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ORIGINAL ARTICLE

Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish

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Abstract

Aims: To isolate, select and evaluate *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish.
Methods and Results: Natural isolates obtained from mud sediment and *Cyprinus carpio* were purified and assessed *in vitro* for efficacy based on the inhibition of growth of pathogenic *Aeromonas hydrophila* and the decrease in concentrations of ammonium, nitrite, nitrate and phosphate ions. Based on suitability to predefined characteristics, the isolates B001, B002 and B003 were selected and evaluated *in vitro* in the presence of *Aer. hydrophila* and in a preliminary *in vivo* trial with *C. carpio*. The inhibitory effect on pathogen growth and the decrease in concentrations of waste ions was demonstrated. Based on 16S RNA sequence homology, the isolates were identified as *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis*, respectively. Isolate B002 did not contain the anthrax virulence plasmids pOX1, pOX2 or the *B. cereus* enterotoxin.
Conclusions: Selected isolates effected synergistic reduction in pathogen load and the concentrations of waste ions *in vitro* and *in vivo* and are safe for use in ornamental aquaculture.
Significance and Impact of the Study: A new approach for assessment of biological agents was demonstrated and has yielded putative isolates for development into aquaculture products.

Introduction

Culture of fish in reticulated systems results in waste accumulation, disease proliferation and negative environmental impact (Liao and Mayo 1974; Boyd 1985; Shimeno *et al.* 1997). Interaction between the host, environmental stress and disease-causing agents contributes to the onset of disease (Paperna 1991; Jeney and Jeney 1995; Austin and Austin 1999; Moriarity 1999), resulting in usage of chemicals and anti-microbials, which alter natural populations, damage the environment and increase resistance and virulence of pathogenic micro-organisms (de Kinkelin and Michel 1992; Gatesoupe 1999; Moriarity 1999; Skjermo and Vadstein 1999; Sze 2000; Jana and Jana 2003). Alternative methods for disease control and enhancement of water quality are therefore required (de Kinkelin and Michel 1992; Barker 2000; Sze 2000). Bacterial amendments have potential to improve fish health by improving water quality and reducing pathogen load (Fast and Menasveta 2000; Gomez-Gil *et al.* 2000; Jana and Jana 2003; Hong *et al.* 2005). However, the usefulness of biological agents is dependant on their survival in the environment (Gross *et al.* 2003). Spores of the genus *Bacillus* have advantages over vegetative cells, because they are stable for long periods, can be formulated into useful commercial products, are widely used as biological agents, possess antagonistic effects on pathogens and are naturally ingested by animals (Hong *et al.* 2005). *Bacillus* spp. are furthermore unlikely to use genes for antibiotic resistance or virulence from Gram-negative micro-organisms such as *Aeromonas* spp. (Moriarity 1999). The objectives of this study were to isolate microbes of the genus *Bacillus* from

natural environments in South Africa and to determine their suitability towards enhancement in water quality, using *Cyprinus carpio* (ornamental carp) as a model species, because the specimen value of ornamental carp is high and survival of ornamental carp is important for both hobbyists and culturists. Ulcerative erythrodermatitis caused by *Aeromonas hydrophila* is a disease prevalent in this species, which can result in damage to the appearance of the specimen and mortality (Jeney and Jeney 1995; Austin and Austin 1999). Furthermore, nitrogen- and phosphorous-based waste accumulations pose a threat to fish health and the environment (Jana and Jana 2003) and result in stress which aggravates infestation by parasites and pathogens (Liao and Mayo 1974; Jeney *et al.* 1992; Ng *et al.* 1992; Grommen *et al.* 2002; Gross *et al.* 2003; Jimenez-Montealegre *et al.* 2005). In the present study, selection criteria were based on the ability of isolated microbes of the genus *Bacillus* to decrease concentrations of both pathogenic bacteria and the ions of waste metabolism, typically produced by *C. carpio*. Selected putative biological agents were evaluated under simulated pond water conditions *in vitro* and *in vivo* and subjected to identification and safety tests. Safety assessment in selection studies of biological agents is limited (Verschuere *et al.* 2000; Hong *et al.* 2005; Balcázar *et al.* 2006) and there is thus an impetus for thorough evaluation of the required characteristics and appropriate safety assessment of putative biological agents prior to development into commercial products.

Materials and methods

Isolation of putative biological agents of the genus *Bacillus*

A method was developed based on previous research (Holt *et al.* 1994) and was validated using known organisms inoculated into sterile soil samples, wherein a full recovery of test organisms was achieved (data not presented). Mud samples and live *C. carpio* were obtained from dams used for the rearing of this fish species in the Gauteng Region, South Africa. Mud sediment was collected from the bottom of the dam in a presterilized McCartney bottle. Mucus was collected from the skin layer by scraping with a sterile loop and gut contents were collected by squeezing the abdomen of the specimen to release the excrement from the gut. Each of the mud sediment samples, skin mucus samples or gut content samples (1 g suspended into 3 g of 0·9% m/v NaCl solution) was added into a presterilized McCartney bottle containing nutrient broth (9 ml) and incubated for 24 h at 30°C followed by incubation at 45°C for 10 min in a convection oven to activate the sporulation process. Ethanol (50% v/v) was added to a volume of 20 ml to each of the bottles which were incubated at 20°C for 1 h. The contents were centrifuged at 10 000 ***g***, the supernatants decanted and the resultant pellets incubated at 105°C in a convection oven for 5 min. The dry pellets were reconstituted into 20 ml of sterile physiological saline and serially diluted to 10^{-4} in 10^{-1} increments. Thereafter, aliquots (0·1 ml) of each of the serial dilutions were spread onto nutrient agar plates supplemented with polymyxin B (10 mg l^{-1}; Merck, Darmstadt, Germany). The plates were incubated for 24 h at 30°C. Single colonies isolated from these plates, were purified and subjected to Gram staining, API identification (API 50 CHB/CHE, Biomérieux, Marcy-l'Etoile, France) and an assessment of catalase activity (Washington 1981).

Isolate and pathogen storage

Isolates were grown in culture media (0·8% m/v yeast extract, 0·005% m/v $MnSO_4$, 0·01% m/v $CaCl_2$ and 0·03% m/v $MgSO_4{\cdot}7H_2O$). The pathogenic organism, *Aer. hydrophila* (ATCC 7966), was obtained from the American Type Culture Collection (www.atcc.org) and grown according to Kielwein *et al.* (1969) and Kielwein (1971). Cultures were cryo-preserved using sterile glycerol (25% v/v) as described by Meza *et al.* 2004. Media components were obtained from Merck (Darmstadt, Germany). These cryo-preserved cultures were used as starter inocula for all experiments.

Isolate selection based on *in vitro* assessments

The *in vitro* tests comprised inhibition of pathogenic *Aer. hydrophila*, determination of specific growth rate and determination of the decrease in the concentrations of ammonium, nitrite, nitrate and phosphate ions in water. Inhibition of *Aer. hydrophila* by each of the isolates was assessed by plate well inhibition assays (Bauer *et al.* 1959). Whole broth of each isolate was preincubated in Tryptic Soy Broth (Foldes *et al.* 2000) at 30°C for 24 h. Aliquots (0·1 ml) were added per well to nutrient agar plates prespread with *Aer. hydrophila* and incubated at 30°C for 24 h, followed by measurement of zones of inhibition. Specific growth rate (μ) and rate of decrease in ion concentration were determined by inoculation of each of the isolates into synthetic pond water (0·0085% m/v KNO_3, 0·006% m/v $NaNO_2$, 0·0093% m/v $(NH_4)_2SO_4$, 0·0038% m/v H_3PO_4, 0·1% m/v yeast extract and 0·1% m/v glucose) which was composed to amplify detection of effects that would typically be found in conventional pond water, and was prepared by dissolution of the ingredients in tap water, pH adjustment to 7·00 using NH_4OH (25% m/v) and sterilization through a 0·22 μm sterile

filter. Synthetic pond water (100 ml) was decanted into presterilized 500 ml Erlenmeyer flasks. Each flask was inoculated with one cryovial of each isolate and incubated at 30°C on a rotary shaker at 220 rev min^{-1} (Innova 2300, New Brunswick Scientific, Edison, NJ, USA). Each flask was aseptically sampled (5 ml) prior to inoculation and thereafter two hourly up until the stationary growth phase was observed. The specific growth rate (μ) was determined from $OD_{660\ nm}$ measurements (Genesys 20 spectrophotometer, Spectronic, USA) for data points conforming to high linearity ($r^2 > 0{\cdot}9$) of a plot of $\ln(OD_{660\ nm})$ against time. Samples were analysed for ion concentrations of ammonium (Reflectoquant, Cat. No. 1·16892·0001, Merck, Darmstadt, Germany), and nitrate, nitrite and phosphate which were measured by ion exchange chromatography (Morales *et al*. 2000) using Ion Chromatography (Dionex, Sunnyvale, USA) with an anion precolumn and anion separator column (Dionex AG14 and AS14, Sunnyvale, USA). The rate of decrease in the concentrations of ammonium, nitrite, nitrate and phosphate ions by each isolate in synthetic pond water was determined by using data points conforming to high linearity ($r^2 > 0{\cdot}9$) of a plot of ion concentration against time. Statistical comparison of data was by two-tailed *t*-tests assuming equal variance.

Verification of performance of selected isolates by *in vitro* co-cultivation with pathogenic *Aeromonas hydrophila*

Selection criteria for isolates were grouped into growth rate, inhibition of pathogen and decrease of waste ion concentration, and the response values normalized as a relative percentage of the maximum for each grouping to provide an overall suitability index, which indicated the cumulative desirability for each of the isolates for the criteria tested. Each of the selected isolates (B001, B002 and B003) and a control organism (B007; selected on the basis of low growth, low ion removal rates, and inability to inhibit pathogen growth), were co-inoculated with *Aer. hydrophila* into synthetic pond water and cultivated as described previously. Combinations of the selected isolates (B001 + B002, B001 + B003, B002 + B003, B001 + B002 + B003) were similarly tested. The viable cell count of *Aer. hydrophila* was determined by serial dilution and plating on selective agar (Kielwein *et al.* 1969; Kielwein 1971), whereas the viable cell count of *Bacillus* spp. was assessed on Nutrient Agar plates supplemented with Polymyxin B (Donovan 1958) as described previously. All trials were conducted in triplicate. Specific growth rate (based on viable cell count) and rate of decrease in ion concentration were determined as previously described.

Identification of selected isolates by genetic evaluation – 16S RNA sequences

Amplification of the 16S gene of each selected isolate (B001, B002 and B003) was performed (University of Cape Town, DNA Sequencing Laboratory, Cape Town, South Africa) using five sets of forward and reverse overlapping sequence primers, which allowed sequencing of the entire length of the double-stranded DNA (approx. 1423 bp). The five sets of forward (^{f}) and reverse (^{r}) primers (5′–3′) were as follows (Alm *et al*. 1996).

^{f1}AGAGTTTGATCTGGCTCAG ^{r1}GTATTACCGCGGCTGCTGGCAC

^{f2}ACTCCTACGGGAGGCAGCAG ^{r2}GGACTACCGGGTATATCTAATCC

^{f3}GCCAGCAGCCGCGGTAATAC ^{r3}CACGAGCTGACGACACCATGC

^{f4}GGATTAGATCCCGGTAGTCC ^{r4}CCATTGTAGACGTGTGAGCCC

^{f5}GCATGGTGTCGTCAGCTCGTG ^{r5}ACGGTACCTTGTTACGACTT

Sequencing was carried out using a DYEnamic ET Dye Terminator Cycle Sequencing kit for MegaBACE based on traditional dideoxynucleotide chain termination chemistry (Lane 1991). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). PCR cleanup was performed using Qiaquick PCR purification kits (Qiagen, Cat. No. 28104, Hilden, Germany), High Pure PCR Product purification kits (Roche Applied Science, Cat. No. 1 732 668, IN, USA) or using Post Reaction Purification columns (Sigma, Cat. No. S-5059, St Louis, MO, USA) as per manufacturer's instructions. Sequence alignments were performed using BLASTN, available on the NCBI server (http://www.ncbi.nlm.nih.gov).

Safety assessment of selected isolates

Anthrax detection was performed using a BioThreat Alert™ kit (Tetracore Inc, Rockville, USA) according to manufacturer's instructions. Additionally, presence of *B. anthracis* virulence plasmids (pOX1 and pOX2) in isolate B002 was examined using the LightCycler *Bacillus anthracis* Detection kit (Roche Applied Science, Cat. No. 03303411001, Basel, Switzerland), which allows specific amplification of *capB* and *pagA* genes. Total genomic DNA extraction of *B. cereus* was performed using a High Pure PCR Template Preparation kit (Roche Applied Science, Cat. No.11796828001, Basel, Switzerland). Real-Time PCR was performed using a LightCycler 2·0 (PerkinElmer, MA, USA) according to the manufacturer's instructions which enabled on-line evaluation of

amplification efficiency as well as melting curve analysis to access the accuracy of individual PCR preparations and reactions. Positive controls contained *capB* and *pagA* amplicons as template DNA (50–100 ng) and the negative controls contained PCR water. Two separate reactions were performed using specific primers targeting amplification of genes encoding capB and pagA proteins and using template DNA (50–100 ng) from the *B. cereus* isolate (B002). Evaluation of *B. cereus* enterotoxin production was performed using the Oxoid *Bacillus cereus* diarrhoeal toxin kit (BCET-RPLA, Oxoid Ltd, UK) according to manufacturer's instructions.

Preliminary assessment of efficacy of selected isolates *in vivo*

A combination of the isolates B001, B002 and B003 were tested *in vivo* to verify the effects observed *in vitro*, whereby pathogen inhibition and decreases in the concentrations of waste ions excreted by *C. carpio* were investigated. The experiment was carried out in glass aquaria (100 l) comprising triplicate control and test systems. Each aquarium was equipped with three air diffusers, a base filter and a reticulated filter chamber. Filter chambers were packed with biological filter matting (1·22 g l^{-1}). The water recirculation rate and air flow rate were 6·0 and 1·8 l per litre of tank volume per hour, respectively. Each aquarium was purged every 3 days (5% v/v) and tap water was added to maintain the starting volume. Each aquarium was stocked with 11 juvenile *C. carpio* with an average mass of 3·49 ± 0·13 g and average length of 59·80 ± 1·07 mm per specimen. The stocking density in each aquarium was 0·38 ± 0·01 g l^{-1}. Fishes were fed commercial koi pellets (3 mm diameter, ∼35% m/m protein) twice daily at a rate of 5% of the initial total body mass, per day, for the duration of the trial. The pH was maintained between 7·0 and 8·0 and was adjusted with either HCl (1% m/m) or $CaCO_3$ (2 mol l^{-1}). The temperature was maintained in the range 20 ± 1°C using an aquarium heater (150 W). Temperature and dissolved oxygen were measured daily using a Multi 350i multimeter (WTW, Weilheim, Germany). The system was not cleaned for the duration of the trial. Control aquaria were dosed with a water placebo, whereas test aquaria were treated with a mixture (1 ml each) of equal proportions of the isolates B001, B002 and B003 at a dosage rate of 1×10^5 CFU l^{-1} at 7-day intervals. *Aeromonas hydrophila* was dosed at the same level to all aquaria. The dosage rates of the isolates and *Aer. hydrophila* were based on previous studies examining dosage and efficacy response *in vitro* (data not presented), wherein 1×10^5 CFU l^{-1} was determined to be a suitable dosage. The dosage regime could furthermore be realistically applied in commercial systems for *Bacillus*-based products. The control and test aquaria were sampled every second day and the water samples analysed for total count of *Bacillus* spp., *Aer. hydrophila* and oxygen, ammonium, nitrite, nitrate and phosphate concentrations. The test and control treatments were compared over the 80-day period by examining the frequency of occurrences, where the concentrations of key measurables were significantly lower ($P < 0·05$) in the test than the control treatment because an overall analysis of averages is unreliable in naturally fluctuating systems. At the end of the trial period, the fishes were removed from the aquaria, the sludge and slime contents washed into the water phase and homogenized prior to the collection of three randomly selected samples. All samples were analysed as described previously.

Results

Isolation, selection and evaluation of isolates *in vitro*

Nine isolates belonging to the genus *Bacillus* were isolated by the procedure described. Six of the isolates were obtained from mud sediment of a dam used for the rearing of *C. carpio*, two from the intestinal content and one from the skin mucus layer of *C. carpio*. All isolates obtained were spore forming, Gram-positive and catalase positive rods and identified as *Bacillus* spp.

Results for evaluation of growth rate, pathogen inhibition and decrease in the concentrations of waste ions *in vitro* are tabulated in Table 1. The highest growth rates were observed for the isolates B001, B002 and B003, with the remaining isolates demonstrating substantially lower growth rates. Only the isolates B002 and B003 inhibited the growth of *Aer. hydrophila* in plate well inhibition assays. The isolate B001 reduced the concentrations of all four of the ions measured, whereas the isolates B002 and B003 reduced the concentrations of all the ions except phosphate. The remaining isolates reduced the concentrations of two or less of the four ions measured. The suitability index, based on growth rate, pathogen inhibition and rate of decrease in ion concentration indicated that the isolates B002, B001 and B003 displayed suitability indices of 84·1%, 44·1% and 41·6%, respectively, which were significantly higher ($P < 0·01$) than any of the other isolates tested. Based on these results, the isolates B001, B002 and B003 were selected as candidate putative biological agents and subjected to further study. The isolates B001, B002 and B003 were therefore deposited at the Netherlands Culture Collection of Bacteria as NCCB 100131, 100132 and 100133, respectively. Isolate B007 resulted in the lowest suitability index and was selected as a control organism for these studies.

Table 1 Summary of responses of isolates against criteria measured *in vitro*

Isolate No.	Specific growth rate μ (h^{-1})	Inhibition zone diameter	Ion reduction rate (mg l^{-1} h^{-1})			
			Ammonium	Nitrite	Nitrate	Phosphate
Control	0·000	NI	NR	NR	NR	NR
B001	1·400	NI	1·000	0·606	3·550	7·205
B002	1·609	+(16 mm)	1·371	2·025	8·575	NR
B003	1·211	+(18 mm)	0·514	1·138	1·900	NR
B004	0·702	NI	0·486	NR	NR	5·750
B005	0·659	NI	0·429	NR	NR	NR
B006	0·616	NI	1·329	NR	NR	9·275
B007	0·162	NI	0·565	NR	NR	NR
B008	0·235	NI	0·619	NR	NR	NR
B009	0·181	NI	1·071	0·988	NR	NR

NI, no inhibition; NR, no reduction.

Table 2 Comparison of combinations of isolates against single isolates selected *in vitro*

Isolate No.	Pathogen growth rate (h^{-1})	Ion reduction rate (mg l^{-1} h^{-1})			
		Ammonium	Nitrite	Nitrate	Phosphate
Control B007	0·437	0·077	0·056	0·014	0·230
B001	0·240	0·860	2·314	1·440	3·412
B002	−0·199	1·160	5·300	2·860	1·188
B003	−0·173	0·594	1·944	2·622	1·146
B001 + B002	−0·571	1·900	5·667	2·183	1·888
B001 + B003	−0·465	1·145	4·830	1·683	1·457
B002 + B003	−0·572	1·000	4·910	2·467	1·322
B001 + B002 + B003	−0·622	2·313	6·730	3·150	3·430

Co-cultivation of the isolates B002 or B003 with pathogenic *Aer. hydrophila* in synthetic pond water resulted in complete growth inhibition of the pathogenic bacteria (Table 2). The growth rate of *Aer. hydrophila* was −0·199 and −0·173 when co-cultivated with isolates B002 and B003, respectively. All the test isolates grew favourably. Isolate B001 attenuated pathogen growth rate (0·238 h^{-1}) in comparison with the control organism B007 (0·606 h^{-1}) (Table 2). Isolate B002 resulted in the highest rate of decrease of ammonia, nitrate and nitrite concentrations, whereas isolate B001 demonstrated the highest rate of decrease of phosphate concentration. All the three selected isolates had a significantly higher ($P < 0·01$) rate of decrease of all ions, in comparison with the control isolate B007, which demonstrated a negligible decrease in concentration of any of the ions measured (Table 2). Additionally, the combination of all three of the selected isolates (B001 + B002 + B003) resulted in a significantly lower ($P < 0·01$) pathogen growth rate and higher rate of decrease in ion concentration when compared with single and paired combinations of isolates (Table 2). The percentage composition of B001, B002 and B003 was 33%, 28% and 29%, respectively, at the end point of this test.

Identification and safety assessment of selected isolates

Based on the preliminary identification by API 50 CHB/CHE, the selected isolates B001, B002 and B003 were classified as *B. subtilis*, *B. mycoides* and *B. licheniformis*, respectively. As a result of inaccuracies at species level in the API system, the identification of the isolates was verified by 16S RNA sequence homology. The results of the 16S RNA identification of the isolates B001 and B003 matched the tentative identification by API 50 CHB/CHE and were classified as *B. subtilis* (99%) and *B. licheniformis* (98%), respectively. Isolate B002 was reclassified as *B. cereus* based on the results of the BLAST search which corresponded to a match of 100%.

The close relationship between *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides* is well documented (Leonard *et al.* 1998). Identical 16S rRNA sequences for *B. anthracis* and *B. cereus* have also been reported (Ash and Collins 1992). Results of the rapid qualitative test (Bioalert™) revealed that none of the three isolates selected were positive for anthrax toxins (data not show). Molecular level differentiation (Real-time PCR) specifically targeting amplification of plasmid-borne virulence factor genes clearly showed that the *B. cereus* isolate (B002) did not contain the genes encoding any of the virulence plasmids (pOX1 and/or pOX2). Amplification of these virulent genes in positive control reactions using *capB* and *pagA* amplicons as template DNA was observed. Furthermore, results of the melting curve analysis were positive for DNA unfolding and folding, thus indicating successful PCR reactions. This result further confirmed the identification of the isolate B002 as *B. cereus*, which warranted further confirmation that the isolate did not produce the *B. cereus* diarrhoeal enterotoxin. The isolate

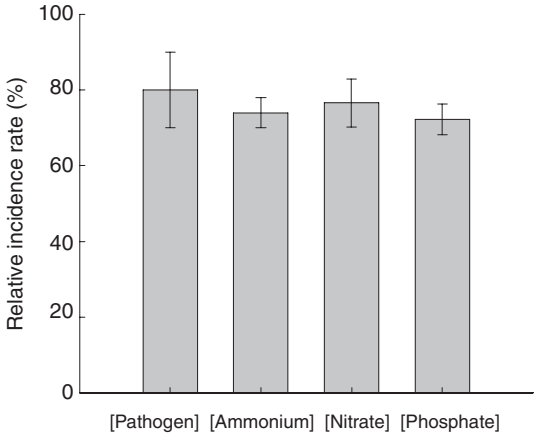

Figure 1 Incidence rate (%) where concentrations of key responses were lower in test than in control treatments *in vivo*.

B002 was found to be negative for the presence of this enterotoxin (data not shown).

Preliminary assessment of efficacy of selected isolates *in vivo*

Based on the results of the *in vitro* study and the biosafety assessment, the isolates B001, B002 and B003 were examined *in vivo*. The data analysed over the 80-day time period are presented as a frequency distribution of occurrences in the test treatments in comparison with the control treatments in Fig. 1. Pathogen, ammonium, nitrite and nitrate concentrations were significantly lower in test treatments when compared with control treatments in 80%, 74%, 77% and 72% of the occurrences measured ($n = 120$, $P < 0{\cdot}05$), respectively. Nitrite was not detectable in any of the treatments for the duration of the trial. There were no mortalities or observable incidents of disease and there were no significant differences in oxygen concentration ($P = 0{\cdot}391$), fish mass ($P = 0{\cdot}522$) or length ($P = 0{\cdot}276$) gain. The results of pathogen and ion concentrations measured in control and test treatments at the terminal point of the *in vivo* trial (Fig. 2) were similar to the frequency distribution data over the 80-day period, wherein pathogen, ammonium, nitrate and phosphate concentrations were significantly lower in test treatments when compared with the control treatments ($P < 0{\cdot}001$). The cumulative concentration of *Bacillus* spp. was significantly higher in the test treatment ($P < 0{\cdot}001$), whereas the pathogen concentration was correspondingly lower when compared with the control treatment. In all cases, the percentage composition of the isolates B001, B002 and B003 approximated that observed in the *in vitro* study.

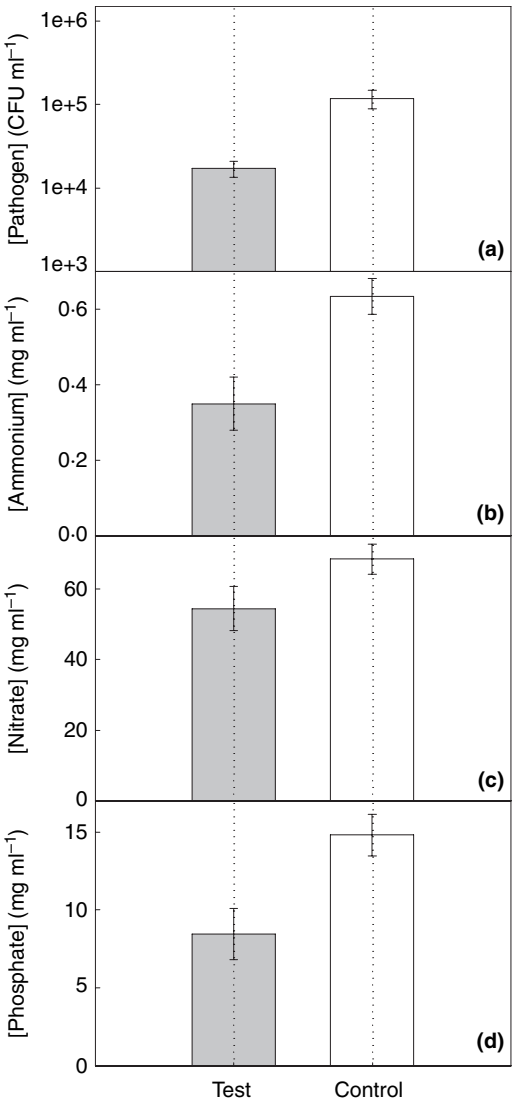

Figure 2 End point comparisons of test and control concentrations of key responses *in vivo* (a) pathogen, (b) ammonium, (c) nitrate and (d) phosphate.

Discussion

Application of spore-forming bacteria as biological agents for improving water quality and reducing disease offers a number of advantages (Sanders *et al.* 2003; Wolken *et al.* 2003) and a number of spore-forming biological agents are sold worldwide for animal use (Sanders *et al.* 2003). The isolation procedure yielded nine *Bacillus* isolates and similar approaches based mainly on resistance of endospores to elevated temperatures have elsewhere been reported for isolation of *Bacillus* spp. (Foldes *et al.* 2000).

Attenuation of growth of *Aer. hydrophila* by the isolate B001, inhibition of growth by the isolates B002 and B003 and a decrease in pathogen cell number when a mixed

culture (B001 + B002 + B003) was tested in synthetic pond water (Table 2) could potentially be ascribed to the mechanism of competitive exclusion (Sanders *et al.* 2003; Hong *et al.* 2005), because the average growth rates of the selected isolates were in excess of 1·5 times than that of *Aer. hydrophila* (data not shown), when compared with the control treatment (B007). These isolates therefore demonstrate potential for reduction of *Aer. hydrophila* in aquaculture systems (Vanbelle *et al.* 1990; Matoyama *et al.* 1999; Moriarity 1999). Furthermore, the isolates B001, B002 and B003 decreased the concentrations of ammonia, nitrite and nitrate ions in synthetic pond water (Table 2). The isolate B001 decreased the ammonia concentration more rapidly than the nitrite concentration, whereas the isolates B002 and B003 decreased the concentration of nitrite more rapidly than that of ammonia, indicating that application of B002 and B003 could prevent nitrite accumulation. All three isolates were capable of decreasing the concentrations of nitrates at rates exceeding that of ammonia and nitrite, thus indicating a potential synergistic benefit for use of all three isolates as biological agents. In this study, the selected isolates demonstrated significantly higher rates of decrease in waste ion concentrations ($P < 0{\cdot}01$) than the control organism and this phenomenon was also previously observed in *B. subtilus*, *B. cereus* and *B. licheniformis* (Kim *et al.* 2005). The mixed culture of the selected isolates may potentially exert these effects via a multitude of mechanisms such as bioaccumulation, bio-assimilation, nitrification and dissimilatory nitrate reduction. Although nitrogen removal is classically predominated by autotrophic bacteria in natural systems, there have been several reports suggesting a contribution by heterotrophic bacteria in this regard (Abou Seada and Ottow 1985; Robertson and Kuenen 1990; Sakai *et al.* 1996; Sakai *et al.* 1997; Martienssen and Schöps 1999; Su *et al.* 2001; Kim *et al.* 2005; Lin *et al.* 2006). Isolate B001 decreased the concentration of phosphate ions in primary studies (Table 1), but all the selected isolates decreased phosphate concentration in the presence of the pathogen (Table 2), which may have been caused by improved bio-availability of bound phosphate, through solubilization (Illmer and Schinner 1995). This attribute is important as phosphate and nitrate accumulation can result in algal blooms in culture systems (Kaushik 1995).

In addition to quantification of the efficacy of the selected isolates, the identification and safety assessment is an important requirement fulfilled by this study. The identification of organisms within the *B. cereus* group is difficult because of the genetic similarity between *B. cereus*, *B. anthracis* and *B. thuringiensis* (Carlson *et al.* 1994; Phelps and McKillip 2002; Helgason *et al.* 2004; Rasko *et al.* 2004). In the present study, 16S rRNA-based identification of *B. cereus* was limited by the nearly identical 16S rRNA sequences of the group members (Ash *et al.* 1991; Ash and Collins 1992). Definitive identification and safety of B002 was therefore based on the absence of key virulence genes, such as the *B. anthracis* lethal toxin complex (pOX1) and poly-D-glutamic acid capsule (pOX2) (Okinaka *et al.* 1999). Furthermore, the absence of anthrax toxin and strain dependent *B. cereus* enterotoxin in isolate B002 (Turnbull 1999) confirms the safety of this isolate for use as a potential biological agent.

The findings of the preliminary *in vivo* trial were similar to those of the *in vitro* tests, wherein there was an attenuation of prevalence of *Aer. hydrophila* and a decrease in the concentrations of ammonium, nitrate and phosphate ions by the mixed culture of the three selected isolates over the 80-day trial period (Fig. 1) and at the end point of the trial (Fig. 2). The treatment did not result in a negative impact on oxygen sufficiency, growth or health of the test specimens, which is an important consideration for potential application of the isolates. Although the interaction of the putative biological agents with the larger bio-community when tested *in vivo* could have resulted in a complex ecological system, the observations were similar to those in the *in vitro* studies. The selection of isolates *in vitro* based on grouped characteristics through suitability indices have resulted in synergistic holistic improvements in water quality *in vivo*. Other researches have also reported that the addition of beneficial bacteria can enhance the health of animals by effecting a holistic improvement in waste ion removal and pathogen reduction (Larmoyeux and Piper 1973; Liao and Mayo 1974; Jeney and Jeney 1995; Shimeno *et al.* 1997; Boyd and Tucker 1998; Frances *et al.* 1998; Frances *et al.* 2000). Results have clearly indicated the synergistic positive effect of the selected isolates of *Bacillus* spp. on pathogen inhibition and water quality *in vitro* and *in vivo*. The selected isolates should be further evaluated in larger scale *in vivo* trials and an in-depth assessment of the mode of action regarding pathogen inhibition and removal of waste ions should be made, prior to progress towards commercial products.

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