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Catherine D. Luyt, S. M. M. Khamanga, W. J. Muller, Roman Tandlich*

Survival of Bifidobacteria and their usefulness in Faecal Source Tracking

Abstract: Bifidobacteria have long since been recommended as indicators of human and animal pollution. Concentration ratio (tracking ratio) of the sorbitol-utilising bifidobacteria (SUB) and the total bifidobacteria (TB) can be used to distinguish between animal and human sources of faecal water contamination. The cut-off value needs to be calibrated in a given geographical area. Seven sites with permanent faecal contamination were selected in South Africa. Concentrations of SUB ranged from 10-50000 cells/100 mL, while TB ranged from 0-8000 cells/100 mL. The tracking ratio ranged from 0.10 to 6.25, but no clear cut-off value could be established. The YN-17 agar was replaced for TB with the modified Beerens medium with pH = 5.70, to suppress the growth of faecal streptococci. Tracking ratios observed are most likely the results of different survival rates of SUB and TB. Bifidobacteria die-off due to nutrients was not found to be significant using design of experiment. Thus a lack of continuous input or oxygen levels in water may be major factors. This would limit the ratios used as a faecal source tracking method.

Keywords: bifidobacteria, faecal source tracking, water quality

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Abbreviations:

B. bifidum - *Bifidobacteria bifidum*
CFU - Colony Forming Units
CO_2 - Carbon dioxide
SUB - Sorbitol Utilising Bacteria
°C - Degrees Celcius
E. coli – *Escherichia coli*
HCl - Hydrochloric acid
HBSA– Human Bifid Sorbitol Agar
L- litre
MBM – Modified Beerens Medium
mg - milligram
mL - millilitre
MRS agar - de Man, Rogosa and Sharpe Agar
NaOH - Sodium Hydroxide
ND - Not determined
OG - overgrown membrane filter
PCR - Polymerase chain reaction
TB - Total Bifidobacteria
NMMP - South African National Microbial Monitoring Programmes for surface water
TTC - all dilutions made were overgrown thus accurate counts could not be established

1 Introduction

Many people in South Africa still lack access to safe drinking water. This is due to pipe breaks, water shortages or lack of infrastructure (Lewin et al., 2007, PMG, 2009, Luyt et al., 2012). Some alternative water sources include boreholes, springs and rivers. The lack of sanitation can increase faecal pollution in alternative water sources. Screening rivers and alternative water sources for microbial concentrations is essential to decrease diarrhoeal diseases. The South African National Microbial Monitoring Programmes for surface water (NMMP) is designed to monitor microbial water quality in areas which are highly prone to faecal contamination using the concentration of *E. coli*, pH and turbidity (Murray et al., 2004). The NMMP

***Corresponding author: Roman Tandlich:** Environmental Health and Biotechnology Research Group, Division of Pharmaceutical Chemistry, Faculty of Pharmacy, Rhodes University, South Africa, E-mail: roman.tandlich@gmail.com
Catherine D. Luyt: Environmental Health and Biotechnology Research Group, Division of Pharmaceutical Chemistry, Faculty of Pharmacy, Rhodes University, South Africa
S. M. M. Khamanga: Division of Pharmaceutics, Faculty of Pharmacy, Rhodes University, South Africa
W. J. Muller: Amatole Waterboard, East London, South Africa

is unable to identify faecal contamination sources, while up to 30 % of surface water resources are excluded from any form of regular monitoring of microbial water quality (Rivett et al., 2009). The use of faecal source tracking methods could increase the information about the source of the pollution and help identify the public health risk of the water body (Hagedorn et al., 2011). Especially as human faecal pollution is more likely to contain human pathogens than animal contamination (Sinton et al., 1998).

Source tracking is a process of identifying the source faecal contamination in a water source (Scott et al., 2002, Meays et al., 2004, Ahmed et al., 2007, Cimenti et al., 2007, Hagedorn et al., 2011, Tandlich et al., 2012). Many different methods have been proposed. South Africa needs a low cost method with minimal equipment, which does not require highly skilled technicians (Luyt et al., 2012). Two methods proposed for South Africa are bacterial antibiotic resistance and the tracking ratio of bifidobacteria. Antibiotic resistance has been proposed, however it still needs to be calibrated in South Africa. The attempt to calibrate and identify problems with bifidobacteria tracking ratio will be looked at here. First a short summary of the available literature data on the bifidobacteria in faecal source tracking are provided below.

Bifidobacteria have been proposed as a relatively cost-effective library independent method for source tracking (Mara and Oragui, 1983, Carrillo et al., 1985, Jagals and Grabow, 1996, Blanch et al., 2006, Bonjoch et al., 2009, Ballesté and Blanch, 2011). Bifidobacteria are anaerobic and Gram-positive members of the microflora of the human and agricultural animal intestines (Bonjoch et al., 2005). These bacteria are excreted in substantial concentrations in the faeces of these organisms and are present in a water sample if there is faecal contamination (Blanch et al., 2006). Multiplication in the water environment is unlikely as optimally growth temperaturesare between 37 and 38 °C in humans and higher in animals (Ballongue, 2004, Wilson, 2005). Conflicting reports have reported growth between 20 and 49.5°C (Ballongue, 2004, Wilson, 2005). In South Africa, surface water temperatures seldom reach 30°C (Carrillo et al., 1985, Sinton et al., 1998, Nebra et al., 2002). Their growth in river or oligotrophic environments would be further limited by their nutritional requirements. These include ammonium salts or organic nitrogen and for *B. bifidum* requires magnesium, manganese and iron (Ballongue, 2004).

Enumeration of bifidobacteria is cost-effective and requires minimum labour intensity, i.e. competent personnel can be trained quickly for analyses (Blanch et al., 2006). The majority of laboratories in South Africa can do microbiological analysis, thus a bacterial approach is more likely to be accepted than tracking based on viral or chemical methods and have accessible laboratories. Still the number of accredited laboratories in South Africa who are approved to conduct enumeration of indicator bacteria is small due to cost constraints and other factors (Balfour et al., 2011). The NMMP laboratories are designed for *E. coli* enumeration, thus with minimal modification bifidobacteria can be enumerated. This makes the process more feasible. However it should be noted that the media used to enumerate the bifidobacteria is very complex and contains multiple antibiotics which could inhibit stressed bacteria and has the potential to hindered growth. The other problem is the selectivity of the media, which will influence the rate of false positives and overgrowth by other bacteria.

Faecal source tracking using bifidobacteria is based on the enumeration of sorbitol-fermenting bifidobacteria (SUB) and total bifidobacteria (TB) (Blanch et al., 2006). Animal and human sources of faecal contamination are distinguished based on the ratio of the two concentrations in a water sample (referred to as tracking ratio in further text; Blanch et al., 2006). There is always a cut-off point, which for Bonjoch et al (2005) was 0.2, while for Blanch et al (2006) was 3.2. Thus a tracking ratio below the cut-off point indicates animal sources of the faecal contamination, while higher values indicate human source (Bonjoch et al., 2005; Blanch et al., 2006).

The tracking ratio is the ratio of sorbitol utilising bifidobacteria (SUB) to the total bifidobacteria (TB) concentrations and is calculated as shown in equation 1.
Equation 1:

$$\textbf{Tracking Ratio} = \frac{[\textbf{Sorbitol Utilising bifidobacteria (SUB)}]}{[\textbf{Total Bifidobacteria (TB)}]}$$

(Bonjoch et al., 2005)

Using this information and an inventory of the potential contamination sources in a given area, the most likely one can be identified. Calibration is required in each geographical region due to changes in the cut-off values (Blanch et al., 2006).

Survival experiments have been performed by a couple of different authors, but all seem to have different values, depending on the water used for the survival experiment, e.g. sterilised ground water, surface water and in phosphate buffered saline; the conditions and enumeration media (Gyllenberg et al., 1960, Resnick and Levin, 1981, Carrillo et al., 1985). The most recent study by Ottoson (2009) found the die-off rate to be 3.9 CFU/ mL/day in surface water at 22°C, and 1.4 CFU/ mL day at 4°C. This was higher than the die-off rate of *B. adolescentis* reported

by Resnick and Levin (1981). The effect of changing the chemical constituents or concentrations of them in river/surface water has not been studied in detail to date. Most studies just test survival in ground water or river water in a particular area, however the chemical parameters of the water is not detailed. Thus an identification of possible chemical parameters which may influence the survival rates in different environments was undertaken. This would help the survival rate to be adjusted for different rivers.

Data from dairy product survival experiments have alluded to the bifidobacteria being sensitivity to pH, redox potential and oxygen concentrations(Shah et al., 1995, Shah and Lankaputhra, 1997, Hansen et al., 2002, Wang et al., 2002, Bolduc et al., 2006, Jayamanne and Adams, 2006, Jayamanne and Adams, 2009, Saarela et al., 2010). This was taken into account when designing the model water.

2 Methods

Bifidobacteria were isolated from the rivers in Grahamstown and grown overnight in tryptic soy broth (Sigma, Johannesburg, South Africa), with reduced oxygen by nitrogen being bubbled through it before being autoclaved at 37°C in a Shaker (Labcon shaking water bath, Laboesign engineering Pty, Maraisburg, South Africa). The inoculum was placed into model water.

2.1 Model water

The survival experiments were based on factorial design and thus the model water was adapted to represent all the experiments proposed by the DOE++ program. The model water contained sodium sulphate (0.06416 g/L), glucose (0.0285 g/L), calcium carbonate, sodium chloride, potassium phosphate, sodium nitrate and bovine serum albumin and hydrochloric acid (HCl) or sodium hydroxide (NaOH) were used to adjust the pH. The concentrations varied according to the run number of the experiment as shown in Table 1.

The basic model water contained glucose (5.7 mg GE/L), sodium sulphate (43 mg/L sulphate), ammonium chloride (1.08 mg/L ammonium), and then protein (Bovine serum albumin), sodium nitrate, potassium dihydrogen phosphate sodium chlorides and sediments (Pool filter sand) was added and the pH modified and temperature at which it was incubated set according to experiment design tables (). The basic model water is shown in Table 1. Inclusion of glucose was to provide a sugar source for the bacteria. Sorbitol was also added to the model water. The sediment (particulate matter) was represented by course pool filter sand (100 g) (Sparrow Pools, Grahamstown, South Africa) which was washed twice in water and dried before being added to the model water. Calcium carbonate was added to some of the model waters especially for the enterococci and bifidobacteria survival experiments.

Table 1: Initial concentrations of nutrients did not change during the different experiment runs.

Parameter	Basic Model water
Turbidity (NTU)	23
Salinity (mS/m)	82
DO (mg/L)	6.01
Sulphates (mg/L)	43
Glucose (mg GE/L)	5.7
Sorbitol (mg/L)	4
NH4+ (mg/L)	1.08
TSS (mg/L)	0

Model water was prepared using solutions of the salts and then water added to MilliQ water (800 mL). The MilliQ water is prepared using a MilliQ RO® purification system (Millipore Co., Massachusetts, United States of America).

2.2 Survival experiments

The survival experiments were performed in Schott bottles, covered with foil. The model water was autoclaved for 15 minutes (Model RAU-53Bd REX MED, Hirayama Manufacturing, Tokyo, Japan). The solution was allowed to cool to room temperature before bacteria were inoculated into the water. The model water was swirled twice a day and the lid opened once a day to allow oxygen replacement under laminar flow (Model LA1200 BII;Labeair laboratory and air purification systems cc, Midrand, South Africa). The bottles were either stored at 8°C in a fridge or at 32°C in an Labcon incubator (Model FSIM B; Labmark, Johannesburg, South Africa) covered in foil to exclude sunlight.The model water was analysed at 0, 1, 3, 12, 24, 48, 72 hours and in regular intervals until the end of the experiment.

2.3 Experiment design

The design of factorial or fractional factorial models were performed using DOE++ version 1.0.7. (ReliaSoft Corparation, 2011). The survival rate of bifidobacteria under these different chemical and environmental conditions were determined in a factorial design (5 ×2 ×1) and then fractional factorial design (5×2). The factorial design had two storage temperatures (8°C, 32°C), pH (5.3, 9.85), protein (0, 140mg/L), nitrate (0, 6.8mg/L) and chlorides (0, 302 mg/L) were used. The ranges were

chosen from physical chemical water quality data in the surrounding rivers and literature (Zuma, 2010, Tandlich et al., 2012). All experiments were done in duplicate and the average recorded. The designed experiments are shown in Table 2, Table 3 and Table 4. Table 3 shows the coding of the experimental runs.

Table 2: The factorial design of bifidobacteria survival experiments in model water.

Run Number		pH	Temperature (°C)	Nitrates (mg/ L)	Chloride (mg/ L)	Protein (mg/ L)
24	1	9.8	32	6.8	0	140
22	2	9.8	8	6.8	0	140
21	3	5.3	8	6.8	0	140
26	4	9.8	8	0	302	140
14	5	9.8	8	6.8	302	0
13	6	5.3	8	6.8	302	0
20	7	9.8	32	0	0	140
1	8	5.3	8	0	0	0
5	9	5.3	8	6.8	0	0
15	10	5.3	32	6.8	302	0
16	11	9.8	32	6.8	302	0
8	12	9.8	32	6.8	0	0
17	13	5.3	8	0	0	140
28	14	9.8	32	0	302	140
27	15	5.3	32	0	302	140
18	16	9.8	8	0	0	140
23	17	5.3	32	6.8	0	140
32	18	9.8	32	6.8	302	140
12	19	9.8	32	0	302	0
6	20	9.8	8	6.8	0	0

Table 3: The fractional factorial design for the bifidobacteria survival experiments after calcium carbonate addition for pH stabilisation.

Experiment number	Run Order	X_1	X_2	X_3	X_4	X_5	X_6	X_7
8	2	-1	-1	-1	1	1	1	-1
7	4	-1	-1	1	1	-1	-1	1
6	7	-1	1	-1	-1	1	-1	1
5	6	-1	1	-1	1	-1	1	-1
4	1	1	-1	-1	-1	-1	1	1
3	8	1	-1	-1	1	1	-1	-1
2	3	1	1	1	-1	-1	-1	-1
1	5	1	1	1	1	1	1	1

Table 4: The coding and real values of the factorial factors for the pH stabilised experiment.

Factors	Levels	
	-1	+1
X_1 A:pH of the solution adjusted with NaOH or HCl	5.3	9.8
X_2B:Temperature incubated at in degrees Celsius	8	32
X_3C:Nitrates concentrations of the model water in milligrams per litre	0	6.8
X_4D:Chlorides concentrations of the model water in milligrams per litre	0	302
X_4E:Phosphate concentrations of the model water in milligrams per litre	0	1
X_5F:Protein concentrations of the model water in milligrams per litre	0	140
X_6 G:Sediment as washed and sterilised pool sand per litre	0	5 g

2.4 Statistical analysis

The data were analysed by the Pareto Chart and the analysis of variance (ANOVA) was applied to examine the statistical significance of factors. A level of the probability of 0.1 was considered a statistically significant. Fractional factorial experiment design using DOE++ version 1.0.7 was applied for statistical analysis (ReliaSoft Corparation, 2011).

2.5 Enumeration of bifidobacteria

The samples were serially diluted using quarter-strength Ringer's solution. The bifidobacteria were plated onto HBSA agar (Mara and Oragui, 1983) and Beerens media (MBM) (pH increased to 5.7 by decreasing the NaOH concentration) (Beerens, 1990). The samples were spread plated or membrane filtered. The plates were incubated anaerobically (using CO_2 generating kit (Oxoid gas generating kits, EC labs, Port Elizabeth, South Africa) in gas jars (Merck, Johannesburg/Cape Town, South Africa) at 37°C for 48 hours. Colony numbers were calculated by undiluting and expressed as CFU/ml. The inactivation rate is calculated by the log of the CFU/20ml per hour. The survival rates were calculated using the log CFU of the initial minus the final divided by the time elapsed time period.

3 Results and Discussion

The river water chemical parameter range from Grahamstown sampling sites are shown in Table 5. The results of the Grahamstown water were analysed using PRIMER 6+PERMANOVA(Plymouth Marine laboratory, 2009). The data was analysed for correlation between multiple variables simultaneously, using DISTLM (Distance-based linear models) with Bray Curtis and Akaike Information Criterion (AIC) as measurement parameters. The main chemical parameters were thus identified from these statistics. The significant chemical parameters were chlorides and pH as well as temperature. The dissolved oxygen may have been significant, however lower dissolved oxygen rates may have not been a significant influence on the survival of facultative and obligate anaerobes should not significantly influence their die-off (Rolfe et al., 1977, Talwalkar and Kailasapathy, 2004). Testing water dissolved water and keeping it constant was not possible, thus it was not included in the design. The chemical water quality results were compared to known chemical parameters which affect microbes and their metabolic requirements. From these ranges the model water was designed. It should be noted that Grahamstown is not a coastal area and thus the salt levels are not particularly high.

The tracking ratio did not always identify the difference between human and animal faecal

Table 5: Ranges of the different chemical parameters in the sampled Grahamstown rivers and surrounding sites over a period from May until September 2011.

Parameter	Range for all of the sampling sites
Bifidobacteria on HBSA agar	0 – 26512 CFU/100ml
Bifidobacteria on Beerens Modified agar	0 – 3600 CFU/100ml
Chlorides	2.03 - 283.19 mg/L
Ammonium	0 - 20.21 mg/L
Phosphate	0 - 12.14 mg/L
Nitrates	0 - 47.3 mg/L
Sulphates	0.56 - 90.01 mg/L
pH	7.06 - 9.85 mg/L
EC	9 – 238 mS/m
Hardness	0 - 403.52 mg $CaCO_3$/L
Temperature	13.5 - 28.6 °C
Escherichia coli	0 – 80 CFU/100 mL
Faecal coliforms	0 – 1600 CFU/100 mL

contamination in the sampling sites. This is shown in Table 6. The cut-off value for the tracking ratio, used by Blanch et al (2006) was 3.2 , while Bonjoch et al (2005) used 0.2 in a smaller study. Neither of these ratios clearly distinguished the contamination of the water in comparison to the site description and input sources identified. Thus the survival rate of the bifidobacteria was tested to identify if this may be the cause of the ratio not working.

The bifidobacteria were isolated from river water in order to use the bifidobacteria present in the environment for more realistic data. The bifidobacteria were cultured overnight in tryptic soy broth with sorbitol added to prevent sorbitol selectivity being compromised. The first factorial design run was started and the results are shown in Table 7. Theinactivation rates varied from 0.54 to 0.82 log CFU/20ml/hour. However the pH of the model water decreased significantly due to acid production and the lack of stabilising minerals. Bifidobacteria are known to produce lactic and acetic acid during growth and thus this problem needed to be addressed, as it would not occur naturally in river water due to stabilising minerals and continuous changes in the water. Higher pHs could lead to a faster die-off rate as bifidobacteria are reported to die-off below pH 4 (Wilson, 2005). However acid tolerance will differ between species due to the differing use of the H^+-ATPase (ATP-Adenosine triphosphate enzyme) activity, which act as a pH maintenance pump (Matsumoto et al., 2004).

The use of calcium carbonate was investigated to identify if it could be used as a buffer for the model water. The survival experiments were run with one model water and varying concentrations of calcium carbonate to ensure that the calcium carbonate did not injure or kill the bacteria alone. The pH change did not vary by more than 0.63, thus calcium carbonate was used. The pH changed as the nutrients were used and the acid increased overtime. Nonetheless,the pH change was not substantial thus it calcium carbonate was used as a buffer at 1g/L concentration. The increased calcium carbonate also

Table 6: The concentration of bifidobacteria and the tracking ratio at selected sites near to and around Grahamstown.

Sites	Sampling date in March 2010	Site description	Sorbitol Utilising Bacteria (SUB) (CFUs/100 mL)	Total Bifidobacteria (TB) (CFUs/100 mL)	Tracking Ratio
Site 9	27	Dam with mainly human faecal input, however cattle may also contribute	55	36	1.5
Site 10	27	Dam with mainly human faecal input, however dogs may also contribute	0	7	0
Site 11	27	Sewage and cattle influence	23000	8000	2.9
Site 12	27	Cattle mainly and humans	0	2500	0
Site 13	31	Mainly wild animals, however human pollution possible from upstream	123	81	1.5
Site 14	31	Mainly wild animals, however human pollution possible from the dam	24	39	0.6
Site 15	31	Dam with mainly wild animal faecal pollution	95	0	ND[a]

ND – Not determined

Table 7: The average concentration of total bifidobacteria (CFU/20mL) on modified Beerens Media from the factorial design model water according to their survival time.

Design number	0 hour	1 hour	3 hours	24 hours	72 hours	Inactivation rate	Initial pH	Final pH
5	258	140	86.5	200	0	0.5458	9.8	3.01
6	264	140	136	217	0	0.7532	5.3	3.56
7	424	420	400	299	0	0.8216	9.8	6.4
8	400	138	313.33	155	42	0.5981	5.3	2.93
9	456	366	286	250	0	0.7586	5.3	3.33
10	457	407	349	276	0	0.799	5.3	4.59
11	300	289	200	154	0	0.7532	9.8	6.73

increased the turbidity of the solution at the beginning of the experiment, while at the end of the experiment a considerable amount of calcium carbonate had formed lumps and was no longer free flowing or in suspension, even after swirling. The model water was never shaken as this would have caused a huge difference in dissolved oxygen concentration. Thus the bottle with model water was swirled well before any sample was taken.

The new survival experiments were done in model water with an initial 1g/L calcium carbonate concentration. The results were also examined on MRS agar as this media was reported to be less inhibitory to stressed bifidobacteria.

As shown previously by Ottoson(2009), the survival rates were lower at 32°C compared to 8°C. The effect of sediment was added to the equation as faecal coliforms were reported in literature to be highly influenced by sediment (LaLiberte and Grimes, 1982, Burton et al., 1987, Sherer et al., 1992). The sediment used was pool sand which had been washed and autoclaved. Conversely, sediment did not seem to significantly affect the survival of bifidobacteria.

Figure 1 shows that none of the different factors investigated significantly affected the survival of bifidobacteria alone. The study shows that the combination of sediment and temperature had the most synergistic effect on the survival of bifidobacteria. The obtained data did not fit any of the GInaFit survival models (Geeraerd, 2012).

The problem with tracking ratios is not necessarily attributable to the survival rate, but the fact that their survival rate is so short, unless there is very recent faecal pollution it may be lower just due to die-off. There did not seem to be a difference in the die-off of the total bifidobacteria compared to the sorbitol utilising bacteria and these are in agreement to the work done by Ottoson(2009). On the other hand, the bifidobacteria did not grow well under stressed conditions on the modified

Table 8: The average bifidobacteria concentration (CFU/ 20mL) on HBSA agar from the different factorial design model water according to the number of hours they survived. The inactivation rate is calculated by the log of the CFU/20ml per hour.

Experimentnumber	0 hours	1 hours	3 hours	24 hours	72 hours	152 hours	Initial pH	Final pH	Inactivation rate (log CFU/20ml/hour)
5	552	222	226	220	210	>500	9.8	3.01	0.8853
6	471	100	60	59	50	3	5.3	3.56	0.0776
7	368	233	230	51	36	0	9.8	6.4	0.9974
8	726	526	230	81	150	0	5.3	2.93	0.7982
9	298	195	100	24	10	0	5.3	3.33	0.5957
10	295	270	133	75	0	0	5.3	4.59	0.8318
11	296	283	176	134	0	0	9.8	6.73	0.7631

OG – overgrown membrane filter

Table 9: shows the number of bifidobacteria enumerated from the model water and the various time periods, with the calculated inactivation rates

Experiment number	Day1	day2	day3	day4	day 13	Inactivation rate (log CFU/20ml/hour)
	0 hours	24 hours	48 hours	96 hours	300 hours	
1	165	36.4	31.6	0	30	0
2	220	29.2	86.8	18.4	17.2	0.083636
3	500	TTC	TTC	110.4	38.4	0.2208
4	260	TTC	TTC	81.6	22.8	0.313846
5	200	38.4	26.4	20	6.6	0.1
6	165	36.8	71.2	26	19.2	0.157576
7	105	44	27.2	137.6	17.4	1.310476

TTC – all dilutions made were overgrown thus accurate counts could not be established.

Beerens media. The survival of bifidobacteria was not significantly different in the presence of faecal coliforms. The enterococci and faecal coliforms survived far longer than the bifidobacteria and this has also been noted by Ottoson(2009). The inclusion of enterococci survival with bifidobacteria is important as Enterococci produce a range of different bacteriocins which can inhibit the growth of other bacteria (Salminen et al., 2004). However they did not seem to affect the survival rates of bifidobacteria.

The study indicates that X_1, X_2, X_3, X_4, X_5 are not significant under these concentrations and conditions thus have no significant effect on the inactivation rate of the bifidobacteria.

The Pareto Chart shows the statistically significant factors or lack thereof. The bar graph lengths are proportional to the absolute values of the estimated effects. A bar graph which crosses the vertical (critical line, blue line) can be interpreted as having a significant effect on Y (inactivation rate).

The Pareto Chart depicted in Figure 1enables the identification of the statistically significant effects. The vertical line represents the critical value (14.928) indicates that none of the variables or combinations thereof were significant at a 90% significance level.

A commonly method for evaluating the model is Analysis of variance (ANOVA) as shown in Table 10. It provides some statistical parameters such as the coefficient of determination, R^2 and the F-value, which compare regression and residual variance (Box and William, 2005, Pontes et al., 2011).

The F-value for the model is higher than the tabulated one, indicating model reliability at the 90% level of confidence. Moreover the R^2 approaches one which reinforces the good fit of the model. Consequently, 1 is therefore able to navigate the design space in order to determine the response Y. These parameter are shown in Table 10 and Table 11.

4 Conclusions

The tracking ratio needs to be calibrated in entirely different climatic and environmental region within a country. The lack of culturability on Beerens media sooner than HBSA media may affect the tracking ratio calibration. The tracking ratio has not been shown to be significant in South Africa, in spite of this the use of human sorbitol utilising bacteria for pollution identification is still worthwhile. The SUB do not replicate in the environment and die-off rapidly, thus for tropical areas it maybe more reliable to identify human faecal contamination than *E. coli*, but it is best used in conjunction with *E. coli* enumeration. This has been stated previously by Mushi et al (2010). The other possible reason for tracking ratio calibration problems could be the lack of continuous

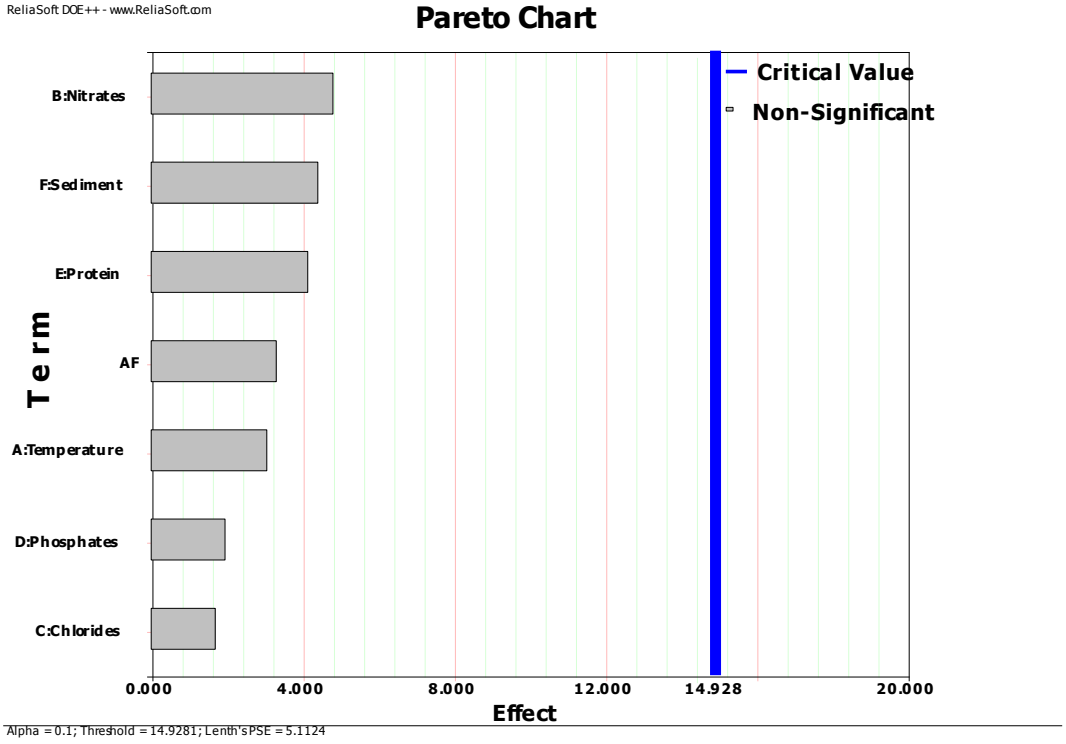

Figure 1: The Pareto Chart of the bifidobacteria survival rate versus the different environmental parameters using DOE++ version 1.0.7.

Table 10: ANOVA table of the effects of the inactivation rate of the bifidobacteria due to the conditions established.

ANOVA Table					
Source of Variation	**Degrees of Freedom**	**Sum of Squares [Partial]**	**Mean Squares [Partial]**	**F Ratio**	**P Value**
Model	6	328.534	54.7557	10.346	0.2336
Main Effects	5	324.0148	64.803	12.2445	0.2135
2-Way Interaction	1	80.9406	80.9406	15.2937	0.1594
Residual	1	5.2924	5.2924		
Lack of Fit	1	5.2924	5.2924		
Total	7	333.8264			

Table 11: The standard effects for the different parameters.

Regression Information							
Term	**Effect**	**Coefficient**	**Standard Error**	**Low CI**	**High CI**	**T Value**	**P Value**
Intercept		12.3744	-	-	-	-	-
A:pH	-3.5242	-1.7621	-	-	-	-	-
B:Temperature	-4.2806	-2.1403	-	-	-	-	-
C:Nitrates	9.056	4.528	-	-	-	-	-
D:Chlorides	-1.8822	-0.9411	-	-	-	-	-
E:Phosphate	6.5194	3.2597	-	-	-	-	-
F:Protein	2.8958	1.4479	-	-	-	-	-
G:Sediment	2.8306	1.4153	-	-	-	-	-

faecal input. The media for bifidobacteria still needs to be optimised to prevent the lack of culturability under stressed conditions or PCR (Polymerase chain reaction) and enzymatic methods should be considered. However in South Africa this would pose a cost and limit the laboratories in which this enumeration could occur. The chemical water quality of the rivers does not seem to be the significant contribution factor to the die-off rate of bifidobacteria, thus oxygen sensitivity and other factor would need to be investigated.

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