



Streptophage-mediated control of off-flavour taint producing streptomycetes isolated from barramundi ponds



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ABSTRACT

Off-flavour taint of aquaculture products is a global issue reducing consumer confidence in the farmed produce as they are taken up via the gills of fish, and deposited in the lipids of the animal. If the fish are not purged, resulting undesirable muddy earthy flavour taint can be tasted by consumers. These undesirable flavour and odour is caused by the terpenoid compounds namely geosmin and 2-methylisoborneol, produced as secondary metabolites by certain bacteria including the cyanobacteria and actinomycetes. Current strategies to remediate the problem rely on treating the symptoms not the cause and involve the use of time consuming purging methods and costly chemicals.

Biological control using bacteriophages, specific to the problem causing bacteria, offers a natural alternative to chemical control, which might reduce further complications of copper based algacides and its subsequent implications on water quality. In an adaptation of such biological control approach streptomycetes isolated from barramundi ponds were tested for their susceptibility to streptophages to understand whether host destruction via phage lysis would subsequently eliminate off-flavour taint productions by these isolates.

Following the determination of the streptophage susceptibility of the isolates one of the most odourous streptomycete species (USC-14510) was selected to be tested further using different pond simulations resembling real-life applications. Geosmin was tested as the indicator of off-flavour taint production and as it has been previously reported that the cyanobacteria-actinomycete interactions occurring in ponds result in even greater levels of geosmin and 2-methylisoborneol, the geosmin levels for the isolate in the presence of cyanobacteria and streptophages were also tested. Findings indicated that the highly odourous *Streptomyces* species (USC-14510) once infected with streptophages, can lose its capacity to produce off-flavour taints. Pond simulation studies also revealed geosmin production was significantly reduced when streptophages were introduced into the pond water where streptomycete species were grown. The bacteriophage control method developed in the presented study might again confirm significant potential for the bacteriophage-mediated remediation strategy to be adapted by the aquaculture industry.

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1. Introduction

Barramundi (*Lates calcarifer*) is an indigenous bony fish species of northern Australia and Asia, currently valued at \$25 million with

28% of the total aquaculture produce in Australia [1]. Due to the demand through both the domestic and international markets, wild-captured barramundi supplies are not able to satisfy current market requirements. This demand has pushed for an increase in the production of the fish via terrestrial aquaculture ponds and barramundi aquaculture at present is valued at around \$20 million for Queensland alone, with envisaged further increase [2].

However, one of the main issues faced by aquaculturists is the off-flavour taints that affects many freshwater aquaculture

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Table 1

Testing for geosmin and 2-methylisoborneol production by the isolate USC-14510 when treated with and without the phage suspension on agar slants.

Sample type	Phage Application and Fibre exposure
Fibre Blank	Run of blank fibre
Control	Fibre exposed to agar only for 10 min
Streptomycete isolate USC-14510 and daily streptophage inoculation	Daily addition of 100 μ L of phage suspension to 100 μ L of spread and dried streptomycete suspension, Fibre exposed for 10 min
Streptomycete isolate USC-14510 and single streptophage inoculation	Once only application at Day 0 of 100 μ L of phage suspension to 100 μ L of spread and dried streptomycete suspension, Fibre exposed for 10 min
Streptomycete isolate USC-14510 only	100 μ L streptomycete suspension spread and dried only. Fibre exposed for 10 min

products worldwide. These off flavours are the bio-accumulation of primarily two compounds that are geosmin and 2-methylisoborneol. Geosmin (GSM) and 2-methylisoborneol (2-MIB) are semi-volatile, terpenoid compounds which are secondary metabolites produced by wide range of microorganisms [3] but mainly by cyanobacteria [4] and actinomycetes [5]. The off-flavour taint is a problem for not only barramundi farmers, but for many other freshwater aquaculture species internationally such as catfish and trout, as well as for drinking water supplies [6]. The fish can be unmarketable if the off-flavour taints the flesh, and purging is the only method of removing the off-flavour [7]. This has severe economic implications for the industry due to costs [8].

Queensland has one sea cage operation [2] and six to eight pond based barramundi aquaculture systems, which are freshwater, estuarine and salt systems [9]. Most ponds are unlined, earth ponds, and are located north of the Mackay area (21.1411°S, 149.1861°E), above the tropic of Capricorn, an area with average temperatures of 13–23 °C in July and 23–30 °C in December and January [10]. With such high temperatures and excessive nutrients from both fish waste and fish meal feedstock, and direct soil contact, barramundi ponds are conducive to actinomycete growth and cyanobacterial blooms.

When aquaculture ponds are constructed in Australia as bare earth ponds, with no lining, metabolically active component of the pond microflora produce the GSM and 2-MIB that are released into the pond water. As both GSM and 2-MIB are lipophilic, they are taken up via the gills of fish, and deposited in the lipids of the animal [11,12]. If the fish are not purged, consumers can taste these compounds, recognised as an undesirable muddy earthy flavour taint [13]. Purging adds a delay of 3–5 days before the fish are marketable, plus the need to have the area for a depuration pond and availability of clean water, free of the impurities and their producers [7].

Current strategies to remediate the problem rely on treating the symptoms not the cause as well as the use of time consuming purging methods and costly chemicals. Other methods of prevention of tainting the fish include applications of copper sulphate as an algacide, the use of recirculation tanks where the pond water is continually filtered and the mechanical methods used for the removal of cyanobacteria after a bloom occurs [14]. Yet such human intervention still is not effective in preventing the release of the metabolites to the pond impacting product quality. Preventing the formation of the taint is therefore the ideal solution, however, the

dynamic environment of the aquaculture ponds is conducive to high algal and bacterial growth, and the use of products harmful or able to be accumulated by the fish, to control the algae or bacteria is limited. One environmentally-friendly approach might be the use of bacteriophages specifically targeting the odour causing microorganisms since bacteriophages have been proven effective in different environmental setting to control targeted bacteria [15–17]. Currently, the use of bacteriophages in aquaculture industry is gaining importance such as their application in the fishery industry for infection control in fish and shellfish [18–20]. Actinophages are viruses that infect actinomycetes and they were reported to comprise six morphological types and belong to three different viral families: *Myoviridae*, *Siphoviridae* and *Podoviridae*. Streptophages are the ones specific for the members of the family *Streptomycetaceae* and mostly belong to the *Siphoviridae* group with a long and non-contractile tail [21] and they usually show a high degree of polyvalency within family [22,23].

In the light of above presented information the following research study was designed to investigate the potential use of streptophages as biological control agents to eliminate odourous streptomycetes in laboratory and simulated settings with a long-term objective to reduce or eliminate off-flavour taints produced by these bacteria in barramundi ponds.

2. Methods and materials

2.1. Isolation and characterization of the streptomycete isolates

Streptomycetes were isolated from barramundi ponds using mud samples collected from barramundi ponds located in northern Queensland, Australia using the methods described by Küster and Williams [24] and stored at the University of the Sunshine Coast, Microbial Library at –25 °C in frozen glycerol suspension [25].

Isolates were identified using molecular sequencing method [26]. The QIAGEN HotStarTaq® Multiplex PCR Kit and the primers B27F and 1492R were used to obtain PCR products, which were subsequently sequenced at Macrogen Inc. (South Korea, <http://www.macrogen.com/>). 16S rRNA sequences were subsequently deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). 16S rRNA gene sequences were aligned in the same orientation with the online implementation of the SINA aligner (<http://www.arb-silva.de/aligner>) [27]. The sequences were then de-gapped and subsequently de-replicated by clustering at 99.9%

Table 2

Experimental design details of the pond simulation tests.

1	2	3	4	5	6
Sand	Sand	Sand	Sand		
Pond water	Pond water	Pond water	Pond water	Pond water	Pond water
Streptomycete isolate	Streptomycete isolate	Streptomycete isolate in sand	Streptomycete isolate in sand	Streptomycete isolate	Streptomycete isolate
Phage		Phage in sand		Phage	
		Rubber lining	Rubber lining	Rubber lining	Rubber lining

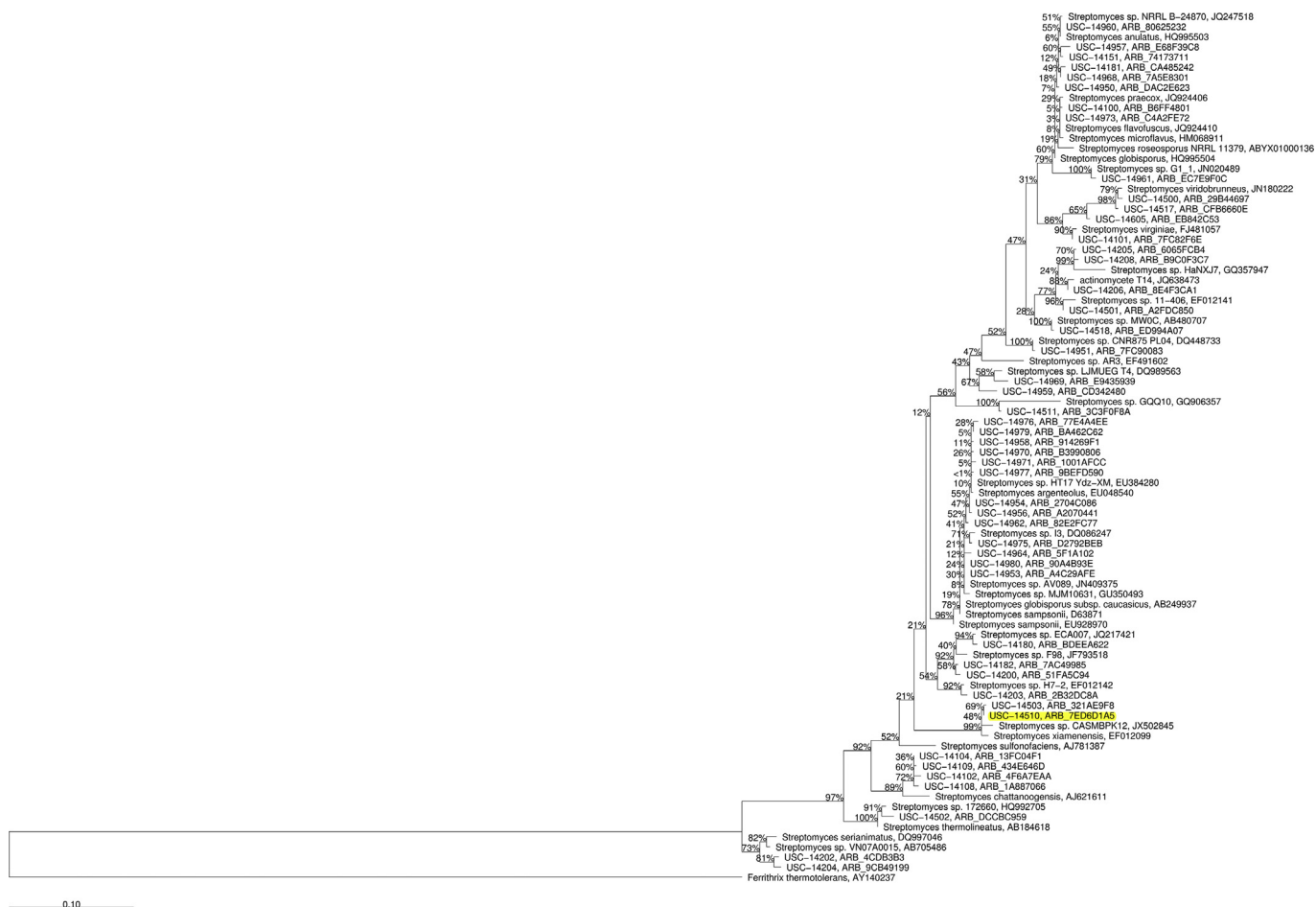


Fig. 1. Phylogenetic tree of the odorous streptomycete isolates in relation to their closest relatives of the family Streptomycetaceae. *Ferrithrix thermotolerans* was used as an out-group sequence to root the tree.

sequence identity using the online implementation of CD-HIT-EST [28]. Representative sequences from each cluster were then realigned with SINA online and imported into the ARB software environment version 6.0.3 [29]. Imported sequences were added to the main reference tree supplied within ARB via the parsimony method to identify and select close relatives of the imported sequences. With these additional reference sequences and the imported query sequences, phylogenetic trees were constructed *de novo* using the RAxML algorithm [30] as implemented within ARB, with 100 bootstrap tests.

2.2. Isolation and characterization of the streptophages

Ten different streptophages were isolated and purified using single plaque dilution method [31] from various pond-associated mud and soil samples in Queensland, Australia against the streptomycete isolates as hosts. They were characterized by their morphology using a Transmission Electron Microscopy (TEM) and ability to lyse streptomycete isolates used in this study per the methods described by El-Tarabily et al. (1995) [23]. They were further tested for their virulence against the streptomycete type strains to further confirm their identity.

Titre of the streptophages per ml of suspension (pfu/ml) was determined using the method described by Bradley et al. (1961) [32]. A high titre of suspension of all phages ($\times 10^{10}$ pfu/ml) was subsequently prepared by mixing all streptophages and used to lyse the odorous streptomycetes using the confluent lysis method by

placing two drops of the phage suspension in the middle of the host lawn on the Peptone Yeast Extract Calcium (PYCa) agar plate [32]. The following criteria were used to determine the largest confluent lysis zone: (1) +++: Highly susceptible (complete lysis of the host), (2) ++: Susceptible (complete partial lysis), (3) +: Moderately susceptible (lysis and single plaques), (4) \pm : Low susceptibility (lysis but regrowth of the host), (5) -: Not susceptible.

2.3. Odour determination and headspace gas chromatography studies

From the streptomycete isolates, a selected subset with the evidence of musty-earthly smell production on the agar plates as well as susceptible to the streptophages were selected for the detection of geosmin and 2-MIB levels at the Department of Agriculture and Fisheries, Queensland Government. One of the most odorous isolate USC-14510 was subsequently selected to study further to produce these compounds when treated with the streptophage suspension.

Headspace-gas chromatography mass spectroscopy (HS-GC/MS) was used (PerkinElmer Clarus 580 Gas chromatograph, PerkinElmer Clarus SQ 8 S Mass spectrometer). This method was chosen as a way of identifying the mass spectroscopy peaks of geosmin and 2-methylisoborneol, and comparing the peak areas as a method of comparison for each sample, allowing evaluation of increasing or decreasing concentration. Whilst this method was unable to quantify the absolute concentration present, the comparison of the

Table 3
Details of the odorous streptomycetes isolated from barramundi ponds.

Isolates	GenBank Accession Number	Phage Susceptibility	Geosmin Peak Area	2-MIB Peak Area
USC-14100	KX358624	++		
USC-14101a	KX358625	–		
USC-14101b	KX358626	±		
USC-14102	KX358627	++		
USC-14104	KX358628	+++		
USC-14106	KX358629	+		
USC-14108	KX358630	++		
USC-14109	KX358631	+		
USC-14151	KX358632	+		
USC-14181	KX358634	+		
USC-14182	KX358635	+		
USC-14200	KX358636	++	865332	447338
USC-14201	KX358637	±	573208	186515
USC-14202	KX358638	+++	374194	164924
USC-14203	KX358639	++	134239	352857
USC-14204	KX358640	–		
USC-14205	KX358641	±	99818	150086
USC-14206	KX358642	±	665933	149262
USC-14207	KX358643	±		
USC-14208	KX358644	±	451078	158817
USC-14500	KX358645	–		
USC-14501	KX358646	±	175280	222033
USC-14502	KX358647	±	225622	207677
USC-14503	KX358648	++		
USC-14510	KX358649	+++	2804783	267244
USC-14511	KX358650	±	2597848	180380
USC-14514	KX358651	–		
USC-14515	KX358652	–		
USC-14516	KX358653	–		
USC-14517	KX358654	±	87420	392802
USC-14518	KX358655	++	309388	171351
USC-14519	KX358656	–	191429	486395
USC-14520	KX358657	±	75537	197640
USC-14605	KX358658	++		
USC-14707	KX358659	++	16902	44151
USC-14950	KX358660	++		
USC-14951	KX358661	+		
USC-14952	KX358662	++		
USC-14953	KX358663	+		
USC-14954	KX358664	±		
USC-14955	KX358665	+		
USC-14956	KX358666	+		
USC-14957	KX358667	++		
USC-14958	KX358668	±		
USC-14959	KX358669	+		
USC-14960	KX358670	±		
USC-14961	KX358671	±		
USC-14962	KX358672	+		
USC-14963	KX358673	++		
USC-14964	KX358674	++		
USC-14965	KX358675	++		
USC-14966	KX358676	++		
USC-14967	KX358677	±		
USC-14968	KX358678	+		
USC-14969	KX358679	+		
USC-14970	KX358680	+		
USC-14971	KX358681	++		
USC-14972	KX358682	+		
USC-14973	KX358683	+		
USC-14974	KX358684	+++		
USC-14975	KX358685	++		
USC-14976	KX358686	–		
USC-14977	KX358687	+		
USC-14978	KX358688	+		
USC-14979	KX358689	++		
USC-14980	KX358690	+++		
USC-14981	KX358691	++		
USC-14982	KX358692	++		
USC-14983	KX358693	++		

+++ : Highly susceptible (complete lysis of the host), ++ : Susceptible (complete partial lysis), + : Moderately susceptible (lysis and single plaques), ± : Low susceptibility (lysis but regrowth of the host), – : Not susceptible.

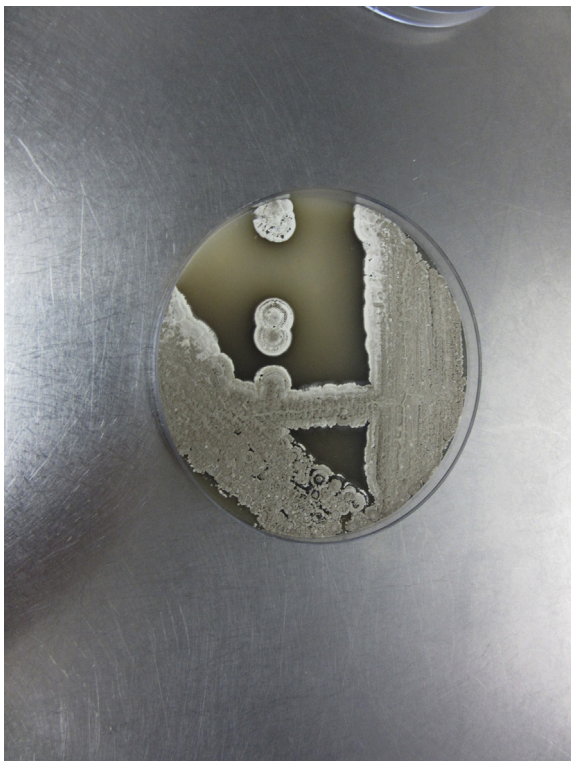


Fig. 2. Growth characteristics of the streptomycete isolate USC-14510 on oatmeal agar.

peak areas provided evidence of variation in concentration. This was performed by the TurboMass software on the GC/MS and is calculated by measuring the area of the peak of interest, as the peak is proportional to the volume of compound present.

Slant oatmeal agar (OAM) in McCartney bottles were prepared to grow the isolate USC-14510 using six replicates for each

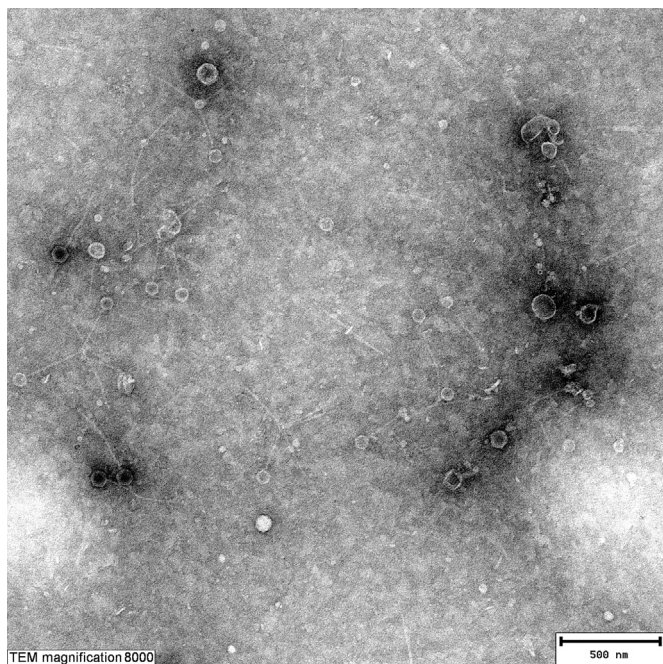


Fig. 3. Electron micrograph of streptophage suspension composed of all different phages (bar indicates 500 nm).

treatment. Details of the treatments used are given in Table 2. A Perkin Elmer Clarus 580 Gas Chromatograph and Clarus SQ 8 S Mass spectrometer were used. The oven temperature was set at 40 °C for 2 min and was ramped 10 °C per minute to 210 °C and held for 1.5 min. The GC injection port temperature was set to 220 °C. Compound ionisation was at 70 eV electron impact analysing over mass range m/z 40–250. The fibre was exposed to each McCartney bottle for 10 min. The fibre was then exposed in the injection port for 2 min for the control blank or 1 min for the four treatments (Table 1).

For the control treatments, standards of geosmin (Aldrich Lot#LB74037) and 2-methylisoborneol (Aldrich Lot# 0001445519) were run. Each standard (1 μ L) was placed in a small vial and this was placed in a larger clean, sterile glass vial sealed with aluminium foil, and allowed to equilibrate for 10 min. The fibre was exposed in the vial with geosmin for one minute, before being run through the HS-GC/MS. These were used to confirm the presence of geosmin and 2-methylisoborneol in the headspace of the McCartney bottles as a metabolite of the streptomycete.

2.4. Pond simulation studies

Pond simulation studies were conducted to determine (1) whether odorous compounds would be absorbed into the sand, (2) whether AlgaBoost™ (F/2 1000 \times concentrate) would trigger cyanobacterial growth which might subsequently be subject to streptomycete colonization and result in the increased odour production, and (3) whether rubber lining material used to line commercially operated ponds would lower the odour levels by providing a barrier against the mud dwelling streptomycetes.

The first two pond simulations, where sand and AlgaBoost™ were used, yielded no geosmin detection by the GC-MS. This could be due to either AlgaBoost™ not being conducive to the growth of streptomycetes, or the sand was adsorbing the geosmin. Accordingly, a third trial was conducted where AlgaBoost™ was replaced by sterile pond water (Table 2).

Synthetic rubber lining (1.5 mL thickness, Clark's Rubber, Australia), that had the same composition of rubber lining that is used in the commercially operated ponds was used in the third test. However, prior to the testing potential toxic effects of the rubber lining on the streptomycete isolate (USC-14510) was determined. Squares of pond lining rubber (1.5 cm²) were cut and sterilised using UV and disinfectants. Spores of the streptomycete isolate (USC-14510) (100 μ L) suspended in sterile MilliQ water and placed onto each rubber square. Spore suspensions on the rubber pieces were then incubated for 7 days at 28 °C. Following the incubation, the streptomycete inoculum was swabbed and placed into PYCa broth and again incubated at 28 °C for 5 days to determine the viability of the streptomycetes. After determination of the lack of toxic effect of the rubber lining material on the streptomycete isolate (USC-14510) the following treatments were tested for the geosmin production by the streptomycete (Table 2).

In a final test to determine whether geosmin production was the highest when cyanobacterium was colonized by the streptomycete isolate a loopful of *Microcystis aeruginosa* (obtained from Innovative Food Technologies, Department of Agriculture & Fisheries, QLD) culture scraped from an agar plate and inoculated into 150 mL filtered pond water in a 250 mL Schott bottle. The suspension was then incubated at 28 °C and after 4 days, 0.5 mL of streptomycetes spore suspension was added together with 100 μ L of the polyvalent phage suspension. Control treatment excluded the phage application. The Schott bottles were left in the incubator until ready to expose the fibre for the GC-MS. The fibre was exposed to the headspace for 10 min. This was then run in the same manner as previously described.

3. Results

3.1. Isolation and characterization of the streptomycete isolates

Molecular characterization of the isolates confirmed that they belonged to the genus *Streptomyces* (Fig. 1). Their GenBank accession numbers are listed in Table 3. Majority of the isolates were found to be susceptible to the polyvalent streptophages and the isolates USC-14104, 14202, 14510, 14974 and 14980 showed the highest level of susceptibility to the phages (Table 3).

USC-14510 was selected to be tested further in the *in vitro* and pond simulation studies due to its phage susceptibility and higher levels of geosmin production (Fig. 2, Table 3). Growth characteristics of the isolate is illustrated in Fig. 2.

3.2. Isolation and characterization of the streptophages

TEM studies confirmed that the isolated phages belonged to the *Siphoviridae* family, of the order *Caudovirales* [21,33] (Fig. 3). Most numerous presented phage particles in the suspension of all phages had a head diameter of ~70 nm, with long non-contractile tails of ~200 nm. Phages were also found to be polyvalent and streptomycete susceptibility results are given in Tables 3 and 4 against the streptomycete isolates as well as the type strains of the genus *Streptomyces*.

3.3. Odour determination and headspace gas chromatography studies

3.3.1. Slant agar test

Phage application test results when streptomycetes were grown in slant oatmeal agar in McCartney bottles indicated that phage-treated streptomycete isolate (USC-14510) was found to produce significantly lower amounts of geosmin (Table 5). Greatest reduction in GSM was observed with the daily phage treatment of the streptomycete isolate (USC-14510) with a ratio of 8.5 compared to the control.

3.3.2. Pond simulation test

Geosmin was detected in the final pond simulation test where AlgaBoost™ and sand were omitted which were found to mask the compound, and was detected at the retention time of around 14 min 35–40 s (Fig. 4). A peak area comparison was made to compare the geosmin production, however, no 2-mib was detected, as was the case when tested on the OAM slants.

Cyanobacteria-streptomycete combination indicated that the geosmin production was significantly higher and however, when the phage was incorporated in the culture system solution with the streptomycetes, the geosmin production was the lowest. Geosmin

Table 5

Geosmin peak area values for streptomycete isolate (USC-14510) treated with and without phage.

Treatment	Peak Area	Ratio
Single addition of phage to USC-14510 on Day 0	1 472 205	2.4
Streptomycete isolate (USC-14510) only	5 233 687	8.5
Daily addition of phage to the streptomycete isolate (USC-14510)	617 681	1

reduction ratio in the final test was 1:3:4 for (1) streptomycete and phage (2) to streptomycete only and (3) to streptomycete and cyanobacteria production of geosmin respectively. However, no 2-mib was detected, as was the case when tested on the slant agar.

4. Discussion

Molecular identification results confirmed that all odorous isolates deriving from the samples obtained from several aquaculture ponds in north Queensland, Australia, where off-taint flavours impacting the sale value of the farmed fish, were the members of the genus *Streptomyces*. Odours in the environments are primarily reported to be caused by cyanobacteria [7] and actinomycetes [34] and the members of the genus *Streptomyces* being the most odorous [35,36]. As an example studies by Rabe et al. [37] in their determination of linking the production of terpenes to genomic data, list *S. xiamenensis* as a species that release large amounts of terpenes. Again, in another example Yamada et al. [38] used genomic comparisons of over 250 *Streptomycetaceae* species to identify the species capable of producing terpene metabolites, stating that many *Streptomyces* species displayed the ability to produce geosmin. Findings of the presented study therefore agree with previous findings. Moreover, during the pond simulation, one treatment combined the streptomycete isolate (USC-14510) with a sample of cyanobacterium *M. aeruginosa* and the peak area for geosmin as measured by the GC-MS, was 25% greater than the streptomycete alone. It has been reported that around one quarter of actinomycetes isolated from soil have cyanobacterial lysing properties [39–42]. The anti-algal compounds produced by certain streptomycetes, such as L-lysine [40] and 2'-deoxyadenosine causes loss of cell integrity, and release of the intracellular contents [41,42]. Correlation between geosmin production and avermectin-type antibiotic production was investigated by Řezanka et al. (1994) [43] who reported with the increase production of the total of avermectins, the synthesis of geosmin is also enhanced by more than one order of magnitude. Accordingly, increased in the geosmin levels produced by the streptomycete species USC-14510 might be due to the anti-algal compound production which, might in turn resulting in increased geosmin levels produced by the species.

Table 4

Streptophages activity spectra against *Streptomyces* type strains.

Phage code	φ-1	φ-2	φ-3	φ-4	φ-5	φ-6	φ-7	φ-8	φ-9	φ-10
Streptomyces Type Strains										
<i>S. albidoflavus</i> (ATCC 8663)	+	+	–	+	–	+	–	–	–	+
<i>S. antibioticus</i> (ATCC 8663)	+	+	+	–	–	+	–	–	+	–
<i>S. coelicolor</i> (ATCC 23899)	–	+	+	–	–	+	+	–	+	+
<i>S. griseus</i> (ATCC 10137)	+	+	+	+	+	+	+	+	–	–
<i>S. halstedii</i> (ATCC 10897)	+	+	+	+	+	+	–	+	–	–
<i>S. hydroscopicus</i> subsp. <i>hydroscopicus</i> (ATCC 27438)	–	+	–	+	+	–	+	+	+	–
<i>S. lividans</i> (ATCC 19844)	+	–	–	–	+	–	+	–	–	+
<i>S. luteoreticuli</i> (ATCC 27446)	–	–	+	–	–	–	+	–	–	+
<i>S. rimosus</i> (ATCC 23955)	+	–	+	+	–	+	–	–	+	+
<i>S. spectabilis</i> (ATCC 27465)	–	+	+	+	–	+	–	–	+	+

+: susceptible; –: resistant.

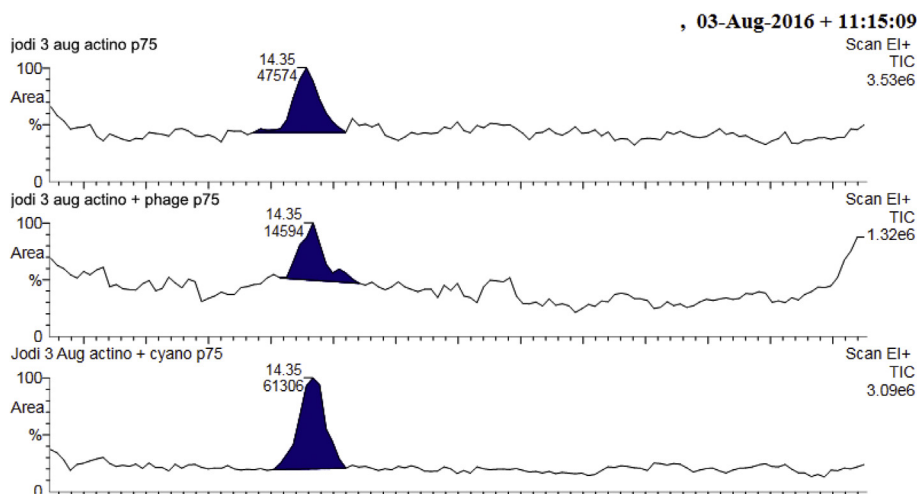


Fig. 4. Geosmin peak areas of the third pond simulation test.

Failure to detect 2-mib might also be due to the masking effect of the high geosmin levels in both agar slant and the pond simulation tests that require further investigation.

The potential of streptophages to be used as preventative agents to control odour production in aquaculture ponds might be significant, since more than 90% of the streptomycete isolates tested showed susceptibility to the lytic activity of these phages. Findings agree with the previous reports that the use of phage suspensions containing different phage types increases the host range spectra [44,45]. The use of composite phage suspension was also reported to reduce the probability of phage resistance in bacteria [44] as different phage infections on the same species would reduce bacterial mutation frequency [46]. Accordingly, the use of polyvalent phages in a composite suspension would provide a broader activity spectra against different species of the genus *Streptomyces* and thus eliminating odorous compounds produced by these species that cause the off-flavour taints [47]. In the long term, revenue losses for the industry can therefore be reduced or fully eliminated, as well as the prevention of the detrimental environmental impact of the chemicals currently used to control the problem.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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