Biolog for the determination of microbial diversity in activated sludge systems

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Introduction

Functional diversity, which is being measured with substrate utilization assays such as Biolog (Bochner, 1989; Garland and Mills, 1991; Haack *et al.,* 1994; Winding, 1994; Zak *et al.,* 1994; Bossio and Scow, 1995; Ellis *et al.,* 1995; Wünsche *et al.,* 1995; Guckert *et al.*, 1996; Insam *et al.*, 1996; Smalla *et al.*, 1996; Garland, 1997; Glimm *et al.*, 1997; Heuer and Smalla, 1997; Insam and Ranger, 1997; Kersters *et al.,* 1997; Engelen *et al.*, 1998; Sakano and Kekhof, 1998), can be determined in terms of the presence, absence or rate of substrate utilization (Griffiths *et al.,* 1997). Griffiths *et al.* (1997) indicated that there could be changes in microbial community structure with no changes in function, but that function was affected below a certain level of species diversity (microbial capacity). The objective was to relate the microbial capacity of the community to utilization of certain selected substrates. The hypothesis being that the more substrates utilized, the higher the diversity due to the collective action of individual species.

A specific organism will not necessarily utilize all the available substrates in a system, nor does the utilization of some of the substrates suggest that this is the complete set of substrates which the particular organism can use, due to:

- competition, which might suppress the activity of a particular organism
- dominance where one organism utilizes all the substrates in such a way that the contribution of the organisms to substrate utilization is overshadowed and goes unnoticed
- substrates might not match the metabolic activity of a specific organism (meaning that it will not show up on the analysis)
- the system might be selective, i.e. it would only allow the metabolic activities of aerobic or faculatively anaerobic, heterotrophic and copiotrophic microorganisms capable of growing at sufficient rates on the substrates
- depending on the evenness of each species, i.e. the organisms present in higher numbers will be able to utilize the carbon sources easier than organisms present in low numbers
- inoculum density has an influence on the tempo and occurrence of colour development due to the growth rates of each organism on different substrates incubation time influences substrate utilization of a community due to individual growth rates of each organism on different substrates (Wüsche *et al.,* 1995).
- antagonistic interactions might occur, where one organism inhibits another organism’s growth and therefore its ability to utilize the carbon sources and
- toxic effects of the redox dyes may inhibit the growth of some organisms (Ullrich *et al.*, 1996).

The aim is therefore not to try to detect each and every metabolic reaction of all the species in the community, but the collective utilization pattern for a specific community. Since, (1) a high species diversity should lead to a higher relative number of substrates utilized, because there are more possibilities and (2) upon dilution, some organisms will be lost (causing a decrease in species diversity) from the community, depending on their abundance and the relative contribution (perhaps only one metabolic reaction in the system), reducing the number of possibilities. The extent of the reduction of the possibilities upon dilution, should theoretically reflect something about the community structure. The key, therefore lies in the interpretation of the results.

The Biolog system unlike traditional culture-dependent methods, which are generally selective for the component of the community that has to be cultured, can reflect the activities of a broad range of bacteria (Zak *et al.,* 1994). The Biolog system is therefore not considered as a culture-dependent

method, but rather as a collection of metabolic tests (database) used for the purpose of generating a recognizable pattern for a specific community. Therefore, this technique could be useful for understanding the biological phosphorus removal phenomenon in activated sludge systems. Previous work has indicated that phosphorus removal was not as a result of a specific microbial community composition, but rather related to the total biomass in the aerobic zone of a system (Mills and Wassel, 1980; Ehlers, 1998).

Methods

Biolog plates. Biolog GN microplates (Biolog Inc., Hayward, CA) were used in this study.

Sample sources and inoculum preparation. Mixed liquor grab samples (1l) were drawn from the aerobic anoxia and anaerobic zones of the following activated sludge plants: Heidelberg (3 stage), Vlakplaats (3 stage), Tsakane (3 stage) and Hartebeesfontein (2 stage), Gauteng, South Africa (ERWAT). Chemical analyses for the anaerobic, anoxic and aerobic zones of Heidelberg, Flakplaats, Tsakane and Hartebeesfontein systems were obtained from East Rand Waterboard (ERWAT) (Table 4).

Inoculation and incubation of Biolog microplates. Biolog microplates were inoculated with undiluted and diluted activated sludge samples from Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein water works. All the microbial activated sludge suspensions were diluted with sterile saline (0.85% NaCl) and used as inoculum for Biolog GN microwell plates. Biolog GN microplate wells were inoculated with 150 |il of the activated sludge suspensions. Microplates were incubated in the dark at 21°C without agitation. Colour formation in the individual cells of the microtiter plates was measured with an Anthos reader 2001 (Anthos Labtec Instruments) at 620 nm. Readings of the microplates were made in duplicate after 24 h, 48 h and 72 h of incubation and microwells were also visually studied.

Data handling. Carbon source utilization patterns were obtained by determining the percentage carbon sources utilized after a 72 h incubation period. The number of carbon sources utilized was divided by 95 and expressed as a percentage value, which represented carbon source profiles/utilization. Data from each well was directly stored on a computer in the Bionum version 1.1 for Windows (Applied Maths, Kortrijk, Belgium) at "0" (negative) and "1" (positive) to be calculated according to the Simple matching coefficient (S_{SM}) used for determining the correlation between samples (data sets) in the Gelcompare 4.0 program. Analyses and dendrogram constructions were done with the GelCompar 4.0 program (Applied Maths, Krotrijk, Belgium). The program clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA).

Results and discussion

The dendrogram in Figure 1 can be divided into two sections. Section I and II were less than 40% similar. Section I could be divided into three groups with a 64% similarity. Group A consists of the 10^{-3} and 10^{-4} dilutions of Heidelberg, which were 86% similar and the 10^{4} dilution of Tsakane, which was 69% similar to the rest of the group. Group B contained the 10^{2} to 10^{4} dilutions of Hartesbeesfontein, which were 87% similar and the 10^{3} dilution of Vlakplaats with a 75% similarity to the rest of the group. Group C contained the 10^{-1} and 10^{-2} dilutions of Heidelberg, Vlakplaats and Tsakane being 81% similar. This indicated that there were no differences amongst these dilutions. Section II contained the 10^{-4} dilution of the Vlakplaats system.

The dendrogram in Figure 2 can be divided into two sections, which were less than 4% similar. Within Section I there were three groups which were 59% similar. Group A contained the 10^{-1} and 10^{-2} dilutions of Heidelberg with a 93% similarity and the 10^{-3} dilution of Heidelberg being 69% similar to the rest of the

group. Group B contained the 10^{-1} and 10^{-2} dilutions of Tsakane and the 10^{-1} and 10^{-2} dilutions of Vlakplaats with a 79% similarity. Group C contained the 10^{-3} and 10^{-4} dilutions of Tsakane with a 73% similarity.

Section II contained the 10^{-3} dilution of Vlakplaats (62% similar to the rest of section) and both the 10^{-4} dilutions of the Vlakplaats (VLKP) and Heidelberg (HDLB) activated sludge systems with a 98% similarity, which indicated that these two dilutions were closely related.

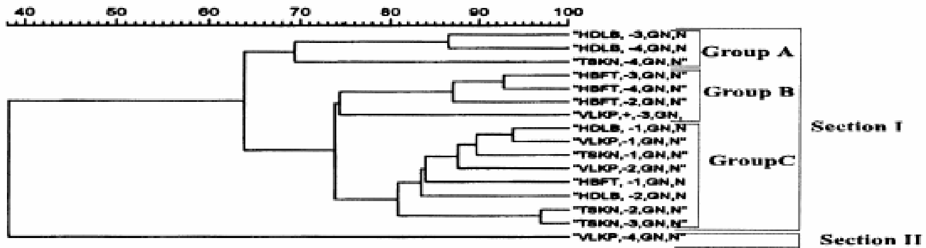

Figure 1 Dendrogram indicating the microbial community structure of the anaerobic zone (N) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) water works

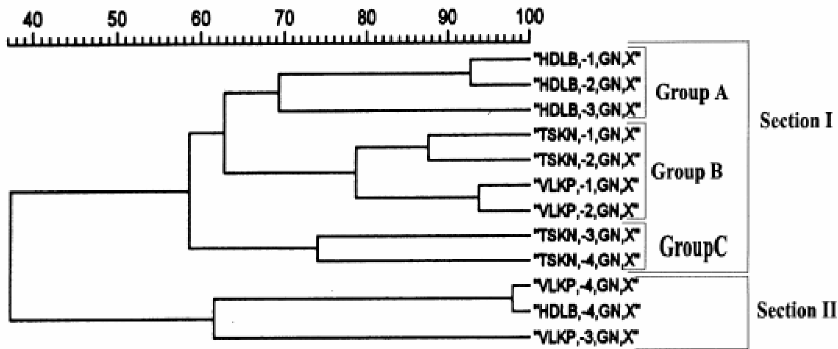

Figure 2 Dendrogram indicating the microbial community structure of the anoxic zone (X) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) water works

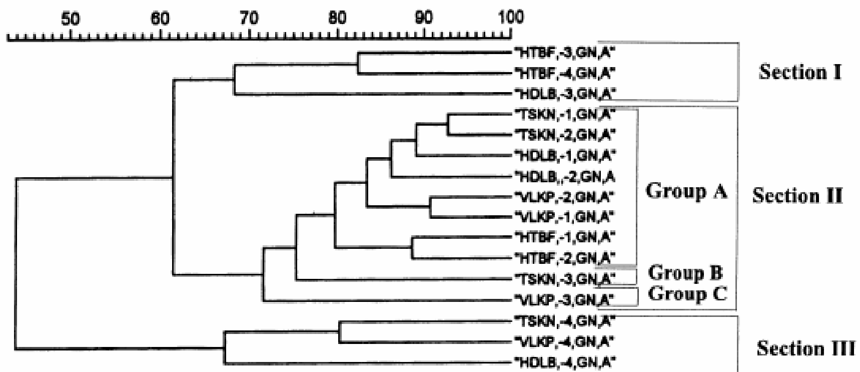

Figure 3 Dendrogram indicating the microbial community structure of the aerobic zone (A) of the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) water works

Table 1 Comparison of similarity amongst the dilutions of the anaerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) activated sludge system inoculated into Biolog GN microplates

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	95%	65%	85%
	VLKP	95%	100%	65%	85%
	TSKN	65%	65%	100%	65%
	HBFT	85%	85%	65%	100%
10^{-2}	HDLB	100%	84%	81%	74%
	VLKP	84%	100%	81%	75%
	TSKN	81%	81%	100%	75%
	HBFT	74%	75%	75%	100%
10^{-3}	HDLB	100%	64%	64%	64%
	VLKP	64%	100%	74%	75%
	TSKN	64%	74%	100%	74%
	HBFT	64%	75%	74%	100%
10^{-4}	HDLB	100%	38%	64%	64%
	VLKP	38%	100%	38%	38%
	TSKN	64%	38%	100%	74%
	HBFT	64%	38%	74%	100%

The dendrogram in Figure 3, could be divided into three sections. Sections I, II and III were less than 50% similar. Section I contained the 10^{-3} and 10^{-4} dilutions of the Hartebeesfontein system, with an 82% similarity and the 10^{-3} dilution of the Heidelberg system being 68% similar to the rest of the section. Section II can be divided into three groups: group A contained all the 10^{-1} and 10^{-2} dilutions of the four activated sludge systems with an 80% similarity. Group B contained the 10^{-3} dilution of Tsakane, which was 75% similar to group A. Group C contained the 10^{-3} dilution of Vlakplaats being 72% similar to group A and B. Section III contained the 10^{-4} dilutions of Vlakplaats and Tsakane, which were 82% similar to each other and the 10^{-4} dilution of Heidelberg, which was 67% similar to the latter.

Table 2 Comparison of similarity between the dilutions of the anoxic one within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) activated sludge system inoculated into Biolog GN microplates

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	63%	63%	NA
	VLKP	63%	100%	79%	NA
	TSKN	63%	79%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-2}	HDLB	100%	63%	63%	NA
	VLKP	63%	100%	79%	NA
	TSKN	63%	79%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-3}	HDLB	100%	37%	59%	NA
	VLKP	37%	100%	37%	NA
	TSKN	59%	37%	100%	NA
	HBFT	NA	NA	NA	NA
10^{-4}	HDLB	100%	98%	38%	NA
	VLKP	98%	100%	38%	NA
	TSKN	38%	38%	100%	NA
	HBFT	NA	NA	NA	NA

NA = Hartebeesfontein (HBFT) does not have an anoxic zone

Table 3 Comparison of similarity between the dilutions of the aerobic zone within the Heidelberg (HDLB), Vlakplaats (VLKP), Tsakane (TSKN) and Hartebeesfontein (HBFT) activated sludge system inoculated into Biolog GN microplates

Dilutions		HDLB	VLKP	TSKN	HBFT
10^{-1}	HDLB	100%	84%	89%	79%
	VLKP	84%	100%	84%	79%
	TSKN	89%	84%	100%	79%
	HBFT	79%	79%	79%	100%
10^{-2}	HDLB	100%	84%	84%	79%
	VLKP	84%	100%	83%	79%
	TSKN	84%	83%	100%	79%
	HBFT	79%	79%	79%	100%
10^{-3}	HDLB	100%	61%	61%	68%
	VLKP	61%	100%	71%	61%
	TSKN	61%	71%	100%	61%
	HBFT	68%	61%	61%	100%
10^{-4}	HDLB	100%	68%	68%	43%
	VLKP	68%	100%	80%	43%
	TSKN	68%	80%	100%	43%
	HBFT	43%	43%	43%	100%

Our hypothesis was that differences in microbial community structure in activated sludge systems may exist, but that this had no bearing on the effectiveness with which these systems removed phosphate. If PO_4^{3-} removal was related to the microbial community composition, a high correlation of the Biolog patterns would be expected amongst PO_4^{3-} removing systems. However, this was not the case in our study. All the different zones of systems tested indicated a high initial diversity (10^{-1} to 10^{-2} dilutions), due to the high number of substrates utilized. No specific patterns could, however be identified for PO_4^{3-} removing systems, indicating that PO_4^{3-} removal was not community structure specific. This agrees with previous studies (Dold *e/a/.*, 1980; Kersters *e/a/.*, 1997).

The results in Table 4 indicate that effective COD removal, effective nitrification and a sufficient MLSS concentration in the aerobic zone were required for effective PO_4^{3-} removal.

Table 4 Chemical analysis of the Heidelberg, Vlakplaats, Tsakane and Hartebeesfontein activated sludge water system at the time of sampling obtained from ERWAT

System	pH	COD mg.1^{-1}	NH_3/N mg.1^{-1}	NO_3/ NO_3 mg.1^{-1}	PO_4P mg.1^{-1}	MLSS mg.1^{-1}
Heidelberg						
Anaerobic	7.4	102	12.7	0.1	8.7	ND
Anoxic	7.5	98	0.4	14.8	0.3	ND
Aerobic	7.5	10	0.2	14.2	0.1	5862
Vlakplaats						
Anaerobic	7.4	ND	14.7	1.4	9.9	ND
Anoxic	7.4	ND	8.6	1.5	7.9	ND
Aerobic	7.4	ND	1.4	6.1	5.2	33.7
Tsakane						
Anaerobic	6.9	77	17.0	ND	9.7	ND
Anoxic	7.1	44	18.8	ND	7.9	ND
Aerobic	7.1	83	10.6	6.2	0.1	4166
Hartebeesfontein						
Anaerobic	ND	ND	19.7	0.4	2.7	962
Aerobic	ND	ND	19.6	1.5	4.3	271

ND = Not determined

This was in agreement with previous studies (Buchan, 1980; Bosch, 1992; Schwieger and Tebbe, 1998). This also suggested that the biomass concentration was more important for effective PO_4^{3-} removal than the specific microbial community structure, as was previously indicated (Mills and Wassel, 1980; Kersters *et al.*, 1997).

Our results support the notion that biomass concentration is more important in PO_4^{3-} removal (Bosch, 1992; Momba, 1995; Muyima, 1995), than community composition. In terms of PO_4^{3-} removal, research should focus on the role of biomass concentration and its relationship to PO_4^{3-} removal and methods to determine the biomass accurately. Future research on using Biolog for community-level carbon source utilization patterns should focus on a range of diverse environments including, for example, hot water springs, rivers, etc. in order to further validate the method.

Conclusions

- Metabolic profiles of different zones in different water treatment systems reflected a pattern resembling unevenness and a high microbial diversity.
- PO_4^{3-} removal was not community structure specific.

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