Bacterial Species Diversity as an Indicator of Biocide Efficacy

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Abstract

Biological control of microbiology micro-organisms in water systems is conventionally controlled by the use of biocides and biodispersants. There is, however, no simple means of determining the efficacy of the chemical dosage. At present this is conducted by complex bacterial culturing in an analytical microbiology laboratory.

The concept of survival of the fittest is not new and can be readily applied to the bacterial community. If a stress is applied to a bacterial environment it is therefore likely that the numbers and bacterial species diversity will change.

Biolog Ecoplates® have been used to detect changes in bacterial population in waste water plants, natural rivers and soils. These plates comprise of 31 different carbon substrates, repeated in triplicate, on a microtitre plate. Once the sample is added to the wells, the bacteria capable of metabolising the carbon source respire the substrate and cause the water in the well to turn purple. This is indicative of a positive result.

It is hypothesized that the addition of a biocide to an aquatic environment should cause the resident bacterial population to change both in terms of numbers and species diversity.

1. Introduction

South African power plants are mostly wet cooled, obtaining their make-up water from various surface water sources (dams and rivers) (Pather, 2004). The quality of water, in these rivers, is on a downward trend, due to increasing pollution, which necessitates chemical treatment of this water (Pather, 2004). Treatment includes the dosing of biodispersants and biocides to minimise and control the microbiological population within the system (Gericke, 2002).

Although several biocides are utilised throughout the South African power generation industry, Isothiazalone and Dibromonitrilopropionamide (DBNPA) are the most common. These biocides are usually applied in an alternating pattern so as to avoid possible development of biocidal resistance (Eskom Standard).

Historically, microbiological control dosing was conducted on a two weekly cycle with a continuous biodispersant dosage. Biocide efficacy testing was completed by conducting classical plate counts on the planktonic population in the cooling water (Eskom Standard).
However, many bacterial species are not culturable by classical microbiology means, thus an inaccurate indication of the bacterial population in the treated water is possible (Hill et al., 2000).

DBNPA is an inert, fast acting, non-oxidising biocide that inactivates enzymes, destroying metabolic pathways responsible for energy production and transfer of wastes and nutrients. It is neutral and degrades to relatively harmless by-products viz. carbon dioxide, ammonia and bromide ions (Exner et al. 1973). Due to its lack of persistence it is most often used as an alternate biocide. It has an unpleasant smell and becomes deactivated above pH 8.5 or in the presence of sulphide or sulphite contamination (Huber et al., 2010).

Biolog Ecoplates® have been successfully used to monitor changing populations in water (Lekhanya, 2010), sewage (van Heerden, 2002) and soil (Stephan et al., 2000). Different bacterial species use various carbon substrates for nutrition. If the bacterial species, in a certain environment, are killed or altered by the addition of a stress to the environment (biocide), the number of substrates utilised should decrease or change also (Brözel and Cloete, 1993).

This is not necessarily linked to the bacterial count in the environment i.e. although the bacterial count in the environment may decrease, species diversity does not have to follow suit. There may be a few individuals of each species present. Bacteria, like all organisms, are affected by stresses on their environment (Nannipieri et al., 2003). It is theorised that the addition of a biocide (stress) to the environment should decrease the number of species present – the species diversity. Dosage concentration and the type of chemical dosed should also affect this decrease.

2. Materials and Methods

A 500 litre cooling water system rig simulator (CW Rig) (Figure 1) was used to evaluate changes in bacterial species diversity after an addition of DBNPA biocide at concentrations of 8mg/L and 20mg/L. The CW rig is filled with cooling water, from an operational power plant, and the water level is maintained by stopcock to ensure that any water losses incurred, either by evaporation or windage, are made up.

The water in the CW rig is allowed to circulate, without treatment, for 3 days prior to any chemical dosage. Ten millilitres (20mg/L) or 4ml (8mg/L) of DBNPA biocide was added after taking an initial pre dose sample (time 0). Samples were then taken after 15 and 30 minutes, and 1, 2, 3, 4 and 6 hours post dosing. The classical microbiology and Biolog Ecoplate® analyses were completed by Eskom RT&D Microbiology and the molecular work submitted to MicroSci Consulting.
Total aerobic bacteria plate counts were conducted by completing ten fold serial dilutions in sterile Ringers solution. One millilitre of each dilution was then aseptically transferred into a sterile 90mm petri-dish. Approximately 15ml of cooled, molten Plate Count Agar was added to the petri-dish. The petri-dish was swirled gently to mix the sample into the agar and was then allowed to set.

The petri-dishes were then incubated, inverted, at 37°C for 48 hours. All colonies that formed on the agar were counted, and this count was multiplied by the dilution factor of the initial dilution in order to determine the final count.

Substrate Utilisation – Biolog Ecoplates®

One hundred and fifty microlitres of, tenfold serial dilution samples was added to each of the 96 wells on the Biolog Ecoplate®, containing a triplicate set of 31 carbon substrates, with a blank in each set. The Biolog Ecoplates® were then incubated at 35°C and examined after 24 and 48 hours. Any purple colouration, regardless of intensity, was marked and counted as a positive reaction. These reactions were logged in a binary fashion and used to construct a digital graphic image of the plates for statistical analysis.

Molecular Analysis

Three hundred millilitres of sample was filtered through a 0.45μm sterile filter which was aseptically transferred into 20ml of sterile saline solution.

DNA extraction - bead beating

Total DNA was extracted using the BIO101 Fast DNA Spin kit (Soil) (Qbiogene Molecular Biology products, Pretoria, South Africa).
One gram/1ml of sample was added to the Lysing Matrix E tube. Sodium Phosphate Buffer (978 µl) and MT Buffer (122 µl) were added, the tube vortexed for 30 s then centrifuged at 14 000 rpm for 10 min.

The supernatant was transferred to a clean tube, 250 µl PPS reagent was added and mixed by inversion. The suspension was then centrifuged for 5 min at 14 000 rpm to pellet the precipitate. The supernatant was transferred to a clean tube and 1ml of Binding Matrix suspension added.

After settling, approximately 500 µl of supernatant was removed and discarded. The Binding Matrix was then resuspended in the remaining supernatant. 600 µl of the suspension was added to a SPIN™ Filter and centrifuged at 14000 rpm for 1 minute. The catch tube was emptied and the remaining supernatant added to the SPIN™ Filter and recentrifuged.

Five hundred microlitres of SEWS-M was added to the SPIN™ Filter and centrifuged at 14 000 rpm for 1 minute. The flow-through was decanted and the SPIN™ Filter replaced in the catch tube. The pellet was centrifuged at 14000 rpm for 2 minutes to dry the matrix.

The SPIN™ Filter was placed in a fresh Catch tube and air dried for 5 minutes at room temperature. Fifty µl DES (DNase/Pyrogen Free water) was added to the matrix and gently stirred with a pipette tip. The resuspension was centrifuged at 14000 rpm for 1 minute to transfer the eluted DNA to the catch tube.

**PCR amplification**

A portion of the bacterial 16S rRNA gene was amplified by means of PCR using K and M primers

- **K (PRUN518R)** : 5’ATT-ACC-GCG-GCT-GCT-GG3’ (Siciliano et al. 2003)
- **M (pA8f-GC)** : 5’CGC-CCG-CCG-CCG-GCG-GGG-GCG-GGA-GCA-CGG-AGG-AGT-TTG-ATC-CTG-GCT-CAG3’ (Fjellbirkeland et al. 2001)

A reaction with no template DNA was included as a negative control. Each PCR tube contained a total volume of 20 µl: 10.8 µl sterile SABAX water, 2.5 µl PCR buffer (10x), 2 µl MgCl₂ (25mM), 2 µl dNTPs (2.5 µM), 1 µl primer K (50 µM), 1 µl primer M (50 µM), 1 µl template DNA (27 ng/µl), 0.2 µl Taq polymerase (5 U/µl). Prokaryotic DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, followed by 10 min at 72°C, and then held at 4°C. PCR product was analysed on a 1.5 % TAE agarose gel.

**DGGE**

PCR products were subjected to DGGE according to the method described by Muyzer et al. (1993). Ten microlitres (ca. 250 ng) of each PCR product was loaded per lane onto a 25-55% urea/formamide denaturing gradient gel. Gels were run at 70V for 17hrs at a constant temperature of 60°C. Image analysis was performed using the Gel2K programme (Norland 2004). The fingerprint comparisons were analysed using CLUST (Norland 2004). Dominant bands were compared and analysed to determine population diversity. Several comparison options are available but Jaccard and Simple indices (matchings) were used in this study.
These comparisons differ in how they compare the results obtained. Simple matching involves the comparison of the number (diversity) of species within a sample (DGGE lane) in comparison to other samples (lanes). Jaccard matching (Jaccard index/similarity coefficient) is a statistical comparison of similarity and diversity of sample sets. It is defined as the size of the intersection divided by the size of the union of the sample sets. (Wikipedia)

3. Results and discussion

Substrate utilisation and classical microbiology

The total aerobic bacterial count and the Biolog Ecoplate® substrate utilisation results are given in Figures 2 and 3 for the 20mg/l and 8mg/L trials respectively. In both cases the planktonic count decreased by log 3, however, the change in the before and after dosage counts were greater and achieved more rapidly in the 20mg/l trial than in the 8mg/l trial. This stands to reason due to the increased concentration of the active ingredient dosed. The higher dosage also maintained the low counts for a longer period and slowed the relative increase in growth over the 48 hour trial.

The lack of carbon substrate utilisation at 15min, 30min and 1 hour after the 20mg/l dose (Figure 2), is indicative that the bacterial species, capable of utilising the substrates were all eliminated. There were however, a small number of bacteria still present in the system at these times. The lack of substrate utilisation during test times indicated that the Biolog Ecoplate® was not sensitive enough to detect these low bacterial numbers or possibly that the substrates tested on the plates did not include other energy sources present that bacteria had access to in the cooling water (a variable composition medium). It may also be due to the high concentrations of biocide acting as a bacteriostat on the remaining bacteria in the system. After 2 hours, when the concentration of the biocide active ingredient began to reduce, the static and still present bacteria again began to metabolise and multiply, showing a rapid increase in the total aerobe count and substrate utilisation.

Carbon substrate utilisation results for the 8mg/l trial (Figure 3) were extremely erratic, indicating that, although the DBNPA was effective at reducing the planktonic counts, there were still a variety of bacterial species remaining within the few individuals, present in the water that were capable of utilising the various carbon sources.

While the substrate utilisation returned to the initial, time zero, point within 48 hours during the 20mg/l DBNPA trial, the results in the 8mg/l DBNPA trial initially decreased slightly although the number of substrates did not decrease completely and fluctuated throughout the trial. This indicates that, due to the decreased numbers, there were changes in the bacterial species diversity, possibly due to the availability of excess nutrients (fresh make up water and remnants of dead microbes).

Tables 1 and 2 show substrate utilisation, over the duration of the DBNPA trial, for the 20mg/l and 8 mg/l trials, respectively. In order to generate a statistical comparison between the substrate utilisation at each time, simulated gel diagrams were developed for analysis with Gel2K.
Figure 2: Biolog Ecoplate® substrate utilisation versus aerobic bacterial counts for the duration of the 20mg/L DBNPA trial.

Figure 3: Biolog Ecoplate® substrate utilisation versus aerobic bacterial counts for the duration of the 8mg/L DBNPA trial.
Table 1: Biolog Ecoplate® substrate utilisation over the duration of the 20mg/L DBNPA trial

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Table 2: Biolog Ecoplate® substrate utilisation over the duration of the 8mg/L DBNPA trial.

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Figures 4 and 5 show the Gel2K statistical relationships (simple matching, group average) between the different substrate utilisation, at the various times, throughout the 20 mg/l and 8 mg/l trials respectively. These figures show the percentage differentiation (scale on the left) between the samples at different times. Figure 4 indicates that the samples taken after 15 and 30 minutes, 1, 2 and 3 hours are 60% different to the samples from time 0 and time 48hrs. It is interesting to note that the samples after 4, 6 and 24 hours are also separated from the time 0 and 48hrs but not to the same extent as the earlier samples.

The results indicated in Figure 5, however, display a completely different scenario. Species diversity between time 0 and 48 hours are statistically 40% different. The samples after 15 minutes and 1 hour align with the 48 hour sample, while the other samples align more closely with the time 0 sample. This indicates that there is little effect on the species diversity when the lower concentrations (8mg/l) of DBNPA are dosed. There is normal variation of the cooling water system. The change in the species diversity after 48 hours may be indicative of the more dominant bacterial species becoming more prevalent in the system through metabolic competition.
Figure 4: Gel2K statistical relationship between the different substrate utilisation at the various times throughout the 20 mg/l DBNPA trial.

Figure 5: Gel2K statistical relationship between the different substrate utilisation at the various times throughout the 8 mg/l DBNPA trial.
Molecular analysis

Figure 6 shows the computerised gel pattern for Gel2K analysis related to the 20 mg/l trial molecular DGGE comparison and Figure 7 shows the Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 20 mg/l trial. Figure 7 indicates that the species present in the time 24 and 48 hr samples are 80% different from those present in the remainder of the trial. The species in the time 0 and 15 min samples are slightly (45%) different from the species detected in the other early trial samples.

Figure 6: Computerised gel pattern for Gel2K analysis related to the 20 mg/l DBNPA trial molecular DGGE comparison

Figure 7: The Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 20 mg/l DBNPA trial

Figure 8 shows the computerised gel pattern for Gel2K analysis related to the 8 mg/l trial molecular DGGE comparison and Figure 9 shows the Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 8 mg/l trial. Figure 9 indicates that the species present in the 24 and 48 hr samples are the most different from the time 0 sample although this difference is only 45 and 40% respectively. There is no logical progression of the species variation in this trial. This means that although there is a decrease in the bacterial counts in the samples, the species diversity is not changing significantly.
Figure 8: Computerised gel pattern for Gel2K analysis related to the 8 mg/l DBNPA trial molecular DGGE comparison

Figure 9: The Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 8 mg/l DBNPA trial

4. Conclusion

The results of this trial show that dosing the correct concentration of biocide does cause the desired changes in the bacterial population, by decreasing the numbers of bacteria in the system. It also indicates that the species diversity (indicated by substrate utilisation) is significantly affected and can be used to predict the biocide efficacy.

The comparison between the results for the 20 and 8mg/l trials shows that the Biolog Ecoplate® could identify the biocide efficacy at a higher, more effective dosage but did not correlate with the 8mg/l dose. This may be due to a less effective concentration of active ingredient being present.
This means of determining biocide efficacy can therefore only be utilised when the biocide is being dosed at optimal levels for the system being tested. It is therefore essential that the supplier's recommendations be correctly followed.

The effect of other biocides used in cooling water treatment plants must be evaluated, and the accuracy tested on an operational plant. This will determine which other external factors may affect the biocidal efficacy.

5. References


