potentially available carbon.
- The pH of all the un-steamed grass treatments increased rapidly to an alkaline pH in excess of 9. However, the origin of the alkaline conditions remains unknown. The pH of the steamed grass did not increase.
- The microbial community structure was very dynamic. This was attributed to the changes in the substrate composition over time and possibly interspecific competition.

- The fact that the digestibility was not increased, is not necessarily a negative factor. In AMD treatment a sustainable substrate is required, an a rapid increase in digestibility would reduce availability of substrate over an extended period of time. The leachate, as a by-product could actually be more significant, if it can be sustained. In this study, leachate was produced throughout the 6 week experimental period.

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CHAPTER 4

SULPHATE REDUCTION USING FUNGAL HYDROLYSIS BY-PRODUCTS (LEACHATE) OF *CENCHRUS CILIARIS* CV. MOLOPO (BUFFELSGRASS)

ABSTRACT

Removal of sulphate from sulphate-rich waters through biological sulphate reduction has the potential to be an effective procedure for bioremediation of acid mine drainage (AMD). A suitable carbon source that can sustain sulphate reducing bacteria (SRB) growth remains a major obstacle in using SRB in biotechnological applications. Previous studies indicated that organic carbon was possibly leached from *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by chemical and microbiological mechanisms. Using a filter paper disk assay, the leachate did not exhibit antimicrobial activity when tested against Gram negative or Gram positive organisms. The effect of lignocellulose leachates on sulphate reduction by SRB was investigated using drip bag bioreactors. An SRB-enriched inoculum was produced by incubating anaerobic digester sludge with artificial AMD. Reactors containing artificial AMD, 3% inoculum and 3% or 6% of the leachates were constructed. The chemical complexity of the substrate and its biodegradation influenced sulphate reduction. Metalsulphides contributed to fluctuations in the chemical oxygen demand (COD). Overall sulphate reduction varied from 27.7% to 44.9% and long-term sulphate reduction rates of 9.4 mg/l/d to 15 mg/l/d were observed. Sulphate removal efficiencies (in terms of mg SO₄²⁻ reduced / mg COD consumed) of 27% to 52% were obtained. No biological sulphate reduction was observed in the control reactors. Sulphate removal was comparable to literature values, but sulphate reduction rates were considerably lower in this study. Near neutral pH was maintained in all leachate amended reactors, unlike leachate-free controls. Both total anaerobic bacteria and sulphate reducing bacteria benefited from leachate addition. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.

1. INTRODUCTION

1.1 The carbon source dilemma

Removal of sulphate from sulphate-rich waters through biological sulphate reduction (BSR) has the potential to be an effective and cost-effective procedure for bioremediation of acid mine drainage (AMD). Sulphate reducing bacteria (SRB) will form the core component of such an approach. A suitable carbon source that can sustain SRB growth remains a major obstacle in using SRB in biotechnological applications (Béchar *et al.*, 1994). Various carbon sources have been evaluated for use in passive treatment of AMD under laboratory and field conditions (Table 4.1).

Defined carbon substrates do not provide a cost-effective means of stimulating sulphate reduction by sulphate reducing bacteria. Cost of acquirement, transport, handling difficulties etc. prevent these carbon substrates from being applied on-site in large-scale passive AMD treatment systems. Undefined carbon substrates typically are agricultural residues and other lignocellulose wastes. These carbon substrates are cheap sources of fermentable organic carbon. However, in its natural form, lignocellulose is a highly recalcitrant heteropolymer. The intricate associations between the dominant polymers (cellulose, hemicellulose and lignin) creates a physical and chemical barrier that prevents the rapid and complete degradation of the organic carbon. Therefore, efforts to improve lignocellulose digestibility aim at removing the lignin barrier, thereby releasing carbohydrate polymers for hydrolysis and fermentation.

Table 4.1: Defined and undefined carbon sources evaluated for use in AMD treatment systems.

Carbon source	References
Wood dust	Tuttle <i>et al.</i> , 1969a; Tuttle <i>et al.</i> , 1969b
Wood dust and various defined organic nutrients	Wakao <i>et al.</i> , 1979
Digester slurry of cattle waste	Ueki <i>et al.</i> , 1988
Spent mushroom compost	Dvorak <i>et al.</i> , 1992
Alfalfa hay, timothy hay, and cereal straw	Bécharde <i>et al.</i> , 1994
Whey and cow manure	Christensen <i>et al.</i> , 1996
Rice stalks and cow manure	Cheong <i>et al.</i> , 1998
Lactate	Elliott <i>et al.</i> , 1998
Cow manure, sawdust, and cheese whey	Drury <i>et al.</i> , 1999
Methanol, lactate, and horse manure	Tsukamoto and Miller, 1999
Various undefined carbon sources	Chang <i>et al.</i> , 2000

1.2 Lignocellulose leachate: characteristics and significance

Leaching of nutrients from crop residues left on the field after harvest is a natural phenomenon (Schreiber, 1999). Previous studies indicated that organic carbon was possibly leached from Buffelsgrass by chemical and microbiological mechanisms (Chapter 3)

Our knowledge on the leachate can be summarised as follows:

- *The chemical composition of the leachate is complex.* Hot water extracts of wheat straw contained phenolic compounds and carbohydrates (Galletti *et al.*, 1990; Milstein *et al.*, 1981; Vered *et al.*, 1981). Larger and more complex lignocellulose degradation products like lignin-carbohydrate complexes (LCC) might also be present (Cornu *et al.*, 1994; Jeffries, 1990). Other compounds including proteins, nucleic acids and lipids or

Jeffries, 1990). Other compounds including proteins, nucleic acids and lipids or degradation intermediates of these compounds might be present in the leachate. Therefore, a wide range of compounds might be present in the leachate but their effect on sulphate reduction by sulphate reducing bacteria remains uncertain.

- *Leachate might stimulate bacterial growth due to the presence of nutrients.* Water extract from wheat straw residues had no inhibitory effects on the tested microbes (Abdel-Nasser *et al.*, 1983). High bacterial cell yields were obtained when *Arthrobacter* spp. was grown on wood leachate produced from pentachlorophenol (PCP) treated wood poles (Mollah and Allen, 1999). In this leachate PCP contributed to about 5% of the total COD. Bacterial growth on this leachate was attributed to the presence of other carbon sources in the leachate. Therefore, leachate might be advantageous by stimulating sulphate reduction by sulphate reducing bacteria.
- *Microbial consortia might neutralise toxicity of the leachate.* Extractions of wheat straw with hexane, ethyl ether and ethanol indicated the presence of antibacterial substances (Abdel-Nasser *et al.*, 1983). Based on *Daphnia magna* toxicity tests, the biodegradation process detoxified the wood leachate produced from pentachlorophenol (PCP) treated wood poles (Mollah and Allen, 1999). Biodegradation resulted in an 18-fold reduction in toxicity. No phytotoxicity of field leachates (leachates that originated from decomposing agricultural material left on the ground after harvest) was observed (Breakwell and Turco, 1989). Chang *et al.* (2000) found that compounds inhibitory to sulphidogenesis contained in raw agricultural biomass was degraded under anaerobic conditions. SRB can utilise an impressive number of carbon compounds, and their diverse metabolic capabilities enhances their survival in natural and man-made environments (Hansen, 1994). Therefore, SRB can overcome any inhibitory effects contained in the leachate by cooperative degradation within an anaerobic environment. The question of whether SRB can benefit from lignocellulose leachate remains unanswered.

Akhtar *et al.* (1997) reduced the amount of fungal inoculum required for biopulping by adding low cost nutrients in the form of corn steep liquor (CSL). These nutrients stimulated

fungal growth and colonisation of wood chips. CSL is produced during the wet milling process when dry corn is soaked in a warm dilute acid solution. CSL and lignocellulose leachate are similar in the following aspects:

1. Both originate from natural and/or aided breakdown of lignocellulose.
2. Both are by-products from a primary process – leaching occurs during natural degradation of plant material and CSL is produced during wet corn milling.
3. Both offer cheap sources of nutrients

Therefore, lignocellulose leachate can be a source of readily available organic carbon and other growth factors.

1.3 Objectives

The effect of the leachate on aerobic bacteria was investigated, but the effect of the leachate on sulphate reduction by sulphate reducing bacteria remained a primary concern. This project was divided into three phases (Figure 4.1). The first phase comprised the production of the inoculum to be used in the follow-up phases. The objective of the first phase was to ensure that the SRB depleted the residual carbon contained within the digester sludge. The content of this drip bag was used as the inoculum to evaluate the effect of the leachate as the sole carbon source on sulphate reduction by the SRB. The second phase consisted of a trial run using only one leachate in a drip bag experiment. The objective of the second phase was to ensure that the experimental setup would yield the expected results. The third phase started once the trial run was successfully completed. The objective of the third phase was to evaluate all the available leachates in a similar experimental setup as described for phase two.

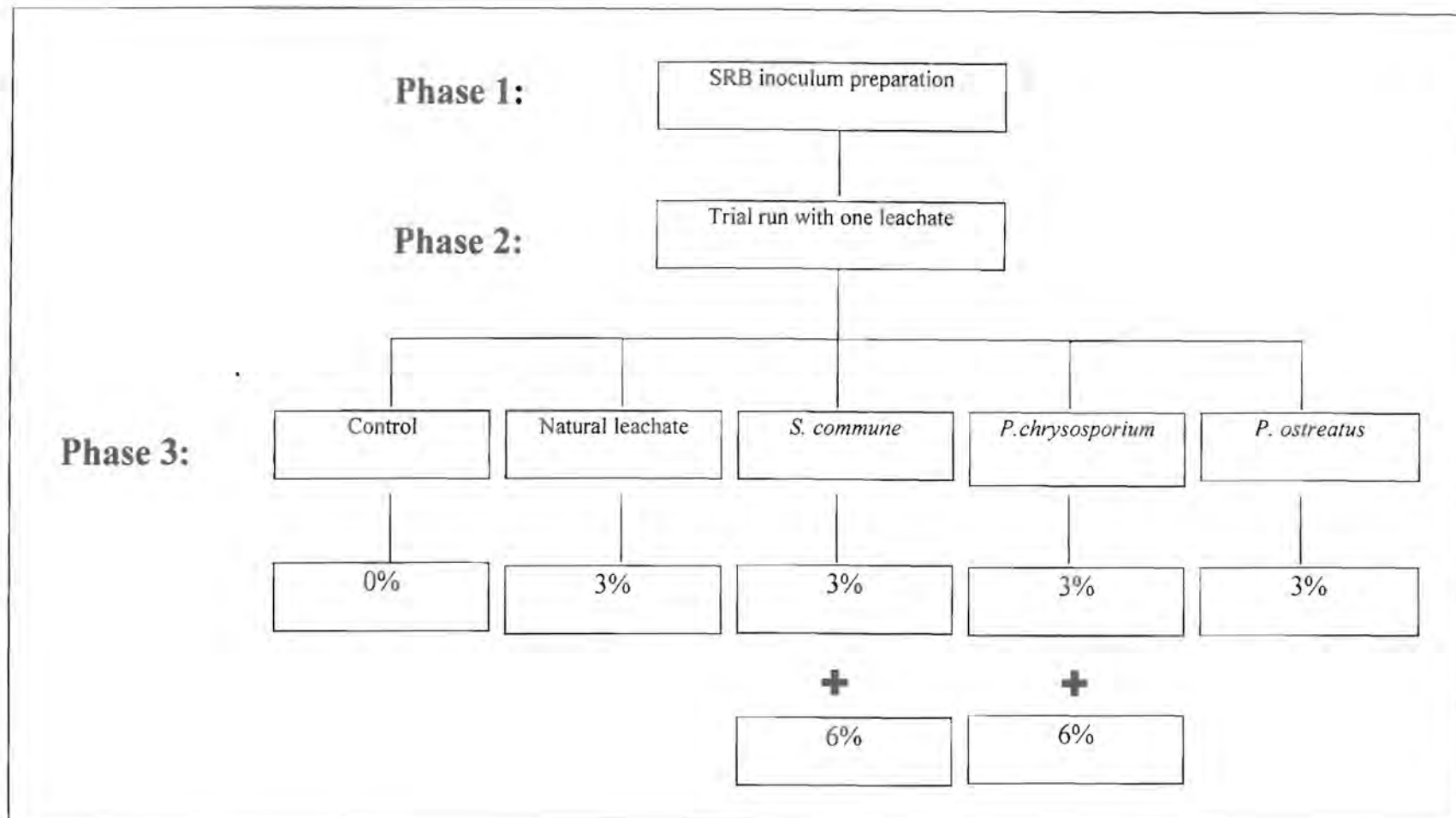


Figure 4.1: Summary of the experimental layout for evaluating sulphate reduction using the leachate as carbon source.

2. MATERIALS AND METHODS

2.1 Leachate antimicrobial activity assay

The antimicrobial activity of the leachates from fine natural grass treatments (weeks 1, 3, and 5) was tested against 2 Gram positive bacteria species (*Bacillus subtilis*, *Staphylococcus aureus*) and 2 Gram negative bacteria species (*Pseudomonas putida*, *Pseudomonas alcaligenes*). A number of pretreatment methods were evaluated to determine the best leachate sterilisation procedure (Table 4.2). Raw leachate (no pretreatment) was also evaluated. Sterile water containing chloramphenicol (250 mg/l) was included as a control.

Table 4.2: Pretreatment methods evaluated to determine the best leachate sterilization procedure.

- Ultracentrifugation for 10 min at 2 500 rpm, 5 000 rpm and 10 000 rpm
- Filter sterilisation through 0.2 μm porosity membranes
- Autoclaving

0.1 ml of an actively growing bacterial culture was inoculated into an empty petri dish. Nutrient Agar at 45°C was poured into the petri dish, rotated to ensure good mixing and distribution of bacterial cells, and allowed to cool and set. Pretreated leachate was pipetted onto sterilized filter paper disks (10 mm diameter) until the disks were saturated. Leachate impregnated filter paper disks were transferred aseptically onto the agar plates. Two disks were placed on each plate. The plates were incubated at 37°C and monitored after 24, 48 and 72 h for the formation of inhibition zones.

2.2 Preparation of inoculum

Anaerobic digester sludge, obtained from Daspoort Water Treatment Facility in Pretoria, was used as inoculum. 600 ml of digester sludge was incubated with 1400 ml of artificial acid mine drainage (AMD) without the addition of leachate (Table 4.3).

Table 4.3: Chemical composition of artificial acid mine drainage.

Component	Final concentration (mg/l) or volume added per liter (ml/l)
H ₃ PO ₄ (85%)	0.02 ml/l
MgSO ₄ .7H ₂ O	1310 mg/l
H ₂ SO ₄	519 mg/l
FeSO ₄ .7H ₂ O	4557 mg/l
NH ₄ Cl	190 mg/l
The final pH was adjusted to 7.0 using 1M NaOH.	

The reactor was filled with the AMD/digester mixture and sealed. Excess gas inside the reactor was removed with a sterile syringe and needle. The inoculum reactor was incubated at room temperature (17°C - 23°C) for 21 d. After 21 d, the incubation temperature was increased to 30°C. The reactor was shaken every day and excess gas was removed daily with a sterile syringe and needle. Samples were taken aseptically (using a sterile syringe and needle) from the reactor once a week until the desired SRB / total anaerobic bacteria ratio was obtained.

Following sulphate depletion, 1310 mg/l MgSO₄.7H₂O and 4557 mg/l FeSO₄.7H₂O were added to the inoculum reactor after 63 d. Samples were taken once a week and analysed for sulphate (Spectroquant SQ 118, Merck), total anaerobic bacteria, SRB and pH. COD was not determined.

The vitality of the inoculum was tested using 2 reactors containing 1 l artificial AMD and 30 ml (3%) of inoculum reactor content. 30 ml (3%) of a 3 month old Buffelsgrass leachate was added to one of the reactors. Samples were collected once a week and sulphate (Spectroquant SQ 118, Merck), pH, total anaerobic bacteria and SRB were determined.

2.3 Leachate origin and application strategy

A dark brown leachate was collected from plastic-tubing reactors containing moist Buffelsgrass incubated for 6 weeks with natural or added fungi (Chapter 3). The leachates from fine and medium cut natural grass (unsteamed) were pooled for each treatment. The volume of available leachate dictated the amount of replicates that could be included in this experiment and the concentrations at which the leachates could be applied (Table 4.4).

Table 4.4: Leachate application strategy.

Treatment	Concentration	No. of replicates
1. Control	None	2
2. Natural leachate	3%	1
3. <i>P. ostreatus</i>	3%	2
4. <i>S. commune</i>	3%	2
5. <i>P. chrysosporium</i>	3%	2
6. <i>S. commune</i>	6%	1
7. <i>P. chrysosporium</i>	6%	1

Therefore, 4 leachate pools were evaluated: leachate from uninoculated unsteamed Buffelsgrass (1), and leachates from unsteamed Buffelsgrass inoculated with *Schizophyllum commune* (2), *Pleurotus ostreatus* (3) or *Phanerochaete chrysosporium* (4).

2.4 Leachate amended experiments

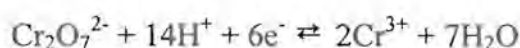
A total of eleven 2 l reactors were constructed for the final phase of the study (Figure 4.1; Table 4.4). All reactors contained 1800 ml of artificial acid mine drainage (AMD) (Table 4.3) and 60 ml (3%) of the inoculum. The appropriate volume of leachate was added to yield a final concentration of either 3% or 6% (Table 4.4). Reactors were sealed, excess gas removed and incubated at 30°C. The contents were mixed daily and excess gas was removed aseptically using sterile syringes and needles.

2.5 Physical and chemical analyses

Unless otherwise specified, 100 ml sample was removed from the reactors once weekly and sent to Waterlab Research (Pty) Ltd for chemical oxygen demand (COD) (dichromate method; APHA-AWWA-WPCF, 1995) and sulphate determinations (turbidimetric method; APHA-AWWA-WPCF, 1995). The ratio of H₂S and HS⁻ in the samples depended on the pH and were calculated using the following equation:

$$H_2S = [1 + 1.02 \times 10^{(pH-7)}]^{-1}$$

From these fractions, the total COD was corrected by subtracting the COD equivalent of the FeS content. During the determination of the COD, the oxidation reaction using Cr₂O₇²⁻ as oxidant occurred as follows:



The remaining dichromate was determined titrimetrically and the amount of oxidation was determined by difference. Stoichiometrically, 3.5 moles O₂ is required to oxidise 1 mole HS⁻. Therefore, 3.39 g of O₂ is required to oxidise 1 g of HS⁻.

During the third phase of the experiment, 5 d biochemical oxygen demand (BOD₅) was determined for all samples after 42 d until the end of the experiment after 70 d. The pH was determined using a Ross model 8102 electrode (Orion Research).

2.6 Microbiological analyses

1 ml sample was taken from the reactor once a week and used for the microbiological analyses. The total anaerobic bacteria were enumerated on nutrient agar (Biolab) incubated under anaerobic conditions in an anaerobic jar (48 h at room temperature). Gas generating kits (Oxoid) were used to generate hydrogen and carbon dioxide in the sealed anaerobic jar. Anaerotest^R test strips (Merck) were included as indicators of anaerobiosis in the anaerobic jar. The total anaerobic plate counts (TAPC) were conducted in duplicate. SRB were enumerated in duplicate using modified Postgate's medium B (Table 4.5). SRB were enumerated after 21 d incubation at 30°C.

Table 4.5: Composition and preparation of modified Postgate's medium B for the enumeration of sulphate reducing bacteria (SRB).

Solution A		Solution B		Solution C	
	900 ml distilled H ₂ O		100 ml distilled H ₂ O		50 ml distilled H ₂ O
KH ₂ PO ₄	0.5 g	MgSO ₄ .7H ₂ O	2.0 g	Tri-sodium citrate	2.94 g
NH ₄ Cl	1.0 g	FeSO ₄ .7H ₂ O	0.5 g	Titanium-chloride	5 ml
Yeast extract	1.0 g				
Resazurin	0.01 g				
Agar	10 g				
Sodium lactate (50%)	7 ml				
Adjust pH to 7.0 with 1 M NaOH prior to the addition of the agar. Autoclave at 121°C for 20 minutes.		Filter sterilise into solution A after it has been autoclaved.		Adjust to pH 7.0 with NaHCO ₃ . Autoclave at 121°C for 20 minutes. Add 200 µl to the A+B mixture already aseptically dispensed into sterile Hungate tubes.	

3. RESULTS

3.1 Leachate antimicrobial activity

None of the different pretreatment methods (Table 4.2) influenced the outcome of the antimicrobial activity experiment. Leachate pretreated with ultrafiltration through 0.2µm porosity membranes, high-speed centrifugation (2500 rpm, 5000 rpm and 10000 rpm for 10 min), and autoclaving showed no antimicrobial activity. Instead, the opposite occurred with zones of increased bacterial density within the leachate diffusion zone. No inhibition was caused by the leachates recovered from week 1, 3 and 5 of all treatments of fine natural grass (Figure 4.2).

3.2 Vitality of inoculum

Visual observations

Gas was removed from the inoculum reactor on a daily basis to prevent the build-up of inhibitory volatile compounds. However, gas production ceased after 21 days and no black precipitate formed. It was decided to incubate the reactor at 30°C for the remainder of the experimental period.

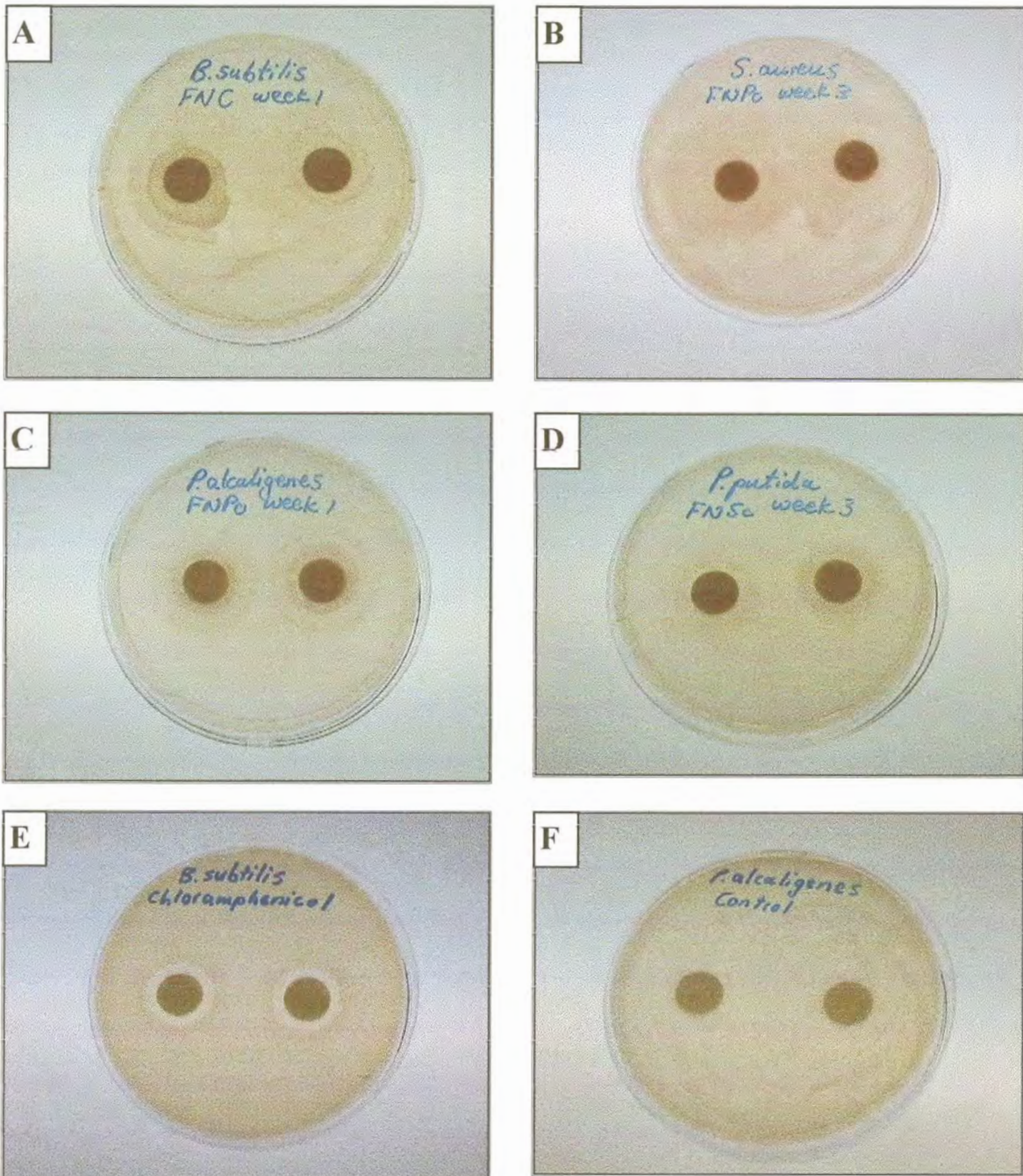


Figure 4.2: Antimicrobial activity assay for leachates derived from different weeks of fine natural grass treatments: A) Control (FNC), B) *Phanerochaete chrysosporium* (FNPc), C) *Pleurotus ostreatus* (FNPo), and D) *Schizophyllum commune* (FNSc). Heavy bacterial growth was observed within close proximity of the leachate saturated disks (A and B). Diffusion zones were clearly visible in some cases (C) and bacterial growth was visible within these zones. The controls contained either chloramphenicol (E) or distilled water (F) and showed clear inhibition zones or none respectively.

The entire reactor blackened after 2 days at 30°C. Therefore, the elevated incubation temperature had a positive effect on the overall sulphate reduction process due to the rapid formation of the black precipitates. H₂S was also detected by smell in subsequent samples.

Physical and chemical analyses

Active biological sulphate reduction occurred in the inoculum reactor. The reduced sulphate was converted to H₂S (odour detectable after 19 d) and metal sulphides (formation of black precipitates). The production of metal sulphides influenced COD determinations. The organic COD content was sufficient to facilitate complete sulphate reduction after 56 d at 31.2 mg SO₄²⁻/l/d (Figure 4.3).

Stoichiometrically, 1171.8 mg/l COD was required for complete sulphate reduction. Experimentally, 1624 mg/l COD was consumed which represented a COD demand of 452.1 mg/l. The increase in the pH from 6.97 to 8.09 after 56 d was evidence of sulphate reducing bacterial activity. Sulphate was reintroduced into the reactor after 63 d and decreased over 14 d from 1778 mg/l to 1428 mg/l at 25 mg/l/d (Figure 4.3). Theoretically, H₂S accumulated to 405.3 mg/l in the inoculum reactor after 56 d. Reis *et al.* (1992) found that H₂S produced from sulphate reduction had a direct and reversible toxicity effect on SRB. A H₂S concentration of 547 mg/l completely inhibited culture growth. However, H₂S toxicity at concentrations lower than 200 mg/l has been reported (Hilton and Oleszkiewicz, 1988; Hulshoff Pol *et al.*, 1998). Unionised H₂S is toxic to microorganisms because neutral molecules can permeate well through the bacterial cell membrane. Inside the cell, H₂S can react with proteins to form new disulfide bonds and can also affect the intracellular pH (Hulshoff Pol *et al.*, 1998).

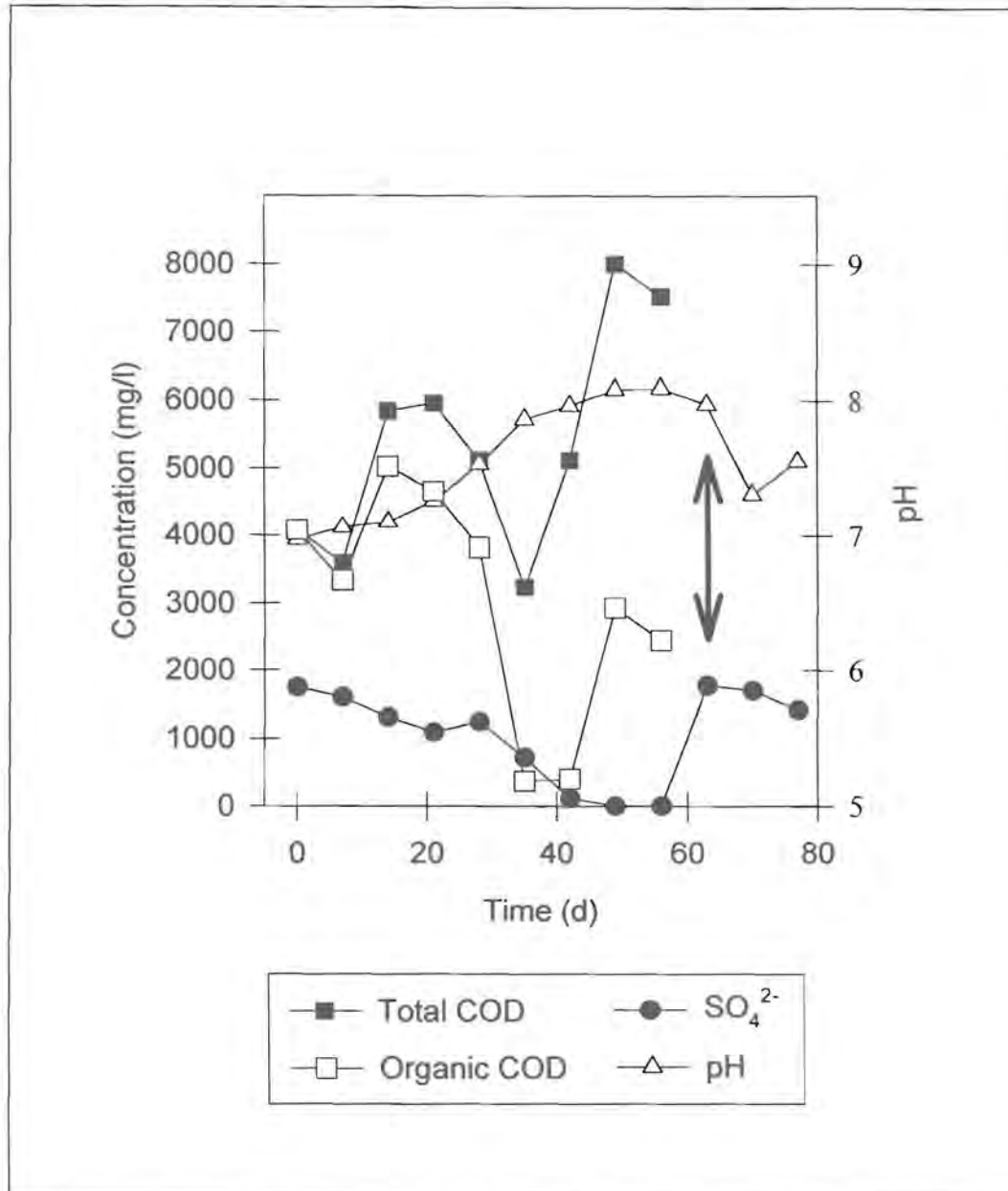


Figure 4.3: Change in chemical parameters monitored during inoculum preparation. The arrow indicates when sulphate was supplemented. COD was not determined after 63 d.

Microbiological analyses

Chemical analyses indicated that active sulphate removal occurred in the inoculum reactor (Figure 4.3). Microbiological results indicated that an active consortium of SRB was present in the inoculum reactor. The total number of anaerobic bacteria increased over 21 d from 10^3 colony forming units per ml (cfu/ml) to 10^7 cfu/ml (Figure 4.4). Anaerobic bacteria proliferated during the hydrolysis of the anaerobic

digester sludge. However, once the hydrolysis stage was completed, SRB effectively outcompeted the fermentative bacteria for nutrients in the form of fermentation intermediates. This was evident in the decline of the total number of anaerobic bacteria during the following 3 weeks to 10^5 cfu/ml. SRB maintained their dominance in the inoculum reactor at SRB/TAPC ratios of 0.18 to 2.96 (after 35 to 56 d) until addition of unsterile sulphate after 56 d (Figure 4.4). We concluded that an active consortium of SRB was dominant in the reactor. Hence, phase 2 of this study was initiated to determine the vitality of this inoculum.

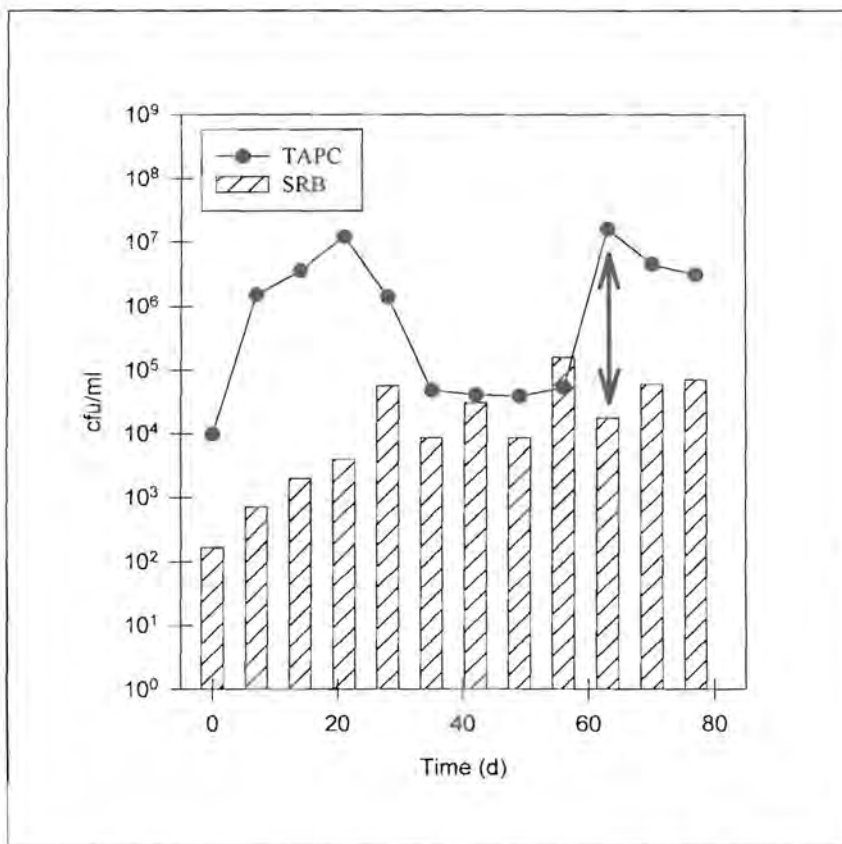


Figure 4.4: Development of microbial population structure during inoculum preparation. The arrow indicates when sulphate was supplemented. TAPC = total anaerobic bacteria count; SRB = sulphate reducing bacteria

Inoculum evaluation experiment

The inoculum responded well when it was reintroduced into fresh artificial AMD (pH \pm 7) with a 3-month old Buffelsgrass leachate as carbon source. The greatest difference between the reactors was evident when they were compared visually

between the reactors was evident when they were compared visually (Figure 4.5). The sulphate concentration in the drip bag with the 3% leachate decreased over 14 d from 2833 mg/l to 1763 mg/l at 76.4 mg/l/d (Table 4.6). The leachate contained appreciable amounts of sulphate (2324 mg/l) and this facilitated the onset of biological sulphate reduction (Ueki *et al.*, 1988). Artificial AMD was unstable and this was reflected by the pH decrease and the release of sulphate over 14 d (Table 4.6). SRB attained dominance within 14 d in the leachate amended reactor, but SRB numbers decreased in the control reactor (Table 4.6). The pH decrease in the control reactor contributed to the decline in SRB numbers since optimum SRB growth occurred at pH 5-9 (Postgate, 1979).



Figure 4.5: Comparison of the reactor that received 3% leachate (left) with the control reactor (right). Both reactors were inoculated with the inoculum produced in this study. Gas production was detected after 7 d in the leachate amended reactor, but not in the leachate-free control reactor.

Table 4.6: Chemical and microbiological results from preliminary drip bag experiment evaluating effectiveness of inoculum in the presence and absence of leachate.

Time (d)	3% leachate ^a				Leachate-free control			
	pH	SO ₄ (mg/l)	TAPC ^b (cfu/ml)	SRB ^c (cfu/ml)	pH	SO ₄ (mg/l)	TAPC (cfu/ml)	SRB (cfu/ml)
0	6.98	2833	0	0	6.94	1977	2150	0
7	6.67	1957	3.0x10 ⁴	1.9x10 ⁵	5.42	2388	1.2x10 ⁶	3.2x10 ⁴
14	6.90	1763	1050	8.5x10 ⁴	4.64	2127	2.2x10 ⁷	900

a. 3 month old leachate produced from *Cenchrus ciliaris* cv. Molopo. Initial sulphate concentration: 2324 mg/l; b. Total anaerobic plate count; c. Sulphate reducing bacteria.

3.3 Leachate amended experiments

Visual observations

Contents of all 11 reactors were dark blue-green at the start of the experiment. The content of the control reactors became red-brown after 3 d while the leachate amended reactor contents turned black after 3 d. H₂S odour were not detected in the samples over the experimental period of 70 d. One of the control reactors turned dark grey after 49 d.

Characteristics of biological sulphate removal

Biological sulphate reduction took place in all the leachate amended reactors (Figures 4.6-4.8 and Table 4.7). Overall sulphate reduction rates of 9.4 mg/l/d (Natural leachate) to 15.1 mg/l/d (6% *S. commune* leachate) were observed (Table 4.7). However, when the sulphate increases after 63-70 d were ignored, the sulphate reduction rates improved to a maximum of 17.7 mg/l/d (natural leachate) (Table 4.7).

No sulphate reduction occurred in the control reactors. Again this can be attributed in part to low pH. However, once the pH increased to around 6 after 42 d, sulphate reduction could still not commence due to the absence of sufficient organic carbon. Near-neutral pH values were maintained within leachate amended reactors. Lignocellulose leachate probably has a buffering mechanism which facilitate decomposition of lignocellulose in any environment.

Closer inspection of the sulphate reduction curves revealed that sulphate reduction was dependent upon the release/bioavailability of organic carbon (Figures 4.6-4.8). The first phase involved rapid utilisation of organic matter (simple sugars, proteins, etc.) by fermentative microorganisms. The pH in all the leachate amended reactors decreased slightly by 0.1 - 0.5 pH units within the first 7 d, but increased thereafter. This adaptation phase was characterised by sulphate reduction within the first 3 to 7 d in some of the leachate amended reactors.

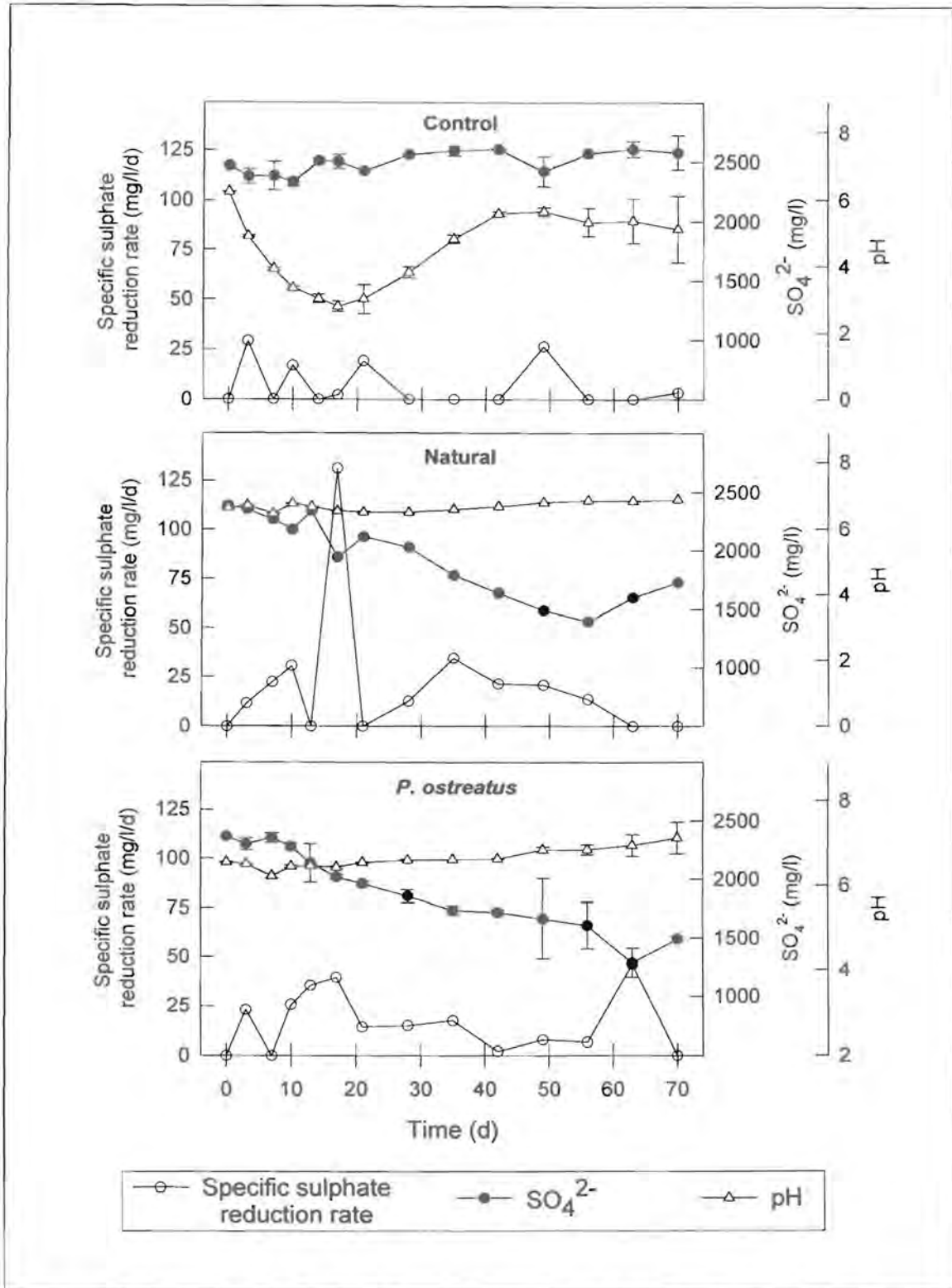


Figure 4.6: Comparison of pH values and specific sulphate reduction rates between sampling events for leachate-free controls, natural and *P. ostreatus* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

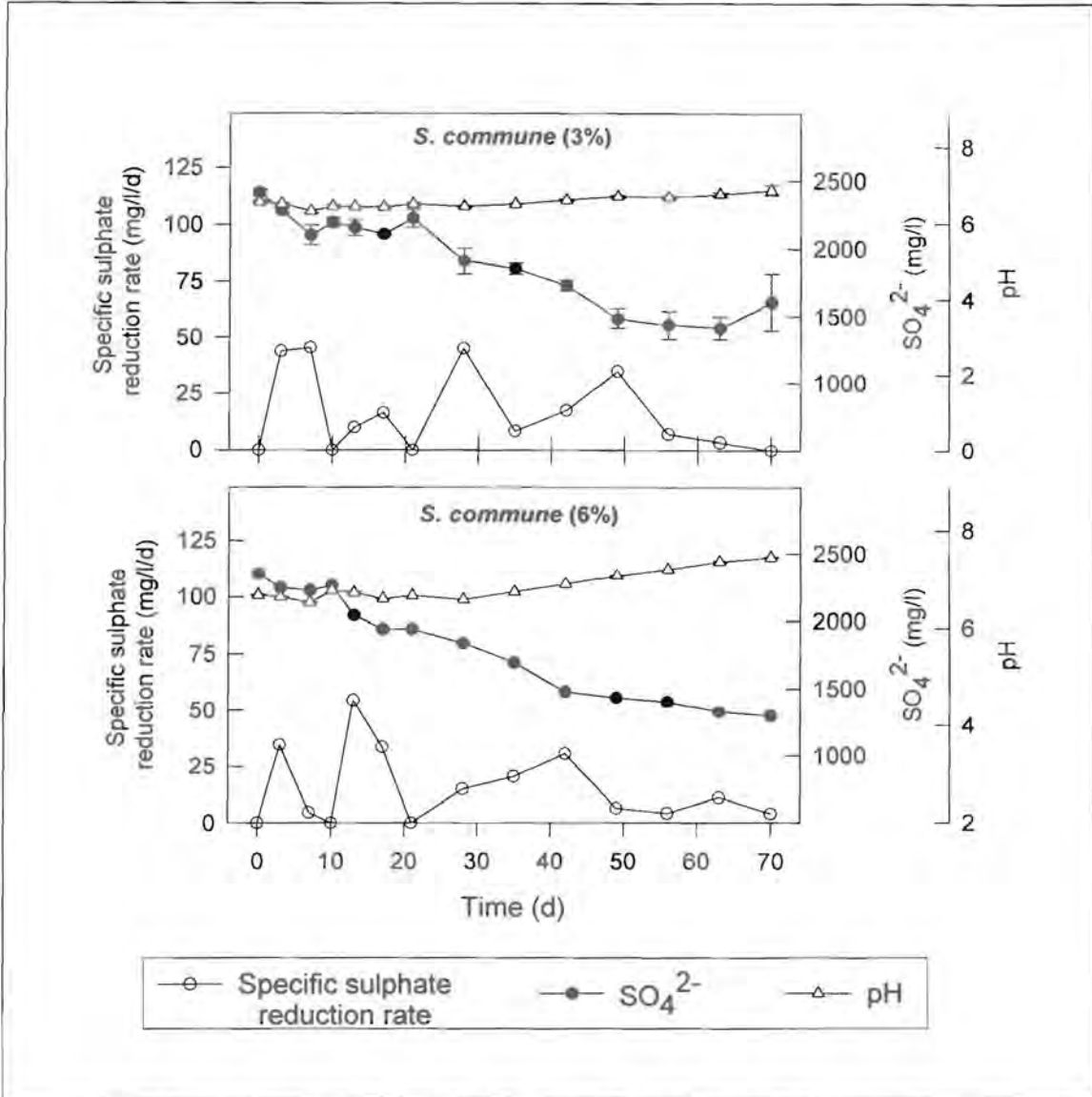


Figure 4.7: Comparison of pH values and specific sulphate reduction rates between sampling events for *S. commune* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

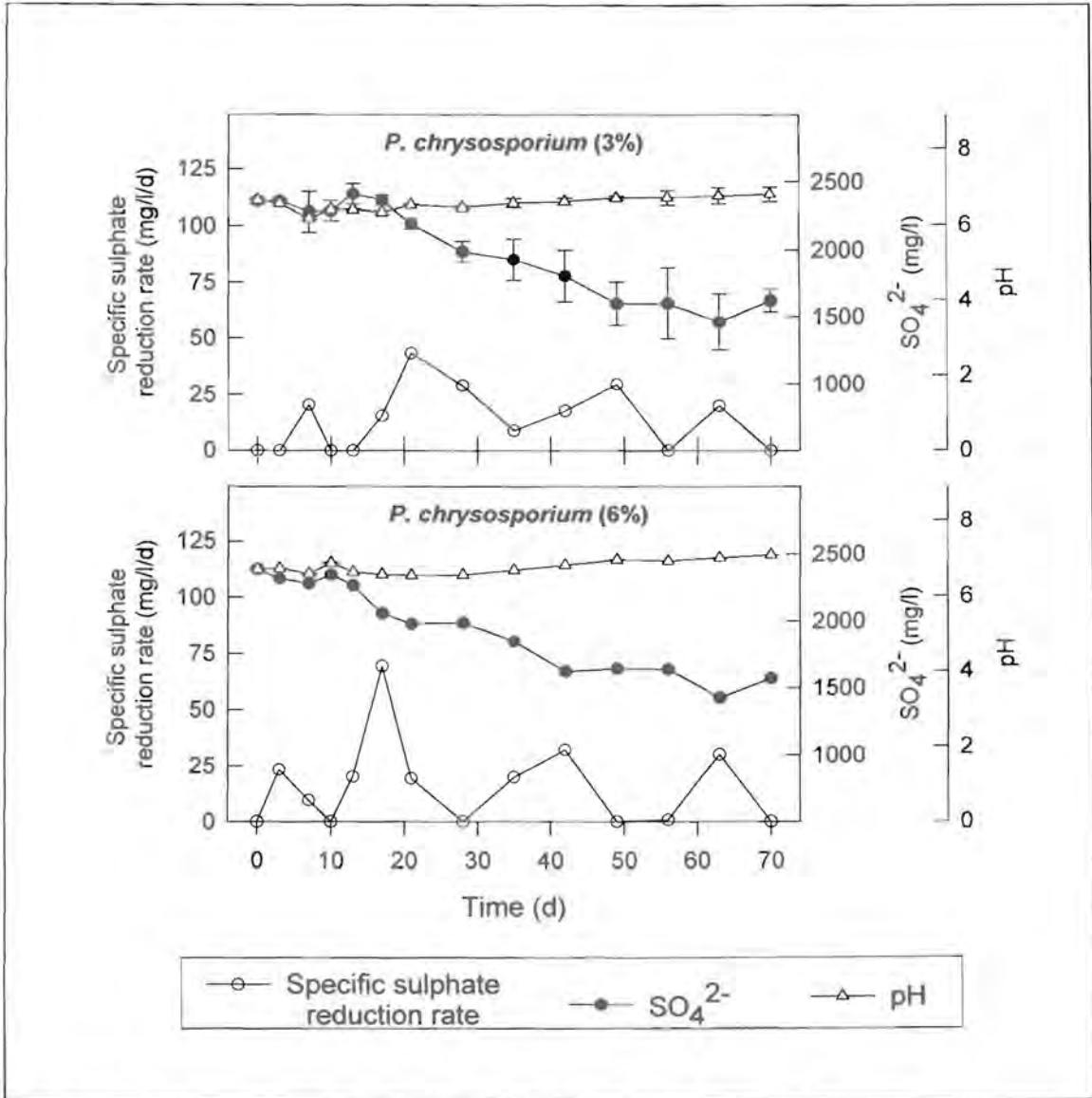


Figure 4.8: Comparison of pH values and specific sulphate reduction rates between sampling events for *P. chrysosporium* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

Table 4.7: Overall sulphate removal efficiencies for leachate amended treatments after 70 d. Most of the sulphate values increased after 56 or 63 d. Therefore, the best sulphate reduction rates and percentage sulphate removed are indicated in brackets.

Treatment	Initial SO ₄ ²⁻ (mg/l)	Final SO ₄ ²⁻ (mg/l)	SO ₄ ²⁻ reduction rate (mg/l/d)	SO ₄ ²⁻ reduction rate after 35 d (mg/l/d)	% SO ₄ ²⁻ removed
Control	2466	2579	0	0	+ 4.6
Natural	2386	1725 (1395 after 56 d)	9.4 (17.7)	17.2	27.7 (41.5)
<i>P. ostreatus</i>	2368	1493 (1287 after 63 d)	12.5 (17.2)	18.1	36.9 (45.7)
<i>S. commune</i> (3%)	2413	1604 (1411 after 63 d)	11.6 (15.9)	16.1	33.5 (41.5)
<i>P. chrysosporium</i> (3%)	2350	1621 (1461 after 63 d)	10.4 (14.1)	12.3	31.0 (37.8)
<i>S. commune</i> (6%)	2359	1299	15.1	18.7	44.9
<i>P. chrysosporium</i> (6%)	2386	1573 (1429 after 63 d)	11.6 (15.2)	15.3	34.0 (40.1)

Following the adaptation phase, the rate of sulphate reduction decreased after 10 to 15 d. The duration of this lag phase differed between treatments. Sulphate reduction resumed after the lag phase and introduced a period of sulphate reduction characterised by sequential starts and stops in terms of the rate of sulphate reduction (Figures 4.6-4.8).

At least two periods of sulphate reduction separated by a lag phase can be distinguished in the data of all leachate treatments (Figures 4.6-4.8). The trends observed in the variable phase are reminiscent of diauxic bacterial growth (Figure 4.9) (Caldwell, 1995). When *E. coli* was transferred to a medium containing glucose and lactose, glucose was utilised first and lactose was utilised only after glucose exhaustion. Therefore, once the monosaccharides were exhausted, a short stationary phase followed during which the enzymatic systems needed to utilise the disaccharides were induced. The exponential growth phase continued albeit at a slower rate, because more metabolic energy was required to utilise the now abundant, but more complex carbon source.

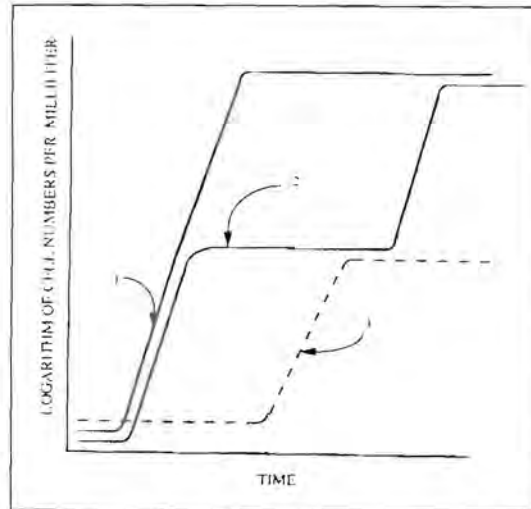


Figure 4.9: Diauxic growth of *E. coli*. (1) Lactose-grown cells transferred into a medium containing a mixture of glucose and lactose. (2) Glucose-grown cells transferred into a medium containing glucose and lactose. (3) Glucose-grown cells transferred into a medium containing only lactose as a carbon source (Caldwell, 1995)

Therefore, the specific sulphate reduction rates indicated that complex organic compounds are being degraded under anaerobic conditions, yielding degradation intermediates that could be utilised by the sulphate reducing consortium to sustain sulphate reduction. The bioavailability of these degradation intermediates regulated the initiation and rate of sulphate reduction.

Relative biodegradability potential

In this study, the BOD₅ results were an indicator of the relative potential of the reactor contents to be biodegradable. The BOD₅ analysis was performed using an aerobic sludge seed. Under aerobic conditions, a COD:BOD₅ ratio of 2:1 is considered as optimal. However, this relationship might not be relevant for anaerobic environments. In this study, and under anaerobic conditions, the COD:BOD₅ ratio varied approximately between 1:0.13 to 1:4 (Figure 4.10-4.12). The worst ratio was 1:4 (Control after 56 d, *P. ostreatus* after 49 d) and the best ratio was 0.5 (*P. ostreatus* after 63 d). For all treatments in general, these ratios decreased from 1:3 to 1:1 or less, indicating that biodegradability increased over time.

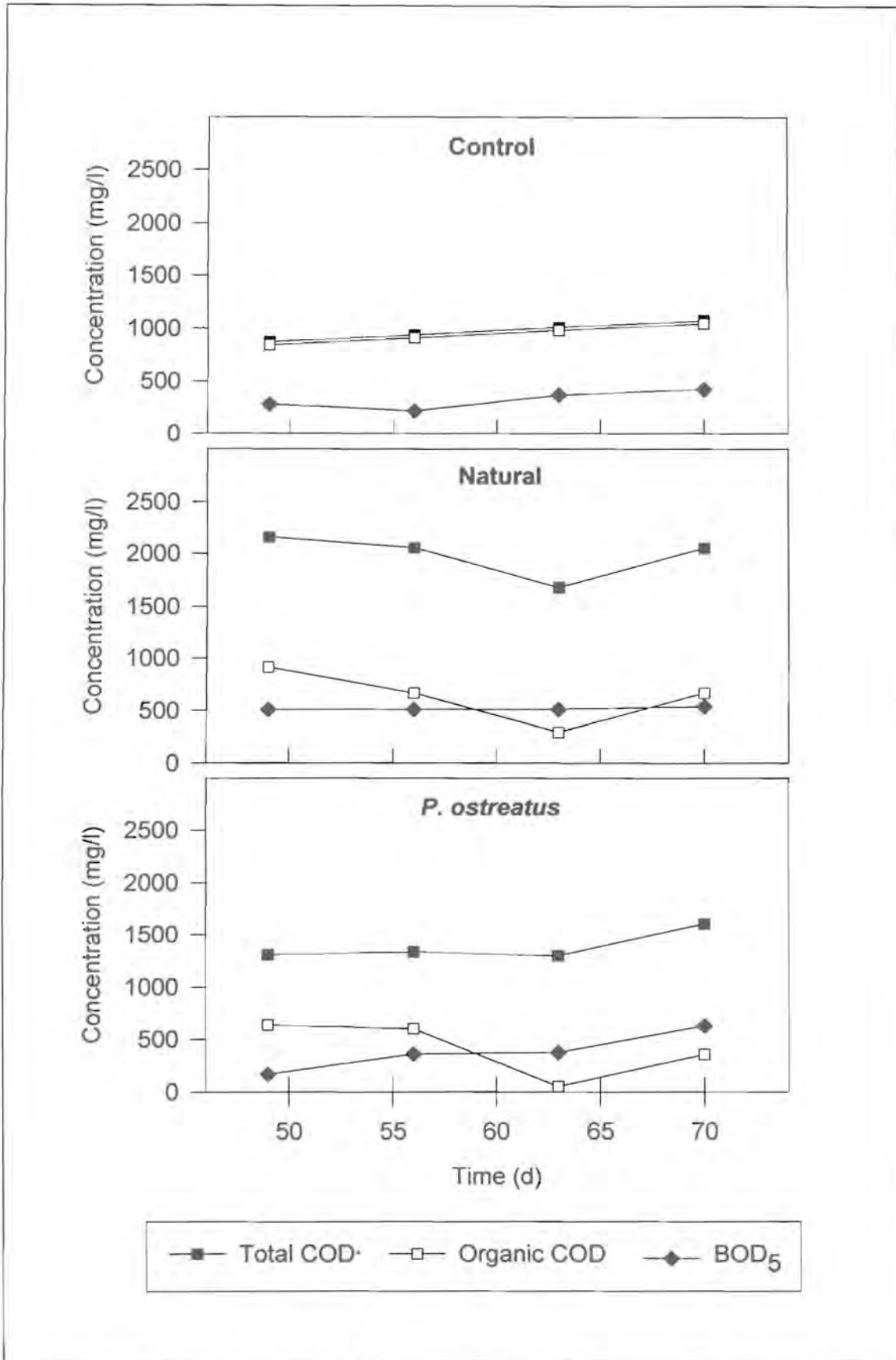


Figure 4.10: Comparison of BOD₅ results from the control, and natural and *P. ostreatus* leachate treatments.

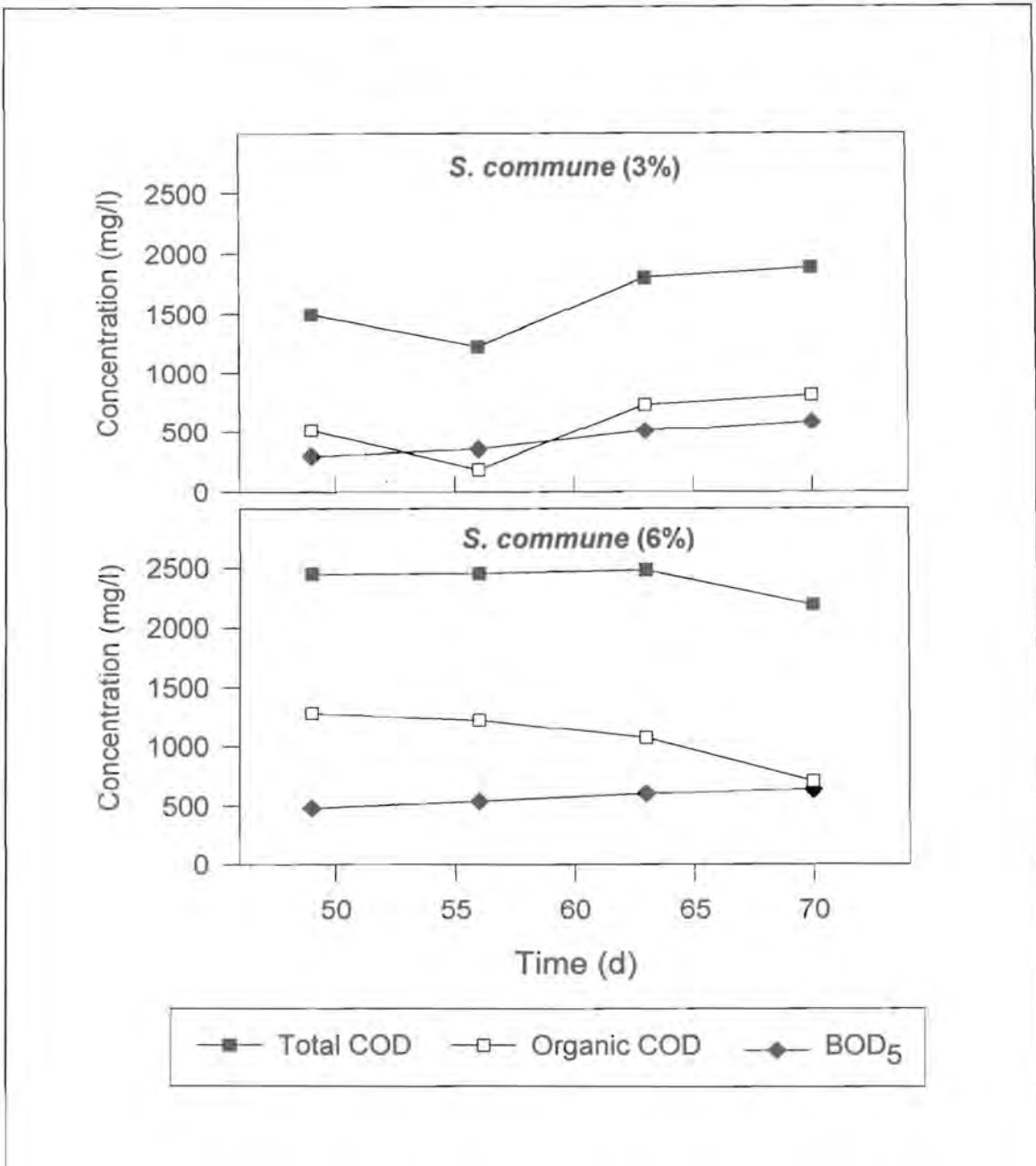


Figure 4.11: Comparison of BOD₅ results from the *S. commune* leachate treatments.

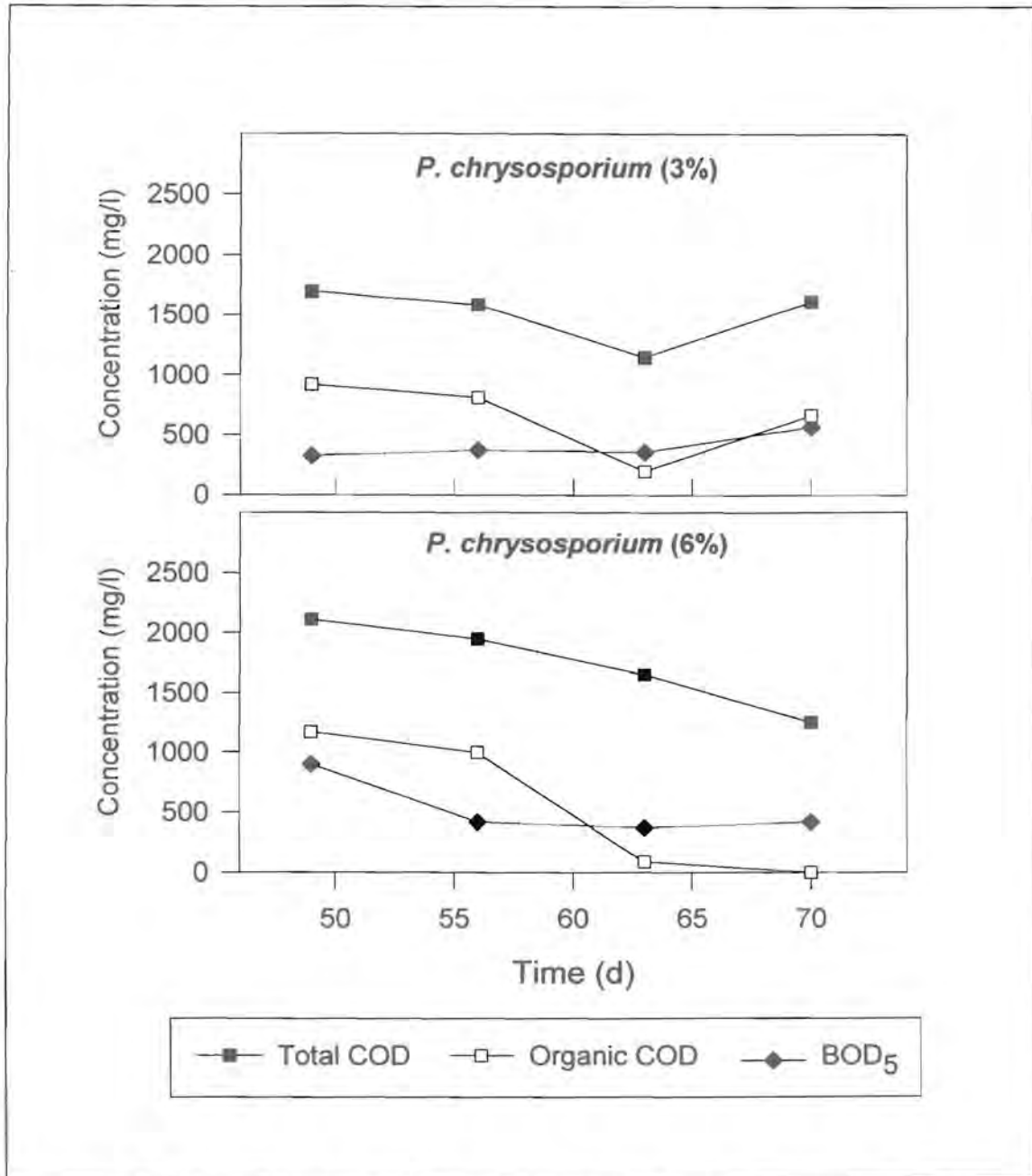


Figure 4.12: Comparison of BOD₅ results from the *P. chrysosporium* leachate treatments.

Sulphide toxicity

Predicting H₂S production stoichiometrically and theoretically, based on the solution pH, we have concluded that H₂S toxicity did pose a threat to the viability of SRB in this study (Figures 4.13-4.15). In this study, the total sulphide theoretically present in the control reactors after 70 d, 98.7% was in the form of un-ionised H₂S. Stoichiometrically, 424.1 mg/l H₂S accumulated in the control reactors after 70 d. However, process failure in the control reactors can also be attributed to insufficient organic carbon concentration, low pH, and low SRB numbers.

In the leachate amended treatments, stoichiometric H₂S concentrations varied from 648 mg/l (*S. commune*, 6%) to 920 mg/l (natural). Specific sulphate reduction rates decreased over time (35 d to 70 d) in most of the leachate amended reactors with the theoretical accumulation of H₂S (Figures 4.6-4.8, 4.13-4.15).

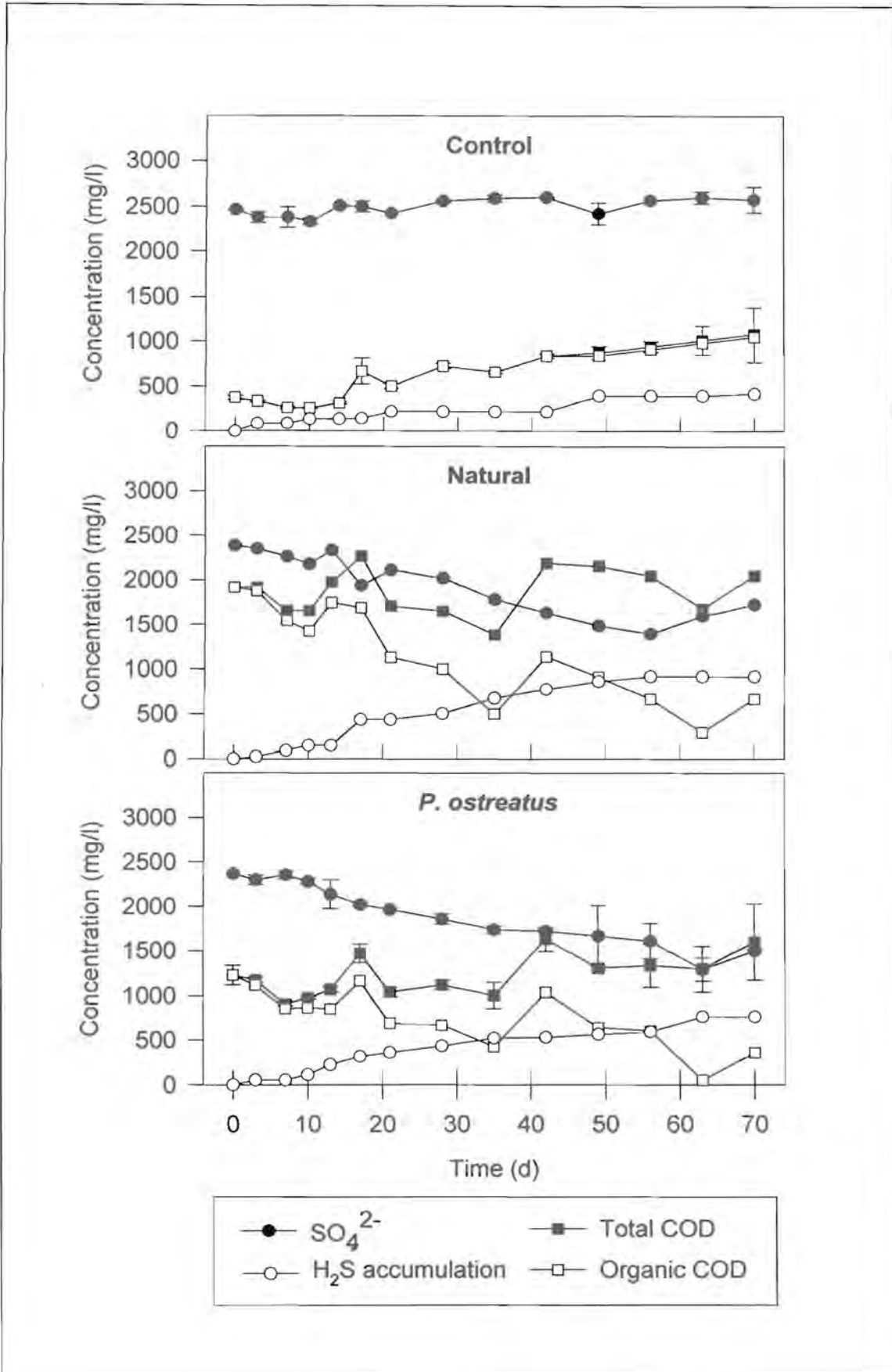


Figure 4.13: Relationship between observed sulphate reduction and theoretical $\text{FeS}/\text{H}_2\text{S}$ accumulation for the controls, and natural and *P. ostreatus* leachate.

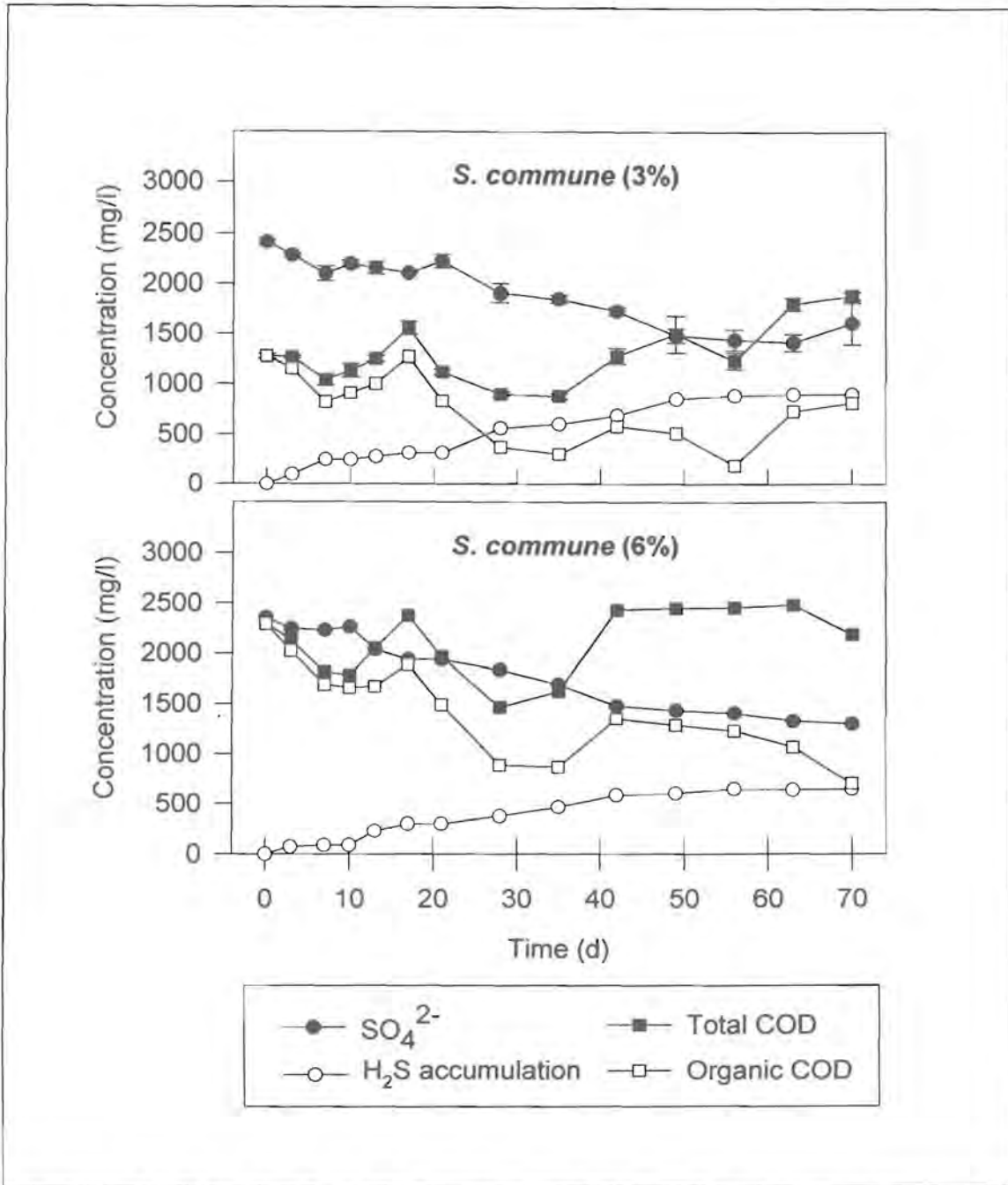


Figure 4.14 : Relationship between observed sulphate reduction and theoretical FeS/H₂S accumulation for *S. commune* leachate treatments.

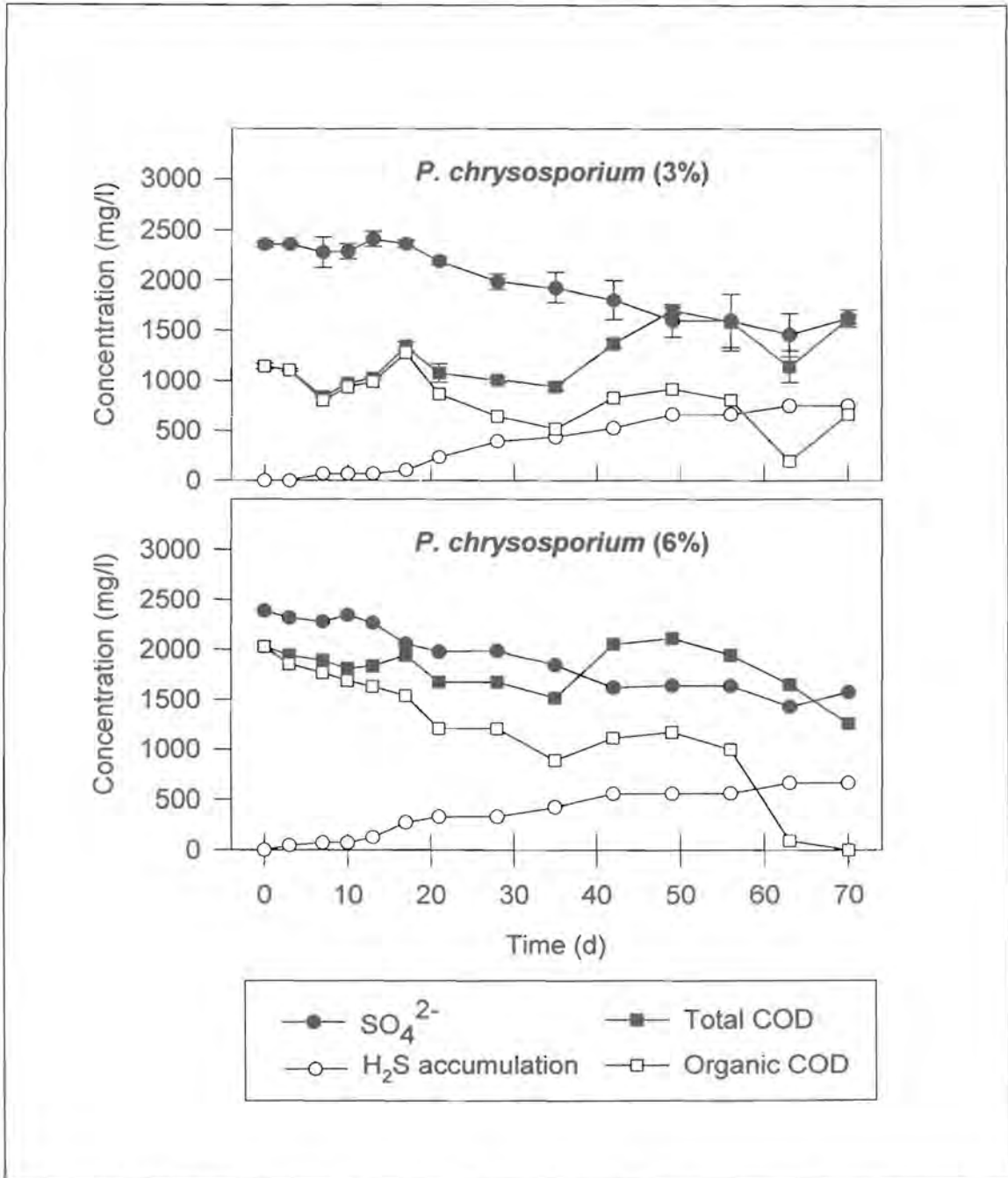


Figure 4.15: Relationship between observed sulphate reduction and theoretical $\text{FeS}/\text{H}_2\text{S}$ accumulation for *P. chrysosporium* leachate treatments.

Microbiological results

The leachate not only sustained SRB activity in the leachate amended units, but the anaerobic microbial population in general also benefited (Figures 4.16-4.18). Relatively high numbers of total anaerobic bacteria was maintained in the leachate amended units throughout the experimental period.

SRB numbers increased from 10^4 cfu/ml to more than 10^5 cfu/ml within 7-20 d (Figures 4.16-4.18). These numbers remained constant for the remainder of the experiment between 10^4 and 10^5 cfu/ml in most of the leachate amended treatments, reaching 10^5 to 10^6 cfu/ml in the units amended with 3% of *S. commune* and *P. ostreatus* leachates (Figures 4.17 and 4.18). However, a gradual decrease in the SRB numbers was observed after 30-40 d in the treatments amended with 3% of *S. commune* and *P. ostreatus* leachates. It appears as if SRB viability is decreasing in the 3% *S. commune* treatment because sulphate reduction rates decreased over the same time period (Figure 4.7). The reasons for this could be greater sensitivity to sulphide inhibition or poor biodegradability of the remaining COD (Figure 4.11).

The numbers of total anaerobic bacteria also remained constant around 10^6 cfu/ml during the experimental period (Figures 4.16-4.18). The number of total anaerobic bacteria and SRB declined in the control treatments from the outset of the experiment. However, these numbers increased after 30 d (Figure 4.16). This sudden proliferation of microorganisms was directly related to the increase in pH. Even though SRB numbers increased to 10^4 cfu/ml, no continuous sulphate reduction occurred. The increasing differences between the duplicate control reactors were evident in the increasing standard deviations by the end of the experiment (Figures 4.6 and 4.16). One reactor remained red-brown while the other was dark-grey in colour. Reactor A (final pH 4.10) supported 5.7×10^4 cfu/ml total anaerobic bacteria while reactor B (final pH 6.12) supported 1.26×10^7 cfu/ml total anaerobic bacteria at the end of the experiment. Both reactors showed a sulphate increase of 7.9% (A) and 3.3% (B) respectively (Figure 4.6).

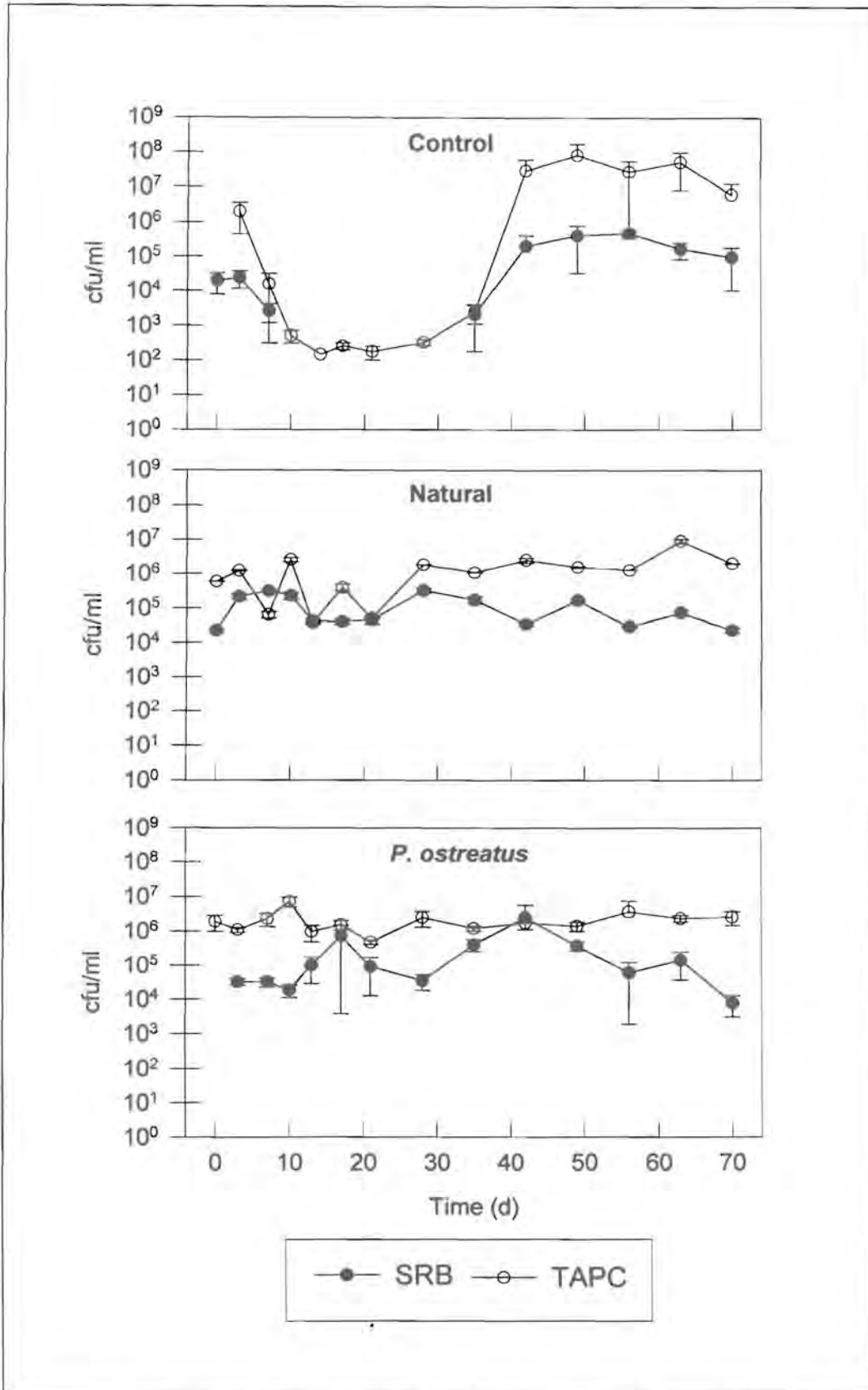


Figure 4.16: Change in microbiological parameters of leachate-free controls, natural and *P. ostreatus* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

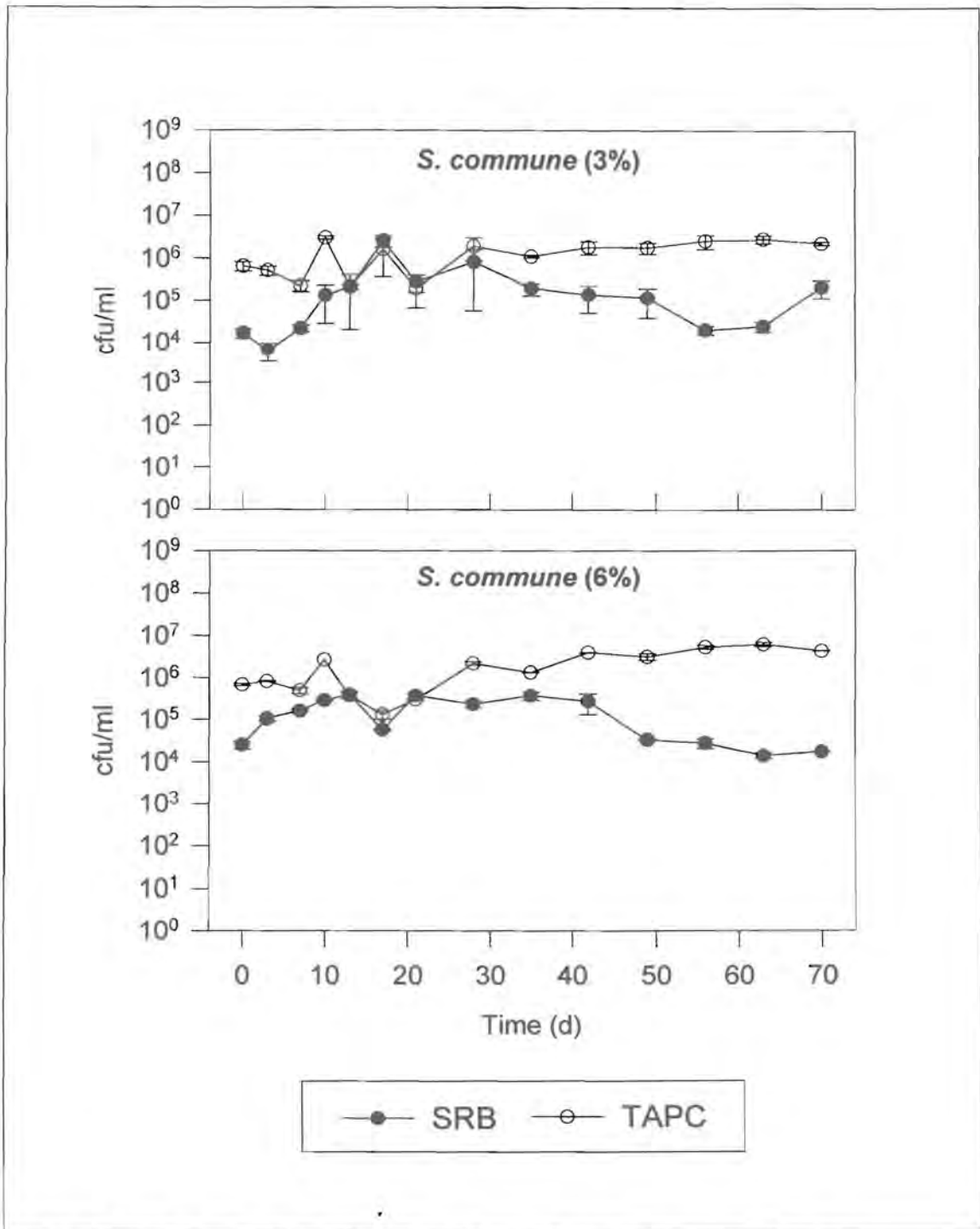


Figure 4.17: Change in microbiological parameters of *S. commune* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

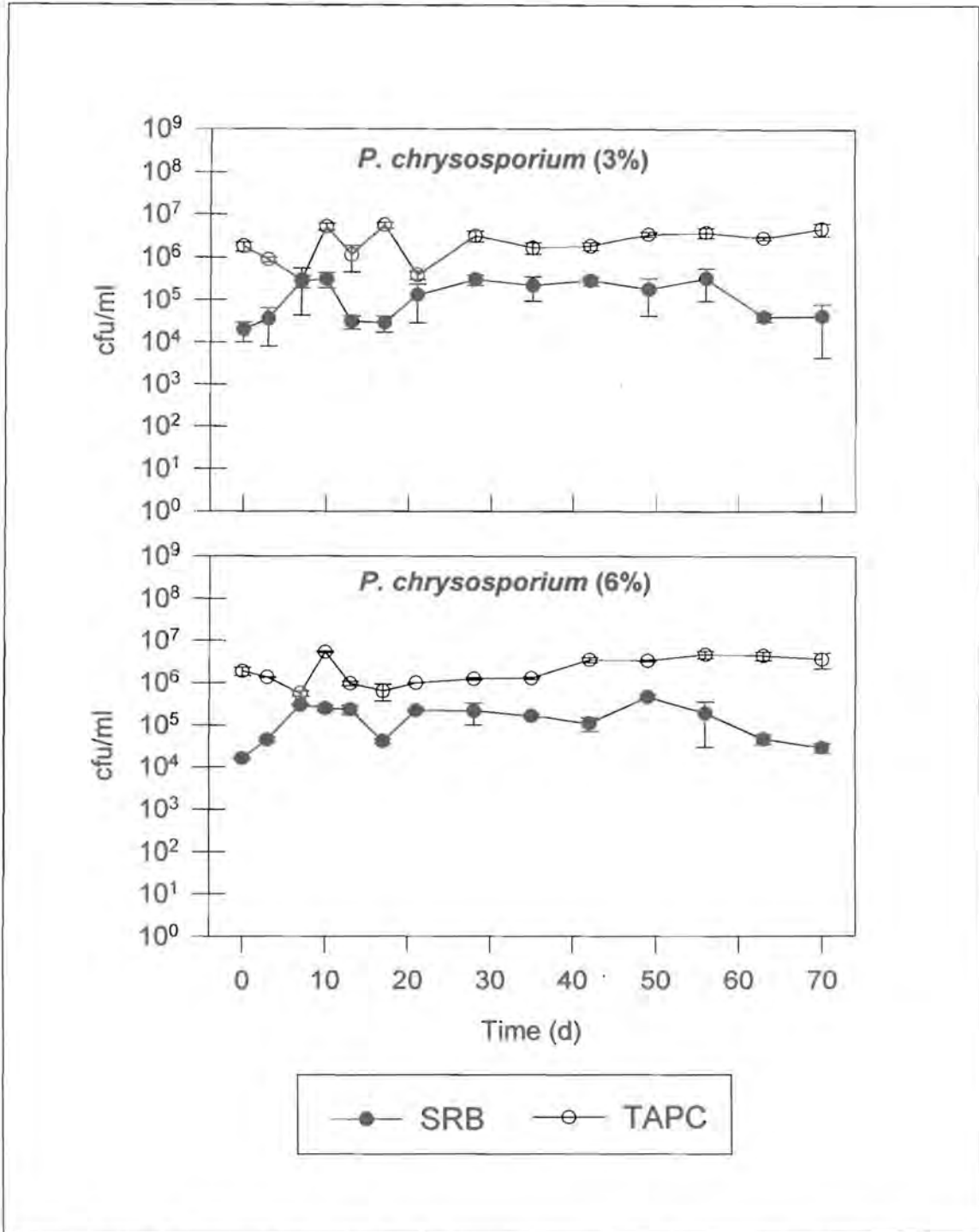


Figure 4.18: Change in microbiological parameters of *P. chrysosporium* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

Overall process performance

Comparison of experimental to theoretical sulphate removal efficiencies indicated that biological sulphate reduction was supported to different degrees (Table 4.8). Sulphate removal efficiencies in excess of 100% were calculated for *S. commune* (3%) and *P. chryso sporium* (3%) treatments. Although the total COD data were corrected for the sulphide content, COD accumulation was still observed (Figures 4.13-4.15). If the lowest COD values at the end of the experiment were used to calculate the sulphate removal efficiency, *S. commune* (3%) and *P. chryso sporium* (3%) treatments showed 50% and 52% efficiency respectively, while natural and *P. ostreatus* treatments showed 27% and 50% efficiency respectively.

The COD increases at the end of the experiment could be attributed to accumulation of SRB metabolic end-products and the failure of the methanogens to remove this COD (Polprasert and Hass, 1995). However, reported sensitivity of methanogens to sulphide toxicity varies (Hilton and Oleszkiewicz, 1988; Hulshoff Pol *et al.*, 1998).

Table 4.8: Efficiency of biological sulphate removal process based on lignocellulose leachate carbon source. Efficiencies calculated using the lowest COD value are indicated in brackets.

Treatment	SO ₄ ²⁻ removed (mg/l)	COD consumed (mg/l)	Theoretical COD requirement (mg/l)	SO ₄ ²⁻ removed (mg SO ₄ ²⁻ / mg COD)	% SO ₄ ²⁻ removal efficiency
Control	0	0	0	0	0
Natural	661	1253	443	0.53	35 (27)
<i>P. ostreatus</i>	875	870	586	1.01	67 (50)
<i>S. commune</i> (3%)	809	463	542	1.75	117 (50)
<i>S. commune</i> (6%)	1051	1589	704	0.66	44
<i>P. chryso</i> (3%)	738	481	494	1.53	103 (52)
<i>P. chryso</i> (6%)	813	2027	545	0.40	27

4. DISCUSSION

4.1 Process performance

The ultimate aim of this work was to determine whether lignocellulose leachate could be used to stimulate full-scale biological sulphate reduction. A comparison of the current process performance to theoretical yields will determine process efficiency and ultimately cost-effectivity.

We have provided possible explanations for the accumulation of COD in all of treatments. Standard methods and literature cite several sources of interference (APHA-AWWA-WPCF, 1960; Methods for the examination of waters and associated materials, 1986). We have indicated that FeS accumulation influenced the COD results. Other researchers made similar observations and corrected COD values for sulphide and other reduced inorganic species (Maree and Strydom, 1987; Polprasert and Haas, 1995). After correction of our data for sulphide interference, an overall COD decrease was observed.

Other sources of interference emanate from the chemical complexity of the leachate. The brown colour of the leachate was an indication of a high phenolic content in the form of lignin or lignin degradation products (Eaton *et al.*, 1980). The organic contents can therefore interfere with the COD analysis, especially organic molecules with ring structures (humic substances, lignin degradation intermediates, and phenolic compounds). Accumulation of SRB metabolic end-products and the failure of methanogens to remove this COD could be an explanation for the COD accumulation at the end of the leachate experiment (Polprasert and Hass, 1995).

Process performance was estimated by comparing experimental and theoretical sulphate reduction efficiencies. If the COD accumulation at the end of the experiment was ignored, sulphate removal efficiencies of 27% to 52% were calculated. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachates performed better compared to the other leachate treatments. These fungi have powerful polymer degrading enzymes (notably cellulases, hemicellulases and lignin

degrading enzymes) that facilitate lignocellulose degradation. Lignocellulose degradation intermediates released by the action of these enzymes are probably present in the leachates in greater quantities compared to the leachate obtained from natural fungal degradation of Buffelsgrass.

The overall sulphate reduction rates obtained in this study were lower than rates reported in literature (Compare Table 4.7 and Table 4.9). Sulphate reduction rates or efficiencies found in literature were not reported in terms of sulphate removed per reducing equivalent of substrate (COD). Overall long-term sulphate reduction rates of 9.4 mg/l/d to 15.1 mg/l/d were obtained in this study (Table 4.7), which was lower than the rates obtained by Dvorak *et al.* (1992). The short-term sulphate reduction rates observed in this study were considerably lower than rates reported in Table 4.9 and by Zdyb (1999) (Table 4.10). However, the final sulphate reduction rates reported in this study compare favourably with the final rates obtained by Drury (1999) (Table 4.9).

In this study, the overall sulphate removal varied from 27.7% to 44.9% (Table 4.7). Comparing the results from Table 4.7 with the literature values in Table 4.9, overall sulphate removal percentages comparable to all undefined carbon source-based systems were achieved in this study (Bécharde *et al.*, 1994; Chang *et al.*, 2000; Tsukamoto and Miller, 1999).

4.2 Characteristics of organic COD

According to Hulshoff Pol *et al.* (1998), addition of organic carbon to a sulphate treatment system should be cost effective and not contribute to pollution of the receiving waters. Cost effectivity of undefined carbon sources could be easily estimated, but the down-stream effects could be easily overlooked. The characterisation and conversion of inert COD or non-biodegradable COD is becoming more important to industry because of ever increasing effluent discharge limits set by water control agencies (Archibald *et al.*, 1998). These inert COD fractions have been identified as humic substances (Namour and Müller, 1998) and soluble microbial products (Barker and Stuckey, 1999).

Table 4.9: Examples of defined and undefined carbon sources evaluated for use in active or passive AMD treatment systems.

Carbon sources	Laboratory/ Field experiment	Significance and/or mean sulphate reduction rates (mg/l/d) / efficiencies (%)	Duration of study (d)	References
Wood dust	Both	Origin of concept of treating AMD through biological sulphate reduction. Lab study utilising partially degraded wood dust: <ul style="list-style-type: none"> • 53.2 mg/l/d (acid mine water) @ 37°C • 58.3 mg/l/d (artificial AMD) @ 37°C 	Lab study: 14 d	Tuttle <i>et al.</i> , 1969a; Tuttle <i>et al.</i> , 1969b
Wood dust and various defined organic nutrients	Lab	<ul style="list-style-type: none"> • Glucose (0.1%) ◇ 22.8 mg/l/d • Acetic acid (0.5%) ◇ 6.5 mg/l/d • Na-lactate (0.5%) ◇ 58 mg/l/d 	Overall: 30 d	Wakao <i>et al.</i> , 1979
Digester slurry of cattle waste	Lab	<ul style="list-style-type: none"> • 48.95 mg/l/d Rate of sulphate reduction was enhanced by increasing the sulphate concentration.	Overall: 20 d	Ueki <i>et al.</i> , 1988
Spent mushroom compost	Field	<ul style="list-style-type: none"> • Pittsburgh system ◇ 32 mg/l/d • Palmerton system ◇ 21 mg/l/d 	Pittsburgh system: 350 d Palmerton system: 112 d	Dvorak <i>et al.</i> , 1992
Alfalfa hay, timothy hay, and cereal straw	Lab	4.7% (cereal straw), 31% (timothy hay), and 34.2% (alfalfa hay) overall sulphate reduction. Long-term stability could not be achieved. Supplementation with sucrose and urea was required.	Overall: 107 d Process failure after 21 d	Bécharard <i>et al.</i> , 1994
Whey and cow manure	Lab	Sulphate increased in reactors inoculated with SRB for reasons unknown. Oversaturation with ferric ion minerals caused rapid chemical precipitation during setup and reduced starting concentrations of metals as well as sulphate.	Overall: 203 d	Christensen <i>et al.</i> , 1996
Rice stalks and cow manure	Field	Good initial metal removal, but operating problems affected metal removal efficiency. No data on sulphate removal available.	Unknown	Cheong <i>et al.</i> , 1998
Lactate	Lab	<ul style="list-style-type: none"> • 38.3% sulphate removal at pH 3.25 • 14.4% sulphate removal at pH 3.0 Viable SRB could be isolated after 64 d from pH 3.0 environment.	⇒ 42 d ⇒ 64 d	Elliott <i>et al.</i> , 1998
Cow manure, sawdust, and cheese whey	Lab	Whey addition increased effluent alkalinity, sulphate removal (98% vs. 60%), and metals removal. Sulphate removal rate decreased over time from 24mg/l/d to 12 mg/l/d (with whey) or 24 mg/l/d to 4 mg/l/d (without whey).	Overall: 644 d (23 months)	Drury, 1999
Methanol, lactate, and horse manure	Both	Field study: <ul style="list-style-type: none"> • Spent manure ◇ 7% sulphate removal • Spent manure + methanol ◇ 31% " 	⇒ Process failure after 230 d ⇒ Overall: 140 d	Tsukamoto and Miller, 1999
Various undefined carbon sources	Lab	32% sulphate removal on average for all carbon sources.	Overall: 35 d Greater sulphate removal efficiency after 14 d than 35 d	Chang <i>et al.</i> , 2000

Table 4.10: Summary of results obtained from AMD treatment experiments using undefined carbon sources (Zdyb, 1999). Reactors were inoculated with 30% anaerobic digester sludge.

Carbon sources	Experimental period (d)	Sulphate reduction rate (mg/l/d)	% sulphate removal
Digester sludge ^a	10	0	0
Molasses	10	52.6	12.02
Cow manure (composted)	10	2.0	0.76
Hay	22	103.4	98.96
Fly ash	25	86.3	86.67
Mushroom compost	25	95.2	92.18
Cow manure (fresh) ^b	28	104.6	89.05
Kikuyu (fresh) ^b	28	138.3	97.84
Whey ^b	28	82.7	77.87
Chicken manure (composted)	30	41.8	66.19
Citrus compost	30	45.7	52.75
Silage	30	92.7	93.71

a. Net sulphate increase was reported.

b. Reactors were recharged with the same amount of carbon source after 19 d of operation. Stationary phase in sulphate reduction after 19 d prompted carbon recharge. Sulphate reduction resumed and continued until end of experiment.

The leachate is chemically complex and can contain a mixture of carbohydrates, phenolic compounds, and complex combinations of both. Also, these inert fractions might be insignificant in the beginning of the experiment, but accumulate when biodegradation of readily available residues takes place. Lignin and lignin degradation intermediates can be degraded in anaerobic environments at slow but environmentally significant rates (Zehnder, 1988). Ziomek and Williams (1989) found that *Desulfotribrio desulfuricans* modified lignins under anaerobic conditions with a release of low molecular weight phenolic compounds. Therefore, in this study the organic matter in the COD was biodegradable at first, but recalcitrant residues accumulated with time. These inert fractions can be degraded by anaerobic consortia, albeit at a slow rate. However, accumulation of inert COD would indirectly result in organic carbon depletion and inevitable process failure. This could explain why the reactors amended with 6% leachate managed lower sulphate removal efficiencies (Table 4.8).

4.3 Implications of leachate buffering capacity

A problem with biological treatment of AMD is that extremely acidic pH can inhibit biological sulphate reduction (Elliott *et al.*, 1998). The optimum pH for SRB growth is between 5 and 9 (Postgate, 1979). However, viable SRB have been isolated from a very acidic environment (pH 3.0) (Elliott *et al.*, 1998). The initiation of sulphate reduction in AMD treatment systems required an increase in pH to a threshold level of 4-5.2, either by activity of other fermentative bacteria or addition of neutralizing agents (Wakao *et al.*, 1979). A higher initial pH increased the sulphate reduction rate by decreasing the lag phase (Ueki *et al.*, 1988; Wakao *et al.*, 1979). That is why laboratory experiments are conducted using artificial AMD formulations that have been neutralized (Chang *et al.*, 2000). However, pre-adjusting the pH of AMD chemically is not realistic and reproducible in large scale operations. In this study, addition of lignocellulose leachate to the artificial AMD/inoculum mixture prevented a decrease in pH compared to the control reactors.

5. CONCLUSIONS

- Sulphate removal efficiencies of 27% to 52% (mg SO₄²⁻ reduced / mg COD consumed) were obtained in leachate amended reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachates performed better compared to the other leachate treatments.
- Overall sulphate removal varied from 27.7% to 44.9%, and long-term sulphate reduction rates of 9.4 mg/l/d to 15.1 mg/l/d were observed. Although sulphate removal was comparable to literature values, the sulphate reduction rates were considerably lower.
- Accumulation of inert COD in the form of lignin degradation intermediates could affect process performance of future studies using lignocellulose leachate as carbon source for biological sulphate reduction.

- Near-neutral pH were maintained in all leachate amended reactors. Therefore, leachate buffering capacity/potential could enhance SRB survival and consequently expedite biological sulphate reduction.
- The analytical approach toward following biological sulphate reduction should incorporate additional chemical analyses (iron and sulphide) to accurately model the dynamics of FeS formation and dissolution.

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CHAPTER 5

GENERAL CONCLUSIONS

- The biological treatment of acid mine drainage using sulphate reducing bacteria (SRB) is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction.
- Biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by white rot fungi was investigated to enhance the biodegradability of this lignocellulose substrate. *In vitro* dry matter digestibility was used to measure the effectiveness of fungal delignification. Although the initial digestibility was high ($\pm 60\%$), none of the inoculated or control treatments increased the digestibility of the natural substrate.
- A brown leachate was produced in all treatments over the experimental period. Literature indicated that such a leachate could contain inhibitors or stimulants of bacterial growth. An antimicrobial activity assay indicated that the leachate did not inhibit aerobic Gram negative or Gram positive bacteria.
- The effect of the leachate on sulphate reduction by SRB was investigated at 3% and 6% concentrations, using 3% preconditioned inoculum. No biological sulphate reduction was observed in the control (leachate-free) reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachate performed better compared to other leachate treatments. Sulphate removal was comparable to literature values (27.7% to 44.9%), but the sulphate reduction rates obtained in this study (9.4 mg/l/d to 15 mg/l/d) were considerably lower. Near-neutral pH was maintained in all leachate amended reactors, unlike the controls. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.

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CHAPTER 5

GENERAL CONCLUSIONS

- The biological treatment of acid mine drainage using sulphate reducing bacteria (SRB) is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction.
- Biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by white rot fungi was investigated to enhance the biodegradability of this lignocellulose substrate. *In vitro* dry matter digestibility was used to measure the effectiveness of fungal delignification. Although the initial digestibility was high ($\pm 60\%$), none of the inoculated or control treatments increased the digestibility of the natural substrate.
- A brown leachate was produced in all treatments over the experimental period. Literature indicated that such a leachate could contain inhibitors or stimulants of bacterial growth. An antimicrobial activity assay indicated that the leachate did not inhibit aerobic Gram negative or Gram positive bacteria.
- The effect of the leachate on sulphate reduction by SRB was investigated at 3% and 6% concentrations, using 3% preconditioned inoculum. No biological sulphate reduction was observed in the control (leachate-free) reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachate performed better compared to other leachate treatments. Sulphate removal was comparable to literature values (27.7% to 44.9%), but the sulphate reduction rates obtained in this study (9.4 mg/l/d to 15 mg/l/d) were considerably lower. Near-neutral pH was maintained in all leachate amended reactors, unlike the controls. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.